ABSTRACT To fully understand vascular transport of plant viruses, the viral and host proteins, their structures and functions, and the specific vascular cells in which these factors function must be determined. We report here on the ability of various cDNA-derived coat protein (CP) mutants of tobacco mosaic virus (TMV) to invade vascular cells in minor veins of Nicotiana tabacum L. cv. Xanthi nn. The mutant viruses we studied, TMV CP-O, U1mCP15-17, and SNC015, respectively, encode a CP from a different tobamovirus (i.e., from odontoglossum ringspot virus) resulting in the formation of non-native capsids, a mutant CP that accumulates in aggregates but does not encapsidate the viral RNA, or no CP. TMV CP-O is impaired in phloem-dependent movement, whereas U1mCP15-17 and SNC015 do not accumulate by phloem-dependent movement. In developmentally-defined studies using immunocytochemical analyses we determined that all of these mutants invaded vascular parenchyma cells within minor veins in inoculated leaves. In addition, we determined that the CPs of TMV CP-O and U1mCP15-17 were present in companion (C) cells of minor veins in inoculated leaves, although more rarely than CP of wild-type virus. These results indicate that the movement of TMV into minor veins does not require the CP, and an encapsidation-competent CP is not required for, but may increase the efficiency of, movement into the conducting complex of the phloem (i.e., the C cell/sieve element complex). Also, a host factor(s) functions at or beyond the C cell/sieve element interface with other cells to allow efficient phloem-dependent accumulation of TMV CP-O.

The movement of plant viruses through vascular tissue is essential to allow their maximum accumulation and symptom development in the host plant (1). In crops infected with viruses, the greatest yield reductions are due to vascular-dependent movement (2). Although a considerable amount of information is known about the cell-to-cell movement of plant viruses (3–5), much less is known about vascular-dependent movement (5–8). The majority of plant viruses, including tobacco mosaic virus (TMV), move through the phloem (1). The coat protein (CP) of TMV is critical for phloem-dependent accumulation of this virus. Mutant strains of TMV that produce CP, but are defective in virion assembly, spread as labile infectious entities from cell-to-cell, but do not accumulate in tissue accessed by phloem-dependent movement (9). More recently, it has been possible to delete or modify the CP open reading frame of cDNA clones of TMV, and infectious transcripts from these clones yielded virus that moved cell-to-cell but did not accumulate in upper uninoculated leaves unless complemented by CP expressed in transgenic plants (10–13). Saito et al. (14) mutated the nucleotide sequence within the origin of capsid assembly and observed that inefficient assembly led to inefficient accumulation of the virus in upper leaves. Results from this and other work indicate that the virus moves either in an encapsidated form (i.e., as a virion) or as a viral ribonucleoprotein (14, 15). Both forms require functional CP for the phloem-dependent accumulation of virus. Many other plant viruses require their CP for phloem-dependent accumulation (e.g., refs. 16–21). Recently, Dolja et al. (20, 22) determined that the N-terminal 29 residues and the C-terminal 18 residues of the tobacco etch poytivirus CP were necessary for phloem-dependent accumulation. Also, it has been suggested that the capsid of red clover necrotic mosaic dianthovirus, and not some other CP-containing form, is necessary for the long distance movement of the virus (23). Results from all these studies indicate the necessity of the CP for phloem-dependent accumulation, but in no instance has the accumulation of a CP mutant within the vasculature of the inoculated leaves been studied. Invasion of minor veins by mutant viruses is only now coming under study. Cronin et al. (24) have recently determined that the helper component–proteinase of tobacco etch potyvirus functions within the vein to allow phloem-dependent accumulation.

Phloem-dependent accumulation of virus requires that the virus invade the vein cells from the mesophyll cells of the inoculated leaf. Minor veins (class IV and V; ref. 25) account for approximately 90% of the veins in sections from mature tobacco leaves (ref. 26; X.S.D. and R.S.N., unpublished data) and are likely the first veins spreading virus would contact from a site of inoculation. These veins are generally sheathed by long bundle sheath (BS) cells and contain various cell types including xylem tracheary elements, phloem or vascular parenchyma (VP) cells, companion (C) cells, and sieve elements (SE) (5, 8, 26). Hilf and Dawson (27) have shown that host factors different from those required for cell-to-cell movement affect phloem-dependent accumulation of toamoviruses. The location of these host factors within the veins has not been determined. In regard to virally encoded movement factors, cell-to-cell movement of TMV is facilitated by at least one specific viral protein; the 30-kDa or movement protein (28, 29). The movement protein interacts with the plasmalemma and the plasmodesmata (30, 31), and can cause a significant increase in the molecular size exclusion limits of plasmodesmata (30). However, the movement protein expressed by transgenic tobacco was unable to alter the size exclusion limits of plasmodesmata between BS cells and VP cells (32). Based on this observation and the evidence that the CP of TMV is necessary for phloem-dependent accumulation of this virus, it was suggested that the CP may be the additional viral factor required for TMV to move from BS to VP cells (32, 33).

We have addressed this hypothesis using TMV CP mutants unable to accumulate via the phloem due to an altered or

Abbreviations: BS, bundle sheath; C, companion; CP, coat protein; cpbs, coat protein bodies; DPI, days post-inoculation; ORSV, odontoglossum ringspot virus; SE, sieve element; TMV, tobacco mosaic virus; VP, vascular parenchyma; PB, phosphate buffer.

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absent CP. We also determined the location where a host factor(s) must function to allow efficient phloem-dependent accumulation of a TMV mutant encapsidated with a non-native CP. The accumulation of these mutants in vein cells was monitored through immunocytocchemical studies during periods when rapid phloem-dependent movement would be expected.

**MATERIALS AND METHODS**

**Viruses, Plants, and Virus Inoculation of Plants.** U1-TMV was obtained and purified as described (34). U1mCP15-17, a CP mutant of U1-TMV, was made from an infectious cDNA clone of U1-TMV p35SU1R (refs. 15 and 35) using a slightly modified protocol described in the Altered Sites mutagenesis kit (Promega; ref. 35). The mutagenic primer used was UB-stSac (5'-CCGGCCACCGCGGTGGCAAGAACGAAC-TGAG-3'), which introduces unique BsrXI and SacII sites in the vicinity of nucleotide 5756 of the TMV genome. The CP of U1mCP15-17 was thus altered at positions 15–17 (i.e., from Ser-Ser-Ala to Pro-Pro-Arg) in the CP sequence. SNC015, a CP mutant of U1-TMV containing the CP coding sequence but lacking an initiation codon (12), was produced by *in vitro* transcription of a cDNA clone behind a T7 promoter as described (35). TMV CP-O was produced by exchanging the U1 strain CP gene with the CP gene from odontoglossum ringspot tobamovirus (ORSV; ref. 27).

*Nicotiana tabacum* L. cv. Xanthi nn, a systemic host for TMV, was used in this study. The plants were grown, both prior to and after inoculation with virus, as described (26). At one month after planting, the 5th leaf of each plant was mechanically inoculated with purified TMV CP-O virus (20 µg/ml, 20 µl/leaf) or with total RNA (~4 µg/leaf) isolated from leaves of *Nicotiana benthamiana* infected with mutant viruses or from leaves of *N. tabacum* L. cv. Xanthi nn infected with the U1 strain of TMV (U1-TMV). Total RNA was isolated by phenol extraction and ethanol precipitation. Plants were inoculated with viral RNA as described (35) or with TMV CP-O as described for purified virus (35).

**Antiserum.** Antiserum against TMV was prepared by injecting a rabbit with purified TMV and harvesting blood after the first booster injection. Antiserum against the 126-kDa protein of TMV was obtained from Hal S. Padgett and Roger N. Beachy (Scripps Research Institute, La Jolla, CA). Antiserum against ORSV was obtained from William O. Dawson (University of Florida, Lake Alfred). All antiserum were used without further purification.

**Reverse Transcription-PCRs.** At 10 or 30 days post-inoculation (DPI), the inoculated leaf and stem nodes of plants inoculated with U1mCP15-17 or U1-TMV were harvested individually and stored at −70°C before extraction of RNA. Three plants were harvested for each virus analyzed. Two plants inoculated with 0.1 M sodium phosphate buffer (PB) (pH 7.0) were harvested as controls for each experiment.

Total RNA was isolated from individual samples via phenol extraction and ethanol precipitation. First strand cDNAs were synthesized with Superscript reverse transcriptase (Life Technologies, Gaithersburg, MD) using Primer B (5'-ATGCATCT-TGACTACCCCGTTGAGAGCA-3') complementary to nucleotides 6176–6207 of the TMV genome and the cDNAs then amplified by PCR using the B primer and Joint primer (5'-CGATGATGATTCGGAGGCTACTG-3', which corresponds to nucleotides 5664–5687 of the TMV genome) as described (35). The resulting PCR products were electrophoresed in 1% agarose gels and stained with EtBr.

**Tissue Fixation and Embedding.** Individual chlorotic lesions were randomly sampled from Xanthi nn leaves inoculated with U1mCP15-17 or SNC015 at 3 and 6 DPI, respectively. Xanthi nn leaves inoculated with U1-TMV in PB or PB alone were sampled at the same time as those for the mutant viruses. Sampled tissues were fixed for 3 h as described (36) except

**RESULTS**

**Symptom Induction by U1mCP15-17, SNC015, TMV CP-O, and U1-TMV.** U1mCP15-17 induced chlorotic lesions on in-
occulated Xanthi nn leaves by 3 DPI. On occasion, chlorotic lesions adjacent to major veins expanded slowly along the veins in a fashion similar to that observed for TMV CP mutants studied previously (i.e., TMV mutants PM1 and PM2; ref. 9). SNC015 induced the formation of chlorotic lesions on inoculated leaves of Xanthi nn by 4 DPI. No systemic symptoms were observed in plants inoculated with UlmCP15-17 or SNC015. TMV CP-O induced chlorotic lesions on inoculated Xanthi nn leaves by 3 DPI. At approximately 7 DPI, expanding lesions at the top of the plant began to show mild vein-clearing symptoms followed by a mild mosaic at 12 DPI. U1-TMV induced chlorotic lesions in inoculated leaves of Xanthi nn by 3 DPI and systemic symptoms (e.g., vein-clearing) by 5 DPI. Vein-clearing was followed by the formation of a severe mosaic at 7 DPI. These results suggest that UlmCP15-17 and SNC015 do not move in a phloem-dependent manner and indicate that TMV CP-O is impaired in phloem-dependent movement as first shown by Hilf and Dawson (27).

**Analysis of UlmCP15-17 Infection Over Time.** Inoculated leaves and stem nodes at and above the inoculated leaves were harvested and analyzed individually at 10 DPI for UlmCP15-17 and U1-TMV accumulation to verify that UlmCP15-17 does not move in a phloem-dependent manner. Total RNA was isolated from each sample and subjected to reverse transcription-PCRs to detect viral RNA. UlmCP15-17 RNA was detected only in the inoculated leaves and the attached nodes (i.e., node 0), whereas U1-TMV RNA was present in all the nodes, including those at the shoot apex (Fig. 1). A second experiment was conducted where tissue was sampled at 30 DPI and UlmCP15-17 RNA was detected in the same tissues as determined at 10 DPI and in the first node above node 0 (data not shown). No viral RNA was detected in tissue from mock-inoculated plants (Fig. 1). Biological assays were conducted on extracts from nodal tissue by inoculation onto the hypersensitive host, *N. tabacum* L. cv. Xanthi NN, to determine if the presence of infectious virus positively correlated with the presence of a virus-dependent PCR product. Necrotic lesions were observed on leaves only after inoculation with extract that had yielded a viral-specific PCR product (data not shown). These results indicated that UlmCP15-17 spreads through plant tissue in a manner consistent with cell-to-cell movement and not phloem-dependent movement.

**Immunocytochemical Detection of UlmCP15-17 and TMV CP-O in Inoculated Leaves.** UlmCP15-17 produces a CP, but is defective in virion assembly as determined by Western blot analysis and immunosorbent electron microscopy (not shown). Thin sections of tissue prepared from chlorotic lesions induced by UlmCP15-17 on inoculated leaves revealed numerous dark-staining bodies within cells similar to the coat protein bodies (cpbs) observed for another CP mutant of TMV (i.e., CP 10; ref. 37). As observed for CP 10 in that study, the dark-staining bodies we observed often were associated with X-bodies; cytoplasmic structures containing TMV-encoded 126 kDa- and 183 kDa-proteins known to be associated with viral replication (38–43). Sections of mesophyll cells probed with antisera against TMV showed that the dark-staining bodies contained UlmCP15-17 CP (Fig. 2A). Therefore, the dark-staining bodies we observed appeared to be analogous to the cpbs seen previously (37). When semi-thin sections pre-
pared from U1mCP15-17-infected leaf tissues were subjected to double-sided labeling immunocytochemistry using antisera against TMV and then examined under a light microscope, black spots, representative of cpbs, were seen in the cytoplasm of infected mesophyll cells (data not shown). When 14 infected typical class V veins in 8 sections from 8 different chlorotic lesions were examined under the light microscope, 40% of the VP and 5% of the C cells were found to have immunogold-labeled cpbs. Similar results were obtained when thin sections containing typical class V veins were immunolabeled and examined under the electron microscope (Fig. 2 B and C; Table 1). No immunogold-labeled cpbs were seen in SEs in typical class V veins (Fig. 2 B and C and data not shown). Also no cpbs and no signal were observed in sections from plants inoculated with U1-TMV or PB, respectively (Fig. 2 D and E).

TMV CP-O accumulated in VP cells within class V veins of inoculated leaves (Fig. 3). Immunocytochemical analyses of 8 semi-thin sections containing 12 class V veins showed that by 4 DPI, TMV CP-O had accumulated in 94% of the VP cells and 6% of the C cells.

**Cytological and Immunocytochemical Detection of SNC015 in Inoculated Leaves.** Thin sections prepared from chlorotic lesions induced by SNC015 and U1-TMV at 6 DPI were analyzed by immunocytochemistry using antisera against the TMV 126-kDa protein. Many X-bodies were observed in the cytoplasm of mesophyll cells infected with U1-TMV, and these X-bodies were specifically labeled with gold particles (Fig. 4A). X-bodies in VP cells from tissue infected with SNC015 or U1-TMV were also labeled (Fig. 4 B and C). Examination of typical class V veins infected with SNC015 showed that the virus accumulated in VP cells, but not in C cells unlike U1-TMV (Table 1). No SEs or xylem tracheary elements contained X-bodies or were labeled (Fig. 4 B and C; data not shown). When sections from SNC015-infected plants were incubated with antisera against TMV, no labeling was detected (data not shown).

**DISCUSSION**

Ding et al. (32) found that TMV movement protein could significantly increase the size exclusion limits of plasmodesmata between mesophyll cells and mesophyll and BS cells in mature tobacco leaves, but was unable to modify the size exclusion limits of plasmodesmata between BS and VP cells. This observation, and the fact that TMV CP is required for phloem-dependent accumulation in tobacco, led to a suggestion that TMV CP may be required to allow the transport of TMV across this boundary (5, 32, 33). In this study we have determined that, like U1-TMV, a virion defective mutant (U1mCP15-17) and a coat proteinless mutant of TMV (SNC015) could accumulate in VP cells in inoculated tobacco leaves (Figs. 2 B and C and 4B, Table 1). Therefore, neither the virion nor the CP is required for TMV to enter VP cells, the potential first step in the process of phloem-dependent movement.

We have recently suggested that a viral factor other than the CP potentiates TMV movement into VP cells (26). We determined that a strain of TMV (M1C-TMV), whose attenuated phenotype maps to the 126-kDa protein open reading frame (34, 35, 44), was deficient in its ability to accumulate in VP and C cells within the inoculated leaf. The CP of M1C-TMV cannot be involved in the lack of phloem-dependent accumulation because the CP sequence of this virus and its accumulation in inoculated leaves were identical to that of U1-TMV (34, 44). Other researchers (45–47) have also determined that, for several plants viruses, sequences analogous to the TMV 126-kDa protein open reading frame have an influence on cell-to-cell and phloem-dependent accumulation. de Zeeuw and Gaard (48), studying pea enation mosaic virus, found that a vesicular material, containing RNA-dependent RNA polymerase and virus-specific double-stranded RNA, is likely transported from VP or C cells to SEs. Therefore, the involvement of virally encoded proteins other than the CP in phloem-dependent movement has support and requires further study.

Host factors also have a role in the phloem-dependent movement of viruses (reviewed in refs. 5 and 7). Several studies have demonstrated that although some TMV CP mutants could produce virion-like particles they were still defective in phloem-dependent accumulation in plants (11, 49). Hilf and Dawson (27) replaced the native CP of U1-TMV with the CP from ORSV, and found that the chimeric virus (TMV CP-O) produced nuclease-resistant particles, but the virus moved very poorly from leaf to leaf in tobacco. They suggested that the virion produced by the chimeric virus was unable to interact with host factors to allow efficient phloem-dependent accumulation. In our study, using the same chimeric virus, we determined that the virus could accumulate in the VP cells of minor veins in inoculated leaves. Therefore, if the hypothesis of Hilf and Dawson (27) is correct, the host factor(s) required for this chimeric virus to efficiently accumulate systemically function at the C cell interface with other cells, within the C cells, or beyond.

Numerous studies have shown that the transport of photosynthetic in plants encounters a specialized barrier between the VP cells and the C cell/SE complexes, and this barrier allows the maintenance of extremely different osmotic and electrical potentials in the VP cell and the complex (for review see ref. 46). In this study the VP cells of SMV-infected leaves were shown to be hypertonic (46). Furthermore, in order to prevent the accumulation of too much virus in the inoculated leaf, the inoculum concentration of TMV was chosen to be low enough to avoid necrosis in the inoculated leaf (Fig. 1). Once this criterion was met, the inoculation procedure was shown to be safe for the plant host and did not cause lesions in any of the leaves examined in this study (Fig. 1).

**Table 1.** Percentage of cells infected for three cell types around and within typical class V veins of tobacco leaves inoculated with CP mutants of U1-TMV or U1-TMV

<table>
<thead>
<tr>
<th>Virus</th>
<th>Exp.</th>
<th>No. of veins analyzed</th>
<th>Percentage of cells infected</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>BS</td>
</tr>
<tr>
<td>U1mCP15-17</td>
<td>1</td>
<td>35 (18)*</td>
<td>78</td>
</tr>
<tr>
<td>U1-TMV</td>
<td>2</td>
<td>2 (2)</td>
<td>100</td>
</tr>
<tr>
<td>SNC015</td>
<td>2</td>
<td>17 (9)</td>
<td>27</td>
</tr>
<tr>
<td>U1-TMV</td>
<td>6</td>
<td>6 (4)</td>
<td>100</td>
</tr>
</tbody>
</table>

Xanthi nn plants were inoculated in separate experiments with total RNA (4 µg/ml) containing U1mCP15-17, SNC015, or U1-TMV. At 3 DPI (U1mCP15-17, experiment 1) or 6 DPI (SNC015, experiment 2), tissue containing chlorotic lesions were randomly sampled and analyzed, respectively, for TMV CP or 126-kDa protein accumulation by immunocytochemistry and electron microscopy. For each experiment, sections from Xanthi nn plants inoculated with U1-TMV were analyzed for comparison. BS, Bundle sheath cell; VP, vascular parenchyma cell; C, companion cell.

*Numbers in parentheses equal total number of lesions analyzed.
meric virus are required for invasion of C cells. However, the ratios of VP to C cells infected with U1mCP15-17 and TMV CP-O (8 to 40:1 and 16:1) were greater than those observed in this study and previously for U1-TMV at a similar stage of infection (4 to 5:1, ref. 26 and Table 1). Also, we have not observed any C cells infected with the CP-less mutant, SNC015, although in theory we should have if the ratio of VP to C cell infection for this virus is similar to that of U1-TMV (Table 1). The results suggest that a native encapsidation-competent CP of TMV may aid in the efficient transport of the virus into the C cell/SE complexes, although it is not essential. The results also suggest that a CP is necessary to allow virus movement into the C cell/SE complex; however, the lack of signal in C cells of tissues infected by SNC015 may be due to an insufficient number of samples analyzed or a lack of sensitivity in the detection method employed. Both U1mCP15-17 and SNC015 accumulated in fewer BS or vascular bundle cells within chlorotic lesions than the parental U1-TMV (Table 1). Free RNA viruses are known to accumulate to only low steady-state levels and this trait has been attributed to the instability of the unprotected RNA (10, 13, 51, 52). It has been suggested that a general impairment of cell-to-cell movement and accumulation of virus may disproportionately inhibit phloem-dependent accumulation (7). Mise and Ahlquist (53) found that a movement protein mutant of cowpea chlorotic mottle bromovirus capable of cell-to-cell spread stops moving and accumulating before it reaches a novel cell boundary, i.e., its cell-to-cell movement is confined to epidermal cells through which the corresponding wild-type virus moves unimpeded. Also, as noted, M10C-TMV accumulates in fewer vascular cells than U1-TMV and this characteristic correlates with the impaired systemic accumulation of this virus (26, 34). Whether the general decrease in infection observed for U1mCP15-17 and SNC015 contributes to their lack of phloem-dependent accumulation remains to be determined.

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**Fig. 4.** Immunodetection of 126/183-kDa protein in vein cells of *N. tabacum* cv. Xanthi nn leaves inoculated with a coat proteinless TMV mutant, SNC015, or the parental U1-TMV. Sections prepared from chlorotic lesions harvested at 6 DPI were labeled using an antiserum against the TMV 126-kDa protein followed by a goat anti-rabbit IgG gold (20 nm) conjugate. (A) An immunogold-labeled X-body adjacent to a virus aggregate in an infected mesophyll cell. X, X-body; R, virus aggregate. (×30,000; bar = 0.3 μm.) (B) X-bodies (denoted by arrowheads) containing 126/183-kDa protein detected in two VP cells of a class V vein from a section infected with SNC015. *Inset* (indicated by arrow) is a magnification of the boxed area within the vein and shows the deposition of gold particles over an X-body. C, companion cell; VP, vascular parenchyma cell, XT, xylem tracheary element. (C) An X-body containing 126/183-kDa protein detected in a VP cell within a class V vein from a section infected with U1-TMV. *Inset* (indicated by arrow) is a magnification of the boxed area within the vein and shows the deposition of gold particles over an X-body. (Veins, ×1,300; bars = 4 μm; *Insets*, ×25,000; bars = 0.4 μm.)

50. We have shown that transport of TMV between these cell types is also controlled, because U1-TMV and M10C-TMV accumulate in more VP cells than C cells in inoculated tobacco leaves both early and late after infection (Table 1; ref. 26). In this study we have shown that neither a virion nor host factors essential for phloem-dependent movement of a specific chi-