Expression of polygalacturonases and evidence to support their role during cell separation processes in Arabidopsis thaliana

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Abstract
Polygalacturonases (PGs) have been proposed to play an important role in the process of cell separation. The Arabidopsis thaliana genome contains 69 annotated genes that by amino acid homology and transcript organization could be classified as putative PGs and these can be grouped into multiple clades. An analysis of five members located in two separate clades, using reporter fusion constructs and reverse transcription-PCR, revealed that whilst these PGs exhibit high sequence similarity they have distinct patterns of spatial and temporal expression. Sites of expression include the aleurone and endosperm cells surrounding the emerging radicle in a germinating seed, the cortical cells adjacent to the developing lateral root, the abscission zones of floral organs, the dehiscence zone of anthers and siliques, and pollen grains. Silencing of an abscission-related PG (At2g41850), using a T-DNA insertion strategy, delayed the time-course of floral organ loss but did not prevent shedding from taking place. These observations are discussed with regard to the contribution that PGs may play during the life cycle of a plant.

Key words: Abscission, Arabidopsis thaliana, cell separation, dehiscence, gene expression, lateral root development, phylogenetic tree, polygalacturonase, seed germination.

Introduction
Most plant cells are surrounded by a rigid wall that serves to provide both protection and support. The wall comprises a matrix of complex carbohydrates and proteins bonded together to provide considerable tensile strength, whilst at the same time having the flexibility to be distended so that growth and development can take place (Carpita and Gibeaut, 1993). A number of models have been proposed to account for wall structure; however, there is still not a complete understanding of its construction and, in particular, the key differences that may exist between the walls surrounding cells from different organs or tissues (O’Neill and York, 2003).

Although the architecture of the cell wall provides developmental flexibility there are times during the life cycle of a plant where extensive modifications are necessary to reduce, or remove entirely, the constraints that it imposes (Cosgrove, 1999). For instance, prior to the shedding of a plant organ sufficient wall remodelling must be achieved to enable cell separation to take place (Roberts et al., 2002) whilst during the ripening of a fleshy fruit a spectrum of cellular and biochemical events bring about softening of the pericarp or mesocarp tissues (Rose et al., 2003).

The activity of a number of cell wall-degrading enzymes, including polygalacturonases (PGs) and β-1,4-glucanases, has been shown to increase during cell separation (Peterson et al., 1995; Lashbrook et al., 1998; Rose et al., 2003). Other proteins, such as expansins (Brummell et al., 1999; Anjanasree and Bansal, 2003; Belfield et al., 2005), have also been proposed to play a role in wall breakdown. Expression profiles of genes encoding these wall-softening agents have revealed that transcript accumulation precedes cell separation processes such as abscission, ripening, and dehiscence both at a temporal and a spatial level (Jenkins et al., 1996; González-Carranza et al., 2002; Rose et al., 2003). Such correlations suggest that it may be possible to identify sites where cell wall disassembly is taking place.
by studying the expression profiles of key cell wall-degrading enzymes.

In silico analysis of the Arabidopsis genome sequence has revealed that there are a substantial number of putative PGs based on sequence similarity (Henrisat et al., 2001; Girke et al., 2004; Kim et al., 2006; http://bioinfo.unc.edu/projects/Cellwall/index.pl). Whilst it is not known whether all of these peptides are expressed, or even have polygalacturonase activity, their predicted amino acid sequences are sufficiently homologous to suggest that PGs comprise a substantial gene family and this raises the question of why so many members exist and what function(s) they may have during Arabidopsis development. Expression analysis using reverse-transcription-polymerase chain reaction (RT-PCR) has demonstrated that transcript accumulation of PG family members can be detected in roots, leaves, pollen tubes, flowers, and siliques throughout the development of an Arabidopsis plant (Torki et al., 1999, 2000; Sander et al., 2001). A semi-quantitative RT-PCR analysis has been published recently of 66 PG gene family members in Arabidopsis (Kim et al., 2006). Whilst this comprehensive study has generated a wealth of valuable information it does not provide a cellular characterization of the sites of PG expression. Data on this scale are currently provided by the GUS/GFP reporter gene approach carried out by González-Carranza et al. (2002) who revealed that At2g41850 (PGAZAT) and At3g57510 (PGDZAT) are up-regulated within the dehiscence zone cells of both anthers and pods, and at the site of seed abscission (Jenkins et al., 1999; Roberts et al., 2002). The correlation between the cellular site of expression of these two genes and the onset of organ separation supports a role for these PGs in wall breakdown, however, this has yet to be proven. By contrast, it has been shown that T-DNA silencing of At4g20050 (the QUARTET3 gene) prevents degradation of the pollen mother cell wall, moreover, when the QRT3 peptide is expressed in yeast it exhibits PG activity (Rhee et al., 2003).

The work described in this paper examines whether five individual members of the PG gene family are expressed at multiple locations within a plant and whether genes that exhibit close sequence similarity have overlapping patterns of expression. The consequence of silencing an individual family member is also explored to identify the role that this PG may play during Arabidopsis development.

Materials and methods

Plant materials and growth conditions

Seeds of Arabidopsis ecotypes Columbia-0 or Wassilewskija were grown in 3:1 v/v Levington compost (Levington professional F2 fine structure-medium nutrients standard pH without sand): vermiculite mix. Plants were grown under greenhouse conditions with supplementary lighting to generate a photoperiod of 16 h of light at 22 °C.

Phylogenetic tree

A multiple protein sequence alignment was generated with the available amino acid sequences from putative polygalacturonases from Arabidopsis thaliana (from TAIR site: http://arabidopsis.org/) using the program ClustalW from the European Bioinformatics Institute http://www.ebi.ac.uk/clustaw (Higgins et al., 1994) and the guide tree information was imported to generate a Phylogenetic tree using the program Phylodendrum (DG Gilbert version 0.8d Software which was developed at the Genome Informatics Lab of Indiana University Biology Department). Bootstrap values were generated using the ClustalW Multiple Sequence Alignment Program (version 1.83XP) as described by Chenna et al. (2003).

Prediction of the structure of PG genes was inferred from the available information from the genome sequence from Arabidopsis and from previous studies on At2g41850 (PGAZAT) and At3g57510 (PGDZAT). The tomato gene structure of PGA4 (Accession No AF001002) was obtained from the ENTREZ site.

Plasmid construction and plant transformation

Details of the promoter fusion from At2g41850 and At3g57510 genes were described previously (Jenkins et al., 1999; González-Carranza et al., 2002). The promoters from PGs At2g43860 and At1g80170 were amplified by polymerase chain reaction (PCR) from genomic DNA using Platinum PfX DNA polymerase from Invitrogen following the manufacturer's instructions. A 1495 bp fragment containing the promoter of from At2g43860 and a 1478 bp fragment of from the promoter of from At1g80170 were amplified with primers containing the restriction sites HindIII and BamHI. For At2g43860: PG64020F-HindIII 5'-GGTAAGCTTTGTCAGCCCTAATAC-3' and PG64020R-BamHI 5'-CCTGGGCTGGATCCACATTCGTTTCGAG-3', for At1g80170: 55489HindIIIIF 5'-CCCAACAAAGCTTGTCTCCGCGCGTGACCC-3' and 55489BamHI 5'-CTAGGGATCCGAACGGAATGGAATTAG-3'. The PCR parameters used were: 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 60 °C for 1 min, and 72 °C for 1.5 min, and a final elongation step at 72 °C for 7 min. The PCR products were sub-cloned in P-GEM T-Easy from Promega, digested, and fused to the binary vectors pBI101 or pBI101.2, respectively. The β-glucuronidase gene is downstream of the multiple cloning site. The integrity of plasmids generated was confirmed by sequencing, electroporation into the Agrobacterium tumefaciens C58 strain, and growing to an OD600 of 0.5–0.8. Arabidopsis plants from ecotype Columbia-0 (for At2g43860) or Wassilewskija (for At1g80170) wild type were transformed using the ‘floral dip’ method described by Clough and Bent (1998).

Selection of transformants was carried out in a growth room at 22 °C using Petri dishes containing 4.33 g l⁻¹ of Murashige and Skoog basal salt mixture (MS) pH 5.9, 0.8% (w/v) agar, and kanamycin 40 μg ml⁻¹. Transformation was confirmed by PCR using a primer in the GUS gene and the forward primer from the promoter sequence. T2 seeds were collected from individual lines and screened for kanamycin resistance to identify homozygous lines.

GUS analysis

Different stages of development including germinating seeds, seedlings, roots rosette leaves, cauline leaves, senescent leaves, buds, young flowers, green siliques, mature siliques, senescent siliques, and drying seeds were collected and incubated at 37 °C for 18 h in GUS substrate. Material was then placed in 100% (v/v) ethanol to remove chlorophyll. The GUS staining buffer contained 200 mM phosphate buffer pH 7.2, 0.1% (v/v) Triton X-100, and...
GUS analysis
Gene expression in the ProAt2g41850::GFP lines was examined using a Leica (TCS SP2) laser scanning confocal microscope equipped with argon krypton and green HeNe lasers and anAOBS scan head system (Leica Microsystems, Bannockburn, IL). GFP was excited at 488 nm with the argon ion laser. Images were recorded using the Leica CONFOCAL software.

Tissue preparation from microscopy
Some GUS-stained tissue was infiltrated with LR White Acrylic resin (London resin Co Ltd) and stored at 4°C. Tissue was embedded in gelatine capsules and the resin polymerized at 60°C for 24 h. Sections of approximately 10 µm were cut using a Reichert OMU2 ultramicrotome with a glass knife and were floated on 30% (v/v) acetone solution before drying onto a microscope slide. Sections were mounted permanently in Eupler mountant and visualized under dark field using a Nikon microscope and Lucia G software (Laboratory Imaging, Cambridge, UK Ltd) for image capture.

RT-PCR analysis of gene expression in promoter::GUS fusion plants
To confirm that the expression of the GUS gene was acting as a reliable indicator of endogenous expression, total RNA was extracted from different frozen tissues from both Columbia-0 wild-type plants and homozygous transgenic lines for each gene studied including cauline leaves, abscission zones excised from floral organs, roots, germinating seeds, young flowers, and siliques. Tissues (0.25–0.5 g) were homogenized using the SV Total RNA isolation system from Promega, following the manufacturer’s instructions and quantified with a Nanodrop ND-1000 Spectrophotometer and visualized on a 1% (w/v) agar plate, with emergence (12 h) were excised from plants grown under controlled conditions and immediately placed onto a 1% (w/v) agarose gel. Two micrograms of total RNA were used to make cDNA using SuperScript II RNase H–RT (Invitrogen) following the manufacturer’s instructions. One-step RT-PCRs were performed and each 50 µl reaction contained 1.75 mM MgCl2, 0.8 mM dNTPs, 5 mM DTT, RT-PCR buffer, 10 units RNase inhibitor, 15 units of MultiScribe reverse transcriptase, 2.5 units AmpliTaq Gold DNA Polymerase, and At2g41850-specific primers, AZPGRF (5’-ATGCCGGTTAACATCTATACGGTC-3’) and AZPGRTR (5’-GCTTTACACATCCCAAGTGTCAC-3’) at a final concentration of 0.15 µM (these primers amplify products of the following size: genomic DNA: 950 bp and cDNA: 450 bp). The amplification conditions used were 42°C for 2 min, 95°C for 10 min, then 43 cycles of 94°C for 20 s, 62°C for 1 min, followed by 72°C for 7 min. Control reactions without the addition of any MultiScribe reverse transcriptase were also performed using exactly the same conditions, to confirm the absence of any contaminating genomic DNA in the reactions.

Screening of the Sainsbury Laboratory Arabidopsis thaliana dSpm transposants (SLAT) population and isolation of an At2g41850 knock out
Seeds for the SLAT line pools were obtained from the Nottingham Arabidopsis Stock Centre (NASC, http://arabidopsis.org.uk). The screening of the SLAT population was performed by Inverse PCR (iPCR) and products were fixed in a filter. Hybridization of the filter was performed with a gene-specific probe using the PCR DIG probe synthesis kit (Boehringer Mannheim) as instructed by the manufacturer. Semi-nested PCR was employed with genomic DNA isolated from positive pools and single plant lines containing the insertion were isolated using transposon-and gene-specific primers. Azygous (negative segregant) control plants were also isolated from the population.

Phenotypic analysis of knockout plants
Plants used for phenotypic analysis were grown in controlled growth rooms as described above. Branches of individual plants (both At2g41850 knockout and azygous controls) were tagged and the time taken for approximately 400 individual flowers to progress from petal emergence to floral organ abscission was recorded by assessing the plants at 12 h intervals. Plants were rotated within the growth room to eliminate any positional effects. In each of the above experiments, the analysis was repeated on three separate occasions.

Ethylene and air-treatment of excised flowers
Flowers of the same developmental stage (immediately after petal emergence) were excised from plants grown under controlled conditions and immediately placed onto a 1% (w/v) agar plate, with the pedicel of each flower being pushed into the agar. Two plates were used, each made up of three sections with each section containing flowers from the At2g41850 knockout or the azygous line. One plate was placed into a sealed glass chamber containing 10 µl 10−1 ethylene, the other plate being incubated in a sealed container with potassium permanganate to provide an air (without ethylene) environment. The number of flowers that had undergone floral abscission was recorded independently for each different plant line at 3 h intervals. The percentage of flowers that had undergone abscission at each time point was then calculated separately for the
knockout and azygous plants. This experiment was repeated on four separate occasions to check the consistency of the results.

Results

Generation of a polygalacturonase phylogenetic tree

To generate the phylogenetic tree, putative predicted protein sequences from PG genes were retrieved from The Arabidopsis Information Resource (TAIR http://arabidopsis.org/) after interrogating the entire genome database with the amino-acid sequence of At2g41850 (PGAZAT). The sequences were aligned using the ClustalW program from the European Bioinformatics Institute http://www.ebi.ac.uk/clustalw (Higgins et al., 1994), and the inferred amount of evolutionary change was used to draw a phylogenetic tree with the PhyloPendulum program (Genome Informatics Lab of Indiana University Biology Department) (Fig. 1).

The phylogenetic tree groups the sequences into a number of clades based on amino acid homology and sequence divergence and concurs with that generated by the Cell Wall Navigator Database (http://bioinfo.ucr.edu/projects/Cellwall/index.pl). The abscission-related (At2g41850) and the dehiscence-related (At3g57510) PGs cluster into a distinct group with At3g07970 and At1g80170 and all four family members share a common gene structure comprising nine exons and eight introns (Kalaitzis et al., 1997) have a contrasting gene structure comprising four exons and three introns. The most homologous Arabidopsis PGs to these tomato genes fall into a different clade with At2g43860 exhibiting the greatest amino acid sequence homology (56%) to TAPG4 (see Supplementary Fig. S1A–D at JXB online). The abscission-related PGs identified in tomato TAPG1, 2, 3, and 4 (Kalaitzis et al., 1999) have a contrasting gene structure comprising four exons and three introns. The most homologous Arabidopsis PGs to these tomato genes fall into a different clade with At2g43860 exhibiting the greatest amino acid sequence homology (56%) to TAPG4 (see Supplementary Fig. S1E, F at JXB online).

Spatial and temporal expression patterns of Arabidopsis PGs

In order to address the question of whether members that have close homology might have a similar function or be expressed in comparable tissues, the expression of At2g41850 (PGAZAT), At3g57510 (PGDZAT), At3g07970, and At1g80170 was examined by fusing the promoter of these genes to GUS and studying the accumulation of this reporter protein in three different homoyzous transgenic lines for each construct. Promoter activity of At3g57510 had previously been shown to be restricted to the dehiscence zone of anthers and pods and the seed:funiculus abscission zone when visualized in Brassica napus through the GUS reporter gene (Jenkins et al., 1999). The spatial and temporal expression pattern of At2g43860 was also examined as an example of a PG located in a distinct clade but sharing sequence homology with a gene from another species (i.e. tomato) that is expressed during abscission.

At2g41850 (PGAZAT) expression

Fusion of 1476 bp of the promoter of At2g41850 to GUS revealed expression of this reporter gene at the base of cauline leaves, anther filaments, petals, and sepal at the time of shedding (Fig. 2A–C). Further examination of this material identified that expression was also consistently observed at the site of lateral root emergence (Fig. 2D–H). A detailed analysis of ProAt2g41850::GFP lines revealed that expression was restricted to the cortical and epidermal cells surrounding the emerging lateral root (Fig. 2G, H). Accumulation of GFP could also be found in the basal cell of trichomes from senescing leaves (Fig. 2I).

At3g57510 (PGDZAT) expression

Fusion of 1408 bp of the promoter of At3g57510 to GUS was sufficient to drive expression of this reporter gene in the dehiscence zone cells of anthers and siliques (Fig. 3A, B) thus confirming the observations made by Jenkins et al. (1999) using B. napus.

At3g07970 expression

When a 1492 bp fragment of the promoter of this gene was fused to GUS no consistent expression of the reporter gene could be located at any specific location even after a detailed analysis of a substantial number of transgenic lines was undertaken.

At1g80170 expression

A 1478 bp fragment of the promoter from At1g80170 when fused to GUS resulted in reporter expression in young, mature, and dehiscing anthers and siliques (Fig. 3C–E). Expression was not found within roots, or the abscission zone tissues of floral organs. However, GUS-stained pollen grains were invariably retained at the sepal:petal junction at the base of the flower until the floral organs were shed (Fig. 3E) and on the stigmatic surface (Fig. 3F). Sections of young flowers revealed that GUS staining could be found not only in the pollen grains but also in some tissues of the anther locules (Fig. 3G).

At2g43860 expression

To identify sites of At2g43860 expression 1492 bp of the promoter of this gene was fused to GUS and used to transform plants. Analysis of independent homozygous lines showed that expression could be detected in developing and germinated seeds (Fig. 4A, B). A more detailed anatomical examination of the latter revealed that expression took place in the chalazal region immediately adjacent to the site of penetration of the radicle through the testa and that the aleurone and endosperm cells...
Fig. 1. Phylogenetic tree generated using amino acid sequences of putative polygalacturonases from Arabidopsis thaliana. The branch lengths are proportional to the amount of inferred evolutionary change and boot strap values are included. Scale indicates 0.1 distance from the root of the tree. A tick indicates genes whose expression has been examined in this study. QRT, QUARTET3 gene.
showed signs of undergoing separation from one another to facilitate root emergence (Fig. 4C, D).

**RT-PCR analysis of gene expression in promoter:GUS fusion plants**

In order to confirm that the transcripts of the different PG genes were accumulating in the tissues identified by the reporter gene experiments, RT-PCR analysis was performed with primers designed to amplify either the specific PG of interest or the GUS gene from transgenic lines. The PG primers were designed either close to, or within, the untranslated regions of the transcript to eliminate the chances of amplification from other PG family members. Moreover, sites of primer binding were located outside introns so that contamination by genomic DNA could be detected in the RT-PCR amplifications.

Using *At2g41850*-specific primers a transcript of 1.36 kb could be amplified from cDNA generated from RNA extracted from AZ tissues of cauline leaves and floral organs, and root tissues. Using primers designed to detect expression of *At2g43860* a band of 1.14 kb was amplified from RNA extracted from germinating seeds, while expression of *At1g80170* was confirmed by carrying out RT-PCR using specific primers on cDNA made from RNA isolated from young flowers. A band of 1.3 kb was also amplified from RNA extracted from floral AZ tissues and mature siliques. Expression in transgenic plants expressing GUS was confirmed using primers specific for the β-glucuronidase gene in tissues extracted from AZ for *At2g41850*, germinating seeds from *At2g43860* and young flowers from *At1g80170*. All the amplified products were sequenced to confirm that the transcripts corresponded to the PG under scrutiny (Fig. 5).
Isolation and characterization of a knockout line of At2g41850

In order to examine the consequences of silencing At2g41850 on Arabidopsis development, a knockout (KO) line of this gene was isolated from the SLAT collection (Sainsbury Laboratory Arabidopsis thaliana dSpm Transposants) (Tissier et al., 1999).

A SLAT filter containing iPCR products representing the transposon flanking sequences from approximately 50 550 individual Arabidopsis transposants was screened and a pool representing 50 plants was shown to have an insertion into the gene of interest. Seeds from this pool were obtained and out of 200 plants tested four individuals were found to contain the insertion. Further analyses revealed that the insertion was located within the ORF in Exon 5, 1048 bp downstream of the ATG (see Supplementary Fig. S1A at JXB online). As these plants were heterozygous they were allowed to self and a homozygous KO line along with an azygous (negative segregant) line were identified and studied in subsequent experiments.

In order to confirm that silencing of At2g41850 had taken place, total RNA was extracted from young, pre-abscising, and abscising flowers of both the KO and azygous lines. RNA was also extracted from ethylene (10 μl l⁻¹) and ‘air’ (–ethylene) treated excised flowers that had been collected when approximately 75% of the flowers had undergone abscission. The primers designed to perform this analysis amplified a transcript of the predicted size (450 bp) in the cDNA of excised flowers from azygous but not in the KO plants undergoing either ‘natural’ or ethylene-promoted abscission, confirming that the At2g41850 gene had been silenced (Fig. 6).

When grown in a controlled environment growth room the At2g41850 KO lines exhibited a small but consistent delay in floral organ shedding compared with the negative segregant control. However, due to natural variation both within and between samples, it was necessary to examine the time-course of shedding under more synchronized conditions by studying the process in excised flowers in the presence or absence of ethylene.
Flowers of the same developmental stage were excised from the At2g41850 KO line and an azygous control and the time-course of floral organ abscission determined in an environment in the absence or presence (10 μl l⁻¹) of ethylene. In both treatments, the At2g41850 KO line showed a delay in floral organ abscission when compared with the negative segregant (Fig. 7). On average, the delay in each treatment was approximately 12 h, with between 50% and 60% of flowers from the KO line showing abscission, when the process was complete (100% of floral abscission) in the azygous line. These observations were found to be consistent, with similar delays being seen on four separate occasions. Ethylene-treatment was found to promote abscission in both the At2g41850 KO and wild-type plants, confirming a role for ethylene in accelerating the process.

As At2g41850 was also expressed strongly at the site of lateral root development and emergence an analysis of both the number of lateral roots and the rate at which they emerged in At2g41850 KO and azygous plants was carried out. No consistent phenotypic difference could be identified in lateral root development between the two genotypes.

Discussion

Polygalacturonases have been hypothesized to play important roles throughout the life cycle of a plant including germination, cell expansion, pollen grain maturation, anther dehiscence, abscission, fruit ripening, and pod shatter (Roberts et al., 2002; Kim et al., 2006). It has been proposed that these hydrolytic enzymes play a contributory role in the breakdown of adhesion between neighbouring cells by bringing about middle lamella degradation (Rose et al., 2003). Indeed constitutive overexpression of a fruit-specific PG in apple plants has been shown to bring about abnormal cell separation within the leaf resulting in changes in leaf morphology, stomatal function, and, ultimately, plant water relations (Atkinson et al., 2002).

This analysis of the Arabidopsis genome indicates that there are 69 putative members of the PG gene family based on sequence homology. The phylogenetic arrangement broadly concurs with that generated by both the Cell Wall Navigator database (Girke et al., 2004) and that published by Kim et al. (2006) although the latter authors failed to include the QUARTET3 gene, and two recently annotated members (At4g32375 and At5g49215) in their phylogenetic analysis. Gene family members are sited throughout all five chromosomes, although some of them...
ProAt2g41850:GUS detailed analysis of independent abscission (González-Carranza et al., 2002). A more detailed analysis of independent ProAt2g41850:GUS and ProAt2g41850:GFP transgenic lines has revealed that expression is also apparent at the base of senescing cauline leaves and at the site of emergence of the lateral root. Although the rosette leaves of Arabidopsis plants are not shed, loosening of the cauline leaves at their attachment point to the inflorescence stem does take place when they undergo senescence and expression of At2g41850 is correlated with this event. It had been previously reported that, during the development of lateral roots in Allium porrum, an increase in PG activity had been detected, however, the suggestion made by the authors, based primarily on antibody staining, was that the emerging lateral secreted the enzyme to loosen cortical cells ahead of the growing radicle tip (Peretto et al., 1992). These data show clearly that it is the cells adjacent to the emerging root tip that accumulate the At2g41850 transcript. This strategy would ensure that the growing root apex is emerging into an environment that has already undergone some cell wall loosening and indicates that co-ordination of events between a developing lateral and the surrounding tissues must exist. It has recently been discovered that the expression of At2g41850 is up-regulated in root tissues by IAA (Z González-Carranza, K Elliot, JA Roberts, unpublished data) and this observation raises the possibility that auxin might act as a signal in both the induction of the site where a lateral root forms (Casson and Lindsey, 2003) and the facilitation of its emergence. The discovery that At2g41850 is expressed strongly in the basal cells of trichomes on the surface of senescing leaves may suggest that these structures, like other organs, have the capacity to abscise. The shedding of trichomes has been previously documented (Valkama et al., 2004), however, it is far from clear whether this is an active process or just a natural consequence of abrasive events occurring on a leaf surface.

The gene with closest homology to At2g41850 is At3g57510. This gene is an orthologue of SAC66, a PG whose transcript has been shown to accumulate at the site of anther and pod dehiscence in Brassica napus (Jenkins et al., 1999; Roberts et al., 2000). Expression of this gene can also be observed at the seed–funiculus junction where seed shedding takes place. Although At2g41850 and At3g57510 share close sequence similarity and may have comparable functions in bringing about cell separation at a predetermined site, their patterns of expression do not overlap. Attempts to localize the site(s) of expression of the third member of this clade, At3g07970, by fusing the promoter of this gene to GUS, proved unsuccessful. The transcript of this gene could be amplified from RNA extracted from rosette leaves (Z González-Carranza, K Elliot, JA Roberts, unpublished data), however, no consistent patterns of GUS accumulation could be identified in this material suggesting that the level of expression may be low. In silico analysis of At3g07970 expression using the Genevestigator (Zimmermann et al., 2004) or AtEnsembl databases has confirmed this assertion. Although Kim and Patterson (2006) have detected expression of this gene in floral abscission zone tissue, it has not been possible to confirm this observation. It is possible that expression takes place under certain conditions.
environmental conditions or at specific stages in the life cycle of the plant. An alternative strategy that might be used to identify the site of expression of At3g07970 would be to fuse its promoter to barnase and look for evidence of tissue ablation (Jenkins et al., 1999).

Analysis of the expression of another member of this clade (At1g80170), which exhibits close sequence homology to At2g41850 (45%), reveals that promoter activity in ProAt1g80170-GUS plants is restricted to the anthers with GUS accumulation being largely, but not exclusively, associated with pollen grains. Expression of PGs during anther development has been reported previously in Brassica species (Robert et al., 1993; Hong et al., 1997) and a substantial amount of wall modification takes place during pollen development and release (Twell, 2002). RT-PCR analysis has confirmed that At1g80170 is expressed in developing flowers, however, expression can also be detected in abscission zone tissue and even in young siliques. This observation can now be explained in that both tissues would be heavily contaminated with pollen grains which would contribute to the pool of RNA extracted from this site. Thus the use of RT-PCR (Kim et al., 2006), and even microarray analysis, as a means of determining site of expression of a specific gene should be used with some caution as RNA from other tissues might provide a form of contamination.

The gene structure of the abscission-related polygalacturonases in tomato (TAPG1-4) comprises four exons and three introns (Hong and Tucker, 1998). By contrast, both At2g41850 and At3g57510, the only two Arabidopsis PGs that have been shown by reporter gene analysis to be expressed during organ shedding, are made up of nine exons and eight introns. This arrangement is similar to that observed in the PG that has been shown to contribute to pectin disassembly in tomato (Giovannoni et al., 1989). To assess whether this gene structure might be important in defining the role or site of expression of the PGs, the expression was analysed of At2g43860, the Arabidopsis gene with closest homology to TAPG4 that is expressed strongly in both the abscission zones and pistils of tomato flowers (Hong et al., 2000). Our studies have revealed that At2g43860 is expressed within the endosperm cells of the seed adjacent to the site of the emerging radicle. A role for cell wall-degrading enzymes, including PGs, in seed germination has been proposed previously (Sitrit et al., 1999) and tissue print studies of germinating tomato seeds has revealed that transcripts of both an expansin and an XTH gene accumulate precisely in the endosperm region adjacent to the expanding radicle (Chen and Bradford, 2000; Chen et al., 2002). These observations, and our own, once again indicate that a signalling mechanism must exist between tissues of the root and those of adjacent tissues through which it has to emerge. No evidence can be found for expression of At2g43860 at the sites of organ abscission in Arabidopsis, however, GUS analysis indicates that it is expressed in developing seeds, although its role at this developmental stage is unclear.

The expression pattern observed for At2g41850 indicates that the PG may play a key role in organ abscission in Arabidopsis. To investigate this, the process of floral abscission was compared between At2g41850 knockout and azygous (negative segregant) plants maintained under controlled conditions in a growth room. Initial observations of the At2g41850 knockout lines showed that shedding was still taking place and that disruption of this PG had not prevented organ separation. However, there was considerable variability in the material and this did not allow us to conclude whether preventing the expression of At2g41850 had any impact on the time-course of abscission.

Excised flowers from the At2g41850 KO line and an azygous (negative segregant) line were maintained in the presence (10 μl−1) or absence of ethylene and the timing of floral abscission determined. Flowers from both KO and azygous lines exhibited accelerated abscission in the presence of ethylene compared with ‘air’ controls. However, in each treatment flowers from At2g41850 KO plants exhibited a 12 h delay when compared with the azygous control. This delay clearly supports a role for At2g41850 in regulating the timing of cell separation during floral abscission in Arabidopsis and further evidence to support this assertion comes from the recent report by Kim and Patterson (2006) that another T-DNA disruption line of At2g41850 exhibited a slightly attenuated time-course of organ shedding. The action of this PG cannot be critical for shedding as At2g41850 KO flowers continue to undergo abscission and it is possible that either additional PG family members contribute to the process or that a cocktail of wall-loosening proteins, including β-1,4-glucanases (Roberts et al., 2002) and expansins (Belfield et al., 2005) are involved in the process.

In this paper we have identified that the PG gene family comprises a substantial number of putative members based on sequence similarity. An analysis of different family members has revealed that the spatial patterns of expression of this sample are distinct and that transcript accumulation can be correlated with sites where cell separation takes place. However, a spatial and temporal transcriptional analysis of all members would be necessary before it would be possible to exclude any substantial overlap in expression patterns. Further evidence to support a role for these enzymes in regulating cell separation comes from the demonstration that the time-course of floral organ shedding is delayed in plants where an abscission-related PG is silenced. Although the efficacy of down-regulating this PG on cell separation appears to be limited, this discovery identifies a strategy whereby the contribution of different PG family members in bringing about wall degradation could be explored. By
complementing knockout lines with chimeric constructs containing the promoter of one family member fused to the open reading frame of another, it may be possible to examine whether PGs can act only on substrates in specific tissues. Such information might help to explain why there are so many members of this important gene family in plants.

Supplementary data
Supplementary Fig. S1 shows the predicted structure of polygalacturonases genes from Arabidopsis thaliana and tomato and can be found at JXB online.

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References


