

# Overexpression of Pectin Methylesterase Inhibitors in *Arabidopsis* Restricts Fungal Infection by *Botrytis cinerea*<sup>1[C][W]</sup>

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Pectin, one of the main components of plant cell wall, is secreted in a highly methylesterified form and is demethylesterified in muro by pectin methylesterase (PME). The action of PME is important in plant development and defense and makes pectin susceptible to hydrolysis by enzymes such as endopolygalacturonases. Regulation of PME activity by specific protein inhibitors (PMEIs) can, therefore, play a role in plant development as well as in defense by influencing the susceptibility of the wall to microbial endopolygalacturonases. To test this hypothesis, we have constitutively expressed the genes *AtPMEI-1* and *AtPMEI-2* in *Arabidopsis* (*Arabidopsis thaliana*) and targeted the proteins into the apoplast. The overexpression of the inhibitors resulted in a decrease of PME activity in transgenic plants, and two PME isoforms were identified that interacted with both inhibitors. While the content of uronic acids in transformed plants was not significantly different from that of wild type, the degree of pectin methylesterification was increased by about 16%. Moreover, differences in the fine structure of pectins of transformed plants were observed by enzymatic fingerprinting. Transformed plants showed a slight but significant increase in root length and were more resistant to the necrotrophic fungus *Botrytis cinerea*. The reduced symptoms caused by the fungus on transgenic plants were related to its impaired ability to grow on methylesterified pectins.

Pectin is a structurally complex polysaccharide that accounts for nearly 35% of the dicot and nongraminaceous monocot primary cell wall. A main component of pectin is homogalacturonan (HGA) consisting of a backbone of 1,4-linked  $\alpha$ -D-GalUA units, with variable amounts of methylester in the C<sub>6</sub> position. Pectins are secreted into the cell wall in a highly methylesterified form and, soon thereafter, are deesterified in muro by pectin methylesterase (PME; Brummell and Harpster, 2001; Willats et al., 2001). Demethylesterification produces free carboxyl groups and modifies the pH and

charge of the wall, allowing the aggregation of polyuronides into a calcium-linked gel structure and increasing the wall firmness (Willats et al., 2001). In addition, the action of PMEs makes HGA susceptible to degradation by hydrolases such as endopolygalacturonases (endoPGs), contributing to the softening of the cell wall (Brummell and Harpster, 2001; Wakabayashi et al., 2003).

Plant PMEs are involved in important physiological processes such as microsporogenesis, pollen growth, pollen separation, seed germination, root development, polarity of leaf growth, stem elongation, fruit ripening, and loss of tissue integrity (Tieman and Handa, 1994; Wen et al., 1999; Micheli et al., 2000; Pilling et al., 2000, 2004; Micheli, 2001; Bosch et al., 2005; Jiang et al., 2005; Bosch and Hepler, 2006; Francis et al., 2006; Tian et al., 2006). They may also have a role in resistance to fungal and bacterial pathogens (McMillan et al., 1993; Boudart et al., 1998; Wietholter et al., 2003) and are required for the systemic spread of *Tobacco mosaic virus* through the plant (Dorokhov et al., 1999; Chen et al., 2000; Chen and Citovsky, 2003). Spatial and temporal regulation of PME activity during plant development is complex, due to the presence of a large family of isoforms (Markovic and Janecek, 2004). In the *Arabidopsis* (*Arabidopsis thaliana*) genome, 67 genes encode putative

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PMEs (Arabidopsis Genome Initiative, 2000; Markovic and Janecek, 2004). Nearly all the Arabidopsis PME genes are predicted to encode pre-pro-proteins (Micheli, 2001). The pre-region is required for protein targeting to the endoplasmic reticulum (Micheli, 2001; Dorokhov et al., 2006) and the pro-peptide is thought to play an autoinhibitory role of the enzyme during its secretion to the apoplast, where only the mature part of the protein is found (Giovane et al., 2004). In addition, a mechanism of regulation of PME activity is played by individually expressed specific protein inhibitors discovered in kiwi fruits (*Actinidia chinensis*; Balestrieri et al., 1990; Giovane et al., 1995) and recently was found in Arabidopsis (Wolf et al., 2003; Raiola et al., 2004). PME inhibitors named PME1 are encoded in Arabidopsis by a small gene family of two members, *AtPMEI-1* and *AtPMEI-2*, located in different chromosomes. *AtPMEI-1* and *AtPMEI-2*, which share about 47% identity at the amino acid level, consist of 151 amino acids

**Table 1.** Quantification of PME activity in *AtPMEI-1*, *AtPMEI-2*, and wild-type plants with radial gel diffusion assay

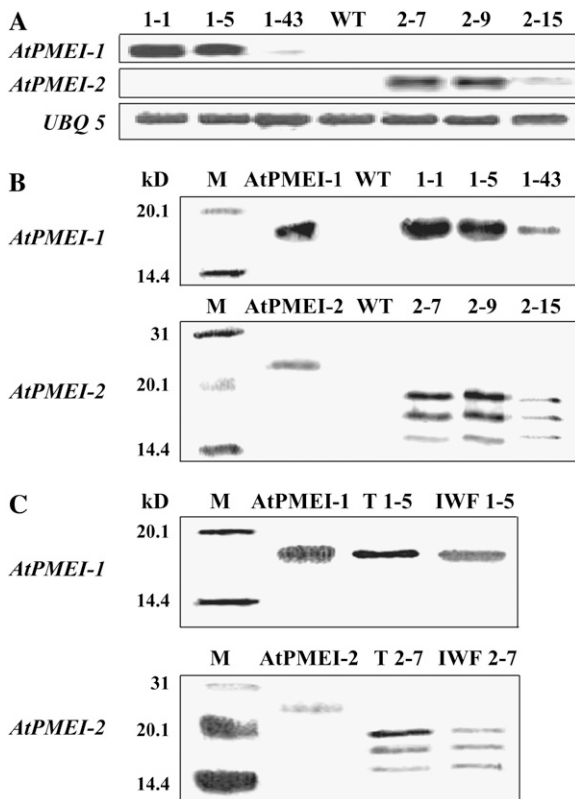
PME activities (%) were determined from leaves of *AtPMEI-1* and *AtPMEI-2* transgenic lines relative to wild type. Data represent average  $\pm$  SE of five biological replicates. The PME activity of the wild-type leaves (0.013 units) was set to 100%.

Line	PME Activity
Wild type	100
1-43	85.9 $\pm$ 0.7
1-1	61.6 $\pm$ 2.3
1-5	46.7 $\pm$ 1.1
2-15	83.8 $\pm$ 0.2
2-7	52.4 $\pm$ 0.3
2-9	44.7 $\pm$ 0.8

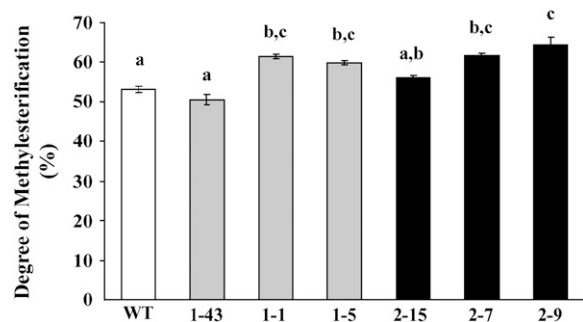
(molecular mass of 16,266 D, predicted pI = 7.7) and 148 amino acids (molecular mass 15,615 D, predicted pI = 9.0), respectively (Wolf et al., 2003; Raiola et al., 2004). PMEIs from Arabidopsis and kiwi exhibit an  $\alpha$ -helix up-and-down four-helical bundle fold and five strictly conserved Cys residues, four of which engaged in disulfide bridges for the maintenance of the protein structure (Hothorn et al., 2004; Di Matteo et al., 2005). PMEIs inhibit PMEs of plant origin by forming a noncovalent stoichiometric 1:1 complex and typically do not inhibit PMEs produced by plant pathogenic microorganisms (Mattei et al., 2002; D'Avino et al., 2003; Giovane et al., 2004; Di Matteo et al., 2005).

The specificity of PME1 toward plant PME suggests a physiological role in the modulation of endogenous PME activity during development and growth. However, given the effect of pectin methylesterification on the physicochemical properties of the walls, they may also have a role in defense against pathogens by influencing the susceptibility of the wall to cell wall-degrading enzymes, which in some cases have a major role in pathogenesis (Clark and Lorbeer, 1976; Collmer and Keen, 1986; Cole et al., 1998; De Lorenzo et al., 2001; D'Ovidio et al., 2004). Pectin hydrolysis often occurs in the diseases caused by soft rot bacteria and fungi and appears to be the prerequisite for degradation of other cell wall components (D'Ovidio et al., 2004). For example, *Botrytis cinerea*, a ubiquitous necrotrophic fungus, possesses an efficient pectinolytic machinery including a variety of endoPGs, some of which are important virulence factors (ten Have et al., 1998; Kars et al., 2005). Possibly for this reason, the host preference of *B. cinerea* is generally considered to be restricted to plants with relatively high pectin content (van Kan, 2006).

To investigate the role of pectin methylesterification in plant growth and in plant-pathogen interactions, we have constitutively expressed the genes *AtPMEI-1* and *AtPMEI-2* (Raiola et al., 2004) in Arabidopsis plants. We report here that the transgenic expression of the two inhibitors decreases the plant PME activity and alters the level of pectin methylesterification of the wall. The transformed plants show an altered growth response and a lower susceptibility to *B. cinerea*.



**Figure 1.** Expression of *AtPMEI-1* and *AtPMEI-2* in Arabidopsis transgenic plants. Numbers indicate the different *AtPMEI-1* or *AtPMEI-2* transformed lines; WT, untransformed wild-type plants. A, RNA gel-blot of T2 independent lines; UBQ 5, ubiquitin. B, Immunodetection of *AtPMEI-1* and *AtPMEI-2* in leaf extracts of transgenic lines using polyclonal antibodies generated against recombinant *AtPMEI-1* or *AtPMEI-2*. M, Protein markers with relative molecular mass indicated at left; as standard, 30 ng of purified *AtPMEI-1* or 20 ng of *AtPMEI-2* expressed in *P. pastoris* was used (Raiola et al., 2004). C, Immunodetection of *AtPMEI-1* and *AtPMEI-2* in IWFs from rosette leaves. T, Total proteins from rosette leaves (9  $\mu$ g); IWF, protein from intercellular washing fluids (3  $\mu$ g).



**Figure 2.** Determinations of the DM of cell wall from transgenic plants transformed with *AtPMEI-1* (lines 1-43, 1-1, 1-5), *AtPMEI-2* (lines 2-15, 2-7, 2-9), and wild type. The different letters indicate data sets significantly different according to Tukey's Student range test ( $P < 0.01$ ). Values represent the average  $\pm$  SE of three independent experiments with  $n = 4$  plants each.

## RESULTS

### AtPMEI Reduces PME Activity in Transgenic Arabidopsis

To explore the physiological role of AtPMEIs in Arabidopsis, transgenic plants constitutively expressing *AtPMEI-1* or *AtPMEI-2* genes were generated. To target the inhibitors into the apoplast, the sequence encoding the predicted N-terminal signal peptide for secretion was included in the coding sequence of each gene and both genes were placed under the control of the cauliflower mosaic virus 35S promoter. Strand-specific probes were used to analyze the accumulation of *AtPMEI-1* or *AtPMEI-2* mRNA in transformed rosette leaves, where the genes are expressed at low levels in untransformed plants (Wolf et al., 2003; Raiola et al., 2004). Thirty T2 transgenic plants exhibited a wide range of *AtPMEI-1* or *AtPMEI-2* mRNA levels (data not shown). Three transgenic lines transformed with *AtPMEI-1* (1-1, 1-5, and 1-43) and three lines transformed with *AtPMEI-2* (2-7, 2-9, and 2-15) were selected for analysis (Fig. 1A). Segregation analysis of the marker gene *bar* for resistance to the herbicide Basta in the self-crossed T2 progeny showed either a 3:1 Mendelian segregation ratio (1-1, 2-7, 2-9, and 2-15 lines) or a 15:1 segregation ratio (1-5 and 1-43 lines). These ratios correspond to one and two independent insertions of the transgene, respectively, and suggest a normal fitness of transformed pollen grains during fertilization (Bosch and Hepler, 2006). Lines 1-1 and 1-5 and lines 2-7 and 2-9 expressed levels of *AtPMEI-1* or *AtPMEI-2* transcripts about 90 and 200 times higher, respectively, than wild-type plants. These lines exhibited high levels of proteins as shown by immunoblotting analysis using polyclonal antibodies raised against AtPMEI-1 or AtPMEI-2 expressed in *Pichia pastoris* (Raiola et al., 2004; Fig. 1B). AtPMEI-1 was produced as a single band with an apparent molecular mass of 17 kD. AtPMEI-2 showed three bands with apparent molecular masses of 20, 18, and 16 kD. Upon treatment with *N*-glycosidase A, the two bands with

the higher molecular mass disappeared with a concomitant increase of the 16-kD band, i.e. the band corresponding to the protein with the predicted molecular mass (15,615 D) of the nonglycosylated form. Glycosylation of AtPMEI-2 was also observed in the recombinant protein expressed in *P. pastoris*. Lines expressing AtPMEI-1 did not show any band when the immunoassay was performed with the antibodies raised against AtPMEI-2 and, vice versa, lines expressing AtPMEI-2 did not show any band when AtPMEI-1 antibodies were used (data not shown). Lines 1-43 and 2-15 expressed levels of *AtPMEI-1* or *AtPMEI-2* transcripts 5 and 20 times higher than wild-type plants and exhibited low levels of transgenic proteins, whereas no AtPMEI-1 or AtPMEI-2 proteins were detected in untransformed plants (Fig. 1B). The accumulation of the inhibitors was detected in transformed seedlings grown either under light and in the dark (data not shown). In lines 1-1 and 2-9, the proteins were detected in extracellular fluids isolated from mature leaves, indicating that both inhibitors have been correctly targeted to the apoplast (Fig. 1C).

We assessed whether the overexpression of *AtPMEI-1* or *AtPMEI-2* resulted in a decrease of PME activity in transgenic Arabidopsis. Total protein extracts from rosette leaves were assayed using the quantitative PME activity radial gel diffusion assay (Downie et al., 1998). Lines 1-1 and 1-5 and lines 2-7 and 2-9 showed 45% to 62% of PME activity as compared with the wild-type plants, whereas PME activity of lines 1-43 and 2-15 was only slightly lower than that detected in wild-type plants (Table I).

To detect whether the inhibitors interact with endogenous PME(s), total proteins extracted from mature leaves of *AtPMEI* transformed lines 1-5 and 2-7 were separated by gel-filtration chromatography (Supplemental Fig. S2, A and B) and subjected to SDS-PAGE followed by western-blot analysis using polyclonal antibodies against AtPMEI-1 or AtPMEI-2 (see "Materials and Methods"). AtPMEI-1 and AtPMEI-2, expressed in *P. pastoris* and purified to homogeneity, eluted in fractions with a molecular mass of about 15 to 25 kD (i.e. 25–27), consistently with their molecular mass, and PME inhibitory activity was associated with the

**Table II.** Quantification of uronic acid content in *AtPMEI-1* and *AtPMEI-2* transformants and in wild-type cell walls

The same letter indicates not significant differences according to Tukey's Student range test ( $P < 0.05$ ). Data represent average  $\pm$  SE of four biological replicates.

Line	Uronic Acids $\mu\text{mol mg}^{-1}$ cell wall
Wild type	0.39 $\pm$ 0.06a
1-43	0.49 $\pm$ 0.04a
1-1	0.41 $\pm$ 0.04a
1-5	0.45 $\pm$ 0.04a
2-15	0.40 $\pm$ 0.1a
2-7	0.42 $\pm$ 0.02a
2-9	0.41 $\pm$ 0.03a

presence of the immunodetected bands (Supplemental Fig. S2D). Instead, AtPMEI-1 and AtPMEI-2 from transformed leaf extracts eluted in the fractions 22 to 24, which contain proteins with an estimated molecular mass of about 50 to 60 kD (Supplemental Fig. S2, C and E); no PME inhibitory activity was associated to the presence of the immunodetected bands. Moreover, PME activity was also eluted in fractions 25 and 26, which contained proteins with a molecular mass of about 30 to 40 kD. The chromatographic behavior of AtPMEI-1 and AtPMEI-2 from transformed plants and the absence of detectable inhibitory activity indicate that both inhibitors are eluted as inactive complexes with endogenous PME(s). On the other hand, the reduced enzymatic activity in the transformed tissues and the elution of free PME activity from the gel-filtration chromatography indicate that the enzyme is present in excess as an unbound active form.

Proteins were analyzed by SDS-PAGE and after staining were reduced, alkylated, digested with trypsin, and analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Two PMEs were recognized in both AtPMEI-1- and AtPMEI-2-containing fractions: the most abundant was identified as the isoform AtPME3 (SwissProt accession Q9LUL7; locus At3g14310) and the less abundant second one was identified as a putative PME (SwissProt accession Q9SKX2, locus At2g43050; Supplemental Table S1).

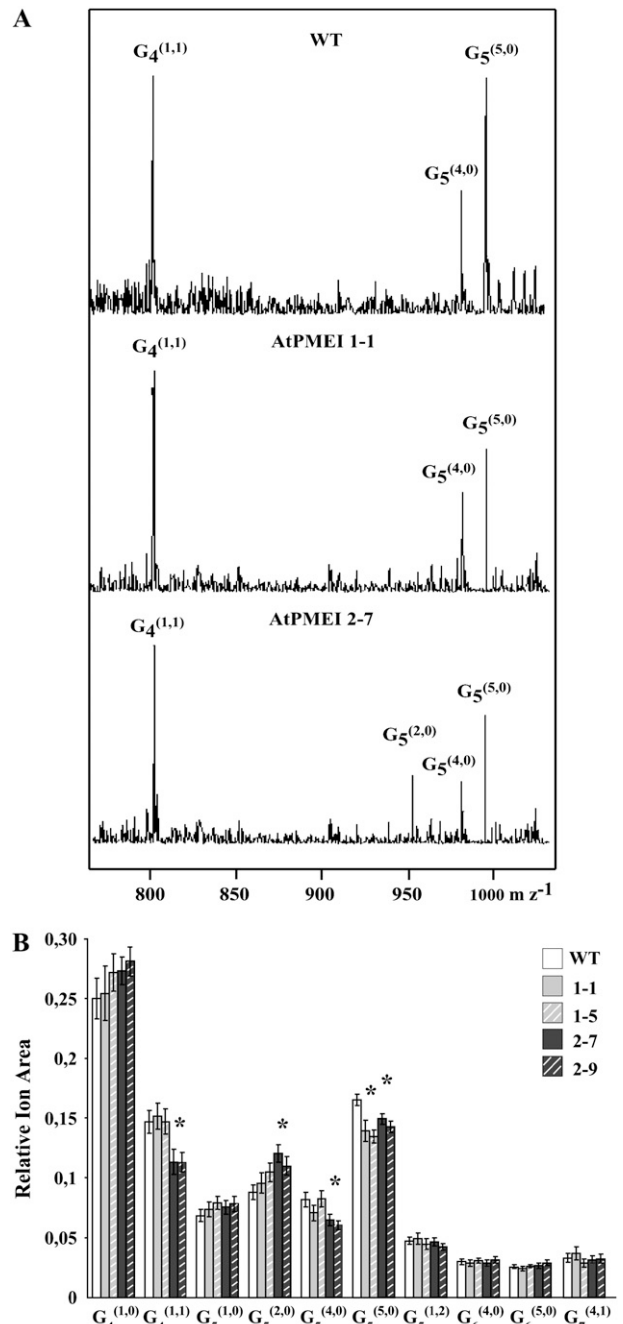
### Overexpression of AtPMEI in Arabidopsis Affects the Degree of Pectin Methylesterification

The degree of pectin methylesterification (DM) was determined in cell walls extracted from rosette leaves of transformed *AtPMEI* plants. As compared with the

**Table III.** Nominal masses, composition, and significance of ions generated by MALDI-TOF MS of the various *AtPMEI* transgenic lines

Nominal Mass <sup>a</sup>	Composition <sup>b</sup>	1-1 <sup>c</sup>	1-5 <sup>c</sup>	2-7 <sup>c</sup>	2-9 <sup>c</sup>
759	G <sub>4</sub> <sup>(1,0)</sup>				
<b>801</b>	<b>G<sub>4</sub><sup>(1,1)</sup></b>			*	*
935	G <sub>5</sub> <sup>(1,0)</sup>				
<b>949</b>	<b>G<sub>5</sub><sup>(2,0)</sup></b>			*	*
<b>977</b>	<b>G<sub>5</sub><sup>(4,0)</sup></b>			*	*
<b>991</b>	<b>G<sub>5</sub><sup>(5,0)</sup></b>	*	*	*	*
1033	G <sub>5</sub> <sup>(1,2)</sup>				
1153	G <sub>6</sub> <sup>(4,0)</sup>				
1167	G <sub>6</sub> <sup>(5,0)</sup>				
1386	G <sub>7</sub> <sup>(4,1)</sup>				

<sup>a</sup>Nominal mass (M+Na<sup>+</sup>)<sup>+</sup> of pectic oligosaccharides released by endoPG/PME digestion of Arabidopsis etiolated seedling cell wall material. <sup>b</sup>G<sub>n</sub><sup>(m,a)</sup> fragment; "G<sub>n</sub>" denotes the number of galacturonic acid residues in the backbone; and "m" and "a" the number of methylesterified and acetylated residues, respectively. <sup>c</sup>*AtPMEI-1* or *AtPMEI-2* transformed lines. \* and bold, ion areas significantly different compared with wild type by *t* test (*P* = 0.01; data were obtained from 30 independent samples for wild type and 20 independent samples for each transformed line; 10 seedlings were used for each sample).



**Figure 3.** Comparison of the relative abundance of pectic oligosaccharides released by endoPG/PME digestion from transformed and wild-type seedlings. A, Positive-ion MALDI-TOF mass spectra of endoPG/PME-generated pectic fragments from cell wall of wild-type, *AtPMEI-1*, and *AtPMEI-2* etiolated seedlings. B, The area of ion signals obtained from MALDI-TOF MS spectra of the different pectic fragments were integrated. Data represent the average  $\pm$  sd of *n* = 30 independent samples for wild type and *n* = 20 independent samples for each transformed line. Ten seedlings were used for each sample. Asterisks indicate the significant differences (*P* < 0.05).

wild-type plants, a significant increase of DM of about 16% was detected in lines 1-1 and 1-5 and lines 2-7 and 2-9 expressing high levels of *AtPMEI-1* and *AtPMEI-2* (Fig. 2). Lines 1-43 and 2-15, both expressing a low level of inhibitor, showed no differences. The content of uronic acids in transformed plants was not significantly different from that of wild-type plants (Table II).

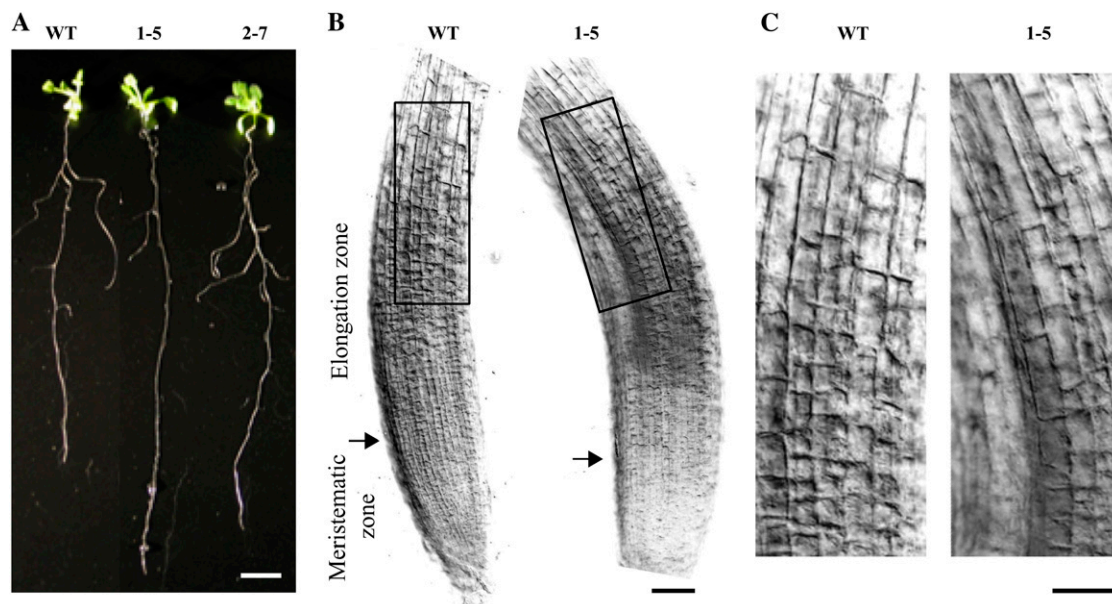
The fine structure of pectins of transformed plants was also analyzed by enzymatic fingerprinting (Lerouxel et al., 2002). Cell walls from wild-type and transformed seedlings were treated with a combination of fungal PME and endoPG, which releases methyl-esterified or *O*-acetylated HGA fragments (Obel et al., 2006). The water-soluble oligosaccharides were successively analyzed by matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF MS). The standard symbols reported by van Alebeek (van Alebeek et al., 2003), with some modifications, were used to define the pectic fragments. In particular, in the  $G_n^{(m,a)}$  fragment, " $G_n$ " denotes the number of GalUA residues in the backbone, and " $m$ " and " $a$ " indicate the number of methyl-esterified and of acetylated residues, respectively. The main ions detected by MALDI-TOF MS analysis were assigned to  $(M+Na)^+$  adducts on the basis of their  $M_r$  and literature data (Daas et al., 1998; Table III). Differences in MALDI-TOF mass spectra were observed in both highly expressing *AtPMEI-1* and *AtPMEI-2* lines in comparison with wild type (Fig. 3A). The areas of ion signals were integrated, allowing the establishment of the relative abundance profile of pectic fragments in the cell wall digest. Figure 3B shows that  $G_4^{(1,0)}$ ,  $G_4^{(1,1)}$ , and  $G_5^{(5,0)}$  were the main fragments released. Compared with the

wild type, all the *AtPMEI* transformed plants analyzed showed a significantly reduced level of fully methyl-esterified  $G_5^{(5,0)}$  fragments. In addition, the decrease of  $G_4^{(1,1)}$  and  $G_5^{(4,0)}$  and the increase of  $G_5^{(2,0)}$  ions were detected in lines 2-7 and 2-9 with respect to the wild-type plants. The differences in oligosaccharide profiles observed among plants indicate alteration in pectic structure/accessibility possibly due to differences in degree and/or pattern of methylesterification. On the other hand, no significant differences among *AtPMEI-1*, *AtPMEI-2*, and wild-type seedlings were observed when xyloglucan structure was analyzed by enzymatic oligosaccharide profiling using a xyloglucan-specific endo  $\beta$ -(1  $\rightarrow$  4)-D-glucanase (Lerouxel et al., 2002).

#### Transgenic Arabidopsis Plants Exhibit Altered Growth and Reduced Susceptibility to *B. cinerea*

Transformed plants grown in greenhouse did not show any obvious differences in growth, development, and plant fertility in comparison with the untransformed ones. However, when seedlings were vertically grown on solid Murashige and Skoog medium, a significant 20% increase in root length was observed in lines 1-5 and 2-7 with respect to wild-type plants (Fig. 4A; Table IV). Root cells of the expanding zone, the region in which cell elongation mainly occurs, were elongated with respect to the controls (Fig. 4, B and C).

Resistance of transgenic plants to the necrotrophic fungus *B. cinerea* was assessed. Leaves of wild-type and transgenic plants were inoculated with fungal conidia and the lesion size was determined 3 d postinoculation



**Figure 4.** Morphological analysis of vertically grown *AtPMEI* and wild-type plants. A, Wild type, *AtPMEI-1* (line 1-5), and *AtPMEI-2* (line 2-7) plants; bar = 10 mm. B, Optical microphotographs of epidermal root cells. The starting point of root elongation zone is indicated by the arrow. Corresponding root areas are delimited by black boxes. C, Magnification of the boxed areas. Bars = 50  $\mu$ m. [See online article for color version on this figure.]

**Table IV.** Average lengths of *AtPMEI-1*, *AtPMEI-2*, and wild-type roots

Root lengths were measured in seedlings after 12 d of vertical growth. Data represent average  $\pm$  SE of three independent experiments with  $n = 10$  plants each. Significant differences by Student's *t* test for  $P < 0.001$  are indicated by asterisks.

Line	Root Length
	<i>mm</i>
Wild type	83.7 $\pm$ 1.2
1-43	88.2 $\pm$ 3.6
1-5	107.7 $\pm$ 1.5*
2-15	88.1 $\pm$ 4
2-7	98.1 $\pm$ 2.6*

(Fig. 5A). No differences in the ratio between the number of expanding lesions and the number of inoculated spots were observed among transformed lines and wild-type plants (data not shown). Instead, lines 1-5, 1-1, 2-7, and 2-9 displayed a reduced radial lesion size ( $P < 0.01$ ) compared with wild type and lines 1-43 and 2-15 (Fig. 5B). No inhibitory activity of both purified *AtPMEI-1* and *AtPMEI-2* was observed against PME activity of *B. cinerea*, excluding a direct mechanism of these inhibitors in limiting fungal growth.

To ascertain if the reduced symptoms produced by *B. cinerea* on the *AtPMEI* lines are related to an impaired ability of this fungus to grow on methylesterified pectins, we examined the fungal growth on liquid synthetic media containing polygalacturonic acid (PGA) or 81% methylesterified pectin (E81) as carbon source. *B. cinerea* grew significantly better on PGA (average milligram of mycelium dry weight  $\pm$  SD, 30.2  $\pm$  2.7;  $n = 6$ ) than on E81 (12.6  $\pm$  1.2;  $n = 6$ ), indicating that the fungus prefers unesterified pectins as carbon source.

To determine if the cell wall composition of transformed plants affects *B. cinerea* growth, we measured the fungal growth on liquid medium containing cell walls isolated from wild type or from *AtPMEI* lines as a carbon source. A reduction of 14.3% and 28.5%, respectively, was observed on cell walls of lines 1-1 and 2-7 in comparison with the untransformed plants.

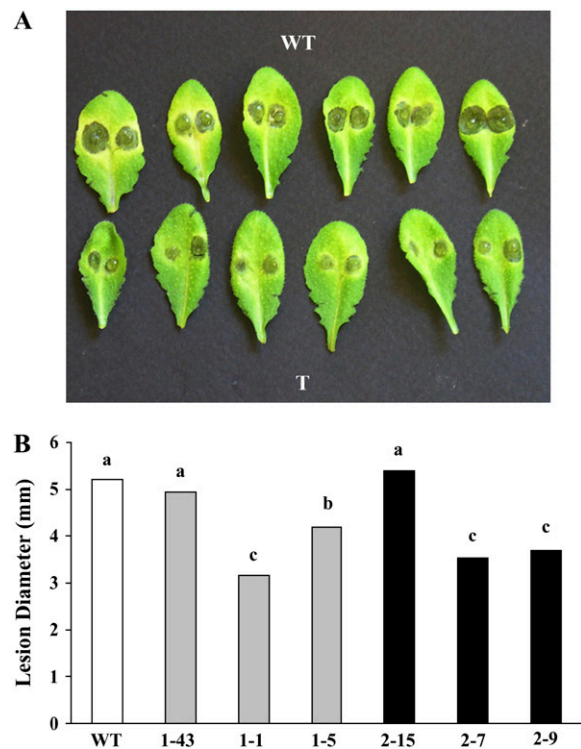
## DISCUSSION

In this study we have generated *Arabidopsis* plants constitutively expressing the PME inhibitors *AtPMEI-1* and *AtPMEI-2* (Wolf et al., 2003; Raiola et al., 2004). Among the transformed lines obtained, the lines 1-1, 1-5, 2-7, and 2-9 showed the highest level of *AtPMEI* transcripts and proteins and a concomitant lower level of PME activity. The reduction of PME activity was likely due to the interaction between the overexpressed inhibitor(s) and endogenous enzyme(s). In gel-filtration experiments, the lack of detectable PME activity and the shift of the PME elution volume toward a higher  $M_r$  suggested that both PMEIs were engaged in the formation of a complex with endogenous PME. All three glycoforms of *AtPMEI-2* pres-

ent in the transformed plants may form a complex with endogenous PME, consistent with the previous observation that the fully glycosylated recombinant *AtPMEI-2*, expressed in *P. pastoris*, inhibits plant PMEs (Raiola et al., 2004). It is relevant that the two *N*-glycosylation sites are spatially located outside the contact interface identified in the PME/PMEI complex (Di Matteo et al., 2005).

PMEs interacting with *AtPMEI-1* and *AtPMEI-2* were identified as *AtPME3* (At3g14310), shown to be mainly expressed in *Arabidopsis* hypocotyls, leaves, and roots (Micheli et al., 1998; Pina et al., 2005; Louvet et al., 2006) and *At2g43050* mainly expressed in siliques and stems (Micheli et al., 1998; Pina et al., 2005; Louvet et al., 2006; see also the gene expression database: <http://csbdb.mpimp-golm.mpg.de/>). The finding that both PMEs were associated with both inhibitors is consistent with the large spectrum of recognition played by the inhibitors (Mattei et al., 2002; Ciardiello et al., 2004; Raiola et al., 2004; Di Matteo et al., 2005).

The DM in transformed plants was significantly higher than in wild-type plants. On the other hand, the total content of uronic acids of *AtPMEI-1* and *AtPMEI-2* plants was not significantly different from that of



**Figure 5.** Reduction of *B. cinerea* symptoms in *Arabidopsis* plants overexpressing *AtPMEI-1* and *AtPMEI-2*. A, Detached leaves from wild type and *AtPMEI* transformed lines (T) were inoculated with *B. cinerea* conidia and the lesion size was determined 3 d postinoculation. B, The average diameter of the expanding lesions is shown, and different letters indicate data sets significantly different at  $P < 0.01$  (LSD 0.01). The experiment was repeated three times with different plant batches, and statistical analysis of the results was performed by randomized-blocks ANOVA. [See online article for color version on this figure.]

wild-type plants, suggesting that the susceptibility of pectin to endogenous hydrolases is not affected. However, qualitative difference in the fine structure of pectins could be detected by enzymatic fingerprinting (Lerouxel et al., 2002), and the pattern of alteration was highly reproducible within the same class of independent transformants (i.e. *AtPMEI-1* or *AtPMEI-2*). Differences between *AtPMEI-1* and *AtPMEI-2* transformants suggest that the two inhibitors may differently influence PMEs acting on various pectic subdomains.

It is known that pectin methylesterification plays a crucial role in plant growth: it is maximal during the cell expansion phase and decreases as cell elongation ceases (Goldberg, 1984; McCann and Roberts, 1994). Transgenic plants whose specific PME activities were inhibited, using antisense mRNA, exhibited predictable effects on pectin chemistry but little or no alterations on plant growth (Tieman et al., 1992; Tieman and Handa, 1994), likely because the silenced PMEs are not involved in growth. Our transgenic *AtPMEI-1* or *AtPMEI-2* plants showed longer roots mainly due to an increase in cell elongation when seedlings were grown on agar in vertical position. The analysis of roots is considered an ideal system to study cell growth (Scheres et al., 2002), and our results are consistent with the notion that an increased DM promotes cell expansion and positively affects growth.

*AtPMEI-1* and *AtPMEI-2* are expressed in flowers and pollen, while *AtPMEI-1*, which shows a considerably higher expression level than *AtPMEI-2*, is also expressed in roots, as well as in seedlings, stems, and mature leaves (Wolf et al., 2003; Raiola et al., 2004). Due to the large number of PME members in Arabidopsis and to the specificity of their expression in different tissues and at different developmental stages, it is difficult to define the contribution of these inhibitors in the regulation of PME activity. However, the tissue distribution of PMEs in Arabidopsis (Supplemental Fig. S1) indicates a lower activity in flowers with respect to the other tissues, and this well correlates with the high expression level of the inhibitors in flower (Wolf et al., 2003; Raiola et al., 2004).

Methylesterification of pectin may correlate with a lesser accessibility to pectin-degrading enzymes and therefore with an increased resistance to pathogens (McMillan et al., 1993; Marty et al., 1997; Boudart et al., 1998). Arabidopsis plants expressing high level of *AtPMEI-1* or *AtPMEI-2* showed a reduced spreading of disease symptoms after inoculation with *B. cinerea* as compared with inoculated wild-type plants. Since endoPG prefer PGA rather than methylesterified pectin (Kars et al., 2005), it is likely that the higher DM of pectins of *AtPMEI* plants hampers the activity of endoPGs produced by this fungus, thus delaying its colonization of the plant tissue. EndoPGs are the main cell wall-deconstructing enzymes secreted by *B. cinerea*, and two isoenzymes (BcPG1 and BcPG2) are required for its virulence on several hosts (van Kan, 2006). On the other hand, fungal PME activity, which is not inhibited by *AtPMEI*s, is thought to facilitate the

activity of PG during plant infection. However, growth data in liquid cultures indicate that the fungal biomass on methylesterified pectin is lower than on PGA, suggesting that *B. cinerea* PME does not perform an efficient pectin demethylesterification. The possible induction of plant PMEs during fungal infection (Boudjeko et al., 2005; Abuqamar et al., 2006) can also be considered. In this case, the increase of PME content in transformed plants would be effective to impede the de-esterification of the pectic polymer by plant PME and, consequently, the accessibility of the fungal PG to its substrate.

A role of the DM of the cell wall pectins in plant disease resistance has been reported in several pathosystems. For example, highly methylesterified pectin has been related to resistance of potato (*Solanum tuberosum*) cultivars to Erwinia soft rot (McMillan et al., 1993; Marty et al., 1997). On the other hand, the DM and the distribution of methylesters along the pectic polymer can also influence the type of oligogalacturonides (OGs) released from the host cell wall. These, in turn, may affect the plant resistance to disease because of their elicitor activity. For example, pectic fragments with higher DM and superior elicitor activity have been reported to accumulate in bean (*Phaseolus vulgaris*) cultivars resistant to *Colletotrichum lindemuthianum* (Boudart et al., 1998). Also, a different methylester distribution was found in HGA of near-isogenic wheat (*Triticum aestivum*) lines resistant and susceptible to the stem rust fungus *Puccinia graminis* f. sp. *tritici*, and was considered to affect the type of OG elicitors released during the plant-fungus interaction (Wietholter et al., 2003). The possibility that *AtPMEI* affects the pattern of distribution of methyl esters within GalA polymer and, consequently, the type of OGs released and their elicitor activity remains to be investigated.

## MATERIALS AND METHODS

### Plant Growth

Arabidopsis (*Arabidopsis thaliana*) accession Columbia-0 was obtained from G. Redei and A.R. Kranz (Arabidopsis Information Service, Frankfurt). Arabidopsis plants were grown in a controlled environmental chamber maintained at 22°C, 70% relative humidity, with a 16-h photoperiod (100  $\mu\text{mol m}^{-2} \text{s}^{-1}$  fluorescent light). For vertical growth, seeds were sterilized and germinated in vertical position for 12 d on agar plates containing Murashige and Skoog medium (Sigma-Aldrich) containing 0.8% agar and 1% Suc with 16-h photoperiod (100  $\mu\text{mol m}^{-2} \text{s}^{-1}$  fluorescent light). Root elongation zones were microscopically examined, in vertically grown seedlings, using an Axiophot microscope (Carl Zeiss), and photomicrographs were taken using a Canon Powershot G3 camera. For MALDI-TOF MS analysis, seedlings were grown for 4 d in the dark to minimize environmental influences (Santoni et al., 1994; Lerouxel et al., 2002).

### Pathogen Growth and Infection

*Botrytis cinerea* strain SF1 obtained from Dr. S. Ferrari (University of Padua, Italy) was grown for 15 d on malt extract agar at 24°C and 12-h photoperiod before spore collection. Conidia at the density of  $5 \times 10^5$  conidia  $\text{mL}^{-1}$  were germinated in 12 g  $\text{L}^{-1}$  potato dextrose broth (Difco) at room temperature for 3 h. Fully developed leaves of 6-week-old Arabidopsis plants were detached

and placed in petri dishes with petioles embedded in 0.7% agar. Two droplets of spore suspension (5  $\mu\text{L}$  each) were placed on the surface of each leaf and incubated at 23°C and 12-h photoperiod and lesion diameter was measured after 3 d. The experiment was repeated three times with different plant batches, and statistical analysis of the results was performed by randomized-blocks ANOVA.

## Fungal Growth on Different Pectic Substrates

*B. cinerea* was cultured on Czapeck Dox medium amended as sole carbon source, either with 0.5% (w/v) PGA (Sigma) or 0.5% (w/v) lime pectin with 81% degree of methylesterification (E81; DANISCO) or cell walls (40 mg of dried material dispersed in 50 mL of medium) isolated from transformed and wild-type leaves. The flasks were inoculated with 1 mL of conidia ( $4 \times 10^5$  conidia  $\text{mL}^{-1}$ ) and incubated on a rotary shaker at 100 rpm at 23°C for 2 d. Mycelium was harvested by filtration through tared crucibles and oven-dried at 65°C to a constant weight. Three flasks were collected for each medium.

## Plant Transformation

For transgenic expression in Arabidopsis, the coding sequences of *AtPMEI-1* and *AtPMEI-2* were amplified from genomic DNA isolated from Arabidopsis seedlings with the NucleoSpin Plant kit (Macherey-Nagel) using Pfu DNA polymerase (Promega). The primers pairs used were *AtPMEI-1*/Fw (5'-TTG-TCCATGGCTGCGAATCTAAG-3') and *AtPMEI-1*/Rv (5'-CGGGAGCTCTT-AATTACGTGGTAACAT-3') for *AtPMEI-1*, and *AtPMEI-2*/Fw (5'-TTGTCC-ATGGCAGCATACCTGAC-3') and *AtPMEI-2*/Rv (5'-CGGGAGCTCTCAC-ATCATGTTGAGATGAC-3') for *AtPMEI-2*. The amplicons were subcloned between the *NcoI* and *SacI* sites (underlined in the above primer sequences) in the pJD301 plasmid (Luehrsen and Walbot, 1991) and sequenced. The cassettes, comprising the 35S promoter of *Cauliflower mosaic virus*, the  $\Omega$  leader of *Tobacco mosaic virus*, the *AtPMEI* open reading frame, and the nopaline synthase 3' sequence, were inserted into the *XbaI* site of the pCAMBIA 3300 vector (Cambia). Transgenic Arabidopsis plants were generated by *Agrobacterium tumefaciens*-mediated transformation as described previously (Clough and Bent, 1998). Following transformation, transgenic lines were selected in T1 generation on agarized Murashige and Skoog medium containing phosphinothricin (8 mg  $\text{L}^{-1}$ ). T2 lines resistant to phosphinothricin were selected for subsequent analysis.

## RNA Gel-Blot Analysis

Total RNA was extracted from rosette leaves of 6-week-old plants, using the Trizol reagent (Life Technologies) and following manufacturer's instructions. Samples of 12  $\mu\text{g}$  of total RNA were separated on a 1.2% agarose gel containing 1.2% formaldehyde and transferred onto Hybond-N+ membrane (Amersham). Prehybridization and hybridization reactions were performed at 45°C in the presence of DIG Easy Hyb solution (Roche Diagnostics). Filters were hybridized with a digoxigenin-labeled probe, incubated in CSPD (Roche Diagnostics), and exposed to x-ray film (X-Omat AR; Kodak). The probe used to detect the *AtPMEI-1* and *AtPMEI-2* mRNA was a cDNA corresponding to coding region of each *AtPMEI* gene labeled with digoxigenin-11-dUTP by PCR, using the following primers: Fw 5'-ATGGCTGCGAATCTAAG-3' and Rv 5'-TTAATTACGTGGTAACAT-3' for *AtPMEI-1*; and Fw 5'-ATGGCAGCA-TACCTGAC-3' and Rv 5'-TCACATCATGTTTGGATGAC-3' for *AtPMEI-2*. Specific UBQ5 probe was prepared as described (Rogers and Ausubel, 1997).

## Protein Purification and Analysis

Total protein extracts were obtained by homogenizing leaves of 6-week-old Arabidopsis plants in the presence of 1 M NaCl, 12.5 mM citric acid, 50 mM  $\text{Na}_2\text{HPO}_4[0]$ , pH 6.5 (1 mL per g of tissue). The homogenate was then shaken for 1 h at 4°C, centrifuged at 15,000g for 15 min, and the supernatant collected. Protein concentration was determined according to Bradford (1976) using Bio-Rad reagents and bovine serum albumin as standard. SDS-PAGE was performed as described by Laemmli (1970); for each sample, 9  $\mu\text{g}$  of protein extract was loaded into the gel. Immunoblot analysis was performed as described previously (Desiderio et al., 1997) using rabbit-specific antibodies raised against recombinant *AtPMEI-1* or *AtPMEI-2* purified to homogeneity (Raiola et al., 2004).

To partially purify the transgenic inhibitors, total proteins were precipitated with ammonium sulfate up to 80% saturation. The precipitate was suspended in 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, loaded onto a Superdex75 (HR10/30), eluted with the same buffer at a flow rate of 0.5  $\text{mL min}^{-1}$ ,

and fractions of 0.5 mL were collected. For comparison, *AtPMEI-1* and *AtPMEI-2* expressed in *Pichia pastoris* and purified to homogeneity were run under the same conditions. SDS-PAGE and immunoblot analysis were performed using rabbit-specific antibodies raised against recombinant *AtPMEI-1* and *AtPMEI-2*. To detect the presence of *AtPMEI-2* glycoforms, Superdex fractions containing the *AtPMEI-2* were boiled for 15 min and digested with 0.4 munits of *N*-glycosidase A (Roche Applied Science). The sample was run on SDS-PAGE and protein bands were detected by immunoblot analysis.

For LC-MS/MS analysis, 3 mg of total proteins isolated from *AtPMEI-1* and *AtPMEI-2* leaves was run on a Superdex75 preparative column (HR26/60) in the same buffer as above at a flow rate of 2  $\text{mL min}^{-1}$ , and fractions of 3 mL were collected and analyzed by immunoblot to select the fraction containing the inhibitor. The selected fractions were analyzed by SDS-PAGE and stained with Coomassie Brilliant Blue. After staining the protein bands were excised from the gel, reduced, alkylated, and digested with trypsin according to Hellman et al. (1995). Tryptic peptides were analyzed by LC-MS/MS (LC-ESI-quadrupole iontrap MS 1100 Series; Agilent Technologies) equipped with a column Zorbax SB-C18 5- $\mu\text{m}$  150  $\times$  0.5 mm. Protein identification was performed by searching the Mass Spectrometry Protein Sequence DataBase (Imperial College London) using the Mascot search engine, which uses a probability-based scoring system. The following parameters were used for database searches: peptide and MS/MS mass tolerance of 1.5 D; peptide charge of +1, +2, or +3; trypsin as digesting enzyme with one missed cleavage allowed; and carbamidomethylation of Cys as a fixed modification.

## Intercellular Washing Fluid Isolation

Intercellular washing fluids (IWFs) were collected from Arabidopsis leaves by centrifugation as described previously (Salvi et al., 1990) and according to Lohaus et al. (2001). Rosette leaves were excised from 6-week-old *AtPMEI-1* and *AtPMEI-2* plants. Leaves were stacked on a 105- $\mu\text{m}$  nylon mesh in the bottom of a 20-mL plastic syringe. The packed leaves were washed with 12.5 mM citric acid, 50 mM  $\text{Na}_2\text{HPO}_4$ , pH 6.5, for 5 min. Afterward, they were vacuum-infiltrated for 5 min with the same buffer. IWF was recovered by centrifuging the vacuum-infiltrated stems at 500g for 5 min at 4°C. The amount of IWF obtained from 1 g of tissue (fresh weight) was 0.2 to 0.3 mL. Contamination of IWFs by cytoplasmic components was ruled out by measuring Glc-6-P dehydrogenase activity according to Takahama (1993), which accounted for less than 1% of total extractable activity.

## Determination of PME Activity

PME activity was quantified by the radial gel diffusion assay as described by Downie et al. (1998) with some modifications. A gel was prepared with 0.1% (w/v) of 81% methylesterified lime pectin (E81; Danisco A/S), 1% (w/v) agarose, 12.5 mM citric acid, and 50 mM  $\text{Na}_2\text{HPO}_4$ , pH 7.0. The gel was cast into agar plates (13 mL per plate) and allowed to polymerize at room temperature. Wells with a diameter of 4 mm were made and the protein samples (2  $\mu\text{g}$  in 20  $\mu\text{L}$ ) were loaded in each well. Plates were incubated at 30°C for 16 h. The gels were stained with 0.05% (w/v) ruthenium red for 45 min, destained with water, and the diameter of the red-stained areas, resulting from the hydrolysis of esterified pectin in the gel, was measured. PME activities were determined in protein extracts from rosette leaves carefully chosen at the same stage of development isolated by 6-week-old *AtPMEI-1*, *AtPMEI-2*, and wild-type plants. Each value was obtained from five independent biological replicates. PME activity of *B. cinerea* was determined by agar diffusion assay by testing aliquots of culture filtrate after 3 d of fungal growth in liquid Czapeck Dox medium containing 0.5% (w/v) commercial apple pectin (Sigma-Aldrich) as the sole carbon source. A standard curve was prepared using commercial orange peel PME (Sigma-Aldrich) showing a log-linear relationship between the stained zone diameter and the activity of PME applied to the well within a PME activity range going from 0.001 units (staining zone diameter = 5 mm) to 5 units (staining zone diameter = 25 mm). PME activity was calculated based on this standard curve.

## Isolation of Cell Walls and Determination of the Degree of Methylesterification

Leaves were frozen in liquid nitrogen and homogenized using a Retschmill machine (model MM200; Retsch) at 25 Hz for 1 min. The ground tissue was washed twice in 70% ethanol, vortexed, and pelleted by centrifugation at 10,000g for 10 min. The pellet was suspended with a chloroform:methanol



mixture (1:1, v/v). After centrifugation and evaporation of the solvent, 1 mg of sample was saponified and suspended in 0.25 M NaOH. The solution was incubated at room temperature for 1 h and afterward neutralized with HCl. After centrifugation at 10,000g, aliquots of the supernatant (20  $\mu$ L) were loaded in microtiter plate (96-well cod.9018 from Costar) filling up with water to a total volume of 50  $\mu$ L. Alcohol oxidase (50  $\mu$ L) was added to each well (0.03 units in 0.1 M sodium phosphate, pH 7.5; Sigma), and this mixture was incubated at room temperature for 15 min on shaker. Thereafter, 100  $\mu$ L 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 absorbance was measured at 412 nm in microplate reader (ETI-System Reader; Sorin Biomedica Cardio S.p.A.). The methanol content was estimated as the amount of formaldehyde produced from methanol by alcohol oxidase, according to Klavons and Bennett (1986), by comparison with a standard calibration curve.

For the determination of uronic acid content, the saponified samples were treated with 200  $\mu$ L of 2 M TFA for 1 h at 121°C in screw-cap tube properly closed. After washing three times with 2-propanol, uronic acids were quantified by colorimetry using the automated sulfamate/*m*-hydroxy diphenyl assay (Filisetti-Cozzi and Carpita, 1991). Uronic acid concentration was estimated by comparison with a standard calibration curve using D-GalUA. The degree of methylesterification was expressed as methanol to uronic acid molar ratio (%).

### Oligosaccharide Mass Profiling MS Analysis

Cell walls were isolated from etiolated seedlings as above described. Thirty or 20 independent samples, each originating from 10 individual seedlings, were used for wild type or each transformed line, respectively. Fifty millimolar ammonium formate, pH 4.5, containing 0.02 units of xyloglucan-specific endoglucanase (Pauly et al., 1999) or a combination of 0.05 units of PME from *Aspergillus aculeatus* and 0.15 units of endoPG from *Aspergillus niger* (Megazyme), was added to cell walls and incubated for 17 h at 37°C. The combination of these pectic enzymes released methylesterified or *O*-acetylated HGA fragments from cell walls (Obel et al., 2006). After digestion, the suspension was centrifuged and the entire supernatant, containing the solubilized oligosaccharides, was dried in a speed-vac concentrator.

MALDI-TOF MS of the pectic oligosaccharides released after enzymatic digestions were recorded on a Voyager DE-Pro MALDI-TOF MS (Applied Biosystems) in positive mode with an acceleration voltage of 20,000 V and a delay time of 350 ns. Mass spectra were obtained in the reflectron mode using 2,5-dihydroxybenzoic acid (10 mg mL<sup>-1</sup>) as matrix mixed with the solubilized sugars 1:1 (v/v). The spectra were manually recorded and output-data files were analyzed, compared, and statistically evaluated by a Student's *t* test using the PERL program (Lerouxel et al., 2002). Pectic fragments were not detected in cell wall preparation without enzymes, and no detectable pectic fragments were observed by replacing the two pectic enzymes with endoPG alone (data not shown). In addition, pectic fragments generated from cell walls of Arabidopsis plant transformed with pCambia 3300 plasmid alone showed no significant differences with respect to the wild type. Ions having 42 additional mass units were assigned to fragments containing *O*-acetyl substitutions on the Gal residues.

### Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Quantification of PME activity in different tissues from wild-type plants with radial gel diffusion assay.

**Supplemental Figure S2.** Immunodetection of AtPMEI-1 and AtPMEI-2 in transformed leaf extracts after gel filtration.

**Supplemental Table S1.** Sequences of tryptic fragments obtained by LC-MS/MS analysis.

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