PECTIN METHYLESTERASE34 Contributes to Heat Tolerance through Its Role in Promoting Stomatal Movement[1][OPEN]

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Pectin, a major component of the primary cell wall, is synthesized in the Golgi apparatus and exported to the cell wall in a highly methylesterified form, then is partially demethylesterified by pectin methylesterases (PMEs; EC 3.1.1.11). PME activity on the status of pectin methylesterification profoundly affects the properties of pectin and, thereby, is critical for plant development and the plant defense response, although the roles of PMEs under heat stress (HS) are poorly understood. Functional genome annotation predicts that at least 66 potential *PME* genes are contained in Arabidopsis (*Arabidopsis thaliana*). Thermotolerance assays of *PME* gene T-DNA insertion lines revealed two null mutant alleles of *PME34* (At3g49220) that both consistently showed reduced thermotolerance. Nevertheless, their impairment was independently associated with the expression of HS-responsive genes. It was also observed that *PME34* transcription was induced by abscisic acid and highly expressed in guard cells. We showed that the *PME34* mutation has a defect in the control of stomatal movement and greatly altered PME and polygalacturonase (EC 3.2.1.15) activity, resulting in a heat-sensitive phenotype. *PME34* has a role in the regulation of transpiration through the control of the stomatal aperture due to its cell wall-modifying enzyme activity during the HS response. Hence, PME34 is required for regulating guard cell wall flexibility to mediate the heat response in Arabidopsis.

Acquired thermotolerance is the ability of cells to cope with lethal high temperatures as a consequence of pretreatment at an elevated but sublethal temperature. The accumulation of heat shock proteins (HSPs) is one of the best-characterized heat stress (HS) responses under the control of heat stress factors (HSFs) and plays an important role in the acquisition of thermotolerance (Alexandrov, 1994). Like other organisms, plants have developed extraordinary strategies to adapt to HS through the synthesis of HSPs, including HSP100, HSP90, HSP70, HSP60, and small HSPs (Lindquist and Craig, 1988; Wang et al., 2004). In addition, the heat-inducible class A HSFs, namely HSFA1s and HSFA2, are important for inducing and maintaining acquired thermotolerance in Arabidopsis (*Arabidopsis thaliana*; Schramm et al., 2006; Charng et al., 2007; Liu et al., 2011; Yoshida et al., 2011). Apart from HSP induction, abscisic acid (ABA), salicylic acid, reactive oxygen species (ROS), and Ca^{2+}/calmodulin are necessary for the establishment of thermotolerance in plants (Kotak et al., 2007).

Plant cell walls are complex extracellular structures and highly sophisticated mixtures of polysaccharides, proteins, and other polymers; these are assembled into a rigid but flexible and dynamically organized network (Wolf et al., 2009a). When the cell is challenged with stress conditions that affect the cell wall, such as specific transcriptional responses, some profitable cell wall proteins are induced and crucial changes in cell wall architecture occur (Klis et al., 2006). Hence, plant cell walls provide a physical barrier and are also essential for plant development and plant-pathogen interactions. In addition, the maintenance and modification of cell wall integrity are achieved by modifying the cell wall to relieve stress (Hamann et al., 2009; Hongo et al., 2012).

Pectin, one of major components of the plant cell wall, is synthesized in the Golgi apparatus before it is secreted into the cell wall as a methylester; it then can be

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demethylesterified subsequently by the action of pectin methylesterases (PMEs; EC 3.1.1.11). PMEs belong to carbohydrate esterases class 8 in the CAZy database (http://www.cazy.org; Cantarel et al., 2009). They are large multigene families: 66 open reading frames have been annotated as putative full-length PMEs in Arabidopsis. During cell wall formation, the homogalacturonan (HGA) fraction (the major component of pectins) is secreted and demethylesterified by PMEs to release both protons and methanol, thus creating negatively charged carboxyl groups (Caffall and Mohnen, 2009). The process of pectin demethylesterification contributes to the stiffening of the cell wall by producing blocks of unesterified carboxyl groups that interact with Ca^{2+} to promote the formation of Ca^{2+}-pectate gel (Liners et al., 1989). The demethylesterified HGA can be targeted by pectin-degrading enzymes such as endopolygalacturonases (PGs; EC 3.2.1.15), which affect the texture and rigidity of the cell wall (Micheli, 2001; Pelloux et al., 2007).

PMEs have attracted significant attention because of their importance in various aspects of plant physiology, including cellular separation, fruit ripening, internodal stem growth, elongation of the root tip and pollen tube, as well as plant defense against pathogens (Levesque-Tremblay et al., 2015b). Microarray-based analyses revealed that about 75% of the Arabidopsis *PME* genes exhibited distinct profiles of tissue-specific and stress-specific expression, with various expression levels corresponding to simultaneous abiotic and biotic stresses (Pelloux et al., 2007). This finding reflects the diversity of their roles in cell wall modification during plant development and in response to environmental stresses.

In higher plants, *PME* genes encode the so-called PRE-PRO proteins that have peptide motifs considered to be the signature of PMEs. The PRE domain, which leads to the export of PMEs to the cell wall, is formed from a common signal peptide and a transmembrane (TM; or signal anchor) domain. The mature protein, the active part of the protein (PME domain), is preceded by an N-terminal extension known as the PRO region, which shares similarities with PME inhibitors (PMEIs). The PRO region is involved in subcellular targeting and is an intramolecular inhibitor of PME activity, preventing the premature demethylesterification of pectins prior to secretion (Bosch et al., 2005; Bosch and Hepler, 2006; Wolf et al., 2009b). PMEs can be subdivided on the basis of the presence or absence of the PRO region into type I and type II: type I PMEs contain PRO regions, whereas type II PMEs do not (Micheli, 2001). Both types of PME proteins were found in Arabidopsis and tobacco (*Nicotiana tabacum*) pollen tubes (Li et al., 2002; Jiang et al., 2005; Tian et al., 2006). PME antibodies are only able to detect the fully processed mature PME that lacks the PRO region, indicating that the region is cleaved off either during or after secretion (Micheli, 2001; Wolf et al., 2009b).

The degree and pattern of methylesterification of HGA mediated by PMEs were found to be essential for eliciting defense responses in wild strawberry (*Fragaria vesca*; Osorio et al., 2008). Arabidopsis *PME41* and *PME48* participate in chilling/freezing tolerance through the regulation of brassinosteroid signaling and in pollen grain germination, respectively (Qu et al., 2011; Leroux et al., 2015). Arabidopsis PME3 has been found to interact directly with the cellulose-binding protein of the cyst nematode (*Heterodera schachtii*), reducing the level of pectin methylesterification in the cell wall to aid cyst nematode parasitism (Hewezi et al., 2008). Overexpression of Arabidopsis *PME5* and *PMEI3* resulted in softer and harder shoot apical meristem cell walls, respectively, compared with wild-type plants (Peaucelle et al., 2011). PME35 is responsible for the demethylesterification of pectins and is involved in regulating the mechanical strength of the supporting tissue in Arabidopsis inflorescence stems (Hongo et al., 2012). In addition, Arabidopsis PME activities contribute to the regulation of seed germination by altering the mechanical properties of the cell wall in micropylar endosperm and radicle cells (Müller et al., 2013). *HIGHLY METHYL ESTERIFIED SEEDS* (*PME6*) is abundant during mucilage secretion, acting on embryo morphology and mucilage extrusion, both of which are involved in embryo development (Levesque-Tremblay et al., 2015a). Pectin content, PME activity, and pectin demethylesterification also are involved in hydrogen peroxide (H_2O_2)-induced cell expansion and root diameter in the cell wall of rice (*Oryza sativa*) root tips (Xiong et al., 2015). Nevertheless, whether PMEs play a regulatory role in the heat response remains largely unknown.

In Arabidopsis leaves, the cell wall contains approximately 50% pectic polysaccharides; however, the proportion of pectin varies depending on the tissue, species, and environmental changes (Zablackis et al., 1995). Guard cell walls also are rich in pectic polymers, even though the roles of these polymers remain unclear (Zablackis et al., 1995; Jones et al., 2003; Harholt et al., 2010). Guard cells are important for modulating stomatal aperture and gas exchange in response to external environmental stimuli such as CO_2 concentration, light intensity, and humidity. Furthermore, they may be involved in endogenous hormonal stimuli, such as auxin and ABA, and in intracellular Ca^{2+} signaling transduction (Kim et al., 2010). Oligogalacturonides are fragments of pectin that act as signal molecules by inducing ROS from guard cells to reduce the stomatal aperture in both *Commelina communis* and tomato (*Solanum lycopersicum*; Lee et al., 1999). The different pectic polymers within the guard cell wall are essential for promoting or retarding stomatal movement (Jones et al., 2003). Arabinanase is a wall hydrolytic enzyme that degrades the arabinan chains between pectins and, therefore, maintains the fluidity within the pectin network to set the flexibility of the guard cell wall for stomatal aperture (Jones et al., 2003). *AsEXP1*, a heat-responsive expansin gene, was up-regulated during HS to increase cell wall elasticity, which is positively associated with

thermotolerance in *Agrostis* grass species (Xu et al., 2007). An Arabidopsis guard cell-expressed expansin, EXPA1, plays a role in regulating stomatal movement by altering the structure of the guard cell wall in a nonenzymatic and short-term cell wall extension manner (Zhang et al., 2011). Transpiration occurs through the stomatal aperture in aiding the dissipation of heat by cooling through water evaporation. The regulation of the stomatal aperture by water status and by internal CO_2 availability is well documented; even so, the specific cell wall proteins involved in modifying the guard cell wall response for stomatal movement under elevated temperatures still have not been fully addressed.

In a previous study, we verified that maintaining apoplastic Ca^{2+} homeostasis through PME activity has a pronounced effect on plant growth and heat response (Wu et al., 2010; Wu and Jinn, 2010). In this study, we used a genetic approach to dissect acquired thermotolerance by screening Arabidopsis *PME* gene T-DNA knockout lines to determine the contribution of *PMEs* to thermotolerance. We identified and characterized the type I PME *PME34* (At3g49220), which encodes a plasma membrane-localized and cell wall-deposited protein and functions during guard cell wall modification. Analysis of *PME* gene T-DNA insertion mutants revealed that the *PME34*-null mutants were hypersensitive to heat but independent of HSF-mediated transcriptional activation. The transcript accumulation of *PME34* was induced by exogenous ABA treatment, and the *PME34* promoter mediated strong *GUS* reporter expression in guard cells. *PME34*-deficient mutants showed greatly altered PME and PG activity as well as affected stomatal movement in response to heat. Thus, *PME34* plays an important role in controlling the stomatal aperture to regulate the rate of transpiration during the heat response.

RESULTS

Isolation and Characterization of the *PME34* Mutant with Altered Phenotype in Thermotolerance

To determine whether *PME* genes were involved in regulating the HS response, we tested 53 Arabidopsis homologous T-DNA insertion lines corresponding to 32 *PME* genes (Supplemental Table S1) for both acquired and basal thermotolerance. The corresponding *PME* gene designation was in accordance with the UniProt database (http://www.uniprot.org). Seeds of wild-type Columbia (Col), *HSP101*-mutant (*hsp101*, a heat-sensitive mutant, as reference; Hong and Vierling, 2000), and *PME*-defective (*pme*) plants were grown on the same plate and then treated with different HS conditions (Hsu et al., 2010; Wu et al., 2012).

No phenotypic differences were observed in 29 of the 32 *PME* gene T-DNA insertion mutants compared with Col under acquired thermotolerance analysis with the HS regime of 1-h 37°C sublethal HS → 22°C recovery for 2 h → 44°C lethal heat stress (LHS) for 160 min. Two defective *PME34* alleles (*pme34-1* and *pme34-2* as null mutants; Supplemental Fig. S1, A and B) and *PME28* (At5g27870; *pme28*) were greatly impaired under the acquired thermotolerance assay (Fig. 1A). *pme34* and *pme28* mutants showed substantially reduced survival at 50% compared with Col plants (Fig. 1B). No major phenotypic differences between *pme34*, *pme28*, and Col plants were observed during the basal thermotolerance test (Supplemental Fig. S2), whereas

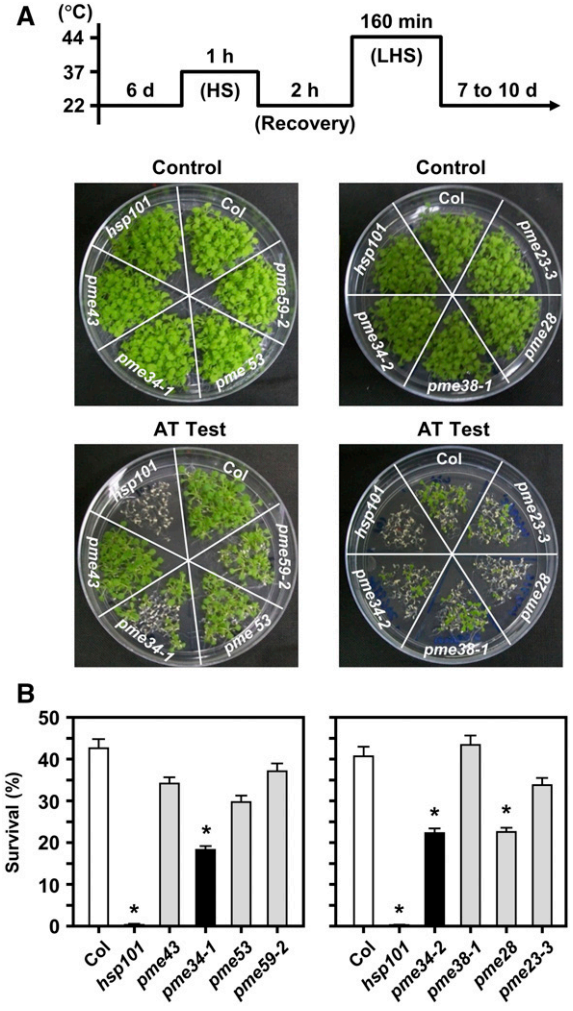

Figure 1. Phenotype characterization of *PME* T-DNA insertion lines under acquired thermotolerance (AT). Six-day-old seedlings of Arabidopsis wild type (Col), the *HSP101* mutant (*hsp101*), and *PME*-defective (*pme*) lines were treated with 22°C for 2 h as a control and treated with 37°C, 1 h (sublethal HS [HS]) → 22°C, 2 h (recovery from HS; recovery) → 44°C, and 160 min (LHS) for the AT test. The pictogram shows the HS regime. A, Photographs were taken after 7 to 10 d from the end of treatment. B, Seedlings that survived were counted and survival rate was calculated (%). Two alleles of *PME34*-null mutants, *pme34-1* and *pme34-2*, were hypersensitive toward the AT test. Data are means ± SD from three independent replicates (n = 150 seedlings). *, Significant at $P < 0.05$ compared with Col.

defective *PME7* (At1g02810; *pme7*) showed decreased survival under basal thermotolerance. After two rounds of screening, the rest of the *PME* T-DNA insertion lines showed no significant difference in thermotolerance from Col, as shown in Supplemental Figure S3.

The *PME* gene expression profiles retrieved from the Arabidopsis microarray database (AtGenExpress consortium; http://www.arabidopsis.org/info/expression/ATGenExpress.jsp) suggested that *PME34* is expressed ubiquitously in many tissues (Supplemental Table S2); in addition, both *PME28* and *PME7* were expressed specifically in pollen grains and roots, respectively. Here, we aimed to determine the function of *PME34* with respect to acquired thermotolerance.

Three independent T-DNA transgenic lines (*PME34-C1* to *PME34-C3*) containing the full-length *PME34* gene and its 2-kb potential promoter region generated in a *pme34-1* background were obtained (Supplemental Fig. S4A), and *PME34* complementation lines were restored to the Col phenotype of acquired thermotolerance during heat treatment (Supplemental Fig. S4B). Thus, the approaches of genetic screening and complementary lines confirmed that *PME34* is required for heat responses.

Expression of *PME34* under Abiotic Stress and Hormone Treatments

To determine whether *PME34* is regulated by abiotic stresses, 6-d-old seedlings underwent treatments with HS, mannitol, NaCl, $NaAsO_2$, and $CdCl_2$, among others. The expression levels were analyzed using reverse transcription (RT)-PCR (Fig. 2, A and B) or real-time quantitative (qRT)-PCR (Fig. 2, C–E). The expression of *PME34*, *PME28*, and *PME7* was strongly reduced with 3-h 37°C HS and then reversed after a 3-h recovery from the HS (Fig. 2, A, C, and D). Other treatments, such as NaCl, H_2O_2, mannitol, $NaAsO_2$, and $CdCl_2$, had a moderate effect on the expression of

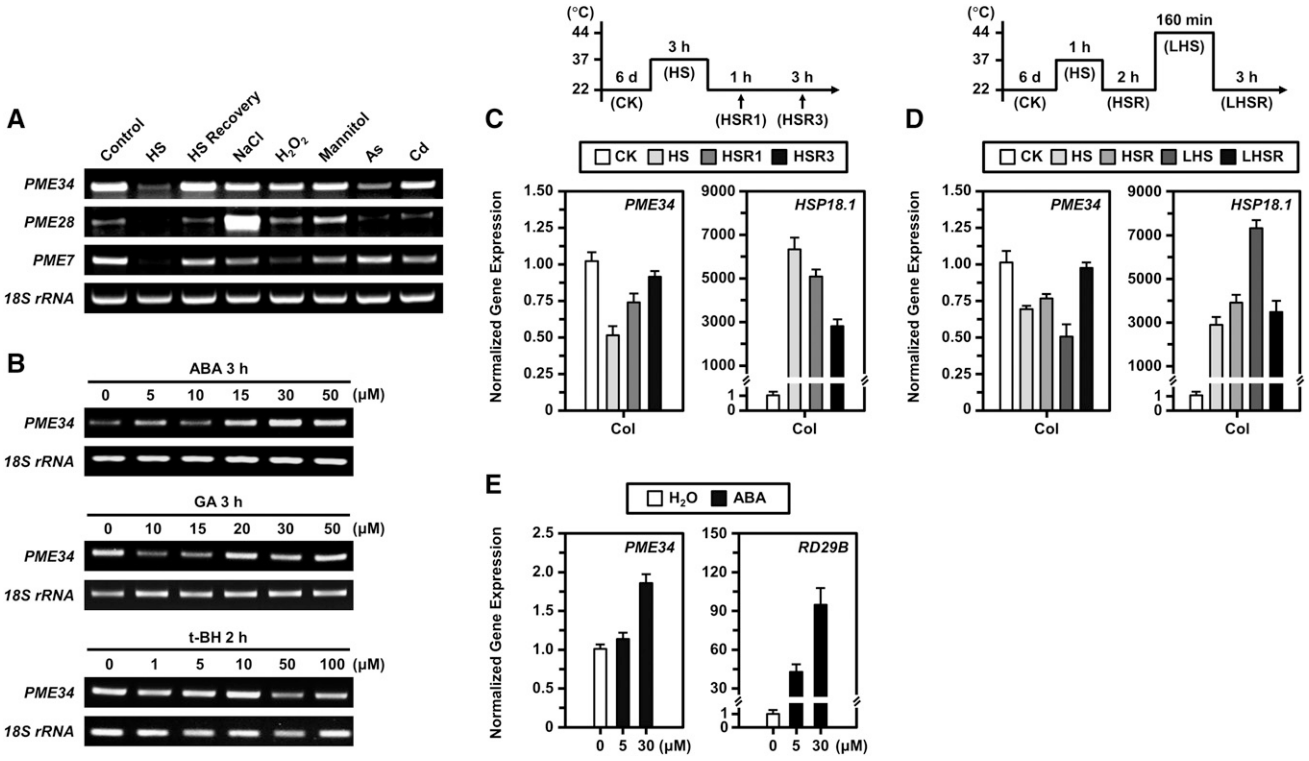

Figure 2. *PME* gene response to abiotic stresses and hormone treatments. A, The expression levels of *PME34*, *PME28*, and *PME7* in response to abiotic stresses were analyzed by RT-PCR. Six-day-old seedlings were treated with 37°C HS for 3 h (HS) and then with HS with recovery at 22°C for 3 h (HS recovery), 150 mM NaCl for 8 h, as well as 6-h treatments of 300 mM D-mannitol, 0.5 mM H_2O_2, 250 μM $NaAsO_2$ (As), and 500 μM $CdCl_2$ (Cd). Treatment at 22°C with water was used as a control. The transcript level of *PME34* was highly restored at the recovery phase after 37°C HS. B, The expression of *PME34* was analyzed with the application of different concentrations of ABA, gibberellic acid (GA), and *tert*-butyl hydroperoxide (t-BH) as indicated. *18S rRNA* was used as a loading control. C to E, Six-day-old Col seedlings were analyzed by basal (C) and acquired (D) thermotolerance tests as well as by different concentrations of ABA for 3 h (E). Gene expression was analyzed by qRT-PCR, and pictograms show the HS regime. The heat-responsive gene *HSP18.1* (At5g59720) and the ABA-responsive gene *RD29B* (At5g52300) were used as references. The fold expression was normalized relative to 22°C control (CK) or water treatment. Data are means ± SD of three biological replicates. *PP2AA3* (*PP2A*) was used as an internal control.

PME34 and *PME7*; nevertheless, NaCl treatment markedly enhanced *PME28* expression (Fig. 2A). The expression of *PME34* was elevated with an increase of ABA content (Fig. 2, B and E), whereas GA and *tert*-butyl hydroperoxide (a lipid peroxidation inducer) had no major effect on *PME34* expression.

Furthermore, ABA response (*abi1-1*) and biosynthesis (*aba2-1*) mutants of Arabidopsis were used to investigate the expression of *PME34* in the presence and absence of ABA as well as in the response to HS. Under ABA treatment, the expression of *PME34* was impaired significantly in the *abi1-1* mutant compared with wild-type Landsberg *erecta* (L*er*) plants, but expression remained unaffected in the *aba2-1* mutant plants (Fig. 3A). *RD29B*, a master gene in the ABA response, served as a reference (Fig. 3B). Notably, the expression levels of *PME34* were significantly affected in the *abi1-1* mutant under heat treatments (Fig. 3, C and D) compared with L*er*. Therefore, ABA signaling is required for proper *PME34* expression.

Plasma Membrane Localization and Cell Wall Deposition of PME34

PME34 (UniProt no. Q9M3B0) belongs to the type I PMEs, as it contains a signal peptide containing a TM region (amino acids 46–66), a PMEI domain (amino acids 81–232), predicted subtilisin-like proteases processing basic motifs (amino acids 250–253 and 271–274), and a PME domain (amino acids 284–582), according to the UniProt database (Supplemental Fig. S5). The full-length PME34 was fused to the N- or C-terminal end of GFP (PME34-GFP or GFP-PME34), cotransformed with an mCherry-tagged plasma membrane localization marker (PM-RFP), and transiently expressed in Arabidopsis protoplasts and onion (*Allium cepa*) epidermal cells (Fig. 4).

PME34-GFP (or GFP-PME34) was colocalized with the PM-RFP marker to the plasma membrane (Fig. 4A). To determine the topology of the PME34 TM, a fluorescence protease protection assay was used to restrict the proteolytic digestibility of the PME34-GFP-tagged

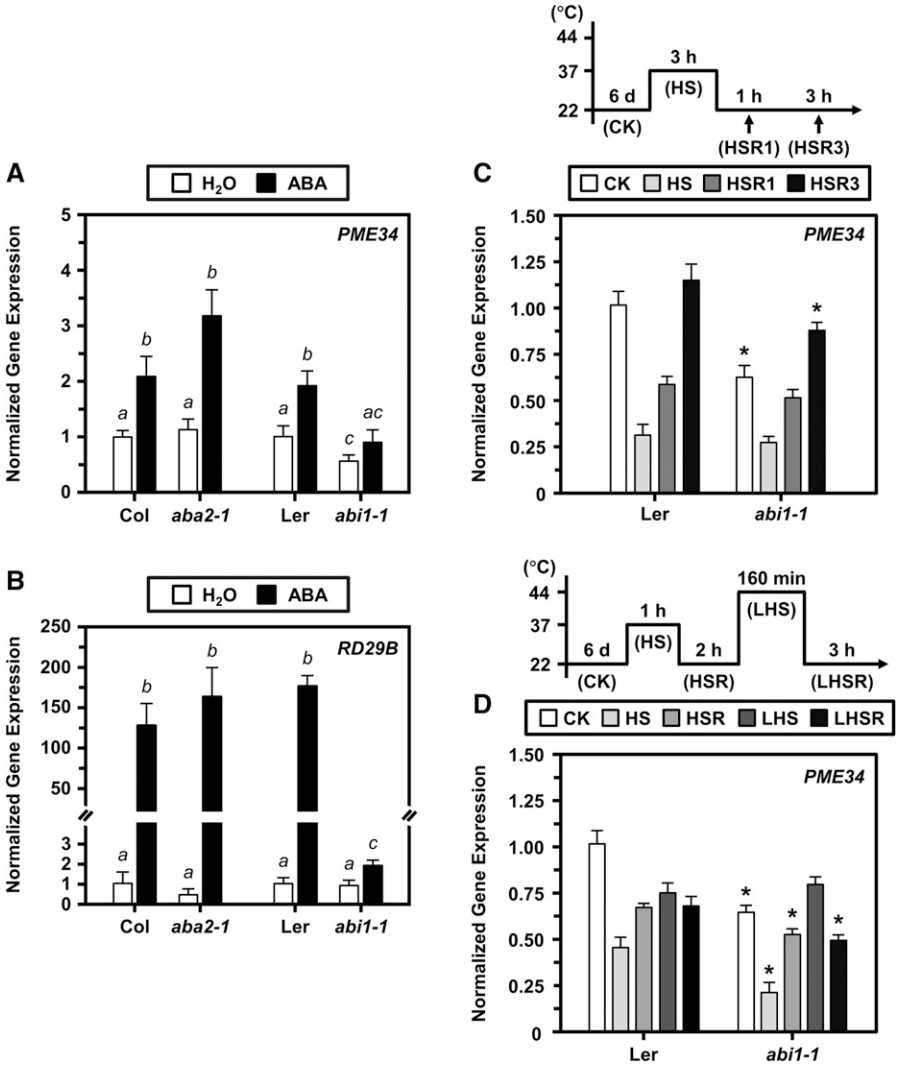

Figure 3. Transcriptional profiles of *PME34* in ABA-deficient (*aba2-1*) and ABA-insensitive (*abi1-1*) plants in response to ABA and heat. Six-day-old seedlings of *aba2-1* and *abi1-1* were incubated in water containing 30 μM ABA for 3 h (A and B) and then analyzed by basal and acquired thermotolerance tests (C and D) as indicated in Figure 2. The expression levels of *PME34* and *RD29B* were analyzed by qRT-PCR. The fold expression was normalized relative to their corresponding wild-type plants (Col or L*er*) in water or 22°C control (CK) treatment. Data are means ± SD of three independent replicates. *a*, *b*, *c*, and * represent significantly different values compared with wild-type plants given water or 22°C control treatment ($P < 0.05$). *PP2A* was used as an internal control.

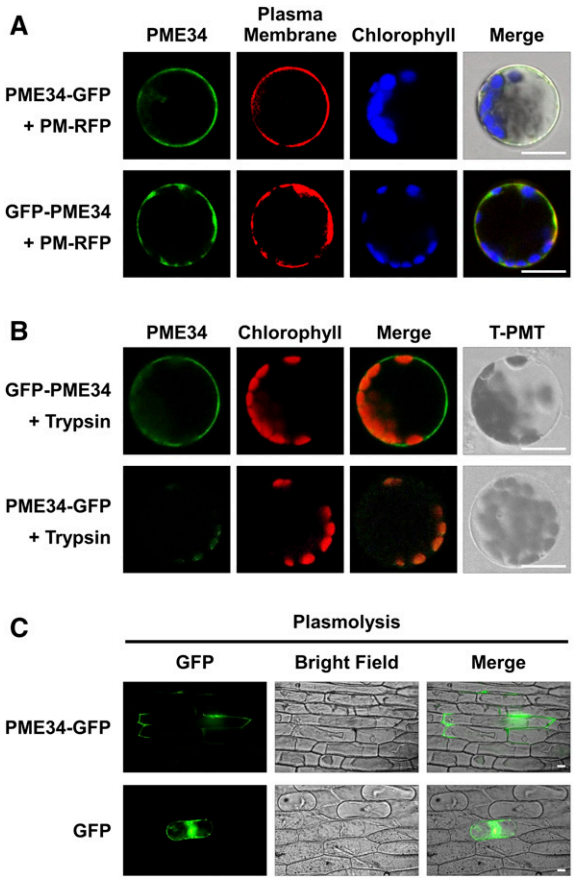

Figure 4. Subcellular localization analyses of PME34 in Arabidopsis mesophyll protoplasts and onion epidermal cells. A, PME34 was fused to N- or C-terminal GFP and coexpressed with the mCherry red fluorescent protein-labeled plasma membrane marker (RFP-PM) in protoplasts, as indicated. Blue shows chlorophyll using autofluorescent light. B, The protoplast fluorescence protease protection assay was performed with trypsin treatment. T-PMT, Transmitted light channel. C, Onion epidermal cells were treated with mannitol for the plasmolysis assay. GFP was used as a control. Bars = 20 μm.

TM (Fig. 4B). The trypsin treatment confirmed that PME34 seems to be protease sensitive on the membrane domain facing the extracellular space. Plasmolysis analysis of onion epidermal cells by treatment with mannitol confirmed that PME34 was deposited into the cell wall matrix (Fig. 4C). Thus, we suggest that a posttranslational modification of the plasma membrane-localized PME34 allows it to deposit to the cell wall.

PME34 Overexpression Shows Increased PME Activity But No Effect on Thermotolerance

So far, there are few examples of a protein sequence annotated as a PME contributing to PME activity in vivo. To explore the physiological role of PME34, we identified three independent cauliflower mosaic virus (CaMV) *35S* promoter::*GFP-PME34* transgenic lines (*PME34-OE1* to *PME34-OE3*) with increased accumulation of *PME34* mRNA transcripts using RT-PCR (Fig. 5A). Under normal conditions, total PME activity of *PME34-OE* lines was 2-fold greater than that detected in Col plants (Fig. 5B). Thus, PME34 protein can function as an active PME. However, glutathione *S*-transferase (GST)-tagged PME34 formed cytoplasmic inclusion bodies in *Escherichia coli*, and PME34 activity did not recover after resolubilization from this insoluble fraction (Supplemental Fig. S6). Therefore, PME34 protein activity is better suited to be analyzed in plant cells.

In addition, no significant difference in thermotolerant response was observed between *PME34-OE* lines and Col plants (Fig. 6, A and B).

Temporal Regulation of PME and PG Activity Is Required for Acquired Thermotolerance

The integrated actions of PME and PG activity are important for cell wall modifications that contribute to the maintenance of cell wall integrity, which is important for relieving HS (Moustacas et al., 1991; Jones et al., 2003; Bellincampi et al., 2004). Heat treatment was applied as in Figure 1 to assess the actives of PME and PG. Samples were collected at each step of the treatment, as indicated at the top of Figure 7, and then protein extracts were analyzed for PME (Fig. 7A) and PG (Fig. 7B) activity as described previously (Wu et al., 2010).

When compared with Col plants at 22°C as a control (treatment 1), the induction of PME activity in Col

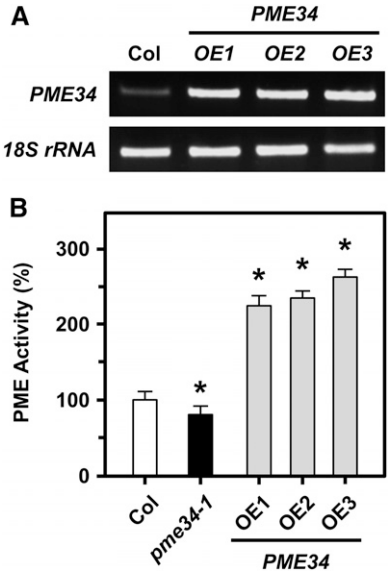

Figure 5. PME activity in *PME34* overexpression lines. Six-day-old seedlings of Col, *pme34-1*, and three independent CaMV *35S* promoter::*GFP-PME34* lines (*PME34-OE1*, *PME34-OE2*, and *PME34-OE3*) were analyzed. A, *PME34* expression levels were analyzed by RT-PCR. *18S rRNA* was used as a loading control. B, PME activity (%) was normalized to that of Col. Data are means ± SD of three independent replicates. *, Significant at $P < 0.05$ compared with Col.

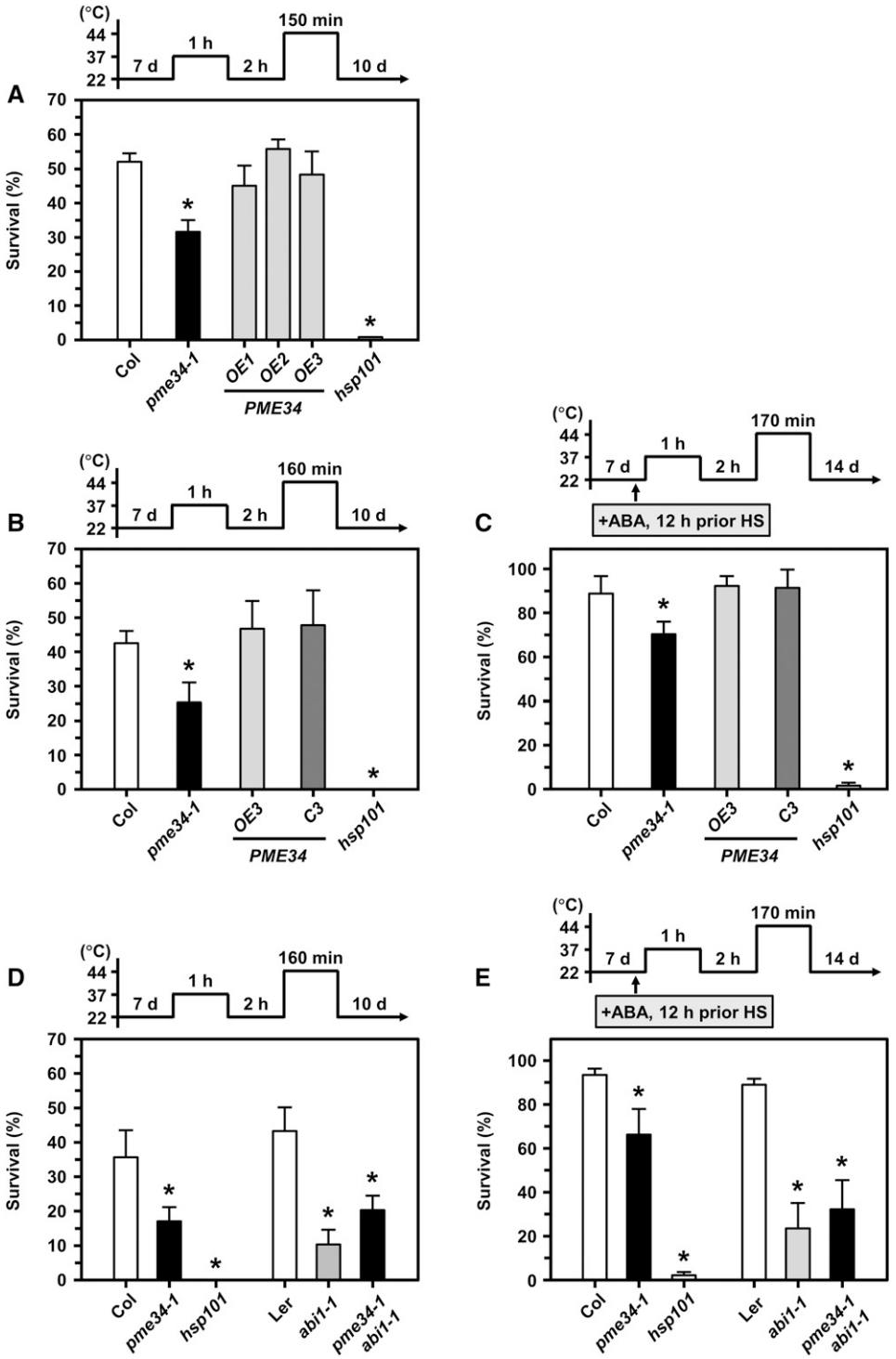

Figure 6. Thermotolerance test of *pme34-1*, *PME34* overexpression (*PME34-OE1*, *PME34-OE2*, and *PME34-OE3*), *PME3* complementation in a *pme34-1* background (*PME34-C3*), and *pme34-1 abi1-1* mutants. Acquired thermotolerance analysis was performed in 7-d-old seedlings, and pictograms show the HS regime. Plates were incubated without (A, B, and D) or with (C and E) 2.5 mL of 10 μM ABA for 12 h before heat at 37°C for 1 h. *hsp101* was used as a reference. Survival was measured after 10 or 14 d from the end of treatment. Data are means ± SD of three biological replicates (n = 150 seedlings). *, Significant at $P < 0.05$ compared with wild-type plants, Col or L*er*.

increased to 1.26-fold after 1-h 37°C mild HS treatment (treatment 2) but was 32.3% lower than the control after 2-h recovery from the 37°C HS (treatment 3). Remarkably, Col PME activity was 1.77-fold of that under treatment 3 following the treatment of 44°C LHS for 160 min (treatment 4). In the 22°C and 37°C HS treatments, PME activity in *pme34* plants was 21% and 40% lower than that of the respective Col plants. Interestingly, PME activity of *pme34* was greatly enhanced to 1.36-fold of the respective Col following 44°C LHS treatment (Fig. 7A).

The PG activity in Col plants showed a similar trend in response to 37°C treatment and recovery from 37°C HS (treatments 2 and 3) compared with the 22°C control (treatment 1). Notably, following the 44°C LHS treatment (treatment 4), activity in Col decreased to 17% of

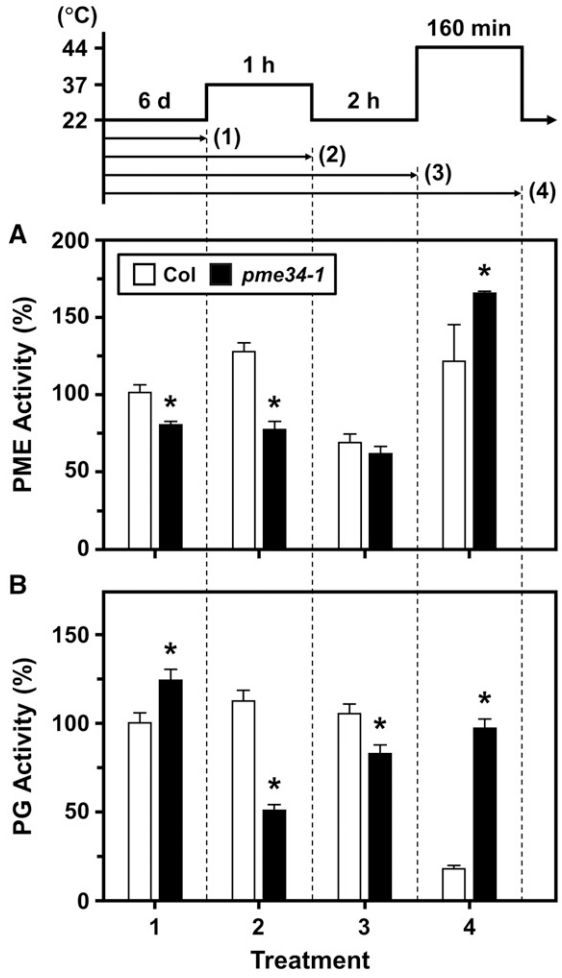

Figure 7. PME and PG activity in Col and *pme34* plants in response to HS. Six-day-old seedlings of Col and *pme34-1* were analyzed. The pictogram shows the HS regime. The activity (%) of PME (A) and PG (B) was normalized to the control (Col at 22°C treatment). Data are means ± SD of three independent replicates. *, Significant at $P < 0.05$ compared with Col.

treatment 3. PG activity in *pme34* plants was higher than that of Col plants, at 1.24-fold Col activity under normal conditions. It was also 55% and 21% lower than the respective Col activity under 37°C HS and 2-h recovery from the 37°C HS. Noticeably, PG activity in *pme34* plants increased to 5.41-fold of the respective Col following 44°C LHS treatment (Fig. 7B).

In summary, the *PME34* mutation resulted in abnormal actions of PME and PG in response to heat, which is associated with its heat-sensitive phenotype.

The Heat-Sensitive Phenotype of *pme34* Is Independent of Impaired *HSP* Gene Expression

The mechanisms of plant thermotolerance have been well studied, mainly through the regulatory network of HSF-mediated transcriptional activation. To examine the association with the heat-sensitive phenotype of *pme34*, we investigated the pattern of HS up-regulated marker gene expression in *pme34* plants (Fig. 8).

There was no difference observed in the mRNA transcript levels of *HSP18.1*, *HSP70*, *HSP90*, and *HSP101* (Fig. 8A) or in the protein accumulations of class I small HSPs and HSP70 (Fig. 8B) in *pme34* compared with that of Col plants under normal or 37°C HS treatment. The patterns of heat-responsive *HSF* gene expression in *pme34*, such as *HSFA1a*, *HSFA2*, *HSFB1*, and *HSFC1*, were shown to be similar to those in Col plants (Supplemental Fig. S7A). HSFA1s and HSFA2 function as master regulators of the HS response and are essential for downstream HS-related gene expression (Schramm et al., 2006; Charng et al., 2007; Liu et al., 2011; Yoshida et al., 2011). Compared with Col plants, there were no observable differences in the expression of *PME34* in either the quadruple mutant *hsfa1a/b/d/e* or *hsfa2* (Supplemental Fig. S7B). Therefore, it can be concluded that the heat-sensitive phenotype of *pme34* occurs independently of HSF-mediated transcriptional activation.

PME34 Is Involved in ABA-Mediated Downstream Responses

ABA-deficient and -insensitive mutants are sensitive to HS, whereas the ABA-signaling master effector ABA-RESPONSIVE ELEMENT-BINDING PROTEIN1-overexpressed plants have been found to show

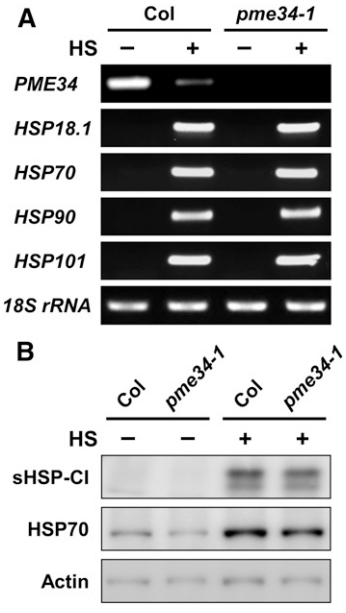

Figure 8. Transcriptional levels and protein accumulations of major heat response genes in Col and *pme34* plants in response to heat. Six-day-old seedlings of Col and *pme34-1* were treated without (−) or with (+) heat at 37°C for 1 h (HS). A, The expression of the heat up-regulated marker genes *HSP18.1*, *HSP70*, *HSP90*, and *HSP101* was analyzed by RT-PCR. B, The accumulation of class I small HSPs (sHSP-CI) and HSP70 was analyzed by western blotting. *18S rRNA* and actin were used as loading controls.

enhanced thermotolerance; thus, ABA plays a role in the thermotolerance response (Larkindale and Knight, 2002; Kim et al., 2004; Larkindale et al., 2005; Huang et al., 2016; Suzuki et al., 2016).

We used a 12-h ABA pretreatment before testing ABA-mediated thermotolerance, and the 44°C 160-min LHS period was extended to 170 min (Fig. 6, C and E). *pme34-1* showed ABA enhanced survival under thermotolerance, as found previously in Col (Huang et al., 2016); however, *pme34-1* plants still displayed significantly reduced thermotolerance in comparison with Col plants (Fig. 6, B and C). Notably, *abi1-1* and *pme34-1 abi1-1* mutants did not show significant difference under thermotolerance treatments, either pretreated with ABA or untreated (Fig. 6, D and E). Thus, ABA signaling is required for the regulation of *PME34* expression and required for the ABA-mediated HS response.

PME34 Expression in Guard Cells Was Increased by ABA Treatment

Using guard cell-specific microarray analyses side by side with mesophyll cell-specific microarray data to search putative strong guard cell-specific promoters (Yang et al., 2008), 59 *PME* genes were analyzed under ABA treatment. ABA-induced *PME34* expression increased to 13.1-fold in guard cells but not in mesophyll cells (Supplemental Fig. S8; Supplemental Table S3). Based on the microarray analysis, the upstream 2-kb potential promoter region (contains four potential abscisic acid-responsive elements [ABREs]; Supplemental Fig. S9) of *PME34* was used for a tissue-specific expression assay.

Histochemical analysis of the *PME34* promoter::*GUS* expression showed that *PME34* was highly expressed in vascular tissues and guard cells (Fig. 9, A and B; Supplemental Fig. S10) and was strongly up-regulated following 3 h of 20-μm ABA treatment in guard cells (Fig. 9, C and D). Therefore, *PME34* has a potential role in the regulation of ABA-mediated stomatal movements.

PME34 Affects Transpiration and Stomatal Movement

We examined the rapidity of the stomatal response in *pme34* by analyzing water transpiration rates. The measurement of water loss from collected leaves treated without or with heat was recorded every 20 min for 120 min at room temperature (Figs. 10 and 11).

At 22°C normal growth condition (Fig. 10A), *pme34* plants had a significantly greater rate of water loss compared with Col plants. Conversely, Col lost more water than *pme34* after 37°C HS for a 1-h treatment (Fig. 10B). Thus, the water loss of *pme34* was little different between without or with HS, as shown in Figure 10C (merged data of Fig. 10, A and B), which indicated that the transpiration control of *pme34* was

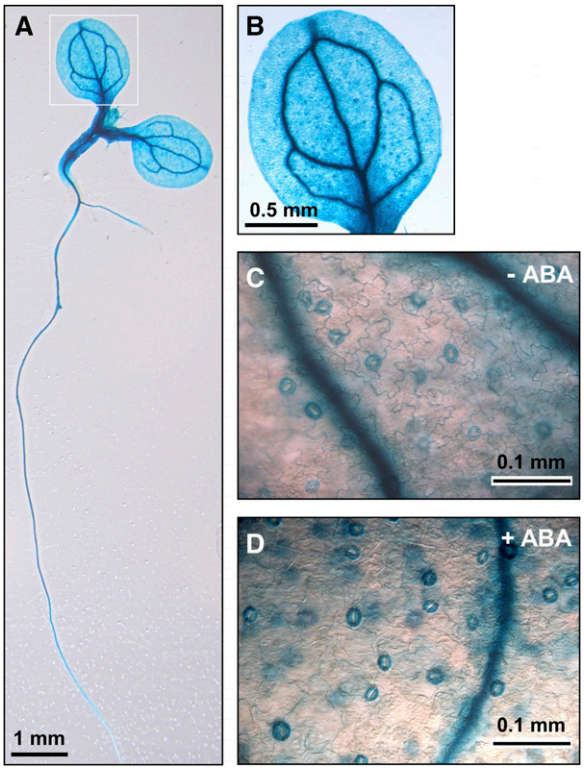

Figure 9. Histochemical analysis of *PME34* promoter::*GUS* expression. Seven-day-old seedlings were analyzed for GUS activity. A and B, GUS expression of *PME34* in a seedling (A) and cotyledon magnification of A (B). C and D, Cotyledons of the same seedling were treated without (C; −) or with (D; +) 30 μm ABA for 3 h, and then specimens were immersed together in staining solution.

affected. This suggests that the stomatal apertures of *pme34* plants may not be managed properly under the heat response because of locked guard cell wall movement preventing stomata from opening or closing.

Next, the transpiration rate of detached leaves was measured following the further treatment of 44°C LHS from 30 to 160 min, as indicated at top right of Figure 11. The *PME34*-deficient line lost more water than Col (Fig. 11, B and C); in addition, stomatal apertures of *pme34* were opened wider than those of Col plants, which were able to restrict the aperture over time (Fig. 12; Supplemental Fig. S11). Furthermore, we analyzed the stomatal morphology, density, and leaf vein networks (Roschzttardtz et al., 2014) of *pme34*; however, there were no significant correlations with the plants lacking *PME34* gene expression (Supplemental Figs. S11–S13).

Due to differences in stomatal apertures in *pme34* after HS treatments as indicated in Figures 10 and 11, we used thermal imaging by an infrared camera to analyze leaf surface temperature change (Supplemental Fig. S14). After 1-h 37°C HS treatment, leaves collected from Col showed cooler surface temperatures than those observed from *pme34* following a 60-min

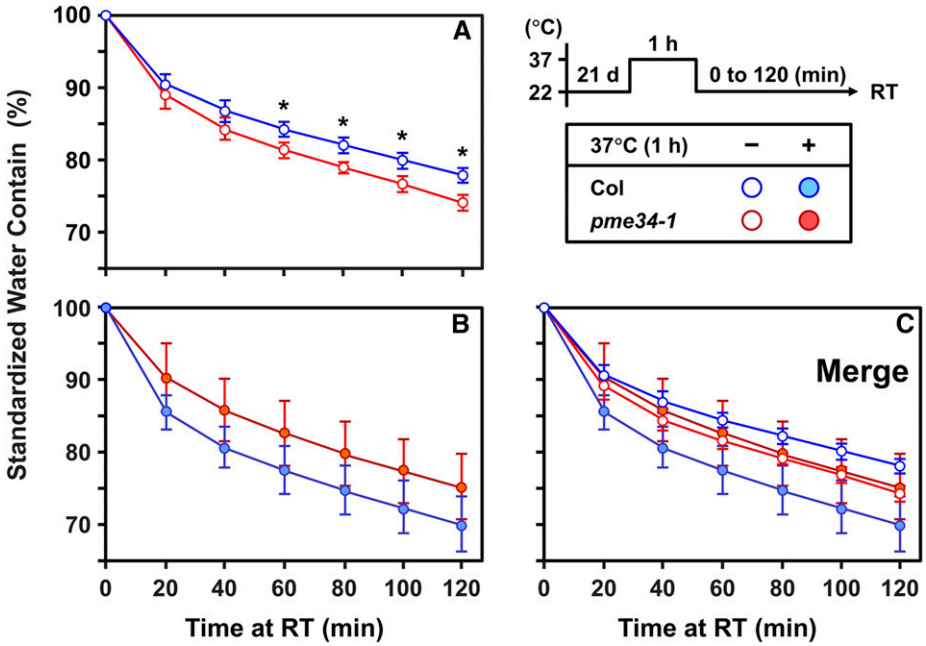

Figure 10. Transpiration rate in Col and *pme34* plants after mild heat treatment. A and B, Leaves of Col and *pme34-1* plants were collected following treatment without (A; −) or with (B; +) heat at 37°C for 1 h. Transpiration rate was examined by measuring water loss (% of initial fresh weight) and recorded every 20 min for 120 min at room temperature (RT; 24°C–26°C and 55%–70% humidity), as indicated. The pictogram shows the HS regime. C, Merged data from A and B. Data are means ± SD of three independent replicates (n = 50 seedlings). *, Significant at $P < 0.05$ compared with Col.

exposure at room temperature (Supplemental Fig. S14A). In addition, after the 80-min 44°C LHS treatment, the *pme34* leaves showed lower temperatures and a more dehydrated phenotype than those of Col plants (Supplemental Fig. S14B). This observation supports the notion that mutation of *PME34* influences changes in stomatal movement and inflexible stomatal apertures in response to heat.

Consequently, we suggest that Arabidopsis *PME34* controls stomatal movement through the modification of the guard cell wall with its enzymatic activity and plays an important role in the regulation of heat responses.

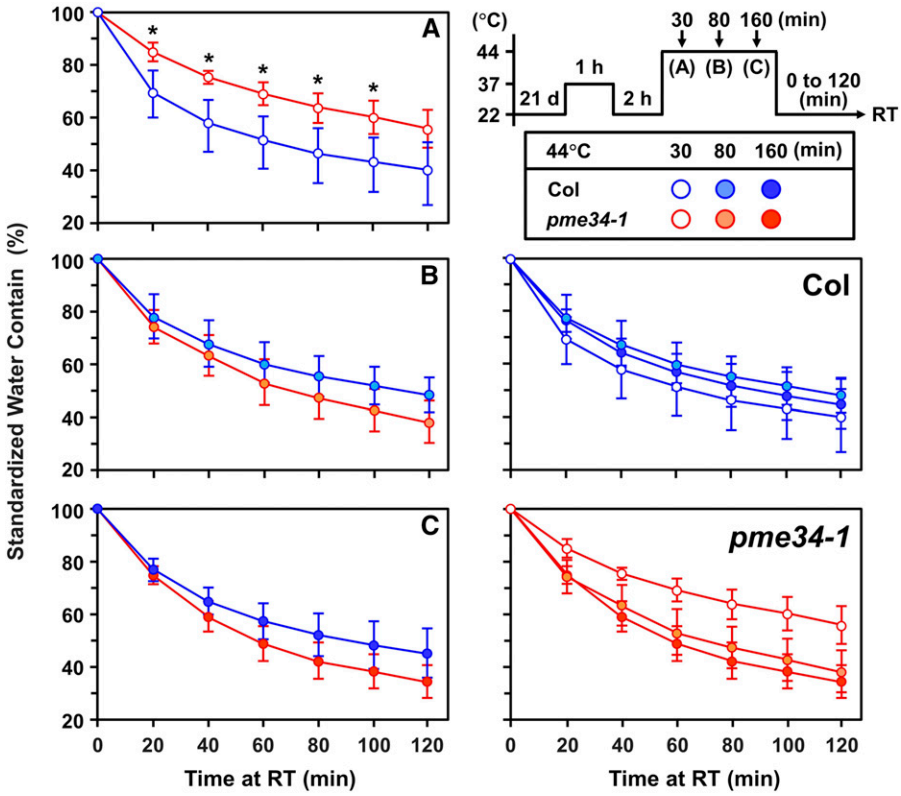

Figure 11. Transpiration rate in Col and *pme34* plants after lethal heat treatment. A to C, Leaves of Col and *pme34-1* plants were collected following 30 (A), 80 (B), or 160 (C) min of 44°C lethal heat treatment, and then the transpiration rates were measured at room temperature (RT), as indicated in Figure 10. The pictogram shows the HS regime. The graphs at right depict the merged transpiration rates of Col and *pme34-1* from A to C. Data are means ± SD of three independent replicates (n = 50 seedlings). *, Significant at $P < 0.05$ compared with Col.

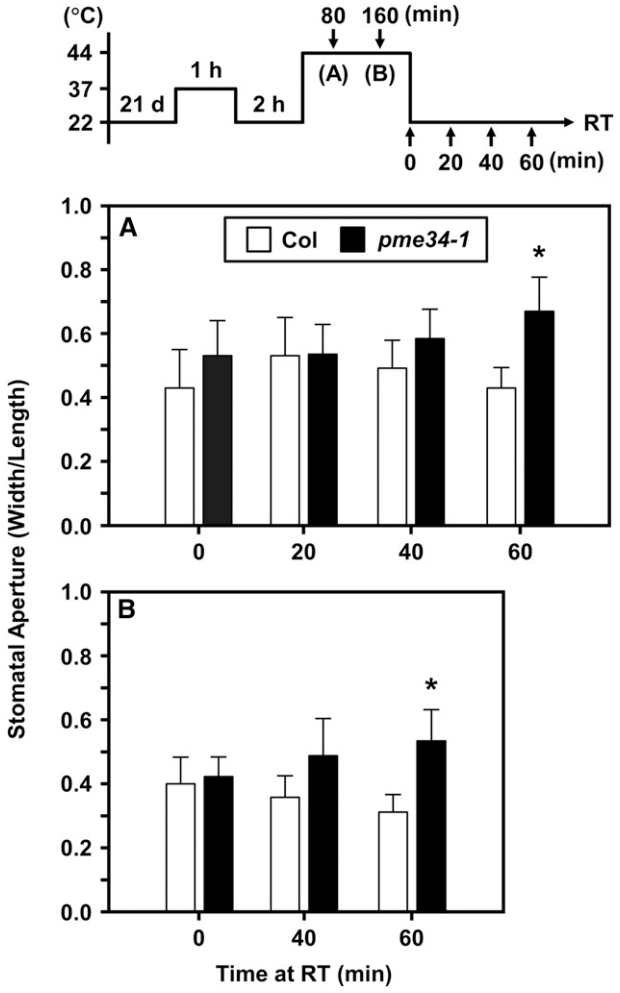

Figure 12. Stomatal aperture in Col and *pme34* plants after lethal heat treatment. A and B, Leaves of Col and *pme34-1* plants were collected following 80 (A) or 160 (B) min of 44°C lethal heat treatment, and then stomatal apertures were recorded every 20 min at room temperature (RT), as indicated Figure 10. The pictogram shows the HS regime. Stomatal apertures were measured in terms of width-length ratio. Data are means ± SD of three independent replicates (n = 150 stomata). *, Significant at $P < 0.05$ compared with Col.

DISCUSSION

Plant cell walls form a flexible structural barrier; they can rapidly remodel in response to biotic or abiotic stimuli. Genes encoding enzymes of synthesizing or hydrolyzing components of the cell wall display differential expression after subjection to different stress conditions (Houston et al., 2016). This suggests that they may moderate stress responses through changes in cell wall composition to maintain cell wall integrity. Using the comprehensive microarray polymer profiling has revealed that changes in the activities of PME, PG, and cellulose synthase are associated with particular modifications in the physical properties and composition of cell walls (Moller et al., 2007). The cell wall enzymatic modification of the pectin network in guard cell walls has a profound effect on stomatal function; for example, arabinanase blocks the movement of guard cells, whereas PME and PG cause an increase in stomatal aperture opening (Jones et al., 2003). Here, we examined the genetic basis for heat sensitivity-associated phenotypes, in particular a member of the Arabidopsis *PME* family, *PME34*. The novel functions of *PME34* are involved in distinct cellular processes, including the modification of the guard cell wall and appropriately adjusting the capability for transpiration, in addition to being a positive regulator of the heat response.

PME34 Is Required for Acquired Thermotolerance

To further validate our previous observations (Wu et al., 2010; Wu and Jinn, 2010), a genetic screening approach of Arabidopsis *PME* T-DNA insertion mutants was used to define *PME* function in response to heat. We found that *pme7*, *pme28*, and *pme34* each exhibited thermosensitive phenotypes at different levels (Fig. 1; Supplemental Fig. S2). The expression profiles of *PME7*, *PME28*, and *PME34* showed them to be tissue specific in roots and pollen grains and ubiquitously expressed in many tissues, respectively, by comparative analysis of microarray data in Arabidopsis (Supplemental Table S2). *PME34* is preferentially expressed in young leaves and stems compared with senescing leaves and roots; in particular, *PME34* has the ability to respond to various stresses, including *Pseudomonas* spp. infection and wounding as well as treatments with ABA and cold. *PME7* (encoding a protein without a potential TM domain) and PME28 showed sequence identities of 44% and 38% with PME34, respectively (Supplemental Fig. S15); three of these PMEs possess a conserved PME domain. According to the Affymetrix ATH1 genome array database, there are at least 14 Arabidopsis *PME* isoforms expressed in pollen (Bosch and Hepler, 2005). *VANGUARD1* (*VGD1* [*PME5*]) encodes a PME and is required to enhance the growth of pollen tubes in the style and transmitting tract tissues. Two PME isoforms, *PME4* (At2g47030) and *VGDH2* (At3g62170), are highly homologous to VGD1, with 85% and 69% sequence identity, respectively; however, the *vgd1* mutant phenotype could be restored to the wild type solely by *PME4* (Jiang et al., 2005). Although most Arabidopsis *PME* genes encode similar proteins, they do not necessarily reflect the functional redundancy within the family of *PME* genes (Pelloux et al., 2007).

PME and PG Activities Are Differentially Regulated by Heat

We demonstrated that *PME34* transcription was reduced by heat while it was restored to normal levels during recovery (Fig. 2, A, C, and D). The cell wall modification enzyme has been reported to be involved in the regulation of the heat response in *Brassica napus*.

The expression of *B. napus PME35* (EV193389) also was down-regulated nearly 10-fold under HS (Yu et al., 2014). We found a 21% decrease in total PME activity of *pme34* compared with Col under normal conditions (Fig. 7A). This result is consistent with the finding of the defective PME *vgd1* mutant, which exhibits 18% less overall PME activity than the wild type (Jiang et al., 2005). Bosch and Hepler (2005) found that the high transcriptional level of *PME* did not automatically translate and accumulate a higher protein level; thus, enzymatic activity regulation is likely more crucial than translation/protein accumulation. Notably, PME plays a role in pathogen defense; however, it was found that it is a specific effect of PME, such as a change in the status of pectin methylesterification, that determines immunity, rather than total PME activity (Bethke et al., 2014). That there are 66 PME genes in Arabidopsis may suggest an extended genetic redundancy; nevertheless, selected *pme* mutants, such as *pme3* and *pme12* plants, showed increased *Pseudomonas syringae* pv *maculicola* ES4326-induced PME activity, possibly due to overcompensation for loss of these PMEs (Bethke et al., 2014). Here, we showed that the *PME34* transcript decreased during HS but the total PME activity was increased. Thus, apart from the transcriptional regulation of *PME34* expression, the posttranslational modification of PME34 also is implicated in the regulation of enzymatic activity for its function in heat responses.

PME activity is spatially and temporally regulated during plant growth and in response to environmental cues. PME activity also depends on pH, the degree of pectin methylesterification (Micheli, 2001; Pelloux et al., 2007), as well as the heat activation response (Wu et al., 2010, 2012). The demethylesterification activities of PME isoforms can act linearly at alkaline pH but randomly at acidic pH. When PMEs act randomly on the HGA, the demethylesterification releases protons that promote PG activity and contribute to cell wall loosening (Moustacas et al., 1991). Previously, guard cell wall expansion and separation have been associated with PME and PG activity. Through the identification of *quartet* (*qrt*) mutants, it was suggested that PME QRT1 (Jones et al., 2005; Francis et al., 2006) and PG QRT3 (Rhee and Somerville, 1998; Rhee et al., 2003) may function in the same way, leading to pectin degradation and cell wall loosening. In addition, different PME isoforms can exhibit action patterns distinct to their specific pectic substrates (Jiang et al., 2005). Compared with Col, both PME and PG activity in *pme34* showed a substantial decrease in response to 37°C mild HS but increased noticeably after 44°C LHS treatment (Fig. 7). The irregular PME and PG activity in *pme34* might allow for pectin degradation, causing stomata to open wider (Figs. 10–12) and further influencing the plant thermotolerance response. This observed change in the activity of pectin-modifying enzymes is believed to play a vital role in remodeling cell wall extensibility to mitigate the effects of stress.

PMEIs have been shown to inhibit PMEs; therefore, they also should be taken into account when studying PME-related cell wall modification (Pelloux et al., 2007). PME activity can be regulated by specific endogenous PMEIs (Sénéchal et al., 2015; Hocq et al., 2017), and PMEIs inhibit the activity of PME by forming a PME/PMEI complex whose stability is pH dependent and higher in acidic conditions, typical of the apoplastic environment (Hothorn et al., 2004, 2010; Di Matteo et al., 2005). We suggest that specific PMEIs and/or the PMEI domain of PME34 might be involved in the regulation of PME34 activity during the HS response.

PME34 Contributes to the Control of Stomatal Movement

Screening of HS-sensitive phenotypes of Arabidopsis mutants indicated that HSP induction and accumulation are correlated with thermotolerance. Besides HSPs, other pathways, such as ABA, salicylic acid, and ROS, are involved in acquired thermotolerance (Larkindale et al., 2005). These secondary effects may be transported from the stress perception site to other parts of plants to coordinate the whole-plant response to the stresses. The control of stomatal movement is a rapid adaptive response to drought stress and is regulated by a complex signaling pathway. ABA is an important phytohormone that mediates plant adaptation to stress. It can trigger the elevation of cytosolic Ca^{2+} to activate a signaling cascade of guard cells to regulate stomatal movement. ABA regulates several types of ion channels, activating the signaling cascade of Ca^{2+} influx and K^+ anion efflux from guard cells. This results in a reduction in guard cell turgor and stomatal closure and, eventually, a decrease in water loss via transpiration.

ABA biosynthesis, catabolism, signaling, and transport are involved in the activation of numerous heat-responsive genes (Baron et al., 2012; Huang et al., 2016). HS-induced stomatal movement is fully reversible; however, ABA produced in the roots can reach the leaves via transpiration and cause stomatal closure. It has been observed that ABA shifts heat-induced stomatal opening toward a higher threshold temperature and, therefore, that ABA acts in an opposite manner to the heat response on stomatal movement (Feller, 2006; Reynolds-Henne et al., 2010). Using the Arabidopsis Promoter Analysis Navigator (PlantPAN2.0; http://plantpan2.itps.ncku.edu.tw), a database for transcription regulatory networks (Chow et al., 2016), four potential ABREs in the 2-kb promoter region of *PME34* were discovered (Supplemental Fig. S9). We showed that the expression of *PME34* was activated by ABA (Figs. 2, B and E, and 3A), and *PME34* exhibited a tissue-specific pattern that was highly ABA mediated in guard cells but not in mesophyll cells (Fig. 9; Supplemental Fig. S8; Supplemental Table S3). However, *pme34* displayed no effects in stomatal morphology, density, and vein networks (Supplemental Figs. S11–S13). The Arabidopsis transcription factor SCAP1 positively regulates *MYB60*, K^+ channel protein (*GORK*), and *PME6* gene expression in maturing guard cells (Negi et al., 2013). The *scap1* mutant, which affects

pectin demethylesterification in guard cell walls, shows the aberrant appearance of stomata and impairs stomatal opening and closing. However, while the *pme6* mutant did not show notable morphological defects of stomata, its stomatal CO_2 and light sensitivity were lower compared with the wild type. *pme34* showed a clear impairment in the control of stomatal movements (Figs. 10–12), with a concomitant effect on leaf temperature (Supplemental Fig. S14). *pme34* also showed an impaired ABA-mediated heat response (Fig. 6, B and C), whereas the *abi1-1* and *pme34-1 abi1-1* mutants showed no significant difference in response (Fig. 6, D and E). Thus, these results highlight the important role of *PME34* in the ABA-associated response; it affects transpiration through the control of stomatal movement and also is required for heat responses.

The extension of the 44°C LHS treatment caused more water loss in *pme34* than in Col (Fig. 11, B and C), whereas PME and PG activities in *pme34* were greatly increased after 44°C treatment (Fig. 7). Consequently, we showed that *PME34* deficiency has profound effects on a stomatal function by enzymatic modification of the guard cell wall to increase stomatal aperture opening (Fig. 12). We suggest that the enzymatic modification of the guard cell wall with a combination of PME and PG activity possibly caused the stomata to be flexible enough to open far wider than normal and, therefore, increased bending of the guard cell wall. Results from our studies are consistent with the findings of Jones et al. (2003). Arabinanase-induced locking of the guard cell wall depends on the presence of HGA; therefore, the disruption or removal of HGA in or from the cell wall is sufficient to overcome the locking effects of arabinanase (Jones et al., 2003). The arabinan side chains, in rhamnogalacturonan I domains associated with HGA, provide steric hindrance to render HGA polymers unable to coalesce and form rigid calcium-linked polymers. The overexpression of PG, which cleaves pectins, was disabled in stomatal closure (Atkinson et al., 2002). Thus, PME, PG, and arabinanase act in concert to maintain the flexibility of guard cell walls in response to change in cell turgor pressure.

The mild HS-activated PME activity has a positive effect on cell wall-localized Ca^{2+} influx as well as on retaining plasma membrane integrity to prevent cellular content leakage (Wu et al., 2010). Accordingly, the absence of *PME34*, which results in affected stomatal aperture, may be due to the signaling of Ca^{2+} and/or oligosaccharides released during cell wall modification.

In conclusion, the change of cell wall metabolism and cell wall-modifying enzyme activity in controlling cell wall plasticity is an important physiological mechanism of plants in response to HS. We suggest that PME34 functions in controlling stomatal movements and in regulating the flexibility of the guard cell wall required for the heat response.

MATERIALS AND METHODS

Plant Materials and Transformation

Arabidopsis (*Arabidopsis thaliana*) Col homologous T-DNA insertion lines of *HSP101* (At1g74310; *hsp101*, SALK_066374C) and *PMEs* were obtained from the Arabidopsis Biological Resource Center (Ohio State University). Seeds were placed on one-half-strength Murashige and Skoog medium containing 1% Suc and 0.8% phytoagar incubated at 22°C with 16 h of light at 60 to 100 μmol m^{-2} s^{-1}. Transgenic plants were generated in an Arabidopsis Col background with *Agrobacterium tumefaciens* GV3101-mediated transformation by the floral dip method (Clough and Bent, 1998) and then were selected by spraying seedlings with 0.4% Basta herbicide.

RNA Isolation, cDNA Synthesis, and qRT-PCR

Total RNA was prepared from 6- to 9-d-old seedlings with TRIZOL reagent (Invitrogen) and the TURBO DNA-free Kit (Applied Biosystems) and then subjected to cDNA synthesis with the Ready-To-Go Kit (GE Healthcare Life Sciences). PCR products were separated by agarose gel electrophoresis and stained with ethidium bromide for quantification (ImageQuant 5.2; GE Healthcare Life Sciences). qRT-PCR results were analyzed with the MyiQ thermocycler (Bio-Rad) with reagents containing the PCR mix of iQ SYBR Green Supermix (Bio-Rad). The data obtained were analyzed using iQ5 Optical System Software (Bio-Rad) and normalized to an internal control, *PP2A* (At1g13320; Czechowski et al., 2005).

Constructs

The open reading frame of *PME34* was amplified, digested, and cloned into *Eco*RI/*Nco*I sites of pRTL2-GFP (Carrington et al., 1990) or into the Gateway vector pCR8/GW/TOPO and recombined into the destination vector p2FGW7 (Karimi et al., 2002) to generate an N- or C-terminal protein fusion with GFP. The constitutive CaMV *35S* promoter was used for transient expression in Arabidopsis protoplasts and onion (*Allium cepa*) epidermal cells. The *GFP-PME34* fragment was subcloned into pCAMBIA3300 (CAMBIA) to generate Arabidopsis transgenic plants carrying the CaMV *35S* promoter::*GFP-PME34*. A 2-kb potential *PME34* promoter region was subcloned into pCAMBIA1391Z (CAMBIA) for analysis of the *PME34* promoter in transient assays using a *GUS* reporter, and the GUS staining method was performed as described (Weigel and Glazebrook, 2002).

Thermotolerance Test and Western-Blot Analysis

Six- to 7-d-old seedlings were used for the thermotolerance test as described previously (Charng et al., 2006; Hsu et al., 2010; Huang et al., 2016). Plates with seedlings were sealed with plastic electrical tape and submerged in a water bath at 44°C for 30 min for the basal thermotolerance test. For the acquired thermotolerance test, plates were preheated at 37°C for 1 h and allowed to recover at 22°C for 2 h prior to 44°C LHS for 150 to 160 min. Healthy growing seedlings were counted after 7 to 14 d from the end of the heat treatment. Western-blot analysis was as described previously (Hsu et al., 2010).

Protoplast Preparation, Transfection, and Protease Treatment

Arabidopsis protoplast preparation and transfection were as described (Yoo et al., 2007). An amount of 10 to 20 μg of plasmid DNA and 2×10^4 protoplasts were used for transfection, then incubated at 26°C for 16 to 24 h and examined by confocal microscopy (LSM780; Zeiss). In colocalization experiments, PME34-GFP or GFP-PME34 and the mCherry-labeled plasma membrane marker (CD3-1007; Nelson et al., 2007) were expressed simultaneously in the same cells. The protoplast fluorescence protease protection assay was conducted with a treatment of 8 mM trypsin protease (Lorenz et al., 2006). The GFP signal was detected with 488-nm excitation and 505- to 530-nm emission. mCherry was observed using 543-nm excitation and 560- to 615-nm emission. Chlorophyll autofluorescence was detected in the range of 630 to 680 nm.

Subcellular Localization in Onion Epidermal Cells

Particle bombardment and transient expression in onion epidermal cells were as described previously (Guan et al., 2004). Approximately 2.5 μg of DNA was

coated onto 0.6-μm gold particles (Bio-Rad) and transiently introduced into onion epidermal cells with a helium biolistic particle-delivery system (PDS-1000; Bio-Rad). The cells bombarded with GFP fusion constructs was kept at 26°C in the dark for 16 h and then examined by microscopy. The plasmolysis of onion cells was induced by 0.8 M mannitol treatment for 30 min.

PME and PG Activity Assay

Six-day-old seedlings were ground with a phosphate citrate buffer (0.1 M citric acid to 0.2 M Na_2HPO_4, 1 M NaCl, pH 5) at 4°C and centrifuged for 15 min at 12,000*g*, then the supernatant was collected. PME activity quantification was as described (Ren and Kermode, 2000; Francis et al., 2006). A 5-μg protein extract was added to 1 mL of 0.1% esterified pectin (Sigma-Aldrich; P9561) in 50 mM sodium phosphate buffer (pH 7). Samples were incubated at 37°C for 1 h, and 0.2 mL of 0.02% Ruthenium Red (Sigma-Aldrich; R2751) was added to each sample. They were briefly vortexed and incubated for 15 min at room temperature. The demethylesterified pectin bound to Ruthenium Red was precipitated by adding 0.2 mL of 0.6 M $CaCl_2$. The mixture was centrifuged at 12,000*g* for 10 min, and the supernatants were collected and measured for absorbance at A_{534}. A standard curve was established using control samples with known concentrations of purified PME (Sigma-Aldrich; P5400). All samples were replicated four times.

PG activity was measured spectrophotometrically by forming reduced sugar groups from polygalacturonic acid (Sigma-Aldrich) with 2-cyanoacetamide (Honda et al., 1980; Wu et al., 2010). Protein extracts were incubated with 0.1% polygalacturonic acid in 40 mM sodium acetate buffer (pH 4.4) at 35°C for 10 min. The reaction was stopped by the addition of 0.1 M borate buffer (pH 9) and 1% 2-cyanoacetamide and then incubated at 100°C for 10 min. The liberated reducing sugar was determined at A_{276}.

Water Loss, Leaf Surface Temperature, and Stomatal Aperture Measurement

To eliminate the variability resulting from dry weight or plant size, transpiration (water loss) measurement was standardized (%) and then calculated as $[(FW_i - DW)/(FW_o - DW)] \times 100$, where FW_i and FW_o are fresh weight for any given interval and original fresh weight, respectively, and DW is dry weight (Fujita et al., 2005). Leaf thermal images were obtained using an infrared thermography camera (Thermo Gear G100; NEC Avio Infrared Technologies) with resolution of 0.08°C and accuracy of ±2%, mounted on a tripod. To analyze stomatal aperture and density on the surface of abaxial epidermis, the sequential replica method for in vivo imaging was used as described (Williams and Green, 1988; Elsner et al., 2012). Briefly, silicon polymer molds were taken from the surface of individual leaves at a similar region and filled with epoxy resin. The obtained replicas were sputter coated and observed by scanning electron microscopy (S-520; Hitachi). Stomatal apertures were measured using ImageJ software (http://rsb.info.nih.gov/ij).

Statistical Analysis

Data were analyzed by Student's *t* test or Fisher's LSD test. $P < 0.05$ was considered statistically significant.

Accession Numbers

Primers used and accession numbers are given in Supplemental Table S4.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Schematic representation of the *PME34* locus with characterized mutations and the characterization of *pme34-1* and *pme34-1 abi1-1* mutants.

Supplemental Figure S2. Analysis of *PME* T-DNA insertion lines under the basal thermotolerance test.

Supplemental Figure S3. Thermotolerance test in *PME* homologous T-DNA insertion lines.

Supplemental Figure S4. *PME34* complementation in the *pme34-1* background restored to the Col phenotype under the acquired thermotolerance test.

Supplemental Figure S5. Analysis of PME34 structural motifs and amino acid sequence.

Supplemental Figure S6. Affinity purification of GST-tagged PME34.

Supplemental Figure S7. Transcriptional profiles of *HSF* genes in Col and *pme34* plants, and *PME34* gene expression in both *hsfa1a/b/d/e* quadruple knockout and *hsfa2* mutants response to heat treatment.

Supplemental Figure S8. Transcriptional profiles of *PME* genes in guard cells and mesophyll cells in response to ABA treatment.

Supplemental Figure S9. Potential ABRE cis-regulatory elements in a 2-kb promoter region of *PME34*.

Supplemental Figure S10. Histochemical analysis of *PME34* promoter::*GUS* expression.

Supplemental Figure S11. Stomatal aperture measurement in Col and *pme34* plants after lethal heat treatment.

Supplemental Figure S12. Stomatal density measurements in Col and *pme34* plants.

Supplemental Figure S13. Cotyledon vein network analysis in Col and *pme34* plants.

Supplemental Figure S14. Leaf temperature measurements of Col and *pme34* plants in response to heat treatment.

Supplemental Figure S15. Amino acid sequence comparison of PME34, PME28, and PME7.

Supplemental Table S1. List of 53 Arabidopsis *PME* homologous T-DNA insertion lines for the thermotolerance assays.

Supplemental Table S2. Transcriptional profiles of *PME7*, *PME28*, and *PME34* genes.

Supplemental Table S3. Transcriptional profiles of *PME* genes in both guard cells and mesophyll cells in response to ABA treatment.

Supplemental Table S4. List of primers and accession numbers.

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