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Pollen-specific pectin methylesterase involved in pollen tube growth

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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

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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