The Plant Cell, Vol. 11, 535–548, April 1999, www.plantcell.org © 1999 American Society of Plant Physiologists

Viral Movement Proteins as Probes for Intracellular and Intercellular Trafficking in Plants

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INTRODUCTION

The ability of viruses to cross the cellulosic cell wall to propagate infection throughout a plant has been a long-standing puzzle in plant cell biology and virology. Contemplated from the perspective of the topology of the plant cell and the plant as an integrated structure of the differentiated tissue types, and given the existence of apoplastic and symplastic domains, the solution was not apparent. Plasmodesmata were the obvious conduits for moving between cells—or were they? Conventional wisdom held that plasmodesmata were too small for the transport of nucleic acids, no less virus particles. Consideration of this problem was advanced once two major biases were discarded: (1) the static view of plasmodesmata as simply being boring holes in the plant cell wall (Robards and Lucas, 1990; Beebe and Turgeon, 1991; Lucas et al., 1993); and (2) the assumption, despite the fact that moving cell to cell across a wall is a fundamentally unique aspect of plant virus infection that, as shown for animal viruses, encapsidated virus particles would move from cell to cell within the plant.

Our first insights into this process came from studies on the movement protein (MP) encoded by the RNA-containing tobacco mosaic virus (TMV; the 30-kD protein) and red clover necrotic mosaic virus (RCNMV; Deom et al., 1987; Wolf et al., 1989; Citovsky et al., 1990; Ding et al., 1992a; Fujiwara et al., 1993; Giesman-Cookmeyer and Lommel, 1993; Waigmann et al., 1994). These MPs could increase the size exclusion limits (SEL) of plasmodesmata when expressed in transgenic plants or introduced by microinjection into leaf mesophyll cells, and it appeared that cell-to-cell spread of infection could occur in the absence of virus particles. Based on these findings and additional data, it was suggested that complexes of viral RNA and MP move from cell to cell. As described in a number of excellent recent reviews (Hull, 1991; Lucas and Gilbertson, 1994; Maule, 1994; Carrington et al., 1996; Lartey and Citovsky, 1997; McLean et al., 1997), these findings focused much attention and a flurry of activity on the ability of different viral-encoded MPs to affect plasmodesmal gating, the potential existence of plasmodesmal targeting sequences within these MPs, and the use of MPs as tools for identifying components of plasmodesmata.

The continued investigation of the function of MPs, encoded by a number of very different viruses, from the perspective of what the virus must accomplish—namely, to coordinate replication of the virus genome and the vectorial movement of the progeny genomes from the infected cell into adjacent cells—has expanded this focus to include using MPs to unravel the fundamental mechanisms by which macromolecular transport is directed and integrated within and between plant cells. MPs not only use plasmodesmata but they can modify and at times can even intercept these intercellular channels, depending on whether they function in fully differentiated or developing cells (Thomas and Maule, 1995; Kasteel et al., 1996; Ward et al., 1997). It is not merely coincidental that quite specific and distinct interactions occur between the cortical endoplasmic reticulum (ER) and the MPs encoded by TMV or the DNA-containing bipartite geminivirus squash leaf curl virus (SqLCV) (Sanderfoot and Lazarowitz, 1995; Ward et al., 1997; Heinlein et al., 1998; Mas and Beachy, 1998; Reichel and Beachy, 1998). These distinct interactions provide new approaches for identifying and investigating the functional importance of subdomains within the ER (Reuzeau et al., 1997b; Staehelin, 1997). The discovery that there are specific associations of the TMV 30-kD protein with microtubules and actin filaments complements studies on the role of the cytoskeleton in directing transcytoplasmic movement and regulating plasmodesmal function (Heinlein et al., 1995; McLean et al., 1995). Finally, studies of the MPs encoded by SqLCV, which replicates in the nucleus, provide the additional dimension of MP function in nucleocytoplasmic transport and afford a unique opportunity to study the regulation of nuclear shuttling and the mechanism of nuclear export in plant cells (Sanderfoot and Lazarowitz, 1996; Sanderfoot et al., 1996; Ward and Lazarowitz, 1999).

This review addresses the unique potential of viral MPs for understanding how macromolecules are transported within cells in a directed and integrated manner and for defining

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the signal transduction pathways involved in these processes.

"KNEADED OUT OF THE SAME DOUGH BUT NOT BAKED IN THE SAME OVEN"—THE BASICS OF MOVEMENT

To infect a susceptible host successfully, plant viruses must move locally from cell to cell and enter the phloem, through which they will move long distance to establish a systemic infection and produce disease. This process requires that plant viruses overcome the barrier posed by the plant cell wall, a feat mediated by virus-encoded MPs. That such proteins exist was first inferred from genetic studies. A plant virus having a null mutation in MP function can still replicate and encapsidate progeny virus particles. However, these progeny virions do not move out of the inoculated leaf to produce systemic infection (Atabekov and Dorokhov, 1984; Atabekov and Taliansky, 1990; Hull, 1991). Thus, the active expression of MPs eliminates the cell wall barrier to plant virus movement, which suggests that MPs must somehow function to fundamentally alter cell architecture and thereby remove this hurdle.

The prediction of the genetic studies, namely, that MPs would alter cell structure to overcome the cell wall barrier to virus movement, has been borne out by studies on the cell-to-cell movement of a number of diverse plant viruses. These studies demonstrate that most viral MPs (with one notable and biologically relevant exception in the geminiviruses) act to channel the viral genome through the cell wall (Lucas and Gilbertson, 1994; Carrington et al., 1996; McLean et al., 1997). We now appreciate that distinct viruses attack this problem in different, although not necessarily unrelated, ways, depending on the cell types infected. Furthermore, the final act of crossing the cell wall is preceded by regulated interactions of MPs and other viral components with the endomembrane system and cytoskeletal network. These specific interactions are designed to identify the viral genome and move it in a directed manner to the cell wall for transport.

A Single MP but No Coat Protein

TMV and RCNMV represent the simplest case of RNA viruses that infect mesophyll cells, encode a single MP, and do not need coat protein (CP) for cell-to-cell movement, although CP is needed for systemic infection (Vaewhongs and Lommel, 1995; X. Ding et al., 1996). The MP encoded by each virus can bind RNA in vitro (Citovsky et al., 1990, 1992; Giesman-Cookmeyer and Lommel, 1993) and increase the SEL of plasmodesmata in mesophyll cells. This latter finding was originally shown both by dye-coupling studies in transgenic plants expressing the MP (Deom et al., 1987; Wolf et al., 1989) and by direct microinjection of the MP together with dye-coupled dextrans into tobacco leaf mesophyll cells (Fujiwara et al., 1993; Waigmann et al., 1994). The TMV MP has been localized to plasmodesmata in immunogold-labeling studies of transgenic plants (Atkins et al., 1991; Ding et al., 1992a; Moore et al., 1992). In addition, in plants infected with a recombinant TMV virus or potato virus X (PVX) vector that expresses the 30-kD protein as a fusion to the green fluorescent protein (GFP), the MP–GFP fusion protein is localized to spots in the cell wall that appear to be plasmodesmata when imaged by confocal and fluorescence microscopy (Oparka et al., 1996a, 1996b; Padgett et al., 1996). Notably, the plasmodesmata do not appear to be grossly altered in their physical appearance by the 30-kD MP.

These studies have led to a model, shown in Figure 1, whereby the TMV MP acts as a molecular chaperone to bind the viral genome and target it to plasmodesmata, where the MP then increases the SEL and thus facilitates movement of the viral genome into adjacent uninfected cells (Lucas and Gilbertson, 1994; McLean et al., 1997). Subsequent studies of MPs 3a, encoded by cucumber mosaic virus (CMV), and MPB (for MP encoded by the B component; formerly BL1 or BC1), encoded by the geminivirus bean dwarf mosaic virus (BDMV), using similar approaches of in vitro nucleic acid binding assays and/or microinjection of dye-coupled dextrans or nucleic acids along with the MP or into transgenic plants that expressed the MP (Noueiry et al., 1994; Ding et al., 1995; Li and Palukaitis, 1996), led to the suggestion that all MPs would act in a similar manner (Lucas and Gilbertson, 1994).

The approach of microinjecting MPs expressed in and isolated from *Escherichia coli* into leaf mesophyll cells has been useful for assaying certain properties of these viral-encoded proteins and providing a more dynamic view of plasmodesmata. A more complete picture of MP action and the details of virus movement, particularly in relation to the regulation of MP function and the integrated interactions of MPs with viral and host components in different cell types to bring about a successful infection, are now emerging from the investigation of MP function in the context of virus infection using a variety of techniques for expressing and visualizing MPs in living cells and plants (Heinlein et al., 1995; Sanderfoot and Lazarowitz, 1995; Oparka et al., 1996a, 1997a, 1997b; Canto et al., 1997; Ward et al., 1997; Blackman et al., 1998; Reichel and Beachy, 1998; Santa Cruz et al., 1998; Ward and Lazarowitz, 1999). Examination of plants infected with CMV recombinant viruses that express GFP–3a fusions or free GFP substituted for 3a or CP, and immunolocalization studies show that CP, in addition to the 3a MP, is needed for cell-to-cell movement of the viral genome and have further revealed the role of MP in viral entry into the phloem in minor veins (Canto et al., 1997; Blackman et al., 1998). Studies using the TMV GFP–MP fusion protein show that effects on plasmodesmal gating are restricted to the leading edge of infected cells (Canto et al., 1997; Oparka et al., 1997a).

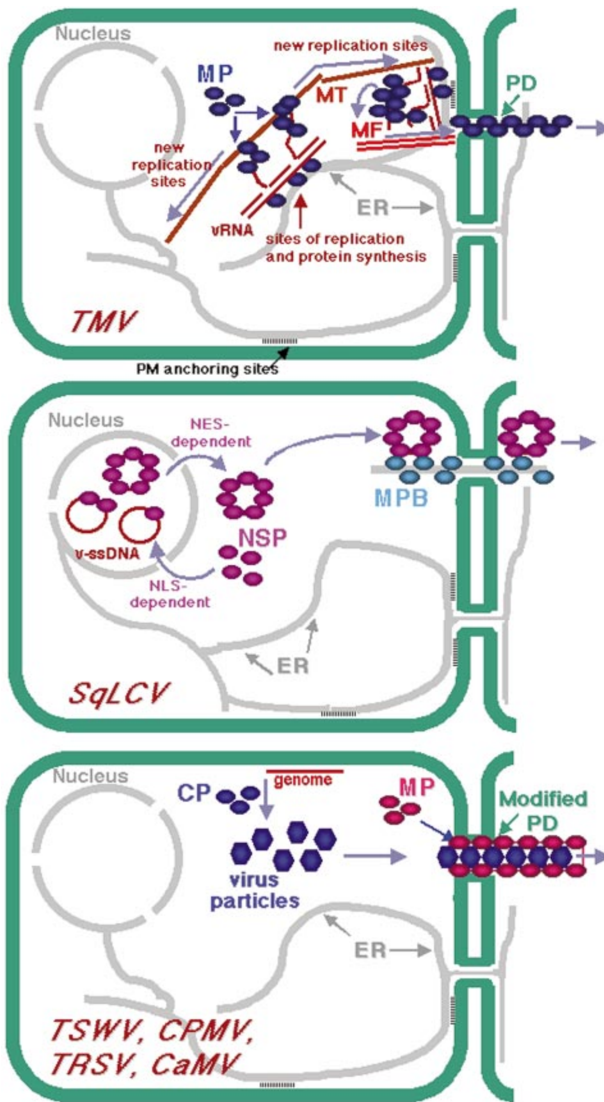

Figure 1. Models for Plant Virus Intracellular Movement.

(Top) TMV: MP complexed with viral genomic RNA is proposed to move along microtubules from ER-associated sites of viral replication and protein synthesis ("viral factories") to establish additional viral factories at other ER sites. From viral factories associated with cortical ER, actin microfilaments may deliver MP–genome complexes to putative cell wall adhesion sites and plasmodesmata.
(Middle) SqLCV: NSP is a nuclear shuttle protein that moves newly replicated viral ssDNA genomes from the nucleus to the cytoplasm. MPB, associated with ER-derived tubules, traps the NSP–ssDNA complexes in the cytoplasm and is proposed to guide these along the tubules and through the cell wall into adjacent cells. In these adjacent cells, NSP–ssDNA complexes would be released and target the viral DNA to the nucleus to initiate a new round of replication and infection.
(Bottom) TSWV, CPMV, TRSV, and CaMV: These viruses require CP in addition to MP for cell-to-cell movement. The viral genome, encapsidated in virus particles, moves through MP-containing tubules that appear to emerge from highly modified plasmodesmata.

A Requirement for CP

RNA viruses from a diverse range of families that infect mesophyll cells encode a single MP and require CP for cell-to-cell movement. It appears that an encapsidated form of the viral RNA genome (a subviral or virus particle) moves from cell to cell, and this movement is accommodated by the formation of large tubular structures that extend from the cell wall to adjacent uninfected cells (Figure 1; Kasteel et al., 1993, 1996; Weiczorek and Sanfacon, 1993; Kormelink et al., 1994; Ritzenthaler et al., 1995).

Immunogold labeling of thin sections from infected plants localizes the MP encoded by a number of viruses, including tomato spotted wilt virus (TSWV; tospovirus), cowpea mosaic virus (CPMV; comovirus), cauliflower mosaic virus (CaMV; caulimovirus), and tomato ringspot and grapevine fanleaf viruses (nepovirus), to such large tubules. Furthermore, these tubular structures also appear to contain virus-like particles (Linstead et al., 1988; Kasteel et al., 1993; Weiczorek and Sanfacon, 1993; Kormelink et al., 1994; Ritzenthaler et al., 1995). The tubules extend from the walls of infected mesophyll cells at or near what appear to be modified plasmodesmata that lack a central desmotubule. Although virions in the comovirus, caulimovirus, and nepovirus families are icosahedral, TSWV as a typical bunyavirus has three genomic components encapsidated in flexuous helical nucleocapsids (Prins and Goldbach, 1998). A number of helical viruses, such as potexvirus and potyvirus, also require CP for cell-to-cell movement (see below) but do not move via tubules. Thus, there is no simple correlation between tubule formation and the transport of icosahedral virus particles. Whether particle diameter is the major determinant, as has been suggested in studies of PVX (Cruz et al., 1998), remains to be seen. It is relevant to note that TSWV as a negative-sense RNA virus would have to transport its replicase (L) protein associated with viral nucleocapsids from cell to cell to initiate replication upon entering uninfected cells. Perhaps tubules are required to accommodate these TSWV replicase–nucleocapsid complexes.

Studies using transiently transfected protoplasts and/or insect cells demonstrate that the MP of TSWV, CaMV, and CPMV (Perbal et al., 1993; Wellink et al., 1993; Storms et al., 1995; Kasteel et al., 1996, 1997) appears to be necessary and sufficient to induce the formation of these tubules and that neither CP, nor apparently plasmodesmata or unique plant cell factors, are required for tubule formation. However,

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CP, coat protein; ER, endoplasmic reticulum; MF, microfilament; MP, movement protein; MT, microtubule; NES, nuclear export signal; NLS, nuclear localization signal; PD, plasmodesmata; TRSV, tomato ringspot virus; vRNA, viral RNA genome; v-ssDNA, viral single-strand DNA genome.

CP is required for the formation of the virus-like particles within the tubules (Kasteel et al., 1997). Studies in which plants are inoculated with RNA components of CPMV that contained GFP substituted for CP and/or MP show that CP as well as MP are required for the cell-to-cell spread of the virus infection (Verver et al., 1998). Based on all of these studies, it is proposed that virus particles of TSWV, CPMV, and these other viruses associated with tubules move cell to cell through MP-containing tubules and modified plasmodesmata. Curiously, attempts to identify cellular components that are associated with the MP-containing tubules for these viruses have failed to show any association with ER, actin, or other cell components (Kasteel et al., 1997). This contrasts with studies on the MPs encoded by TMV and SqLCV (Heinlein et al., 1995, 1998; McLean et al., 1995; Ward et al., 1997; Reichel and Beachy, 1998; see below).

A group of diverse viruses, namely, the potexviruses, hordeiviruses, furoviruses, and carlaviruses, encodes a clustered so-called triple gene block of three proteins that are required for cell-to-cell and systemic movement (Petty et al., 1990; Beck et al., 1991; Gilmer et al., 1992). Some of these viruses, such as PVX, also require CP for cell-to-cell as well as systemic movement. The functions of the triple gene block proteins have been studied in most detail for PVX (the 25-, 12-, and 8-kD proteins). Examination of plants inoculated with wild-type or mutant PVX viruses that can each express either β-glucuronidase (GUS) or GFP, including a mutant containing a *GFP* replacement for the *CP* gene, establishes that CP, in addition to the triple gene block–encoded proteins, is required for cell-to-cell movement (Baulcombe et al., 1995; Santa Cruz et al., 1996, 1998; Morozov et al., 1997; Verchot et al., 1998).

Immunolocalization studies in infected plants and protoplasts, and confocal imaging of plants inoculated with PVX expressing a GFP–CP fusion, show that CP localizes to plasmodesmata; however, dye-coupling studies in transgenic plants that express CP show that CP does not affect plasmodesmal gating properties (Oparka et al., 1996b; Santa Cruz et al., 1996, 1998). Using viruses that express a *GUS* reporter gene, dye-coupling studies in trichomes microinjected with wild-type PVX or a mutant deleted in the gene encoding the 25-kD protein show that the 25-kD protein affects plasmodesmal SEL, although partial modification of SEL was observed in the absence of the 25-kD protein (Angell et al., 1996). However, immunolocalization studies demonstrate that rather than localizing to plasmodesmata, the 25-kD protein is found in viral inclusion bodies in the cytoplasm (Davies et al., 1993). Immunoelectron microscopy using a virion-specific antiserum further identifies virions within the plasmodesmata of all cell types from the epidermis to the bundle sheath, and during infection GFP–CP fusion protein can be seen to be transported between cells (Santa Cruz et al., 1998).

Thus, encapsidated PVX particles move cell to cell through what appear to be modified plasmodesmata, but in the absence of tubules. The 25-kD protein, perhaps in concert with the 12-kD protein, whose function in movement has yet to be demonstrated, plays a role in affecting SEL to facilitate this movement without being localized to plasmodesmata per se (Angell et al., 1996). Intriguingly, in the minor veins, virions (based on staining with the anti-virion antiserum) are found in plasmodesmata between bundle sheath cells and phloem parenchyma or companion cells but not in plasmodesmata that connect companion cells and the sieve elements, although CP is required for phloem transport of the virus (Cruz et al., 1998). It was suggested that either PVX enters sieve elements in a nonvirion form or that the SEL of these particular plasmodesmata is sufficiently large that a bottleneck of virus particles is not observed at these junctions (Cruz et al., 1998). Relevant to this point, immunolocalization studies on the 3a MP and CP of CMV, which requires CP for cell-to-cell movement, led to the conclusion that CMV enters minor vein sieve elements as a ribonucleoprotein complex of MP, CP, and viral RNA and that viral assembly occurs in the sieve element parietal layer (Blackman et al., 1998). CP is required for the long-distance phloem transport of most plant viruses, and it will be of interest to see whether, for reasons that are not immediately clear, assembly of virus particles in the sieve elements turns out to be a general rule.

A Nuclear Circuit

The bipartite geminiviruses require two MPs, but not CP, for cell-to-cell or systemic infection. These single-stranded DNA (ssDNA) viruses replicate through double-stranded DNA intermediates in the nucleus, and it is this nuclear aspect that necessitates two MPs (MPB and NSP [for nuclear shuttle protein, formerly BR1 or BV1]) with distinct functions to cooperatively move the viral genome (Sanderfoot and Lazarowitz, 1996). NSP binds the viral genome and transports it between the nucleus and cytoplasm; MPB cooperates with NSP in transcytoplasmic movement and acts to move the viral genome across the cell wall and into adjacent uninfected cells (Figure 1). The MPs encoded by the phloem-limited SqLCV have been studied in some detail. And the cooperative interaction of NSP and MPB has been demonstrated in vivo in tobacco protoplasts, as has the action of NSP as a nuclear shuttle protein (see below; Sanderfoot and Lazarowitz, 1995; Sanderfoot et al., 1996; Ward and Lazarowitz, 1999).

SqLCV NSP has been immunolocalized to nuclei of phloem parenchyma and immature phloem cells (procambial cells) in systemically infected leaves of pumpkin and squash, and also targets to the nucleus when transiently expressed in tobacco protoplasts (Pascal et al., 1994; Sanderfoot and Lazarowitz, 1995; C.M. Laukaitis, R. Medville, and S.G. Lazarowitz, unpublished results). Consistent with its proposed role in moving the viral genome, NSP also has the properties of a typical ssDNA binding protein in vitro (Pascal et al., 1994). Recent studies in which the MPs encoded by BDMV were microinjected into tobacco leaf mesophyll cells along with

fluorescently tagged nucleic acids further support the conclusion that ssDNA moves cell to cell (Rojas et al., 1998). Microinjection of BDMV MPB into tobacco leaf mesophyll cells suggests that it acts similarly to the TMV 30-kD protein and other MPs in increasing plasmodesmal SEL and rapidly moving cell to cell (Noueiry et al., 1994). As with TMV, examination of SqLCV MPB in the context of virus infection provides a somewhat different, although not necessarily wholly inconsistent, picture: MPB is immunolocalized to unique tubules that extend from and cross the walls of developing phloem cells in pumpkin and squash, and these do not look like plasmodesmata where they cross the cell wall (Ward et al., 1997).

The sum of these studies suggests that NSP acts to shuttle the viral ssDNA genome between the nucleus and cytoplasm and that MPB traps NSP–ssDNA complexes in the cytoplasm and directs these to and across the cell wall, and into adjacent uninfected cells, where the NSP–ssDNA complexes would be released and target the viral genome back to the nucleus to initiate new rounds of replication and infection (Sanderfoot and Lazarowitz, 1996).

"CONFRONTED WITH INSURMOUNTABLE OPPORTUNITIES"

Interactions with the Cytoskeleton

The cytoskeleton is a dynamic network. Microtubules and actin filaments, together with their associated proteins, conduct a variety of essential functions within growing and interphase cells, from the anchoring of cellular organelles to the directed trafficking of protein, cell wall, and membrane components. In addition, cortical microtubules can be seen to undergo both global and regional rearrangements (Lloyd, 1994; Staehelin, 1997; Boevink et al., 1998; Marc et al., 1998). Guidance of the phragmoplast during cell division and growth of the cell plate, cell wall deposition in interphase cells, nuclear import, rearrangements of ER subdomains, and the translocation of Golgi stacks are only some of the processes shown to involve microtubules and actin filament arrays (Staehelin and Hepler, 1996; Staehelin, 1997; Boevink et al., 1998; Smith and Raikhel, 1998). The cytoskeleton is also involved in trafficking the genomes of animal viruses that replicate in the nucleus (e.g., influenza, herpes simplex, and adenovirus) to the nucleus (Ben-Ze'ev et al., 1983; Topp et al., 1994; Avalos et al., 1997; Li et al., 1998). Therefore, it seems logical that specific associations between viral MPs and the cytoskeleton might be important in the transport of viral genomes.

That elements of the cytoskeleton may be involved in directing MP, as well as virus and viral genomes, to and/or through plasmodesmata was first described for TMV. As shown in Figure 2, fusion proteins between GFP and the TMV MP coaligned with microtubules in infected tobacco protoplasts derived from the BY-2 cultured cell line. This coalignment is disrupted by low temperature and treatment of the protoplasts with oryzalin and propizamide, two agents known to disrupt microtubules (Heinlein et al., 1995; McLean et al., 1995). Furthermore, evidence was presented to demonstrate that MP associates with F-actin in vitro (McLean et al., 1995). It had earlier been shown that plasmodesmata contain actin (White et al., 1994), and dye-coupling studies in the presence of agents that disrupt microtubules further suggested that actin filaments may be involved in affecting plasmodesmal gating (B. Ding et al., 1996). Based on these results, it was logical to suggest that microtubules and filamentous actin may deliver complexes of MP with viral RNA to and through plasmodesmata (Heinlein et al., 1995; Carrington et al., 1996).

In plant cells, including BY-2 cells, microtubules are observed in the cortical cytoplasm at the cell periphery (cortical microtubules) and in association with ER (Allen and Brown, 1988; Hepler et al., 1990; Reuzeau et al., 1997a).

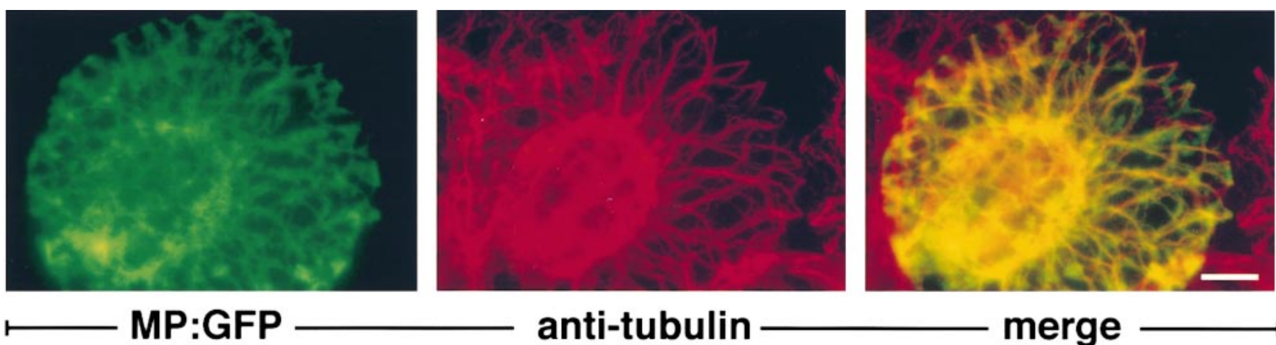

Figure 2. Colocalization of TMV MP with Tubulin in Infected Protoplasts.

Fusion proteins between GFP and the TMV MP can be seen to coalign with microtubules in infected tobacco protoplasts derived from the BY-2 cultured cell line. See text for details. Bar = 10 μm.

Similarly, both TMV MP and the MP–GFP fusion protein are aligned with cortical and noncortical microtubules, as demonstrated by confocal and optical section fluorescence microscopy (Heinlein et al., 1998; Mas and Beachy, 1998). Interestingly, the association of MP with microtubules was most pronounced during the mid to late stages of infection and subsequent to its association with elements of the ER (see below; Heinlein et al., 1998; Mas and Beachy, 1998). In TMV-infected leaves, MP–GFP was first detected in or adjacent to plasmodesmata and subsequently in association with microtubules, suggesting that association with microtubules was not directly related to the targeting of MP to plasmodesmata (Oparka et al., 1997a). Furthermore, following its association with microtubules, MP–GFP is rapidly destroyed, leading to the suggestion that association with microtubules may reflect a pathway to degradation of MP during late stages of infection (Padgett et al., 1996; Heinlein et al., 1998).

Associations with the Cortical ER

The cortical ER in plants is involved in a number of specialized functions in cell growth and development, as suggested by specific structural features of the ER and growing evidence for direct contacts between the ER and various cellular organelles, the cytoskeleton, and elements of the plant cell plasma membrane and wall (Allen and Brown, 1988; Hepler et al., 1990; Reuzeau et al., 1997a; Staehelin, 1997; Boevink et al., 1998; also see Vitale and Denecke, 1999, in this issue). An emerging picture of animal and plant cells is that the ER is not a homogeneous membrane reticulum but contains functionally specialized subdomains (Reuzeau et al., 1997b; Staehelin, 1997). Of particular relevance to the role of the ER in virus movement between cells is the apparently direct association between cortical ER and both cortical actin and the desmotubule that forms the central structure of the plasmodesmata (Hepler, 1982; Overall et al., 1982; B. Ding et al., 1992b, 1996). The nuclear envelope is continuous with the ER, and there are extensive contacts between the ER and the underlying actin cytoskeleton in plant cells (Staehelin, 1997; Boevink et al., 1998). Couple this with the dynamic changes that the ER can undergo during vesicular transport, plant cell division and growth, and associated with specialized plant cell functions, and the ER is too tempting a target for any virus to pass up. The TMV 30-kD protein and SqLCV MPB each associates with the cortical ER in a very specific but distinct manner (Ward et al., 1997; Heinlein et al., 1998; Mas and Beachy, 1998; Reichel and Beachy, 1998). The distinct ER associations for each MP would appear to reflect the different cell types infected by each virus.

The TMV 30-kD Protein and the Cortical ER

It has long been appreciated that endomembranes, including the ER, play an important role in RNA virus replication. Indeed, many studies of the replication proteins of RNA-containing plant as well as animal viruses have revealed an integral association of virus replication with cell membranes (Richards and Ehrenfeld, 1990; Buck, 1996; Lai, 1997). Functional RNA virus replicase complexes, including those for TMV and PVX, have been isolated from infected tissues or cells in association with cellular membranes (Young et al., 1987; Doronin and Hemenway, 1996; Osman and Buck, 1996). In vivo and in vitro studies have established that poliovirus replication occurs in association with ER membranes (Richards and Ehrenfeld, 1990), and recent studies on tobacco etch virus provide evidence for the targeting of the replication complex of this potyvirus to ER membranes (Schaad et al., 1997). It is thus logical to suggest that the association of replication complexes and membranes, and in particular the ER, may play a role in viral movement within and between plant cells. In this context, it is important to consider the involvement of viral MPs with cellular membranes.

In both TMV-infected tissues and transgenic plants that express the TMV 30-kD protein, the MP is found in subcellular fractions that contain plasma membrane and ER (Moore et al., 1992). In more recent studies, the subcellular accumulation of the MP–GFP fusion protein was followed by fluorescence microscopy of infected protoplasts and leaves. The MP–GFP was observed to accumulate at a number of subcellular locations in the cell. These sites included the cortical and noncortical ER (Heinlein et al., 1998; Reichel and Beachy, 1998) as well as plasmodesmata (Padgett et al., 1996; Oparka et al., 1997a). MP–GFP also localized to punctate structures at or near the surface of infected cells, which were proposed to be sites where the ER is anchored to the plasma membrane (Heinlein et al., 1998; Reichel and Beachy, 1998).

MP is also found in large aggregates of ER that contain replicase. Furthermore, association of MP–GFP with different types of membranes appears to change through the infection cycle (Heinlein et al., 1998). The nature of the association of the TMV MP with the ER is not fully defined, but it has been convincingly demonstrated that this association is more than peripheral: treatments with high concentrations of NaCl, urea, or CHAPS do not remove MP from ER membranes (Reichel and Beachy, 1998). Solubilizing the ER with high concentrations of Triton X-100 disrupts the membranes, and the MP is found to partition with the hydrophobic solvent (Fenczik, 1994). Partial proteolysis of microsomes isolated from infected tissues shows that the C terminus of the MP is accessible to digestion (Reichel and Beachy, 1998). All of these observations lead to the conclusion that MP is tightly associated with the ER, with the C terminus of the protein being exposed on the cytosolic side of the membranes.

A unique approach to investigate the association of TMV infection with ER membranes has involved the inoculation of transgenic tobacco plants in which GFP is targeted to the lumen of the ER, thus allowing imaging of the ER by confo-

cal microscopy (Boevink et al., 1996). Infection of these plants by TMV causes severe disruption of the ER in the early to mid stages of infection; during this period, large aggregates of ER are formed (Reichel and Beachy, 1998). During the same time period, MP–GFP is found at or near the cell wall, adjacent to plasmodesmata. The ER-associated sites grow larger with time, forming amorphous electron-dense bodies, similar to the X-bodies reported >30 years ago (Esau and Cronshaw, 1967) that contain MP as well as replicase (Szecsi et al., 1999). Remarkably, in the later stages of infection, the large aggregates dissipate and the ER network returns to the preinfection state of a well-organized cortical reticulum (Reichel and Beachy, 1998). Thus, there is clear evidence of association of MP with ER throughout the cell, including on ER membranes within or near plasmodesmata.

Is the TMV MP targeted to and through the plasmodesmata via the ER? This question has yet to be fully addressed, yet the implications of experiments to date indicate that this may be the case. It is clear that MP is located within plasmodesmata, on cortical and noncortical ER, at punctate structures near the ER, and in bodies that are appressed to the plasma membrane and plasmodesmata. It is not clear that association of MP with ER is directly related to the cell-to-cell spread of infection. Nevertheless, based on these observations, it is tempting to suggest that the spread of TMV from one cell to another occurs as a result of attachment of the replication complex, or complexes comprising MP and viral RNA, to ER and transport of the complexes to adjacent cells.

SqLCV MPB and the Cortical ER

SqLCV infection is restricted to the phloem in pumpkin and squash plants (Pascal et al., 1994; Ward et al., 1997; C.M. Laukaitis and S.G. Lazarowitz, unpublished results). Immunogold-labeling studies localize MPB to unique tubules that extend from and cross the walls of developing phloem cells (procambial cells) in systemically infected leaves of pumpkin. Confocal microscopy of sections from pumpkin and squash leaves systemically infected with a recombinant SqLCV that expresses free GFP further demonstrates that the virus is moving in immature phloem. The MPB-associated tubules resemble the cortical ER where it is appressed to form the desmotubule, and these tubules also specifically stain with antisera against the ER lumenal binding protein (BiP) (Ward et al., 1997). These findings led to the suggestion that these MPB-associated tubules are derived from the ER. In contrast to tubules reported for other plant viruses, no virus-like particles are seen within or associated with the SqLCV MPB-containing tubules, consistent with SqLCV CP not being required for systemic infection of pumpkin and squash (Ingham et al., 1995).

This association of MPB with the ER has been further established in cell fractionation studies and transient expression assays in tobacco protoplasts. Using differential centrifugation and the two-phase partitioning method, MPB is found to cofractionate with ER membrane– and BiP-containing fractions from infected plants (Ward et al., 1997). Curiously, when transiently expressed from a baculovirus vector in Sf9 insect cells or as a transcriptional fusion to the 35S promoter in tobacco protoplasts, MPB, as shown in Figure 3, is localized to the cortical cytoplasm (Pascal et al., 1994; Sanderfoot and Lazarowitz, 1995). What targets MPB to the cortical cytoplasm? The answer appears to lie in an association with cortical ER, as shown in Figure 4 by coimmune labeling of MPB-expressing protoplasts with anti-MPB antisera and antisera against BiP. Whereas BiP is localized to ER throughout the cytoplasm, superimposition of the confocal images suggests that MPB does colocalize with BiP, but only in the cortical ER. This association of MPB with the cortical ER is distinct from those observed for the TMV MP with ER membranes, as discussed above. Another distinction from the TMV 30-kD protein is apparent when one looks at microtubule and actin filament associations. In coimmune and fluorescence labeling studies, MPB does not colocalize with either tubulin or phalloidin (actin filaments) in protoplasts nor does it copurify with microtubules or actin filaments from infected plants or protoplasts (B.M. Ward and S.G. Lazarowitz, unpublished results).

Are the MPB-associated tubules the functional analog of the desmotubule? More studies, including the use of GFP–MPB fusion proteins in living cells, are needed to answer this question. In the context of this geminivirus moving in developing phloem cells, the infected plant and protoplast studies suggest that SqLCV MPB may take advantage of being in dividing cells to intercept the pathway by which plasmodesmata are normally formed, interacting with the cortical ER to form channels in the wall that are now virus specific by virtue of their containing MPB. If MPB tubules are analogous to the desmotubule, this would address a puzzling question in the topology of MPB–NSP associations in the cytoplasm. SqLCV MPB has the properties of a peripheral membrane protein (E. Pascal and S.G. Lazarowitz, unpublished results) and lacks an apparent signal sequence. Virus containing an N-terminal His-tagged form of MPB is infectious and retains the His tag, and mutational studies identify two internal regions of MPB as required for ER association (Sanderfoot and Lazarowitz, 1995, 1996; B.M. Ward and S.G. Lazarowitz, unpublished results). Thus, the data suggest that MPB may associate with the cytoplasmic tail of one or more ER-resident membrane proteins. Viewed this way, we suggest that NSP–genome complexes would interact with adjacent MBP molecules along the tubules, with translocation toward the wall occurring by a ratcheting mechanism driven by changing affinities in these interactions, similar to the way proteins cross nuclear pores. This presumably would be an energy-requiring process. What could regulate this process? Like most MPs, NSP and BMP are phosphoproteins (Pascal et al., 1994), and mutational studies are consistent with post-translational changes in the phosphorylation state of either or

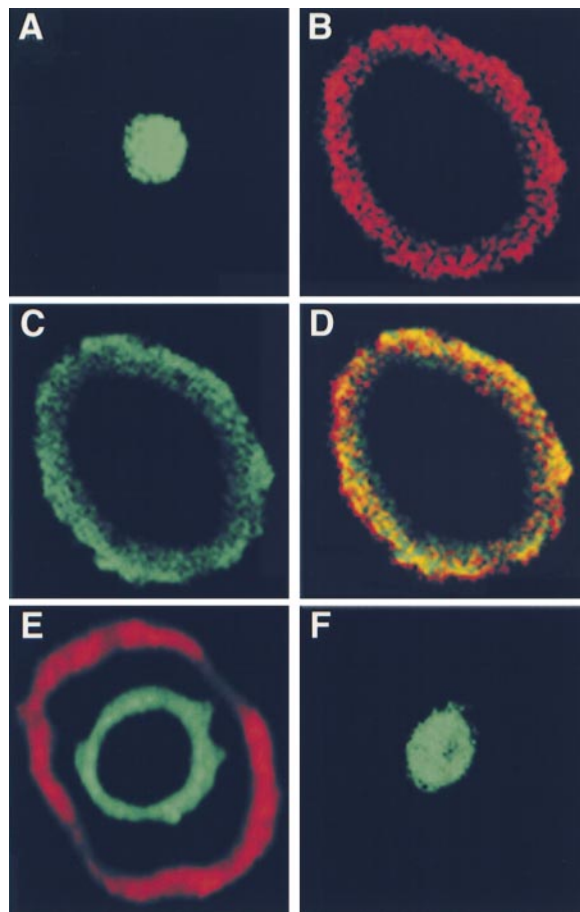

Figure 3. Interaction of SqLCV NSP and MPB in Tobacco Protoplasts.

NSP and MPB were transiently expressed in tobacco protoplasts (*Nicotiana tabacum* cv Xanthi) as transcriptional fusions to the CaMV 35S promoter. Cells were fixed at different times after transfection, and NSP and MPB were visualized by indirect immunofluorescence staining and confocal microscopy. NSP was stained with a fluorescein- or Oregon Green–conjugated secondary antibody (green channel), and MPB was stained with a Texas Red–conjugated secondary antibody (red channel).
(A) NSP expressed alone localizes to the nucleus.
(B) to **(D)** NSP and MPB coexpressed in the same cell at 5 days after transfection. MPB **(B)** remains localized to the cortical cytoplasm, the same subcellular location in which it is found when expressed alone (see Sanderfoot and Lazarowitz, 1995). NSP **(C)** is redirected from the nucleus (see **[A]**) to the cortical cytoplasm when coexpressed with MPB. Superimposing **(B)** and **(C)** shows that NSP and MPB colocalize in the cortical cytoplasm **(D)**.
(E) NSP and MPB coexpressed in the same cell at 7 days after transfection. Superimposed images of the cell stained for NSP (green) and MPB (red). NSP no longer interacts with MPB but is found in the perinuclear region of the cell surrounding the nucleus.
(F) Coexpression of MPB and an NSP mutant protein in which three leucine residues within the NES were mutated to alanine residues. Cells were only stained to show NSP, using Texas Red–conjugated secondary antibody. This NES-defective NSP mutant remains in the nucleus when coexpressed with MPB colorized green for ease of comparison.

both proteins regulating their interaction (Sanderfoot and Lazarowitz, 1995, 1996).

"EVERY EXIT IS AN ENTRY SOMEWHERE"—NUCLEOCYTOPLASMIC TRANSPORT

A fundamental aspect of cell growth and development is the regulation of nucleocytoplasmic transport. Such transport occurs through nuclear pore complexes (NPCs; Forbes, 1992; Davis, 1995). Although NPCs can allow for passive diffusion of small (<30 kD) molecules, most proteins and nucleic acids (the latter as nucleoprotein complexes) are transported across these channels by an active process that is energy dependent and saturable, and involves the recognition of specific signal sequences on the cargo being transported (Görlich and Mattaj, 1996; Corbett and Silver, 1997; Görlich, 1997).

Nuclear import involves a growing list of nuclear localization signals (NLSs) on the cargo molecule that interact with the importin α/β heterodimer or transportin, and additional import receptors continue to be identified (Corbett and Silver, 1997; Görlich, 1997; Truant et al., 1998). These interactions, which lead to docking at and transport across the NPCs, have been well studied biochemically and genetically in animal cells and yeast and are now being investigated in plant cells (Hicks and Raikhel, 1995; Corbett and Silver, 1997). Our understanding of nuclear export has lagged behind that of import in large part due to the difficulty of demonstrating export: at equilibrium, proteins that shuttle in and out of the nucleus appear to be nuclear localized, apparently because the rate of nuclear import exceeds that of export (Gerace, 1995). Thus, demonstrating nuclear export requires perturbing this equilibrium. This has generally been accomplished by using heterokaryons of animal cells or by microinjecting labeled test substrates into the nuclei of HeLa cells or *Xenopus* oocytes and assaying for cytoplasmic accumulation in a time-dependent fashion (Goldstein, 1958; Fischer et al., 1995; Wen et al., 1995; Fridell et al., 1996a).

Nuclear export signals (NESs) have been recently identified in a number of rapidly shuttling nuclear proteins from animals and yeast. First characterized in the Rev protein encoded by the human immunodeficiency virus (HIV), in the transcription factor TFIIIA from *Xenopus* and toads, and in protein kinase A inhibitor protein (Fischer et al., 1995; Wen et al., 1995; Fridell et al., 1996b), these NESs are hydrophobic, leucine-rich sequences of 10 to 13 amino acids in which the leucines are essential for export function (Fischer et al., 1995; Wen et al., 1995; Fridell et al., 1996b). Similar to NLSs,

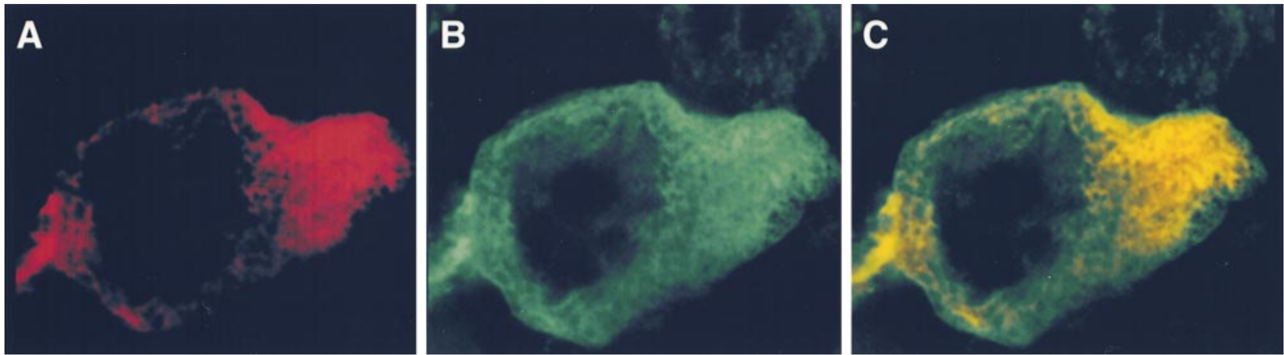

Figure 4. MPB Localization to the Cortical ER.

Tobacco protoplasts expressing MPB were stained for MPB and BiP (as an ER marker; see Vitale and Denecke, 1999, in this issue) by indirect immunofluorescence and visualized by confocal microscopy.
(A) MPB, stained using a Texas Red–conjugated secondary antibody.
(B) BiP, stained using an Oregon Green–conjugated secondary antibody.
(C) Superimposed images of **(B)** and **(C)** show that MPB appears to colocalize with BiP (ER) in the cortical cytoplasm.

NESs appear not to possess extensive secondary structure because they can confer the ability to be exported from the nucleus when fused to the N or C terminus of a test substrate. Nuclear export has not been studied in plant cells. However, the nuclear site of replication for the geminiviruses with their ssDNA genomes necessitates an MP in the form of a nuclear shuttle protein (NSP) to move the replicated viral genomes from and to the nucleus. The interaction of SqLCV NSP and MPB has recently been used to develop a novel cell-based assay for investigating nuclear export in plant cells (Sanderfoot and Lazarowitz, 1996; Sanderfoot et al., 1996; Ward and Lazarowitz, 1999).

SqLCV NSP was first shown to shuttle in transient expression assays in both insect cells and tobacco protoplasts (Sanderfoot and Lazarowitz, 1995; Sanderfoot et al., 1996). When expressed as a transcriptional fusion to the 35S promoter, NSP targets to nuclei in transfected tobacco protoplasts (Figure 3). However, coexpression with MPB redirects NSP from the nucleus to the cortical cytoplasm: MPB interacts with NSP and perturbs the equilibrium, trapping NSP in the cytoplasm and directing it to the cell periphery (Figure 3; Sanderfoot and Lazarowitz, 1995; Sanderfoot et al., 1996). The same was found to be true when NSP and MPB were coexpressed in insect cells. Thus, NSP does shuttle between the nucleus and cytoplasm, MPB provides directionality to intracellular movement through a specific protein–protein interaction and its localization to the cortical ER, and the two SqLCV MPs cooperatively interact to move the viral genome (Pascal et al., 1994; Sanderfoot and Lazarowitz, 1995, 1996; Rojas et al., 1998). This interaction is regulated; at later times after transfection of protoplasts, NSP no longer interacts with MPB, but is found in the perinuclear region that surrounds the nucleus (Figure 3) (Sanderfoot and Lazarowitz, 1996). This regulation of the NSP–MPB interaction appears to be plant cell specific because it does not occur in insect cells (Sanderfoot and Lazarowitz, 1996; Sanderfoot et al., 1996).

Site-directed mutations in SqLCV NSP and MPB have been analyzed both in protoplasts and when reincorporated into the SqLCV genome. These studies and the analysis of GUS–NSP fusion proteins have led to the development of detailed functional domain maps for both MPB and NSP (Sanderfoot and Lazarowitz, 1996). Not unexpectedly, NSP was shown to contain NLSs—two, in fact, both located in the N-terminal half of the protein: a bipartite NLS located between residues 22 and 41, and a basic SV40 T antigen–type NLS between residues 89 and 93, both of which are required for full function (Sanderfoot et al., 1996). Mutational studies also showed that the C terminus of NSP contains a domain essential for NSP to interact with MPB (Sanderfoot et al., 1996). That this interactive domain lies within the last 57 residues of NSP has been directly demonstrated by fusing C-terminal fragments of NSP to GUS and showing, by indirect immunofluorescence staining with anti-GUS antibody and confocal microscopy, that these cytoplasmically localized fusion proteins are redirected to the cortical cytoplasm when coexpressed with MPB in tobacco protoplasts (Sanderfoot et al., 1996; Ward and Lazarowitz, 1999).

Recently, this GUS-fusion assay has been used to identify an NES within NSP, located just upstream of the interactive domain, that resembles the NES found within HIV Rev and TFIIIA (Ward and Lazarowitz, 1999). A series of C-terminal truncations of NSP fused to GUS was constructed, and each GUS–NSP fusion protein was tested for its interaction with MPB when the fusion was expressed in the cytoplasm or nucleus of tobacco protoplasts. The rationale was that those fusion proteins with an intact interactive domain (assayed from the cytoplasmic location) would be relocalized

from the nucleus to the cortical cytoplasm by MPB only if they also contained an NES (Ward and Lazarowitz, 1999). These studies identified a 22–amino acid peptide located just upstream of the interactive domain in NSP as being required for export of NSP from the nucleus. This peptide contains the sequence SLEKDLLIDLH, which resembles the NESs found in other rapidly shuttling nuclear proteins. Mutation of the leucine residues in this sequence abolished both SqLCV infectivity and the relocation of NSP from the nucleus to the cortical cytoplasm of tobacco protoplasts in the presence of MPB (Figure 3; Ward and Lazarowitz, 1999). Furthermore, the NES from the *Xenopus* transcription factor TFIIIA was shown to restore fully NSP interaction with MPB and partially restore SqLCV infectivity (Ward and Lazarowitz, 1999).

Thus, nuclear shuttling of NSP is essential for its function in SqLCV movement, and NSP can be added to the growing list of rapidly shuttling nuclear proteins that contain a leucine-rich NES. The finding that NSP can shuttle between the nucleus and cytoplasm of insect cells, and that an NSP–NES mutant that contained the TFIIIA NES could be exported from nuclei in tobacco protoplasts demonstrates that the basic nuclear export machinery is highly conserved among plants, animals, and yeast. Yet, differences do exist because the TFIIIA NES did not fully restore viral infectivity. Importantly, the interaction of NSP and MPB now provides a novel in vivo assay for investigating nuclear export in both plant and animal cells.

"FROM THE NOTES EMERGE CHORDS AND PHRASES AND TEMPOS AND MELODIES, AND FROM THESE EMERGE THE SONATA"—FUTURE DIRECTIONS

The tenet of molecular virology—that viruses and their components are powerful tools for investigating fundamental cellular and developmental processes in the organisms they infect—has been amply demonstrated in investigations of bacterial and animal viruses. The advent of reverse genetics has had an immense impact on plant virology, inevitably leading to the current exciting focus on using plant viruses as a means to investigate plant cell structure and function. Given their essential role in a process that is unique to plant viruses, namely, the systemic infection of a multicellular host when faced with the barrier of the cell wall, MPs in particular are allowing the examination of fundamental aspects of intracellular and intercellular trafficking of macromolecules, some of which may be unique to plant cells.

Microinjection combined with dye-coupling studies in leaf mesophyll cells has been a useful approach to examine certain properties of MPs. However, understanding the complex regulated cascade of events that takes place in virus-infected cells and realizing the full potential of viral-encoded MPs as models for probing plant cell structure and function require the application of techniques that allow one to investigate MP function both biochemically and in living cells in the context of the virus life cycle. In the past few years, researchers have begun to unravel some of the details of how viral genome replication and movement are coupled. It is these studies that have directed and continue to direct our attention to the cytoskeletal network and ER, and in the case of nuclear replicating viruses, to nuclear import and export receptors and nuclear pore interactions. A number of the basic interactions between viral and cell components have been identified, and the challenge now—in terms of both virology and cell biology—is to determine how these processes are regulated and demonstrate the mechanisms involved in recognition and targeted trafficking. The continued application of new technologies in fluorescence microscopy and optical sectioning, including fluorescence recovery after photobleaching, use of the multiphoton confocal microscope to investigate energy resonance transfer in living cells, and in-block sectioning using confocal laser scanning, coupled with biochemical and structural studies, will allow us to address these intriguing and important questions.

Many gaps remain in our understanding of the process of virus movement and the implications for plant cell biology. Are there plasmodesmal targeting sequences in viral MPs? Based on analogies to nuclear import and the involvement of import receptors, there was clearly a hope that such plasmodesmal targeting sequences would exist. If they do, they remain to be identified as such. Alternatively, it may be that viral replication complexes and MPs form specific associations with subdomains of the cortical ER and/or cortical microtubules and actin filaments, which themselves are associated with plasmodesmata and act to guide components toward these intercellular channels. In this context, defining the roles of microtubule and microfilament associated proteins, including molecular motors, and identifying functionally relevant ER subdomains and ER–cytoskeleton interactions become important. The elucidation of protein–protein and protein–membrane interactions between viral replication or movement complexes and the ER should provide new insights into the existence and function of ER subdomains within the cell. Nuclear import and export targeting sequences clearly do exist in the geminiviral-encoded NSP, and further investigation of NSP function and its interactions with nuclear components and MPB provides a novel approach for investigating the mechanism of nuclear export in plant cells, and potentially the biogenesis of channels in the plant cell wall. Do tubule-producing viruses in mesophyll cells recruit the desmotubule from plasmodesmata to form the conduit for cell-to-cell movement of viral particles? Are the SqLCV MPB-associated tubules the analog of the desmotubule? If so, these processes may simply represent the two extremes of the same process, with the movement of viruses such as TMV, RCNMV, and PVX falling in between. Thus, these may all be interconnected, representing viral solutions to the "movement problem" based on the cellular milieu in which the virus finds itself. It is a future of exciting cell biological problems waiting to be addressed.

ACKNOWLEDGMENTS

We thank (in order) centuries of wisdom for the Yiddish proverb, Pogo, and Tom Stoppard for the quotations, and Ursula Goodenough for writing a book as enjoyable and insightful as *The Sacred Depths of Nature* (Oxford University Press, 1998), from which we borrowed a sentence for our concluding remarks. We share her wonder at the workings of nature and her love of music. We also thank the members of our laboratories, past and current, for all of their contributions and lively discussions over the years. In particular, we acknowledge Manfred Heinlein for Figure 2 and Tony Sanderfoot and Brian Ward for Figures 3 and 4. Work in our labs has been supported by National Science Foundation Grants No. MCB-9417664 (to S.G.L.) and No. MCB-9631124 (to R.N.B.).

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