Transgenic expression of pectin methylesterase inhibitors limits tobamovirus spread in tobacco and Arabidopsis

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SUMMARY

Plant infection by a virus is a complex process influenced by virus-encoded factors and host components which support replication and movement. Critical factors for a successful tobamovirus infection are the viral movement protein (MP) and the host pectin methylesterase (PME), an important plant counterpart that cooperates with MP to sustain viral spread. The activity of PME is modulated by endogenous protein inhibitors (pectin methylesterase inhibitors, PMEIs). PMEIs are targeted to the extracellular matrix and typically inhibit plant PMEs by forming a specific and stable stoichiometric 1:1 complex. PMEIs counteract the action of plant PMEs and therefore may affect plant susceptibility to virus. To test this hypothesis, we overexpressed genes encoding two well-characterized PMEIs in tobacco and Arabidopsis plants. Here, we report that, in tobacco plants constitutively expressing a PMEI from *Actinidia chinensis* (AcPMEI), systemic movement of *Tobacco mosaic virus* (TMV) is limited and viral symptoms are reduced. A delayed movement of *Turnip vein clearing virus* (TVCV) and a reduced susceptibility to the virus were also observed in Arabidopsis plants overexpressing AtPMEI-2. Our results provide evidence that PMEIs are able to limit tobamovirus movement and to reduce plant susceptibility to the virus.

INTRODUCTION

Viruses, as obligate parasites, have acquired the ability to utilize host factors to accumulate and diffuse in plants. The initial entry of viruses into the plant tissue occurs through mechanically damaged walls of leaf hairs and epidermal cells, as well as wounds made by insects, mites or nematodes. Thereafter, viruses accumulate and spread into the host epidermis and mesophyll cells through plasmodesmata (PD), and move systemically through the vascular system to reach noninoculated distal tissues where they continue cell-to-cell spread. Both cell-to-cell and systemic movement are processes that require interaction between viral and host components (Niehl and Heinlein, 2011). To favour infection, plant viruses

induce extensive host cell remodelling, such as the modification of PD structure (Laliberté *et al.*, 2013). For example, they have the capability to transiently increase the size exclusion limit (SEL) of PDs using virus-encoded movement proteins (MPs), which allows viral passage into adjacent cells (Carrington *et al.*, 1996; Ghoshroy *et al.*, 1997; Lazarowitz and Beachy, 1999). MPs can also form ribonucleoprotein complexes with the viral genome to allow the intracellular and intercellular transport of the virus through PDs (Kehr and Buhtz, 2008; Schoelz *et al.*, 2011).

Tobacco mosaic virus (TMV), the most important member of tobamovirus, causes serious losses in the yield and quality of several economically important crops, including tobacco and tomato (Scholthof *et al.*, 2011). Multiple interactions of TMV with host components contribute to the viral infection cycle (Liu and Nelson, 2013; Pallas and Garcia, 2011). TMV replication is believed to occur mainly in an endoplasmic reticulum (ER)-associated virus replication complex containing the replication proteins, MP, vRNA and host proteins. The intra- and intercellular transport of TMV requires a 30-kDa MP (MP^{TMV}), which has been shown to perform multiple functions and interacts with a variety of host proteins to promote virus movement (Liu and Nelson, 2013; Niehl and Heinlein, 2011; Pallas and Garcia, 2011). The interactions of MP^{TMV} with cortical ER, microfilaments and microtubules assist the subcellular distribution of TMV (Liu and Nelson, 2013). Interactions of MP^{TMV} with the plasma membrane calreticulin (Chen *et al.*, 2005), the cytoplasmic ankirin repeat-containing protein ANK (Ueki *et al.*, 2010), the microtubule-associated protein MPB2C (Kragler *et al.*, 2003) and the end-binding protein 1 EB1A (Brandner *et al.*, 2008) facilitate the cell-to-cell movement of TMV. Furthermore, binding of MP^{TMV} with PD-associated casein kinase1 (PAPK) is known to phosphorylate MP, thereby modulating its movement activity (Lee *et al.*, 2005), and the interaction of MP^{TMV} with F-actin has been shown to disrupt actin polymerization, possibly required to increase SEL of PD (Su *et al.*, 2010). It has been reported recently that MP^{TVCV}, the MP encoded by *Turnip vein clearing virus* (TVCV), in addition to the host ER membranes and PDs, is associated with F-actin-containing nuclear filaments and chromosomes into the nucleus, indicating novel host interactions affecting tobamovirus movement and infection (Levy *et al.*, 2013). Moreover, the interaction of MP^{TMV} with cell wall-associated pectin methylesterase (PME) has been found to be involved in the local

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and systemic movement of TMV (Chen and Citovsky, 2003; Chen *et al.*, 2000; Dorokhov *et al.*, 1999). A PME isoform from *Nicotiana tabacum* leaves (NtPME; EMBL accession AJ249786) has been found to interact with MP^{TMV} and is necessary for TMV cell-to-cell movement (Chen *et al.*, 2000; Dorokhov *et al.*, 1999). Tobacco plants, in which the expression of a phloem-specific PME isoform was reduced by antisense suppression, was shown to exhibit slower TMV systemic movement and reduced viral outcome from the vascular system (Chen and Citovsky, 2003). Tomato PME1 (Gaffe *et al.*, 1997) and citrus PME3 (Nairn *et al.*, 1998) also interact *in vitro* with MPs of TMV, TVCV and *Cauliflower mosaic virus* (CaMV), a virus belonging to the genus caulimovirus (Chen *et al.*, 2000). Recently, the interaction between the MP of the furovirus *Chinese wheat mosaic virus* (CWMV) and PME from *Nicotiana benthamiana* has been reported to occur in the cell walls of epidermal cells (Andika *et al.*, 2013). Overall, this evidence suggests that viruses of different genera could share mechanisms of local and systemic translocation mediated by the interaction of MP with plant PMEs, and that PME is a host factor affecting the development of symptoms and susceptibility.

PMEs are a large class of cell wall-remodelling enzymes, some of which are ubiquitously expressed during the entire plant life cycle, whereas others are induced during growth and on pathogen infection (Lionetti *et al.*, 2012; Wolf *et al.*, 2009). PMEs catalyse the demethylation of pectin and release negatively charged carboxyl groups and methanol (Mohnen, 2008) which accumulate in the intercellular spaces and mainly diffuse out of the leaves after stomatal opening (Fall and Benson, 1996; Huve *et al.*, 2007). The activity of PME is modulated by endogenous protein inhibitors (pectin methylesterase inhibitors, PMEIs), which have been identified in many plant species (An *et al.*, 2008; Hong *et al.*, 2010; Raiola *et al.*, 2004; Reca *et al.*, 2012; Zhang *et al.*, 2010). PMEIs are targeted to the extracellular matrix and typically inhibit plant PMEs by forming a specific and stable stoichiometric 1:1 complex (Di Matteo *et al.*, 2005). PMEIs counteract the action of plant PMEs and therefore may affect plant susceptibility towards viruses. To test this hypothesis, we overexpressed genes encoding two well-characterized PMEIs in tobacco and Arabidopsis plants. As functional PMEIs have not yet been identified in tobacco, we constitutively expressed a PMEI from *Actinidia chinensis* (AcPMEI) in *N. tabacum*. We report that the ectopic expression of AcPMEI delays the spread of TMV in tobacco, and that the overexpression of AtPMEI-2 reduces the susceptibility of Arabidopsis to TVCV.

Table 1 Pectin methylesterase inhibitor (PMEI) activity against total PMEs from different plant leaves.

	AcPMEI	AtPMEI-1	AtPMEI-2
Tobacco	5	20	10
Arabidopsis	70	50	10

Data represent the quantity in nanograms of inhibitor required to reduce by 50% the halo of 1 cm produced by PME from crude leaf extracts.

RESULTS

Expression of AcPMEI in tobacco inhibits PME activity and increases cell wall methylesterification

To determine whether PMEI, by interacting with plant PMEs, interferes with MP-dependent cell-to-cell movement and viral systemic diffusion, we generated tobacco plants constitutively expressing the inhibitor. As functional PMEIs have not yet been identified in tobacco, the best characterized PMEIs from Arabidopsis (Hothorn *et al.*, 2004; Raiola *et al.*, 2004) and kiwi (AcPMEI) (Di Matteo *et al.*, 2005) were tested against PME activity from tobacco leaves (Table 1). As a result of its greater inhibitory activity against tobacco PMEs, AcPMEI from kiwi was chosen and constitutively expressed in tobacco by *Agrobacterium*-mediated leaf disc transformation. The sequence encoding the N-terminal signal peptide of the polygalacturonase-inhibiting protein (PGIP) from *Phaseolus vulgaris* was included upstream of the coding sequence of AcPMEI (Fig. S1, see Supporting Information) to target the inhibitor into the apoplast (Desiderio *et al.*, 1997). The chimeric gene was placed under the control of the CaMV 35S promoter for constitutive expression, with the Ω leader as translation enhancer (Fig. 1a). After selection with kanamycin, 15 independent transformants exhibiting *AcPMEI* transcripts were obtained. The level of expression of AcPMEI in transformed lines was determined by immunoblot analysis using polyclonal antibodies generated against purified AcPMEI. The *AcPMEI* lines 3 and 9, both exhibiting a 3:1 Mendelian segregation ratio for the transgene, were isolated. The AcPMEI 3-2 and 9-4 lines homozygous for the transgene were selected for subsequent analysis. Line 3-2 showed a 30% higher level of the inhibitor than line 9-4 (Fig. 1b,c). AcPMEI appeared as a single band with an apparent molecular mass of 16.5 kDa (Fig. 1b), consistent with the predicted molecular mass and the absence of glycosylation sites (Di Matteo *et al.*, 2005). When compared with wild-type (WT) plants, PME activity was reduced by 40% in leaves of line 3-2 and by 20% in leaves of line 9-4 (Fig. 2a). Consistently, PME activity in the midveins and leaf blade, i.e. the tissues in which viral movement mainly occurs, was reduced by 55% and 30% in lines 3-2 and 9-4, respectively, indicating the ability of AcPMEI to affect tissue-specific PME isoforms (Fig. 2b). As a consequence, the degree of methylesterification of the cell wall was increased by 70% and 45% in lines 3-2 and 9-4, respectively, with respect to the WT (Fig. 2c). No differences in morphology and growth were observed between transformed plants and untransformed controls.

Expression of AcPMEI limits cell-to-cell and systemic movement of TMV in tobacco

Leaves of transformed plants were inoculated with TMV-green fluorescent protein (TMV-GFP) and the cell-to-cell translocation in

Fig. 1 SP^{PGIP} -AcPMEI gene construct and expression of *Actinidia chinensis* pectin methylsterase inhibitor (AcPMEI) in transgenic tobacco lines. (a) Schematic diagram of AcPMEI with the secretory signal peptide of bean polygalacturonase inhibitor protein 1 (SP^{PGIP}) and schematic representation of the expression construct, pBI121 Ω - SP^{PGIP} -AcPMEI, used for plant transformation. Expression of SP^{PGIP} -AcPMEI was driven by the *Cauliflower mosaic virus* (CaMV) 35S promoter (35S), the Ω leader translation enhancer and the nopaline synthase (nos) terminator. (b) Representative Western blot analysis performed using polyclonal antibodies generated against purified AcPMEI; M, molecular weight marker; WT, wild-type. (c) Level of AcPMEI expression in transgenic lines determined by Western blot analysis using ImageJ software. The fold change was relative to line 3-2. Bars represent the average $\pm$ standard deviation of three independent experiments. Asterisk indicates significant difference according to Student's *t*-test ($P < 0.05$).

leaf blades was quantified by examining the size of the bright GFP foci visible under UV light (Niehl and Heinlein, 2011). Local spread of fluorescence was reduced significantly in transgenic lines when compared with the WT. Lines 3-2 and 9-4 exhibited a significant reduction in the area of bright spots of about 60% and 18%, respectively (Fig. 3). Bright spots were absent in mock-inoculated leaves of both WT and transgenic plants. The systemic spread was analysed by the appearance of visible disease symptoms and by the immunodetection of TMV-coat protein (TMV-CP) in leaves at different days post-infection (dpi) using anti-TMV-CP antibodies (Chen and Citovsky, 2003; Ghoshroy *et al.*, 1997). At 8 dpi, although WT plants developed typical TMV symptoms, no symptoms appeared in line 3-2 transformed plants (Fig. 4a). The mosaic was visible in line 3-2 with a delay of 2 days. Notably, TMV-CP accumulation in WT leaves was detected at 6 dpi, whereas a

significant delay of 2 days, as well as a reduction in viral protein, was observed in line 3-2, and a delay of 1 day was observed in line 9-4 (Fig. 4b,c).

To determine whether TMV alters PMEs in tobacco, the activity of the enzyme was monitored in uninfected WT and AcPMEI plants, and in plants inoculated with the virus. PME activity in both WT and transgenic plants was induced by rubbing (mock) and by TMV infection (Fig. 5). In WT leaves, PME activity was induced early by rubbing and declined to basal levels at 8 h post-treatment. On TMV infection, the induction was markedly higher. The level of PME activity in transgenic plants was lower than in WT plants and, notably, a significantly lower level of PME activity was induced by TMV infection (Fig. 5).

Overexpression of AtPMEI-2 reduces susceptibility of *Arabidopsis* to TVCV

The analysis was extended to the TVCV-*Arabidopsis* pathosystem. As a result of the lack of evidence for binding between MP from TVCV and PMEs from *Arabidopsis thaliana*, we verified, by affinity chromatography, whether the viral MP interacts with the enzyme. For this purpose, a recombinant histidine-tagged TVCV-MP (MP_{His}) was expressed in *Escherichia coli*. Purification of MP_{His} gave a prevalent band with an apparent molecular mass of about 35 kDa which was detected by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis (Fig. 6a,b). Other bands with a lower molecular mass were detected and represented truncated forms of the protein, probably as a result of the presence in the coding sequence of several arginine codons (AGG and/or AGA), rarely used in *E. coli*, and known to have a negative effect on the bacterial protein translation process (Brill *et al.*, 2000; Rosenberg *et al.*, 1993). Purified MP_{His} was immobilized on a Sepharose matrix and tested for binding to PMEs from *Arabidopsis* leaves. Intercellular washing fluids (IWFs) were loaded onto the MP-Sepharose column and, following an extensive wash, the bound proteins were eluted at a high ionic strength. PME activity was recovered in the eluted fractions, indicating the ability of the MP of TVCV to bind PMEs from *Arabidopsis* (Fig. 6c). No PME activity was detected in fractions going through a blank Sepharose column prepared as control (Fig. 6d).

The systemic infection of TVCV was analysed in *Arabidopsis* plants overexpressing AtPMEI-2 (Lionetti *et al.*, 2007; Raiola *et al.*, 2004), and was monitored by quantifying the accumulation of TVCV-CP within uninoculated rosette leaves. As specific anti-TVCV antibody is not commercially available, the accumulation of TVCV-CP was quantified by SDS-PAGE (Lartey *et al.*, 1997). TVCV-CP was detected as a band of 18 kDa at 5 dpi, whereas a smaller amount was found in transgenic leaves, with a significant delay at 7 dpi (Fig. 7a,b). PME activity was induced significantly in WT leaves by rubbing and, on TVCV treatment,

Fig. 2 Pectin methylesterase (PME) activity and cell wall methylesterification in leaves of wild-type (WT) and transformed tobacco plants. PME activity in leaves (a) and in leaf blade and midvein (b) of WT and transformed plants. (c) Degree of cell wall methylesterification in leaves of WT and transformed plants. Data represent average $\pm$ standard deviation ($n = 6$). The experiments were repeated three times with similar results. The different letters indicate datasets significantly different according to analysis of variance (ANOVA) followed by Tukey's test ($P < 0.05$).

Fig. 3 Accumulation of *Tobacco mosaic virus*-green fluorescent protein (TMV-GFP) in leaves of wild-type (WT) and transformed plants. (a) GFP fluorescence of infection foci in inoculated TMV-GFP tobacco leaves at 9 days post-inoculation (dpi). Inoculated leaves were photographed under illumination by ultraviolet light. (b) The average area of TMV-GFP infection foci $\pm$ standard error at 9 dpi. At least 100 spots from 10 leaves were measured in each of three independent experiments. The different letters indicate datasets significantly different according to analysis of variance (ANOVA) followed by Tukey's test ($P < 0.01$).

the induction was markedly higher. In plants overexpressing AtPMEI-2, the level of PME activity was lower than in WT plants, and a significantly lower activity was induced on viral infection (Fig. 8). At the early stages of infection, AtPMEI-1 and AtPMEI-2, the best characterized Arabidopsis inhibitors (Hothorn *et al.*, 2004; Lionetti *et al.*, 2007; Raiola *et al.*, 2004), were not detected by immunoblot analysis in TVCV-treated WT leaves (Fig. S2, see Supporting Information).

DISCUSSION

The ability of a virus to move within a plant is a prerequisite for a successful infection. The interaction of MP with host PMEs is required for cell-to-cell movement and for the systemic spread of TMV (Burch-Smith and Zambryski, 2012; Chen and Citovsky, 2003; Chen *et al.*, 2000; Dorokhov *et al.*, 1999; Pallas and Garcia, 2011). The suppression of PME isoforms involved in virus infection represents a possible strategy to counteract viral diseases. We generated tobacco plants constitutively expressing a PME from kiwi (AcPMEI) which efficiently interacts and strongly inhibits PMEs from several plant species (Di Matteo *et al.*, 2005; Jolie *et al.*, 2010; Volpi *et al.*, 2011). The expression of AcPMEI in tobacco caused a significant reduction of PME activity in both vascular and nonvascular tissues, and an increase in cell wall methylesterification, indicating an efficient interaction of the inhibitor with PME in both tissues. As a consequence, the local and systemic translocation of TMV in transgenic tobacco plants was reduced significantly. We extended our study to *A. thaliana*. As TMV poorly infects Arabidopsis, we used TVCV, the tobamovirus of choice, for studies in this model plant. We showed that MP^{TVCV} binds *in vitro* PMEs from Arabidopsis, and the overexpression of AtPMEI-2 in Arabidopsis reduces the plant susceptibility to TVCV systemic movement and infection. Overall, our results show that PMEs act on different tobamovirus–host interactions.

The importance of PME in plant susceptibility to virus is also highlighted by its induction kinetics during viral infection. Analysis of Arabidopsis gene expression on infection with *Turnip mosaic virus* (TuMV), *Cabbage leaf curl virus* (CaLCuV) and *Cucumber mosaic virus* (CMV) showed a number of PME isoforms up-regulated by the infection (Ascencio-Ibanez *et al.*, 2008; Marathe *et al.*, 2004; Yang *et al.*, 2007). A PME isoform (NbPME;

Fig. 4 Delayed systemic movement of *Tobacco mosaic virus* (TMV) and symptoms in wild-type (WT) and transgenic tobacco plants. (a) TMV symptoms in systemic leaves of WT and *Actinidia chinensis* pectin methyltransferase inhibitor (AcPMEI)-transformed plants (line 3-2) at 8 days post-inoculation (dpi). (b) Representative Western blot analysis indicating TMV-CP accumulation in the upper systemic leaves of infected WT and transformed lines at the indicated days post-inoculation. (c) Coat protein (CP) accumulation expressed as a percentage of the maximal amount of CP measured in WT plants. Data represent average $\pm$ standard error ($n = 3$); the experiment was repeated three times with similar results.

Fig. 5 Pectin methyltransferase (PME) activity in wild-type (WT) and transgenic tobacco plants at early stages of *Tobacco mosaic virus* (TMV) infection. The PME activity was quantified at the indicated hours post-inoculation (hpi); M, mock-inoculated plants; TMV, virus-inoculated plants. Data represent average $\pm$ standard deviation ($n = 6$). The experiment was repeated twice with similar results. The different letters indicate datasets significantly different according to analysis of variance (ANOVA) followed by Tukey's test ($P < 0.05$).

accession AA085706) is up-regulated in *N. benthamiana* plants after TMV infection (Dorokhov *et al.*, 2012). We found here that PME activity is induced at the early stages of infection in tobacco and *Arabidopsis* leaves inoculated with TMV and TVCV, respectively. The induction of PMEs probably increases the possibility of the host enzyme to cooperate with viral MPs to enlarge the PD pore and facilitate virus spread. PMEs in both tobacco and *Arabidopsis* transgenic plants not only affect the existing PMEs, but also inhibit the induction of PME activities by the virus and further prevent the pathogen from exploiting a host susceptibility factor required for infection. PME activity locally modulates the degree of methylesterification known to affect cell wall mechanical properties (Mohnen, 2008; Willats *et al.*, 2006). As PD channels are embedded in specialized cell wall regions containing cellulose, callose and nonesterified pectin (Dahiya and Brewin, 2000; Roy *et al.*, 1997; Sutherland *et al.*, 1999; Zavaliev *et al.*, 2013), their gating capacity during virus infection may be affected by changes in the cell wall structure and properties modulated by PME action. The co-localization of TMV MPs with PME in the cell wall that embeds PDs in tobacco leaves (Andika *et al.*, 2013) suggests that PME influences the cell wall mechanical properties at the level of PDs to favour viral diffusion. Our results, demonstrating that the overexpression of PME increases the cell wall methylesterification of leaves and limits the viral diffusion in leaf tissues, are also

Fig. 6 Detection of recombinant *Turnip vein clearing virus* (TVCV)-MP_{His} after nickel affinity column chromatography by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (a) and Western blot analysis (b). M, molecular weight marker; lane 1, proteins from *Escherichia coli* M15 (negative control); lane 2, proteins from *E. coli* M15 transformed with TVCV-MP_{His}. Arrows indicate the purified movement protein (MP). Pectin methylesterase (PME) activity in fractions eluted from affinity chromatography on a Sepharose column conjugated with TVCV-MP_{His} (c) and from a blank Sepharose column (d): 1, intercellular washing fluids; 2, flow-through; 3, washing; 4, proteins eluted from the columns.

consistent with this hypothesis. However, it cannot be excluded that, during viral infection, the overexpression of PME reduces the PME-dependent release of methanol, which has been proposed to enlarge PDs and to facilitate viral spread (Dorokhov *et al.*, 2012). Recently, methanol has been shown to induce the expression of a putative PME in *N. benthamiana* (Dorokhov *et al.*, 2012). Our results indicate that AtPMEI-1 and AtPMEI-2 expression is not induced by TVCV treatment. However, it is possible that the induction of specific PME isoforms is a natural response exploited by the host to restrict PME-mediated viral infection. Consistent with this hypothesis, a high level of *PMEI* gene expression has been shown to be induced in potato at the early stage of infection with *Potato virus Y* (Kogovsek *et al.*, 2010).

This work provides the first evidence that the overexpression *in planta* of biochemically characterized PMEs limits the movement of TMV in tobacco and also delays the systemic spread of TVCV in Arabidopsis. The approach applied may possibly be extended to other important crop species. The action of PMEs in

Fig. 7 Systemic accumulation of *Turnip vein clearing virus*-coat protein (TVCV-CP) in rosette leaves of wild-type (WT) and pectin methylesterase inhibitor-overexpressing Arabidopsis (AtPMEI-2) plants. (a) Representative sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) indicating TVCV-CP accumulation (arrow) in systemic leaves of infected WT and AtPMEI-2 plants at the indicated days post-inoculation. (b) CP accumulation is expressed as a percentage of the maximal amount of CP measured in WT plants. Data represent average $\pm$ standard error ($n = 3$). The experiment was repeated three times with similar results.

limiting viral spread suggests that this class of inhibitor may also be utilized in breeding programmes of plant varieties less susceptible to tobamoviruses.

EXPERIMENTAL PROCEDURES

Plant transformation, selection and growth

The DNA sequence containing the coding region of the *AcPMEI* gene (Di Matteo *et al.*, 2005; Volpi *et al.*, 2011), fused to the signal peptide of the bean polygalacturonase-inhibiting protein 1 (Pvpgip1) for apoplastic targeting (Desiderio *et al.*, 1997; Volpi *et al.*, 2011) (Fig. S1), was inserted into the *Bam*HI and *Sac*I site of the pBI121(Ω) vector under the control of the CaMV 35S promoter and translation enhancer Ω leader (Hansen *et al.*, 1994) (Fig. 1a). The pBI121 Ω -SP^{PGIP} *AcPMEI* was mobilized into *Agrobacterium tumefaciens* strain LBA4404 by electroporation. Leaf explants from tobacco (*N. tabacum*, cv SR1) were transformed, and

Fig. 8 Pectin methylesterase (PME) activity in wild-type (WT) and transgenic *Arabidopsis* plants at early stages of *Turnip vein clearing virus* (TVCV) infection. PME activity was quantified at the indicated hours post-inoculation (hpi); M, mock-inoculated plants; TVCV, virus-inoculated plants. Data represent the average $\pm$ standard deviation ($n = 6$). The experiment was repeated twice with similar results. The different letters indicate datasets significantly different according to analysis of variance (ANOVA) followed by Tukey's test ($P < 0.05$).

kanamycin-resistant plants were regenerated (Horsch *et al.*, 1985). Primary transformants were allowed to self-fertilize, and T0 seeds were collected and germinated on Murashige and Skoog (MS) medium with 300 $\mu\text{g}/\text{mL}$ kanamycin. Selected seedlings were transferred into soil. T1 and T2 progeny were subjected to the same selection, and lines showing resistance to kanamycin were selected for subsequent analysis.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis was performed as described previously (Raiola *et al.*, 2011) using the following oligonucleotide primers: *AcPMEI* Fw, 5'-GGATCTTAAGGGCTTGGTCA-3'; *AcPMEI* Rv, 5'-CACCATCAAAAGCAGCAGAA-3'. The homologue of *ubiquitin 5* (*UBQ5*; At3g62250) was amplified using the following primers: *UBQ5* Fw, 5'-GTAAAGCTCGCTGTCTTCAGT-3'; *UBQ5* Rv, 5'-TCAAGC TTCAACTCTCTTTC-3'. Tobacco plants were grown in soil in an insect-free controlled environment growth chamber maintained at 24 °C and 70% relative humidity, with a 16-h/8-h day/night cycle and a photosynthetically active radiation (PAR) level of 230 $\mu\text{mol}/\text{m}^2/\text{s}$. *Arabidopsis* plants were grown in a growth chamber maintained at 22 °C and 70% relative humidity, with a 12-h/12-h day/night cycle and a PAR level of 100 $\mu\text{mol}/\text{m}^2/\text{s}$.

Total protein extraction and PME expression analysis

Total protein extracts were obtained by homogenizing leaves of adult tobacco plants in the presence of 1 M NaCl, 20 mM sodium acetate, 0.02% sodium azide, protease inhibitor 1:100, pH 5.5 (2 mL of extraction buffer per gram of tissue). The homogenate was shaken for 1.5 h at 4 °C, centrifuged at 15 000 g for 15 min, and the supernatant was collected. The protein concentration was determined using Bradford reagent and bovine serum albumin as standard (Bradford, 1976). SDS-PAGE was performed by

loading 5 μg of protein extract for tobacco and 10 μg for *Arabidopsis* tissues into the gel. Immunoblot analysis was performed as described previously using polyclonal rabbit-specific antibodies raised against recombinant AcPMEI, AtPMEI-1 and AtPMEI-2, produced by Primm Biotech (Milan, Italy), and purified to homogeneity (Di Matteo *et al.*, 2005).

Determination of PME activity and degree of methylesterification of the cell wall

PME activity in leaves of WT and AcPMEI plants was quantified by the alcohol oxidase/acetyl acetone procedure (Klavons and Bennett, 1986). Four micrograms of total proteins extracted from leaf tissues in 50 μL of protein extraction buffer were incubated with 50 μL of 0.05% apple pectin (Sigma-Aldrich, St Louis, MO, USA; cat. no 76282; 70%–75% esterification) in 0.1 M sodium phosphate, pH 7.5. The reaction was incubated at 30 °C for 30 min and blocked by boiling at 100 °C for 10 min. The solution was incubated in a 96-microwell plate and, after the addition of 50 μL of alcohol oxidase (0.03 units in 0.1 M sodium phosphate, pH 7.5; Sigma), the samples were incubated at room temperature for 15 min on a shaker. Thereafter, 100 μL of a mixture containing 0.02 M 2,4-pentanedione in 2 M ammonium acetate and 0.05 M acetic acid was added. After 10 min of incubation at 68 °C, samples were cooled on ice and the absorbance was measured at 412 nm in a microplate reader (Cary 50 MPR microplate reader, Varian, Palo Alto, CA, USA). The methanol content was estimated as the amount of formaldehyde produced from methanol by alcohol oxidase, by comparison with a methanol standard calibration curve. AcPMEI [accession number P83326, National Center for Biotechnology Information (NCBI) database], AtPMEI1 (accession number NP-188348) and AtPMEI2 (accession number NP-188348), expressed in *Pichia pastoris* and purified as described previously (Di Matteo *et al.*, 2005; Raiola *et al.*, 2004), were used. The efficiency of PME inhibition and PME activity in Table 1 and Fig. 8 were quantified by the radial gel diffusion assay, as described previously (Lionetti *et al.*, 2007). A commercial orange peel PME (Sigma-Aldrich) was used to calculate the units of PME activity. The isolation of alcohol-insoluble solids (AIS) and the analysis of uronic acid content and degree of methylesterification were performed as described previously (Lionetti *et al.*, 2007, 2010).

Expression and purification of recombinant TVCV-MP

TVCV-MP was expressed as a histidine-tagged protein using the expression vector pQE30 (The QIAexpressionist™, Qiagen, Valencia, CA, USA). The coding sequence of TVCV-MP was amplified from pTVCV50 plasmid (containing the full-length TVCV cDNA; (Lartey *et al.*, 1995) with *pfu* DNA polymerase (Promega, Madison, WI, USA) using the following primer pairs: 5'-ATGCGGATCCATGTCGATAGTCTCGTACGA-3' and 5'-ATGCGGTACCTT AAGCATTGGTATGGGCTC-3'. PCR was performed in 50 μL of total reaction volume with 10 ng of plasmid pTVCV50 as follows: one cycle at 94 °C for 2 min, 30 cycles at 94 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min; and a final step at 72 °C for 7 min. The amplification product was isolated and cloned between the *Bam*HI and *Kpn*I sites (in italic in the above primer sequences) into the N-terminal six-histidine tag expression vector pQE30 (Qiagen). The resulting pQE30 construct (henceforth called TVCV-MP_{His}-pQE30), containing the full-length MP gene, was used to transform the *E. coli* strain M15[pREP4] (Qiagen). Ni-NTA agarose resin (His-Select® HF

Nickel Affinity Gel; Sigma) was used for purification of MP_{His}, and the released proteins were analysed by SDS-PAGE and Western blot analysis. The amino acid sequence of the recombinant protein was confirmed by LTQ Orbitrap mass spectrometry. After staining, the protein band was excised from the gel, reduced, alkylated and digested with trypsin according to Hellman *et al.* (1995). Tryptic peptides were analysed by liquid chromatography-tandem mass spectrometry (LC-MS/MS) (LC-ESI-quadrupole iontrap MS 1100 Series; Agilent Technologies, Palo Alto, CA, USA) using a Zorbax SB-C18 column (0.5 × 150 mm; particle size 5 µm), as described previously (Lionetti *et al.*, 2007).

Isolation of PME activity by TVCV-MP affinity chromatography

IWFs were collected from Arabidopsis rosetta leaves, as described by Lionetti *et al.* (2007). TVCV-MP_{His} (1 mg) was covalently linked to 1 mL CNBr-activated Sepharose (CNBr-Sepharose® 4B, Sigma) according to the manufacturer's instructions. The column was washed with equilibration buffer (EB) containing 25 mM Tris/HCl (pH 8.0), 1 mM ethylenediaminetetraacetic acid (EDTA) and 50 mM LiCl. IWFs (6 mL) were dialysed in EB, loaded onto the affinity MP_{His}-Sepharose column and incubated for 2 h at room temperature. The proteins that were not bound to the column (i.e. flow through, FT) were collected. The column was washed with 10 volumes of EB and the retained proteins were eluted with 25 mM Tris/HCl (pH 8.0) containing 1 mM EDTA and 1 M LiCl, as described previously (Dorokhov *et al.*, 1999). A blank Sepharose column was also prepared as a control.

Tobamovirus infection of tobacco and Arabidopsis plants

TVCV (strain PV-0361) (DSMZ GmbH, Braunschweig, Germany) and TMV (strain U1) were propagated in Arabidopsis and tobacco plants, respectively, and purified as described by Gooding and Hebert (1967) and Lartey *et al.* (1993). Fully expanded basal rosette (source) leaves of Arabidopsis plants at the eight-leaf stage (three leaves for each plant) were rub inoculated with 5 µL of TVCV suspension (20 µg/mL in 50 mM potassium phosphate buffer, pH 7.0, and Celite at 4 mg/mL). At the indicated days post-inoculation, two uninoculated rosette leaves (just above those inoculated) were harvested from infected plants (Lartey *et al.*, 1997). Leaf tissue was extracted and analysed for the presence of CP by SDS-PAGE and colloidal Coomassie Blue G staining (Sigma), as described by Lartey *et al.* (1997).

TMV was mechanically inoculated onto one mature lower (source) leaf located at the same basal internode of each 7-week-old plant by rubbing 20 µL of TMV suspension (5 µg/mL) containing 4 mg/mL carborundum as an abrasive on each leaf. At the indicated days post-inoculation, the fourth uninoculated upper leaf, usually located three to four leaves from the top of the plant, was harvested. Systemic viral infection was monitored using the CP assay, as described by Chen and Citovsky (2003). Tissue samples were collected, extracted and analysed for the presence of CP by SDS-PAGE. Western blot analysis was performed using anti-TMV-CP antibodies (1:200) (LOEWE Biochemica, Sauerlach, Germany) and alkaline phosphatase-conjugated goat anti-rabbit IgG as secondary antibody (1:2500) (Sigma), followed by immunodetection using 5-bromo-4-

chloroindol-3-yl phosphate/nitroblue tetrazolium (BCIP/NBT) substrate, according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). The amounts of CP were quantified by ImageJ software (W. S. Rasband, ImageJ; US National Institutes of Health, Bethesda, MD, USA). To analyse AtPMEI-1 and AtPMEI-2 expression in Arabidopsis WT plants during TVCV treatment, SDS-PAGE was performed as described by Laemmli (1970); for each sample, 10 µg of total proteins isolated from Arabidopsis leaves were loaded onto the gel. Immunoblot blot analysis was performed, as described previously (Lionetti *et al.*, 2007), using polyclonal antibodies generated against purified recombinant AtPMEI-1 or AtPMEI-2 (Raiola *et al.*, 2004).

Detection of fluorescence in tobacco leaves inoculated with TMV expressing GFP

RNA transcripts were synthesized from TMV vector expressing GFP (Toth *et al.*, 2002), linearized with *KpnI* and transcribed *in vitro* in the presence of cap analogue (m7G(5')ppp(5')G) (Promega) using RiboMAX™ Large-Scale RNA Production Systems (Promega), according to the manufacturer's instructions. The transcripts were used to rub inoculate tobacco leaves with a solution containing 50 mM of KH₂PO₄, pH 7.5, and 0.04% macaloid, using Celite as abrasive, and the TMV-GFP suspension was extracted from tobacco leaves expressing GFP. Fully expanded tobacco leaves were rub inoculated with a suspension of TMV-GFP. Inoculated leaves were photographed with a digital camera under UV illumination at 9 dpi, and the area of TMV-GFP foci was measured by ImageJ software (W. S. Rasband, ImageJ; US National Institutes of Health).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1 DNA sequence containing the coding region of the SP^{PGIP}-*AcPMEI* gene used for plant transformation. The sequence of the signal peptide of *PGIP* is highlighted in bold.

Fig. S2 Accumulation of AtPMEI-1 and AtPMEI-2 in *Arabidopsis* wild-type (WT) plants after *Turnip vein clearing virus* (TVCV) treatment. Representative Western blot analysis performed at the indicated hours post-inoculation (hpi) of TVCV on WT leaves using polyclonal antibodies generated against purified AtPMEI-1 (a) or AtPMEI-2 (b). C₁ and C₂, crude extracts from AtPMEI-1- and AtPMEI-2-overexpressing plants, respectively, were used as control; molecular mass of protein markers is indicated on the left (top panel). Coomassie staining of total proteins isolated from WT leaves after sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) is shown as a loading control (bottom panel).