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Pectin Methylesterases Modulate Plant Homogalacturonan Status in Defenses against the Aphid *Myzus persicae*[OPEN]

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Because they suck phloem sap and act as vectors for phytopathogenic viruses, aphids pose a threat to crop yields worldwide. Pectic homogalacturonan (HG) has been described as a defensive element for plants during infections with phytopathogens. However, its role during aphid infestation remains unexplored. Using immunofluorescence assays and biochemical approaches, the HG methylesterification status and associated modifying enzymes during the early stage of Arabidopsis (*Arabidopsis thaliana*) infestation with the green peach aphid (*Myzus persicae*) were analyzed. Additionally, the influence of pectin methylesterase (PME) activity on aphid settling and feeding behavior was evaluated by free choice assays and the Electrical Penetration Graph technique, respectively. Our results revealed that HG status and HG-modifying enzymes are significantly altered during the early stage of the plant-aphid interaction. Aphid infestation induced a significant increase in total PME activity and methanol emissions, concomitant with a decrease in the degree of HG methylesterification. Conversely, inhibition of PME activity led to a significant decrease in the settling and feeding preference of aphids. Furthermore, we demonstrate that the PME inhibitor AtPMEI13 has a defensive role during aphid infestation, since *pmei13* mutants are significantly more susceptible to *M. persicae* in terms of settling preference, phloem access, and phloem sap drainage.

INTRODUCTION

Phytophagous insects have developed different strategies to extract nutrients from plants to complete their life cycle, resulting in a direct impairment of host health and performance. Of the phytophagous insects that affect commercial crops, aphids have a greater impact due to the nutrient losses caused by colonies draining plants and promoting saprophytic fungal growth, thus significantly decreasing crop yields (Östman et al., 2003; Dedryver et al., 2010). Moreover, viruses transmitted by aphids are the most relevant risk factor for the target crop. Indeed, aphids function as vectors for ∼50% of the 700 known insect-borne viruses (Hooks and Fereres, 2006; Dedryver et al., 2010). Consequently, aphids are one of the most costly pests in terms of pesticide treatments (Murray et al., 2013).

The aphid feeding process starts when the stylet penetrates the host and then moves toward the phloem through intercellular pathways, such as cell wall matrices, middle lamellae, and gas spaces, until sieve elements are reached (Kimmins, 1986; Tjallingii and Esch, 1993). Most cells along the stylet pathway are briefly punctured (typically for 5–10 s), but the stylets are always withdrawn from the cells and then continue along the intercellular route until sieve elements are found (Tjallingii and Esch, 1993).

Intercellular cell wall polysaccharides are a main component of the intercellular stylet pathway. These macromolecules share common features among vascular plants and consist of cellulose microfibrils anchored to the cell membrane, cross-linked by and embedded in matrices of hemicellulose and pectic polymers (Ridley et al., 2001; Wolf and Greiner, 2012). In this context, homogalacturonan (HG) has been found to participate in different plant developmental and defensive processes (Ridley et al., 2001; Gramegna et al., 2016). HG is a homopolymer of galacturonic acid (GalA) residues, which can be methylesterified at C-6 and may carry acetyl groups on O-2 and O-3 (Ridley et al., 2001). According to the current model of HG synthesis, it has been established that HGs are synthesized in the Golgi apparatus in a highly methylesterified state and then secreted into the cell wall (Ibar and Orellana, 2007). In the cell wall, the methylesterification status may be modified by the action of pectin methylesterases (PMEs), which

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remove the methylester groups (EC 3.1.1.11). In turn, these reactions of HG demethylesterification are regulated by the proteinaceous PME inhibitors (PMEIs) (Hothorn et al., 2004; Caffall and Mohnen, 2009; Saez-Aguayo et al., 2013; Levesque-Tremblay et al., 2015).

The degree and pattern of HG methylesterification are key factors influencing the mechanical properties of cell walls, and hence in controlling plant development (Peaucelle et al., 2008; Levesque-Tremblay et al., 2015). In fact, depending on the methylesterification degree, HG domains can be directed into different fates: (1) polymer breakdown by polygalacturonases (PGs; EC 3.2.1.15) and pectate lyase (PL; EC 4.2.2.2), causing cell wall loosening, and (2) ionic cross-linking with other demethylesterified HG chains through calcium ion bridges, creating the so-called "egg box" structures leading to cell wall stiffening and reduced matrix porosity (Braccini et al., 1999; Willats et al., 2001; Levesque-Tremblay et al., 2015).

HG modification and degradation are important factors during the attack of pathogens or phytophagous insects possessing cell wall-degrading enzymes such as PMEs, PGs, and PLs as virulence factors (Cantu et al., 2008; Malinovsky et al., 2014). The evidence linking HG to the defensive responses of plants includes the broad spectrum of pathogen resistance or susceptibility phenotypes that are created by altering HG-modifying enzymes in different plant species (Cantu et al., 2008). Although the evidence relating to HG metabolism during aphid feeding is limited, it is thought that the presence of HG-modifying enzymes such as PMEs and PGs, in the saliva of aphids, could facilitate stylet penetration through the intercellular matrix (McAllan and Adams, 1961; Dreyer and Campbell, 1987; Ma et al., 1990). Additionally, by exploring the transcriptional profiles of Arabidopsis (*Arabidopsis thaliana*) plants attacked by different pathogens and phytophagous insects, De Vos et al. (2005) found that the *PECTIN METHYLESTERASE13* (*AtPMEI13*) gene was specifically upregulated during *Myzus persicae* feeding yet was unchanged during the interaction with other attackers studied. Despite this valuable information, there still exists a lack of detailed mechanistic understanding about the role of HG during plant-aphid interactions.

The focus of the present work was to characterize the dynamics of HG and its modifying enzymes during the early stage of aphid infestation and how these changes could influence the aphid settling and feeding behavior. To this end, the globally distributed aphid *M. persicae* and Arabidopsis were used as the plant-aphid interaction model. Here, we show that during early aphid infestation, total PME activity and methanol emission increase with a concomitant decrease in the degree of HG methylesterification. Exogenous inhibition of total PME activity leads to a significant decrease in aphid settling preference in wild-type Col-0 plants. Furthermore, by exploiting the results obtained by De Vos et al. (2005), the inhibitory activity of AtPMEI13 and its defensive role during aphid infestation were isolated and characterized. Due to the marked preference of *M. persicae* to settle on *pmei13* plants, concomitant with longer phloem sap ingestions on these mutants compared with the wild-type genotypes, it has been demonstrated that AtPMEI13 is a resistance factor during aphid colonization in Arabidopsis.

RESULTS

Determination of Early Infestation Stage during *M. persicae* Arabidopsis Interaction

Prior to the experiments and analysis, in order to determine the time scales of the early infestation stages, the proper sampling time for the experiments was established. We decided to establish as the early aphid infestation stage the time that aphids took to perform the first sustained phloem ingestion from the first contact with the Arabidopsis leaf (aphid landing). To this, we used the Electrical Penetration Graph (EPG) technique, which creates distinct fluctuating voltage patterns referred to as EPG waveforms, which in turn has been experimentally related to different feeding processes or activities performed by the insect, in this case the aphid *M. persicae* (Figure 1A).

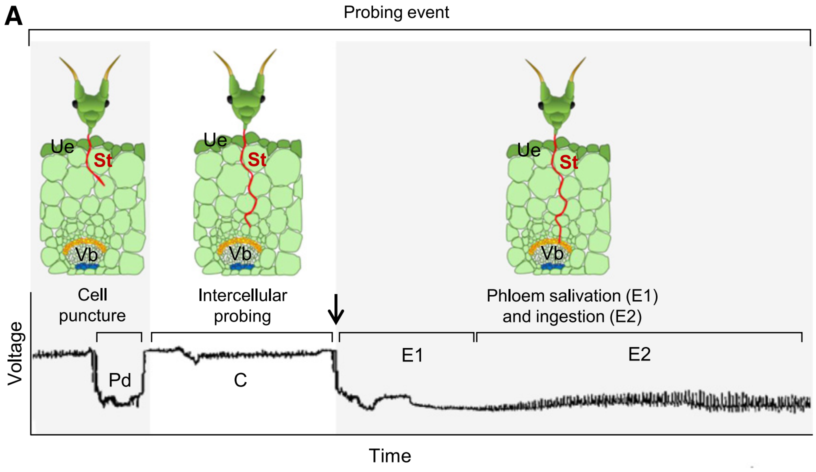
The EPG results showed that wingless adult *M. persicae* aphids settled on wild-type leaves and achieved the first sustained phloem ingestion after 271.2 ± 34.8 min (4.5 ± 0.5 h) from landing (Figure 1B). Thus, considering that aphids took ~5 h to perform the first sustained phloem ingestion (and adding 1 h to cover possible variations), we established as an early infestation stage the first 6 h of plant aphid interaction, and based on this timing, further experiments were done. Additionally, EPG analysis revealed that after the first host penetration, performed 4.2 min after landing, host tissues were probed by aphids ~35 times (35 probes), and within these probing events, *M. persicae* performed an average of 145 membrane punctures, visualized as potential drops (Figures 1A and 1B). Moreover, during the first 360 min (6 h), *M. persicae* spent just 85.1 min in nonprobing activities (i.e., with their stylets out of the host plant) (Figure 1B). Therefore, this confirmed that aphids perform an exhaustive examination by constantly probing the host tissues during the early infestation stage (6 h of plant-aphid interaction).

Early Stage of Aphid Infestation Increases Total PME Activity, Methanol Emissions, and Abundance of Demethylesterified HG

Considering that PME activity and the HG methylesterification status have been described as defense-related elements during pathogen attack (Cantu et al., 2008; Osorio et al., 2008; Raiola et al., 2011; Bethke et al., 2014), it was decided to measure the total PME activity and its consequent effects on the HG methylesterification degree and methanol emissions (Figure 2A) during the early stage of plant-aphid interaction. Our results showed that total PME activity increased ~20 in aphid-infested leaves of Arabidopsis with respect to the control plants (i.e., noninfested; Figure 2B). Consequently, the degree of methylesterification of HG decreased significantly by 19 (Figure 2C), concomitant with a threefold increase in the methanol emissions in the aphid-infested plants compared with the control condition (Figure 2D).

To visualize the cell wall modifications that occurred as a result of the increase in PME activity, immunofluorescence assays on infected and control Arabidopsis leaves were performed. The immunofluorescence assays that were done to visualize the HG methylesterification status, in situ, support the

A



B

	Duration (min)	
Sequential variables	Mean	SE
Time from start of EPG to 1st sustained phloem ingestion (> 10 min) (time for host acceptance)	271.2	34.8
Time from start of EPG to first probe	4.2	1.1
Non-sequential variables	Mean	SE
Number of probes	35.2	3.0
Duration of non-probing	85.1	10.2
Number of potential drops	145.1	15.2

Figure 1. Determination of Sampling Time for Early Aphid Infestation Stage.

EPG was performed to evaluate the feeding behavior of *M. persicae* using Arabidopsis (wild-type Col-0) as a host with the aim of determining the proper sampling time related to the early infestation stage.

A) Schematic representation of the biological activities of the aphid stylet inside the host plant and its corresponding EPG waveform. The arrow points to the potential drop related to the stylet entry into the sieve elements. C, intercellular probing; E1, phloem salivation; E2, phloem ingestion; Pd, cell puncture (potential drop); St, stylet; Ue, upper epidermis; Vb, vascular bundle.

B) EPG variables analyzed to define the timing of the early aphid infestation stage. Mean and SE were calculated from $n = 20$ (20 independent EPG recordings).

results obtained in Figures 2B. and 2C The LM19 monoclonal antibody, which targets the demethylesterified domains of HG, showed a doubling of the signal in the parenchyma tissue and lower epidermis of infested leaves, with respect to the control condition (Figure 3; Supplemental Figures 1). and 2 Additionally, some replicates with LM19 antibody revealed HG demethylesterification zones localized close to aphid bodies and stylets (Supplemental Figure 1), suggesting that HG modifications could be occurring as a consequence of stylet penetration through the pectic matrix. On the other hand, a significant 30 reduction in the signal of the LM20 antibody, which recognizes highly methylesterified HG, was observed in the aphid-infested leaves compared with the noninfested plants (Figure 3; Supplemental Figure 3). Additionally, HG epitopes were measured by enzyme-linked immunosorbent assay (ELISA); however, no differences were detected during aphid infestation by this method (Supplemental Figure 4). Therefore, these results showed that early aphid infestation induced an increase in the total PME activity, with the consequent demethylesterification of HG and methanol release.

Early Stage of Aphid Infestation Increases the Calcium Cross-Linked HG and Alters the Total PL Activity

Once HG chains are demethylesterified in cell walls, they may be directed to two different fates: (1) polymer breakdown by PGs and/or PLs or (2) interact ionically with other demethylesterified HG chains through calcium bridges, creating the so-called egg box structures (Figure 4A; Braccini et al., 1999; Willats et al., 2001). Then, as the early *M. persicae* infestation process induces HG deesterification, which of these two subsequent steps (PG/PL breakdown or egg box arrangement) could be occurring in early aphid-infested plants was investigated. To achieve this, total PG and PL activities were measured. The results revealed that the total PG activity remains unchanged (Figure 4B), concomitant with a significant increase in total PL activity (Figure 4C), in the aphid-infested plants with respect to the control condition.

Since the other possible fate of demethylesterified HG is the ion cross-linking, we visualized these epitopes by using the monoclonal antibody 2F4. Interestingly, it was found that the egg box arrangement of HG is significantly more abundant in infested

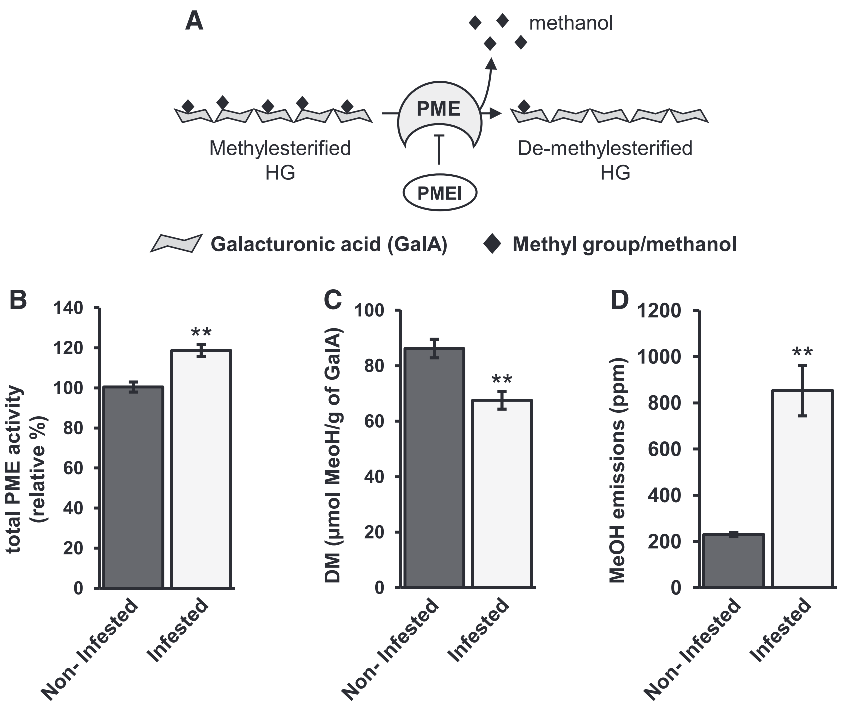


Figure 2. Early Plant-Aphid Interaction Increases Global PME Activity and Decreases the Degree of HG Methylesterification.

(A) Schematic representation of the HG demethylesterification process. PME is catalyzed by HG demethylesterification, and their activity is regulated by their proteinaceous inhibitor (PMEIs).

(B) Total PME activity measured after 6 h of *M. persicae* Arabidopsis interaction with 4-week-old wild-type Col-0 plants. Total protein extracts from rosette leaves of wild-type Col-0 plants were used to measure global PME activity. Values are expressed as relative PME activity and normalized to the average wild-type Col-0 activity (noninfested). Error bars represent SE from $n = 3$ (three individual plants).

(C) Degree of methylesterification (DM) after 6 h of *M. persicae* Arabidopsis interaction. Error bars represent SE from $n = 3$ (three individual plants).

(D) Methanol (MeOH) emissions measured after 6 h of *M. persicae* Arabidopsis interaction with 4-week-old wild-type Col-0 plants by full evaporation headspace gas chromatography. Values correspond to ppm of methanol in 1 µL of collected transpiration vapor. Error bars represent SE from $n = 4$ (four individual plants).

(B) to **(D)** Asterisks represent significant differences determined by Student's *t* test (**, $P < 0.005$).

plants, since a significant tripling in the signal of 2F4 antibody was measured mainly in the lower epidermis and parenchyma tissue of the aphid-infested leaves compared with the control condition (Figures 4D and 4E; Supplemental Figure 5). This suggests that, during early *M. persicae* infestation, changes in HG structure lead to an increase in the abundance of both demethylesterified and ion cross-linked HG.

Exogenous Modulation of PME Activity and Methanol Emissions Influence the Aphid Settling Preference

The above results revealed that HG methylesterification status is significantly altered during the early plant-aphid interaction (Figures 2 and 3; Supplemental Figures 1 to 3). However, we cannot distinguish whether these HG alterations correspond to a defensive mechanism of the host plant or to the consequences of the aphid infestation/feeding process. In order to gain an insight into this question, it was decided to investigate how different levels of PME activity of the host plant could influence the aphid behavior in terms of settling preference.

The first approach was to exogenously modulate the total PME activity of wild-type Col-0 plants and then subject these plants to a free choice assay, which reveals the preference of the aphids to settle on the most suitable host to establish a new colony (Poch et al., 1998). This was achieved by infiltrating one group of plants with 1 mg/mL epigallocatechin gallate (EGCG; Sigma-Aldrich), which has been described as a specific chemical inhibitor of global PME activity (Lewis et al., 2008). Then, a second group of plants was infiltrated with 15 units/mL orange peel PME (Sigma-Aldrich; Figure 5A). After 1 h of the infiltration procedure, treated plants plus a water-infiltrated control group (mock) were subjected to the free choice assay. The results show that treatment with the chemical PME inhibitor EGCG resulted in $\sim$10 reduction in total PME activity (Figure 5B) concomitant with a 2.7-fold reduction of methanol emissions (Figure 5D) compared with the infiltration control (mock). On the other hand, infiltration with the commercial orange peel PME cocktail increased the total PME activity by 15 (Figure 5B) compared with the mock-infiltrated plants, while methanol emissions showed no differences between both conditions (Figure 5D). Free choice assays on these treated plants

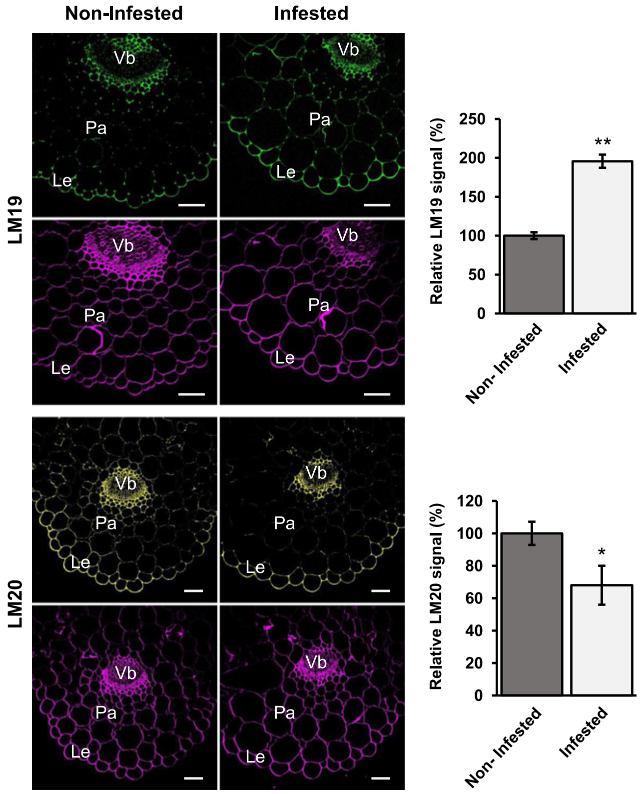


Figure 3. Early Aphid Infestation Increases the Abundance of Demethylesterified HG Epitopes.

Representative transverse sections of 4-week-old wild-type Col-0 Arabidopsis leaves were immunolabeled with LM19 and LM20 monoclonal antibodies to target demethylesterified HG (green) and highly methylesterified HG (yellow), respectively. Calcofluor White was applied to stain all cell walls (magenta). The images show closeups of the lower epidermis and parenchyma surrounding the main vascular bundle of leaves of noninfested and infested plants. Le, lower epidermis; Pa, parenchyma; Vb, vascular bundle. Bars = 50 m. The graphs at right show the relative fluorescence signal of each antibody. Values were normalized with respect to the noninfested condition. Error bars represent the SE obtained from four biological replicates (four leaves from different plants, from different culture batches). Asterisks represent significant differences determined by Student's *t* test (*, P < 0.05 and **, P < 0.005).

showed no significant differences in aphid preference when compared with the increased PME activity group of plants (PME infiltrated) with the control condition (mock; Figure 5C). Interestingly, a significant reduction in aphid settling was observed for the reduced PME activity plants (EGCG infiltrated), since only 20 of the total aphid population preferred those plants as host, compared with 38 and 42 of the aphid population that preferred to settle on mock-treated and orange peel PME-treated plants, respectively (Figure 5C).

Moreover, it is known that methanol is a critical volatile defense signal emitted during phytophagous insect feeding (Baldwin et al., 2006; von Dahl et al., 2006), and considering that our results show increased methanol emissions in aphid-infested plants (Figure 2D), it was decided to investigate how methanol emissions could influence the host settling preference of *M. persicae*. To accomplish this and based on methanol emissions from infested plants, which averaged 0.09 (v/v) (900 ppm; Figure 2D), a methanol solution of 0.1 (v/v) was prepared to infiltrate wild-type Col-0 leaves, and then these plants were subjected to an aphid free choice assay using water-infiltrated plants as controls (mock). As shown in Figure 5E, the results revealed that aphids significantly prefer to settle on methanol-infiltrated plants, since 60 of the total aphid population chose those plants as host compared with the 40 of insects that chose the mock plants. These results suggest that both exogenous modulation of PME activity and methanol emission in Arabidopsis leaves could influence the *M. persicae* settling preference. However, considering that the infiltration procedure could lead to unknown changes in the plant physiology and consequently alter the aphid behavior, a second approach was designed in order to determine the influence of the PME activity over the settling behavior of aphids.

PMEI13 Possesses in Vitro and in Vivo Inhibitory Activity of PMEs, and *pmei13* Mutant Lines Are More Susceptible to *M. persicae* Settling

Expression analysis using a microarray published by De Vos et al. (2005) showed that a PME inhibitor (PMEI13) is specifically upregulated during *M. persicae* infestation of Arabidopsis. Considering this finding, the potential role of PMEI13 during the plant-aphid interaction was evaluated. Two T-DNA insertional mutant lines were identified in the locus *At5g62360/PMEI13* and were designated as *pmei13 1* (background Col-0) and *pmei13 2* (background WS4; Supplemental Figure 6A). Expression analysis using RT-PCR and RT-qPCR were done on *pmei13 1* and *pmei13 2* mutant plants. Amplification of the full-length coding sequence of *PMEI13* in both *pmei13 1* and *pmei13 2* mutant lines confirmed that both mutants are knockdown lines, with decreases of 65.5 and 57.1 in *PMEI13* transcript accumulation in comparison with their corresponding wild-type genotypes, respectively (Supplemental Figures 6B and 6C). Then, in order to characterize the PME-inhibiting capacity of PMEI13, the inhibitory effect of recombinant PMEI13 on global PME activity of wild-type plants by using a gel diffusion assay was determined, as described by Saez-Aguayo et al. (2013, 2017). The results presented in Supplemental Figure 6D show that the induced bacterial culture containing the recombinant PMEI13 (PMEI13x6 his IPTG) has 30 and 23 less global PME activity than cultures containing the empty vector (EV IPTG) and the noninduced PMEI13 construct (PMEI13x6 his –IPTG), respectively. Thus, these results confirm that PMEI13 is an inhibitor of pectin methylesterase activity.

To confirm the in vivo inhibitor activity of PMEI13 in Arabidopsis, total PME activity was measured in 4-week-old *pmei13 1* and *pmei13 2* plants. The results show that both *pmei13* mutant lines possess higher total PME activity compared with the wild-type genotypes. *pmei13 1* showed 14 more PME activity compared with wild-type Col-0, while *pmei13 2* exhibited 11 more PME activity compared with wild-type WS4 (Supplemental Figure 7A). These significant increases in total PME activity observed in *pmei13* mutants were consistent with the increased