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Developmental Biology 294 (2006) 83–91
DEVELOPMENTAL BIOLOGY
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Pollen-specific pectin methylesterase involved in pollen tube growth

Guo-Wei Tian, Min-Huei Chen, Adi Zaltsman, Vitaly Citovsky *

Department of Biochemistry and Cell Biology, State University of New York, Stony Brook, NY 11794-5215, USA

Received for publication 29 September 2005; revised 15 February 2006; accepted 16 February 2006
Available online 27 March 2006

Abstract

Pollen tube elongation in the pistil is a crucial step in the sexual reproduction of plants. Because the wall of the pollen tube tip is composed of a single layer of pectin and, unlike most other plant cell walls, does not contain cellulose or callose, pectin methylesterases (PMEs) likely play a central role in the pollen tube growth and determination of pollen tube morphology. Thus, the functional studies of pollen-specific PMEs, which are still in their infancy, are important for understanding the pollen development. We identified a new *Arabidopsis* pollen-specific PME, AtPPME1, characterized its native expression pattern, and used reverse genetics to demonstrate its involvement in determination of the shape of the pollen tube and the rate of its elongation.

Keywords: *Arabidopsis*; Native PME expression; Pectin; Pollen grain; Pollen tube

Introduction

Pollen tube elongation in the pistil is a crucial step in the sexual reproduction of plants. The pollen tubes invade the stigmatic tissue, penetrate the style, and eventually deposit the two sperm cells in the embryo sac where they fuse with the egg and central cell to form the zygote and endosperm, completing the fertilization process (reviewed in Lord and Russell, 2002; Palanivelu and Preuss, 2000; Preuss, 2002). While the pollen tube growth is known to occur exclusively at the tip with the new tube wall continuously forming at the growing tip (Taylor and Hepler, 1997), the molecular mechanism of this growth process remains obscure. The wall of the pollen tube tip is composed of a single layer of pectin and, unlike many other plant cell walls, does not contain cellulose or callose (Ferguson et al., 1998; Li et al., 1994). Thus, pectin metabolism and modification likely play a central role in the pollen tube growth.

Pectins are polymerized in the Golgi, methylesterified and modified with side chains, and subsequently released into the apoplastic space as highly methylesterified polymers. The homogalacturonan component of pectin can later be demethylesterified by pectin methylesterases (PMEs) (Micheli, 2001). This enzymatic activity of PMEs can lead either to cell wall loosening or to cell wall stiffening, depending on the apoplastic pH (Catoire et al., 1998; Denés et al., 2000; Micheli, 2001). In higher plants, pectin demethylesterification is catalyzed by a number of PME isoenzymes which can express their activities in response to certain developmental or environmental cues and/or in a tissue-specific fashion. For example, while some PMEs are ubiquitously present (Gaffe et al., 1997), others are specifically expressed during root development (Wen et al., 1999), fruit ripening (Brummell and Harpster, 2001; Frenkel et al., 1998), or stem elongation (Bordenave et al., 1996; Pilling et al., 2000). Furthermore, recent analysis of pollen-specific transcriptome of *Arabidopsis* indicated that several PMEs are specifically expressed in floral buds, including pollen (Pina et al., 2005). However, despite the apparently major role that PMEs may play in the growth of the pollen tube – the cell wall of which is composed mainly of pectins – the functional studies of pollen-specific PMEs are still in their infancy.

To date, only two studies examined the PME function during pollen development and pollen tube growth; specifically, exogenously added tobacco PME has been shown to inhibit pollen tube growth by thickening the apical cell wall (Bosch et al., 2005) whereas the inactivation of VANGUARD1 (VGD1), the only *Arabidopsis* PME with a demonstrated function in the pollen tube growth, resulted in unstable and poorly growing pollen tubes (Jiang et al., 2005). The diversity of *Arabidopsis* pollen-expressed PMEs (Pina et al., 2005) suggests that

* Corresponding author. Fax: +1 631 632 8575.
E-mail address: vitaly.citovsky@stonybrook.edu (V. Citovsky).

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doi:10.1016/j.ydbio.2006.02.026

additional members of this protein family may be involved in pollen tube growth, potentially affecting different aspects of this process.

Here, we identified a new *Arabidopsis* pollen-specific PME, AtPPME1, characterized its native expression pattern, and used reverse genetics to demonstrate its involvement in determination of the growth rate and the shape of the pollen tube.

Materials and methods

Plant material

All *Arabidopsis thaliana* plants used in this study were in the Col-0 background. Plants were grown in soil at 22°C under a light cycle of 16 h light/8 h dark.

YFP tagging and native expression of AtPPME1

Transgenic *Arabidopsis* plants expressing YFP-tagged AtPPME1 from its native regulatory sequences were generated using the fluorescent tagging of full-length proteins (FTFLP) technique (see http://aztec.stanford.edu/gfp/ and Tian et al., 2004). We constructed a transgene containing the native *AtPPME1* 5′ UTR with the promoter, coding region with introns, and the 3′ UTR sequences; because most regulatory sequences in *Arabidopsis* are contained in relatively small (2 kb) regions (The Arabidopsis Genome Initiative, 2000), we included 1516 bp upstream of the *AtPPME1* translation initiation codon and 1000 bp downstream of the STOP codon in our constructs. First, *AtPPME1* was amplified from genomic DNA – purified from leaves of 6-week-old plants using the DNeasy Plant Mini Kit (Qiagen, Valencia, CA) – as two fragments using two sets of primers, P1/P2 and P3/P4. The sequences of these primers were P1: 5′-gctcgatccacctaggctcatgtaaacaaatagcag-3′, P2: 5′-cacagctccacctccacctccaggccggcctccttggatgtagccgag-3′, P3: 5′-tgctggtgctgctgcggccgctggggcctccaagtggcttctcccacc-3′, and P4: 5′-cgtagcgagaccacaggaagcagagagagtaacacc-3′. For the second PCR reaction, a pair of gene–non-specific Gateway (Walhout et al., 2000) primers were designed. The forward primer, 5′-ggggacaagtttgtacaaaaaagcaggctgctcgatccacctaggct-3′, contained the attB1 sequence, and the reverse primer, 5′-ggggaccactttgtacaagaaagctgggtcgtagcgagaccacagga-3′, contained the attB2 sequence. These primers were combined with three templates, i.e., the *YFP* sequence derived from pRSET$_B$-Citrine (Griesbeck et al., 2001) and two *AtPPME1* fragments, and a triple template PCR (TT-PCR) was performed to produce the full-length *AtPPME1* gene with the YFP coding sequence inserted into its last exon 30 bp upstream of the STOP codon (Tian et al., 2004). All PCR reactions were performed using the ExTaq DNA polymerase (TaKaRa, Japan) which, under our conditions, exhibited the highest fidelity while still capable of efficiently amplifying long DNA fragments (Tian et al., 2004). The resulting TT-PCR product was recombined into the Gateway donor vector pDONR207 (Invitrogen), verified by DNA sequencing, and transferred by Gateway recombination into the binary destination vector pBIN-GW (Tian et al., 2004). Finally, the binary construct was introduced into the *Agrobacterium tumefaciens* strain GV3101, and the resulting bacterial cultures were used to transform *Arabidopsis* plants by the modified flower dip method (Kim et al., 2003). Seven kanamycin-resistant T1 transformants were selected for analysis of the YFP-tagged AtPPME1 expression.

Identification of AtPPME1 homozygous lines

T-DNA insertion line SALK_077776 of *Arabidopsis* (Alonso et al., 2003) was received from the *Arabidopsis* Biological Resource Center (Ohio State University, Columbus). Plants homozygous for T-DNA insertion in the *AtPPME1* gene were identified by PCR using genomic DNA extracted with the DNeasy Plant Mini Kit, two gene-specific primers, 5′-atcacccgctttgtgttccct-3′ and 5′-tgtcctcttgattgcagcttcttc-3′, and a T-DNA left border-specific LBb1 primer, 5-gcgtggaccgcttgctgcaact-3′ as described by the Salk Institute Genomic Analysis Laboratory (SIGNAL, http://signal.salk.edu/tdnaprimers.html).

Genetic complementation of the atppme1 mutant

To produce the full-length *AtPPME1* transgene, we utilized a modified FTFLP technique (Li et al., 2005; Tian et al., 2004). The full-length *AtPPME1* gene with its native regulatory elements was amplified using the P1/P4 primers (see above), and then the attB1 and attB2 recombination sites were added using the Gateway primers as described above. The amplified *AtPPME1* gene was recombined into pDONR207, transferred to the binary destination vector pSAT6-DEST, a derivative of pSAT6-DEST-EGFP-C1 (Tzfira et al., 2005) without the GFP sequence, and then subcloned into the pRCS2-*hpt* binary vector (Tzfira et al., 2005). The resulting binary construct was used to produce transgenic plants as described above, except that the transformants were selected on a hygromycin-containing medium.

RT-PCR

Total RNA was extracted from 2.0 g of plant tissues, using TRI REAGENT (Molecular Research Center, Inc., Cincinnati, OH), treated with RQ1 RNase-free DNase (Promega, Madison, WI), and reverse-transcribed with Moloney murine leukemia virus (M-MLV) reverse transcriptase using the dT23VN primer (New England Biolabs, Beverly, MA). The resulting cDNAs were PCR-amplified (Kang et al., 1995; Ni et al., 1998) using a mixture of *AtPPME1*-specific primers and primers specific for the *Arabidopsis ACT8* actin gene (At1g49240, An et al., 1996). *AtPPME1*-specific forward 5′-cggcaccagatggtaaaact-3′ and reverse 5′-tggatgtagccgagggatag-3′ primers generated a 559-bp product from the *AtPPME1* transcript, while actin-specific forward 5′-accttgctggtcgtgacctt-3′ and reverse 5′-gatcccgtcatggaaacgat-3′ primers generated a 632-bp product from the *ACT8* transcript.

In vitro pollen tube growth

Fully open flowers with freshly dehiscent anthers were collected and air-dried for 2–4 h. Pollen grains were germinated on the glass slides with agarose pads containing 10% sucrose, 0.01% boric acid, 3 mM $Ca(NO_3)_2$ (Thorsness et al., 1993), and 1% agarose in a moist kimwipe box incubated at 28°C (Johnson-Brousseau and McCormick, 2004). The growing pollen tubes were examined and their length measured 6 h and 24 h after beginning of germination. For length measurements, 150 pollen tubes of each sample were chosen randomly.

Quantification of the PME enzymatic activity

PME activity was quantified by a gel diffusion assay (Bourgault and Bewley, 2002; Downie et al., 1998) as described (Chen and Citovsky, 2003) with modifications. Briefly, 100 mg of pollen grains was collected using a vacuum cleaner method (Johnson-Brousseau and McCormick, 2004) from the wild-type, mutant, and genetically complemented plants, placed in a 1.5-ml microfuge tube containing one 3-mm glass bead and 400 μl of extraction buffer [1 M NaCl, 2.5 mM phenylmethylsulfonyl fluoride (PMSF), 10 μM leupeptin, 0.1 M citrate/0.2 M sodium phosphate, dibasic, pH 7.0] and homogenized for 10 s at 4°C in a TPC M250 Amalgamator (TPC Advance Technology, CA). The homogenate was cleared (20,000 g for 10 min at 4°C), and the protein content of the supernatant was quantified by the Bradford method (Ausubel et al., 1987). Protein samples (50 μg) were loaded into 2-mm round wells in a 2% (w/v) agarose gel containing 0.1% of 90% esterified citrus fruit pectin (Sigma-Aldrich) and 0.5 mg/ml EIA-grade gelatin (BioRad, CA) in a Petri dish (pectin served as PME substrate and gelatin was used to further stabilize the tested pollen extracts; Bourgault and Bewley, 2002; Downie et al., 1998). The gels were incubated for 16 h at 30°C, rinsed with water, stained for 45 min at 25°C with 0.05% (w/v) ruthenium red (Sigma-Aldrich) which stains deesterified pectin (Downie et al., 1998), and the diameter of each stained zone was measured to the nearest 0.1 mm with calipers. The PME activity in nanokatals (nkatal) was calculated based on the standard curve of log-transformed enzyme activity versus stained zone diameter generated using a commercial-grade orange peel PME (Sigma-Aldrich).

Microscopy

For imaging of pollen in transgenic plants expressing YFP-tagged AtPPME1, mature anthers or pistils from fully open flowers were collected on

Table 1
Arabidopsis PMEs

Group	AGI code	PMEI domain present	No of amino acids	Estimated molecular mass (x10^3)	Estimated theoretical pI
Group I (23)	At1g05310	No	393	43.3	8.2
	At1g11370	No	288	31.4	6.4
	At1g44980	No	246	27.7	5.5
	At1g69940[a]	No	361	39.1	8.7
	At2g19150	No	339	37.6	8.7
	At2g21610	No	352	39.1	8.1
	At2g36700	No	333	37.1	8.8
	At2g36710	No	407	45.0	6.1
	At2g47280	No	320	35.1	5.3
	At3g10720	No	263	29.5	6.1
	At3g17060	No	344	38.8	8.9
	At3g24130	No	335	37.3	8.8
	At3g29090	No	317	35.5	6.6
	At4g33220	No	404	45.3	9.0
	At5g07410	No	361	39.3	8.7
	At5g07420	No	361	39.7	9.2
	At5g07430	No	361	39.9	9.0
	At5g18990	No	330	36.5	9.2
	At5g19730	No	383t	42.5	9.2
	At5g26810	No	293	32.4	6.2
	At5g47500	No	362	40.0	9.0
	At5g55590	No	380	42.5	6.3
	At5g61680	No	338	37.3	9.1
Group II (43)	At1g02810	Yes	579	64.0	8.9
	At1g11580	Yes	557	61.7	9.0
	At1g11590	Yes	524	58.7	7.0
	At1g23200	Yes	554	61.3	9.2
	At1g53830	Yes	587	64.2	9.1
	At1g53840	Yes	586	64.1	8.5
	At2g26440	Yes	547	60.4	5.1
	At2g26450	Yes	614	68.6	8.6
	At2g43050	Yes	518	56.6	8.6
	At2g45220	Yes	511	56.0	9.2
	At2g47030	Yes	588	64.1	9.2
	At2g47040	Yes	595	64.7	9.1
	At2g47550	Yes	560	61.5	9.0
	At3g05610	Yes	669	73.0	6.0
	At3g05620	Yes	543	60.8	8.9
	At3g06830	Yes	568	61.9	8.9
	At3g10710	Yes	561	62.1	9.4
	At3g14300	Yes	968	105.6	8.4
	At3g14310	Yes	592	64.2	9.0
	At3g27980	Yes	497	55.5	7.5
	At3g43270	Yes	527	57.6	8.9
	At3g47400	Yes	594	65.5	9.9
	At3g49220	Yes	598	65.3	8.8
	At3g59010	Yes	529	57.5	9.0
	At3g60730	Yes	519	57.9	9.4
	At3g62170	Yes	588	63.0	8.8
	At4g00190	Yes	474	52.4	5.8
	At4g02300	Yes	532	59.8	5.6
	At4g02320	Yes	518	57.9	5.3
	At4g02330	Yes	573	63.9	8.7
	At4g03930	Yes	535	59.9	6.5
	At4g15980	Yes	701	78.6	9.0
	At4g33230	Yes	609	67.7	9.3
	At5g04960	Yes	564	62.2	8.9
	At5g04970	Yes	624	68.1	8.4
	At5g09760	Yes	551	60.4	5.9
	At5g20860	Yes	512	56.7	9.4
	At5g27870	Yes	732	78.5	6.2
	At5g49180	Yes	571	62.8	8.8
Group II (43)	At5g51490	Yes	536	58.9	9.6
	At5g51500	Yes	540	59.6	9.7
	At5g53370	Yes	587	64.2	7.6
	At5g64640	Yes	602	65.5	5.7

(a) Corresponds to the *AtPPME1* gene.

glass slides and mounted in water. Plant cell walls and pollen grain walls were visualized by staining with 10 μg/ml propidium iodide. For imaging of in vitro pollen tube growth, the pollen tube samples were mounted in the medium composed of glycerol/water (1:1 v/v). Confocal images were collected with a Zeiss LSM 5 Pascal laser scanning confocal microscope system. A 514 nm argon laser was used to excite YFP, and a reflected light coupled with DIC condenser was used for phase contrast digital imaging. The length of the pollen tubes was determined using the semi-automatic "Profile" function of the LSM Zeiss Pascal 5 software, and the average rate of pollen tube elongation determined over 24 h of growth.

Results

Arabidopsis PMEs

The *Arabidopsis* genome encodes 66 PME-related proteins (CAZY database, http://afmb.cnrs-mrs.fr/CAZY/). Amino acid sequence analysis of these proteins using the SMART tool (Letunic et al., 2004; Schultz et al., 1998) showed that 23 of them contain only the catalytic PME domain (Markovic and Jornvall, 1992; Plastow, 1988; Ray et al., 1988), while 43 also contain a PME inhibitor (PMEI) domain (Camardella et al., 2000; Giovane et al., 1995) (Table 1). Thus, *Arabidopsis* PMEs can be subdivided into two general groups based on their domain structure (Table 1); group 1 includes smaller proteins with 250–400 amino acid residues and molecular mass of 30–45 kDa that contain a PME domain, and group 2 comprises larger proteins with 500–700 amino acid residues and molecular mass of 55–105 kDa that include, in addition to a PME domain, 1–3 PMEI domains.

Because the one *Arabidopsis* PME, VGD1, known to function in pollen tube growth belongs to group 2 (Jiang et al., 2005), we decided to focus on the members of group 1, the role of which in pollen tube development has not been examined. Fig. 1A shows an unrooted phylogenetic tree for full-length amino acid sequences of the group 1 PMEs which revealed the presence of three clusters containing 13, 6, and 4 genes, respectively. One of these genes, At1g69940 – which encodes a 361-aa protein containing a conserved PME domain and an N-terminal signal peptide (Fig. 1B) – was not included in the recent *Arabidopsis* pollen transcriptome studies (Pina et al., 2005). Thus, we selected it for detailed expression and reverse genetics analyses.

Pollen-specific expression of AtPPME1

To examine potential tissue specificity of the At1g69940 gene expression, we utilized the Fluorescent Tagging of Full-Length Proteins (FTFLP) technique (Tian et al., 2004). In this approach, which has been shown to faithfully reproduce the

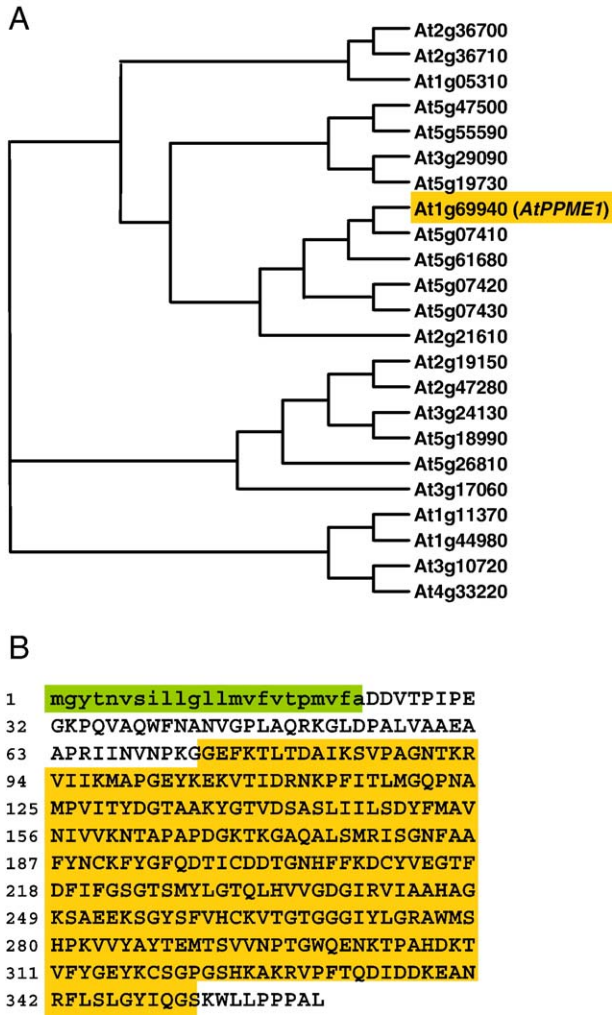

Fig. 1. Phylogenetic analysis of group 1 *Arabidopsis* PMEs and amino acid sequence of AtPPME1 and its protein product. (A) Phylogenetic tree constructed by the neighbor-joining method (Saitou and Nei, 1987) using the ClustalW algorithm (http://www.genebee.msu.su/clustal/advanced.html). The gene for AtPPME1 is highlighted in orange. (B) Amino acid sequence of AtPPME1. The N-terminal signal peptide is shown in lowercase and highlighted in green and the PME domain is highlighted in orange. Numbers on the left indicate positions of amino acid residues.

native level and pattern of gene expression as well as subcellular localization of the fluorescently tagged gene product (Chen et al., 2005; Tian et al., 2004), the At1g69940 gene was amplified to include its complete intergenic regions – i.e., the 5′ UTR and promoter sequences and the 3′ UTR and downstream sequence – and the coding region with introns and with a YFP tag inserted 30 bp (10 amino acids) upstream of the stop codon (Tian et al., 2004). The location of the tag was chosen outside of the PME domain and distant from the signal peptide (see Fig. 1B). This expression construct was introduced into *Arabidopsis* by *Agrobacterium*-mediated transformation, and several lines of the resulting transgenic plants were analyzed by confocal microscopy.

We observed no expression of the full-length At1g69940 gene driven by its native promoter and tagged with *YFP* in any of the plant organs and tissues (data not shown) except for mature pollen (Fig. 2). The YFP signal was detected only in the pollen grains, but not in the numerous surrounding cells of the anther (Figs. 2A, B), indicating highly restrictive expression of this gene (Figs. 2A, B), which we therefore named *AtPPME1* (*A. thaliana* pollen-specific *PME 1*). Within the expressing pollen grains, AtPPME1 accumulated mainly around the cell periphery and in intracellular vesicles, potentially representing the ER and/or Golgi endomembrane system (Fig. 2C); this subcellular localization is consistent with the presence of the signal peptide in AtPPME1 and the known secretion pathways of PMEs in plant cells (Micheli, 2001).

During fertilization, pollen grains are deposited on the stigma, and the compatible interaction between pollen and stigmatic cells triggers growth of the pollen tube which invades the stigmatic tissue, ultimately reaching the ovule (reviewed in Lord and Russell, 2002; Palanivelu and Preuss, 2000; Preuss, 2002). Fig. 3 shows that the growing pollen tube exhibited high

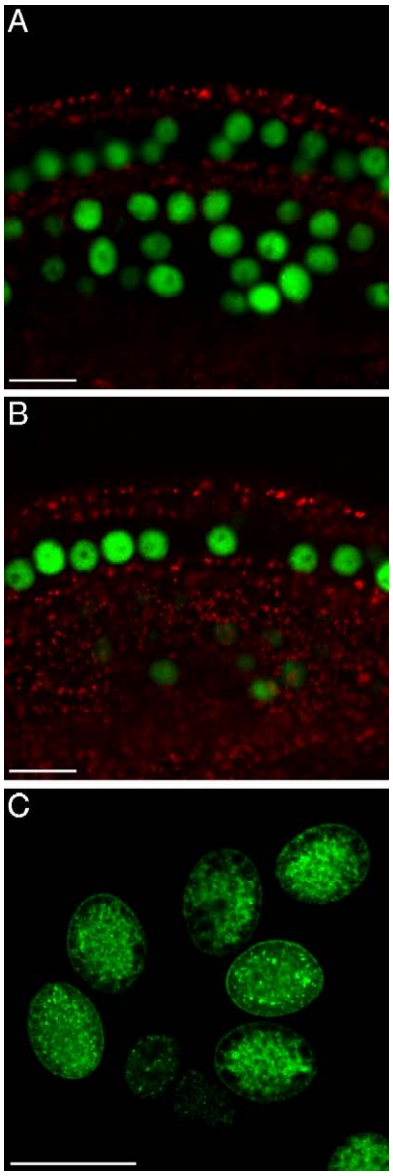

Fig. 2. Pollen-specific expression of YFP-tagged AtPPME1. All panels show mature pollen grains in anther sacs at different magnifications. YFP signal is in green, and plastid autofluorescence is in red. All images are single confocal sections. Scale bars = 30 μm.

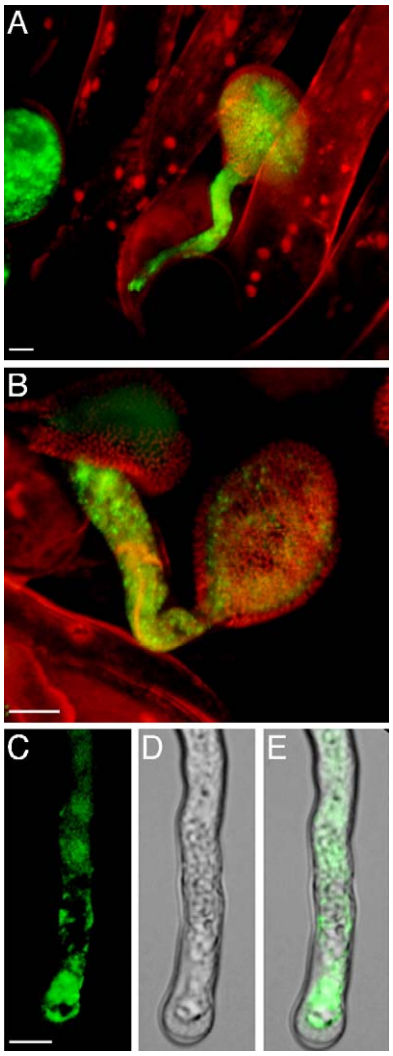

Fig. 3. Expression of YFP-tagged AtPPME1 in pollen grains and pollen tubes. (A, B) Pollen grains and pollen tubes growing between stigmatic papillae on stigma in a living pistil. Cell walls were stained with propidium iodide. (C) YFP-AtPPME1 expression in a pollen tube germinated and grown for 24 h in vitro. (D) Phase contrast image of the pollen tube shown in panel C. (E) Merged images from panels C and D. YFP signal is in green, and plastid autofluorescence and propidium iodide signal are in red. Images in panels A and B are projections of several confocal sections, image in panel C is a single confocal section. Scale bars = 10 μm.

levels of the *AtPPME1* expression on stigma (panels A, B) and in isolated pollen grains germinated in vitro (panels C–E). Consistent with the previous observations with the VGD1 PME (Jiang et al., 2005), the YFP-tagged AtPPME1 was distributed throughout the whole pollen tube (Figs. 3C–E). Again, confirming its pollen-specific expression, the YFP-tagged AtPPME1 was found only in the growing pollen, but not in the adjacent stigmatic papillae (Figs. 3A, B).

AtPPME1 is involved in pollen tube growth

The highly specific expression pattern of AtPPME1 suggests that this protein exerts its main biological effects during pollen development and/or pollen tube growth. This notion was confirmed by our reverse genetics analyses of an *AtPPME1* knockout plant line. To this end, we mined the SALK collection of *Arabidopsis* mutants (Alonso et al., 2003) and selected a mutant with a T-DNA insertion(s) in the second exon of the *AtPPME1* gene. The mutant plants were self-pollinated, the homozygous line was identified by PCR analysis, and the location of the 5′-end of the T-DNA insert in this line, designated *atppme1*, was confirmed by DNA sequencing (not shown).

We then used RT-PCR analysis to confirm that the *atppme1* plants do not contain the transcripts of the mutated gene. Fig. 4A shows that wild-type *Arabidopsis* plants accumulated the 559-bp RT-PCR product corresponding to the *AtPPME1* transcript (lane 1, bottom band) in their floral tissues. This expression was not observed in other plant tissues, such as roots (not shown), leaves (lane 2), or stems (lane 3), consistent with the pollen-specific pattern of expression of the YFP-tagged AtPPME1 (see Figs. 2 and 3). In contrast, no RT-PCR products specific for the *AtPPME1* transcript were detected in *atppme1* plants (Fig. 4, lanes 4–6), indicating that this mutant line indeed represented a null, knockout mutant for the *AtPPME1* gene. In control experiments, analysis of actin-specific transcripts generated similar amounts of RT-PCR products in all samples, indicating equal efficiencies of the RT-PCR reactions (Fig. 4A, upper bands in all lanes).

The lack of the AtPPME1 expression resulted in reduction of the overall PME activity in pollen grain of the *atppme1* plants. Fig. 4C shows that the ability of mutant pollen to demethylestrify pectins (PME activity = 680 nkatal/mg protein) was decreased by 20% as compared to the wild-type pollen (PME activity = 850 nkatal/mg protein). This reduction was comparable to that obtained in a knockout mutant of another pollen-expressed PME, *VGD1* (Jiang et al., 2005), and it supported the notion that the *AtPPME1* gene indeed encodes a functional PME enzyme.

Next, we investigated the effects of the absence of AtPPME1 on pollen growth and morphology in the *atppme1* plants. To this end, we germinated and cultured pollen grains in vitro. This approach allowed us to clearly characterize the morphology of the growing pollen tubes, and to precisely determine the rate of the growth. Three types of *Arabidopsis* plants were used in these experiments: wild-type plants, transgenic plants expressing YFP-tagged AtPPME1, and the *atppme1* knockout mutant.

Wild-type pollen tubes grew straight (Fig. 5A) and were indistinguishable from those of the YFP-AtPPME1 plants (not shown). In contrast, already early in the growth process, i.e., 6 h after germination, pollen tubes of the *atppme1* mutant, germinated under the same conditions, exhibited curved, irregular morphology and were dramatically stunted (compare Figs. 5B–D to Fig. 5A). The mutant pollen tubes remained dramatically shorter than the wild-type ones as the growth continued, i.e., 24 h after germination (compare Fig. 5F to Fig. 5E).

Higher magnification of the growing pollen tube tip showed that, in the *atppme1* knockout mutant, it has lost the characteristic relatively regular shape (compare Figs. 5H to I). The shorter length of pollen tubes in the *atppme1* plants was reflected by the reduced rate of the pollen tube growth. Specifically, quantification of the pollen tube growth showed that the average rate of tube elongation in the *atppme1* mutant

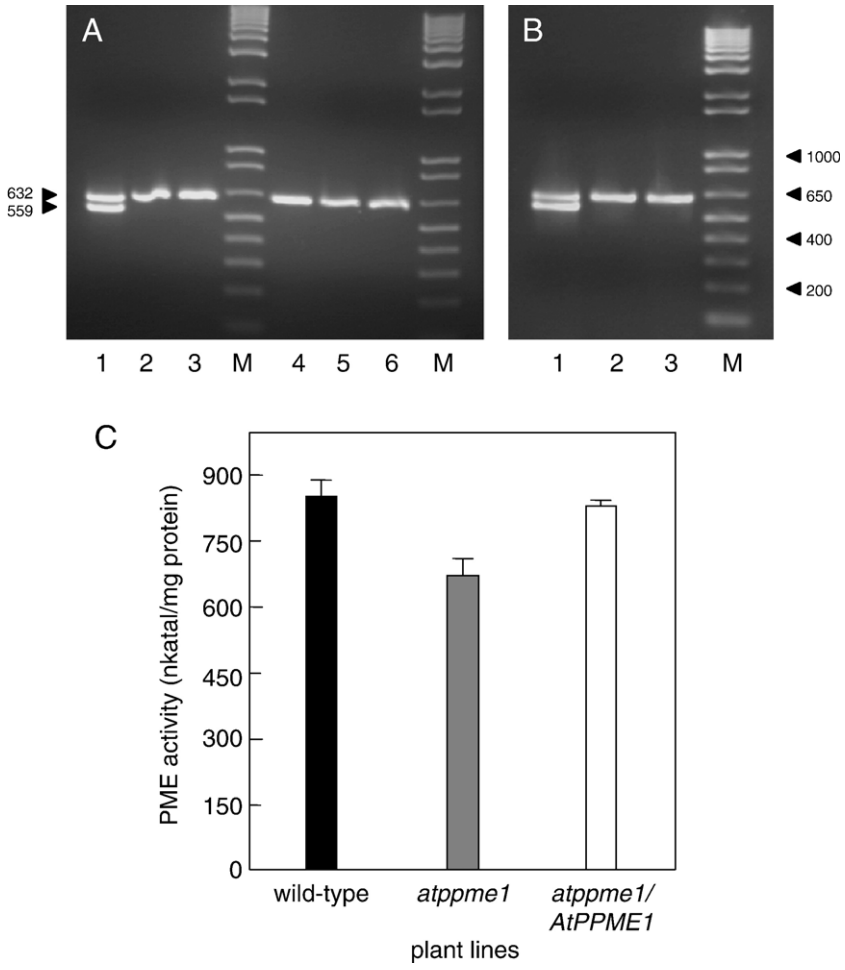

Fig. 4. RT-PCR detection of *AtPPME1* transcripts and quantification of PME activity in pollen. (A) Wild-type (lanes 1–3) and mutant *atppme1* plants (lanes 4–6). (B) Mutant *atppme1* plants genetically complemented with the wild-type *AtPPME1* gene. Lanes 1 and 4, flower; lanes 2 and 5, leaves; lanes 3 and 6, stems; lane M, molecular size markers. RT-PCR products specific for the *AtPPME1* transcript (559 bp) and for the *ACT8* transcript (632 bp) are indicated on the left. Molecular size markers in base pairs (bp) are indicated on the right. (C) PME enzymatic activity in pollen. Black, gray, and white bars indicate PME activity in the wild-type and *atppme1* plants and in *atppme1* plants genetically complemented with the wild-type *AtPPME1* gene, respectively. All data represent average values of three independent measurements with indicated standard deviation values.

(10.4 ± 3.3 μm/h, n = 150) was significantly lower than that in the wild-type plants (31.8 ± 5.0 μm/h, n = 150).

Interestingly, AtPPME1 specifically affected the pollen tube growth, length, and morphology, but it had no apparent effect on the morphology of ungerminated pollen grains and their cell walls pattern (Fig. 6) or on the efficiency of pollen germination in vitro. Similarly, we observed no differences between the wild-type and the homozygous *atppme1* mutant plants in their seed production and fertility. Specifically, both plant lines contained similar numbers of seeds in their siliques, i.e., 37.8 ± 2.9 (n = 50) per silique for the wild-type plants and 37.6 ± 3.1 (n = 50) per silique for the homozygous *atppme1* plants. When heterozygous *atppme1* male plants were genetically crossed with wild-type female plants, the resulting F1 progeny contained an approximately 1:1 mixture (14:13, n = 27) of heterozygous to wild-type seed genotypes in their siliques. Consistently, when heterozygous *atppme1* plants were self-pollinated, their F1 progeny segregated as 1:2:1 (6:13:6 *atppme1* homozygous to heterozygous to wild-type ratio, n = 23). Collectively, these data suggest that, although irregular and stunted, the *atppme1* pollen tubes largely retain their functionality during fertilization.

To exclude this possibility that another, unrelated mutation may have contributed to the *atppme1* phenotype, we performed a genetic complementation analysis. The *atppme1* mutant was transformed with a transgene corresponding to the full-length genomic sequence of *AtPPME1* with its native regulatory elements. The resulting plants expressed the *AtPPME1* transgene, accumulating its transcripts as detected by RT-PCR (Fig. 4B). This expression occurred in floral tissues (Fig. 4B, lane 1, lower band), but not in leaves (lane 2), stems (lane 3), or roots (not shown), mirroring expression of the wild-type *AtPPME1* (see Fig. 4A). Analysis of actin-specific transcripts produced similar amounts of PCR products in all samples, indicating equal efficiencies of the RT-PCR reactions (Fig. 4B, upper bands in all lanes).

We then examined the morphology and growth rate of the pollen tubes derived from the *atppme1* mutant plants that expressed the *AtPPME1* transgene from its native regulatory sequences. These transgenic plants developed pollen grains that, when germinated in vitro, formed pollen tubes indistinguishable

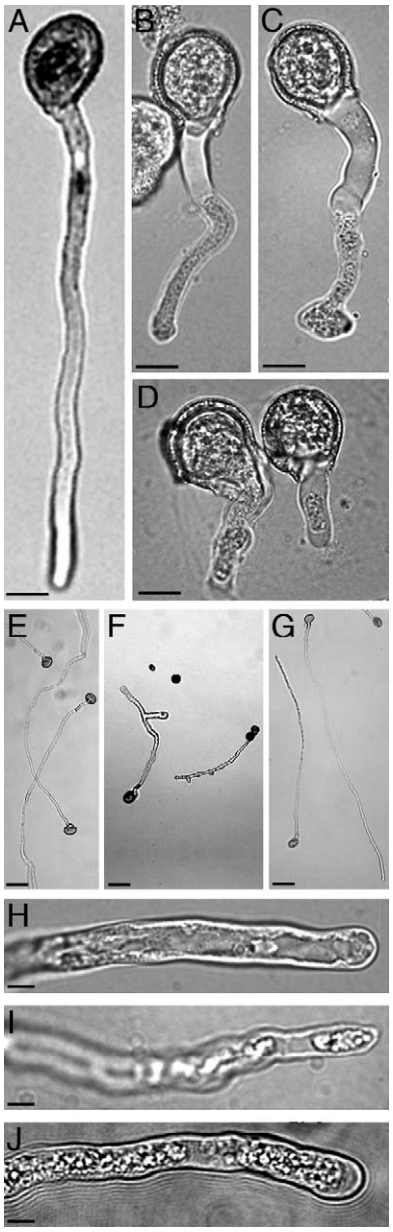

Fig. 5. Effects of AtPPME1 on morphology of in vitro germinated pollen tubes. (A) A wild-type pollen tube 6 h post-germination. (B–D) Unusually shaped and stunted *atppme1* pollen tubes 6 h post-germination. Scale bars = 10 μm. (E) Wild-type pollen tubes 24 h post germination. (F) Stunted *atppme1* pollen tubes 24 h post germination. (G) Pollen tubes from *atppme1* plants genetically complemented with the wild-type *AtPPME1* gene 24 h post-germination. Scale bars = 50 μm. (H) A wild-type pollen tube tip 24 h post-germination. (I) Unusually shaped *atppme1* pollen tube tip 24 h post-germination. (J) A pollen tube tip from *atppme1* plants genetically complemented with the wild-type *AtPPME1* gene 24 h post-germination. Scale bars = 5 μm.

from the wild-type pollen tubes (compare Figs. 5G to E and Figs. 5J to H). The average growth rate of these transgenic pollen tubes (33.4 ± 5.7 μm/h, n = 150) was also similar to that of the pollen tubes from the wild-type plants. Furthermore, the total PME activity in the pollen grains of *atppme1*/*AtPPME1* plants was restored to the wild-type levels (Fig. 4C). Thus, the *atppme1* phenotype is most likely due to disruption of the native AtPPME1 function by the mutation in this gene.

Discussion

PMEs must play a central role in growth of pollen tube wall which is composed mainly of pectin (Ferguson et al., 1998; Li et al., 1994). Indeed, PME-related genes are expressed in pollen of many diverse plant species, e.g., *Brassica* (Albani et al., 1991), alfalfa (Qiu and Erickson, 1995), maize (Wakeley et al., 1998), tobacco (Bosch et al., 2005; Lacoux et al., 2003; Rogers et al., 2001), *Salix* (Futamura et al., 2000), *Medicago* (Rodríguez-Llorente et al., 2004), and *Arabidopsis* (Jiang et al., 2005; Pina et al., 2005). Exploration of the specific effects of PMEs on the pollen tube growth, however, is just the beginning, with one study showing the requirement for the endogenous VGD1 PME for growth of *Arabidopsis* pollen tubes (Jiang et al., 2005) and another reporting inhibition of growth of pollen tubes of *Lilium formosanum* by exogenously added tobacco PME, NtPPME1 (Bosch et al., 2005).

One possible complication in interpretation of the PME function in pollen is that both PMEs with a demonstrated role in the pollen tube growth, VGD1 and NtPPME1, contain, in addition to their catalytic PME domain, a PMEI domain. This domain, which is similar to the kiwi (Camardella et al., 2000; Giovane et al., 1995) and *Arabidopsis* PME inhibitors (Raiola et al., 2004; Wolf et al., 2003), PMEIs, most likely impairs the enzymatic activity of the PME domain by intramolecular

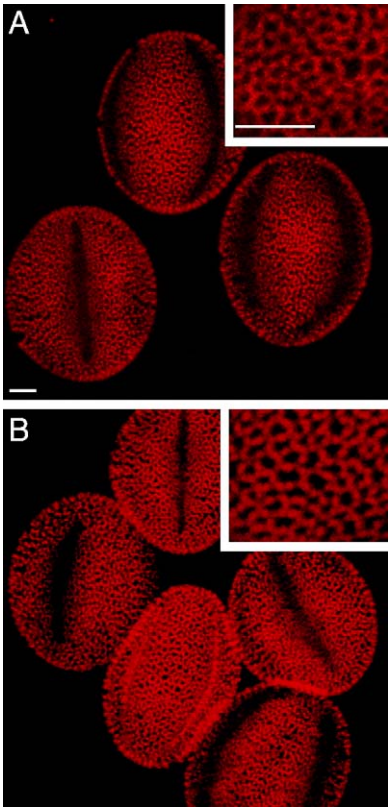

Fig. 6. Similar morphology of wild-type and *atppme1* pollen grains. (A) Wild-type pollen grains. (B) Pollen grains from *atppme1* plants. Insets show higher magnification of the pollen grain cell wall. Cell walls were stained with propidium iodide, the signal of which is in red. All images are projections of several confocal sections. Scale bars = 5 μm.

inhibition (Bosch et al., 2005). Thus, the apparent activity of PMEI-containing PMEs and their biological effects most likely represents a result of complex interplay of both the enzyme and its inhibitor. To circumvent this difficulty and to obtain a direct insight into the role of PME in pollen tube growth, it would be useful to study a PME gene that encodes only the PME domain, but not the PMEI domain. Our analysis of the *Arabidopsis* genome sequence identified 23 such PMEs among the 66 members of the PME-like protein family. We selected one of these genes, designated it *AtPPME1*, and demonstrated that its expression is pollen-specific.

The specificity of AtPPME1 expression was determined using the FTFLP approach which offers two significant advantages for studies of gene expression: first, it yields information about the tissue specificity of gene expression by the use of native promoters, and second, it produces internally tagged full-length proteins that are likely to exhibit native intracellular localization (Tian et al., 2004). Indeed, our results demonstrated tight restriction of the *AtPPME1* gene expression to pollen grains and pollen tubes and showed that the AtPPME1 protein accumulates in the cell periphery and intracellular structures most likely representing the endomembrane system known to be involved in secretion of PMEs (e.g., Bosch et al., 2005; Gaffe et al., 1997). Thus, AtPPME1 represented a new pollen-specific PME which has not been discovered by the previous study of the *Arabidopsis* pollen transcriptome (Pina et al., 2005).

The biological function of AtPPME1 was then studied by reverse genetics using a T-DNA insertional mutant of the *atppme1* gene. The *atppme1* mutation is a novel mutation which compromises the shape and growth rate of *Arabidopsis* pollen tubes, but, unlike the *vgd1* mutation (Jiang et al., 2005), does not affect the pollen tube stability, silique morphology, and plant fertility. Thus, AtPPME1 may have a more specific, narrowly defined function in pollen tube growth than VGD1, indicating functional differences between different pollen-specific PMEs.

The slow growth and irregular shape of the *atppme1* pollen tubes are most likely due to a reduced rigidity of their cell walls. This, in turn, may result from the reduction of the loss of the AtPPME1 activity in the mutant. Generally, PMEs demethylesterify pectin chains either randomly or processively. The mode of the PME action is thought to depend on the isoelectric point (p*I*) of the enzyme, with acidic PMEs acting randomly and basic PMEs functioning processively or linearly (Micheli, 2001). Because AtPPME1 is a basic molecule with a predicted p*I* of 8.7 (Table 1), it is most likely a processive enzyme, demethylesterifying stretches of homogalacturonan molecules in the pollen tube walls and creating regions of free carboxyl groups. These can interact with calcium cations, altering their local concentration around the growing pollen tubes. Because normal pollen tube growth is associated with a specific concentration of Ca^{2+} (Franklin-Tong, 1999; Malho et al., 2000; Messerli et al., 2000; Trewavas and Malho, 1998), alterations of this concentration due to the lack of the AtPPME1 enzymatic activity in the *atppme1* mutant may compromise the structure and elongation rates of the mutant pollen tubes. Thus, AtPPME1 likely plays an important and specific role in normal growth and morphological development of pollen tubes in *Arabidopsis*. The biological significance of this role is underscored by the observations that a relatively small effect of the lack of AtPPME1 on the total PME activity of the pollen causes profound functional consequences, bringing about dramatic changes in the pollen tube growth and development.

Acknowledgments

Our laboratory is supported by grants from the National Institutes of Health, the National Science Foundation, the National Research Initiative of the U.S. Department of Agriculture, the U.S.–Israel Binational Agricultural Research and Development Fund (BARD), and the U.S.–Israel Binational Science Foundation to V.C., and by Vaadia-BARD Postdoctoral Fellowship Award to A.Z.

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