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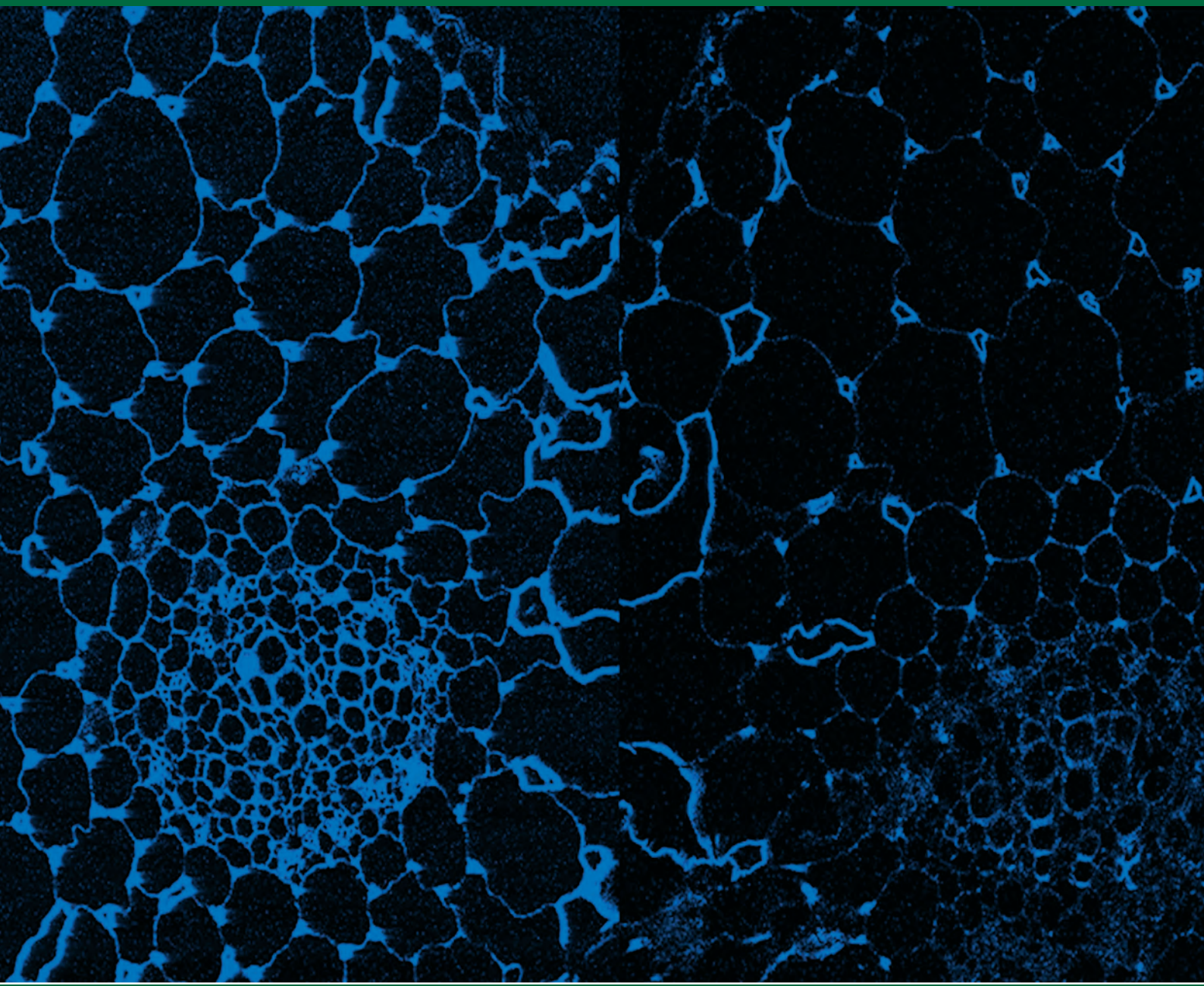

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Systemic movement of a tobamovirus requires host cell pectin methylesterase

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Summary

Systemic movement of plant viruses through the host vasculature, one of the central events of the infection process, is essential for maximal viral accumulation and development of disease symptoms. The host plant proteins involved in this transport, however, remain unknown. Here, we examined whether or not pectin methylesterase (PME), one of the few cellular proteins known to be involved in local, cell-to-cell movement of tobacco mosaic virus (TMV), is also required for the systemic spread of viral infection through the plant vascular system. In a reverse genetics approach, PME levels were reduced in tobacco plants using antisense suppression. The resulting PME antisense plants displayed a significant degree of PME suppression in their vascular tissues but retained the wild-type pattern of phloem loading and unloading of a fluorescent solute. Systemic transport of TMV in these plants, however, was substantially delayed as compared to the wild-type tobacco, suggesting a role for PME in TMV systemic infection. Our analysis of virus distribution in the PME antisense plants suggested that TMV systemic movement may be a polar process in which the virions enter and exit the vascular system by two different mechanisms, and it is the viral exit out of the vascular system that involves PME.

Keywords: tobamovirus, pectin methylesterase, systematic movement, antisense suppression, tobacco.

Introduction

Tobacco mosaic virus (TMV), one of the best studied plant viruses, begins its infection by moving locally between cells of the initially inoculated non-vascular tissues of the host plant. When the spreading virions reach the vascular system, they enter it and are systemically transported throughout the plant. While the mechanism of the local, cell-to-cell movement of TMV has been extensively studied, the molecular requirements for its systemic transport remain relatively unexplored. It is this systemic movement of plant viruses through the host vasculature, however, that is essential for maximal viral accumulation and development of disease symptoms (Hull, 1991). Systemic movement of most plant viruses, including TMV, occurs through the phloem (Hull, 1991) and follows the flow of photoassimilates from source to sink organs (Leisner and Turgeon, 1993). This phloem-dependent viral movement requires that virions invade the vein cells from the adjacent non-vascular cells of the inoculated leaf, spread throughout the phloem system, and exit it within systemic organs to infect their non-vascular tissues. The host plant proteins required for these events of the systemic transport process have not been identified. We addressed this question by examining the role of pectin methylesterase (PME) in TMV systemic infection.

Previously, PME was shown to interact with TMV movement protein (MP; Chen *et al.*, 2000; Dorokhov *et al.*, 1999), which is responsible for the cell-to-cell movement of the virus (reviewed in Heinlein, 2002; Lucas and Gilbertson, 1994). Inhibition of this interaction using a mutant MP unable to bind PME blocked the local spread of the virus (Chen *et al.*, 2000). It remained unknown, however, whether or not PME is also involved in the systemic movement of TMV. Here, we utilized a reverse genetics approach, in which PME expression was inhibited by antisense suppression, to obtain direct support for PME involvement in viral systemic spread. Our data suggest that reduced levels of PME in the plant vasculature compromise TMV egress from the infected vasculature, resulting in a significant delay in TMV systemic infection.

Results

Pectin methylesterase (PME) antisense plants

To directly study the biological role of PME in TMV infection *in planta*, we generated transgenic tobacco plants constitutively expressing the tobacco PME cDNA in the antisense orientation. A total of 40 independently transformed antisense lines were produced, two of which, designated PME AS8 and PME AS36, were characterized in detail. First, PME expression was analyzed using confocal immunofluorescence microscopy. Figure 1 shows immunostaining of wild-type and PME AS8 tobacco leaves with anti-PME antibodies followed by an inodicarbocyanine (Cy5)-conjugated secondary antibody. Cy5 is excited near 650 nm and fluoresces near 670 nm, thus substantially circumventing the autofluorescence usually associated with the plant cell cytoplasm. In confocal optical sections of the wild-type leaves, PME was found within vascular bundles as well as in the surrounding non-vascular tissues (Figure 1a,b). The immunofluorescence staining was PME-specific because it was not detected in leaf samples probed with pre-immune serum (Figure 1c,d). In the PME AS8 leaves, however, PME expression differed between the vascular bundles and the non-vascular cells (Figure 1e,f). Whereas the amounts of PME expressed in non-vascular tissues were not significantly reduced as compared to the same tissues in the wild-type leaves, only low levels of PME were detected in the vascular bundles of the PME AS8 leaves. Similar reduced levels of PME expression within the vasculature but not in the non-vascular tissues were detected in the PME AS36 line (data not shown).

That antisense suppression of PME was most prominent in the plant vasculature was confirmed using the quantitative PME activity assay (Downie *et al.*, 1998). Figure 2 shows that, in the wild-type leaves, their classes I and II veins exhibited high PME activity, whereas, in the primarily non-vascular areas of the leaf blade, this enzymatic activity was relatively low. Both PME AS8 and PME AS36 lines possessed dramatically lower levels of the PME activity in the classes I and II veins; specifically, the PME activity was reduced in the class I veins to 13 and 23% and in the class II veins to 31 and 47% of the wild-type level, for each antisense line, respectively. The PME enzymatic activity in the non-vascular areas of the leaf blades from both the AS8 and AS36 lines, however, was only slightly lower than that detected in the wild-type plants (Figure 2). Collectively, these results suggest that, in our PME antisense plants, PME suppression occurred preferentially within the vascular tissues.

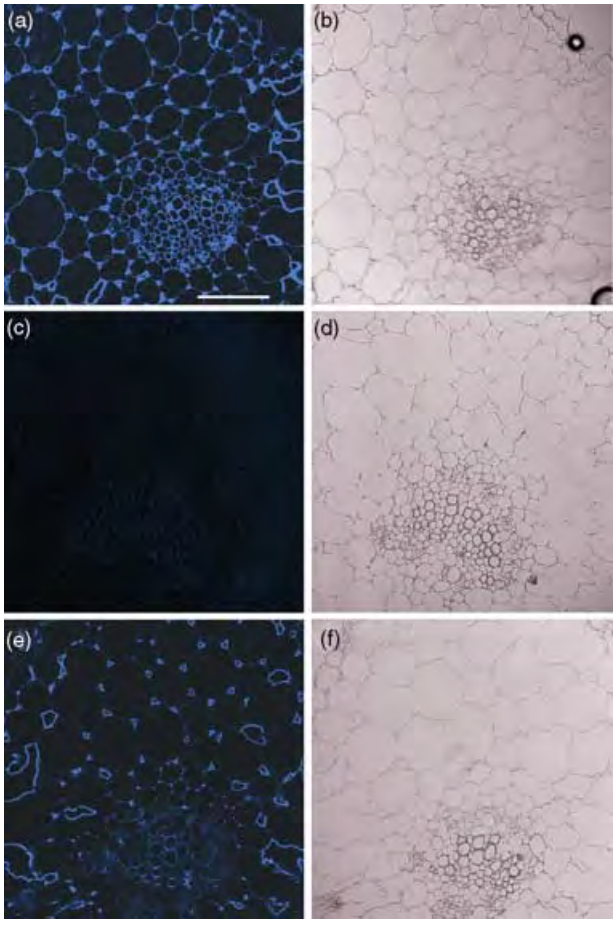

Figure 1. PME expression in leaves of the wild-type and PME antisense plants.
(a, b) Leaf sections of wild-type plants probed with anti-PME antibody.
(c, d) Leaf sections of wild-type plants probed with pre-immune antibody.
(e, f) Leaf sections of PME AS8 plants probed with anti-PME antibody.
Panels a, c, and e represent Cy5 confocal fluorescence images, and panels b, d, and f represent phase contrast images. Note that vascular tissue is seen as smaller cells arranged in bundles whereas non-vascular tissue is represented by larger cells surrounding the vascular bundles. Bar = 100 μm.

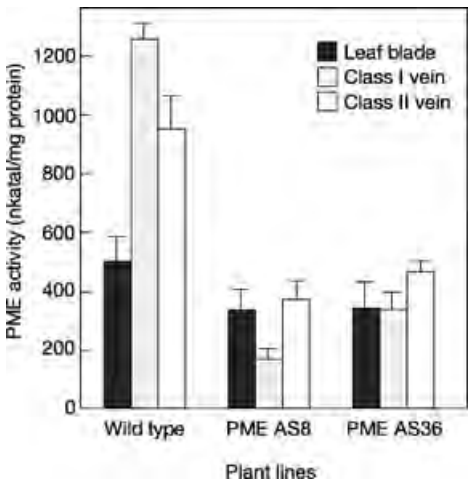

Figure 2. PME enzymatic activity in vascular and non-vascular leaf tissues of the wild-type and PME antisense plants.
Black, gray, and white bars indicate PME activity in the leaf blade, and class I and class II vein portions of the leaf, respectively. All data represent average values of three independent measurements with indicated standard deviation values.

Effects of antisense suppression of PME on viral systemic movement

Next, the PME antisense plants were tested for their ability to support TMV infection. Because these plants exhibited reduced PME levels mainly within their vascular tissues, we hypothesized that the main effect of this PME suppression would be on the viral systemic transport, which occurs through the plant vasculature (reviewed in Heinlein, 2002; Lucas and Gilbertson, 1994). Thus, wild-type and PME AS8 and PME AS36 plants were inoculated with TMV, and viral systemic movement was assessed by detection of TMV CP, a known hallmark of tobamoviral infection (Lartey *et al.*, 1997), within uninoculated, systemic leaves (Citovsky *et al.*, 1998; Ghoshroy *et al.*, 1998). CP was identified by Western blot analysis using anti-TMV CP antibodies (Figure 3). To better analyze TMV systemic movement, the kinetic studies were performed, in which the viral presence was assayed on a daily basis following inoculation; each sample was harvested from the third and/or fourth top leaf at the day of the sampling.

Figure 3 shows that the wild-type plants efficiently supported systemic movement of TMV, allowing the entire process of systemic infection to be accomplished within 7–8 days after inoculation. This kinetics of TMV systemic spread in tobacco is consistent with that reported for systemic movement of another tobamovirus, TVCV, in *Arabidopsis* (Lartey *et al.*, 1997). In contrast, by the time when the TMV systemic spread in the wild-type plants had already been completed, the PME AS8 and PME AS36 lines still showed no CP in their uninoculated leaves (Figure 3). Only 17–18 days after inoculation, the AS36 plants showed the first signs of the systemic infection whereas the AS8 plants first exhibited the viral CP in their systemic leaves 24–25 days after inoculation (Figure 3). Thus, in the PME antisense plants, TMV systemic movement occurred 5–12 times slower than that in the wild-type plants, in which the systemic appearance of TMV CP was first detected 2–3 days after inoculation with the virus. Once the systemic movement in the PME plants had begun, however, its rate was comparable to that of the wild-type plants (see slopes of the kinetics curves in Figure 3), requiring about 5 days to reach saturation at the wild-type CP levels. These results implicate PME as a cellular factor that is, at least partly, required for development of TMV systemic infection.

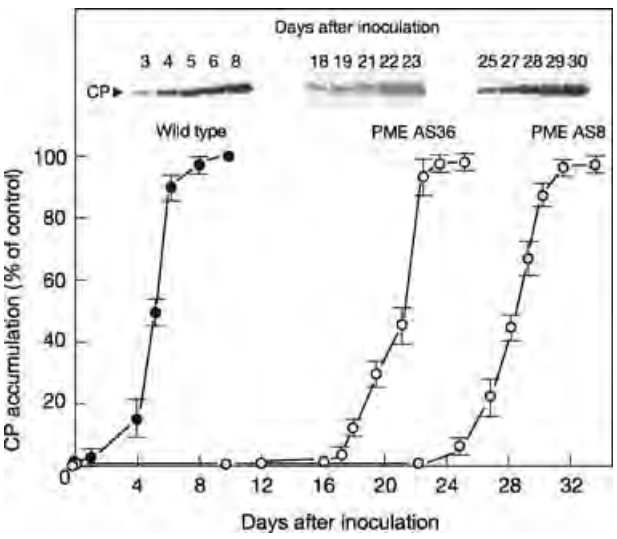

Figure 3. Delayed systemic movement of TMV in PME antisense plants. At the indicated periods after inoculation, CP levels were determined in uninoculated, systemic leaves of TMV-infected wild-type plants (black circles) and in PME AS8 and PME AS36 lines (gray and white circles, respectively). CP accumulation is expressed as percent of the maximal amount of CP within uninoculated leaves of the wild-type control plants. All data represent average values of four independent measurements with indicated standard deviation values. Insets demonstrate representative results of the Western blot analysis of CP content in each plant line.

It was then important to examine whether or not the delays in TMV systemic movement in the PME antisense tobacco were simply because of non-specific repression of phloem loading and/or unloading in these plants. To this end, we utilized 6(5)-carboxyflyorescein (CF) diacetate, the impermeant CF moiety known to be translocated from source to sink leaves through the phloem; this translocation is followed by CF unloading into the sink tissues (Roberts *et al.*, 1997). Importantly, the developmental stage and the position on plant of the leaves sampled for CF phloem loading and unloading were identical to those of the leaves analyzed for the viral movement (see above). When the vasculature of mature source leaves of the control, wild-type plants was loaded with CF, the dye was translocated through the phloem to the upper, still developing leaves, resulting in labeling of classes I–III veins (Figure 4a). This CF vascular translocation was detected within 30–90 min, consistent with the properties of the phloem transport rather than the much faster (<3 min) transport through the xylem (Roberts *et al.*, 1997). Also consistent with the previous observations (Roberts *et al.*, 1997), in CF-importing leaves, CF was unloaded from the veins to the non-vascular, mesophyll tissues; this unloading was visualized as 'bleeding' of the dye out of the leaf veins (Figure 4b). When these phloem labeling assays were performed on PME AS8 line, they detected no significant differences in the rate or extent of CF loading, transport, and unloading (Figure 4c,d). Thus, the reduction in PME expression within the vascular tissues of our PME antisense plants most likely did not detectibly affect phloem transport of solutes.

Tobacco mosaic virus (TMV) systemic movement arrests in vascular tissues of PME antisense plants

The delayed systemic movement of TMV in PME antisense plants may be because of the inability of the virions to

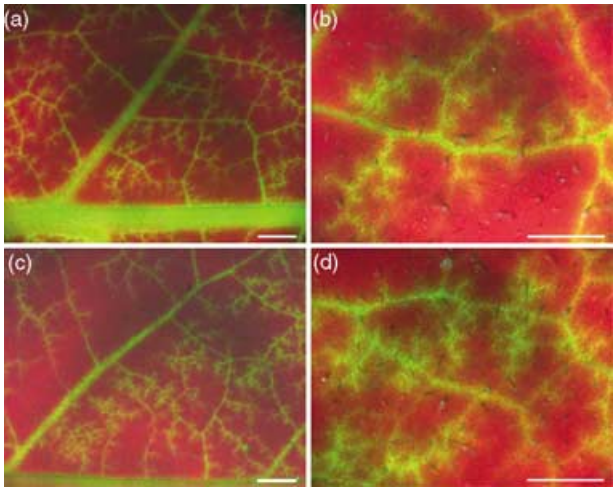

Figure 4. Phloem unloading of the fluorescent dye CF in intact sink leaves of the wild-type and PME antisense plants. The veinal classes I–III network is labeled with CF.
(a) CF-importing leaf of a wild-type tobacco plant.
(b) Enlarged region of the leaf shown in (a).
(c) CF-importing leaf of the PME AS8 line.
(d) Enlarged region of the leaf shown in (c).
CF unloading is indicated by dye contained in several adjoining mesophyll cells around the veins in panels b and d. Bars = 2.5 mm.

either enter the plant vasculature from the inoculated tissue or to exit the vascular system into uninoculated tissues. Immunofluorescence confocal microscopy was used to distinguish between these two possibilities and, thus, to identify the target tissue of the inhibitory effect of PME suppression. Figure 5 shows the representative results of an experiment in which five wild-type plants and five PME AS8 plants were inoculated with TMV. Cross-sections were prepared from the inoculated and uninoculated leaves of these plants at two locations: predominantly non-vascular tissues of the leaf blade and predominantly vascular tissues of the petiole area of the same leaf. The sections were stained with anti-TMV antibody followed by a Cy5-conjugated secondary antibody.

In TMV-infected wild-type plants, the virus was found both in non-vascular tissues (Figure 5a) and in vascular bundles of the petioles of the inoculated leaves (Figure 5b). Similarly, TMV was detected within vascular and non-vascular tissues of the systemic, uninoculated

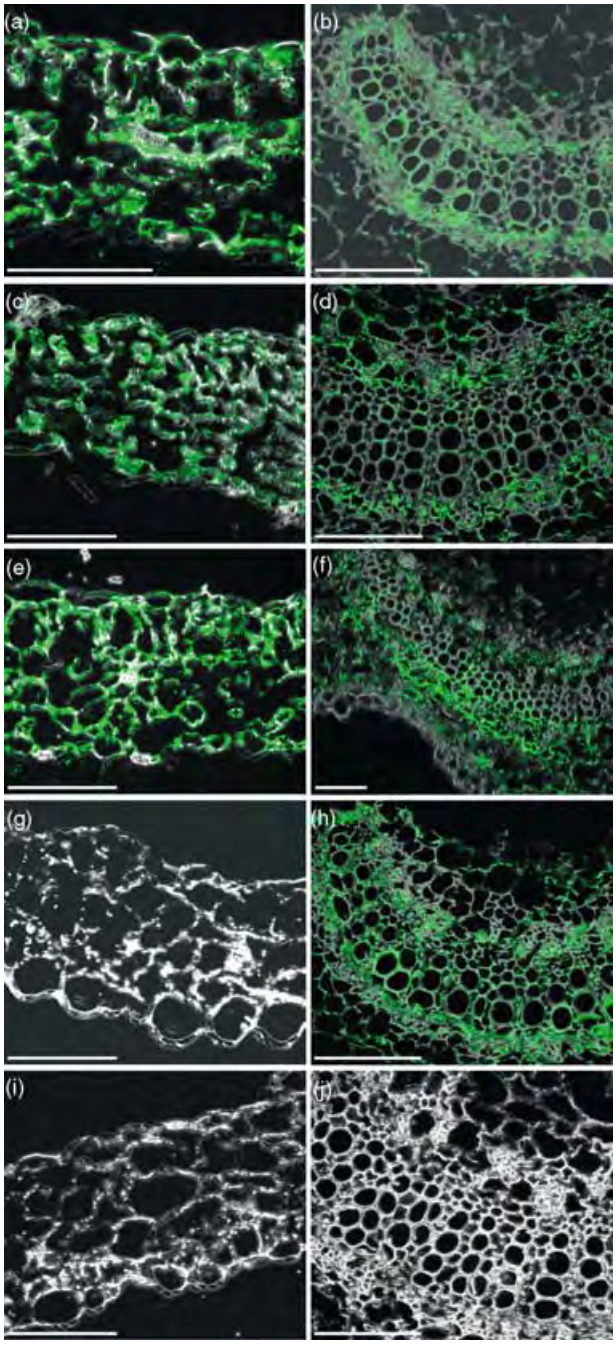

Figure 5. TMV accumulation in vascular tissues of uninoculated leaves of PME antisense plants. Plant samples were harvested 3 weeks after inoculation and analyzed by confocal immunofluorescence microscopy using anti-TMV antibodies.
(a) Section through non-vascular tissues of an inoculated leaf from a wild-type plant.
(b) Section through the petiole of the same leaf.
(c) Section through non-vascular tissues of an uninoculated leaf from a wild-type plant.
(d) Section through the petiole of the same leaf.
(e) Section through non-vascular tissues of an inoculated leaf from a PME AS8 plant.
(f) Section through the petiole of the same leaf.
(g) Section through non-vascular tissues of an uninoculated leaf from a PME AS8 plant.
(h) Section through the petiole of the same leaf.
(i) Section through non-vascular tissues of a leaf from an uninfected, control PME AS8 plant.
(j) Section through the petiole of the same leaf.
All panels represent the Cy5 immunofluorescent images (detecting TMV virions and shown in false green color) superimposed on phase images of the same tissues (shown in gray scale). Bars = 100 μm. Each panel is a representative image of five independent PME AS8 plants analyzed in this experiment.

leaves of these plants (Figure 5c,d), consistent with the efficient systemic movement of the virus in the wild-type tobacco (see Figure 2). In PME AS8 plants, TMV virions accumulated within non-vascular and vascular tissues of the inoculated leaves and their petioles (Figure 5e,f) at the levels comparable to those observed in the wild-type plants (Figure 5a,b). In contrast, the systemic, uninoculated leaves of PME AS8 plants accumulated the virus only within their vascular petiole tissues but not in the non-vascular tissues of the leaf blade (Figure 5g,h, respectively). As expected, control, uninoculated wild-type (data not shown) and PME antisense plants did not show TMV-specific fluorescent signal in any of their tissues (Figure 5i,j). In addition, no signal was observed in all plant tissues with pre-immune antibody (data not shown). These results, i.e. vascular arrest of TMV, were observed in all PME AS8 plants tested. Thus, the reduced expression of PME in the PME antisense plants likely delayed the TMV egress from the vascular system, and had little or no effect on viral entry into the plant vasculature. However, as mentioned above (see Figure 3), once the delayed viral systemic movement had begun (4–5 weeks after inoculation), the viral distribution between the vascular and non-vascular cells in the PME AS8 plants was essentially identical to that observed in the wild-type plants (data not shown).

Discussion

Pectin methylesterase (PME), an enzyme present in all plant tissues and species, is considered to play a multifunctional role in cell wall metabolism and participate in plant growth and development (Li *et al.*, 2002; Wen *et al.*, 1999). Thus, global antisense suppression of PME may not result in a viable phenotype; indeed, previously, inhibition of PME expression has been achieved mainly in an organ-specific fashion (Gaffe *et al.*, 1997). Similarly, in our PME antisense plants that were able to regenerate following transformation, PME suppression did not occur equally throughout the plant but was most pronounced in the vascular tissues. Furthermore, even within the vasculature, the suppression was not complete; for example, the class I vein portions of the antisense leaves retained 10–20% of the PME expression as compared to the wild-type plants.

That PME suppression took place mainly in the vascular system of the antisense plants allowed us to specifically examine the role of PME in TMV systemic movement, which also occurs via the vasculature. Our data indicate that the reduced levels of PME resulted in a dramatic delay, by up to an order of magnitude, in viral systemic spread. That this movement was significantly retarded but not blocked is consistent with the substantial but incomplete antisense suppression of PME. Non-invasive imaging of CF demonstrated that its phloem transport in PME antisense plants was not compromised. Thus, the observed delays in TMV systemic infection are probably not because of non-specific effects of the reduced levels of PME on the normal phloem loading and unloading of solutes.

Interestingly, only the onset of the systemic movement was affected. Once the viral presence was detected in the uninoculated leaves, the systemic infection proceeded rapidly, at the same rate as that in the wild-type plants. Immunofluorescence analysis of TMV accumulation in the inoculated and uninoculated leaves indicated that the virus moved within the inoculated leaf, entered into and spread through the host vasculature but was unable to efficiently exit into the non-vascular tissues (e.g. mesophyll and epidermis) of the systemic leaves, resulting in the delay of the systemic infection. Viral systemic movement may therefore be a polar process in which the virions enter and exit the vascular system by two different mechanisms, and it is the viral exit out of the vascular system that requires the presence of PME within the host cells. Similar inhibition of viral movement out of but not into the plant vasculature was observed using non-toxic concentrations of cadmium and over-expression of a cadmium-induced glycine-rich protein (cdiGRP; Citovsky *et al.*, 1998; Ghoshroy *et al.*, 1998; Ueki and Citovsky, 2002). The differences in viral entry into and exit out of the host phloem were also evident from the previous observations of functional equivalence of different vein classes for TMV entry and their lack of equivalence for TMV exit (Cheng *et al.*, 2000). Furthermore, transport of numerous cellular proteins in the plant vasculature often is also polar; specifically, proteins synthesized within the companion cells move into the sieve elements but fail to be transported into the surrounding phloem parenchyma, bundle sheath, or mesophyll cells (reviewed in Lucas and Gilbertson, 1994). Collectively, these observations suggest that macromolecular transport into the vasculature may be more promiscuous, e.g. occurring by diffusion or by a loosely regulated process, whereas transport out of the vasculature may be selective and/or tightly regulated.

Because our PME antisense plants did not display significant suppression of PME in non-vascular tissues, we were unable to directly measure the effect of the lack of PME on TMV cell-to-cell movement. Our previous results, however, indicate that the interaction between PME and the TMV MP is required for this movement (Chen *et al.*, 2000). Thus, it is tempting to speculate that the TMV MP–PME interaction may at least in part underlie the requirement for PME for the viral systemic spread. Indeed, whereas the direct role of MP in the systemic movement has not been demonstrated directly, it has been previously suggested that MP may function in concert with CP during the long distance movement of TMV (Ding *et al.*, 1992; Lucas and Gilbertson, 1994).

Experimental procedures

Generation of PME antisense plants

Tobacco PME cDNA (a kind gift from Drs Atabekov and Dorokhov, Moscow State University, Russia) was first inserted in reverse orientation as a PCR-amplified *Eco*RI fragment into a plant expression vector, pCd, containing the 35S promoter of cauliflower mosaic virus, tobacco mosaic virus translational enhancer (Gallie *et al.*, 1989), and the nopaline synthase polyA signal. Then, the entire antisense expression cassette was subcloned as a *BamH*I–*Xba*I fragment into the binary vector pBIN19 (Frisch *et al.*, 1995), carrying a kanamycin selection marker, and introduced into the disarmed *Agrobacterium* strain C1C58, which was then used to transform tobacco plants (*Nicotiana tabacum* cv. Turk) as described by Horsch *et al.* (1985). The resulting transgenic plants were selected on a kanamycin-containing medium and maintained for 1 month under sterile conditions on an MS basal medium (Murashige and Skoog, 1962) with no exogenous growth regulators. Plants were then transferred to soil in a greenhouse, allowed to set seed, and the transgenic progeny were selected by germinating the seeds on MS agar in the presence of kanamycin and then maintained on soil. All PCR reactions were performed using a high-fidelity proofreading Pfu DNA polymerase (Promega Corp., Madison, WI, USA), and their products were verified by dideoxynucleotide sequencing (Kraft *et al.*, 1988).

Pectin methylesterase (PME) enzymatic assay

Tobacco leaves were surgically dissected to isolate their class I veins (mid-ribs) and class II veins (Roberts *et al.*, 1997), and the remaining parts of the leaf blade as described by Cheng *et al.* (2000). PME activity in these tissue samples was quantified by a gel diffusion assay as described by Bourgault and Bewley (2002) and Downie *et al.* (1998). Briefly, the tissue samples were flash-frozen in liquid nitrogen and homogenized in 1 ml of extraction buffer (1 M NaCl, 2.5 mM phenylmethylsulfonyl fluoride (PMSF), 10 μM leupeptin, 0.1 M citrate/0.2 M sodium phosphate, dibasic, pH 7.0). The homogenate was centrifuged at 16 000 ***g*** at 4°C, and the protein concentration of the recovered supernatant was determined by the Bradford method (Ausubel *et al.*, 1987) and adjusted to the same value for all samples. These cell extracts (10 μl) were then loaded into 2-mm round wells in a 2% (w/v) agarose gel containing 0.1% of 90% esterified pectin (Sigma-Aldrich, St. Louis, MO, USA) and 0.5 mg ml^{-1} EIA-grade gelatin (Bio-Rad Life Science, Hercules, CA, USA) in a Petri dish (pectin served as PME substrate and gelatin was used to further stabilize the tested cell extracts (Bourgault and Bewley, 2002; Downie *et al.*, 1998)). The gels were incubated for 16 h at 30°C, rinsed with water, stained for 45 min at room temperature with 0.05% (w/v) ruthenium red (RR) dye (Sigma), which stains de-esterified pectin (Downie *et al.*, 1998), and the diameter of each stained zone was measured to the nearest 0.1 mm with calipers. The amount of PME activity in nanokatals (nkatal) was calculated based on the standard curve of log-transformed enzyme activity versus stained zone diameter generated using a commercial-grade orange peel PME (Sigma).

Preparation of virus, inoculation of plants, and CP assay

Tobacco mosaic virus (TMV) was purified as described for turnip vein clearing virus (Lartey *et al.*, 1993). For one experiment, we utilized three to five plants per line, and from each plant, three to four samples were taken per treatment; each experiment was repeated at least three times. TMV was mechanically inoculated onto one mature, lower (source) leaf of each plant by rubbing 20 μl of TMV suspension (5 μg ml^{-1} of viral protein) on each leaf. Viral infection was monitored using the TMV CP assay. To this end, uninoculated, upper leaves were harvested; usually, these leaves were located on the same side of the plant as the inoculated leaf three to four leaves from the top of the plant and three to four leaves from the inoculated source leaf. Tissue samples (0.2 g FW) were collected, extracted, and analyzed for the presence of CP by SDS–polyacrylamide gel electrophoresis (PAGE) as described by Citovsky *et al.* (1998) and Ghoshroy *et al.* (1998) followed by Western blot analysis using anti-TMV CP antibodies and the ECL Western blotting kit (Amersham BioSciences, Piscataway, NJ, USA). Although our data (see Figure 3) show only those blot areas that correspond to the CP band, no other, non-specific bands were detected in these analyses (data not shown). The amounts of CP were quantified by scanning densitometry (Bio-Rad model GS-670, Bio-Rad Life Science) of the Western blot bands.

Vascular transport

The procedures were performed as described by Roberts *et al.* (1997). Briefly, for phloem loading, and unloading the adaxial surface of a mature source leaf was gently abraded with fine sandpaper, and 20 μl of CF diacetate (50 μg ml^{-1}) was applied to the abraded area, which was then covered with thin polythene film to evenly spread the dye across the leaf surface and to minimize evaporation. Every 30 min, phloem unloading was examined in the upper, developing leaves using a Leica MZ FLIII stereoscope equipped for epifluorescence; this imaging was non-invasive, utilizing intact leaves. For this examination of CF unloading, we chose the leaves of the same developmental stage and location on the plant as those used for assaying the systemic movement of TMV.

Confocal immunofluorescence microscopy

Antibodies were produced in rabbits against purified TMV CP and against purified tobacco PME as described by Chen *et al.* (2000). Pre-immune serum was used in all immunolocalization experiments as negative control. For immunofluorescence microscopy, tissue samples (0.5–1.0 cm) were harvested, fixed with a mixture of formaldehyde (4%) and glutaraldehyde (0.5%) in a sodium phosphate buffer, pH 7.2, and dehydrated in a series of diluted ethanol. The samples were then embedded in Paraplast Plusô (Fisher Scientific, Hampton, NJ, USA), sectioned (8 μm), and reacted with polyclonal anti-PME antiserum or anti-TMV CP antibodies as described by Citovsky *et al.* (1998). The sections were then stained with Cy5-conjugated secondary antibody (Jackson ImmunoResearch, Inc., Westgrove, PA, USA) and examined using a Nikon Diaphot inverted microscope with a Bio-Rad MRC 600 krypton/argon laser scanning confocal attachment.

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