Research Article

Running head: Role of PME48 in pollen grain germination

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PECTIN METHYLESTERASE48 is involved in Arabidopsis pollen grain germination.

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Summary: Arabidopsis PECTIN METHYLESTERASE48 modifies homogalacturonans in the intine cell wall during maturation of the pollen grain and is central for a proper germination.
Footnotes

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Abstract

Germination of pollen grains is a crucial step in plant reproduction. However, the molecular mechanisms involved remain unclear. We investigated the role of PECTIN METHYLESTERASE48 (PME48), an enzyme implicated in the remodeling of pectins in Arabidopsis thaliana pollen. A combination of functional genomics, gene expression, in vivo and in vitro pollen germination, immunolabeling and biochemical analyses was used on wild-type and AtPme48 mutant plants. We showed that AtPME48 is specifically expressed in the male gametophyte and is the second most expressed PME in dry and imbibed pollen grains. Pollen grains from homozygous mutant lines displayed a significant delay in imbibition and germination in vitro and in vivo. Moreover, numerous pollen grains showed two tips emerging instead of one in the wild-type. Immunolabeling and FT-IR analyses showed that the degree of methylesterification of the homogalacturonan (HG) was higher in pme48/- pollen grains. In contrast, the PME activity was lower in pme48/- partly due to a reduction of PME48 activity revealed by zymogram. Interestingly, the wild-type phenotype was restored in pme48/- with the optimum germination medium supplemented with 2.5 mM calcium chloride suggesting that in the wild-type pollen, the weakly methylesterified HG is a source of Ca^{2+} necessary for pollen germination. Although pollen specific PMEs are traditionally associated with pollen tube elongation, this study provides strong evidence that PME48 impacts the mechanical properties of the intine wall during maturation of the pollen grain which, in turn, influence pollen grain germination.

Key words: Arabidopsis thaliana, cell wall, pollen grain, imbibition, germination, pectin methylesterase48, intine, homogalacturonan, methylesterification.
Introduction

Sexual plant reproduction requires the growth of tip-polarized pollen tubes through the female tissues in order to deliver the two sperm cells to the embryo sac. Despite the importance of this crucial step that leads to the seed production, the molecular mechanisms implicated in the spatial and temporal controls of the pollen tube growth are not fully known (Johnson and Lord, 2006; Palanivelu and Tsukamoto 2012). However, it has been proposed that the modulation of the stiffness of the pollen tube was important during pollen tube growth (Parre and Geitmann, 2005a; Fayant et al., 2010; Vogler et al., 2013). Pollen tube elongation is highly polarized with the growth area being restricted to the apex of the tube. New membranes and cell wall materials are rapidly secreted at the tip providing the building material necessary to sustain the fast pollen tube growth (Bove et al., 2008; Guan et al., 2013).

The pollen tube cell wall of many species, including tobacco (Li et al., 1995; Ferguson et al., 1998), lily (Roy et al., 1997) and Arabidopsis (Lennon and Lord, 2000; Dardelle et al., 2010) is characterized by one layer in the tip region and two distinguishable layers back in the shank. Back from the tip, the inner layer is mainly composed of callose, a (1,3)-β-glucan, which is not detectable in the tip region in normal growth conditions (Ferguson et al., 1998; Derksen et al., 2002; Parre and Geitmann, 2005b; Dardelle et al., 2010; Chebli et al., 2012). Moreover, callose is also deposited periodically within the pollen tube to form plugs that maintain the tube cell in the expanding apical region. The outer cell wall in the tip and back from the tip is mostly composed of pectins, xyloglucan, cellulose and proteoglycans such as arabinogalactan proteins (Geitmann and Steer, 2006; Dardelle et al., 2010; Chebli et al., 2012; Nguema-Ona et al., 2012).

In eudicot species, pectins constitute a major portion of the primary cell wall. Pectins are complex polysaccharides consisting of homogalacturonan (HG), rhamnogalacturonan-I,
rhamnogalacturonan-II and xylogalacturonan (Vincken et al., 2003; Dardelle et al., 2010; Fry, 2011; Dumont et al., 2014). HG is a polymer consisting of repeating units of (1,4)-α-D-galacturonic acid (GalA) that is synthesized in the Golgi apparatus and may be deposited in the cell wall under a highly methylesterified form (Zhang and Staehelin, 1992).

Immunolabeling of HG on Arabidopsis pollen tubes showed a dominant localization of the highly methylesterified HG in the tip and of partially methylesterified HG behind the tip region (Dardelle et al., 2010; Chebli et al., 2012). This labeling pattern is also observed in pollen tubes from plants possessing a solid style such as the Solanaceae (potato, tobacco, petunia), Oleaceae (jasmine), and Poaceae (corn) (Li et al., 1994; Qin et al., 2007; reviewed by Mollet et al., 2013) and a hollow style such as lily (Jauh and Lord, 1996). The methylesterified HG in the apical dome of the pollen tube is thought to provide sufficient plasticity of the cell wall for sustaining the pollen tube growth (Chebli and Geitman, 2007). In the sub-apical region, methylesterified HG is processed by pectin methylesterases (PMEs). Two modes of action have been described for PMEs (Micheli, 2001). If PMEs remove contiguous methylester groups, the mode of action is named “blockwise mode of action”. In that case, de-methylesterified HG can form ionic bonds between the negatively charged carboxyl groups of several HG chains and Ca^{2+} ions forming a pectate gel that may provide sufficient stiffness to the pollen tube cell wall (Micheli, 2001; Geitmann and Steer, 2006; Chebli et al., 2012). Alternatively, the partial removal of non-contiguous methylester groups by PMEs which is named “random mode of action” may allow pectin-degrading enzymes (such as polygalacturonases, PGases and pectate lyases, PLs) to cleave the HG backbone thus affecting the rigidity of the cell wall (Micheli, 2001; Bosch and Hepler 2005; Parre and Geitmann, 2005a; Sénéchal et al., 2014). Therefore, the fine control of the modulation of the degree of methylesterification (DM) of the HG by PMEs is of main importance (Wolf et al.,
2009a) in the pollen tube growth dynamics. The *Arabidopsis* genome contains 66 putative PMEs. Most of them display a specific expression pattern, especially for 14 of them that are specifically expressed in pollen grains or pollen tubes (Qin et al., 2009; Wolf et al., 2009a).

PMEs are classified in two distinct groups depending on the presence/absence of an N-terminal extension domain (the PRO region) showing similarity with PME inhibitor (PMEI) domain (Micheli, 2001). PMEs from the group 1 do not have a PMEI domain but can be inhibited by PMEIs (Röckel et al., 2008) whereas PMEs from the group 2 can contain from 1 to 3 PMEI domains (Tian et al., 2006). It is hypothesized that the PRO region is cleaved during the maturation of the PME, as so far, only PMEs lacking this domain were found in the cell wall (Micheli, 2001). Wolf et al., (2009b) have shown that the PRO region of the group 2 PMEs could regulate the release of the mature PME from the Golgi apparatus. It has also been reported that the PRO region could also play an auto-inhibitory role during maturation (Bosch et al., 2005).

The first direct evidence for the crucial roles of PMEs during pollen tube growth was described in two knock-out mutants *vanguard1* (Jiang et al., 2005) and *Atppme1* (Tian et al., 2006). *VANGUARD1* (*VGD1*, At2g47040) encodes a pollen specific group 2 PME. The functional disruption of *VGD1* resulted in bursting of pollen tubes *in vitro* and marked retardation of the *vgd1* pollen tube elongation in the pistil resulting in a strong reduction of the male fertility and seed set. *AtPPME1* (At1g69940), coding for a pollen specific group 1 PME, was also identified to play an important role in pollen tube growth (Tian et al., 2006). The lack of *AtPPME1* transcripts in the knock-out mutant affected the shape and the growth rate of pollen tubes, indicating that AtPPME1 is required for the integrity of the cell wall and the tip-polarized growth of the pollen tube (Tian et al., 2006).
Here, we report the study of a homozygous knock-down mutant for \textit{AtPME48} during imbibition and germination of pollen grains and pollen tube growth. We have investigated \textit{in vitro} and \textit{in vivo} pollen germination, pollen tube morphology and growth as well as the level of PME activity and the degree of methylesterification of the HG by immunolabeling and FT-IR in mutant and wild-type plants. Our results show that the group 1 PME48, the second most expressed \textit{PME} gene in pollen, plays a major role in remodeling the HG of the intine cell wall during \textit{Arabidopsis} pollen grain maturation resulting, after rehydration, in a normal pollen grain germination.

\textbf{Results}

\textbf{Expression of pollen specific PMEs assessed by Quantitative RT-PCR}

The expression of the 14 pollen specific PME genes was analyzed by qRT-PCR on total RNA extracted from dry and imbibed for 1 h in liquid germination medium pollen grains and 6 h-old pollen tubes. Most PME genes were expressed in dry pollen grains with a strong expression of PME4 (\textit{VGD1} homolog), PME5 (\textit{VGD1}), PME37 and PME48 (Fig. 1A). During imbibition and \textit{in vitro} grown pollen tubes, PME4, PME5, PME50 as well as PME48 were strongly expressed compared to the ten other PMEs (Fig. 1, B and C). PME48 and PME50 belong to the group 1 PMEs, whereas PME4 and PME5, known as \textit{VANGUARD1 HOMOLOG} and \textit{VANGUARD1} (Jiang et al., 2005), belong to the group 2 PMEs.

\textbf{Tissue specific expression of \textit{AtPME48}}

In order to check whether \textit{PME48} is specifically expressed in pollen, the activity of the promoter of \textit{PME48} was assessed using \textit{pPME48::GUS} and \textit{pPME48::YFP} constructions. Fluorescence of the YFP protein was observed in dry (Fig. 1D) and imbibed (Fig. 1E) pollen.
grains and in 6 h-old *in vitro* grown pollen tubes (Fig. 1F). GUS staining showed that the promoter activity of *PME48* was restricted to the male gametophyte (Fig. 1, G-I). GUS activity was detected in dry pollen grains (Fig. 1G) and in 6 h-old *in vitro* grown pollen tubes (Fig. 1H). *In vivo* staining showed also a strong GUS activity in pollen grains within the anther and in germinated pollen grains deposited on the stigma (Fig. 1I). GUS staining was also clearly visible in pollen tubes growing through the transmitting tract (Fig. 1I insert). Staining was neither observed in the vegetative organs of the transformed plants nor in pollen grains or pollen tubes from wild-type plants (not shown).

**Isolation and characterization of pme48-/^- homozygous mutant line**

We have selected a T-DNA insertion line from the SALK library. SALK #122970 contained a T-DNA insert in the *PME48* coding sequence. The T-DNA insertion was predicted to be located in the last exon of the sequence (Fig. S1A). The homozygous mutant line (*pme48-/^-*) has been isolated, two copies of the insert were amplified with the gDNA (Fig. S1B) and *PME48* transcript was not detectable by RT-PCR (Fig. S1C). The vegetative organs of *pme48-/^-* did not display any visible phenotype or growth defect compared to wild-type plants (not shown).

The level of *PME48* transcript was then analyzed by qRT-PCR in dry, imbibed pollen grains and 6 h-old pollen tubes. Unlike the data obtained by RT-PCR (Fig. S1C), qRT-PCR revealed that *pme48-/^-* was not a knock-out but a knock-down mutant (Fig. 2). *PME48* was lightly expressed in dry (9%) (Fig. 2A) and imbibed (10%) (Fig. 2B) pollen grains and in 6 h-old pollen tubes (19%) (Fig. 2C) compared to wild-type pollen grains and pollen tubes. DAPI staining showed that *pme48-/^-* pollen grains contained the two sperm cells and the vegetative nucleus as observed in wild-type pollen grains (Fig. S2A). Using fluorescein diacetate (FDA), the viability assays showed that most of *pme48-/^-* pollen grains were viable (72.3% ± 3.7) but
slightly lower than wild-type pollen grains (79% ± 4.6) (P<0.0001, n>1,000 for each sample) (Fig. S2, B and C). In addition, the length of wild-type dry pollen grains was also slightly higher (29.8 ± 0.15 µm) than the one of pme48-/- pollen grains (28.8 ± 0.12 µm) (P<0.0001, n=550 for each sample) (Fig. S2, D and E). Similarly, the length of the siliques of the mutant was 1.4 mm shorter than that of the wild-type (P<0.0001, n=210 for each sample) (Fig. S2 F). The silique of the mutant contained a reduced number of seeds (40.3 ± 1.33 seeds per silique) compared to the wild-type (48.6 ± 0.5 seeds per silique) (P<0.0001, n=210 for each sample) (Fig. S2 G).

In vitro and in vivo pollen grain germination and pollen tube growth of pme48-/-

In the optimal solid germination medium described by Boavida and McCormick (2007), 65% and 90% of wild-type pollen grains were germinated after 6 h and 24 h of culture, respectively (Fig. 3A). In contrast, the percentage of germinated pme48-/- pollen grains was drastically reduced as only 10% of the pollen grains were germinated after 24 h of culture (P<0.0001, n>10,000 for each sample) (Fig. 3A). In liquid medium, 71 and 73% of germination was observed after 6 h and 24 h of culture for the wild-type pollen grains (Fig. S3A). In contrast, the levels of pme48-/- pollen grain germination did not exceed 43.8% after 6 h of culture and reached only 60% after 24 h (P<0.0001, n>10,000 for each sample) (Fig. S3A). As pme48-/- pollen grains displayed a delay in germination, the speed of imbibition of the pollen grains was assessed by calculating the ratio length/width. Dry pollen grains have ellipsoid shapes and the length is approximately twice as much as the width. During imbibition, pollen grains become spherical and the length is nearly equal to the width, the length/width ratio is then approximately 1. On the optimal solid medium, the imbibition was faster for the wild-type pollen grains compared to the mutant. The smallest ratio was obtained after 24 h with 1.16 and 1.39 for the wild-type and pme48-/-, respectively (Fig. 3B). After 24 h of culture, pollen...
grains from the mutant lines were less imbibed than the wild-type pollen grains imbibed for 6 h (P<0.0001, n>500 for each sample). The same result was observed in liquid medium (P<0.0001, n>500 for each sample) (Fig. S3B). Based on these observations, it appeared that the ability of the pollen grain to rehydrate was affected in pme48/- mutant lines. The delay in germination was also observed in vivo. Hand-pollination of emasculated wild-type pistils with wild-type or pme48/- pollen grains showed that the wild-type pollen tubes have travelled 2/3 of the transmitting tract after 6 h (Fig. 3C, left panel) and completed their journeys by reaching the bottom of the ovary after 24 h (Fig. 3D, left panel). In contrast, pme48/- pollen tubes have reached only 1/2 of the transmitting tract after 6 h (Fig. 3C, right panel), but after 24 h, pme48/- pollen tubes have also reached the base of the ovary (Fig. 3D, right panel).

In addition to lower germination rates, pme48/- displayed remarkable phenotype as shown on the representative images obtained by time-lapse video during the germination of pollen grains and the growth of pollen tubes (Fig. 3E). Wild-type pollen grains have already germinated 90 min after immersion in the liquid medium (Fig. 3E, upper panel), the proportion of burst tubes was around 10% (Fig. 3F), the growth rate was about 1.5µm min⁻¹ during the first 90 min of growth (Fig. 3H, Fig. S4) and pollen tubes displayed a normal phenotype (Fig. 3E; movie S1). On the other hand, pme48/- pollen tubes appeared to be more unstable with 32% of burst tubes (P<0.0001, n>500 for each sample) (Fig. 3F). Moreover, a significant number of pme48/- pollen grains (18%) displayed two tips emerging from the same pollen grain (P<0.0001, n>500 for each sample) (Fig. 3E, G, lower panel and close up pictures). The first tip emerged from pollen grains and after 4-5 h of culture a second tip emerged from the same pollen grain (circled pollen grains in blue; Fig. 3E lower panel close up picture; movie S2). Once germinated and if pollen tubes were produced, the growth speed of pme48/- pollen tubes was slightly faster compared to wild-type pollen tubes (P<0.0001,
n>35 for each sample) (Fig. 3H; Fig. S4). Another consequence of the PME48 mutation, pollen tubes growing in liquid GM displayed larger diameters than wild-type pollen tubes (Fig. 3I; Fig. 4A and H). The pme48-/- pollen tube diameters were significantly wider (5.51 ± 0.05 µm) compared to wild-type pollen tubes (5.18 ± 0.06 µm) (P<0.0001, n = 126 for wild-type and n = 172 for pme48-/- pollen tubes) (Fig. 3I).

**Immunolabeling of highly methylesterified HG in pollen grains and pollen tubes**

Cell surface immuno-localization of highly methylesterified HG with the mAb LM20 revealed that the epitopes were almost exclusively restricted to the tip region of wild-type pollen tubes (Fig. 4, A-E). Pollen grains from wild-type plants showed a very weak, almost no labeling of the intine wall (Fig. 4, C-E). Similar results were obtained on semi-thin sections (Fig. 4, F and G). In contrast, the labeling of pme48-/- pollen tubes was not restricted to the tube tip but extended to the sub-apical region of the tube but with the strongest fluorescence at the tip (Fig. 4, H-K). Finally, pme48-/- pollen grains displayed a more intense fluorescence of the intine wall by cell surface immunolabeling (Fig. 4, J-L) and on semi-thin sections (Fig. 4, M and N) compared to the wild-type.

Immunolabeling with the mAb LM19 that recognizes weakly methylesterified HG epitopes did not reveal any noticeable difference between wild-type and pme48-/- (not shown). LM19 and LM20 show overlapping binding capabilities to different levels of methylesterification except for totally de-esterified HG epitopes that are only recognized by LM19 (Verhertbruggen et al., 2009). Therefore, these results suggest that highly methylesterified HG epitopes were more abundant in the intine wall of pme48-/- pollen grains compared to the wild-type.

**PME activity and degree of methylesterification of HG in pme48-/-**
In order to assess further the biochemical differences between the wild-type and pme48-/-, we investigated the total PME activity in pollen grains by using enzymatic assays. The data showed a 50% reduction of the total PME activity in pme48-/ pollen grains compared to the wild-type (Fig. 5A). Moreover, zymogram after isoelectrofocusing (IEF) of total proteins extracted from pollen grains revealed a disappearance of a diffuse band in the pI range between 8.2 and 9 in pme48-/ (Fig. 5B) that may correspond at least in part to PME48 which has a predicted pI of 8.3. Two spots, at pI ranging from 9.8 to 10.2 and from 9.5 to 9.7, in the range of predicted pIs of other pollen PMEs did not show any visible change in the activity (Fig. 5B). These biochemical data support the qRT-PCR results that clearly showed a significant reduction of PME48 expression. Finally, the degree of methylesterification (DM) of HG was estimated by FT-IR in hot water soluble pectin-enriched fractions extracted from dry pollen grains (Fig. 5C-D). A marked difference was observed at 1740 cm\(^{-1}\) assigned to the vibration of methylester groups of HG (Fig. 5C). The relative absorbance at this wavenumber was higher in pme48-/ pollen grains. The DM of the HG was consistently higher in the pme48-/ (32.5 ± 1.7 %) compared to the wild type (13.4 ± 1.1 %) pollen grains (Fig. 5D). As de-esterified HG binds more Ca\(^{2+}\) than esterified HG, our data may suggest that HGs in the intine wall of pme48-/ are less associated with Ca\(^{2+}\) compared to wild-type pollen grains.

**Supplementation of Ca\(^{2+}\) to the GM restores the phenotype of pme48-/-**

To assess if the germination defect of pme48-/ pollen grains was related to the possible lower Ca\(^{2+}\) sink in the intine wall due to the higher DM of the HG, the optimum culture medium containing 5 mM CaCl\(_2\) was supplemented with 2.5 mM CaCl\(_2\) to reach a final concentration of 7.5 mM or with the Ca\(^{2+}\) chelator, EDTA. In the presence of 1 mM EDTA, none of the wild-type and pme48-/ pollen grains germinated, even after 24h of culture (n>1,000) (Fig. 6A-B). In the supplemented medium with Ca\(^{2+}\), pme48-/ pollen grain germination rates were
restored reaching the levels of the ones observed with wild-type pollen grains (n>1,000). The percentage of pollen germination reached 52% and 90% after 6 h and 24 h, respectively (Fig. 6A-B). Moreover, the speed of imbibition of pme48/- pollen grains was as fast as the wild-type pollen grains (Fig. 6C). In addition, a significant reduction of the abnormal phenotypes such as burst tubes (from 33% to 13%) (Fig. 3F, 6D) and the double-tipped tubes (from 18% to 2%) (Fig. 3G, 6E) was observed in pme48/- pollen grains cultured in the supplemented GM. Finally, in the CaCl2 supplemented medium, the rates of burst tubes increased in wild-type pollen grains (32%) (Fig. 6D) compared to that observed when pollen grains were grown in the optimum GM (~10%) (Fig. 3F).

Discussion

The regulation of the DM of HG has been implicated in many aspects of plant development (Wolf et al., 2009a) including cell adhesion (Tieman and Handa, 1994; Wen et al., 1999; Mollet et al., 2000; Bouton et al., 2002; Mouille et al., 2007; Durand et al., 2009), adventitious rooting (Guénin et al., 2011), primordia emergence at the shoot apical meristem (Peaucelle et al., 2008; Peaucelle et al., 2011), fruit ripening (Brummell and Harpster, 2001; Phan et al., 2007) and plant defence (Bethke et al., 2014). Although data have shown an important role for the pectin de-methylesterification process in pollen tetrad separation (Francis et al., 2006) and pollen tube growth (Jiang et al., 2005; Tian et al., 2006), the involvement of PMEs during pollen germination is still poorly understood. Among the 66 predicted PME genes in the genome of Arabidopsis, 14 are specifically expressed in the male gametophyte (data from Genevestigator (Hruz et al., 2008) and EFP Browser (Winter et al., 2007). Two of them have already been studied using functional genomics approaches. The first one is VANGUARD1 (At2g47040), the alteration of which led to unstable pollen tubes...
and retarded growth of the pollen tube in the style and transmitting tract, resulting in a significant reduction of male fertility (Jiang et al., 2005). The second pollen specific PME characterized to date is PPME1 (At1g69940) (Tian et al., 2006). The homozygous mutant atppme1 displayed reduced growth and irregular shape of pollen tube grown in vitro. In the present study, we show that the alteration of the expression of PME48, the second most expressed PMEs in pollen grains, results in a strong delay in imbibition and in germination both in vivo and in vitro as well as altered phenotypes with abnormal rates of burst tubes and two pollen tube tips emerging from the same pollen grain. The phenotype is rescued by supplementing the optimum GM (Boavida and McCormick, 2007) with 2.5 mM CaCl₂ to reach 7.5 mM. In 7.5 mM CaCl₂, pme48-/− pollen grains imbibed and germinated normally. These data suggest that PME48 is mainly involved in the remodeling of the intine cell wall during maturation of the pollen grain. The reduction of PME48 activity has also consequences later on pollen tube morphology. The pollen tubes are slightly wider in the mutant, it may reflect the role of HG in the mechanical properties of the cell wall (Parre and Geitmann, 2005a; Palin and Geitmann, 2012) and corroborates the predictions made by mechanical simulations (Chebli and Geitmann, 2007; Zerzour et al., 2009; Fayant et al., 2010). The more abundant highly methylesterified HG in the intine wall of pme48-/− pollen grains and at the tip and in the sub-apical region of pme48-/− pollen tubes provides more visco-elasticity and less rigidity of the cell wall, thus promoting several tips emerging from the pollen grain and wider diameters of pollen tubes probably due to the internal turgor pressure (Parre and Geitmann, 2005a, Winship et al., 2010).

Mature pollen grains are surrounded by two cell walls: the outer exine, and the inner intine. In many species such as Nicotiana tabacum (Li et al., 1995), Arabidopsis thaliana (Van Aelst and Van Went, 1992; Rhee and Somerville, 1998), Lilium hybrid (Aouali et al.,
Euphorbia peplus (Suarez-Cervera et al., 2002), Zygophyllum fabago (Castells et al., 2003) and Larix decidua (Rafinska et al., 2014), the intine of mature pollen grains is mostly composed of weakly methylesterified HG, or a mix of highly and weakly methylesterified HGs, the latest being more abundant. The almost absent labelling of weakly methylesterified HG with JIM5 at the pollen mother cell and tetrad stages (Rhee and Somerville, 1998) but its strong detection in the intine at the late microspore stage and mature dry Arabidopsis pollen grains (Van Aelst and Van Went, 1992, Rhee and Somerville, 1998) indicates an early action of PMEs during pollen formation and maturation (Fig. 8). HG polymers are synthesized in the Golgi apparatus and may be secreted under a highly methylesterified form and then processed in the cell wall by PMEs (Zhang and Staehelin, 1992; Caffall and Mohnen, 2009; Harholt et al., 2010). This is consistent with our qRT-PCR results showing that pollen-specific PMEs, including PME48, are strongly expressed in dry pollen grains. A similar pattern of expression of these PMEs was previously obtained in two transcriptomic analyses of dry pollen grains and pollen tubes (Wang et al., 2008; Qin et al., 2009). Moreover, proteomic analyses of Arabidopsis revealed the presence of at least 3 PMEs in mature pollen grains: PME37, PPME1 and PME48 (Holmes-Davis et al., 2005; Ge et al., 2011). Similarly, at least 2 PMEs were found in the proteome of rice (Dai et al., 2006) and maize (Zhu et al., 2011) dry pollen grains.

The early de-methylesterification of HG by PME48 during the pollen formation and maturation (Fig. 7) may have three major consequences. First, the removal of hydrophobic methylester groups can enhance the hydrophilic properties of the cell wall, thus promoting pollen grain hydration (Fig. 7). The DM of HG can affect the water holding capacity of the pectate gel (Willats et al., 2001) leading to a water loss and changes of the physical properties of the pectate gel which appeared to collapse under pressure (Willats et al., 2001). The mum2
mutant (impaired in the expression of a β-galactosidase) failed to hydrate properly the seed mucilage (Macquet et al., 2007). Interestingly, in the mucilage of mum2, the content of methylesterified HG increased compared to the wild-type suggesting that i) β-galactosidase may be required for PME activity (Western et al., 2001; Walker et al., 2011) and ii) PME activity is required for the hydration of HGs. This observation may explain why in the pme48-/mutant lines the imbibition and germination of pollen grains were strongly delayed, as the DM of the HG in the intine of pme48-/ is almost 2.5-fold higher than in the wild-type.

Second, upon the block-wise action of PMEs, de-esterified blocks of HG chains can be cross-linked with calcium (Micheli, 2001). During the wild-type pollen grain formation and maturation, the activity of PME48 promotes the release of negative charges of the carboxylic groups allowing the interaction of calcium with the HG chains. The main Ca\(^{2+}\) sink in the plant cell wall is the de-methylesterified HG (Wolf et al., 2009a; Hepler et al., 2012). Thus, the unesterified HG in the maturing pollen grain may act as a reservoir of Ca\(^{2+}\) that will be used later during rehydration and germination (Fig. 7). The reduction of PME activity in pme48-/ may locally affect the binding capacity of calcium ions by HGs. This possibly may explain the reverse phenotype observed in the calcium-supplemented GM. In vitro pollen germination of many species requires Ca\(^{2+}\) (Brewbaker and Kwack, 1963; Ge et al., 2007) and Ca\(^{2+}\) plays a central role during pollen germination (Brewbaker and Kwack, 1963; Ge et al., 2007; Hepler et al., 2012) acting putatively as a signal to activate the germination process and/or acting directly on the physical property of the intine. Ca\(^{2+}\) dynamics have been investigated in Arabidopsis pollen grains during germination (Iwano et al., 2004). These authors have shown that, during pollen grain hydration, cytoplasmic Ca\(^{2+}\) increased at the future site of protrusion of the pollen tube tip but not exclusively as, often, an increase was also observed at the opposite site (Fig. 7). The early action of PME48 during the maturation and dehydration of pollen grains and the accumulation of Ca\(^{2+}\) in the intine may help to
prepare the future protrusion of the pollen tube tip by weakening the intine wall (Fig. 7).

Recently, Rafińska et al., (2014) suggested that the cell wall of the female tissues can also act as a reservoir of Ca\(^{2+}\) in order to ensure correct germination and pollen tube growth in \emph{Larix decidua}. Upon pollination, an increase of Ca\(^{2+}\) concentration was also observed in the stigma and transmitting tract of lily (Zhao et al., 2004) and tobacco (Ge et al., 2009). This observation may explain that the impact of the \emph{PME48} mutation on the \emph{in vivo} pollen germination and tube growth was not as dramatic as \emph{in vitro} in the optimum solid GM. However, we cannot rule out that PMEs originating from the stigma may also participate in this process or that pollen grains cultured in \emph{in vitro} and semi-\emph{in vivo} conditions display a different gene expression pattern (Qin et al., 2009).

As discussed by Jolie et al. (2010), the pH, the degree of methylesterification and the pattern of methylesterification are known to modify the mode of action of PMEs (Catoire et al., 1998, Denès et al., 2000; Sénéchal et al., 2014). Thus, we can also hypothesized that upon random action of the PME (PME48 or others from the 13 other pollen specific PMEs), the partial removal of methylester groups may allow other pectin-degrading enzymes such as PGases and/or PLs to cleave the HG affecting the rigidity of the cell wall (Micheli, 2001; Sénéchal et al., 2014). In the \emph{Arabidopsis} genome, 69 annotated genes can be classified as putative PGases (González-Carranza et al., 2007). A semi-quantitative RT-PCR analysis investigating 66 among the 69 genes coding for PGases showed that 32 genes were strongly expressed in flower tissues (Kim et al., 2006). Other studies have also shown that PGases were present in ungerminated pollen grains of \emph{Brassica napus} (Deamley and Daggard, 2001) and during hydration of \emph{Platanus acerifolia} pollen grains (Suárez-Cervera et al., 2005). In addition, in \emph{Brassica campestris}, the alteration of the expression of \emph{BcMF2} (coding for a putative PGase resulted in an overdeveloped intine and abnormal germination (Huang et al.,
The transcriptome analysis of Arabidopsis pollen grains has revealed that 6 genes coding for putative PGases and 4 genes encoding putative PLs are strongly expressed in dry pollen grains and pollen tubes (reviewed by Mollet et al., 2013). Taken together, these observations suggest that the early action of PME48 during maturation of the pollen grain may also be required to ensure the future degradation of the intine by PGases and PLs (Fig. 7a). Enzymes originating from the stigma may also participate in the degradation of HGs in the intine. Such enzymes including PGases have been found in the exudates from lily and olive stigmas (Rejon et al., 2013).

Although pollen specific PMEs are traditionally associated with pollen tube elongation, the present study provides substantial evidence that PMEs, especially PME48, are also directly implicated in the changes of the mechanical properties of the intine wall during maturation promoting the correct germination of mature pollen grains.

Material and Methods

Plant materials, growth conditions and mutant genotyping

Arabidopsis (Arabidopsis thaliana ecotype Columbia-0) wild-type and mutant seeds, stored at 4°C, were spread on the surface of sterile soil and cultured in a growth chamber with a photoperiod of 16 h of light/8 h of dark at 20°C during the light phase and at 16°C in the dark phase with 60% humidity with daily watering.

pme48/- (mutant of the PME48 gene, At5g07410) seeds originated from the Arabidopsis Biological Resource Center (ABRC) and corresponded to the SALK_122970 (T-DNA 1403 bp downstream ATG). Homozygous plants for the T-DNA insertion in the PME48 gene were identified by PCR. Genotyping PCR reactions were performed with primer pairs
Quantitative RT-PCR analyses

Gene expression of pollen specific PMEs

Total RNA were extracted from pollen of 6-week-old wild-type and pme48/- plants using the NucleoSpin® RNA Plant kit (Macherey-Nagel) as described by the supplier. After RNA quantification using NanoDrop spectrophotometry, and a DNase treatment, 100 ng of RNA were converted into single cDNAs with QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA) following the instructions of the supplier. Real time quantitative PCR (RT-qPCR) analyses were performed on 1/5 diluted cDNA. For RT-qPCR, the LightCycler® 480 SYBR Green I Master (Roche, Cat.No.04887352001) was used in 384-well plates in the LightCycler® 480 Real-Time PCR System (Roche). The CT values for each sample (crossing threshold values are the number of PCR cycles required for the accumulated fluorescence signal to cross a threshold above the background) were acquired with the LightCycler 480 software (Roche) using the second derivative maximum method. Primers used are shown in Table S1. Stably expressed reference genes (At3g28750, At3g57690, At5g59370), specifically designed for this study on pollen grains, were selected using GeNorm software (Vandesompele et al., 2002). They were used as internal controls to calculate relative expression of target genes, according to method described by Gutierrez et al. (2009). Each amplicon was first sequenced to ensure the specificity of the amplified sequence.

Analysis of PME48 transcripts in mutant lines
The lack of \textit{PME48} transcript in \textit{pme48-/} was checked by RT-PCR using PME48-F2 (CTGGAACTGGAGGAGGAACT) and PME48-R2 (CATAAATCTCAACTCCTCCATG). PCR was performed on the cDNA generated from total RNA extracted from 6 h-old pollen tubes grown \textit{in vitro}. Primers used for the RT-PCR are in bold and underlined in Figure S1D.

\textbf{Analysis of promoter activity}

\subsection*{GUS Staining}

Mutant seeds containing 1-kb of the promoter of \textit{At5g07410} upstream of the GUS coding sequence were generated as described by Louvet et al., (2006).

GUS staining was performed on dry pollen grains, \textit{in vitro} grown pollen tubes and \textit{in vivo} on self-pollinated flowers. Flower and dry pollen grains samples were fixed in cold acetone for 20 min and rinsed three times in 50 mM phosphate buffer pH 7.0 containing 2 mM potassium ferricyanide, 2 mM potassium ferrocyanide and 0.2\% triton X100. \textit{In vitro} grown pollen tubes samples were not incubated in acetone. Samples were then incubated for 16 h in the dark at 37°C in 50 mM phosphate buffer pH 7.0 containing 1 mM X-Gluc, 2 mM potassium ferricyanide, 2 mM potassium ferrocyanide and 0.2\% triton X100. Flower samples were then cleared with several washes in 70\% ethanol.

\subsection*{Analysis of the activity of the promoter of \textit{PME48} (pPME48) using YFP protein}

The promoter of the \textit{PME48} gene (\textit{At5g07410}) was PCR-amplified from a plasmid construct containing this sequence cloned upstream of the GUS coding sequence, in the pBI101.3 binary vector (Louvet et al., 2006). A ligation independent cloning (LIC) method was used to insert the promoter of the \textit{PME48} gene (pPME48) upstream of the super YELLOW FLUORESCENT PROTEIN (sYFP, Kremers et al., 2006) in a LIC binary vector, named
pPLV06 (GenBank accession number JF909459) and belonging to the set of plant LIC vectors made by De Rybel et al., (2011). This vector also contains the SV40 nuclear localization signal. The PCR amplified promoter insert contained in the plasmid construct was subsequently verified by sequencing and the plasmid was used to transform Agrobacterium tumefaciens GV3101::pSOUP (Hellens et al., 2000). A. thaliana plants were transformed by floral dip as described by Zhang et al. (2006) and selected on kanamycin (25 mg l⁻¹).

In vitro pollen tube growth

Pollen grains were grown in vitro in a liquid medium according to the method described by Boavida and McCormick, (2007). Briefly, flowers (40 per 1.5 mL tube) were submerged in 1 mL of germination medium (GM) containing 5 mM CaCl₂ 2H₂O, 0.01% (w/v) H₃BO₃, 5 mM KCl, 1 mM MgSO₄ 7H₂O, and 10% (w/v) sucrose, pH 7.5. Tubes were shaken with a vortex to release the pollen grains from the anthers. Flowers were removed with a pair of tweezers and the pollen suspension was then centrifuged at 4,000g for 7 min. New GM (250 µL) was added to the pellet and pollen grains were transferred into glass vials (14 × 45 mm) and grown in a growth chamber in the dark at 22°C. For solid medium culture, 1.5% agarose (Sigma) was added to the GM and deposited on a microscope slide. After polymerization, wild-type or mutant open flowers were gently brushed on the surface of the agarose pad to release the pollen grains. Glass slides were then placed in a growth chamber in the dark at 22°C under 100% relative humidity. Before any further manipulation, pollen germination and pollen tubes growth were assessed under microscope.

Twenty images per sample were acquired after 2, 4, 6, 8, 14, 16 and 24 h of culture and the percentage of germination (n > 10,000) and the speed of growth of pollen tubes (n > 35) were measured using ImageJ software (Abramoff et al., 2004). Germination assays and the speed of growth of pollen tubes were repeated six times. For the measurement of the
diameter of pollen tubes, wild type and \textit{pme48/-} pollen grains were harvested by dabbing flowers onto silane coated microscope slides. Slides were incubated at 30°C for 30 min in a moist chamber and subsequently covered with liquid GM and grown for 4 h in a growth chamber in the dark at 22°C. The diameter of pollen tubes was determined on the pictures with ImageJ (n=126 and n=172 for the wild type and \textit{pme48/-} pollen tubes, respectively from 4 independent experiments).

Pollen grains were also cultured in the GM supplemented with 1 mM EDTA or 2.5 mM CaCl$_2$ 2H$_2$O to reach the final concentration of 7.5 mM CaCl$_2$.

\textbf{In vivo pollen tube growth}

Emasculated mature flowers from wild-type plants were hand-pollinated with wild-type or mutant (\textit{pme48/-}) pollen grains. The pollinated pistils were collected 6, 12 and 24 hours after pollination (hap) and fixed in an ethanol:acetic acid (3:1, v/v) solution. The fixed pistils were rehydrated with successive baths of ethanol (70%, 50% and 30%), washed three times with distilled water and treated overnight in a softening solution composed of 8 M NaOH. After several washes with distilled water, the pistil tissues were stained with decolorized aniline blue solution (DAB) (0.1% aniline blue in 100 mM K$_3$PO$_4$ buffer, pH 11) for 2 h in the dark (Johnson-Brousseau and McCormick, 2004).

\textbf{Viability test and DAPI staining}

The viability of pollen grains was assessed using fluorescein diacetate (FDA) dissolved in acetone at 10 mg mL$^{-1}$ and stored at -20°C. Prior to each experiment, FDA was diluted in a 10% sucrose solution to a final concentration of 0.2 mg mL$^{-1}$. Hydrated pollen grains were dipped in 250 µL of the FDA solution on a glass slide and kept in the dark for 5 min. A minimum of 1000 pollen grains were analyzed.
The nuclei of the pollen grains were stained with 10 µg mL⁻¹ DAPI (4',6-diamidino-2-phenylindole) for 15 min in the dark at room temperature. At least 250 pollen grains were analyzed for each mutant or wild-type.

**Immunolocalization of highly methylesterified HG epitopes**

Immunolocalization was performed by cell surface labelling as previously described by Dardelle et al., (2010) or on semi-thin sections. For the cell surface immunolabeling, pollen grains or pollen tubes in GM were mixed (v/v) with a fixation medium containing 100 mM PIPES buffer, pH 6.9, 4 mM MgSO₄ 7H₂O, 4 mM EGTA, 10% (w/v) sucrose, 5% (w/v) formaldehyde and incubated for 90 min at room temperature. Pollen grains or pollen tubes were rinsed 3 times by centrifugation with 50 mM PIPES buffer, pH 6.9, 2 mM MgSO₄ 7H₂O, 2 mM EGTA and 3 times with phosphate-buffered saline (PBS) (100 mM potassium phosphate pH 7.4, 138 mM NaCl, 2.7 mM KCl).

For the immuno-localization on semi-thin sections, dry pollen grains were fixed in ethanol:acetic acid (3/1 v/v) and rinsed in 75% ethanol. After centrifugation, the pellet of pollen grains was embedded in a block of 2% agarose. The block was then incubated in 75, 95 and 100% ethanol for 1 h 30 each at room temperature. Pollen grains were then transferred in increasing concentrations of methacrylate (25, 50, 75 and 100%) according to Baskin et al., (1992). The resin was polymerized at 4°C for 24 h under UV light. Semi-thin sections (1 µm) were obtained with the EM UC6 ultramicrotome (Leica) and deposited on Poly-L-Lysine coated microscope slides.

Samples were then incubated overnight at 4°C with the LM20 antibody that recognizes methylesterified HG epitopes (Verhertbruggen et al., 2009) (diluted at 1/5 with PBS + 3% milk). Samples were rinsed three times with PBS and incubated for 3h at 30°C with the
secondary antibody, a goat anti-rat IgG (whole molecule)-FITC, diluted at 1/100. Controls were carried out by incubation of pollen grains or pollen tubes with the secondary antibody only.

**Image acquisition**

Pollen grains and pollen tubes were observed under bright-field and fluorescence illumination on a Leica DLMB microscope equipped with the FDA filter (absorption, 470; emission, 520-560 wavelength), under UV illumination for DAPI and DAB staining (absorption 358; emission 461 wavelength) or FITC filter (absorption, 490; emission, 520 wavelength). Images were acquired with a Leica DFC300FX camera. Pollen grains and pollen tubes expressing the *pPME48::YFP* were observed with a laser scanning confocal microscopy (Leica TCS SP2 AOBS). YFP was visualized using the 488 nm laser line of an argon laser, with a 510–530 band-pass filter. Pollen grains were also observed by scanning electron microscope with the Hitachi TM3000 tabletop in analytical mode. Time lapse imaging was performed with the inverted Leica DMI 6000B microscope equipped with the Leica DFC450C camera.

**Fourier transform infrared (FT-IR) spectroscopy.**

Eight mg of pollen grains from wild-type or mutant plants were collected with the vacuum-pollen method described by Johnson-Brousseau and McCormick (2004). After removing the flower debris with tweezers, pollen grains were placed into micro-centrifuge tubes containing ethanol 96%. After several washes, the samples were dried overnight under a fume hood. Pollen grains were then incubated for 1 h in 200 µL of sterilized water at 100°C. After a speed vacuum treatment to eliminate water, samples were oven-dried for 48 h to obtain a pectin-enriched fraction. This fraction was stored in a sealed container with silica gel prior to FT-IR analysis. Dry samples were analyzed with an OMNI-Sampler FT-IR spectroscope (version...
5.2a) at 4 cm\(^{-1}\) resolution. The DM was calculated using the absorbance intensities at 1630 and 1740 cm\(^{-1}\) assigned to the vibration of carboxyl groups (COO\(^{-}\)) of GalA and its methylester form, respectively (Gribaa et al., 2013), using the equation described by Gnanasambandam and Proctor (2000) and Manrique and Lajolo (2002): \[ DM = \frac{A_{1740}}{A_{1740} + A_{1630}}. \]

Commercial pectins with determined DM were used as controls. Using this method, the DM of pectin from citrus fruit with 85% DM (Sigma) gave 75.1 ± 2.5% DM, pectin from citrus fruit with 55–70% DM (Sigma) gave 60.5 ± 1.5% DM, pectin from citrus fruit with 20–30% DM (Sigma) gave 26.9 ± 3.4% DM, and finally pectate Na pectin from citrus with a DM = 8.6% (Sigma) gave 10.8 ± 0.9% DM. Three biological replicates, for the wild type and the mutant were analyzed and 10 acquisitions were acquired per biological sample.

**Protein extraction and enzymatic assay**

Total proteins were extracted by grinding 2 mg of dry pollen grains in 50 mM sodium phosphate buffer pH 7.5, 12.5 mM citric acid, 1 M NaCl, 0.2 % (w/v) PVPP (PolyVinylPolyPyrrolidone) and 0.01 % (w/v) Tween 20 and 1 tablet of protease inhibitor cocktail (Roche) for one night at 4 °C. Cellular fragments were discarded by centrifugation at 10,000 g for 45 min. The crude protein extract was concentrated by ultrafiltration on Amicon PM10 membranes (Millipore) in 50 mM sodium phosphate buffer, pH 7.5. Proteins were quantified by the micromethod of Bradford (Bradford 1976), with the Biorad kit and bovine serum albumin as a standard. PME activity was measured using the alcool oxidase method according to Klavons and Bennett (1986). One IU of PME activity induces the production of 1 µmol of methanol per min.
Isoelectric focalisation and zymogram

Isoelectric focalisation (IEF) and zymogram were performed as described by Paynel et al. (2014). Briefly, IEF of native proteins was performed on ultrathin polyacrylamide gel with 6–10.5 pH range (Pharmalytes) in 5 % acrylamide according to the manufacturer’s procedure with a Multiphor II system (LKBPharmacia). The pH gradient was measured with a contact electrode (pH Inlab 426, VWR International), along a central gel strip. Samples (20 µL) were loaded at the anodic side. After IEF, gels were washed for 15–30 min in 20 mM Tris–HCl pH 8.5, 5 mM EDTA. Activity of PME was then monitored in gel (zymogram) by using a sandwich of 1 % (w/v) citrus pectin with a DM of 85 % (Sigma) and 1 % (w/v) agar according to Bertheau et al. (1984). The gel was incubated for 1 h at 25 °C and the demethylated pectins resulting from PME activities were precipitated with 0.1 M malic acid and stained with 0.02 % (w/v) ruthenium red.

Statistical analysis

Data were statistically treated using the graphpad software (www.graphpad.com).

Supplemental material

Table S1. List of primer pairs used for the qRT-PCR analyses.

Figure S1. Genomic organization of the PME48 gene, location of the T-DNA, PME48 expression in the mutant and transcript sequence of PME48.

Figure S2. Viability and phenotypic characteristics of wild-type and mutant lines.

Figure S3. Germination rate of wild-type and pme48/- pollen grains in liquid medium.
**Figure S4.** Estimation of the growth speed in liquid medium of wild-type (black bars) and *pme48-/-* (grey bars) pollen tubes.

**Movie S1.** Time-lapse imaging of germination and tube growth of wild-type pollen grains.

**Movie S2.** Time-lapse imaging of germination and tube growth of *pme48-/-* pollen grains.

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Figure 1. A-C, Relative gene expression of the 14 pollen specific PMEs in wild-type dry pollen grains (A), imbibed pollen grains for 1h (B) and 6 h-old pollen tubes (C) was measured using stably expressed reference genes (At3g28750, At3g57690, At5g59370) in three biological samples with similar results. Only the results obtained with At3g28750 are shown. The locus and the corresponding UniProt name of the proteins are indicated. The data correspond to the mean ± sd of three technical replicates of a biological sample. D-F, Analyses of the expression of the promoter of PME48 using pPME48::YFP construction by confocal microscopy in dry pollen grains (D), imbibed pollen grains for 1 h (E) and during pollen tube growth (F). Images are the overlay of the bright field and fluorescent pictures. G-I, Analyses of the expression of the promoter of PME48 using pPME48::GUS construction in dry pollen grains (G), in 3 h-old pollen tube (H), in anthers (I) and in self-pollinated pistil (close-up picture in I). a, anther; ov, ovary; ovu, ovule; pt, pollen tube; s, stigma; st, style; tt, transmitting tract.

Figure 2. Relative gene expression of PME48 in pme48/- dry pollen grains (A), imbibed pollen grains for 1h (B) and 6h-old pollen tubes (C) was measured using stably expressed
reference genes (At3g28750, At3g57690, At5g59370) in three biological samples with similar results. Only the results obtained with At3g28750 are shown. The data correspond to the ratio of the expression in wild-type or pme48/- compared to the wild-type and are the mean ± sd of three technical replicates of a biological sample.

Figure 3. In vitro and in vivo germination of wild-type and pme48/- pollen grains. A, Germination rate of wild-type (black square) and pme48/- (white circle) pollen grains in the optimum solid medium. Pollen grains were considered germinated when the length of the tube was equal to the diameter of the pollen grain. B, Estimation of the imbibition rate by measuring the ratio length/width of wild-type (black bars) and pme48/- (grey bars) pollen grains. C-D, in vivo growth of wild-type and pme48/- pollen tubes in wild-type pistil 6 h (C) and 24 h (D) after hand-pollination revealed by aniline blue staining. Arrows indicate the location that most of the pollen tubes have reached in the transmitting tissue. E, Time-lapse images of wild-type and pme48/- pollen germination in liquid medium showing two emerging tips from the pollen grain (blue circle) and burst tubes (*). The number in the close up picture indicates which tubes emerge first. Close up pictures of abnormal phenotypes observed in the mutant line compared to the wild-type. F, Estimation of the rate of burst pollen tubes in the wild-type (black bars) and pme48/- (grey bars). G, Estimation of the rate of pollen grains with two emerging tips in wild-type (black bars) and pme48/- (grey bars). H, Estimation of the growth speed of wild-type (black bars) and pme48/- (grey bars) germinated pollen tubes. I, Comparison of the pollen tube diameters between wild-type (black bars) and pme48/- (grey bars) grown in liquid GM for 4h. Different letters indicate statistically significant differences between the wild-type and pme48/- lines, determined by the Student’s t-test (P<0.0001; n> 10 000 in A; n> 500 in B, F and G; n> 35 in H; n = 126 for the wild-type
and n= 172 for pme48/- from 4 independent experiments in I. Scale bars represent 50 µm (E) and 100 µm (C, D).

**Figure 4.** Immunolocalization of highly methylesterified HG epitopes probed with LM20 in wild-type and pme48/- pollen grains and pollen tubes. A-E, Cell surface immunolabeling in wild-type pollen grains and pollen tubes. F,G, Immunolabeling on semi-thin sections of wild-type dry pollen grains. H-L, Cell surface immunolabeling in pme48/- pollen grains and pollen tubes. M,N, Immunolabeling on semi-thin sections of pme48/- dry pollen grains. * locates the pollen grain and the arrow points to the pollen tube tip; Ex, Exine; In, Intine.

**Figure 5.** Biochemical analyses of wild-type and pme48/- dry pollen grains. A. Enzymatic assay of the total PME activity contained in wild-type (black) and pme48/- dry pollen grains (grey). B. Zymogram after IEF of PMEs contained in wild-type and pme48/- dry pollen grains. A diffuse band corresponding to the pI (8.2) of PME48 is lacking in pme48/- dry pollen grains. C-D, determination of the degree of methylesterification of the HG in dry pollen grains by the FT-IR. C, Representative FT-IR spectra of pectin-enriched fractions extracted from wild-type (black) and pme48/- dry pollen grains (grey). D, Quantification of the DM of HG in wild type (black bar) and in pme48/- (grey bars) pollen grains. Data are the mean of three biological replicates ± sd.

**Figure 6.** Effect of calcium on the germination and phenotype of pollen tubes. A-B, Germination percentage of wild-type (black bars) and pme48/- (grey bars) pollen grains in the optimal solid medium (CaCl2 5 mM), in the optimal solid medium supplemented with 1mM EDTA, and in the optimal medium supplemented with 2.5 mM calcium (i.e. final concentration CaCl2 7.5 mM) after 6 (A) and 24 h (B) of growth. Pollen grains were considered germinated when the length of the tube was equal to the diameter of the pollen grain. C, Estimation of the imbibition rate by measuring the ratio length/width of wild-type
(black bars) and \textit{pme48-/-} (grey bars) pollen grains in the solid medium containing 7.5 mM of CaCl\textsubscript{2}. D, Estimation of the rate of burst pollen tubes in the wild-type (black bars) and \textit{pme48-/-} (grey bars) in the solid medium containing 7.5 mM of CaCl\textsubscript{2}. E, Estimation of the rate of pollen grains with two emerging tips in wild-type (black bars) and \textit{pme48-/-} (grey bars) in the solid medium containing 7.5 mM of CaCl\textsubscript{2}. Different letters indicate statistically significant differences between the wild-type and \textit{pme48-/-} lines, determined by the Student’s t-test (P<0.0001; n > 1000 in A and B; n> 500 in C; n> 250 in D and E).

**Figure 7.** Model showing the possible mode of action of PME48 during pollen dehydration, imbibition and germination. 1, PME48 may be secreted in the intine wall during the maturation of the pollen grain and start to act on the methylesterified form of the HG by random or block wise actions (1). Methylesterified HG of the intine (black) is then transformed in weakly methylesterified HG (orange). The dry pollen grain contains mostly weakly esterified HG that could form pectate gel with calcium. 2, imbibition of the pollen grain is enhanced by the presence of the more hydrophilic weakly methylesterified HG. 3, the source of calcium creating the calcium influx in the pollen grain may originate, at least partially, from the release of Ca\textsuperscript{2+} from the pectate gel (3) thus weakening the mechanical properties of the intine. The intine may be degraded by polygalacturonases and/or pectate lyases (4) and/or break under the turgor pressure (4'). 5, These processes may promote the emergence of the pollen tube tip and allow the germination of the pollen grain. sc, sperm cells; veg n, vegetative nucleus; pm, plasma membrane; GalA, galacturonic acid.

**Supplemental data**

**Table S1.** List of primer pairs used for the qRT-PCR analyses.
**Figure S1.** A, Genomic organization of the *PME48* (At5g07410) gene and location of the T-DNA insertion of *pme48/-* mutant line. The white boxes indicate the positions of the exons and the line the position of the introns (both exons and introns are drawn to scale). Grey boxes indicate the untranslated regions (UTR) in 3’ and 5’. Primers used for the genotyping are indicated with arrows and labelled F1, R1 and LB. B, Amplification of the T-DNA insert in the homozygous *pme48/-* mutant line. gDNA was extracted from leaves of wild-type and mutant plants. C, RT-PCR amplification products of *PME48* mRNA transcripts in 6 h-old pollen tubes from wild-type and *pme48/-*. D, Transcript sequence of *PME48*. Primers used for the RT-PCR are in bold and underlined, primers used for the qRT-PCR are highlighted in grey. The T-DNA is located in a black box. Letters in grey are UTR. ^ Location of an intron in the genomic DNA.

**Figure S2.** Viability and phenotypic characteristics of wild-type and mutant lines. A, DAPI staining of pollen grains showing the nuclei of the two sperm cells and the vegetative nucleus. Scale bars = 10 µm. B, C, Pollen viability test using FDA. Viable pollen grains fluoresce under UV. Scale bars = 30 µm. D, SEM of wild-type and *pme48/-* dry pollen grains. Scale bars = 30 µm. E, Measurements of the length of wild-type and *pme48/-* dry pollen grains. F, G, Quantification of the size of the siliques and the number of seeds per silique in wild-type and *pme48/-* plants. Different letters indicate statistically significant differences as determined by the Student’s t-test (P<0.0001). n> 1000 (B,C), n=550 (D,E), n=210 (F,G).

**Figure S3.** A, Germination rate of wild-type (black Square) and *pme48/-* (white circle) pollen grains in the optimum liquid GM. Pollen grains were considered germinated when the length of the tube was equal to the diameter of the pollen grain. B, Estimation of the imbibition rate by measuring the ratio length/width of wild-type (black bars) and *pme48/-* (grey bars) pollen grains. Different letters indicate statistically significant differences among
the wild-type and \textit{pme48/-} lines, as determined by the Student’s t-test (P<0.0001; n >10 000 in A; n>500 in B).

**Figure S4.** Estimation of the growth speed of wild-type (black bars) and \textit{pme48/-} (grey bars) pollen tubes in liquid medium.

**Movie S1.** Time-lapse imaging of germination and tube growth of wild-type pollen grains. Images were acquired every 5 minutes during 6 hours in liquid medium.

**Movie S2.** Time-lapse imaging of germination and tube growth of \textit{pme48/-} pollen grains. Images were acquired every 5 minutes during 6 hours in liquid medium.
Relative expression of PME48 in wild-type and pme48-/-
During maturation of the pollen grain, PME partially demethyllesterifies the HG of the intine wall.