

# ARABIDOPSIS DEHISCENCE ZONE POLYGALACTURONASE1 (ADPG1), ADPG2, and QUARTET2 Are Polygalacturonases Required for Cell Separation during Reproductive Development in *Arabidopsis*<sup>W</sup>

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Cell separation is thought to involve degradation of pectin by several hydrolytic enzymes, particularly polygalacturonase (PG). Here, we characterize an activation tagging line with reduced growth and male sterility caused by increased expression of a PG encoded by *QUARTET2* (*QRT2*). *QRT2* is essential for pollen grain separation and is part of a small family of three closely related endo-PGs in the *Arabidopsis thaliana* proteome, including ARABIDOPSIS DEHISCENCE ZONE POLYGALACTURONASE1 (ADPG1) and ADPG2. Functional assays and complementation experiments confirm that ADPG1, ADPG2, and *QRT2* are PGs. Genetic analysis demonstrates that ADPG1 and ADPG2 are essential for silique dehiscence. In addition, ADPG2 and *QRT2* contribute to floral organ abscission, while all three genes contribute to anther dehiscence. Expression analysis is consistent with the observed mutant phenotypes. *INDEHISCENT* (*IND*) encodes a putative basic helix-loop-helix required for silique dehiscence, and we demonstrate that the closely related *HECATE3* (*HEC3*) gene is required for normal seed abscission and show that *IND* and *HEC3* are required for normal expression of *ADPG1* in the silique dehiscence zone and seed abscission zone, respectively. We also show that jasmonic acid and ethylene act together with abscisic acid to regulate floral organ abscission, in part by promoting *QRT2* expression. These results demonstrate that multiple cell separation events, including both abscission and dehiscence, require closely related PG genes.

## INTRODUCTION

Cell separation events that lead to organ abscission or dehiscence play important roles in plant development, particularly during reproductive processes. Examples include the abscission of leaves and outer floral organs and several processes related to pollination and seed set, fruit maturation, and seed dispersal (Lewis et al., 2006). Depending on individual plant species, entire flowers can abscise in the absence of fertilization and seed set or in unfavorable conditions. Abscission generally occurs at the completion of fruit maturation, often as an aid to seed dispersal, but can also occur during the early stages of fruit development even if seed set is successful. Undesirable abscission events are a major issue in the commercial production of many crops, including fruit loss from premature abscission (e.g., in many perennial horticultural crops) and seed loss from field crops (e.g., canola [*Brassica napus*]).

Pod shatter is a specialized type of fruit dehiscence event in which the fruit breaks into parts and, because it can be studied in

*Arabidopsis thaliana*, represents one of the best understood types of cell separation. In the family Brassicaceae, which includes *Arabidopsis* and canola, cell separation generally occurs along the sites of fusion between carpels that compose the fruit. A second separation event then occurs to allow the seed to detach from the maternal plant. Dehiscence of the *Arabidopsis* silique is very similar to the process in canola (Spence et al., 1996) and has been used as an effective model in which to study pod shatter. During ovary and fruit development, cell fate specification must occur to form the dehiscence zone (DZ), a specialized layer in which cell separation occurs to allow the silique to open. In *Arabidopsis*, the DZ consists of a few cell layers separating the replum from the edges of the two fused carpels (Spence et al., 1996). Genetic approaches have revealed that several genes encoding transcription factors are required for DZ differentiation (Ferrándiz et al., 2000; Liljegren et al., 2000; Rajani and Sundaresan, 2001; Liljegren et al., 2004). Once the DZ is correctly specified and established, other essential processes include secondary wall formation at the valve margins (Mitsuda and Ohme-Takagi, 2008) and degradation of cell walls, including the middle lamella, in the separation layer. This is thought to occur by the action of proteins involved in cell wall loosening, including polygalacturonases (PGs),  $\beta$ -1,4-glucanase, and expansin (Bonghi et al., 1993; Taylor et al., 1993, 1994; Lashbrook et al., 1994; del Campillo and Bennett, 1996; Cho and Cosgrove, 2000), although this hypothesis is supported by only limited direct evidence.

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Other important cell separation events occur in male flower organs during flower development. During pollen development, a separation event is required after meiosis of the pollen mother cell to separate the four microspores. The three quartet (*qrt*) mutants (*qrt1*, *qrt2*, and *qrt3*) of *Arabidopsis* are defective in this process and produce tetrad pollen in which microspores fail to separate during pollen development (Preuss et al., 1994). Immunohistochemical analyses suggest that QRT1 and QRT2 are required for pectin degradation of the cell wall surrounding the pollen mother cell during pollen development (Rhee and Somerville, 1998). Recent molecular studies have revealed that QRT1 and QRT3 encode a pectin methylesterase (PME) and an atypical PG, respectively (Rhee et al., 2003; Francis et al., 2006). Although QRT2 has been mapped to the top of chromosome 3 (Preuss et al., 1994), the affected gene has not yet been identified.

Pollen function also requires a second cell separation event in which the mature anthers dehisce to release functional pollen grains at anthesis. Anther dehiscence requires breakdown of the stomium, specialized cells that keep the anther locules closed, and genetic analysis has shown that the plant hormone jasmonic acid (JA) is required for this process. For example, plants lacking ALLENE OXIDE SYNTHASE (AOS) are JA deficient and do not shed pollen (Park et al., 2002; von Malek et al., 2002). At the cellular level, anther dehiscence is similar to silique dehiscence and, like microspore separation, is thought to involve similar cell wall degrading enzymes (Roberts et al., 2002). Later in *Arabidopsis* flower development, additional cell separation events occur in the floral tissues of the outer three whorls. Several days after anthesis, the sepals, petals, and stamens detach from the flower base to reveal either an unfertilized ovary or a developing silique containing immature seeds.

The cell separation events described above are all thought to involve the degradation of pectin by PGs, although this hypothesis has not been confirmed by genetic evidence. It is also not clear to what extent, if any, the same PGs function in different abscission/dehiscence events, and this uncertainty has contributed to the complex naming system, based on expression in different abscission zones (AZs) and DZs, sometimes used for PGs. Homogalacturonan-rich pectin is commonly found in the middle lamella region of the cell wall where two adjacent cells abut and pectin integrity is important for cell adhesion (MacDougall et al., 1996; Ridley et al., 2001). Endopolygalacturonases (endo-PGs) catalyze random hydrolysis of  $\alpha$ -1,4-glycosidic linkages in polygalacturonic acid (GalUA), a polymer that constitutes the main chain of the homogalacturonan region of pectin (Biely et al., 1996). Although there is only limited direct genetic evidence for the physiological importance of individual PGs, correlations have been reported between increasing PG activity and cell separation in fruit ripening and in the shedding of leaves, flowers, and fruit (Taylor et al., 1993; Kalaitzis et al., 1995; Brown, 1997; Kalaitzis et al., 1997). More recently, silencing of tomato (*Solanum lycopersicum*) abscission-related PGs was shown to increase the break strength of the leaf abscission zone and delay abscission in explants treated with ethylene (Jiang et al., 2008), and a putative *Arabidopsis* PG has been shown to promote floral organ abscission (González-Carranza et al., 2007). The importance of PG is also illustrated by the 'Flavr savr' tomato (Hadfield and Bennett, 1998) and peach (*Prunus persica*) lacking a functional *Melting flesh/Freestone* locus

(Peace et al., 2005), both of which have reduced expression of a fruit ripening-associated PG and delayed fruit softening.

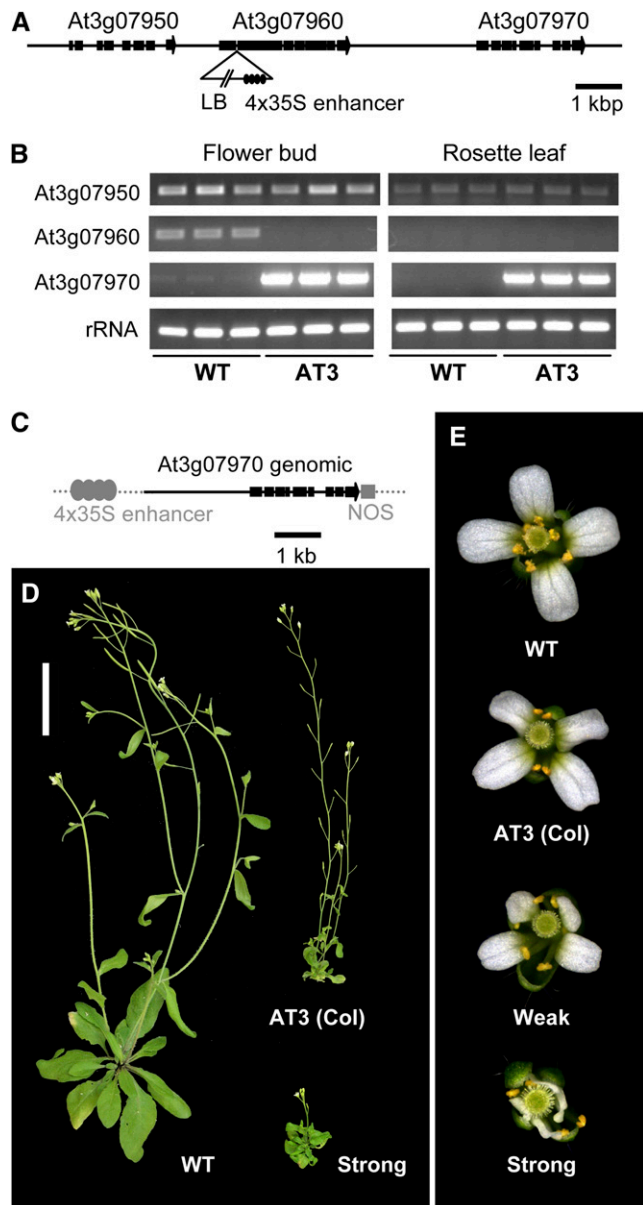
The best-characterized (fungal) endo-PG enzyme requires four to five consecutive runs of unesterified GalUA residues for cleavage (Benen et al., 1999; Pařenicová et al., 2000). However, little is known about the enzyme activity and substrate specificity of most plant PGs. There are at least 69 and 59 predicted PGs in the *Arabidopsis* and rice (*Oryza sativa*) genomes, respectively (Kim et al., 2006; González-Carranza et al., 2007), and it has been suggested that one group of related PGs tend to be expressed in flowers and flower buds, while PGs expressed in vegetative tissues generally belong to other groups (Torki et al., 2000; Kim et al., 2006). The implication is that the diverse potential physiological roles of PGs may be a consequence of differential expression in specific tissues rather than or in addition to differences in enzyme substrate specificity. These questions have been investigated to a limited extent using transgenic plants with altered expression of endo-PGs. For example, overexpression of an apple (*Malus domestica*) endo-PG in transgenic apples resulted in silvery colored leaves and premature leaf shedding due to reduced cell adhesion in leaves and in the leaf abscission zone (Atkinson et al., 2002). By contrast, ectopic expression of a tomato fruit-specific endo-PG (pTOM6) in tobacco (*Nicotiana tabacum*) revealed no leaf phenotype or detectable alterations in cell wall pectins, although the pTOM6 protein was properly processed and localized in the cell wall of tobacco leaves (Osteryoung et al., 1990). While these results suggest that individual PGs may have different substrate specificities or requirements for activity, it is not clear how overall PG activity is regulated in different plant tissues in vivo.

Here, we report on the characterization of an activation tagging line with reduced growth and male sterility caused by increased expression of a PG encoded by At3g07970. This gene has been previously named QRT2 based on tetrad pollen production in mutant *qrt2* plants (Preuss et al., 1994). QRT2 is part of a small family of three closely related endo-PGs in the *Arabidopsis* proteome, and our genetic analysis demonstrates that two of these PGs are required for silique dehiscence (ARABIDOPSIS DEHISCENCE ZONE POLYGALACTURONASE1 [ADPG1] and ADPG2), two are required for normal floral organ abscission (ADPG2 and QRT2), and all three are required for normal anther dehiscence. Expression analysis based on transcriptional  $\beta$ -glucuronidase (GUS) constructs is consistent with the observed mutant phenotypes. Based on the transcriptional regulation of all three genes by JA, and of ADPG2 by ethylene, we show that JA and ethylene act together with abscisic acid (ABA) to regulate floral organ abscission, in part by promoting QRT2 expression.

## RESULTS

### Activation Tagging of the At3g07970 Gene

From a population of several hundred independent activation tagging lines, we identified one line, designated activation tagging line no. 3 (AT3), in which most anthers failed to shed pollen and the majority of flowers did not set seeds (see Supplemental Figure 1 online). The selectable marker (the *bar* gene)



**Figure 1.** Activation Tagging of *At3g07970*.

**(A)** Genomic region of the T-DNA insertion (activation tagging) site in line AT3. Boxes and lines represent exons and introns/intergenic regions, respectively. LB indicates left border sequence of the activation tagging vector (pSKI015; Weigel et al., 2000), and the four closed ovals represent the 4x 35S enhancer sequence.

**(B)** RT-PCR analysis, using RNA extracted from flower buds and rosette leaves, of genes flanking the T-DNA insertion site in AT3. rRNA was used as the control.

**(C)** Schematic diagram of the 35S enhancer-ProQRT2:QRT2 construct used to determine if the AT3 phenotype is caused by overexpression of the *At3g07970* gene. The solid and dotted lines 5' of the 1st exon indicate the presumed *At3g07970* promoter sequence (2085 bp) and sequence (630 bp) from the pMN19 vector (Weigel et al., 2000), respectively. The four gray ovals and box represent the 4x 35S enhancer sequence (from pMN19) and nopaline synthase terminator (NOS) from the pGWB1 vector, respectively.

cosegregated with the mutant phenotype through several generations, suggesting that there was a single T-DNA insertion site that might be causing activation of an adjacent gene. The T-DNA insertion site adjacent to the left border was identified using thermal asymmetric interlaced-PCR (TAIL-PCR) (Liu et al., 1995) and found to be located within the second exon of *At3g07960*, which encodes a predicted protein similar to 1-phosphatidylinositol-4-phosphate 5-kinase (Figure 1A). The genes closest to the T-DNA enhancer sequence were *At3g07950* (predicted to encode an unknown protein) and *At3g07970* (predicted to encode a PG), and the T-DNA was located ~3.5 and 5.7 kb from their putative translational start codons, respectively (Figure 1A). RT-PCR analysis of these three genes revealed that compared with the wild-type, only *At3g07970* was clearly overexpressed in flower buds and rosette leaves of AT3 plants (Figure 1B). The reduced expression of *At3g07960* in AT3 flower buds is presumably due to the T-DNA insertion in this gene. Since the AT3 phenotype is genetically dominant, it is likely that reduced expression of *At3g07960* does not contribute to the AT3 phenotype (see below). Quantitative real-time RT-PCR (qRT-PCR) revealed that the transcript level of *At3g07970* in AT3 flower buds was ~350 times higher than in wild-type flower buds (Table 1).

To determine if the AT3 mutant phenotype is caused by activation of the *At3g07970* gene, we generated transgenic plants with the 4x 35S enhancer sequence fused to a genomic fragment including introns, exons, and the putative promoter (2085 bp 5' from the predicted translation initiation site) of the *At3g07970* gene (Figure 1C). Of 30 independent transgenic lines, 14 exhibited anther defects and a dwarf rosette with curled leaves similar to the AT3 line and failed to set seeds (Figure 1D). These lines could be further divided into those with either weak (seven lines) or strong (seven lines) phenotypes. The weak lines had a less severe rosette phenotype and flowers similar to those of AT3. The strong lines had severely dwarfed rosettes with extremely curled leaves and defective flowers that often failed to fully open with abnormal petals and completely indehiscent anthers (Figure 1E).

To confirm overexpression of *At3g07970* in these transgenic plants, we performed qRT-PCR using flower buds from selected lines and found that relative to wild-type flower buds, *At3g07970* expression in lines with a strong phenotype was >2000 times higher, while expression lines with a weak phenotype was between 10 and 40 times higher (Table 1). For the original AT3 line, the increase in *At3g07970* expression was intermediate between the weak and strong lines (Table 1). Thus, *At3g07970* expression was strongly correlated with the observed phenotypes, demonstrating that the AT3 phenotype is caused by activation of the *At3g07970* gene.

**(D)** Phenotype of 6-week-old plants. Wild-type Columbia, AT3 (backcrossed into Columbia [Col-0] twice), and a representative strong line containing the construct in **(C)** are shown. Bar = 3cm.

**(E)** Flower bud phenotype at floral developmental stage 14 (Smyth et al., 1990) of the wild-type, AT3 (backcrossed into Col-0 twice), and representative examples of the weak and strong phenotype in plants containing the construct in **(C)**.

**Table 1.** Change in *QRT2* Transcript Levels in Flower Buds of AT3 and 35S Enhancer-Pro*QRT2*:*QRT2* Transgenic Plants Relative to the Wild-Type

Line	Expression	Abnormal Phenotypes		
	(Fold Increase) <sup>a</sup>	Rosette	Flower	Anther
AT3 ( <i>Ler</i> )	352 ± 35	++	+	++
Weak a	10	+	+	++
Weak b	24	+	+	++
Weak c	42	+	+	++
Strong d	3818	+++	+++	+++
Strong e	2451	+++	+++	+++
Strong f	3526	+++	+++	+++

<sup>a</sup>The change in expression (fold increase) was calculated relative to the expression level in wild-type plants.

Other than AT3, all lines are in the *Col* background. The mean ± SE is shown for the AT3 line. Weak (a to c) and strong (d to f) indicates lines containing 35S enhancer-Pro*QRT2*:*QRT2* (Figure 1C). The extent of abnormal phenotypes for each line relative to the wild-type is indicated by the following: none detected (–), weak (+), medium (++) or strong (+++).

### At3g07970 Is *QRT2*

To examine the biological role of At3g07970, we identified loss-of-function alleles of At3g07970 using T-DNA insertion lines obtained from the ABRC (Figure 2A). These two mutant lines are likely to represent null or near-null alleles because expression could not be detected by qRT-PCR using 40 cycles and gene-specific primers on either side of the T-DNA insertion sites (see Supplemental Figures 2 and 3 online). Plants homozygous for either of the two independent mutant alleles of At3g07970 had no apparent phenotype compared with the wild-type, with the exception that mature pollen grains were arranged in a tetrad similar to the phenotype of the *qrt* mutants (Preuss et al., 1994). Although the *QRT2* gene was not identified, previous mapping experiments revealed that this locus is linked to *GAPC* at the top of chromosome 3 (Preuss et al., 1994), in the same region as At3g07970. In addition, microscopy observations using an antibody that recognizes pectin indicated that *QRT2* may be required for cell type-specific pectin degradation to separate microspores (Rhee and Somerville, 1998). As these results are consistent with At3g07970 encoding *QRT2*, we performed allelism tests by crossing the original *qrt2-1* allele with the two At3g07970 T-DNA insertion mutants (hereafter called *qrt2-2* and *qrt2-3*). Pollen grains of all F1 progeny exhibited the tetrad phenotype, confirming that At3g07970 is *QRT2*. Consistent with this result, F1 progeny from control crosses between *qrt2-2* and the wild-type or between *qrt2-3* and the wild-type produced only monad pollen.

The *qrt2-1* allele was generated using ethyl methanesulfonate (Preuss et al., 1994). To identify the mutation present in *qrt2-1*, a 2735-bp genomic fragment, corresponding to the *QRT2* open reading frame with 359 bp upstream of the translational start codon and 10 bp downstream of the stop codon, was cloned from *qrt2-1* and wild-type Landsberg *erecta* (*Ler*) plants and sequenced. A single nucleotide mutation was found in *qrt2-1* that changed the predicted amino acid sequence from Val (GTG) to

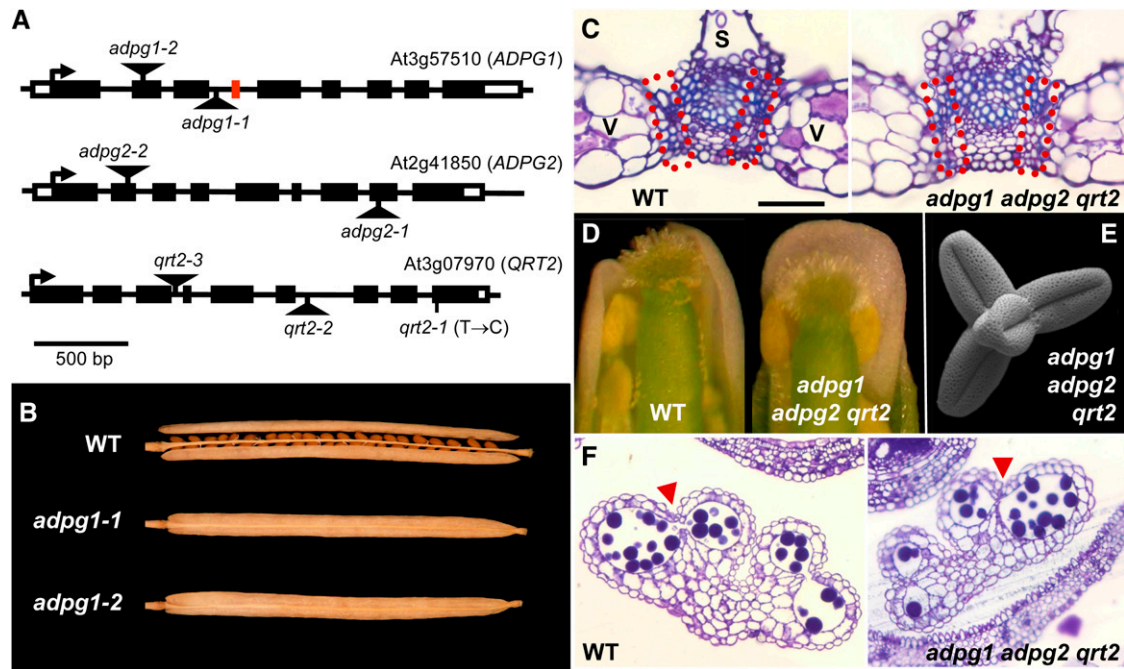
Ala (GCG) at position 372 (Figure 2A; see Supplemental Figure 2A online). Val is a hydrophobic amino acid, and sequence analysis revealed that a hydrophobic amino acid (Val, Leu, or Ile) in this position is highly conserved in PGs from plants and fungi (Markovic and Janecek, 2001; Kim et al., 2006). qRT-PCR from flower buds revealed no detectable change in *QRT2* expression level between wild-type *Ler* and *qrt2-1* (see Supplemental Figure 2B online), consistent with the *qrt2-1* mutant phenotype being caused by a single amino acid substitution that decreases protein activity.

### Loss of PG Function Prevents Pod Shatter

Phylogenetic analysis of predicted *Arabidopsis* PGs revealed that there are two PGs closely related to *QRT2*, encoded by At3g57510 and At2g41850 (Kim et al., 2006). The putative PG encoded by At3g57510 has previously been given several names, including ADPG1, PGA9, pga1;6, and SAC70 (Jenkins et al., 1999; Sander et al., 2001). Although the putative PG encoded by At2g41850 has been referred to as PGAZAT (González-Carranza et al., 2002), we refer to this protein as ADPG2 based on its expression pattern and biological role (see below).

The similarity between *QRT2* and ADPG1 is potentially revealing because the latter has previously been identified as a putative ortholog of RAPESEED DEHISCENCE ZONE POLYGALACTURONASE1 (RDPG1) from canola. RDPG1, also called SAC66, has been proposed to be involved in breakdown of the middle lamella of the separation zone during pod shatter, although functional analysis of ADPG1 or RDPG1 in planta has not been reported (Jenkins et al., 1996, 1999; Petersen et al., 1996; Sander et al., 2001). Based on the similarities between the predicted proteins and the observation that *ADPG1* is also expressed in the silique DZ, a similar role has been proposed for ADPG1 in *Arabidopsis* (González-Carranza et al., 2007). Consistent with possible roles in fruit abscission processes, expression data also revealed that *ADPG1* and *ADPG2* are expressed predominantly in floral tissues (Grennan, 2006; Kim et al., 2006; González-Carranza et al., 2007; see below).

To examine the biological role of ADPG1 and ADPG2, we identified two independent loss-of-function T-DNA insertion mutant alleles for each gene obtained from ABRC (SALK lines) and the Max-Planck Institute (GABI line) (Figure 2A). With the possible exception of *adpg1-1*, these lines are likely to represent null or near-null alleles because expression could not be detected by qRT-PCR using 40 cycles and gene-specific primers on either side of the T-DNA insertion sites (see Supplemental Figure 3 online). For *adpg1-1*, the presence of the T-DNA inserted into the third intron did not completely prevent expression based on a similar analysis. However, qRT-PCR revealed that the transcript level in *adpg1-1* was 18 times less than the wild-type (see Supplemental Figure 4A online). In addition, sequence analysis of RT-PCR products revealed that the entire 4th exon, consisting of 21 nucleotides and encoding seven amino acids, was missing from the *adpgp1-1* mRNA (Figure 2A, missing exon in *adpg1-1* in red; see Supplemental Figure 4B online). Since this sequence is still present in genomic DNA of *adpg1-1*, it is likely that the T-DNA insertion in the third intron causes incorrect



**Figure 2.** *adpg1*, *adpg2*, and *qrt2* Loss-of-Function Phenotypes.

**(A)** Position of the T-DNA insertions in At3g07970 (*QRT2*) and the related genes, *ADPG1* and *ADPG2*. Boxes and lines represent exons and introns, respectively. Triangles indicate positions of T-DNA insertions, and the position of the point mutation in *qrt2-1* is shown. Open boxes represent predicted 5' and 3' untranslated regions. There is no information available regarding the 5' untranslated region of *QRT2*. The exon shown in red is missing in transcript from the *adpg1-1* allele.

**(B)** Silique indehiscence phenotype of the *adpg1-1* and *adpg1-2* mutants. Fully matured wild-type siliques break easily, while those of the *adpg1* mutants require external mechanical stress

**(C)** Transverse sections of wild-type and *adpg1 adpg2 qrt2* triple mutant siliques (stage 17) stained with Toluidine Blue. The red boxes indicate where the DZs form in the wild-type and fail to form in the mutant. V, silique valve; S, septum. Bar = 50  $\mu$ m.

**(D)** Flowers of the wild-type (stage 14: pollination) and the *adpg1 adpg2 qrt2* triple mutant with delayed anther dehiscence.

**(E)** Scanning electron microscopy image of a mature pollen tetrad from the *adpg1 adpg2 qrt2* triple mutant.

**(F)** Transverse sections of wild-type and *adpg1 adpg2 qrt2* triple mutant anthers (stage 13) stained with Toluidine Blue. Arrowheads indicate where stomium separation has occurred in the wild-type anther and has not yet occurred in the *adpg1 adpg2 qrt2* anther.

splicing which results in exon 3 being joined directly to exon 5. The effect, if any, that this has on protein activity is not known.

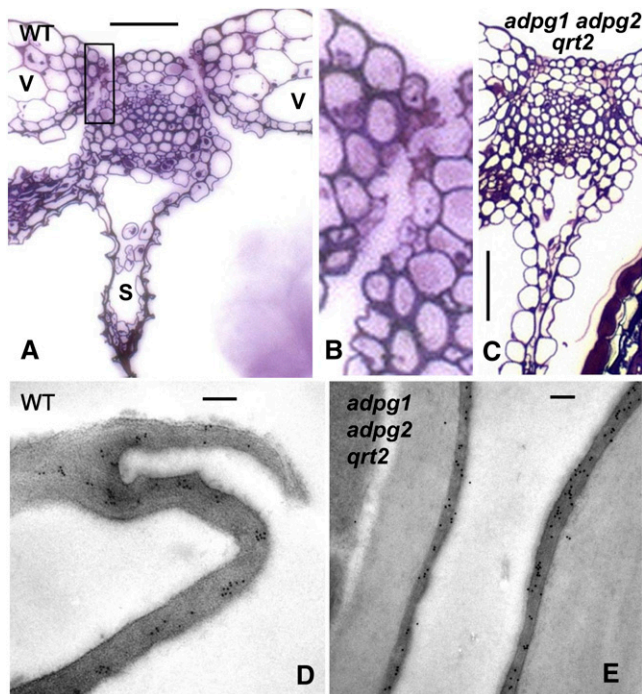
Plants homozygous for mutant alleles *adpg1-1* or *adpg1-2* were indistinguishable from the wild-type, including the release of monad pollen, with the exception of impaired pod shatter due to failure of the valve to properly detach from the central part of the silique (Figure 2B). However, pod shatter did occur when mature, dry *adpg1* siliques were gently compressed to increase pressure on the valve DZs. Once opened in this way, seed abscission appeared normal. By contrast, under normal growing conditions, single *adpg2* mutants appeared similar to wild-type plants in terms of pod shatter and also produced monad pollen. However, reduced pod shatter was observed in *adpg2-1* and *adpg2-2* plants (and in double mutants with *qrt2*) when watering was ceased before overall plant senescence was complete (see Supplemental Table 1 online). To determine if the *ADPG1*, *ADPG2*, and *QRT2* genes are functionally redundant, double and triple mutants were generated by crossing. Siliques of the double mutants *adpg1-1 adpg2-1* and *adpg1-2 adpg2-2* exhibited

a more severe phenotype than did those of the *adpg1* single mutants and failed to dehiscence even if compressed. When siliques were cut open, seed abscission again appeared normal, as did pollen grain separation. Triple mutants were also constructed with the two *qrt2* T-DNA alleles. In terms of pod shatter and seed abscission, *adpg2 qrt2* double mutants were similar to *adpg2* single mutants, double mutants lacking both *ADPG1* and *QRT2* appeared identical to *adpg1* single mutants, and the *adpg1-1 adpg2-1 qrt2-2* and *adpg1-2 adpg2-2 qrt2-3* triple mutants resembled the *adpg1 adpg2* double mutants (see Supplemental Table 1 online). These results suggest that *ADPG1* and *ADPG2* have partially redundant roles in *Arabidopsis* pod shatter. As expected, all plants homozygous for *qrt2* produced tetrad pollen (Figure 2E).

As pod shatter represents a well-characterized cell separation event (Roberts et al., 2002; Lewis et al., 2006), the silique DZ was used to investigate pectin levels associated with reduced PG function. Consistent with a role for *ADPG1* and *ADPG2* in cell separation in the final stages of pod shatter, transverse sections



of maturing siliques just prior to pod shatter (stage 17b; Roeder and Yanofsky, 2006) revealed that *adpg1 adpg2 qrt2* triple mutants were morphologically similar to the wild-type (Figure 2C). At stage 18, when silique dehiscence normally takes place (Roeder and Yanofsky, 2006), cell separation occurred in wild-type siliques but not in *adpg1 adpg2 qrt2* triple mutant siliques (Figure 3). Although it has been reported previously that cell separation occurs between cells without cell rupture (Spence et al., 1996), we observed separation between intact cells in addition to breaking of nearby cells (Figure 3B) in the DZ of wild-type siliques. Pectic polysaccharides, including potential PG substrates, can be visualized using monoclonal antibodies that recognize unesterified (JIM5) and esterified (JIM7) regions of pectin (e.g., Francis et al., 2006). For example, JIM5 has been used previously to show that, in contrast with the wild-type, the primary cell wall that surrounds the microspores is not degraded in *qrt2-1* anthers (Rhee and Somerville, 1998). If ADPG1 and



**Figure 3.** ADPG1 and ADPG2 Are Required for Pod Shatter.

(A) to (C) Toluidine Blue–stained transverse sections of wild-type (A) and (B) and *adpg1 adpg2 qrt2* triple mutant (C) siliques at stage 18. V, silique valve; S, septum. (B) shows an enlarged image of the section indicated by the rectangle in (A). The wild-type silique has begun to dehisce, and broken cell walls are visible where the valves have separated from the replum. By contrast, the replum and valve remain intact in the triple mutant.

(D) and (E) TEMs of transverse sections of comparable wild-type (D) and *adpg1 adpg2 qrt2* triple mutant (E) siliques, immediately after pod shatter in wild-type siliques, labeled with the JIM5 monoclonal antibody against the unesterified region of pectin. A broken cell wall from the wild-type is shown in (D), while cell separation has not occurred in the triple mutant (E). Bars = 50  $\mu\text{m}$  in (A) and (C) and 0.2  $\mu\text{m}$  in (D) and (E).

ADPG2 degrade the majority of pectin present in the silique DZ, clear differences would be expected in the pectin levels of wild-type and mutant siliques. However, comparison between transmission electron microscopy (TEM) sections of wild-type and *adpg1 adpg2 qrt2* triple mutant siliques at stage 18 did not reveal any obvious differences in the overall levels of pectin (Figures 3D and 3E), suggesting that the degradation of only a small proportion of the total JIM5-recognized pectin is required for pod shatter.

### Loss of PG Function Delays Anther Dehiscence

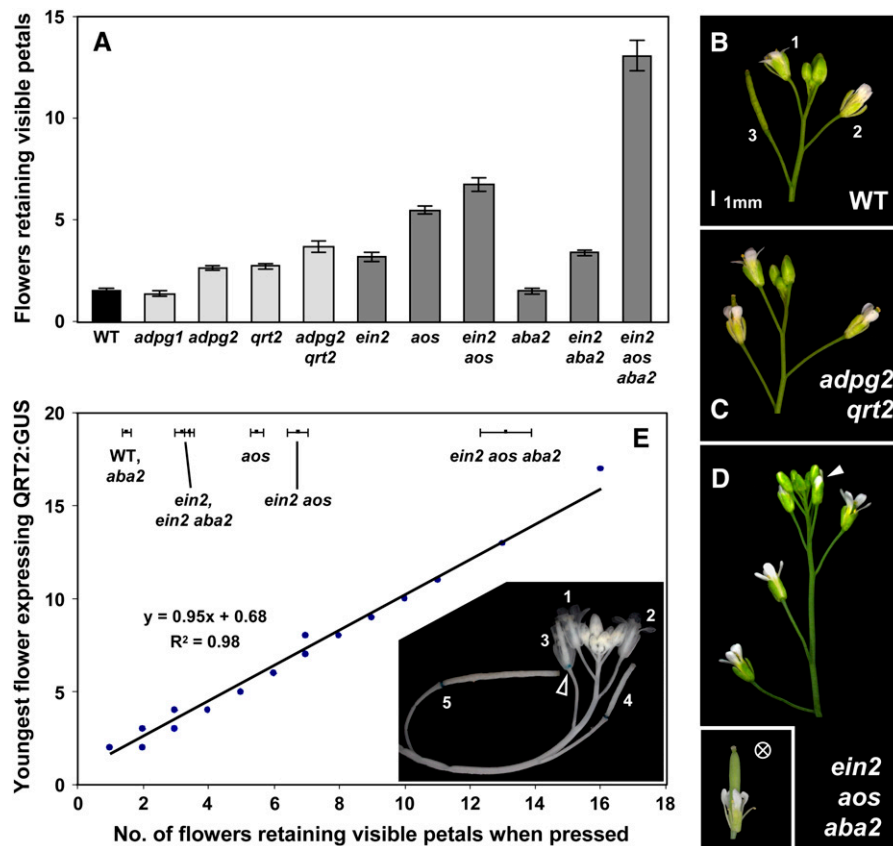
In contrast with the single and double mutants, the *adpg1 adpg2 qrt2* triple mutants consistently exhibited delayed anther dehiscence in early flowers (Figure 2D) due to failure of the stomium to separate at stage 13 (Figure 2F; Sanders et al., 1999). The anther phenotype progressively became weaker as the plant continued to grow and produced more flowers. The delay in pollen release and pollination in early flowers did not prevent seed set in the triple mutants, although it appeared that a small proportion of flowers were not pollinated. These results suggest that functional redundancy exists between ADPG1, ADPG2, and QRT2 in anther dehiscence. Furthermore, it is clear that a common PG-dependent mechanism is involved in cell separation events in the anther and silique DZ and during microspore development.

### Loss of PG Function Delays Floral Organ Abscission

After investigating several methods to assess floral organ abscission, firmly pressing each flower on intact plants grown under standard conditions was chosen as the most reliable and consistent (see Supplemental Figure 5 online). There was a clear delineation between younger flowers with floral organs that did not detach when pressed and older flowers that lost their floral organs easily. In particular, this approach resolved the issue of floral organs being lost during plant growth and handling. Analysis of the *adpg1 adpg2* and *qrt2* single mutants in this manner revealed that ADPG2 and QRT2 promote floral organ abscission (Figure 4), consistent with previous reports that ADPG2 promotes this cell separation event (González-Carranza et al., 2007). The *adpg2 qrt2* double mutant exhibited a slightly greater delay than either single mutant, while the *adpg1 adpg2 qrt2* triple mutant appeared similar to the *adpg2 qrt2* double mutant, consistent with no role for ADPG1 in this process.

### ADPG1, ADPG2, and At1g48100 Encode PGs

Based on the similarity of their predicted amino acid sequence to plant PGs for which functional assays have been reported (Hadfield et al., 1998; Degan et al., 2001), ADPG1, ADPG2, QRT2, and a less closely related putative PG encoded by At1g48100 are expected to encode PGs. However, as there is no direct evidence of PG activity for these proteins, we attempted to directly measure PG activity of heterologous proteins in vitro. Truncated versions of these four proteins, lacking the N-terminal hydrophobic region predicted to function as a signal peptide, were expressed in *Escherichia coli* as His-tagged recombinant



**Figure 4.** ADPG2, QRT2, Ethylene, JA, and ABA Are Required for Normal Floral Organ Abscission.

**(A)** The number of flowers on a single inflorescence with visible petals and floral organs remaining attached after firm pressing (at least nine plants per genotype; means  $\pm$  SE are shown).

**(B)** Typical wild-type inflorescence with two flowers with visible petals that retained floral organs after pressing. Flowers were numbered down the main inflorescence starting from the youngest with visible petals for scoring floral organ abscission.

**(C)** Typical *adpg2 qrt2* inflorescence with three flowers with visible petals that retained floral organs after pressing.

**(D)** Typical *ein2 aos aba2* inflorescence showing the youngest four flowers with visible petals that retained floral organs after pressing. All floral organs remained turgid and healthy while still attached. The arrowhead indicates the youngest flower (flower 1) with visible petals: more immature flowers (not counted) are present compared with AOS inflorescences because JA is required for normal flower maturation. Self-pollination of *ein2 aos aba2* triple mutant flowers (with pollen from JA-treated triple mutants) failed to restore floral organ abscission (inset).

**(E)** Relationship between the number of flowers retaining visible petals after firm pressing and the youngest flower with QRT2:GUS expression in the floral AZ. Plants with a range of floral organ abscission phenotypes were selected from the F2 progeny of a cross between a representative QRT2:GUS line and the *ein2 aos aba2* triple mutant. The values at the top of the graph show the mean  $\pm$  SE for the different genotypes as shown in **(A)**. Flowers were numbered as shown in the inset image, which shows a plant with three flowers retaining visible petals after firm pressing. Flower 3 is the youngest flower with detectable GUS expression in the floral AZ (arrowhead). For all genotypes, GUS staining was first observed in either the youngest flower that lost its floral organs or in the next youngest flower, just about to shed its organs (as in the inset). Floral organ abscission was not observed prior to GUS expression in any plant. Thirty-two F2 progeny were examined, of which 26 contained the QRT2:GUS transgene.

fusion proteins. His-GFP (green fluorescent protein), His-ADPG1, His-ADPG2, and His-At1g48100 proteins of the expected size were successfully expressed in *E. coli* based on SDS-PAGE followed by Coomassie blue staining. These His-tagged recombinant proteins were purified using a nickel-nitrilotriacetic acid agarose (Ni-NTA) column and their activity assayed using polygalacturonic acid as the substrate (Table 2). All three proteins possessed PG activity, and the specific activity of His-ADPG2 was  $\sim$ 30 times higher than that of His-ADPG1, whereas activity of His-GFP could not be detected. The activity

of the His-At1g48100 recombinant protein was 40 and 100 times higher than that of His-ADPG2 and His-ADPG1, respectively.

#### Expression of ADPG1, ADPG2, and QRT2

Previous work has investigated the expression pattern of both ADPG1 and ADPG2. In *Arabidopsis*, ADPG1 expression has previously been localized to the DZ of anthers and maturing siliques by immuno-electron microscopy with an anti-RDPG1 antibody, by comparison with an RDPG1:GUS reporter (Sander

**Table 2.** Specific Activity of PGs in Vitro

Protein	Activity (Units/mg)	SE
GFP	ND	–
ADPG1	0.025	0.006
ADPG2	0.679	0.060
At1g48100	2.713	0.247

His-tagged GFP or PG recombinant protein was expressed in *E. coli* and purified using a Ni-NTA column. The specific activity was calculated from three independent samples. ND, not detected.

et al., 2001) and by using an ADPG1:GUS reporter (González-Carranza et al., 2007). The expression domain for ADPG2 was examined using transcriptional constructs in which either GUS or GFP was under the control of the presumed ADPG2 promoter (González-Carranza et al., 2002, 2007). Based on this analysis, ADPG2 has been reported to be expressed in roots and in the abscission zone of the sepals, petals, and stamens of flowers and is upregulated by ethylene in these tissues (González-Carranza et al., 2007). ADPG2 expression has not been reported in anthers or silique DZs. Previous attempts to monitor At3g07970 (*QRT2*) expression using GUS were not successful (González-Carranza et al., 2007), although this gene is expressed in flowers undergoing floral organ abscission (Kim and Patterson, 2006).

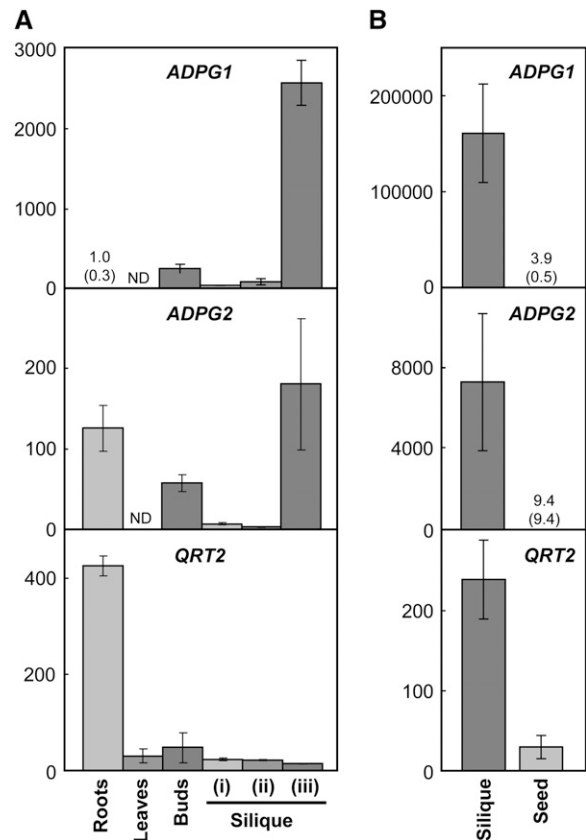
Expression of ADPG1, ADPG2, and QRT2 was initially examined using qRT-PCR with RNA from different plant tissues (Figure 5). Consistent with previous reports, ADPG1 was the most highly expressed of the three genes and was detected predominately in flower buds (which included anthers prior to anthesis) and siliques just before pod shatter. ADPG2 was also expressed in flower buds and mature siliques as well as in roots. QRT2 was expressed predominantly in roots with lower expression levels in rosette leaves, flower buds (see also Figure 1B), and siliques. In addition, in mature siliques harvested just after they turned yellow, but before pod shatter, the seeds were removed from the remaining fruit tissue and RNA extracted separately. For all three genes, the majority of expression occurred in the non-seed tissue (Figure 5B).

To further investigate the expression patterns of ADPG1, ADPG2, and QRT2, promoter:GUS transcriptional constructs were transformed into wild-type plants. Initially, we tested GUS staining from 8 to 12 independent transgenic lines for each construct to confirm that GUS activity was consistently detected in the same tissues. At least two representative lines were selected for further analysis.

Consistent with the loss-of-function phenotypes and the expression analysis described above, the ADPG1:GUS reporter was predominantly expressed in silique DZs (Figure 6A) and in anthers just prior to anthesis (Figure 6D). Expression was also observed in seed abscission zones (Figure 6A), although we have not observed any obvious defect in seed abscission in *adpg1* mutants. GUS staining in the silique DZ was first detected in fertilized pistils and increased throughout silique development until reaching a maximum in fully matured siliques just prior to pod shatter, consistent with the results obtained by qRT-PCR (Figure 5). In elongating siliques, GUS staining was first detected

in the DZ at the base of siliques and spread along the entire DZ as the silique matured. Cross sections of anthers from ADPG1:GUS lines confirmed that ADPG1-GUS was expressed in stomium cells, which allow anther dehiscence to occur (Keijzer, 1987), the apical tip of the anther filament (Figure 6J), and the DZ of siliques (Figure 6K).

To further investigate the expression of ADPG1 in the silique DZ and the seed AZ, mutants with defects in these processes were examined. Several genes that are required for correct

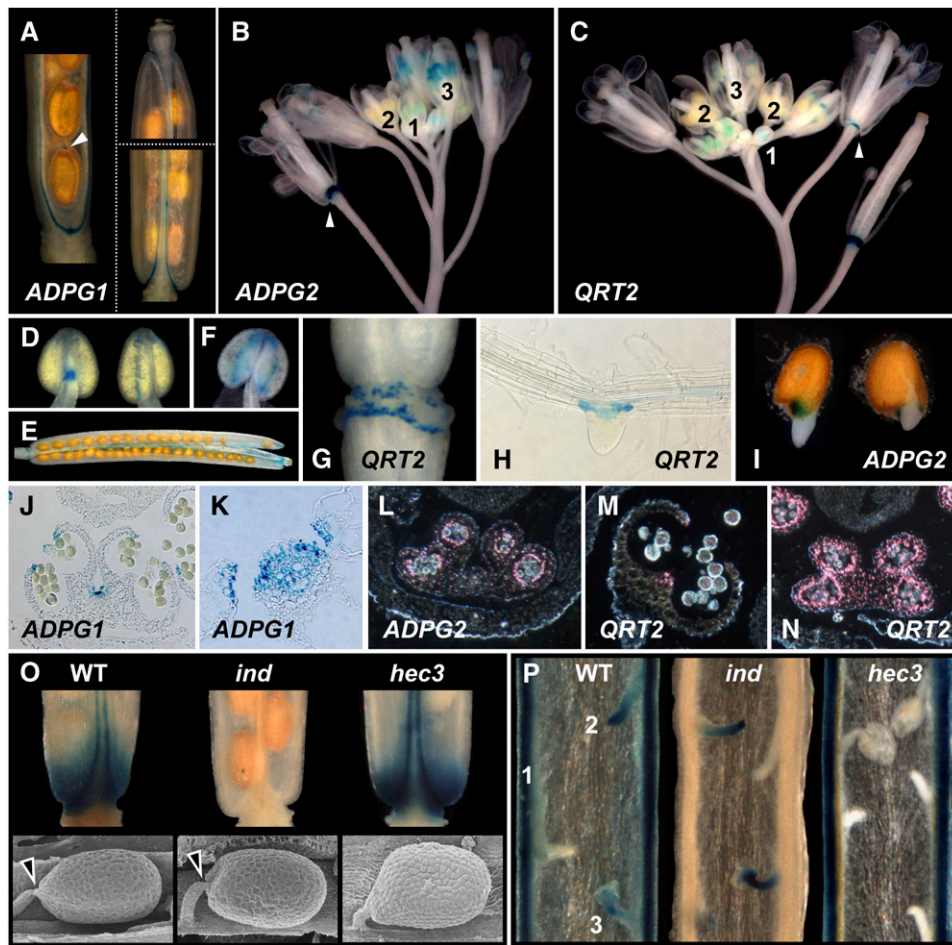
**Figure 5.** Tissue-Specific Expression of ADPG1, ADPG2, and QRT2.

The relative expression levels of ADPG1, ADPG2, and QRT2 in different tissues were determined using qRT-PCR and gene-specific primers. The lowest expression level (ADPG1 in roots) was adjusted to 1, and the relative expression levels of each gene in all tissues tested was calculated relative to this value. Data represent the means and SE from three independent biological samples and are shown on the figure (with SE in parentheses) where too small to see. ND, not detected.

(A) RNA was extracted from roots and rosette leaves from 3-week-old plants just before bolting, from flower buds, and from siliques at three developmental stages: (i) young siliques (1 to 3 d after pollination), (ii) developing siliques (about 1 week after pollination), and (iii) fully matured siliques just after turning yellow but before pod shatter (2 to 3 weeks after pollination).

(B) RNA was also isolated separately from entire siliques with all seeds removed, but including the receptacle, placenta, replums, funiculi, and style (Silique) and the removed seeds (Seed) from mature siliques just after turning yellow (2 to 3 weeks after pollination).





**Figure 6.** *ADPG1*, *ADPG2*, and *QRT2* Are Expressed at Sites of Cell Separation.

- (A) *ADPG1*:GUS expression in the silique DZ and seed AZ (arrowhead) from mature siliques immediately prior to pod shatter.
- (B) *ADPG2*:GUS expression in anthers and the floral organ AZ (arrowhead) prior to floral organ abscission. In anthers, expression occurs during early development (1), is absent at later stages (2), and returns just prior to dehiscence (3).
- (C) *QRT2*:GUS expression in anthers and in the floral organ AZ (arrowhead) prior to floral organ abscission. In anthers, expression occurs during early development (1), is absent at later stages (2), and returns just prior to dehiscence (3).
- (D) *ADPG1*:GUS expression at the apical tip of the stamen filament and in the anther DZ.
- (E) *ADPG2*:GUS expression in the silique DZ at pod shatter.
- (F) *QRT2*:GUS expression and anther dehiscence was restored after *aos* plants were sprayed to runoff with 1 mM methyl jasmonate. No GUS activity was detected in untreated *aos* anthers.
- (G) *QRT2*:GUS expression in the floral organ AZ at the abscission scars of the stamens, petals, and sepals.
- (H) *QRT2*:GUS expression surrounding an emerging lateral root.
- (I) *ADPG2*:GUS expression at the site of radicle emergence in germinating seeds 24 h after stratification. No expression was observed immediately after stratification finished.
- (J) Transverse section of a flower at stage 13 showing *ADPG1*:GUS expression where stomium separation has occurred and at the apical tip of the stamen filament.
- (K) Transverse section of a dehiscing silique showing *ADPG1*:GUS expression where the valves have separated from the replum.
- (L) Dark-field image showing *ADPG2*:GUS expression in the anther tapetum at flower stage 11.
- (M) Dark-field image showing *QRT2*:GUS expression in the anther DZ at stage 13.
- (N) Dark-field image showing *QRT2*:GUS expression in the anther tapetum at flower stage 10, when microspore separation occurs.
- (O) *ADPG1*:GUS expression in the valve margin is present in the wild-type and *hec3* but absent in the nonshattering *ind* mutant (top). Scanning electron micrograph of mature wild-type, *ind*, and *hec3* seeds showing the constrictions (arrowheads) in the wild-type and *ind* funiculi where abscission will occur. This structure is absent in *hec3* plants, and seed abscission does not occur (bottom).
- (P) *ADPG1*:GUS expression in mature siliques with the valve removed. *IND* is required for expression in the silique DZ (1), while *HEC3* is required for expression in the seed AZ (2) and ovule funiculus (3).

formation of the silique DZ have been identified, including *INDEHISCENT* (*IND*). *IND* encodes a putative basic helix-loop-helix transcription factor and forms part of a large gene family in *Arabidopsis*. Siliques on *ind* mutants do not shatter due to a failure to develop specialized cells at the junction between the carpels and the replum that later form the DZ (Liljegren et al., 2004). Compared with wild-type siliques, *ADPG1*:GUS expression was not detectable at the junction between a carpel and replum, where the DZ fails to form in this mutant (Figures 6O and 6P). By contrast, *ADPG1*:GUS expression was still present at the site of the seed AZ, consistent with normal seed abscission in the *ind* mutant. Unfertilized ovules also expressed *ADPG1*:GUS in the funiculus, despite the fact that only fertilized seeds abscise.

*HECATE3* (*HEC3*) encodes a protein closely related to *IND* that is required for normal functioning of female reproductive tissues (Gremski et al., 2007). In addition to these phenotypes, *hec3* mutants also fail to form a seed AZ, and seed abscission does not occur (Figure 6O). Consistent with a role for *ADPG1* in seed abscission, *ADPG1*:GUS was not expressed in the funiculus of fertilized seeds or unfertilized ovules in *hec3* plants (Figure 6P). However, pod shatter occurs normally in *hec3* plants, and *ADPG1*:GUS was expressed in the DZ of *hec3* siliques (Figures 6O and 6P). These results suggest that *IND* and *HEC3* are required for normal expression of *ADPG1* in the silique DZ and seed AZ, respectively.

In agreement with previous reports and qRT-PCR analysis (Figure 5), *ADPG2*:GUS was expressed in the AZs of sepals, petals, and stamens in flowers just prior to floral organ abscission (Figure 6B) and at the site of lateral root emergence (Kim and Patterson, 2006; González-Carranza et al., 2007). *ADPG2*:GUS was also expressed early in anther development, at around the time of microspore separation (Figure 6L), later in the anther DZ just prior to dehiscence (Figure 6B), and in the DZ of maturing siliques (Figure 6E). These observations, obtained using less-stringent GUS staining conditions to increase sensitivity, are consistent with the observed role of *ADPG2* in anther dehiscence and pod shatter and the qRT-PCR analysis (Figure 5) described above but are inconsistent with previously published results (González-Carranza et al., 2007). A likely explanation for this discrepancy is differences in the presumed promoter sequence used. We used 2177 bp upstream from the predicted translation initiation site, whereas González-Carranza et al. (2007) used 1476 bp of the *ADPG2* promoter which lacked the 25 bp of 5' untranslated *ADPG2* sequence. Based on GUS analysis, *ADPG2* was also expressed in germinating seeds, at the point at which the radicle broke through the seed coat during germination (Figure 6I), a process that involves cell separation (Roberts et al., 2002). No defect in seed germination was observed, presumably because other PGs are also expressed in this tissue (González-Carranza et al., 2007).

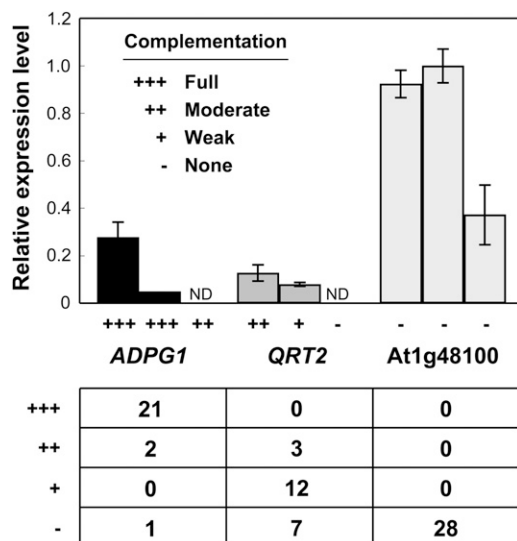
The *QRT2*:GUS reporter was predominantly expressed in the abscission zones of sepals, petals, and stamens in flowers just prior to floral organ abscission (Figure 6C), similar to the reported expression pattern of *ADPG2* and consistent with previous results obtained from a time course of floral organ abscission (Kim and Patterson, 2006). A previous attempt to drive GUS expression by the presumed *At3g07970* (*QRT2*) promoter did not result in detectable GUS activity (González-Carranza et al.,

2007), presumably because this construct used 1492 bp, while we used 2085 bp of 5' sequence. *QRT2*:GUS was also expressed early in anther development, at around the time of microspore separation (Figure 6N), and later just prior to anther dehiscence (Figure 6M). GUS staining was also observed in mature siliques at pod shatter, consistent with qRT-PCR analysis (Figure 5). In contrast with *ADPG1* and *ADPG2* expression in mature siliques, and consistent with no detectable role for *QRT2* in pod shatter, *QRT2*:GUS was expressed only in the vascular bundles in the replum and at the central region of the valve. These data are consistent with the roles for *QRT2* in microspore separation, anther dehiscence, and floral organ abscission described above.

Although expression of both *ADPG2* (González-Carranza et al., 2007) and *QRT2* (Figure 6H) was detected in at the site of lateral root emergence, where cell separation occurs (Roberts et al., 2002), no obvious root phenotype was observed in either single mutant or in the *adpg2 qrt2* double mutant. Finally, the expression of *QRT2* throughout the plant, albeit in very localized regions, is consistent with the defects observed in AT3 flowers and vegetative tissues (Figure 1; see Supplemental Figure 1 online).

### Complementation of the Pod Shatter Defect

To further confirm the biological role of the PG encoded by *ADPG1*, we introduced a construct containing the putative *ADPG1* promoter fused to the *ADPG1* cDNA into the *adpg1-1 adpg2-1 qrt2-2* triple mutant. As the *adpg2 qrt2* double mutant does not have a detectable pod shatter phenotype under normal conditions, complete complementation of the *adpg1* lesion will restore pod shatter to the wild-type. Twenty-one of 24 independent transgenic lines showed normal pod shatter (Figure 7), suggesting that the promoter region used is sufficient for functional expression of the *ADPG1* cDNA. This result also suggests that the intron and 3' untranslated sequences of *ADPG1* (Figure 2A) are not essential for gene function. To investigate possible functional specificity between PGs, we used the same *ADPG1* promoter sequence to drive expression of either the *QRT2* cDNA or the cDNA of *At1g48100*, which encodes a putative PG from a related clade to *ADPG1*/*ADPG2*/*QRT2* (BLAST score for *ADPG1* versus the *At1g48100* gene product is  $6 \times 10^{-68}$ ; Kim et al., 2006). Both constructs were transformed into the *adpg1-1 adpg2-1 qrt2-2* triple mutant and assayed for their ability to restore pod shatter (Figure 7). In 15 of 22 independent *ADPG1*:*QRT2* transgenic lines, the pod shatter phenotype of the *adpg1 adpg2 qrt2* triple mutant was partially complemented. By contrast, none of the 28 *ADPG1*:*At1g48100* transgenic lines assayed were complemented for pod shatter. To eliminate the possibility that the level of complementation was limited by the transcript level of *QRT2* or *At1g48100* driven by the *ADPG1* promoter, we performed qRT-PCR using transgene-specific primers. Compared with transcript levels of *ADPG1* in the triple mutant, those of *QRT2* and *At1g48100* were similar or higher (Figure 7). For the two functional constructs, the transcript levels of *ADPG1* and *QRT2* were correlated with the degree of complementation of the pod shatter phenotype, although a lower level of *ADPG1* expression was required for complementation compared with



**Figure 7.** Complementation of the Pod Shatter Defect.

The cDNAs of *ADPG1*, *ADPG2*, and *At1g48100* were each placed under the control of the presumed *ADPG1* promoter and transformed separately into the *adpg1 adpg2 qrt2* triple mutant. The three constructs were tested for their ability to complement the pod shatter defect due to reduced *adpg1* and *adpg2* activity. The bottom panel shows the number of independent lines for each construct in each complementation category: full (+++), moderate (++), weak (+), and none (-). Transgene-specific primers were used to determine the expression levels of the different cDNAs in three lines of each construct with various degrees of complementation (top panel). Values represent the mean  $\pm$  SE from triplicate qRT-PCR reactions. ND, not detected.

*QRT2*. Thus, based on this assay for pod shatter, *ADPG1* is more active than *QRT2* and *At1g48100* is not active.

### Hormonal Regulation of Floral Organ Abscission

Cell separation events are highly controlled processes, and plant hormones have often been implicated in regulating organ abscission. Consistent with previous reports that ethylene mutants have delayed floral organ abscission (Bleecker and Patterson, 1997; Chao et al., 1997; Patterson, 2001; Patterson and Bleecker, 2004), *ADPG2* expression is increased by exogenous ethylene in the AZ of floral organs (González-Carranza et al., 2002) and genetic analysis revealed that *ADPG2* promotes floral organ abscission (Figure 4; González-Carranza et al., 2007).

Genetic analysis of JA biosynthesis and signal transduction mutants has shown that this hormone plays an important role in anther development and dehiscence. Using a JA-deficient mutant, a recent microarray study revealed that the transcript levels of *ADPG1*, *ADPG2*, and *QRT2* were upregulated  $\sim$ 10-fold after JA treatment of anthers (Mandaokar et al., 2006). This result, together with the delayed anther dehiscence phenotype of the *adpg1 adpg2 qrt2* triple mutant and induction of *QRT2*:GUS expression in *aos* anthers treated with 1 mM methyl jasmonate (Figure 6F), suggests that the defective anther phenotype of JA mutants is partly due to reduced expression of these PG genes.

The results described above raise the possibility that JA and ethylene may act together to regulate different cell separation events, such as floral organ abscission, in part by promoting expression of *ADPG1*, *ADPG2*, or *QRT2*. To test this hypothesis, we examined floral organ abscission in mutants lacking *ETHYLENE INSENSITIVE2* (*EIN2*; Alonso et al., 1999) and *AOS* as described above (Figure 4). Floral organ abscission was delayed in the JA-deficient *aos* and ethylene-insensitive *ein2-1* single mutants and in the *ein2 aos* double mutant, confirming that both hormones promote this cell separation process. The fact that floral organ abscission eventually occurred in the *ein2 aos* double mutant demonstrates that other signals can also promote abscission in the absence of ethylene and JA. Because of its proposed role in a range of senescence/abscission events, the hormone ABA is a good candidate for at least a component of this signal. ABA-deficient mutants, such as those that lack *ABA DEFICIENT2* (*ABA2*) (Cheng et al., 2002; González-Guzmán et al., 2002), display normal floral organ abscission, consistent with important roles for ethylene and JA (Figure 4). To test the relative contributions of these three hormones, we generated the *ein2-1 aba2-2* double and *ein2-1 aos aba2-2* triple mutants. Floral organ abscission was delayed in the double mutant (similar to the *ein2-1* single mutant) and extremely delayed in the triple mutant (Figure 4), demonstrating partially redundant roles for all three hormones in this abscission event. Apart from delayed floral organ abscission, flowers of the *ein2*, *aba2*, and *ein2 aba2* double mutant appeared to develop and senesce normally. By contrast, flower development and senescence was delayed in the *aos*, *ein2 aos*, and *ein2 aos aba2* mutants compared with wild-type plants (Park et al., 2002; von Malek et al., 2002; see Supplemental Figure 5 online).

To determine if ethylene, JA, and ABA can regulate PG expression in floral organ AZs, a segregating F2 population from a cross between the *ein2 aos aba2* triple mutant and a representative *QRT2*:GUS line was examined. Consistent with segregation of the three hormone-related genes, plants in this population exhibited floral organ abscission phenotypes ranging from similar to wild-type to similar to the *ein2 aos aba2* parent (Figure 4E). Approximately 75% of the progeny chosen for analysis carried the *QRT2*:GUS reporter, consistent with the segregation of a single transgene locus. This approach enabled the relationship (if any) between *QRT2* expression and abscission to be determined over a wide range of floral organ abscission phenotypes. A very strong correlation was observed between the position of the youngest flower expressing *QRT2*:GUS in the floral AZ and the number of flowers retaining floral organs after firm pressing (Figure 4E). In all plants examined, *QRT2*:GUS was first expressed either in the floral AZ of the youngest flower in which floral organ abscission occurred or in the AZ of the next-youngest flower just prior to abscission of the floral organs. Supporting a causal role for *QRT2* in cell separation, no plants were observed in which floral organ abscission preceded expression of *QRT2*:GUS in the floral organ AZ. These results suggest that all three hormones are required for normal *QRT2* expression in floral organ AZs. While ethylene may directly regulate *QRT2* expression in the floral organ AZ, JA and ABA may act indirectly via roles in promoting flower senescence.

## DISCUSSION

PGs have been suggested to play critical roles in cell separation in plants during a number of physiological processes involving abscission or dehiscence of plant organs and tissues. For example, an increase in the activity and protein level of PGs can be detected in the cell separation zone just prior to pod dehiscence (Sander et al., 2001). However, despite a range of indirect evidence, relatively little experimental data using loss-of-function alleles of genes known to encode PGs have been reported (Lewis et al., 2006). In addition to 'Flavr Savr' tomato, which has reduced PG expression and delayed fruit softening (Dellapenna et al., 1986; Sheehy et al., 1988; Smith et al., 1990), *QRT3* has been shown to encode a PG required for separation of microspores during pollen development (Rhee et al., 2003). Based on a single loss-of-function allele, González-Carranza et al. (2007) have also recently reported that *ADPG2* promotes floral organ abscission, although these authors did not confirm PG activity for *ADPG2*.

Using recombinant proteins, we have demonstrated that *ADPG1*, *ADPG2*, and At1g48100 encode functional PGs. The ability of an *ADPG1:QRT2* construct to partially complement the *adpg1 adpg2 qrt2* pod shatter phenotype also confirms that *QRT2* is a PG, consistent with its proposed role in pectin degradation (Rhee and Somerville, 1998). Supporting an important role for pectin in plant growth, overexpression of *QRT2* causes dwarfism and male sterility. The simplest explanation for this result is that increased *QRT2* expression leads to abnormal cell-cell adhesion, which inhibits growth and flower function.

As overexpression studies provide only limited information on normal gene function, we have analyzed loss-of-function alleles for *ADPG1*, *ADPG2*, and *QRT2* and identified important physiological roles for these genes in several cell separation events during reproductive development (Table 3). In addition, Jiang et al. (2008) have recently shown that PGs are also involved in leaf abscission in tomato. Taken together, these results support the conclusion that multiple, and possibly all, cell separation/abscission/dehiscence events in plants use a common PG-dependent mechanism. In *Arabidopsis*, combinations of *ADPG1*, *ADPG2*, and *QRT2* appear to be involved in all cell separation processes associated with reproductive development so far examined.

## PGs Are Required for Microspore Separation

Previous genetic analyses have shown that like *QRT1* and *QRT3*, *QRT2* is required during microsporogenesis for the separation of the developing pollen grains (Preuss et al., 1994; Rhee and Somerville, 1998), and we have confirmed this result with two additional loss-of-function *qrt2* alleles. *QRT1* and *QRT3* encode a PME and a PG relatively distantly related to *QRT2*, respectively (Rhee et al., 2003; Francis et al., 2006). Pectins are synthesized in Golgi bodies and secreted from cells in a highly methylesterified form (Micheli, 2001; Schols and Voragen, 2002). PME catalyzes pectin demethylesterification and appears to increase the ability of PG to cleave pectin (Tucker and Seymour, 2002). Consequently, an attractive model for microspore separation is that pectin is first demethylated by *QRT1* and subsequently degraded by PGs, such as *QRT2* and *QRT3*. Although both *QRT2* and *QRT3* encode PGs, they are not functionally redundant because loss of either gene prevents microspore separation. Structural comparison of pollen grain tetrads using scanning electron microscopy of the *qrt* mutants has revealed that *qrt1* and *qrt2* are structurally similar to the wild-type, with the exception of the pollen fusion phenotype. By contrast, *qrt3* pollen grains frequently had a layer of material deposited on the surface of the distal region of the pollen grain tetrads (Rhee et al., 2003), suggesting that *QRT2* and *QRT3* have distinct roles during pollen development. Since the structure of *qrt1* pollen is similar to that of *qrt2*, it is possible that pectin demethylesterified by *QRT1* may be used as a substrate by *QRT2* rather than by *QRT3*.

The *qrt2-1* allele is predicted to encode a mutant protein with a single amino acid substitution (Val to Ala) at position 372 compared with the wild-type *QRT2* protein, suggesting that this amino acid is important for enzyme activity. Val is a highly hydrophobic and aliphatic amino acid, as are Leu and Ile. Phylogenetic analysis reveals that Val, Leu, or Ile at the position corresponding to the *qrt2-1* lesion is highly conserved in PGs from plants and fungi, supporting an important role for this residue in enzyme function. Although the three-dimensional structure of PGII from the phytopathogenic fungus *Aspergillus niger* has been determined, and site-directed mutagenesis used to identify several critical amino acid residues required for activity and substrate binding (van Santen et al., 1999; Armand et al., 2000; Páges et al., 2000), the role of this Val/Leu/Ile residue has not yet been clarified.

**Table 3.** Distinct and Overlapping Roles of *ADPG1*, *ADPG2*, and *QRT2* in Separation Events during *Arabidopsis* Development

Organ	Separation Event	Expression			Mutant Phenotype
		<i>ADPG1</i>	<i>ADPG2</i>	<i>QRT2</i>	
Young anthers	Microspore separation	x	√	√	Tetrad pollen
Mature anthers	Anther dehiscence	√	√	√	Delayed anther dehiscence
Mature flowers	Floral organ abscission	x	√	√	Delayed floral organ abscission
Mature siliques	Pod shatter	√	√	x	Indehiscent silique
Dry siliques	Seed abscission	√	x	x	None observed <sup>a</sup>
Imbibed seeds	Radical emergence	x	√	x	None observed
Roots	Lateral root emergence	x	√	√	None observed

<sup>a</sup>*ADPG1:GUS* expression is not detected in the *hec3-1* mutant in which normal seed abscission does not occur.

√, Expression detected; x, expression not detected.

### PGs Are Required for Pod Shatter and Normal Anther Dehiscence

The two *Arabidopsis* proteins most closely related to QRT2 are encoded by the duplicate genes *ADPG1* and *ADPG2* (Kim et al., 2006). Based on its expression pattern in the DZ of maturing siliques, *ADPG1* has previously been suggested to be involved in pod shatter (González-Carranza et al., 2007). Genetic analysis revealed that both *ADPG1* and *ADPG2* are partially functionally redundant for silique dehiscence (Figures 2 and 3), a conclusion supported by the observation that *ADPG2* is also expressed in the silique DZ prior to pod shatter (Figure 6E). A role for *ADPG1* in pod shatter is also supported by the absence of detectable expression of this gene at the valve margin of the nonshattering *ind* mutant. In addition, although no defect in seed abscission was observed in the *adpg1 adpg2 qrt2* triple mutant, *ADPG1* was not detected in funiculi of the seed abscission-defective *hec3* mutant. This result suggests that *ADPG1*, along with PGs other than *ADPG2* and *QRT2*, are required for seed abscission.

Functional redundancy between related PGs is also observed in anther dehiscence such that the *adpg1 adpg2 qrt2* triple mutant has delayed pollen release, while single and double mutants have no detectable anther defect. The fact that anthers of the triple mutant do eventually dehisce suggests that other PGs may also contribute to this process. Based on phylogenetic and expression analyses (Grennan, 2006; Kim et al., 2006; González-Carranza et al., 2007), *At1g80170* is a good candidate to encode an additional PG involved in anther dehiscence.

### PGs Are Required for Normal Floral Organ Abscission

Floral organ abscission was delayed, but not completely prevented, in the *adpg2* and *qrt2* single mutants and in the *adpg2 qrt2* double mutant (Figure 4), consistent with the expression of both genes in the floral organ AZ (Figure 6). By contrast, *ADPG1* is not expressed in this tissue and does not appear to be required for this abscission process. Based on expression analysis, *At2g43890* and *At2g43880* (Kim et al., 2006) may also be involved in floral organ abscission together with *ADPG2* and *QRT2*.

### Hormones and Floral Organ Abscission

Plant hormones have a number of important roles during flower development, fertilization, and seed development and throughout fruit set, growth, and senescence. Based on available data for the regulation of *ADPG1/ADPG2/QRT2* by JA and ethylene, we investigated the role of these two hormones in organ abscission using the ethylene-insensitive *ein2* and JA-deficient *aos* mutants. Analysis of the *ein2 aos* double mutant revealed that these plant hormones act in a partially redundant manner to promote floral organ abscission. This conclusion is consistent with the expression of *ADPG2* (Figure 6; González-Carranza et al., 2002, 2007) and *QRT2* (Figure 6; Kim and Patterson, 2006) in the floral organ abscission zone, delayed flower organ abscission in plants with reduced *ADPG2* expression (Figure 4; González-Carranza et al., 2007), and the known role of ethylene in promoting floral organ senescence and detachment (Bleecker

and Patterson, 1997; Chao et al., 1997; Patterson, 2001). ABA also promotes floral organ abscission in combination with JA and ethylene as this process was further delayed in an *ein2 aos aba2* triple mutant (Figure 4). As floral organ abscission eventually occurred in the *ein2 aos aba2* triple mutant, and other cell separation events appeared normal in these plants, additional signals that promote cell separation must also exist. Auxin is a good candidate for this signal as it has been suggested to be involved in many, and perhaps all, abscission events. For example, application of auxin or auxin-like molecules can inhibit pod shatter in canola (Chauvaux et al., 1997), and auxin analogs have been shown to inhibit *RDPG1* activity by blocking its secretion into the cell wall (Degan et al., 2001). Finally, auxin mutants have been reported to have delayed floral organ abscission and silique dehiscence in *Arabidopsis* (Ellis et al., 2005; Okushima et al., 2005). Thus, floral organ abscission is controlled by the combined action of at least four different plant hormones that act in part by regulating PG expression.

### Functional Specificity of *Arabidopsis* PGs

Many genes encoding putative PGs have been identified in a number of species, including *Arabidopsis*, tomato, and rice (Hadfield et al., 1998; Kim et al., 2006; González-Carranza et al., 2007). Other cell wall proteins, such as expansin and xyloglucan endotransglycosylase, also consist of multiple gene families in plants (Rose et al., 2002; Sampedro and Cosgrove, 2005). However, in the absence of comprehensive biochemical analyses, it is not known whether members of these gene families, which often display different expression patterns, possess different substrate specificities (Rose et al., 2002; Sampedro and Cosgrove, 2005). For example, cytochrome P450 monooxygenases are also encoded by multiple gene families, with individual genes encoding enzymes with strict substrate specificity (Schuler and Werck-Reichhart, 2003). Our cDNA swapping experiments using the *ADPG1* promoter in the *adpg1 adpg2 qrt2* triple mutant background suggest that closely related PGs can also exhibit substrate specificity (Figure 7). While the *ADPG1:ADPG1* construct was able to fully restore pod shatter, *ADPG1:QRT2* (BLAST score for *ADPG1* versus the *QRT2* gene product is  $10^{-104}$ ) only partially restored pod shatter in *adpg1 adpg2 qrt2* plants. Furthermore, when the same promoter was used to drive expression of the *At1g48100* cDNA (BLAST score for *ADPG1* versus the *At1g48100* gene product is  $6 \times 10^{-68}$ ), no restoration of pod shatter was observed. Although we cannot formally exclude defects in translation or posttranslational processing in these experiments, these results suggest the existence of strict substrate specificity or substrate accessibility for closely related PGs. Consistent with limited pectin substrates for *ADPG1* and *ADPG2* in planta, it appears that the majority of *JIM5*-recognized pectin in cell walls of the silique DZ is not degraded during pod shatter (Figure 3).

### Agricultural Importance of Abscission Events

The roles for PGs and plant hormones described here have important practical implications for agriculture and horticulture. For example, preventing or delaying floral organ abscission is of



great potential interest to the cut flower industry. More generally, abscission/dehiscence associated with the harvest of seeds and/or fruit is also a major production issue in a wide range of crops. One of the best known examples is pod shatter, which can cause seed loss prior to harvest and is an important problem for several crops, particularly canola (oil seed rape). Reducing the activity of canola genes closely related to *ADPG1* and *ADPG2* (Sander et al., 2001; González-Carranza et al., 2002) may lead to siliques that do not shatter as readily as normal siliques, reducing seed losses.

## METHODS

### Plant Materials and Growth Conditions

*Arabidopsis thaliana* ecotypes Col-0 or *Ler* were used in this study. The *aos* (accession number CS6149) and *ein2-1* (CS3071) mutant seeds were obtained from the ABRC (<http://www.biosci.ohio-state.edu/~plantbio/Facilities/abrc/abrchome.htm>). The *aba2-2* seeds were obtained from Eiji Nambara (University of Toronto, Canada). Plants were grown under long-day conditions (16 h light/8 h dark) in a 22°C growth room either on soil (equal volumes of seed raising mix [Debco] and perlite) with standard *Arabidopsis* nutrient solution (<http://www.biosci.ohio-state.edu/pcmb/Facilities/abrc/handling.htm>), on Growool (Swain et al., 2004), or on agar medium consisting of 0.5× MS salts, 1% (w/v) sucrose, and 0.8% agar for selection of transgenic plants or 0.6% phytigel (Sigma-Aldrich) for detection of GUS staining in roots.

### Activation Tagging Mutant Screening and TAIL-PCR

We generated several hundred activation tagging lines in the *Ler* background using *Agrobacterium tumefaciens*-mediated transformation and the vacuum infiltration method (Bechtold et al., 1993) with the activation tagging vector pSKI015 (Weigel et al., 2000). Seeds from infiltrated plants were sown on Growool and sprayed with Basta (Swain et al., 2004) to identify transgenic seedlings that were screened for novel phenotypes. To identify the T-DNA insertion site, TAIL-PCR was performed according to Liu et al. (1995) using genomic DNA extracted from AT3 inflorescences with the PhytoPure plant DNA extraction kit (Amersham Life Sciences) and primers SKIL1 (5'-ACGACGGATCGTAATTTGTGCG-3'), SKIL2 (5'-TTC-ATTTTATAATAACGCTGCGG-3'), or SKIL3 (5'-CTTCTTTCTCCAT-ATTGACC-3') for the pSKI015 left border sequence and the AD2 degenerate primer (Liu et al., 1995).

### Identification of T-DNA Insertion Mutants

T-DNA insertion mutants were obtained from the ABRC for SALK lines (Alonso et al., 2003) or the Max-Planck institute for the GABI-Kat line (Rosso et al., 2003). Alleles were named as follows: *adpg1-1* (SALK\_034714), *adpg1-2* (SALK\_057704), *adpg2-1* (SALK\_035098), *adpg2-2* (GABI\_289C07), *qrt2-2* (SALK\_132478), and *qrt2-3* (SALK\_031337). Homozygous T-DNA insertion mutants were identified using PCR with gene-specific primers (see Supplemental Table 2 online). The primer complementary to the T-DNA was SALK Lba1 (5'-TGG-TTCACGTAGTGGGCCATCG-3') for SALK lines and GABI LB (5'-CCC-ATTTGGACGTGAATGTAGACAC-3') for the GABI-Kat line. Genomic DNA was isolated by standard methods (Neff et al., 1998). PCR was performed using rTaq DNA polymerase (Invitrogen).

### RNA Extraction and Analysis

RNA was extracted using the phenol/SDS method (Naito et al., 1994). Total RNA (1 μg) was treated with DNase I (Promega) to eliminate

genomic DNA contamination. After inactivation of DNA polymerase I activity by adding EDTA (2.5 mM final concentration) and heating at 65°C for 10 min, first-strand cDNA was synthesized from DNase I (Amplification Grade; Invitrogen) treated total RNA with random hexamers using a SuperScript III reverse transcriptase according to the manufacturer's instructions (Invitrogen). For RT-PCR, PCR was performed using first-strand cDNA as a template with gene-specific primers (see Supplemental Tables 3 and 4 online) followed by agarose gel electrophoresis to quantify the amount of PCR product. qRT-PCR with the SYBR Green I dye method was performed using the first-strand cDNA as a template with gene-specific primers (see Supplemental Tables 3 and 4 online) on a sequence detector system (My iQ single color real-time PCR detection system Model: MyiQ Optical Module; Bio-Rad). The mean value of three biological replicates was normalized using an 18S rRNA as the internal control with primers 18S rRNA For (5'-GGGACAGTCGGGGGCATTTCG-3') and 18S rRNA Rev (5'-TCCGCTGATCCCTGGTCCGGC-3').

### Plasmid Construction and Transformation

We used the Gateway system (Invitrogen) for plasmid construction, and the sequences of all plasmids used in this study were examined to ensure that no mutations were introduced during plasmid construction.

The *QRT2* cDNA and *ADPG1* cDNA were cloned into the pENTR vector (Invitrogen) to create pENTR-QRT2 and pENTR-ADPG1, respectively. The 35S enhancer sequence was fused upstream of the *QRT2* genomic fragment consisting of 2085 bp of *QRT2* promoter sequence from the predicted translational initiation site and the *QRT2* gene with introns and exons to create pENTR-35S enhancer-ProQRT2:QRT2. The *ADPG1* promoter, 2433 bp upstream from the predicted translational initiation site, was fused upstream of the *ADPG1* cDNA, *QRT2* cDNA, and At1g48100 cDNA to create pENTR-ProADPG1:ADPG1, pENTR-ProADPG1:QRT2, and pENTR-ProADPG1:At1g48100, respectively. The GUS coding region was fused downstream of the *ADPG1* promoter (2433 bp upstream from the translation initiation site), the *ADPG2* promoter (2177 bp upstream from the translation initiation site), and the *QRT2* promoter (2085 bp upstream from the predicted translation initiation site) to create pENTR-ProADPG1:GUS, pENTR-ProADPG2:GUS, and pENTR-ProQRT2:GUS, respectively. Precise procedures for constructing these plasmids are described in the Supplemental Methods online.

To generate binary vectors for plant transformation, an LR reaction was performed with the binary vector for the Gateway system, pGWB1, in which a target gene from pENTR can be introduced before the nopaline synthase terminator. The resulting binary vectors, 35S enhancer-ProQRT2:QRT2, ADPG1:GUS, ADPG2:GUS, QRT2:GUS, ADPG1:ADPG1, ADPG1:QRT2, and ADPG1:At1g48100, were transformed into wild-type Col-0 or the *adpg1-1 adpg2-1 qrt2-2* mutant using the *Agrobacterium*-mediated floral dip method (Clough and Bent, 1998). Plants carrying a T-DNA insertion were selected on agar plates containing 20 mg/L kanamycin (Sigma-Aldrich), 20 mg/L hygromycin (Sigma-Aldrich), and 50 mg/L carbenicillin (Sigma-Aldrich).

### Scanning Electron Microscopy of Pollen Tetrad

Pollen from the *adpg1 adpg2 qrt2* triple mutant was mounted on double-sided carbon tabs, coated with ~20 nm gold using an Emitech K550X sputter coater, and viewed at 15 kV accelerating voltage in a Zeiss EVO LS15 scanning electron microscope.

### Light Microscopy

Tissue was fixed in 2% glutaraldehyde in 25 mM sodium phosphate buffer at pH 6.8, vacuum infiltrated, and incubated at 4°C overnight. Tissue was briefly rinsed in 25 mM sodium phosphate buffer, pH 6.8, and dehydrated

in an ethanol series (25, 50, 70, 95, and 100%), with 2 h in each solution. Tissue was run through an LR white resin/ethanol mix (25:75, 50:50, 75:25, 100:0  $\times 2$ ) and finally embedded in LR white resin. Using a microtome, 2- $\mu\text{m}$  cross sections were cut and placed on glass slides. These were stained with 0.3% Toluidine Blue in 1% sodium tetraborate, pH 9.0, for 30 s or in 0.02% ruthenium red, then water mounted and covered with a cover slip for observation by light microscopy.

For GUS assays, plant material was gently fixed by incubation in 90% acetone on ice for 15 min, and the tissue washed in sodium phosphate buffer, pH 7.2. The tissue was then incubated in a 3 mM GUS staining solution consisting of 3 mM  $\text{K}_3\text{Fe}(\text{CN})_6$ , 3 mM  $\text{K}_4\text{Fe}(\text{CN})_6$ , 2 mM 5-bromo-4-chloro-3-indolyl- $\beta$ -D-glucuronic acid, and 50 mM  $\text{NaPO}_4$ , pH 7.2, vacuum infiltrated, and incubated at 37°C for 16 h. For detection of ADPG2:GUS and QRT2:GUS in anthers and siliques, 0 mM  $\text{K}_3\text{Fe}(\text{CN})_6$ /0 mM  $\text{K}_4\text{Fe}(\text{CN})_6$  was used to increase sensitivity. After staining, tissues were fixed in 50% ethanol, 5% acetic acid, and 3.7% formaldehyde and then washed several times with 70% and then 85% ethanol. For making cross sections after staining, the tissues were processed through an ethanol series (30, 50, 70, 85, 95, and 100%), with the 50% step incorporating a 1-h fixative wash (50% ethanol, 5% acetic acid, and 3.7% formaldehyde). Tissue was run through a Lemosol/ethanol gradient (25:75, 50:50, 75:25, 100:0  $\times 2$ ) and finally embedded in Paraffin. Using a microtome, 7- $\mu\text{m}$  cross sections were cut and placed on glass slides. The tissue was examined using a microscope.

#### Preparation of Siliques for Light and Electron Microscopy

*Arabidopsis* siliques were fixed in 2.5% glutaraldehyde (EM Grade) in PBS, pH 7.2, for 2 h at room temperature and stored overnight at 4°C. Siliques were washed three times in distilled water and then dehydrated in a graded ethanol series. Over several days, the samples were infiltrated with LR White resin, and then individual siliques were placed in gelatin capsules, filled with fresh resin, and polymerized in a 55°C oven overnight.

#### Sectioning and Toluidine Blue Staining of Dehiscing Siliques

Individual siliques in resin blocks were sectioned (1  $\mu\text{m}$ ) transversely on a Leica Ultracut R microtome. The sections were collected and dried onto glass microscope slides and stained with a 2% (w/v) aqueous Toluidine Blue solution for 30 s, washed, and dried on a hotplate.

#### Immunolabeling with Gold-Labeled Pectin Antibodies for TEM

Ultrathin sections (80 nm) were cut on a Leica Ultracut R microtome using a diamond knife and collected on 100 mesh formvar-coated gold grids. The grids were preincubated in a blocking buffer (1% BSA in PBS) for 30 min at room temperature to prevent nonspecific antibody binding. Two monoclonal antibodies raised against pectic polysaccharides, JIM5 and JIM7 (Knox et al., 1990; Plant Probes) were diluted 1:50 in blocking buffer. The grids were incubated in drops of the diluted primary antibody for 1 h at room temperature and then overnight at 4°C. The grids were washed three times in PBS and two times in blocking buffer and then incubated in a 1:20 dilution of an anti-rat secondary antibody conjugated to 18-nm colloidal gold (Jackson ImmunoResearch). Grids were washed in PBS and several times in water before poststaining in 2% (w/v) aqueous uranyl acetate. The sections were viewed on a Philips BioTwin transmission electron microscope and images captured on a Gatan multiscan digital camera. In control experiments, the primary antibody was omitted and no labeling was observed.

#### Functional Assay of PG Proteins

Truncated versions of the PG proteins, lacking the hydrophobic N-terminal region encoding a signal peptide predicted by the SOSUI program (Hirokawa et al., 1998; <http://bp.nuap.nagoya-u.ac.jp/sosui/>)

were expressed as His-tagged fusion proteins in *Escherichia coli*. GFP and N-terminal truncated versions of ADPG1, ADPG2, QRT2, and At1g48100 were cloned into the pENTR vector (Invitrogen) to create pENTR-GFP, pENTR-tADPG1, pENTR-tADPG2, pENTR-tQRT2, and pENTR-tAt1g48100, respectively. GFP was used as a negative control. Details of these plasmid constructions are provided in the Supplemental Methods online. A Gateway LR reaction was performed using pENTR-GFP, pENTR-tADPG1, pENTR-tADPG2, pENTR-tQRT2, and pENTR-tAt1g48100 with pDEST17, which is Gateway *E. coli* expression vector that provides a 6x Histidine tag at the N terminus of the expressed target gene driven by the T7 promoter (Invitrogen). The resulting plasmids, pDEST17-His-GFP, pDEST17-His-tADPG1, pDEST17-His-tADPG2, pDEST17-His-tQRT2, and pDEST17-His-tAt1g48100, were transformed into *E. coli* strain origami B (DE3) (Novagen) that is able to provide a soluble and active enzyme with proper disulfide bond formation (Shimizu et al., 2005). Soluble PG protein was purified using a Ni-NTA column (Ni-NTA agarose; Qiagen) according to the manufacturer's protocol. Purified PG solution was dialyzed against a 150 mM sodium chloride/50 mM sodium acetate, pH 5.0, solution. The standard curve was determined by the use of galacturonic acid (Sigma-Aldrich), and PG activity was measured according to Gross (1982) using polygalacturonic acid from citrus fruit (Sigma-Aldrich) as the substrate. One unit of the endo-PG activity was defined as the amount of enzyme that liberated 1  $\mu\text{mol}$  of reducing end groups per 1 min at pH 5.0 and 30°C. Specific activity was calculated from three independent samples.

#### Accession Numbers

Sequence data for the genes described in this article can be found in the Arabidopsis Genome Initiative under the following accession numbers: *ADPG1* (At3g57510), *ADPG2* (At2g41850), *QRT2* (At3g07970), and *GAPC* (At3g04120).

#### Supplemental Data

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

**Supplemental Figure 1.** Phenotype of the AT3 Line.

**Supplemental Figure 2.** *qrt2-1* Causes a Single Amino Acid Substitution in the Predicted QRT2 Protein without Altering the Transcript Level.

**Supplemental Figure 3.** Reduced Transcript Levels of *ADPG1*, *ADPG2*, and *QRT2* in the *adpg1 adpg2 qrt2* Triple Mutants.

**Supplemental Figure 4.** *adpg1-1* Reduces Transcript Levels and Causes an In-Frame Deletion.

**Supplemental Figure 5.** Delayed Abscission of Floral Organs in PG and Hormone Mutants.

**Supplemental Table 1.** Phenotypes of *adpg1*, *adpg2*, and *qrt2* Single, Double, and Triple Mutants.

**Supplemental Table 2.** Primers for Genotyping T-DNA Insertion Mutants.

**Supplemental Table 3.** Primers for RT-PCR to Examine the Transcript Level in T-DNA Insertion Mutants.

**Supplemental Table 4.** Primers for RT-PCR to Examine Target Gene Expression.

**Supplemental Methods.** Plasmid Construction.

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