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The Ins and Outs of Nondestructive Cell-to-Cell and Systemic Movement of Plant Viruses

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Table of Contents

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Propagation of viral infection in host plants comprises two distinct and sequential stages: viral transport from the initially infected cell into adjacent neighboring cells, a process termed local or cell-to-cell movement, and a chain of events collectively referred to as systemic movement that consists of entry into the vascular tissue, systemic distribution with the phloem stream, and unloading of the virus into noninfected tissues. To achieve intercellular transport, viruses exploit plasmodesmata, complex cytoplasmic bridges interconnecting plant cells. Viral transport through plasmodesmata is aided by virus-encoded proteins, the movement proteins (MPs), which function by two distinct mechanisms: MPs either bind viral nucleic acids and mediate passage of the resulting movement complexes (M-complexes) between cells, or MPs become a part of pathogenic tubules that penetrate through host cell walls and serve as conduits for transport of viral particles. In the first mechanism, M-complexes pass into neighboring cells without destroying or irreversibly altering plasmodesmata, whereas in the second mechanism plasmodesmata are replaced or significantly modified by the tubules. Here we summarize the current knowledge on both local and systemic movement of viruses that progress from cell to cell as M-complexes in a nondestructive fashion. For local movement, we focus mainly on movement functions of the 30 K superfamily viruses, which encode MPs with structural homology to the 30 kDa MP of *Tobacco mosaic virus*, one of the most extensively studied plant viruses, whereas systemic movement is primarily described for two well-characterized model systems, *Tobacco mosaic virus* and *Tobacco etch potyvirus*. Because local and systemic movement are intimately linked to the molecular infrastructure of the host cell, special emphasis is placed on host factors and cellular structures involved in viral transport.

Keywords coat protein, host factors, intercellular transport, movement protein, phloem transport, plasmodesmata

Moveo ergo sum.

—From musings of a Descartesvirus

1. INTRODUCTION

Studies of tobacco plants infected with *Tobacco mosaic virus* (TMV) led to the recognition of viruses as plant pathogens more than a century ago (Beijerinck, 1898), laying the foundation for the modern science of virology. Since then, a huge variety of plant viruses have been isolated and characterized from a multitude of agronomically important plant species. Although genomic structures and sequences of individual plant viruses are very diverse, the essential principles of the infection process are conserved: viruses replicate within and spread from an initially infected cell to neighboring cells by utilizing naturally occurring intercellular bridges, the plasmodesmata. This process, termed local or cell-to-cell movement, takes place primarily in mesophyll and epidermal tissues of the host plant leaves. Next, viruses

cross the boundary into the vascular system of the plant to exploit the phloem stream for so-called long-distance or systemic movement throughout the plant. Finally, in tissues distant from the initially inoculated leaf, viruses unload from the vascular system to yet again invade the systemic mesophyll and epidermal cells by cell-to-cell movement mechanisms.

The majority of studies of molecular mechanisms underlying plant viral spread have focused on the cell-to-cell movement process. This research of the cell-to-cell movement has been pioneered using TMV, a tobamovirus, but studies of model viruses from numerous and diverse viral genera (in this review, plant virus genera are according to Brunt *et al*., 1996 onwards), including caulimoviruses, dianthoviruses, alfamoviruses, tospoviruses, bromoviruses, cucumoviruses, fabaviruses, sobemoviruses, carmoviruses, necroviruses, tombusviruses, geminiviruses, hordeiviruses, potexviruses, pomoviruses, luteoviruses, and umbraviruses, have followed suit. Many principles of cell-to-cell movement first elucidated for the TMV paradigm have proven to be true for other, even unrelated, viral genera as well, even though different viruses have evolved many variations on these common themes. The central role in cell-to-cell movement is played by the viral movement proteins (MPs). MP functions are encoded by all viruses, but their number, their interaction with cellular structures, and their detailed mode of action vary depending on the viral group. During local movement, viruses spread by one of the two clearly distinct mechanisms: viral MPs either (1) interact with the viral genomes to form nucleoprotein complexes thought to represent the movement intermediates (M-complexes) that cross through plasmodesmata into adjacent cells, or (2) become a part of virus-induced tubules that extend through the cell wall, presumably replacing plasmodesmata and serving as a conduit for the spread of virus particles. In the first mechanism, plasmodesmata are not destroyed or damaged by the movement process, whereas in the second mechanism pathological structures—the tubules—are clearly apparent within or in place of plasmodesmata.

Our knowledge about viral systemic movement, which for many years received less attention than the cell-to-cell movement, is now rapidly expanding with studies focusing on the model systems of TMV and *Tobacco etch potyvirus* (TEV). The viral determinants involved in systemic spread, frequently not as clearly defined as those involved in the cell-to-cell movement, include MPs, coat proteins (CPs), replicases, and, in the case of potyviruses, HC-Pro proteinase and the viral genome-linked protein (VPg). The diversity of viral factors, together with the involvement of numerous highly specialized vascular cell types that viruses must invade to gain entrance into the phloem stream, have posed an experimental challenge that is not easily overcome. Therefore, understanding of the molecular mechanisms involved in viral systemic movement is still less advanced than that of the local movement. Furthermore, because many viral proteins involved in systemic movement also may act to suppress host defenses, it is difficult to separate this latter indirect effect on movement from the movement function *per se*.

This review summarizes our knowledge of both cell-to-cell and systemic movement of plant viruses, including a detailed description of the events at the interface between these two mechanistically distinct types of viral spread. We begin the review with a description of the structure and composition of plant intercellular connections—the plasmodesmata—that viruses exploit as physical passageways to spread between the host cells (Section 2). Then, the viral cell-to-cell movement is discussed (Section 3), with a special focus on the 30 K superfamily of plant viruses, which are characterized by MPs with structural similarity to the 30 kDa MP of TMV (Melcher, 2000). Viruses belonging to this group move between cells either as nucleoprotein M-complexes or as viral particles through transplasmodesmal tubules. Because these two transport mechanisms are radically different, we have limited this review to viruses within the 30 K superfamily that travel as M-complexes through plasmodesmata in a nondestructive way, *i.e*., without irreversibly damaging the plasmodesmal structure and/or integrity. Dominant within the cell-to-cell movement section is an in-depth description of MPs, the key functional molecules of the cell-to-cell movement process (Sections 3.1 to 3.4). To highlight similarities as well as distinctions between MPs, this section is organized by structural features (Section 3.1) and biological activities of MPs rather than by viral groups. The biological activities of MPs range from nucleic acid binding, one of the most ubiquitous of all MP functions (Section 3.2), to gating of plasmodesmata (Section 3.3), to the complex and often difficult-to-interpret patterns of subcellular localization (Section 3.4). The biological function of MPs is intricately dependent on their cellular partners, *i.e*., the host factors that interact with MPs; however, the nature of these plant factors has remained elusive until recent years and is only now gradually unraveling. Therefore, one of the features of this review is a comprehensive description of host proteins that bind MPs (Section 3.5), regulate their functions (Section 3.6), or both. Integrating these data, a host factor-based model for TMV MP functions during the viral life cycle is presented (Section 3.7). To present a more complete picture of the MP functions, we complement the discussion of the 30 K superfamily MPs by comparing and contrasting their properties with those of MPs of viruses outside of this group.

Our review of cell-to-cell movement is followed by the discussion of viral systemic movement (Section 4), which details the current knowledge about viral factors involved in this process (Section 4.1) as well as knowledge about host factors thought to either facilitate or inhibit the systemic movement (Section 4.2). Interestingly, a block in systemic spread is frequently the cause of plant resistance to viral diseases; thus, the studies of cellular pathways for inhibition of systemic movement may lead to development of novel antiviral strategies. The discussion of systemic movement is completed by a description of the intricate chain of events occurring at the interface between the cell-to-cell and systemic movement pathways (Section 4.3). In addition, and although it is beyond the scope of this review, the role of virus-induced suppression of host defense responses such as

posttranscriptional gene silencing (PTGS) in facilitating movement is briefly discussed when directly relevant (Section 4).

Due to its powerful genetics and numerous, publicly available genomics tools, arabidopsis has become the system of choice for most plant-based research. Studies on plant viruses, on the other hand, have traditionally relied on agronomically prominent plant species such as tobacco, potato, or wheat. To integrate plant viral research with the advantages of the arabidopsis experimental system, several viral species capable of infecting arabidopsis are receiving increased attention. We therefore conclude this review article by summarizing the status of research on viral movement using arabidopsis as a model system (Section 5), an approach likely to become the source of major advances towards better understanding of the molecular events of the cell-to-cell and systemic transport of plant viruses.

2. STRUCTURE AND COMPOSITION OF PLASMODESMATA, THE INTERCELLULAR CONDUITS FOR VIRAL MOVEMENT

Cell-to-cell communication in plants takes place through intercellular connections, the plasmodesmata. Plasmodesmata can be classified morphologically as unbranched (simple) and branched, or developmentally as primary and secondary. Primary plasmodesmata are generally simple, unbranched channels that form at the cell plate during cytokinesis. Secondary plasmodesmata are frequently branched and form through preexisting cell walls, allowing the symplastic integration of cells that are not immediately clonally related (reviewed by Kragler *et al*., 1998; Lucas *et al*., 1993).

The ultrastructure of plasmodesmata was first revealed in 1963 (Robards, 1963), and since then it has been refined by numerous electron microscopy studies (*e.g*., Botha, 1992; Ding *et al*., 1992b) and extensively reviewed (Lucas, 1995; Overall and Blackman, 1996; Zambryski and Crawford, 2000). Plasmodesmata contain two types of membranes, the plasma membrane and the membrane of the endoplasmic reticulum (ER). The plasma membrane, which is continuous between adjacent cells, defines the outer surface of the plasmodesmal pore. The inner leaflet of the plasma membrane facing the pore contains electron dense particles about 3 nm in diameter (Ding *et al*., 1992b). The axial center of plasmodesmata is traversed by appressed ER membranes termed the *desmotubule* (Botha *et al*., 1993; Ding *et al*., 1992b; Hepler, 1982; Robards, 1963; Tilney *et al*., 1991), which is also densely covered with globular particles (Ding *et al*., 1992b). The region between the plasma membrane and the desmotubule—the cytoplasmic sleeve—is segmented by the globular particles into 8 to 10 transport channels, each approximately 2.5 nm in diameter (Ding *et al*., 1992b). Spokelike extensions may interconnect the particles of the desmotubule and the plasma membrane (Ding *et al*., 1992b; Overall and Blackman, 1996). Each plasmodesma generally begins and ends with the neck region, which is speculated to act as a sphincter in the control of molecular traffic through plasmodesmata (Olesen, 1979; Overall and Blackman, 1996; White *et al*., 1994). For some types of plasmodesmata, an area of electron-lucent material, termed *collar*, is localized around the neck regions of plasmodesmata (Turner *et al*., 1994; Waigmann *et al*., 1997). Most likely the collar is composed of callose, a sugar polymer (Hughes and Gunning, 1980; Northcote *et al*., 1989; Turner *et al*., 1994) (see also Section 4.2.2).

In contrast to their ultrastructure, the molecular composition of plasmodesmata is still largely unknown. To identify plasmodesmal protein composition, a number of strategies has been employed. Classic biochemical approaches, presumably the most direct strategy to purify proteins, are challenging because of the technical difficulties in isolating pure plasmodesmata without cellular contaminants. Nevertheless, several attempts have been made to purify plasmodesmal proteins biochemically. Two putative plasmodesma-associated proteins (PAPs) from maize mesocotyl, designated PAP26 and PAP27, were identified by crossreaction with antibodies directed against connexins, gap-junctional proteins (Yahalom *et al*., 1991). Gap junctions are connective structures between animal cells that mediate intercellular communication and transport of water, ions, and small molecules (reviewed by Kumar and Gilula, 1996). Immunogold labeling of maize (*Zea mays)* mesocotyl tissue with anti-PAP26 antibody decorates plasmodesmata of cortical cells along their entire length, whereas the antibody against PAP27 mainly stains the neck region of plasmodesmata (Yahalom *et al*., 1991). Thus, despite structural dissimilarities between gap junctions of animal cells and plasmodesmata of plant cells, some plasmodesmal components may be immunologically related to the connexin protein family. On the other hand, an arabidopsis protein initially reported to represent a gap junction homolog (Meiners *et al*., 1991) was later annotated as a protein kinase-like protein (Mushegian and Koonin, 1993).

Another potential plasmodesmal component, a 41 kDa protein, was purified from isolated maize mesocotyl cell wall fractions by high-salt and high-pH extraction and localized by immunogold labeling to plasmodesmata and to electron dense cytoplasmic structures representing Golgi membranes (Epel *et al*., 1996). Maize mesocotyl plasmodesmata may also contain a calcium-dependent protein kinase (CDPK) (Yahalom *et al*., 1998). This protein kinase activity, identified by crossreaction between an arabidopsis CDPK antiserum and cell wall fractions containing embedded plasmodesmata, has an apparent molecular mass of 51 and 56 kDa and is autophosphorylated in the presence of calcium. The identified maize CDPK is similar to other plant CDPKs (Roberts and Harmon, 1992) in its activation by calcium (but not by phospholipids or calmodulin) and in its ability to autophosphorylate (Yahalom *et al*., 1998). Immunoelectron microscopy using antibodies against arabidopsis CDPK localized the maize CDPK to isolated maize mesocotyl plasmodesmata, suggesting that this kinase as well as its substrate proteins are likely associated to plasmodesmata and may be involved in regulating plasmodesmal permeability (Yahalom *et al*., 1998).

In another attempt to isolate plasmodesmal components, proteins were extracted from cell wall preparations of maize root

tips (Turner *et al*., 1994). Antibodies were raised against the total protein fraction, and two monoclonal antibodies, JIM67 and JIM64, were selected for further studies (Turner *et al*., 1994). Using electron microscopy, both JIM67 and JIM64 decorated mesophyll and trichome plasmodesmata in *Nicotiana clevelandii* (Waigmann *et al*., 1997), suggesting that these antibodies recognize structural components of plasmodesmata that are conserved not only between different plant tissues but also between species.

Overall, the biochemical approach has identified several proteins as potential plasmodesmal components, but their encoding genes have not yet been isolated. Furthermore, the biological function of most of these proteins and/or their relevance to intercellular transport and communication have not yet been demonstrated.

Complementing biochemical approaches, another strategy was pursued that relyied on antibodies against known proteins likely to reside at plasmodesmata. Specifically, cytoskeleton components, such as actin and myosin, and calcium-binding proteins, such as centrin and calreticulin, were selected for these studies. Several cytoskeletal proteins have been identified to associate with plasmodesmata (reviewed by Aaziz *et al*., 2001; Heinlein, 2002a). Antibodies to animal or plant actin decorate plasmodesmata in young and mature cell walls of barley (*Hordeum vulgare*) (Overall *et al*., 2000), *Nicotiana* (White *et al*., 1994), maize and cress (*Lepidium sativum*) (Reichelt *et al*., 1999), and in the green alga *Chara corallina* (Blackman and Overall, 1998). Actin may be localized spirally around the desmotubule or line the cytoplasmic sleeve between the desmotubule and plasma membrane of the plasmodesma (White *et al*., 1994), thereby linking cytoskeletons of adjacent cells (Overall *et al*., 2000). Depolymerization of F-ctin induced by treatment or microinjection of plant cells with cytochalasin D (Ding and Kwon, 1996; White *et al*., 1994) or profilin (Ding and Kwon, 1996) induces an apparent dilation of the plasmodesmata, supporting the idea that actin may play a role in regulating the size of permeable plasmodesmal channels.

Also, a myosin-like protein has been identified as a plasmodesmal component in onion (*Allium cepa*), barley, and maize roots (Radford and White, 1998) using an antibody against animal myosin. Myosin antibodies also label the plasmodesmata in cells of the alga *Chara*, indicating that myosin may represent a component of plasmodesmata in higher and lower plants (Overall *et al*., 2000). In an independent study, the plant-specific unconventional myosin VIII protein was found to localize to plasmodesmata in root tissues from arabidopsis, cress, and maize (Reichelt *et al*., 1999). Myosin VIII from arabidopsis was the first plant myosin to be identified and sequenced (Knight and Kendrick-Jones, 1993), and based on its N-terminal motor domain and C-terminal sequences it was assigned into its own class VIII (Reichelt *et al*., 1999). Since then, the several myosins of this plant-specific class that have been described are thought to participate in the site-specific anchoring of actin filaments at the cell periphery (Volkmann *et al*., 2003). Microinjection of polyclonal antibodies against the tail domain of myosin VIII into epidermal cells of arabidopsis roots and mesophyll cells of tobacco (*N. tabacum*) results in nonspecific opening of plasmodesmata and an increase in their permeability (Volkmann *et al*., 2003), suggesting that myosin VIII is required for preserving the structural integrity of plasmodesmata.

From the group of calcium-binding proteins, centrin or a centrin-like protein was found to localize to plasmodesmata of higher plants (Blackman *et al*., 1999). Centrin is a major component of calcium-sensitive contractile nanofilaments that undergo rapid contraction in response to elevated intracellular calcium levels in green algae *Tetraselmis striata* and *Chlamydomonas reinhardii* (Salisbury and Floyd, 1978; Sanders and Salisbury, 1989), and phosphorylation is involved in relaxation of these filaments (Martindale and Salisbury, 1990). Immunofluorescence and immunoelectron microscopy localized centrin or a centrin-like protein to the neck regions of plasmodesmata in onion and cauliflower root tips, and an especially rich content of centrin was found associated with primary plasmodesmata during formation of the new cell wall (Blackman *et al*., 1999). Thus, centrin may be a component of calcium-sensitive nanofilaments in the neck region of plasmodesmata and may play a role in calcium-induced regulation of intercellular transport (Blackman *et al*., 1999).

Calreticulin is a conserved ER-based protein that is thought to act as chaperone and regulator of calcium levels (Michalak *et al*., 1992, 1999). Immunogold electron microscopy showed that antibodies against maize calreticulin localize to cortical ER connected to the plasmodesmata in the maize-root apex (Baluska *et al*., 1999). Cytoskeletal and calcium-binding proteins may function in a cooperative fashion; for example, by regulating calcium levels, calreticulin may influence the activity of myosin VIII and centrin, and thereby participate in elevating plasmodesmal permeability.

A third strategy aimed at identification of plasmodesmal components is genomics-based and employs genome-wide expression of plant cDNAs fused to green fluorescent protein (GFP) and subsequent selection of potential plasmodesmata-localizing proteins by their characteristic punctate localization patterns within cell walls. Recently, expression from a viral vector of a GFP-tagged cDNA library derived from *Nicotiana benthamiana* roots revealed 11 GFP fusion proteins (PD01-04 and PD06-12) that localize to cell-wall–associated puncta and thus may represent potential plasmodesmal proteins (Escobar *et al*., 2003). PD02 and PD04 have no significant similarity to known sequences, whereas PD01, PD03, and PD08 are homologous to sequences already present in databases but without assigned function. Several other identified proteins represent homologs of plant proteins with assigned functions; for example, PD06 is a homolog of berberine bridge enzyme from *Papaver somniferum*, PD11 is related to 1-aminocyclopropane-1-carboxylate deaminase from *Arabidopsis thaliana*, PD07 is homologous to an enzyme involved in the nicotine biosynthetic pathway from *N. tabacum*, PD10 protein has homology to the Ras-related protein Rab11 from *N. tabacum*, and PD09 and PD12 show similarity to proteins from tomato (*Lycopersicon esculentum*) and arabidopsis

involved in "redox"-based signaling reactions (Pastori and Foyer, 2002). Immunogold labeling with anti-GFP antiserum confirmed plasmodesmal localization of the PD01:GFP fusion protein. However, plasmodesmal localization of some of these GFP-tagged proteins may represent a plant response to viral systemic infection used for transient expression of the GFP-tagged cDNA clones and, thus, the biological significance of the identified proteins requires further experimentation (Escobar *et al*., 2003).

Composition of plasmodesmata can be altered in response to pathogen attack and cold stress; thus, stress-response proteins may be associated with plasmodesmata. For example, the maize pathogenesis-related PRms protein was shown by immunoelectron microscopy to localize to plasmodesmal regions in response to fungal infection of maize radicles. It remains unclear, however, whether PRms represents an integral component of plasmodesmata or this protein is simply deposited to the cell wall (Murillo *et al*., 1997).

Dehydrins are proteins produced in response to stress associated with the water status of plant cells, such as salinity, drought, and freezing stress. Localization of dehydrins was studied in cold-acclimated vascular cambium cells of red-osier dogwood (*Cornus sericea*) (Karlson *et al*., 2003). Immunogold electron microscopy localized a 24 kDa dehydrin-like protein predominantly to the neck and collar regions of plasmodesmata of cold-acclimated vascular cambium cells, whereas no signal was detected in nonacclimated tissues (Karlson *et al*., 2003). Thus, association of dehydrins with plasmodesmata may play a role in minimizing membrane damage during freezing stress (Karlson *et al*., 2003).

Overall, just under twenty potential plasmodesmata-associated proteins have been identified, although whether or not they indeed function as plasmodesmal structural or functional components, or both, remains unknown. In any case, are we close to elucidating the protein composition of plasmodesmata? On the one hand, plasmodesmata have been likened to gap junctions (Meiners and Schindler, 1987; Meiners *et al*., 1991), which are composed of only one type of protein (Kumar and Gilula, 1996); on the other hand, plasmodesmal transport has also been compared to transport through the nuclear pore (Citovsky and Zambryski, 1993; Lee *et al*., 2000). Clearly, plasmodesmata are much more complex than gap junctions in animals, but are they as complex as nuclear pores with their more than 100 structural proteins (Rout and Aitchison, 2001)? Considering that plasmodesmata vary in structure and permeability depending on the function and tissue type, the plasmodesmal complexity may indeed be close to that of the nuclear pore. If so, the number of known plasmodesmal components most likely represents only the beginning of a list that awaits completion. Expanding and improving the protein identification approaches should help to elucidate further the composition of plasmodesmata. To reveal the biological function of each identified plasmodesmal component, genetic screens for mutants in plasmodesmata-encoding genes (see Section 5) as well as studies on intercellular protein trafficking and plasmodesmal permeability are an absolute must.

3. CELL-TO-CELL TRANSPORT OF PLANT VIRUSES: HAVE MOVEMENT PROTEIN, WILL TRAVEL

3.1. MP Structure: Are Common Functions Supported by Common Structures?

Based on their amino acid sequence comparison, MPs of plant viruses can be divided into four main groups: small (less than 10 kDa) MPs encoded by carmoviruses, large (69 to 85 kDa) MPs of tymoviruses, MPs encoded by the triple gene block group of viruses, and the 30 K superfamily related to the 30 kDa MP of TMV. Interestingly, besides these classical MPs other viral proteins such as TMV replicase (Hirashima and Watanabe, 2001, 2003) may participate in cell-to-cell movement, illustrating the complexity of viral factors involved in this transport process.

The 30 K superfamily is the largest group of viral MPs, and their structure and function are relatively well characterized. The members of the 30 K superfamily share only low sequence similarity, with only one identified conserved motif, $LXDX_{50-70}G$ (Koonin *et al*., 1991; Melcher, 1990). The poor sequence conservation suggests that the 30 K superfamily members may instead possess a common three-dimensional conformation. Therefore, a study predicting secondary structure elements based on the genus consensus sequences of MPs from 18 virus genera was performed (Melcher, 2000). This analysis revealed a common core structure flanked by variable N-terminal and C-terminal domains. The common core structure consists of four α-helices (α-A-D) and seven β-elements (β-1-7). The N-terminal region was generally variable in length; however, MPs of viruses that form tubules as a conduit for viral cell-to-cell movement are characterized by longer N-termini containing numerous α-helices. In contrast, the C-terminal region was predicted to be a predominantly random coil (Melcher, 2000). For TMV MP, the common core region overlaps with the two single-stranded nucleic acid binding-domains (Citovsky *et al*., 1992; see Sections 3.2 and 3.4.1) and the domain involved in plasmodesmal targeting, increasing plasmodesmal permeability (Boyko *et al*., 2000c; Waigmann *et al*., 1994; see Section 3.3), or both, whereas the C-terminal random coil is dispensable for cell-to-cell movement (Berna *et al*., 1991) but is involved in negative regulation of the MP function (Waigmann *et al*., 2000; see also Section 3.6). Potentially, the unstructured C-terminus acts as a flexible tail that regulates access to those functional domains. Indeed, a regulatory role for the C-terminus is supported by experimental evidence: the C-terminus of TMV MP harbors three phosphorylation sites (Citovsky *et al*., 1993), which have been shown to play a role in downregulation of the MP biological activity (Waigmann *et al*., 2000; see also Section 3.6). In addition, five regions of moderate sequence conservation within the 30 K superfamily were determined; four of them are located in the common core, and the fifth motif, consisting of variations of the

tripeptide SIS, is located close to the C-terminus in 14 of the genera (Melcher, 2000).

Data collected for recombinant TMV MP purified from *Escherichia coli* are in good agreement with the model obtained by secondary structure predictions (Brill *et al*., 2000). Circular dichroism (CD) spectroscopy used to estimate the secondary structure of recombinant TMV MP overexpressed in *E. coli* and solubilized in urea and sodium dodecyl sulfate (SDS) demonstrated a high α-helical content, suggesting an ordered tertiary structure. Trypsin digestion followed by mass spectroscopy revealed two cleavage-resistant regions that contained two highly hydrophobic domains between amino acid residues 58 to 85 and 145 to 175. These domains may have been shielded from tryptic digestion by integration into lipid-like detergent micelles. Thus, these domains constitute two putative membrane-spanning regions that could cause MP to behave as an integral membrane protein (Reichel and Beachy, 1999; see also Section 3.4.3). The C-terminal part of TMV MP (amino acids 250 to 268) was highly sensitive to trypsin treatment, indicating that this part of the protein is accessible to the solvent. Based on these data, a topological model for TMV MP was proposed (Brill *et al*., 2000). The two potential transmembrane domains of TMV MP span the membrane, thereby imposing a U-shaped conformation on the protein, whereas the shorter N-terminal and longer C-terminal regions are exposed to the cytosol (N_{cyt}-C_{cyt} topology). The highly trypsin-sensitive C-terminus, which is not required for the MP function (Berna *et al*., 1991; Gafny *et al*., 1992), would protrude into the cytosol and become easily accessible for regulatory modifications such as phosphorylation (see Section 3.6.). It is important to note, however, that the TMV MP structure determined based on the analysis of the protein solubilized using SDS (Brill *et al*., 2000)—an ionic detergent extremely difficult to remove once bound to protein—may not in all aspects faithfully reflect the conformation of native MP.

The presence of potential transmembrane domains is not only a feature of TMV MP but has also been observed in MPs of other viral groups. For example, carmoviruses encode two small proteins involved in viral cell-to-cell movement (Hacker *et al*., 1992; Li *et al*., 1998). Although these MPs do not share amino acid motifs with MPs from the 30 K superfamily (Melcher, 2000), they may share structural similarity. Recent data based on a combination of CD and nuclear magnetic resonance (NMR) spectroscopy revealed the secondary structure of p7, one of the two MPs of *Carnation mottle virus* (CarMV) (Vilar *et al*., 2001). This MP, CarMV p7, is an RNA-binding protein (Marcos *et al*., 1999) that consists of three domains: a variable and unstructured N-terminus, a highly conserved C-terminus that folds into a stable β-sheet, and a central domain that folds into an α-helix upon binding to RNA (Vilar *et al*., 2001). The second MP, CarMV p9, is an integral membrane protein with two transmembrane helices. The orientation of these transmembrane helices within the membrane is such that the protein topology resembles a U-shaped conformation with a short N-terminus and long C-terminus exposed into the cytosol. It was proposed that the cytosolic C-terminal region may interact with its soluble RNA-binding partner MP, CarMV p7, thereby providing membrane localization to the CarMV p7-RNA complex, which may be important for the viral cell-to-cell movement (Vilar *et al*., 2002). The combined topology proposed for the two MPs of CarMV is remarkably similar to that proposed for TMV MP (Brill *et al*., 2000), suggesting that despite sequence differences some conserved structural and topological elements exist that may be important for the MP function.

Furthermore, in the triple gene block (TGB) group of viruses, whose cell-to-cell movement is mediated by three proteins generally referred to as TGBp1, TGBp2, and TGBp3 (Morozov and Solovyev, 2003), MPs may also behave as integral membrane proteins. All three TGB MPs were shown to be essential for viral movement (reviewed by Morozov and Solovyev, 2003). However, only TGBp2 and TGBp3 contain hydrophobic sequences (Morozov *et al*., 1987, 1989; Solovyev *et al*., 1996a) and are likely integral membrane proteins. The molecular organization of TGBp2 is uniform within viruses of the TGB group and consists of two internal hydrophobic sequences separated by an extremely conserved central region (Morozov *et al*., 1987; Skryabin *et al*., 1988; Solovyev *et al*., 1996a). In contrast, the molecular organization of TGBp3 is more variable and may either contain one hydrophobic sequence at the N-terminus (Morozov *et al*., 1991), or one integral and one C-terminal transmembrane segment separated by a central region (Koenig *et al*., 1998; Solovyev *et al*., 1996a). Experimental evidence suggests that both TGBp2 and TGBp3 are associated with ER membranes (reviewed by Morozov and Solovyev, 2003) (see Section 3.4.3). The predicted topology for integration into the ER membrane would be that TGBp2 inserts in a U-shaped confirmation with both N- and C-termini exposed to the cytosol. In contrast, TGBp3, containing one hydrophobic sequence, would face the ER lumen with its N-terminus, whereas the C-terminus would face the cytoplasm. For TGBp3 containing two hydrophobic regions, both the N-terminus and the C-terminus would be exposed to the ER lumen (Morozov and Solovyev, 2003; Solovyev *et al*., 1996a).

In contrast to TGBp2 and TGBp3, TGBp1 is a soluble protein lacking transmembrane domains. TGBp1 is an RNA-binding protein (Bleykasten *et al*., 1996; Cowan *et al*., 2002; Donald *et al*., 1997; Kalinina *et al*., 1996, 2001; Lough *et al*., 1998; Rouleau *et al*., 1994; Wung *et al*., 1999) with an RNA helicase activity (Kalinina *et al*., 2002). Its NTPase/helicase sequence domain displays similarity to replicative helicases belonging to superfamily I (Caruthers and McKay, 2002). The secondary structure prediction for TGBp1 is based on modeling the TGBp1 sequence to the known crystal structure of the PcrA helicase, a member of superfamily I helicases (Caruthers and McKay, 2002). TGBp1 is predicted to fold into two domains with a characteristic arrangement of α-helices and β-elements (Kalinina *et al*., 2002). TGBp3 directs subcellular targeting of TGBp2 from the ER network to the sites of TGBp3 localization, which are peripheral bodies located in close association with the cell

wall and connected to the cortical ER network (Solovyev *et al*., 2000; see also Section 3.4.3). TGBp2 and TGBp3 may therefore bind to and deliver a TGBp1-viral RNA complex to plasmodesmata (see Section 3.2). This cooperative role of all three TGB proteins is supported by the observations that TGBp1 expressed alone, without its TGBp2 and TGBp3 partners, often is not targeted to the cell periphery (Erhardt *et al*., 1999, 2000; Lawrence and Jackson, 2001).

Our as yet incomplete comprehension of the MP conformation would hugely benefit from the solution of the crystal structure of a viral MP. One of the reasons that no crystallography data are yet available is the difficulty purifying the necessary amounts of highly soluble MP. Nevertheless, secondary structure predictions in conjunction with biochemical analyses and CD spectroscopy have revealed an unexpected degree of structural similarity between MPs of different groups of viruses that share no sequence homology. Thus, these approaches provide a valuable basis for understanding the relation between MP structure and function.

3.2. Movement Complexes as Cell-to-Cell Transport Intermediates

The ultimate goal of the MP function is to transport the viral genome from the infected cell to its healthy neighbors. The most direct way for MP to achieve this objective would be to associate with the viral nucleic acid molecule and chaperone it through plasmodesmata to adjacent cells. Thus, MP must interact with viral genomes which, in many genera of plant viruses, are single-stranded RNA or DNA molecules. Indeed, TMV MP was the first viral MP shown to bind single-stranded (ss) but not double-stranded (ds) RNA and DNA (Citovsky *et al*., 1990). Based on the electron microscopy and atomic force microscopy data, TMV MP–nucleic acid complexes were calculated to have a diameter of 1.5 to 3.5 nm (Citovsky *et al*., 1992; Kiselyova *et al*., 2001), which is compatible with the 3.2- to 4.3-nm size exclusion limit of dilated plasmodesmata (Waigmann *et al*., 1994); note that these plasmodesmal channels, even following their dilation by MP (Waigmann *et al*., 1994; Wolf *et al*., 1989; see Section 3.3), are too small to transport protein-free, folded TMV RNA with a calculated diameter of 10 nm (Gibbs, 1976).

Mutational analysis of TMV MP demonstrated that its single-stranded, nucleic-acid–binding activity is mediated by two independently active domains located between amino acid positions 112 to 185 and 186 to 268 of this 268-residue–long MP (Citovsky *et al*., 1992). MP binding to ssDNA and ssRNA is strong, cooperative, and sequence nonspecific (Citovsky *et al*., 1990, 1992); these characteristics are typical for ssDNA binding proteins (SSBs) (Chase and Williams, 1986), suggesting that TMV MP belongs to this large protein family. That MP binding to single-stranded nucleic acids is not limited to specific nucleotide sequences is biologically important because (1) it allows MP to coat the entire sequence of the viral genomic RNA, and (2) it explains the observations that TMV infection allows cell-to-cell movement of other, often unrelated viruses, including those that do not spread through plasmodesmata in nature (*e.g*., Atabekov *et al*., 1999; Atabekov and Taliansky, 1990; Carr and Kim, 1983; Malyshenko *et al*., 1989). Furthermore, even MPs of several evolutionary-distant viruses complement cell-to-cell movement of an unrelated movement-defective virus when transiently expressed in plant tissues (Morozov *et al*., 1997, and transgenically expressed MPs allowed cell-to-cell movement of an insect RNA virus, *Flock house virus* (FHV), in plant tissues (Dasgupta *et al*., 2001). In addition, when MPs are genetically swapped between different viruses, the resulting chimeric viruses are able to move cell to cell, indicating that the MP function does not depend on the nucleotide sequence of the transported viral genome (De Jong and Ahlquist, 1992; Deom *et al*., 1994; Giesman-Cookmeyer *et al*., 1995; Nejidat *et al*., 1991; Solovyev *et al*., 1996b, 1997, 1999; Wu and Zhou, 2002).

But how does TMV MP find the viral RNA molecules within the infected cell? The most likely scenario is that MP, due to its high affinity to single-stranded nucleic acids (Citovsky *et al*., 1990, 1992), will attach to any ssDNA or ssRNA molecule in its vicinity. Because TMV replication and translation spatially overlap each other, occurring within "viral factories" in the cell cytoplasm (Heinlein *et al*., 1998), TMV MP has a high probability of encountering TMV RNA immediately following its *de novo* synthesis. Of course, MP may bind and transport other nucleic acid molecules such as genomes of other viruses or even cellular RNA. In fact, TMV MP was shown to form complexes with endogenous, cellular RNA following its expression in bacterial cells (Brill *et al*., 2000). Furthermore, TMV RNA has been shown to form ribonucleoprotein complexes, termed vRNP, in TMV-infected plants (Dorokhov *et al*., 1983), although the exact protein composition of these *in vivo*-formed particles remains unknown.

Interestingly, numerous studies demonstrated that nucleic acid binding is not limited to TMV MP but may represent one of several functional hallmarks of MPs encoded by members of many diverse plant virus genera, such as tobamoviruses, caulimoviruses, dianthoviruses, alfamoviruses, tospoviruses, umbraviruses, bromoviruses, cucumoviruses, fabaviruses, sobemoviruses, carmoviruses, necroviruses, tombusviruses, geminiviruses, hordeiviruses, potexviruses, pomoviruses, and luteoviruses (Table 1). Specifically, MP of the Cr-TMV isolate of *Turnip vein clearing tobamovirus* (TVCV) (Melcher, 2003) binds the viral genomic RNA stably but without sequence specificity (Ivanov *et al*., 1994). MP encoded by the gene I of *Cauliflower mosaic virus* (CaMV), a caulimovirus, associates with both ssDNA and ssRNA (Citovsky *et al*., 1991; Thomas and Maule, 1995). CaMV MP binding affinity for ssRNA is higher than that for ssDNA, suggesting that MP-CaMV RNA complexes may be involved in the cell-to-cell spread of this pararetrovirus (Citovsky *et al*., 1991). This TMV-like mechanism for cell-to-cell movement may coexist with the better characterized spread of CaMV in the form of a whole viral particle through MP-induced tubules (Huang *et al*., 2000, 2001b; Perbal *et al*., 1993) that may span plasmodesmata. Cooperative binding to ssRNA and ssDNA but not to dsDNA was also shown

TABLE 1
Nucleic acid binding properties of plant viral MPs

Genus	Virus	MP	ssRNA binding	ssDNA binding	dsRNA binding	dsDNA binding	Cooperativity	Sequence specificity	Reference
Tobamovirus	TMV	30 kDa	++	++	N.R.	—	++	No	Citovsky *et al.*, 1990, 1992
	TVCV (Cr-TMV)	29 kDa	++	N.R.	N.R.	N.R.	N.R.	No	Ivanov *et al.*, 1994
Caulimovirus	CaMV	P1	++	+	N.R.	—	++	No	Citovsky *et al.*, 1991; Thomas and Maule, 1995
Dianthovirus	RCNMV	35 kDa	++	++	N.R.	—	++	No	Giesman-Cookmeyer and Lommel, 1993; Osman *et al.*, 1992, 1993
Alfamovirus	AMV	P3	++	++	—	—	+	No	Schoumacher *et al.*, 1992a, 1992b, 1994
Tospovirus	TSWV	NSm	++	N.R.	—	N.R.	N.R.	No	Soellick *et al.*, 2000
Umbravirus	GRV	ORF4	++	++	N.R.	—	—	No	Nurkiyanova *et al.*, 2001
Bromovirus	BMV	3a	++	++	—	—	++	No	Fujita *et al.*, 1998; Jansen *et al.*, 1998
Cucumovirus	CMV	3a	+	+	—	—	+	No	Li and Palukaitis, 1996; Nurkiyanova *et al.*, 2001; Vaquero *et al.*, 1997
Fabavirus	BBWV-2	VP37	+	+	—	—	++	No	Qi *et al.*, 2002
Sobemovirus	CoMV	P1	+	N.R.	N.R.	—	N.R.	No	Tamm and Truve, 2000
Carmovirus	TCV	p8	++	N.R.	N.R.	N.R.	++	No	Akgoz *et al.*, 2001; Wobbe *et al.*, 1998
	CarMV	p7	++	N.R.	N.R.	N.R.	++	No	Marcos *et al.*, 1999
Necrovirus	TNV	p7a	++	++	N.R.	—	N.R.	N.R.	Offei *et al.*, 1995
Tombusvirus	TBSV	P22	++	N.R.	N.R.	N.R.	+	N.R.	Desvoyes *et al.*, 2002
Bipartite	SLCV	BV1/BC1	+/±	++/±	N.R.	—	N.R.	No	Pascal *et al.*, 1994
geminivirus	BDMV	BV1/BC1	N.R.	++/−	N.R.	++/++	N.R.	2–9 kb open circles	Rojas *et al.*, 1998
Hordeivirus	PSLV	63 kDa TGBp1	++	N.R.	N.R.	N.R.	+	No	Kalinina *et al.*, 2001
	BSMV	58 kDa TGBp1	++	—	++	—	N.R.	No	Donald *et al.*, 1997
	BNYVV	42 kDaTGBp1	++	++	++	++	N.R.	No	Bleykasten *et al.*, 1996
Potexvirus	PVX	25 kDa TGBp1	+	N.R.	N.R.	N.R.	+	No	Kalinina *et al.*, 1996, 2001
	BaMV	28 kDa TGBp1	+	N.R.	N.R.	N.R.	N.R.	No	Wung *et al.*, 1999
	FoMV	26 kDa TGBp1	++	N.R.	N.R.	N.R.	N.R.	No	Rouleau *et al.*, 1994
	WClMV	26 kDa TGBp1	++	N.R.	N.R.	N.R.	—	No	Lough *et al.*, 1998
Pomovirus	PMTV	51 kDa TGBp1	++	N.R.	N.R.	N.R.	N.R.	No	Cowan *et al.*, 2002
		13 kDa TGBp2	++	N.R.	N.R.	N.R.	N.R.	No	Cowan *et al.*, 2002
Luteovirus	PLRV	17 kDa	++	++	N.R.	—	N.R.	No	Tacke *et al.*, 1991

++, binding comparable to that of TMV MP; +, binding weaker than that of TMV MP; ±, very weak binding;—, no binding; N.R., not reported. Plant virus genera are according to Brunt *et al.* (1996).

for MPs of *Red clover necrotic mosaic dianthovirus* (RCNMV) (Giesman-Cookmeyer and Lommel, 1993; Osman *et al*., 1992, 1993) and *Alfalfa mosaic virus* (AMV), an alfamovirus (Schoumacher *et al*., 1992a, 1992b, 1994), although AMV MP exhibited binding cooperativity slightly lower (Schoumacher *et al*., 1992a) than that of RCNMV MP (Osman *et al*., 1992) or TMV MP (Citovsky *et al*., 1990, 1992).

Sequence-nonspecific binding to ssRNA but not to dsRNA was also demonstrated for NSm, MP of *Tomato spotted wilt tospovirus* (TSWV) (Soellick *et al*., 2000). Umbraviral MP, the ORF4-encoded 28 kDa protein of *Groundnut rosette virus* (GRV) also binds ssDNA and ssRNA, but not dsDNA, albeit without binding cooperativity, resulting in only partial coating of the nucleic acid molecules (Nurkiyanova *et al*., 2001). The 3a MP of *Brome mosaic bromovirus* (BMV) does not bind dsRNA but binds both ssRNA and ssDNA cooperatively and sequence nonspecifically (Fujita *et al*., 1998; Jansen *et al*., 1998). Notably, MPs of AMV, TSWV, GRV, and BMV induce formation of tubules on the cell surface of plant protoplasts (Kasteel *et al*., 1997; Nurkiyanova *et al*., 2001; Storms *et al*., 1995; Zheng *et al*., 1997), suggesting that these viruses, similarly to CaMV, may also employ the cell-to-cell movement mechanism involving formation of transplasmodesmal tubules. However, MPs of the viruses, such as *Cowpea mosaic virus* (CPMV) (reviewed by Pouwels *et al*., 2002), that are thought to move between cells exclusively as viral particles through tubular structures have not been shown to possess nucleic-acid–binding activities; instead, they may interact with the whole virions via MP-CP binding (Carvalho *et al*., 2003; Lekkerkerker *et al*., 1996).

Cucumber mosaic cucumovirus (CMV), a cucumovirus, encodes a 3a protein with cell-to-cell movement function (Ding *et al*., 1995a; Vaquero *et al*., 1994) which cooperatively binds ssRNA and ssDNA but not dsRNA or dsDNA (Li and Palukaitis, 1996; Nurkiyanova *et al*., 2001; Vaquero *et al*., 1997). Although CMV MP also forms tubules in CMV-infected plant protoplasts, the role of these tubules in viral movement remains unresolved (Canto and Palukaitis, 1999). VP37, the putative MP of *Broad bean wilt fabavirus 2* (BBWV-2), binds ssDNA and ssRNA cooperatively and without sequence specificity (Qi *et al*., 2002). MP of *Cocksfoot mottle sobemovirus* (CoMV), P1, also binds ssRNA in a sequence-nonspecific manner and does not bind dsDNA (Tamm and Truve, 2000). Nucleic acid binding of CMV, BBWV-2, and CoMV MPs, however, is weaker than that observed with TMV MP (Li and Palukaitis, 1996; Qi *et al*., 2002; Tamm and Truve, 2000). MPs of two carmoviruses, the p8 protein of *Turnip crinkle virus* (TCV) (Akgoz *et al*., 2001; Wobbe *et al*., 1998) and the p7 protein of CarMV (Marcos *et al*., 1999), bind ssRNA cooperatively and sequence nonspecifically. Also, P22, an MP of *Tomato bushy stunt tombusvirus* (TBSV), exhibits binding cooperativity in its interaction with ssRNA (Desvoyes *et al*., 2002). Also, p7a, one of the two MPs of *Tobacco necrosis necrovirus* (TNV, strain D) binds ssRNA and ssDNA, but not dsDNA, with moderately high affinity (Offei *et al*., 1995).

Single-stranded DNA genomes of bipartite geminiviruses, such as *Squash leaf curl virus* (SLCV) and *Bean dwarf mosaic virus* (BDMV), encode two types of movement proteins, BV1 and BC1 (formerly, BR1 and BL1, respectively); BV1 functions as a nuclear shuttle protein to export viral genomes from the host cell nucleus, in which they replicate, and BC1 acts as true MP, mediating cell-to-cell transport, potentially with the assistance of the cortical ER (reviewed by Gafni and Epel, 2002; Hanley-Bowdoin *et al*., 2000; Lazarowitz and Beachy, 1999; Mansoor *et al*., 2003; see also Sections 3.4.3 and 3.4.4). Both BV1 and BC1 bind single-stranded nucleic acids. In the case of SLCV, BV1 binds ssDNA better than ssRNA, whereas BC1 binds single-stranded nucleic acids much weaker than BV1 (Pascal *et al*., 1994). Thus, BC1 may form a complex with the SLCV genome indirectly, through its association with BV1, that is directly bound to the viral genomic ssDNA (Pascal *et al*., 1994); indeed, BC1 and BV1 have been reported to interact with each other (Sanderfoot *et al*., 1996; Sanderfoot and Lazarowitz, 1995, 1996). In contrast, BC1 of BDMV binds dsDNA, whereas its BV1 partner binds both ssDNA and dsDNA with comparable affinity and without sequence specificity; furthermore, BC1 and BV1 preferentially bind to open circles of DNA in the size range of 2 to 9 kb (Rojas *et al*., 1998). Thus, BDMV BV1 and BC1 may transport a replicative dsDNA form of the virus (Noueiry *et al*., 1994; Rojas *et al*., 1998), whereas SLCV BV1 and BC1 likely mediate movement of the true viral genomic ssDNA (Pascal *et al*., 1994).

MPs of hordeiviruses, potexviruses, and pomoviruses, all of which belong to the group of TGB viruses, also exhibit nucleic-acid–binding activity. Among hordeiviruses, the 63 kDa TGBp1 of *Poa semilatent virus* (PSLV) cooperatively binds ssRNA (Kalinina *et al*., 2001), while the 58 kDa TGBp1 (the βb protein) of *Barley stripe mosaic virus* (BSMV) binds both ssRNA and dsRNA without sequence specificity (Donald *et al*., 1997), and the 42 kDa TGBp1 of *Beet necrotic yellow vein virus* (BNYVV) binds single- and double-stranded DNA and RNA molecules (Bleykasten *et al*., 1996). Among potexviruses, the 25 kDa TGBp1 of *Potato virus X* (PVX) interacts with ssRNA sequence nonspecifically and cooperatively (Kalinina *et al*., 2001) but relatively weakly (Kalinina *et al*., 1996), the 28 kDa TGBp1 of *Bamboo mosaic virus* (BaMV) binds ssRNA also with a relatively low affinity, while the binding of the 26 kDa TGBp1 of *Foxtail mosaic virus* (FoMV) to ssRNA is stronger and occurs in a sequence-nonspecific fashion (Rouleau *et al*., 1994; Wung *et al*., 1999). Interestingly, the FoMV TGBp1-ssRNA interaction requires the presence of magnesium ions (Rouleau *et al*., 1994). The 26 kDa TGBp1 of *White clover mosaic potexvirus* (WClMV) binds ssRNA noncooperatively and without sequence specificity, and it requires association with the viral CP for cell-to-cell movement of RNA (Lough *et al*., 1998). In addition, the 51 kDa TGBp1 and the 13 kDa TGBp2 of *Potato mop-top pomovirus* (PMTV) bind ssRNA irrespective of sequence specificity (Cowan *et al*., 2002). It is tempting to speculate that biological activities of single MPs, such as TMV MP, are divided

among the three TGB proteins so that one of them, usually TGBp1, fulfills the nucleic-acid–binding function. In the case of PMTV, binding of TGBp2 to the RNA component of the TGBp1-PMTV RNA complex may help direct it to the cell periphery for subsequent movement (Cowan *et al*., 2002).

Finally, even MPs of luteoviruses, which spread only within the phloem and do not move beyond the host vascular system (Mayo and Ziegler-Graff, 1996; Smith and Barker, 1999), have been shown to bind single-stranded nucleic acids; specifically, the 17 kDa MP of *Potato leaf roll luteovirus* (PLRV) (Schmitz *et al*., 1997) binds ssDNA and ssRNA (Tacke *et al*., 1991). These observations suggest that nucleoprotein complexes participate not only in cell-to-cell movement but also in the systemic transport of plant viruses.

Although most viral MPs bind nucleic acids, the detailed features of this binding may vary between viral groups. For example, TMV MP unfolds ssDNA and ssRNA molecules (Citovsky *et al*., 1992) whereas RCNMV MP does not (Fujiwara *et al*., 1993). Also, identification of nucleic acid-binding domains in many viral MPs (*e.g*., Akgoz *et al*., 2001; Citovsky *et al*., 1992; Donald *et al*., 1997; Fujita *et al*., 1998; Giesman-Cookmeyer and Lommel, 1993; Kalinina *et al*., 2001; Morozov and Solovyev, 2003; Morozov *et al*., 1999; Osman *et al*., 1993; Schoumacher *et al*., 1994; Sokolova *et al*., 1997; Tacke *et al*., 1991; Thomas and Maule, 1995; Vaquero *et al*., 1997; Wung *et al*., 1999) did not reveal conserved amino acid sequence motifs. In all cases, however, complexes between MPs and viral genomes most likely represent the intermediates of the movement process and thus can be designated movement (M) complexes. Viral genomic RNA or DNA molecules packaged in M complexes are likely protected from cellular nucleases, shaped in a transferable form that is compatible with the permeability of MP-dilated plasmodesmata (Citovsky *et al*., 1992; Kiselyova *et al*., 2001), and interacting with the host protein components of the cell-to-cell transport machinery. Similar multifunctional protein–ssDNA transport (T) complexes, have been described for nuclear import of the agrobacterium T-DNA in plant cells (reviewed by Tzfira and Citovsky, 2002; Zupan and Zambryski, 1997). Furthermore, viral genomes sequestered into M-complexes may be diverted from translation and replication to cell-to-cell movement. Indeed, *in vitro*-formed M complexes between TMV MP and TMV RNA are nontranslatable and nonreplicable *in vitro* and in isolated plant protoplasts; this inhibition of translation and replication of TMV RNA is relieved in plant tissues, suggesting that M-complexes are at least partly uncoated during their passage through plasmodesmata (Karpova *et al*., 1997); this uncoating was proposed to occur following TMV MP phosphorylation (Karpova *et al*., 1999) by a cell-wall–associated protein kinase (Citovsky *et al*., 1993).

3.3. Viral MPs Gate Plasmodesmata and Themselves Move Between Cells

Studies on the permeability of plasmodesmata were first initiated in the beginning of the last century (Plowe, 1931). Since then, size exclusion limit (SEL) of plasmodesmata has been determined for numerous types of plasmodesmata by microinjection of fluorescently labeled tracer molecules of various sizes, such as low-molecular-weight dyes and peptides (Goodwin, 1983; Iglesias and Meins Jr., 2000; Oparka and Prior, 1992; Terry and Robards, 1987; Tucker, 1982) or dextrans (Derrick *et al*., 1990; Ding *et al*., 1995a; Fujiwara *et al*., 1993; Lough *et al*., 1998; Nguyen *et al*., 1996; Noueiry *et al*., 1994; Oparka *et al*., 1997; Poirson *et al*., 1993; Rojas *et al*., 1997; Santa Cruz *et al*., 1998; Vaquero *et al*., 1994; Waigmann *et al*., 1994, 2000; Waigmann and Zambryski, 1995; Wolf *et al*., 1989). In the last few years autofluorescent proteins, such as GFP or fusion proteins composed either of GFP oligomers or of GFP fused to a protein considered inert with respect to plasmodesmal trafficking, have been used for this purpose (Crawford and Zambryski, 2000; Oparka *et al*., 1999; Wymer *et al*., 2001). Intercellular trafficking of these fluorescent molecules is considered a diffusion-driven process dependent on a concentration gradient between the adjacent cells and the size of the transported molecule, usually characterized by the hydrodynamic radius or the Stokes' radius (Terry and Robards, 1987; Wymer *et al*., 2001). Therefore, cell-to-cell movement of a tracer molecule provides information on the effective pore size of the plasmodesmal channel. Since the Stokes' radius depends both on the molecular mass and the chemical nature of the molecule, the Stokes' radii of different types of molecules cannot be directly compared based only on their respective molecular masses. For example, the Stokes radius of a 10 kDa dextran has been experimentally determined as approximately 2.2 nm and is similar to that of a 23 kDa globular protein (Jorgensen and Moller, 1979; le Maire *et al*., 1986). The Stokes radius of the 27 kDa barrel-shaped GFP with a predicted diameter of 2.4 nm (Ormo *et al*., 1996), therefore may not be all that different from the Stokes radius of a 10 kDa dextran, even though GFP has nearly triple the molecular mass.

Historically, plasmodesmata were believed to limit exchange between cells to molecules with a molecular mass up to 1 kDa (Cleland *et al*., 1994; Derrick *et al*., 1990; Goodwin, 1983; Terry and Robards, 1987; Tucker, 1982; Tucker *et al*., 1989). Although the basal SEL of 1 kDa is still generally true for several types of plasmodesmata, for example, those connecting mesophyll and epidermal cells in mature leaves (Crawford and Zambryski, 2001; Oparka *et al*., 1997; Waigmann *et al*., 1994; Wolf *et al*., 1989), it has become increasingly clear that plasmodesmal SEL is flexible and depends on tissue type, developmental stage, and environmental conditions (Cleland *et al*., 1994; Crawford and Zambryski, 2001; Kim *et al*., 2002; Oparka *et al*., 1999; Waigmann and Zambryski, 1995). For example, plasmodesmata within the phloem system connecting companion cells and sieve elements support movement of at least 10 kDa dextrans (Kempers and van Bel, 1997), those connecting the cells of leaf hairs (trichomes) have a basal SEL for dextrans of approximately 7 kDa (Angell *et al*., 1996; Waigmann and Zambryski, 1995), and plasmodesmata in sink leaves are permeable for GFP fusion proteins with a molecular mass up to 50 kDa (Oparka *et al*., 1999).

That viral MPs possess the ability to increase plasmodesmal SEL, *i.e.*, to "gate" plasmodesmata, was first reported for TMV MP expressed in transgenic tobacco plants (Wolf *et al.*, 1989) and has since then become one of the hallmark functions of viral MPs. Leaf mesophyll plasmodesmata of the TMV MP transgenic plant show an elevated SEL, permitting diffusion of 10 kDa dextrans (Wolf *et al.*, 1989). This plasmodesmal gating by TMV MP is developmentally regulated, being more pronounced in mature leaves, concomitantly with the increased amounts of MP in the cell walls of these leaves (Deom *et al.*, 1990). Similarly, transgenic tobacco plants expressing the MPs of AMV, CMV, PLRV, or TGBp1 of WClMV elevate plasmodesmal permeability and permit cell-to-cell diffusion of 4 to 20 kDa fluorescently labeled dextrans in their trichomes, epidermis, or mesophyll (Ding *et al.*, 1995a; Hofius *et al.*, 2001; Lough *et al.*, 1998; Poirson *et al.*, 1993; Vaquero *et al.*, 1994).

Studies of MP function in transgenic plants are inherently flawed because due to constitutive expression of MP they do not afford insights into the dynamics of interaction between MP and plasmodesmata. This difficulty was circumvented by direct microinjection of recombinant MPs purified from *E. coli* into mesophyll cells of wild-type plants. Comicroinjection of TMV MP (Waigmann *et al.*, 1994), RCNMV MP (Fujiwara *et al.*, 1993), CMV MP (Ding *et al.*, 1995a), WClMV TGBp1 (Lough *et al.*, 1998), and BDMV BC1 (Noueiry *et al.*, 1994), together with fluorescently labeled dextrans of various sizes, revealed the potential of these MPs to interact with and gate mesophyll plasmodesmata within minutes after microinjection. Gating frequently extended beyond plasmodesmata of the injected cell, facilitating trafficking of dextrans to multiple cells not directly connected to the injected cell (Ding *et al.*, 1995a; Fujiwara *et al.*, 1993; Nguyen *et al.*, 1996; Noueiry *et al.*, 1994; Waigmann *et al.*, 1994). Analysis of a series of deletion mutants of TMV MP for their gating capacity identified a region between amino acid positions 126 and 224, designated domain E, as an MP domain essential for gating plasmodesmata (Waigmann *et al.*, 1994).

MP-induced plasmodesmal gating is not observed in all tissues; for example, leaf trichome cells of *N. clevelandii*, which are interconnected by plasmodesmata with an elevated basal SEL of 7 kDa, are not further gated by microinjected TMV MP (Waigmann and Zambryski, 1995). Potentially, the already high plasmodesmal SEL in this cell type may be sufficient for viral movement to proceed without additional MP gating (Waigmann and Zambryski, 1995). Together, the fast onset of movement and the spread of dextrans into cells distant from the injected cell suggested that MPs move themselves between cells by pirating pre-existing endogenous pathways for cell-to-cell transport of macromolecules (Waigmann and Zambryski, 1994). Using microinjection of recombinant MPs purified from *E. coli* and chemically labeled with fluorescent dyes, direct confirmation of MP intercellular trafficking between leaf mesophyll cells has been provided for TMV MP (Nguyen *et al.*, 1996), RCNMV MP (Fujiwara *et al.*, 1993), BDMV BC1 (Noueiry *et al.*, 1994) CMV MP (Ding *et al.*, 1995a; Nguyen *et al.*, 1996) and TGBp1 of WClMV (Lough *et al.*, 1998). Also, cell-to-cell movement of an intact, unlabeled recombinant TMV MP has been demonstrated using its microinjection into leaf trichome cells followed by immunohistochemical detection (Waigmann and Zambryski, 1995). Furthermore, TMV MP mediates movement of a β-glucuronidase (GUS) reporter as a 90 kDa GUS-TMV MP fusion protein, whereas the 60 kDa GUS alone provided in *trans* cannot move between cells. Thus, unlike the diffusion of inert tracer molecules (see above), the size *per se* of the transported molecule may not represent the major selection criterion for MP-mediated cell-to-cell movement. Instead, MPs likely contain a plasmodesmal transport signal that confers selectivity and is essential for an active transport mechanism (Waigmann and Zambryski, 1995). In this respect, the MP plasmodesmal transport may closely resemble nuclear transport, which is also mediated by specific targeting signals in the transported molecule (reviewed by Damelin *et al.*, 2002; Dingwall and Laskey, 1991).

The capacity of several MPs to mediate transport of the corresponding viral nucleic acids was also revealed in conjunction with microinjection techniques. The MPs of TMV (Nguyen *et al.*, 1996), RCNMV (Fujiwara *et al.*, 1993), and CMV (Ding *et al.*, 1995a; Nguyen *et al.*, 1996), as well as TGBp1 of WClMV, are able to traffic genomic viral RNA between cells, consistent with the idea that MPs promote viral movement by guiding M complexes through plasmodesmata. In contrast, BDMV BC1 was found to mediate movement selectively of double stranded BDMV DNA, which constitutes the replicative form of this ssDNA virus (Noueiry *et al.*, 1994). Thus, for BDMV, the replicative form rather than the viral genome may represent the cell-to-cell movement intermediate (see also Section 3.2).

Increasing plasmodesmal permeability, trafficking between cells, and mediating intercellular transport of viral nucleic acids are among the signature functions characterizing a viral MP. Therefore, microinjection-based tests for these functions were used as a means to identify MPs of viruses in which a dedicated MP had not yet been identified by genetic or sequence analysis-based approaches. For example, the V1 and C4 proteins of a monopartite *Tomato yellow leaf curl geminivirus* (TYLCV) (Rojas *et al.*, 2001) as well as CP and HC-Pro proteins of potyviruses *Bean common mosaic necrosis virus* (BCMNV) and *Lettuce mosaic virus* (LMV) (Rojas *et al.*, 1997) fulfill all three functions and are therefore considered MPs.

More recently, microinjection approaches have been complemented by movement studies involving transient expression of MP-GFP translational fusions *in planta*. Plasmids encoding these fusions are introduced by biolistic delivery into single epidermal cells of leaves. Thus, in contrast to microinjection techniques, this method detects movement of plant-produced rather than bacterially expressed MP. Movement is generally scored by detecting GFP fluorescence in a halo of cells surrounding the expressing, biolistically transformed cell. With this technique, several MPs, including TMV MP (Crawford and Zambryski, 2001; Kotlizky *et al.*, 2001), CMV MP (Itaya *et al.*, 1998), MP of *Apple chlorotic leaf spot trichovirus* (ACLSV) (Satoh *et al.*, 2000), AMV MP (Huang and Zhang, 1999) TYLCV V1 and C4 (Rojas *et al.*, 2001), and TGBp2 of PVX, have been shown to

move cell to cell (see also Section 3.4). Interestingly, untagged PVX TGBp2 gates plasmodesmata to allow movement of coexpressed free GFP in *N. benthamiana* (Tamai and Meshi, 2001), and the GFP-tagged TGBp2 of PVX moves from cell to cell in *N. benthamiana* leaves but not in the leaves of *N. tabacum* (Mitra *et al*., 2003). However, GFP-TGBp2 movement does occur in TGBp1-expressing transgenic *N. tabacum*, indicating that TGBp2 requires TGBp1 to promote its movement in *N. tabacum* (Mitra *et al*., 2003; see also Section 3.4.1).

Finally, gating of plasmodesmata has also been observed during viral infections. *Tobacco rattle tobravirus* (TRV) and PVX increase the permeability of leaf trichome plasmodesmata in *N. clevelandii* (Angell *et al*., 1996; Derrick *et al*., 1992). Furthermore, during PVX and TMV infection, plasmodesmata connecting epidermal cells of infected *Nicotiana* plants are also gated (Oparka *et al*., 1997; Santa Cruz *et al*., 1998). For TMV, plasmodesmal gating is restricted to the leading edge of infection and not apparent at later stages of infection, even though TMV MP is still present in those cells (Oparka *et al*., 1997). Thus, the plasmodesmata-gating activity of TMV MP is negatively regulated during infection (see Section 3.6).

3.4. Subcellular Localization of Viral MPs

The following subsections will provide an overview of identification of subcellular structures, such as the cell wall, cytoskeleton, ER, and the cell nucleus, with which viral MPs are associated, as well as the functional relevance of this intracellular localization. Due to the large amount of information, a representative subset of studies most pertinent to the subject was selected.

3.4.1. Localization to the Cell Wall and Plasmodesmata

Early studies indicated that viruses utilize plasmodesmata for their MP-mediated intercellular spread and, not surprisingly, that MPs localize to the cell wall and to plasmodesmata. For example, using cell fractionation, TMV MP was found to localize to the plant cell wall in virus-infected tobacco plants (Berna *et al*., 1991; Deom *et al*., 1990). TMV MP localization to the cell wall does not depend on the presence of its C-terminal 55 amino acid residues but is lost when 73 amino acids are deleted from the C-terminus (Berna *et al*., 1991). This loss of cell wall localization is correlated to a loss in infectivity, suggesting that localization of TMV MP to the host plant cell wall is important for its function (Berna *et al*., 1991; Gafny *et al*., 1992). TMV MP location within the cell wall was determined by immunoelectron microscopy, which established that TMV MP targets to plasmodesmata both during TMV infection (Tomenius *et al*., 1987) and in transgenic plants expressing TMV MP (Atkins *et al*., 1991; Ding *et al*., 1992a). Interestingly, in transgenic plants, TMV MP was exclusively associated with secondary plasmodesmata of older leaves, filling their central cavities, but it was absent from primary plasmodesmata predominantly found in younger leaves (Ding *et al*., 1992a). These findings correlate with the MP-induced increase in plasmodesmal permeability in TMV MP-expressing transgenic plants, which is observed in older but not in younger leaves (Deom *et al*., 1990; Ding *et al*., 1992a). Nonetheless, TMV MP expressed in transgenic plants complements movement of a movement-deficient TMV strain in both older and younger leaves (Deom *et al*., 1987), raising serious doubts whether TMV MP deposited in the central cavities of secondary plasmodesmata indeed represents the functional form of this protein (Citovsky *et al*., 1993; see also Section 3.6).

With the advance of GFP (Chalfie *et al*., 1994) and its variants as markers of protein localization in living cells (reviewed by Cubitt *et al*., 1995; Tsien and Miyawaki, 1998), a wealth of detailed information on subcellular localization of TMV MP as a GFP fusion protein (TMV MP-GFP) has become available in various biological systems such as whole plants, suspension culture cells, and plant protoplasts. Adding to the complexity of this information, TMV MP-GFP was either expressed in the context of the virus (in plants or protoplasts) or by itself both stably (in transgenic plants, transgenic suspension culture cells) and transiently (in plant epidermal cells). Since the TMV MP-GFP fusion protein promotes TMV spread (Heinlein *et al*., 1995; Padgett *et al*., 1996), complements infection of a movement-deficient TMV mutant (Roberts *et al*., 2001), and itself moves between cells in transient expression studies in the absence of the virus (Crawford and Zambryski, 2001; Kotlizky *et al*., 2001), the addition of GFP to the C-terminus of TMV MP does not interfere with essential cell-to-cell transport functions of this protein.

Localization of TMV MP-GFP to the plant cell wall manifests in highly fluorescent punctate structures during TMV infection (Boyko *et al*., 2000a; Heinlein *et al*., 1995; Oparka *et al*., 1997), upon transient expression of the TMV MP-GFP fusion protein in epidermal cells of plant leaves (Crawford and Zambryski, 2001; Kotlizky *et al*., 2001), and in transgenic plants expressing TMV MP-GFP (Roberts *et al*., 2001). Does punctate fluorescence at cell walls represent plasmodesmal localization of the TMV MP-GFP? To address this question callose, a sugar polymer known to surround plasmodesmal neck regions (Hughes and Gunning, 1980; Northcote *et al*., 1989; Turner *et al*., 1994) and thought to be involved in controlling plasmodesmal permeability (Botha *et al*., 2000; Bucher *et al*., 2001; Delmer *et al*., 1993; Iglesias and Meins Jr., 2000; Northcote *et al*., 1989; Ueki and Citovsky, 2002), was used as a marker for plasmodesmata-enriched regions of the cell wall. Colocalization of TMV MP-GFP fluorescence and immunofluorescence-detected callose was observed (Oparka *et al*., 1997), indicating that the cell-wall–associated TMV MP-GFP puncta most likely reside in plasmodesmata-enriched areas of the cell wall. TMV MP-GFP expressed as a transgene in tobacco plants also localizes to cell-wall–associated puncta; this localization is developmentally controlled, with high expression levels in source leaves and low expression levels in sink leaves (Roberts *et al*., 2001). Interestingly, TMV MP-GFP labeling of walls interconnecting trichome cells was also developmentally controlled, with a gradient of fluorescence from the base to the tip of the trichome (Roberts *et al*., 2001), closely paralleling the development of complex secondary plasmodesmata in trichome cells, which also progresses from base (exclusively

branched, most likely secondary, plasmodesmata with large central cavities) to tip (only simple, unbranched structures, likely still representing primary plasmodesmata) (Waigmann *et al*., 1997). Based on these expression patterns, cell-wall–associated TMV MP-GFP puncta may exclusively label complex secondary plasmodesmata (Roberts *et al*., 2001), as observed for TMV MP expressed in transgenic plants as an unfused, free protein (Ding *et al*., 1992a). Overall, taking into account that immunoelectron microscopy has clearly established the plasmodesmal localization of TMV MP (Atkins *et al*., 1991; Ding *et al*., 1992a; Tomenius *et al*., 1987), the cell-wall–associated punctate fluorescence most likely corresponds to TMV MP-GFP accumulated in or around plasmodesmata.

An even more intriguing question is whether the cell wall-associated TMV MP-GFP is a protein form that functionally interacts with plasmodesmata, for example, by gating or moving through these channels, or the puncta simply represent deposits of inactive TMV MP, potentially sequestered within the central cavities of branched plasmodesmata (Citovsky *et al*., 1993). During infection, TMV MP-GFP localizes to cell-wall–associated puncta in cells at the leading edge of an infection ring as well as in the trailing edge and the center of the ring, whereas closer to the leading edge TMV MP-GFP also localizes to the ER-associated sites or the microtubules (Boyko *et al*., 2000a; see below). Importantly, it is TMV MP-GFP in the cell walls of cells close to the infection front that gates plasmodesmata as assessed by microinjection of Texas Red-conjugated 10 kDa dextran. In contrast, cell-wall–associated TMV MP-GFP in cells in the center of the expanding infection ring does not affect plasmodesmal permeability (Oparka *et al*., 1997), indicating that TMV MP-GFP has been inactivated in those cells. Thus, during infection, the activity of cell wall-associated TMV MP-GFP is controlled, depending on the position within the infection site and hence the temporal and spatial progression of the infection process. The relationship between subcellular localization and functionality in TMV spread was also addressed by utilizing GFP-tagged TMV MP mutants in the context of the infectious virus (Boyko *et al*., 2000c, 2002; Kotlizky *et al*., 2001). All movement-competent TMV MP-GFP mutants accumulated in the cell-wall–associated puncta at the infection front, whereas deletion of 5 or 30 N-terminal amino acids as well as deletion of the C-terminus starting from the amino acid residue at position 188 interfered with the efficient plasmodesmal targeting, rendering these mutants movement deficient (Boyko *et al*., 2000c). Interestingly, a nonfunctional TMV MP mutant lacking amino acid residues 203 to 213 accumulated in the cell-wall–associated puncta only at the trailing edge of infection but not at the infection front, suggesting that targeting of TMV MP-GFP is differentially regulated in early and late stages of infection (Boyko *et al*., 2000c). Overall, even though several aspects positively correlate cell-wall–associated TMV MP-GFP with TMV spread, the detailed functionality of these TMV MP-GFP puncta remains unresolved.

Correlation between the visible pattern of TMV MP-GFP expression and its movement function, while very useful, may sometimes be misleading. For example, in sink tissues of TMV MP-GFP transgenic plants, the fluorescent signal is detected only in trichomes, but functionally this fusion protein complements spread of a movement-deficient TMV mutant also in nontrichome cells of sink tissues (Roberts *et al*., 2001). Potentially, this expanded functionality in sink leaves is provided by very low, undetectable levels of TMV MP-GFP associated with primary plasmodesmata.

Transient expression of TMV MP-GFP in the absence of the virus is usually achieved by biolistic delivery of the corresponding plasmid DNA into single cells of leaf epidermis. This system presents the advantage that cell-to-cell movement of the GFP fusion protein to the neighboring cells can be easily monitored by fluorescence microscopy. The efficiency of cell-to-cell transport of TMV MP-GFP is measured by the percentage of expressing cells that permit movement of TMV MP-GFP into adjacent cells and depends on the plant species, growth conditions, and leaf age (Crawford and Zambryski, 2001). In the expressing cell, TMV MP-GFP displays a variety of subcellular patterns such as cytoplasmic, localization at cortical ER and microtubules, and accumulation in cell-wall–associated puncta. Interestingly, in cells that TMV MP-GFP has moved to, only one type of subcellular localization pattern, namely the cell-wall–associated puncta, is observed (Crawford and Zambryski, 2001; Kotlizky *et al*., 2001). Similar to TMV MP, MP of a closely related tobamovirus *Ob* also accumulates to cell-wall–associated puncta when expressed as a GFP fusion protein in the context of the virus (Padgett *et al*., 1996).

Several MPs of other viruses, such as CMV, AMV, and BMV are also observed in cell-wall–associated puncta. It should be noted that for these viruses the mode of cell-to-cell transport is not yet clear: both transport through transplasmodesmal tubules and movement as ribonucleoprotein complexes are discussed (see Section 3.2). The formation of cell-wall puncta by MP-GFP fusions, however, lends support to the idea of the TMV-like cell-to-cell transport as an MP-RNA complex. For example, CMV MP forms cell-wall–associated puncta when fused to GFP and expressed in the context of the virus (Canto *et al*., 1997). Viral spread promoted by CMV MP-GFP is limited to a few cells around the inoculated cell; however, 10 kDa dextran microinjected into these cells shows extensive intercellular spreading, indicating different requirements for cell-to-cell movement of dextrans and viral genomes (Canto *et al*., 1997). CMV MP-GFP transiently expressed in wild-type tobacco also exhibits punctate localization and capacity for intercellular trafficking; both of these functions correlate with leaf maturation and therefore are not detected in young leaves (Itaya *et al*., 1998). The fluorescent puncta represent MP association with central cavities of plasmodesmata as revealed by immunoelectron microscopy studies of CMV MP-GFP produced from a PVX vector (Blackman *et al*., 1998). Similarly, the intact CMV MP produced in CMV-infected *N. clevelandii* leaves (Blackman *et al*., 1998) or in transgenic tobacco plants (Itaya *et al*., 1998) targets the central cavities of the plasmodesmal channels. In transgenic plants, accumulation of CMV MP-GFP is limited to complex secondary plasmodesmata

and closely follows their appearance during leaf development (Itaya *et al*., 1998); however, such plants are not able to complement an MP-deficient CMV, indicating that the transgenically produced CMV MP-GFP is not fully functional (Canto and Palukaitis, 1999).

BMV MP exhibits a different pattern of association with plasmodesmata in BMV-infected barley leaves; immunoelectron microscopy localized BMV MP to the neck region and along the length of plasmodesmata but did not indicate its accumulation in central cavities of plasmodesmata (Fujita *et al*., 1998). AMV MP also accumulates in cell-wall–associated puncta believed to represent plasmodesmata when expressed as a GFP fusion protein in epidermal cells of tobacco leaves or in onion bulb scales, and is functional in intercellular trafficking in both systems (Huang and Zhang, 1999; Sánchez-Navarro and Bol, 2001). AMV MP localization to plasmodesmata is clearly demonstrated by immunoelectron microscopy in mesophyll cells of virus-infected young leaves of *N. benthamiana*; this localization is exclusive to plasmodesmata at the infection front, many of which appear as simple, unbranched structures (van der Wel *et al*., 1998). Interestingly, these plasmodesmata in AMV-infected tissues are structurally modified so that their desmotubule is removed and the diameter increased as compared to noninfected cells or cells further within the infection site. Such modifications are consistent with a tubule-guided cell-to-cell transport mechanism of AMV, but no tubules were observed in this study (van der Wel *et al*., 1998). Studies with unfused AMV MP, on the other hand, detected tubular structures in plant protoplasts (Kasteel *et al*., 1997; Zheng *et al*., 1997).

The 17 kDa MP of PLRV, a phloem-limited luteovirus (reviewed by Mayo and Ziegler-Graff, 1996; Smith and Barker, 1999) and not a member of the 30 K superfamily of viruses, targets secondary plasmodesmata when expressed either by itself or as a GFP fusion protein in transgenic tobacco plants: PLRV MP and PLRV MP-GFP are located in the central cavities of branched plasmodesmata in vascular and nonvascular tissue of source leaves, but they are absent from primary plasmodesmata in sink leaves. There only trichomes contained PLRV MP-GFP, most likely because they had already developed secondary plasmodesmata (Hofius *et al*., 2001). Thus, the expression patterns of PLRV MP-GFP and TMV MP-GFP in transgenic plants are strikingly similar, even though PLRV MP, belonging to a phloem-limited virus, is ectopically expressed in nonvascular tissues of transgenic plants. On the functional level, both PLRV MP and PLRV MP-GFP increase permeability of mesophyll plasmodesmata, as evidenced by microinjection of fluorescently labeled dextrans (Hofius *et al*., 2001), and influence the carbohydrate content of source leaves in a dose-dependent manner, *i.e*., low amounts of PLRV MP lead to a decrease in carbohydrate levels of source leaves, whereas high expression levels of this MP result in accumulation of carbohydrates, suggesting a complex connection between the MP gating ability, levels of MP expression, and its metabolic impact (Hofius *et al*., 2001).

For several other viral groups, MP-GFP fusion proteins localize to cell-wall–associated puncta. For example, this pattern is observed for GRV MP and the putative 50 kDa MP encoded by ORF2 of ACLSV when transiently expressed as GFP fusion proteins in *Nicotiana occidentalis* and *N. benthamiana* plants (Ryabov *et al*., 1998; Satoh *et al*., 2000; Yoshikawa *et al*., 1999). The cell-wall–associated puncta formed by GRV MP-GFP expressed from PVX or TMV vectors in *N. benthamiana* plants are found in close proximity to callose deposits, suggesting targeting of GRV MP-GFP to plasmodesmata (Ryabov *et al*., 1998). Transiently expressed ACLSV MP-GFP that localizes to cortical filaments (see also Section 3.4.2) and cell-wall–associated puncta in epidermal leaf cells of *N. occidentalis* is competent for gating plasmodesmata and cell-to-cell movement, and complements local movement of an MP-deficient strain of ACLSV (Satoh *et al*., 2000), indicating that the GFP fusion protein is functional. As observed with other MPs, gating plasmodesmata and cell-to-cell trafficking by ACLSV MP are more pronounced in mature leaves than in young leaves (Satoh *et al*., 2000). Immunoelectron microscopy of ACLSV-infected *Chenopodium quinoa* leaves confirms localization of ACLSV MP to plasmodesmata along their entire length and in the cytoplasm in close proximity to the neck regions, but does not indicate MP accumulation in the central cavities of secondary plasmodesmata (Yoshikawa *et al*., 1999). In the same plant host, CarMV p7 also associates with the cell wall, in particular at later stages of infection as judged by biochemical fractionation of the infected *C. quinoa* leaves (Garcia-Castillo *et al*., 2003); however, whether or not the cell-wall–associated CarMV p7 resides in plasmodesmata remains unknown.

Among the triple gene block viruses, TGBp1 of *Peanut clump pecluvirus* (PCV) copurifies with the cell-wall fraction derived from the inoculated leaves and from uninoculated, systemically infected apical leaves of *N. benthamiana* plants. In addition, in the inoculated leaves, PCV TGBp1 is also detected in the P30 fraction, which contains, among other organelles, ER and Golgi bodies (Erhardt *et al*., 1999). When PCV TGBp1 localization was more precisely resolved by immunogold electron microscopy, the protein was found in close association with neck or collar regions of plasmodesmata in the infected leaves (Erhardt *et al*., 1999). In contrast, TGBp1 expressed as a transgene in *N. benthamiana* was not associated with plasmodesmata, but plasmodesmal localization could be restored by infection with a TGBp1-defective PCV, indicating that additional viral products may be required for plasmodesmal targeting of TGBp1 (Erhardt *et al*., 1999). Similar results were obtained for BNYVV TGBp1, which when expressed as a GFP fusion from a BNYVV replicon localizes to punctate, occasionally paired bodies apposed to the cell wall at the infection front (Erhardt *et al*., 2000). GFP-BNYVV TGBp1 colocalized to callose, indicating that the TGBp1 was targeted to plasmodesmata-rich regions. In the absence of either BNYVV TGBp2 or TGBp3, GFP-BNYVV TGBp1 was cytoplasmically localized, suggesting that TGBp2 and TGBp3 are required for plasmodesmal localization of TGBp1 (Erhardt *et al*., 2000) (see also Section 3.4.3). Interestingly, PVX CP localizes to plasmodesmata (Oparka *et al*., 1996; Rouleau *et al*., 1995) and can fulfill

some of the movement functions because cell-to-cell movement of a CP-less PVX mutant is rescued by transient coexpression with TMV MP (Fedorkin *et al*., 2001). However, PVX CP does not gate plasmodesmata (Oparka *et al*., 1996); instead, PVX TGBp2, which enables cell-to-cell movement of free GFP when transiently coexpressed in epidermal cells of *N. benthamiana,* fulfills this function (Tamai and Meshi, 2001).

3.4.2. Association with Microtubules

Although interaction of plant viral MPs with cytoskeletal components was initially observed for the Hsp70-related MP of *Beet yellows closterovirus* (BYV) (Karasev *et al*., 1992), the topic gained widespread recognition only after TMV MP was reported to interact with the cytoskeleton (Heinlein *et al*., 1995; McLean *et al*., 1995). Interaction of actin and tubulin with TMV MP was detected biochemically *in vitro* (McLean *et al*., 1995) and by immunofluorescence in TMV-infected as well as TMV MP-transfected protoplasts derived from a *N. tabacum* cell suspension culture (McLean *et al*., 1995). In addition, in *N. tabacum* and *N. benthamiana*, the spread of recombinant TMV or Ob tobamoviruses expressing their MPs as GFP fusion proteins is characterized by a highly fluorescent ring of cells with MP-GFP accumulated at numerous cortical filaments (Heinlein *et al*., 1995; Padgett *et al*., 1996). A similar subcellular localization pattern was observed (Gillespie *et al*., 2002) with TMV expressing MP fused to a red fluorescent protein reporter DsRed (Matz *et al*., 1999). Using immunofluorescence, MP-GFP–decorated filaments were shown to coalign with microtubules, but not with actin filaments, in BY2 protoplasts infected with the TMV or Ob construct. Consistently, treatment of the infected protoplasts with oryzalin, a microtubule disassembling drug, destroyed the MP-GFP–labeled filaments, whereas treatment with cytochalasin D, an F-actin–disrupting drug, did not (Heinlein *et al*., 1995). Thus, association between TMV MP and microtubules is detected using MP fusions with GFP and DsRed (Gillespie *et al*., 2002; Heinlein *et al*., 1995; Padgett *et al*., 1996) as well as with the unfused MP (McLean *et al*., 1995), while association between TMV MP and actin was observed only with the unfused MP (McLean *et al*., 1995).

Since TMV MP directly interacts with tubulin or actin, both cytoskeletal proteins can be regarded as host factors of TMV MP (see also Section 3.5). But what is the molecular basis of this interaction? Studies on the subcellular localization of a series of TMV MP mutants fused to GFP and expressed in the context of TMV vectors implicate core regions of the MP molecule, around amino acid residues 49 to 51 and 88 to 101, in binding to microtubules (Kahn *et al*., 1998) and the C-terminal protein region between amino acid positions 203 and 268 in modulating this association (Boyko *et al*., 2000c). Furthermore, sequence comparison between tobamoviral MPs and tubulins indicates a conserved motif between amino acids 151 and 158 of TMV MP that resembles a tubulin motif mediating lateral contacts between microtubule protofilaments (Boyko *et al*., 2000a). Therefore, tobamoviral MPs may coassemble with tubulins and become an integral part of the microtubular lattice. Point mutations within this motif confer temperature sensitivity to microtubular association, providing experimental evidence for involvement of this MP region in interaction with microtubules (Boyko *et al*., 2000a). The complex between TMV MP-GFP and microtubules is highly stable and cannot be dissociated under high salt or cold conditions, both of which are standard treatments for the depolymerization of microtubules (Boyko *et al*., 2000a). Thus, TMV MP-GFP may display some functional similarity to microtubule-associated proteins (MAPs) involved in stabilizing the microtubular network in animal cells (reviewed by Garcia and Cleveland, 2001). TMV MP-GFP also accumulates into similarly stable complexes on microtubules of animal tissue culture cells, which indicates that no plant-specific factors are required for this association (Boyko *et al*., 2000a).

Accumulation of TMV MP-GFP, TMV MP-DsRed, or Ob MP-GFP at microtubules is under spatiotemporal control during infection, being most pronounced in cells with high TMV MP-GFP expression levels located several cell layers behind the outer edge of the expanding infection site. In the center of the infection site, the MP-associated fluorescence is significantly lower and is limited to cell-wall–associated puncta, even though these same cells have contained high levels of fluorescently tagged MP associated with microtubules earlier in the infection process, when they were at the expanding edge of infection (Boyko *et al*., 2000a; Gillespie *et al*., 2002; Padgett *et al*., 1996). This loss of MP association with microtubules is linked to degradation of MP as shown for Ob MP-GFP in the infected BY2 protoplasts (Padgett *et al*., 1996). Such degradation of MP may proceed via the proteasome pathway, because treatment with clasto-lactacystin-b-lactone, a proteasome inhibitor, resulted in increased stability of TMV MP in infected BY2 protoplasts (Reichel and Beachy, 2000). When TMV MP-GFP is expressed in epidermal cells of *N. benthamiana* by biolistic transformation in the absence of viral infection, its association with microtubules is also transient and is followed, concurrently with its cell-to-cell movement, by relocation to the cell-wall–associated puncta (Kotlizky *et al*., 2001). This transient interaction of TMV MP-GFP with microtubules is stabilized in a nonfunctional TMV MP deletion mutant lacking amino acids 3 to 5, which remains associated with microtubules during the observation time and does not form cell-wall–associated puncta (Kotlizky *et al*., 2001).

The functional significance of the transient association between MP and microtubules is not fully understood. Since microtubules are involved in cellular transport processes, for example, RNA transport (reviewed by Bloom and Beach, 1999), a function in MP-mediated transport of viral genomes presents an attractive hypothesis. A number of studies correlate TMV MP-GFP binding to microtubules with efficiency of TMV spread in *N. benthamiana*. For example, by elevating temperature, spatiotemporal control of MP association with microtubules was changed so that MP localizes to microtubules throughout the infection ring of a MP-GFP–tagged TMV construct, except in the first and second cell at the very leading edge of infection, where

TMV MP-GFP accumulates in the cell-wall–associated puncta and ER-associated cortical bodies (Boyko *et al*., 2000b). This shift in subcellular distribution towards microtubular association is accompanied by accelerated viral spread. It was proposed that at higher temperature MP is more efficiently targeted from the ER-associated sites to the microtubules, which—assuming that microtubules indeed serve as a transport route to plasmodesmata, would promote accumulation of MP at plasmodesmata and therefore lead to the increased viral spread (Boyko *et al*., 2000b).

Several nonfunctional mutants of TMV MP-GFP, such as deletion mutants lacking 66 or 81 C-terminal amino acids, a mutant lacking amino acids 49 to 51, or a point mutant Pro81Ser, as well as temperature-sensitive mutants at the restrictive temperature, are characterized by their lack of localization to microtubules when expressed in the context of the virus, suggesting that TMV MP association with microtubules plays a role in cell-to-cell transport of TMV RNA (Boyko *et al*., 2000a, 2002; Kahn *et al*., 1998). When expressed in the context of the virus, these nonfunctional MP mutants can be studied only in plants transgenic for the wild-type TMV MP, which complements viral infection; thus, the subcellular localization patterns of the mutant TMV MP-GFP may be modulated by the unlabeled wild-type MP, significantly complicating interpretation of the results of these experiments (Boyko *et al*., 2002; Kahn *et al*., 1998). The time course of intracellular localization of a GFP-tagged TMV MP mutant lacking amino acid residues 49 to 51 was also analyzed in virus-infected single BY2 protoplasts. The mutant MP targeted to and remained at the ER-associated bodies near the cell nucleus, whereas the wild-type TMV MP-GFP moved from the ER-associated bodies to microtubules and then redistributed to the cell periphery. These observations support a role of microtubules in intracellular distribution of TMV MP (Mas and Beachy, 2000).

Microtubules and microfilaments have also been implicated in subcellular distribution of TMV RNA. TMV RNA colocalizes with microtubules in TMV-infected protoplasts, and disrupting microtubules by treatment with oryzalin results in accumulation of TMV RNA in the ER-associated bodies close to the cell nucleus and prevents distribution of the viral RNA throughout the host cell cytoplasm and to the cell periphery (Mas and Beachy, 1999). Although colocalization of TMV RNA with actin filaments has not been documented due to technical difficulties, treatment of infected BY2 protoplasts with cytochalasin D, a drug that depolymerizes F-actin, alters the pattern of TMV RNA localization, suggesting involvement of actin filaments in intracellular distribution of TMV RNA (Mas and Beachy, 1999). Obviously, because protoplasts constitute a single cell system, the functional implications of these observations for the cell-to-cell spread of the virus are still obscure.

Even though the biological significance of MP association with microtubules is frequently discussed in relation to targeting of viral genomes to the cell periphery, several alternate functions, *e.g*., involvement in MP storage, turnover, and degradation, or even a mechanism to interfere with plant defense responses such as RNA silencing, have been suggested (Boyko *et al*., 2002; Gillespie *et al*., 2002; Padgett *et al*., 1996). In fact, increasing evidence supports these alternative interpretations and argues against the involvement of TMV MP–microtubule interaction in the movement process *per se*. For example, TMV MP association with microtubules is generally not apparent in cells at the front of infection, where active viral RNA cell-to-cell transport is required for progression of viral spread (Boyko *et al*., 2000a; Oparka *et al*., 1997; Padgett *et al*., 1996). Moreover, disruption of microtubules in *N. benthamiana* either by drug treatment or by silencing of α-tubulin does not interfere with the spread of fluorescently tagged TMV vectors, suggesting that intact microtubules are not required for viral transport (Gillespie *et al*., 2002). Also, a Leu72Val mutant of TMV MP (MP^{R3}), which is generated by DNA shuffling and selected based on its enhanced ability to mediated TMV spread (Toth *et al*., 2002), failed to accumulate on microtubules of epidermal leaf cells when tagged with GFP or DsRed and expressed either from the TMV vector (Gillespie *et al*., 2002) or alone (Kragler *et al*., 2003). Because the MP^{R3} mutant gates plasmodesmata, supports TMV RNA spread, and itself traffics between cells, MP accumulation at microtubules most likely is not an essential prerequisite for movement (Gillespie *et al*., 2002; Kragler *et al*., 2003). The TMV MP interaction with microtubules plays a role in a process other than movement, which is suggested by transient coexpression of GFP-tagged TMV MP with its interacting host factor MPB2C, a novel microtubule-associated protein (see Section 3.5). Upon coexpression, subcellular distribution of TMV MP-GFP shifts from the cell wall-associated puncta toward accumulation at microtubules; this shift correlates with a reduction rather than an increase in cell-to-cell trafficking of TMV MP-GFP (Kragler *et al*., 2003). This inhibitory effect of MPB2C on TMV MP transport is not due to nonspecific effects on plasmodesmal transport, because MPB2C does not interfere with cell-to-cell movement of the GFP-tagged MP^{R3} mutant or of CMV MP-GFP (Kragler *et al*., 2003). Together with the observations that neither accumulation of TMV MP at microtubules nor intact microtubules are required for viral spread (Gillespie *et al*., 2002), the decrease in TMV MP cell-to-cell movement upon its enhanced accumulation at microtubules (Kragler *et al*., 2003) further supports the view that microtubular accumulation of TMV MP does not play a direct role in the movement process. Potential functions of the MPB2C-TMV MP interaction are discussed in Section 3.5.

Besides TMV MP and Ob MP, MP of another tobamovirus, *Tomato mosaic virus* (ToMV), localizes to intracellular filaments, presumably microtubules (Kawakami *et al*., 1999, 2003). Detailed functional studies addressing the biological relevance of this microtubular association of ToMV MP are not yet available. Except tobamoviruses, MPs of few other plant viruses have been linked to the cytoskeleton. ACLSV MP was reported to localize to cortical filaments resembling microtubules when expressed as a GFP fusion in epidermal cells of *N. occidentalis*

leaves (Satoh *et al*., 2000). However, no definitive evidence for colocalization of ACLSV MP-GFP with microtubules was obtained (Satoh *et al*., 2000). TSWV MP was shown to interact with plant homologs of kinesin and myosin (von Bargen *et al*., 2001), and the Hsp70-related MP of BYV binds microtubules *in vitro* (Karasev *et al*., 1992). The biological significance of these interactions for viral cell-to-cell movement remains to be addressed.

3.4.3. Association with the ER

Early biochemical fractionation experiments of TMV-infected tissues detected TMV MP, in addition to the cell wall and the soluble fraction, also in the endomembrane fraction (Deom *et al*., 1990), indicating membrane-associated subcellular localization sites for MP that are distinct from plasmodesmata. Indeed, in plant tissues infected with TMV expressing TMV MP-GFP, cortical bodies sometimes referred to as inclusion bodies are the dominant subcellular localization pattern in cells close to the expanding infection front (Boyko *et al*., 2000a; Heinlein *et al*., 1998; Padgett *et al*., 1996; Reichel and Beachy, 1998). To elucidate the nature of these cortical structures, infection sites of the TMV MP-GFP–tagged virus were studied in transgenic *N. benthamiana* plants expressing an ER-targeted variant of GFP (erGFP) (Reichel and Beachy, 1998); selective excitation allows us to distinguish partially between the fluorescent signals of TMV MP-GFP and erGFP. Small cortical TMV MP-GFP–tagged bodies form early in the infection process in conjunction with the ER network; later, large cortical TMV MP-GFP aggregates containing erGFP appear. The appearance of the large cortical aggregates, which most likely represent collapsed ER structures, is followed by TMV MP-GFP association with microtubules concurrently with recovery of the cortical ER network. Thus, the smaller cortical bodies that contain TMV MP-GFP are associated with the ER network, while the larger aggregates likely associate with disrupted ER; and TMV MP-GFP association with the ER precedes its association with microtubules (Reichel and Beachy, 1998). Similarly, DsRed-tagged TMV MP produced from a recombinant virus first accumulates in bodies associated with ER vertices at the front of an infection site and then is transferred to underlying microtubules (Gillespie *et al*., 2002). Biochemical fractionation of leaf tissues derived from TMV-infected *N. benthamiana* plants transgenic for erGFP confirmed the ER localization of TMV MP and indicated that the protein is partially exposed at the cytosolic face of the ER (Reichel and Beachy, 1998). As described in Section 3.1, CD spectroscopy and tryptic digest analyses indicate that TMV MP contains two α-helical transmembrane domains and a flexible C-terminal tail (Brill *et al*., 2000). These transmembrane domains may span the ER membrane, whereas the flexible C-terminal tail is exposed to the cytosol and accessible to tryptic digestion (Brill *et al*., 2000).

Association with cortical ER is also observed when TMV MP-GFP is transiently expressed in epidermal cells of *N. tabacum* leaves, but the localization pattern appears more network-like than punctate (Crawford and Zambryski, 2000). In addition, fine cortical bodies, sometimes connected by a cortical network, as well as irregular-shaped cortical structures, are observed in BY-2 protoplasts infected with TMV vector expressing TMV MP-GFP (Heinlein *et al*., 1998). These cortical structures that accumulate TMV MP-GFP are located in close proximity to the cortical ER and microtubules, as determined by labeling with the ER-specific dye $DiOC_6$ and by coimmunolocalization with antibodies against the ER-luminal protein BiP or tubulin. Because the irregular-shaped cortical structures also contained viral replicase, they may correspond to sites of viral replication (Heinlein *et al*., 1998). Supporting this notion, the viral RNA was found to partially colocalize to the ER-derived structures in the infected BY-2 protoplasts (Mas and Beachy, 1999). The fine cortical structures observed in infected protoplasts may correspond to cell-wall adhesion sites, the areas where the ER and the plasma membrane attach to the cell wall (Heinlein *et al*., 1998). It remains to be determined which, if any, of these structures correspond to the cortical bodies observed in the infected plants (Boyko *et al*., 2000a; Gillespie *et al*., 2002; Heinlein *et al*., 1998; Padgett *et al*., 1996; Reichel and Beachy, 1998).

Mutational analysis of GFP-tagged TMV MP expressed *in planta* in the context of the virus implicates regions around amino acids 9 to 11 and 88 to 101 (Kahn *et al*., 1998) and amino acid residues 214 to 233 (Boyko *et al*., 2000c) in accumulation of TMV MP-GFP at the cortical ER bodies. This accumulation is stabilized by several mutations within TMV MP, such as a Leu72Val point mutation and N-terminal deletions of 5 or 30 amino acids. These TMV MP tagged with GFP exhibit association with the ER cortical bodies throughout the infection site, preventing disappearance of MP in the center of the expanding infection area, thereby changing the shape of the infection site from a ring to a disc (Boyko *et al*., 2000c; Gillespie *et al*., 2002). Conversely, the C-terminally truncated TMV MP mutant lacking amino acids 214 to 268 fails to accumulate at cortical bodies. Because this mutant remains functional in cell-to-cell transport (Berna *et al*., 1991; Boyko *et al*., 2000c), detectable accumulation of TMV MP in the ER bodies is not essential for viral spread (Boyko *et al*., 2000c).

Besides TMV MP, the MPs of the closely related tobamoviruses Ob and ToMV also accumulate in cortical bodies, which, by analogy to TMV MP, most likely represent the ER-associated sites (Kawakami *et al*., 1999; Padgett *et al*., 1996). Also, AMV MP, tagged with GFP and expressed in epidermal cells of onion scales, associates with the ER, colocalizing with ER-specific dyes (Huang and Zhang, 1999).

Association of viral MPs with the ER is not limited to the members of the 30 K superfamily of viruses. For example, TGBp2 and TGBp3, two of the three MPs of the TGB group of viruses, are associated with ER-derived structures (reviewed by Morozov and Solovyev, 2003). Both TGBp2 and TGBp3 are characterized by hydrophobic sequences that may represent transmembrane segments involved in interaction with the ER (Solovyev *et al*., 1996a). Microscopy studies localize transiently expressed and GFP-tagged PSLV TGBp2 to the cortical ER

network and to small motile vesicles also associated with the cortical ER (Solovyev *et al*., 2000). Similarly, GFP-tagged TGBp2 of PVX is arrayed in a polygonal pattern that resembles the ER network both when expressed in transgenic plants or upon transient expression. Treatment with an ER-disrupting drug severely affects the localization pattern of GFP-TGBp2, supporting potential association of this protein with the ER (Mitra *et al*., 2003).

Transiently expressed PVX TGBp2 tagged with GFP moves between cells in *N. benthamiana* leaves and in *N. tabacum* transgenic for TGBp1, but not in the wild-type *N. tabacum*, indicating a host-dependent requirement for the presence of TGBp1 to support the TGBp2 cell-to-cell transport (Krishnamurthy *et al*., 2002). Furthermore, cell-to-cell movement of GFP-tagged TGBp2 in TGBp1 transgenic tobacco depends on the developmental stage of the leaf and is predominantly detected in source leaves but not in sink leaves (Mitra *et al*., 2003). Mutations within the predicted transmembrane domains of PVX TGBp2 (see Section 3.1) abolished ER localization and cell-to-cell movement of transiently expressed GFP-tagged TGBp2, as well as the spread of PVX (Mitra *et al*., 2003). Interestingly, a mutation outside the transmembrane domains, which does not effect association of TGBp2 with the ER, selectively inhibits movement of GFP-tagged TGBp2 in TGBp1-expressing *N. tabacum* but not in *N. benthamiana,* whereas PVX movement was abolished in both plant species. Thus, ER association of TGBp2 may be required but not sufficient for virus movement (Mitra *et al*., 2003). PVX TGBp3 is also associated with a cortical network resembling the ER (Krishnamurthy *et al*., 2003), whereas PSLV TGBp3 accumulates in membrane bodies at the cell periphery that are connected to the cortical ER and in close proximity to plasmodesmata-enriched pitfields (Gorshkova *et al*., 2003; Solovyev *et al*., 2000; Zamyatnin Jr. *et al*., 2002). Immunodetection experiments support the ER association of PMTV TGBp2, TGBp3, and PSLV TGBp3 (Cowan *et al*., 2002; Gorshkova *et al*., 2003). TGBp3 is thought to redirect TGBp2 to peripheral bodies (Solovyev *et al*., 2000; Zamyatnin Jr. *et al*., 2002), and a combination of TGBp2 and TGBp3 likely recruits TGBp1 to these bodies (Solovyev *et al*., 2000; Zamyatnin Jr. *et al*., 2002). Since TGBp1 proteins form M-complexes with viral RNA (see Section 3.2), the role of TGB2 and TGB3 may be primarily to deliver these M complexes to plasmodesmata for subsequent cell-to-cell movement (Morozov and Solovyev, 2003; see also Section 3.1).

Finally, MPs of some geminiviruses, such as the BC1 proteins of *Abutilon mosaic virus* (AbMV) and SLCV, as well as the V1 protein of TYLCV, may also associate with ER-derived cellular structures (Aberle *et al*., 2002; Rojas *et al*., 2001; Ward *et al*., 1997). Also, CarMV p9 contains two potential hydrophobic membrane-spanning domains that insert the protein into ER-derived microsomes in an *in vitro* transcription/translation assay (Vilar *et al*., 2002). The potential significance of the ER association for the biological activity of these MPs, which act in concert with additional viral factors that localize to different subcellular compartments, *i.e*., the cell nucleus for the geminiviral BV1 proteins (reviewed by Hanley-Bowdoin *et al*., 2000; Lazarowitz and Beachy, 1999; Mansoor *et al*., 2003) or plasma membrane for the CarMV p7 protein (Vilar *et al*., 2002), will be further discussed in Section 3.4.4.

3.4.4. Association with the Nucleus, Plasma Membrane and Other Cellular Structures

Nuclear localization has been observed for MPs of geminiviruses and tombusviruses. Unlike many other plant viruses, geminiviruses contain DNA genomes that replicate in the host cell nucleus and thus face the task of moving their genomes out of the nucleus to the cell periphery and through plasmodesmata. Therefore, two types of movement functions, one that facilitates nuclear export and one that mediates cell-to-cell transport, are required. Geminiviruses belonging to the subgroup of begomoviruses are considered members of the 30 K superfamily (Melcher, 2000) and contain either bipartite or monopartite ssDNA genomes. Bipartite geminiviruses encode two MPs: BV1 (formerly, BR1), which is a nucleic-acid–binding protein (see Section 3.2), and BC1 (formerly, BL1; reviewed by Gafni and Epel, 2002; Hanley-Bowdoin *et al*., 2000; Lazarowitz and Beachy, 1999; Mansoor *et al*., 2003). BV1 proteins are implicated in the nuclear transport activity by their subcellular localization. For example, SLCV BV1 localizes to the cell nucleus in the infected plants (Pascal *et al*., 1994) while the BV1 proteins of BDMV (Rojas *et al*., 2001) and AbMV (Zhang *et al*., 2001) exhibit nuclear accumulation upon their transient expression as GFP fusion proteins. Furthermore, BV1 proteins also possess a nuclear export activity, representing a true nucleocytoplasmic shuttle protein (Lazarowitz and Beachy, 1999; Sanderfoot *et al*., 1996; Sanderfoot and Lazarowitz, 1995, 1996; Ward and Lazarowitz, 1999). During geminiviral infection, BV1 is thought to bind viral genomes inside the host cell nucleus (see Section 3.2) and export them into the cytoplasm for cell-to-cell movement, which is mediated by BC1 (reviewed by Hanley-Bowdoin *et al*., 2000; Lazarowitz and Beachy, 1999; Mansoor *et al*., 2003). Interestingly, the nuclear export activity of BV1 manifests only in the presence of BC1 (Lazarowitz and Beachy, 1999; Sanderfoot *et al*., 1996; Sanderfoot and Lazarowitz, 1995, 1996; Ward and Lazarowitz, 1999), thereby physically coupling nuclear export of the viral genomes to their intercellular transport.

The BC1 protein of AbMV, a phloem-limited geminivirus, whose subcellular localization pattern was analyzed by ectopic expression of AbMV BC1-GFP in epidermal cells of *N. benthamiana,* accumulates either in cortical flecks at the cell periphery or around the nucleus (Zhang *et al*., 2001, 2002). When expressed in yeast, AbMV BC1 localized to the plasma membrane and ER-derived vesicles (Aberle *et al*., 2002), suggesting that BC1 exploits the cellular membrane flow from the ER to plasma membrane for targeting to the cell periphery (Zhang *et al*., 2002). That the BC1 protein retains functionality even in nonphloem tissue is indicated by its ability to mobilize GFP-AbMV BV1 out of the cell nucleus to the plasma membrane and into neighboring cells (Zhang *et al*., 2001). Also, SLCV BC1 localizes to the plasma membrane in Sf9 insect cells and tobacco

protoplasts (Pascal *et al*., 1994; Sanderfoot and Lazarowitz, 1995). Conversely, in virus-infected pumpkin sink leaves, the SLCV BC1 protein predominantly associates with unique ER-derived tubules that penetrate the cell wall and are found only in undifferentiated vascular tissues (procambium), and only in lesser amounts with the plasma membrane. The BC1-containing tubules have been proposed to serve as a conduit for intercellular transport of the viral M complexes composed of the genomic ssDNA and BV1 (Ward *et al*., 1997). Similarly, tubule-like structures containing viral particles were reported for several other geminiviruses, such as a field isolate of *Euphorbia mosaic geminivirus* (EuMV; Kim and Lee, 1992) and *Indian cassava mosaic geminivirus* (ICMV; Roberts, 1989).

MPs of monopartite geminiviruses share no significant sequence homology with BV1 or BC1 (Rojas *et al*., 2001); instead, as many as four proteins—V1, V2 (coat protein), C2, and C4—may be involved in viral movement (Jupin *et al*., 1994; Wartig *et al*., 1997). CP of TYLCV, a ssDNA-binding protein (Palanichelvam *et al*., 1998), localizes to the cell nucleus when transiently expressed in petunia protoplasts as fusion with GUS reporter or when chemically labeled with a fluorescent dye and microinjected into drosophila embryos (Kunik *et al*., 1998). These observations were confirmed by microinjection of chemically labeled fluorescent TYLCV CP into *N. benthamiana* mesophyll cells or by transient expression of GFP-tagged TYLCV CP in *N. tabacum* suspension culture protoplasts, in epidermal cells of bean hypocotyls, and *N. benthamiana* leaves (Rojas *et al*., 2001). Besides its nuclear import, TYLCV CP also functions in nuclear export (Rhee *et al*., 2000a). These activities suggest that TYLCV CP is a monopartite geminiviral functional homolog of the bipartite geminiviral BV1; indeed, microinjected TYLCV CP facilitates transport of fluorescently labeled DNA into and out of the plant cell nucleus (Rojas *et al*., 2001). Similarly, CP of another monopartite geminivirus, MSV, is a karyophilic protein (Liu *et al*., 1999a). The intricate subcellular localization pattern of the CP of *African cassava mosaic virus* (ACMV), a bipartite geminivirus, implicates the protein both in nuclear shuttling and transport of the viral genome towards the cell periphery (Unseld *et al*., 2001). Whereas the full length ACMV CP tagged with GFP is localized to the cell nucleus upon transient expression in epidermal cells of *N. benthamiana*, various deletion mutants of CP are also targeted to the cell periphery and accumulate there in puncta and bodies (Unseld *et al*., 2001), a pattern reminiscent of TMV MP-GFP association with the cell wall (see Section 3.4.1).

The TYLCV V1 and C4 proteins are potential functional homologs of BC1. In epidermal cells of *N. benthamiana* leaves and bean hypocotyls, TYLCV V1-GFP localizes around the cell nucleus, in the cytoplasm, and at the cell periphery; costaining with the ER-specific dye rhodamine B hexyl ester suggests association of the protein with the ER (Rojas *et al*., 2001). In contrast, TYLCV C4-GFP is consistently observed at the cell periphery, suggesting its association with the plasma membrane (Rojas *et al*., 2001). Both V1-GFP and C4-GFP display a limited capacity to move from cell to cell, consistent with their ability to gate mesophyll plasmodesmata of *N. benthamiana* leaves (Rojas *et al*., 2001).

Nuclear localization has also been observed for MP of TCV, which does not belong to the 30 K superfamily. TCV encodes two small proteins, p8 and p9, associated with cell-to-cell movement (Hacker *et al*., 1992). GFP-tagged TCV p8 is confined to the cell nucleus following its transient expression in protoplasts and in leaves of arabidopsis and *N. benthamiana*, as well as upon stable expression in transgenic arabidopsis, or when expressed from movement-incompetent TCV replicons. Nuclear localization was also observed for TCV p8 tagged with the antigenic peptide FLAG. Nuclear import of TCV p8 depends on two nuclear localization signals (NLS) and is not required for cell-to-cell movement (Cohen *et al*., 2000). The ability of TCV p8 to accumulate in the plant cell nucleus is unique among carmoviruses and also surprising given that TCV is an RNA virus that is not expected to have a nuclear stage during its life cycle; it cannot be excluded that tagging of TCV p8 with GFP or FLAG disrupts the native conformation of this viral protein, fortuitously exposing NLS signals normally hidden in the native protein. Alternatively, nuclear localization of the TCV p8 may relate to a novel, yet unexplored, function of this protein (Cohen *et al*., 2000). When subjected to a similar analysis, the second MP of TCV, p9, tagged with GFP distributes evenly throughout the cytoplasm, offering no insights into its function (Cohen *et al*., 2000). Unlike TCV p8, its CarMV counterpart, p7, is mainly cytosolic as determined by biochemical fractionation of infected *C. quinoa* leaves, but, at later stages of the infection process, increasing amounts of p7 are detected in the cell wall fraction (Garcia-Castillo *et al*., 2003). Due to its twin hydrophobic domains, CarMV p9 associates with the ER-derived microsomes *in vitro* (Vilar *et al*., 2002; see Section 3.4.3). The two CarMV MPs may act in concert, with p7 forming an M complex with the viral RNA and p9, through its interaction with p7, anchoring this complex to the cell membrane to facilitate the cell-to-cell movement (Vilar *et al*., 2002).

BMV MP is found in cytoplasmic inclusions in infected barley leaves (Fujita *et al*., 1998). Since the replicase-related BMV 1a and 2a proteins also localize to these inclusions, they may represent the sites of the viral RNA synthesis. Potentially, BMV MP detected in these electron-dense inclusions may be a *de novo* synthesized protein (Dohi *et al*., 2001). PVX TGBp1 tagged with GFP at its C-terminus, TGBp1-GFP, was also detected in cytoplasmic inclusions when transiently expressed in sink or source leaves of tobacco plants, whereas the N-terminal fusion, GFP-TGBp1, localized in the cytoplasm but did not form inclusions (Yang *et al*., 2000). In this study, only the cytoplasmically localized GFP-TGBp1 moved from cell to cell when transiently expressed in tobacco plants (Yang *et al*., 2000). In another study, however, PVX TGBp1-GFP, but not GFP-TGBp1, complemented movement of a TGBp1-deficient PVX in *N. benthamiana* and exhibited punctate localization patterns within or adjacent to the host cell wall (Morozov *et al*., 1999).

3.4.5. Subcellular Localization Patterns of MPs: A Doorway to Function?

Specific subcellular localization patterns generally can provide valuable information about protein function. But what are the insights into the functional mechanisms of plant viral cell-to-cell movement that can be gleaned from the wealth of studies of association between MPs and various subcellular structures described above, and how reliable is our interpretation of the results of these studies? Many viral MPs form M complexes with the transported viral genomes and translocate them through plasmodesmata. Thus, localization of MPs to plasmodesmata within the host cell walls would make biological sense and is implicitly expected. Indeed, MPs from many viral groups, such as tobamoviruses, bromoviruses, geminiviruses, trichoviruses, umbraviruses, luteoviruses and the viruses of the triple gene block group, target to the plant cell periphery. For some MPs, the specific sites of this targeting have been definitively identified as plasmodesmata by immunoelectron microscopy, while other MPs were tagged with GFP and localized to cell wall-associated puncta frequently assumed to indicate plasmodesmal localization. Because this punctate cell-wall–associated GFP fluorescence has been directly linked by electron microscopy to plasmodesmal localization of several viral MPs in transgenic plants (Hofius *et al*., 2001; Itaya *et al*., 1998), it most likely indeed indicates plasmodesmal targeting of MP in most, if not all, cases. Interestingly, the biological activity of plasmodesmata-associated MPs may be modulated in the course of the infection process. For example, TMV MP-GFP at the front of the expanding infection area gates plasmodesmata, whereas TMV MP-GFP in the center of the same area does not (Oparka *et al*., 1997). Potentially, MP within the plant cells located behind the infection front may have been inactivated by host responses based on posttranslational modification such as phosphorylation (see Section 3.6). Thus, to observe localization of MP to plasmodesmata *per se* is not sufficient for drawing conclusions regarding the activity of MP.

Not quite as frequently observed among viral MPs is their association with the ER, which has been reported for MPs of tobamoviruses, bromoviruses, geminiviruses, tombusviruses, and viruses of the triple gene block group. This ER localization pattern is consistent with structural predictions and experimental evidence, which point to an intrinsic capability of several MPs to integrate into or associate with the ER membrane (see Section 3.4.3). For some viruses, viral replication and translation is thought to occur in close association with the ER (Carette *et al*., 2000; Restrepo-Hartwig and Ahlquist, 1996; Schaad *et al*., 1997a), suggesting that their MPs may integrate into the ER membrane shortly after their production. On a functional level, MP association with the ER may be linked to transport of the M complex from the site of its assembly, *i.e*., "viral factories" where viral genomes are replicated and translated (Heinlein *et al*., 1998) toward plasmodesmata (Morozov and Solovyev, 2003; Vilar *et al*., 2002), through the ER-derived transplasmodesmal tubules into neighboring cells (Lazarowitz and Beachy, 1999), or both Also, MP may be involved in distribution or establishment of replication sites by anchoring replication complexes at the ER membrane (Heinlein, 2002b; Heinlein *et al*., 1998; Lazarowitz and Beachy, 1999).

MP localization to the cell nucleus is rare, and it has been reported only for MPs of geminiviruses, and one carmovirus, TCV. Nuclear localization of geminiviral MPs is linked to the nuclear replication stage in the life cycle of these viruses such that these karyophilic MPs have evolved to enter the host cell nucleus and shuttle viral DNA into the cytoplasm for subsequent intercellular transport (reviewed by Lazarowitz and Beachy, 1999). The biological significance of nuclear localization of the p8 MP of TCV remains unexplained.

Microtubular localization is firmly established only for MPs of tobamoviruses, exemplified by TMV, and may in fact constitute a specialization of these viruses. Whether or not the mere association of tobamoviral MPs with microtubules implicates these cytoskeletal elements in intracellular transport toward plasmodesmata is still unresolved. Potentially, microtubules may play more than one role in the TMV life cycle. At the expanding edge of infection, microtubules may indeed provide an intracellular transport route to the cell periphery, but the amounts of TMV MP actively engaged in this transport along microtubules may be below detection with the GFP-tagged protein. This hypothesis is apparently contradicted by experiments indicating that intact microtubules are not required for viral spread (Gillespie *et al*., 2002). However, these experiments were performed in transgenic plants expressing GFP-tagged tubulin, and disruption of microtubules was monitored by fluorescence microscopy; thus, it cannot be completely excluded that some microtubules composed predominantly of the wild-type, untagged tubulin may have escaped disruption and, while remaining undetected, were sufficient to maintain viral movement. In the host tissues with already established infection, MP binding to microtubules probably is not required for movement, as suggested by studies using MP mutants (Gillespie *et al*., 2002) and MP-interacting host factors (Kragler *et al*., 2003); instead, it may be involved in MP degradation (Gillespie *et al*., 2002; Mas and Beachy, 1999; Padgett *et al*., 1996; Reichel and Beachy, 1998). This movement-unrelated microtubular association of MP occurs at high levels and is easily detected. Thus, subcellular localization of MP to cytoskeletal networks may represent different, even mutually exclusive, functions of this protein.

Distribution of MPs between plant cells as a result of their cell-to-cell transport also represents a form of localization pattern, albeit intercellular and not subcellular. Direct studies of MP movement gained powerful impetus since the advance of autofluorescent proteins such as GFP (reviewed by Cubitt *et al*., 1995; Tsien and Miyawaki, 1998) or DsRed (Matz *et al*., 1999), as real-time reporters of protein localization in living cells that may provide an opportunity to catch a glimpse of MP actively engaged in transport. Thus, not surprisingly a large part of the above-described database on subcellular localization of MPs has been generated by analyzing protein fusions between MP and

autofluorescent reporters. But how likely is it that a GFP-based approach will faithfully reflect the actual cell-to-cell transport event? Apart from the general consideration that the 27 kDa GFP tag may interfere with or modulate the activity of the fusion protein, there are several more specific concerns that may interfere with achieving this goal. First, to detect GFP fluorescence with epifluorescence or confocal microscopy, a minimal number of molecules needs to accumulate at a certain cellular location to generate a signal above the detection limit. This requirement is not fully compatible with the natural characteristics of the MP cell-to-cell transport, which may involve only minute amounts of MP (Arce-Johnson *et al*., 1995). Indeed, when TMV MP-GFP is expressed in single cells, only a small fraction of the produced protein traffics into neighboring cells (Gillespie *et al*., 2002; Kotlizky *et al*., 2001; E. Waigmann *et al*., unpublished observations). Second, the known MP localization patterns detected using GFP fusions either are not dynamic at all or change very slowly, in the time-range of hours or even days (*e.g*., Kotlizky *et al*., 2001). It is difficult to reconcile these essentially static images with an active transport process; in comparison, RNA transport along cytoskeletal elements occurs in small particles that move rapidly through the cell (reviewed by Jansen *et al*., 2001). Potentially, some of the MP-GFP images reflect a steady state that has been reached as a consequence of host responses to the various activities of MP. For example, the exclusive accumulation of transgenically expressed MPs in the central cavities of secondary plasmodesmata (Hofius *et al*., 2001; Itaya *et al*., 1998; Roberts *et al*., 2001) or the very stable accumulation of large amounts of tobamoviral MPs at microtubules (Boyko *et al*., 2000a) may result from the host plant response rather than reflect participation of these subcellular structures in the movement process.

Finally, a significant proportion of GFP-based data has been assembled from experiments in epidermal leaf cells of the host plant *N. benthamiana*. This system is technically convenient because epidermal cells are ideal for fluorescence microscopy, as they are easily accessible and contain relatively low numbers of chloroplasts, and *N. benthamiana* is a highly promiscuous host that imposes fewer restrictions on viral spread compared to many other plant species (Waigmann *et al*., 2000). As a consequence, however, the resulting data are biased for this particular system, and caution should be exercised in generalizing conclusions.

3.5. Partners-in-Movement: Host Proteins that Interact with MP

During infection, pathogens often adapt the host cellular processes for their own need. Thus, MPs, the molecular pirates of plasmodesmata, most likely insinuate into the endogenous pathways for intercellular transport and utilize them to spread viral genomes throughout the host plant. While the biological activities of MPs have been extensively investigated and are relatively well-understood, the studies of their cellular partners are just beginning. Maintaining the reputation of TMV as the virus of "many firsts" (Creager *et al*., 1999), from the first virus to be discovered and visualized by electron microscopy to the first viral CP to be sequenced (reviewed by Creager, 2002; Scholthof *et al*., 1999), TMV MP is the first viral MP for which a cellular-interacting protein was isolated and identified. Using renatured gel blot overlay binding assays, TMV MP was shown to interact with a 38 kDa protein from cell walls of tobacco leaves; based on the amino acid sequence analysis, the purified TMV MP-interacting protein was identified as a mature, processed form of a cell-wall enzyme pectin methylesterase (PME) (Chen *et al*., 2000; Dorokhov *et al*., 1999). Binding between TMV MP and mature and unprocessed forms of PME was confirmed using the yeast two-hybrid protein–protein interaction system (Chen *et al*., 2000). Furthermore, a TMV MP mutant that does not interact with PME also fails to support the cell-to-cell transport of the virus (Chen *et al*., 2000). In addition to TMV MP, MP of another tobamovirus, TVCV, as well as CaMV MP also interact with PME (Chen *et al*., 2000), although the biological role of this interaction has not been not examined. In contrast, a maize homeodomain protein KNOTTED1 (KN1), which is known to traffic through plasmodesmata in tobacco leaves (Lucas *et al*., 1995), does not interact with PME in the two-hybrid system (Chen *et al*., 2000), suggesting that this protein moves between cells by a pathway that does not involve PME.

The mechanism by which PME participates in TMV cell-to-cell movement remains unknown. One possibility is that PME acts as a host cell receptor for TMV MP (Dorokhov *et al*., 1999). Indeed, immunoelectron microscopy studies demonstrated that, in tobacco, PME localizes throughout the cell wall, including plasmodesmata (Chen *et al*., 2000). Furthermore, in several plant species, such as flax, tomato, and apple, pectin is found in cell-wall microdomains surrounding plasmodesmata (Morvan *et al*., 1998; Orfila and Knox, 2000; Roy *et al*., 1997), and PME has been proposed to function within these plasmodesmata-rich areas (Roy *et al*., 1997). Thus, binding to PME may target and/or anchor TMV MP to the host cell wall and, if this event occurs in the vicinity of plasmodesmata, the movement process is initiated, whereas binding to PME in the cell wall areas that do not contain plasmodesmata results in abortive movement, with TMV MP either being degraded or redirected back into the cell cytoplasm (see also Section 3.7 and Figure 1a).

Also, PME may facilitate TMV MP transport to the host cell ER. Although TMV MP copurifies with the ER enzyme markers (Moore *et al*., 1992) and likely utilizes the ER for transport from the site of viral synthesis to plasmodesmata (Heinlein *et al*., 1998), which also contain the ER membranes (Ding *et al*., 1992b), it lacks an apparent ER signal sequence (Atkins *et al*., 1991; Deom *et al*., 1991) and does not associate with membranes when expressed *in vitro* (Heinlein *et al*., 1998). Potentially, TMV MP may be transported to the ER with the help of unprocessed PME, which contains the ER translocation signal (Gaffe *et al*., 1997). In this scenario, the secreted PME molecule may span the ER membrane so that it can interact with TMV MP and attach it to the cytoplasmic face of the ER; following transport to the cell wall, PME may be secreted while TMV MP is retained at the cell wall.

Finally, PME may play a more active role in viral movement. The enzymatic activity of PME modulates pH and ion balance and alters cell wall porosity (*e.g.*, Nairn *et al.*, 1998; Pressey, 1984), affecting many aspects of plant growth and development (reviewed by Micheli, 2001) and plant response to pathogen attack (Markovic and Jornvall, 1986). TMV MP binding may interfere with the PME activity, altering the cell wall ion balance and thereby inducing changes in plasmodesmal permeability and facilitating viral cell-to-cell movement (see also Section 3.7 and Figure 1a). Interestingly, PME is also involved in TMV systemic movement, participating in the viral exit from the phloem into non-vascular tissues of uninfected systemic organs (Chen and Citovsky, 2003) (see Section 4.2).

In addition to PME, TMV MP interacts with a tobacco microtubule-associated protein MPB2C (Kragler *et al.*, 2003). MPB2C was isolated in a membrane-based yeast interaction screen (SOS recruitment system; Aronheim *et al.*, 1997) with TMV MP as bait, and interaction between MPB2C and TMV MP was confirmed biochemically by renatured gel blot overlay assay. Binding between MPB2C and TMV MP is specific, because MP from an unrelated virus CMV does not interact with the MPB2C protein (Kragler *et al.*, 2003). MPB2C is a previously uncharacterized 36 kDa protein harboring a N-terminal hydrophobic region and a coiled coil domain with similarity to the myosin/kinesin superfamily (Kragler *et al.*, 2003). Homologs of MPB2C are found in all major monocotyledonous and dicotyledonous plant species, including arabidopsis. Immunofluorescence analysis of subcellular localization patterns of endogenous MPB2C as well as confocal microscopy analysis of MPB2C tagged with DsRed revealed punctate microtubular localization, suggesting that MPB2C recognizes specific sites on microtubules (Kragler *et al.*, 2003). Punctate microtubular localization has been previously observed, for example, with the CLIP proteins, a group of microtubule plus end-targeted proteins involved in endosomal transport (Perez *et al.*, 1999) or kinesins, which are microtubular motor proteins (Cai *et al.*, 2000). The punctate pattern of MPB2C association with microtubuli may indicate a transport-related endogenous function. Consistent with binding of TMV MP to MPB2C, both proteins colocalize at microtubule-associated sites. Furthermore, transient coexpression studies to elucidate the MPB2C function in the cell-to-cell movement of TMV MP suggest that the MPB2C protein is involved in TMV MP accumulation at microtubules (see Section 3.4.2), and that elevated levels of TMV MP at microtubules negatively interfere with its cell-to-cell movement. In contrast, intercellular transport of a movement-enhanced mutant of TMV MP, MP^{R3} (Gillespie *et al.*, 2002; Toth *et al.*, 2002) which does not accumulate at microtubules, is not affected by MPB2C. Furthermore, MPB2C shows reduced binding affinity to MP^{R3} as compared to the wild-type TMV MP *in vitro* (Kragler *et al.*, 2003). Thus, interaction between TMV MP and the MPB2C protein may not be required for TMV MP cell-to-cell transport; instead, the MPB2C protein may be part of a pathway negatively affecting cell-to-cell movement by redirecting TMV MP from its default cell-to-cell transport route towards accumulation at microtubules (see also Section 3.7 and Figure 1b). In this regard, it is interesting to note that the biological significance of the frequently observed accumulation of TMV MP at microtubules is still controversial; interpretations range from positive requirement for TMV MP-RNA complex transport towards plasmodesmata (Boyko *et al.*, 2000a) to negative regulation of movement by directing TMV MP towards a degradation pathway (Gillespie *et al.*, 2002; Mas and Beachy, 1999; Padgett *et al.*, 1996) (for more details see Sections 3.4.2 and 3.4.5). The MPB2C involvement in TMV MP accumulation at microtubules, together with its negative impact on TMV MP transport activity, support the latter interpretation. However, so far the studies of MPB2C function have focused on the MPB2C role in intercellular transport of the TMV MP protein in the absence of the viral context; thus, the function of TMV MP-MPB2C interaction in the spread of TMV virions remains to be addressed.

TMV MP must also interact with cellular protein kinases, which have been shown to specifically phosphorylate this protein *in vivo* (Haley *et al.*, 1995; Waigmann *et al.*, 2000; Watanabe *et al.*, 1992) and *in vitro* (Citovsky *et al.*, 1993; Karpova *et al.*, 1999). At least two types of such kinases are predicted: a calcium-independent serine/threonine kinase that phosphorylates the C-terminus of TMV MP and is located in the cell wall (Citovsky *et al.*, 1993; Waigmann *et al.*, 2000), and one or more serine/threonine kinases that phosphorylate N-terminal and internal TMV MP residues (Haley *et al.*, 1995; Karger *et al.*, 2003; Kawakami *et al.*, 1999, 2003; Matsushita *et al.*, 2003; Watanabe *et al.*, 1992) and probably are not associated with the plant cell wall. Phosphorylation of some amino acid residues, *e.g.*, Ser-258, Thr-261, Ser-267, and Thr-104, is thought to negatively regulate TMV MP activity (Karger *et al.*, 2003; Waigmann *et al.*, 2000), whereas the biological significance of other phosphorylation sites within this protein remains obscure. Furthermore, TMV MP phosphorylation, potentially following the passage of the M complexes through plasmodesmata, may partially dissociate the M complex, allowing translation and replication of the viral RNA (Karpova *et al.*, 1997, 1999). Detailed discussion of TMV MP phosphorylation as well as that of other viral MPs, e.g., ToMV MP (Matsushita *et al.*, 2000), CMV MP (Matsushita *et al.*, 2002b), TBSV MP (Desvoyes *et al.*, 2002), PLRV MP (Sokolova *et al.*, 1997), and others, is presented in Section 3.6. Also, in Sections 3.4.1 and 3.4.5 we discuss possible biological roles in viral movement and TMV MP turnover played by interactions between TMV MP and plant cytoskeletal elements, *i.e.*, tubulin and actin, which have been demonstrated both *in vivo* and *in vitro* (Gillespie *et al.*, 2002; Heinlein *et al.*, 1995; McLean *et al.*, 1995; Padgett *et al.*, 1996).

In addition to TMV MP, several other viral MPs have been used as ligands to discover and isolate their interacting proteins. For example, gold-conjugated CMV MP was shown to bind to tobacco cell-wall proteins, but because protein binding was assayed on dot blots, the size or identity of the CMV

MP-interacting proteins were not determined (Kragler *et al*., 1998). CaMV MP, previously shown to interact with PME (Chen and Citovsky, 2003; see above), was also reported to bind to three arabidopsis proteins, designated MPI1, MPI2, and MPI7, in the yeast two-hybrid system (Huang *et al*., 2001a). Only MPI7, a member of a rab receptor protein family, was further characterized for its interaction with CaMV MP. Fluorescently-tagged MPI7 and CaMV MP colocalize with each other in transiently transfected arabidopsis leaf protoplasts and induce fluorescence resonance energy transfer (FRET) between their fluorophore tags, indicating close-range interaction between MPI7 and CaMV MP (Huang *et al*., 2001a). Also, a CaMV MP mutant unable to bind MPI7 is noninfectious (Huang *et al*., 2001a). Interestingly, the same mutations in CaMV MP that interfere with its binding to MPI7 also affect formation of CaMV MP-induced tubules in plant protoplasts (Huang *et al*., 2001a, 2001b), although MPI7 is not present in such tubules (Huang *et al*., 2001a). How MPI7 may participate in CaMV cell-to-cell movement is still unknown, but an animal homolog of MPI7, rat PRA1, was proposed to be involved in the vesicle transport machinery (Martincic *et al*., 1997). On the other hand, rat PRA1 does not interact with CaMV (Huang *et al*., 2001a), making it difficult to extrapolate the mechanism of MPI7 action from that of PRA1.

When MP of another tobamovirus, ToMV, was used as probe in the gel blot overlay assay to screen a *Brassica campestris* cDNA expression library, a putative transcriptional coactivator KELP, which binds ToMV MP as well as MPs of CMV and a crucifer-infecting wasabi strain of tobamovirus CTMV-W, was isolated (Matsushita *et al*., 2001). Moreover, similar screening of a tobacco cDNA expression library with ToMV MP as probe identified another putative transcriptional coactivator, MBF1, that interacts with ToMV MP and CTMV-W MP but not with CMV MP (Matsushita *et al*., 2002a). Arabidopsis homologs of this tobacco MBF1 protein were also shown to bind ToMV MP (Matsushita *et al*., 2002a). As yet there is no biological evidence that KELP and/or MBF1 function in viral movement. Such function may be carried out indirectly, by modulating host gene expression, for example, to suppress plant defense responses to the moving virus (Matsushita *et al*., 2002a).

Another example of interaction between a viral MP and a cellular transcription factor is binding of TBSV MP to a tobacco homeodomain leucine-zipper protein HFi22 (Desvoyes *et al*., 2002). This binding was detected in the two-hybrid assay and confirmed by *in vitro* coimmunoprecipitation. Importantly, two movement-deficient mutants of TBSV MP did not interact with HFi22, lending strong support to the involvement of this plant protein in TBSV movement (Desvoyes *et al*., 2002). Similarly to KELP and MBF1, binding of HFi22 to MP was proposed to suppress expression of the host defense genes (Desvoyes *et al*., 2002). A more intriguing model for HFi22 action is based on the observations that plant transcription factors, such as KN1, DEFICIENS, LEAFY, and APETALA1, move from cell to cell (Lucas *et al*., 1995; Perbal *et al*., 1996; Sessions *et al*., 2000) and even transport their own mRNA molecules between cells (Lucas *et al*., 1995). By analogy to these proteins, HFi22 may also move between cells and, when bound to TBSV MP-TBSV RNA complexes, shuttle them through plasmodesmata (Desvoyes *et al*., 2002).

MP of TSWV interacts with two DnaJ-like proteins from tobacco and arabidopsis. This interaction was initially detected in the two-hybrid system and confirmed by coprecipitation *in vitro* (Soellick *et al*., 2000). While no direct evidence exists to date to involve DnaJ proteins in viral movement, they have been proposed to mediate interaction between TSWV MP-TSWV RNA complexes and cellular Hsp-70 chaperone, recruiting the latter to assist transport through plasmodesmata (Soellick *et al*., 2000). Indeed, Hsp-70 is known to interact with DnaJ proteins (reviewed by Kelley, 1998), and a Hsp-70 homolog encoded by BYV facilitates viral transport between cells (Alzhanova *et al*., 2001). In a separate study, TSWV MP was shown to interact not only with DnaJ-like proteins but also with plant homologs of kinesin and myosin (von Bargen *et al*., 2001); the biological significance of these interactions for viral cell-to-cell movement, however, is unknown.

One of the two MPs of TCV, p8, interacted with an arabidopsis protein designated Atp8 in the two-hybrid system and in slot-blot binding assays (Lin and Heaton, 2001). One of the hallmarks of Atp8 is two RGD sequences (Lin and Heaton, 2001); previously, RGD motifs have been implicated in interactions with integrins during attachment of extracellular proteins to cell surfaces (Ruoslahti, 1996) as well as in integrin-mediated internalization of animal viruses (Wickham *et al*., 1993). How interaction of TCV p8 with plant RGD proteins can potentiate viral movement remains obscure.

BV1 proteins of two geminiviruses, *Cabbage leaf curl virus* (CLCV) and SLCV interact with an arabidopsis acetyltransferase, designated AtNSI (McGarry *et al*., 2003). AtNSI resides in the cell nucleus where BV1 functions to bind viral genomes and export them into the cytoplasm for BC1-mediated cell-to-cell movement (reviewed by Hanley-Bowdoin *et al*., 2000; Lazarowitz and Beachy, 1999; Mansoor *et al*., 2003). Overexpression of AtNSI in arabidopsis enhances the efficiency of infection by CLCV. Unlike many other eukaryotic acetyltransferases, AtNSI does not function as a transcriptional coactivator; instead, it may regulate cell differentiation. Specifically, AtNSI was proposed to acetylate plant proteins that induce cell differentiation, whereas BV1 binding to AtNSI was reported to downregulate AtNSI-mediated histone acetylation *in vitro*; thus, BV1–AtNSI interaction may maintain the host cells in a dedifferentiating state, which is more susceptible to infection by some geminiviruses (McGarry *et al*., 2003).

Studies of interactions of TGB proteins of PVX with tobacco proteins (Fridborg *et al*., 2003) uncovered an interesting link between viral MPs and callose, a cell-wall component known to restrict plasmodesmal permeability and prevent viral spread (Bucher *et al*., 2001; Delmer *et al*., 1993; Iglesias and Meins Jr., 2000) (see also Section 4.2.2). In the two-hybrid system, P12,

which represents the TGBp2 protein of PVX, interacts with three tobacco proteins, designated TIP1-3; binding of all three TIPs to PVX TGBp2 was specific because it did not occur with TGBp1 or TGBp3 proteins of the virus (Fridborg *et al*., 2003). TIP1-3 belong to the same protein family and share sequence homology with the tobacco ankyrin repeat-containing protein HBP1. Importantly, all three TIPs also interacted with β-1,3-glucanase, the enzyme that degrades callose (Fridborg *et al*., 2003). These observations link viral MPs with a potential mechanism to reduce callose deposits in the host cell walls, most likely leading to an increase in plasmodesmal permeability. In this model, MP-TIP complexes may activate β-1,3-glucanases or, alternatively, free TIPs may inhibit the β-1,3-glucanase activity and MP binding to TIPs may relieve this inhibition. Whether or not TIPs or MP-TIP indeed affect the enzymatic activity of β-1,3-glucanases remains to be determined.

Recent years have seen a virtual explosion of studies identifying cellular interactors of MPs encoded by a variety of plant viruses. As described above, some of these studies provided initial solid evidence that the identified proteins may indeed be involved in viral movement, while others did not. The "smoking gun," however, is still missing; potentially, such definitive evidence for the biological role of cellular partners of viral MPs will come from future reverse genetics studies.

3.6. Regulation of MP Functions by Phosphorylation

Viral MPs are multifunctional proteins whose activities need to be tightly coordinated with the demands of the viral life cycle and the physiology of the host plant. For example, the M complexes, after successful transport through plasmodesmata, should release the viral genome for translation and replication; this M complex disassembly likely requires changes in MP activity. Also, during their intra- and intercellular transport, MPs interact with a changing set of host cellular structures and factors in an ordered spatial and temporal sequence (see Sections 3.4 and 3.5). Most likely, MP affinity to host factors is modulated in a controlled fashion during the infection cycle. Finally, MPs, due to their interaction with plasmodesmata, interfere with the intercellular communication and affect the metabolism of the host plant. Thus, for survival of the host it may be desirable to terminate the MP activity once it has fulfilled its tasks in the viral movement process. One possible effective mechanism to modulate and/or regulate different MP activities would be a series of phosphorylation/dephosphorylation events executed by cellular kinases and phosphatases. Indeed, increasing evidence indicates that MPs exist as phosphoproteins in plant cells, and that the phosphorylation status of MPs specifies some of their functions.

The most detailed picture on sites and effects of phosphorylation is presently available for the MPs of tobamoviruses, in particular the closely related TMV MP and ToMV MP. Phosphorylation of TMV MP has been examined *in vitro* using enriched cell-wall preparations, ER fractions, and total soluble protein extracts, all of which were derived from tobacco leaves, as well as *in vivo* in tobacco plants and in protoplasts from *N. clevelandii* leaves. Not surprisingly, different assay systems revealed different phosphorylation sites: 1) Deletion and substitution mutagenesis analyses reveal three phosphorylation sites very close to the C-terminus of the 268 amino-acid–long TMV MP, Ser-258, Thr-261, and Ser-267 (Citovsky *et al*., 1993; Waigmann *et al*., 2000). Since these sites have been identified *in vitro* for recombinant TMV MP incubated with cell-wall–enriched fractions from tobacco leaves (Citovsky *et al*., 1993) and *in vivo* for TMV MP associated with the cell walls of MP-expressing transgenic plants (Waigmann *et al*., 2000), it is likely that the corresponding host kinase is located at the cell wall, perhaps in association with plasmodesmata. 2) *In vivo* experiments in protoplasts derived from *N. clevelandii* leaves indicate multiple phosphorylation events involving Ser residues in TMV MP regions between amino acid positions 61 to 114 and 212 to 231, but not at the very C-terminus of TMV MP (Haley *et al*., 1995). Phosphorylation in one of these internal regions was confirmed *in vitro* using an ER-associated kinase activity from *N. tabacum* leaves, which phosphorylates TMV MP at Thr-104 (Karger *et al*., 2003). Incubation with total soluble protein extract from tobacco leaves also leads to phosphorylation of TMV MP, but the corresponding phosphorylation sites have not been determined (Ivanov *et al*., 2001). The closely related ToMV MP is also phosphorylated both at internal sites and at the C-terminus. Specifically, incubation of purified recombinant ToMV MP with soluble cellular protein extracts from BY2 suspension culture cells results in C-terminal phosphorylation of the 264 amino-acid–long ToMV MP at the residues Thr-256 and Ser-261 (Matsushita *et al*., 2000, 2003) and at one or more unidentified internal Ser sites (Matsushita *et al*., 2003). In contrast, ToMV MP produced from viral RNA in infected BY2 protoplasts is phosphorylated at two internal residues, Ser-37 and Ser-238, but not at the C-terminal sites (Kawakami *et al*., 1999).

Cellular protein kinases responsible for MP phosphorylation have not been definitively identified; nonetheless, some of the features of these enzymes have been revealed by biochemical studies using MPs as specific substrates. All cellular protein kinases that phosphorylate MP are active in the presence of Mg^{2+} and do not require Ca^{2+}. These biochemical properties distinguish the tobacco cell-wall–associated kinase phosphorylating the C-terminus of TMV MP (Citovsky *et al*., 1993) from known plant cell-wall–associated kinases (He *et al*., 1996; Yahalom *et al*., 1998). The kinase activities in total soluble protein extracts or ER fractions derived from *N. tabacum* leaves are both further stimulated upon replacement of Mg^{2+} by Mn^{2+} (Ivanov *et al*., 2001; Karger *et al*., 2003), and may in fact be identical. The kinase activity phosphorylating C-terminal sites of ToMV MP is also active in the absence of Ca^{2+}, and, upon treatment with various protein kinase inhibitors, exhibits enzymatic properties characteristic of a casein kinase 2 (CK2)-like kinase (Matsushita *et al*., 2000). CK2-like protein kinases may also be responsible for *in vitro* phosphorylation of TMV

MP coincubated with the cell-wall fractions of *N. tabacum*, *N. glutinosa*, and *N. benthamiana* (Karpova *et al*., 2002). CK2 kinases control diverse aspects of metabolism and development in plants and act as heterotetramers composed of catalytic α subunits and regulatory β subunits encoded by gene families (Riera *et al*., 2001). A recombinant form of one of the α subunits of *N. tabacum* CK2 phosphorylates ToMV MP *in vitro*, albeit only at one C-terminal residue, Ser-261, and at one or more internal Ser residues, but not at Thr-256 (Masushita *et al*., 2003). In the *in vitro* reactions, the differences in site recognition between the BY2 cell-derived kinase activity, which recognizes Thr-256 and Ser-261 (Matsushita *et al*., 2000), and the recombinant α subunit of the tobacco CK2, which recognizes only Ser-261 (Matsushita *et al*., 2003), may reflect differences between the activities of the complete enzyme and its isolated catalytic subunit. The kinase activity responsible for phosphorylation of the internal Ser-37 and Ser-238 residues of ToMV MP *in vivo* (Kawakami *et al*., 1999) has not been characterized with regard to its biochemical properties or subcellular localization; however, because the *in vivo* study was performed in BY2 protoplasts, this protein kinase is most likely not associated with cell walls.

The functional relevance of some of these phosphorylation sites was examined using two types of MP mutants: those that cannot be phosphorylated and those that mimic phosphorylation. Nonphosphorylatable MP mutants are created by deletion of the phosphorylated Ser/Thr residues or their substitution with Ala residues that cannot be phosphorylated, whereas phosphorylation-mimicking mutants are generated by introducing Asp or Glu residues at the appropriate sites. Negatively charged Asp and Glu are known to reproduce the electrostatic effects of phosphorylation and mimic its functional impact (Dean and Koshland, 1990; Thorsness and Koshland, 1987). This approach was used to elucidate the biological function of the C-terminal phosphorylation of TMV MP (Waigmann *et al*., 2000). Since the C-terminal 55 amino acids of TMV MP encompassing the complete C-terminal phosphorylation site are dispensable for TMV spread (Berna *et al*., 1991; Boyko *et al*., 2000c; Gafny *et al*., 1992), the C-terminal phosphorylation is clearly not essential for the TMV MP function. Instead, it was proposed to play a negative role and inactivate TMV MP (Citovsky *et al*., 1993). To test this idea, a TMV MP mutant mimicking the C-terminal phosphorylation was examined for various TMV MP functions, such as RNA binding, interaction with PME, stability, gating of plasmodesmata, and viral spread. Binding to RNA (Citovsky *et al*., 1993), stability in *N. tabacum* and *N. benthamiana* protoplasts, and binding to PME are not affected by mimicking the C-terminal phosphorylation (Waigmann *et al*., 2000).

In contrast, the ability of TMV MP to gate mesophyll plasmodesmata is severely reduced, and its capacity to promote viral cell-to-cell and systemic movement is abolished in *N. tabacum* (Waigmann *et al*., 2000). Interestingly, this negative effect of mimicking the C-terminal phosphorylation of TMV MP on its transport function is observed in *N. tabacum*, but not in the more promiscuous host *N. benthamiana* (Waigmann *et al*., 2000), suggesting that the C-terminal phosphorylation represents a host-dependent inactivation mechanism (Rhee *et al*., 2000b; Waigmann *et al*., 2000) (see also Section 3.7 and Figure 1a). Thus, the C-terminal part of TMV MP contains a true regulatory domain that is not required for function but that modulates the MP activity, depending on its phosphorylation status. The regulatory function of the C-terminus of TMV MP may have a mechanistic basis in the secondary structure of this protein. Secondary structure predictions of the 30 K superfamily MPs define a common core with a conserved fold, followed by a predominantly random coil at the C-terminus (Melcher, 2000). This random coil structure may facilitate formation of a flexible tail well-suited to regulate access to the common core domain (Melcher, 2000).

What is the biological rationale for inactivating the TMV MP transport function during infection? Although TMV MP is synthesized only transiently, the produced protein persists in the infected cells, accumulating within their plasmodesmata (Ding *et al*., 1992a; Tomenius *et al*., 1987). Continuous presence of active MP may elevate plasmodesmal permeability and alter intercellular communication, an important biological process. Potentially, interference with plasmodesmal transport during viral infection may be minimized by phosphorylating TMV MP after it has performed its cell-to-cell transport function. This model suggests that MP is most active in freshly infected cells, while MP within cells at later stages of infection becomes permanently inactivated (Rhee *et al*., 2000b; Waigmann *et al*., 2000). Indeed, plasmodesmal gating is restricted to the outer edge of an expanding TMV infection site even though TMV MP itself is present also in plasmodesmata of cells located towards the center of the infected area (Oparka *et al*., 1997).

TMV MP phosphorylation may also control other events in the viral life cycle, such as translation and replication of the viral RNA. *In vitro*-formed M complexes between TMV MP and TMV RNA are nontranslatable and nonreplicable *in vitro* and in isolated plant protoplasts (Karpova *et al*., 1997), presumably due to shielding of the RNA molecule by the cognate MP. However, TMV RNA regains infectivity, and by implication its capacity for translation and replication, in plant tissues (Karpova *et al*., 1997), where the M complexes can become phosphorylated during their passage through plasmodesmata. Indeed, the M complexes are converted into a translatable and infectious form following phosphorylation of their MP component by cell-wall–enriched fractions from tobacco (Karpova *et al*., 1999). Although not conclusively shown in this study, the use of cell-wall–enriched fractions as kinase source suggests that they phosphorylate MP at its C-terminal sites, known to be recognized by the cell-wall–associated protein kinase activity (Citovsky *et al*., 1993; Waigmann *et al*., 2000). Thus, it is tempting to speculate that the C-terminal phosphorylation of TMV MP may act as a molecular switch between viral spread and translation/replication (Rhee *et al*., 2000b) (see also Section 3.7 and Figure 1a).

Since TMV spreads and replicates even when the C-terminus of TMV MP is deleted, phosphorylation events at other sites may act as functional substitutes of the C-terminal phosphorylation. Indeed, similar relief of translational repression was observed after incubation of M complexes with protein kinase C, which, based on its site specificity, is unlikely to recognize the C-terminal phosphorylation sites. Alternatively, unlike the full-length MP, C-terminal deletion mutants may bind TMV RNA without inhibiting its translation and replication abilities, requiring no phosphorylation for infectivity.

Phosphorylation of the internal residue Thr-104 of TMV MP, which also is not essential for function, may constitute another mechanism for MP inactivation: while eliminating Thr-104 phosphorylation by replacing this residue with neutral Ala does not affect viral movement, mimicking MP phosphorylation by substituting Thr-104 with negatively charged Asp strongly inhibits cell-to-cell spread of the mutant virus in *N. tabacum* cv. Xanthi nc plants (Karger *et al*., 2003).

Functional analysis of ToMV MP phosphorylation has focused on the internal sites Ser-37 and Ser-238, demonstrating that whereas substitution of Ser-238 with Ala does not affect the infectivity of the virus, substitution of Ser-37 with Ala renders the virus nonfunctional both in local lesion and systemic hosts (Kawakami *et al*., 1999). This functional impairment of the Ser37Ala ToMV MP mutant may be due to its improper subcellular localization, because the corresponding GFP fusion protein exhibits low levels of dispersed fluorescence and lacks localization to puncta, irregular patches, and filaments, which are observed with the wild-type ToMV MP-GFP expressed in BY2 protoplasts (Kawakami *et al*., 1999). Surprisingly, replacement of Ser-37 with another phosphorylatable amino acid residue Thr or phosphorylation-mimicking Glu also abolishes infectivity, even though the Ser37Thr mutant MP is phosphorylated, and both mutant MPs localize to similar subcellular structures as the wild-type ToMV MP. In addition, naturally occurring revertants of the virus mutant Ser37Ala/Ser238Ala arise from complementing mutations at other, nonphosphorylated residues of MP, and one of the revertant MPs is not even phosphorylated *in vivo* (Kawakami *et al*., 2003). However, the MPs of the revertant viruses are less efficient in promoting cell-to-cell movement than the wild-type ToMV MP. For some of these revertants, the partial restoration of the cell-to-cell transport function is linked to an increase in their stability and for others to re-establishment of the wild-type pattern of subcellular localization in BY2 protoplasts (Kawakami *et al*., 2003). Thus, the presence and phosphorylation of the Ser residue at position 37 in the ToMV MP molecule are important for the optimal function of ToMV MP, but they do not represent essential prerequisites for this function *per se* (Kawakami *et al*., 2003).

TMV and ToMV are by far not the only viruses whose MPs undergo phosphorylation within the host cells. For example, ACLSV MP is detected in a phosphorylated form in the infected *C. quinoa* tissues (Sato *et al*., 1995), and CMV MP is phosphorylated at one or more as-yet unidentified serine residues *in vivo* when expressed in transgenic tobacco plants (Matsushita *et al*., 2002b). Recently, *in vitro* phosphorylation of TBSV MP by a cell-wall–enriched fraction from *N. benthamiana* was also reported (Desvoyes *et al*., 2002). Also, PLRV MP is phosphorylated in the infected potato plants or when expressed in transgenic potato plants (Tacke *et al*., 1993). *In vitro* studies with crude membrane preparations from potato leaves suggest that phosphorylation of PLRV MP occurs within its nucleic-acid–binding domain and is carried out by a Ca^{2+}-dependent, membrane-associated protein kinase C-like activity (Sokolova *et al*., 1997). It was speculated that phosphorylation of the nucleic-acid–binding domain of PLRV MP may weaken its binding to PLRV RNA and therefore lead to dissociation of the PLRV M complex (Sokolova *et al*., 1997). Similarly, phosphorylation inhibits the RNA-binding activity of CP of *Potato potyvirus A* (PVA) (Ivanov *et al*., 2001, 2003. PVA CP is not phosphorylated when packaged into virions, supporting the idea that its phosphorylation and RNA-binding domains overlap each other and thus are not exposed on the surface of the virion (Ivanov *et al*., 2001). PVA CP is phosphorylated both *in vivo* in virus-infected plants and *in vitro* by tobacco cell extracts (Ivanov *et al*., 2001) and by the purified α subunit of the tobacco CK2 and maize CK2 (rmCK2a) (Ivanov *et al*., 2003). Phosphorylation of PVA CP *in vitro* by rmCK2a occurs mainly at the Thr-242 residue, and blocking PVA CP phosphorylation at this site by mutagenizing its CK2 consensus sequences inhibits both cell-to-cell and systemic movement of GFP-tagged PVA virions (Ivanov *et al*., 2003). This inhibition may reflect the regulatory role of PVA CP phosphorylation, which has been suggested to allow more efficient replication and translation of the viral genomic RNA by preventing premature assembly of viral particles (Ivanov *et al*., 2003). As mentioned above, similar effect on TMV RNA replication and translation has been proposed for the phosphorylated TMV MP (Karpova *et al*., 1997; Karpova *et al*., 1999).

For many viral MPs that undergo phosphorylation, identification of the phosphorylation sites, isolation of the corresponding cellular protein kinase activities, and functional evaluation of the phosphorylation events in the context of the MP activity represent the major question waiting to be solved. Nevertheless, a continuously increasing number of MPs from diverse groups of plant viruses are discovered to serve as phosphorylation substrates *in vitro* or, even more importantly, are shown to exist as phosphoproteins *in planta*, which highlights the functional importance of MP phosphorylation in the viral life cycle. Therefore, studies addressing MP phosphorylation are rapidly gaining attention in the field of viral cell-to-cell movement.

3.7. Multiple Roles of TMV MP in the Viral Life Cycle: A Host Factor-Based Model

Numerous models have been proposed for the roles of MPs in the life cycles of most of the viruses discussed in the previous sections (see, for example, reviews by Carrington *et al*., 1996; Ding, 1998; Lazarowitz and Beachy, 1999; Lucas and

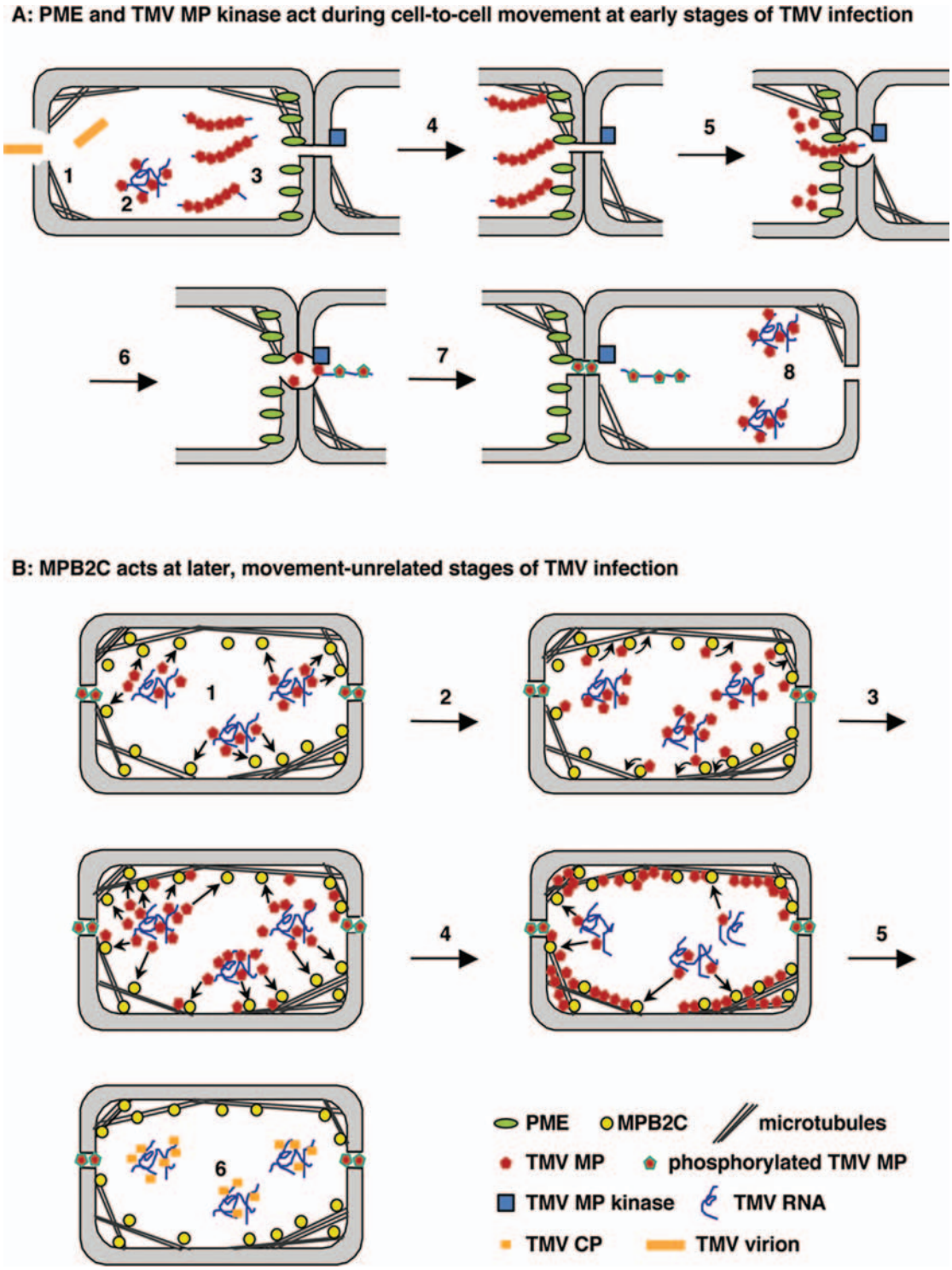

FIG. 1. A host factor-based model for TMV MP functions during the viral life cycle. (A) Host factors PME and TMV MP kinase act during cell-to-cell movement at early stages of infection. Steps 1 to 5 depict events taking place in the initially infected cell: (1) entry of virions; (2) establishment of translation/replication sites; (3) formation of M-complexes composed of MP and TMV RNA; (4) targeting to and docking at cell wall-localized PME; (5) gating of plasmodesmata. Steps 6 to 8 depict events taking place in the newly infected, neighboring cell; (6) translocation of M complexes into the neighboring cell is coupled to partial uncoating of the M complex and MP phosphorylation by a plasmodesmata-associated host cell kinase; (7) phosphorylated MP stored within plasmodesmata is gating incompetent, and plasmodesmal permeability returns to its unperturbed level; (8) new viral translation/replication sites are established. Because step 8 is essentially identical to step 2, the cell-to-cell movement cycle can be perpetuated. (B) Host factor MPB2C acts at later, movement-unrelated stages of TMV infection. (1) Numerous translation/replication sites produce large amounts of MP. (2) MP binds to microtubule-associated host factor MPB2C. (3) MPB2C directs MP from ER-associated sites to microtubules. (4) High levels of MP accumulate at microtubules. (5) Microtubule-associated MP is degraded. (6) MP translation is replaced by production of CP. Degradation of MP coupled with termination of MP synthesis effectively eliminate MP from the infected cells; only phosphorylated MP stored within plasmodesmata persists. All symbols for Figure 1 are explained in lower right corner of Figure 1b.

Gilbertson, 1994; Morozov and Solovyev, 2003; Taliansky and Robinson, 2003). This is particularly true for TMV, which for the last two decades has served as a paradigm for viral intercellular spread. Models of TMV movement have evolved from the initial, simple schemes that focused on MP binding to TMV RNA to form M complexes, followed by MP-mediated targeting of these complexes to plasmodesmata and MP-induced gating of plasmodesmal channels (Citovsky and Zambryski, 1991; Waigmann and Zambryski, 1994), to more complicated designs incorporating hypothetical host factors (Ding, 1998) or a potential intracellular targeting routes of M complexes along microtubules towards plasmodesmata (Ghoshroy *et al*., 1997; Lazarowitz and Beachy, 1999; Waigmann *et al*., 1998). Only recently have TMV movement models begun to discern between MP functions at various stages of the viral life cycle and to incorporate regulatory events and MP-interacting host factors (Haywood *et al*., 2002; Heinlein, 2002b; Rhee *et al*., 2000b; Tzfira *et al*., 2000).

Here, we present an updated model for MP functions in the TMV life cycle that highlights our current knowledge about the potential role of MP-interacting host factors PME (Chen *et al*., 2000; Dorokhov *et al*., 1999), MP-phosphorylating kinase (Citovsky *et al*., 1993; Waigmann *et al*., 2000), and MPB2C (Kragler *et al*., 2003; see also Figure 1). In this model, both PME and the MP kinase act at early stages of infection, such as those occurring at the leading edge of the expanding infection site, to ensure cell-to-cell movement and translation/replication of the TMV genome (Figure 1a). Initial infection of plant cells by TMV virions usually occurs by mechanical inoculation, probably through breached host cell walls (Figure 1a, step 1). After uncoating, TMV RNA is translated to produce viral proteins, such as replicase (not shown) and MP (Figure 1a, step 2). Translation and replication of viral RNA likely take place in *viral factories* that are spatially restricted areas of the cell cytoplasm closely connected to the ER (Heinlein *et al*., 1998). Due to its high local concentration within the viral factories, and even though it binds single-stranded nucleic acids without sequence specificity (Citovsky *et al*., 1990), MP preferentially forms M complexes with the newly replicated TMV RNA (Figure 1a, step 3; see also Section 3.2). M complexes are then targeted towards the cell wall, potentially by a cytoskeletal or ER-based route (see also Section 3.4), and dock onto PME molecules distributed throughout the cell wall (Chen *et al*., 2000; see also Figure 1a, step 4). Binding of M complexes may modulate the PME enzymatic activity and, in so doing, induce changes in cell-wall porosity (*e.g*., Nairn *et al*., 1998; Pressey, 1984; see also Section 3.5). If M complexes dock at PME molecules located at or in the vicinity of plasmodesmata, the cell-wall structure surrounding plasmodesmata might be loosened, thereby facilitating MP-mediated gating of plasmodesmata (Figure 1a, step 5). Thus, only docking of M complexes at PME near plasmodesmata progresses into the productive cell-to-cell transport, whereas interaction with PME located at other cell-wall sites aborts movement, perhaps by disintegration of M-complexes and recycling or degradation of their components (Figure 1a, step 5; see also Section 3.5). Plasmodesmata with enhanced permeability support translocation of M complexes into the adjacent cell (Figure 1a, step 6).

During passage through plasmodesmata, MP may become phosphorylated at its C-terminus by a plasmodesmata-localized protein kinase (Figure 1a, step 6; see also Section 3.6), and the M complex may be partially stripped from MP by an as-yet unknown mechanism that may also involve phosphorylation (Karpova *et al*., 1999) (Figure 1a, step 6). MP that has been removed from the complex probably is retained within plasmodesmata in its phosphorylated form (Figure 1a, step 7). Since the C-terminal phosphorylation of MP likely inactivates its plasmodesmata-gating activity in *N. tabacum* (Waigmann *et al*., 2000; see also Section 3.6), plasmodesmal permeability is restored to the basal, "ungated" levels, even though the phosphorylated MP is still present within plasmodesmata (Figure 1a, step 7). The M complex, altered in its conformation by partial uncoating and phosphorylation of the bound MP, is now competent for translation, replication, and establishment of "viral factories" in the newly infected cell (Figure 1a, step 8).

In contrast, the function of MPB2C likely is not connected to cell-to-cell movement (Figure 1b). MPB2C might act at later stages of TMV infection, such as those taking place in cells several layers behind the leading edge of the expanding infection site, an area characterized by high levels of fluorescence in infection rings tagged with TMV MP-GFP (see Section 3.4). Numerous "viral factories" have been established in the cytoplasm of these cells, producing high levels of viral RNA and MP (Figure 1b, step 1); however, at that stage it is not the cell-to-cell movement of TMV that is required to maintain the viral life cycle, but rather the production of CP and assembly of virions. This program switch may be coupled to elimination of MP from the plant cell cytoplasm, a process potentially aided by the host factor MPB2C (see Section 3.5). MPB2C is a poorly characterized host protein that associates with microtubules at discrete sites and binds directly to TMV MP (Figure 1b, step 2). Potentially, MPB2C acts as a shuttle receptor, redirecting MP from ER-associated sites towards microtubules (Figure 1b, step 3), where MP forms stable complexes, perhaps by integrating into the microtubular lattice (Boyko *et al*., 2000a). MPB2C mediates efficient accumulation of TMV MP at microtubules, culminating in extensive decoration of the cortical microtubular network with MP molecules (Figure 1b, step 4). MP accumulation at microtubules is likely followed by its degradation (Figure 1b, step 5); at the same time, MP translation ceases and is replaced by production of CP (Figure 1b, step 6). How may accumulation of MP at microtubules lead to MP degradation? Potentially, the exceptionally stable complexes between MP and microtubules (Boyko *et al*., 2000a) may interfere with the dynamic nature of the microtubular network and render it nonfunctional. As a consequence, the microtubular network and its cognate proteins, among them MP, may be eliminated by proteasomal degradation (Gillespie *et al*., 2002; Reichel and Beachy, 2000). A functional microtubular network is then quickly rebuilt, but, since the MP

synthesis has ceased, it cannot become reassociated with TMV MP. Only phosphorylated TMV MP stored within plasmodesmata (Figure 1a) escapes degradation and persists within the infected plant.

This model was developed specifically to include the recent information on TMV MP-interacting host factors, and therefore represents yet another step forward in understanding the dynamic role of TMV MP during the viral life cycle. No doubt, models of cell-to-cell viral movement will continue to be refined as MP functions are elucidated in even more detail and additional host factors are identified.

4. SYSTEMIC TRANSPORT OF PLANT VIRUSES: LONG-DISTANCE RUNNERS

Cell-to-cell movement of most viruses is a prelude to massive systemic invasion of the entire host plant, which begins when the moving virions reach the host vasculature. At that time, five distinct and consecutive steps must occur to allow long-distance, systemic transport (Figure 2): (1) viral entry into the vascular parenchyma (VP) through the bundle sheath (BS), (2) penetration into the companion cell/sieve element (CC/SE) complex from VP, (3) rapid transport to other plant organs through SE, (4) unloading from the CC/SE complex into uninfected VP, and (5) viral egress from VP through BS and into the mesophyll (ME) cells of systemic plant organs. Involvement of these highly specialized host tissues and multiple viral factors in the systemic transport have impeded direct experimental approaches, such as protein microinjection or transient gene expression by microbombardment, to study this transport process on the molecular level. Thus, in comparison with the cell-to-cell movement, the mechanisms of the systemic movement are still poorly understood.

4.1. Viral Factors Involved in Systemic Movement

Viral factors known to be involved in systemic movement are summarized in Table 2. In some virus genera, such as tobamoviruses, cell-to-cell movement and systemic movement are mediated by distinct proteins that often act by different mechanisms and independently of each other. Other viruses, such as geminiviruses and cucumoviruses, have evolved to utilize the same proteins for both cell-to-cell and systemic movement, intricately intertwining the two transport processes and making it more difficult to distinguish between their activities and to analyze their mechanisms.

Generally, viral CPs are thought to be involved in the systemic movement process of many viruses, including tobamoviruses, *e.g.*, TMV (Dawson *et al.*, 1988; Holt and Beachy, 1991; Osbourn *et al.*, 1990; Saito *et al.*, 1990; Siegal *et al.*, 1962; Takamatsu *et al.*, 1987); dianthoviruses, *e.g.*, RCNMV (Vaewhongs and Lommel, 1995; Xiong *et al.*, 1993) and *Carnation ring spot virus* (CRSV; Sit *et al.*, 2001); tombusviruses, *e.g.*, TBSV (Desvoyes and Scholthof, 2002); Scholthof *et al.*, 1993), *Cucumber necrosis virus* (CuNV; McLean *et al.*, 1993), and *Cymbidium ring spot virus* (CymRSV; Dalmay *et al.*, 1992; Huppert *et al.*, 2002; monopartite geminiviruses, e.g., *Maize streak virus* (MSV; Boulton *et al.*, 1989,1993; Liu *et al.*,1999b, 2001), TYLCV (Noris *et al.*, 1998), and *Bean yellow dwarf virus* (BeYDV; Liu *et al.*, 1998), bipartite geminiviruses, *e.g.*, *Bean golden mosaic virus* (BGMV; Pooma *et al.*, 1996) and *Tomato golden mosaic virus* (TGMV; Brough *et al.*, 1988; Gardiner *et al.*, 1988); alfamoviruses, *e.g.*, AMV (Spitsin *et al.*, 1999; van der Kuyl *et al.*, 1991); cucumoviruses, *e.g.*, CMV (Takeshita *et al.*, 1998; Taliansky and Garcia-Arenal, 1995); bromoviruses, *e.g.*, BMV (Rao and Grantham, 1996); luteoviruses, *e.g., Beet western yellows virus* (BWYV; Mutterer *et al.*, 1999; Ziegler-Graff *et al.*, 1996); potexviruses, *e.g.*, WClMV (Lough *et al.*, 2001); and potyviruses, *e.g.*,TEV (Dolja *et al.*, 1994, 1995), *Tobacco vein mottling virus* (TVMV; Lopez-Moya and Pirone, 1998), and *Pea seed-borne mosaic virus* (PSbMV; Andersen and Johansen, 1998).

Occurrence of particles of such diverse viruses as TMV (Ding *et al.*, 1996; Esau and Cronshaw, 1967), *Cucumber green mottle mosaic tobamovirus* (CGMMV; Simon-Buela and Garcia-Arenal, 1999), *Rice yellow mottle virus* (RYMV) (Opalka *et al.*, 1998), BWYV (Esau and Hoefert, 1972a, 1972b), *Barley yellow dwarf luteovirus* (BYDV; Gill and Chong, 1975; Jensen, 1969), CMV (Blackman *et al.*, 1998), and PLRV (Schmitz *et al.*, 1997; Shepardson and McCrum, 1980) in the samples derived from vasculature of the infected plants suggests that their systemic spread involves vascular transport of whole virions. However, umbraviruses, which do not produce CP, move systemically, possibly by forming a ribonucleoprotein complex between the viral genomic RNA and a viral protein encoded by ORF3 (Taliansky and Robinson, 2003). Thus, formation of the viral capsid is not required for systemic movement of some viruses (Ryabov *et al.*, 1999a, 2001b; Taliansky *et al.*, 2003; Taliansky and Robinson, 2003).

Furthermore, even in a number of viruses that produce CP elimination of this protein—*e.g.*, in RCNMV (Xiong *et al.*, 1993), TBSV (Scholthof *et al.*, 1995), CuNV (McLean *et al.*, 1993), and TGMV (Brough *et al.*, 1988; Gardiner *et al.*, 1988)—or disruption of its encapsidation activity in CP mutants—*e.g.*, in *Cowpea chlorotic mottle bromovirus* (CCMV) (Schneider *et al.*, 1997) and CymRSV (Dalmay *et al.*, 1992; Huppert *et al.*, 2002)—does not abolish systemic infection of some hosts, suggesting that encapsidation is not an absolute prerequisite for viral systemic movement. On the other hand, the systemic movement ability of some CP mutants of several viruses, such as TMV (Culver *et al.*, 1995; Dawson *et al.*, 1988), CRSV (Sit *et al.*, 2001), RCNMV (Xiong *et al.*, 1993), TGMV (Pooma *et al.*, 1996), and TEV (Dolja *et al.*, 1994, 1995), is impaired, although they retain the encapsidation activity. Thus, viral encapsidation and systemic movement can often be uncoupled.

In addition to their major CP component, some viral capsids contain minor constituents that also may play a role in the systemic movement. For example, the capsid of the luteovirus BWYV consists of two protein species; a major 22 kDa component, p3, and a minor 74 kDa component, a read-through

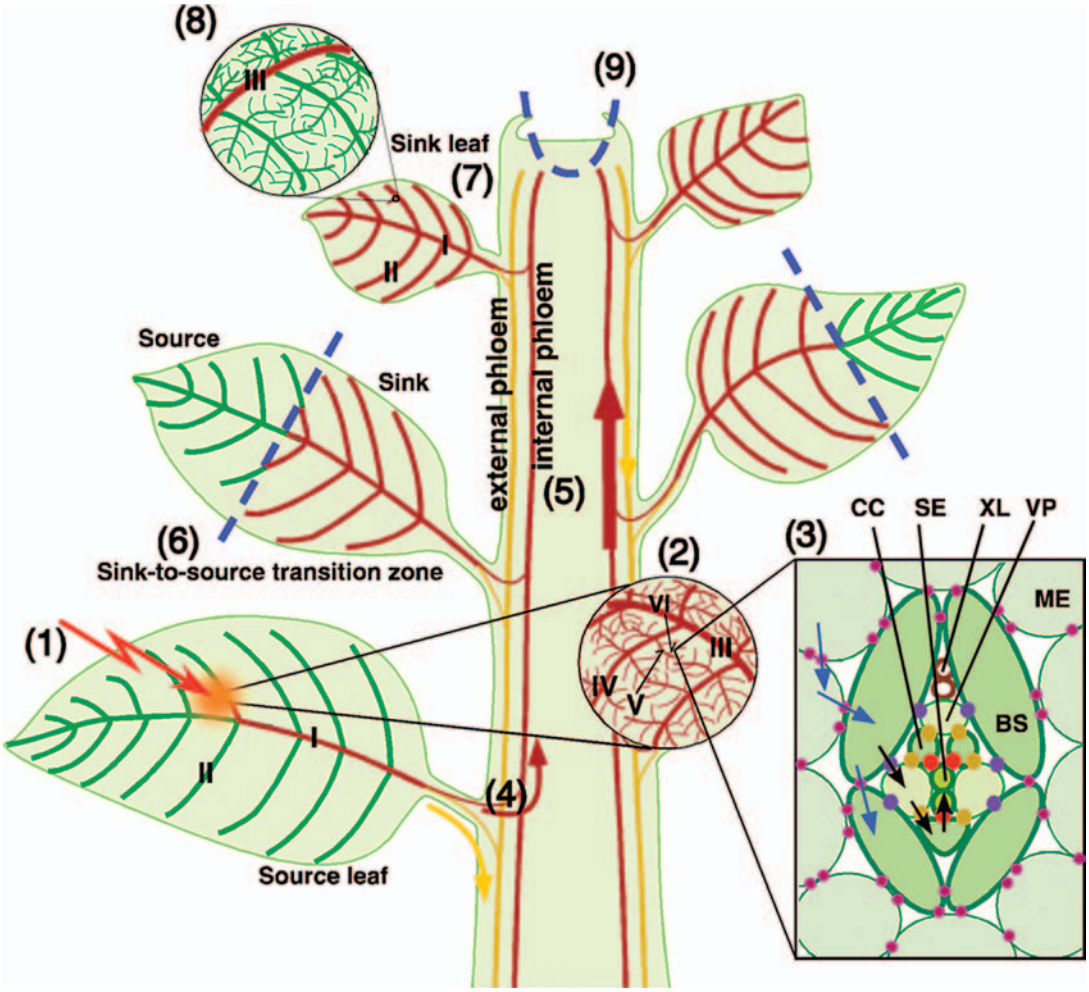

FIG. 2. Cellular routes for systemic movement of plant viruses. Viral long-distance transport through the phloem of a dicotyledonous plant is illustrated. Leaf veins that are utilized by viruses for systemic transport are drawn in red and yellow, and those that are not used are indicated in green. Blue dotted lines indicate cellular boundaries, *i.e.*, leaf sink-to-source transition zones and apical meristem, that viruses are unable to cross. (1, 2) Viral infection initiates with mechanical inoculation (red jagged arrow) of mesophyll cells of a lower, source leaf. The virus spreads from cell to cell and reaches the vascular system, into which it enters through all classes of veins, major and minor (indicated by roman numerals I to V), of the source leaf. In this leaf, the veins (indicated in green) traffic the virus toward the systemic organs but do not promote its phloem transport within the inoculated source leaf itself. (3) To enter into the phloem, the virus has to cross from mesophyll cells (ME) through bundle sheath cells (BS) and phloem parenchyma (VP) into companion cells (CC) and then into sieve elements (SE). Systemic movement of most, but not all, viruses does not involve xylem (XL). Movement from ME to ME, ME to BS, and BS to BS requires only viral MPs and is indicated by blue arrows; plasmodesmata connecting these cells are indicated as pink circles. Movement from BS to VP, VP to CC, and CC to SE requires additional viral factors and is indicated by black arrows. Furthermore, plasmodesmata between BS and VP and between VP and CC represent separate barriers for viral movement potentially requiring different viral factors, whereas plasmodesmata between CC and SE represent specialized deltoid-shaped structures; these functionally different plasmodesmata are indicated as purple, yellow, and orange circles, respectively. (4) Once within SE tubes, virus moves out of the inoculated leaf using adaxial (indicated in red) and abaxial phloem (indicated in yellow) of leaf veins, which connects to the internal phloem and external phloem, respectively, of the stem. (5) Internal phloem mediates the faster upward movement of the virus (longer dark red arrow), whereas external phloem mediates the slower downward movement (shorter yellow arrow). (6) In leaves undergoing sink-to-source transition during their maturation, virus cannot cross the cellular boundary at the transition zone, which is indicated by a blue dotted line separating the viral-trafficking (red) and nontrafficking phloem (green). (7, 8) To complete systemic infection, virus unloads from the phloem into the sink leaves. This unloading occurs only from major veins (classes I to III indicated in red) but not from minor veins (indicated in green), which are eventually infected by cell-to-cell movement of the virus. (9) The apical meristem is separated from the rest of the plant by a boundary (blue dotted line) that does not permit transport of viruses or even low molecular weight tracer molecules, suggesting that the tissue is symplastically isolated.

protein (RT) p74; p74 is synthesized by suppressing translational termination of p3, which allows the translation to continue to the adjacent ORF 5, thereby producing an additional read-through protein domain (RTD; Bahner *et al.*, 1990; Brault *et al.*, 1995; Filichkin *et al.*, 1994; Martin *et al.*, 1990; Wang *et al.*, 1995). BWYV mutants that do not produce RTD are still encapsidated, forming virions, but they exhibit reduced systemic infection in *N. clevelandii*, suggesting that RTD is required for the efficient systemic transport of the virus (Mutterer *et al.*, 1999).

TABLE 2
Viral proteins involved in systemic transport

Genus	Virus	Viral factors	Host range determinant	Dispensable in some hosts	Reference
Tobamovirus	TMV	CP	+	N.R.	Dawson *et al*., 1988; Holt and Beachy, 1991; Osbourn *et al*., 1990; Saito *et al*., 1990; Siegal *et al*., 1962; Takamatsu *et al*., 1987
		126-kDa	+	N.A.	Bao *et al*., 1996; Chen *et al*., 1996; Ding *et al*., 1995b; Holt *et al*., 1990; Lewandowski and Dawson, 1993; Nelson *et al*., 1993; Nishiguchi *et al*., 1985; Shintaku *et al*., 1996
Potyvirus	TEV	CP	N.R.	N.R	Dolja *et al*., 1994, 1995
		HC-Pro	N.R.	N.A.	Cronin *et al*., 1995; Kasschau *et al*., 1997
		VPg	+	N.A.	Schaad and Carrington, 1996; Schaad *et al*., 1997b
	TVMV	CP	N.R.	N.R.	Lopez-Moya and Pirone, 1998
	PPV	HC-Pro	+	N.A.	Saenz *et al*., 2002
	PSbMV	CP	+	N.R.	Andersen and Johansen, 1998
	PVA	VPg	+	N.A.	Rajamaki and Valkonen, 1999, 2002
		6K2	+	N.A.	Rajamaki and Valkonen, 1999
Dianthovirus	RCNMV	MP	N.R.	N.A.	Wang *et al*., 1998
		CP	N.R.	+	Vaewhongs and Lommel, 1995; Xiong *et al*., 1993
	CRSV	CP	N.R.	N.R.	Sit *et al*., 2001
Tombusvirus	TBSV	CP	N.R.	+	Desvoyes and Scholthof, 2002; Scholthof *et al*., 1993; Scholthof *et al*., 1995
		p19	N.R.	+	Scholthof *et al*., 1995
	CuNV	CP	N.R	+	McLean *et al*., 1993
	CymRSV	CP	N.R.	+	Dalmay *et al*., 1992; Huppert *et al*., 2002
Caulimovirus	CaMV	ATF	+	N.R.	Qiu *et al*., 1997
		Gene VI	+	N.R.	Wintermantel *et al*., 1993
Monopartite gemnivirus	MSV	V1	N.R.	N.A.	Boulton *et al*., 1989, 1993
		CP	+	N.A.	Boulton *et al*., 1989, 1993; Liu *et al*., 1999b, 2001
	TYLCV	CP	N.R.	N.A.	Noris *et al*., 1998
	BeYDV	CP	+	N.A.	Liu *et al*., 1998, 1999b
		V1	+	N.R.	Liu *et al*., 1998, 1999b
Bipartite geminivirus	BGMV	CP	+	N.A.	Pooma *et al*., 1996
		BV1	N.R.	N.A.	Schaffer *et al*., 1995
		BC1	N.R.	N.A.	Schaffer *et al*., 1995
	TGMV	CP	+	+	Brough *et al*., 1988; Gardiner *et al*., 1988; Pooma *et al*., 1996
		BV1	N.R.	N.A.	Jeffrey *et al*., 1996; Schaffer *et al*., 1995
		BC1	N.R.	N.A.	Jeffrey *et al*., 1996; Schaffer *et al*., 1995
	ACMV	BV1	N.R.	N.A.	von Arnim *et al*., 1993
		BC1	N.R.	N.A.	von Arnim *et al*., 1993
Alphamovirus	AMV	CP	+	N.R.	Spitsin *et al*., 1999; van der Kuyl *et al*., 1991

(Contuinued on next page)

TABLE 2
Viral proteins involved in systemic transport

Genus	Virus	Viral factors	Host range determinant	Dispensable in some hosts	Reference
Cucumovirus	CMV	CP	N.R.	N.A.	Takeshita *et al*., 1998; Taliansky and Garcia-Arenal, 1995
		3a	+	N.A.	De Jong *et al*., 1995; Kaplan *et al*., 1997; Li *et al*., 2001; Sanz *et al*., 2000; Takeshita *et al*., 1998
		2b	N.R.	N.A.	Soards *et al*., 2002
	TAV	CP	+	N.A.	Thompson and Garcia-Arenal, 1998
Bromovirus	BMV	CP	N.R.	N.R.	Rao and Grantham, 1996
Umbravirus	GRV	ORF3	+	N.R.	Ryabov *et al*., 1999a; Taliansky *et al*., 2003
	PEMV-2	ORF3	+	N.R.	Ryabov *et al*., 2001b
	TMoV	ORF3	+	N.R.	Ryabov *et al*., 2001b
Hordeivirus	BSMV	TGBp1	+	N.R.	Solovyev *et al*., 1999
	PSLV	TGBp1	N.R.	N.R.	Kalinina *et al*., 2001
Potexvirus	WClMV	TGBp1	N.R.	N.R.	Lough *et al*., 2001
		CP	N.R.	N.A.	Lough *et al*., 2001
Luteovirus	PLRV	p17	N.R.	+	Lee *et al*., 2002
	BYDV	P17	N.R.	N.R.	Chay *et al*., 1996
	BWYV	P74	N.R.	N.R.	Mutterer *et al*., 1999

N.R., not reported; N.A., not applicable because the protein has also a movement-unrelated function. Plant virus genera are according to Brunt *et al*. (1996).

In some viruses, MPs not only mediate cell-to-cell movement but also play roles in systemic movement. For example, BV1 and BC1 MPs of bipartite geminiviruses, such as BCMV, TGMV, and ACMV, aid systemic transport (Jeffrey *et al*., 1996; Schaffer *et al*., 1995; von Arnim *et al*., 1993), and TGBp1 MPs of hordeiviruses and potexviruses, which function during cell-to-cell movement (see Sections 3.1 and 3.2), are required for the systemic infection as well (Kalinina *et al*., 2001; Lough *et al*., 2001; Solovyev *et al*., 1999). Similarly, CMV MP is involved in both local and systemic movement of the virus (De Jong *et al*., 1995; Kaplan *et al*., 1997; Li *et al*., 2001; Sanz *et al*., 2000; Takeshita *et al*., 1998). In many cases, however, these two activities of MPs are distinct because they can be uncoupled. For instance, point mutations in RCNMV MP prevent viral systemic movement, presumably by inhibiting RCNMV loading into the CC/SE complex, but they do not affected cell-to-cell movement (Wang *et al*., 1998). Also, MPs of some luteoviruses, such as BYDV and PLRV, are involved in systemic transport (Chay *et al*., 1996; Lee *et al*., 2002), associating with plasmodesmata that connect CC with SE (Schmitz *et al*., 1997). In some hosts, however, MP is not required for vascular transport of luteoviruses, such as PLRV and BWYV (Lee *et al*., 2002; Ziegler-Graff *et al*., 1996), suggesting that luteoviral MPs may, at least in part, determine the host specificity of the viral systemic movement.

In addition to CP and MP, other viral factors with different biological activities participate in the systemic movement process. For example, the CMV 2b (Soards *et al*., 2002) and TBSV p19 proteins (Scholthof *et al*., 1995) modulate viral spread, presumably by counteracting posttranscriptional gene silencing (PTGS; Soards *et al*., 2002; Voinnet *et al*., 1999), a known plant defense response against viruses (reviewed by Moissiard and Voinnet, 2004; van der Boogaart *et al*., 1998; Voinnet, 2001). The 18 kDa aphid transmission factor (ATF) and the protein product of gene VI, which is required for viral propagation, are also responsible for light-dependent systemic CaMV infection of *N. bigelovii* (Qiu *et al*., 1997; Wintermantel *et al*., 1993). Also, as described below, the 126 kDa replicase of TMV, HC-Pro proteinase, the 6K2 protein, and the viral genome-linked (VPg) protein of TEV are involved in the process of viral systemic movement. Here, we focus on tobamoviruses and potyviruses, for which detailed information on the roles of their individual proteins in the systemic spread is available, and on umbraviruses, which represent a special and interesting case of systemically moving viruses that do not encode a conventional CP and do not form true virions.

4.1.1. Tobamoviruses

4.1.1.1. TMV CP. Involvement of CP in systemic spread of TMV, a type member of tobamoviruses, is inferred from the inability of CP-deficient viruses to move systemically (Dawson *et al*., 1988; Holt and Beachy, 1991; Osbourn *et al*., 1990; Siegal *et al*., 1962; Takamatsu *et al*., 1987), and from restoration of this movement by CP expressed in transgenic plants (Holt and Beachy, 1991; Osbourn *et al*., 1990). Virion-like structures, observed in tissue extracts from CP-expressing transgenic plants infected with a CP-defective TMV, indicate that CP supplied *in trans* can encapsidate the viral genome (Holt and Beachy,

1991; Osbourn *et al*., 1990). Point mutation in the origin of CP assembly, which allows CP synthesis but blocks formation of virions, diminishes systemic movement, suggesting that it is the encapsidated form of the virus that moves long distances (Saito *et al*., 1990). On the other hand, chimeric TMV RNA expressing, instead of CP, the umbraviral ORF3 protein, which mediates the systemic spread of umbraviruses but cannot form the TMV capsids, moves systemically, possibly due to formation of ribonucleoprotein transport complexes (Ryabov *et al*., 1999a, 2001b; Taliansky *et al*., 2003). Thus, the TMV systemic movement, similar to that of bromovirus CCMV, whose CP is dispensable for systemic infection of some hosts (Schneider *et al*., 1997), may not always involve viral particles. In addition, several TMV CP mutants, still able to form virion-like particles, cannot move systemically (Culver *et al*., 1995; Dawson *et al*., 1988), suggesting that virion formation is not the sole function of CP in viral systemic transport.

Several lines of evidence suggest that CP may interact with host factors to enable virus systemic movement. For example, when recombinant TMV expressing CP and MP of a different tobamovirus, *Odontoglossum ringspot virus* (ORSV), is inoculated onto *N. tabacum* cv. Xanthi, its systemic movement is greatly reduced, resembling the spread of ORSV rather than TMV in this host plant (Hilf and Dawson, 1993). The same chimeric virus moves efficiently in *N. benthamiana*, which represents a susceptible host for both TMV and ORSV (Hilf and Dawson, 1993). Also, chimeric TMV expressing its own CP and ORSV MP (TMV 30K-O) shows delays in both local and systemic spread in *N. tabacum* cv. Xanthi as compared to the wild-type TMV, suggesting that the impaired systemic movement of TMV 30K-O is due to its delayed cell-to-cell movement. Conversely, chimeric TMV expressing its own MP and ORSV CP (TMV CP-O) exhibits the same rate of cell-to-cell spread as TMV, but its systemic spread is delayed, indicating the function of CP in host-specific parameters of viral systemic movement (Hilf and Dawson, 1993).

The role of CP in determination of host specificity of systemic movement was further demonstrated when TMV CP was replaced with CP of an unrelated virus AMV. AMV systemically infects spinach (*Spinacia oleracea*), and its CP is known to be required for virion assembly (Jaspars, 1974; Reusken *et al*., 1997) and viral long-distance movement (van der Kuyl *et al*., 1991). Exchanging TMV CP with that of AMV conferred on the resulting chimeric TMV the ability to systemically infect spinach, which serves only as a local infection host for the wild-type TMV (Spitsin *et al*., 1999).

The mechanism by which CP potentiates viral systemic movement is unknown. Generally, CP may have a dual function: it passively protects the encapsidated viral genome from cellular nucleases of the vascular system and mediates the passage of the spreading virus into the vasculature from the surrounding nonvascular tissues. This latter activity is supported by the detailed immunolocalization studies of three mutant strains of TMV: TMV CP-O, which expresses ORSV CP instead of TMV CP and shows delayed systemic movement; CP(U1mCP15-17), which expresses a TMV CP mutant with three amino acid substitutions, Ser15Pro, Ser16Pro, and Ala17Arg, and is deficient for encapsidation but not for systemic movement; and SNC015, which lacks the CP initiation codon and thus does not express CP (Ding *et al*., 1996). While all three mutant strains enter the VP cells, only TMV CP-O and CP(U1mCP15-17) are found within CC, albeit with lower frequency than the wild-type TMV, suggesting that CP is required for viral transport across the VP-CC boundary. These observations also suggest that CP is not required for viral transport from ME to VP (Ding *et al*., 1996). Potentially, crossing the ME-VP boundary can be accomplished by the viral MP, which remains functional in all three mutant TMV strains. However, previous observations indicate that TMV MP alone, which in nonvascular tissues is known to localize to and gate plasmodesmata (Ding *et al*., 1992a; Tomenius *et al*., 1987; Waigmann *et al*., 1994), also accumulates within plasmodesmata of the vascular cells, such as BS and VP, but fails to increase their permeability (Ding *et al*., 1992a), suggesting that yet another viral factor is required for this function. Potentially, the TMV 126 kDa protein could represent such factor.

4.1.1.2. The 126 kDa Protein of TMV. The TMV 126 kDa protein is encoded by the first open reading frame of the TMV genome and represents an early termination derivative of the viral 183 kDa protein. The 126 kDa protein possesses methyl transferase (MT)- and helicase (HEL)-like domains, whereas the full-length 180 kDa protein also contains an RNA-dependent RNA polymerase domain (Ishikawa *et al*., 1986, 1991a; Lewandowski and Dawson, 2000). The primary function of 126 kDa protein is to form a heterodimer with the 183 kDa protein, increasing the rate of replication. However, the 126 kDa protein may also participate, alongside with the viral MP and CP, in the TMV systemic movement.

Amino acids substitutions in the 126 kDa protein modulate the virulence of TMV, most likely by augmenting its systemic movement. Specifically, the 126 kDa protein is responsible for the differences in symptoms induced by attenuated and normal TMV strains (Holt *et al*., 1990; Lewandowski and Dawson, 1993; Nishiguchi *et al*., 1985). For example, compared to the TMV-U strain, the Holm's masked (TMV-M) strain accumulates only at low levels in vascular tissues in the infected leaves as well as in the systemic leaves of *N. tabacum* cv. Xanthi nn, although its local movement in the same leaves is not compromised (Ding *et al*., 1995b; Nelson *et al*., 1993). Similarly, a chimeric virus strain Mic-TMV, containing a small portion of TMV-U fused to the M strain sequence, which includes the gene encoding the 126/183 kDa proteins, induces the same attenuated symptoms in *N. tabacum* cv. Xanthi nn as the M strain itself (Nelson *et al*., 1993). The TMV-U and M strains differ from each other within their 126 kDa protein-coding sequences in 28 nucleotide substitutions, 20 of which are silent and 8 of which alter the amino acid composition of the 126 kDa protein. Importantly, specific replacements of these residues in the Mic-TMV strain with those that correspond to the TMV-U strain result in severe disease symptoms in *N. tabacum* cv. Xanthi NN (Shintaku *et al*., 1996). Furthermore, replacing only two amino acids of TMV-Mic in

the region in between the putative MT and HEL domains of the 126 kDa protein with the corresponding residues of TMV-U is sufficient to cause the TMV-U–like severity of symptoms (Bao *et al*., 1996), demonstrating that the 126 kDa protein is responsible for variations in the systemic symptoms. A study of the rakkyo strain of TMV, TMV-R, which causes only latent infection in inoculated leaves of *N. tabacum* cv. Bright Yellow (BY), revealed that substituting its 126/183 kDa replicase sequence with that of TMV-U causes the resulting recombinant TMV-R to elicit the TMV-U–like symptoms in BY plants (Chen *et al*., 1996). Finally, the presence of a functional 126 kDa protein was implicated in systemic movement of defective RNAs (dRNAs) of TMV (Knapp *et al*., 2001).

How does the 126 kDa protein exert its effect on the viral systemic movement? Although not essential for TMV replication, the 126 kDa protein modulates this process (Ishikawa *et al*., 1986, 1991a), suggesting that mutations in the 126 kDa protein may reduce systemic infection indirectly, simply by lowering the efficiency of viral replication. Indeed, even in the inoculated leaves of BY plants, TMV-R accumulated to lower levels that TMV-U (Chen *et al*., 1996). However, viral replication rates do not always correlate with the extent of systemic infection (Traynor *et al*., 1991). Also, that TMV still moves systemically, even when its replication within stem phloem is blocked, by maintaining a portion of the stem at 4°C (Susi *et al*., 1999) suggests that reduction in TMV replication, at least in the stem, does not abolish viral systemic movement. Thus, the 126 kDa protein may affect viral systemic movement more directly, for example, by helping the virions to move through the ME-VP boundary; as mentioned above, the activities of MP and CP appear to be insufficient to promote this movement. Alternatively, the 126 kDa protein may act to suppress silencing of the viral genomes, which represents one of the host defense responses against the spreading virus (reviewed by Moissiard and Voinnet, 2004; van der Boogaart *et al*., 1998; Voinnet, 2001). Indeed, a recent study of the 130 kDa protein of ToMV, a close homolog of the TMV 126 kDa protein, demonstrated that this viral replicase suppresses PTGS, potentially, by preventing *de novo* formation of the antiviral PTGS system (Kubota *et al*., 2003).

4.1.2. Potyviruses

Potyvirus single-stranded, positive-sense genomic RNA encodes a single polyprotein cleaved to give as many as 10 viral proteins (reviewed by Revers *et al*., 1999; Urcuqui-Inchima *et al*., 2001). Potyviruses do not encode a dedicated MP; instead, the viral local movement functions are fulfilled by CP (Dolja *et al*., 1994, 1995; Rojas *et al*., 1997), HC-Pro (Rojas *et al*., 1997), and CI (Carrington *et al*., 1998), whereas the systemic movement is mediated by CP, HC-Pro, and VPg proteins.

4.1.2.1. CP. Several mutagenesis studies have shown that CP is involved in potyviral systemic movement and that this function is distinct from the CP encapsidation activity. The potyviral CP possesses distinctive N-terminal, central, and C-terminal domains. The N- and C-terminal domains are located on the surface of the virion, show variability among different potyviruses (Allison *et al*., 1985; Shukla *et al*., 1988), and may be involved in viral systemic movement. For example, TEV mutants with deletions in the CP N-terminal domain produce virions *in vivo* but exhibit defects in their long-distance movement (Dolja *et al*., 1994). Specifically, deleting 25 N-terminal residues of CP of TEV, which expresses GUS as reporter (TEV-GUS), did not affect virion assembly and replication in plant protoplasts. The mutant virus also moved cell to cell, although more slowly than its parental TEV-GUS, but was incapable of moving systemically (Dolja *et al*., 1994). *In situ* GUS staining of the infected plants revealed viral presence in the areas adjacent to the vascular tissue but not within the vasculature itself, suggesting that the 25 N-terminal residues of CP are essential for viral transport through vascular tissues (Dolja *et al*., 1994). The role of the C-terminal part of CP in systemic movement was studied using a TEV mutant, in which 17 C-terminal residues of CP were deleted. Similar to the N-terminal deletion mutant of TEV CP (Dolja *et al*., 1994), its C-terminal deletion mutant does not move systemically even though it retains its abilities to replicate, form capsids, and move locally from cell to cell, albeit more slowly than the parental line (Dolja *et al*., 1995). Interestingly, supplying the wild-type TEV CP *in trans* by expression in transgenic plants rescued the systemic transport of the N-terminal CP deletion mutant but not of the C-terminal CP deletion mutant, although the cell-to-cell transport efficiency was enhanced for both of these mutants (Dolja *et al*., 1995). Thus, N- and C-terminal domains of TEV CP play important and distinct roles in systemic movement of this virus.

Substituting basic amino acids Lys or Arg for the Asp residue in the Asp-Ala-Gly (DAG) motif, which is located in the N-terminal part of CP and is conserved among many potyviruses, disrupts systemic movement of two potyviruses, TVMV and TEV, although their encapsidation activity remains intact (Lopez-Moya and Pirone, 1998). Also, a single amino acid substitution in the N-terminal portion of PSbMV CP limits its systemic movement in some hosts (Andersen and Johansen, 1998). Two isolates of PSbMV, DPD1 and NY, infect pea (*P. sativum*) systemically, but NY cannot move long distances in *Chenopodium quinoa*, accumulating only in the inoculated leaves, whereas DPD1 establishes systemic infection in this host (Andersen and Johansen, 1998). Mutational analysis demonstrated that, among three amino acid differences found between CPs of these two isolates, replacing the Ser residue at position 47 of the NY CP with Pro, which is found at the same position in DPD1, is sufficient to allow systemic spread in *C. quinoa*; and the reverse mutant, in which Pro of the DPD1 CP is substituted with Ser, becomes restricted within the inoculated leaves (Andersen and Johansen, 1998). This role of CP Ser-47 in restricting PsbMV systemic movement is likely specific for the NY and DPD1 isolates because other isolates of this virus that contain Ser-47 in their CPs still exhibit systemic movement in their hosts (Andersen and Johansen, 1998).

In the case of *Zucchini yellow mosaic potyvirus* (ZYMV), substituting 43 N-terminal residues of CP with Myc-tag neither

impairs systemic movement in cucurbits (squash, melon, and cucumber) nor affects encapsidation (Arazi *et al*., 2001). The surface of mutant virions was labeled by anti-Myc antibody and immunogold, supporting the earlier observation that the N-terminal domain of potyviral CP is exposed on the surface of the virion. However, when the N-terminus of ZYMV CP is tagged with the 16-amino-acid–long immunogenic epitope of foot-and-mouth disease virus, the viral systemic movement is suppressed; further addition of the Myc-tag to the N-terminus of this fusion CP rescued the systemic infection (Arazi *et al*., 2001). Thus, unlike TEV CP (Dolja *et al*., 1994) and TVMV CP (Lopez-Moya and Pirone, 1998), the intact N-terminus of ZYMV CP is not essential for systemic movement of ZYMV in the host plants.

4.1.2.2. HC-Pro. Helper component-proteinase (HC-Pro) is a multi-functional potyviral protein that has a papain-like cysteine proteinase activity and is essential for aphid transmission, cell-to-cell movement, suppression of PTGS, and symptom development (reviewed by Revers *et al*., 1999; Urcuqui-Inchima *et al*., 2001). HC-Pro also plays a critical role in potyviral systemic movement, and a highly conserved sequence in the central part of HC-Pro is involved in this activity. Specifically, substituting an RPA amino acid sequence for the conserved protein motif CCE between positions 293 and 295 of HC-Pro of TEV-GUS dramatically suppresses systemic movement while having only minor effects on viral replication or cell-to-cell movement (Cronin *et al*., 1995). The systemic movement ability of this HC-Pro mutant is restored in transgenic plants expressing the wild-type HC-Pro, supporting the role of this protein in viral systemic transport. *In situ* GUS staining reveals that the HC-Pro mutant TEV-GUS virus enters into CC adjacent to the local infected foci, indicating that its systemic movement is likely arrested at the step of entering into or unloading from SE (Cronin *et al*., 1995). These possibilities were further examined in a later study, showing that inoculation of this HC-Pro mutant strain of TEV-GUS on hetero-grafted plants composed of the wild-type stock and HC-Pro–expressing transgenic scion (or vice versa) does not rescue systemic movement, most likely because the functional HC-Pro is required for both viral loading into and unloading from the host vasculature (Kasschau *et al*., 1997).

In another potyvirus, *Plum pox virus* (PPV), HC-Pro may represent a limiting factor for systemic infection of *N. tabacum* (Saenz *et al*., 2002). PPV systemically infects several species of the nicotiana genus including *N. clevelandii* and *N. benthamiana*; PPV also replicates in the inoculated leaves of *N. tabacum* cv. Xanthi-nc, but it fails to infect this host systemically. However, when PPV is inoculated on transgenic *N. tabacum* cv. Xanthi-nc plants that express the HC-Pro, P1, and P5 proteins encoded by the 5′-terminal part of the genome of TEV, which moves systemically in this host species, the systemic movement of PPV also occurs. The ability of these transgenic tobacco plants to allow PPV systemic infection was abolished by mutating the HC-Pro part of the transgene (Saenz *et al*., 2002), indicating that it is the HC-Pro protein that determines the host specificity of PPV and TEV systemic infection.

More recent studies showed that HC-Pro is an efficient suppressor of PTGS (Kasschau and Carrington, 1998; Kasschau *et al*., 2003; Mallory *et al*., 2001). Thus, HC-Pro may function in the systemic movement indirectly by suppressing silencing of the potyviral genomes.

4.1.2.3. VPg. Another potyviral factor involved in systemic movement is the viral genome-linked protein VPg, which is covalently attached to the 5′ end of viral genomic RNA and is essential for viral replication activity (reviewed by Revers *et al*., 1999; Urcuqui-Inchima *et al*., 2001). The role of VPg in systemic infection is inferred from the observations that both TEV-HAT and TEV-Oxnard can replicate and move cell to cell in *N. tabacum* cv. V20, but only TEV-Oxnard can spread systemically in this host (Schaad and Carrington, 1996; Schaad *et al*., 1997b). Comparison of systemic movement of chimeric viruses assembled from TEV-HAT and TEV-Oxnard reveals that the TEV-Oxnard VPg is the key component for infecting *N. tabacum* cv. V20 systemically (Schaad *et al*., 1997b). Within the VPg coding sequence, a 67-nucleotide segment containing 10 nucleotide differences, but only five amino acid differences, between TEV-HAT and TEV-Oxnard is responsible for controlling the viral systemic infection phenotype in *N. tabacum* cv. V20 (Schaad *et al*., 1997b).

Similar observations were obtained for PVA-M and PVA-B11 isolates of PVA in the host solanaceous plant species *Nicanda physaloides* (Rajamaki and Valkonen, 1999). In *N. physaloides*, PVA-M is restricted within the inoculated leaves, whereas PVA-B11 spreads systemically. When the genomic sequence of PVA-B11, which encodes the C-terminal part of CI, VPg, 6K2, and the N-terminal part of NIa-Pro proteins, is replaced with the corresponding region of PVA-M, the resulting chimeric strain B11-M loses its ability to move systemically. The replaced sequence contains four amino acid differences between the two isolates: one in the 6K2 protein and three in VPg, suggesting that both proteins may function in the systemic movement of PVA (Rajamaki and Valkonen, 1999). Because 6K2 exists as polyprotein with VPg and NIa in the infected cells (Restrepo-Hartwig and Carrington, 1994), it may function synergistically and in *cis* with VPg during the process of viral systemic movement.

In a different host, *i.e*., potato (*Solanum commersonii*), it is PVA-B11 that does not move systemically, probably due to its inability to enter the host plant phloem (Rajamaki and Valkonen, 2002). A single amino acid substitution, His118Tyr, in the central domain of the PVA-B11 VPg protein restores the systemic movement of PVA-B11 in potato, potentially allowing the virus to cross into the host vasculature; this mutation also elevates the level of virus accumulation in the infected tissues, including the phloem cells (Rajamaki and Valkonen, 2002). Additional amino acid substitutions in the central (residue 116) and C-terminal domains of VPg (residue 185) and in the N-terminus of the 6K2 protein (residue 5) alter virus accumulation and the rate of systemic infection but are not sufficient, if introduced by themselves, to restore phloem loading of PVA (Rajamaki and Valkonen, 2002). Thus, the central domain of PVA VPg is important for specific virus-host interactions that allow

systemic invasion of plants by PVA (Rajamaki and Valkonen, 2002).

How VPg facilitates the systemic transport remains unknown. A recent histochemical study suggests that VPg may specifically act in CC to facilitate viral unloading into the sink leaves (Rajamaki and Valkonen, 2003). When PVA is inoculated on potato plants, initial systemic infection foci develop in the vicinity of major and minor veins. In these foci, the viral CP, CI, and HC-Pro colocalize with viral genomic RNA in the VP and ME cells, but none of these PVA proteins are found in CC (Rajamaki and Valkonen, 2003). In contrast, VPg is detected within CC in the infection foci, but only at an early stage of virus unloading. Also, VPg (but not other PVA proteins or RNA) localizes exclusively within CC in all vein classes, suggesting that VPg targets to CC at the onset of systemic infection to facilitate virus unloading into the sink leaves of the host plant (Rajamaki and Valkonen, 2003). During this unloading into systemic organs, VPg, which is covalently linked to the viral genome, may direct the viral RNA–protein complex to and through plasmodesmata.

4.1.3. Umbraviruses

Umbraviruses are unusual in that that they do not encode a conventional CP and thus do not form true viral particles in the infected tissues (reviewed by Robinson and Murant, 1999). Nevertheless, umbraviruses rapidly establish systemic movement in the compatible hosts. One of the viral factors, the ORF3 protein of GRV, supports the long-distance transport of both the GRV RNA and the genomic RNA of a CP-less mutant of an unrelated virus, TMV (Ryabov *et al*., 1999b). In addition, when the GRV ORF3 protein is expressed from chimeric TMV, designated TMV(ORF3), in place of TMV CP, it binds the TMV(ORF3) RNA and facilitates its transport through the host plant vasculature, demonstrating that the GRV ORF3 protein can systemically transport heterologous RNA molecules in the form of ribonucleoprotein complexes (Taliansky *et al*., 2003). Moreover, chimeric TMV strains expressing, instead of their CP, the ORF3-encoded proteins from other umbraviruses, such as *Pea enation mosaic virus-2* (PEMV-2) and *Tobacco mottle virus* (TMoV), move systemically in *N. benthamiana* and *N. clevelandii* but not in *N. tabacum* (Ryabov *et al*., 2001b). Because *N. benthamiana* and *N. clevelandii* are systemic hosts for PEMV-2, TMoV, and TMV, while *N. tabacum* is a systemic host only for TMV but not for both of the umbraviruses (Ryabov *et al*., 2001b), the ORF3 protein may also determine the host specificity of the systemic transport process. While the mechanism of this ORF3 protein function is still unclear, simple protection of the viral RNA from cellular nucleases may not play a major role in establishing the host range of systemic transport because the ORF3 protein–RNA complexes are stable in cell extracts of both *N. benthamiana*, in which the ORF3 protein supports systemic infection, and *N. tabacum*, in which the ORF3 protein does not facilitate systemic infection (Ryabov *et al*., 2001b).

Electron microscopic studies showed that *in vivo*, within infected cells, binding of the ORF3 protein to RNA produces filamentous ribonucleoprotein particles with helical structure, but not as uniform as classical virions (Taliansky *et al*., 2003). The ORF3 protein forms oligomers *in vitro* and binds RNA consistent with its RNA binding activity *in vivo* (Taliansky *et al*., 2003). The ORF3 protein–RNA complexes are detected in all types of cells and are abundant in phloem-associated cells, especially in CC and immature SE (Taliansky *et al*., 2003); this accumulation of the ORF3 protein within the host plant vasculature is consistent with the biological role of this protein as a facilitator of umbraviral systemic transport.

4.2. Host Factors Involved in Systemic Movement

Generally, the known cellular factors involved in viral systemic spread belong to two functional groups: those that facilitate movement and those that block or restrict it. Our knowledge about the *facilitators* is less advanced than our knowledge about the *blockers*. Because such host functions are often determined by reverse genetics, using gene knockouts or knockdowns, it is possible that the facilitating factors are more vital for the plant life cycle and, thus, are more recalcitrant to discovery and characterization by this approach.

4.2.1. Cellular Factors that Facilitate Viral Systemic Movement

One of the cellular factors that promote viral systemic movement may be encoded by the *VSM1* gene of arabidopsis. An arabidopsis mutant, designated *vsm1* (virus systemic movement) and isolated from a chemically mutagenized plant population, shows significant delays in the systemic movement of TVCV (Lartey *et al*., 1998). Upon inoculation with TVCV, the *vsm1* plants do not exhibit the symptoms of the viral disease and do not accumulate TVCV virions or viral genomic RNA in their uninoculated, systemic organs. The local viral movement within the inoculated *vsm1* leaves, however, is not affected. TVCV systemic movement within the *vsm1* plants is likely blocked at the step of viral entry into the host plant vasculature from the infected leaf tissue (Lartey *et al*., 1998). Interestingly, the *vsm1* plants also restrict systemic movement of another tobamovirus, TMV, but not of an unrelated caulimovirus, CaMV (Lartey *et al*., 1998). The identity of the *VSM1* gene and its protein product remains unknown.

Another cellular factor shown to be required for systemic transport of tobamoviruses is PME. This cell-wall protein is especially interesting because of its involvement in both local (Chen *et al*., 2000; Dorokhov *et al*., 1999; see Section 3.5) and systemic viral movement (Chen and Citovsky, 2003). The role of PME in viral systemic movement was demonstrated using antisense suppression of its gene in tobacco plants, which preferentially occurs within the vascular tissues (Chen and Citovsky, 2003). TMV accumulation in uninoculated leaves of these PME antisense plants is significantly delayed, indicating impaired systemic transport of this virus. On the other hand, no differences were detected in vascular loading and unloading of a fluorescent solute between the PME antisense plants and wild-type tobacco, indicating that PME is not involved in phloem transport

of solutes. Immunofluorescence confocal microscopy analysis demonstrated that, in the PME antisense plants, TMV virions enter the host vasculature but fail to exit into uninoculated nonvascular tissues (Chen and Citovsky, 2003). Thus, TMV systemic movement may be a polar process in which the virus enters and exits the vascular system by two different mechanisms, and it is the viral egress out of the vascular system that involves PME.

4.2.2. Cellular Factors that Restrict Viral Systemic Movement

Often, plant resistance to viral disease is due to the suppression of viral systemic rather than local movement. For example, impaired viral systemic movement underlies the naturally occurring resistance of pepper to *Pepper mottle potyvirus* (PepMoV) (Guerini and Murphy, 1999; Murphy *et al*., 1998; Murphy and Kyle, 1995), potato to PVX (Hamalainen *et al*., 2000) and PLRV (Derrick and Barker, 1992, 1997), tobacco to the TEV-HAT isolate of TEV (Schaad and Carrington, 1996), barley to CCMV (Goodrick *et al*., 1991), cucumber to the Florida strain of *Tomato aspermy cucumovirus* (TAV) (Thompson and Garcia-Arenal, 1998), and several arabidopsis ecotypes to TEV (Mahajan *et al*., 1998). Thus, restriction of viral systemic movement may represent one of the common plant defense strategies against viral diseases. Below, we focus on several major cellular factors identified to date that are known to restrict systemic movement of plant viruses.

4.2.2.1. RTM Genes. The discovery of the restricted TEV movement (*RTM)* genes was based on the observation that while some arabidopsis ecotypes, such as C24 and Landsberg *erecta* (La-*er*), support both the local and long-distance spread of TEV, other ecotypes such as Columbia-0 (Col-0), Col-3, Wassilewskija-2 (Ws-2), and several others allow only the local, cell-to-cell movement (Mahajan *et al*., 1998; Whitham *et al*., 1999). These latter ecotypes restrict TEV systemic movement by a mechanism different from the classic hypersensitive response (HR) and systemic acquired resistance (SAR) pathways for plant defense against pathogens. HR involvement was ruled out due to the absence of local lesions in the inoculated tissues of the restrictive ecotypes. SAR was excluded because Col-0 plants transgenic for the *NahG* gene, which do not develop SAR due to conversion of salicylic acid to catechol by the *NahG*-encoded salicyalte hydroxylase (Gaffney *et al*., 1993), are still unable to support TEV systemic infection. In addition, TEV does not move systemically in Col-0 plants carrying *npr1* alleles that are unable to activate SAR, and in Col-0 plants with *ndr1* and *pad4* alleles that do not develop R-gene-mediated resistance (Mahajan *et al*., 1998).

Genetic crosses between the susceptible and resistant arabidopsis ecotypes identified and mapped a dominant *RTM1* locus in Col-3 plants that restricts TEV systemic movement (Mahajan *et al*., 1998). A genetic screen was then devised to isolate *RTM1*-suppression mutants by mass inoculating mutagenized arabidopsis plants with airbrush and a genetically engineered TEV strain carrying a *bar* gene (TEV-bar), which confers herbicide resistance on the systemically infected hosts (Whitham *et al*., 1999). Using this approach, another dominant *RTM* locus, *RTM2*, was identified by infecting mutagenized Col-0 plants with TEV-bar followed by screening for herbicide-resistant phenotypes that indicate TEV systemic infection; as expected, this genetic screen also identified mutants in the *RTM1* locus itself (Whitham *et al*., 1999). Thus, the *RTM1* and *RTM2* loci are responsible, potentially in a cooperative fashion, for production of a restricted TEV movement phenotype (Whitham *et al*., 1999). The restrictive effects of *RTM1* and *RTM2* on viral systemic movement in the Col-3 and Ws-2 ecotypes are very specific for TEV because the presence of these loci does not affect systemic spread of other potyviruses, such as *Potato virus Y* (PVY), TVMV, and *Turnip mosaic virus* (TuMV), as well as unrelated viruses such as CMV (Chisholm *et al*., 2000).

Subsequently, *RTM1* and *RTM2* genes were cloned and characterized (Chisholm *et al*., 2000, 2001; Whitham *et al*., 2000). *RTM1* and *RTM2* are expressed exclusively in the phloem-associated cells, and their protein products accumulate in sieve elements (Chisholm *et al*., 2001), consistent with the effect of these loci on TEV systemic rather than local movement. Amino acid sequence analyses of these proteins indicate that RTM1 is similar to a lectin jacalin, suggesting its involvement in plant defense (Chisholm *et al*., 2000), whereas RTM2 contains a heat shock protein (HSP) motif (Whitham *et al*., 2000), suggesting a chaperone-like activity (Chisholm *et al*., 2001). These putative activities, however, may not represent the still unknown mechanisms of RTM-mediated restriction of TEV systemic movement because jacalin-like proteins function in plant defense pathways that are distinct from virus resistance (Chisholm *et al*., 2000), and, unlike most other plant genes encoding small HSPs, expression of *RTM2* is not heat-inducible and does not affect thermotolerance (Whitham *et al*., 2000).

In addition, to *RTM1* and *RTM2*, a third locus, *RTM3*, may be involved in restriction of TEV systemic movement in the Col-0 ecotype (unpublished data cited by Chisholm *et al*., 2001). The identity of this gene has not yet been reported.

4.2.2.2. Cell-Wall Components: cdiGRP, Callose, and β-1,3-Glucanase. The most logical candidates for host factors involved in viral movement between cells may be found in the plant cell wall that contains plasmodesmata, through which viruses actually move. Indeed, increasing evidence implicates cell-wall proteins and polysaccharides in positive and negative regulation of the movement process. For example, as described in Sections 3.5 and 4.2.1, PME, a cell wall enzyme, promotes optimal cell-to-cell and systemic movement of tobamoviruses (Chen and Citovsky, 2003; Chen *et al*., 2000; Dorokhov *et al*., 1999), whereas several other cell-wall components restrict viral systemic movement.

One such negative regulator of viral systemic movement is a cadmium (Cd)-induced, glycine-rich protein, cdiGRP, recently discovered in tobacco plants (Ueki and Citovsky, 2002). Identification of cdiGRP is based on the observations that systemic movement of tobamoviruses, such as TVCV and TMV, is blocked in tobacco plants pretreated with low concentrations of heavy metal cadmium, while local virus movement in these

plants is not affected (Citovsky *et al*., 1998; Ghoshroy *et al*., 1998). This inhibitory effect of cadmium ions is tobamovirus-specific because it is not observed when cadmium-treated plants are inoculated with other, unrelated viruses, such as TEV (Ghoshroy *et al*., 1998); interestingly, however, cadmium treatment also inhibits systemic spread of PTGS in *N. tabacum* and *N. benthamiana* plants (Ueki and Citovsky, 2001). Cadmium-induced inhibition of viral systemic spread occurs by a SAR-independent mechanism because it is also observed in *NahG*-expressing transgenic plants (Citovsky *et al*., 1998), which are unable to accumulate salicylic acid and develop SAR (Gaffney *et al*., 1993). Immunodetection of tobamoviral CP revealed that the virus accumulates in the vasculature of uninoculated, systemic leaves but not in the surrounding mesophyll cells, indicating that in cadmium-treated plants, the spreading virions enter into but do not exit out of the host plant vascular system (Citovsky *et al*., 1998). Because exposure of plants to higher, toxic cadmium ion concentrations restores viral systemic movement (Ghoshroy *et al*., 1998), low levels of cadmium ions most likely induce production of host factors controlling viral systemic transport, whereas cadmium ion poisoning prevents this response. Using PCR-based subtraction cloning, a tobacco *cdiGRP* gene was isolated, the expression of which is induced by low but not by high concentrations of cadmium ions (Ueki and Citovsky, 2002). Cadmium-induced expression of cdiGRP is tissue specific, with the protein found mainly in the cell walls of the plant vasculature. Importantly, constitutive expression of cdiGRP in transgenic plants significantly reduces tobamoviral systemic movement in the absence of cadmium, while antisense suppression of cdiGRP allows virions to spread systemically even in cadmium-treated plants (Ueki and Citovsky, 2002).

cdiGRP does not directly restrict viral movement. Instead, this protein induces—by an as-yet unknown mechanism—callose formation within the cell walls of the phloem; these callose deposits, in turn, most likely reduce viral transport from the phloem into the surrounding nonvascular cells (Ueki and Citovsky, 2002). Callose is a 1,3-β-D-glucan (Stone and Clarke, 1992) deposited between the plasma membrane and the cell wall, often in the vicinity of plasmodesmata (Northcote *et al*., 1989; Ueki and Citovsky, 2002). Degradation of callose is thought to increase plasmodesmal permeability (Botha *et al*., 2000; Northcote *et al*., 1989), whereas its deposition is believed to restrict intercellular transport (Bucher *et al*., 2001; Delmer *et al*., 1993; Iglesias and Meins Jr., 2000). Thus, callose represents a polysaccharide cell wall component that restricts viral movement.

Callose amounts in the cell walls are directly controlled by the balance of two opposing enzymatic activities: callose synthase that produces callose and β-1,3-glucanase that hydrolyzes callose (Kauss, 1985, 1996). Plant callose synthases are still poorly characterized; β-1,3-glucanases are better studied. Plant β-1,3-glucanases are grouped into three classes according to their structure (reviewed by Beffa and Meins Jr., 1996; Leubner-Mezger and Meins Jr., 1999). Class I β-1,3-glucanases are basic proteins localized in the vacuole of mesophyll and epidermal cells, and class II and III β-1,3-glucanases are acidic isoforms secreted into the cell walls. Class II β-1,3-glucanases include pathogenesis-related (PR) proteins PR2, PR N, and PR O, and class III consists of a single member, PR-Q′ (reviewed by Beffa and Meins Jr., 1996; Leubner-Mezger and Meins Jr., 1999). By virtue of their ability to regulate the amount of callose, which then restricts intercellular transport, β-1,3-glucanases can be regarded as cellular factors that control viral movement. Indeed, TMV infection of tobacco plants elevate the β-1,3-glucanase activity, which presumably enables more efficient viral movement (reviewed by Beffa and Meins Jr., 1996). Conversely, antisense suppression of β-1,3-glucanase in nicotiana species results in increased callose deposits in the cell wall (Beffa *et al*., 1996), reduced plasmodesmal permeability (Iglesias and Meins Jr., 2000), and delayed local and systemic movement of such viruses as TMV, TNV, and PVX (Beffa *et al*., 1996; Iglesias and Meins Jr., 2000). Similarly, overexpression the β-1,3-glucanase coding sequence from TMV-based vector facilitates viral movement, whereas antisense expression of the same sequence delays viral movement in the inoculated leaf (Bucher *et al*., 2001). Thus, induction of callose accumulation by an abiotic stimulus, *i.e*., cadmium ions *via* cdiGRP (Ueki and Citovsky, 2002), or by antisense suppression of β-1,3-glucanases (Beffa *et al*., 1996; Iglesias and Meins Jr., 2000) negatively regulates systemic and/or cell-to-cell transport of plant viruses.

In addition to these cellular factors that regulate the viral transport *per se*, apparent restriction of viral systemic movement is achieved by PTGS of the viral genomes (reviewed by Moissiard and Voinnet, 2004; van der Boogaart *et al*., 1998; Voinnet, 2001). Because PTGS as a host antiviral response effectively destroys viral genomic molecules rather than interfering with their intercellular transport, it is beyond the scope of this review.

4.3. Cellular Routes for Systemic Movement

Unlike viral cell-to-cell movement, which occurs relatively uniformly within and between different nonvascular tissues, the systemically moving viruses must cross several discrete cellular boundaries, each of which may potentially impede the movement process. As mentioned in Section 4, to move long distances, plant viruses must invade the host vasculature by crossing through BS into VP, enter the CC/SE complex, spread through SE, and unload within the systemic organs through their CC and VP into the nonvascular ME tissues (Figure 2). This movement parallels the known route of photoassimilate transport from the source-to-sink tissues (Leisner and Turgeon, 1993; Oparka and Santa Cruz, 2000; Santa Cruz, 1999). This transport similarity has been confirmed by the studies that compared systemic movement of a GFP-expressing recombinant PVX with that of a photoassimilate transport tracer CF (Roberts *et al*., 1997). However, although plant viruses and photoassimilates follow the same cellular routes, the mechanisms underlying these two systemic transport processes likely differ, at least in the components of their protein machinery. For example, while TMV systemic movement involves PME, the transport of solutes does

not (Chen and Citovsky, 2003). Also, as described in Section 4.1, viruses require their own proteins (*e.g.*,CP, VPg, HC-Pro, etc.) for systemic movement, while transport of photoassimilates obviously does not require these protein functions.

4.3.1. Invasion of Vasculature Through the BS-VP Cell Boundary

GFP-expressing recombinant TMV was used to characterize the virus vascular invasion routes in the source leaves of *N. benthamiana*, demonstrating viral loading into minor (classes IV and V) as well as major veins (classes III and larger) (for vein classification, see Roberts *et al*., 1997). Thus, all vein classes function equally as gateways for TMV entry into the vasculature of the source leaves (Cheng *et al*., 2000). During the vascular invasion process, the first cell type that the virus encounters is BS. Presumably, viruses enter BS cells by the cell-to-cell movement mechanism, whereas viral transport from BS into VP occurs by a different pathway. Indeed, TMV MP, which in nonvascular tissues is sufficient to gate plasmodesmata (Ding *et al*., 1992a; Tomenius *et al*., 1987; Waigmann *et al*., 1994), accumulates within plasmodesmata between BS and VP but does not increase their permeability (Ding *et al*., 1992a). Thus, the BS-VP boundary in the inoculated leaf represents the first barrier that virus encounters during long-distance movement (Figure 2).

Illustrating the biological relevance of the BS–VP barrier, viral transport can be specifically blocked at this cellular interface in some hosts. For example, CCMV systemic movement is arrested in BS cells of a resistant cultivar of soybean, and this restriction is responsible for the resistance phenotype (Goodrick *et al*., 1991). Transgenic tobacco plants overexpressing CMV replicase do not support CMV systemic infection due to the block in virus translocation from BS to VP (Wintermantel *et al*., 1997). Also, in cucumber cotyledons, a chimeric cucumovirus strain expressing CP of the Florida strain of TAV, whose systemic movement is restricted in cucumber plants, accumulates in BS but does not enter into VP (Thompson and Garcia-Arenal, 1998).

Once viruses have crossed the BS–VP boundary and invaded VP cells, systemic movement is initiated, indicating that VP represent the starting point of this transport process. Indeed, detailed microscopy studies demonstrated that invasion of the vascular system of solanaceous (*N. benthamiana, C. annuum, N. tabacum* cv. Xanthi nn, and *L. esculentum*) and fabaceous host plant species (*Phaseolus vulgaris* and *P. sativum*) by tobamoviruses TMV and *Sunnhemp mosaic virus* (SHMV) and potyviruses PVY and *Peanut stripe virus* (PStV, also known as *Bean common mosaic virus*, BCMV) begins from VP (Ding *et al*., 1998). Interestingly, once in the phloem, some viruses, such as TMV and CMV, do not require replication for their systemic movement (Susi *et al*., 1999; Wintermantel *et al*., 1997).

4.3.2. Entry into the CC/SE Complex Through the VP-CC Boundary

Once in VP, plant viruses continue their journey into the CC/SE complex. To this end, they must first enter the CC cells, crossing the VP–CC boundary. The existence of this boundary is evident from experiments with the CP-less SNC015 mutant of TMV mutant, which is able to cross the BS–VP boundary and accumulate in VP but fails to cross the VP–CC boundary and remains excluded from CC (see Section 4.1; Ding *et al*., 1996). Thus, the VP–CC boundary represents the second barrier for the viral systemic movement (Figure 2).

In the CC/SE complex, only the enucleate SE, which are connected by plasma-membrane–lined pores into tubes, allow virtually unrestricted pressure-driven flow of solutes and macromolecules throughout the plant while CC supplies SE with most cellular maintenance functions (reviewed by Lucas and Gilbertson, 1994; Lucas *et al*., 2001). Thus, to spread systemically, the traveling virions must leave CC and enter SE. Presumably, this transport occurs through specialized deltoid-shaped plasmodesmata that connect CC and SE. Macromolecular transport through these plasmodesmata is polar such that cellular endogenous proteins synthesized within CC move into SE but fail to be transported back into VP, BS, or ME (reviewed by Lucas and Gilbertson, 1994).

MPs of several plant viruses, such as PLRV and CMV, localize to plasmodesmata at the CC–SE boundary (Blackman *et al*., 1998; Hofius *et al*., 2001; Schmitz *et al*., 1997). Furthermore, GFP-tagged CMV expressed from a CC-specific promoter of *Commelina yellow mottle virus* (ComYMV) is transported into SE, indicating its ability to gate the plasmodesmata that connect these cells; this transport is specific because dimeric GFP also expressed from the ComYMV promoter remains confined to CC (Itaya *et al*., 2002). Consistent with the MP role during viral transport from CC into SE, in CMV-infected *N. clevelandii* CMV virions are found in SE but not in CC, suggesting that CMV genomes translocate into SE as MP-RNA complexes and form virions only within SE (Blackman *et al*., 1998). It is tempting to speculate that because TMV MP alone cannot overcome the BS–VP and VP–CC boundaries (Ding *et al*., 1992a, 1996), whereas CMV MP is sufficient to move from CC to SE and from CC to VP (Itaya *et al*., 2002), viral factors involved in systemic but not cell-to-cell movement, e.g., CP, *etc*. (see Section 4.1), are required only to cross from BS to VP to CC but not to enter SE; alternatively, these observations may simply reflect functional differences between MPs of TMV and CMV.

4.3.3. Viruses Move Differently Through Internal and External Phloem

Having penetrated the SE tubes of the inoculated leaves, viruses move throughout the plant. Often, this long-distance transport proceeds at different rates and in two directions: faster, upward movement and slower, downward movement (Andrianifahanana *et al*., 1997; Cheng *et al*., 2000). In several viruses, such as TMV, PepMoV, and PLRV, these two movement modes occur through structurally different types of phloem, external and internal (Figure 2). The external and internal phloem in the transport veins of petioles and stems of such plant families as Solanaceae, Cucurbitaceae and others derive from the

abaxial (facing away from the axis of the plant and located on the underside of the leaf) and adaxial phloem (facing toward the axis of the plant and located on the upper side of the leaf), respectively, of the major leaf veins (Cheng *et al*., 2000; Turgeon, 1989).

In *N. benthamiana* inoculated with a GFP-expressing recombinant strain of TMV, the GFP fluorescence is detected in the external phloem and external phloem-associated cells of the stem internode below the inoculated leaf and exclusively in the internal phloem and internal phloem-associated cells of the stem internode above the inoculated leaf. These two opposing venues of viral transport are almost independent because little transport is detected between the internal and external phloem of the stem (Cheng *et al*., 2000). Similarly, systemic infection of pepper plants by the Florida isolate of PepMoV (PepMoV-FL) follows a defined pattern of downward movement through external phloem and upward movement through internal phloem (Andrianifahanana *et al*., 1997) whereas the virus-resistant pepper cultivar *C. annuum* cv. Avelar allows downward movement of PepMoV-FL through external phloem but restricts the upward movement through internal phloem, resulting in virus-free young stem tissues (Guerini and Murphy, 1999). Finally, differential involvement of internal and external phloem in viral systemic movement was also shown using PLRV-resistant potato plants in which virus is restricted to internal phloem, whereas both internal and external phloem display PLRV accumulation in the susceptible potato plants (Barker and Harrison, 1986; Derrick and Barker, 1992, 1997). Thus, plant viruses may move to the source leaves, downward from the inoculated leaf, through the abaxial phloem of leaves and external phloem of petioles and stems, but utilize the adaxial leaf phloem and internal petiole and stem phloem for their upward movement to the sink leaves. Interestingly, some plant organs, *e.g.*,apical shoot meristem, restrict movement of viruses, such as TMV, remaining permanently virus-free (Cheng *et al*., 2000). Plant roots, on the other hand, usually become infected during virus downward movement; for example, TVCV virions have been found within the root phloem of arabidopsis (Lartey *et al*., 1997).

4.3.4. Virus Unloading from Phloem into Systemic Organs

For most viruses, except those that are phloem-limited, *e.g.*, luteoviruses (Mayo and Ziegler-Graff, 1996; Smith and Barker, 1999) and bipartite geminiviruses (Morra and Petty, 2000; Qin and Petty, 2001), the last step of the process of their systemic movement is unloading from the phloem into the surrounding nonvascular tissues. This unloading of the virus is essential for development of viral diseases, and its inhibition—for example, by exposure of the plants to low concentration of cadmium ions (see Section 4.2.2.2; Citovsky *et al*., 1998; Ghoshroy *et al*., 1998; Ueki and Citovsky, 2002)—prevents formation of the disease symptoms. Perhaps unexpectedly, viral exit from the phloem is not simply a reversed process of their entry into this tissue. Increasing evidence suggests that these processes occur by different mechanisms. For example, while all veins—major and minor—function equally during loading of GFP-expressing recombinant TMV into the phloem of the source leaves (Cheng *et al*., 2000; also see above), only major veins (class III and larger) unload the virus in the sink leaves of *N. benthamiana* (Cheng *et al*., 2000). Similarly, GFP-expressing recombinant PVX unloads predominantly from the class III veins, while the class VI and V veins play no role in unloading of the virus; they eventually became infected simply by cell-to-cell movement of the virus from the mesophyll (Roberts *et al*., 1997).

Virus unloading patterns also change as the leaves undergo sink-to-source transition. In tobacco leaves, this transition occurs basipetally, from apex to base, with the apical part of the leaf already functioning as source and the basal part still as sink (Roberts *et al*., 1997; Turgeon, 1989; Figure 2). In such transition leaves, GFP-expressing recombinant PVX is unable to unload in the source portions of the leaf (Roberts *et al*., 1997). Thus, the sink-to-source transition events likely alter leaf vasculature in an as yet unknown fashion, perhaps by causing its symplastic isolation or by creating an obstacle for viral unloading from these modified veins.

Interestingly, in the dicotyledonous *N. benthamiana* plant, the specific patterns of virus unloading and vein involvement mirror those of the fluorescent solute CF, although viral unloading occurs considerably slower than that of the much smaller CF (Roberts *et al*., 1997). Parallels in unloading of viruses and solutes were also found in monocotyledonous plants, such as barley, in which unloading patterns of CF and GFP-expressing recombinant BSMV display a striking similarity, with both CF and the virus exiting major longitudinal veins and entering mesophyll (Haupt *et al*., 2001).

To date, two host factors have been identified that affect viral unloading: PME and cdiGRP (Chen and Citovsky, 2003; Ueki and Citovsky, 2002). As described in detail in Sections 4.2.1 and 4.2.2, respectively, PME is required for the systemic transport of tobamoviruses, whereas cdiGRP negatively regulates this process. Both proteins, however, appear to affect viral unloading such that reduced levels of PME expression or elevated levels of cdiGRP expression in tobacco vasculature trap the virus within the phloem of the systemic leaves, precluding its unloading into the nonvascular tissues (Chen and Citovsky, 2003; Ueki and Citovsky, 2002). These observations suggest that viral systemic movement may be a directional process employing different molecular pathways for entry into and exit out of the host plant phloem. The differences in vascular loading and unloading of plant viruses are also evident from the above-described observations of functional equivalence of different vein classes for TMV entry and their lack of equivalence for TMV exit (Cheng *et al*., 2000). Thus, macromolecular transport into the plant vasculature may be more promiscuous, *e.g.*,occurring by a loosely regulated process, whereas transport out of the vasculature may be more selective and/or tightly regulated.

4.3.5. Restricted Phloem Unloading and Its Alleviation by Coinoculation with Unrelated Viruses

Some viruses cannot leave the host vascular system either in specific hosts that do not allow viral unloading or simply because they are naturally phloem-limited. For example, BGMV, a bipartite geminivirus, remains largely confined in vascular tissues of *N. benthamiana*, whereas several other bipartite geminiviruses, such as CLCV and TGMV, unload into the surrounding mesophyll (Morra and Petty, 2000; Qin and Petty, 2001). When BGMV is coinoculated with TGMV, BGMV gains the ability to infect mesophyll cells, suggesting that TGMV supplies viral factors that alleviate BGMV phloem limitation (Morra and Petty, 2000). The TGMV determinants that enable BGMV to infect mesophyll cells include a *cis*-acting, noncoding TGMV *BRi* element upstream of the BV1 (formerly, BR1) ORF and at least one of the two *trans*-acting factors, the AL2 protein and BV1/BC1 (formerly, BR1/BL1) (Morra and Petty, 2000). A later study suggested that AL2, in association with host factors, acts through the *BRi* region to enhance the *BV1* gene expression (Qin and Petty, 2001). Because the BV1 and BC1 proteins represent geminiviral MPs, phloem limitation of BGMV may in fact be caused not by its inability to unload *per se* but by failure of the unloaded virus to move from cell to cell in the mesophyll tissues due to insufficient expression levels of its MPs.

Luteoviruses exemplify plant viruses that are naturally phloem-limited. Following direct injection into phloem cells by aphids, these viruses spread within the phloem but do not leave the host vasculature, although they can replicate in protoplasts derived from nonvascular tissues (reviewed by Mayo and Ziegler-Graff, 1996; Smith and Barker, 1999). Coinfection of *N. clevelandii* or *N. benthamiana* with a mixture of PLRV luteovirus and an unrelated PVY potyvirus results in an elevated titer and more frequent occurrence of PLRV within mesophyll (Barker, 1987, 1989), suggesting that potyviral factors facilitate phloem unloading of PLRV. The potyviral determinants that alleviate luteoviral phloem limitation have not been identified. However, they probably do not include HC-Pro, one of the potyviral proteins involved in systemic movement (reviewed by Revers *et al*., 1999; Urcuqui-Inchima *et al*., 2001; see Section 4.1), because transgenic *N. benthamiana* plants expressing PVA HC-Pro do not promote the occurrence of luteoviruses in mesophyll cells (Savenkov and Valkonen, 2001). Phloem unloading of PLRV into mesophyll tissues also can be promoted by coinoculation with PEMV-2 umbravirus or with a cucumovirus CMV(ORF4) strain, which is a chimeric CMV expressing the ORF4-encoded GRV MP instead of CMV MP (Ryabov *et al*., 2001a). The molecular mechanism of PLRV phloem restriction, however, is unclear. Because neither a chimeric potexvirus PVX expressing GRV MP nor a mutated CMV(ORF4) with blocked expression of the PTGS-inhibiting factor 2b support PLRV unloading into the mesophyll (Ryabov *et al*., 2001a), the phloem limitation of PLRV may derive from a combination of two factors: lack of ability to unload from the phloem *per se*, and failure to accumulate in the mesophyll due to the host plant defense reactions.

Another example of complementation of viral systemic transport by coinoculation with an unrelated virus is restoration of movement of a potyvirus isolate PepMoV-FL through internal phloem of *C. annuum* cv. Avelar plants by a cucumovirus strain CMV-KM (Guerini and Murphy, 1999). In this host, however, PepMoV-FL does not move within the internal phloem at all rather than just being restricted in phloem unloading (Guerini and Murphy, 1999). CMV-KM truly promotes the PepMoV-FL phloem movement because it does not enhance PepMoV-FL accumulation in plant protoplasts, indicating that the presence of CMV-KM does not simply block potential host defense reactions against PepMoV-FL (Guerini and Murphy, 1999). In a reverse complementation, systemic spread of a long-distance movement-deficient M strain of CMV (M-CMV) in zucchini squash (*Cucurbita pepo*) is rescued by coinoculation with either ZYMV strain A (ZYMV-A) or its attenuated variant ZYMV-AG (Choi *et al*., 2002). However, whether this complementation of M-CMV spread is due to the restored viral movement *per se* or is simply a result of the PTGS-suppressing activity of potyviral HC-Pro (reviewed by Revers *et al*., 1999; Urcuqui-Inchima *et al*., 2001) remains unclear.

That systemic movement of diverse viruses can be complemented by coinoculation with another, unrelated virus implies that at least some viral factors participating in the long-distance transport are functionally similar and, therefore, interchangeable. This is reminiscent of the virus genome sequence-independent complementation of the cell-to-cell MP function between unrelated viruses (see Section 3.2). Importantly, complementation of systemic movement also reveals the complex interplay between the various steps of systemic movement, such as the viral ability to move within the vasculature, to unload into systemic tissues, and, following unloading, to escape the host defenses, all of which are required for the establishment of systemic infection.

5. *ARABIDOPSIS THALIANA* AS A MODEL PLANT TO STUDY VIRUS MOVEMENT

Historically, studies of plant virus movement have employed a wide spectrum of viruses and host plants. Many of these hosts, however, are slow growing and have very large and uncharacterized genomes, and therefore are poorly amenable to genetics and reverse genetics experimentation. Thus, while benefiting from a significant diversity, the studies of viral movement suffer from the lack of an experimental system capable of taking advantage of such modern research tools as complete genome characterization, proteome characterization, availability of tagged mutant collections, DNA microarrays, and collections of individual full-length cDNA clones. Following two decades of research, a single plant species, *A. thaliana*, has emerged as such a model plant for most research areas of plant biology (Alonso *et al*., 2003; Kaiser, 2000; Lukowitz *et al*., 2000; Meinke *et al*., 1998; Meyerowitz, 1987; Pang and Meyerowitz, 1987; Ruan *et al*., 1998; Seki *et al*., 2002; Simon, 1994; Szabados *et al*., 2002; The *Arabidopsis* Genome Initiative, 2000;

Wortman *et al*., 2003). That arabidopsis is infected by a diverse range of plant viruses (Simon, 1994; Sosnova and Polak, 1975)—such as CaMV (Callaway *et al*., 2000), TCV (Dempsey *et al*., 1993; Simon *et al*., 1992), TEV (Mahajan *et al*., 1998; Whitham *et al*., 1999), TSWV (German *et al*., 1995), CLCV (Hill *et al*., 1998), and crucifer-infecting tobamoviruses TVCV and its isolate Cr-TMV, *Oilseed rape mosaic virus* (ORMV) and its isolates TMV-C, TMV-Cg, *Chinese rape mosaic virus*, and *Youcai mosaic virus*, CTMV-W, and Holmes' *Ribgrass mosaic virus* (RMV) (reviewed by Melcher, 2003), and several others—allows its use as a model system for studies of many aspects of virus–host interactions, including viral cell-to-cell and systemic movement.

One of the first plant viruses, the movement of which in arabidopsis was described in detail, is TVCV (Lartey *et al*., 1997); TVCV infection of arabidopsis was later used to identify an arabidopsis mutant (*vsm1*) with delayed viral systemic movement (Lartey *et al*., 1998). While the identity of the *VSM1* gene remains unknown, three arabidopsis genes, *RTM1*, *RTM2*, and *RTM3*, involved in long-distance transport of TEV have been identified (Chisholm *et al*., 2000, 2001; Mahajan *et al*., 1998; Whitham *et al*., 2000); two of them, *RTM1* and *RTM2*, were cloned and characterized (Chisholm *et al*., 2000, 2001; Mahajan *et al*., 1998; Whitham *et al*., 2000; see Section 4.2.2).

Another plant virus movement-related genetic screen of arabidopsis was developed to identify host genes that suppress an infection-deficient mutation in CaMV MP, which causes significant delays in development of the CaMV disease symptoms (Callaway *et al*., 2000). Two *arabidopsis* mutants were identified that, upon infection with CaMV containing the infection-deficient MP, displayed accelerated symptom production; one of these mutant loci, *asc1*, was mapped to chromosome 1 (Callaway *et al*., 2000). The identity of the *ASC1* gene and the mechanism of its function remain unknown. Infection of *arabidopsis* mutants with the Cr-TMV isolate of TVCV identified two mutations in which the viral CP accumulated to lower levels in the uninoculated leaves (Ishikawa *et al*., 1991b), suggesting that the mutated genes may participate in viral systemic movement. Instead, subsequent studies demonstrated that one of these genes, *TOM1*, is involved in viral multiplication rather than movement (Ishikawa *et al*., 1993; Yamanaka *et al*., 2000, 2002).

In addition to mutant collections, different arabidopsis ecotypes have been used to examine viral movement. For example, in experiments with a Dijon ecotype line Di-17 of arabidopsis bred to develop hypersensitive response to TCV infection (Dempsey *et al*., 1993), a TCV p8 mutant with slightly weaker RNA binding caused enlarged necrotic lesions and systemic disease symptoms, suggesting that weaker interaction of TCV p8 with the viral genome may facilitate uncoating of the M complexes in the newly infected cells (Wobbe *et al*., 1998). Also, examination of 14 arabidopsis ecotypes for their ability to support systemic movement of TMV, which displays no visible symptoms in commonly used arabidopsis ecotypes, identified one ecotype that allows rapid TMV movement accompanied by symptoms, nine ecotypes that allow slower movement without symptoms, and four ecotypes that allow little or no systemic movement (Dardick *et al*., 2000). Genetic analysis of some of these ecotypes suggested that at least two of their genes modulate susceptibility to TMV (Dardick *et al*., 2000).

Finally, a recent study has begun a systematic characterization of arabidopsis factors involved in determination of resistance to LMV (Revers *et al*., 2003). Three dominant arabidopsis LMV resistance genes were tentatively identified: one gene that is involved in blocking LMV replication and/or cell-to-cell movement and two genes that control the systemic movement of this virus (Revers *et al*., 2003). The mechanism by which these genes operate has yet to be determined.

Although these studies made inroads into the use of arabidopsis for genetic studies of viral movement, the tremendous potential of this experimental system has not been fully realized. Significant progress may be made by designing genetic assays to identify the host cell components of the viral movement pathways. For example, transgenic expression of fluorescently tagged MPs driven by cell-specific promoters, such as trichome-specific GL2 promoter (Szymanski *et al*., 1998) or companion cell-specific promoter of CYMV (Itaya *et al*., 2002; Matsuda *et al*., 2002), will likely result in movement of the tagged MP from the expressing cell into the neighboring non-expressing cells; screening for arabidopsis mutants in which the cell-to-cell movement of MP is altered or compromised should identify plant genes involved in this transport process.

6. CONCLUDING REMARKS

This review article provides an in-depth summary of our current knowledge on nondestructive cell-to-cell and systemic spread of viruses in plants with a special focus on the 30 K superfamily of plant viruses. The studies of viral spread have gained an enormous impetus within the last decade, a fact that is reflected by the large scope of this review, surveying more than 500 publications. Despite diversities in morphology of viral particles, genomic structure, number and sequence of movement proteins, and interacting host factors, the plant viruses described here are unified by their ability to move cell-to-cell through plasmodesmata as M complexes composed of MPs and viral genomic nucleic acids. Therefore, the cell-to-cell movement section of this review is largely devoted to a comparison of structural and functional properties of the molecules central to the viral movement process, the MPs, which have been extensively studied for a large number of plant viruses. While studies of the MP-mediated mechanism of viral cell-to-cell movement have a long-standing tradition, dedicated studies of molecular pathways of viral systemic movement is a relatively new field of research that has primarily focused on two viral genera, tobamoviruses and potyviruses. Both cell-to-cell and systemic movement events are intimately connected to the cellular processes of the host plant, with the invading viruses exploiting the infrastructure of the plant cell, *i.e.*, its structural components and functional pathways, for spreading their infection. However, as shown in this review, the studies of the molecular composition of the host cell

structures involved in viral movement, *e.g.*, plasmodesmata, as well as identification and functional characterization of host factors participating in viral spread, are still in their infancy.

Reflecting on the broader meaning of the title of this article, which new and promising directions in the research of viral spread could be considered *in*, and which approaches may have exhausted their potential and thus are *out*? The ins that immediately come to mind include all experiments designed to broaden our understanding of the host contributions to viral movement, from characterization of cellular factors, to regulatory mechanisms, to diverse host-specific and cell type-specific effects that modulate viral transport. In addition, the specific investigation of molecular aspects of the systemic movement can be counted among the ins. This endeavor, which for a long time has been hampered by the intrinsic complexity of the system, is now rapidly advancing, promising to uncover novel mechanistic concepts in plant biology as well as new strategies to block or enhance viral spread. Finally, increasing evidence suggests that viral MPs are involved in the suppression of host defenses against virus infection; Studies of this aspect of the MP function are just beginning.

To identify the potential outs in the virus movement research is less easily done. Even the continuing studies of the so-called *hallmark* functions of viral MPs that have been recognized more than a decade ago, such as nucleic acid binding and localization to plasmodesmata and their gating, are becoming more refined and still prove valuable in assigning transport functions to as-yet uncharacterized viral proteins. Perhaps some experimental approaches that have led to groundbreaking discoveries in earlier phases of viral movement studies but that have now been partially replaced by technically simpler methods, might be considered as outs. For example, microinjection, and to a certain extent immunoelectron microscopy, have been substituted by microbombardment of fluorescently tagged tracer molecules or DNA constructs expressing GFP fusion proteins. Nonetheless, both of these methods still have their place in the field, because only microinjection techniques provide insights into the dynamics of intercellular spread, and the level of spatial resolution provided by electron microscopy remains unsurpassed.

So, where do we stand now on the way towards the understanding of viral movement? Whereas the past was successful in unraveling the global concepts of viral spread, the future will likely be devoted to understanding the molecular details of these global concepts and their multitudes of variations. The progress in achieving those goals will largely depend on how fruitful the different strategies for identification and analysis of host components of the viral movement pathways will prove to be. A promising start has already been made, as evidenced by a considerable number of genes and proteins initially implicated in the movement process by genetic means and by searching for MP interaction partners. Thus, the future will likely hold in store novel and revealing insights into the plant viral spread as well as new avenues towards combating viral diseases and exploiting plant virus components as molecular tools for improving agronomically important plants. And of course, as ever, insights derived from the virus movement research will profoundly advance our understanding of basic aspects of plant biology.

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