

Chapter 2

Role of Capsid Proteins

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Abstract Coat proteins (CPs) of all plant viruses have an early function in disassembly of parental virus and a late function in assembly of progeny virus. Depending on the virus, however, CPs may play a role in many steps of the infection cycle in between these early and late functions. It has been shown that CPs can play a role in translation of viral RNA, targeting of the viral genome to its site of replication, cell-to-cell and/or systemic movement of the virus, symptomatology and virulence of the infection, activation of *R* gene-mediated host defenses, suppression of RNA silencing, interference with suppression of RNA silencing, and determination of the specificity of virus transmission by vectors. These functions are reviewed in this chapter.

Keywords Virus assembly; Virus disassembly; Translation of viral RNA; Replication of viral RNA; Cell-to-cell movement; Long-distance movement; Hypersensitive response; RNA silencing; Vector transmission

1 Introduction

With the exception of umbraviruses, the genomic RNA or DNA of plant viruses is encapsidated by one or more types of coat (or capsid) protein (CP) molecules. In the classical view, CP protects the viral genome from degradation during virus multiplication in the infected plant and transmission of the virus from plant to plant. In the past decades, however, it has become clear that, depending on the virus, CP can be involved in almost every step of the viral infection cycle, including delivery of the virus into the plant cell, disassembly of virus particles, translation of viral RNA, replication of the viral genome, assembly of progeny virus, virus movement in the plant, activation or suppression of host defenses, and transmission of the virus to healthy plants. Recent data indicate that many steps of the infection cycle are tightly linked.

This chapter will briefly review known functions of CP with reference to the methods used to analyze the role of CP in plant virus infection. A more extensive

review has been published by Callaway et al. (1). Emphasis will be on viruses with a positive-strand RNA genome as these represent the majority of plant viruses.

2 Virus Entry and Translation of Viral RNA

Initiation of infection by plus-strand RNA viruses requires uncoating of virus particles and translation of genomic RNA into viral proteins including the RNA-dependent RNA polymerase (replicase) required for viral minus-strand RNA synthesis. It has been proposed that the rigid rod-shaped *Tobacco mosaic virus* (TMV) particles are destabilized after entry into the plant cell by interaction with lipid containing structures, by interaction with a hypothetical subcellular receptor-like component, or by exposing the virus to a low calcium concentration and raised pH. This latter condition would negatively charge carboxylate groups in CP, affecting carboxyl–carboxylate interactions between CP subunits and carboxyl–phosphate interactions between CP and RNA. Elimination of these interactions by mutagenesis of participating Glu and Asp residues to Gln and Asn affects TMV disassembly (2). In vitro, exposure of TMV particles to pH 8.0 results in dissociation of CP from the 5′-terminal 200 nucleotides of the viral RNA and the partially uncoated particle acts as a messenger for translation of the 126kDa and 183kDa replicase proteins in a cell-free system in a process called cotranslational disassembly. Electron micrographs revealed “striposomes” consisting of ribosomes attached to one end of less-than-full-length virus particles. After electroporation of protoplasts with TMV particles, the 5′-terminal region of the viral RNA, including most or all of the 183kDa open reading frame (ORF), became susceptible to ribonuclease within 2–3 min. Uncoating of the 3′ region of the RNA began between 2 and 5 min after electroporation and occurred in the 3′–5′ direction. These observations are compatible with the hypothesis that TMV RNA is cotranslationally uncoated from the 5′ terminus by ribosomes, whereas the 3′ terminus is coreplicationally uncoated by traversing replicase proteins (2). However, a fundamental difficulty of in vivo experiments is that plant cells are exposed to large numbers of virus particles, which may obscure the minor fraction of the inoculum that establishes the infection.

After uncoating, viral plus-strand RNA has to compete with a vast excess of cellular mRNAs for the translational machinery of the host. The translational efficiency of cellular messengers is greatly enhanced by the formation of a closed-loop structure, because of an interaction between the poly(A)-binding protein (PABP), bound to the 3′ poly(A) tail, and the eIF4G subunit of the initiation factor complex eIF4F, bound to the 5′ cap structure (Fig. 1a). Viral messengers without a cap or poly(A) tail use alternative strategies to form a closed-loop structure (3). Viruses from the genera *Alfavirus* (type species *Alfalfa mosaic virus*, AMV) and *Ilarvirus* in the family Bromoviridae require viral CP for efficient translation of the viral RNAs. The 3′ end of the three genomic RNAs and subgenomic CP messenger, RNA 4, of these viruses can adopt two mutually exclusive conformations: a strong CP-binding site (CPB) or a tRNA-like structure (TLS) resembling the TLS of other viruses in the family Bromoviridae. The 5′ termini of the RNAs are capped. A mixture of the three genomic RNAs of AMV has a low intrinsic infectivity (Fig. 1b, panel AMV wt), which is increased 1,000-fold by binding of CP to the 3′

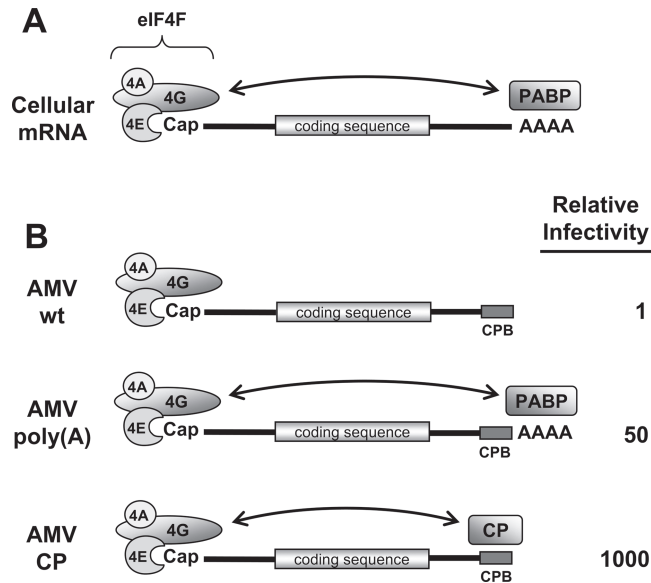


Fig. 1 Coat protein (CP) initiates *Alfalfa mosaic virus* (AMV) infection by mimicking the function of the poly(A) binding protein (PABP). (A) Translational efficiency of cellular mRNAs is strongly enhanced by the formation of a closed-loop structure, because of an interaction between PABP, bound to the 3' poly(A) tail, and the eIF4G subunit of the initiation factor complex eIF4F, bound to the 5' cap structure. (B) The tripartite AMV genome is represented by a single RNA molecule with the 3' terminus folded into the CP-binding (CPB) structure. In the absence of CP the genomic RNAs have a low intrinsic infectivity (panel AMV wt), which is stimulated 50-fold by extension of the RNAs with an artificial 3' poly(A) tail (panel AMV poly(A)) and 1,000-fold by binding of CP to the 3' termini of the RNAs (panel AMV CP). It has been shown that, like PABP, CP specifically interacts with eIF4G and stimulates translation in vivo of AMV RNAs 40-fold (4)

end of the RNAs (Fig. 1b, panel AMV CP). Extension of the 3' termini of the viral RNAs with an artificial poly(A) tail, to allow binding of PABP, increased infectivity 50-fold (Fig. 1b, panel AMV poly(A)) when compared with the CP-free inoculum (4). This suggested that CP mimics the function of PABP in translation of the viral RNAs. Transfection of carrot protoplasts with a transcript containing the luciferase ORF fused with a 3' sequence consisting of the AMV 3' UTR revealed that binding of CP to this UTR enhanced translational efficiency of the reporter construct 40-fold. In GST pull-down assays, a CP-GST fusion specifically pulled down the eIF4F (and eIFiso4F) complex from a wheat germ extract. Far Western analysis of protein blots run with recombinant wheat germ initiation factors revealed that AMV CP specifically interacted with the eIF4G and eIFiso4G subunits of eIF4F and eIFiso4F, respectively (4). These results support the notion that, by analogy to PABP, CP increases translational efficiency of AMV RNAs by the formation of a closed-loop structure through its simultaneous interactions with the 3' end of the viral RNAs and the eIF(iso)4G subunit present in the cap-bound eIF(iso)4F complex. It has been proposed that CP in the inoculum initiates infection by promoting

translation of RNAs 1 and 2 of alfamo- and ilarviruses into the replicase proteins required for viral minus-strand RNA synthesis. Such a mechanism explains why AMV CP is no longer required to initiate infection when RNAs 1 and 2 in the inoculum are extended with an artificial 3' poly(A) tail or when polyadenylated RNAs 1 and 2 are expressed from nuclear genes in transgenic tobacco plants (4).

The genome of DNA viruses has to move to the nucleus of the plant cell to initiate transcription of mRNAs encoding the replicase proteins. Geminiviruses with a monopartite single-stranded DNA genome in the genera *Mastrevirus* and *Begomovirus* encode CPs that act as nuclear shuttles to traffic viral DNA into and out of the nucleus. Trafficking of CP/DNA complexes could be monitored in these experiments by microinjection of protoplasts with *E. coli* expressed GFP-tagged CP or DNA labeled with the fluorescent TOTO-1 dye (5, 6). A similar role of CP in nuclear transport of the double-stranded DNA genome of pararetroviruses from the family Caulimoviridae has been studied by expression of GFP-tagged mutant CP in plasmid-transfected plant protoplasts (7). Thus, CP may promote early events in the initiation of infection by plant DNA viruses.

3 Replication of the Viral Genome

There is growing evidence that translation and replication of positive-strand RNA viruses are tightly coupled. The genomic RNA has to be cleared from ribosomes before initiation of minus-strand RNA synthesis occurs. After translation of AMV RNAs, CP has to dissociate from the 3' termini to allow the formation of the TLS-structure that is required for minus-strand promoter activity. One possibility is that this dissociation is induced by the binding of the newly synthesized replicase proteins to a minus-strand promoter hairpin upstream of the CPB/TLS sequence (4). As dissociation of CP strongly reduces translational efficiency of the viral RNAs, the replicase proteins could trigger the switch from translation to replication. So far, however, a role of CP in the replication of plant viral RNAs or DNAs remains to be demonstrated (4).

4 Virus Assembly

Encapsidation of newly synthesized plant viral RNA has been proposed to occur upon exit of the RNA from vesicles that contain viral replication complexes. A tight link between replication and encapsidation has been suggested for both DNA and RNA viruses. In the yeast two-hybrid system and by using Far-Western assays, CP of the pararetrovirus *Cauliflower mosaic virus* (CaMV) was shown to interact with the viral transactivator protein (TAV), supporting the notion that translation of viral RNA on the surface of cytoplasmic inclusion bodies (viroplasm) and its packaging and reverse transcription in proviral capsids are linked (8). TAV is the main component

of the inclusion body matrix and mediates reinitiation of translation of the polycistronic CaMV RNA through interactions with eIF3 and the 60S ribosomal subunit. Transient expression of BMV (bromovirus) RNAs and CP from a T-DNA vector in agroinfiltrated leaves results in encapsidation of viral RNAs as well as host RNAs. Only upon coexpression of functional replicase proteins, the encapsidation of host RNAs was excluded (9). Probably, a link with replication increases the specificity of the encapsidation process. In the family Bromoviridae, encapsidation of RNAs 1 and 2 by the RNA 3 encoded CP occurs (by definition) in *trans*. However, encapsidation in protoplasts of AMV RNA 3 with a knock-out mutation in the *CP* gene could not be complemented by coreplicating wild-type RNA 3 (4). This observation points to a coupling between RNA 3 replication, synthesis of subgenomic RNA 4, translation of RNA 4 into CP, and encapsidation of RNA 3 (and possibly RNA 4). In view of the evidence that various steps in the viral replication cycle are linked, results from in vitro encapsidation studies should be confirmed by experiments done in vivo.

For a few RNA viruses, the RNA sequence that acts as the origin of assembly (oas) in in vitro packaging assays has been identified. Some of these oas sequences have been inserted into heterologous RNAs to confirm that they direct encapsidation of the RNA by CP in vivo. Most detailed studies have focused on the assembly of the rigid rod-shaped particles of TMV (*vulgare* strain) (2). The TMV oas is composed of one essential hairpin structure and two accessory hairpins located in the movement protein (*MP*) gene between bases 5,290 and 5,527. According to the most widely accepted model, a 20S disk of two layers of 17 CP subunits each binds to the oas and converts to a protohelical form. This RNA–protein complex initiates helical rod elongation in the 5′ direction of the RNA by using 20S disks and in the 3′ direction by using CP monomers or trimers. Potex- and potyviruses have particles with flexuous rod-shaped morphology. In the RNAs of the potexviruses *Papaya mosaic virus* and *Potato virus X* (PVX) and the potyvirus *Tobacco vein mottling virus*, oas sequences have been mapped near the 5′ end in in vitro packaging assays (10, 11). The flexuous rod-shaped particles of closteroviruses contain five viral proteins. The 5′ terminal ~630 nucleotides of the RNA are associated with the minor CP (CPm) to form the tail structure, whereas the remainder of the RNA is associated with the major CP. The tail is extended with segments consisting of the virus-encoded homolog of cellular Hsp70 (Hsp70h) and viral proteins p64 and p20 (12). CP, CPm, Hsp70, and p64 are required for virion assembly. Sequences in the 5′ UTR of closterovirus RNA have been implicated in the formation of virions.

Viruses in the family Bromoviridae have icosahedral symmetry. The 3′ end of the RNAs of bromo- and cucumoviruses contains a tRNA-like structure (TLS) whereas the 3′ termini of alfamo- and ilarvirus RNAs can be folded either in a TLS-structure or in a structure with a high affinity for CP. Surprisingly, this CP-binding structure was found to be dispensable for encapsidation of RNAs 1 and 2 of the alfamovirus AMV. Transient expression of 3′-terminally truncated AMV RNAs 1 and 2 from a T-DNA vector in agroinfiltrated leaves supported replication of RNA 3, and the truncated RNAs were encapsidated by the RNA 3 encoded CP (4).

In RNA 3 of the bromovirus (BMV), the signal for in vitro packaging was found to consist of a 69-nucleotide sequence in the 3' region of the MP ORF and the 3' TLS of 200 nucleotides. The TLS could perform its function in either *cis* or *trans*. When added in *trans* as a 200 nucleotide fragment to 3' terminally truncated RNA 3, the TLS fragment was not copackaged with the truncated RNA 3 in an in vitro assay. Expression of BMV CP and TLS-defective viral RNAs from a T-DNA vector in agroinfiltrated leaves revealed that the TLS was not required for encapsidation of BMV RNAs in vivo, and it was proposed that its function in encapsidation could be taken over by cellular tRNAs (9). Accumulation of nonreplicating AMV and BMV RNAs in protoplasts was increased 20-fold or more by expression of the cognate CP. This illustrates that encapsidation protects the viral RNAs from degradation (4, 9). For another isometric plant virus, *Turnip crinkle virus* (TCV, genus *Carmovirus*), studies done in vivo revealed that a 186-nucleotide region at the 3' end of the CP gene was indispensable for viral RNA encapsidation (13).

5 Virus Cell-to-Cell and Systemic Movement

From primary infected cells, plant viruses move cell-to-cell through plasmodesmata, are transported from mesophyll cells into phloem tissue, and exit from the vasculature to enter the healthy upper leaves of the plant. The role of CP in this process has been recently reviewed (refs. 1, 14; *see also* Chap. 3). Generally, virus movement in plant tissue is monitored by insertion of the *GFP* reporter gene in the viral genome and the effect of mutations in viral genes is analyzed. At the level of cell-to-cell movement, a subdivision can be made into viruses with CP-independent and CP-dependent movement. CP-independent viruses include tobamo-, diantho-, carmo-, hordei- and umbraviruses. Viruses that do require CP for cell-to-cell movement can be further subdivided into those moving as virus particles and those moving by other mechanisms that do not necessarily involve virion formation. Transport of virus particles through plasmodesmata-penetrating tubules made up of viral MP has been observed in plant tissues infected with *Cowpea mosaic virus* (CPMV, *Comovirus*), *Grapevine fanleaf virus* (*Nepovirus*), and the pararetroviruses CaMV (*Caulimovirus*) and *Commelina yellow mottle virus* (*Badnavirus*). By blot overlay assays, a specific interaction between CPMV MP and virions was shown. The interaction involved the large CP subunit in the virion and the C-terminus of MP. CaMV virions may interact with MP through the virion associated protein (VAP) (15). Closteroviruses do not form tubules, yet they are transported as viral particles. The structural proteins CP, CPm, p64, and Hsp70h are required for virion formation and cell-to-cell transport; the p20 protein is dispensable for virion formation and cell-to-cell movement, but is necessary for transport through the vascular system. Flexuous rod-shaped potex- and potyviruses also require CP for cell-to-cell movement, but it is not fully clear whether these viruses are transported as virions or VNP complexes.

In the family Bromoviridae, AMV, BMV, and *Cucumber mosaic virus* (CMV) require CP for cell-to-cell movement, but *Cowpea chlorotic mottle virus* (CCMV)

does not. The MPs of AMV, BMV, and CMV assemble into virion-containing tubular structures at the surface of infected protoplasts, but such structures have not been observed in plasmodesmata in leaf tissue infected with these viruses. Movement of BMV strain M1 requires CP that is encapsidation competent but BMV strain M2 does not require CP for cell-to-cell movement. AMV and CMV require CP for cell-to-cell movement but movement is observed for some CP mutants that are unable to form virions. Moreover, C-terminal point mutations or deletions in the MP of BMV and CMV result in movement of these viruses that is no longer CP-dependent. Probably, viruses in the family Bromoviridae move cell-to-cell as VNP complexes. With the exception of CCMV, CP of these viruses may play an auxiliary role in MP-mediated virus transport, such as suppression of host defense mechanisms. A differential requirement for CP in virus movement is also observed in the family Geminiviridae of viruses with a single-stranded DNA genome. Geminiviruses with a monopartite genome of the genus *Mastrevirus* require CP for cell-to-cell movement whereas bipartite viruses from the genus *Begomovirus* do not. The mastrevirus CP has a functional analogy with the begomovirus BV1 protein. Note that the genus *Begomovirus* contains both monopartite and bipartite geminiviruses (5, 6).

Most viruses require CP for systemic movement through the phloem either as virions or viral nucleoprotein (VNP) complexes. In specific host plants, CP is dispensable for systemic spread of the tombusvirus *Tomato bushy stunt virus*, the hordeivirus *Barley stripe mosaic virus* and for tobnaviruses. Umbraviruses do not encode a CP and move systemically as VNPs consisting of viral RNA and the ORF3-encoded protein. Although TMV generally requires CP for systemic movement, CP deletion mutants can move long distances in *N. benthamiana*. The mechanism of systemic movement is poorly understood.

6 Vector Transmission

In addition to mechanical transmission, plant viruses are transmitted from plant to plant by vectors such as nematodes, fungi, or insects (including leafhoppers, planthoppers, whiteflies, aphids, mealybugs, thrips, beetles, and mites). Generally, transmission requires virion formation in the source plant, and CP is a major determinant of the specificity of the virus-vector interaction (ref. 1; *see also* Chap. 6). CP subunits in the viral capsid may interact directly with putative receptors in the vector or via accessory viral proteins. In transmission of, for instance, the cucumovirus (CMV) by aphids or the tombusvirus *Cucumber necrosis virus* (CNV) by zoospores of the fungus *Olpidium bornovanus*, CP is believed to be the sole virus-encoded determinant. Interaction of CNV particles with the zoospores *in vitro* results in a conformational change of the virus that renders CP in the viral capsids susceptible to digestion with trypsin (16). Luteoviruses are transmitted by aphids in a circulative, nonpropagative manner that requires virions to traverse the aphid hindgut epithelial cells into the body cavity (hemocoel) and then traverse accessory salivary gland cells into the salivary canal. Transmission can be studied by feeding aphids on

purified virus or homogenates of protoplasts infected with wild-type or mutant virus to which sucrose has been added. Efficiency of virus transmission to oat plants can be measured and virus can be detected in various organelles of the aphid by electron microscopy and RT-PCR. In addition to CP, transmission of luteoviruses was shown to be dependent on the presence in virions of a few copies of a readthrough protein (RTP) consisting of the CP sequence fused to a C-terminal extension. The RTP is not required for uptake of virions by the aphid or their trafficking to the hemocoel, but appears to be required for transport of virus through membranes of the aphid salivary gland (1). Umbraviruses do not encode CP and are transmitted by aphids only when encapsidated by CP and RTP of a helper luteovirus. To this goal, the seven definitive umbravirus species are each associated with a specific luteovirus.

Aphid transmission of potyviruses requires the viral helper component, protease (HC-Pro) as an accessory protein. By site-directed mutagenesis, it has been shown that interaction between HC-Pro and potyvirus CP involves a PKT-motif in HC-Pro and a DAG-motif near the N-terminus of CP. Retention of HC-Pro on the aphid's stylet involves a KITC-motif in HC-Pro. Electron microscopic observations revealed an association of potyvirus particles and HC-Pro with the cuticle lining of the mouth parts of aphid vectors. These data support the hypothesis that HC-Pro forms a bridge between virus particles and the aphid food canal (1). It has been proposed that non-structural protein 2b encoded by RNA 2 of tobnaviruses act as accessory proteins in transmission of these viruses by trichodorid nematodes (genera *Trichodorus* and *Paratrachodorus*) in a way that resembles the role of HC-Pro in virus transmission by aphids. *Tobacco rattle virus* (TRV) particles ingested by root-feeding nematodes are retained as clumps associated with the oesophageal cuticle and are released during subsequent feeding on roots of healthy plants. In yeast two-hybrid assays, a specific interaction between TRV CP and its cognate 2b protein was observed and in thin sections of tobnavirus-infected plants the 2b protein colocalized with virus particles (17).

Transmission by aphids of the caulimovirus (CaMV) involves two viral accessory proteins: VAP and the aphid transmission factor (ATF). VAP is bound to virions and associates with MP to permit cell-to-cell movement or with ATF to facilitate aphid transmission of the virus. The interactions between these viral proteins were mapped by Far Western and GST pull-down assays. In transmission of CaMV by aphids, ATF is believed to bridge virion-VAP complexes with the inner lining of the aphid stylet (*see ref. 15*).

7 Plant Response to Virus Infection

Successful infection of a plant requires the virus to overcome host defense mechanisms. Two major defense mechanisms are mediated by plant resistance genes (*R* genes) and the mechanism of RNA interference (RNAi). The gene-for-gene hypothesis predicted that defense mechanisms mediated by *R* genes are activated by an interaction between the product of a viral avirulence (*Avr*) gene, termed as effector, and the product of a plant *R* gene (*see ref. 18*). However, with a few exceptions,

such interactions were not found and the gene-for-gene model was modified into the “guard hypothesis.” This hypothesis predicts that the viral effector targets a key component (guardee) of the basal defense system of the plant in order to invade successfully. A virus-induced change in the structure of the guardee is recognized by an R protein (guard), which subsequently activates defense mechanisms leading to a hypersensitive response of the plant to virus infection. In a susceptible host that lacks the *R* gene, the viral effectors act as virulence factors (18).

The interaction between the carmovirus TCV and *A. thaliana* ecotypes containing the resistance gene *HRT* (the guard) lends support to the guard hypothesis. A yeast two-hybrid screen and in vitro GST pull-down assays revealed that TCV CP interacts with the host transcription factor TIP (the guardee). Confocal microscopy of leaves agroinfiltrated with GFP-tagged TIP showed that TIP localizes to the nucleus. However, coexpression of GFP-TIP and TCV CP prevented the nuclear localization of TIP. The interaction between TIP and CP is required for *HRT*-mediated defense responses (19). Agroinfiltration of *N. benthamiana* transformed with the potato resistance gene *Rx1* with a construct expressing CP of the potexvirus PVX or coexpression of the potato resistance gene *Rx2* and PVX CP in agroinfiltrated *N. tabacum* confirmed that PVX CP is the effector in resistance mediated by resistance genes *Rx1* and *Rx2* in potato. CP of cucumovirus CMV strain Y mediates resistance conferred by the *RCY1* gene of *A. thaliana* (18). Structural studies using site-directed mutagenesis of TMV CP revealed that maintenance of the three-dimensional fold of this CP is essential for elicitation of the *N'*-mediated hypersensitive response in *Nicotiana sylvestris* (2).

Defense mediated by RNA silencing (RNAi) is triggered in virus-infected plants by double-stranded RNA (dsRNA) derived from viral replication intermediates or in the case of plant DNA viruses from annealing of overlapping complementary viral transcripts (ref. 20; see also Chap. 5 for details on RNAi). To overcome this plant defense mechanism, many viruses have evolved suppressors of gene silencing, which interfere with the RNA silencing pathway at different levels. CPs of several plant viruses have been identified as suppressors of gene silencing (18, 20). CP of the carmovirus TCV suppresses RNA silencing possibly by interfering the function of a Dicer-like ribonuclease. This function of TCV CP is not related to its role in *HRT*-mediated resistance. CP of the closterovirus *Citrus tristeza virus* suppresses intercellular silencing. The small CP subunit of the comovirus CPMV has been reported to act as a weak suppressor of gene silencing. CP of the Satellite of *Panicum mosaic virus* (family *Tombusviridae*) acts as a pathogenicity factor. This CP did not suppress gene silencing but interfered with the suppressor activity of the PVX (potexvirus) p25 protein (21).

8 Future Directions

In addition to their structural roles, many novel and unexpected functions of viral CPs have been discovered in the past decades. Further research will undoubtedly shed new light on the role of these multifunctional proteins in virus replication and

their interactions with viral and host components. Plant viral-based vectors have a high potential for the production of safe and cheap vaccines by directing the synthesis of virions that display foreign peptides fused to CP on the surface of viral particles (1). During evolution, CPs have been adapted to the strategy of the virus to evade the activation of host defense mechanisms and almost every man-made change in the CP sequence affects symptomatology of the infection. Further studies on the roles of CP will provide insight in virus–plant interactions.

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Plant Virology Protocols

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