A Novel Approach to Dissect the Abscission Process in Arabidopsis[1][C][W][OA]

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Abscission is the consequence of a specialized layer of cells undergoing a complex series of molecular and biochemical events. Analysis of the specific molecular changes associated with abscission is hampered by contamination from neighboring nonseparating tissues. Moreover, studies of abscission frequently involve the examination of events that take place in isolated segments of tissue exposed to nonphysiological concentrations of ethylene or indole-3-acetic acid for protracted periods (more than 24 h) of time. To resolve these problems, we have adopted the use of a transgenic line of Arabidopsis (*Arabidopsis thaliana*) where the promoter of an abscission-specific polygalacturonase gene (*At2g41850/ARABIDOPSIS DEHISCENCE ZONE POLYGALACTURONASE2*) has been fused to a green fluorescent protein reporter. RNA was extracted from green fluorescent protein-tagged cells, released from abscising floral organs, and used to generate a complementary DNA library. This library was used to probe a microarray, and a population of abscission-related transcripts was studied in detail. Seven novel abscission-related genes were identified, four of which encode proteins of unknown function. Reverse transcription-polymerase chain reaction analyses and promoter fusions to the β-glucuronidase reporter gene confirmed the expression of these genes in the abscission zone and revealed other places of expression during seedling development. Three of these genes were studied further by crossing reporter lines to the abscission mutants *inflorescence deficient in abscission* (*ida*) and *blade-on-petiole1 (bop1)/bop2* and an IDA-overexpressing line. Phenotypic analysis of an *At3g14380* transfer DNA insertion line indicates that this gene plays a functional role in floral organ shedding. This strategy has enabled us to uncover new genes involved in abscission, and their possible contribution to the process is discussed.

Abscission takes place when organs such as leaves, flowers, sepals, petals, fruits, and seeds detach from the body of a plant. This shedding occurs when organs are no longer functional or are diseased, when fruit have ripened, or when seed dispersion takes place (Patterson, 2001; Roberts et al., 2002; Lewis et al., 2006). Abscission is the consequence of a complex series of biochemical and molecular events that lead to a coordinated program of development culminating in cell separation. The abscission zone (AZ) can frequently be identified prior to organ shedding by being composed of a row (or rows) of small isodiametric cells containing dense cytoplasmic contents that are preprogrammed to respond to developmental or environmental cues (Sexton and Roberts, 1982; Roberts et al., 2002).

Shedding is only one of a series of events that occur during abscission. Once the organ is detached from the body of the plant, the remaining cells proximal to the site of abscission undergo changes that protect them against water loss and invasion by pathogens. For example, a spectrum of PATHOGENESIS RELATED (PR) proteins have been shown to be expressed at the fracture face (Eyal et al., 1993; Coupe et al., 1995; Roberts et al., 2000), and remodeling of the cell wall through pectin and hemicellulose-modifying proteins has been documented during abscission (Lashbrook et al., 1998; Brummell et al., 1999; del Campillo, 1999; González-Carranza et al., 2002) to form a protection layer at the scar site (Biggs and Northover, 1985).

[1] This work was supported by the Biotechnology and Biological Sciences Research Council, the University of Nottingham, a postdoctoral fellowship sponsored by the Higher Education Commission of the Government of Pakistan, and a studentship funded by the Government of Thailand.

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www.plantphysiol.org/cgi/doi/10.1104/pp.112.205955

The abscission process has been extensively studied, and many of the analyses have been performed using excised segments of tissue (or explants) exposed to nonphysiological concentrations of plant regulators such as ethylene or auxin for sustained periods of time (from a few hours to several days; Osborne, 1958; LaMotte et al., 1969; Sexton and Roberts, 1982; Coupe et al., 1995; González-Carranza et al., 2002; Belfield et al., 2005; Tucker et al., 2007). Such an approach makes the precise cellular composition of the AZ difficult to establish, and to add to this complication, analyses of molecular changes occurring during the process is potentially confused by "contamination" from neighboring nonseparating tissues.

One strategy that has proved effective to dissect the molecular events that regulate abscission has been to isolate and characterize mutants that exhibit impaired organ shedding. This approach has revealed that AZ differentiation in the tomato (*Solanum lycopersicum*) pedicel requires the expression of *JOINTLESS*, a MADS box-encoding gene (Mao et al., 2000), and *LATERAL SUPPRESSOR*, a VHIID transcriptional regulator gene (Malayer and Guard, 1964; Schumacher et al., 1999), while in Arabidopsis (*Arabidopsis thaliana*), flower expression of the MADS box genes *AGAMOUS-LIKE15* (*AGL15*) and *AGL18* (Fernandez et al., 2000; Adamczyk et al., 2007) and the BTB/POZ domain proteins *BOP1/BOP2* (Hepworth et al., 2005; Norberg et al., 2005; McKim et al., 2008) is necessary for AZ development. Other genes have been shown in Arabidopsis to play key roles in regulating wall dissolution and remodeling, including the membrane-trafficking regulator *NEVERSHED* (Liljegren et al., 2009), the membrane-associated receptor-like kinase *CASTAWAY* (Liljegren et al., 2009; Burr et al., 2011), the peptide ligand *INFLORESCENCE DEFICIENT IN ABSCISSION* (*IDA*; Butenko et al., 2003), and the receptor-like kinases *HAESA* and *HAESA-LIKE2* (Jinn et al., 2000; Cho et al., 2008; Stenvik et al., 2008) that have been shown to interact with *IDA* (Shi et al., 2011).

The sequencing and annotation of genomes have not only revealed the genes that constitute an organism but have also provided a mechanism, through microarray technology, to determine gene expression in both temporal and spatial manners (Duggan et al., 1999; Rensink and Buell, 2005; Galbraith and Birnbaum, 2006). However, when applied to complete organs, the low expression levels of transcripts coupled with low levels of resolution remain as barriers to discover the specific gene transcripts represented in a particular cell type. To address this concern, microarray studies have been coupled with microdissection and cell sorting, and the combination of these technologies has successfully facilitated cataloguing transcripts from individual cell types (Cadman et al., 2006; Cai and Lashbrook, 2006; Galbraith and Birnbaum, 2006; Suwabe et al., 2008; Zhang et al., 2008; Matas et al., 2010). In 2008, the first report of a transcriptome study from Arabidopsis stamen AZ tissues was carried out by Cai and Lashbrook (2008), and transcript profiles were established for five floral stages from cells isolated by laser-capture microdissection. Although laser-capture microdissection and cell sorting provide a considerable advance, some technical problems remain, including the manipulation of tissue samples, the use of hydrolytic enzymes to "release" cell materials, and the use of RNA enrichment methods. These treatments may impact on the cells under focus and their population of transcripts, which could lead to a profile misrepresentation in the samples obtained (Birnbaum et al., 2003; Galbraith and Birnbaum, 2006; Zhang et al., 2008). To overcome some of these issues, the use of genetic markers, such as fluorescent proteins driven by tissue-specific promoters expressed in specific cells (Birnbaum et al., 2003, 2005; Nawy et al., 2005; DeBlasio et al., 2010) or subcellular organelles such as the nuclei (Zhang et al., 2008) coupled with gentle tissue homogenization (Galbraith et al., 1983; Zhang et al., 2005) and flow cytometry (Galbraith et al., 2004; Zhang et al., 2005; Galbraith, 2007), have been employed.

In this article, we describe the use of the GFP marker transgenic line $Pro_{At2g41850}$::*GFP*, previously demonstrated to be expressed specifically during floral organ shedding in Arabidopsis (González-Carranza et al., 2002) as a tool to visualize AZ cells that have undergone natural separation. *At2g41850* is also known as *ADPG2* and has been demonstrated to be involved in both floral organ abscission and silique dehiscence (Ogawa et al., 2009). We took advantage of the cell wall degradation that inherently occurs during the abscission process to avoid altering the transcript levels through processing of the tissue and maintaining manipulation to a minimal level. The mRNA isolated from the collected cells was used to generate a cDNA library that was used as a probe in an Arabidopsis Affymetrix ATH1 chip. We selected and studied the pattern of expression of seven genes, *At1g64405*, *At2g23630*, *At2g44010*, *At3g14380*, *At3g53040*, *At3g56350*, and *At5g50540*, four of which (*At1g64405*, *At2g44010*, *At3g53040*, and *At5g50540*) currently have no assigned role. Phenotypic analysis of a T-DNA insertion line that is a functional knockout of *At3g14380* reveals that silencing of this gene delays the timing of petal abscission. The possible contribution of the other genes to the floral organ-shedding process in Arabidopsis is discussed.

RESULTS

AZ Cells Tagged with GFP Can Be Collected to Generate a Highly Specific cDNA Library

A transgenic Arabidopsis reporter line ($Pro_{At2g41850}$::*GFP*), generated by fusing the promoter of the abscission-related polygalacturonase gene *At2g41850* (*PGAZAT/ADPG2*) to GFP (González-Carranza et al., 2002; Ogawa et al., 2009), was used to visualize using a fluorescence-dissecting microscope AZ cells that have undergone natural separation (Supplemental Video S1). About 30,000 GFP-tagged cells from in excess of 300 sepal, petal, and anther filament AZs were collected using glass capillary

micropipettes, and the mRNA was isolated to generate a cDNA library. To test that genomic DNA contamination was not present in the first-strand cDNA, an internal control was performed using primers from the introns of the *At2g41850* gene (Supplemental Fig. S1). The resulting unamplified libraries were titered using *Escherichia coli* XLI-Blue cells, resulting in 6×10^6 plaque-forming unit μL^{-1} independent clones in one fraction and 10.125×10^6 plaque-forming unit μL^{-1} in the second fraction. The percentage of recombinant clones was determined to be 76.5%, with averages of 258/82 and 224/67 (white/blue colonies).

To test the fidelity of the library, PCR was performed using specific primers to amplify the open reading frame of the *At2g41850* gene (Supplemental Fig. S2). Degenerate primers were also employed to amplify putative AZ products from expansins and metallothionein genes (Supplemental Fig. S2) previously reported to be expressed in AZ cells (Coupe et al., 1995; Cho and Cosgrove, 2000; Belfield et al., 2005; Tucker et al., 2007). The segments amplified were cloned and plasmids from individual colonies sequenced, showing the amplification of the expansin genes *At2g39700* (*EXPANSIN4* [*AtEXP4*]) and *At2g28950* (*AtEXP6*) and the metallothionein gene *At5g02380* (*MATHALLOTHIONEIN2b*); reamplification of the complete open reading frame from these genes was performed from the library, demonstrating that the library contained full-length cDNA transcripts (data not shown).

To confirm that the transcripts from the expansin genes amplified by PCR were expressed in floral AZ tissues, transgenic lines were obtained from $Pro_{At2g39700}$::*GUS* and $Pro_{At2g28950}$::*GUS* (D.J. Cosgrove, unpublished data). GUS analysis confirmed that these expansin genes were expressed specifically during the abscission process. $Pro_{At2g39700}$::*GUS* shows expression in epidermal and subepidermal tissue in the floral pedicel tissue, while $Pro_{At2g28950}$::*GUS* shows a more defined pattern of expression in the epidermal AZ cells of sepals, petals, and anther filaments (Fig. 1).

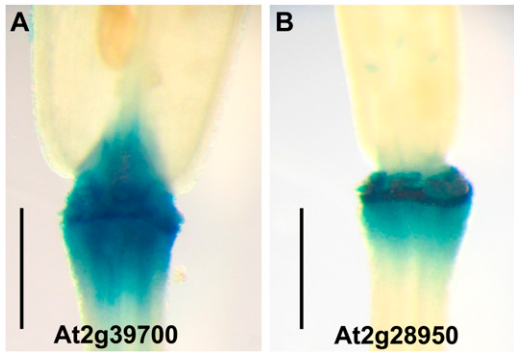

Figure 1. GUS expression in Arabidopsis floral AZs from $Pro_{At2g39700}$::GUS (*AtEXP4*) and $Pro_{At2g28950}$::GUS (*AtEXP6*). Flower position 8 is shown, where position 1 is the flower with the first visible petal. Bars = 1 mm.

A cDNA Library Can Be Used as a Probe in a GeneChip to Identify Abscission-Expressed Genes

A probe was generated using the cDNA library, and an Affymetrix GeneChip (ATH1) was hybridized in duplicate. After normalization, 1,200 genes assigned as being "marginal" or "present" by the Affymetrix treatment of the arrays were selected (Supplemental Table S1). A whole-plant tissue-specific classification of this set of genes using information from Genevestigator (Hruz et al., 2008) was generated, showing expression of these genes in AZs and in other tissues. Among the genes identified in the microarray as having the highest expression was *IDA*, which has been shown to be critical for floral organ abscission to proceed normally (Butenko et al., 2003).

Seven genes, *At1g64405*, *At2g23630*, *At2g44010*, *At3g14380*, *At3g53040*, *At3g56350*, and *At5g50540*, were selected for more detailed analysis on the basis of the following criteria: (1) whole-plant tissue specificity (Genevestigator; Hruz et al., 2008); (2) genes previously reported to be abscission related by Cai and Lashbrook (2008) in stamen AZ tissues; (3) genes not previously reported to be expressed at high levels in Arabidopsis AZs; (4) number of putative family members in the Arabidopsis genome: we initially focused on genes belonging to families with a low number of potential members (less than 15) so that we could probe their role using gene-silencing strategies (Swarbreck et al., 2008); and (5) potential gene function, particularly those genes that have previously been shown to be involved in cell wall remodeling, such as *At2g23630*, which has been annotated as a putative pectin esterase (Swarbreck et al., 2008; Table I).

When the levels of expression detected for each gene in the microarray of the cDNA library were compared with the expression levels reported in AZ tissues in Genevestigator (Hruz et al., 2008), the order of magnification varied from 2.3 to 159 times in the genes studied (Table I).

At1g64405 is proposed to be a unique gene in Arabidopsis, and together with *At3g14380* it has been reported to be expressed in floral AZs (Cai and Lashbrook, 2008). *At2g44010*, *At3g14380*, and *At5g50540* are each members of small gene families comprising two members. *At3g56350* and *At3g53040* are proposed to belong to families of 10 and 13 members, respectively, while *At2g23630* is thought to belong to a family of 20 (Table I).

No gene function has been assigned to the genes *At1g64405*, *At2g44010*, *At3g14380*, and *At5g50540* (Swarbreck et al., 2008). *At2g23630* has been described as SKS16 (Swarbreck et al., 2008). *At3g53040* has been described as a late embryogenesis abundant (LEA) protein and reported to be expressed in seeds (Cadman et al., 2006; Chibani et al., 2006; Hundertmark and Hincha, 2008). The most highly expressed gene in the microarray analysis of the cDNA library (Table I), *At3g56350*, is a member of the iron/manganese superoxide dismutase family, and the protein encoded by this

Table I. *Selection criteria for the genes studied*

Gene	Genevestigator[a] (Means of Experiments)		ATH_1 Signal in Microarray of the cDNA Library	No. of Genes in the Arabidopsis Genome	Gene Description
	Reported Tissue with Highest Level of Expression	Reported Level of Expression in AZs (ATH1:22k Array)			
At1g64405	AZ (13,728)	13,728[b]	3,952.9	Unique gene	Unknown protein
At2g23630	Root tip/root cap (43,477)	136	319.4	20	SKS16 (SKU5 similar16)
At2g44010	Radicle (9,935)	232	3,024.9	2	Unknown protein
At3g14380	Stigma (26,895)	25,892[b]	136.4	2	Uncharacterized protein
At3g53040	Embryo (33,901)	120	5,268.0	13	Late embryogenesis abundant protein
At3g56350	Seed (21,731)	128	20,410.0	10	Iron/manganese superoxide dismutase family protein
At5g50540	Mesophyll cell protoplast (631)	108	574.8	2	Unknown protein

[a]Hruz et al. (2008). [b]From experiments obtained in a stamen AZ transcriptome analysis (Cai and Lashbrook, 2008).

gene has been shown to increase during seed germination (Rajjou et al., 2006).

Spatial and Temporal Expression of Seven Identified Genes Show Specificity in Abscission and Other Cell Processes

The patterns of expression of the seven genes selected for detailed analysis were determined in different Arabidopsis tissues by reverse transcription (RT)-PCR. *At1g64405*, a previously documented abscission-related gene (Cai and Lashbrook, 2008), showed highest transcript levels in flowers and roots, but expression was also detected in buds and cauline leaves (Fig. 2A). RT-PCR confirmed the expression of *At2g23630* in roots as reported in Genevestigator (Hruz et al., 2008), but no expression was detected in flowers, suggesting that the transcript level from this gene is very low and that the RT-PCR is not sensitive enough to detect the transcript (Fig. 2B).

At2g44010, a gene identified as being expressed in radicle tissue according to Genevestigator (Hruz et al., 2008), exhibited highest expression in buds, flowers, and yellow siliques (Fig. 2C). RT-PCR analysis for *At3g14380* showed greatest expression in flowers and buds and lower levels in roots and yellow siliques (Fig. 2D). Two splice variants of this gene were confirmed as being expressed in flowers and buds (Supplemental Fig. S6). *At3g53040* and *At3g56350* share the same pattern of expression, with the highest level of expression being seen in yellow siliques and a lower level in roots, buds, and flowers (Fig. 2, E and F). *At5g50540* showed greatest expression in roots and yellow siliques and trace expression in buds and flowers (Fig. 2G).

The 5′ upstream genomic regions of these genes were amplified from Columbia-0 (Col-0) wild-type genomic DNA and fused to the reporter GUS to undertake a more detailed expression analysis. The sizes of these genomic regions were 1,865, 1,877, 1,303, 1,303, 1,655, 884, and 1,407 bp for *At1g64405*, *At2g23630*, *At2g44010*, *At3g14380*, *At3g53040*, *At3g56350*, and *At5g50540*, respectively. These genomic regions were sufficient to drive the GUS gene in floral AZs (Fig. 3).

Homozygous reporter lines were identified, and expression was determined in independent transgenic lines. In all lines studied, reporter expression could be seen during floral organ abscission (Fig. 3). The expression pattern in $Pro_{At1g64405}$::*GUS* plants revealed that *At1g64405* is highly expressed in AZs from flower positions 1 to 18 both in the epidermal cells and in the subepidermal cells of the pedicel, expanding in a broad area of the silique and pedicel from flower positions 9 to 16. Expression is also strongly apparent in stigmatic tissues, anther filaments, and petals (Fig. 3A). $Pro_{At2g23630}$::*GUS* plants showed discrete expression of the reporter in AZ tissues from flower positions 3 to 18 and some expression in anthers in flowers from positions 1 to 3 (Fig. 3B). Expression in the AZ cells of $Pro_{At2g44010}$::*GUS* plants can be detected in flowers from positions 2 to 18, with GUS accumulation also being observed in stigmatic tissues and anthers (Fig. 3C). Analysis of $Pro_{At3g14380}$::*GUS* plants revealed high levels of expression in epidermal AZ cells from flower positions 1 to 18 in addition to the stigmas, anther filaments, and pollen of flowers from positions 1 to 3 (Fig. 3D). A similar pattern of expression was observed in $Pro_{At3g53040}$::*GUS*, $Pro_{At3g56350}$::*GUS*, and $Pro_{At5g50540}$::*GUS* lines, although expression was not as intense and was not apparent in stigmatic tissue (Fig. 3, E–G).

A detailed analysis of GUS expression in transgenic seedlings at 2, 4, 6, 8, 11, and 15 d old from the lines generated revealed that these genes are also expressed in other tissues during seedling development, including testa (*At2g23630* and *At3g53040*), roots (in all genes studied), vascular tissue of cotyledons (*At1g64405*, *At3g53040*, and *At3g56350*) and of leaves (*At1g64405*, *At2g23630*, *At3g14380*, and *At3g56350*), stems (except *At3g14380*), leaf lamina (except *At1g64405* and *At3g56350*), and young bud primordia (except *At1g64405* and *At3g14380*; Supplemental Fig. S3, A–AP).

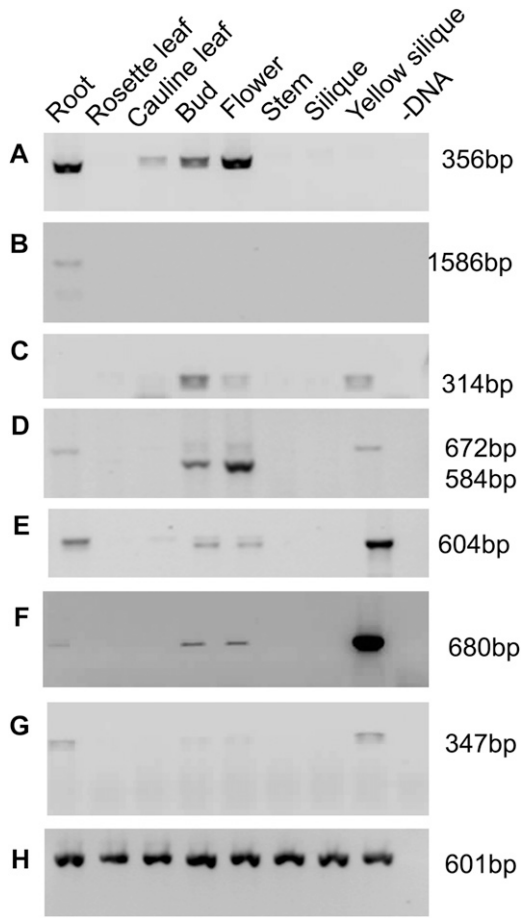

Figure 2. RT-PCR analysis of the expression of *At1g64405* (A), *At2g23630* (B), *At2g44010* (C), *At3g14380* (D), *At3g53040* (E), *At3g56350* (F), *At5g50540* (G), and *AT5G44200* (H; cap-binding protein) as a housekeeping gene. RNA was extracted from Col-0 wild-type roots, rosette leaves, cauline leaves, buds, flowers, stems, siliques, and yellow siliques. For primer information, see Supplemental Table S1.

GUS Reporter Analysis in *ida*, *bop1/bop2*, and Pro_{35S}::*IDA* Mutant Backgrounds Reveals a Spatial and Temporal Correlation with Floral Organ Shedding

To explore the effect of delaying or accelerating the abscission process on the expression of *At2g41850* (used as a positive control), *At1g64405*, and *At3g14380*, crosses were made between homozygous lines of $Pro_{At2g41850}$::*GUS*, $Pro_{At1g64405}$::*GUS*, or $Pro_{At3g14380}$::*GUS* lines with the abscission-related mutants *ida* (Butenko et al., 2003) and *bop1/bop2* (Hepworth et al., 2005; McKim et al., 2008) and with an overexpressing line of the *IDA* gene (Stenvik et al., 2006). F2 progeny were identified with mutant phenotypes and genotyped to confirm the presence of the appropriate GUS transgene. A detailed analysis of reporter expression, during the abscission process, was undertaken from young buds to flower position 14 on the mutant/GUS lines (Figs. 4–6).

The spatial and temporal accumulation of GUS in the $Pro_{At2g41850}$::*GUS* parental line confirmed that the gene is strongly expressed in AZ tissues from flowers at positions 4 to 14, with the levels of expression decreasing as the silique matures (Fig. 4A). Flowers from the $Pro_{At2g41850}$::*GUS* × *ida* line showed delayed and reduced expression, with the first visible signals of GUS accumulation being apparent at flower position 8 and the highest expression at position 14 (Fig. 4B). In contrast, reporter expression in $Pro_{At2g41850}$::*GUS* × Pro_{CaMV}::*IDA* plants could be detected at position 2, with enhanced levels of expression being maintained up to position 14. The characteristic associated with overexpressing IDA of a greatly increased number of AZ cells was observed in these plants (Fig. 4C) as well as ectopic abscission at the base of cauline leaves (data not shown). Some expression of GUS could be seen in the AZ region of $Pro_{At2g41850}$::*GUS* × *bop1/bop2* plants from position 10, but expression was primarily evident in anther filaments from flower position 6 (Fig. 4D).

The $Pro_{At1g64405}$::*GUS* parental line showed expression in flowers from positions 1 to 18 (Fig. 3A). In an IDA background, GUS expression was both reduced in intensity and delayed, with the reporter not being detected until position 8 and declining in intensity by position 14. Expression was also much more discretely localized (Fig. 5A). $Pro_{At1g64405}$::*GUS* × Pro_{CaMV}::*IDA* plants also showed a delay in expression, with GUS detection being seen from flower position 4 and increasing steadily to position 14 (Fig. 5B). This delay in expression was most marked in $Pro_{At1g64405}$::*GUS* × *bop1/bop2* plants, although GUS accumulation was clearly detectable at the base of sepal, petals, and anther filaments from positions 8 to 14 (Fig. 5C).

GUS analysis of the parental $Pro_{At3g14380}$::*GUS* line showed expression in AZ tissues from flower positions 1 to 18 (Fig. 3D). In an *ida* background, reporter expression in $Pro_{At3g14380}$::*GUS* plants was both less intense and more discrete (Fig. 6A), while in an overexpressing *IDA* line, expression in the AZ region was high from flower positions 2 to 14 (Fig. 6B). In $Pro_{At3g14380}$::*GUS* × *bop1/bop2* plants, no GUS expression could be detected in the AZ region (Fig. 6C).

Functional Analysis of *At3g14380*

To determine a role for *At3g14380* during floral organ shedding, a putative knockout (KO) line was studied from the SALK T-DNA collection (SALK_03248; Alonso et al., 2003). This line has a T-DNA insertion in the second exon of *At3g14380* (463 bp downstream from the ATG). Genomic and RT-PCR analyses were performed to identify and confirm silencing of the KO line and expression in the negative segregant (NS) lines (Supplemental Figs. S5 and S6, respectively). As indicated previously, two splice variants of this gene were observed and confirmed as being expressed in flowers and buds.

A time course of flower development in the KO and NS lines (lines 3 and 1, respectively; Supplemental Figs. S5 and S6) established that floral abscission took place

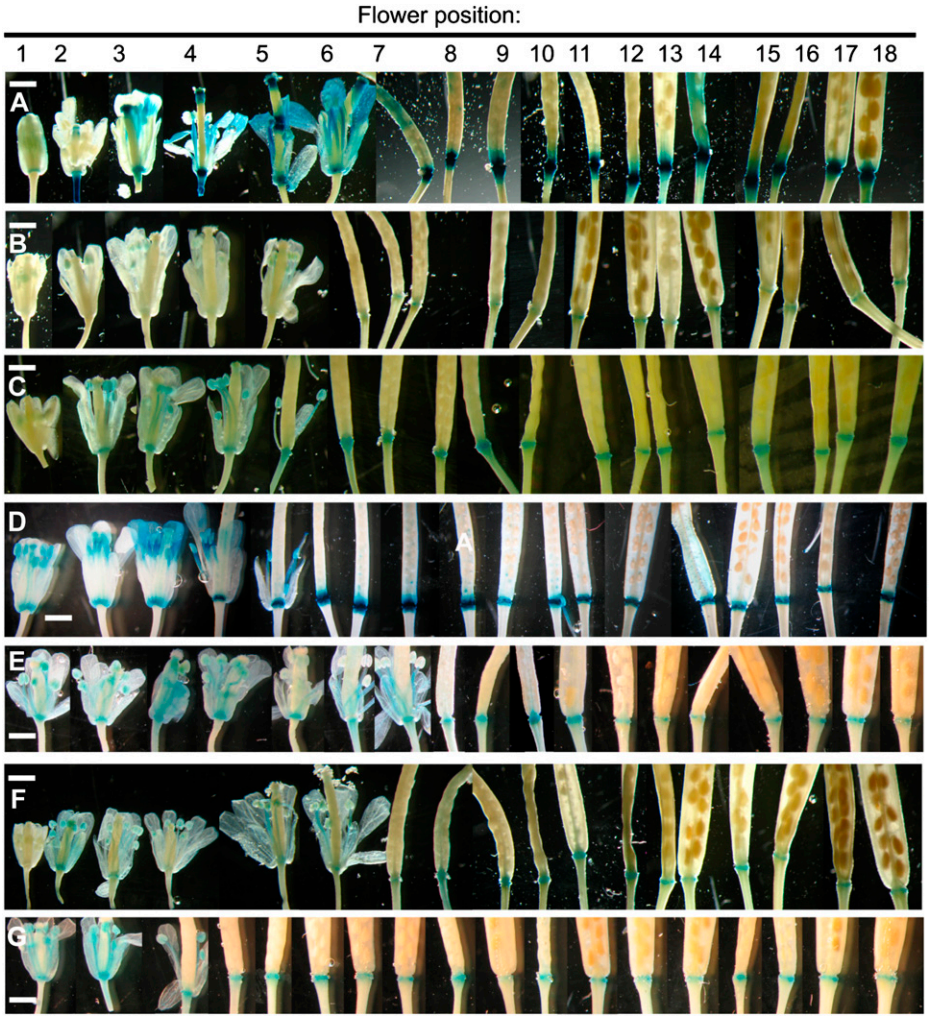

Figure 3. Flower and silique expression from $Pro_{At1g64405}$::*GUS* (A), $Pro_{At2g23630}$::*GUS* (B), $Pro_{At2g44010}$::*GUS* (C), $Pro_{At3g14380}$::*GUS* (D), $Pro_{At3g53040}$::*GUS* (E), $Pro_{At3g56350}$::*GUS* (F), and $Pro_{At5g50540}$::*GUS* (G). Time course of expression is shown from floral development positions 1 to 18, where position 1 is the first flower where petals are visible to the eye. Tissue was GUS stained for a period of 6 h (A and D) or 12 h (B, C, and E–G) of incubation in GUS substrate. Bars = 1 mm.

later in the former (position 9) compared with the latter (position 7; Fig. 7). To provide a quantitative assessment of floral organ abscission, a device to measure break strength was developed (for details, see "Materials and Methods") and the force required to remove a petal from the floral body was determined. Petal break strength in both *At3g14380* KO and NS was assessed at flower positions 3, 4, and 5. The force required to remove a petal from an *At3g14380* KO flower is significant higher, particularly at flower position 5, than that required to remove a petal from a NS flower at an equivalent developmental stage (Fig. 8).

DISCUSSION AND CONCLUSION

A Collection of "Naturally" Generated Protoplasts Tagged with GFP Provides a Highly Specific Probe to Detect AZ-Related Transcripts

In this paper, a method is described to study the abscission process in Arabidopsis that overcomes some of the difficulties that researchers have to face to establish a precise transcript composition of the cells from this zone.

Taking advantage of the specific expression that the polygalacturonase gene *At3g41850* has in floral AZs, a marker line, $Pro_{At2g41850}$::*GFP* (González-Carranza et al., 2002), was utilized to visualize and collect tagged cells (Supplemental Video S1). Polygalacturonase is a cell wall-degrading enzyme that, together with other cell wall-modifying agents, has been proposed to hydrolyze the middle lamella of the cells that constitute the AZ (Bleecker and Patterson, 1997; Patterson, 2001; Roberts et al., 2002; Lewis et al., 2006). It has been proposed that polygalacturonases play a role in the cell separation stage of the abscission process, one of the final events that contributes to organ shedding (Hong et al., 2000; Roberts et al., 2000, 2002; González-Carranza et al., 2002; Cai and Lashbrook, 2008). The capacity of the abscission process to "release" cells from the body of the plant was exploited to avoid the manipulation that is involved in preparing tissues for microdissection and isolation of transcripts (Birnbaum et al., 2003, 2005; Nawy et al., 2005). Furthermore, no application of plant growth regulators was utilized to influence the timing of organ shedding. Due to the timing of expression of *At2g41850* toward the end of

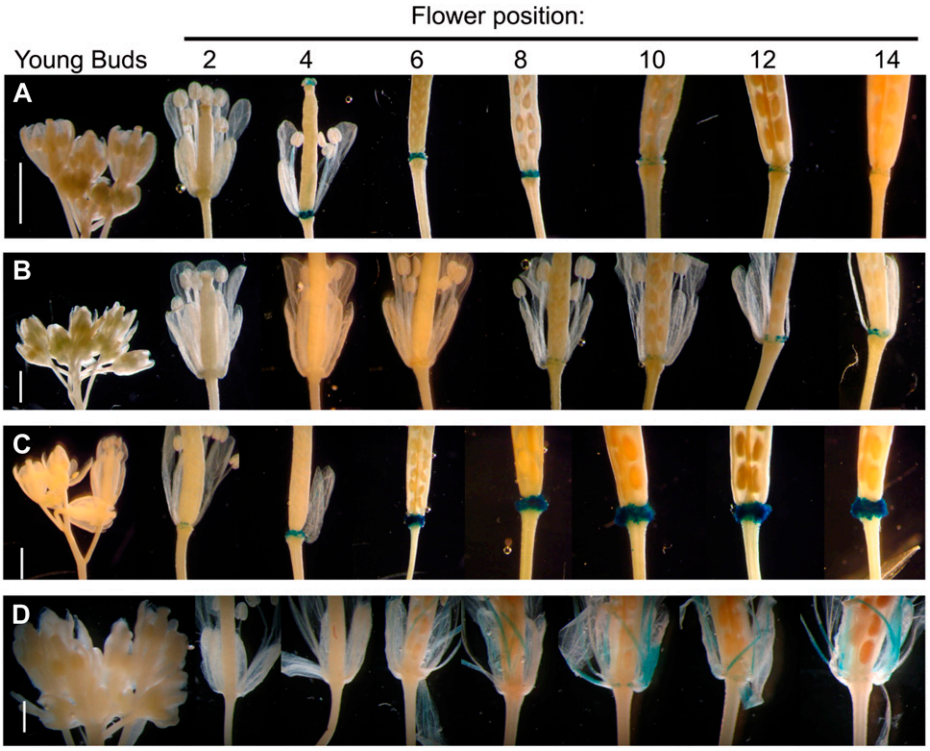

Figure 4. Flower and silique expression from $Pro_{At2g41850}$::*GUS* (A), $Pro_{At2g41850}$::*GUS* crossed with *ida* mutant (B), $Pro_{At2g41850}$::*GUS* crossed with Pro_{35S}::*IDA* (C), and $Pro_{At2g41850}$::*GUS* crossed with *bop1/bop2* mutant (D). Time course of expression is shown from young buds to flower position 14, where position 1 is the first flower where petals are visible to the eye. Tissue was GUS stained for a period of 12 h of incubation in GUS substrate. Bars = 1 mm.

the cell separation process, it is likely that the transcripts identified as being abscission related may be most closely correlated with events that take place toward the end of organ shedding. In addition, it is possible that some unavoidable artifacts due to cDNA construction, including variations of cDNA termini, restriction enzyme-cutting abnormalities, and chimeric sequences (Zhou et al., 2012), may be present in our library.

In this experiment, the cDNA library generated from RNA extracted from a population of separated AZ cells was used to probe an Affymetrix chip to identify transcripts expressed in the cells and determine a measure of their expression levels. To test the fidelity of the library, we amplified two expansin genes, *At2g39700* (*AtEXP4*) and *At2g28950* (*AtEXP6*), and a methallothionein-like gene, *At5g02380* (*MT2b*), using degenerate primers (Supplemental Fig. S2). Analysis of GUS promoter fusion lines of the two expansin genes confirmed that both were expressed in AZ tissues (Fig. 1). To our knowledge, this is the first demonstration of a possible role for these expansin family members in Arabidopsis floral organ abscission, although abscission-related expression has been documented in other plants (Belfield et al., 2005), and Cho and Cosgrove (2000) found that silencing *At1g26770* (*AtEXP10*) increased leaf break strength in Arabidopsis. Expansins are involved in cell wall extension, and although they are not involved in hydrolytic breakdown of the walls, a role in wall loosening has been well documented (McQueen-Mason and Cosgrove, 1995; Cho and Cosgrove, 2000; Cosgrove, 2005). Expansins

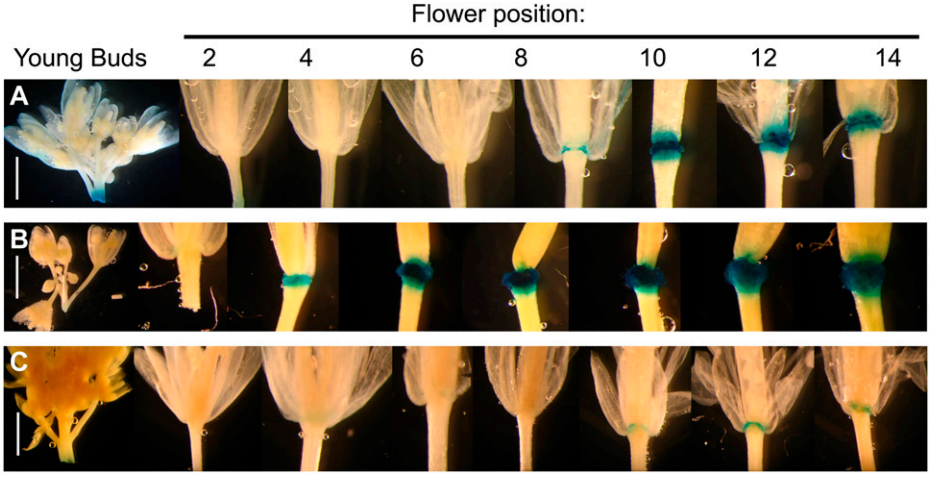

Figure 5. Flower and silique expression from $Pro_{At1g64405}$::*GUS* crossed with *ida* mutant (A), $Pro_{At1g64405}$::*GUS* crossed with Pro_{35S}::*IDA* (B), and $Pro_{At1g64405}$::*GUS* crossed with *bop1/bop2* mutant (C). Time course of expression is shown from young buds to flower position 14, where position 1 is the first flower where petals are visible to the eye. Tissue was GUS stained for a period of 6 h of incubation in GUS substrate. Bars = 1 mm.

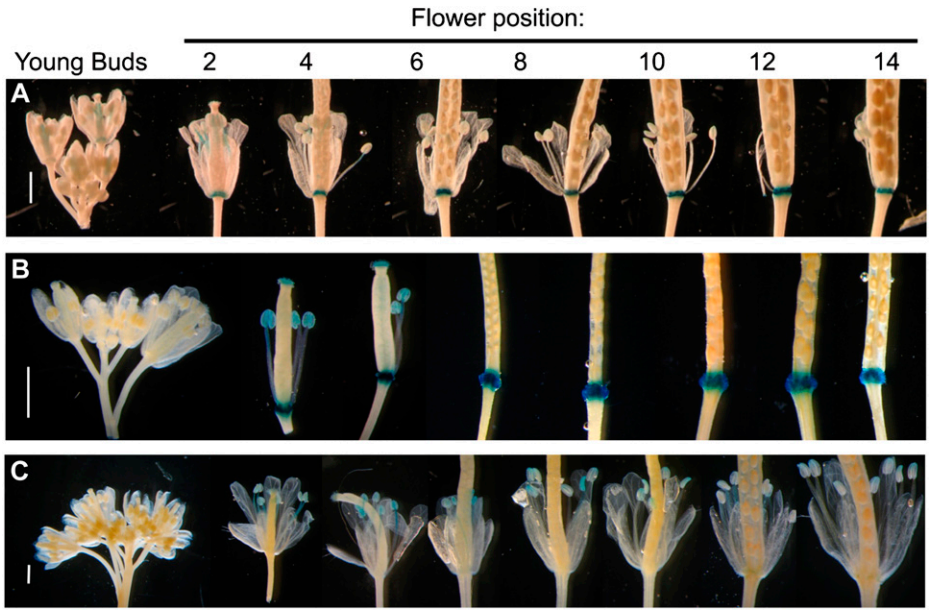

Figure 6. Flower and silique expression from $Pro_{At3g14380}$::*GUS* crossed with *ida* mutant (A), $Pro_{At3g14380}$::*GUS* crossed with Pro_{35S}::*IDA* (B), and $Pro_{At3g14380}$::*GUS* crossed with *bop1/bop2* mutant (C). Time course of expression is shown from young buds to flower position 14, where position 1 is the first flower where petals are visible to the eye. Tissue was GUS stained for a period of 12 h of incubation in GUS substrate. Bars = 1 mm.

may play a role in the growth of cells that are involved in the formation of a protective layer once cell separation has occurred or even in the expansion that takes place in the proximal side of the AZ prior to separation (Patterson, 2001).

Coupe et al. (1995) suggested that methallothionein-like proteins may remove free radicals generated during the abscission process or act as antioxidants in AZ tissues (Michaeli et al., 2001; Guo et al., 2008). It has been demonstrated that *At5g02380* (*MT2b*), together with another three members of the methallothionein family, is expressed in Arabidopsis and *Brassica napus* floral AZs (Guo et al., 2003; Tsuwamoto and Harada, 2010). Although we were unable to amplify segments of MT1a, MT2a, and M3 with our degenerate primers, transcripts for these genes have been detected in our cDNA library (Supplemental Table S1). And a gene similar to phytochelatin synthase has also been identified.

Analyses of Expression from Seven Identified Genes in a Single Abscission cDNA Library May Aid in the Study of Other Cellular Processes

RT-PCR and promoter fusion analyses from the seven abscission-related genes selected in this study were expressed in other plant tissues, including seeds, roots, vascular tissues, cauline leaves, leaf primordia, buds, flowers, and yellow siliques (Figs. 2 and 3; Supplemental Fig. S3). These analyses indicate that

Figure 7. Developmental time course of flowers from 4-week-old plants. Flowers shown represent positions 2 to 15, where position 1 is the first flower where petals are visible to the eye. A, *At3g14380* KO plants. B, *At3g14380* NS plants (SALK_03248). Bars = 1 mm. [See online article for color version of this figure.]

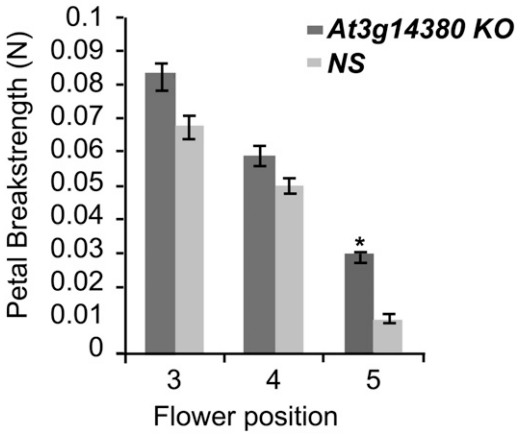

Figure 8. Break-strength analysis of petals from flowers excised from 4-week-old plants of *At3g15380* KO-L3 wild type and NS-L1 (SALK_03248) at positions 3, 4, and 5, where position 1 is the first flower where petals are visible to the eye. Four flowers from each stage were used in a Lloyds LF plus machine to measure the force required to separate the petal from the pedicel of the flower. Measurements were statistically analyzed by ANOVA. The asterisk indicates a difference in the mean at $P < 0.002$ ($n = 12$).

the genes not only play a role during abscission but also in other events that may involve events such as wall remodeling or protection against pathogens (Eyal et al., 1993; Coupe et al., 1995; Lashbrook et al., 1998; Brummell et al., 1999; del Campillo, 1999; Roberts et al., 2000; González-Carranza et al., 2002).

When the levels of GUS expression from $Pro_{At2g41850}$::*GUS* were compared in a floral time course of abscission (Fig. 3), it was noticed that the pattern shown in this paper is more limited than that in Ogawa et al. (2009; Fig. 6B). This may be due to the length of the promoters fused to drive the reporter gene expression (1,476 versus 2,177 bp, respectively).

Proposed Roles for the Abscission-Related Genes Identified

At1g64405 is a unique gene in the Arabidopsis genome with an unknown role. It encodes a small protein of 118 amino acids with a domain rich in Ser, and it is predicted to have a molecular mass of 13.404 kD and a pI of 4.71 (http://web.expasy.org/compute_pi/; Bjellqvist et al., 1993; Supplemental Fig. S4A).

It has been shown that the transcript from *At1g64405* is up-regulated 6.5-fold in an inducible line of *WAX INDUCER1* fused to a hemagglutinin epitope (*WIN1-HA*) by dexamethasone. *WIN1* is a transcription factor of the ethylene response factor family. In addition to *At1g64405*, other genes, including LACS2 (a long-chain acyl-CoA synthetase) and CYP86A7 and CYP86A4 (two cytochrome P450 genes), that were up-regulated in this line, have been identified to have an involvement in cutin biosynthesis. Moreover, the transcript expression of these genes, including *WAX INDUCER1* and *At1g64405*, overlap, and it has been shown in a microarray analysis of epidermal tissue (Suh et al., 2005) that they are expressed in the epidermal layer where cutin biosynthesis also takes place (Kannangara et al., 2007). Furthermore, it has been demonstrated that up-regulation of the *WIN1* gene in conjunction with other transcription factors triggers wax production, improves drought tolerance, and alters cuticle permeability (Aharoni et al., 2004; Broun et al., 2004). In addition, the transcript of *At1g64405* has been reported to be present in the epidermis of the top of the stem in an analysis performed to analyze the cuticular composition in Arabidopsis (Suh et al., 2005). These findings suggest that *At1g64405* may play a role in the cutin biosynthesis pathway, its protein may be located in epidermal tissues, and it may be regulated by WIN1 and ethylene. In the abscission process, it may be contributing to protect the exposed fracture surface from water loss and invasion by pathogens.

It has been proposed that *At2g23630* (*SKS16*) is a gene that has pectin esterase activity and acts as a copper ion-binding protein (Swarbreck et al., 2008). It is predicted that its molecular mass is 61.029 kD, its pI is 9.74 (http://web.expasy.org/compute_pi/; Bjellqvist et al., 1993), and it has a hydrophobic N-terminal peptide of 25 amino acids that is cleavable (http://www.cbs.dtu.dk/services/SignalP/; Nielsen and Krogh, 1998; Bendtsen et al., 2004; Supplemental Fig. S4B), so it is likely that the protein product from this gene may be secreted. Although a role for pectin esterases in abscission was first proposed over 50 years ago (Osborne, 1958), the evidence remains conflicting (Yager, 1960; LaMotte et al., 1969; Ratner et al., 1969; Moline et al., 1972; Roberts and González-Carranza, 2009). In Arabidopsis, it has been demonstrated that three of at least 59 putative pectin esterase genes in Arabidopsis are up-regulated during floral organ abscission (Cai and Lashbrook, 2008). It has been suggested that pectin esterases act during cell separation to remodel the cell walls via the cleaving of methyl groups from (1,4)-α-D-galacturonic acid to allow polygalacturonases to degrade the middle lamella (Roberts and González-Carranza, 2009).

Although the genes belonging to the *SKS* family have been described as putative pectin esterases, it has not yet been proven that they have esterase activity (Sedbrook et al., 2002). The sequences of these proteins share similarities to ascorbate oxidases and laccases, two families of multicopper oxidases that integrate four copper ions into three regions (types 1, 2, and 3); however, the amino acid domains where the copper ions bind are conserved in both of these two families, but in the genes of the *SKS* family, regions type 1 and 3 are missing and motif 2 is not intact, suggesting that *SKS* genes are monocopper oxidases rather than pectin esterases and that their specificity remains to be elucidated (Sedbrook et al., 2002; Jacobs and Roe, 2005).

At2g44010 encodes an unknown protein of 101 amino acids. It is predicted to have a molecular mass

of 11.52 kD and a pI of 10.26. Although it may be targeted to mitochondria, the gene with closest similarity (67%) at the amino acid level (*At3g59880*) is proposed to be cytoplasmic (http://ppdb.tc.vornell.edu/dbsearch/; Heazlewood et al., 2005, 2007; Supplemental Fig. S4C). *At3g59880* was not represented in the Affymetrix chip utilized in this study. Both of these genes along with other unknown proteins have been reported to be exclusively expressed in the rosids clade of flowering plants. *At2g44010* is expressed in anther sacs, anther filaments, and strongly in roots (Fig. 3C; Supplemental Fig. S3, M–R), and further analyses will be required to elucidate its contribution to the abscission process.

At3g14380 has been described as an uncharacterized protein; it has close homology to the gene *At1g17200*, sharing 54.5% homology at the amino acid level (http://www.ebi.ac.uk/Tools/msa/clustalw2/; Larkin et al., 2007; Goujon et al., 2010). Their predicted molecular masses are 19.18 and 21.89 kD and their pI values are estimated at 5.24 and 5.5, respectively (http://web.expasy.org/compute_pi/; Bjellqvist et al., 1993). It is also predicted that both of these proteins have four transmembrane domains and a possible N-terminal signal sequence (http://phobius.sbc.su.se/poly.html [Käll et al., 2005]; http://www.cbs.dtu.dk/services/TMHMM/ [Krogh et al., 2001]; Supplemental Fig. S4D). Integral membrane family proteins play important roles in signal transduction, cell adhesion, cell-cell communication, perception of environmental changes, transport, reception of signals, trafficking, and protein-protein interactions as well as maintaining the integrity, polarity, and size of the cells (Sadowski et al., 2008; Hubert et al., 2010). In has been reported that these proteins show more conservation in the transmembrane domains than the rest of the protein, and the conservation tends to prevail at one particular side of the helix, which suggests that interaction motifs may be conserved (Zviling et al., 2007). Recently, a novel protein family has been identified to play a crucial role in the formation of the Casparian strip in the root endodermis of Arabidopsis. These proteins act as membrane markers, where the Casparian strips will develop to form a diffusion barrier equivalent to animal epithelia (Roppolo et al., 2011). A phylogenetic analysis (Supplemental Fig. S1 in Roppolo et al., 2011) has revealed that both *At3g14380* and *At1g17200* belong to one of the clades of this tree. Some but not all of the conserved amino acids in the CASPs (for Casparian strip membrane domain protein) clade are conserved in *At3g14380* and *At1g17200* (Supplemental Fig. S4D). Roppolo et al. (2011) suggest that multiple interactions of the members of the CASPs can take place to aid in their localization at the Casparian strip membrane domain, and they speculate that they may form a scaffold for the localization and immobilization of cell wall biosynthetic enzymes and suggest that this role could be shared by the members of other clades. In the light of this hypothesis, one possible role for *At3g14380* is that it provides a physical "focus" for cell wall-modifying processes to take place, either for cell separation or for events post shedding of organs. Certainly, some targeted trafficking of proteins must take place to orchestrate the polarity of wall remodeling that is evident during abscission. It is known that abscission cells in the proximal side expand prior to separation (Patterson, 2001), and the proposal is that this expansion is required to create enough tension to provoke a mechanical rupture of the area. It has been reported that *EVERSHED*, a receptor-like kinase gene, plays a role in confining the size of AZ cells (Leslie et al., 2010), and if the role of *At3g14380* is also to contribute to the shaping of separating cells, it may be in the same pathway. In addition to the expression in AZ areas, we also observed expression of this gene in other areas, such as young leaf primordia of seedlings and vascular tissue of leaves (Supplemental Fig. S3, S–X); the role that this gene may play in these regions may be similar to what happens during abscission. Interestingly, during our RT-PCR analyses, we detected two different transcripts from this gene in buds and flowers, strongly suggesting the generation of at least two different protein isoforms of this gene.

Intriguingly, silencing the expression of *At3g14380* using a T-DNA insertion led to a delay in the timing of floral organ shedding, and this was reflected by a reduction in the rate at which the break strength of petals declined during development (Fig. 7). This observation confirms a functional role for *At3g14380* in abscission, and further work is ongoing to determine whether this effect is directly or indirectly on cell wall loosening.

At3g53040 is described as a LEA protein, with a predicted molecular mass of 52.084 kD and a pI of 5.29 (http://web.expasy.org/compute_pi/; Bjellqvist et al., 1993; Supplemental Fig. S4E). The abundance of the protein from this gene in nondormant seeds of the Arabidopsis Cape Verde Islands (Cvi-0) accession varies by a relative abundance of 0.30 ± 0.15 in imbibed seeds treated with 30 μM abscisic acid (ABA) for 1 d compared with untreated seeds, suggesting that this protein may be involved in protection of the seeds against abiotic stress and may be responsive to ABA (Chibani et al., 2006). Interestingly, Cadman et al. (2006) report more than 2-fold higher expression of this gene during the dormant stage of Cvi-0 seeds compared with the after-ripened stage. It is possible that the transcript may be accumulated during dormancy to allow the availability of the protein after this stage. Moreover, Hundertmark and Hincha (2008) reported an extensive study of LEA proteins from Arabidopsis, classifying *At3g53040* into the LEA_4 group. This is the dominant group containing 18 out of 51 genes, and they report that this gene is highly induced by ABA (3,312-fold) and that its transcript accumulates in seeds. It has been suggested that LEA proteins play an important role in tolerance to dehydration (Chibani et al., 2006; Battaglia et al., 2008; Hundertmark and Hincha, 2008) and act as chaperones to protect proteins or membranes, and it has also been suggested that they may act as molecular sponges of water and ion chelators (Cadman et al., 2006; Caramelo and Iusem, 2009). It is possible, therefore, that *At3g53040* acts in

the proximal AZ cells to prevent water loss after organ shedding (Fig. 3E; Supplemental Fig. S3, Y–AD). A role for ABA in abscission remains somewhat contentious (Rinne et al., 1992; Roberts and González-Carranza, 2007); however, it is possible that this hormone may play an important role in regulating the expression of *At3g53040* and other genes that may be involved in the prevention of water loss in the AZ.

At3g56350 is a gene described to be a member of the iron/manganase superoxide dismutase family. It is predicted to encode a protein with a molecular mass of 26.89 kD, a pI of 6.25 (http://web.expasy.org/compute_pi/; Bjellqvist et al., 1993), with a hydrophobic N-terminal peptide of 23 amino acids that is cleavable (http://www.cbs.dtu.dk/services/SignalP/; Nielsen and Krogh, 1998; Bendtsen et al., 2004; Supplemental Fig. S4F). Interestingly, together with the gene *At3g53040*, it has been reported to be expressed 2-fold higher during the dormant stage compared with the after-ripened stage of Cvi-0 seeds (Cadman et al., 2006), suggesting that these genes may be regulated by a similar mechanism. The protein of this gene has also been identified to increase in germinating seeds from the Arabidopsis Landsberg ecotype in the presence of 0.5 μM salicylic acid for 24 h, suggesting that this gene may play a role in defense (Rajjou et al., 2006). It is possible, therefore, that *At3g56530* encodes a protein that may protect the exposed area in the body of the plant and in other places where cell separation make take place.

At5g50540 is predicted to encode a small protein of 113 amino acids with a molecular mass of 13.025 kD and a pI of 11.06. This gene has an identical nucleotide sequence to *At5g60645*, with the only variability at their untranslated regions, suggesting that that area of chromosome 5 has experienced a very recent event of duplication (Swarbreck et al., 2008; Supplemental Fig. S4G). It is predicted that both genes *At5g50540* and *At5g60645* have an anchor signal in the C terminus with the cleavage site between positions 63 and 64 (http://www.cbs.dtu.dk/services/SignalP/; Nielsen and Krogh, 1998; Bendtsen et al., 2004), and the role of signal anchors is to retain a protein in a target membrane (von Heijne and Gavel, 1988; Sakaguchi et al., 1992). Taji et al. (2008) performed an analysis of cDNAs that were expressed when *Thellungiella halophila*, the halophyte model plant, was subjected to extreme abiotic stresses. *T. halophila* shares 90% homology with Arabidopsis genes. The authors found that *At5g50540* and *At5g50645* shared 30% identity with the RTFL01-03-G04 cDNA clone and classified this gene according to Gene Ontology terms as a sugar transporter with an InterPro identifier of IPR003663. Sugar transporters are responsible for carrying carbohydrates, acids, and organic alcohols in prokaryotes and eukaryotes (Mueckler et al., 1985). The expression of this gene has been also reported during pollen tube growth in Arabidopsis (Wang et al., 2008); it is possible that during this event, At5g50540/At5g50645 proteins aid the degradation of cell walls during the pollination process.

The Expression of *At2g41850*, *At1g64405*, and *At3g14380* Is Altered in *ida* and *bop1/bop2* Mutants and Pro_{35S}::*IDA*

To investigate how the genes *At2g41850*, *At1g64405*, and *At3g14380* may be affected during the abscission process, promoter-GUS fusion lines were crossed to an *ida* KO, an IDA overexpressor line, and a double KO of *bop1/bop2*. It was expected that if the expression of these genes is correlated with the timing of organ shedding, then the expression of the reporter gene would be reduced in the *ida* KO background, elevated in the IDA-overexpressing line, and absent in the double KO of *bop1/bop2*, as this line has been described to lack a differentiated AZ (Hepworth et al., 2005; McKim et al., 2008).

The *At2g41850*, *At1g64405*, and *At3g14380* genes show the spatiotemporal expression that would be predicted from abscission-related genes, with all of them showing reduced expression in *ida* and up-regulation in the IDA-overexpressing background in AZs. Intriguingly, both *At2g41850* and *At1g64405* are expressed in the *bop1/bop2* background, indicating that although the AZ cells in this double mutant are not anatomically identifiable, they still retain some abscission identity (Figs. 4–6).

In conclusion, we have presented a novel strategy to study the abscission process, capitalizing on the cell degradation machinery of the plant. We have isolated RNA transcripts from GFP-tagged cells that have undergone natural abscission, and this has allowed us to obtain an accurate representation of transcripts from AZs. By generating a cDNA library and using this to probe an Affymetrix chip, it has been possible to identify and characterize the expression of genes previously unidentified to play a role in the abscission process. This approach has allowed us to uncover new genes involved in abscission; some of them have been studied in detail, revealing that they are expressed in AZs as well as other events associated with seedling development, and by means of functional analysis, we demonstrated that the *At3g14380* gene plays a role during the floral abscission process. The method described in this paper can also be employed to identify transcripts from other sites where cell separation takes place and could be developed further to undertake proteomic and metabolomic studies. Furthermore, the novel genes identified may play key roles in regulating organ shedding and enable us to manipulate the process in crop plants.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis plants (*Arabidopsis thaliana*) Col-0, $Pro_{At2g41850}$::*GFP*, $Pro_{At2g41850}$::*GUS* (González-Carranza et al., 2002), $Pro_{At2g39700}$::*GUS*, $Pro_{At2g28950}$::*GUS* (D.J. Cosgrove, unpublished data), Pro_{35S}::*IDA* (Stenvik et al., 2006), *ida-2* (Cho et al., 2008), *bop1/bop2* (Col-0; Hepworth et al., 2005; McKim et al., 2008), transformed plants, KO lines (SALK_03248; Alonso et al., 2003), and crosses generated were grown in a growth chamber (with a temperature of 22°C and a 22-h photoperiod at 200 μmol m^{-2} s^{-1}) or in a glasshouse (with a temperature of 23°C ± 2°C and a 16-h photoperiod at 200 μmol m^{-2} s^{-1}) in plastic pots containing Levington M3 (Scotts) compost. To analyze patterns of expression in seedlings at different stages of development, seeds were germinated under sterile conditions as described by González-Carranza et al. (2007),

and plates were maintained in a growth room with a temperature of 22°C ± 2°C and a 24-h photoperiod at 200 μmol m^{-2} s^{-1}.

Generation of a cDNA Library from AZ Cells Tagged with GFP

About 30,000 floral AZ cells from 300 different flowers tagged with the GFP marker from the promoter fusion line *Pro$_{At2g41850}$::GFP* (González-Carranza et al., 2002) that had undergone natural separation were collected using micropipettes, which were generated by heating and pulling 1-mm glass capillary tubes (World Precision Instruments). The visualization and collection of the cells was performed using a fluorescence-dissecting microscope (Olympus SZX9).

The mRNA from the collected cells was isolated using the Dynabeads oligo $(dT)_{25}$ mRNA DIRECT Micro Kit (Dynal). It was estimated that 30,000 cells yield about 0.9 to 75 ng of mRNA. The mRNA isolated was used to generate a cDNA library using the SMART cDNA Library Construction Kit (Clontech Laboratories). The cDNA was synthesized using the long-distance PCR synthesis protocol following the manufacturer's instructions (Clontech Laboratories). The PCR amplification program used was 95°C for 1 min followed by 26 thermal cycles of 95°C for 15 s and 68°C for 6 min.

An internal control to test for genomic DNA contamination in the first-strand cDNA was performed using primers from the introns of the *At2g41850* gene (primer set no. 1; Supplemental Table S2). Once verified that the genomic DNA was not present in the cDNA, digestions in the cDNA amplified were performed using Proteinase K and *Sfi*I, the cDNA was fractionated using a Chroma Spin 400 column (Clontech Laboratories) to separate the desired sizes, and fractions 8 to 12 were pooled. The cDNA was precipitated, concentrated, and ligated to the arms of the λTriplEX2 vector (Clontech Laboratories) using three different concentrations, 0.5, 1.0, and 1.5 μL, from the cDNA in three separated ligation reactions. The packaging and amplification of the library were performed using the Gigapack III Gold Packaging Extract kit and *Escherichia coli* XLI-Blue cells, respectively (Stratagene).

The conversion of clones from the library from the λTriplEX2 clone pTriplEX2 plasmids was performed using *E. coli* BM25.8 cells grown at 31°C as indicated by the manufacturer (Clontech Laboratories). To test the fidelity of the pooled library, PCR was performed using specific primers to amplify the open reading frame of the *At2g41850* gene (primer set no. 2; Supplemental Table S2). Thermal cycling conditions were as follows: 94°C for 2 min, 30 cycles of 94°C for 30 s, 55°C for 1.5 min, 72°C for 1.5 min, followed by one cycle of 72°C for 7 min (GeneAmpPCR System 9700). Degenerate primers were also employed to amplify putative AZ products from expansin and metallothionein genes (primer sets no. 3 and no. 4, respectively; Supplemental Table S2). Thermal cycling conditions were as follows: 94°C for 2 min, 30 cycles of 94°C for 30 s, 55°C for 30 s for expansins and 60°C for 20 s for methallothioneins, 72°C for 30 s, followed by one cycle of 72°C for 7 min, respectively (GeneAmpPCR System 9700). The PCR was performed using 1 μL of first-strand cDNA as a template in a 25-μL reaction containing 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM deoxyribonucleotide triphosphates (Pharmacia), 2.5 units of Red Hot *Taq* polymerase (ABgene Laboratories), and 1 μM of each primer combination for the studied genes. The amplified products were cloned into the pGEM T-EASY vector (Promega). The PCR insert screening from the library was performed using the Advantage 2 PCR Kit and λTriplEx LD-Insert Screening Amplimers (Clontech Laboratories).

Microarray Analysis from a cDNA Library from Single AZ Cells from Arabidopsis

The cDNA library was used as a probe and was labeled and hybridized in duplicate to an Affymetrix GeneChip (ATH1) according to the manufacturer's instructions. The array was scanned and analyzed using Genespring software (version 5.0; Silicon Genetics). Log_2 values of the genes were normalized, and 1,205 genes flagged as being "marginal" or "present" were selected; 500 genes were subselected with either high expression in the cDNA library or showing higher expression than reported in the Genevestigator tool (Hruz et al., 2008). A tissue-specificity classification was performed with the Genevestigator information, and seven genes were selected for further analysis.

Spatial and Temporal Expression from Seven Genes Identified in the Microarray Analysis

To perform RT-PCR analysis, total RNA was isolated from wild-type Arabidopsis plants from roots, rosette leaves, cauline leaves, buds, flowers, stems, siliques, and yellow siliques as described by Han and Grierson (2002), except that 0.5 g of Arabidopsis tissue was used instead of tomato (*Solanum lycopersicum*) fruit tissue.

First-strand cDNA was synthesized from total RNA using the SuperScriptII Reverse Transcriptase Kit (Invitrogen) following the manufacturer's instructions, with each reaction containing 2 μg of tRNA. The PCR was performed using 1 μL of first-strand cDNA as a template in a 25-μL reaction containing 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM deoxyribonucleotide triphosphates (Pharmacia), 2.5 units of Red Hot *Taq* DNA polymerase (ABgene Laboratories), and 1 μM of each primer combination for the studied genes: *At1g64405*, *At2g23630*, *At2g44010*, *At3g14380*, *At3g53040*, *At3g56350*, *At5g50540*, and the nuclear cap-binding protein *At5g44200* as a housekeeping gene as a control (primer names HK-CBPFOR and HK-CBPREV; primer set nos. 5–12, respectively; Supplemental Table S2). Thermal cycling conditions were as follows: 94°C for 2 min, 30 cycles of 94°C for 30 s; and then 60°C/30 s for *At1g64405*, 60°C/2.5 min for *At2g23630*, 56°C/30 s for *At2g44010* and *At3g14380*, 60°C/1.5 min for *At3g53040*, 62°C/1.5 min for *At3g56350* and *At5g44200*, and 62°C/30 s for *At5g50540*; 72°C for 30 s for *At1g64405*, *At2g44010*, *At3g14380*, and *At5g50540*; 72°C for 1.5 min for *At3g53040*, *At3g56350*, and *At5g44200*; and 72°C for 2.5 min for *At2g23630*; followed by one cycle of 72°C of 7 min (GeneAmpPCR System 9700).

Generation of Constructs, Transgenic Plant Crosses, and Identification of KO Lines

Genomic DNA from Arabidopsis Col-0 leaf tissue was extracted using the GenElute Plant Genomic DNA Miniprep Kit (Sigma-Aldrich). DNA from all the constructs generated was amplified using the proofreading PlatinumPfxDNA polymerase (Invitrogen) following the manufacturer's instructions (primers used are the same as sets 13–19, but without the restriction sites, which are highlighted in boldface; Supplemental Table S2). Thermal cycling conditions were as follows: 94°C for 4 min, and 30 cycles of 94°C/15 s and 68°C/2 min for *At1g64405*; 94°C for 4 min, and 30 cycles of 94°C/15 s, 60°C/30 s, and 68°C/2 min for *At2g23630* and *At2g44010*; 94°C for 4 min, and 30 cycles of 94°C/15 s, 65°C/30 s, and 68°C/2, 1.5, 1, and 1.5 min for *At3g14380*, *At3g53040*, *At3g56350*, and *At5g50540*, respectively (GeneAmpPCR System 9700). The PCR products obtained were subcloned in pGEM T-EASY vector (Promega), confirmed by sequencing, reamplified with primers from sets 13 to 19 using the same thermal cycling conditions mentioned earlier, digested, and fused to the binary vectors pBI101.1 or modified pMOG::GUS (González-Carranza et al., 2007) at the multiple cloning sites or at the site of digestion.

Promoter and cloning information is as follows: *At1g64405* (promoter size of 1,865 bp, cloned into pBI101.1 using *Hin*dIII and *Bam*HI restriction sites), *At2g23630* (promoter size of 1,877 bp, cloned into MOG::GUS using *Xho*I and *Xba*I restriction sites), *At2g44010* (promoter size of 1,303 bp, cloned into pBI101.1 using *Hin*dIII and *Bam*HI restriction sites), *At3g14380* (promoter size of 1,303 bp, cloned into pBI101.1 using *Hin*dIII and *Bam*HI restriction sites), *At3g53040* (promoter size of 1,655 bp, cloned into MOG::GUS using *Xba*I and *Bam*HI restriction sites), *At3g56350* (promoter size of 884 bp, cloned into pBI101.1 using *Hin*dIII and *Bam*HI restriction sites), and *At5g50540* (promoter size of 1,407 bp, cloned into MOG::GUS using *Xba*I and *Bam*HI restriction sites).

Escherichia coli DH5α cells were transformed with the ligations; positive colonies were selected by PCR, and the integrity of the constructs was confirmed by sequencing. The plasmids were then electroporated and grown into *Agrobacterium tumefaciens* C58 strain to an optical density at 600 nm of 0.5 to 0.8. Arabidopsis wild-type Col-0 plants were transformed following the protocol described by Clough and Bent (1998).

Transgenic plants were selected by their ability to grow in Murashige and Skoog medium (Sigma; Murashige and Skoog; 1962) containing 40 mg L^{-1} kanamycin and confirmed by PCR using the correct set of primers per construct. The transformed plants were maintained to maturity, and their seeds were collected.

At least six homozygous lines were selected by segregating parental lines to check for the consistency of expression, and one of them was used to perform crosses with the different mutants or KO lines following the protocol by Weigel and Glazebrook (2002) using a GZ6 stereomicroscope (Leica Microsystems).

To down-regulate the expression of the gene *At3g14380*, a T-DNA insertion line was identified and ordered from the Arabidopsis Stock Center (SALK_032428; Alonso et al., 2003). This line has a T-DNA insertion in the second exon of *At3g14380*. To identify homozygous lines, two sets of PCR analyses were performed using two specific primers and one insert and one specific primer (primer sets 8 and 20, respectively; Supplemental Table S2) using the thermal conditions mentioned previously. To confirm the absence of

transcript in the KO lines identified, RT-PCR analysis was performed in floral tissues of 4-week-old plants using the methodology described earlier.

GUS Analysis

For GUS expression analysis, plant tissues from positive transformants were collected at different ages: 2-, 4-, 6-, 8-, 11-, and 15-d-old seedlings.

For analysis of expression during flower abscission, flowers from homozygous lines and from the different crosses obtained were collected. The position was determined from the first flower where petals were visible; from that position, all subsequent flowers were numbered. Flowers from lines $Pro_{At2g39700}$::*GUS* and $Pro_{At2g28950}$::*GUS* (D.J. Cosgrove, unpublished data), correspond to flower position 8.

The plant tissue was incubated in a solution containing 1 mg mL^{-1} 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (Medford Laboratories), 200 mM sodium phosphate, pH 7.0, and 0.06% (v/v) Triton X-100 and then incubated 4 to 12 h at 37°C. After the reaction was complete, the tissues were destained to remove the chlorophyll, using increasing concentrations of ethanol, before storing them in 100% ethanol at 4°C. Tissue was viewed and photographed using a Stemi SV6 stereomicroscope (Carl Zeiss International) with a Power Shot A620 digital camera using the Digital Camera Solution Software (Canon).

Confocal Microscopy

A movie from an Arabidopsis AZ flower was generated using the transgenic line $Pro_{At2g41850}$::*GFP* (González-Carranza et al., 2002); a flower in stage 8 (position determined as described previously) was analyzed using a TCS SP2 laser-scanning confocal microscope equipped with argon/krypton and green HeNe lasers and an AOBS scan head system (Leica Microsystems). The GFP protein was excited at 488 nm with the argon ion laser. The images and movie were recorded using the Leica Confocal software.

Break-Strength Analysis

Arabidopsis flowers from 4-week-old plants of identified KO and NS lines (lines 3 and 1, respectively; SALK_032428; Supplemental Figs. S5 and S6) for the gene *At3g14380* were excised from positions 3 to 5 on the inflorescence, where position 1 was considered the first flower with visible petals to the eye, and the pedicel of the flower was attached to a cocktail stick using superglue. A Lloyds LF plus machine used to measure tomato tensile strength was modified by adding a circular knob at the fixed base of the machine and the moving arm. The cocktail stick attached at the stalk of the flower was fitted in the groove of the knob at the fixed end of the machine, and another cocktail stick was fitted on the moving arm. The moving arm was lowered near the flower, and the petal was attached to the cocktail stick using superglue. NEXYGEN FM software was used to drive the moving arm away from the fixed base at 1 m min^{-1} at an angle of 180° to measure the force required to separate the petal from the stalk of the flower. The raw data obtained from the break-strength analysis of the KO and the control NS flowers was imported into Microsoft Excel. These values were used to perform statistical ANOVA (one-way ANOVA) by means of Genestat 13th Edition, and a graph was generated using Excel 2007.

Floral Phenotypic Analysis of *At3g14380* KO and NS Lines

Six plants from the identified KO and NS lines (lines 3 and 1, respectively; SALK_032428) were grown under the same conditions, and 15 flowers were dissected and analyzed using a Zeiss Stemi SV6 stereomicroscope.

Floral abscission was recorded with a Kodak MDS290 digital camera attached to the microscope. Images were analyzed with Adobe Photoshop software.

The Arabidopsis Information Resource accession numbers for the studied genes in this paper are as follows: *At1g64405*, 505006202; *At2g23630*, 2046763; *At2g44010*, 2051794; *At3g14380*, 2091045; *At3g53040*, 2085171; *At3g56350*, 2078356; *At5g50540*, 2159767.

Supplemental Data

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

Supplemental Figure S1. Amplification of a transcript in genomic DNA.

Supplemental Figure S2. Amplification of transcripts from an Arabidopsis AZ cDNA library.

Supplemental Figure S3. Analysis of GUS expression in seedlings.

Supplemental Figure S4. Sequence and protein information of genes studied.

Supplemental Figure S5. Identification of KO plants.

Supplemental Figure S6. Analysis of expression from KO and NS plants.

Supplemental Table S1. Selected genes from hybridization experiments.

Supplemental Table S2. Set of primers used in the experiments.

Supplemental Video S1. Confocal movie from a floral AZ from Arabidopsis.

ACKNOWLEDGMENTS

We thank Reidunn B. Aalen and Melinka A. Butenko for the *ida* mutant and the Pro_{35S}::*IDA* seeds; Sarah McKim for the *bop1/bop2* seeds; Daniel Cosgrove for the $Pro_{At2g39700}$::*GUS* and $Pro_{At2g28950}$::*GUS* seeds; Manojit Basu for developing the technique to determine petal breakstrength; and Philippe Lamesch for help with the information from the duplication in chromosome 5.

Received August 21, 2012; accepted September 16, 2012; published September 19, 2012.

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