Secondary Plasmodesmata Are Specific Sites of Localization of the Tobacco Mosaic Virus Movement Protein in Transgenic Tobacco Plants

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Expression of the tobacco mosaic virus 30-kD movement protein (TMV MP) gene in tobacco plants increases the plasmodesmatal size exclusion limit (SEL) 10-fold between mesophyll cells in mature leaves. In the present study, we examined the structure of plasmodesmata as a function of leaf development. In young leaves of 30-kD TMV MP transgenic (line 274) and vector control (line 306) plants, almost all plasmodesmata were primary in nature. In both plant lines, secondary plasmodesmata were formed, in a basipetal pattern, as the leaves underwent expansion growth. Ultrastructural and immunolabelling studies demonstrated that in line 274 the TMV MP accumulated predominantly in secondary plasmodesmata of nonvascular tissues and was associated with a filamentous material. A developmental progression was detected in terms of the presence of TMV MP; all secondary plasmodesmata in the tip of the fourth leaf contained TMV MP in association with the filamentous material. Dye-coupling experiments demonstrated that the TMV MP-induced increase in plasmodesmatal SEL could be routinely detected in the tip of the fourth leaf, but was restricted to mesophyll and bundle sheath cells. These findings are discussed with respect to the structure and function of plasmodesmata, particularly those aspects related to virus movement.

INTRODUCTION

Plasmodesmata are cytoplasmic connections between plant cells, and they play potentially important roles in intercellular transport, cell-to-cell communication, cell differentiation, plant growth, and development (Gunning and Robards, 1976; Robards and Lucas, 1990). In addition, they are major routes for cell-to-cell spread of plant viruses (Hull, 1989; Robards and Lucas, 1990). Therefore, a basic knowledge of plasmodesmal structure and function is fundamental to the understanding of many biological processes related to plants.

In recent studies, we have used the fact that plant viruses interact with, and move cell to cell through, plasmodesmata to probe plasmodesmal structure and function (Wolf et al., 1989, 1991; Deom et al., 1990, 1991; Lucas et al., 1990; Berna et al., 1991). The smallest physical dimension of known plant viruses, when measured at any given angle, is larger than 10 nm, and even the free-folded viral nucleic acid is thought to be at least 10 nm in size (Gibbs, 1976). However, a recent high-resolution electron microscopic study of freeze-substituted plasmodesmata, performed on tobacco leaves, established that the most obvious transport channels within plasmodesmata are physically about 2.5 nm in diameter (Ding et al., 1992). These dimensions are consistent with plasmodesmatal size exclusion limits (SEls) of 800 to 900 D determined by dye-coupling experiments (e.g., Goodwin, 1983; Terry and Robards, 1987; Wolf et al., 1987). Hence, it is unlikely that any known plant virus can move through plasmodesmata without modifying the physical dimension, and thus the SEL, of the plasmodesmatal transport channels. Indeed, it is now known that the genomes of many plant viruses encode a specific movement (or transport) protein (Melcher, 1990; Koonin et al., 1991), which facilitates viral movement through plasmodesmata (Atabekov and Taliensky, 1990), and the mechanisms involved are the focus of current investigations (Citovsky and Zambrayski, 1991; Citovsky et al., 1992).

The 30-kD tobacco mosaic virus movement protein (TMV MP) is currently one of the most intensively studied proteins involved in virus movement, and its role in potentiating cell-to-cell movement of TMV has been conclusively established (Deom et al., 1987; Meshi et al., 1987). In an investigation on the functional mechanism of the TMV MP, Wolf et al. (1989) demonstrated that the 30-kD protein modifies the plasmodesmatal SEL between mesophyll cells from the normal 0.9 kD...
to about 10 kD in mature leaves of transgenic tobacco that express the TMV MP gene. Later studies indicated that this modification is a function of leaf age; i.e., the TMV MP is expressed in leaves of all ages, but increases the plasmodesmal SEL only in mature leaves (Deom et al., 1990).

To further understand the mechanism of the MP-plasmodesma interaction and the developmental aspects of this phenomenon in particular, we conducted a systematic examination of the structure of plasmodesmata between various cell types in leaves of successive ages from two transgenic tobacco (cv Xanthi) lines: one was TMV MP expressing (274), and the other was a nonexpressing vector control (306). Furthermore, we utilized immunogold labeling to localize the TMV MP and dye coupling to probe the plasmodesmal SEL between various cell types within the tobacco leaf. The cumulative results from these studies have provided a better understanding of several fundamental issues regarding MP-plasmodesma interactions.

RESULTS

Primary versus Secondary Plasmodesmata

Before presentation of our data, it is necessary to define the nomenclature we have used to describe the plasmodesmata we observed. Two basic categories of plasmodesmata have been observed in our material: simple plasmodesmata that exist as discrete, plasma membrane-lined entities, as shown in Figures 1A and 1B, and plasmodesmata that consist of multiple branches united via a central cavity at the region of the cell wall middle lamella, as shown in Figures 1C and 2A. In this study, all the simple plasmodesmata that occur as discrete, linear entities have been referred to as primary plasmodesmata, whereas all the branched forms have been termed secondary plasmodesmata. However, we are aware of the limitations of this nomenclature because the terms primary and secondary, when used to describe plasmodesmata, refer to ontogeny not morphology. Specifically, plasmodesmata that are formed during cytokinesis are referred to as primary plasmodesmata (Jones, 1976; Robards and Lucas, 1990); plasmodesmata that are formed through other processes, e.g., cell fusion or perforation of an existing cell wall, are termed secondary plasmodesmata (Jones, 1976; Robards and Lucas, 1990).

As shown in Figure 1, at an early developmental stage of the tobacco leaf, all plasmodesmata appeared as discrete simple strands. They are most likely formed during cytokinesis and therefore are primary in origin. Branched plasmodesmata appeared only at later stages of leaf development (Figures 1C and 1E), and the new plasmodesmal branches are likely to have been formed across the existing cell wall. However, uncertainty exists regarding the nature of some plasmodesmata that are "H-shaped," as shown on the right of Figure 1C. One possibility is that this form may represent plasmodesmata of secondary origin; alternatively, this could well represent two primary plasmodesmata that are fusing in the vicinity of the middle lamella. In the latter case, these H-shaped, modified primary plasmodesmata would then undergo structural development, via the addition of further protoplasmic bridges, to give rise to bona fide secondary plasmodesmata. Figure 2E illustrates the difficulty of determining the origin of simple plasmodesmata that are present in mature leaves. Such plasmodesmata could either be secondary or primary that have retained their structural and functional integrity throughout leaf development. Examples of simple secondary plasmodesmata have been found in graft unions (Kollmann and Glocmann, 1985, 1991); however, in this situation they occur at very low frequencies and are interspersed among irregularly branched secondary plasmodesmata. Using the plant graft system as the model for secondary plasmodesma, we presume that the majority of single-stranded plasmodesmata in mature tobacco leaves are likely to be primary in origin.

In the absence of additional structural and functional data to establish the identity of single-stranded plasmodesmata in fully mature tissue, we will provisionally use the term "primary" plasmodesmata to include all the single-stranded plasmodesmata in our system. Importantly, this usage will not interfere with our interpretation of data because, as we will show, the TMV MP only accumulates in branched, truly secondary plasmodesmata.

Development of Secondary Plasmodesmata as a Function of Leaf Maturation

We systematically examined the structure of plasmodesmata in leaves of sequential developmental stages, from the first to the sixth leaf, in plants of both lines 274 and 306. Photosynthetic 14CO2 labeling and whole leaf autoradiography experiments established that the first leaf (defined as the youngest leaf to attain a length of 5 cm) was undergoing the sink-to-source transition (data not shown; see also Turgeon, 1984, 1989). The basal region of this leaf still functioned as a sink for photoassimilates, while the tip region had developed sufficient photosynthetic competence to be able to export photoassimilates to sink regions. These observations are consistent with established sink-to-source transition patterns, whereby leaf maturation starts at the tip and progresses basipetally (Turgeon, 1989). In tobacco plants used in this study, the second and third leaves were still undergoing expansion growth, while the fourth leaf was nearing full size, and later leaves were fully expanded and mature. Clearly, each leaf represents a more developmentally mature state compared with the preceding leaf. In our study, samples were taken from the tip and basal regions of each leaf from the first through the sixth. Thus, potential changes in plasmodesmal structure/function associated with leaf development could be closely followed.

In the basal region of the first leaf, all plasmodesmata observed appeared to be primary in nature (Figure 1A). In the tip region, plasmodesmata were still predominantly primary,
but a majority appeared to have developed a central cavity between the desmotubule and the plasma membrane in the region of the middle lamella, whereas the two ends remained unchanged (Figure 1B; see also Ding et al., 1992). A small fraction of the plasmodesmata in this region (tip of the first leaf) exhibited a more complex structure, and, based on our operational definition, these were classified as secondary plasmodesmata (Figure 1C).

The frequency of secondary plasmodesmata was found to increase as a function of leaf development. By the third leaf, secondary plasmodesmata between nonvascular cells were found to represent 40 to 50% of the total at the base and 60
Figure 2. Plasmodesmata between Various Cell Types in Mature Leaves of Control and TMV MP Transgenic Tobacco Plants.
to 70% at the tip. At the tip of the fourth leaf, and throughout the entire fifth and sixth leaves, more than 90% of all plasmodesmata observed were secondary in form. In this analysis, more than 500 plasmodesmata were examined in each case. Secondary plasmodesmata within the tip of the second and third leaves were generally doubly, but sometimes triply, branched on either or both ends, and the size of the central cavity was relatively small (Figures 1D and 1E). Starting in the fourth leaf, secondary plasmodesmata consisting of three or more branches on either or both ends were commonly observed, and their central cavities were usually larger than those in younger leaves (Figures 2A and 2B).

During leaf development, secondary plasmodesmata also developed between bundle-sheath and phloem-parenchyma cells, as shown in Figure 2F, and between various cell types of the phloem tissue, as shown in Figure 3. However, whereas all plasmodesmata between sieve elements and companion cells and between sieve elements and phloem-parenchyma cells were secondary in mature leaves, only a small fraction appeared to be secondary among other phloem cells (Figure 3).

These patterns of plasmodesmal development were similar in both the 274 and 306 lines. However, as discussed below, an electron-dense material gradually accumulated exclusively in secondary plasmodesmata in the 274 line.

Electron-Dense Filamentous Material Present Exclusively in Secondary Plasmodesmata of the TMV MP Transgenic Plants

In the 274 line of tobacco (expressing TMV MP), an electron-dense filamentous material of 16- to 18-nm diameter was first observed in the central cavities of a few secondary plasmodesmata in nonvascular tissues at the tip of the second leaf (Figure 1D). At the tip of the third leaf, this filamentous material was observed in more than 50% of secondary plasmodesmata (Figure 1E). In the tip of the fourth, and throughout the entire area of the fifth and sixth leaves, the filamentous material was present in every secondary plasmodesma in nonvascular tissues (Figure 2B).

The accumulation of this filamentous material was closely associated with the development of central cavities: as they enlarged, the amount of filamentous material also increased, filling the cavity (compare Figures 1E and 2B). Secondary plasmodesmata between bundle-sheath and phloem-parenchyma cells always contained this filamentous material (Figure 2F). In contrast, it was rarely found between bundle-sheath and companion cells (Figure 2G). Furthermore, little of this filamentous material was observed in plasmodesmata between various vascular cells (Figure 3). Although we did observe similar material in a few secondary plasmodesmata between some vascular cells (Figure 3C), the quantity was always very low and its presence was sporadic. Many secondary plasmodesmata in this tissue were clearly devoid of the material (Figures 3A, 3D, and 3F). In this regard, it is important to note that these filaments were never found in any primary plasmodesmata, including those in the mature leaves of the 274 plants (Figure 2E). Finally, this material was absent from every plasmodesma of control line 306 plants, irrespective of leaf age (Figure 2A).

TMV MP Immunolocalized Exclusively in Secondary Plasmodesmata in Nonvascular Tissue

For immunolocalization of the TMV MP in transgenic tobacco plants, samples from the tip regions of the third, fifth, and sixth leaves were cryofixed and freeze substituted. The advantage of these techniques is that they preserve protein antigenicity much better than conventional chemical fixation procedures (Kandasamy et al., 1991). Moreover, these techniques have been successfully used to elucidate the substructure of plasmodesmata (Ding et al., 1992). Polyclonal antibodies raised against the TMV MP were used to localize the MP on the freeze-
Figure 3. Plasmodesmata between Vascular Cells of Mature Leaves of TMV MP Transgenic Tobacco Showing Sparse Presence of the Filamentous Material in Plasmodesmata in this Tissue.

(A) Secondary plasmodesma between sieve element (SE) and companion cell (CC). Dt, desmotubule. ×68,200.
(B) Primary plasmodesma between two companion cells. Dt, desmotubule. ×68,200.
(C) Electron-dense filamentous material (F) in a secondary plasmodesma between two companion cells. The occurrence of this material was sporadic and in low amount in the vascular tissue. ×105,400.
(D) Plasmodesma between sieve element (SE) and phloem-parenchyma cell (PP). ×68,200.
(E) Primary plasmodesmata between phloem-parenchyma cells. Dt, desmotubule. ×68,200.
(F) Secondary plasmodesma between phloem-parenchyma cells. Unlabeled arrow indicates what is probably a dilated desmotubule. ×106,400.

substituted leaf samples by indirect immunogold labeling (see Methods). In the 274 line, the antibody was found to bind exclusively to secondary, but not primary, plasmodesmata between various nonvascular cells, and between bundle-sheath and phloem-parenchyma cells, as shown in Figure 4.

Interestingly, in the labeled secondary plasmodesmata, the antibody most heavily decorated the filamentous material present in the central cavity (Figures 4A, 4B, and 4C). This suggests an association between the TMV MP and the filamentous material. No plasmodesmata within the vascular tissue were
positively labeled (data not shown). Plasmodesmata in the 306 line of tobacco plants also showed no labeling (data not shown).

These results demonstrate that the presence of the filamentous material in the central cavities of secondary plasmodesmata is directly associated with the localization of the TMV MP.

Plasmodesmatal SEL

Previous experiments have established by microinjection of lucifer yellow CH (LYCH) and fluorescein isothiocyanate-labeled dextrans (F-dextrans) that the TMV MP modulation of the plasmodesmatal SEL is leaf age dependent and occurs only in mature leaves (Deom et al., 1990). Our present study refined those experiments by microinjecting fluorescent probes into both the tip and base regions of the third, fourth, and fifth leaves of the 274 line. In general, enhanced dye coupling was first detected between mesophyll cells in the tip of the fourth leaf, as shown in Table 1. Thus, the fourth leaf represents a transition stage for expression of the increased SEL induced by the TMV MP.

We extended the dye-coupling experiments to other cell and tissue types and found that the coupling of the 3.9- and 9.4-kD F-dextrans also occurred between mesophyll and bundle-sheath cells, and among bundle-sheath cells themselves in mature leaves of the 274 line, as shown in Table 2 and Figure 5. Surprisingly, in mature leaves of the 274 line, the 3.9- and 9.4-kD F-dextrans were never observed to move from bundle-sheath cells into phloem-parenchyma cells (Figure 5D), despite the presence of TMV MP in the secondary plasmodesmata between these two cell types (Figures 2F and 4B). In contrast, LYCH (molecular mass of 457 D) moved freely between these cell types (Figure 5C).

When F-dextran of any size was injected into phloem-parenchyma cells, no dye movement out of the injected cell was ever observed (Table 2). Although the F-dextran may have been injected into the vacuole in some cases, which would definitively prevent dye movement, many injections were clearly cytoplasmic.

As previously established by Wolf et al. (1989), no movement of F-dextran of any size was observed between any cell types in the 306 (control) line of tobacco (data not shown).

DISCUSSION

Secondary Plasmodesmata Are Exclusive Sites of TMV MP Localization

Our results demonstrated that in tobacco, the transgenically expressed TMV MP (1) accumulates only in secondary plasmodesmata; (2) is associated with an electron-dense filamentous material present in the central cavity of these plasmodesmata; (3) is present in but ineffective toward increasing the SEL of secondary plasmodesmata between bundle-sheath and phloem-parenchyma cells; and (4) is rarely detected in plasmodesmata between vascular cells.

Although TMV MP is present in the secondary plasmodesmata in the tip of the third leaf, our dye-coupling experiments detected no modification of the plasmodesmatal SEL. There are several possible explanations for this finding. First, the TMV MP present in these secondary plasmodesmata may be below a threshold level necessary to exert an effect on the SEL. Second, any change in the plasmodesmatal SEL induced by the low level of TMV MP in these leaves may be too small to detect. Third, it is possible that another, subsequent and unobserved, developmental event of maturation is necessary for the TMV MP to functionally alter the SEL such that large dextrans may freely pass. Thus, it is not until the tissue reaches a stage equivalent to that of the base of the fourth leaf, when the TMV MP is present in the majority of secondary plasmodesmata, that the mesophyll enters a transitional state in which plasmodesmata are competent for modification to their SEL (Table 1). At the tip of the fourth leaf, the TMV MP is present in every secondary plasmodesma and at high levels, and modification of plasmodesmatal SEL can be routinely detected (Table 1).

Although we have shown that MP accumulates only in secondary plasmodesmata and increases the SEL in mature leaves, it is well known that young tobacco leaves are susceptible to TMV infection. At present, we have no clear explanation for this apparent discrepancy. Whether the formation of secondary plasmodesmata in young leaves is accelerated or other cellular processes are involved in the TMV infection process awaits further characterization.

Given these considerations, our results demonstrated a close temporal correlation between the modification of plasmodesmatal SEL and localization of TMV MP in secondary plasmodesmata in the MP transgenic tobacco plants. This correlation suggests that secondary, not primary, plasmodesmata are the exclusive sites of TMV MP localization and function.

Examination of published micrographs of plasmodesmata decorated by anti-TMV MP antibody in TMV-infected tobacco (Tomenius et al., 1987), those that were tagged by the anti-TMV MP antibody in TMV MP transgenic tobacco (Atkins et al., 1991) and those that were shown to contain filamentous material in mature leaves of such plants (Moore et al., 1992), clearly indicates the involvement of secondary plasmodesmata.

Functional Differences between Primary and Secondary Plasmodesmata

The transgenic TMV MP gene in line 274 has been shown to be expressed in both young and mature leaves, with the level of MP in mature leaves accumulating to approximately three times that detected in the youngest leaf assayed (Deom et al., 1990). Thus, the specific targeting of the TMV MP to secondary plasmodesmata, at a particular stage of leaf maturation, represents a fascinating developmental event. It is possible
Figure 4. Immunogold Labeling of TMV MP in Transgenic Tobacco from the Tip Region of the Fifth Leaf.
Table 1. TMV MP Modification of SEL of Plasmodesmata between Mesophyll Cells as a Function of Leaf Developmental Stage in Transgenic Plants Expressing the TMV MP Gene

<table>
<thead>
<tr>
<th>Leaf No. Region</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
<th>Experiment 3</th>
<th>Experiment 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Three Tip</td>
<td>3.9</td>
<td>3.9</td>
<td>3.9</td>
<td>3.9</td>
</tr>
<tr>
<td>Base</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Four Tip</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Base</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Five Tip</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Base</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

Dye-coupling experiments were performed on tobacco line 274, and all plants used had 11 to 12 true leaves. Increase in SEL caused by the TMV MP was indicated by movement of 3.9- and 9.4-kD F-dextrans. + indicates dye movement with five to seven injections being performed at each location; - indicates no movement.

Table 2. Mobility of Fluorescent Probes through the Symplasm of Leaf Cells in Transgenic Tobacco Expressing the TMV MP Gene

<table>
<thead>
<tr>
<th>Injected Cell Type</th>
<th>Probe</th>
<th>Molecular Mass (daltons)</th>
<th>% of Injections Expressing Movement</th>
<th>Dye-Coupled Cell Types</th>
</tr>
</thead>
<tbody>
<tr>
<td>MC</td>
<td>LYCH</td>
<td>457</td>
<td>75 (4)</td>
<td>+ + +</td>
</tr>
<tr>
<td></td>
<td>F-dextran 3,900</td>
<td>60 (5)</td>
<td></td>
<td>+ + -</td>
</tr>
<tr>
<td></td>
<td>F-dextran 9,400</td>
<td>100 (2)</td>
<td></td>
<td>+ + -</td>
</tr>
<tr>
<td>BS</td>
<td>LYCH</td>
<td>457</td>
<td>75 (4)</td>
<td>+ + +</td>
</tr>
<tr>
<td></td>
<td>F-dextran 3,900</td>
<td>46 (13)</td>
<td></td>
<td>+ + -</td>
</tr>
<tr>
<td></td>
<td>F-dextran 9,400</td>
<td>100 (2)</td>
<td></td>
<td>+ + -</td>
</tr>
<tr>
<td></td>
<td>F-dextran 17,200</td>
<td>0 (2)</td>
<td></td>
<td>+ + -</td>
</tr>
<tr>
<td>PP</td>
<td>LYCH</td>
<td>457</td>
<td>57 (7)</td>
<td>+ + +</td>
</tr>
<tr>
<td></td>
<td>F-dextran 3,900</td>
<td>0 (6)</td>
<td></td>
<td>+ + -</td>
</tr>
</tbody>
</table>

Data are percentage of injections that showed movement of the specific probe, determined 1 to 2 min after injection. + indicates movement; - indicates no movement of the dyes. Values in parentheses represent number of injection experiments performed; MC, mesophyll cells; BS, bundle-sheath cells; PP, phloem-parenchyma cells.

Figure 4. (continued).

(A) Central cavity of a secondary plasmodesma between mesophyll cells. Arrows indicate visible filaments that are labeled. CW, cell wall; Cyt, cytoplasm. x131,500.
(B) Two secondary plasmodesmata between the bundle-sheath (BS) and the phloem-parenchyma (PP) cell. Arrow indicates one of the labeled filaments. CW, cell wall. x87,700.
(C) High-magnification view of a secondary plasmodesmatal central cavity between mesophyll cells showing decoration of the filamentous material by antibody-linked gold particles. One of the filaments is outlined by the small arrows. x30,000.
(D) Primary plasmodesma (Pd) between mesophyll cells that shows no antibody labeling. CW, cell wall. x131,000.
(E) Control labeling experiment performed on a secondary plasmodesma connecting two mesophyll cells within a mature TMV MP transgenic tobacco plant. This section was incubated in secondary antibody only. Filamentous material is indicated by arrows. CW, cell wall. x153,400.
Figure 5. Dye-Coupling Experiments Performed to Determine Plasmodesmatal SEL within the Vasculature of TMV MP Transgenic Tobacco.
plasmodesmata and modifies their transport channels and what role the C terminus plays in this process are unclear. However, we postulate that to do so, the TMV MP must interact with the desmotubular and/or plasma membrane proteins at the neck regions of secondary plasmodesmata (Ding et al., 1992).

The specificity of the TMV MP for secondary and not primary plasmodesmata strongly suggests that these two plasmodesmal types may differ in some functional aspects in situ. Further support for this suggestion comes from the finding that formation of secondary plasmodesmata is severely inhibited by high activity of a yeast acid invertase in tobacco plants transgenic for this enzyme, whereas primary plasmodesmata remain apparently normal in structure and function (B. Ding, J. S. Haudenshield, L. Willmitzer, and W. J. Lucas, unpublished results).

**Virus Movement and Macromolecular Trafficking through Plasmodesmata**

The presence of “TMV particles” in mesophyll plasmodesmata in TMV-infected tobacco, as reported by Weintraub et al. (1976), has been used as evidence to argue that TMV moves from cell to cell as an intact virion. Based on the striking morphological and dimensional similarity between the TMV MP–associated filaments and those putative TMV particles, we interpret those putative TMV particles present in the plasmodesmata described by Weintraub et al. (1976) to be MP-associated filaments. Consequently, there is no solid evidence to suggest intercellular movement of TMV particles. It is noteworthy that the plasmodesmata that were shown to contain filaments (Weintraub et al., 1976) were also secondary in nature. Recently, Citovsky et al. (1990, 1992) showed that the TMV MP has the capability to bind single-stranded nucleic acids, which is consistent with earlier reports that unencapsidated TMV forms filamentous virus ribonucleoprotein complexes (Dorokhov et al., 1983). Based on this finding, Citovsky and coworkers postulated that the TMV MP could function as a molecular chaperone, which, by binding to the viral nucleic acid, forms an extended and unfolded protein–nucleic acid complex (viral ribonucleoprotein) that targets modified plasmodesmata (Citovsky et al., 1990, 1992; Citovsky and Zambryski, 1991).

The ability of some plant viruses, e.g., TMV, to move symptomatically in an unencapsidated form (Meshi et al., 1987; Dawson et al., 1988) indicates that macromolecular trafficking (protein or mRNA) through plasmodesmata is possible and, therefore, raises the very important question as to whether such trafficking occurs naturally in plants.

**Bundle-Sheath/Vascular-Parenchyma Boundary**

Although the TMV MP accumulates in secondary plasmodesmata between bundle-sheath and phloem-parenchyma cells, the SEL of these plasmodesmata is not modified, as shown by the nonmovement of F-dextrans between these two cell types. If an increase in the plasmodesmatal SEL is a general prerequisite for intercellular TMV movement, our results immediately suggest that additional plant- and/or virus-encoded protein(s) are needed to modify the plasmodesmatal SEL at the bundle-sheath/phloem-parenchyma boundary for phloem invasion by the virus. One candidate that may fulfill this function is the TMV coat protein (CP). Specific mutations in the TMV CP either disable or greatly impair systemic virus infection while not affecting local movement between mesophyll cells (Siegal et al., 1962; Dawson et al., 1988; Culver and Dawson, 1989; Osborn et al., 1990; Saito et al., 1990). The simplest explanation for this phenomenon is that the mutant virus cannot invade the phloem. Given our observation that little TMV MP is present in the plasmodesmata between vascular cells, movement of the virus through such plasmodesmata may also be facilitated by CP activity. Furthermore, there are plant viruses (e.g., the luteoviruses and geminiviruses) that are phloem limited. A recent study has demonstrated that vascular localization of beet western yellows virus (a member of the luteovirus group) is within the vascular-parenchyma/bundle-sheath boundary (B. Ding and W. J. Lucas, unpublished results).

Figure 5. (continued).

(A) and (B) Light micrographs of a portion of fresh leaf tissue (fifth leaf) to show the cell types for microinjection. (B) is a magnified view of the labeled cells in (A). MS, mesophyll cell; BS, bundle-sheath cell; PP, phloem-parenchyma cell. (A) x210; (B) x380.

(C) Movement of LYCH between cells shown in (A) and (B) after microinjection. The dye was injected into the bundle-sheath cell (BS) and moved into adjacent mesophyll cells (MS) and phloem-parenchyma cells (PP). x240.

(D) Intercellular movement of the 39-kD F-dextran. The dye was injected into a mesophyll cell (MS) adjoining a bundle-sheath cell (BS). Although the 39-kD F-dextran moved into surrounding mesophyll cells and the adjacent bundle-sheath cell, it did not move into the neighboring phloem-parenchyma cells (PP). x240.

(E) and (F) Light micrographs of longitudinal and transverse sections, respectively, of vascular bundles from the fifth leaf showing spatial relationships between various cell types involved in the vascular dye-coupling experiments. MS, mesophyll cell; BS, bundle-sheath cell; PP, phloem-parenchyma cell. x150.
Collectively, these results establish that the bundle-sheath/vascular-parenchyma boundary is a special barrier to virus movement. Some viruses (e.g., TMV) have apparently evolved a mechanism that allows them to cross this boundary to initiate long-distance systemic infection.

Finally, we emphasize that TMV MP transgenic tobacco has value beyond the study of virus-plasmodesma interaction. It is also useful in the study of general plasmodesmal biology. For example, the system has recently been used to study the effect of changes in plasmodesmal SEL on photosynthate partitioning in plants (W. J. Lucas, A. Olesinski, R. J. Hull, J. S. Haudenshield, C. M. Deom, R. N. Beachy, and S. Wolf, unpublished results).

METHODS

Plant Material

Two lines of transgenic tobacco (Nicotiana tabacum cv Xanthi), as described by Deom et al. (1990), were used in the present study. The 274 line expresses the TMV MP gene constitutively. The 306 line is a transgenic vector control lacking the MP gene.

Seeds were sown on the surface of greenhouse potting mix and covered to 1 mm with fine sand. At 4 weeks, the plantlets were transplanted to 4-inch-diameter plastic pots. Plants were grown in an insect-free greenhouse until they were transferred to a controlled-environment growth chamber at least 5 days before use in microinjection studies. Plants sometimes remained in the growth chamber for prolonged periods (months) while experimentation progressed. The temperature regime in the growth chamber was 24°C/18°C (day/night) with a 16-hr photoperiod at a PAR level of 280 to 230 pmol m−2sec−1.

For immunolocalization of the TMV MP, the primary antibody was a rabbit-derived polyclonal antibody (IgG) raised against the TMV MP (Deom et al., 1987). A goat-derived and 5-nm-diameter gold-conjugated anti-rabbit IgG antibody (Sigma) was used as the secondary antibody.

Cryofixation

The propane jet freezing method of Ding et al. (1991) was followed. Briefly, leaf samples of about 1 mm² were immersed in a 20 mM Mes buffer solution, pH 5.5, containing 2 mM CaCl₂, 2 mM KCl, and 0.2 M sucrose for 5 to 10 min. As discussed in Ding et al. (1991), this treatment increases the depth of good freezing, but does not introduce any detectable artifacts. After incubation, the samples were frozen in a propane jet freezer (model 7200; Research and Manufacturing Company, Tucson, AZ) for details, see Ding et al., 1991).

Freeze Substitution

The procedure of Ding et al. (1991) was followed with the following modifications. The frozen samples were freeze substituted with 2% glutaraldehyde in acetone (diluted from a commercial 10% solution in acetone; Electron Microscopy Sciences) at –90°C for approximately 36 hr, at –20°C for 7 to 9 hr, and at 4°C for 12 hr. (These substitutions took place over molecular sieves prebaked for 6 hr in a 400°F oven.) The samples were then substituted in anhydrous ethanol at 4°C for 2 to 3 hr and brought to room temperature. After two more rinses in ethanol, the samples were infiltrated with, and then embedded in, LR White resin (Electron Microscopy Sciences). The resin was polymerized at 50°C for 20 hr.

Immunogold Labeling

For immunolocalization of the TMV MP, the primary antibody was a rabbit-derived polyclonal antibody (IgG) raised against the TMV MP (Deom et al., 1987). A goat-derived and 5-nm-diameter gold-conjugated anti-rabbit IgG antibody (Sigma) was used as the secondary antibody.

Electron Microscopy

All sections were examined with a Philips EM410LS electron microscope operating at 80 kV.
Microinjection

The molecular size exclusion limit (SEL) of the plasmodesmata in the two lines was determined by microscopic observation of intercellular dye coupling of microinjected probes. Lucifer Yellow CH dilithium salt (LYCH; Sigma) with a molecular mass of 457 D and fluorescein isothiocyanate-conjugated dextrans (F-dextrans; Sigma and Molecular Probes, Inc., Eugene, OR) with molecular masses ranging from 3.9 to 17.2 kD were used as fluorescent probes. All probes were dissolved in 5 mM KHCO₃, filtered through a 0.5-μm-pore nylon syringe filter, and stored at 4°C.

Intact plants were placed with the target leaf abaxial side up, positioned on the injection microscope stage, and held in place on a glass slide with double-sided cellophane tape. Alternatively, a 12- to 16-cm² section was cut from the target leaf and the entire section submerged under water in a plastic Petri dish on the microscope stage. A 20- to 50-mm² section of epidermis was carefully peeled to expose the target tissue, and the site was immediately bathed with distilled water. Glass micropipettes having an outer tip diameter of <1 μm were fabricated from 1-mm-diameter borosilicate glass capillaries (World Precision Instruments, Sarasota, FL) in a Flaming/Brown micropipette puller (model No. P-87; Sutter Instrument Co., Novato, CA). The probe was backloaded into the micropipette, and impalement of the target cell was facilitated by a hydraulic micromanipulator (model No. MO-104N; Narishige Scientific Instrument Lab, Greensville, NY).

In some species, the tonoplast quickly moves along the glass to exclude the tip of the pipette from the vacuole, thereby enabling direct delivery of probes to the cytoplasm. This method was adopted in the present study because it has the advantage over the liposome delivery protocol (Madore et al., 1988; Wolf et al., 1989) of reducing the problem of tip clogging. In addition, vacuolar versus cytoplasmic delivery is easily identified by following the fate of the injected fluorescent molecule. Direct delivery into the vacuole results in permanent fluorescent labeling of the cell, whereas cytoplasmic delivery gives rise to a characteristic peripheral fluorescence pattern that dissipates as the probe moves through plasmodesmata into the neighboring cells. Pressure microinjection via a pneumatic Picopump (model PV830; World Precision Instruments) was viewed by epifluorescence. Dye movement was monitored through an epifluorescence microscope (Leitz Orthoplan; Ernst Leitz GMBH, Wetzler, Germany) equipped with a blue (BP 390-490) excitation filter, a chlorophyll cutoff filter, and a video-intensified microscopy system (model No. VO-5800 U-matic; Sony) in real time and displayed in false color on a Sony Trinitron monitor (model No. PVM-127Q1/137Q1QM) for direct photography to 35-mm color slide film. For bright-field images of the live tissue, the Leitz Vario Orthomat photography system was used.

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