Movement-Associated Proteins of *Potato Virus A*: Attachment to Virus Particles and Phosphorylation

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ACADEMIC DISSERTATION

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IV. Gabrenaite-Verkhovskaya R, Puustinen P, Lomma J, Eskelin K, Rönstrand L, Mäkinen K. 2007. Thr132/Ser133 and Thr168 of *Potato virus A* genome-linked protein VPg regulate the course of infection putatively via phosphorylation. Submitted.

SUMMARY

The particles of *Potato virus A* (PVA; genus *Potyvirus*) are helically constructed filaments that contain multiple copies of a single type of coat-protein (CP) subunit and a single copy of genome-linked protein (VPg), attached to one end of the virion. Examination of negatively-stained virions by electron microscopy revealed flexuous, rod-shaped particles with no obvious terminal structures. It is known that particles of several filamentous plant viruses incorporate additional minor protein components, forming stable complexes that mediate particle disassembly, movement or transmission by insect vectors. The first objective of this work was to study the interaction of PVA movement-associated proteins with virus particles and how these interactions contribute to the morphology and function of the virus particles. Purified particles of PVA were examined by atomic force microscopy (AFM) and immuno-gold electron microscopy. A protrusion was found at one end of some of the potyvirus particles, associated with the 5' end of the viral RNA. The tip contained two virus-encoded proteins, the genome-linked protein (VPg) and the helper-component proteinase (HC-Pro). Both are required for cell-to-cell movement of the virus. Biochemical and electron microscopy studies of purified PVA samples also revealed the presence of another protein required for cell-to-cell movement – the cylindrical inclusion protein (CI), which is also an RNA helicase/ATPase. Centrifugation through a 5-40% sucrose gradient separated virus particles with no detectable CI to a fraction that remained in the gradient, from the CI-associated particles that went to the pellet. Both types of particles were infectious. AFM and translation experiments demonstrated that when the viral CI was not present in the sample, PVA virions had a "beads-on-a-string" phenotype, and RNA within the virus particles was more accessible to translation.

The second objective of this work was to study phosphorylation of PVA movement-associated and structural proteins (CP and VPg) *in vitro* and, if possible, *in vivo*. PVA virion structural protein CP is necessary for virus cell-to-cell movement. The tobacco protein kinase CK2 was identified as a kinase phosphorylating PVA CP. A major site of CK2 phosphorylation in PVA CP was identified as a single threonine within a CK2 consensus sequence. Amino acid substitutions affecting the CK2 consensus sequence in CP resulted in viruses that were defective in cell-to-cell and long-distance movement. The CK2 regulation of virion assembly and cell-to-cell movement by phosphorylation of CP was possibly due to the inhibition of CP binding to viral RNA. Four putative phosphorylation sites were identified from an *in vitro* phosphorylated recombinant VPg. All four were mutated and the spread of mutant viruses in two different host plants was studied. Two putative phosphorylation site mutants (Thr45 and Thr49) had phenotypes identical to that of a wild type (WT) virus infection in both *Nicotiana benthamiana* and *N. tabacum* plants. The other two mutant viruses (Thr132/Ser133 and Thr168) showed different phenotypes with increased or decreased accumulation rates, respectively, in inoculated and the first two systemically

infected leaves of *N. benthamiana*. The same mutants were occasionally restricted to single cells in *N. tabacum* plants, suggesting the importance of these amino acids in the PVA infection cycle in *N. tabacum*.

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Rasa

When a distinguished but elderly scientist states that something is possible, he is almost certainly right. When he states that something is impossible, he is very probably wrong.

Arthur C. Clarke (Clarke's first law)

CONTENTS

ABBREVIATIONS

AFM	atomic force microscopy
BYSV	*Beet yellow stunt virus,* family *Closteroviridae*
BYV	*Beet yellows virus,* family *Closteroviridae*
CD	circular dichroism
CI	cylindrical inclusion protein
ClYVV	*Clover yellow vein virus,* family *Potyviridae*
CMV	*Cucumber mosaic virus*, family *Bromoviridae*
CP	coat protein
CPm	minor coat protein
CTV	*Citrus tristeza virus,* family *Closteroviridae*
EM	electron microscopy
ER	endoplasmic reticulum
Gfp	green fluorescent protein gene
GFP	green fluorescent protein
HC-Pro	helper component proteinase
IGEM	immuno-gold electron microscopy
LMV	*Lettuce mosaic virus,* family *Potyviridae*
MALDI-TOF	matrix-associated laser desorption/ionization – time of flight
MP	movement protein
NIa	nuclear inclusion protein a
NIb	nuclear inclusion protein b
NTP	nucleotide triphosphate
NTR	nontranslated region
OAS	origin of assembly
PPV	*Plum pox virus,* family *Potyviridae*
PSbMV	*Pea seed-borne mosaic virus,* family *Potyviridae*
PVA	*Potato virus A,* family *Potyviridae*
PVBV	*Pepper vein banding virus,* family *Potyviridae*
PVIP	VPg-interacting protein
PVX	*Potato virus X,* family *Flexiviridae*
PVY	*Potato virus Y,* family *Potyviridae*
Rluc	*Renilla* luciferase gene
RLUC	*Renilla* luciferase protein
RNP	ribonucleoprotein
RSS	RNA silencing suppressor
RT-PCR	reverse transcription polymerase chain reaction
SA	salicylic acid
SL	stem-and-loop
SMV	*Soybean mosaic virus,* family *Potyviridae*
TEV	*Tobacco etch virus,* family *Potyviridae*

TGB	triple gene block
TMV	*Tobacco mosaic virus,* genus *Tobamovirus*
ToMV	*Tomato mosaic virus*, genus *Tobamovirus*
TuMV	*Turnip mosaic virus,* family *Potyviridae*
TVMV	*Tobacco vein mottling virus,* family *Potyviridae*
VLP	virus-like particle
VPg	viral genome-linked protein
WSMV	*Wheat streak mosaic virus,* family *Potyviridae*
WT	wild type

1 INTRODUCTION

Viruses infect all major groups of organisms: animals, plants, fungi, bacteria, and archaea. The host range of a virus can be determined first by the ability of the virus to enter the host cell. When the virus is inside the host cell, the infection also depends on whether there is appropriate cellular machinery available for the virus to replicate. Finally, the virus usually needs to get out of the cell and to spread the infection. This thesis concentrates on plant virus (*Potato virus A*) movement-dedicated particle structure and possible regulation of virus spread in plants by structural protein phosphorylation. In this work the novel morphology of the particle movement form is presented. Also the association of two additional movement-related viral proteins to the potyvirus particle is analyzed, and their possible roles in the virus infection cycle are discussed. Finally a new model of the potyvirus movement-competent form is presented, together with a possible interaction scheme between virion structural and associated proteins.

1.1 Basic virion forms

The main function of virion particle formation is to protect of the genome from the hostile surroundings of a host cell. Virus particles (virions) are also very important in virus transfer between host organisms, tissues, and cells. The main structure in any virion is a protein shell, called the protein coat or capsid, surrounding the viral genome (Flint *et al*., 2000; Hull *et al*., 2002). The viral capsid is usually composed of one to several different types of viral proteins. Capsid proteins can also act as enzymes needed for virus genome replication or as mediators in virus-host interactions. In general, five basic structural forms of virus particles can be found: icosahedral virions, helical virions, enveloped icosahedral virions, enveloped helical virions, and complex particles with capsid structures that are still not well understood (Flint *et al*., 2000).

In helically symmetric viruses the protein subunits interact with each other and with the nucleic acid to form a coiled structure. Helical capsids may be rigid or flexible, depending on the arrangement of the protein subunits with respect to one another (Flint *et al*., 2000). A helical virion can be defined mathematically by two parameters: the amplitude (diameter) and the pitch (the distance covered by each complete turn of the coat protein units). Helical symmetry is very common in plant viruses that have a single-stranded, positive-sense RNA genome. Some single-stranded, negative-stranded RNA plant viruses also have helical capsids (Hull *et al*., 2002; Fauquet *et al*., 2005). The best studied virus

with helical symmetry is the non-enveloped plant virus *Tobacco mosaic virus* (TMV).

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The next sections of the introduction will focus on the structure and assembly of viral movement forms of filamentous plant viruses, and on the description of their structural and non-structural movement-associated proteins.

1.2 Movement forms of helical plant viruses

Cell-to-cell movement mechanisms of different plant viruses have some common principles, but their exact nature varies between virus taxa (Carrington *et al*., 1996; Lazarowitz, 1999). There are at least two distinct mechanisms for viral cell-to-cell movement (Carrington *et al*., 1996; Lazarowitz and Beachy, 1999; Lucas, 2006).

The first strategy is found in the virus families *Bromoviridae* and *Caulimoviridae*, the genus *Tospovirus* from the *Bunyaviridae* family, and the genera *Tobamovirus*, *Dianthovirus* and *Tombusvirus*. It is coat protein (CP)-independent (Siegel *et al*., 1962; Takamatsu *et al*., 1987) and requires a single movement protein (MP) (reviewed by Lucas, 2006). MPs are virus proteins involved exclusively in cell-to-cell transport (Waigmann *et al*., 1994). MPs increase the cell plasmodesmata size exclusion limit allowing intercellular virion transport (Lucas, 2006).

A second, well described movement strategy is CP-dependent and is used by comoviruses and nepoviruses (Lucas, 2006). Although this strategy requires CP for cell-to-cell movement, the transport of virions occurs through virus MP-induced tubules that span the cell walls of adjacent cells (Wieczorek and Sanfacon, 1993; Bertens *et al*, 2000).

In spite of the significant sequence divergence between MPs used by the aforementioned virus families, and the different mechanisms of how they modify plasmodesmata, they belong to the same 30K superfamily of proteins (Bertens *et al*., 2000; Melcher, 2000).

Other plant viruses, including members of the family *Closteroviridae*, and genera of *Potyvirus* and *Potexvirus* also depend on the CP for cell-to-cell movement, but they code for two or more additional MPs (Dolja *et al*., 1994; Lazarowitz and Beachy, 1999; Alzhanova *et al*., 2000). In addition to CP, several replication-associated proteins have also been implicated in virus movement (Carrington *et al*., 1996, 1998).

If CP is involved in virus movement, do viruses need virions for movement? The cell-to-cell movement strategy of *Potato virus X* (PVX, family *Flexiviridae*) involves the transport of filamentous virions through plasmodesmata (Cruz *et al*., 1998). An analysis of PVX CP mutants showed that mutations preventing particle assembly also prevented virus accumulation in inoculated

leaves (Chapman *et al*., 1992). Virion formation is also required for cell-to-cell movement of closteroviruses (Peremyslov *et al*., 1999; Satyanarayana *et al*., 2000; Alzhanova *et al*., 2000; Alzhanova *et al*., 2001; Napuli *et al*., 2003) and possibly also of Potyviruses (Dolja *et al*., 1994, 1995; Ivanov *et al*., 2001).

1.3 Structure and assembly of helical plant virus particles

The best example of helical architecture is provided by the rigid, rod-shaped virions of TMV that are formed from a single RNA molecule and more than 2,000 copies of the CP, with the RNA winding in a helical path that follows the arrangement of the CP subunits (reviewed by Klug, 1999; Stubbs, 1999). Similar structures can be found in many families of rod-shaped and filamentous plant RNA viruses (Hull, 2002). Little is known about the assembly mechanisms of helical plant viruses, except for TMV.

1.3.1 Rod-shaped viruses

TMV is the type member of the genus *Tobamovirus*. TMV virion is a rigid rod (18 nm × 300 nm) consisting of 2130 identical CP subunits stacked in a helix around a single strand of the plus-sense RNA, 6395 nucleotides in length. Forty nine CP subunits are arranged in three turns of the viral helix. Each protein subunit binds three nucleotides of viral RNA. Although TMV is one of the simplest viruses known, virus particle assembly is complex. TMV components contain all the information required for virion self-assembly (Fraenkel-Conrat and Williams, 1955; Caspar, 1963; Ohno *et al*., , 1972). TMV assembly was recently reviewed by Butler, Klug, and Stubbs (Butler, 1999; Klug, 1999; Stubbs, 1999, Culver, 2002). Under cellular conditions the 20S helix and/or disks make up the predominate CP aggregates (Diaz-Avalos and Caspar, 1998). These 20S aggregates are required for efficient nucleation with the viral RNA. Virion assembly initiates at the RNA origin of assembly (OAS), a stem-loop structure located 876 nucleotides from the 3' end of the RNA (Okada, 1986). The association of the RNA with CP is predicted to occur by insertion of the OAS stem-loop into the central hole of the 20S aggregate. In the absence of RNA, the structure of the inner loop is disordered so as to allow access of the RNA to its binding site within the 20S aggregate. However, RNA binding results in the structural ordering of residues within the inner loop, locking the nucleoprotein complex into a virion-like helix and allowing assembly to proceed. Assembly

elongation occurs in both the 5' and 3' directions of the RNA (Schuster *et al*., 1980). Assembly in the 5' direction occurs rapidly and is consistent with the addition of 20S aggregates to the growing nucleoprotein rod. The 5' end of the RNA is pulled up through the central hole of the helix. Assembly in the 3' direction is much slower and is thought to involve the addition of either single subunits or protein aggregates (reviewed in Butler, 1999; Klug, 1999; Stubbs, 1999, Culver, 2002).

1.3.2 Filamentous viruses

Closteroviruses

Differently from rigid TMV virions (300 nm), virions of closteroviruses are flexous and exceptionally long filamentous particles (1500-2200 nm) that mediate protection and active transport of the genomic RNA within infected plants. These virions are composed of a long "body" and a short "tail" (Fig. 1). Five of the ten encoded proteins are incorporated into the virion (Agranovsky *et al*., 1995; Napuli *et al*., 2000; Prokhnevsky *et al*., 2002; Napuli *et al*., 2003). The main body of the virus particle (15- to 20-kb positive-strand RNA genome) is encapsidated by the major capsid protein (CP) (Karasev, 2000; Dolja, 2003). The virion tail is composed of a CP homolog - a minor capsid protein (CPm) (Agranovsky *et al*., 1995; Boyko *et al*., 1992; Tian *et al*., 1999) and covers about 650 bases of the 5' terminus of the genomic RNA (Satyanarayana *et al*., 2004). Both CP and CPm share conserved amino acid sequence motifs with other filamentous plant viruses (Boyko *et al*., 1992). Two additional proteins are present in the virion tails, Hsp70h and a 64kD homolog (p64 in *Beet yellows virus* (BYV), p61 in *Citrus tristeza virus* (CTV)) that contains a CP-like C-terminal domain and a unique N-terminal domain (Tian *et al*., 1999; Napuli *et al*., 2000, 2003). These two proteins assist in virion formation or stabilization (Satyanarayana *et al*., 2000; Alzhanova *et al*., 2001; Napuli *et al*., 2003). Tail disassembly may promote RNA uncoating. The fifth virion protein of BYV is a 20 kDa protein (p20) that interacts with Hsp70h (Prokhnevsky *et al*., 2002). The efficient assembly and subsequent cell-to-cell movement of closteroviruses requires CP, CPm, HSP70h, and 64 kDa homolog (Peremyslov *et al*., 1999; Satyanarayana *et al*., 2000; Alzhanova *et al*., 2000, 2001; Napuli *et al*., 2003; Alzhanova *et al*., 2007), although a low level of assembly was observed when only CP or CPm was present (Satyanarayana *et al*., 2000).

The formation of the tailed virions is a prerequisite for intercellular trafficking. The p20 protein is dispensable for BYV virion assembly and cell-to-cell movement, but it is necessary for transport through the plant vascular system (Prokhnevsky *et al*., 2002). Atomic force microscopy (AFM)

analyses revealed that the tail was thinner than the rest of the virion and that it was subdivided into three segments (Fig. 1, Peremyslov *et al*., 2004). Very similar tail morphology was observed (Peremyslov *et al*., 2004, supplementary material) for a BYV relative, *Grapevine leafroll-associated virus 2* (Zhu *et al*., 1998), indicating that the tail structure is conserved at least among two members of the genus *Closterovirus.* Because the p20-deficient virions contain all other virion proteins but lacked the tail end-segment, it was proposed that this segment was formed by p20 (Peremyslov *et al*., 2004).

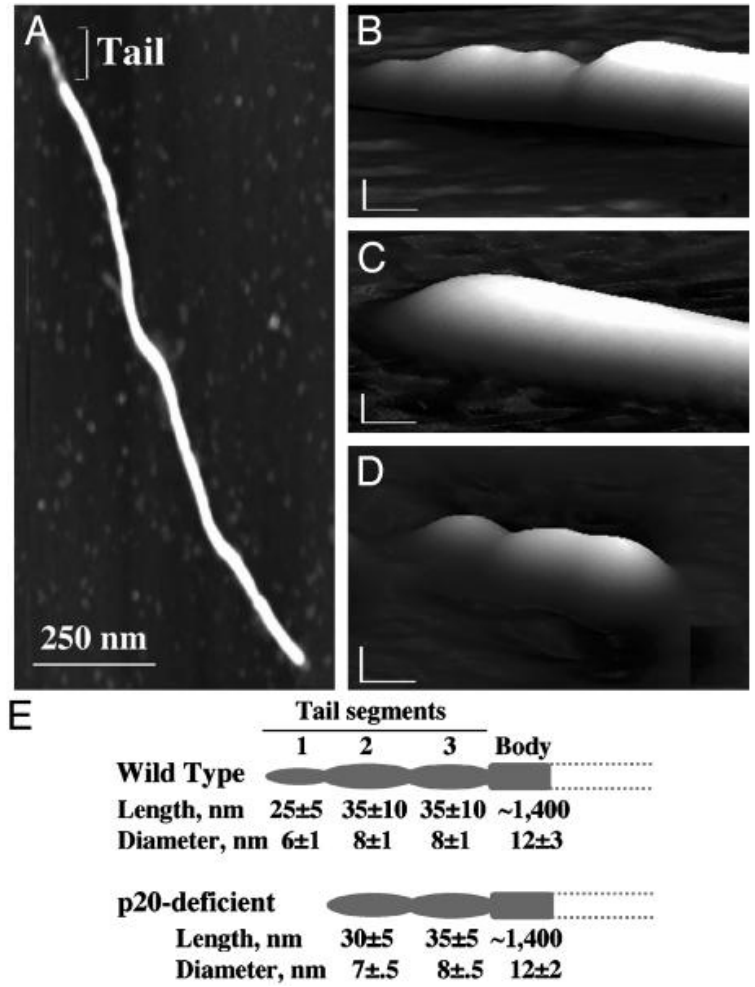

Figure 1. Atomic force microscopy picture of BYV particle body and tail (Peremyslov *et al*., 2004). (A) A full-length virion. (B) A three-segmented virion tail. (C) An opposite blunt end of the virion. (D) Three-segmented tail isolated after sonication of the virions. B-D are 3D images; horizontal bars = 25 nm and vertical bars = 5 nm. (E) Models of the tail regions for the wild-type virions (Upper) and p20-deficient virions (Lower). ©PNAS

The closterovirus virion packaging signal is located at the 5' end of the genome. It was shown that in BYV, CPm alone can initiate virion assembly (Peremyslov *et al*., 2004). The CPm of CTV can encapsidate and protect the entire genomic RNA, albeit at reduced levels. However, the combination of HSP70h, p61, and CPm restricted encapsidation to the 5' ~630 nucleotides (Satyanarayana *et al*., 2004). Two conserved stem-and-loop (SL) structures within the 5' nontranslated region (NTR) (Lopez *et al*., 1998), which are *cis*-acting elements necessary for replication, are involved in virion formation of CTV (Gowda *et al*., 2003). The 5' NTR sequences varied considerably in different CTV isolates, and sequence identity was as low as 42% (Albiach-Martí *et al*., 2000), but all strains were predicted to retain two similar adjacent SL structures (Lopez *et al*., 1998) in the positive strands (Gowda *et al*., 2003). The element required to initiate assembly of the CTV CPm overlaps with putative SL structures required for RNA replication (Satyanarayana *et al*., 2004). The CPm OAS was larger and less mutation-tolerant than the overlapping replication element (Satyanarayana *et al*., 2004). The encapsidation efficiency of the Turnip yellow mosaic virus depended on coat protein binding to protonated cytosines in the 5' proximal hairpin (Bink *et al*., 2002) and later it was shown that the stability of the 5' proximal hairpin of the 5' NTR regulated translation efficiency and initiation of encapsidation (Bink *et al*., 2003).

The roles of Hsp70h and 64kD in tail formation are not clear. It seems that, together, these proteins may act as molecular rulers for determining the length of the tail or as connectors between tail and body and/or individual tail segments. Tail assembly possibly requires an active Hsp70h ATPase domain and ATP hydrolysis coupled to substrate binding (Alzhanova *et al*., 2001). The assembly of virion body could start by attachment of the CP subunits to the surface at one end of a preformed tail.

Potexviruses

Potexvirus virions are flexous rods, 470-580 nm long and about 13 nm in diameter (Hull, 2002), with a deeply grooved surface (Parker *et al*., 2002), which is highly hydrated – bound water molecules help to maintain the surface structure of the virion (Baratova *et al*., 2004).

PVX is a filamentous virus (modal length of about 515 nm and diameter of 13.5 nm). PVX particles consist of a positive sense, single-stranded RNA molecule coated with approximately 1300 identical CP subunits. PVX has a helical structure, with the CP N-terminus exposed on the viral surface (Baratova *et al*., 1992a, b; Parker *et al*., 2002). Subunits form a helical array (3.6 nm pitch), with the 6.4 kb viral RNA packed between the turns of the helix (Parker *et al*., 2002). Tritium planigraphy (Bogacheva *et al*., 1998) revealed that the N-terminal

region of the CP was exposed on the virion surface, whereas the C-terminal region in intact virus particles was almost inaccessible (Baratova *et al*., 1992). There are 8.9 subunits per turn of the primary helix in PVX (Parker *et al*., 2002) and 7.8 subunits per turn in the case of narcissus mosaic virus (Kendall *et al*., 2007). The 25 kDa CP of PVX exists as a 1.8S monomer in the presence of disaggregating agents (Miki and Knight, 1968; Dementjeva *et al*., 1970). After removal of the disaggregating agent, the 3–5S aggregate was a major component of PVX protein preparations and a minor, 10–15S component was also revealed (Dementjeva *et al*., 1970). Further aggregation of protein with production of a limited number of single- and double-layered discs and short stacks of discs was shown by electron microscopy (EM) and optical-diffraction patterns (Kaftanova *et al*., 1975).

It has been proposed that cell-to-cell movement of potexviruses involves either virions (Cruz *et al*., 1998) or a particular type of ribonucloprotein (RNP) complex different from native virions (Lough *et al*., 1998, 2000). Microinjection studies provided evidence of intercellular movement of an *in vitro*-assembled RNP consisting only of potexvirus RNA, CP and triple gene block (TGB) protein p1 (Lough *et al*., 1998, 2000).

In vitro assembly experiments with the potexvirus, *Papaya mosaic virus*, have identified 38–47 nucleotides at the 5' terminus required for initiation of virion assembly (Sit *et al*., 1994). Initiation of *Clover yellow mosaic virus* and PVX assembly, unlike those of TMV were suggested to be sequence-specific rather than secondary structure-specific (Sit *et al*., 1994).

Potyviruses

Potyvirus coats are thought to consist of about 2000 copies of a single type of CP subunit (Varma *et al*., 1968). Recent EM and fiber diffraction studies of *Wheat streak mosaic virus* (WSMV) have confirmed the helical structure of the virions (Parker *et al*., 2002) and showed that the helical pitch of the virus was about 3.3 nm with 6.9 (5.9-7.8) CP subunits per turn of the helix. The mechanism of assembly of potyviruses is poorly understood. Recombinant CP of several potyviruses assembles into virus-like particles (VLP) when expressed in *Escherichia coli* (Jagadish *et al*., 1993; Jacob and Usha, 2002). The two charged residues R194 and D238 of *Johnsongrass mosaic virus* CP were shown to be required for the assembly process. These amino acids were previously thought to be involved in the formation of a salt bridge crucial for the assembly process (Dolja *et al*., 1991), but mutational analysis showed that the salt bridge might not be necessary (Jagadish *et al*., 1993). The C-terminal part of *Soybean mosaic virus* (SMV) CP contained two small regions (amino acids 190-212 and 245-249) required for CP-CP interaction and virus assembly

(Kang *et al*., 2006). In *Plum pox virus* (PPV) three amino acids, (R(3015), Q(3016) and D(3059), amino acid numbering from polyprotein), are required for virion formation in infected *N. benthamiana* cells (Varrelmann and Maiss, 2000).

Assembly of virus particles was shown to begin with the interaction of CP subunits within the 5' terminal region of progeny viral RNA molecules (Wu and Shaw, 1998). Anindya and Savithri (2003) provided evidence that disassembly and reassembly of *Pepper vein banding virus* (PVBV) proceeded via a ring-like intermediate (Anindya and Savithri, 2003). They showed that electrostatic interactions could be pivotal in stabilizing the particles. The N-terminal 53 and C-terminal 23 amino acids were found to be crucial for the intersubunit interactions involved in the initiation of virus assembly. These segments are surface exposed in the ring-like intermediate and indispensable for further interactions that result in the formation of the VLPs.

1.4 Structural proteins of filamentous viruses

1.4.1 Coat proteins (CP)

In addition to the assembly, cell-to-cell and long-distance transport functions of plant virus CPs, they have roles in replication, vector transmission, cross-protection, and symptom development (Callaway *et al*., 2001). Amino acid sequence analyses (Dolja *et al*., 1991) have demonstrated the existence of two families of the CPs that assemble into helical capsids of plant viruses: the capsid proteins of rod-shaped viruses (tobamo-, tobra-, hordei-, and furoviruses) and those of filamentous viruses (poty-, bymo-, potex-, carla-, and closteroviruses). (Callaway *et al*., 2001). Both families might have diverged from separate protein ancestors (Dolja *et al*., 1991; Callaway *et al*., 2001).

Closterovirus CP and Cpm

Systematic analysis of 38 CP and CPm mutants of BYV clearly indicated that assembly of the tailed virion is a prerequisite for movement (Alzhanova *et al*., 2001). Each mutation introduced into CP or CPm that prevented assembly of the virion body or tail resulted in complete arrest of virus translocation (Alzhanova *et al*., 2001). However, those authors suggested that the virion body and tail had different roles in virus infection. The major role of the body may be to prevent RNA degradation during its translocation inside and between cells. An additional role of the

body would be to provide a structural platform for tail attachment. However the studies of CTV suggested that the tails were formed prior to the body (Satyanarayana *et al*., 2004). Although either CP or Cpm alone was sufficient for encapsidation of entire viral genome (Alzhanova *et al*., 2001; Satyanarayana *et al*., 2004), assembly of fully functional virions required involvement of additional viral proteins (Peremyslov *et al*., 2004; Alzhanova *et al*., 2006). CPm is also required for the plant-to-plant transmission of closteroviruses by insects (Tian *et al*., 1999).

Potexvirus CP

Participation of CP in the potexviruses infection cycle is not limited to its role in virion formation. Cell-to-cell movement and encapsidation functions of *White clover mosaic virus* CP were clearly separated: the movement-deficient C-terminally truncated CP mutant was still able to form virions (Lough *et al*., 2000). Potexvirus CP is also responsible for induction of the Rx resistance system in potato plants (Kohm *et al*., 1993; Goulden and Baulcombe, 1993), and has been shown to play a major role in regulation of virion translational activity at different stages of the infection process (Atabekov *et al*., 2000, 2001; Kozlovsky *et al*., 2003; Rodionova *et al*., 2003). The N-terminal region of potexvirus CP is surface-located (Baratova *et al*., 1992a), highly sensitive to the action of plant sap proteases (Koenig *et al*., 1978), and can be easily removed by mild trypsin treatment without disruption of the virion structure (Tremaine and Agrawal, 1972).

CP is essential for PVX movement (Baulcombe *et al*., 1995) through both plasmodesmata and phloem (Cruz *et al*., 1998; Fedorkin *et al*., 2001). Although CP was shown to localize to plasmodesmata (Rouleau *et al*. 1995; Oparka *et al*. 1996), PVX CP did not play an active role in their modification (Cruz *et al*., 1998). An increase in the plasmodesmal size exclusion limit was dependent on one or more of the TGB proteins and was independent of CP (Cruz *et al*., 1998). PVX mutants lacking either a functional CP or TGB were restricted to single epidermal cells (Cruz *et al*., 1998).

Potyvirus CP

A tritium planimetry-based 3D model of *Potato virus A* (PVA) CP showed that the C-terminal region was not exposed (Baratova *et al*., 2001). The model predicted three regions of tertiary structure : (a) the surface-exposed N-terminal region, consisting of an unstructured N-terminus of 8 amino acids and two β-strands, (b) a C-terminal region including two α-helices, as well as three β-strands that form a two-layer

structure called an abCd unit, and (c) a central region comprising a bundle of four α-helices in a fold similar to that found in TMV CP (Baratova *et al*., 2001). A central region of PVA CP was implicated in the inter-subunit interactions (Baratova *et al*., 2001), while 53 N-terminal and 23 C-terminal amino acids of PVBV were crucial for CP-CP interactions and virion assembly (Anindya and Savithri, 2003).

The N-terminal domain of potyvirus CP is very important for potyvirus cell-to-cell movement and virion assembly. In *Tobacco etch virus* (TEV), the N-terminus (amino acids 131-175) was responsible for CP oligomerization (Voloudakis *et al*., 2004). Asp198 was necessary to maintain protein secondary structure required for CP subunit interactions, but not for *in vitro* protein-RNA interactions (Voloudakis *et al*., 2004). The C-terminal region of SMV CP was shown to contain a domain(s) or amino acids required for CP-CP interaction and virus assembly (Kang *et al*., 2006). Deletion of the C-terminal region of the CP caused loss of the CP-CP self-interaction ability.

Kimalov *et al.* (2004) have shown that interaction between potyviral CP subunits did not depend on the net charge of the CP N-terminus. Although the study showed that altering the surface-exposed N-terminal domain charge of *Zucchini yellow mosaic virus* CP resulted in systemically non-infectious or replication-deficient viruses, the charge change had no effect on the formation of VLPs in bacteria (Kimalov *et al*., 2004).

Potyviral CP interacts with several viral proteins. These interactions are important for virus cell-to-cell movement and transmission by vectors. CP-helper component proteinase (HC-Pro) interaction is required for aphid transmission (Pirone and Blanc, 1996). Although coordinated functions of HC-Pro and CP are also required for accumulation and movement of PVA (Andrejeva *et al*., 1999), no interactions were found between PVA HC-Pro and CP in the yeast two-hybrid system (Guo *et al*., 1999, 2001). However, an interaction between HC-Pro and viral CP was demonstrated in extracts of *Lettuce mosaic virus* (LMV) -infected leaves, as well as for two other potyviruses, PPV and *Potato virus Y* (PVY) (Roudet-Tavert *et al*., 2002). Electron microscopy indicated that HC-Pro probably does not interact with the CP in the form of assembled virions or VLPs (Roudet-Tavert *et al*., 2002). Atreya *et al*. (1991) characterized the Asp-Ala-Gly (DAG) motif in the N-terminus of potyviral CP as essential for aphid transmissibility (Atreya *et al*., 1991). The specificity between an aphid vector and a potyvirus depended not on the DAG domain but either on the affinity between the aphid species and the HC-Pro protein used or on the affinity between the HC-Pro and the virions (Dombrovsky *et al*., 2005). HC-Pro and virion affinity depended on the whole N-terminus of potyviral CP.

Potyvirus CP also interacts with host factors. In *Turnip mosaic virus* (TuMV) infection of several plant species, interaction was described between CP and a 37 kDa protein, which was localized in chloroplasts (McClintock *et al*., 1998).

1.4.2 Viral genome-linked protein (VPg)

A VPg can be found at the 5' end of the RNA genome of 10 genera of plant viruses (Table 1). In this list, the *Potyviridae* is the only family of filamentous viruses where VPg was so far detected to be exposed in the virion. Hence, only in this family, VPg is involved in virus movement in addition to its replication functions.

The role of VPg in virus replication is probably determined by its interactions with viral and host proteins. For example, the yeast two-hybrid system revealed that VPg interacts with itself, and with nuclear inclusion protein a (NIa) and the NIa proteinase domain in TVMV (Hong *et al*., 1995), ClYVV (Yambao *et al*., 2003), PVA (Guo *et al*., 2001), PSbMV (Guo *et al*., 2001) and SMV strain G7HVPg (Kang *et al*., 2004). TVMV VPg also interacts with nuclear inclusion protein b (NIb), the RNA polymerase, and this interaction also requires a functional RNA attachment site in VPg (Hong *et al*., 1995).

The VPg of PVA was found to be uridylylated by NIb (Puustinen and Mäkinen, 2004) without requiring an RNA template. PVA VPg was also found to contain a putative 7-amino acid-long nucleotide triphosphate (NTP)-binding site. Deletion of this site from VPg reduced its nucleotide-binding capacity and debilitated the uridylylation reaction (Puustinen and Mäkinen, 2004). Similar template-independent VPg uridylylation by NIb was found in another potyvirus, PVBV (Anindya *et al*., 2005). N- and C-terminal deletion analysis of VPg revealed that the N-terminal 21 and C-terminal 92 residues of PVBV VPg were dispensable for in vitro uridylylation. The amino acid residue uridylylated by PVBV NIb was identified to be Tyr 66 by site-directed mutagenesis (Anindya *et al*., 2005). Poliovirus VPg is also uridylylated by the poliovirus RNA polymerase and primes the transcription of polyadenylate RNA (Paul *et al*., 1998). Poty- and picornaviruses share similar genome organizations and polyprotein processing strategies. Possibly by analogy to picornaviruses, potyvirus replication also begins with uridylylation of VPg which acts as a primer for progeny RNA synthesis (Puustinen and Mäkinen, 2004).

VPg is also important for potyvirus movement. It has been shown that VPg forms a "movement complex" with other viral proteins and/or host factors in infected plants (Yambao *et al*., 2003). A strong HC-Pro-VPg interaction in ClYVV was detected both by the yeast two-hybrid system and by *in vitro* far-Western blot analysis (Yambao *et al*., 2003). The VPg C-terminal region (38 amino acids) was important for the VPg-VPg interaction and the central 19 amino acids were needed for the HC-Pro-VPg interaction (Yambao *et al*., 2003).A similar interaction was found in LMV (Roudet-Tavert *et al*., 2007), where interaction between LMV VPg and the lettuce translation initiation factor 4E, the cap-binding protein (eIF4E), was also demonstrated *in vitro*.

Table 1. List of the families and genus of plant viruses that contain genome-bound VPg. According to the 8th report of the ICTV on virus taxonomy (Fauquet *et al*., 2005).

Family	**Genus**	**Examples**
Potyviridae	*Rymovirus*	*Ryegrass mosaic virus*
	Potyvirus	*Clover yellow vein virus (ClYVV),* *Pea seed-borne mosaic virus (PsbMV),* *PVA, PVY, PPV, TEV, TuMV,* *Tobacco vein mottling virus (TVMV)*
	Ipomovirus	*Sweet potato mild mottle virus*
	Tritimovirus	*WSMV*
Comoviridae	*Comovirus*	*Cowpea mosaic virus*
	Nepovirus	*Grapevine fanleaf virus* *Tomato black ring virus* *Tomato ringspot virus*
Luteoviridae	*Enamovirus*	*Pea enation mosaic virus*-1
	Polerovirus	*Potato leaf roll virus*
Sequiviridae	*Sequivirus*	*Parsnip yellow fleck virus*
	Waikavirus	*Rice tungro spherical virus*

In addition, the VPg protein of TuMV and TEV interacted with the eIF4E (Léonard *et al*., 2000; Schaad *et al*., 2000). Interaction with eIF4E and HC-Pro both involved the same LMV VPg central domain (Roudet-Tavert *et al*., 2007). The structure of this domain in the VPg context was predicted to include an amphiphilic α-helix, with the amino acids related to biological functions in various potyviruses exposed on the hydrophilic side (Roudet-Tavert *et al*., 2007). The fact that the same VPg domain is responsible for interactions with replication and movement factors suggests that some regulation mechanism is needed for the transition between these two functions of VPg.

Another factor, potyvirus VPg-interacting protein (PVIP), a plant-specific protein possibly involved in transcriptional control through chromatin remodeling, was found to interact with TuMV VPg (Dunoyer *et al*., 2004). The VPg N-terminal 16 amino acids were both necessary and sufficient for the interaction with at least one PVIP protein and the amino acid at position 12 was directly implicated in the interaction. The interaction with PVIP affected systemic symptoms in infected plants and virus cell-to-cell and systemic movement. Reduced

expression of PVIP genes also reduced susceptibility to virus infection (Dunoyer *et al*., 2004).

Several studies demonstrated that amino acid sequence variations in the VPg protein allow potyviruses to overcome resistance in plants (Nicolas *et al*., 1997; Schaad *et al*., 1997b; Redondo *et al*., 2001; Kuhne *et al*., 2003). Recent studies demonstrated that plant resistance to potyvirus infection also depended on the eIF4E sequence. *Arabidopsis* mutants that lacked eIF(iso)4E were resistant to TuMV and TEV infections (Lellis *et al*., 2002). A natural recessive resistance gene of pepper against PVY corresponded to amino acid sequence variation in eIF4E (Ruffel *et al*., 2002). The eIF4E as a resistance gene against TEV was also found in Capsicum, however the disrupted cap binding of the eIF4E was not required for potyvirus resistance (Kang *et al*., 2005).

1.5 Virus tail (movement complex) proteins

1.5.1 Closterovirus HSP70 homologs

Homologs of HSP70h and p61 have been shown to be tightly attached to virions of members of the *Closteroviridae* family such as *Lettuce infectious yellows virus* (Tian *et al*., 1999), CTV (Satyanarayana *et al*., 2000) and BYV (Agranovsky *et al*., 1997; Napuli *et al*., 2000; Napuli *et al*., 2003). Closterovirus HSP70 functions in two basic processes of the virus life cycle: virion assembly and intercellular translocation (Alzhanova *et al*., 2001).

The N-terminal domain of closterovirus BYV HSP70h had ATPase activity, as expected for an HSP70 chaperone, but its C-terminal region did not bind to unfolded proteins (Agranovsky *et al*., 1997). Mutation of the CTV HSP70 putative ATPase active site also inhibited virion assembly (Satyanarayana *et al*., 2000). One or both proteins of HSP70h and p61 could bind to the transition zone between nucleotides 611 and 631 to prevent progression of encapsidation by CPm (Satyanarayana *et al*., 2004).

The binding of HSP70h to actin microfilaments was necessary for closterovirus trafficking in cytosol and its targeting to plasmodesmata (Prokhnevsky *et al*., 2005). HSP70h-mediated ATP hydrolysis could also provide energy for virus translocation. Inactivation of the Hsp70h gene by replacement of the start codon ATG with ATA (Peremyslov *et al*., 1998) or by deletion of 493 codons resulted in complete arrest of BYV translocation from cell to cell. Identical movement-deficient phenotypes were observed in BYV mutants where the HSP70h lacked either the C-terminal domain or computer-predicted ATPase domain, and also if there were point mutations in the putative catalytic site of the ATPase domain (Peremyslov *et al*., 1999). HSP70h also provided a docking site for the long-distance transport factor p20 (Prokhnevsky *et al*., 2002).

1.5.2 Closterovirus 64kD homologs

The 64kD protein homolog, which is conserved among the members of the *Closteroviridae*, has been shown to be associated with virion preparations (Tian *et al*., 1999). The corresponding genes of CTV (p61), BYV (p64), and *Grapevine leaf roll-associated virus-2* (p63) share a conserved motif with ~120-residue-long domain of cellular chaperone Hsp90 (Koonin *et al*., 1991; Pappu *et al*., 1994; Zhu *et al*., 1998).

The p64 protein of BYV contains a CP-like domain (Napuli *et al*., 2003). The virion-embedded, CP-like domain of p54 may fit into the helical assembly of the CP and/or Cpm subunits. One of the possible functions of p64 is formation of the connector between the body and the tail of closterovirus virions. It seems likely that, in addition to its structural role, the unique N-terminal domain of p64 provides additional activities required for the cell-to-cell movement of BYV (Napuli *et al*., 2003).

1.5.3 Closterovirus p21 homologs

In contrast to nuclear localization of the cucumovirus RNA silencing supressors (RSS) (Lucy *et al*., 2000) and peroxisomal localization of the *Peanut clump virus* RSS (Dunoyer *et al*., 2002), p21 of BYV (Reed *et al*., 2003) and p20 of CTV (Gowda *et al*., 2000) are found in cytoplasmic inclusions. The p20 protein has a high affinity for itself (Gowda *et al*. 2000). The crystal structure showed that p21 formed an octameric ring with an outer diameter of 130 Å, an inner diameter of 90 Å, and a thickness of 20 Å (Ye and Patel, 2005). A monomer of p21 is composed of nine α helices and can be divided into amino- and carboxy-terminal domains. Conservation and charge analysis suggested that the inner surface of the ring could be involved in RNA binding. The p21 protein showed no strict binding specificity for siRNA duplexes (Ye and Patel, 2005).

BYV p21 has RSS activity and suppresses RNA silencing by inhibition of a step after Dicer-mediated cleavage of dsRNA (Reed *et al*., 2003). Biochemical studies showed that p21 bound siRNA duplexes both *in vitro* and *in vivo*, but not single-stranded miRNA (Chapman *et al*., 2004), so p21 could function by inactivating siRNA duplexes. Experiments with p22 of *Beet yellow stunt virus* (BYSV) (Reed *et al*., 2003) and p20 of CTV (Lu *et al*., 2004, Reed *et al*., 2003) indicate that the RSS function was conserved in other closteroviruses as well. Multiple alignments revealed a pattern of sequence conservation between p21-like proteins of BYV, BYSV, CTV, and *Grapevine leafroll-associated virus-2* (Reed *et al*., 2003; Ye and Patel, 2005).

1.5.4 Potexvirus TGB proteins

TGBp1 protein of potexviruses induces plasmodesma gating, binds viral RNA, has ATPase activity, and forms inclusion bodies in virus-infected cells (Rouleau *et al*., 1994; Angell *et al*., 1996; Donald *et al*., 1997; Liou *et al*., 2000; Lough *et al*., 200;1 Kalinina *et al*., 2002; Howard *et al*., 2004; Hsu *et al*., 2004). PVX TGBp1 also suppressed RNA silencing, and this activity was necessary for virus cell-to-cell movement (Voinnet *et al*., 2000; Bayne *et al*., 2005).

The PVX TGBp2 and TGBp3 proteins are associated with the endoplasmic reticulum (ER) (Solovyev *et al*., 2000; Zamyatnin *et al*., 2002; Krishnamurthy *et al*., 2003; Mitra *et al*., 2003; Ju *et al*., 2005; Schepetilnikov *et al*., 2005;). PVX TGBp2 has two transmembrane segments and a central domain that is conserved among TGB-containing viruses (Mitra *et al*.. 2003). Mutations disrupting the transmembrane domain inhibited virus cell-to-cell movement, indicating that membrane association of TGBp2 was necessary for virus movement. Electron microscopic analysis showed that the PVX TGBp2 protein induced formation of ER-derived vesicles during virus infection (Ju *et al*., 2005). A deletion mutation in the central domain of the TGBp2 protein caused TGBp2 to accumulate in enlarged vesicles and in the ER network. The same mutation inhibited virus movement. Specific granular vesicles induced by PVX TGBp2 may drive virus cell-to-cell movement (Ju *et al*., 2007). TGBp2 and TGBp3 of *Potato mop-top* virus from the genus *Pomovirus* increased the plasmodesmata size exclusion limit and were involved in the endocytic pathway in viral intracellular movement (Haupt *et al*., 2007).

1.6 Potyvirus cell-to-cell movement proteins

The potyvirus genome consists of a single-stranded RNA molecule of about 10 kb, which is translated in a polyprotein that is proteolytically processed by three virus-encoded proteinases, resulting into up to ten final protein products, many of which are multifunctional (Fig. 2. reviewed by Riechmann *et al*., 1992). So far, four potyvirus proteins have been shown to have functions in virus cell-to-cell movement: CP (Dolja *et al*., 1994), VPg (Nicolas *et al*., 1997), HC-Pro (Rojas *et al*., 1997), and cylindrical inclusion protein (CI) (Carrington *et al*., 1998). The role of CP and VPg in virus movement has already been introduced in sections 1.4.1 and 1.4.2, and the involvement of CI and HC-Pro is summarized below.

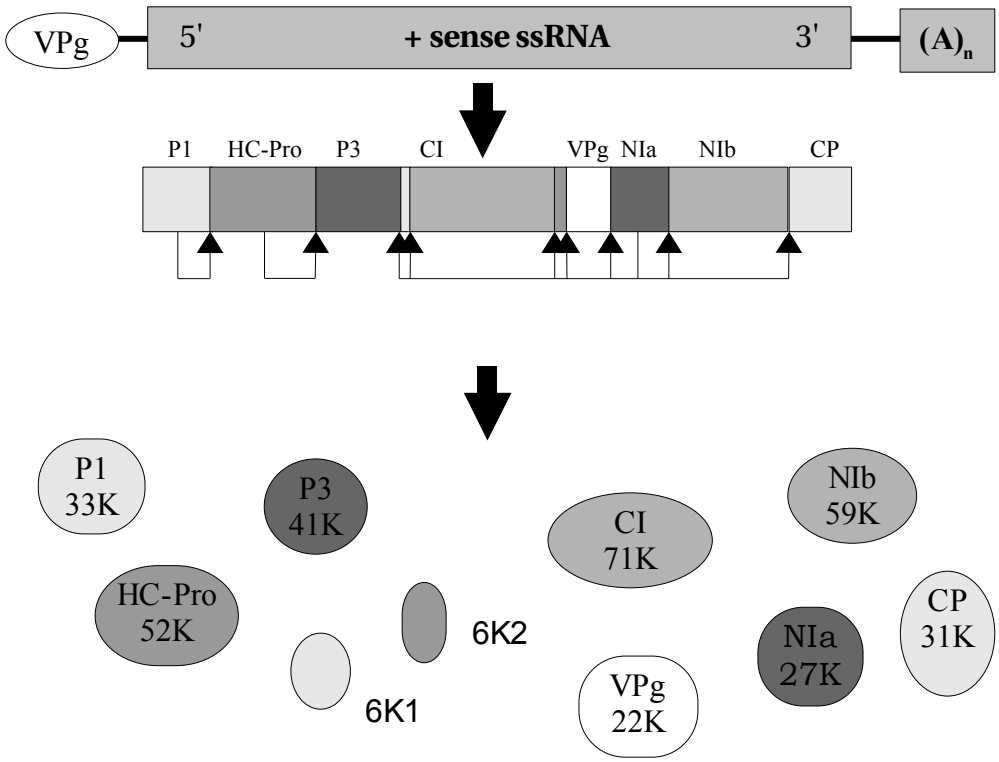

Figure 2. Schematic representation of potyvirus polyprotein processing by viral proteinases and resulting 10 mature proteins (adapted from Riechmann *et al*., 1992).

1.6.1 CI helicase

Potyviral CI was shown to have RNA helicase and ATPase activities (Lain *et al*., 1990); it belongs to the DEXH/D group of helicases (Fernandez *et al*., 1995, 1997; Fernandez and Garcia, 1996) with the ability to unwind RNA duplexes in the 3'-5' direction in PPV (Lain *et al*., 1990) and *Tamarillo mosaic virus* (Eagles *et al*., 1994). An RNA binding domain was identified in the most conserved motive (VI) of potyvirus CI (Fernandez *et al*., 1995). The second RNA binding domain was located in the N-terminal region of CI (Fernandez and Garcia, 1996), which contains helicase motives (I) and (Ia). Although NTP hydrolysis was not an essential component for RNA binding of the PPV CI protein (Fernandez *et al*., 1995), it was still required for potyvirus RNA helicase activity (Fernandez *et al*., 1997). NTPase activity was located in helicase motif (V) and it is essential for virus RNA replication (Fernandez *et al*., 1997).

In addition to its function in virus replication, CI is also essential for virus cell-to-cell movement (Carrington *et al*., 1998). In several potyviruses CI form cones anchored to the cell wall or plasma

membrane near the plasmodesmata (Rodriguez-Cerezo *et al*., 1997; Roberts *et al*., 1998). The ability of CI to form pinwheel structures and to aid in virus movement depends on its ability to interact with itself. The self-interaction domain of CI was located in the N-terminal part of the protein (Lopez *et al*., 2001). Efficient potyvirus transport through plasmodesmata requires specific interactions between CI and CP. In double virus infections the corresponding proteins of the co-infected virus strain were not able to rescue the movement of the first virus strain with a defective CI or CP (Langenberg, 1993). Plasmodesmata-associated CI structures were shown to contain both CP and viral RNA (Rodriguez-Cerezo *et al*., 1997; Roberts *et al*., 1998). In addition to CP, PVA CI also strongly interacted with HC-Pro in the yeast two-hybrid system (Guo *et al*., 2001). According to the present models of potyvirus infection (Roberts *et al*., 1998; Carrington *et al*., 1998), CI protein guides newly formed virions to the CI structures, formed around plasmodesmata. The soluble CI protein interacts with plasmodesmatal CI structures, thus, mediating the passage of a virus into the next cell. This occurs only during the phase of active virus replication in the cell, after which the inclusion bodies disassociate from the cell wall, accumulate in the cytoplasm, and begin to degrade (Roberts *et al*., 1998).

In addition to its interaction with viral proteins, CI from PPV was found to interact with the photosystem I PSI-K protein, the product of the gene psaK, of *N. benthamiana* (Jimenez *et al*., 2006). Experiments with RNA silencing and knockout plants demonstrated that downregulation of the psaK gene leads to higher PPV accumulation, suggesting a role for the CI-PSI-K interaction in PPV infection. Recently CI from TuMV was shown to act as a determinant for a genotype-specific resistance interaction (Jenner *et al*., 2006). Mutations that determined viral resistance were found in the C terminal domain, outside any of the conserved sequences reported to be associated with helicase or cell-to-cell transport activities (Jenner *et al*., 2006).

1.6.2 HC-Pro

Potyviral helper-component protease is a multifunctional protein exerting its cellular functions in interaction with putative host proteins (Carrington *et al*., 1989). HC-Pro plays an important role in suppression of RNA silencing in infected plants (reviewed in Roth *et al*., 2004). HC-Pro is also required for virus transmission by aphids (Atreya *et al*., 1992; Huet *et al*., 1994, Llave *et al*., 2002).

According to predictions of the secondary structure, HC-Pro can be divided into two compact structural domains, connected by a less structured domain (Plisson *et al*., 2003). All functions of HC-Pro, except the self-cleavage function that is fully contained in the C-terminus domain, involve more than one structural domain.

The transmission function needs the N-terminus and the PKT motif in the hinge domain. RNA silencing, virus movement and genome amplification are associated with the C-terminus and hinge domains (Plisson *et al*., 2003). Although the presence of at least four oligomeric species of the hisHC-Pro of TEV was detected (Ruiz-Ferrer et al., 2005), size exclusion chromatography (Thornbury *et al*., 1985) and gel filtration (Plisson *et al*., 2003, Wang and Pirone, 1999) indicated that the functional HC-Pro in transmission was a dimer. It is not clear whether the N terminus (Urcuqui-Inchima *et al*., 1999) or both the N- and C-terminus (Guo *et al*., 1999) are involved in HC-Pro self-interaction. However the N-terminus of HC-Pro is not essential for oligomerisation (Plisson *et al*., 2003).

The N-terminal part seemed to be involved only in the transmission process, because full infectivity was still retained after the deletion of this region (Dolja *et al*., 1993, Plisson *et al*., 2003). The present model suggests that HC-Pro forms a "bridge" between the virus particles and the aphid mouthparts (Ammar *et al*., 1994, Martin *et al*., 1997, Roudet-Tavert *et al*., 2002, Wang *et al*., 1996) when HC-Pro binds to the aphid vector's stylets through the N-terminal KITC motif (Blanc *et al*., 1998).

The PTK motif in the C-terminal proteinase domain contributes to binding of HC-Pro to the N-terminal region of viral CP. This CP region overlaps with a highly conserved DAG motif that is also essential for the transmission process (Blanc *et al*., 1997; Peng *et al*., 1998; Lopez-Moya *et al*., 1999). The PTK motif of PPV is also indispensible for HC-Pro's activity in inducing synergistic reaction to PVX virus infection (Yang and Ravelonandro, 2002). The C-terminus also has a cell-to-cell movement domain, as in *Bean common mosaic necrosis virus* HC-Pro, a C-terminal deletion partially or totally abolished cell-to-cell movement of heterogeneously expressed protein in microinjection studies (Rojas *et al*., 1997).

The central region of HC-Pro is generally assumed to be important in genome amplification (IGN motif), synergism with other viruses, and systemic movement within the host plant (Kasschau *et al*., 1997; Cronin *et al*., 1995). The central region of HC-Pro is implicated in suppressor activity and overlaps with the region identified for genome amplification and viral movement (Kasschau and Carrington, 2001).

HC-Pro does not interfere with the spreading of the silencing signal in plant tissues but inhibits accumulation of short interfering RNA (Mallory *et al*., 2001). There are also indications that HC-Pro might block the function of miRNAs (Kasschau *et al*., 2003). In grafting experiments, HC-Pro was unable to block spreading of the silencing signal (Mallory *et al*., 2001). However transient expression by agroinfiltration of HC-Pro and GFP in transgenic plants expressing GFP was shown to prevent the systemic spread of silencing (Hamilton *et al*., 2002; Pfeffer *et al*., 2002). HC-Pro was unable to block the systemic propagation of the silencing signal successfully in advance of the virus infection (Simon-Mateo *et al*., 2003).

Potyvirus HC-Pro interacts with several plant factors. For example, HC-Pro of LMV interferes with the 20S proteasome ribonuclease (Ballut *et al*., 2005). This interaction possibly plays a role in defense and counter-defense interplay in the course of interaction between potyviruses and their hosts. PVA HC-Pro was shown to interact with RING finger protein (Guo *et al*., 2003).

1.7 Posttranslational modifications of movement-related proteins

Among the plant viruses, the phosphorylation of MP of viruses from genus *Tobamovirus* has been studied most intensively. Phosphorylation of the 30-kDa protein of TMV has been shown in tobacco protoplasts (Watanabe *et al*., 1992) and in transgenic plants (Citovski *et al*., 1993). P30 expressed in transgenic plants was phosphorylated *in vitro* at Ser258, Thr261, and Ser265. Phosphorylation of these amino acids was also reported *in vivo* (Waigmann *et al*., 2000), and the phosphorylation was required for efficient MP interaction with plasmodesmata in *N. tabacum* but not in *N. benthamaina* (Waigmann *et al*., 2000). The host-dependent regulation of MP phosphorylation and TMV cell-to-cell movement was also confirmed later (Trutnyeva *et al*., 2005). Phosphorylation of Thr261 did not interfere with P30 binding to single-stranded DNA (ssDNA) (Citovski *et al*., 1993), however phosphorylation of MP in general was responsible for a transition of MP-RNA complexes between non-translatable and translatable forms (Karpova *et al*., 1999). Phosphorylation of another residue, Thr104, was shown to be involved in regulation of virus cell-to-cell movement (Karger *et al*., 2003). In another tobamovirus, *Tomato mosaic virus* (ToMV), serines 37 and 238 of MP were identified as amino acids phosphorylated *in vivo* in *N. tabacum* protoplasts and *N. benthamiana* plants (Kawakami *et al*., 1999). Phosphorylation of Ser37 was shown to stabilize MP. However, the presence of the serine amino acid itself was required for MP stability. Recombinant MP of ToMV was shown to be phosphorylated *in vitro* by casein kinase II (CKII)-related enzyme. Threonines and serines in the C-terminal amino acid domain and not Ser37 and Ser238 were phosphorylated by this kinase activity (Matsushita *et al*., 2000). In another study Ser37 and Ser338 were again reported to be phosphorylated and the deletion of these two serines had a severe impact on virus propagation in plant leaves (Kawakami *et al*., 2003). Matsushita *et al*. suggested that most probably CKII-like kinase activity is not responsible for the phosphorylation of these two serines (Matsushita *et al*., 2000), which they later proved by showing the phosphorylation of Ser261 and, probably, Ser256 by a recombinant CK2 catalytic subunit from tobacco (Matsushita *et al*., 2003). The Ser37 could possibly be phosphorylated by another

kinase, for example, protein kinase Rio1p homolog from *N. tabacum* (Yoshioka *et al*., 2004).

Movement-related proteins are phosphorylated not only in the genus *Tobamovirus*. It was shown that the C-terminal domain of a 17 kDa protein (pr17), the phloem-limited movement protein (MP) of *Potato leafroll virus* (genus *Luteovirus*) is phosphorylated by a PKC-related, membrane-associated protein kinase activity (Sokolova *et al*., 1997). The nonstructural 69-kD protein (69K) of *Turnip yellow mosaic virus* (genus *Tymovirus*, family *Tymoviridae*), which is necessary for systemic spread of the virus within the plant, was also phosphorylated *in vitro* (Seron *et al*., 1996). Movement protein 3a of *Cucumber mosaic virus* (CMV, genus *Cucumovirus*, family *Bromoviridae*) expressed in transgenic tobacco plants was phosphorylated at serine residues (Matsushita *et al*., 2002). In addition to that, *in vitro* phosphorylation of another CMV protein, 2a (polymerase) was also reported (Kim *et al*., 2002). Phosphorylation was shown to regulate an interaction of 2a with 1a protein, essential for the replication of the virus (Kim *et al*., 2002). Phosphorylation of replication complex-associated proteins was reported also for *Cauliflower mosaic virus* (genus *Caulimovirus*, family *Caulimoviridae*) (Geldreich *et al*., 1989; Champagne *et al*., 2007) and *Cucumber necrosis virus* (genus *Tombusvirus*, family *Tombusviridae*) (Shapka *et al*., 2005; Stork *et al*., 2005). Phosphorylation was also described for plant viruses from the family *Rhabdoviridae*, such as *Rosette stunt virus* (Xie *et al*., 2003) and *Sonchus yellow net virus* (Jackson *et al*., 2005). In both cases a component of viral nucleocapsid core and the nuclear-associated polymerase complex (protein P) was phosphorylated.

Among the virus families described earlier in the introduction, phosphorylation was so far reported for coat proteins of PVX, PPV, and PVA viruses (Atabekov *et al*., 2001; Ivanov *et al*., 2001; Fernandez-Fernandez *et al*., 2002). The N-terminal peptide of PVX CP represents the major phosphorylation site(s) for Thr/Ser-specific protein kinases (Atabekov *et al*., 2001). CP phosphorylation resulted in a translational activation of viral RNP (Atabekov *et al*., 2001). Removal of the N-terminal peptide from CP abolished the possibility for phosphorylation and consequently activation of virus translation (Atabekov *et al*., 2001). Members of the *Potyviridae* were shown to be phosphorylated *in vivo* by plant-derived Ser/Thr protein kinase activities (Ivanov *et al*., 2001; Fernandez-Fernandez *et al*., 2002). In the case of PVA CP, the phosphorylating kinase exhibited a strong preference for Mn^{2+} over Mg^{2+}, was inhibited by micromolar concentrations of Zn^{2+} and Cd^{2+}, and was not Ca^{2+}-dependent (Ivanov *et al*., 2001). No *in vitro* phosphorylation of PVA CP was detected when CP was packaged in virions (Ivanov *et al*., 2001). The phosphorylation sites of purified PVA virus CP could have been already modified by plant kinases *in vivo*. Indeed, phosphorylation of CP was detected by western blot analysis with anti-phosphoserine and anti-phosphothreonine antibodies in purified PPV virions (Fernandez-Fernandez *et al*., 2002).

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In addition to phosphorylation, the glycosylation of several helical plant virus CPs was reported. Almost all CP subunits in the wild-type PVX virion contained sugar residues in their N-terminal peptides (Baratova *et al*., 2004), attracting many ordered water molecules (water shell). The structure of a whole virion may be changed by a signal molecule binding to the virion surface or by changes in glycosylation, phosphorylation and other types of modification of the virus CP surfaces (Baratova *et al*., 2004). Labelling PPV CP with UDP-[^{3}H]galactose in a reaction catalyzed by galactosyltransferase indicated that the CP was a glycoprotein with N-acetylglucosamine terminal residues. Mass spectroscopy analysis of different PPV isolates and mutants revealed O-linked N-acetylglucosamination of serine and/or threonine residues near the N-terminus of PPV CP (Fernandez-Fernandez *et al*., 2002). An O-linked N-acetylglucosamine (O-GlcNAc) transferase of Arabidopsis called SECRET AGENT (Chen *et al*., 2005), modified three threonines and a serine located near the N-terminus of PPV-CP (Scott *et al*., 2006; de Jesus Perez *et al*., 2006). Also, *in vivo* glycosylation of the coat and the readthrough proteins that constitute the capsid of *Beet western yellows virus* (genus *Polerovirus*, family *Luteoviridae*) was recently shown (Seddas and Boissinot, 2006). The modification appeared to play an important role in virus capsid protein interaction with aphid gut (Seddas and Boissinot, 2006).

1.8 Potato virus A

In the last section of the introduction, I will briefly summarize the features of Potato virus A, to put its component proteins described in comparison with other viruses into context for the reader.

Potato virus A (PVA; genus *Potyvirus*, family *Potyviridae*) infection occurs in potato fields woldwide and may reduce tuber yields as much as 40%. Its host range consists of six experimental hosts (*Lycopersicon pimpinellifolium*, *Nicandra physaloides*, *Nicotiana tabacum*, *Solanum demissum*, *S. demissum x S. tuberosum*, and *Nicotiana debneyi*) and two natural hosts (*S. tuberosum* and *S. betaceae*) (Brunt, 2001). PVA has also been detected in Black Nightshade (*Solanum nigrum*) and Hairy Nightshade (*Solanum sarrachoides*) weeds expressing mild mosaic symptoms and growing in PVA-infected potato fields (Thomas, 2004) .

The RNA genome of PVA is 9565 nucleotides long and contains one open reading frame of 9177 bases encoding a large polyprotein of 3059 amino acids (Puurand *et al*., 1994). The 5' NTR is 161 nucleotides long and the termination codon is followed by a 227-nucleotide sequence (Puurand *et al*., 1994). Overall nucleotide

sequence identity compared with several completely sequenced potyvirus genomes is between 53 and 58%, with overall amino acid sequence identity between 65 and 71% (Puurand *et al*., 1994). The capped *in vitro* transcripts from cloned full-length cDNA with bacteriophage T7 RNA polymerase promoter were infectious in mesophyll protoplasts and intact plants of *Nicotiana tabacum* (Puurand e*t al*., 1996). Most parts of the polyprotein amino acid sequence showed over 95% sequence similarity between 5 PVA isolates from potato (*S. tuberosum*) and tamarillo (*S. betacea*) (Kekaranen *et al*., 1999). Although the tamarillo isolate was the most different from the others, the first 60 nt of the 5'-NTR and the entire 3'-NTR were highly similar in all five isolates (Kekaranen *et al*., 1999). Recombination of virus genomes from closely relative PVA isolates can give rise to new viral strains with novel virulence and symptom phenotypes (Paalme *et al*., 2004).

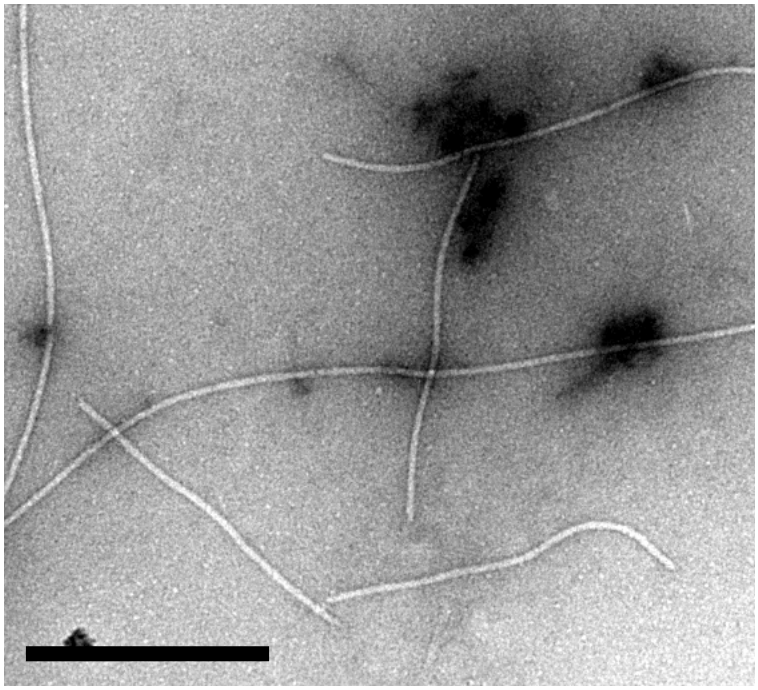

Figure 3. Electron microscopy picture of PVA particles. Bar represents 500 nm.

After translation, PVA polyprotein is processed into 10 mature viral proteins: P1, HC-Pro, P3, 6K1, CI, 6K2, VPg, NIa, NIb, and CP (Puurand *et al*., 1994; Merits *et al*., 2002). Seven potential proteinase NIa, one HC-pro and one P1 proteinase recognition sites were found in the PVA polyprotein by searching for cleavage site consensus sequences amongst the potyvirus group (Fig. 2, Puurand *et al*., 1994). The sites at the P3/6K1, CI-6K2 and VPg/NIa junctions were processed slowly, in contrast to other proteolytic cleavage sites which were processed at a high rate (Merits *et al*., 2002).

The majority of the proteolytic protein intermediate CI-6K2 was found in the membrane fraction. Both 6K1 and 6K2 were required for PVA infectivity, however the presence of 6K2 in the polyprotein sequence was not necessary for efficient polyprotein processing (Merits *et al*., 2002).

Seven PVA proteins (P1, HC-Pro, CI, VPg, NIa, NIb, and CP) bind RNA in a sequence-nonspecific manner (Merits *et al*., 1998). Proteins P1 and P3 interacted with each other and with proteins of the putative replication complex of potyvirus (CI, VPg, NIa, and NIb). P1 also interacted with HC-Pro. No P1 or P3 interaction with CP was detected (Merits *et al*., 1999). In the yeast two-hybrid system interactions were readily detected between HC-HC, HC-CI, VPg-VPg, NIa-NIb and CP-CP, while weaker interactions were detected for the combinations P1-CI, P3-NIb, NIaPro-NIb, VPg-NIa, VPg-NIaPro, NIaPro-NIa and Nia-NIa (Guo *et al*., 2001).

Sequence comparisons of a NIb of potyviruses, bymoviruses, and potexviruses suggested the presence of a putative retinoblastoma protein (pRb) binding motif (Oruetxebarria *et al*., 2002). However mutagenesis of the putative pRb-binding motif in NIb showed no detectable effect on replication of PVA. No interaction of NIb with maize or tobacco RBR proteins was detected in the yeast two-hybrid system (Oruetxebarria *et al*., 2002).

VPg and 6K2 are avirulence determinants in *N. physaloides* (Rajamäki and Valkonen, 1999) and in *S. commersonii (*Rajamäki and Valkonen, 2002), and control vascular movement and systemic infection of PVA . However 6K2 protein affects viral long-distance movement and symptom induction independently and in a host-specific manner (Spetz and Valkonen, 2004).

2 AIMS OF THE STUDY

The first objective of this work was to study PVA movement-associated protein (CI and HC-Pro) interactions with virus particles and how these interactions contribute to the morphology of the virion. The second objective was to study phosphorylation of PVA movement-associated and structural proteins (CP and VPg) *in vitro* and, if possible, *in vivo*.

The specific aims of the work were:

To determine the morphology of purified PVA virions with AFM.

To determine viral proteins that associate with PVA virions.

To study the difference between PVA particles associated with the RNA helicase, PVA CI, and those not connected to CI.

To study the possible effects of CP and VPg phosphorylation on virus infection.

To determine the biologically important phosphorylation sites within PVA CP and VPg.

To identify the tobacco kinase that phosphorylates PVA CP.

3 MATERIALS AND METHODS

The plants, viruses, bacterial strains, antibodies, and plasmids and DNA constructs used in this study are listed in Tables 2, 3, 4, 5 and 6, respectively. All of the mutant and tagged viruses and infectious DNA constructs are based on the wild type PVA B11 isolate. Experimental methods used by the author in this study are described in the original publications and manuscripts and are summarized in Table 7.

Table 2. Plants used in this study.

Plants	Used in articles
Nicotiana tabacum cv SR1	I, II, III, IV
Nicotiana benthamiana	I, II, III, IV

Table 3. Viruses used in this study.

Viruses	Relevant property	Phenotype	Used in articles
PVA strain B11	wild type virus		I, II
PVA-*gfp*$^{NIb/CP}$	*gfp* cloned in to the viral polyprotein between the NIb and CP proteolytic sites	GFP expression	III, IV
PVA-*Rluc*$^{NIb/CP}$	*Rluc* cloned in to the viral polyprotein between the NIb and CP proteolytic sites	Rluc expression	II, IV
PVA-*gfp*$^{NIb/CP}$-VPg45$^{T/A}$	Mutation of putative phosphoamino acid Thr45 to alanine in VPg	GFP expression	IV
PVA-*gfp*$^{NIb/CP}$-VPg49$^{T/A}$	Mutation of putative phosphoamino acid Thr49 to alanine in VPg	GFP expression	IV
PVA-*gfp*$^{NIb/CP}$-VPg132/133$^{TS/AA}$	Mutation of putative phosphoamino acids Thr132 and Ser133 to alanines in VPg	GFP expression	IV
PVA-*gfp*$^{NIb/CP}$-VPg168$^{T/A}$	Mutation of putative phosphoamino acid Thr168 to alanine in VPg	GFP expression	IV
PVA-*Rluc*$^{NIb/CP}$-VPg132/133$^{TS/AA}$	Mutation of putative phosphoamino acids Thr132 and Ser133 to alanines in VPg	Rluc expression	IV
PVA-*Rluc*$^{NIb/CP}$-VPg168$^{T/A}$	Mutation of putative phosphoamino acid Thr168 to alanine in VPg	Rluc expression	IV

Viruses	Relevant property	Phenotype	Used in articles
PVA-*gfp*$^{NIb/CP}$-CP242-244$^{TTS/AAA}$	Mutation of CP phosphorylation site	GFP expression	III
PVA-*gfp*$^{NIb/CP}$-CP242-244$^{TTS/DDD}$	Mutation of CP phosphorylation site	GFP expression	III
PVA-*gfp*$^{NIb/CP}$-CP242-244$^{TTS/YYY}$	Mutation of CP phosphorylation site	GFP expression	III
PVA-*gfp*$^{NIb/CP}$-CP245-247$^{EED/RRR}$	Mutation of CP phosphorylation site	GFP expression	III

Table 4. Bacterial strains used in this study

Escherichia coli strains	genotype/phenotype	Used in articles
M15[pREP4]	Nal^S, Str^S, Rif^S, Thi$^-$, Lac$^-$, Ara$^+$, Gal$^+$, Mtl$^-$, F$^-$, RecA$^+$, Uvr$^+$, Lon$^+$	I, II, III, IV
DH5α	F$^-$, ø80d*lac*ZΔM15, Δ(*lacZYA-argF*)U169, *deo*R, *rec*A1, *end*A1, *hsd*R17(rK$^-$, mK$^+$), *pho*A, *sup*E44, λ-, *thi*-1, *gyr*A96, *rel*A1	II, III, IV
SURE	e14$^-$ (*Mcr*A$^-$) D(*mcr*CB-*hsd*SMR-*mrr*)171, *end*A1, supE44, *thi*-1, *gyr*A96, *rel*A1, *lac, rec*B, *rec*J, *sbc*C, *umu*C::Tn5 (Kanr), *uvr*C [F' *pro*AB l*ac*IqZDM15 *Tn1*0 (*Tetr*)]	II, III, IV

Table 5. Antibodies and antisera used in this study

Antibodies	organism	Used in articles
Polyclonal antiserum against recombinant CP*	rabbit	I, II, III, IV
Polyclonal, affinity-purified IgG against recombinant CI*	rabbit	II
Polyclonal, affinity-purified IgG against recombinant HC-Pro*	rabbit	I
Polyclonal, affinity-purified IgG against recombinant VPg*	rabbit	IV
Peroxidase-conjugated IgG against rabbit IgG	mouse	II
Alkaline phosphatase-conjugated IgG against rabbit (Sigma)	goat	I, II, III, IV

- proteins were purified in denaturing conditions and dialysed against water

Table 6. Plasmids and DNA constructs used in this study

Plasmids and DNA constructs	Description	Used in articles
pQE30(His)$_6$	T5 promotor, *bla*(Ap^R), 6xHis tag, RBSII	III, IV
pGEM-T	*LacZ*, T7, SP6, *bla*(Ap^R)	III, IV
pUC18	*rep*(pMB1) , *bla*(Ap^R), *lacZ*	III, IV
pQE30(His)$_6$VPg	6xHis-tagged recombinant VPg, wild type	IV
pQE30(His)$_6$VPg45$^{T/A}$	6xHis-tagged recombinant VPg, Thr45 mutant	IV
pQE30(His)$_6$VPg49$^{T/A}$	6xHis-tagged recombinant VPg, Thr49 mutant	IV
pQE30(His)$_6$VPg132/133$^{TS/AA}$	6xHis-tagged recombinant VPg, Thr132/Ser133 mutant	IV
pQE30(His)$_6$VPg168$^{T/A}$	6xHis-tagged recombinant VPg, Thr168 mutant	IV
pQE30(His)$_6$HC-Pro	6xHis-tagged recombinant HC-Pro	I
pQE30(His)$_6$CI	6xHis-tagged recombinant CI	II
pQE30(His)$_6$CP	6xHis-tagged recombinant CP, wild type	III
pQE30(His)$_6$tCK2α	6xHis-tagged recombinant α-catalytic subunit of tobacco protein kinase CK2	III
pQE30(His)$_6$CP-242$^{T/D}$	6xHis-tagged recombinant CP, CK2 consensus site mutant	III
pQE30(His)$_6$CP-243$^{T/D}$	6xHis-tagged recombinant CP, CK2 consensus site mutant	III
pUC18-35S-PVA-*gfp*$^{NIb/CP}$	Infectious PVA cDNA under 35S promotor, with *gfp* cloned between the NIb and CP genes	III, IV
pUC18-35S-PVA-*Rluc*$^{NIb/CP}$	Infectious PVA cDNA under 35S promotor, with *Rluc* cloned between the NIb and CP genes	IV
pUC18-35S-PVA-*gfp*$^{NIb/CP}$-CP242-244$^{TTS/AAA}$	Infectious PVA cDNA under 35S promotor, with *gfp* gene and mutations in CP phosphorylation site	III
pUC18-35S-PVA-*gfp*$^{NIb/CP}$-CP242-244$^{TTS/DDD}$	Infectious PVA cDNA under 35S promotor, with *gfp* gene and mutations in CP phosphorylation site	III
pUC18-35S-PVA-*gfp*$^{NIb/CP}$-CP242-244$^{TTS/YYY}$	Infectious PVA cDNA under 35S promotor, with *gfp* gene and mutations in CP phosphorylation site	III
pUC18-35S-PVA-*gfp*$^{NIb/CP}$-CP245-247$^{EED/RRR}$	Infectious PVA cDNA under 35S promotor, with *gfp* gene and mutations in CP phosphorylation site	III
pUC18-35S-PVA-*gfp*$^{NIb/CP}$-VPg45$^{T/A}$	Infectious PVA cDNA under 35S promotor, with *gfp* cloned between the NIb and CP genes, with mutation in VPg putative phosphorylation site	IV
pUC18-35S-PVA-*gfp*$^{NIb/CP}$-VPg49$^{T/A}$	Infectious PVA cDNA under 35S promotor, with *gfp* cloned between the NIb and CP genes, with mutation in VPg putative phosphorylation site	IV

Plasmids and DNA constructs	Description	Used in articles
pUC18-35S-PVA-*gfp*$^{NIb/CP}$-VPg132/133$^{TS/AA}$	Infectious PVA cDNA under 35S promotor, with *gfp* cloned between the NIb and CP genes, with mutation in VPg putative phosphorylation site	IV
pUC18-35S-PVA-*gfp*$^{NIb/CP}$-VPg168$^{T/A}$	Infectious PVA cDNA under 35S promotor, with *gfp* cloned between the NIb and CP genes, with mutation in VPg putative phosphorylation site	IV
pUC18-35S-PVA-*Rluc*$^{NIb/CP}$-VPg132/133$^{TS/AA}$	Infectious PVA cDNA under 35S promotor, with *Rluc* cloned between the NIb and CP genes, with mutation in VPg putative phosphorylation site	IV
pUC18-35S-PVA-*Rluc*$^{NIb/CP}$-VPg168$^{T/A}$	Infectious PVA cDNA under 35S promotor, with *Rluc* cloned between the NIb and CP genes, with mutation in VPg putative phosphorylation site	IV

Table 7. Methods used in this study

Method	Described and used in articles
Recombinant protein expression and purification	I, II, IV
In vitro protein kinase assays	IV
Immunoprecipitation	II, III
Tryptic phosphopeptide mapping	IV
Electron microscopy	I, II, III
Immunogold labelling	I, II
SDS-PAGE and immunoblotting	I, II, III, IV
ATPase assays	II
In vitro translation in wheat germ extracts	II
Luciferase activity measurement and quantification	II, IV
Tungsten particle coating with DNA and bombardment	III, IV
Fluorescence microscopy	III, IV
In vitro phosphorylation	III, IV
Preparation of *N. tabacum* protoplasts	III, IV
Immunocapture reverse transcription PCR	III, IV
CD spectroscopy	IV
Clonings and mutagenesis	II, IV
Plant infections	I, II, III, IV
Virus purification	I, II

4 RESULTS

4.1 PVA virion morphology

As the structures of potexvirus PVX (Kiselyova *et al*., 2003) and closterovirus BYV (Peremyslov *et al*., 2004) particles were investigated using AFM, we also employed this high-resolution technique to study the structure of potyvirus particles. In the present work, AFM imaging of intact PVA virions revealed that approximately 10% of particles (24 of 253 examined) were polar in nature, as there was a protruding tip at one virion end (I, Fig. 1). Reconstructed 3D images of the virion tips consistently showed a tapered end morphology (I, Fig. 1c, d). However, both ends of other particles were blunt and did not differ in their appearance, suggesting that the tip structures either were unstable or were present in only a sub-population of particles, or the particles were damaged during purification and/or sample preparation for AFM imaging. AFM analysis of VLPs, formed by *Escherichia coli*-expressed PVA CP, revealed that both ends looked blunt without any tip structures (I, Fig. 1e).

The size of intact virion bodies were measured to have a modal length of ~ 730 nm (II, Fig. 5), and the tips were visible as 41±6 nm segments that make about 5.5% of virion length. Statistical evaluation of the tail measurements in cross-sections (I, Fig. 1) indicated that the tip had a diameter of 2.3±0.5 nm, in contrast to the constant 9.0±1.2 nm diameter along the entire length of the virion body.

4.2 HC-Pro association with potyvirus virions

The existence of several potyvirus movement-related proteins such as VPg, CI, and HC-Pro, and especially the requirement of HC-Pro for virus transmission by aphids (see Introduction) led us to hypothesize that HC-pro could be part of the potyvirus tip structure. In order to analyze whether HC-Pro comprised part of the tips seen in AFM images, the structure of PVA particles was studied by immuno-gold electron microscopy (IGEM) using antibodies against HC-Pro. In these experiments, PVA particles were trapped on grids and incubated with anti-HC-Pro antibody followed by incubation with anti-rabbit gold conjugate (Roberts, 1986). In these experiments, a few PVA particles (12 of 600 particles screened, 2%) were labeled with gold at one extremity (I, Fig. 2d). In control experiments, no label at virion ends was detected in the presence of the pre-immune serum, or on VLPs formed by recombinant

PVA CP containing no HC-Pro incubated with anti-HC-Pro antibody. A similar labelling pattern was obtained when an antiserum to HC-Pro was used in IGEM experiments with PVY particles. One-extremity gold-labelling was detected on 17 of 600 particles (3%) and no gold was associated elsewhere on the particle bodies. These results confirmed the specificity of labelling the potyvirus particles with HC-Pro antibodies at one virion end.

To study HC-Pro association with potyviral particles in more detail, PVY virions were labeled with anti-HC-Pro and anti-VPg antibodies and visualized with AFM. After the incubation of particles with specific anti-VPg antibodies, large antibody aggregates formed on the majority of the protruding tips (15 of 20 tipped particles, study I, Fig. 3). No such spots were visible in association with the blunt ends or bodies of tipped virions. Similarly, the antibody aggregates were detected on 20 of 29 PVY tips and not on the blunt ends or virion bodies after incubation of virus particles with specific anti-HC-Pro antibodies (I, Fig. 3). Anti-HC-Pro antibody did not label non-tipped PVY particles, however 19 from 30 tested particles with no apparent tip structure were labeled with anti-VPg antibody.

To compare, about 2-3% of potyvirus particles were labeled with gold while using anti-HC-Pro antibodies in IGEM experiments, while AFM immunolabelling was more efficient: about 7-8% of all virions were labeled with anti-VPg or anti-HC-Pro antibodies.

4.3 CI association with PVA virions

In order to further study association of PVA virions with potyvirus movement-related proteins, we performed a Western blot analysis of purified PVA particles using specific anti-CP and anti-CI antibodies. Additionally to CP, the blots showed the presence of CI in the virus preparation (II, Fig. 1). Matrix-assisted laser desorption/ionization- time of flight (MALDI-TOF) mass spectroscopy analysis was performed to characterize the peptide content of the CI-containing band that was visible in the Coomassie-stained SDS-PAGE (II, Fig. 1b). 21 peptide sequences matching with the peptides of CI, four with CP, two with HC-Pro, and two with NIa/VPg region were identified (II, Table 1).

Since CI can form pinwheel-shaped cytoplasmic inclusion bodies we wanted to find out if the CI was bound to virions or whether the CI co-purified in the form of inclusion bodies together with the PVA particles. For that purpose PVA particles (30% sucrose cushion pellet, P1) were further purified through a 5-40% sucrose gradient (II, Fig. 1a). After centrifugation, PVA virions formed a gradient fraction (F), clearly visible under visible light, and a pellet (P2, see II, Fig. 1a). All three virus preparation types (P1, F, P2) were fully infectious (II, Fig. 1d), suggesting that

sucrose gradient purification did not disturb virus structural integrity, required for virus infectivity. However no CI was detected in the F sample (II, Fig. 1c), suggesting that CI is not required for virus ability to infect plants in general. To further rule out the possibility that CI is co-purified with PVA virions only as an impurity in P1 and P2 samples, we immunoprecipitated PVA particles with rabbit anti-VPg IgG bound to magnetic Dynabeads®. CI was detected in both, the purified PVA sample and the immunoprecipitated sample (II, Fig. 2a), indicating that CI is associated with PVA virions. To study whether CI is partially integrated into a virion structure, or is fully exposed on the particle surface, purified PVA virions were subjected to trypsin treatment. All detectable CI in the virus sample was degraded completely in 15 minutes. The results of virion-bound CI trypsination (II, Fig. 2b) suggested that CI is not an integral part of the PVA virion. In order to study the possible role of CI in the morphology of PVA particle, purified PVA samples (P1, F, and P2) were subjected to AFM analysis. Virus samples containing CI were represented by smooth filamentous particles (II, Fig. 5a) and approximately 10% of them contained the tip structures. However, some particles (approximately 5 %) had beaded structures along the whole virion bodies (II, Fig. 5b). AFM analysis of the sample without detectable CI revealed that the majority of the virions (about 80%) had beaded structure (II, Fig. 5c). Due to this beaded structure it was not possible to clearly determine if any of these particles contained apparent tips. To determine the possible location for CI binding to virus particles, purified PVA virions were labeled with protein A-gold conjugate using purified polyclonal CI antibodies. Although CI was found to associate with the virion ends, only 5 particles from several hundreds observed had one-end labelling (II, Fig. 4). Due to the gold labelling on virus particle bodies and very poor one-end labelling, IGEM data was statistically insignificant and inconclusive.

Because potyviral CI is known to be an RNA helicase, we measured ATPase activity in purified virus particles (study II). The ATPase activity was detected in all purified PVA samples and recombinant CI (II, Fig. 3), however the activity was four times weaker in the the gradient fraction virions that contain no detectable CI. We could not measure the concentration of CI in virus preparations, thus it was not possible to compare ATPase activity for recombinant and virus-associated CI. However the ATPase assay measurements suggested that either viral CI had much lower ATPase activity compared to that of recombinant protein, or only a small fraction of virus-associated CI was enzymatically active. To study the possible role of CI ATPase activity in virus uncoating we decided to compare the translatability of PVA particles that were or are were not associated with detectable amounts of CI. *In vitro* translation was performed with the purified *Renilla* luciferase gene (*Rluc*)-tagged PVA particles, and purified *Rluc*-containing viral RNA in wheat germ extracts. The translation efficiency was determined by measuring *Renilla* luciferase (RLUC) activity in the reaction mix. We observed a clear difference between translations of CI-containing and CI-negative virus particle

samples (II, Fig. 6). The RNA within particles in the sample with no CI was 5-10 times more efficiently translated. Addition of purified recombinant CI, which was shown to exhibit ATPase activity, to this sample increased the amount of translation (II, Fig. 6).

4.4 Identification of the tobacco kinase that phosphorylates PVA CP

Ivanov *et al*. (2001) previously demonstrated that the CK2-like kinase activity from tobacco phosphorylates PVA CP. We tested the phosphorylation of PVA CP in the presence of ATP and GTP as phosphoryl group donors (Allende and Allende, 1995; Niefind *et al*., 1999) and heparin as the CK2 inhibiting agent (Hathaway *et al*., 1980). The fact that PVA CP was phosphorylated in the presence of both GTP and ATP and that this reaction was inhibited by heparin, suggested CK2 as a possible kinase for PVA CP phosphorylation. Recombinant PVA CP and TMV MP were used as substrates for i*n vitro* kinase reactions with the isolated α-catalytic subunit of CK2 from maize (rmCK2α ; Boldyreff *et al*., 1993). The results showed that PVA CP and TMV MP were both phosphorylated by rmCK2α in the presence of ATP and GTP. The kinase, isolated from tobacco with affinity purification, was also able to use GTP as a phosphoryl donor and efficiently phosphorylated PVA CP and TMV MP, whereas virtually no phosphorylation of PVA VPg was observed (III, Fig. 2). The affinity column fractions containing the highest kinase activity were used in an in-gel kinase assay to determine the size of the enzyme. The in-gel kinase assay revealed one major radioactive band with an apparent molecular mass of 39 kD. The molecular mass of the identified kinase (39 kD) was identical to that of the recently reported α-catalytic subunit of CK2 from tobacco (tCK2α; Salinas *et al*., 2001). Two unique peptide sequence tags matching the α-catalytic subunit of tobacco CK2 were identified by liquid chromatography–tandem mass spectrometry (LC-MS/MS). Thus, we identified tobacco CK2 as the kinase responsible for the phosphorylation of PVA CP.

4.5 Identification of CP phosphorylation sites

Sequences in PVA CP containing the CK2 phosphorylation site motif (S/T)XX(D/E) (reviewed by Meggio and Pinna, 2003) were identified using the ScanProsite program tool at the ExPASy molecular biology server of the Swiss Institute of Bioinformatics (http://www.expasy.ch). A particularly strong consensus

sequence (242-TTSEED-247) containing one Ser and two Thr residues available for CK2 phosphorylation was identified in the tryptic peptide Asn-229 to Arg-250. Recombinant CP was *in vitro* phosphorylated, trypsinated, and the resulted phosphopeptides were resolved by reverse-phase HPLC. MALDI-TOF mass spectrometric analysis was performed to characterize the peptide content of the main radioactive peak. Mass spectrometry, radioactive phosphate-release sequencing, and two-dimensional phosphoamino acid analysis revealed the presence of phosphorylation in a single peptide of Mr 2430.7, which was identified as 229-NSNTNMFGLDGNVTTSEEDTER-250 with a calculated mass of 2430 kD (III, Fig. 5A). Thus, Thr-242 was determined to be the major site of PVA CP phosphorylation by CK2.

4.6 Effect of CP phosphorylation on PVA infection

Previously it has been shown that PVA CP binds single-stranded RNA with no sequence specificity (Merits *et al*., 1998), and that binding can be inhibited by *in vitro* phosphorylation of CP by the plant Ser/Thr protein kinase activity (Ivanov *et al*., 2001). The affinity changes of PVA CP for RNA was studied upon CK2 phosphorylation. For this purpose gel retardation (electrophoretic mobility shift assay) and UV cross-linking were used. Increasing amounts of PVA CP were incubated in kinase buffer containing unlabeled GTP with or without the addition of the purified rmCK2α. The kinase reactions were allowed to proceed, and two identical electrophoretic mobility shift assays were performed to compare the binding of nonphosphorylated and phosphorylated PVA CP to a radiolabeled RNA transcript (III, Fig. 9). The non-phosphorylated PVA CP exhibited higher affinity for RNA than the CK2-phosphorylated protein. In UV cross-linking experiments it was shown that the phosphorylated protein was not in a close molecular association with RNA (III, Fig. 9). Together, these results demonstrated that the phosphorylation of PVA CP by CK2 reduces the affinity of the protein for RNA.

To see if phosphorylation itself and not the presence of consensus sequence amino acids would effect virion/RNP complex formation, a structural comparison of wild-type PVA CP and its CK2 phosphorylation-deficient mutant was done. For this purpose, the recombinant His-tagged CP mutant having Thr-242, Thr-243, and Ser-244 substituted by nonphosphorylatable Ala residues was expressed and purified. The overall secondary structure of this mutant was compared with that of the wild-type PVA CP using circular dichroism (CD) spectroscopy (III, Fig. 8). The CD spectra of wild-type PVA CP and its mutant were almost identical, indicating that the proteins had equivalent conformations. Thus, we concluded that the CK2 phosphorylation-negative mutations did not induce conformational changes in the CP. To study

any effect of the CK2 phosphorylation-negative mutations on the subunit interactions of PVA CP in bacterial cells, we performed an EM analysis of VLPs formed in bacterial cells. No major difference in the morphology of VLPs formed from the wild-type PVA CP and its CK2 phosphorylation-deficient mutant was detected. Thus, CK2 phosphorylation-negative PVA CP mutation did not disturb the intersubunit interactions between the CP monomers required for oligomerization, indicating that the mutant and wild-type CPs had similar conformations.

To study how the phosphorylation of CP effects viral RNA binding and virion formation *in vivo*, we constructed a full-length infectious cDNA clone of PVA tagged with *gfp* (35S-PVA-*gfp*$^{NIb/CP}$, Table 3 and 6 in section 3). Amino acid exchange mutations were introduced into 35S-PVA-*gfp*$^{NIb/CP}$ by site-directed mutagenesis. Amino acids Thr-242, Thr-243, and Ser-244 (III, Fig. 7) were substituted by nonphosphorylatable Ala residues, and *N. benthamiana* leaves were bombarded with the mutant virus cDNA construct (pUC18-35S-PVA-*gfp*$^{NIb/CP}$-CP242-244$^{TTS/AAA}$). At 7 days after inoculation, plant tissue surrounding single fluorescent cells was analyzed by immunocapture reverse transcription polymerase chain reaction (RT-PCR) in a similar approach to that of Fedorkin *et al.* (2000), using monoclonal anti-PVA antibodies and virus-specific primers. A single RT-PCR product of the correct size was obtained, indicating that the fluorescing cells contained viral RNA that was encapsidated by the mutant CP. Mutant recombinant CP was also able to form VLP particles in *E. coli*. From this result, we concluded that the CK2 phosphorylation-negative mutations do not prevent CP binding to RNA and/or virion (or RNP complex) formation *in vivo*.

To examine the role of PVA CP phosphorylation by CK2 in virus spread in host plants, amino acids Thr-242, Thr-243, and Ser-244 of *gfp*-tagged virus CP were additionally substituted by Asp or Tyr residues (III, Fig. 7), mimicking the electrostatic and steric effects of phosphorylation (Dean and Koshland, 1990). *N. benthamiana* plants were infected with the resulting pUC18-35S-PVA-*gfp*$^{NIb/CP}$-CP242-244$^{TTS/DDD}$, pUC18-35S-PVA-*gfp*$^{NIb/CP}$-CP242-244$^{TTS/YYY}$, and already mentioned pUC18-35S-PVA-*gfp*$^{NIb/CP}$-CP242-244$^{TTS/AAA}$ mutant cDNAs. The mutant viruses were completely unable to spread both cell to cell and systemically. At seven days after inoculation, all of the mutants showed the same defective phenotype, with GFP fluorescence restricted to single cells (III, Fig. 7C-7E). At 20 days after inoculation with the Ala mutant, weak GFP fluorescence appeared in the upper leaves. RT-PCR followed by nucleotide sequencing revealed a reverse mutation from Ala-243 to Thr-243, indicating that the virus had restored the redundant CK2-site of PVA CP to remain viable. This finding also proved that the mutant was able to replicate in individual cells. It was important to verify that the defective phenotypes were caused by CK2 phosphorylation and not by the amino acid substitutions *per se*. A mutant virus was constructed in which Thr-242, Thr-243, and Ser-244 were left intact but the downstream cluster of acidic residues was

replaced with basic Arg residues (III, Fig. 7) to disrupt the acidic context required for the CK2 consensus sequence. The mutant virus showed the same defective phenotype upon the infection in *N. benthamiana* plants. Starting from seven days after inoculation, GFP fluorescence was detected only in single bombarded cells (III, Fig. 7F). Together these results demonstrated that PVA CP phosphorylation by CK2 strongly effected the ability of virus to move from cell to cell in *N. benthamiana*.

4.7 Mapping VPg phosphorylation sites

Five putative phosphorylation sites from an *in vitro* phosphorylated recombinant PVA VPg were mapped using Edman radioactive amino acid release sequencing and MALDI-TOF analysis (study IV). Two-dimensional amino acid analysis revealed that one phosphopeptide contained phosphoserine while others contained phosphothreonine. The results from MALDI-TOF analysis revealed that VPg was not completely digested by trypsin and several intermediate peptides were present in the trypsinated VPg sample (IV, Table 1). Taking into account that the PVA VPg digestion was not complete, it was possible to find combinations of peptides having Thr at positions one and seven as well as Ser at the second position (IV, Table 2). The putative phosphorylation sites interpreted from the results were Thr 45, 49, 132 and 168 and Ser 133. Although the NetPhos (Blom *et al*., 1999) analysis of the VPg amino acid sequence predicted a high probability (score = 0.942) of phosphorylation only for the sequence surrounding Thr168, all four potential phosphorylation sites were subjected to further virus infection analysis.

4.8 Effect of phosphorylation deficient mutations on virus infection

We constructed *gfp*- and *Rluc*-tagged viruses having the mapped amino acids substituted with alanines to study whether they and/or their phosphorylation played any role in virus infection. Infection with the resulting mutant viruses was monitored in *N. benthamiana* and *N. tabacum* plants. All mutant viruses spread in the bombarded leaves and systemically in *N. benthamiana* (IV, Table 3). The systemic infection of these viruses started at 5-6 dpi, depending on the experiment. At 8-9 dpi, the upper leaves of *N. benthamiana* were fully infected (IV, Fig. 3A, column X). However only the WT and PVA-VPg^{T49A} and PVA-VPg^{T45A} mutant viruses spread efficiently in bombarded *N. tabacum* plants (IV, Table 3). Both PVA-VPg$^{TS132/133AA}$ and PVA-VPg^{T168A} were occasionally able to cause systemic infection in *N. tabacum*. PVA-VPg$^{TS132/133AA}$

caused systemic infection in 3 plants out of 15 and PVA-VPg^{T168A} in 3 plants out of 9. Viral particles of infecting viruses were immunocaptured with specific anti-CP antibodies and subjected to RT-PCR reaction. Sequencing of the RT-PCR products revealed the original mutation with no additional mutations in the VPg gene, except in one case where reversion back from PVA-VPg^{T168A} to WT sequence was observed. When *N.tabacum* plants were mechanically inoculated with plant sap from *N. benthamiana* leaves systemically infected with the WT and all mutant viruses, fluorescence was detected in the upper leaves of all the plants on the 11th dpi (IV, Fig. 3A, column Z). The experiment demonstrated that amino acid substitutions in potential phosphorylation sites of VPgs did not interfere with the essential functions of VPg or the capability of the virus to establish an infection in either plant species. However the overall infectivity of the PVA-VPg$^{TS132/133AA}$ and PVA-VPg^{T168A} viruses was reduced in *N. tabacum.* To ensure that amino acid substitutions did not alter VPg structure and, thus, function, CD spectra of recombinant VPg TS132/133AA, VPgT168A and WT PVA VPg were compared (IV, Fig. 4). The spectra were very similar indicating that reduced infectivity of the virus carrying TS132/133AA and T168A substitutions was not caused by clear structural changes in VPg.

To find out if TS132/133AA and T168A mutations had any affect on virus replication in *N. tabacum* cells, *Rluc*-tagged PVA icDNA constructs were electroporated into *N. tabacum* protoplasts. Quantification of virus-derived RLUC-expression revealed that VPg-TS132/133AA and VPg-T168A mutations did not reduce the ability of the viruses to replicate and to form new virus particles. The same constructs were used to infect *N. benthamiana* plants in order to obtain a better understanding of the infection process of the mutant viruses. The RLUC activity in the PVA-VPg$^{TS132/133AA}$ infection was approximately ten times higher than in WT in the bombarded and the first two systemically infected leaves of *N. benthamiana*. PVA-VPg^{T168A} infection gave a ten times lower RLUC activity compared with that of WT virus in similar leaves (IV, Fig. 5A). Differences in the spread rates of RLUC activities in the first upper leaves for both mutants were also observed, showing better accumulation in inoculated leaf and more efficient spread in the first two systemically infected leaves with the PVA-VPg$^{TS132/133AA}$ virus. However, in spite of the initial difference, WT and both mutant viruses were able to accumulate to the same levels in the infected leaves of *N. benthamiana* after 12 days post infection.

5 DISCUSSION

5.1 Virion morphology and stability

This work demonstrated for the first time that potyviral virions are not simple flexous filaments. AFM imaging clearly showed that at least a subpopulation of potyviral particles exhibit a protruding tip at one particle end (study I). The tip measurements revealed differences in the geometry and/or physical properties between the virion tips and the rest of the virus particles. Keeping in mind that a whole potyvirus particle contains about 2000 copies of CP, virion tip dimensions should be equal to a virion body part composed of approximately 100 CP subunits. However its morphology allowed us to suggest that either CP subunit arrangement is different from that of the virion body, or other virion (or host) proteins are involved in formation of the tip structure. Similar virion tip morphology was observed in particles of PVY (study I), indicating that the tip structure is conserved, at least in two members of the *Potyvirus* genus. The presence of VPg in the tip structure (study I) indicates that potyviral tails are formed at the 5' end of the virus particle, similarly to the closterovirus tails (see Introduction).

The low number of tipped virions could be the result of particles loosing tips during virus purification or/and sample preparation for AFM analysis. It is, however, possible, that only some virus particles do have the tip structures in plant cells due to an inefficient particle/tip assembly.

The protruding tips were not obvious when potyvirus particles were examined by EM. One reason for this difference in appearance could be that the PVA tip is less efficiently coated with negative stain due to the different protein composition in virus body and tip structure. Unlike EM, AFM does not require staining. Nevertheless, no terminal tips were observed in some PVA particles nor in any VLP's produced in *E. coli* from recombinant CP (study I). This result confirms that the potyviral terminal tips visualized by AFM are not artifacts resulting from structural differences between concave and convex ends of virus filaments. Moreover, both particle ends of another filamentous virus, PVX (in spite of the presence of TGBp1 protein at its 5' end) or of the rod-shaped TMV analyzed by AFM were blunt and did not differ in their appearance (Peremyslov *et al*., 2004; Kiselyova *et al*., 2003).

AFM imaging also revealed the presence of beaded particles in purified PVA virus preparations (study II). As the lengths of beaded and normal particles were the same, the altered particle morphology could be caused by the loss of CP subunits along the entire length of the virion bodies after the sample preparation for AFM imaging. The beaded particles may be not as stable as other virions, which is then exaggerated by the AFM conditions and sample preparation technique. Similar

particle morphology changes and particle disassembly were observed after the binding of TGBp1 helicase to the PVX virions.

The results from study II also demonstrated that PVA virion stability may depend on the presence of virus-associated CI in the sample (study II). The RNA helicase/ATPase NPH-II of Vaccinia virus also belongs to the DEXH/D group of helicases and, independently of RNA duplex unwinding (Fairman *et al.*, 2004), it can displace proteins from an RNA substrate (Jankowsky *et al.*, 2001). Potyviral CI was reported to belong to the same helicase group (Fernandez *et al.*, 1995, 1997; Fernandez and Garcia, 1996) with the ability to unwind RNA duplexes to 3'-5' direction in PPV (Lain *et al.*, 1990). Similar RNA helicase activity was described for PVX movement protein TGBp1, but in that case RNA unwinding was bi-directional (Kalinina *et al.*, 2002). In the PVX system TGBp1 binding to virions initiates particle destabilization and translation activation of PVX RNA (Atabekov *et al.*, 2000). AFM studies with PVX have also revealed TGBp1-mediated degradation of PVX particles (Kiselyova *et al.*, 2003). Similarly one could think that the ATPase and possibly RNPase activities of PVA CI could be required for virus particle disassembly and/or translation initiation in new plant cells. If CI acts as a RNPase and helicase, we can speculate that the translation of viral particles is enhanced by the presence of CI. However the results from translation experiments showed that in PVA virus preparations the presence of viral CI did not induce the translation of viral particles. The majority of particles present in virus samples with co-purified CI were not in a translation-competent form in spite of the presence of a high concentration of CI. The presence of CI *per se* added as recombinant protein seemed not to inhibit translation. Interestingly, the structural protein p20 in closterovirus BYV tail is not required for tail formation (Prokhnevsky *et al.*, 2002). However there are indications that p20-deficient virions are less stable compared with that of the WT virions (Peremyslov *et al.*, 2004), which is similar to our results with CI.

Together, AFM and translation results show CI as a virus particle stabilizing agent rather than an enzyme needed for virus disassembly. However the transition from stable particle to translatable particle can occur by switching between the stabilizing and translational functions of potyviral CI.

5.2 Association of movement-related proteins with potyvirus particle

The existence of several potyvirus movement-related proteins such as VPg, CI, and HC-Pro, and especially the requirement of HC-Pro for virus transmission by aphids (see Introduction) led us to hypothesize that HC-pro could be part of the potyvirus tip

structure. Previously, both HC-Pro and VPg were shown to self-interact, and HC-Pro was shown to interact with VPg in the yeast two-hybrid system (Yambao *et al*., 2003; Guo *et al*., 2001). VPg associated with the 5'-end of viral RNA in particles was shown to be available for protein–protein interactions on the surface of the particles (Puustinen *et al*., 2002). Taking these observations together, it is possible to suggest that HC-Pro in association with VPg may bind to the end of the virion containing the 5'-RNA terminus forming the tip. Possibly some additional proteins are involved as well. In order to analyze whether HC-Pro comprised part of the tips seen in AFM images, the structure of PVA particles was analyzed by IGEM and AFM. AFM imaging of potyvirus particles incubated with specific antibodies against HC-Pro not only confirmed the specific association of HC-Pro to one end of potyviral particles (study I), but also clearly demonstrated that the association occurred in the tip structure (study I). Although the immunolabelling AFM results confirm specificity of labelling of the PVY tips with anti-VPg and anti-HC-Pro antibodies, it is difficult to explain why fewer virus particles were labeled with anti-HC-Pro antibody in the IGEM experiments. A possible reason could be the reduced stability of PVY and PVA tips subjected to the IGEM procedure.

Binding of HC-Pro to VPg possibly forms a part of the tip, which may serve as a bridge between virus particles and aphid mouthparts, determining virus transmission by aphids. Potyviral HC-Pro interaction with the N terminus of CP, which is exposed in the assembled particle may take place during the formation of the virion tip containing the 5' end of RNA and VPg, rather than along the entire length of the particles.

Other flexuous rod-shaped viruses belonging to distinct evolutionary and taxonomic groups such as the closteroviruses (Peremyslov *et al*., 2004), potexviruses (Atabekov *et al*., 2000; Kiselyova *et al*., 2003), and pomoviruses (Cowan *et al*., 1997), also contain additional virus-encoded proteins or their complexes at the same particle extremity, demonstrating a general tendency among viruses sharing filamentous virus particle morphology and suggesting that the architecture of simple filamentous viruses may be more complex than previously thought. The structural supplements at the virion end containing the 5'-end of RNA may play essential roles in different virus-encoded functions, such as virus assembly/disassembly, movement and vector transmission.

In addition to HC-Pro, according to our biochemical and EM studies, PVA virus particles associate with CI (study II). It is not clear if CI is co-purified in the form of inclusion bodies from infected cells as reported by Lain *et al*. (1991), or as CI protein molecules or oligomers specifically bound to the virions or to the tip structure as HC-Pro (study I). Biochemical studies of sucrose gradient purified virus particles revealed that not all virions are associated with CI. The associated CI proteins may direct intracellular translocation of the virion or transport complex to the plasmodesmata-associated structures through protein-protein interactions between the transport complex-associated CI protein

and the plasmodesmatal structure-associated CI subunits (Carrington *et al*., 1998). The immunoprecipitation results allow us to propose that there is a direct physical link between the CI and virus particle. However it is not clear if CI binds to CP, VPg, or to some other virion-associated protein. There are few reports on potyviral CI and CP interaction *in vivo* (Rodriguez-Cerezo *et al*., 1997; Riedel *et al*., 1998; Guo *et al*., 2001), and so far no interaction between CI and VPg has been reported. However strong CI interaction with HC-Pro has been reported for PVA (Guo *et al*., 2001) and WSMV, a member of the *Potyviridae* family, genus Tritimovirus (Choi *et al*., 2000). These observations allowed to suggest that virus-co-purified CI can actually be associated not with CP but rather with virus-bound HC-Pro. Our IGEM data (study II) also give some support to this hypothesis. Interestingly, all MALDI-TOF identified proteins (study II) are essential for potyvirus movement. They may be part of the particle-attached complex responsible for directional transport of PVA, and at least HC-Pro and VPg were found in the potyviral tip structure (study I).

If CI and virion association is specific, what is its purpose and/or function in potyvirus infection? The ATPase activity results (study II) demonstrate that at least some of the co-purified CI is enzymatically active. However it is not possible to suggest from the available results, whether the active CI is specifically associated with virion ends, or that the protein that just co-purifies with the particles and is derived from cytoplasmic inclusion bodies. Although it was not possible to determine the amount of CI in virus preparations, the comparison of ATPase activity for recombinant and virus-associated CI suggested that either viral CI had much lower ATPase activity compared to that of recombinant protein, or only a small fraction of virus-associated CI was enzymatically active. The presence of some ATPase activity in F samples (study II) suggests that there was still some CI protein left in the virus samples, but the amount was too low to detect it by Western blot. Possibly the detected ATPase activity was from CP. It was shown that CPs of PVX and PVA exhibit ATP-binding and ATPase activities (Rakitina *et al*., 2005). Recombinant PVX and PVA CPs produced in *E. coli* showed Mg^{2+}-dependent ATPase and UTPase activities inhibited by antibodies against virus particles. Deletion of the C-terminal regions of these proteins diminished their ATPase activity (Rakitina *et al*., 2005).

One possible reason for CI association with virions is to aid in virus intercellular or cell-to-cell movement. Recently it was demonstrated that the actin cytoskeleton interacts with closterovirus Hsp70 homolog and targets it to plasmodesmata (Prokhnevsky *et al*., 2005). Although it is known that potyviral CI associates with plasmodesmata, the CI and cell cytoskeleton association has not been shown. It is also not clear if CI ATPase activity is required for the cell-to-cell movement. However, CI mutations that abolish PPV cell-to-cell movement did not disturb the ATPase activity of CI (Gomez de Cedron *et al*., 2006). Keeping in mind that CI interacts with HC-Pro, we speculate that CI associates with the potyvirus tip structure

during its cell-to-cell movement. As a highly multi-functional protein, potyviral CI can possibly have a role not only in virus replication, but also in particle stabilization, similar to BYV p20, and in virus particle delivery to and through plasmodesmata, similar to the BYV p64 Hsp70 homolog.

5.3 Phosphorylation of potyvirus structural proteins

Protein kinase CK2 is a highly conserved, ubiquitously expressed, acidophilic Ser/Thr kinase present in all eukaryotic cells (Allende and Allende, 1995). Multiple alignment of CPs from 28 members of the Potyvirus genus (Shukla *et al*., 1994) revealed a conserved potential CK2 phosphorylation site suggesting the importance of CP phosphorylation in the potyvirus life cycle. The majority of the compared sequences contained Thr or Ser residues within a CK2 consensus motif corresponding to the 242-TTSEED-247 sequence in PVA CP. The insertion of five amino acids next to Glu-245 destroyed PVA ability to replicate in *N. tabacum* protoplasts (Kekarainen *et al*., 2002) and, thus, strengthened the evidence for phosphorylation of PVA CP by CK2 and its importance in virus infection. The results of study III indeed demonstrated that CP was phosphorylated by *N. tabacum* CK2.

Virion assembly and cell-to-cell movement require CP–RNA interactions; therefore, they can be regulated by CK2 phosphorylation. In agreement with this possibility, the results obtained with *gfp*-tagged mutant viruses demonstrated that the amino acid substitutions affecting the CK2 consensus sequence in PVA CP produced movement-deficient phenotypes (study III). The movement-deficient phenotype of mutant virus with substituted acidic residues downstream of the phosphoacceptor site confirmed the regulation of the PVA infection by acidophilic CK2. The detection of partially rescued virus movement by spontaneous reversion of a nonphosphorylatable Ala mutant restoring Thr-243 also supports the suggested role of CK2 in PVA movement.

As there are several reports of CK2 involvement in regulation of protein–nucleic acid interactions (Allende and Allende, 1995; Riera *et al*., 2001), it is possible that CK2 also regulates PVA infection by inhibiting binding of CP to viral RNA. Phosphorylation may change the structure of the CP nucleic acid binding domain. In the proposed structural model of PVA CP (Baratova *et al*., 2001), the CK2 phosphorylation site Thr-242 is located in an interconnecting loop immediately after the β7 strand (amino acids 235 to 241) inside the structural unit commonly found in RNA binding proteins. The phosphorylation at Thr-242 should affect the charge and, possibly, the structure of this region, making regulation of CP-RNA interaction possible. In addition to the phosphorylation sites in the core of the CP, which are inaccessible in assembled virions, other phosphorylated residues may exist on the virion surface. The intact virions of another potyvirus, PPV,

have been shown to interact specifically with anti-phosphoserine and anti-phosphothreonine antibodies (Fernandez-Fernandez *et al*., 2002). The phosphorylation of these residues *in vivo* could explain why they were not identified when PVA particles were phosphorylated *in vitro* (Ivanov *et al*., 2001). Modification of PPV CP by Ser/Thr-linked N-acetylglycosamine (Fernandez-Fernandez *et al*., 2002) suggests that the functions of viral CP are regulated not only by phosphorylation, but also by glycosylation. The coordinated actions of O-glycosylation and phosphorylation can play an important role in controlling fundamental cellular events (Comer and Hart, 2000).

According to the hypothesis, presented in study III, phosphorylation of the RNA binding domain of PVA CP by CK2 regulates the amount of viral template RNA available for replication. At early stages of infection, phosphorylation of PVA CP could be carried out by the ER–associated form of CK2 (Faust *et al*., 2001). This may prevent premature particle assembly, thereby allowing efficient viral RNA replication to proceed. Phosphorylation may also inhibit the formation of nonspecific cellular RNPs during the first stages of PVA infection. The activation of the assembly/movement pathway can be later initiated by dephosphorylation of CP and its restored binding to viral RNA. A similar model has been proposed for the regulation of Rubella virus encapsidation (Law *et al*., 2003). Therefore, CP phosphorylation/ dephosphorylation may represent a general regulatory mechanism used by both animal and plant viruses.

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Phosphorylation, as a regulatory mechanism, may be an important switch between the different functions of PVA VPg during virus infection cycle. The recombinant VPg of PVA (Ivanov *et al*., 2001) and the virion-encapsidated VPg (Puustinen *et al*., 2002) are phosphorylated *in vitro* by cellular protein kinase activities from the host plant. The *in vitro* phosphorylation pattern of recombinant VPg and virus systemic movement of different PVA strains depends on the primary amino acid sequences of their VPg, and on the host plant kinases (Puustinen *et al*., 2002; Rajamäki *et al*., 2002). Thus we hypothesized that VPg phosphorylation may play a role in the level of virus accumulation and in the regulation of the establishment of systemic infection. Very recently a method was developed to demonstrate that PVA VPg is a phosphoprotein *in vivo*. The technical problem of viral VPg purification from infected plant tissues was solved by inserting an affinity tag in to the C-terminus of VPg. A His/HA-tag encoding sequence was cloned in to the 3' end of VPg sequence in the 35S-PVA-*gfp*$^{NIb/CP}$. A 2D electrophoresis (isoelectric focusing combined with SDS-PAGE) followed by western blot analysis of affinity-purified VPg demonstrated the presence of several forms of posttranslationally modified VPg.

Phosphatase treatment reduced the number of modified VPg forms, indicating that PVA VPg is a phosphoprotein *in vivo* (Hafren and Mäkinen, personal communication). It is not yet clear at what stage and which form of VPg is phosphorylated. It could be VPg from assembled virus particles or from the virus replication complex.

Prior to developing the method to study the *in vivo* phosphorylation status of PVA VPg, Edman sequencing and MALDI-TOF analyses of *in vitro* phosphorylated VPg resulted in the identification of potential VPg phosphorylation sites. We decided to study these sites *in vivo* by constructing phosphorylation deficient mutant viruses and analyzing the impact of these mutations on the virus infection cycle. No biological effects were observed by substituting Thr45 or Thr49 with Ala in PVA VPg (study IV). Substitution of Thr47 and Thr48 to Ala at the very same region of TEV VPg did not have any detectable effect on replication or movement (Schaad *et al*., 1996). It seems unlikely that Thr residues at this region would have any general regulatory role in potyvirus infection, although the surrounding Lys residues take part in several essential functions, *e.g.* nuclear localization, replication, and NTP-binding (Puustinen and Mäkinen, 2004; Schaad *et al*., 1996). Thr168 and Thr132/Ser133 were found to be essential amino acids for infectivity, and possible candidates for phosphorylation. However, neither Thr132 nor Ser133 is conserved between potyviruses, whereas the amino acid position corresponding to Thr168 seems in general to be occupied either by Thr or Ser in potyvirus VPgs (study IV). Conservation of a phosphorylatable amino acid at Thr168 site suggests the general importance of phosphorylation of this residue in potyvirus infection.

$VPg^{TS132/133AA}$ and VPg^{T168A} mutations are both located in amino acid clusters that have an essential role either in PVA replication or in particle assembly (Kekarainen *et al*., 2002). The TS132/133AA substitution was beneficial for the virus at a single cell level in *N. tabacum* (study IV) and also in the inoculated and first systematically infected leaves of *N. benthamiana*. PVA-VPg^{T168A} virus had lower capability to accumulate in single cells. However, it was still capable of infecting *N. benthamiana* systemically and to finally reach the levels of WT virus accumulation. The changes in mutant virus accumulation rates and systemic movement in one host plant and blocked systemic infection in another host species point to the possible importance of Thr168 and Thr132/Ser133 amino acids in interactions with host proteins. The newly available technology to study VPg phosphorylation *in vivo* will confirm whethe or not the biological effects and infection phenotypes demonstrated in this work were due to the phosphorylation or only due to the amino acid changes in the protein sequence.

VPg-host interactions that require these amino acids can occur at the level of plant resistance against virus infection. *N. benthamiana* was found to lack the activity of a salicylic acid (SA) -inducible endogenous RNA-dependent RNA polymerase (Yang *et al*., 2004), which makes it unusually susceptible to virus infection. In agreement with this, the

endogenous activity of RNA-dependent RNA polymerase in tobacco increased during virus infection as well as via SA-treatment (Xie *et al*., 2001). This difference between *N. benthamiana* and *N. tabacum* could explain the difference in the behavior of PVA-VPg$^{TS132/133AA}$ and PVA-VPg^{T168A} viruses in both hosts. VPg mutations possibly disturbed the interaction of PVA with the plant SA-mediated defense mechanism, which was shown with PPV to cooperate with RNA-silencing defense during potyvirus infection (Alamillio *et al*., 2006). Possibly the cooperative functioning of these defense mechanisms in *N. tabacum* during PVA infection is powerful enough to stop the infection caused also by either PVA-VPg$^{TS132/133AA}$ or PVA-VPg^{T168A}.

5.4 Model of PVA movement form

Taking together all of the AFM and biochemical data from studies I and II, we propose the following working model for the role of the potyviral tip containing the VPg protein covalently-linked with the 5' terminus of viral RNA, HC-Pro and likely CI (Figure 4). HC-Pro in this model is shown bound to VPg. It is possible that HC-Pro binds VPg only after virion assembly. Before that, theVPg domain that binds HC-Pro is most probably occupied with eIF4E (Roudet-Tavert *et al*., 2007). All the proteins potentially interacting with VPg can compete with each other for such an interaction. Thus, the end of the potyviral particles containing VPg may be a regulatory switch modulating involvement of virus particles in different virus functions. The different terminal supplements may not be an integral part of the potyviral virions, but be associated with them at different stages of the virus infection. The switch may be controlled by, for example, either the phosphorylation status of VPg or through HC-Pro, CI, and/or other proteins located at the end of the virus particles and possibly controlling interactions with various translation factors, PABP or PVIP. One hypothetical reason for the relatively small proportion of tipped particles (approximately 10%) is that they may represent a sub-population of all the potyviral particles that are actively involved in the different virus-specific processes, as suggested above.

Study III demonstrated the capability of phosphorylation and cell-to-cell movement deficient mutant virus to form virions. This finding suggests that potyviral CP and its phosphorylation is needed in potyvirus movement, and possibly in virion uncoating, in addition to its role in preventing virion formation. The dissociation of CI after the transport of virus particle through plasmodesmata possibly destabilizes the arrangement of CP subunits, which may allow their phosphorylation by CK2 and, thus, particle uncoating and viral RNA translation. The phosphorylation of VPg could also act as a virion destabilization trigger. The disturbance of such events should lead to the same consequences in all PVA hosts, however our results from study IV clearly show the

difference in mutant virus (PVA-VPg$^{TS132/133AA}$ and PVA-VPg^{T168A}) behavior in *N. tabacum* and *N. benthamiana*. There is a possibility that VPg is phosphorylated by several plant kinases in different stages of infection. This would partially explain the indication of the *in vivo* phosphorylation of VPg at multiple sites and the dependence of mutant virus infection phenotype on the plant host.

In the proposed model in figure 4, CI is binding to HC-Pro at the very end of the virion tail. In this study we did not get direct evidence for CI-HC-Pro binding. This position is however suggested on the bases of the available protein interaction data (see Introduction) and our AFM and translation experiment data (study II). It is not clear if CI is associated only with those particles that already have bound HC-Pro. Although there are no indications of CI-VPg interaction in the literature at the moment, we can not exclude this possibility in native PVA virions. However in this case, CI and HC-Pro probably would compete with each other for VPg binding. HC-Pro-bound virions are subjects for aphid transmission and that requires that virions are as stable as possible. CI could serve this stabilization purpose, as our results suggest (study II).

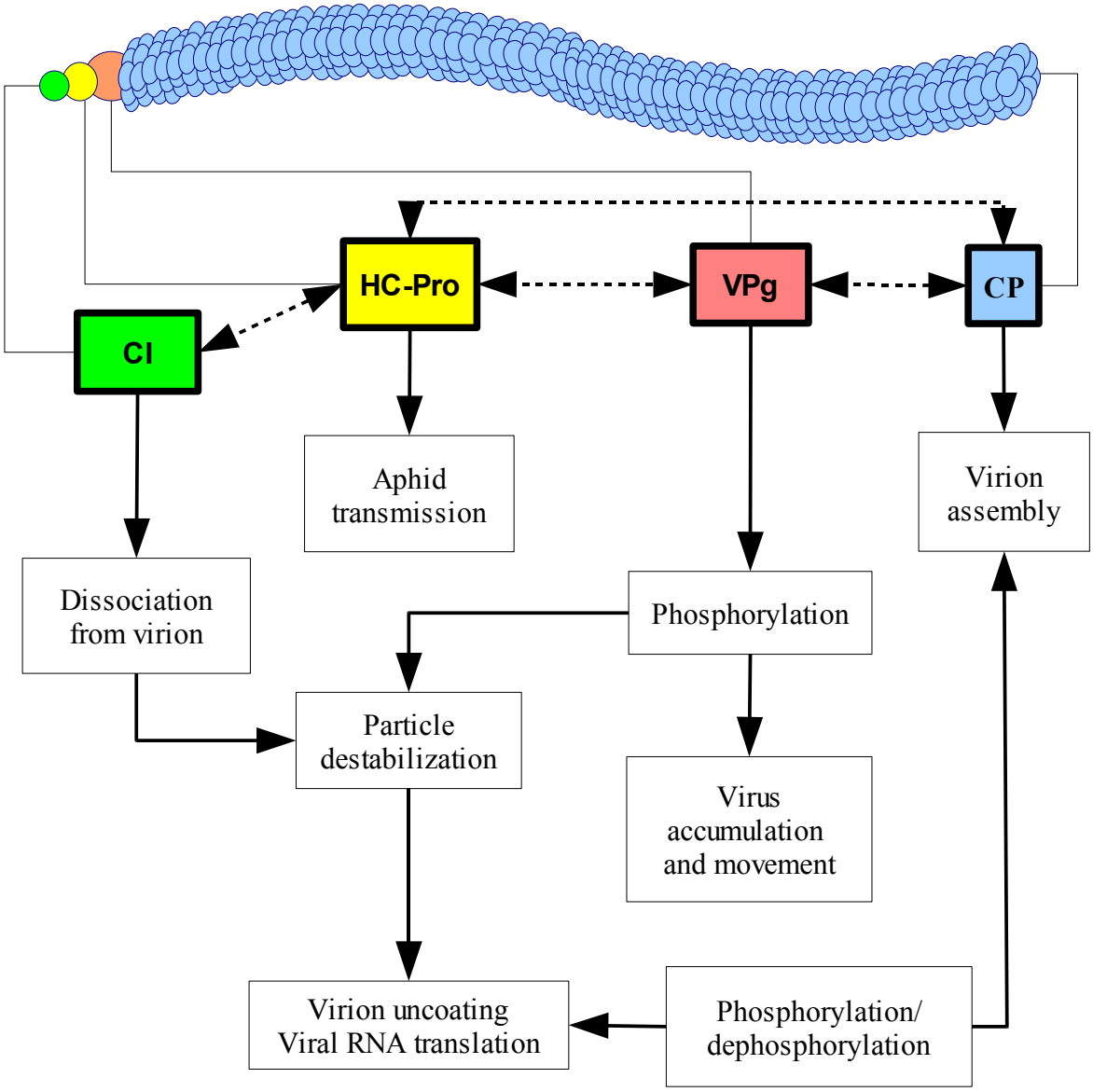

Figure 4. A model of the PVA virion structure and the suggested roles of different virion-associated proteins in the infection process. The viral tip drawn consists of viral proteins VPg, HC-Pro, and, putatively, of CI. The dotted connectors indicate the suggested viral protein-protein interactions.

6 CONCLUDING REMARKS

This work demonstrated for the first time the unique morphology of the potyvirus particle. Approximately 10% of purified PVA and PVY particles contained a tip structure at one virion end. The tip structure was similar to that of closterovirus tails and contained viral proteins having functions in the virus life cycle, possibly similar to closterovirus tail proteins. The PVA HC-Pro protein and VPg were both detected in the tip structure indicating that tips formed at the 5' end of viral RNA. PVA CI was shown to co-purify and co-immunoprecipitate with virus particles. The purified virus sample had ATPase activity, which was probably derived from CI. AFM and translation studies showed that RNA within the particles devoid of CI was more accessible for protein synthesis. The hypothesis for further studies is that CI associates with the tip structure during potyvirus cell-to-cell movement.

In this work *Nicotiana tabacum* CK2 was identified as the kinase phosphorylating PVA CP both *in vitro* and *in vivo*. The CP phosphorylation reduced the affinity of phosphorylated CP to viral RNA. This finding led to a hypothesis that CK2, by phosphorylating potyvirus CP, may regulate virion assembly. The importance of CP phosphorylation by CK2 in PVA infection was also demonstrated, since cell-to-cell movement was not detected. Mutation preventing CP phosphorylation did not effect virion/RNP complex formation in *N. tabacum* cells. However, the mutant virus was not able to move from cell-to-cell, similarly to phosphorylation mimicking mutants, that presumingly could not form virions/RNP complexes. Successful PVA cell-to-cell movement possibly requires either the presence of both, phosphorylated and non-phosphorylated forms of CP in the infected cell, and/or the possibility to switch between the two forms.

Four possible phosphorylation sites were mapped in recombinant VPg of PVA and four mutant viruses were constructed, carrying the mutations that prevent the putative VPg phosphorylation at those sites. Infection of *N. benthamiana* and *N. tabacum* with mutant viruses demonstrated that mutations of amino acids Thr 45 and Thr 49 had no effect on virus spread in both host plants. Viruses harboring $VPg^{TS132/133AA}$ and VPg^{T168A} mutations could infect *N. benthamiana* plants systemically, but a difference in virus accumulation and spread rate was observed in the first systemically infected leaves compared to WT virus infection. The $VPg^{TS132/133AA}$ mutation enhanced virus infection, while the VPg^{T168A} mutation slowed down virus spread. Both mutant viruses could only occasionally cause systemic infection in *N. tabacum*. The mutations did not alter the secondary structure of recombinant VPg proteins, and did not affect the ability of the mutant viruses to replicate and to form new virus particles in *N. tabacum* protoplasts. Tight conservation of Thr168 in VPg proteins of different members of *Potyviridae* family,

and the prediction of putative phosphorylation sites suggest that phosphorylation-deficiency in PVA-VPg^{T168A} may be responsible for the altered infection phenotype whereas in the case of PVA-VPg$^{TS132/133AA}$ the primary amino acids themselves may also be important to retain the WT infection phenotype. In order to further study the molecular mechanism by which these mutations exert their effect, the next important experiment will be to find out whether these mutations indeed affect the phosphorylation status of VPg *in vivo*.

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