Separation of Arabidopsis Pollen Tetrads Is Regulated by *QUARTET1*, a Pectin Methylesterase Gene[1][W]

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Arabidopsis (*Arabidopsis thaliana*) *QUARTET* (*QRT*) genes are required for pollen separation during normal floral development. In *qrt* mutants, the four products of microsporogenesis remain fused and pollen grains are released as tetrads. In Arabidopsis, tetrad analysis in *qrt* mutants has been used to map all five centromeres, easily distinguish sporophytic from gametophytic mutations, and accurately assess crossover interference. Using a combination of forward and reverse genetics, we have identified the gene responsible for the *qrt1* phenotype. Annotation predicts that *QRT1* encodes a pectin methylesterase (PME), and enzymatic assays of QRT1 expressed in *Escherichia coli* indicate that QRT1 has PME activity. Promoter and transcription analysis demonstrate *QRT1* is expressed in anther tissues shortly after meiosis is complete. Unexpectedly, the *QRT1* promoter is also active in a variety of developmentally unrelated tissues, including developing guard cells, the hypocotyl-root transition zone, areas of lateral root emergence, and floral nectaries. PMEs constitute a large gene family in Arabidopsis, are involved in cell wall loosening, and have been implicated in various aspects of floral development and pollen tube elongation. The identification of QRT1 as a PME contributes to our understanding of pollen development and may help to provide valuable genetic tools in other plant species.

The Arabidopsis (*Arabidopsis thaliana*) *quartet* (*qrt*) mutants, particularly *qrt1*, have been a valuable resource to the plant research community for over a decade. *qrt* mutants are particularly interesting because they can be used to provide a powerful genetic tool—tetrad analysis—in plants, as well as insight into pollen development, cell-cell adhesion mechanisms, and plant cell wall biosynthesis and degradation (Rhee and Somerville, 1998; Copenhaver et al., 2000; Schnurr et al., 2006). Whereas *qrt1* mutant plants have been used extensively as a tool in Arabidopsis research (Johnson-Brousseau and McCormick, 2004), the precise identity and function of *QRT1* have not been reported. Identification of *QRT1* will enhance our understanding of pollen development and may also enable the recapitulation of the QRT phenotype, and thus tetrad analysis, in other plant species.

Pollen development in Arabidopsis and other plant species has been well characterized and involves the generation and degradation of numerous specialized cell wall layers (Fig. 1; Bedinger, 1992; Owen and Makaroff, 1995; Brett and Waldron, 1996; Ma, 2005). Pollen development begins with division of an initial cell that gives rise to a pollen mother cell (PMC). The PMC is surrounded by a primary cell wall composed mainly of cellulose, hemicellulose, and pectin (Carpita and Gibeaut, 1993; Reiter, 1994; Brett and Waldron, 1996). Between the plasma membrane and the primary cell wall, the PMC also develops a specialized secondary cell wall composed almost entirely of callose (Dong et al., 2005; Nishikawa et al., 2005). Meiotic division of the PMC results in four microspores. These microspores develop within the surrounding callose layer and are then encased in an additional wall layer called the primexine. As the microspores mature, the primary cell wall and the secondary callose layer, which surround the microspores, degrade, releasing individual microspores into the locules of the anther. Within the locules, the microspore primexine matures to form the exine, and the outermost pollen wall, which is primarily composed of sporopollenin, is secreted by the tapetum and encapsulates the developing pollen grains. Finally, an inner cellulose wall, called the intine, develops between the exine and the plasma membrane to complete pollen wall development (Owen and Makaroff, 1995; Boavida et al., 2005).

Arabidopsis *QRT1*, *QRT2*, and *QRT3* genes are required for normal pollen development (Preuss et al., 1994; Rhee et al., 2003). In wild-type Arabidopsis, the proper degradation of the PMC cell walls and subsequent separation of microspores results in the release of individual microspores as single pollen grains. Loss of function of any one of the three *QRT* genes disrupts separation of pollen grains following meiosis and results in the release of meiotically related mature pollen tetrads. The *qrt1* mutant properly deposits (Preuss et al., 1994) and degrades (Rhee and Somerville, 1998) the secondary PMC cell wall composed of callose; however, the primary PMC cell wall remains partially intact following meiosis (Rhee and Somerville, 1998). The

[1] This work was supported by start-up funds from the University of North Carolina Biology Department, the Carolina Center for Genome Sciences, and the National Science Foundation (grant no. 0517464).

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[W] The online version of this article contains Web-only data.
www.plantphysiol.org/cgi/doi/10.1104/pp.106.085274

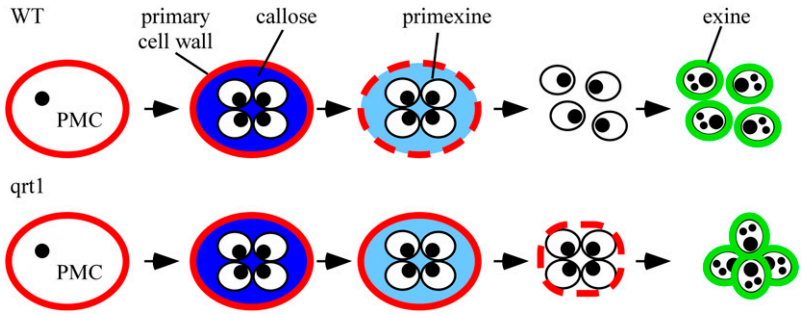

Figure 1. Arabidopsis pollen development. In wild-type pollen development, the PMC divides meiotically to form four microspores surrounded by a specialized callose secondary cell wall. The PMC cell walls degrade, releasing immature microspores into the locule. Exine is then deposited by the tapetum. In *qrt1* pollen development, the PMC cell walls fail to completely degrade. Subsequently, *qrt1* microspores remain grouped together as exine is deposited, resulting in fully viable pollen tetrads.

phenotype of *qrt1* is sporophytic and recessive. Pollen in *qrt2* mutants develops similarly, but retains patchy callose around the microspores (Preuss et al., 1994).

Most plant primary cell walls, including those of PMCs, are composed mainly of cellulose, hemicellulose, and pectins (Reiter, 1994; Brett and Waldron, 1996). The PMC primary cell wall pectins are particularly interesting in the context of *qrt* pollen tetrads because they have been proposed to play a major role in cell-cell adhesion mechanisms (Rhee and Somerville, 1998). Primary cell wall pectins consist mainly of homogalacturan (a polymer of β-1,4-galacturonic acid [GalUA]) and rhamnogalacturonan I and rhamnogalacturonan II (branching polymers of GalUA, Ara, and Rha; Brett and Waldron, 1996; Schols and Voragen, 2002; Tucker and Seymour, 2002). As pectin is synthesized, the GalUA backbone is in a methylesterified state (Schols and Voragen, 2002). Pectin can then be demethylesterified by pectin methylesterases (PMEs) and subsequently cleaved by endo-polygalacturonases (PGs), a process that can result in cell wall loosening (Micheli, 2001). Immunolocalization using an antibody against unesterified pectin shows that, in *qrt1* mutants, the PMC walls that remain around the microspores do not stain for unesterified pectins (Rhee and Somerville, 1998). This result suggests that *QRT1* is involved in pectin demethylesterification. Interestingly, *QRT3* has recently been identified as a PG (Rhee et al., 2003), further supporting a role for pectin degradation in microspore separation. Specifically, Rhee et al. (2003) suggest that QRT3 degrades the pectic polysaccharides of the PMC. Failure to degrade these components in *qrt3* mutants presumably mechanically constrains the developing pollen grains, leading to fusion of their developing walls.

Using a combination of forward and reverse genetics, we have identified *QRT1* as encoding a PME that likely functions upstream of *QRT3* in PMC primary wall degradation. Additionally, we confirm that QRT1 has PME activity by using an enzyme assay with heterologously expressed protein.

RESULTS

Identification of *QRT1*

Previous mapping experiments mapped the *qrt1* locus to chromosome 5 between the markers DFR and LFY3 (Preuss et al., 1994) in a region containing approximately 2,000 predicted genes. We narrowed the list of candidate genes by focusing on those that were annotated as having putative cell wall functions (The Arabidopsis Information Resource; http://www.arabidopsis.org) and are expressed in anther tissues (Wellmer et al., 2004). Five *QRT1* candidate genes were identified and appropriate T-DNA insertion lines were obtained from the SALK (Alonso et al., 2003) and Wisconsin (Krysan et al., 2002) collections. Progeny from those lines were visually screened using a light microscope for tetrad pollen. Lines containing T-DNA inserts in or near At5g55590 (SALK_024104, SALK_051214, and SALK_043429) segregated the pollen tetrad phenotype in ratios consistent with a single Mendelian locus and were provisionally designated *qrt1-4*, *qrt1-5*, and *qrt1-6*, respectively. Other lines containing inserts further upstream or downstream of At5g55590 (SALK_017414 and WiscDsLox453-456-J19) and lines containing inserts in other *QRT1* candidate genes produced only monad pollen.

We performed genetic complementation tests to verify that At5g55590 was *QRT1*. Tetrad-producing SALK_024104 plants (hereafter referred to as *qrt1-4*) were crossed to *qrt1-2*. Only tetrad-producing plants (*qrt1-2*/*qrt1-4*) were observed in the F_1 progeny (Fig. 2). The observed failure to complement demonstrates that *qrt1-4* is allelic to *qrt1-2* and that At5g55590 is QRT1. Progeny from control crosses between *qrt1-4* and *qrt3-3* (Rhee et al., 2003) demonstrated complementation (*qrt1-4*/+; *qrt3-3*/+) and produced only monad pollen (Fig. 2).

We characterized the nature of the original *qrt1-2* ethyl methanesulfonate allele by cloning and sequencing the mutant gene (Preuss et al., 1994). A 4.2-kb genomic fragment corresponding to the At5g55590 open reading frame (ORF) with 2 kb upstream of the translational start codon and 400 bp downstream of the stop codon was cloned from the *qrt1-2* background. Nucleotide sequencing of this region revealed two point mutations not present in the wild-type allele. The first mutation (A to G) is located 10 bp upstream of the translational start codon. The second mutation is a deletion of a thymidine at position 18, causing a frame shift.

To confirm the identity of *QRT1*, we also performed transcomplementation tests by transforming *qrt1-2* lines with a copy of the wild-type locus. A 4.2-kb

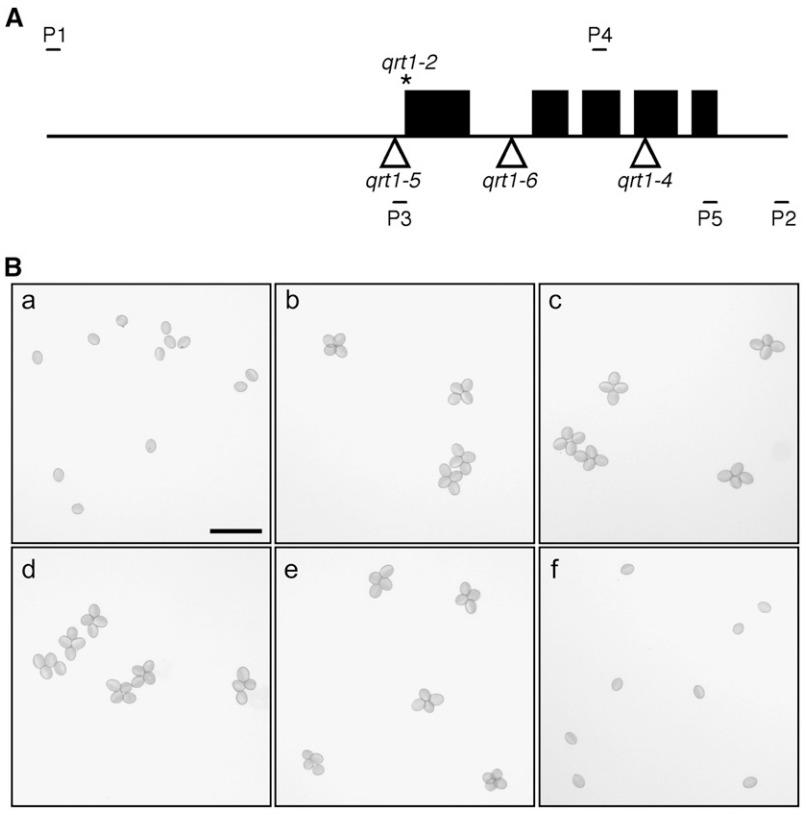

Figure 2. A, Identification of *QRT1*. A map of At5g55590 (*QRT1*) and the surrounding regions is presented. Black boxes represent exons drawn to scale. SIGnAL T-DNA insertion lines with tetrad pollen phenotypes are represented as white triangles and the location of the point mutations in *qrt1-2* is represented as an asterisk. B, Approximate locations of primers P1 to P5 used in this study are also indicated. T-DNA insertions into At5g55590 fail to complement *qrt1-2*. Pollen from wild-type Columbia-0 plants (a), *qrt1-2* homozygotes (b), *qrt3-3* homozygotes (c), *qrt1-4* (SALK_024104) homozygotes (d), *qrt1-4* crossed to *qrt1-2* (*qrt1-4*/*qrt1-2*; e), and *qrt1-4* crossed to *qrt3-3* (*qrt1-4*/+, *qrt3-3*/+; f).

fragment containing the full predicted At5g55590 ORF with 2 kb upstream of the translational start codon and 400 bp downstream of the stop codon was cloned from wild-type plants and introduced into the *qrt1-2* background via Agrobacterium-mediated transformation. The presence of both mutant *qrt1-2* and wild-type alleles in positive transformants was confirmed using cleaved-amplified polymorphic sequence (CAPS; Konieczny and Ausubel, 1993) and derived CAPS (dCAPS; Michaels and Amasino, 1998; Neff et al., 1998) markers specific for the two *qrt1-2* mutations (Fig. 3, A and B). Nearly all T1 transformants analyzed produced only monad pollen (44 monad producing, two tetrad producing; Fig. 3, C–F), demonstrating complementation of *qrt1-2* with At5g55590.

Expression of *QRT1*

A survey of public databases (SIGnAL, The Arabidopsis Information Resource, and National Center for Biotechnology Information) failed to identify existing cDNA clones corresponding to *QRT1*. To determine whether transcription of *QRT1* occurs in Arabidopsis, reverse transcription (RT)-PCR was performed on unopened flowers (stage 12 or younger) from wild-type, *qrt1-2*, and *qrt1-4* plants (Fig. 4) using primers spanning predicted exons 3 to 5 (Smyth et al., 1990). Transcription of *QRT1* was detected in wild-type and *qrt1-2* flowers, but no transcript was detected in the T-DNA insertion mutant *qrt1-4* (SALK_024104). RT-PCR was also used to amplify the full-length *QRT1* transcript. The full-length transcript was sequenced and the five predicted exon splice junctions were verified (GenBank DQ979876).

To better understand the expression pattern of *QRT1*, total RNA was isolated from wild-type tissues and analyzed for *QRT1* expression by RT-PCR. The *QRT1* transcript was detected in unopened, but not opened, flowers, an expression pattern consistent with a role in pollen development (Fig. 4). The transcript was also detected in extracts from whole 5- and 9-d-old seedlings and 14-d-old roots, but not in mature rosette leaves (Fig. 4). Whereas these results are not intended to be quantitative, it should be noted that the transcript was very difficult to detect in any tissue using multiple primer combinations (data not shown). This may suggest that *QRT1* is a low-abundance transcript, which may explain why a full-length *QRT1* cDNA has not been previously identified.

To visualize the expression pattern of *QRT1*, wild-type plants were transformed with β-glucuronidase (GUS)-green fluorescent protein (GFP) fused to the *QRT1* promoter (QRT1pro::GUS-GFP). The same promoter was used to drive expression of *QRT1* in the transcomplementation of *qrt1-2* described above. Twenty independently transformed plants were analyzed for GUS expression and the patterns described below were consistent in each line (Figs. 5 and 6). GUS expression was detected in unopened flowers (Fig. 5B). Specifically, expression was observed in stage 9, 10, and

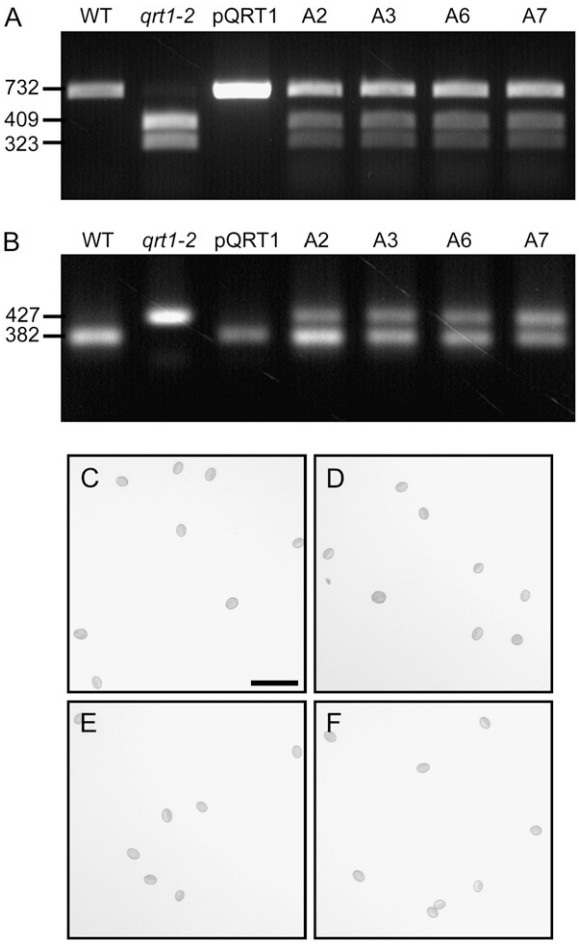

Figure 3. Transcomplementation of *qrt1*. CAPS and dCAPS markers resulting in unique banding patterns for wild-type (WT) and mutant *qrt1-2* alleles were generated for both *qrt1-2* point mutations. The CAPS marker in A yields a single 732-bp product for *QRT1* and 409- and 323-bp products for *qrt1-2*. The dCAPS marker in B yields 382- and 45-bp (data not shown) products for *QRT1* and a single 427-bp product for *qrt1-2*. Only wild-type *QRT1* alleles are present in plasmid pKQRT1, and both wild-type and *qrt1-2* alleles are present in lines A2, A3, A6, and A7 generated by transforming *qrt1-2* with pKQRT1. Pollen from lines A2, A3, A6, and A7 is presented in C, D, E, and F, respectively.

11 flowers, corresponding to the developmental stages immediately following meiosis and associated with tetrad separation (Smyth et al., 1990; Bowman, 1994; Fig. 5C). Closer analysis revealed that floral expression was limited to anther tissues (Fig. 5, D and E). Following microspore separation, GUS expression was significantly reduced and appeared to be primarily limited to the surrounding tapetal cells (Fig. 5, F and G).

Analysis of QRT1pro::GUS-GFP expression in non-anther tissues revealed a variety of precise and developmentally regulated expression patterns. The earliest expression appeared in two distinct tissues by 3 d after germination. Expression was observed in a cluster of cells at the root-hypocotyl junction (Fig. 6A). Also, at this stage, expression was observed in developing guard cells on the expanding cotyledons (Fig. 6, A–C). Epidermal peels indicated that the QRT1 promoter was not active in meristemoid or guard mother cells (GMCs; data not shown). The QRT1 promoter becomes active shortly after the GMCs divide to produce two guard cells, but before stomatal pore opening. In cotyledons, GUS staining in cotyledon guard cells is most visible from 3 to 5 d after germination, and then expression rapidly declines by 7 d (Fig. 6C) and is not detectable by 10 d. A similar pattern of expression is seen in guard cells of developing leaves (data not shown).

Expression of the QRT1 promoter is also detected in the root. Roots older than 7 d often demonstrate patchy expression in epidermal and cortex cells; however, QRT1 promoter expression in younger roots appears to be associated with lateral root emergence. The earliest root expression is observed in 4-d-old roots in single or small clusters of endodermal cells (Fig. 6D). These zones of endodermal expression are nearly always adjacent to or surrounding regions of pericycle cell division (Fig. 6D, arrows) consistent with lateral root initiation. As lateral roots develop and push through the outer root layers, QRT1 promoter expression is often observed as a ring surrounding the lateral root (Fig. 6E). After lateral root emergence, expression is very strong at the junction with the primary root (Fig. 6F). Expression was never observed in the root cap or in the region between the root cap and the nearest lateral root.

QRT1 promoter expression in flowers younger than stage 12 is clearly restricted to developing anther tissues; however, analysis of older flowers reveals expression in nectaries (Fig. 6, G and H) and at the stigmatic surface (Fig. 6I). Nectary expression is only

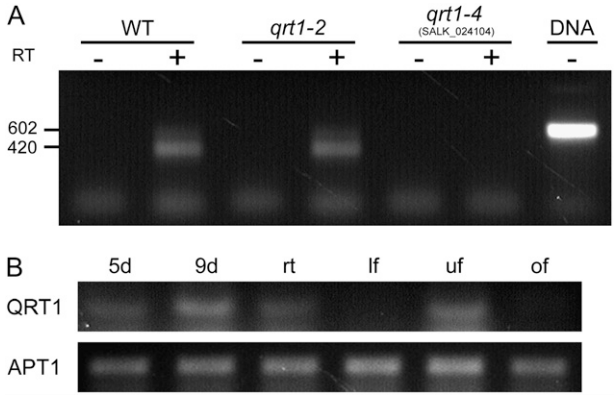

Figure 4. Transcription of *QRT1*. Transcription of *QRT1* was verified by RT-PCR with primers P4 and P5 (see Fig. 2). A, *QRT1* transcript was detected in wild-type and *qrt1-2* lines; however, no transcript was detected in the T-DNA knockout mutant *qrt1-4* or in control reactions lacking reverse transcriptase (RT). B, The unspliced 602-nucleotide DNA product was easily distinguished from the spliced 420-nucleotide mRNA product. *QRT1* and control *APT1* (Moffatt et al., 1994) transcription was evaluated in different tissues. *QRT1* transcript was detected in 5-d seedlings (5 d), 9-d seedlings (9 d), roots (rt), and unopened flower buds (uf), but not in rosette leaves (lf) or opened flowers (of). Transcription of *APT1* was observed in all samples.

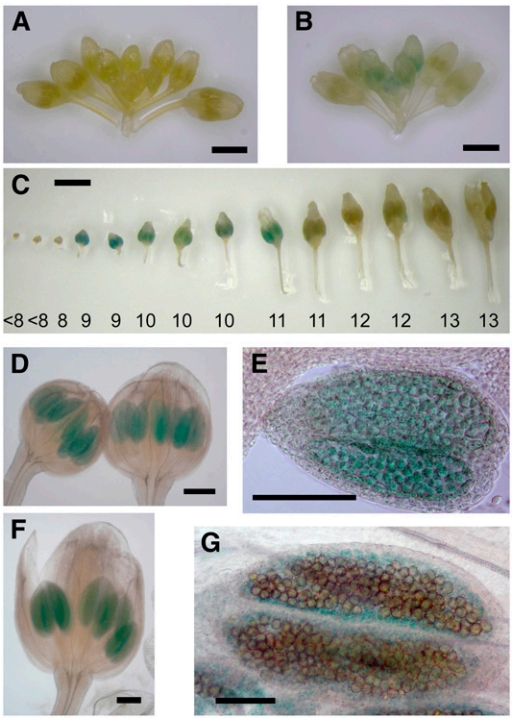

Figure 5. *QRT1* promoter activity in flowers. The presence of GUS driven by the *QRT1* promoter was detected by X-gluc staining in floral tissues from plants transformed with pKQRT1proFS7. GUS activity was observed in developing flowers (B), but only in floral development stages 9 through 11 (C). In stage 9 flowers, GUS activity was observed in the developing pollen and the surrounding tissues (D and E); however, by stage 11, staining was only observed in the tissues surrounding the pollen (F and G). No GUS activity was detected in wild-type flowers (A). Bars = 1 mm (A–C); 100 μm (D–G).

observed in mature flowers before floral organ dehiscence (stages 16 and 17) and is observed in both medial and lateral outgrowths. QRT1 promoter expression is observed in nectary guard cells, but expression is not limited to those cells (Fig. 6H). Following pollination, diffuse staining is often visible at the stigmatic surface (Fig. 6I). Staining is never observed prior to pollination and was seen after self pollination and pollination using wild-type pollen. Analysis of GFP expression in the same plants revealed floral and vegetative expression patterns consistent with those of GUS; however, due to autofluorescence, GFP expression was difficult to extensively characterize.

The Phenotype of *qrt1* Plants

Previous characterizations of *qrt1* plants have described them as phenotypically wild type, except for microspore development (Preuss et al., 1994). Because our QRT1 promoter experiments revealed expression patterns in multiple tissues, *qrt1* plants were reevaluated for phenotypic differences from wild-type plants. Analysis of stomatal patterning, density, and size found no differences between *qrt1* and wild-type plants. Similarly, no differences from wild type were observed in root, nectary, or stigma development or architecture under standard growth conditions.

PME Activity in *qrt1* Mutants

QRT1 contains a conserved domain (pfam01095) associated with PME activity. PMEs have been previously implicated in cell wall modifications during pollen development (Albani et al., 1991; Mu et al., 1994; Wakeley et al., 1998; Amagai et al., 2003; Lacoux et al., 2003) and pollen tube growth (Bosch et al., 2005; Jiang et al., 2005). To determine whether *qrt1* mutants were deficient in PME activity, PME assays were performed on 10-d-old seedlings and stage 9 to 11 flowers. Repeated analyses using a variety of assays (Downie et al., 1998; Grsic-Rausch and Rausch, 2004; Jiang et al., 2005) were unable to demonstrate a significant reduction in total PME activity in *qrt1* tissues (data not shown). This inability to detect a reduction in PME activity may be due to other members of the Arabidopsis PME gene family, creating high levels of background activity.

PME Activity in Heterologously Expressed QRT1

To establish that QRT1 functions as a PME, the *QRT1* cDNA was cloned and expressed in an *Escherichia coli*

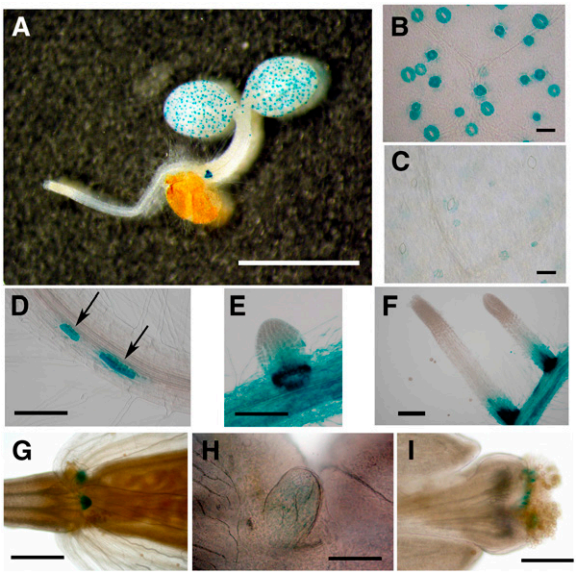

Figure 6. *QRT1* promoter activity in nonanther tissues. The presence of GUS driven by the *QRT1* promoter was detected by X-gluc staining in plants transformed with pKQRT1proFS7. GUS activity was observed in newly developed guard cells (A–C), in the root-hypocotyl transition zone (A), in roots, especially at sites of lateral root emergence (D–F), in nectaries (G and H), and at the stigmatic surface after pollination (I). A, 3-d in vitro seedling. B, 3-d in vitro cotyledon. C, 7-d in vitro cotyledon. D, 4-d in vitro primary root. E and F, 7-d in vitro primary and lateral roots. G, Base of stage 16 flower. H, Higher magnification of nectary from G. I, Stage 15 stigma. Bars = 1 mm (A); 200 μm (G); 100 μm (B–F, and I); 50 μm (H).

T7 RNA polymerase expression system under the control of the *lac* promoter. Crude extracts from QRT1-expressing bacterial cultures showed significant increases in PME activity following induction by isopropylthio-β-galactoside (IPTG; Fig. 7). Total protein was extracted from cultures and added to a solution of commercially obtained methylesterified pectin. Under assay conditions, PME removes methylester groups from methylesterified pectin. Ruthenium red, which binds demethylesterified pectin (Downie et al., 1998), was added to the reaction and pectin was then precipitated, along with the bound ruthenium red, with calcium chloride. The degree of demethylesterification could then be determined by measuring the supernatant for A_{534} (Jiang et al., 2005). Levels of activity in 1 mg of total protein from QRT1-expressing cultures were similar to those observed in positive enzymatic (0.05 units PME) and chemical (50 mM NaOH) control samples, indicating that QRT1 has PME activity.

DISCUSSION

PMEs play a vital role in cell separation. Primary cell wall pectins are composed of the polysaccharides homogalacturan, rhamnogalacturonan I, and rhamnogalacturonan II. GalUA acid is the primary component of all three of these forms of pectin. As pectins are synthesized, it is believed that GalUA residues are added to the polymers in a methylesterified state, resulting in fully methylesterified pectins (Schols and Voragen, 2002). Later in development, as cell walls need to expand, as in guard cell expansion (Nadeau and Sack, 2002), or degrade, as in fruit ripening (Giovannoni, 2001), pectin chains may need to be cleaved. Pectin chain cleavage is initiated by random demethylesterification by PMEs. The resulting demethylesterified pectin is then cleaved by PGs (Micheli, 2001; Tucker and Seymour, 2002). PMEs may also function in a different pathway that can lead to cell wall stiffening (discussed below).

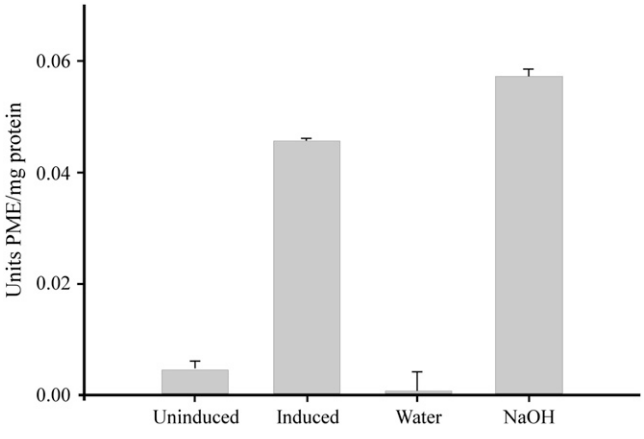

Figure 7. PME activity. PME activity was assayed in extracts from *E. coli* containing pDEST14-QRT1. Cultures were sampled prior to induction with IPTG (uninduced) and 4 h after induction (induced). PME activity in control samples containing either water or NaOH (50 mM final concentration) is also reported. Error bars = SDs.

PMEs constitute a large Arabidopsis gene family characterized by a conserved PME domain (pfam 01095). Plant PME genes have been described as being either type I or type II (Micheli, 2001). Type I PME genes possess a proregion containing a highly conserved PME inhibitor (PMEI) domain (pfam 04043) and typically have two or three exons. Type II PME genes lack a PMEI-containing proregion, typically have five exons, and are often described as being more similar to PME genes of phytopathogenic bacteria and fungi (Micheli, 2001). Despite the obvious role of PMEs in various stages of plant development, relatively few PME genes from Arabidopsis have been structurally or functionally characterized (Richard et al., 1994, 1996; Micheli et al., 1998; Jiang et al., 2005; Tian et al., 2006). Nearly all of the previously described Arabidopsis PME genes are type I PME genes. A phylogenetic analysis of the 66 members of the Arabidopsis PME gene family clearly distinguishes type I from type II PME genes (Supplemental Fig. S1). QRT1 falls within the type II clade of the phylogenetic tree.

PME expression patterns are highly regulated. All previously characterized PMEs from Arabidopsis and other plant species appear to have highly regulated patterns of gene expression at the level of transcription (Bosch and Hepler, 2005). An additional level of PME regulation exists in the complex processing of PMEs. The majority of plant PMEs are expressed as pre-pro-PMEs (Micheli, 2001). The preregion is involved in the extracellular transport of PMEs (Dorokhov et al., 2006). The proregion has homology to PMEIs (discussed below), and the propeptide may play an autoinhibitory role (Giovane et al., 2004). The processing of pre-pro-PME into pro-PME and eventually into functionally mature PME has been suggested to be potential levels of regulation (Micheli, 2001; Bosch and Hepler, 2005). A further level of regulation for some PMEs may be provided by individually expressed PMEIs. PMEIs are capable of binding to the active site of PMEs (Hothorn et al., 2004; Di Matteo et al., 2005). Two Arabidopsis PMEIs (*AtPMEI1* and *AtPMEI2*) have been identified, and their expression patterns and abilities to inhibit PME activity in vitro suggest that they may have roles in specifically inhibiting PME activity in a tissue-specific manner (Wolf et al., 2003; Raiola et al., 2004).

Previous analyses of pollen development in *qrt* mutants have suggested that *QRT1* may play a role in pectin degradation within the PMC primary cell wall (Rhee and Somerville, 1998). The recent identification of *QRT3* as a PG provided further evidence that proper pectin degradation is essential for microspore separation (Rhee et al., 2003). Cleavage of pectin by PGs is stimulated by demethylesterification by PMEs (Tucker and Seymour, 2002). It is therefore likely that the PME QRT1 and the PG QRT3 function in the same

pathway, leading to the loosening and degradation of the PMC primary cell wall (Fig. 8).

The expression pattern of *QRT1* as indicated by RT-PCR and analysis of *QRT1* pro::GUS fusions suggests a complex, yet precise pattern of *QRT1* transcription. The *QRT1* promoter is active in the anthers of developing flowers at stage 9, stage 10, and early stage 11 (Fig. 5). This is consistent with the model that QRT1 is involved in the degradation of the PMC primary cell wall. In Arabidopsis, male meiosis is complete by the end of stage 9, and tetrad separation in wild-type anthers is first detected at stage 10 (Bowman, 1994; Ma, 2005). By stage 11, the *QRT1* promoter appears to be expressed primarily in the tissues surrounding the maturing pollen grains and only weakly in the pollen itself (Fig. 5, F and G). This may suggest a role of QRT1 in tapetum degeneration.

Expression of *QRT1* in nonanther tissues was not anticipated; however, all tissues observed to have *QRT1* expression are associated with specialized cell wall loosening or expansion. For example, stomatal development requires both the separation of guard cells and the expansion of those guard cells (Nadeau and Sack, 2002). Guard cell expansion has been previously associated with PME activity (Jones et al., 2003, 2005); however, because *QRT1* expression was never detected in individual cotyledons or leaves older than 7 d (data not shown), it is unlikely that QRT1 plays a role in the opening and closing of mature stomata. The QRT1 promoter appears to be most active in guard cells shortly after (or perhaps concurrent with) division of the GMC and therefore may be involved with guard cell separation or initial guard cell expansion. *QRT1* expression was also detected in roots. It is well established that root cell elongation is an integral part of root development (Scheres et al., 2002). *QRT1* expression in roots was especially strong at sites of lateral root emergence. Lateral roots develop from the pericycle layer within the primary root and must push through the endodermis, cortex, and epidermis layers to emerge from the root (Bowman, 1994). It is therefore plausible that PMEs may be involved in loosening the outer layers of cells during lateral root emergence to reduce the stress or possibility of damage to the lateral root tip. Considering the expression pattern of the *QRT1* promoter, potential roles for PMEs are also likely at the stigmatic surface and in nectaries.

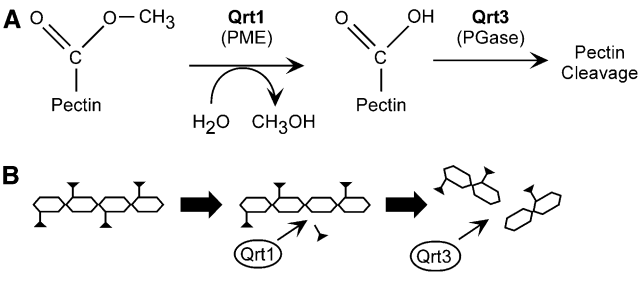

Figure 8. A model for pollen tetrad separation. A, Pectin methylester groups are liberated as methanol by the PME *QRT1*. This stimulates the PG *QRT3* to cleave the demethylesterified pectin polymers. B, *QRT1* and *QRT3* likely work together to cleave pectin in the PMC primary wall, contributing to its degradation.

With such a complex pattern of *QRT1* expression in various tissues, it is curious that the only phenotype associated with *qrt1* mutants is tetrad pollen. Position, size, and numbers of stomata and lateral roots were extensively examined, but no phenotypes distinguishing *qrt1* plants from wild-type plants were identified. Similarly, no differences were observed in floral organ development or dehiscence (other than tetrad pollen). If QRT1 is serving an important role in these tissues, a phenotype might be expected in *qrt1* mutants. It is, however, important to consider the size of the Arabidopsis PME family, and the various levels of PME regulation. At least 66 PMEs are predicted in the Arabidopsis genome (Bosch and Hepler, 2005), suggesting the possibility of genetic redundancy in the tissues where *QRT1* is expressed. A recent analysis of the Arabidopsis PME family indicates that at least 30 PMEs are transcribed in seedlings and at least 50 PMEs are transcribed in flowers (Bosch and Hepler, 2005).

Whereas redundancy may help explain why *qrt1* does not have a phenotype in most tissues, it raises the question of why any phenotype is seen. Querying the expression data from the 22k ATH1 chip at Genevestigator (www.genevestigator.ethz.ch) shows that 35 of 63 PME family members are expressed in either the stamen or pollen (Zimmermann et al., 2004). Only one PME (At3g24130) has its highest relative transcription in the stamen. *QRT1* is highly expressed in the stamen, but its relative expression is slightly higher in hypocotyls, roots, and lateral roots. These analyses must be interpreted with caution, however, because all of the stamen-specific experiments at Genevestigator use transcripts from stamens at stage 12 or older, whereas *QRT1* is expressed most strongly in stages 9 through 11. Thus, the lack of redundancy between *QRT1* and other PME family members could be due to temporal rather than spatial regulation. Alternatively, the QRT1 protein may have some characteristic that distinguishes it from other PMEs and enables it to interact more specifically with the pectin components of the developing pollen walls. Another possibility is that the tissue-specific regulation of PME family members may be achieved though the action of PMEIs. Further genetic and biochemical analysis of *QRT1* and the other members of the PME family in Arabidopsis will be necessary to distinguish between these possibilities.

PMEs catalyze the demethylesterification of pectin and the pattern of demethylesterification within a pectin chain can result in distinctly different physiological consequences (Micheli, 2001; Tucker and Seymour, 2002). When demethylesterification occurs randomly, protons are released that promote the activity of PGs, which, in turn, cleave pectin chains and result in cell wall loosening. Alternatively, demethylesterification of pectin residues can occur linearly. The resulting large blocks of negatively charged pectin residues are

thought to interact with calcium ions (Ca^{++}) to form a pectate gel, thus resulting in cell wall stiffening (Goldberg, 1996; Jiang et al., 2005). A number of factors have been shown to affect which mode of demethylesterification a particular PME undertakes, including pH, ion concentration, and the existing state of pectin methylesterification (Catoire et al., 1998; Denes et al., 2000; Tucker and Seymour, 2002); however, certain PMEs are thought to have a general propensity toward randomized or linear demethylation. In general, PMEs with acidic pH optima, such as most phytopathogenic fungal PMEs, are thought to randomly demethylesterify pectin and result in cell wall loosening. Whereas PMEs with alkaline pH optima, such as most plant PMEs (Bosch and Hepler, 2005), are thought to demethylesterify pectin in blocks and result in cell wall stiffening (Tucker and Seymour, 2002), QRT1 has a slightly acidic predicted pH optimum of 6.3. This is consistent with the apparent role of QRT1 in the degradation of the PMC primary wall. Other recently characterized pollen-expressed PMEs from Arabidopsis (Jiang et al., 2005; Tian et al., 2006) and tobacco (*Nicotiana tabacum*; Bosch et al., 2005) have alkaline pH optima and appear to be involved in maintaining the rigidity of elongating pollen tube walls. It is of interest that different PMEs expressed in developmentally related tissues can have substantially different functions.

Apparent orthologs of *QRT1* exist in a wide range of plant species, including rice, poplar (*Populus* spp.), and tobacco. In our lab, we have successfully amplified sequences with high similarity to *QRT1* from *Arabidopsis arenosa*, *Brassica oleracea*, *Capsella rubella*, and *Olimarabidopsis pumila* (data not shown). This strongly suggests that orthologs of *QRT1* exist in several plant species closely related to Arabidopsis. If similar biochemical pathways leading to microspore separation are present in other plant species, the ability to transfer tetrad analysis to these species may be achieved through RNA interference strategies targeting QRT1 orthologs.

MATERIALS AND METHODS

Lines and Vectors Used

Seeds for *qrt1-2* were kindly provided by Daphne Preuss at the University of Chicago. All T-DNA insertion lines evaluated in the pollen screen and *qrt3-3* (SALK_052045) were obtained from the Arabidopsis Biological Resource Center at Ohio State University. For the analysis of *QRT1* promoter activity, pKQRT1proFS7 was constructed by amplifying *QRT1*—2,000-bp upstream region with primers P1 (5′-CACCGATCGTCTTGGTTGAATGACTAAACCG-3′) and P3 (5′-GGGGAATGATGTCTCCTGCACAAGG-3′), inserting that PCR product into the Gateway entry vector pENTR (Invitrogen), and recombining the entry vector with the destination binary vector pKGWFS7 (Karimi et al., 2002). For *QRT1* complementation, pKQRT1 was constructed by amplifying QRT1 with primers P1 and P2 (5′-CGTGCCACATCTCTGGAAACGTTG-3′), inserting that PCR product into the Gateway entry vector pENTR (Invitrogen), and recombining the entry vector with the destination binary vector pKGW (Karimi et al., 2002). Vectors pKQRT1proFS7 and pKQRT1 were transformed into *Agrobacterium tumefaciens* strain GV3101:pMp90 (Koncz and Schell, 1986) via electroporation. Agrobacterium cultures were grown to mid-log phase, pelleted by centrifugation (10 min at 1,000 relative centrifugal force), resuspended in 5% Suc with 0.05% Silwet, and applied to flowering plants as a fine mist using a spray bottle. Transgenic T1 seedlings were identified on solidified Murashige and Skoog medium containing 50 mg/L kanamycin and 200 mg/L timentin, and transferred to potting mix for analysis.

Characterization of *qrt1-2*

To identify the nature of the mutations in At5g55590 present in the *qrt1-2* background, the entire ORF, including 2 kb upstream of the start codon and 200 bp downstream of the stop codon, was amplified by PCR and sequenced to at least 4 times coverage by the University of North Carolina-Chapel Hill Genome Analysis Facility. The two point mutations identified were then analyzed with dCAPS finder 2.0 (Neff et al., 2002). The upstream mutation resulted in the generation of an *Aat*II site. Therefore, *Aat*II digestion of the 732-bp P8 (5′-GATGATGGTAATGTTTATGTTTGAGG-3′)/P9 (5′-CAAACACGGTTTATGTCTTAAATC-3′) fragment resulted in 409- and 323-bp products only for the mutant *qrt1-2* allele. For the mutation in codon 6, amplification with primers P10 (5′-TTTCCTTGTGCAGGAGACATCATTCCCCATGAAAGTCGAAGAATT-3′) and P9 results in a 427-bp fragment containing an *Xmn*I site only for the wild-type *QRT1* allele. Therefore, *Xmn*I digestion of the P9/P10 fragment resulted in 382- and 45-bp products only for the wild-type *QRT1* allele.

QRT1 Expression

Lines containing pKQRT1proFS7 were analyzed for GUS expression by staining with 5-bromo-4-chloro-3-indolyl-β-glucuronic acid (X-gluc; Jefferson et al., 1987). For analysis of *QRT1* transcription, total plant RNA was isolated using Tri-Reagent (Molecular Research Center) and following the manufacturer's instructions. RT-PCR was achieved with the AccessQuick RT-PCR system (Promega). Characterization of *QRT1* transcription in various tissues was achieved with primers P4 (5′-GCTATATCCAAGGCAATGTAG-3′) and P5 (5′-CTCCATATATGAACTCTCTTCCC-3′). APT1 was used as a control with primers APT1-F (5′-TCCCAGAATCGCTAAGATTGCC-3′) and APT1-R (5′-CCTTTCCCTTAAGCTCTG-3′). Primers P6 (5′-CACCTTTCCTTGTGCAGGAGACATCATTC-3′) and P7 (5′-ATTTAGAGTCTCAGCCATTG-3′) were used to amplify the full cDNA ORF for sequencing. All sequencing was carried out by the University of North Carolina-Chapel Hill Genome Analysis Facility.

Heterologous Expression of *QRT1* and PME Assay

Full-length *QRT1* cDNA was generated using Thermoscript RNase H-reverse transcriptase (Invitrogen) and the reverse primer P7 and amplified with Accuzyme DNA polymerase (Bioline) using primers P6 and P7. The *QRT1* cDNA fragment was introduced into the Gateway entry vector pENTR (Invitrogen) and recombined into the *Escherichia coli* expression vector pDEST14 (Invitrogen). The resulting vector, pDEST14-QRT1, was transformed into the *E. coli* expression strain BL21(DE3). Expression was induced by adding IPTG to a final concentration of 1 mM. Cultures were grown for an additional 4 h. One milliliter of each culture was pelleted and resuspended in 0.1 mL PME extraction buffer (50 mM citric acid, 100 mM sodium phosphate dibasic, 500 mM sodium chloride, and one tablet Complete Mini protease inhibitor [Roche]/100 mL). Each sample was subjected to six freeze-thaw cycles and centrifuged for 5 min at 12,000*g* at 4°C, and the supernatant was collected. Protein concentration was determined using Bio-Rad protein assay. PME activity was assayed essentially as described by Jiang et al. (2005). For each sample, 500 ng of protein extract were added to 0.8-mL pectin solution (0.1% esterified pectin [Sigma P9561] in 50 mM sodium phosphate buffer, pH 7.0). Samples were incubated at 37°C for 18 h. To each sample, 0.2 mL 0.05% ruthenium red (Sigma R2751) was added. Samples were briefly vortexed and incubated at room temperature for 15 min. Pectin was precipitated by adding 0.2 mL of 0.6 M calcium chloride and mixed by inversion. Samples were centrifuged for 10 min at 12,000*g* and the supernatants were collected and measured for A_{534}. Control samples containing known concentrations of purified PME (Sigma P5400) were similarly assayed to establish a standard curve. All samples were replicated four times.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number DQ979876.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Phylogenetic analysis of Arabidopsis PME family.

ACKNOWLEDGMENTS

We would like to thank Daphne Preuss for providing *quartet* lines and helpful discussions. We would also like to thank Stephanie Hartmann for assistance with the phylogenetic analysis of the Arabidopsis PME family and three anonymous reviewers for constructive comments.

Received June 16, 2006; accepted August 31, 2006; published September 15, 2006.

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