Pectin Methylesterase Is Induced in *Arabidopsis* upon Infection and Is Necessary for a Successful Colonization by Necrotrophic Pathogens

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The ability of bacterial or fungal necrotrophs to produce enzymes capable of degrading pectin is often related to a successful initiation of the infective process. Pectin is synthesized in a highly methylesterified form and is subsequently de-esterified in muro by pectin methylesterase. De-esterification makes pectin more susceptible to the degradation by pectic enzymes such as endopolygalacturonases (endoPG) and pectate lyases secreted by necrotrophic pathogens during the first stages of infection. We show that, upon infection, *Pectobacterium carotovorum* and *Botrytis cinerea* induce in *Arabidopsis* a rapid expression of *AtPME3* that acts as a susceptibility factor and is required for the initial colonization of the host tissue.

The plant cell wall, the first line of defense against fungal and bacterial pathogens, is mainly composed of a cellulose-hemicellulose strength-conferring network embedded in a cohesive pectin matrix cross-linked to lignin and proteins by ionic and covalent bonds. Although the infection strategies may largely vary from biotrophic to necrotrophic pathogens, the majority of them have evolved the ability to cleave the structural polymers of the cell wall and release nutrients to sustain their growth and tissue colonization. Although extensive tissue maceration is a typical feature of necrotrophs that secrete large quantities of cell-wall-degrading enzymes (CWDE), plant degradation is also operated by biotrophs that secrete less CWDE (Glazebrook 2005).

The correlation between cell wall disassembly, production of CWDE, and disease symptoms has been extensively studied (Casasoli et al. 2009; van Kan 2006); in general, many virulence-associated CWDE are involved in digestion of pectin. For example, the inactivation of endopolygalacturonase (endoPG) genes in *Botrytis cinerea*, *Alternaria citri*, and *Aspergillus flavus* and in the bacterial pathogen *Xylella fastidiosa* reduced their virulence on host plants (Isschiki et al. 2001;

Roper et al. 2007; Shieh et al. 1997; ten Have et al. 1998). Pectic enzymes are invariably the first CWDE to be secreted by fungi grown on cell wall material, followed by cellulases and hemicellulases (De Lorenzo et al. 1997, 2001). Pectin depolymerization weakens the entire cell wall and exposes other polymers to microbial-associated cellulases and hemicellulases (Berlin et al. 2007; Lionetti et al. 2010; Walton 1994).

A main component of pectin is homogalacturonan (HGA), a linear polymer of α -1,4-linked galacturonic acid that is synthesized and secreted into the cell wall in a highly methylesterified form and, soon thereafter, de-esterified in muro by pectin methylesterases (PME) (EC 3.1.1.11). De-methylesterification produces free carboxyl groups, modifies the pH of the wall, and allows the aggregation of polyuronides into calcium-linked gel structures, thus affecting the porosity of the wall (Willats et al. 2001). De-esterification makes HGA susceptible to the degradation operated by pectic enzymes such as endoPG and pectate lyases (PEL) (Limberg et al. 2000). Genetic and molecular evidence indicates the critical role of pectin esterification in plant defense and is consistent with the concept that the pectin structure may determine the outcome of the interaction between a given host and a pathogen (Hahn et al. 1989). For example, potato genotypes resistant to *Erwinia* spp. have a degree of pectin methylesterification (DM) higher than that of susceptible genotypes (Marty et al. 1997). The DM of pectin in bean cultivars resistant to *Colletotrichum lindemuthianum* is higher than in near-isogenic susceptible lines (Boudart et al. 1998). Similarly, *Arabidopsis* plants overexpressing a pectin methylesterase inhibitor (PMEI) exhibit a higher pectin DM and are more resistant to *B. cinerea* (Lionetti et al. 2007).

The fungus *B. cinerea* and the bacterium *Pectobacterium carotovorum* (formerly *Erwinia carotovora* subsp. *carotovora*) are two necrotrophic pathogens that cause serious losses in a number of crop species. They typically exhibit a coordinated production of large quantities of pectic enzymes: two of the six endoPG genes are virulence factors of *B. cinerea* (ten Have et al. 1998) while one endoPG gene (*pehA*) and at least four PEL genes (*pelA* to *pelD*) are virulence factors of *P. carotovorum* (Flego et al. 2000). Because endoPG and PEL genes cannot efficiently depolymerize highly methylated pectins, a previous de-esterification by PME may facilitate the action of these enzymes. We show here that *AtPME3* is induced in *Arabidopsis* by both pathogens and is required for the colonization of the host tissue.

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Fig. 1. Expression of *AtPME3* in wild-type (WT) plants in response to pathogens. **A**, Expression of *AtPME3* in rosetta leaves after inoculation with *Botrytis cinerea*. **B**, Expression of *AtPME3* in rosetta leaves after inoculation with *Pectobacterium carotovorum*. mRNA expression levels were evaluated by quantitative real-time reverse-transcription polymerase chain reaction in infected and mock-inoculated rosetta leaves from WT plants harvested at the indicated hours postinoculation (hpi). Fold change was calculated from transcript levels in pathogen inoculated leaves compared with mock-inoculated controls. Data are the average $\pm$ standard deviation of three independent experiments, each consisting of three technical replicates. Mean values significantly different from control according to Student's *t* test ($P < 0.01$) are indicated by an asterisk.

RESULTS

Expression of *AtPME3* is induced upon infection and is required for tissue colonization by *B. cinerea* and *P. carotovorum*.

The expression of *AtPME3* (At3g14310), a gene encoding an isoform of PME that, in *Arabidopsis*, is under control of specific inhibitors called PME1 (Lionetti et al. 2007; Raiola et al. 2004), was quantified after inoculation of *Arabidopsis* leaves with *B. cinerea* or *P. carotovorum*. Quantitative real-time polymerase chain reaction (PCR) was performed using gene-specific primers designed to discriminate between the different *PME* genes. A significant and steady increase of *AtPME3* transcript levels was observed up to 72 h postinoculation (hpi) upon *B. cinerea* infection (Fig. 1A). A significant transient increase of *AtPME3* expression was also observed at 14 hpi upon inoculation with *P. carotovorum* (Fig. 1B).

In order to investigate the functional significance of the *AtPME3* induction during infection and how the encoded enzyme influences the outcome of the interactions between *Arabidopsis* and *B. cinerea* or *P. carotovorum*, an *AtPME3* knock-out mutant (*pme3*) was characterized and subjected to pathogenicity tests. An *AtPME3* insertional mutant was identified in the Gabi-Kat mutant collection (Gabi-Kat line 00210) and its sequence analysis revealed a T-DNA insertion located in the first of the three exons at 242 nucleotides downstream from the start codon. Reverse-transcription (RT)-PCR analysis of mRNA levels in *pme3* homozygous plants failed to detect any transcript after a high number of PCR amplification cycles

Fig. 2. *AtPME3* expression influences pectin methylesterification of the cell walls. **A**, *AtPME3* mRNA level in wild-type (WT), *pme3* heterozygous, and *pme3* homozygous (*pme3*) plants. **B**, Quantification of pectin methylesterase (PME) activity by radial gel diffusion assay. PME activity of the WT leaves (0.013 units) was set to 100%. Values were reported as percentage of PME activities in mutant lines relative to WT. Different letters indicate data sets significantly different according to analysis of variance followed by Tukey's test ($P < 0.001$). Data represent average $\pm$ standard deviation of six replicates. **C**, Quantification of degree of pectin methylesterification (DM) in cell walls extracted from leaves of *pme3* and WT plants. Asterisk indicates data sets significantly different according to Student's *t* test ($P < 0.001$). Values represent the average $\pm$ standard error of six replicates. Analysis was repeated twice with similar results. **D**, Immunodot analysis of Chelating agent soluble solid (ChASS) fraction extracted from leaves of *pme3* and WT plants. Indicated amounts of ChASS fractions were applied at a single point to a nitrocellulose membrane. Specific homogalacturonan epitopes were detected using PAM1 and JIM5 monoclonal antibodies.

(Fig. 2A). By contrast, specific amplifications were detected in both *pme3* heterozygous and wild-type (WT) plants. Significantly, disruption of *AtPME3* resulted in a 50% reduction of the overall PME activity in the *pme3* homozygous plants whereas no significant differences in PME activity were observed in *pme3* heterozygous plants compared with WT plants (Fig. 2B). A significant increase of DM of approximately 40% was detected in the cell wall pectin extracted from rosette leaves of *pme3* plants compared with WT plants (Fig. 2C). Immunodot assays using the monoclonal antibodies PAM1, which specifically recognize large (at least 30 continuous galacturonic acid units) demethylesterified blocks of HGA (Willats and Knox 1999), revealed a lower level of PAM1-binding epitopes in *pme3* plants compared with WT plants (Fig. 2D). Immunodot assays using JIM5 antibodies, which bind pectin with a low DM (Willats et al. 2000), consistently showed that *pme3* plants have a higher DM of HGA compared with the WT plants (Fig. 2D). The monosaccharide composition of cell walls from leaves of *pme3* plants compared with WT plants showed a substantial similarity, with slight but not significant reduction of rhamnose and uronic acids and a concomitant slight but not significant increase of xylose (Table 1) Cellulose-derived glucose (percentage of micrograms of glucose per milligram of cell-wall material $\pm$ standard deviation with respect to WT) of *pme3*, *AtPMEI-1*, and *AtPMEI-2* plants was 109 ± 32 , 108 ± 21 , and 110 ± 31 , respectively, and the values were not significantly different.

Susceptibility of *pme3* mutants to *B. cinerea* and *P. carotovorum* was assessed by inoculating leaves with fungal conidia or bacterial cell suspensions and subsequently evaluating symptoms 3 days and 16 h postinoculation, respectively. No differences in the ratio between the number of expanding lesions and the number of inoculated spots were observed

among transformed lines and WT plants (data not shown). Instead, *pme3* homozygous plants displayed reduced symptoms compared with *pme3* heterozygous (data not shown) and WT plants (Fig. 3A to D). This indicates that both fungal and bacterial colonization is reduced on the tissue of *pme3* plants, in agreement with our previous observation showing that *Arabidopsis* plants overexpressing *PMEI*, which drastically reduce the activity of *AtPME3*, exhibit an increased resistance to *B. cinerea* (Lionetti et al. 2007; Raiola et al. 2004). Similarly, pathogenicity tests performed by inoculating *P. carotovorum* on plants overexpressing *AtPMEI-1* or *AtPMEI-2* showed a lower susceptibility of the transformed plants, characterized by a reduced lesion area and a reduced bacterial growth compared with WT plants (Fig. 4). Instead, no differences in the ratio between the number of expanding lesions and the number of inoculated spots were observed among transgenic and WT plants. All in all, our experiments indicate that plants having a more methylated form of HGA, caused by either the lack of expression of *AtPME3* or the overexpression of the *PMEI* inhibitors, are less susceptible to *B. cinerea* and *P. carotovorum* which, consequently, have a reduced capability of invading their tissue. Both pathogens induce in *Arabidopsis* a rapid induction of the *AtPME3* expression and, by doing so, force the plant to cooperate in disease (Hok et al. 2010).

Reduced susceptibility of *pme3* plants correlates with an impaired ability of pathogens to grow on methylesterified pectin.

To assess whether the reduced susceptibility of *pme3* plants observed in our tests is a consequence of the constitutive activation of defense responses, leaves were stained with 3-3'-diaminobenzidine (DAB) that reveals the accumulation of H_2O_2 . We also measured H_2O_2 accumulation in leaves of plants over-

Fig. 3. *Botrytis cinerea* and *Pectobacterium carotovorum* symptoms were reduced in *pme3* plants. **A** and **B**, Detached leaves from wild-type (WT) and *pme3* plants were inoculated with *B. cinerea* conidia and lesion area was determined 72 h postinfection. Bar represents the average lesion area $\pm$ standard error (SE) of 24 expanding lesions. Similar results were obtained from three independent experiments. **C** and **D**, Detached leaves were inoculated with *P. carotovorum* cells and lesion area was measured 16 h postinoculation. Bar represents the average lesion area $\pm$ SE of 12 expanding lesions. Similar results were obtained from three independent experiments. Asterisk indicates data sets significantly different, according to Student's *t* test ($P < 0.01$). Bacterial growth in planta was quantified by determining the number of bacteria isolated from leaves by plate count. Average of logarithm of CFU per leaf $\pm$ standard deviation are the following : WT, 6.92 ± 0.09 ; *pme3*, 6.43 ± 0.10 . Data are significantly different according to Student's *t* test ($P < 0.01$).

expressing *AtPMEI-1* or *AtPMEI-2* that also display a reduced susceptibility to *B. cinerea* and *P. carotovorum*. No significant staining was observed in all cases (Supplementary Fig. S1A). Moreover, leaves of *pme3* plants as well as those of plants overexpressing *AtPMEI-1* or *AtPMEI-2* did not exhibit any fluorescence when illuminated with UV light, indicating no accumulation of secondary metabolites (data not shown). It was concluded that the observed reduced susceptibility to *B. cinerea* and *P. carotovorum* likely does not depend on a constitutive activation of defense responses.

To determine whether the *pme3* mutant as well as plants overexpressing *AtPMEI-1* and *AtPMEI-2* are characterized by an enhanced activation of the salicylic acid (SA) (Ferrari et al. 2003; Zimmerli et al. 2001) and jasmonic acid-ethylene (JA/ET) pathways (Glazebrook 2005), we measured, upon infection, the levels of gene *PR1* (i.e., a marker for SA-dependent defense responses) and gene *PDF1.2*, encoding a plant defensin used as indicator for the activation of the JA/ET pathway (Penninckx et al. 1996). We also analyzed the expression of the *WR3* gene encoding a high-affinity nitrate transporter involved in the JA-independent wound signal transduction (Rojo et al. 1999) and of the *JR1* gene involved in the JA-dependent wound signal transduction (Titarenko et al. 1997). Moreover, we measured the expression of the *PAD3* gene that encodes a putative cytochrome, P450 monooxygenase, required for camalexin biosynthesis (Glawischnig et al. 2004) and shown to be induced by elicitors and infection by *B. cinerea* (Ferrari et al. 2003; Galletti et al. 2008). Expression of *PR1*, *PDF1.2*, *WR3*, *JR1*, and *PAD3* in the *pme3* mutant and in plants overexpressing *AtPMEI-1* (or *AtPMEI-2*) (data not shown) was similar to that of WT plants, being the different genes induced at different times and levels by the two pathogens (Fig. 5).

Because growth of *B. cinerea* is reduced when the fungus is cultured on methylated pectins rather than on unmethylated HGA (Lionetti et al. 2007), to ascertain whether the DM of pectin also affects the growth of *P. carotovorum*, the bacterium was cultured in liquid synthetic media containing unmethylated polygalacturonic acid (PGA) as a sole carbon source or E81 and P60 pectins with DM of 81 and 60%, respectively. After 10 h of culture, *P. carotovorum* had grown significantly better on PGA (optical density at 600 nm [OD₆₀₀] 0.086 ± 0.0014) than on E81 (OD₆₀₀ 0.043 ± 0.0035) or P60 (OD₆₀₀ 0.040 ± 0.0035), indicating that demethylated or less-methylated pectins, as in the case of *B. cinerea* (Lionetti et al. 2007),

are better substrates for the bacterium. No PME activity was detected in the three different culture filtrates of *P. carotovorum* (not shown), in agreement with the report that PME activity is not produced by *P. carotovorum* grown on synthetic media or on pepper and carrot tissues (El-Hendawy et al. 2002). *P. carotovorum* was also grown on liquid medium containing cell wall material isolated from leaves of *pme3* plants and of plants overexpressing *AtPMEI-1* and *AtPMEI-2*. After 12 h of culture, the growth of the bacterium was reduced of approximately 60% compared with the growth in cultures containing cell wall material from WT plants (Fig. 6).

DISCUSSION

Microorganisms usually produce pathogen-derived effectors as a strategy to successfully attack and invade plant tissues but also utilize host-derived molecules that favor the pathogenicity process. To overcome the plant cell wall barrier and render the structure more prone to degradation, some microorganisms force the host enzyme machinery to work in concert with their own arsenal of hydrolytic enzymes. For example, this may be the case with *PMR6*, a putative pectate lyase, and *PMR5* that affects pectin composition; both genes are required for susceptibility of *Arabidopsis* to some powdery mildew species (Vogel et al. 2002, 2004). In *Arabidopsis*, the pectin methylesterase activity is required for susceptibility to *B. cinerea* (Lionetti et al. 2007) and, in tomato, the cooperative action of polygalacturonase (LePG) and expansin (LeExp1) facilitates the fruit-ripening-associated susceptibility to *B. cinerea* (Cantu et al. 2008).

In this article, we have identified a host enzyme, AtPME3, as a key factor of the interaction of *Arabidopsis* with the necrotrophs *B. cinerea* and *P. carotovorum*. Upon infection, the enzyme is induced by both pathogens, while its targeted suppression in planta reduces the pathogen growth and the plant susceptibility to necrotrophs. Expression profile studies indicate that *AtPME3* is ubiquitously expressed in *Arabidopsis*

Table 1. Percentage of neutral sugars and uronic acid composition in cell walls extracted from rosette leaves of *pme3* and *Arabidopsis thaliana* pectin methylesterase inhibitor (*AtPMEI*) plants compared with the wild type (WT)^a

Monosaccharide	Transformant mol%/WT mol% × 100		
	<i>pme3</i>	<i>AtPMEI-1</i>	<i>AtPMEI-2</i>
Rha	74 ± 26	94 ± 38	99 ± 20
Fuc	126 ± 18	96 ± 15	104 ± 11
Ara	112 ± 19	109 ± 33	103 ± 34
Xyl	115 ± 13	92 ± 19	107 ± 11
Man	103 ± 19	81 ± 16	100 ± 22
Gal	110 ± 16	97 ± 7	105 ± 13
Glc	102 ± 36	105 ± 25	95 ± 18
UA	86 ± 24	106 ± 29	95 ± 12

^a Molecular percentage of *pme3* and *AtPMEI* cell wall monosaccharides was normalized with respect to WT. Data represent percent cell wall glycosyl residue composition ± standard deviation (WT *n* = 8, *pme3* *n* = 11, *AtPMEI* *n* = 6). Rha, rhamnose; Fuc, fucose; Ara, arabinose; Xyl, xylose; Man, mannose; Gal, galactose; Glc, glucose; UA, uronic acids. Differences in monosaccharides of *pme3* and *AtPMEI* plants compared with WT were not statistically different according to analysis of variance followed by Tukey's test.

Fig. 4. *Pectobacterium carotovorum* symptoms were reduced in *Arabidopsis* plants overexpressing *AtPMEI-1* or *AtPMEI-2* (*AtPMEI-1* or *AtPMEI-2* plants). Bar represents the average lesion area ± standard error of 12 expanding lesions determined 16 h postinoculation. The experiment was repeated three times with similar results. Different letters indicate data sets significantly different according to analysis of variance (ANOVA) followed by Tukey's test (*P* < 0.01). Bacterial growth in planta was quantified by determining the number of bacteria isolated from leaves by plate count. Average of logarithm of CFU per leaf ± standard deviation are the following: wild-type (WT), 6.98 ± 0.08; *AtPMEI-1*, 6.62 ± 0.07; *AtPMEI-2*, 6.48 ± 0.05. Reduction of bacterial growth on *AtPMEI-1* and *AtPMEI-2* leaves with respect to WT leaves is significantly different according to ANOVA followed by Tukey's test (*P* < 0.01).

rather than being involved in specific developmental processes; the enzyme belongs to a class of housekeeping PME mainly involved in the maintenance of the wall integrity and cell-to-cell cohesion (Micheli et al. 1998).

The induction of *AtPME3* observed in *Arabidopsis* leaves in response to *B. cinerea* and *P. carotovorum* is probably due to the presence of regulatory elements in the promoter region of the gene (NIAS DNA bank) (Higo et al. 1999) (Table 2). These include several consensus-binding sites for GT-1-like factors that influence the SA-inducible gene expression (Buchel et al. 1999); a number of type II MYB consensus sequences involved in the response to pathogens, wounding, and elicitors (Jin and Martin 2000; Rushton and Somssich 1998; Sugimoto et al. 2000); W boxes (TTGAC) often found upstream of pathogen-

responsive genes (Rushton and Somssich 1998; Singh et al. 2002); and a RAV1 element (CAACA) (Sohn et al. 2006) shown to be functionally activated by the CARAV1 transcription factor in pepper after bacterial infection (An et al. 2009). In *Arabidopsis*, *AtPME3* is induced not only by *B. cinerea* and *P. carotovorum* but also by the other necrotroph, *Alternaria brassicicola* (Narusaka et al. 2005). Moreover, *AtPME3* is induced by JA (Jung et al. 2007) and is not induced by *B. cinerea* in *ein2* and *coi1* *Arabidopsis* mutants, defective in JA/ET signaling pathways (Abuqamar et al. 2006). All this evidence suggests a link between *AtPME3* induction, activation of the JA signaling pathway, and infection by necrotrophs.

Mutants lacking a functional *AtPME3* gene (*pme3* plants) have less PME activity and, subsequently, a high level of pec-

Fig. 5. Time course of *PDF1.2*, *PR1*, *PAD3*, *JRI*, and *WR3* expression upon *Botrytis cinerea* and *Pectobacterium carotovorum* infection in wild-type (WT), *pme3*, and AtPMEI-1 plants. Gene induction was assayed in WT (white bars), *pme3* (light gray bars), and AtPMEI-1 (dark gray bars) plants at the indicated hours postinoculation. Expression was assayed by quantitative reverse-transcription polymerase chain reaction. Fold change following infection was relative to mock sample. Bars represent the average $\pm$ standard deviation of three replicates. This experiment was repeated twice with similar results.

tin esterification. Consistently, a high DM has been observed in plants overexpressing *AtPMEI-1* or *AtPMEI-2* that efficiently inhibit *AtPME3* (Lionetti et al. 2007). The monosaccharide composition of cell walls from *pme3* plants, from plants overexpressing *AtPMEI-1* or *AtPMEI-2*, and from WT plants is substantially very similar (Table 1 and 2). This indicates that, in all three cases, it is the high DM of pectin itself and not the sugar composition of the cell wall responsible for the less-susceptible plant phenotype. *B. cinerea* and *P. carotovorum*, cultured in the presence of cell walls from *pme3* and *AtPMEI*-overexpressing plants as sole carbon sources, grew slowly, indicating that the reduced susceptibility is probably related to impaired ability of these microorganisms to metabolize or use a highly methylated pectin. On the other hand, pectin methyl-esterase activity has been reported in some cases to affect the resistance to pathogens through the activation of defense responses. For example, wild strawberry plants overexpressing *FaPEI*, a fruit-specific PME isoform, are more resistant to *B. cinerea*, likely due to the release of oligogalacturonides that constitutively activate defense responses (Osorio et al. 2008). In our case, instead, the absence of accumulation of UV-fluorescent metabolites or H_2O_2 in unchallenged *pme3*- or *AtPMEI*-overexpressing plants indicates that resistance is not dependent on a constitutive activation of defense responses. Moreover, the absence of enhanced activation of *PAD3*, *WR3*, *JR1*, *PR1*, and *PDF1.2* upon infection indicates that resistance is not caused by a better perception of the pathogens and, at the same time, does not involve the synthesis of camalexin and is independent of defense pathways induced by wounding, SA, and JA/ET.

Therefore, *AtPME3* is per se a susceptibility factor required for rapid colonization of the host tissue by *B. cinerea* and *P. carotovorum* because its activity decreases the DM of pectin. Evidence that the *AtPME3* transcripts are higher upon inoculation of *Arabidopsis* with *A. brassicicola* rather than with *A. alternata* is noteworthy (Narusaka et al. 2005) and supports the notion that this enzyme may act as a susceptibility factor and facilitate infection. It is known that, whereas *Arabidopsis* is a host of *A. brassicicola*, it is, instead, a nonhost of *A. alternata* (Narusaka et al. 2005).

The identification of PME isoforms involved in pathogen susceptibility opens new possibilities for selecting plant cultivars more resistant to necrotrophs.

Fig. 6. Reduction of bacterial growth on cell walls isolated from transformed *AtPMEI-1*, *AtPMEI-2*, and *pme3* plants. Bars represent relative percentage of growth and different letters indicate data sets significantly different ($P < 0.01$) according to analysis of variance followed by least significant difference range test. Number of bacterial colonies was determined by 1,000-fold dilution from three independent experiments, with $n = 3$.

MATERIALS AND METHODS

Plant growth.

Arabidopsis thaliana Columbia-0, WT plants, or transgenic lines overexpressing *AtPMEI-1* (line 1-1) or *AtPMEI-2* (line 2-7) or *pme3* mutants were grown in a growth chamber maintained at 22°C and 70% relative humidity, with a 12-h photoperiod (100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ fluorescent light).

Bacterial growth and infection.

P. carotovorum subsp. *carotovorum* (formerly *E. carotovora* subsp. *carotovora*) was obtained from DSMZ GmbH Germany (strain DSMZ 30169). After growth in Luria-Bertani (LB) liquid medium, bacteria were suspended in 50 mM potassium phosphate buffer (pH 7.0) and inoculated at 5×10^7 CFU/ml. In each experiment, 12 fully developed leaves were detached from four 4-week-old *Arabidopsis* plants (3 leaves/plant), placed on a moist filter paper in petri dishes, inoculated with 5 μl of the bacterial suspension, and maintained at 22°C and a 12-h photoperiod. Symptoms were scored by measuring the area of macerated tissue after 16 hpi. Areas were measured by ImageJ software (W. S. Rasband, ImageJ; United States National Institutes of Health, Bethesda, MD, U.S.A.). The experiment was repeated three times with different plant batches, and statistical analysis of the results was performed by one-way analysis of variance (ANOVA) followed by Tukey's or Student's range tests.

Quantification of the bacterial growth after 16 hpi was determined using the procedure previously described (Katagiri et al. 2002).

Fungal growth and infection.

B. cinerea, strain SF1 (Lionetti et al. 2007), was grown for 15 day on malt extract agar at 24°C with a 12-h photoperiod before spore collection. Conidia at the density of 5×10^5 conidia ml^{-1} were germinated in potato dextrose broth (Difco Laboratories, Detroit) at 12 g liter^{-1} and room temperature for 3 h. In each experiment, 12 fully developed leaves were detached from four 6-week-old *Arabidopsis* plants (3 leaves/plant) and placed in petri dishes with petioles embedded in 0.7% agar. Two droplets of spore suspension (5 μl each) were placed on the surface of each leaf and incubated at 23°C with a 12-h photoperiod and lesion diameter was measured after 3 days.

The experiment was repeated three times with different plant batches, and statistical analysis of the results was performed by one-way ANOVA followed by Tukey's or Student's range tests.

Table 2. Putative pathogen-related *cis*-acting elements in the *AtPME3* promoter

Category	<i>cis</i> element name	Sequence	Position
GT sites	GT-1 SCAM4	GRAAAA	-110/-115
	...	...	-429/-434
	...	...	-667/-672
	...	...	-442/-447
Myb sites	MYB1AT	WAACCA	-490/-495
	MYB2AT	TAACTG	-263/-268
	...	...	-415/-420
	MYBCORE	CNGTTR	-263/-268
	...	...	-277/-282
WBox	...	...	-553/-557
	WBOX	TTGAC	-775/-779
	...	...	-589/-593
RAV	RAV1	CAACA	-758/-762

Alcohol-insoluble solids extraction and determination of the degree of methylesterification.

Alcohol-insoluble solids (AIS) were obtained as previously described (Lionetti et al. 2010). AIS (1 mg) were saponified by suspending them in 0.25 M NaOH. The solution was incubated at room temperature for 1 h and afterward neutralized with HCl. After centrifugation at $10,000 \times g$, aliquots of the supernatant (20 μ l) were loaded in microtiter plates (96-well cod.9018 from Costar, Cambridge, MA, U.S.A.) and filled with water to a total volume of 50 μ l. Alcohol oxidase (50 μ l) was added to each well (0.03 units in 0.1 M sodium phosphate, pH 7.5) (Sigma, St. Louis), and this mixture was incubated at room temperature for 15 min on a shaker. Thereafter, 100 μ 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 a microplate reader (ETI-System reader; Sorin Biomedica Cardio S.p.A., Saluggia, Italy). The amount of methanol was estimated as described (Klavons and Bennett 1986). Uronic acid content in saponified AIS was quantified colimetrically using the automated sulfamate/m-hydroxy diphenyl assay (Filisetti-Cozzi and Carpita 1991) with galacturonic acid (Fluka 48280) as standard. The degree of methylesterification was expressed as methanol to uronic acid molar ratio (%).

Monosaccharides composition.

AIS (approximately 2 mg) were treated two times with α -amylase (Sigma A6380) in 0.1 M phosphate buffer (pH 7.0) at 37°C overnight (Zabackis et al. 1995). The material was washed one time with buffer, followed by removal of the methyl groups from the PGA with 0.1 M NaOH at 7°C overnight in order to improve hydrolysis of the pectin. Aliquots were neutralized with 0.1 M HCl and the cell wall material was washed one time with water and one time with acetone before drying. The destarched cell-wall material was hydrolyzed with 2 M trifluoroacetic acid (TFA) for 1 h at 121°C. The released sugars were converted into their alditol acetates according to Englyst and Cummings (Englyst et al. 1982). Analysis of alditol acetates was carried out by gas-liquid chromatography using an Agilent Technologies 7890A GC system equipped with a J&W DB-225MS capillary column (15 m by 0.25 mm, 0.25- μ m film thickness) (Agilent Technologies, Santa Clara, CA, U.S.A.) with the following temperature program: 80°C for 2 min, followed by a linear gradient of 80 to 170°C at 30°C/min, then 170 to 240°C at 4°C/min. The alditol acetates were detected using a flame ionization detector. The uronic acids were determined by a biphenyl assay (Filisetti-Cozzi and Carpita 1991) with galacturonic acid (Fluka 48280) as standard. The crystalline cellulose remaining in the pellet after TFA hydrolysis of the cell wall powder was determined as previously described (Updegraff 1969). The glucose content of the cellulose was determined by an anthrone colorimetric assay (Scott and Melvin 1953) with glucose (Sigma G8270). Statistical analysis of the results was performed by one-way ANOVA followed by Tukey's test.

Immunodot assay.

Chelating agent soluble solid fractions (1 mg ml⁻¹) were applied as 1- μ l aliquots to a nitrocellulose membrane (Amersham, Bucks, U.K.) in a threefold dilution series and allowed to air dry. Membranes were blocked with 3% membrane-blocking reagent powder (Amersham) in phosphate-buffered saline (PBS) (Bio-Rad, Segrate, Milan, Italy) for 1 h prior to incubation for 1.5 h with primary antibodies. Specific epitopes were detected by using PAM1ScFv and JIM5 monoclonal antibodies. After extensive washes in PBS, membranes were incubated

with anti-rat secondary antibody conjugated to horseradish peroxidase (Amersham) for JIM5, and with anti-His conjugated to horseradish peroxidase (Sigma) for PAM1. Membranes were washed as described above prior to detection with ECL detection reagent (Amersham).

Bacterial growth on different pectic substrates.

P. carotovorum was grown in minimal medium (MM) supplemented as sole carbon sources with either 0.2% (wt/vol) PGA (Sigma) or 0.2% (wt/vol) lime pectin with 81% degree of methylesterification (E81; DANISCO, Dupont, Wilmington, DE, U.S.A.) or 0.2% (wt/vol) lime pectin with 60% degree of methylesterification (plant PME de-esterified pectin P60; DANISCO). Bacterium was also grown on AIS isolated from transformed and WT leaves suspended in MM at a final concentration of 1 mg/ml. Cultures were inoculated at a final concentration of 4×10^6 CFU ml⁻¹ and incubated at 28°C on a rotary shaker at 250 rpm. Bacterial growth on PGA, E81, or P60 was measured spectrophotometrically (OD₆₀₀) at different hours postinoculation. The bacterial growth on different cell wall preparations was determined 12 hpi by plating serial dilutions on LB agar plates and counting colonies after 24 hpi at 28°C.

Gene expression analysis.

Total RNA was extracted from 100 mg of leaf tissues with TRIzol reagent (1 ml) using a Mixer Mill Homogenizer (type MM300; Qiagen, Basel, Switzerland). After grinding in an Eppendorf tube with one stainless steel bead (5 mm in diameter) for 1 min at 25 Hz, the homogenate was centrifuged at $12,000 \times g$ for 10 min at 4°C to remove the insoluble material. After transferring the clear supernatant to a fresh tube, 0.5 ml of isopropanol was added and RNA precipitated by centrifugation at $12,000 \times g$ for 10 min at 4°C. After removing the supernatant, the RNA pellet was washed by adding 75% ethanol and resuspended in 30 μ l of RNase/DNase-free water. RNA concentrations were determined both by spectrophotometer and densitometric analysis of RNA bands following agarose gel electrophoresis. Total RNA (2 μ g) was DNase-treated by adding 2 μ l of RQ1 DNase Reaction buffer 10 $\times$ (Promega Corp., Madison, WI, U.S.A.), 2 μ l of RQ1 RNase-free DNase 1 μ /ml (Promega Corp.), and nuclease-free water to a final volume of 20 μ l and incubated 30 min at 37°C. The reaction was stopped with 1 μ l of RQ1 DNase Stop Solution (Promega Corp.) at 65°C for 10 min. For cDNA synthesis, DNase-treated RNA (1 μ g) was reverse-transcribed using the Improm-II reverse transcription system according to the manufacturer's instructions. Reactions were brought to a final volume of 40 μ l containing random hexamer primers to a final concentration of 0.5 μ M. cDNA synthesis conditions were 5 min at 25°C for primer annealing, 60 min at 42°C for first-strand cDNA synthesis, and 15 min at 70°C to inactivate reverse transcriptase. Semiquantitative RT-PCR analysis was performed in a MyCycler thermal cycler (Bio-Rad) and amplification conditions consisted of 30 cycles as follows: 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min.

Primers specific for *AtPME3* and T-DNA were designed to amplify the 3' junction of the *ATPME3::T-DNA* insertion and to select the *pme3* heterozygous and *pme3* homozygous mutants using a PCR-based screening.

Real-time quantitative PCR analysis was performed using an iCycler Real-Time PCR detection system (CFX96; Bio-Rad) according to the manufacturer's guide. A total of 10 μ l of a cDNA dilution (corresponding to 30 ng of total RNA) was amplified in 30 ml of reaction mix containing SYBR Green JumpStart Taq ready mix (S4438; Sigma) and 0.4 mM each primer. AGI number and primer sequences are as follows:

AtPME3 (At3g14310), sense 5'-GAAACTTGTTTTGCTA TCCGC-3' and antisense 5'-GCAGTTACCACGGCAGAGAT-3' for; *PAD3* (At3g26830), sense 5'-TCGCTGGCATAACACT ATGG-3' and antisense 5'-TTGGGAGCAAGAGTGGAGTT-3'; *PDF1.2* (At5g44420), sense 5'-CGCACCCGCAATGG TGG-3' and antisense 5'-ATCCATGTTTGGCTCCTTCG-3'; *PR-1* (At2g14610), sense 5'-GTAGGTGCTCTTGTCTTCCC-3' and antisense 5'-CACATAATCCCACGAGGATC-3'; *JR1* (At3g16470), sense 5'-TGGAACAGAGGAGTTTGTGG-3' and antisense 5'-GTCAGACCACCTGATACCG-3'; and *WR3* (At5g50200), sense 5'-GACCTGCCCCACACAAGATCA-3' and antisense 5'-TGGAGGCAATATCTAGGGACG-3'. The following PCR program was used for all PCR reactions: 95°C for 2 min, followed by 45 cycles of 95°C for 10 s, 60°C for 30 s, 72°C for 10 s, and 95°C for 1 min. Data were analyzed using CFX manager software (version 1.6; Bio-Rad). Each sample reaction was run in triplicate for each gene assay. Cycle threshold (C_T) values were normalized to the C_T value of *UBQ5* (At3g62250), sense 5'-GTAAAGCTCGCTGTTCTTCA GT-3' and antisense 5'-TCAAGCTTCAACTCCTTCTTTC-3'. Normalized transcript levels of each gene in pathogen-infected samples were compared with levels in mock-treated samples and the fold changes in expression level was calculated. Relative expression of the RT-PCR products was determined using the $\Delta\Delta C_T$ method (Livak and Schmittgen 2001).

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AUTHOR-RECOMMENDED INTERNET RESOURCES

National Institute for Agrobiological Sciences (NIAS) DNA bank:
www.dna.affrc.go.jp
 National Institutes of Health ImageJ website: rsb.info.nih.gov/ij