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NUCLEIC ACID TRANSPORT IN PLANT-MICROBE INTERACTIONS: The Molecules That Walk Through the Walls

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■ **Abstract** Many microbes "genetically invade" plants by introducing DNA or RNA molecules into the host cells. For example, plant viruses transport their genomes between host cells, whereas *Agrobacterium* spp. transfer T-DNA to the cell nucleus and integrate it into the plant DNA. During these events, the transported nucleic acids must negotiate several barriers, such as plant cell walls, plasma membranes, and nuclear envelopes. This review describes the microbial and host proteins that participate in cell-to-cell transport and nuclear import of nucleic acids during infection by plant viruses and *Agrobacterium* spp. Possible molecular mechanisms by which these transport processes occur are discussed.

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INTRODUCTION

One of the most fascinating aspects of plant-microbe interactions is the ability of some pathogens to genetically insinuate into their hosts. The two best examples of such "genetic invaders" are plant viruses and *Agrobacterium tumefaciens*, a soil phytopathogen. Plant viruses physically enter plant cells and spread systemically, delivering their DNA or RNA genetic material to most plant organs and tissues. *Agrobacterium* spp., on the other hand, remain outside the infected cell but transfer a DNA segment, called transferred DNA (T-DNA), from its tumor-inducing (Ti) plasmid into the plant cell nucleus, in where the T-DNA integrates into the plant genome. This transport of viral genomes and *Agrobacterium* spp. T-DNA involves passage of the transferred nucleic acids through such barriers as cell walls, plasma membranes, and nuclear envelopes.

In this review, we focus on plant virus cell-to-cell movement as an example of nucleic acid transport across cell walls and plasma membranes. Tobacco mosaic virus (TMV), whose discovery led eventually to the establishment of the science of virology (reviewed in 31), is used as a paradigm for viral movement between plant cells. We also discuss nuclear import and intranuclear transit of *Agrobacterium* spp. T-DNA as an example of transport of nucleic acids across plant nuclear envelopes and within the cell nucleus. Based on our current knowledge of the microbial and host cell factors involved in intercellular and nuclear transport of nucleic acids, models for both transport processes are proposed.

Because pathogenic microorganisms often adapt existing cellular machinery for their own needs, plant viruses and *Agrobacterium* spp. likely use endogenous cellular pathways for cell-to-cell movement and nuclear import of nucleic acids. Thus, this review may improve understanding of the general mechanisms by which DNA and RNA molecules are transported between and within eukaryotic cells.

CELL-TO-CELL MOVEMENT OF VIRAL NUCLEIC ACIDS IN PLANTS

The plant cell wall is perhaps the toughest and thickest intercellular boundary in living nature. Yet, plant viruses exhibit a remarkable ability to cross this lignified border during their spread from the infected cell into the neighboring healthy cells. To this end, many plant viruses have evolved the ability to use plasmodesmata, the naturally occurring intercellular connections that span the cell walls and connect plant cells into a symplastic continuum.

Virus spread usually occurs on two different levels: (*a*) local, cell-to-cell movement through plasmodesmata, and (*b*) systemic spread through the host vascular

system. Whereas viral systemic movement is thought to be a passive process, occurring with the flow of photoassimilates (95), cell-to-cell movement is considered to be an active event that requires specific interaction among the virus, plasmodesmata, and, potentially, other plant cellular components. In most plant viruses, a nonstructural movement protein (MP) is the only virus-encoded component that is responsible for interaction with plasmodesmata and, consequently, for cell-to-cell movement. The first viral MP to be identified was the 30-kDa protein of TMV (reviewed in 16, 133). Since its discovery, TMV MP has continued to be the protein of "many firsts" (31). All known biological activities of plant viral MPs have been first described using TMV MP as a model system. Specifically, TMV MP was shown to (*a*) bind single-stranded (ss) nucleic acids, (*b*) associate with cytoskeleton and endoplasmic reticulum, (*c*) bind to cell wall pectin methylesterases, (*d*) increase plasmodesmal permeability, and (*e*) be negatively regulated by phosphorylation in a host-dependent fashion. These biological functions of TMV MP, as well as a model for MP-mediated intercellular transport of TMV RNA, are presented in the following sections.

MP-Nucleic Acid Complexes as Intermediates of Cell-to-Cell Movement

During TMV cell-to-cell movement, MP may function either in *trans* or in *cis* to the transported viral genomic RNA molecule. Function in *trans* implies that free MP alters plasmodesmata to allow subsequent diffusion of TMV RNA through these channels. MP function in *cis*, on the other hand, would involve formation of MP-RNA complexes that actively interact with and move through plasmodesmata. The fact that TMV MP was found to tightly bind nucleic acids in vitro (20, 25) favors the notion that this protein acts in *cis* to the transported RNA and that MP-TMV RNA complexes may represent cell-to-cell movement intermediates of the virus.

TMV MP specifically bound ssDNA and RNA but not double-stranded (ds) DNA. Binding to ssDNA and RNA, however, occurred without sequence specificity (20, 25). This latter observation may provide the molecular basis for a well-established but poorly understood cross-complementation of viral infection (reviewed in 3). Indeed, some plant viruses, normally unable to spread from cell to cell, gain this ability upon coinfection with an unrelated cell-to-cell moving virus, such as TMV (4, 11, 102). Potentially, TMV MP may associate with the heterologous genomic RNA of the "nonmoving" virus and supply it with the cell-to-cell movement capability.

TMV MP binding to RNA and ssDNA is cooperative (20, 25), which indicates that MP molecules interact with each other upon binding to the nucleic acid lattice (12). Ultrastructural analysis of TMV MP-RNA complexes has revealed that the MP packages RNA molecules into thin and extended complexes with a diameter of 2.0–2.5 nm (25). When compared with the predicted 10-nm diameter of the globule-shaped free TMV RNA (57), TMV MP-RNA complexes

appear better suited for transport through the narrow channels of plasmodesmata (27).

Association with nucleic acids is not unique for TMV MP, and numerous studies have demonstrated that most other plant viral MPs possess various levels of nucleic acid binding activity (reviewed in 56). The MP of cauliflower mosaic virus (CaMV), for example, was shown to interact with both ssDNA and RNA (21, 151, 152), yet it has a higher affinity toward RNA molecules (21). Because CaMV is a pararetrovirus that replicates through an RNA intermediate (82), MP-RNA complexes may be involved in the local movement of that virus. Similar to what occurred with TMV MP, CaMV MP formed long, thin, and unfolded complexes with ss nucleic acids (21). However, the MP of red clover necrotic mosaic virus (RCNMV), which also associated with RNA and DNA, did not significantly alter the conformation of the bound nucleic acid molecules (53).

Although most known viral MPs function as transporters of the viral genomes, their degree of transport selectivity varies. Whereas TMV MP most likely transports both ssDNA and RNA, RCNMV MP is more specific, transporting RNA but not dsDNA or ssDNA (53). On the other hand, the bean dwarf mosaic geminivirus (BDMV) MP BL1 specifically transports dsDNA but not ssDNA or RNA (119). It is interesting that BDMV BL1 transport preference toward dsDNA is radically different from that of the BL1 of another bipartite geminivirus, squash leaf curl virus (SqLCV), which is thought to transport only ssDNA molecules (124). Furthermore, unlike BDMV BL1, which binds dsDNA (134), SqLCV BL1 binds ssDNA (124). SqLCV BL1 binding to ssDNA, however, is weak; formation of functional SqLCV cell-to-cell movement complexes is thought to occur with the help of the second geminiviral MP, BR1, which binds ssDNA very tightly (124). In the current model for SqLCV cell-to-cell movement, BR1 exports viral ssDNA from the site of its replication within the host cell nucleus to the cytoplasm, where BL1 interacts with BR1 and directs the resulting BL1-BR1-ssDNA complexes to and through plasmodesmata (92, 137, 164).

Movement Protein Interactions with Cytoskeleton and Endoplasmic Reticulum

After synthesis in the cell cytoplasm, probably in the vicinity of the endoplasmic reticulum (ER) (70), TMV MP and/or its complexes with TMV RNA must journey to the cell wall and its cognate plasmodesmata. Although this intracellular transport of TMV MP represents one of the central events in viral cell-to-cell movement, in-depth investigations of this process are just beginning.

One possible model for intracellular transport of TMV MP involves interactions with the cytoskeleton. TMV MP has been reported to bind microtubules and, to a lesser extent, actin microfilaments (69, 107). In vivo studies have demonstrated that TMV MP tagged with the green fluorescent protein (GFP) of *Aequorea victoria* colocalized with tubulin as well as with actin filaments in virus-infected protoplasts (69, 107) or in protoplasts that transiently express TMV MP-GFP (107).

Binding of TMV MP to actin and tubulin was also detected in vitro (107). Because cytoskeletal elements, especially actin, are likely connected to plasmodesmata (42, 169), TMV MP and, by implication, its complexes with viral RNA may use them for intracellular movement. That TMV MP "walks" on or is "guided" by cytoskeletal tracks is supported by the kinetics of changes in TMV MP-GFP intracellular localization in transfected protoplasts: 18–20 h after transfection, TMV MP-GFP localized in filamentous arrays, whereas 48–72 h after transfection, most of TMV MP-GFP had migrated to the cell periphery (107).

Recent studies suggest that interactions between tobamoviral MPs and cytoskeleton may have additional or even alternative biological functions. One such function may involve a host-dependent defense response to viral infection (122). Specifically, in *Nicotiana tabacum*, MP of the Ob tobamovirus interacted with the cytoskeleton and formed a characteristic pattern of MP-GFP filaments within cells at the leading edge of infection, where viral cell-to-cell movement occurs. Conversely, in *N. benthamiana*, MP-GFP filaments were observed only in the inner areas of the infected tissue. Furthermore, loss of MP-GFP fluorescence was coincident with MP degradation, which suggests that, in *N. benthamiana*, cytoskeletal components may target MP for degradation (122). Indeed, microtubules have been shown to participate in the induced degradation of cellular proteins in cultured mammalian cells (2). Consistent with this idea, disruption of microtubules by oryzalin did not affect accumulation of TMV MP-GFP in the punctate sites in the cell periphery, which indicates that microtubules do not play a direct role in TMV MP targeting to these presumably plasmodesmal locations (70). Another potential function of MP-cytoskeleton interaction is to negatively regulate TMV MP movement by anchoring this protein to the cell cytoplasm. Similar mechanisms of cytoplasmic anchoring have been described for regulation of protein nuclear import (reviewed in 118).

Intracellular transport of TMV MP may also involve the cellular ER. Indeed, TMV MP-GFP (as well as viral replicase) has been shown to localize to cortical ER in virus-infected *N. benthamiana* leaves and tobacco protoplasts (70). Because its disruption by brefeldin A also blocked transport of TMV MP to the cell periphery (70) in protoplasts, this TMV MP-ER interaction appeared to be required for subsequent localization of TMV MP to punctate sites at the plasma membrane. Studies of TMV MP association with the ER in virus-infected *N. benthamiana* plants demonstrated that TMV MP behaved as an integral membrane protein with its carboxyl terminus exposed to the cytoplasmic face of the isolated ER microsomes (130). Consistently, earlier cell-fractionation studies also showed that TMV MP copurified with enzyme markers common to the ER (111). The molecular mechanism by which TMV MP associates with the ER is still unknown. However, the fact that TMV MP lacks an apparent ER signal sequence (5, 39) and does not associate with membranes when expressed in vitro (70) suggests that a host cell factor may be needed to direct TMV MP to ER. Finally, increasing evidence indicates that other viral MPs, such as alfalfa mosaic virus (AMV) MP and ScLCV BL1, may also associate with the host cell ER (79, 165).

Movement Protein Binding to Cell Wall–Associated Pectin Methyl Esterases

Because TMV MP has been shown to localize to plasmodesmata within plant cell walls (41, 157), it may directly interact with a cell wall-associated receptor. To identify and characterize such cell wall protein(s), recent studies used a renatured blot overlay assay in which tobacco cell wall proteins are separated by gel electrophoresis and then probed with free TMV MP (14, 46, 133). By this approach, TMV MP was found to bind to cell wall-associated proteins with a molecular mass of 33–38 kDa. Sequence analysis of these TMV MP-interacting proteins, after their purification on MP-affinity columns, identified them as isoforms of tobacco pectin methylesterase [PME (14, 46)]. Based on the amino acid sequence of the purified PME, the 3′-terminal part of a tobacco gene encoding this protein was cloned (46).

PME proteins are known to undergo post-translational processing (114). For example, size estimates for most plant PMEs are in the range of 32–42 kDa, whereas the size of the predicted translation products is >50 kDa (114 and references therein). Thus, PME maturation probably involves a post-translational cleavage that separates the conserved carboxyl terminus of these proteins from their more variable amino-terminal region. Indeed, the amino-terminal sequence of a mature tomato PME (103) does not align with the amino terminus of the predicted translation product of the cloned PME gene (65); instead it aligns with the conserved carboxyl terminal PME domain (114). The 33- to 38-kDa relative electrophoretic mobility of the MP-interacting PME suggests that it represents a post-translationally processed, mature protein.

TMV MP-PME interaction was confirmed and further studied in the yeast two-hybrid system (14), in which TMV MP was shown to bind both unprocessed and mature forms of PME from different plant species, such as tomato and orange. Furthermore, an MP domain that is necessary and sufficient for binding to PME was identified and localized to amino acid residues between positions 130 and 185 of the protein. It is important that TMV genomic RNA carrying an MP derivative lacking this protein region was unable to spread within tobacco tissue in vivo. The same viral RNA remained infectious in transgenic tobacco hosts that express intact TMV MP, which indicates that blocking PME binding did not interfere with replication and assembly activities of the viral genomes (14). Thus, association with cellular PME may contribute to the ability of TMV MP to transport the viral genomic RNA between the host plant cells. This hypothesis is further supported by the fact that removal of the PME-binding domain blocked the ability of TMV MP to increase plasmodesmal permeability (161).

The members of the PME multigene family are involved in cell wall turnover and appear to have a role in plant growth and development. PME activity is thought to modulate pH and ion balance and affect cell wall porosity (114 and references therein; 129). In addition, PME has been implicated in more specialized cellular processes, such as plant response to pathogen attack (103). PME interaction with TMV MP suggests that this cell wall-associated enzyme may also play a role in plant viral movement (14, 46).

TMV MP binding to PME may facilitate viral movement by several mechanisms. First, MP may bind unprocessed PME, which carries the ER translocation signal and is destined to be transported to the cell wall. As mentioned above, TMV MP copurifies with ER membranes (111, 130) but does not itself carry ER signal sequences. TMV MP association with unprocessed PME may provide the ER signal in *trans*, resulting in a "piggyback" transport of TMV MP through the ER secretory pathway. After localization to the cell wall, PME may become secreted, whereas TMV MP is retained at the cell wall.

Second, PME may simply function as a cell wall receptor for TMV MP (46). Immunoelectron microscopy studies have suggested that PME is localized throughout the cell wall, including plasmodesmata (14). Potentially, binding to PME may initially target and/or anchor TMV MP to the host cell wall. In this scenario, TMV MP association with PME in the vicinity of plasmodesmata will commence the cell-to-cell transport process. Conversely, binding to PME in the cell wall areas that do not contain plasmodesmata will result in abortive movement, with TMV MP either being degraded or redirected back into the cell cytoplasm. This model assumes that TMV MP targeting to the cell periphery may occur irrespective of the presence of plasmodesmata. Indeed, recent data suggests that TMV MP expressed in tobacco protoplasts that do not possess plasmodesmata forms protrusions on the cell surface (70). In these cells, TMV-MP may recognize the cell surface via binding to PME likely present within cell wall.

Finally, a more active role for PME in viral movement cannot be excluded. For example, TMV MP binding may interfere with the PME activity, altering the cell wall ion balance and, consequently, inducing changes in plasmodesmal permeability. It is noteworthy that purified tobacco PME also has been shown to bind RNA in vitro (46); the biological significance of this activity, however, remains unknown. Regardless of the exact molecular mechanism by which PME acts, it probably is not limited to the cell-to-cell movement of TMV. Indeed, MPs of two other plant viruses, turnip vein clearing virus and CaMV, also bound PME. On the other hand, a maize homeodomain protein, Knotted 1, which is known to move through plasmodesmata in tobacco leaves (100), did not interact with PME in the two-hybrid system (14). Thus, Knotted 1 may move between cells by a pathway that does not involve PME. Previously, cell wall extracts were shown to bind gold-conjugated Knotted 1 and MP of cucumber mosaic virus (90). Because protein binding was assayed on dot blots, no determination was made regarding the size or identity of the putative receptor protein(s) (90); it is possible, however, that the MP-binding protein may also represent PME, whereas the Knotted 1-binding activity may be derived from a different protein present on the same dot blot.

Movement Protein-Induced Increase in Plasmodesmal Permeability

Once the MP-RNA complex has arrived to a plasmodesma, it must travel through this channel to penetrate the neighboring cell. The estimated 2.0- to 2.5-nm meter of the TMV MP-RNA complex (25) would not allow it to passively diffuse

through a smaller, 1.5-nm-wide plasmodesmal opening (171). To mediate cell-to-cell movement, therefore, TMV MP must dilate plasmodesmata. This MP-induced increase in plasmodesmal permeability was first demonstrated by microinjecting fluorescently labeled dextran molecules into leaf mesophyll cells of transgenic tobacco plants that express TMV MP (171). This study showed that, unlike wild-type tobacco plasmodesmata that traffic dextran molecules of 0.75–1 kDa, TMV MP transgenic plants allowed movement of dextran molecules with a molecular mass of $\leq$10 kDa (171). It is surprising, however, that this effect of TMV MP on plasmodesmata was observed only in mature leaves, not in young, upper leaves (38, 171). Because in nature TMV infects both young and mature leaves, TMV MP transgenic plants may modify or down-regulate this protein during their development (27).

To avoid potential artifacts associated with transgenic plants, purified TMV MP was mixed with fluorescently labeled dextrans of increasing molecular weights and directly microinjected into the mature and young leaves of wild-type tobacco plants (161). In both tissues, TMV MP promoted a relatively fast (3- to 5-min) increase in plasmodesmal permeability, allowing cell-to-cell movement of 20-kDa dextrans. It is interesting that the increased plasmodesmal size exclusion limit of 10–20 kDa (161, 171) corresponds to a dilated channel diameter of 5–9 nm, potentially allowing easy passage of 2.0- to 2.5-nm-wide TMV MP-RNA complexes (25) but not of 10-nm-wide free TMV RNA (57).

Dextran spread between 20–50 cells away from the injected cell, rather than only to the adjacent cells, which suggested that microinjected TMV MP also traffics through plasmodesmata to exert its effects within cells distant from the site of injection (161, 171). Subsequent immunolocalization experiments directly confirmed this conclusion and showed that microinjected TMV MP itself moves between plant cells (162). Direct microinjection of TMV MP also allowed the mapping of the domain required for interaction with plasmodesmata to amino acid residues between positions 126 and 224 (161).

Elevation of plasmodesmal permeability appears to represent a common feature of many plant viral MPs. For example, MPs of RCNMV, AMV, cucumber mosaic virus, tobacco rattle virus, potato virus X, and BL1 of BDMV have all been shown to mediate transport of large fluorescent dextrans between plant cells (1, 40, 53, 119, 127, 159). RCNMV, cucumber mosaic virus, BDMV, and AMV MPs were also shown to move from cell to cell themselves, either freely or translationally-fused to reporter proteins such as GFP or ß-glucuronidase (43, 53, 79, 84, 119, 162).

Movement Protein Activity Is Regulated by Phosphorylation

During viral spread within plant tissues, prevention of virus reentry into the already infected cells would be a good strategy for optimal infection efficiency and fastest cell-to-cell movement. Moreover, TMV MP is apparently not subjected to rapid turnover; instead, it persists in the infected cells, accumulating within their plasmodesmata (41, 157). Such a continuous presence of active TMV MP may

elevate plasmodesmal permeability and disrupt intercellular communication. Thus, TMV MP activity should be negatively regulated to prevent TMV virions from reentering the already infected tissues, as well as to minimize interference with the host plant intercellular communication. Indeed, a recent study demonstrated that, although TMV MP is present within plasmodesmata of all infected cells, it increases the plasmodesmal permeability only at the leading edge of the expanding infection site (120).

What is the molecular basis for this regulation of the TMV MP activity? Potentially, it may involve phosphorylation, which is known to occur at multiple sites within the TMV MP molecule (22, 64, 87, 167; M-H Chen, S Ghoshroy, E Waigmann, V Citovsky, submitted for publication). The best characterized TMV MP phosphorylation site comprises the carboxyl terminal serine-258, threonine-261, and serine-265 residues, which are specifically phosphorylated by a host cell wall-associated protein kinase activity both in vitro (22) and in vivo (M-H Chen, S Ghoshroy, E Waigmann, V Citovsky, submitted for publication). TMV MP phosphorylation in vitro did not require Ca^{2+} cations (M-H Chen, S Ghoshroy, E Waigmann, V Citovsky, submitted for publication), which distinguished this activity from several other serine/threonine-specific protein kinases that associate with plant cell walls (68, 172).

The effect of carboxyl terminal phosphorylation on TMV MP function was studied with negatively charged amino acid substitutions within the phosphorylation site; this approach is known to reveal the electrostatic effects of phosphorylation (34, 153, 170). For example, inactivation by phosphorylation of serine-113 in isocitrate dehydrogenase is mimicked when aspartate is substituted at this site (153). Similarly, substituting serine-258, threonine-261, and serine-265 with aspartate residues inactivated the ability of TMV MP to increase plasmodesmal permeability after microinjection (M-H Chen, S Ghoshroy, E Waigmann, V Citovsky, submitted for publication). The negative regulation of TMV MP interaction with plasmodesmata was host dependent. In *N. tabacum*, mimicking phosphorylation blocked the TMV MP capability to dilate plasmodesmata, whereas in *N. benthamiana*, the same TMV MP derivative remained fully active as compared with nonphosphorylated MP (M-H Chen, S Ghoshroy, E Waigmann, V Citovsky, submitted for publication). Thus, *N. benthamiana*, one of the most susceptible hosts for plant viruses (33, 58), may lack the mechanism for TMV MP inactivation by phosphorylation.

Because carboxyl-terminal phosphorylation of TMV MP blocks its interaction with plasmodesmata, it should also inhibit the viral spread from cell to cell in vivo. Indeed, TMV carrying the negatively-charged derivative of MP within its genomic RNA was unable to move locally and systemically within *N. tabacum*. The mutant virus, however, was fully capable of movement in transgenic plants expressing wild-type TMV MP, which indicated that the mutation did not interfere with replication and translation of viral gene products (M-H Chen, S Ghoshroy, E Waigmann, V Citovsky, submitted for publication). The effect of mimicking MP phosphorylation on TMV movement in vivo was also host dependent, occurring

in *N. tabacum* but not in a more promiscuous *N. benthamiana* (M-H Chen, S Ghoshroy, E Waigmann, V Citovsky, submitted for publication).

In addition to regulating interactions with plasmodesmata, TMV MP phosphorylation by cell wall-associated protein kinases may control other events in the viral life cycle. For example, MPs of tobamoviruses act as efficient translational repressors of viral RNAs in MP-RNA complexes in vitro and in isolated plant protoplasts that lack cell walls and plasmodesmata (85). However, these complexes become infectious in plant tissue (85). Also, when TMV MP was phosphorylated in vitro by protein kinase C before or after binding to RNA, the resulting TMV MP-RNA complexes became translatable in vitro and infectious in protoplasts (86). Together, these observations suggest that, in plant tissue, TMV MP-RNA complexes are converted into a translatable form by TMV MP phosphorylation, which may take place in the cell wall during or after passage through plasmodesmata. Thus, TMV MP phosphorylation may act as a molecular switch between viral spread and replication/translation.

Besides its carboxyl-terminal phosphorylation at the plant cell wall, TMV MP is phosphorylated at other serine residues (64, 87, 167), potentially by other protein kinases of the host cell. Although the biological role of these phosphorylation sites is unclear, at least some of them may be critical for TMV MP function. For example, recent studies suggest that serine-37 and serine-238 within tomato mosaic tobamovirus are phosphorylated in tobacco protoplasts. Although mutation of serine-238 did not affect viral infectivity, amino acid changes at position 37 altered intracellular localization of tomato mosaic tobamovirus MP and decreased its stability (87). It is interesting that the substitution of serine-37 with another phosphorylatable residue, threonine, still resulted in a tomato mosaic tobamovirus MP mutant with decreased stability in plant protoplasts (87). Thus, the mere presence of serine-37 rather than its phosphorylation is likely responsible for these effects.

Finally, in addition to phosphorylation, other types of post-translational modification may affect TMV MP function. For example, in *Arabidopsis thaliana*, TMV MP has been shown to be proteolytically processed at its amino terminus. The processed TMV MP was nonfunctional, which suggests that proteolytic cleavage may represent an alternative strategy to deactivate TMV MP (81). Whether other plant viral MPs or endogenous cell-to-cell–moving proteins are also regulated by post-translational modification is unknown. However, AMV MP is post-translationally modified in tobacco cells (60), MP of turnip yellow mosaic virus is phosphorylated in insect cells (139), and MP of potato leafroll virus is phosphorylated in potato (143).

A Model for Viral Cell-to-Cell Movement

Although many key elements of viral transport through plasmodesmata remain unknown, mainly in the area of TMV MP interactions with host cellular factors, we integrated the above knowledge about different TMV MP activities into a general

model for TMV cell-to-cell movement (Figure 1). After physical penetration into the host plant cell (step 1), TMV genomic RNA is likely replicated and translated in "viral factories" associated with the rough ER (70, 130) (step 2). The newly synthesized TMV MP may then cooperatively bind to the viral genomic RNA (20, 25) that is likely located in its proximity within the "viral factories" (70, 105) (step 3). This spatial connection between viral replication and translation may help to prevent sequence-nonspecific binding of TMV MP to cellular RNA molecules. TMV MP binding to RNA shapes it in a transferable form (25) and because viral RNA bound to MP is not translatable and does not replicate (85, 86), TMV MP may also sequester a fraction of the viral genomes from replication and translation and destines them for intercellular movement.

Association of the viral factories with the ER (70) may also allow TMV MP to recognize and bind PME molecules that are being inserted into the ER for their transport to the cell walls (step 4a). Alternatively, TMV MP may bind to the cytoskeletal elements in its vicinity (step 4b). In either case, interaction between TMV MP and PME or cytoskeleton likely targets MP-TMV RNA complexes from the sites of their synthesis and assembly to the cell periphery.

At the cell periphery, TMV MP has been proposed to bind to specific plasmodesmal receptors (90). However, such receptors have not yet been found, despite an extensive search in several laboratories. One possibility is that no specific plasmodesmata-associated sites for TMV MP binding exist. Instead, TMV MP may simply bind to the plant cell wall (steps 5a and 5b) and then enter plasmodesmal channels if they happen to reside in the area of its attachment (step 5a). In this case, TMV MP association with cell walls may be mediated by PME, which is found throughout the cell wall, including plasmodesmata (14). In the areas devoid of plasmodesmata, MP-TMV RNA complexes disassemble and/or TMV MP is degraded (step 6b); TMV MP may also be targeted for degradation after its interaction with the cytoskeleton (122).

Once at the plasmodesmal annulus, TMV MP acts to dilate this channel by an as-yet-unknown mechanism (step 6a). It is tempting to speculate that potential modulation of PME activity by TMV MP binding may contribute to this process, for example by inducing localized changes in ion balance. Increase in plasmodesmal permeability permits MP-TMV RNA complexes to transverse the channel and enter the adjacent host cell (step 7a). Passage through plasmodesmata likely alters MP-TMV RNA complexes (step 7a), allowing them to regain their replication and translation abilities (86) (step 2). These changes to the movement complexes, which are proposed to be caused by TMV MP phosphorylation (86), result in resumption of the infection and movement cycle of the virus (step 8).

While viral genomes continue to invade healthy tissues, TMV MP present within cells behind the leading edge of the expanding infection site is inactivated. At least in some hosts, such as *N. tabacum*, TMV MP is inactivated after its carboxyl-terminal phosphorylation by a cell wall-associated protein kinase (22; M-H Chen, S Ghoshroy, E Waigmann, V Citovsky, submitted for publication). Thus, TMV cell-to-cell movement may involve plasmodesmal gating by the newly

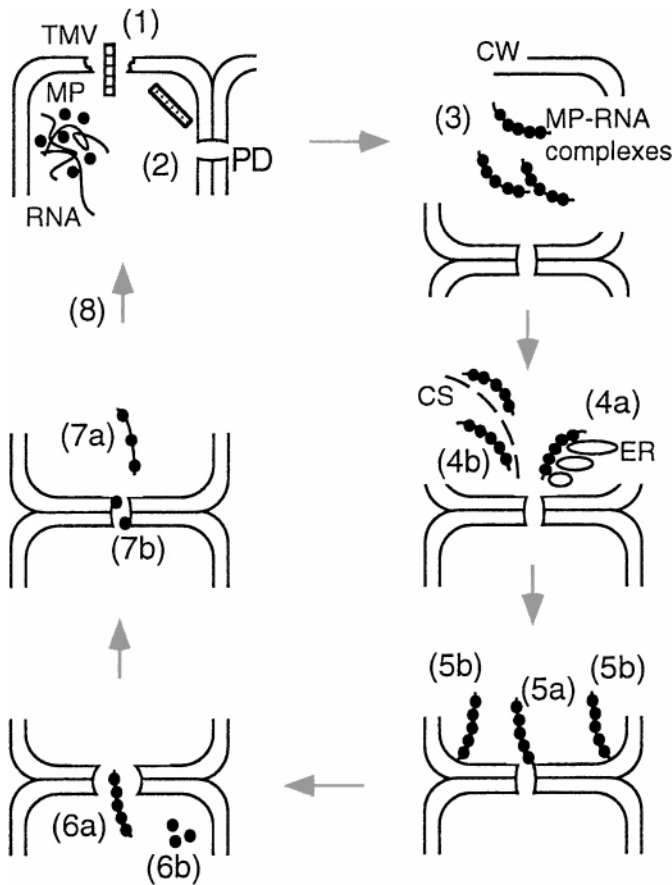


Figure 1 A model for TMV cell-to-cell movement. Steps: *1*, TMV virions enter the host cell by mechanical inoculation; *2*, viral particles uncoat and TMV RNA replicates and translates within "viral factories"; *3*, TMV MP forms complexes with viral RNA and shapes them in a transferable form; *4*, MP-TMV RNA complexes are targeted to the cell periphery by interaction with the cytoskeleton (CS) (*4a*) or ER (*4b*); *5*, MP-TMV RNA complexes bind to PME at the cell wall (CW) either in the vicinity of plasmodesmata (PD) (*5a*) or away from them (*5b*); *6*, MP-TMV RNA complexes localized to plasmodesmata dilate these channels (*6a*), and MP-TMV RNA complexes bound to the cell wall areas devoid of plasmodesmata are disassembled and/or degraded (*6b*); *7*, MP-TMV RNA complexes move into the adjacent cell and are altered to restore their translation and replication abilities (*7a*), while at least some of TMV MP is inactivated by phosphorylation and sequestered within plasmodesmata, which also regain their original permeability (*7b*); *8*, TMV replication, translation, and movement cycle resume.

synthesized, unphosphorylated MP (step 6a), followed by phosphorylation of MP that has already performed its function, preventing it from further action (step 7b). The inactive TMV MP may then remain sequestered within plasmodesmata, which regain their restricted permeability (41, 120) (step 7b).

From Cell-to-Cell Movement to Systemic Infection

Although TMV MP alone is sufficient for cell-to-cell transport of viral RNA genomes to the vicinity of the host plant vascular system, virus entry into the vasculature and its subsequent systemic spread require an additional function generally encoded by viral coat proteins (CPs; reviewed in 56, 92). The exact nature of CP activity in viral systemic movement is still unknown. Potentially, CP, in concert with MP, may act to dilate plasmodesmata at the boundary between vascular and nonvascular tissues. Indeed, although TMV MP specifically accumulates in these plasmodesmata, it does not increase their permeability, which suggests that another factor, such as CP, is required for this function (41).

The molecular pathway by which plant viruses spread systemically is still obscure. Furthermore, it is likely that entrance into the host plant vasculature differs from egress back into nonvascular tissues. For example, exposure of tobacco plants to nontoxic concentrations of the heavy metal cadmium prevented tobamoviral disease (55) by specifically blocking viral exit from the vascular tissue into the noninoculated systemic organs, whereas viral entry into the vasculature was unaffected (18). Potentially, nontoxic levels of cadmium trigger synthesis of cellular factors that interfere with viral systemic spread, and this may represent one of the regulatory mechanisms for systemic RNA transport during viral infection or post-translational gene silencing (29, 88, 160, 168).

NUCLEAR AND INTRANUCLEAR TRANSPORT OF *AGROBACTERIUM* T-DNA

Agrobacterium spp.-plant cell T-DNA transport represents a unique case of transfer of genetic material between kingdoms (147). In nature, *Agrobacterium* spp. T-DNA carries genes involved in synthesis of plant growth regulators and production of opines that represent a major carbon and nitrogen source for this microorganism. Integration and expression of the T-DNA into the host cell genome results in formation of neoplastic growths, known as crown gall tumors, that produce and secrete opines that are then consumed by *Agrobacterium* spp. (reviewed in 75, 140, 175). T-DNA itself is not sequence-specific; any DNA fragment placed between two 25-bp direct repeats (known as T-DNA borders) on the Ti plasmid will be transported to the plant cell and integrated into the host genome. The bacterial machinery necessary for this process of T-DNA transport is provided by proteins encoded by the virulence (*vir*) region located also on the Ti plasmid (reviewed in 23, 140, 175).

The lack of T-DNA sequence specificity implies that the nucleic acid molecule itself does not contain specific signals for nuclear import and integration. Instead, this process is likely mediated by two *Agrobacterium* spp. virulence proteins, VirD2 and VirE2, which are thought to associate directly with the transported T-DNA molecule, forming a transport (T) complex (77, 179). Below, we discuss the molecular structure of the T complex and describe biological functions of VirD2 and VirE2 in T-DNA nuclear and intranuclear transport and integration.

Molecular Structure of *Agrobacterium* T Complex

After induction of *vir* gene expression by small phenolic molecules secreted from the wounded plant cells (144, 146), a transferable molecule of T-DNA is generated. This molecule, designated T-strand (145), is an ss copy of the coding strand of the T-DNA region (reviewed in 140, 179). Evidence to date suggests that the T-strand associates with two *vir* gene products, VirD2 and VirE2. During T-strand production, VirD2 attaches covalently to the 5′ end of the T-strand molecule (71, 78, 166, 173). VirE2, an ssDNA-binding protein, is presumed to coat the T-strand along its length (15, 17, 26, 32, 59). As do most ssDNA-binding proteins (12), VirE2 binds ssDNA cooperatively and without sequence specificity (26), consistent with the sequence nonspecific nature of the T-strand itself. Mutational analysis of VirE2 demonstrated that its carboxyl-terminal portion contained sequences required for ssDNA binding, whereas the amino terminal part was important for binding cooperativity (45).

Although there is little doubt that VirE2 is associated with the T-strand in plant cells (see below), it is still unclear whether this binding occurs already within the bacterial cell or VirE2 and T-strand molecules meet each other only inside the host plant cell. On the one hand, strong cooperative interaction between VirE2 and ssDNA (26) and their potential proximity during VirE2 synthesis and T-strand generation within the same bacterial cell suggest that VirE2 should bind to the T-strand very early in the infection process, especially if both of them are transported into the plant cell through the same channel (as suggested in 8). Indeed, in extracts from *vir*-induced *Agrobacterium* spp., T-strands and VirE2 are coprecipitated by anti-VirE2 antibodies (15). On the other hand, cotransformation of tobacco plants with one *Agrobacterium* strain that contains T-DNA but lacks VirE2 and another strain that lacks T-DNA but contains VirE2 restored infectivity to these individually nonpathogenic bacteria (121), which suggests that T-strands and VirE2 are exported from the bacterial cells independently of each other. Indeed, VirE2 export from *Agrobacterium* spp. into plants can be inhibited without affecting the export of the T-DNA (8, 93). Furthermore, recent studies of another protein product of the *virE* locus, VirE1, suggested that this chaperonelike protein binds to VirE2, prevents VirE2 binding to ssDNA, and facilitates VirE2 but not T-DNA export into plant cells (36, 148, 149, 176). Thus, VirE1 may export VirE2 into the host cell cytoplasm, in which these two proteins should dissociate from each other, allowing VirE2 to bind to the T-strands.

Although it is still unknown whether VirE2 enters the plant cell alone or already associated with the T-strand, this protein most likely binds the T-DNA molecule before nuclear import. This notion is supported by observations that (*a*) tumorigenicity of an avirulent VirE2 mutant of *Agrobacterium* spp. is restored when inoculated on VirE2-expressing transgenic plants (30, 54), and (*b*) fewer T-strands accumulate in plant cells infected with another VirE2 mutant of *Agrobacterium* spp., which suggests that VirE2 associates with the T-strand within the cytoplasm of the host cell and protects it from exonucleolytic degradation (174); this protective activity of VirE2 was also demonstrated in vitro (26). Thus, the T-strand is probably transported from the cytoplasm into the host cell nucleus as a complete T complex.

For a better understanding of how T complexes are transported into the nucleus, it is helpful to know their physical structure. To this end, complexes formed in vitro between purified VirE2 and bacteriophage M13 ssDNA were examined by quantitative scanning transmission microscopy, followed by mass analysis (19). This approach, which uses unstained freeze-dried samples (63, 163), circumvents potential stretching of protein-ssDNA complexes associated with platinum shadowing, used in earlier studies (26). Based on the scanning transmission microscopy data (19), VirE2-ssDNA complexes represent rigid and coiled filaments that are 12.6 nm wide, with a density of 58 kDa/nm. Each turn of the coil contains an average of 3.4 molecules of VirE2 and 63.6 bases of ssDNA, predicting 1176 VirE2 molecules bound to a 22-kb T-strand of the wild-type nopaline-specific *Agrobacterium* spp. (19). The need for active nuclear import is evident from the width of the VirE2-ssDNA complexes. Their outer diameter (12.8 nm) exceeds the orifice of diffusion channels of the nuclear pore (9 nm; reviewed by 51). It is compatible, however, with the size exclusion limit of the nuclear pore, which opens to 23 nm during the process of active nuclear uptake (48, 51). The apparent rigidity of VirE2-ssDNA complexes may also have a role in nuclear transport. Rigid filaments would not easily bend, preventing folding into globular structures with a larger diameter. This feature may be especially important for nuclear import of VirE2 complexes with long ssDNA molecules in some *Agrobacterium* spp. (30).

VirD2 and VirE2 Function in Nuclear Import of the T Complex

The large size of T complexes (19) implies the need for their active nuclear import. Because traffic of the T complex is thought to occur in a polar fashion, starting with its 5′ end (reviewed in 175), VirD2 that is attached to the 5′ end of the T-strand may provide this piloting function. Indeed, in numerous studies using direct immunolocalization as well as translational fusions with reporter enzymes ß-glucuronidase or ß-galactosidase, VirD2 was shown to accumulate specifically in the plant cell nucleus (24, 72, 76, 135, 155). Although VirD2 appears to contain two nuclear localization signals (NLS), one at each end of the molecule (72, 76), only the carboxyl-terminal sequence appears to function during *Agrobacterium*

infection (89, 112, 135, 141). This VirD2 NLS belongs to a bipartite class of such signals (76), characterized by two adjacent basic amino acids, a variable-length spacer region, and a basic cluster in which any three out of the five contiguous amino acids must be basic (44).

The VirD2 role in T-complex nuclear import was confirmed by studies showing that *Agrobacterium* spp. T-DNA expression and tumorigenicity are reduced in NLS-deletion mutants of VirD2 (117, 141). However, the reduced rather than completely blocked infection suggested that another T-complex component, such as VirE2, may participate in the process of nuclear import. Consistently, VirE2 accumulated in the nuclei of plant cells, and this nuclear import was mediated by protein sequences within the middle part of the VirE2 molecule (24, 30). Indeed, mutations in the central region of VirE2 decreased tumorigenicity but did not affect ssDNA binding or stability of this protein (45). Involvement of VirE2 in T-DNA nuclear import was demonstrated directly by cell biological and genetic approaches. First, microinjection of in vitro-formed complexes between VirE2 and fluorescently-labeled ssDNA resulted in efficient accumulation of the labeled ssDNA in the nuclei of stamen hair cells of the flowering plant *Tradescantia virginiana* (178). Microinjection of fluorescent ssDNA alone resulted in clear cytoplasmic fluorescence, yet not in nuclear staining. Furthermore, VirE2 did not import fluorescently-labeled dsDNA, which indicated the requirement for formation of nucleoprotein complexes. Nuclear import of VirE2-ssDNA was blocked by such known specific inhibitors of nuclear import as wheat germ agglutinin and nonhydrolyzable analogs of GTP (178). More recently, a genetic study used an *Agrobacterium* spp. strain lacking the entire VirE2 as well as the specific carboxyl-terminal NLS of VirD2 (54). This *Agrobacterium* spp. double mutant was not infectious on wild-type tobacco but produced tumors on VirE2-expressing transgenic plants. Thus, VirE2 expressed in a plant cell transferred T-strands into the nucleus in the absence of an NLS from any other known T-DNA–associated protein (54).

That both VirD2 and VirE2 actively participate in nuclear import of T-complexes may indicate a certain functional redundancy built into this critical step of *Agrobacterium* spp.-plant cell T-DNA transfer. In addition, the presence of these two proteins may help confer polarity to the T complex during its nuclear import, which may be important for the subsequent integration event. Indeed, genetic studies suggest that T-DNA integration is a polar process; however, it is still unclear whether it begins at the 5′ or the 3′ end of the T-strand molecule (35; 140 and references therein; 154). In either case, there must be a mechanism to differentiate between two ends of the imported T-strand. Potentially, this could be achieved by association of a VirD2 molecule with the 5′ end of the T-strand and the presence of VirE2 molecules attached in the proximity to the 3′ end of the T-strand. Functional variations between the nuclear import activities of these proteins may specify the ends of the T-strand and determine the polarity of its transport.

Because nuclear import of both VirD2 and VirE2 efficiently occurred in plant cells derived from host and nonhost plants for *Agrobacterium* spp. (24), potential functional differences between these proteins were examined in heterologous

nonplant systems. These systems may lack one of the potential plant nuclear transport mechanisms, thereby allowing discrimination between the VirD2 and VirE2 import activities. Using this approach it was demonstrated that, VirD2 possessed an evolutionarily conserved NLS that allowed its nuclear import in *Xenopus* oocytes, *Drosophila* embryos (62), human kidney and HeLa cells (131, 177), and yeast cells (132). Similar to what occurs inplant cells, VirD2 nuclear accumulation in animal cells was blocked by known inhibitors of nuclear import (62).

Unlike VirD2, VirE2 (derived from the nopaline-specific Ti plasmid) was not imported into the nuclei of *Xenopus* oocytes, *Drosophila* embryos (62), HeLa and COS cells (T Tzfira & V Citovsky, unpublished results), and yeast cells (132), which suggests that its nuclear import activity may be plant specific. Although, in another study, VirE2 (derived from the octopine-specific Ti plasmid) accumulated in the nuclei of permeabilized HeLa cells, it failed to mediate nuclear import of fluorescently labeled ssDNA (177). In the same system, VirD2 covalently bound to the labeled ssDNA promoted its nuclear import (177). These functional differences between VirD2 and VirE2 suggest that (*a*) in plant cells, VirE2 and VirD2 may use different cellular factors for their nuclear entry, and (*b*) animal cells may lack a subset of factors that recognize VirE2 and help its nuclear uptake in plant cells.

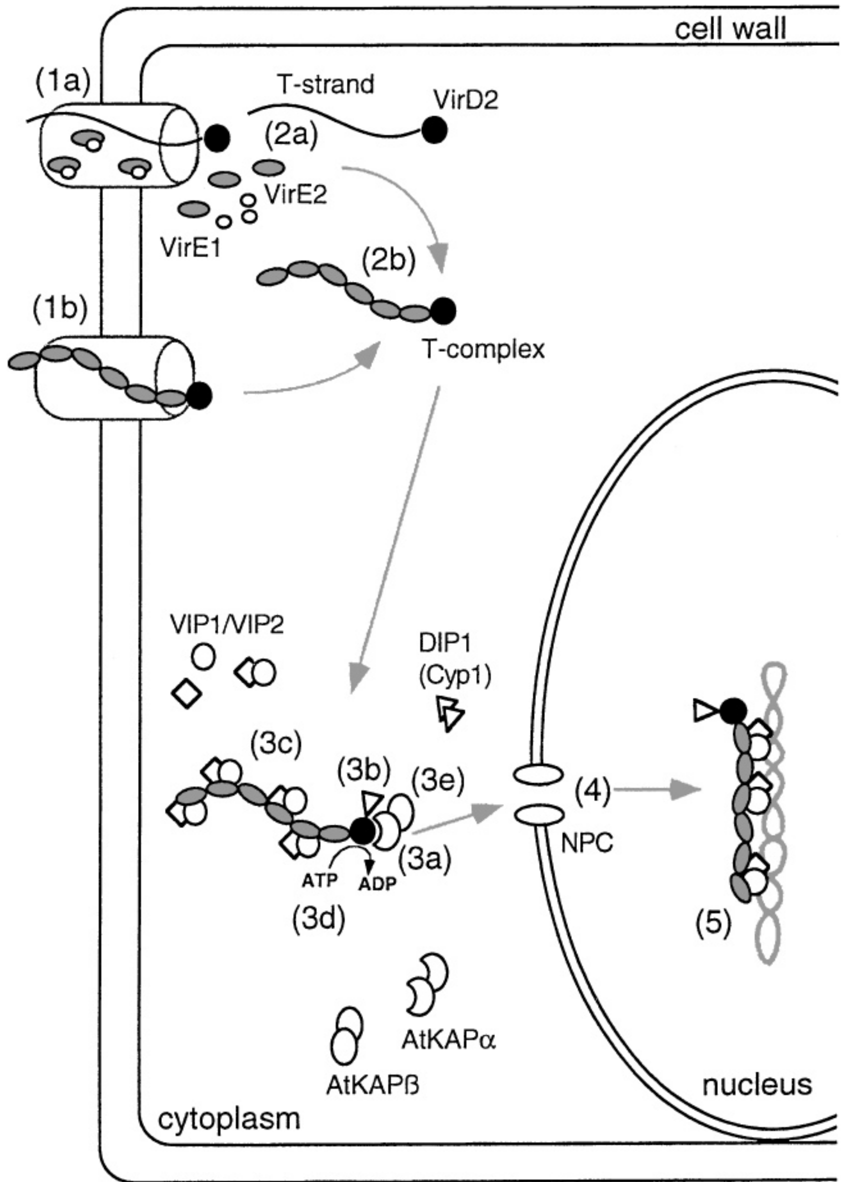
Host Cellular Proteins That Interact with VirD2 and VirE2

It makes biological sense that *Agrobacterium* spp. may use different cellular factors for nuclear import of VirE2 and VirD2. In this strategy, a single molecule of VirD2 at the 5′ end of the T-strand does not directly compete with the more abundant VirE2 and has a better chance to lead the T-complex into the nucleus, specifying its import polarity. Thus, in yeast two-hybrid protein-protein interaction assays (49, 74), a search for plant VirD2- and VirE2-interacting proteins has been initiated. To date, VirD2 has been found to interact with several plant cellular proteins (7, 37). One VirD2-interacting protein, DIP1 of *Arabidopsis* (37), belongs to a large cyclophilin family of peptidyl-prolyl *cis-trans* isomerases, which are highly conserved in plants, animals, and prokaryotes (47, 50, 67, 83, 98, 104, 150). In fact, VirD2 interacted with three isoforms of *Arabidopsis* cyclophilins, Roc1, Roc4 (98), and CypA (67), both in the two-hybrid system and in vitro (37). It is interesting that cyclosporin A, known to bind cyclophilins and block their peptidyl-prolyl *cis-trans* isomerase activity (50, 66, 150), inhibited VirD2-CypA interaction in vitro and abolished *Agrobacterium* spp.-mediated transformation of *Arabidopsis* roots and tobacco cell suspension cultures (37). Although the biological role of cyclophilins in *Agrobacterium* infection is unclear, they were proposed to maintain proper conformation of VirD2 within the host cell cytoplasm and/or nucleus during T-DNA nuclear import and/or integration (37). Indeed, in addition to their enzymatic activity, cyclophilins may act as molecular chaperones aiding protein folding in animal cells (6, 104). DIP2 and DIP3, two additional *Arabidopsis* proteins that bind VirD2 in the two-hybrid assay, have been reported, but no information about their identity or biological role was provided (37).

Another cellular factor encoded by a tomato DIG3 cDNA specifically and strongly interacted with the VirD2 NLS region (Y Tao, P Rao, & S Gelvin, unpublished results). The DIG3 protein was identified as an enzymatically active type 2C serine/threonine protein phosphatase that is homologous to type 2C serine/threonine protein phosphatase from other organisms, including the *Arabidopsis ABI1* gene product (97, 110). An *Arabidopsis abi1* mutant (110) showed a higher sensitivity to *Agrobacterium* spp. transformation than did the wild-type plant, whereas overexpression of DIG3 in tobacco protoplasts inhibited nuclear import of a ß-glucuronidase-VirD2 NLS fusion protein.

Finally, VirD2 was also found to interact with an *Arabidopsis* protein, designated AtKAPα (7), which belongs to a growing family of karyopherins α that are known to mediate nuclear import of NLS-containing proteins (reviewed in 118, 128). AtKAPα contains two features typical of karyopherins α—eight contiguous repeats of the "arm" motif (125) and four amino-terminal clusters of basic amino acids. In animal and yeast karyopherins α, the arm motifs are thought to recognize NLSs, whereas the amino-terminal basic domains probably interact with the karyopherin ß proteins (61). Functionally, AtKAPα was similar to the yeast karyopherin α, Kap60p, or Srp1p, because it complemented this gene function in a temperature-sensitive *srp1-31* yeast mutant (7, 99). AtKAPα-VirD2 interaction, which occurred both in the two-hybrid system and in vitro, required the presence of the VirD2 carboxyl-terminal NLS (7), which supports the potential role of AtKAPα in VirD2 nuclear import. This function was demonstrated directly when AtKAPα promoted nuclear import of fluorescently labeled VirD2 in permeabilized yeast cells (7). The yeast-derived import assay (138) was chosen because no plant experimental system exists in which the role of isolated components of protein nuclear import machinery can be tested in vitro (10, 73, 109). AtKAPα-mediated nuclear import of VirD2 was specific because (*a*) VirD2 lacking its NLS remained cytoplasmic and (*b*) a synthetic peptide corresponding to the VirD2 NLS blocked nuclear import, probably competing with VirD2 for interaction with AtKAPα (7). Thus, during *Agrobacterium* spp. infection, AtKAPα likely promotes nuclear import of VirD2 and its cognate T-strand. In uninfected plants, AtKAPα may function as an NLS-binding protein that mediates transport of the nuclear proteins of the cell.

Unlike VirD2, VirE2 did not interact with AtKAPα in the two-hybrid assay (7), which suggests that another plant protein(s) that recognizes VirE2 may be involved in nuclear import of the T complex. Recently, several VirE2-binding proteins from *Arabidopsis* spp. have been identified (T Tzfira & V Citovsky, unpublished results; see also reference 37). VIP1 and VIP2 interacted with VirE2 but not with known nonspecific activators in the two-hybrid system (T Tzfira & V Citovsky, unpublished results). VIP1 contained a conserved stretch of basic amino acids (basic domain) abutting a heptad leucine repeat (leucine zipper), two structural features characteristic of the basic-zipper proteins that are known to localize to the cell nucleus (158). Interestingly, coexpression of VIP1 with GFP-labeled VirE2 in mammalian COS cells promoted nuclear uptake of VirE2 in these cells. In addition, with the use of a recently developed genetic assay for nuclear import and


cell wall
(1a)
T-strand
VirD2
(2a)
VirE2
VirE1
(2b)
(1b)
T-complex
VIP1/VIP2
DIP1
(Cyp1)
(3c)
(3b)
(3e)
(4)
(3a)
NPC
ATP
ADP
(3d)
(5)
AtKAPα
AtKAPß
nucleus
cytoplasm

export (132), expression of VIP1 was shown to allow nuclear import of VirE2 in yeast cells (T Tzfira & V Citovsky, unpublished results). Thus, VIP1 may interact with VirE2 in the cell cytoplasm and, being a nuclear protein itself, carry it into the cell nucleus. Because VIP1 reconstructs VirE2 nuclear import in nonplant systems and because no animal or yeast homologs of VIP1 were found in protein databases (T Tzfira & V Citovsky, unpublished results), VIP1 may represent the cellular factor involved in the plant-specific nuclear uptake of VirE2.

Another *Arabidopsis* VirE2-interacting protein, VIP2 (T Tzfira & V Citovsky, unpublished results), shared a similarity in its carboxyl-terminal part with the *Drosophila* Rga protein that is thought to mediate interaction between chromatin proteins and the transcriptional complex (52). It is also possible that VIP2 may recognize and associate with the plant chromatin. Unlike VIP1, VIP2 had no effect on intracellular localization of VirE2 when coexpressed in yeast or mammalian cells. However, VIP2 interacted not only with VirE2 but also with VIP1 in the two-hybrid system (T Tzfira & V Citovsky, unpublished results). Because both VIP1 and VIP2 interact with VirE2 and each other, it is tempting to speculate that they may function in a multiprotein complex that performs a dual function; it facilitates nuclear targeting of VirE2 and plays a role in intranuclear transport of VirE2 and its cognate T-strand to the site of integration. A similar dual role in nuclear and intranuclear transport has been suggested for the yeast Kap114p protein that functions to import the TATA-binding protein into the cell nucleus and target it to the promoters of genes to be transcribed (126). Besides VIP1 and VIP2, three other VirE2-interacting proteins have been reported but not identified or characterized (37).

A Model for T-DNA Nuclear Import and Intranuclear Transport

Recent advances in understanding of the biological activities of VirD2 and VirE2, as well as identification of some of their cellular interactors, are synthesized into a working model for nuclear and intranuclear transport of the *Agrobacterium* spp. T complex (Figure 2). This process initiates either with the entry of the T complex into or its assembly within the host cell cytoplasm. If VirE2 is able to associate with the T-strand that is already in the bacterial cell, the entire T complex is transported into the plant cell (step 1b). If, however, VirE2 binding to T-strands is prevented by its association with VirE1 within the bacterium, VirE2-VirE1 complexes and T-strand–VirD2 conjugates are transported separately into the host cell, possibly through the same channels formed by VirB proteins (8) (step 1a). In this case, once VirE2 is in the plant cell, or perhaps even within the transport channel itself, it dissociates from VirE1 by an as-yet-unknown mechanism (step 2a) and attaches to the T-strand molecules, coating their entire length in a cooperative fashion (step 2b). In either scenario, the T-strand probably begins its journey from the cell cytoplasm into the nucleus as a T complex containing both VirD2 and VirE2 proteins. Structurally, the T complex represents a coiled filament in which the T-strand molecule, buried within the VirE2 matrix, is protected from cellular nucleases. During

translocation through the nuclear pore, however, the telephone cordlike T complex may stretch, further reducing its outer diameter and facilitating the import process.

In the host cell cytoplasm, both VirD2 and VirE2 specifically bind to their respective cellular interactors. Most likely, VirD2 associates with AtKAPα, which binds to its carboxyl-terminal NLS signal (step 3a). As do other members of the karyopherin α family, AtKAPα directly mediates nuclear import of NLS-containing proteins. All known animal and yeast karyopherins α function in heterodimer with karyopherin ß; in this complex, the α subunit recognizes the NLS signal in the transported protein molecule, whereas the ß subunit mediates docking of the entire NLS-karyopherin α/ß complex to the nuclear pore and its interaction with the Ran GTPase (reviewed in 61, 118). In a heterologous mammalian in vitro system, however, *Arabidopsis* karyopherin α has been proposed to function alone, independently of karyopherin ß (80). This apparently unique property of *Arabidopsis* karyopherins α is surprising because these proteins are highly homologous to their animal and yeast counterparts, carry the karyopherin ß–binding motif, but do not contain sequences known to be required for binding to the nuclear pore or Ran (7, 73). Furthermore, in rice, karyopherin ß has indeed been isolated and shown to interact with karyopherin α (106). Thus, in vivo, a putative *Arabidopsis* karyopherin ß protein may still be involved in the AtKAPα-mediated nuclear uptake of VirD2 (step 3e). In any case, because VirD2 is attached to the 5′ end of the T-strand, binding of AtKAPα to VirD2 may orient the entire T complex so that its nuclear import will initiate at the 5′ end. In addition to AtKAPα, cellular cyclophilins may also bind VirD2 to maintain its active conformation or perform some other, as yet unknown, functions. Because cyclophilin binding does not involve the NLS region of VirD2 (37), it may occur concurrently with the VirD2-AtKAPα interaction (step 3c). Finally, the VirD2 NLS region may become phosphorylated (step 3d).

Binding of VirE2 to VIP1 further facilitates nuclear import of the T complex. Unlike nuclear import of VirD2, which occurs by a conventional karyopherin α–mediated pathway, VirE2 may be imported as cargo attached to the NLS-containing VIP1 (step 3b). In addition, involvement of other plant factors, such as a hypothetical VirE2-specific karyopherin α or ß, in the VirE2 nuclear import

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Figure 2 A model for nuclear and intranuclear transport of *Agrobacterium* T-DNA. Steps: *1*, T-strand with VirD2 at the 5′ end is transferred through the VirB channel either separately (*1a*) or in complex with VirE2 (*1b*); for separate transport, VirE2 is transferred through the same channel in complex with VirE1 (*1a*). *2*, If transported separately from the T-strand, VirE2 dissociates from VirE1 (*2a*) and binds to the T-strand (*2b*) in the plant cell cytoplasm, forming the T complex. *3*, T complex associates with cellular factors; VirD2 binds to AtKAPα (*3a*) and cyclophilins (*3b*), VirE2 binds to VIP1 and/or VIP2 (*3c*), the VirD2 NLS region is phosphorylated (*3d*), and AtKAPα may interact with the putative AtKAPß (*3e*). *4*, T complex is translocated into the nucleus through the nuclear pore (NPC). *5*, T complex is targeted to the chromosome by VIP1 and/or VIP2.

cannot be excluded. The more direct AtKAPα-mediated import of VirD2, which likely dominates the VIP1-mediated "piggyback" nuclear uptake of VirE2, may be required for nuclear entry of the T complex (step 4) in a polar fashion. Polar translocation may be a common feature of nuclear transport of many naturally occurring nucleic acid-protein complexes (28). Nuclear export of a 75S premessenger ribonucleoprotein particle in *Chironomus tentans*, for example, initiates exclusively at the 5′ end of the RNA (108).

Once in the host cell nucleus, or perhaps even still in the cytoplasm, VirE2 may also associate with VIP2 (step 3b). The fact that VIP1 also recognizes and binds VIP2 may significantly facilitate this association, resulting in stable VirE2-VIP1-VIP2 multiprotein complexes (step 3b). These complexes may first facilitate nuclear import of the T complex and then target it to the host cell chromosome (step 5). This model for intranuclear transport explains how the invading T complex finds its way to the host genome; furthermore, because VIP1 and VIP2 likely interact with chromatin during transcription, they may bring the T complex to chromosomal regions in which the host DNA is more exposed and, thus, better suitable for T-DNA integration (step 5).

From Import to Integration

Nuclear import of the T complex culminates with T-DNA integration into the host genome. The mechanism by which T-DNA integration occurs is largely unknown. Unlike other mobile DNA elements, such as transposons and retroviruses, T-DNA does not encode enzymatic activities required for integration. Thus, T-DNA insertion into the plant DNA must be mediated by proteins transported from *Agrobacterium* spp. themselves, namely VirD2, VirE2, and/or host cell factors.

Indeed, an amino acid sequence at the carboxyl terminus of VirD2, the ω domain (141), is likely involved in T-DNA integration (112, 117). Also, an arginine-to-glycine mutation in the histidine-arginine-tyrosine integrase motif of VirD2 decreases the precision but not the efficiency of T-DNA integration in vivo (155). VirD2 was shown to join ss oligonucleotides in vitro (123). Thus, to complete integration in vivo, VirD2 may also participate in the ligation of the 5′ end of the T-DNA to the genomic DNA, followed by the second-strand synthesis, which is performed by the plant DNA-repair machinery (156). Another study, however, suggested that T strands are converted into a ds form before integration (35). In addition to VirD2, VirE2 may also be involved in the integration process. Specifically, VirE2 may be required for integration fidelity of the 3′ end of the T-strand (136).

Recent studies have also focused on cellular factors involved in the T-DNA integration. In a genetic approach, several *Arabidopsis* spp. Mutant (*rat*) resistant to transformation by *Agrobacterium* spp. were isolated and tested for stable and transient expression of reporter enzymes contained in the *Agrobacterium* spp. T-DNA (116). One mutant, *rat5*, which carried a mutation in the *H2A* histone gene, was deficient in stable but not transient T-DNA expression, indicating the involvement of H2A in T-DNA integration (113). T-DNA integration

deficiency has also been suggested to cause resistance of several ecotypes of *Arabidopsis* spp. to *Agrobacterium* spp. infection (115). Finally, integration deficiencies have also been suggested to underlie the known resistance of maize, and possibly other monocotyledonous plants, to *Agrobacterium* spp. infection (9, 117). Consistent with this hypothesis, nuclear import of VirD2 and VirE2 does not represent the limiting step in *Agrobacterium* spp. infection of maize (24).

FUTURE DIRECTIONS

Recent developments reviewed here illustrate the significant progress achieved in our understanding of nucleic acid transport during plant-microbe interactions. Especially advanced is the knowledge about the microbial proteins (e.g. viral MPs, VirD2, and VirE2) involved in this process. On the other hand, studies identifying the host cellular factors required for transport, although revealing, are just beginning. Thus, future developments in this field will most likely come from identification of additional cellular participants and regulatory components of the transport pathways. For instance, the cell wall-associated protein kinase that phosphorylates TMV MP and regulates its function has yet to be isolated. Cellular proteins that interact with VirD2 and/or VirE2 during T-DNA integration also remain unknown. Perhaps the best way to achieve these goals is to combine biochemical, molecular, and cell biological techniques with a genetic approach aimed at identifying and characterizing plant mutants with altered plasmodesmal functions or susceptibility to *Agrobacterium* spp. infection. For example, *Arabidopsis* mutants that are resistant to systemic spread of tobamoviruses (91) and ecotypes (94, 96, 142) or mutants with altered systemic movement of other plant viruses have been reported (101). In a similar way, *Arabidopsis* mutants and ecotypes deficient in different steps of *Agrobacterium* spp. T-DNA transport and integration have been described (113, 115, 116). Recently, the importance of this genetic approach has been recognized by establishment of a functional genomics initiative by the National Science Foundation, which is aimed at identification of all *Arabidopsis* genes involved in *Agrobacterium* spp.-host cell interactions.

The importance of solving molecular mechanisms of nucleic acid transport during plant-microbe interactions is difficult to overestimate. This fundamental knowledge will have a profound effect on our understanding of intercellular communication in plants, help design new strategies to produce agronomically important plants that are resistant to viruses and *Agrobacterium* spp., and allow development of improved genetic engineering procedures for efficient nuclear delivery and integration of foreign genes.

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