Tobacco mosaic virus (TMV) Replicase and Movement Protein Function Synergistically in Facilitating TMV Spread by Lateral Diffusion in the Plasmodesmal Desmotubule of *Nicotiana benthamiana*

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Virus spread through plasmodesmata (Pd) is mediated by virus-encoded movement proteins (MPs) that modify Pd structure and function. The MP of *Tobacco mosaic virus* (TMVMP) is an endoplasmic reticulum (ER) integral membrane protein that binds viral RNA (vRNA), forming a vRNA:MP:ER complex. It has been hypothesized that TMVMP causes Pd to dilate, thus potentiating a cytoskeletal mediated sliding of the vRNA:MP:ER complex through Pd; in the absence of MP, by contrast, the ER cannot move through Pd. An alternate model proposes that cell-to-cell spread takes place by diffusion of the MP:vRNA complex in the ER membranes which traverse Pd. To test these models, we measured the effect of TMVMP and replicase expression on cell-to-cell spread of several green fluorescent protein-fused probes: a soluble cytoplasmic protein, two ER lumen proteins, and two ER membrane-bound proteins. Our data support the diffusion model in which a complex that includes ER-embedded MP, vRNA, and other components diffuses in the ER membrane within the Pd driven by the concentration gradient between an infected cell and adjacent noninfected cells. The data also suggest that the virus replicase and MP function together in altering Pd conductivity.

To cause disease, a virus must be capable of replicating in host cells, moving into surrounding cells, and then progressing systemically throughout the plant. It is generally accepted that plant viruses traverse the cell wall through plasmodesmata (Pd), membrane-bound tunnels that interconnect the cytoplasm of neighboring cells (Heinlein and Epel 2004). Current models of Pd structure describe a simple unbranched Pd as a wall-embedded plasmalemma-lined cylinder that contains a central axial membrane component, termed appressed endoplasmic reticulum or desmotubule (ER^{DT}), which is derived from and

continuous with cortical endoplasmic reticulum (ER). The plasma membrane (PM) and ER^{DT} define a cytoplasmic sleeve that bridges the cytoplasm of contiguous cells (Heinlein and Epel 2004). The cell wall encasing Pd is modified and apparently plays a role in regulating Pd conductivity (Heinlein and Epel 2004).

The size selectivity of Pd usually is measured in terms of their molecular size exclusion limit (SEL) (Ding et al. 1999). Because the SEL for cytoplasmic spread by diffusion through Pd is much smaller than any known plant virus or viral genome, it generally is considered that virus cell-to-cell spread is an active or facilitated process. For many viruses, cell-to-cell spread is mediated by one or more viral gene products termed movement proteins (MPs). In the absence of MP, most viruses are unable to spread cell to cell (Lucas 2006). The MP of *Tobacco mosaic virus* (TMVMP) and many other plant viruses have been shown to be ER membrane (ER^{mem}) proteins (Brill et al. 2000; Haupt et al. 2005; Ju et al. 2005; Reichel and Beachy 1998; Waigmann et al. 2004). The MP of TMV, which has its N- and C-terminus exposed to the cytoplasm (Brill et al. 2000; Reichel and Beachy 1998), binds single-stranded RNA in vitro, forming a protein:RNA complex with a thin and elongated appearance (Citovsky et al. 1992). It has been proposed that TMVMP chaperones viral RNA (vRNA) through dilated Pd (Citovsky et al. 1992) as an RNA-protein complex or as a membrane complex that also includes vRNA and replicase (Asurmendi et al. 2004; Kawakami et al. 2004; Szecsi et al. 1999). To achieve viral cell-cell spread, MP in some way modifies Pd. Presumably it increases the SEL of Pd (Asurmendi et al. 2004; Deom et al. 1987; Kotlizky et al. 2001; Waigmann et al. 1994, 2004; Wolf et al. 1989) by an as yet unknown mechanism.

Although it is clear that not all viral MPs function similarly, several common features are emerging. Viral MPs can target to Pd autonomously in transgenic plants that express MP genes (Atkins et al. 1991; Kotlizky et al. 2001; Schmitz et al. 1997). Many MPs are ER associated (Haupt et al. 2005; Huang and Zhang 1999; Itaya et al. 1998; Ju et al. 2005; Kawakami et al. 1999; Krishnamurthy et al. 2003; Mitra et al. 2003; Morozov and Solovyev 2003; Padgett et al. 1996; Reichel and Beachy 1998; Waigmann et al. 2004) and bind nucleic acids in vitro (Citovsky et al. 1992; Isogai et al. 2006; Leshchiner et al. 2006), leading to the suggestion that MPs form nucleoprotein complexes with viral genomes to facilitate cell-to-cell move-

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Nucleotide sequence data is available in the GenBank database under the following accession numbers: calreticulin (Atlg56340), calnexin (At5g07340), and ACA2 (At4g37640).

*The e-Xtra logo stands for “electronic extra” and indicates additional information is available online. Figure 1 appears in color and a supplementary appendix is published online.

ment. Furthermore, MP expression promotes callose degradation (Bucher et al. 2001; Fridborg et al. 2003; Iglesias and Meins 2000); thus, it has been suggested that changes in the wall-sleeve surrounding Pd may be of physiological significance and may be involved in potentiating viral spread (Bucher et al. 2001; Fridborg et al. 2003; Heinlein and Epel 2004; Iglesias and Meins 2000; Levy et al. 2007).

A model has been proposed for TMV cell-to-cell spread, termed here the “ER sliding model” (Fig. 1), which hypothesizes that, upon TMV infection, newly synthesized MP inserts into ER and binds newly replicated positive-strand vRNA, forming a membrane-associated complex which also may include viral replicase. Furthermore, it is hypothesized that MP directly or indirectly changes wall properties surrounding Pd, thus allowing the Pd to dilate. The dilation potentiates a cytoskeletal-mediated transport of ER or ER-derived vesicles with associated MP:vRNA cargo through Pd (Boevink and Oparka 2005; Kawakami et al. 2004; Liu et al. 2005; Wright et al. 2007). In the absence of MP, the ER in Pd (the ER^{DT}) is

Fig. 1. Endoplasmic reticulum (ER)-sliding model of plant viral movement. According to this model, the movement protein of *Tobacco mosaic virus* (TMVMP) is an integral ER membrane (ER^{mem}) protein (Reichel and Beachy 1998) that binds viral RNA (vRNA) (Citovsky et al. 1990, 1992). TMVMP also associates with a plasmodesmata-plasma membrane (Pd-PM) wall factor causing the Pd cytoplasmic sleeve to expand (Wolf et al. 1989). Myosin or microtubule motors transport a vRNP complex to Pd-appressed ER or desmotubule (ER^{DT}) (Kawakami et al. 2004; Liu et al. 2005). Due to dilatation of Pd, ER^{DT} is no longer immobile and actin filaments mediate the sliding of the ER through Pd (arrow); in the absence of MP, the ER^{DT} is stationary (i.e., does not move through the Pd). Figure is modified from Figure 4 in Heinlein and Epel (2004). MF = microfilament and CMP = cytoskeleton motor protein.

stationary (Heinlein and Epel 2004). Alternatively, because MP is embedded in ER, one must consider a model in which cell-to-cell spread occurs by diffusion of an MP:vRNA complex in the lipid matrix of the ER^{DT} under the influence of a concentration gradient between adjacent infected and uninfected cells (Lippincott-Schwartz et al. 2000; Runions et al. 2006; Saxton and Jacobson 1997).

In order to test these two models, we transiently expressed a cytoplasmic probe, green fluorescent protein (GFP)^{cyt}; two ER lumen (ER^{lum}) proteins; ER lumen-retained GFP (GFP^{ERlum}) and calreticulin ([GFP:calreticulin]^{ERlum}) (Michalak et al. 1992); and two ER^{mem} proteins, ACA2 ([ACA2:GFP]^{ERmem}) (Hong et al. 1999) and calnexin ([calnexin:GFP]^{ERmem}) (Bergeron et al. 1994; Runions et al. 2006), each fused to GFP as reporter probes (Fig. 2) in wild-type (WT) or transgenic *Nicotiana benthamiana* plants expressing the TMVMP (MP⁺ plants). The spread of probes to adjacent cells was followed by confocal laser scanning microscopy in either the absence or presence of a TMV mutant, TMV^{ΔMPΔCP}, which expresses only the viral replicase. GFP^{ERlum}, a soluble GFP derivative, and calreticulin are proteins that reside in the ER lumen due to an N-terminus signaling sequence and an HDEL ER-retention signal at its C-terminus. Calreticulin binds to misfolded ER luminal proteins and prevents them from being exported from the ER to the Golgi apparatus, functioning as a quality-control chaperone. ACA2 is a multipass ER transmembrane protein with its N terminal side in the lumen of the ER and is a calmodulin-regulated Ca²⁺-ATPases shown by Hong and associates (1999) to be an intrinsic ER^{mem} protein in *Arabidopsis* root cells. Calnexin is a 60-kDa nonglycosylated type I integral protein of the ER. It consists of a large N-terminal calcium-binding luminal domain, a single transmembrane helix, and a short cytoplasmic tail. Two probes of different size and function were chosen for each of the two ER compartments to assure that the data are not a consequence of the particular probe employed.

The ER sliding model predicts that, in the presence of MP, the cytoplasmic and membrane-protein probes should exhibit enhanced cell-to-cell spread. Our data do not support the sliding model because these predictions were not born out. However, the data do support the diffusion model, showing that the ER membrane probes do diffuse cell-to-cell. Furthermore, our data suggest that the virus replicase functions in altering Pd conductivity.

RESULTS

In order to test the working models, we measured the cell-to-cell spread of the cytoplasmic probe GFP^{cyt}, which accumulates as a soluble protein in the cytoplasm and nucleus; two ER luminal protein-probes, GFP^{ERlum} and [GFP:calreticulin]^{ERlum}; and two intrinsic ER membranal protein probes, [calnexin:GFP]^{ERmem} and [ACA2:GFP]^{ERmem} (Figs. 2 and 3A through E, respectively). All probes were expressed following agroinfiltration at very low titers in WT and MP⁺ *N. benthamiana* plants. Probe spread was measured at 48 (data not shown) and 72 h post-agroinfiltration (hpai) (Fig. 4, columns 1 and 3).

The percentage of infection sites that exhibit probe spread (ISEPS) of the various probes from a single infected site to neighboring cells was quantified at 72 hpai (Fig. 5). Similar results were obtained at 48 hpai. In both WT and MP⁺ plants, GFP^{cyt} spreads cell to cell from 100% of transfected leaf epidermal cells (Fig. 5). The extent of spread from an infected cell ($n = 300$) decreased ($P < 0.05$) from 17.7 surrounding epidermal cells in WT plants to 12.7 surrounding epidermal cells in MP⁺ plants.

In WT plants, the spread of both ER^{lum} protein probes to surrounding cells was detectable in only 10% of transfected leaf

epidermal cells (Fig. 5). In contrast, in MP⁺ plants, the lumen probes spread from an average of 80% of transfected leaf epidermal cells (Fig. 5). These results suggest that, in 90% of WT cells, the ER^{DT} lumen is functionally closed to diffusional transport of molecules in the size range employed via the ER-lumen whereas, in transgenic plants expressing TMVMP, the ER^{DT}, is in some manner functionally modified, thus allowing the ER^{lum} probes to spread cell to cell.

In WT plants, the ER^{mem} protein probes spread cell to cell from 80% of transfected leaf epidermal cells, demonstrating that ER^{mem} proteins can spread cell to cell in WT noninfected cells. In contrast, in MP⁺ plants, cell-to-cell spread was significantly reduced, occurring in only 23% of transfected leaf epidermal cells (Fig. 5). These results suggest that, in the absence of replicase (see below), TMVMP expression inhibits cell-to-cell spread of ER^{mem} proteins.

In order to test for the effect of active viral replication on probe spread in WT and MP⁺ plants, each probe was coexpressed together with the TMV^{ΔMPACP}, a TMV mutant lacking both MP and coat protein (CP) that expresses only the TMV

replicase (Fig. 2). The probes were expressed by agroinfiltration employing low titers of *Agrobacterium* while TMV^{ΔMPACP} was coexpressed employing a 750-fold higher titer of *Agrobacterium*. Cell-to-cell spread of each probe was assayed (Figs. 4, columns 2 and 4, and 5).

In WT plants, the fraction of infection sites exhibiting spread of GFP^{cyt} and of both ER^{mem} probes from the expressing cell to surrounding cells was significantly reduced ($P < 0.05$) upon expression of the mutant virus TMV^{ΔMPACP} (Fig. 5). Moreover, in those cells exhibiting spread ($n = 300$), the extent of GFP^{cyt} spread decreased from an average of 17.7 to 2.9 cells ($P < 0.05$). In contrast, the extent of spread of both ER^{mem} probes in those infection sites exhibiting cell-to-cell spread was essentially unaffected.

In MP⁺ plants, TMV^{ΔMPACP} is complemented and the mutant virus spreads cell to cell, producing necrotic lesions on *N. tabacum* cv. Xanthi NN leaves (data not shown). In MP⁺ plants, the expression of TMV^{ΔMPACP} significantly increased ($P < 0.05$) the fraction of infection sites exhibiting cell-to-cell spread of ER^{mem} probes approximately three- to fourfold (Fig. 5). Al-

Fig. 2. Schematic representation of the green fluorescent protein (GFP) fusion proteins and mutant *Tobacco mosaic virus* (TMV)^{ΔMPACP}. All proteins are expressed from the *Cauliflower mosaic virus* (CaMV) 35S promoter. SP, signal peptide; HDEL, endoplasmic reticulum (ER)-retention signal sequence; butterfly box, transmembrane domain; GFP^{cyt}, cytoplasmic enhanced-GFP; GFP^{ER^{lum}}, ER lumen (ER^{lum})-soluble GFP which has an HDEL ER retention signal at its C-terminus; [GFP:Calreticulin]^{ER^{lum}}, ER^{lum} protein which has an SP before GFP and an HDEL ER-retention signal at its C-terminus; [Calnexin:GFP]^{ER^{mem}}, a type I ER membrane (ER^{mem}) protein; [ACA2:GFP]^{ER^{mem}}, an ER^{mem} calmodulin-regulated Ca²⁺-ATPase; TMV^{ΔMPACP}, a TMV mutant lacking both movement protein (MP) and coat protein (CP) expressing only the TMV replicase.

though the expression of TMV^{ΔMPACP} in MP⁺ plants had no effect on the number of sites from which GFP^{cyt} spread (100%), the extent of GFP^{cyt} spread ($P < 0.05$) increased from 12.7 surrounding epidermal cells in noninfected MP⁺ plants to 18.2 surrounding cells in MP⁺ plants expressing TMV^{ΔMPACP}.

In both WT and MP⁺ plants, callose accumulation around Pd significantly increased as a result of agroinfiltration (wound-

ing) (Fig. 6A and B). In WT plants, TMV^{ΔMPACP} expression further induced Pd-associated callose accumulation while the coexpression of TMV^{ΔMPACP} and TMV^{MP} resulted in a significant reduction in callose accumulation, even compared with nonwounded plants (Fig. 6A and B). These results suggest that, in the presence of TMV^{MP}, the expression of TMV^{ΔMPACP} lowers the levels of TMV^{ΔMPACP} stress-induced callose.

Fig. 3. Subcellular localization of cytoplasmic enhanced green fluorescent protein (GFP^{cyt}) and endoplasmic reticulum lumen and membrane (ER^{lum} and ER^{mem}, respectively) proteins. Confocal micrograph showing localization of the GFP fusion proteins after *Agrobacterium* infiltration. **A**, GFP^{cyt} localizes to cytoplasm and nucleus; **B**, GFP^{ERlum}; **C**, [GFP:Calreticulin]^{ERlum}; **D**, [Calnexin:GFP]^{ERmem}; and **E**, [ACA2:GFP]^{ERmem}, all localize to ER in both initially infected cell and adjacent cells into which probe has spread. **F** through **J**, Enlargements of the areas inside the boxes in A–E, respectively. Bar, 50 μm.

Fig. 4. Confocal micrographs showing examples of cell-to-cell spread of cytoplasmic enhanced green fluorescent protein (GFP^{cyt}) and endoplasmic reticulum lumen and membrane (ER^{lum} and ER^{mem}, respectively) probes in epidermal cells of wild-type (WT) and movement protein-expressing (MP⁺) plants in the absence or presence of mutant *Tobacco mosaic virus* (TMV)^{ΔMPACP}. Bar, 50 μm.

DISCUSSION

Most plant viruses spread from cell to cell by a mechanism involving an interaction between MP, virus, and Pd. In the case of TMV, the MP inserts into ER membrane (Boevink and Oparka 2005; Brill et al. 2000; Reichel and Beachy 1998) and binds vRNA (Citovsky et al. 1992), forming an endomembrane:MP:vRNA-complex (Kawakami et al. 2004) that also sequesters replicase, forming a replication complex (Heinlein et al. 1998; Liu et al. 2005; Nelson and Citovsky 2005; Reichel and Beachy 1998; Waigmann et al. 2004). Furthermore, because ER is continuous through Pd, it was suggested that vRNA cell-to-cell spread occurs in association with this membrane compartment (Boevink and Oparka 2005; Mas and Beachy 1999; Nelson and Citovsky 2005; Reichel and Beachy 1998; Szecsi et al. 1999).

The colocalization of MP with ER or ER-derived vesicles and with microtubules (Heinlein et al. 1995) and microfilaments (McLean et al. 1995) led to the suggestion that cytoskeletal elements are involved in Pd targeting and cell-to-cell movement of the viral ribonucleoprotein complex (Heinlein et al. 1995; Mitra et al. 2003). Moreover, because MP purportedly dilated Pd, and because highly mobile ER and ER-derived

vesicles are closely associated with actin filaments (Quader et al. 1987), it was postulated that motility is along actin cables, possibly using myosin or other motor proteins that link the ER to cytoskeleton (Heinlein and Epel 2004; Knebel et al. 1990). Moreover, because actin and myosin are present inside the Pd (Blackman and Overall 1998), it was suggested that, as a result of the MP-induced dilation of Pd, the ER or ER-derived vesicles with the bound MP:vRNA:protein complex now could be transported by microfilament-driven transport, an active process, through the dilated Pd (Boevink and Oparka 2005; Gillespie et al. 2002; Heinlein and Epel 2004; Reichel et al. 1999). It has been estimated that the intracellular movement of viral vesicles on the cytoskeleton in primary infected cells is approximately 160 nm/s (Kawakami et al. 2004). At this rate, if the viral vesicles were to continue moving on the cytoskeleton through a dilated Pd, spread would be on the order of seconds. Such rapid spread is not detected; the movement of a viral replication complex from the first infection site to the second can be detected within 20 h, and from the second infection site to adjacent cells within 4 h (Kawakami et al. 2004). Similar results were obtained in the absence of virus infection (Kotlizky et al. 2001), in which spread of ^{TMV}MP fused to GFP (^{TMV}MP:GFP)

Fig. 5. Spread of cytoplasmic enhanced green fluorescent protein (GFP^{cyt}) and endoplasmic reticulum lumen and membrane (ER^{lum} and ER^{mem}) probes from a source cell to adjacent cells of wild-type (WT) and movement protein-expressing (MP⁺) plants in the absence or presence of mutant *Tobacco mosaic virus* (TMV)^{ΔMPACP}. The infection sites exhibiting probe spread (ISEPS) is reported as the percentage of transfected cells that allow spread of probes into adjacent cells in the absence (-) and presence (+) of TMV^{ΔMPACP} as determined by confocal laser scanning microscopy.

spread was detected at approximately 24 h postbombardment, although subsequent spread was more rapid.

The data presented here do not fit the sliding-ER model. The expression of MP did not stimulate cell-to-cell spread of either GFP^{cyt} (Fig. 5) (Liarzi and Epel 2005) or either of the ER^{mem} probes as predicted by the sliding model but, rather, inhibited spread (Fig. 5). The time course of the cell-to-cell movement of the ER^{mem} probes observed in the present work is similar to that observed for virus spread; therefore, we suggest that the

MP:vRNP complex moves slowly by obstructed diffusion in the lipid membrane of the ER^{DT} to the Pd. The faster viral spread time of approximately 4 h that was obtained following inoculation of a single *N. clelandii* trichome with *Tobacco rattle virus* (Derrick et al. 1992) may be due to differences in tissue and the nature and number of Pd in trichomes (Waigmann et al. 1997). The finding that MP expression inhibits spread of cytoplasmic soluble probes is in agreement with Storms and associates (1998), who reported a decrease of Pd SEL after

Fig. 6. Callose accumulation in wild-type (WT) and movement protein-expressing (MP⁺) plants in response to wounding (agroinfiltration) and to mutant *Tobacco mosaic virus* (TMV)^{ΔMPACP} transient expression. **A**, Fluorescent micrographs of epidermal cells stained with aniline blue of WT (sections A through C) and MP⁺ (D through F) plants: A and D, control cells without treatment; B and E, 24 h post-agroinfiltration (hpai) of *Agrobacterium* containing a blank cassette or C and F, expressing TMV^{ΔMPACP}. **B**, Leaves of WT and MP⁺ plants that were leaf-injected with *Agrobacterium* containing a blank cassette or expressing TMV^{ΔMPACP} were stained with aniline-blue 24 hpai and callose accumulation was determined by quantitative fluorescent microscopy. The mean plasmodesmata callose intensity was expressed in arbitrary units as mean intensity $\pm$ standard error of the mean of three independent experiments. Different letters designate significantly different values at $P < 5\%$, as measured by means and t test.

iontophoretic microinjection of transgenic plants expressing TSWV or TMV MP. This is in contrast to other reports employing various MPs in which fluorescent probes were introduced by a pressure injection technique and which reported that MP increased Pd SEL (Deom et al. 1987, 1990; Ding et al. 1992; Storms et al. 1998; Wolf et al. 1989; 1991). These latter studies reported a SEL of less than 1 kDa in the absence of MP and that MP gated Pd to allow macromolecules >1 kDa to

spread cell to cell. Recent alternate studies now show that Pd do conduct macromolecules in the absence of MP (Crawford and Zambryski 2001; Liarzi and Epel 2005; Martens et al. 2006; Oparka and Cruz 2000; Oparka et al. 1999).

Our data suggest that MP expression functionally alters ER^{DT} conductivity, potentiating spread of ER^{lum} probes. The nature of this change is unclear. Because GFP:calreticulin^{ERlum}, GFP^{ERlum}, and 2× GFP^{ERlum} (data not shown) all spread cell to

Fig. 7. Modified integrated diffusion model of *Tobacco mosaic virus* (TMV) cell-to-cell spread. Stage I: upon viral infection, viral RNA (vRNA) initiates synthesis of replicase with the resultant transcription of plus and minus vRNA stands and subgenomic vRNAs leading to the synthesis of replicase, movement protein (MP), and coat protein (CP) (CP is not required for virus cell spread). MP of TMV (TMVMP) associates with the endoplasmic reticulum (ER) (Mas and Beachy 1999) as an ER membrane (ER^{mem}) protein (Brill et al. 2000; Reichel and Beachy 1998). In response to infection, callose accumulates in the wall region surrounding the plasmodesmata (Pd) restricting the cytoplasmic sleeve. Stage II: the cytoplasmic exposed domain of TMVMP functions as a protein raft and binds vRNA which may complex with replicase forming an ER-virus replication complex (VRC) (Kawakami et al. 2004) which traffics by diffusion within the ER lipids to the appressed ER or desmotubule (ER^{DT}) (squiggle arrow) or by vesicular trafficking to the cortical ER (open arrowheads). Stress-induced class I β-1,3-glucanase accumulates in ER and traffics in lumen of VRC vesicles to plasma membrane with requisite docking protein (Fridborg et al. 2003) (filled arrowheads); vesicular trafficking is probably along actin filaments (not shown). Stage III: cycling vesicles containing β-1,3-glucanase cargo fuse to plasma membrane and deliver β-1,3-glucanase to the cell wall (filled arrowheads). Callose is hydrolyzed, allowing Pd to dilate. Vesicles with attached VRCs recycle back to the cortical ER where vesicles fuse to cortical ER (curved arrow). VRCs diffuse in ER-ER^{DT} continuum through the Pd to adjacent cells by diffusion (squiggle arrow) motivated by the concentration gradient in the ER continuum between a viral infected cell and adjacent noninfected cells.

cell via the ER^{DT} lumen in MP transgenic plants, we suggest that this cell-to-cell spread probably is not the consequence of a reported interaction between calreticulin and TMV^{MP} (Chen et al. 2005) because three different probes all were capable of spreading.

ER^{mem} probes move cell to cell by diffusion in the ER^{DT}.

Our data demonstrate that ER-membrane-intrinsic proteins can spread cell to cell. The ER is a fluid lipid structure in which lipids, embedded proteins, and protein complexes can move by diffusion (Lippincott-Schwartz et al. 2000; Runions et al. 2006; Saxton and Jacobson 1997). Grabski and associates (1993) showed that, in soybean root cells, lipids in the ER but not in the PM can spread cell to cell. The present data demonstrate that proteins can move by diffusion in the ER membrane from cell to cell. These data are consistent with obstructed protein diffusion in the ER-ER^{DT}-ER continuum (Saxton and Jacobson 1997). Thus, ER^{mem}-embedded proteins, such as ER-associated MPs, if not anchored in some manner, should be capable of diffusion from cell to cell in the ER^{DT} lipids under the influence of a diffusion gradient between cells (Lippincott-Schwartz et al. 2000; Runions et al. 2006; Saxton and Jacobson 1997). Our hypothesis that ER^{mem} proteins diffuse cell to cell within the ER^{DT} membrane lipid milieu is supported by the findings of Ohba and associates (2004), who showed that integral ER proteins move from the cytoplasmic ER to the inner nuclear membrane by lateral diffusion through the nuclear pore membrane. Protein diffusion rates within ER membranes also can be affected by several factors, including association with other membrane proteins, protein complexes, and aggregation (Nehls et al. 2000). The reduced rate of spread of ER^{mem} protein probes that we observed in MP⁺ plants may be explained by hindrance of diffusion in the ER lipids due to the presence of TMV^{MP} within the ER^{DT} membrane (Sung and Yethiraj 2006).

A biophysical analysis of protein transport through the Pd in either the cytoplasm, the ER lumen, or ER membranes leads to the conclusion that cell-to-cell probe diffusion is significantly fast to explain observations (Supplementary appendix). In all cases, the estimated rates of passive diffusion across a model Pd are somewhat faster than the observed rates. Although many factors could slow the cell-to-cell spread of the probe proteins, the analysis indicates that active mechanisms are not required to explain their transport.

TMV^{AMPACP} inhibits cell-to-cell spread of GFP^{cyt} and ER^{mem} probes in WT plants and induces callose accumulation at Pd.

The finding that, in WT plants, cell-to-cell spread of either GFP^{cyt} or of ER^{mem} protein probes is significantly inhibited when infected with TMV^{AMPACP}, suggests that, in the absence of TMV^{MP}, the expression of TMV^{AMPACP} induces a stress reaction, possibly callose accumulation, which inhibits cell-to-cell spread (Iglesias and Meins 2000). Our measurements of callose accumulation support this hypothesis (Fig. 6A and B). In MP⁺ plants infected by TMV^{AMPACP}, the level of TMV^{AMPACP} stress-induced callose around Pd (Fig. 6A and B) was lower, presumably due to increased recruitment or activity of class I β -1,3-glucanase (Iglesias and Meins 2000). The lowering of callose levels is dependent on the expression of both MP and replicase (Fig. 6A and B). Similarly, Fridborg and associates (2003) suggested that *Potato virus X* (PVX) infection increased callose deposition in the neck region of Pd and that TGB12K, one of the triple-gene-block proteins, increases the SEL via callose degradation promoting PVX spread. Their data suggest that the PVX MP, TGB12K, directs endomembrane derived vesicles containing class I β -1,3-glucanase, putatively a vacuo-

lar enzyme (Vandenbulcke et al. 1989), to the PM, thus reducing callose accumulation and promoting PVX cell-to-cell spread (Fridborg et al. 2003).

Replicase is involved and necessary for TMV cell-to-cell spread.

Although TMV^{MP} is necessary for cell-to-cell spread of viral infection (Heinlein and Epel 2004), it is not sufficient. Our results are in accord with a previous suggestion that replicase is involved and necessary for cell-to-cell movement of TMV (Hirashima and Watanabe 2003). Our results also are in accord with the suggestion that replicase is part of a virus movement complex that contains all essential components necessary for virus replication (vRNA, replicase, and MP) (Kawakami et al. 2004).

Proposed model.

A modified integrated model of TMV cell-to-cell spread is presented in Figure 7. According to this model, during early infection, the plant cell reacts to the infection leading to callose accumulation and Pd blockage (virus infection stage I). TMV^{MP}, upon synthesis, associates with (Mas and Beachy 1999) and integrates into ER (Brill et al. 2000; Reichel and Beachy 1998), and binds vRNA (Citovsky et al. 1992); replicase, it is suggested, also becomes associated with the complex (virus infection stage II) (Heinlein et al. 1998; Liu et al. 2005; Szecsi et al. 1999). This complex may traffic intracellularly to the ER^{DT} either as vesicles (Fig. 7, open arrowhead) (Liu et al. 2005; Peremyslov et al. 1999) or by diffusion in ER membranes (Fig. 7, squiggle arrow). ER-derived vesicles probably move to Pd along actin filaments (not shown) (Kawakami et al. 2004; Liu et al. 2005; Szecsi et al. 1999). Tagami and Watanabe (2006) present data showing that the ER-Golgi secretory pathway probably is not involved in targeting VRCs to the Pd and in viral intercellular movement because treatment with brefeldin A, which disrupts ER-to-Golgi transport, did not inhibit the targeting of MP to Pd and viral cell-to-cell spread (Tagami and Watanabe 2006). Replicase and MP function synergistically, possibly by targeting class I β -1,3-glucanase via cycling ER-derived vesicles to the wall surrounding the Pd (Fridborg et al. 2003). In the wall domain surrounding the Pd, class I β -1,3-glucanase decreases callose accumulation around the Pd induced by the virus infection, thus allowing unimpeded diffusion of the MP:vRNA:replicase complex in the ER^{DT} membrane (Fig. 7, virus infection stage III arrowhead). The model allows for intracellular but not intercellular trafficking to be mediated by cytoskeletal elements. Recent evidence favors microfilaments because viral spread is blocked by inhibitors of filamentous actin and myosin but not by inhibitors of microtubules (Gillespie et al. 2002; Kawakami et al. 2004; Liu et al. 2005). We suggest that the involvement of cytoskeletal elements is indirect, functioning in either stabilizing the ER (Heinlein et al. 1998), targeting vesicles containing β -1,3-glucanase to the wall surrounding the Pd (Fridborg et al. 2003), or possibly the intracellular trafficking of the MP:endomembrane vesicle to the region of the ER in proximity to Pd (Oparka 2004; Reichel et al. 1999).

Conclusion.

Our modified model proposes that vRNA slowly spreads through the Pd by passive diffusion as an MP:vRNA complex, possibly also associated with replicase in the lipid milieu of the ER^{DT} due to the concentration gradient of the MP:vRNA complex between a viral-infected cell and adjacent noninfected cells. Our data show that ER membrane proteins can move cell to cell by restricted diffusion in the ER via the ER^{DT}. Furthermore, our data and those of others show that both MP and repli-

case are involved in preventing stress-induced callose accumulation at Pd. These data and those of others lead us to propose a modified model which posits that vRNA slowly spreads through the Pd by passive diffusion on an MP raft as an MP:vRNA complex, possibly also associated with replicase in the lipid milieu of the ER^{DT}; diffusion is driven by the concentration gradient of the MP:vRNA complex between a viral-infected cell and adjacent noninfected cells. This transport is dependent on the prevention of callose accumulation in the Pd wall sleeve mediated by the synergetic effect of MP and replicase.

MATERIALS AND METHODS

Plant material.

WT and MP⁺ transgenic *N. benthamiana* plants (Kotlizky et al. 2001) were grown in growth chamber in 10-cm pots in a mix of equal volumes of soil and vermiculite (Pecka Hipper Gan, Rehovot, Israel) at 25°C under long day conditions (cycle of 16h of light and 8 h of darkness).

Construction of plant expression plasmids.

The plasmid pBinGFP:calreticulin (Fig. 2) was constructed in two steps by modifying the plasmid pBinGFP (Sagi et al. 2005). The GFP gene, without its stop codon, first was polymerase chain reaction (PCR) amplified with the calreticulin signal peptide employing forward primer (FP) 5'-CTCTTTGATTCTTTTCGCTCTCGTGGTGATCGTCTCTGCTGAAATGGTGAGCAAGGGCGAGG-3' FP and reverse primer (RP) 5'-GCGGCCGCCTTGTACAGCTCGTCCATGCC-3' containing an *NotI* site. A second PCR was performed on the first PCR product with FP 5'-CCTAGGATGGCGAACTAAACCCTAAATTCATCTCTTTGATTCTTTTCGCTCTCGTG-3' containing an *AvrII* site and RP 5'-GCGGCCGCCTTGTACAGCTCGTCCATGCC-3' containing an *NotI* site. The *AvrII*-SP-GFP-*NotI* fragment was cloned into a unique *XbaI* site present in pBinGFP between the omega enhancer sequence and the GFP start codon and a unique *NotI* site present in pBinGFP between the GFP stop codon and the NOS terminator sequence. The *AvrII*-SP-GFP-*NotI* fragment replaced the GFP and formed a pBinSPGFP plasmid. In the second step, the calreticulin gene was PCR amplified without its signal peptide, with an FP 5'-GCGGCCGCAGTTATCTTCGAGGAGAAATTCGAAGATGG-3' containing an *NotI* site and RP 5'-GCGGCCGCTTAGAGCTCGTCATGGGCGGCATC-3' containing an *NotI* site and cloned into a unique *NotI* site present in pBinSPGFP between the SPGFP last codon and the NOS terminator sequence. PCR was used to verify calreticulin orientation in pBinGFP:calreticulin and the calreticulin gene was amplified with FP 5'-TCTCTTTGATTCTTTTCGCTCTCGTGGTGATCGTCTCTGCTGAAATGGTGAGCAAGGGCGAGG-3' and RP 5'-GCGGCCGCTTAGAGCTCGTCATGGGCGGCATC-3'.

To construct plasmid pBincalnexin:GFP (Fig. 2), the calnexin gene was amplified without its stop codon, by PCR with FP 5'-GTATCCTTAGAGTCCTAGGAATTCATGAGAGAACGGATTATTACATTCGTTTC-3' containing *AvrII* and *EcoRI* sites and RP 5'-GTATCCTTAGAGTCCTAGGACTCTCACGCCTGTCTGC-3' containing an *AvrII* site. The *AvrII*-*EcoRI*-calnexin-*AvrII* fragment was cloned into a unique *XbaI* site present in pBinGFP between the omega enhancer sequence and the GFP start codon. *EcoRI* digestion was used to verify calnexin orientation in pBincalnexin:GFP.

Construction of minimal replicon.

The TMV-based replicon 5' 20-GFP-22 3' (Man and Epel 2004) was digested by *EcoRV* and *NotI*, the sticky end produced by *NotI* was blunted, and the large fragment was self ligated to produce plasmid pTMV^{ΔMPACP}. Plasmid pTMV^{ΔMPACP} contains

the TMV CDNA without nucleotides 4,927 to 6,191 cloned in pBLUESCRIPT KS(+). Plasmid pTMV^{ΔMPACP} was digested by *SplI* and *Cfr9I* and a *SplI*-*Cfr9I* fragment containing the deletion of MP and CP (nucleotides 4927-6191) was moved into p35SU1R (Dagless et al. 1997) digested by the same enzymes, thus replacing the *SplI*-*Cfr9I* fragment from the complete TMV (GenBank accession no. V01408) CDNA of p35SU1R by the *SplI*-*Cfr9I* fragment from pTMV^{ΔMPACP} and producing p35S[TMV^{ΔMPACP}]R. The 35S::TMV^{ΔMPACP}::ribozyme cassette was excised from p35S[TMV^{ΔMPACP}]R by digestion with *PstI* and cloned into the unique *PstI* site of pPZP222 (Hajdukiewicz et al. 1994), producing TMV^{ΔmpACP}, an *Agrobacterium* binary plasmid designed to express a minimal TMV replicon devoid of MP and CP sequences (Fig. 2).

Transient expression.

Transient expression was induced using *Agrobacterium* infiltration as previously described (Sagi et al. 2005). GFP fluorescence was examined in epidermal cells at the lower face of leaves 48 or 72 hpai. All the probes were injected with very low titers of *Agrobacterium* to ensure low-density-separated infection sites (5×10^6 CFU/ml). At this titer, approximately 90% of cells injected with *Agrobacterium* expressing dsRed fluorescence was detected in a single cell only 72 hpai. The TMV mutant lacking both MP and CP (TMV^{ΔMPACP}) was transiently expressed by agroinfiltration at a 750-fold higher titer (4×10^9 CFU/ml) to permit its expression in almost all epidermal cells (Man and Epel 2006). At this titer, approximately 90% of the cells were infected by *Agrobacterium* expressing TMV^{ΔMPACP} (expressing GFP under the CP subgenomic promoter) at 72 hpai.

Callose staining and callose quantification.

Callose staining was performed by immersing whole leaves for 4 h in 85% ethanol and then incubating leaf segments for 16 h in a mixture of 0.1% aniline blue (Fluka, Buchs, Switzerland) in double-distilled water and 1 M glycine, pH = 9.5, at a volume ratio of 2:3, respectively.

Callose quantification was performed by measuring its aniline blue fluorescence intensity. Fluorescence was viewed with a DMRBE fluorescence light microscope (Leica, Wetzlar, Germany) employing a 340- to 380-nm band-pass excitation filter, an RKP 400 dichromatic mirror, and a long-pass 425-nm emission filter.

Analysis of fluorescence intensity associated with Pd of aniline blue was performed under nonsaturation excitation levels with the image processing software ImageJ 1.31v by subtracting the minimum fluorescence value in the area adjacent to the Pd (background) from the maximum fluorescent value of the Pd foci.

Confocal fluorescence microscopy.

Fluorescence microscopy was carried out using a Zeiss R510 confocal laser scanning microscope. GFP excitation was performed with an argon laser set to 488 nm and fluorescence detected with a 505- to 530-nm band-pass filter combination. Images were processed with Photoshop 7.0.

Statistical analysis.

All experimental values were based on at least three independent experiments with each construct for each plant line, and at least 50 replicates were conducted for each combination of construct and plant line. The cell-to-cell movement of the different plant lines was compared by analysis of variance and *t* test analysis according to the statistical program JMP statistical software from SAS (version 4.0.0; SAS Institute Inc., Cary, NC U.S.A.). The analysis of the least significant difference

(LSD) according to the JMP program indicates which means are different from each other at $P < 0.05$.

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AUTHOR-RECOMMENDED INTERNET RESOURCE

National Institute of Health ImageJ 1.31v software: rsb.info.nih.gov/ij