

Arabidopsis PECTIN METHYLESTERASEs Contribute to Immunity against *Pseudomonas syringae*¹[C][W][OPEN]

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Pectins, major components of dicot cell walls, are synthesized in a heavily methylesterified form in the Golgi and are partially deesterified by pectin methylesterases (PMEs) upon export to the cell wall. PME activity is important for the virulence of the necrotrophic fungal pathogen *Botrytis cinerea*. Here, the roles of Arabidopsis PMEs in pattern-triggered immunity and immune responses to the necrotrophic fungus *Alternaria brassicicola* and the bacterial hemibiotroph *Pseudomonas syringae* pv *maculicola* ES4326 (*Pma* ES4326) were studied. Plant PME activity increased during pattern-triggered immunity and after inoculation with either pathogen. The increase of PME activity in response to pathogen treatment was concomitant with a decrease in pectin methylesterification. The pathogen-induced PME activity did not require salicylic acid or ethylene signaling, but was dependent on jasmonic acid signaling. In the case of induction by *A. brassicicola*, the ethylene response factor, but not the MYC2 branch of jasmonic acid signaling, contributed to induction of PME activity, whereas in the case of induction by *Pma* ES4326, both branches contributed. There are 66 *PME* genes in Arabidopsis, suggesting extensive genetic redundancy. Nevertheless, selected *pme* single, double, triple and quadruple mutants allowed significantly more growth of *Pma* ES4326 than wild-type plants, indicating a role of PMEs in resistance to this pathogen. No decreases in total PME activity were detected in these *pme* mutants, suggesting that the determinant of immunity is not total PME activity; rather, it is some specific effect of PMEs such as changes in the pattern of pectin methylesterification.

The plant cell wall determines cell shape, facilitates cell-cell interaction, and provides mechanical strength to plant cells. De Bary (1886) first observed that a plant pathogen, *Sclerotinia sclerotiorum*, degraded host cell walls during infection. Later, it was concluded that plant cell walls act as preformed structural barriers against pathogen entry, because it was noticed that many plant pathogens produced various types of cell wall-degrading enzymes and that some of those were required for optimal infection of host plants (Albersheim et al., 1969).

Arabidopsis mesophyll cells are surrounded by primary cell walls consisting of three major components: cellulose, hemicelluloses, and pectins. Pectins make up approximately 50% of Arabidopsis leaf cell walls (Zablackis et al., 1995; Harholt et al., 2010). They are complex GalA-containing polysaccharides composed of homogalacturonan (HG), rhamnogalacturonan I and II, and xylogalacturonan (Mohnen, 2008). HG is typically the most abundant polysaccharide, constituting approximately 65% of the pectin (Mohnen, 2008; Harholt et al., 2010). HG is a linear homopolymer of 1,4-linked GalA and is synthesized in the Golgi in a highly methylesterified form (Caffall and Mohnen, 2009). Pectin methylesterases (PMEs) demethylesterify HG in the apoplast (Mohnen, 2008; Harholt et al., 2010). Demethylesterification of pectin is considered to be important for mechanical strength of cell walls, because blockwise deesterified pectin can form calcium bonds that promote the formation of so-called egg-box structures and thus supramolecular pectic gels (Jarvis, 1984; Liners et al., 1989). However, there is some controversy. The cell walls of the shoot apical meristem cells of plants overexpressing PME5 appear softer and cell walls of plants overexpressing a pectin methylesterase inhibitor (PMEI), such as PME13, appear harder than wild-type walls when measured using atomic force microscopy (Peaucelle et al., 2011). The degree and pattern of esterification influence the elasticity and porosity of pectic gels, making it likely that PMEs producing HG with

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varying deesterification patterns influence cell wall matrix properties in planta (Willats et al., 2001).

Pectolytic, necrotrophic pathogens, such as the fungus *Botrytis cinerea*, secrete pectin-degrading enzymes, including pectate lyases and polygalacturonases as well as PME early during infection (Espino et al., 2010). Moreover, the *B. cinerea* endopolygalacturonases Bcpg1 and Bcpg2 and the PME Bcpme1 are required for full virulence (ten Have et al., 1998; Valette-Collet et al., 2003; Kars et al., 2005), illustrating the importance of pectin degradation for the success of this pathogen.

In Arabidopsis, microarray experiments showed that the expression of plant PME genes is altered during infection with various pathogens, including the biotrophic pathogen *Golovinomyces cichoracearum*, the hemibiotroph *Pseudomonas syringae*, nonhost pathogens *Phytophthora infestans* and *Blumeria graminis*, as well as the necrotrophic pathogen *B. cinerea* (Lionetti et al., 2012). One PME, PME3, has been implicated in plant immune responses. Mutant *pme3* is more resistant to *B. cinerea* and *Pectobacterium carotovorum* and total PME activity is reduced (Raiola et al., 2011). In addition, ectopic expression of PME1 genes rendered plants more resistant against various necrotrophic pathogens (Raiola et al., 2004; Lionetti et al., 2007; Volpi et al., 2011).

PME genes constitute a family of 66 members in Arabidopsis (Harholt et al., 2010). Many PMEs are encoded as preproteins. The proregion contains a signal peptide and is required for protein targeting to the endoplasmic reticulum. Pro-PMEs are secreted to the apoplast, but only the mature part of the PME (without the proregion) is found in the cell wall (Micheli, 2001). Proregions of PMEs are homologous to PME1 genes from kiwifruit (*Actinidia deliciosa*) and Arabidopsis (Giovane et al., 2004). However, their function is not yet understood (Jolie et al., 2010). Type I PME genes contain the proregion, whereas type II PMEs do not (Pelloux et al., 2007; Jolie et al., 2010). PMEs identified in phytopathogenic organisms also lack a proregion (Markovic and Janecek, 2004).

At one time, plant PMEs were thought to demethylesterify pectin in a processive, blockwise pattern, whereas pathogen PMEs were thought to randomly deesterify pectin (Limberg et al., 2000a, 2000b; Willats et al., 2001). However, studies using monoclonal antibodies recognizing specific methylesterification patterns showed that plant cell wall PMEs can produce HG with nonblockwise and blockwise methylesters in discrete cell wall microdomains (Willats et al., 2001).

Plants recognize molecular patterns that are characteristic of microbes or are released from plant molecules during infection (Jones and Dangl, 2006; Monaghan and Zipfel, 2012), which are referred to as microbe-associated molecular patterns (MAMPs) and damage-associated molecular patterns (DAMPs), respectively. Well-studied MAMPs include flg22, a 22-amino acid peptide derived from bacterial flagellin, and elf18, an 18-amino acid peptide derived from bacterial elongation factor Tu (Felix et al., 1999; Kunze et al., 2004). Plant-derived peptides such as PEP1, a 23-amino acid peptide

isolated from Arabidopsis, are one group of molecular patterns produced during infection that act as DAMPs (Huffaker et al., 2006). Another group comprises small pectic fragments called oligogalacturonides (OGs) (Ferrari et al., 2013). The degree and pattern of pectin methylesterification are important for pectin degradation and hence generation of OGs by pectolytic enzymes (Berger and Reid, 1979; Limberg et al., 2000a, 2000b). The elicitor activity of OGs depends on their length and degree of esterification (Osorio et al., 2008; Ferrari et al., 2013). It has been shown that pectic fragments can be perceived by WALL-ASSOCIATED KINASE1 (WAK1) and WAK2 (Kohorn et al., 2009; Brutus et al., 2010). Recognition of OGs activates downstream defense responses including reactive oxygen species production, ethylene production, and callose deposition (Ferrari et al., 2013). Deesterified OGs show increased elicitor activity and binding to WAK1 when in the egg-box conformation (Cabrera et al., 2008).

Upon recognition of pathogen-derived patterns, plant immune responses are initiated and transduced through signaling events mediated by three plant hormones with major effects on immunity: salicylic acid (SA), jasmonate (JA), and ethylene (ET) (Robert-Seilaniantz et al., 2011; Pieterse et al., 2012). Other plant hormones such as abscisic acid (ABA), cytokinins, and auxins were recently shown to influence pathogen responses (Robert-Seilaniantz et al., 2011; Pieterse et al., 2012). SA and JA signaling processes inhibit each other (Robert-Seilaniantz et al., 2011; Pieterse et al., 2012).

Here, we found that pathogen-induced PME activity is affected by JA signaling. (+)-JA-L-Ile is the biologically active form of JA (Fonseca et al., 2009). It is produced by JASMONATE RESISTANT1, a JA-amino acid synthetase that conjugates the amino acid Ile to jasmonic acid (Staswick and Tiryaki, 2004). JA-Ile is recognized by a receptor complex constituted of the F-box protein CORONATINE INSENSITIVE1 (COI1) and JASMONATE ZIM (JAZ) transcriptional repressor proteins (Xie et al., 1998; Thines et al., 2007; Katsir et al., 2008; Sheard et al., 2010). After JA-Ile binding, JAZ proteins are ubiquitinated by the S-phase kinase-associated protein1 (Skp1), Cullin1, F-box containing (SCF)-COI1 complex and are thus targeted for degradation by the 26S proteasome (Chini et al., 2007; Thines et al., 2007). In the absence of JA, JAZ repressor proteins bind to the basic Helix-Loop-Helix transcription factors MYC2, MYC3, and MYC4 (Pauwels et al., 2010; Fernández-Calvo et al., 2011) and repress MYC2, MYC3, and MYC4-dependent gene expression. These MYC transcription factors have overlapping but distinct effects (Fernández-Calvo et al., 2011) and regulate expression of a subset of JA responsive genes together with ABA. Another branch of JA signaling incorporates ET-dependent responses and requires the ethylene response factor (ERF)-type transcription factors ERF1 and OCTADECANOID-RESPONSIVE ARABIDOPSIS AP2/ERF59 (ORA59; Lorenzo et al., 2003; Pré et al., 2008). ERF-type transcription factors have not been

shown to interact with JAZ repressors. SA suppresses JA signaling downstream of the ET-dependent signaling branch in an ORA59-dependent manner (Van der Does et al., 2013).

PMEs control the esterification status of pectin and have been linked to pathogen success. Here, we aimed to study the role of *PME* genes in plant immunity using a genetics approach. We report that inoculation with the bacterial hemibiotroph *P. syringae* pv *maculicola* ES4326 (*Pma* ES4326) or the fungal necrotroph *Alternaria brassicicola* induces PME activity and decreases pectin methylesterification in *Arabidopsis*. PME activity is also induced after treatment with the MAMPs flg22 and elf18, but not the DAMP PEP1. We show that the pathogen-induced PME activity is host plant derived and dependent on JA signaling. Overexpression of ERF1 promotes PME activity induced by either pathogen. The MYC2 branch of JA signaling is not required for *A. brassicicola*-induced PME activity, but is required for *Pma* ES4326-induced PME activity. Plants with mutations in various *PME* genes are more susceptible to *Pma* ES4326. We did not detect measurable decreases in total PME activity in susceptible *pme* mutants, so distinct patterns of esterification produced by specific PMEs may be more relevant for immunity than total PME activity.

RESULTS

A. brassicicola, *Pma* ES4326, and MAMPs Treatment of *Arabidopsis* Induce Total PME Activity

To test for effects of pathogens with different lifestyles on host PME activity, we monitored total PME activity in *Arabidopsis* after challenge by *A. brassicicola*, a fungal necrotroph to which wild-type accession ecotype Columbia 0 of *Arabidopsis* (Col-0) is resistant, or *Pma* ES4326, a hemibiotrophic bacterial pathogen to which Col-0 is susceptible. We further monitored total PME activity during pattern-triggered immunity initiated by treatment with the MAMPs flg22 or elf18 or the DAMP PEP1. PME activity was determined by extracting cytoplasmic and cell wall-bound proteins from homogenized plant samples and measuring PME activity in these samples using a gel diffusion assay (Downie et al., 1998; Supplemental Fig. S1). First, leaves of Col-0 plants were challenged with *A. brassicicola* or mock. Total PME activity was measured every 24 h for 5 d. Elevated PME activity was detected beginning 48 h after infection with *A. brassicicola*. No increase in PME activity was observed in mock-treated leaves (Fig. 1A). Second, Col-0 plants were inoculated with *Pma* ES4326 or mock. PME activity was measured every 24 h for 4 d. An increase in total PME activity was again detected beginning 48 h after inoculation with *Pma* ES4326 but not after mock treatment (Fig. 1B). Third, Col-0 plants were inoculated with elf18, flg22, PEP1, or mock. PME activity was measured after 2, 4, 8, 24, 32, and 48 h. Increased total PME activity was detected after treatment with the MAMPs at early time points but not after treatment with PEP1 (Supplemental Fig. S2).

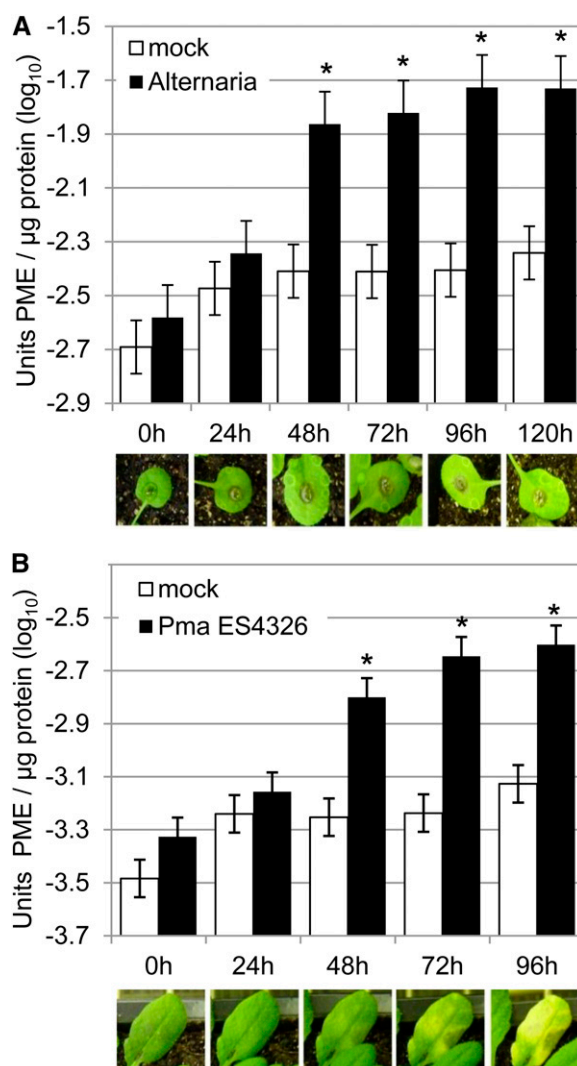


Figure 1. PME activity was induced after pathogen treatment. A, Total PME activity was induced upon challenge with *A. brassicicola*. Wild-type Col-0 plants were inoculated with *A. brassicicola* (*Alternaria*) or mock. Leaf tissue was harvested immediately (0 h) and after 24, 48, 72, 96, and 120 h. Total protein was extracted and PME activity determined using a gel diffusion assay. B, Total PME activity was induced upon challenge with *Pma* ES4326. Wild-type Col-0 plants were inoculated with *Pma* ES4326 ($OD_{600} = 0.002$) or mock. Experiments were performed as in A. Bars represent means and ses of data from four independent experiments, each with three technical replicates analyzed together using a mixed linear model. Asterisks indicate PME activity significantly higher than in mock-treated-plants at the same time point ($q < 0.01$). Photographs show representative leaves infected with *A. brassicicola* (A) or *Pma* ES4326 (B) at the indicated time points. [See online article for color version of this figure.]

Evidently, total PME activity in *Arabidopsis* increases after challenge by *A. brassicicola*, *Pma* ES4326, flg22, or elf18.

Cell Wall Pectin Is Demethylesterified in Response to Pathogen Challenge

Increased PME activity might result in decreased pectin methylesterification in the cell wall. Thus, we tested

for effects of pathogen challenge on pectin methylesterification by immunoblot analysis with the LM19 and LM20 antibodies (Verhertbruggen et al., 2009). LM19 binds more strongly to nonmethylesterified pectin than to methylesterified pectin, whereas LM20 requires methylester groups for binding to pectin and does not bind to non-methylesterified pectin (Verhertbruggen et al., 2009). Plant cell wall pectins of Col-0 plants inoculated with *A. brassicicola* were enriched in nonmethylesterified pectin (Fig. 2A) and contained less methylesterified pectin (Fig. 2B) after infection compared with mock-inoculated plants. Similarly, cell wall pectins from Col-0 plants infected with *Pma* ES4326 contained more non-methylesterified and less methylesterified pectin after infection (Fig. 2, C and D). These data show that challenge by either *A. brassicicola* or *Pma* ES4326 reduces the degree of methylesterification of pectins in the plant cell wall.

The Pathogen-Induced PME Activity Is Plant Derived

PME activity was induced in MAMP-treated Arabidopsis samples, indicating that plants activate PMEs as part of their immune response. However, some pathogens, such as *B. cinerea*, are known to produce PMEs. In principle, the increase in PME activity in response to pathogen challenge could be the result of PMEs produced by the plant or PMEs produced by

the pathogens. We carried out several experiments to distinguish these possibilities. We searched the pathogen genomes for PME genes that could encode PMEs responsible for the measured PME activity. *A. brassicicola* has four genes that have been annotated as PMEs: AB00162.1, AB01108.1, AB01671.1, and AB10201.1 (<http://genome.jgi-psf.org>; Cho et al., 2012; Ohm et al., 2012). Some of the *A. brassicicola* PMEs showed an increase in expression after infection (Supplemental Fig. S3). No PME activity could be detected in *A. brassicicola* grown in rich medium. We reasoned that if the PME activity in *A. brassicicola*-challenged leaves was derived from the fungus, then there should be more PME activity in plant genotypes that allow more growth of the fungus. Arabidopsis wild-type Col-0 plants are resistant to *A. brassicicola* but *phytoalexin-deficient3* (*pad3*) plants lacking the phytoalexin camalexin (Zhou et al., 1999) or *delayed dehiscence2* (*dde2*) plants lacking JA due to absence of allene oxide synthase activity (von Malek et al., 2002) are more susceptible (van Wees et al., 2003; Nafisi et al., 2007). We compared PME activity in *A. brassicicola*-challenged wild-type, *pad3*, and *dde2* plants. The induction of PME activity in *dde2* plants was reduced, whereas that in *pad3* plants was similar to the wild type (Fig. 3A). We conclude that the enhanced PME activity observed during *A. brassicicola* infection is produced by the plant. *Pma* ES4326 does not contain any

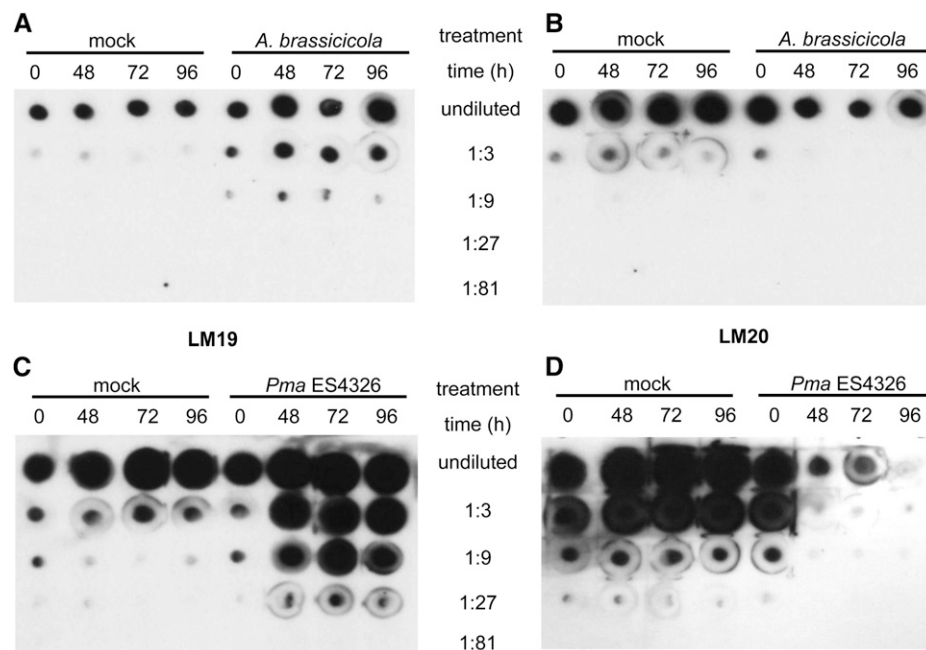


Figure 2. Methylesterification of cell wall pectins was reduced after pathogen challenge. A and B, Nonmethylesterified pectin was enriched (A) and methylesterified pectin was reduced (B) after challenge with *A. brassicicola*. Wild-type Col-0 plants were inoculated with *A. brassicicola* (1×10^6 spores mL^{-1}) or mock. Leaf tissue was harvested immediately (0 h) and after 48, 72, and 96 h. Pectins were extracted and samples were diluted to a final concentration equivalent to $1 \text{ nmol } \mu\text{L}^{-1}$ GalA (undiluted). Samples were serially diluted (1:3 to 1:81) and $1 \mu\text{L}$ each was applied to a nitrocellulose membrane. Membranes were probed with LM19 (A) or LM20 (B) antibodies. C and D, nonmethylesterified pectin was enriched (C) and methylesterified pectin was reduced (D) after challenge with *Pma* ES4326. Experiments were performed as in A and B, but sample concentration was equivalent to $2 \text{ nmol } \mu\text{L}^{-1}$ GalA for the undiluted sample. Two biological replicates were performed and yielded similar results.

genes annotated as *PMEs* (Baltrus et al., 2011). PME activity could not be detected in *Pma* ES4326 grown in either rich King's B medium or in hypersensitive response and pathogenicity-inducing minimal medium. Furthermore, *pad4* and *salicylic acid induction deficient2* (*sid2*) plants are more susceptible to *Pma* ES4326 and thus carry more bacteria 72 h after inoculation, but nevertheless show similar PME activity than wild-type Col-0 plants (Fig. 4A). Hence, the PME activity detected in Arabidopsis leaves after infection with *Pma* ES4326 is also likely of plant origin.

Pathogen-Induced PME Activity Requires JA

The observed pathogen-induced increase in PME activity could be controlled as part of the plant immune response. To test this idea, we determined *A. brassicicola*-induced PME activity in plants with defects in major sectors of the immune signaling network, including *dde2* (blocked in JA signaling), *ethylene-insensitive2* (*ein2*) (blocked in ET signaling; Alonso et al., 1999), *sid2* (blocked in SA signaling; Wildermuth et al., 2001), and *pad4* (*PAD4* is required for SA signaling and many SA-independent gene expression changes after infection by *Pma* ES4326; Jirage et al., 1999; Glazebrook et al., 2003; Wang et al., 2008). *A. brassicicola*-induced PME activity was reduced in *dde2*; however, some residual pathogen-inducible PME activity remained (Fig. 3A). The induction of PME activity in *ein2*, *pad4*, *sid2*, and *pad3* plants was indistinguishable from the wild type (Fig. 3A). No difference from Col-0 could be detected for any genotype in mock-inoculated samples (Supplemental Fig. S4A). These data indicate that JA, but not ET, SA, or *PAD4*, is required for full *A. brassicicola*-induced PME activity.

To determine whether JA signaling is also required for the *Pseudomonas*-induced PME activity, a similar experiment was performed. Mutants *dde2*, *ein2*, *pad4*, and *sid2* were inoculated with *Pma* ES4326 or mock. A *coi1* mutant was also included, because *Pma* ES4326 produces the JA mimic coronatine and hence JA signaling in *dde2*, a biosynthetic mutant, is only slightly altered in *Pma* ES4326-infected plants (Wang et al., 2008). *Pma* ES4326-inducible PME activity was slightly reduced in *ein2* and strongly reduced in *coi1* 72 h after infection (Fig. 4A). No significant difference in *Pma* ES4326-induced PME activity was found in *dde2*, *pad4*, or *sid2*. No difference from Col-0 could be detected for any genotype in the mock-inoculated samples (Supplemental Fig. S5A). These data indicate that similar to *A. brassicicola*-induced PME activity, JA signaling, but not SA or *PAD4* signaling, is required for *Pma* ES4326-induced PME activity. Unlike *A. brassicicola*-induced PME activity, a small effect of ethylene signaling was detected in this particular set of experiments, but was not reproducible in independent sets of experiments (see below).

Both the MYC2- and the ERF1-Dependent Branches of JA Signaling Contribute to Pathogen-Induced PME Activity

Because JA signaling is required for the majority of the pathogen-inducible PME activity, we investigated

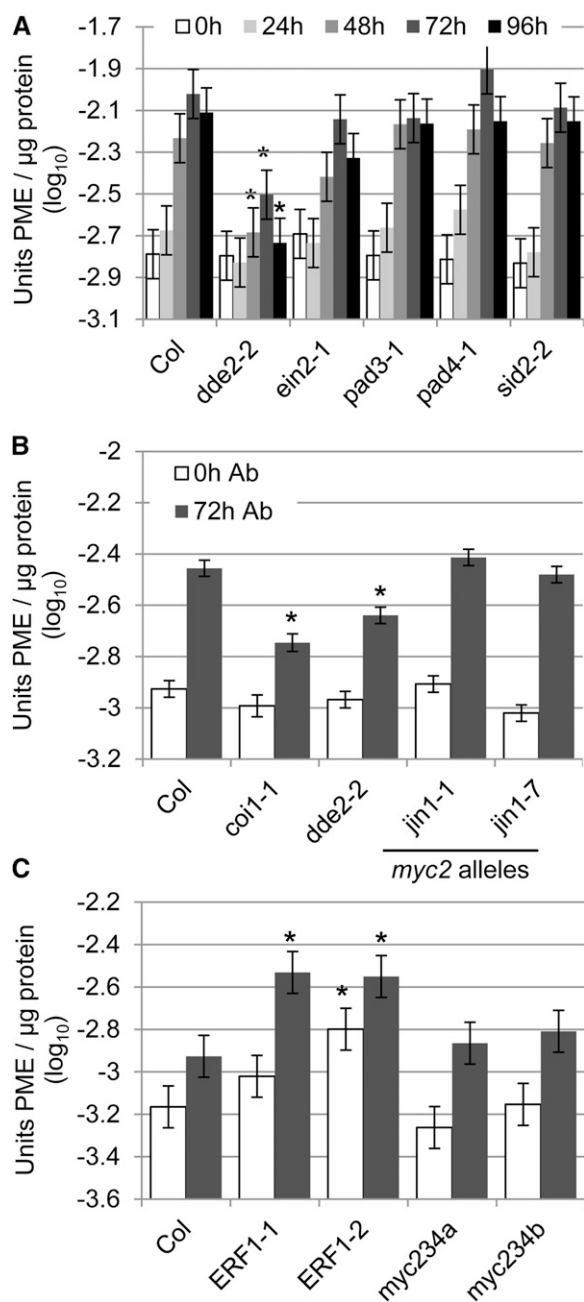


Figure 3. Induction of PME activity by *A. brassicicola* required JA signaling and was promoted by ERF1. A, *A. brassicicola*-induced PME activity required *DDE2*. Wild-type Col-0 (Col) and the indicated mutants were inoculated with *A. brassicicola* (1×10^6 spores mL^{-1}). PME activity was measured in tissue harvested immediately (0 h) and 24, 48, 72, and 96 h after inoculation. B, *A. brassicicola*-induced PME activity was unaltered in *MYC2* mutants (*jin1-1*, *jin1-7*), but reduced in both a JA biosynthesis (*dde2-2*) and a JA coreceptor mutant (*coi1-1*). Experiments were performed as in A but samples harvested only after 0 and 72 h (0 h Ab and 72 h Ab). C, *A. brassicicola*-induced PME activity was unaltered in *myc2 myc3 myc4* mutants (*myc234a* or b), but increased in two *ERF1* overexpression lines (*ERF1-1* and *ERF1-2*). Experiments were performed as in B. Bars represent means and ses of data from three (A and C) or four (B) independent biological replicates each with three technical replicates combined using a mixed linear model. Asterisks indicate significant differences from Col-0 at the indicated time point ($q < 0.01$).

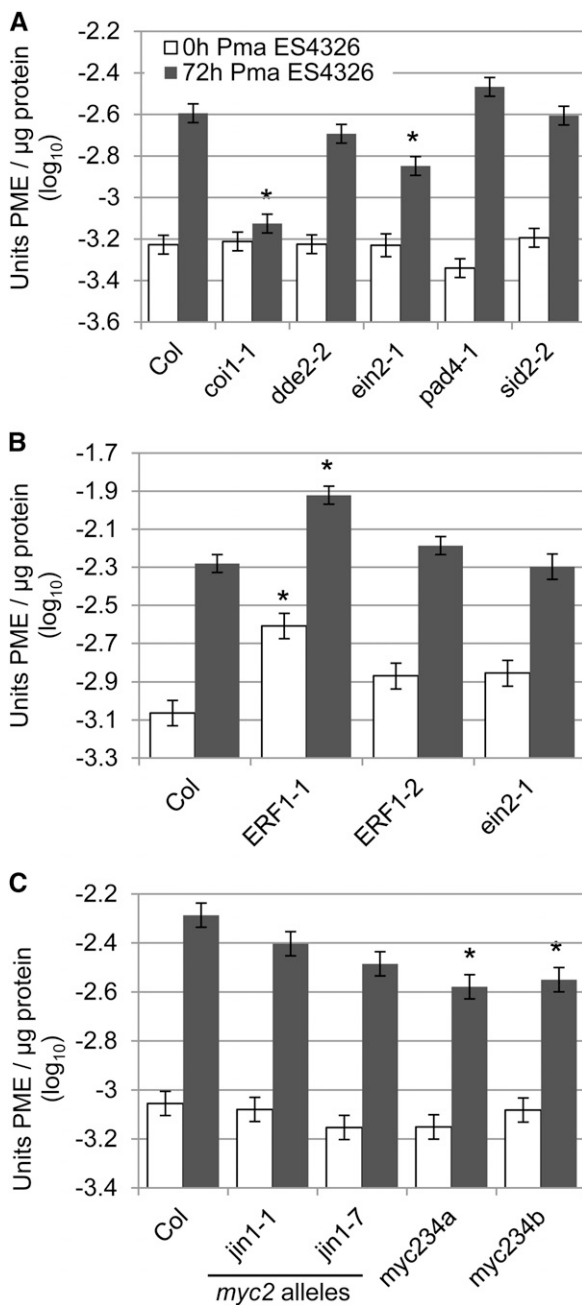


Figure 4. *Pma* ES4326-induced PME activity involved both the ERF1- and the MYC2-dependent branch of JA signaling. A, *Pma* ES4326-induced PME activity was strongly reduced in *coi1*. Wild-type Col-0 (Col) plants and mutants impaired in JA perception (*coi1-1*), JA biosynthesis (*dde2-2*), ET signaling (*ein2-1*), PAD4-dependent signaling (*pad4-1*), and SA biosynthesis (*sid2-2*) were inoculated with *Pma* ES4326 (OD₆₀₀ = 0.002). Leaves were harvested immediately (0 h) and after 72 h (72 h) and total PME activity was determined. B, *Pma* ES4326-induced PME activity was increased in the *ERF1* overexpression line ERF1-1. Experiments were performed as in A. C, *Pma* ES4326-induced PME activity was unaltered in *MYC2* single mutants *jin1-1* and *jin1-7* but reduced in two *myc2 myc3 myc4* triple mutants. Experiments were performed as in A. Bars represent means and s.e.s from two (A and C) or four (B) independent biological replicates with three technical replicates per sample, combined using a mixed linear model analysis. Asterisks indicate significant differences from Col-0 at each time point ($q < 0.01$).

the roles of the two JA signaling branches by analyzing additional JA signaling mutants, including *jasmonate-insensitive1-1* (*jin1-1*) and *jin1-7* (*myc2* mutants; Lorenzo et al., 2004), two *myc2 myc3 myc4* triple mutants (these lines are more completely blocked in JA signaling than *myc2* single mutants; Fernández-Calvo et al., 2011), and two *ERF1* overexpression lines (Solano et al., 1998; Berrocal-Lobo et al., 2002; Lorenzo et al., 2003). We used the *ERF1* overexpression lines because there are 122 *ERF* genes in Arabidopsis (Nakano et al., 2006); *ERF1* has many close homologs with redundant functions, and no phenotypes for any *erf1* null mutants have been described.

We confirmed that *A. brassicicola*-induced PME activity measured 72 h after inoculation was reduced in *dde2* (Fig. 3B). In addition, PME activity was reduced in the *coi1* JA coreceptor mutant (Fig. 3B). No change in *A. brassicicola*-induced PME activity was found in the *MYC2* mutants *jin1-1* or *jin1-7* (Fig. 3B). PME activity was increased in *ERF1-2*, one of two independent *ERF1* overexpression lines tested, immediately after inoculation with mock or *A. brassicicola* (Fig. 3C; Supplemental Fig. S4C). Both *ERF1* overexpression lines, *ERF1-1* and *ERF1-2*, showed increased PME activity 72 h after treatment with mock or *A. brassicicola* (Fig. 3C; Supplemental Fig. S4C). No effect on PME activity could be detected for *myc2 myc3 myc4* triple mutants (Fig. 3C). Collectively, the data show that the *A. brassicicola*-dependent induction of PME activity involves the JA signaling sector and is promoted by *ERF1*, but does not require *MYC2*, *MYC3*, and *MYC4*-dependent responses in the JA and ABA-dependent signaling sector.

Next, *Pma* ES4326-induced PME activity was examined in various JA mutants. No reduction in *Pma* ES4326-induced PME activity could be detected in *ein2*, *jin1-1*, or *jin1-7* (Fig. 4, B and C). This result differs from Figure 4A. The reduction in *Pma* ES4326-induced PME activity in *ein2* plants found there was smaller than the reduction in *coi1*, which was detected in both sets of experiments. The effect of *ein2* might be too small to be reproducibly detected with the assay used. *ERF1-1* showed elevated PME activity 0 and 72 h after inoculation with mock and *Pma* ES4326 (Fig. 4B; Supplemental Fig. S5B). No effect could be detected in the second *ERF1* overexpression line, *ERF1-2*, possibly due to differences in *ERF1* expression levels. *Pma* ES4326-dependent induction of PME activity was significantly reduced in *myc2 myc3 myc4* triple mutants (Fig. 4C; Supplemental Fig. S5C). These data suggest that *Pma* ES4326-induced PME activity involves both branches of the JA signaling sector.

Expression of Arabidopsis *PME* Genes Is Up-Regulated upon Pathogen Challenge

We attempted to identify which of the 66 Arabidopsis *PME* genes are responsible for the observed pathogen-induced increases in PME activity. In publicly available microarray data, expression of three Arabidopsis *PMEs* (*PME3*, *PME17*, and *PME41*) was up-regulated upon

inoculation with *A. brassicicola*. *PME3* expression is up-regulated after infection with two other necrotrophic pathogens: *B. cinerea* and *P. carotovorum* (Raiola et al., 2011). Using quantitative reverse transcription PCR (qRT-PCR), we confirmed up-regulation of these genes in Col-0 and/or *dde2* plants (Supplemental Fig. S6). *PME3* is a good candidate for a plant *PME* responsible for the *A. brassicicola*-induced increase in *PME* activity, because its expression in Col-0 but not *dde2* after *A. brassicicola* inoculation mirrors the *PME* activity measured in these genotypes.

We then measured *A. brassicicola*-induced *PME* activity in the corresponding mutants. Untreated *pme3* plants were previously shown to have reduced total *PME* activity (Raiola et al., 2011). We confirmed this, but found that the increase in *PME* activity in *pme3* plants 72 h after infection with *A. brassicicola* is indistinguishable from the increase in Col-0 (Supplemental Fig. S7A). Therefore, *PME3* cannot be responsible for the change in total *PME* activity observed upon inoculation with *A. brassicicola*. We also measured *A. brassicicola*-induced *PME* activity in *pme17* and *pme41* mutants, which was not different from wild-type Col-0 (Supplemental Fig. S7B).

Microarray data showed that expression of three Arabidopsis *PME* genes (*PME12*, *PME17*, and *At1g11580*, which is one of three Arabidopsis *PMEs* that have not been assigned a number in the UniProt database; Supplemental Table S1) was up-regulated after inoculation with *Pma* ES4326 (Wang et al., 2008). We confirmed this by qRT-PCR (Supplemental Fig. S8). We also measured *Pma* ES4326-induced *PME* activity in the corresponding mutants and in *pme3* plants. As in the *A. brassicicola* experiments, *pme3* plants had lower *PME* activity in the absence of the pathogen (Supplemental Fig. S9A), but the induced level of *PME* activity 72 h after inoculation with *Pma* ES4326 was comparable to wild-type plants; thus, the induction of *PME* activity was increased in *pme3* (Fig. 5A). *Pma* ES4326-induced *PME* activity in the *At1g11580* transfer DNA (T-DNA) line and in *pme17* was unaltered, but increased in two of three independent *pme12* lines (Fig. 5B; Supplemental Fig. S9B).

All together, we identified five Arabidopsis *PME* genes that are differentially expressed after pathogen challenge. No single *PME* mutant line showed a reduction in pathogen-induced *PME* activity, indicating that none of these genes are essential for the pathogen-induced increase in *PME* activity.

***PME* Genes Are Involved in Immunity to *Pma* ES4326**

After establishing that total *PME* activity is enhanced upon pathogen challenge, we studied the role of *PMEs* in immunity by measuring pathogen growth in selected *PME* mutants. It was impractical to test all 66 *PME* genes, so we prioritized candidates using several criteria. First, we measured pathogen growth in mutants with T-DNA insertions in *PME* genes shown to be up-regulated after pathogen challenge. We measured growth of *Pma* ES4326 in *pme3*, an *At1g11580* mutant, *pme12*, and *pme17*.

None of three *pme12* alleles tested showed significant changes in *Pma* ES4326 growth (Fig. 6A). However, two of three *pme3* alleles tested, two of two *pme17* alleles tested, and the *At1g11580* mutant allowed significantly more growth of *Pma* ES4326 (Fig. 6B).

A variety of Arabidopsis T-DNA lines with insertions in genes implicated in cell wall biosynthesis or modification have been screened for cell wall alterations by Fourier transform infrared (FTIR) microspectroscopy (<http://cellwall.genomics.purdue.edu>). In that screen, 12 *pme* mutants displayed altered cell wall compositions (see Supplemental Table S1 for results of the FTIR analysis). This was our second criterion for selecting *PME* genes for pathogen assays. We tested *Pma* ES4326 growth in at least two independent T-DNA alleles of each of these *PME* genes, including the alleles used for FTIR analysis. For six *PME* genes, at least two independent mutations caused small but significant increases in susceptibility to *Pma* ES4326 (*pme35* and *pme39* in Fig. 6A; *ppme1*, *pme17*, *pme31*, and *pme44* in Fig. 6B).

It is likely that there is some level of functional redundancy among *PME* family members. Phylogenetic analysis has grouped the *PME* genes in Arabidopsis into five clades (groups A to E; <http://cellwall.genomics.purdue.edu/>). We decided to create multiply mutant lines and to test pathogen growth. Because the cell wall composition of four *PMEs* in group A (*PPME1*, *PME31*, *PME52*, and *PME55*) and six *PMEs* in group D (*PME12*, *PME22*, *PME32*, *PME35*, *PME39*, and *PME44*) appeared altered in the FTIR screen, we combined T-DNA insertions according to the phylogenetic relationships of these *PME* genes. We combined two *PMEs* of group A (*PME31* and *PME52*) and four *PMEs* of group D (*PME12*, *PME22*, *PME35*, and *PME39*), using the alleles tested in the FTIR screen. The double mutant derived from group A *PMEs* (*pme31 pme52*) was more susceptible to *Pma* ES4326 (Fig. 6B). Enhanced growth of *Pma* ES4326 was also detected in three group D double mutants (*pme12 pme22*, *pme12 pme35*, and *pme35 pme39*), all triple mutants, and the quadruple mutant (Fig. 6A).

We also combined *PME* mutations according to the type of alterations they caused in cell walls. We clustered all 12 *pme* mutants according to their FTIR spectrum differences from Col-0 using uncentered Pearson correlation with complete linkage, to determine which mutations caused similar effects. The results suggested combining *pme35 pme39*, as well as *ppme1* and *pme44* (Supplemental Fig. S10A). We have already stated that the *pme35 pme39* double mutant was more susceptible to *Pma* ES4326 (Fig. 6A). Similarly, a *ppme1 pme44* double mutant showed enhanced growth of *Pma* ES4326 (Fig. 6B).

Third, we selected *PME* genes that underwent changes in expression level in response to pathogen treatments. Expression data for selected *PMEs* were extracted from publicly available microarray experiments that profiled responses to a variety of biotrophic and necrotrophic pathogens and MAMPs. Hierarchical clustering identified *PMEs* with similar responses to infection at the gene expression level. We combined *pme3*, *pme25*, and *pme44* on the basis of their shared suppression by pathogens

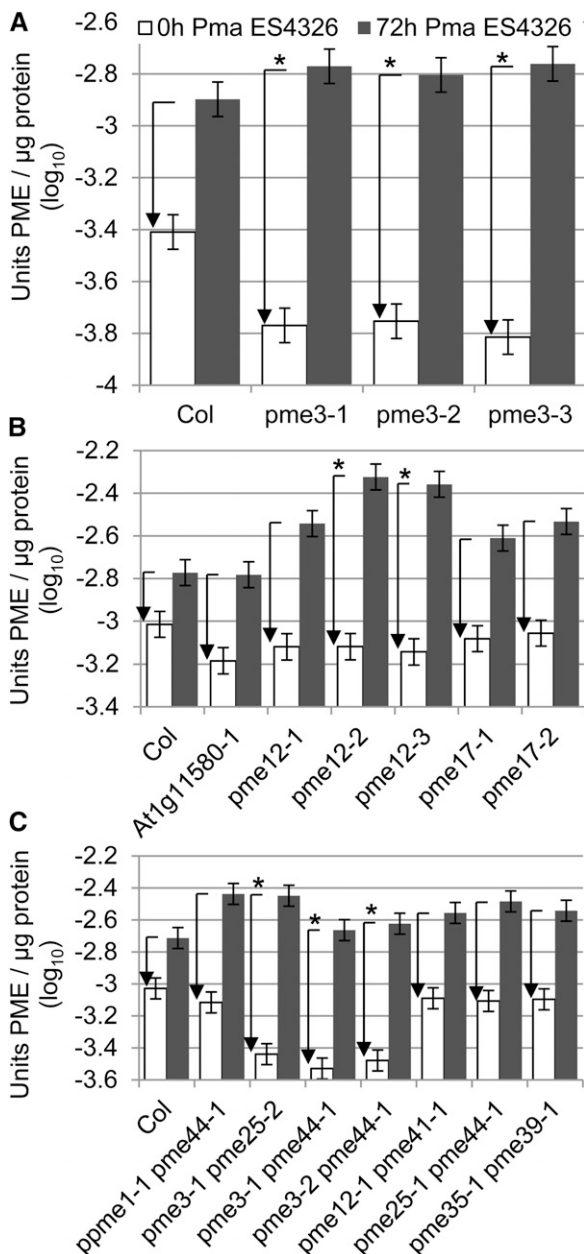


Figure 5. *Pma* ES4326-dependent induction in PME activity was not reduced but increased in some *pme* mutants. A, *Pma* ES4326-dependent induction of PME activity was enhanced in *pme3* mutants. Wild-type Col-0 (Col) and *pme3* plants were inoculated with *Pma* ES4326 (OD₆₀₀ = 0.002). Leaves were harvested immediately (0 h *Pma* ES4326) and after 72 h (72 h *Pma* ES4326) and total PME activity was determined. B, *Pma* ES4326-dependent induction of PME activity was enhanced in *pme12* mutants, but unaltered in *pme17* mutants and a *At1g11580* mutant. Wild-type Col-0 and *pme* mutant plants as specified were inoculated with *Pma* ES4326. Leaves were harvested immediately and after 72 h and total PME activity was determined. C, *Pma* ES4326-dependent induction of PME activity was enhanced in *pme3 pme25* and *pme3 pme44* mutants, but unaltered in *ppme1 pme44*, *pme12 pme41*, *pme25 pme44*, and *pme35 pme39* mutants. Experiment was performed as in B. Bars represent means and *ses* from two independent biological replicates with three technical replicates per sample, combined using a mixed linear

and *pme12* and *pme41*, which were both strongly up-regulated in response to pathogens and MAMPs (Supplemental Fig. S10B). We measured pathogen growth in *pme3 pme25*, *pme3 pme44*, *pme25 pme44*, and *pme12 pme41* double mutants. Each double mutant allowed more growth of *Pma* ES4326 3 d after infection (Fig. 6B).

In summary, many PME single mutants and higher-order mutants were more susceptible to *Pma* ES4326 than wild-type Col-0 (Fig. 6). We tested a total of 17 *PME* genes and mutations in 7 of those caused increased susceptibility in at least two independent T-DNA insertion lines. None of 10 randomly selected SALK T-DNA insertion lines was more susceptible to *Pma* ES4326 (Supplemental Fig. S11). We conclude that many *PME* genes contribute to immunity against *Pma* ES4326.

By contrast, there were no *PME* genes for which two independent mutations caused altered susceptibility to *A. brassicicola* and none of the double, triple, or quadruple mutant lines tested showed any change in *A. brassicicola* growth (Supplemental Figs. S7C and S12). There is no evidence that *PME* genes contribute to resistance to *A. brassicicola*.

Total PME Activity Is Not Reduced in *pme* Mutants with Enhanced Susceptibility to *Pma* ES4326

To determine whether the enhanced susceptibility to *Pma* ES4326 is due to reduced total PME activity, we measured PME activity in a selection of *pme* mutants that showed enhanced susceptibility to *Pma* ES4326. We could not detect any differences in *Pma* ES4326-induced PME activity in any of the group D triple mutants or in the quadruple mutant that had been created according to the phylogenetic relationships of the *PME* genes (Supplemental Fig. S13). In addition, *Pma* ES4326-induced PME activity was unaltered in *pme35 pme39* and *ppme1 pme44* (Fig. 5C; Supplemental Fig. S9C). These double mutants had been created because the corresponding single mutants possessed similar changes in their cell wall, as indicated by clustered FTIR data. We also tested double mutants that had been created because *PME* genes appeared to be similarly expressed upon pathogen challenge. PME activity in *pme12 pme41* and *pme25 pme44* was similar to the wild type (Fig. 5C; Supplemental Fig. S9C). All double mutants containing *pme3* (*pme3 pme25* and *pme3 pme44*) showed reduced PME activity in mock-treated samples and immediately after *Pma* ES4326 inoculation, whereas induction of PME activity 72 h after inoculation with *Pma* ES4326 was higher than in Col-0 (Fig. 5C; Supplemental Fig. S9C). These results are similar to those for *pme3* single mutants (Fig. 5A;

model. Arrows represent the change in PME activity between the 0 and the 72-h time point. Asterisks indicate significant differences to wild-type Col-0 ($q < 0.01$).

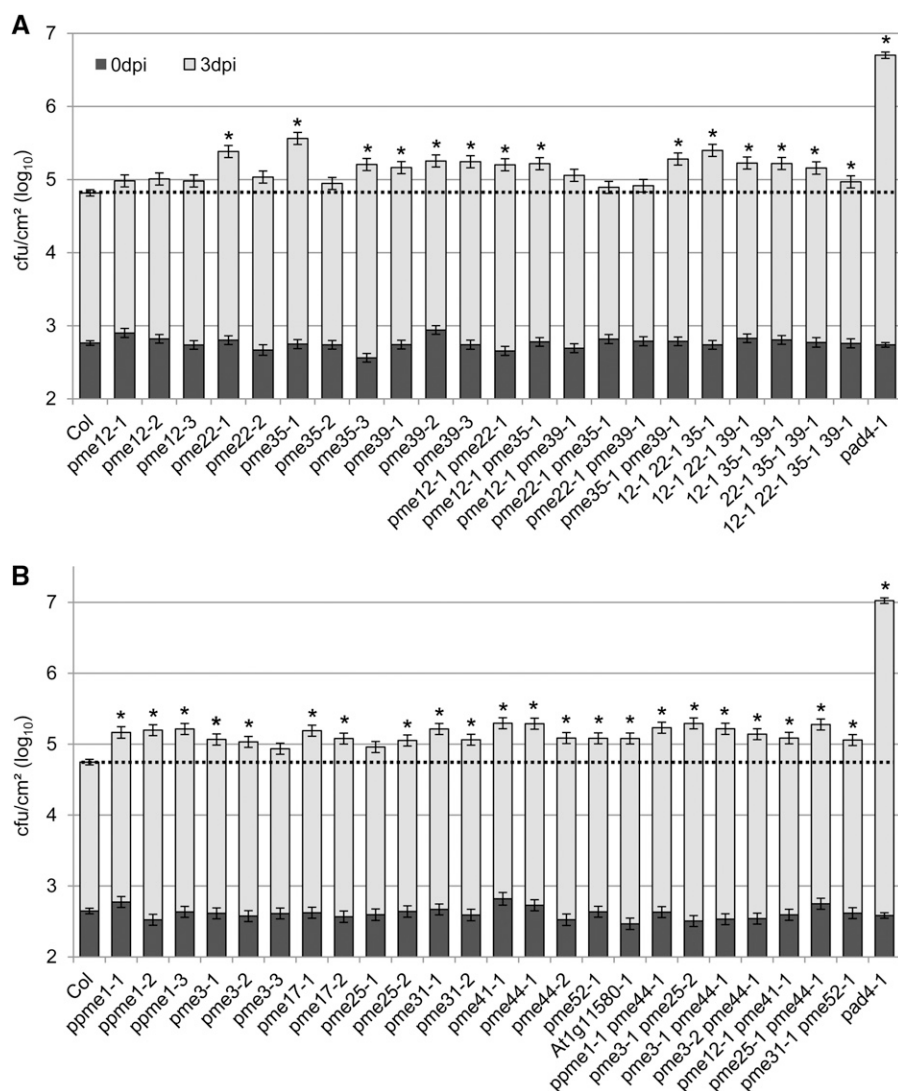


Figure 6. Many *pme* mutants allowed enhanced growth of *Pma* ES4326. A, Single *pme* mutants and multiple mutants of the PME group D are more susceptible to *Pma* ES4326. Plants of the indicated genotypes were inoculated with *Pma* ES4326 ($OD_{600} = 0.0002$). For multiply mutant lines, numbers indicate *PME* gene and allele numbers; for example, 12-1 22-1 35-1 indicates *pme12-1 pme22-1 pme35-1* triple mutant. Bacterial titers were determined immediately (0 dpi) and 3 d later (3 dpi). B, Mutants with defects in *PME* genes that show *Pma* ES4326-dependent expression and mutants with multiple mutations combined according to common cell wall alterations or expression patterns were more susceptible to *Pma* ES4326. Plants of the indicated genotypes were inoculated with *Pma* ES4326 by syringe infiltration. Bacterial titers were determined immediately (0 dpi) and 3 d later (3 dpi). Each bar represents the mean and SE of three independent experiments, each with 4 or 12 biological replicates at 0 and 3 dpi, respectively, combined using a mixed linear model. Asterisks indicate significant differences from Col wild-type ($q < 0.01$). Susceptible *pad4* plants were included as a positive control. Mutants have been plotted in numerical order. Col, Col-0; dpi, days post inoculation.

Supplemental Fig. S9A). Evidently, enhanced susceptibility to *Pma* ES4326 is not correlated with reductions in total PME activity.

DISCUSSION AND CONCLUSION

We showed that two pathogens with very different lifestyles, the necrotrophic fungus *A. brassicicola* and the hemibiotrophic bacterial pathogen *Pma* ES4326, greatly induce PME activity in the host plants. PME activity is also increased during flg22- and elf18-induced pattern-triggered immunity, suggesting that Arabidopsis activates PME activity as part of its immune response. Increased PME activity might render the pectin in the cell wall more demethylated. Hence, pathogens producing pectolytic enzymes might trigger the production of OGs that activate plant defense responses resulting in increased plant immunity. The pathogen-induced increase in PME activity is the likely cause of decreased methylesterification of pectins in pathogen-challenged

leaves. The observed increase in PME activity occurred rather late after pathogen challenge, between 24 and 48 h after inoculation in both cases. By this time, *A. brassicicola* is growing very slowly or not at all, whereas *Pma* ES4326 is growing rapidly, causing visible damage to infected leaves. This raises the question of the effect of induced PME activity on these plant-pathogen interactions. Perhaps the increased PME activity is a response to damage to the cell wall caused by pathogens. Degradation of pectin by pectin-degrading enzymes requires a certain level of pectin deesterification. Pectin fragments with a low degree of esterification and a size between 10 and 15 GalA residues show the highest elicitor activity (Cabrera et al., 2008; Ferrari et al., 2013). Perhaps increased PME activity triggers the production of OGs with more elicitor activity, resulting in increased induction of defense responses.

Pathogen-inducible PME activity required JA signaling, because induction by either pathogen required the JA-Ile coreceptor, COI1. Induction by *A. brassicicola*

also required DDE2, a JA biosynthetic enzyme. As expected, DDE2 was not required for induction by *Pma* ES4326, because this strain produces the JA-Ile mimic, coronatine (Cui et al., 2005; Katsir et al., 2008). We studied diagnostic mutants to distinguish the roles of two branches of JA signaling. We used ERF1 over-expression lines to probe the ethylene branch of JA signaling because no loss-of-function mutants with defects specific to this branch are known. The ERF1-1 line showed higher induced levels of PME activity in response to either pathogen, whereas the ERF1-2 line did so only in experiments with *A. brassicicola*. Over-expression of ERF1 is known to be detrimental, causing plants to be dwarfed with elongated leaves (Solano et al., 1998; Lorenzo et al., 2003). Unsurprisingly, the ERF1 transgene is often silenced. We observed plants of a range of sizes in our experiments. We suspect that the difference in the PME levels between the lines is due to differences in the expression levels of ERF1. We conclude that the ERF-dependent branch of JA signaling, as reported by the effect of ERF1 over-expression, can promote PME activity.

We probed the effect of the MYC2-dependent branch of JA signaling using *myc2* single mutants and *myc2 myc3 myc4* triple mutants, which are blocked in this pathway (Dombrecht et al., 2007; Fernández-Calvo et al., 2011). We found that these mutants had reduced PME activity after *Pma* ES4326 infection, but did not affect activity in *A. brassicicola*-challenged plants. Apparently, both the JA/ET and JA/ABA branches contribute to induction of PME activity by *Pma* ES4326, but only the JA/ET branch contributes to induction by *A. brassicicola*. It seems unlikely that the mechanism underlying PME activation is different after challenge by different pathogens. Rather, the responses to the two pathogens may differ depending on which signaling pathways are activated. *Pma* ES4326 induces robust accumulation of SA, whereas *A. brassicicola* does not (De Vos et al., 2005; Wang et al., 2008). SA inhibits JA signaling by targeting GCC promoter elements in the ERF-dependent branch of JA signaling (Van der Does et al., 2013). Consistently, *Pma* ES4326 induces expression of the SA reporter gene *PATHOGENESIS-RELATED1*, the JA/ABA reporter gene *VEGETATIVE STORAGE PROTEIN2*, but not the JA/ET reporter gene *PLANT DEFENSIN 1.2* (*PDF1.2*) (Wang et al., 2008). *A. brassicicola* does not induce *PATHOGENESIS-RELATED1* or *VEGETATIVE STORAGE PROTEIN2*, but strongly induces *PDF1.2* (De Vos et al., 2005). Thus, the likely reason that we did not observe MYC2 dependence of PME activation is that *A. brassicicola* does not activate the JA/ABA pathway. The ability to activate PMEs by either branch of the JA pathway makes this response robust to possible pathogen repression of one of the branches.

Although challenge with *A. brassicicola* induced PME activity, we did not detect any reproducible differences in disease severity of *A. brassicicola* in any *pme* mutant tested. Two single T-DNA insertion lines (*pme12-3* and *pme22-2*) showed significantly increased disease severity, but this was not detected in allelic T-DNA lines. Thus,

we have no evidence that individual *PME* genes are important for immunity to *A. brassicicola*. However, the *A. brassicicola* disease severity assay shows more variation than *Pma* ES4326 growth assays, and the feasible number of replicates is lower, resulting in less statistical power to resolve small differences. It therefore remains possible that some *pme* mutants have small effects on *A. brassicicola* disease severity.

Many of the *pme* mutants that we tested allowed modestly increased growth of *Pma* ES4326. For most of the *pme* single mutants that showed enhanced susceptibility phenotypes in preliminary bacterial growth assays, we tested additional independent alleles. Five *PME* genes were found to contribute to immunity based on findings that all of two or three alleles tested showed enhanced susceptibility (*PPME1*, *PME17*, *PME31*, *PME39*, and *PME44*), whereas mutations in a further three *PME* genes showed enhanced susceptibility, but only one allele was tested (*PME41*, *PME52*, and *At1g11580*). For four *PME* genes (*PME3*, *PME22*, *PME25*, and *PME35*), some alleles showed enhanced susceptibility, whereas others did not. There are two likely explanations. One is that the *PME* genes mutated in fact contribute to immunity, but with a small effect that is close to the limit of our statistical power to detect differences from the wild type, such that the effect was not detected in some alleles. Another possibility is that the effect observed in some mutants is due to secondary mutations elsewhere in the genome. This latter possibility explains *pme35-1*, which is very susceptible to *Pma* ES4326 (Fig. 6A). The allelic T-DNA line *pme35-2* was no more susceptible than the wild type and *pme35-3* was less susceptible than *pme35-1* but more susceptible than the wild type (Fig. 6A). We noticed that the *pme35-1* single mutant plants showed an altered rosette morphology (Supplemental Fig. S14, A and B). Reverse transcription PCR (RT-PCR) experiments showed that none of the *pme35* alleles produce full-length transcripts, suggesting that they are all null alleles of *PME35* (Supplemental Fig. S14, C and D). The altered leaf morphology and the enhanced susceptibility were lost in a backcrossed line (*pme35-1_bc*) and in various multiply mutant lines containing *pme35-1* (Fig. 6A; Supplemental Fig. S14E). We conclude that secondary mutations in *pme35-1* are responsible for the particularly high susceptibility and altered rosette morphology in this line. Importantly, the presence of secondary mutations compromising immunity is not very common in T-DNA lines, as we did not observe enhanced susceptibility in any of the 10 randomly chosen T-DNA lines that we tested. We conclude that there is a high probability that the five *PME* genes for which all alleles showed enhanced susceptibility in fact contribute to immunity, and it is likely that some of the *PME* genes for which one allele caused enhanced susceptibility also contribute to immunity. Most of the double or higher-order multiple *pme* mutants tested also showed enhanced susceptibility to *Pma* ES4326, strengthening our conclusion that some PMEs are important contributors to immunity.

Generally, PME s can be classified as type I and type II PME s, depending on the presence of PME I domains (Micheli, 2001). The significance of these domains for PME activity is not clear (Micheli, 2001; Di Matteo et al., 2005; Pelloux et al., 2007). We did not detect any correlation between the type of PME mutated and disease susceptibility to *Pma* ES4326 (Supplemental Table S1). Mutants with defects in type I PME s, including *pme39* and *pme44*, as well as mutants with defects in type II PME s, including *ppme1* and *pme31*, exhibited significantly increased susceptibility to *Pma* ES4326.

Single or double mutants containing *pme3* display lower basal levels of PME activity (Supplemental Figs. S7A and S9). A resulting increase in the degree of pectin methylesterification might affect Ca^{2+} -binding and hence formation of egg-box structures in these plants. Such changes in the cell wall structure might render these plants more susceptible to *Pma* ES4326. However, other *pme* mutant plants with no detectable changes in basal PME activity show similar levels of *Pma* ES4326 growth, showing that changes in basal PME activity do not account for all increases in plant susceptibility.

None of the *pme* mutants that are more susceptible to *Pma* ES4326 showed reduced pathogen-induced PME activity. Curiously, *pme3* and *pme12* mutants displayed increased *Pma* ES4326-induced PME activity, possibly due to overcompensation for loss of these PME s. The PME assay we used cannot detect small differences, so it is possible that total PME activity is slightly reduced in some *pme* mutants. For such undetectable differences in PME activity to explain the *Pma* ES4326 growth phenotypes, immunity to this pathogen would have to be very sensitive to reduced PME activity. On the other hand, some PME s might have specific pH or substrate requirements, and may not be active with the commercial methylated pectin and the experimental conditions we used. Such issues could have prevented detection of differences in PME activities critical for disease phenotypes.

An alternative possibility is that the importance of PME s lies not in total activity, but in their effects on the pattern of pectin methylation. We found that many *pme* mutants are susceptible to *Pma* ES4326. This indicates that the PME s do not function redundantly, suggesting that different PME s have specific functions. Certain PME genes are expressed after infection with various pathogens or in a tissue/developmental stage-specific manner (Pelloux et al., 2007; Lionetti et al., 2012). PME activity is affected by a variety of PME I s, 69 in Arabidopsis, as well as pH and ionic strength in the cell wall (Lionetti et al., 2012). Specifically, overexpression of PME I1 and PME I2 in Arabidopsis has been shown to increase resistance to *B. cinerea* (Lionetti et al., 2007). Interestingly, other studies indicate that methylester distribution might be important for defense against some pathogens. For example, carrot cultivars with similar pectin content and degree of esterification show different amounts of pectin release by polygalacturonases and differences in susceptibility to the necrotrophic, pectolytic fungus *Mycocentrospora acerina* (LeCam et al., 1994). The difference in pectin

release by polygalacturonases was diminished after PME treatment (LeCam et al., 1994), suggesting that differences in methylester distribution rather than total degree of esterification affected pathogen success. Moreover, in wheat (*Triticum aestivum*) near-isogenic lines, a blockwise distribution of methylesters was detected in lines susceptible to *Puccinia graminis* compared with a more random distribution in resistant lines (Wiethölter et al., 2003). In addition, the elicitor activity of OGs is dependent on the degree and distribution of methylesters (Cabrera et al., 2008; Osorio et al., 2008). Thus, it is likely that patterns of methylesterification affect plant-pathogen interactions. The PME s required for full immunity to *Pma* ES4326 may affect patterns of methylesterification in a way that impacts immunity to this pathogen. Future studies should aim to elucidate distinct roles of individual PME genes such as by studying methylesterification patterns in *pme* mutant plants.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Wild-type Columbia (Col-0) Arabidopsis and all mutant plants (all in Col-0 background) were grown on autoclaved BM2 germinating mix (Berger Inc) in a controlled environment chamber (Conviron) at a 12-h photoperiod under $100 \mu\text{m}^2 \text{ s}^{-1}$ fluorescent illumination at 22°C and 75% relative humidity. Germplasm used includes *dde2-2* (At5g42650; von Malek et al., 2002), *ein2-1* (At5g03280; Alonso et al., 1999), *pad3-1* (At3g26830; Zhou et al., 1999), *pad4-1* (At3g52430; Jirage et al., 1999), *sid2-2* (At1g74710; Wildermuth et al., 2001), *coi1-1* (At2g39940; Xie et al., 1998), *jim1-1* (At1g32640; Lorenzo et al., 2004), *jim1-7* (SALK_040500), *myc2 myc3 myc4* (MYC3, At5g46760; MYC4, At4g17880; Fernández-Calvo et al., 2011), and ERF1 overexpression lines (At3g23240; Solano et al., 1998; Lorenzo et al., 2003). PME mutants used are listed in Supplemental Table S1. They are T-DNA insertion lines of the SALK (Alonso et al., 2003), SAIL (Sessions et al., 2002), GABI-Kat (Kleinboelting et al., 2012), WiscDsLox (Woody et al., 2007), or SM_3 (John Innes Center) collections and were obtained from the Arabidopsis Biological Resource Center. One *pme3* allele (GK002A10) has been studied previously (Raiola et al., 2011), and this allele was referred to as *pme3-3* in this study.

Pathogen Strains, Growth Conditions, and Pathogen Growth Assays

Prior to each experiment *Alternaria brassicicola* strain ATCC96836 was grown on a modified potato dextrose agar medium (Flors et al., 2008) for 10 d at room temperature. The spores were washed from the surface of the plate with inoculation medium ($1 \times$ Gamborg B5 [Sigma], 10 mM Suc, 10 mM KH_2PO_4 ; Flors et al., 2008), and fungal hyphae were removed from the suspension by filtering through four layers of cheesecloth. The concentration of spores was determined using a hemacytometer and adjusted to 1×10^5 spores mL^{-1} for *A. brassicicola* growth assays and 1×10^6 spores mL^{-1} for PME activity and qRT-PCR assays. Leaves of 3- to 4-week-old Arabidopsis plants were inoculated by placing 10- μL droplets of *A. brassicicola* spore solution or inoculation medium (mock) on the adaxial leaf surface. Inoculated plants were kept at 100% relative humidity. Samples were harvested at various time points by cutting out the infection sites using a cork borer. For *A. brassicicola* growth assays, at least 15 leaf discs from five to six plants were pooled per biological sample. Disease severity, a proxy of fungal growth, was determined as previously described (Tsuda et al., 2009; Botanga et al., 2012). For PME assays, at least four leaf discs from one plant were combined for each biological sample.

Pma ES4326 was grown overnight at room temperature in King's B medium supplemented with 50 $\mu\text{g mL}^{-1}$ of streptomycin. The bacteria were harvested by centrifugation, washed twice, and diluted to the desired density (for bacterial growth assays, $\text{OD}_{600} = 0.0002$ for PME activity; for qRT-PCR assays, $\text{OD}_{600} = 0.002$) in 5 mM MgSO_4 . To measure bacterial growth, bacteria were inoculated into 4- to 5-week-old plants using a needleless syringe and bacterial titers determined immediately or 3 d after infection as previously

described (Tsuda et al., 2008). To measure PME activity, plants were inoculated with bacteria or mock (5 mM MgSO₄) and three leaves from one plant were collected at the indicated time points per biological replicate. To measure PME activity in culture grown bacteria, *Pma* ES4326 was grown overnight at room temperature in either King's B or hypersensitive response and pathogenicity-inducing minimal medium (Kim et al., 2009) using 0.2% Fru as a carbon source.

PME Activity Assay

Leaf tissue was collected at the indicated time points, flash frozen, and pulverized and cytoplasmic and cell wall-bound proteins were extracted by vortexing in cell wall extraction buffer (0.1 M citrate to 0.2 M Na₂HPO₄, 1 M sodium chloride, pH 5.0). Protein concentration was measured using Bradford reagent (BioRad), diluted to equal concentration and used to perform a gel diffusion assay (Downie et al., 1998). For the gel diffusion assay, petri dishes (10-cm diameter) were filled with 13 mL of a gel made of highly esterified pectin (0.1% pectin ≥ 85% esterified [Sigma P9561], 1% agarose, 12.5 mM citric acid, 50 mM Na₂HPO₄, pH 7.0; Lionetti et al., 2007) and four holes per plate were cut out using a number 2 cork borer. Wells were filled with the protein extract (40 μL volume) and plates were incubated at 30°C for 16 h. Plates were then stained with 0.05% Ruthenium Red (MP Biomedicals) for 30 min and destained with water. Plates were scanned and darker colored areas that are indicative of deesterified pectin were measured using ImageJ software. At least two independent biological replicates with three technical replicates each were performed. Known amounts of commercially available PME from orange peel (Sigma P5400) were used as standards. Linear regression was fit to the darker colored area size versus log₁₀PME units of the standards to obtain the standard curve, which was used to calculate the total PME activity of the sample extracts.

Cyclohexane Diamine Tetraacetic Acid Extraction of Cell Wall Pectin

Leaf tissue from inoculated plants was collected at the indicated time points, flash frozen, and pulverized, and cell wall pectin was extracted with extraction buffer (50 mM Trizma, 50 mM cyclohexane diamine tetraacetic acid, pH 7.2) at 95°C for 15 min (Siedlecka et al., 2008). Debris was precipitated by centrifugation for 10 min at 10,000g and the supernatant containing the pectin was divided in two aliquots per sample and freeze dried. One aliquot was used to measure total uronic acids (Filisetti-Cozzi and Carpita, 1991). The concentration of pectin as equivalent to GalA was calculated by comparison with known amounts of GalA. The second aliquot was resuspended in water to achieve equal concentrations of pectin and used for dot blot experiments.

Dot Blot Experiments

Pectin solutions were serially diluted and nitrocellulose membranes were spotted with 1 μL of the diluted pectin solutions. Membranes were dried overnight, blocked with 5% milk in 1 × phosphate-buffered saline (PBS; 8 g L⁻¹ sodium chloride, 0.2 g L⁻¹ KCl, 1.44 g L⁻¹ Na₂HPO₄, 0.24 g L⁻¹ KH₂PO₄, pH 7.4) and probed with LM19 or LM20 antibodies (Verhertbruggen et al., 2009). LM19 and LM20 were diluted 1:250 in 5% milk powder in 1 × PBS. A goat anti-rat horseradish peroxidase-conjugated antibody (Bethyl A110-105P) was used 1:5,000 diluted in 5% milk powder in 1 × PBS as secondary antibody, and membranes were washed with 1 × PBS. Dot blots were developed using the Amersham ECL Prime system (GE Healthcare).

Expression Analysis

Tissue from inoculated plants was harvested at the indicated time points, flash frozen, and pulverized, and RNA was extracted using Trizol (Invitrogen). qRT-PCR was performed as previously described (Truman and Glazebrook, 2012). For *A. brassicicola* gene expression, β-TUBULIN (*AbTUB*, AB07628.1) was used as the internal reference. To verify T-DNA insertion lines, RT-PCR was performed using the One-Step RT-PCR kit (Qiagen) according to the manufacturer's instructions. *Actin2* was used as stably expressed reference gene. The primer sets used are provided in Supplemental Table S2.

Clustering of Microarray Data

Affymetrix full genome (ATH1) microarray data were downloaded from a variety of sources detailed below. The data were normalized using the gcma package of the Bioconductor suite of programs within the R statistical

environment (Gentleman et al., 2004; Wu et al., 2004). Replicates for different treatments, if available, were averaged and the log₂ ratios to control treatments were calculated. Clustering was performed using CLUSTER and visualized using TREEVIEW (Eisen et al., 1998). Prior to clustering, genes and treatments were ordered with the self-organizing maps algorithm and complete linkage hierarchical clustering was performed using an uncentered Pearson correlation metric.

Microarray data from this article can be found in the National Center for Biotechnology Information Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo>) data libraries under accession numbers GSE431, GSE5525, GSE5615, GSE5616, GSE5684, GSE12856, GSE13739, GSE18978, GSE22274, and GSE50526, and in the NASCArrays database under numbers 120 and 447. A list of accession numbers for all Arabidopsis *PME* genes described in this study is provided in Supplemental Table S1. Sequence data for all other Arabidopsis genes from this article can be found in The Arabidopsis Information Resource (<http://www.arabidopsis.org>) under accession numbers At5g42650 (*DDE2*, *AOS*), At5g03280 (*EIN2*), At3g26830 (*PAD3*, *CYP71B15*), At3g52430 (*PAD4*), At1g74710 (*SID2*, *ICS1*), At2g39940 (*COI1*), At1g32640 (*MYC2*), At5g46760 (*MYC3*), At4g17880 (*MYC4*), At3g23240 (*ERF1*), and At3g18780 (*ACTIN2*). Genome sequence data and gene information for *A. brassicicola* strain ATCC96836 can be found in the JGI database (<http://genome.jgi-psf.org>) using the following identifiers AB00162.1, AB01108.1, AB01671.1, AB10201.1, and β-TUBULIN (AB07628.1). Sequence data for larger contigs, each containing multiple genes, can be found in the GenBank data library under accession numbers ACIW01000050.1 (AB00162.1), ACIW01000290.1 (AB01108.1), ACIW01000452.1 (AB01671.1), ACIW01003035.1 (AB10201.1), and ACIW01002235.1 (β-TUBULIN).

Supplemental Data

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

Supplemental Figure S1. PME activity of commercially available purified PME from orange peel.

Supplemental Figure S2. PME activity is induced by treatment with the MAMPs fig22 and elf18 but not the DAMP PEP1.

Supplemental Figure S3. *A. brassicicola* *PME* genes were differentially expressed during infection.

Supplemental Figure S4. PME activity in mock-inoculated samples corresponding to Fig. 3.

Supplemental Figure S5. PME activity in mock-inoculated samples corresponding to Fig. 4.

Supplemental Figure S6. Two Arabidopsis *PME* genes, other than *PME3*, showed increased expression after challenge with *A. brassicicola*.

Supplemental Figure S7. Arabidopsis *PME* genes up-regulated after inoculation with *A. brassicicola* were not required for *A. brassicicola*-induced PME activity or immunity against *A. brassicicola*.

Supplemental Figure S8. Expression of three Arabidopsis *PME* genes was up-regulated after challenge with *Pma* ES4326.

Supplemental Figure S9. PME activity in mock-inoculated samples corresponding to Figure 6.

Supplemental Figure S10. Clustering of *pme* mutants by cell wall differences and of *PME* genes by their pathogen-induced expression.

Supplemental Figure S11. Randomly selected T-DNA lines showed no difference in growth of *Pma* ES4326.

Supplemental Figure S12. Single mutants and multiply mutant lines with known cell wall alterations do not show reproducible differences in *A. brassicicola* disease severity.

Supplemental Figure S13. *Pma* ES4326-induced PME activity was unaltered in plants with multiple mutations in group D *PMEs*.

Supplemental Figure S14. Altered rosette morphology and enhanced susceptibility of *pme35-1* were due to second site mutation(s).

Supplemental Table S1. List of Arabidopsis *PME* genes.

Supplemental Table S2. List of primers used.

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