Stamen Abscission Zone Transcriptome Profiling Reveals New Candidates for Abscission Control: Enhanced Retention of Floral Organs in Transgenic Plants Overexpressing Arabidopsis *ZINC FINGER PROTEIN2*[1][C][W][OA]

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Organ detachment requires cell separation within abscission zones (AZs). Physiological studies have established that ethylene and auxin contribute to cell separation control. Genetic analyses of abscission mutants have defined ethylene-independent detachment regulators. Functional genomic strategies leading to global understandings of abscission have awaited methods for isolating AZ cells of low abundance and very small size. Here, we couple laser capture microdissection of *Arabidopsis thaliana* stamen AZs and GeneChip profiling to reveal the AZ transcriptome responding to a developmental shedding cue. Analyses focus on 551 AZ genes (AZ_{551}) regulated at the highest statistical significance ($P \leq 0.0001$) over five floral stages linking prepollination to stamen shed. AZ_{551} includes mediators of ethylene and auxin signaling as well as receptor-like kinases and extracellular ligands thought to act independent of ethylene. We hypothesized that novel abscission regulators might reside in disproportionately represented Gene Ontology Consortium functional categories for cell wall modifying proteins, extracellular regulators, and nuclear-residing transcription factors. Promoter-β-glucuronidase expression of one transcription factor candidate, *ZINC FINGER PROTEIN2* (*AtZFP2*), was elevated in stamen, petal, and sepal AZs. Flower parts of transgenic lines overexpressing *AtZFP2* exhibited asynchronous and delayed abscission. Abscission defects were accompanied by altered floral morphology limiting pollination and fertility. Hand-pollination restored transgenic fruit development but not the rapid abscission seen in wild-type plants, demonstrating that pollination does not assure normal rates of detachment. In wild-type stamen AZs, *AtZFP2* is significantly up-regulated postanthesis. Phenotype data from transgene overexpression studies suggest that AtZFP2 participates in processes that directly or indirectly influence organ shed.

Abscission zones (AZs) are tiers of small, densely cytoplasmic cells located at sites of organ detachment (Sexton et al., 1985). Within AZs reside one or more cell layers that separate in response to developmental or environmental cues. Shed is typically preceded by AZ cellular rounding coupled with differential enlargement on opposing sides of the future fracture plane (Morre, 1968; Sexton et al., 1985). Accompanying cell enlargement is the activation of multiple pectin and hemicellulose-modifying proteins that modify primary wall structure and reduce adhesion between AZ cells (del Campillo, 1999; Roberts et al., 2002). Accumulation of pathogenesis-related (PR) proteins limits invasion of pathogens into the proximal AZ face until wound sealing is complete (Roberts et al., 2000). Waxy protection layers finally form across the fracture plane.

Functional analyses of abscission-impaired mutants have identified genes that control abscission competence from the time AZ cells differentiate through the time they separate. *LATERAL SUPPRESSOR* (*LS*) encodes a VHIID regulatory gene (Schumacher et al., 1999) required for tomato (*Solanum lycopersicum*) pedicel AZ development (Malayer and Guard, 1964). *JOINTLESS* encodes a MADS-box gene (Mao et al., 2000) also required for AZ differentiation. Lesions in AZ formation in both *ls* and *jointless* mutants are restricted to flower and fruit pedicels; functional AZs are present in leaves and other structures (Butler, 1936; Malayer and Guard, 1964). Thus, AZ differentiation differs between organs. Differentiation must be complete before a plant is competent to respond to abscission signals (Osborne and Sargent, 1976). Although positional and biochemical differentiation may occur early in organogenesis (Kendall, 1918; Osborne and Sargent, 1976; McManus and Osborne, 1990, 1991), morphological features defining AZs may

not be visible. In cotton (*Gossypium hirsutum*), clear AZ structure is not present until before shed, when further AZ cell division may occur (Leinweber and Hall, 1959; Bornman et al., 1967). In Arabidopsis (*Arabidopsis thaliana*) and tomato, clear morphological features define AZs long before abscission.

Genetic studies show abscission capacity to be influenced by disruptions in organ boundaries and other alterations in organ patterning. Partial fusion of sepals in the F-box gene mutant *hawaiian skirt* impairs shedding of Arabidopsis floral parts in which AZs appear to differentiate normally (González-Carranza et al., 2007b). Two BTB/POZ domain proteins, BLADE ON PETIOLE1 (BOP1) and BOP2, are expressed in regions overlapping the floral organ AZs (Ha et al., 2004; Hepworth et al., 2005; Norberg et al., 2005). A *bop1bop2* double mutant exhibited abnormal organ patterning and loss of floral organ abscission (Hepworth et al., 2005; Norberg et al., 2005). Other transcription factors contributing to abscission competence include the structurally related MADS-box domain proteins AGL15 and AGL18; shedding of Arabidopsis floral parts is delayed in plants overaccumulating either protein (Fernandez et al., 2000; Adamczyk et al., 2007). *AGL15* and *AGL18* overexpressors exhibit concomitant slowing of other developmental transitions including flowering time and senescence (Fernandez et al., 2000; Adamczyk et al., 2007). Early flowering time and a slowing of floral senescence also accompany delayed abscission in plants with knocked-down levels of ACTIN-RELATED PROTEIN4 (ARP4; Kandasamy et al., 2005a). Similarly, knocking down *ARP7* expression levels delays abscission of floral parts and alters flower development, impacting fertility (Kandasamy et al., 2005b). Pleiotropic phenotypes of *ARP* RNAi plants have been predicted to arise from aberrant gene transcription patterns caused by altered chromatin structure (Kandasamy et al., 2005b).

In wild-type plants, binding of ethylene to one or more ethylene receptors derepresses the ethylene signal transduction pathway leading to hormone-dependent responses including abscission. Once considered a fundamental abscission signal (Jackson and Osborne, 1970), ethylene is now viewed as an abscission rate regulator (Bleecker and Patterson, 1997; Patterson, 2001). Mutations in the *etr1-1* ethylene receptor gene that render Arabidopsis plants insensitive to ethylene delay, rather than block, floral organ abscission (Patterson and Bleecker, 2004). Ethylene control of abscission is influenced in part via antagonism between ethylene and auxin. Abscission is delayed in plants with suppressed expression of the *ARF2* member of the auxin response family (Ellis et al., 2005). ARF2 controls auxin signaling (Ellis et al., 2005) and ethylene synthesis is altered in *arf2* mutants (Okushima et al., 2005). Nonhormone ligands controlling abscission include that encoded by *INFLORESCENCE DEFICIENT IN ABSCISSION* (*IDA*). Loss-of-function *ida* mutants fail to abscise Arabidopsis sepals, petals, and stamens (Butenko et al., 2003); transgenic lines overexpressing *IDA* rapidly shed all floral organs, as well as additional plant parts that do not normally abscise (Stenvik et al., 2006). The receptor for IDA is unknown but has been proposed to include receptor-like kinases (RLKs). Antisense inhibition of gene expression corresponding to the RLK termed HAESA (HAE) blocks abscission of Arabidopsis stamens, sepals, and petals (Jinn et al., 2000). Expression of *HAE* appears to be independent of ethylene synthesis or perception (Jinn et al., 2000). This is also the case with *AGL15* (Fernandez et al., 2000) and *ARP7* (Kandasamy et al., 2005b). At present, mechanisms by which putative ethylene-independent pathways contribute to abscission are poorly understood.

Many questions remain about abscission signaling. What is the primary abscission cue or cues? What regulators exert earliest control over signal perception and response? Why do some organs abscise in response to a given stimulus whereas others are retained? What genes control ethylene-dependent and independent pathways and how do pathways interact? Functional genomic approaches to addressing these questions have been hindered by an inability to obtain pure populations of AZ cells. Recently, we optimized methods for using laser-capture microdissection (LCM) to harvest highly enriched populations of specialized cells (Cai and Lashbrook, 2006). There, replum cell harvest was linked to ATH1 GeneChip studies of fruit maturation (Cai and Lashbrook, 2006). Here, we reveal dynamic changes in global gene expression taking place in AZs of Arabidopsis stamens progressing from prepollination to organ shed. Functional analyses of one AZ-up-regulated gene, *ZINC FINGER PROTEIN2* (*AtZFP2*), provide evidence that this transcription factor participates in processes that directly or indirectly influence shedding of floral organs.

RESULTS AND DISCUSSION

LCM Facilitates AZ Transcriptome Profiling

Arabidopsis stamens, sepals, and petals abscise postpollination. Detachment is dependent upon separation of AZ cells residing at the bases of floral organs. Small AZ sizes and low numbers of AZ cells in Arabidopsis and other plants have historically confounded efforts to prepare enriched AZ populations for downstream analyses. We chose to isolate AZ cells using LCM, a method that has been successfully used to capture multiple specialized cell types from plants. Sources for laser-captured cells have included, but are not limited to embryos (Casson et al., 2005), the shoot apical meristem (Ohtsu et al., 2007a; Zhang et al., 2007), phloem (Asano et al., 2002; Vilaine et al., 2003; Yu et al., 2007), leaf epidermis (Nakazono et al., 2003; Murata and DeLuca, 2005), leaf mesophyll (Corpas et al., 2006), bundle sheath cells (Kerk et al., 2003), ovules and replums (Cai and Lashbrook, 2006), cell walls (Angeles et al., 2006), roots (Ramsay et al., 2004; Woll et al., 2005; Alkharouf et al., 2006; Dembinsky et al., 2007; Ithal et al., 2007; Klink et al., 2007), and

floral organs (Nakada et al., 2006). LCM and its applications to biological studies in plants have been recently reviewed by Day et al. (2005, 2007), Nelson et al. (2006), and Ohtsu et al. (2007b).

We chose stamens as a source of AZs for LCM studies because there are six stamens instead of four petals or sepals. This elevates the relative incidence of AZs in sectioned tissue and reduces the time required for cell capture. Whole flowers corresponding to five developmental stages linking prepollination to the onset of organ shed were fixed and paraffin-embedded and sectioned tissues were tape-transferred to slides (Cai and Lashbrook, 2006). Figure 1A depicts developmental stages of flowers from which stamen AZs were harvested. Stage numbers are those of Smyth et al. (1990). Anthesis occurs at Stage 13. Stage 15 was divided into substages a to c as defined in "Materials and Methods". Stage 16 tissue was not prepared for LCM after it was determined that floral organs detached at their AZs during early fixation steps.

In Figure 1, B and C, scanning electron microscopy (SEM) visualizes surface features of AZ fracture planes at stages selected for abscission studies. Figure 1B depicts stamen AZ scars left on the parent plant after manual filament removal; Figure 1C shows parental sides of all floral organ fracture planes after organ detachment. The intact rounded AZ cells observed in Figure 1B (stages 15c and 16) and Figure 1C represent proximal AZ cells that have completely separated from contiguous distal AZ cells of the leaving organ (Patterson and Bleecker, 2004). In contrast, torn AZ cells observed in Figure 1B (stages 12–15c) testify that cell wall dissolution processes needed for separation from neighboring cells are not yet complete. In our studies, intact cells present on proximal fracture faces after organ removal are first evident on outermost AZ margins at stage 15c (Fig. 1B). Thus, final stages of separation occurring within a subset of stamen AZ cells have occurred by stage 15c. Cell separation is essentially complete between all stamen AZs of the proximal fracture plane by stage 16, when all AZ cells are rounded and intact (Fig. 1B) and organs detach when lightly touched.

Stamen AZ cells were laser-captured from flowers at stages 12 to 15c. Figure 1, D and E, shows representative flower sections before and after LCM of stamen AZs, respectively. Microdissected AZs included cells from the vascular bundle that passes through all abscission layers. Approximately 10,000 floral organ AZ cells could be captured in approximately 1 d and the amount of RNA subsequently isolated per laser-captured cell was approximately 10 to 15 pg (Cai and Lashbrook, 2006). Total RNA served as the template for preparing hybridization targets for ATH1 Gene-Chips (Cai and Lashbrook, 2006).

Probe Set Signals Regulated at the Highest Level of Statistical Significance Define a 551-Member Slice of the AZ Transcriptome

ATH1 GeneChips contain probe sets representing approximately 24,000 genes of the Arabidopsis genome.

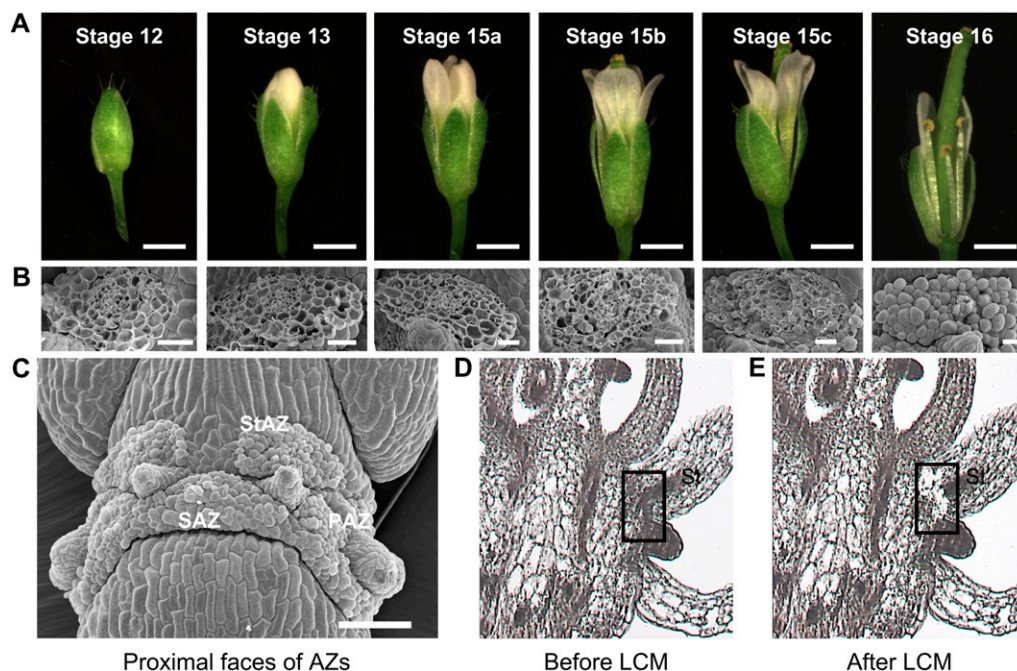**Figure 1.** Developmental stages of Arabidopsis flowers and scanning electron and light micrographs of floral organ AZs. A, Flower stages. Scale bar, 1 mm. B, SEM of stamen AZ fracture planes. Scale bar, 20 μm. C, SEM of proximal faces of AZs from stamens (StAZ), sepals (SAZ), and petals (PAZ). Scale bar, 100 μm. D and E, Sectioned floral tissue before (D) and after (E) LCM of stamen AZs (shown within boxes).

Replicated hybridizations of biotin-labeled aRNAs from AZs at stages 12 to 15c revealed statistically significant changes in probe set signal intensities corresponding to many AZ transcripts. We wished to restrict preliminary analyses to a manageable number of genes whose expression could provide a first glimpse of regulatory processes leading to cell separation. Restricting attention to the most significantly regulated probe sets ($P \leq$ 0.0001) generated a population of 551 transcripts (AZ_{551}) representing the Arabidopsis stamen AZ transcriptome. An approximately 0.2% false discovery rate was determined using the method of Storey and Tibshirani (2003). A complete list of AZ_{551} genes is in Supplemental Table S1. Quantitative real-time PCR (Q-PCR) successfully validated microarray expression data for a subsample of 10 probe sets using stamen AZ RNA from laser-captured cells (data not shown). Supplemental Figure S1 depicts expression patterns of three validated transcripts related to GA metabolism or action. Both Q-PCR and microarray data showed similar trends in mRNA accumulation for the GA-related mRNAs shown in Supplemental Figure S1 and discussed later in the text.

Functional categorization of AZ_{551} using Gene Ontology Consortium (GO) tools (Berardini et al., 2004) on the Arabidopsis Information Resource Web site (http://www.arabidopsis.org) permitted comparisons between predicted functions of AZ transcripts and all Arabidopsis transcripts represented on the ATH1 GeneChip (Table I). Functional classification by the GO cellular component showed that the cell wall and extracellular matrix were represented in AZs at levels exceeding those in ATH1 by approximately 3- to 4-fold. The nucleus was represented in AZs at levels exceeding that of ATH1 by 1.6-fold (Table I). Consistent with AZ cellular component data was the increased representation of GO categories representing molecular function (Table I). Transcription factor activities within the AZ_{551} exceeded that of ATH1 by almost 2-fold. Collectively, the GO cellular component

and molecular function data are consistent with known contributions of transcription factors and cell wall modifying proteins to abscission competence and cell wall disassembly, respectively (Mao et al., 2000; Roberts et al., 2002).

AZ Transcripts in the 551-Member Population Can Be Assigned to Eight Clusters with Both Distinct and Overlapping Functions

Cluster analysis is a statistical procedure that identifies and organizes patterns contained in complex data sets (Spellman, 2003). In genomic studies, clusters may represent common patterns of gene expression observed within a larger gene transcript population. Eisen et al. (1998) and others have shown that functionally related transcripts are often housed in the same cluster. Thus, knowing what biological functions are associated with a subset of transcripts within a cluster can provide important clues about potential roles for noncharacterized transcripts within the same population. As the first step toward establishing what discrete biological processes may collectively facilitate cell separation in AZs, we initiated cluster analyses of the AZ_{551} transcript set. The gap statistic method of Tibshirani et al. (2001) estimated that eight clusters could reasonably represent the gene expression patterns found within this transcript collection (Fig. 2). In brief, the gap statistic procedure assumes that a data set comprises a single component. This assumption serves as a null hypothesis that is rejected when statistical evidence supports the presence of additional clusters separated within expression space (Tibshirani et al., 2001). The K-medoid-based clustering algorithm of Kaufman and Rousseeuw (1990) used a distance metric to group AZ_{551} transcripts sharing similar distributions in expression space. In Figure 2, clusters 1 to 3 represent up-regulated genes within AZ_{551}; clusters 5 to 7 contain down-regulated genes and the remaining

Table I. *Functional categorization of the 551 most significantly regulated transcripts in stamen AZs*

Functional categories in which AZ transcript abundance exceeded that found in the ATH1 by at least 50% are represented. GO categorization data were determined on July 18, 2007. *P*-values were obtained using Fisher's exact test.

| GO Classification by Genes	Percent of Total Transcripts		AZ Overrepresentation	*P*-Value
	ATH1	AZ_{551}		
A. By cellular component			Fold Change	
Cell wall	0.925	3.5	3.8	1.1 e-7
Extracellular	0.72	2.3	3.2	8.0 e-5
Nucleus	7.6	12.1	1.6	1.5 e-5
Other membranes	20.1	31.1	1.5	1.1 e-11
B. By molecular function				
Transcription factor activity	5.8	11.3	1.95	2.9 e-12
C. By biological process				
Response to stress	2.2	4.6	2.1	4.5 e-10
Response to abiotic or biotic stimulus	2.7	5.2	1.9	4.2 e-10
Transcription	3.7	5.4	1.5	2.2 e-6
Signal transduction	2.0	2.9	1.5	9.8 e-4
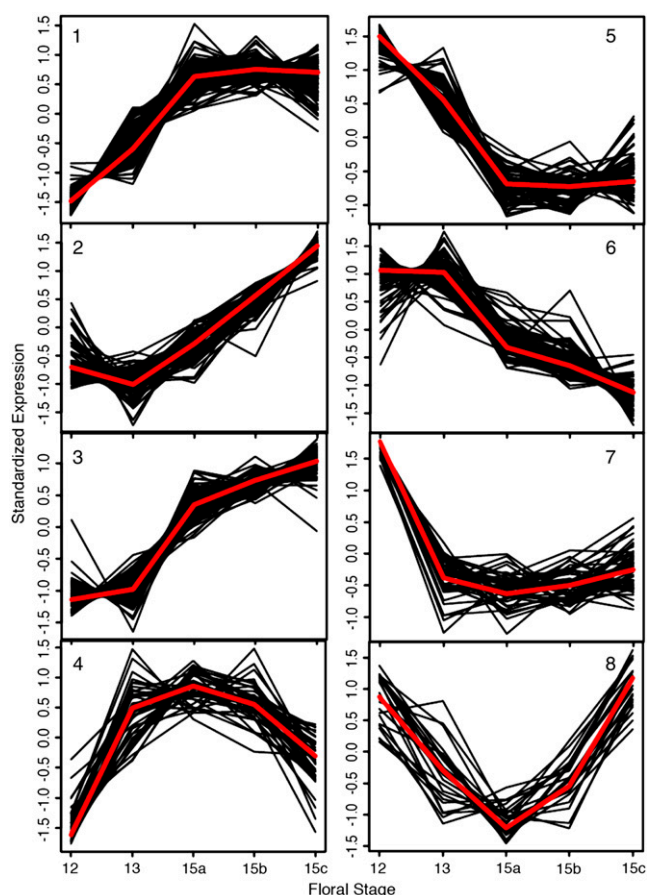
Figure 2. Classification of 551 AZ transcripts into eight clusters with similar patterns of gene expression. Clustering algorithms are described in "Materials and Methods". [See online article for color version of this figure.]

imparted confidence that AZ_{551} would contain functionally uncharacterized transcripts with potentially novel roles in abscission.

In Table I, GO functional categories for AZ_{551} transcripts were compared with Arabidopsis genome transcripts on the ATH1 GeneChip. We similarly wished to establish if certain functions were overrepresented to statistically significant extents in one or more of the eight transcript clusters. In Table II, GO functional categorization of clusters coupled with an assessment of the statistical significance of relative category representation reveals general trends. First, processes occurring in cell walls are significantly overrepresented in clusters with up-regulated transcripts, consistent with known de novo synthesis of wall modifying proteins during abscission (Taylor and Whitelaw, 2001; Roberts et al., 2002). Statistically overrepresented functions within both up- and down-regulated clusters include nuclear functions involving transcription factors and nucleotide binding proteins, and transport functions (Table II). Supplemental Table S2 reveals transcript fold changes between floral stages for a subset of AZ_{551} genes. Annotation conventions within Supplemental Table S2 include those of Kende et al. (2004), Nakano et al. (2006), Palusa et al. (2007), and Rose et al. (2002).
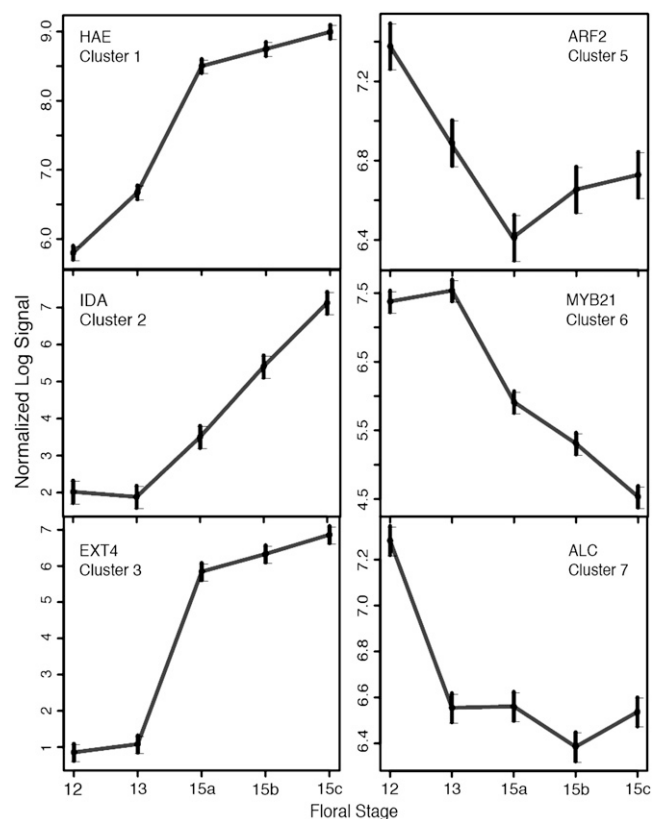
Figure 3. Expression profiles of selected cell separation regulators represented in AZ_{551}. Mean natural log signal intensities are plotted across developmental stages. Gene names are defined in the text.

two clusters represent genes that are both up- and down-regulated. Transcript identities are annotated in Supplemental Table S1.

Figure 3 demonstrates that six clusters within the eight-member cluster collection contain transcripts shown by previous genetic studies to participate in cell separation processes associated with abscission or dehiscence. The effects on abscission of modulating gene expression corresponding to *HAE* (cluster 1), *IDA* (cluster 2), and *ARF2* (cluster 5) were described earlier. mRNA for cluster 3's *AtEXT4* (extensin 4) extensin, previously termed *EXT1*, was previously shown to accumulate in floral AZs and other tissues, where it is responsive to hormonal cues (Merkouropoulos and Shirsat, 2003). *MYB21* gene expression (cluster 6) is required for male fertility; knockout mutants exhibit delayed anther dehiscence (Mandaokar et al., 2006). ALCATRAZ (cluster 7) is required for cell separation during silique dehiscence (Rajani and Sundaresan, 2001). Collectively, data of Figure 3 demonstrate that AZ_{551} contains genes previously defined as being expressed in separating plant cells. Their presence

Table II. *Overrepresented functions in transcript clusters relative to ATH1 probe sets*

GO categorization data were determined on July 18, 2007. *P*-values were obtained using Fisher's exact test.

Cluster No.	By GO Cellular Component	*P*-Value	By GO Molecular Function	*P*-Value
Up-regulated				
1	Nucleus	0.015	Transcription factor activity	0.00002
	Cell wall	0.0009	Nucleotide binding	0.0003
	Other membranes	0.029	Transferase activity	0.02
			DNA or RNA binding	0.03
2	Cell wall	0.0007	Hydrolase activity	0.002
	Extracellular	0.017	Other enzyme Activity	0.0004
	Endoplasmic reticulum	0.007		
3	Nucleus	0.03	Transcription factor activity	0.00001
	Cell wall	0.003	Other enzyme activity	0.008
	Other membranes	0.0019		
Up-regulated early; down-regulated late				
4	Other membranes	0.003	Transporter activity	0.037
	Mitochondria	0.042		
Down-regulated				
5	Extracellular	0.019	Transferase activity	0.008
	Other membranes	0.00003	Hydrolase activity	0.0004
6	Nucleus	0.0004	Transcription factor activity	1.5 e-7
	Cell wall	0.03	DNA or RNA binding	0.001
	Extracellular	0.016		
	Plasma membrane	0.04		
7	Mitochondria	0.03	Transferase activity	0.03
	Other membranes	0.016	Hydrolase activity	0.04
Down-regulated early; up-regulated late				
8	–		Transporter activity	0.025
			Other enzyme activity	0.027

Potential Functions of Up-Regulated Gene Clusters

Up-Regulated Cluster 1 Genes Encode Transcriptional Modulators, RLKs, Cell Wall Modifying Proteins, and Regulators of Hormone Biosynthesis, Action, and Transport

Cluster 1 transcripts increase in abundance between stages 12 and 15a and remain at high levels as abscission commences near stages 15b and 15c (Fig. 2). GO-annotated genes within cluster 1 are assigned to the nuclear compartment at rates surpassing those for other transcripts represented on the ATH1 GeneChip (Table II). Approximately 18% of cluster 1 mRNAs are assigned to a nuclear site versus 7.6% of ATH1 Arabidopsis genes and approximately 12% of AZ_{551} (data not shown). Myb transcription factors are well represented. Supplemental Table S2 reveals transcript fold changes between stages for a subset of AZ_{551} genes including Myb factors. Up-regulated genes include *AtMYB4*, *14*, *45*, *62*, and *75* (Supplemental Table S2). All of these Mybs are modulated to some extent by Suc and nitrogen status with *AtMYB14* also weakly regulated by auxin (Kranz et al., 1998). AtMYB4 and AtMYB75 gene products act as negative and positive transcriptional regulators, respectively, of phenylpropanoid biosynthesis (Borevitz et al., 2000; Jin et al., 2000; Rose et al., 2002). GO molecular function comparisons of cluster 1 transcripts show that transcription factor activities as well as activities for DNA,

RNA, or nucleotide binding are overrepresented (Table II). Up-regulated genes in Supplemental Table S2 include RLKs that translate extracellular signals into cellular responses (Becraft, 2002). AZ_{551} RLKs present in annotations of Shiu and Bleecker (2001) include *HAE* (At4g28490), *HAESA-LIKE2* (*HSL2*; At5g65710), *FLAGELLIN SENSITIVE2* (At5g46330), and others (At1g09970, At3g14840, and At5g59010). *HAE* is noteworthy because Jinn et al. (2000) have reported that antisense inhibition of *HAE* prevented abscission of stamens, sepals, and petals. In our studies, developmental up-regulation of *HAE* at stage 12 (Supplemental Table S2) precedes first visible signs of cell separation at stage 15c (Fig. 1B) by several stages. We conclude that HAE must represent a very early contributor to abscission competence.

HAE appears to mediate steps within an ethylene independent abscission pathway as corresponding antisense mutants have the ethylene sensitivity to evoke a hormone-dependent triple response equivalent to that of wild-type controls (Jinn et al., 2000). Ethylene likely controls the rate of a separate abscission pathway that could communicate with components of the HAE route (Butenko et al., 2006). Our data suggest that both ethylene-dependent and -independent pathways are operational after stage 12 (Supplemental Table S2). Components of the former include ethylene response factors (ERFs) 003 and 072 (At5g25190 and At3g16770) and ethylene insensitive 3 (EIN3)-binding F-box protein 2

(EBF2; At5g25350) that contributes to SCF-dependent ubiquitination and degradation (Potuschak et al., 2003). Modulated ethylene action is accompanied by induction of multiple auxin responsive genes and accumulation of the *PIN4* auxin efflux transcript (At2g01420). BR6OX2 (At3g30180) mediates the last step of brassinolide synthesis. It is up-regulated as is the brassinolide-enhanced target *BEE3* (At1g73830) and GA-regulated *GASA5* (At3g02885; Supplemental Fig. S1). Thus, expression of RLKs like *HAE* and *HSL2* takes place in a complex backdrop of hormone signaling.

Abscission rate is partially controlled by cell wall modifying proteins (Lashbrook et al., 1998; Brummell et al., 1999). Almost 5% of transcripts in cluster 1 are assigned cell wall localization by GO analysis in contrast to almost 1% of all Arabidopsis genes (data not shown). Regulated wall transcripts in Supplemental Tables S1 and S2 include *AtXTH28* (At1g14720), one member of the complex endotransglucosylase/hydrolase (XTH) gene family (Becnel et al., 2006). In current cell wall structural models, xyloglucans coat cellulosic microfibrils and tether microfibril neighbors (Hayashi, 1989; Carpita and Gibeaut, 1993). XTHs have one or both of two activities that can loosen cell walls during growth or strengthen them during assembly (Thompson and Fry, 1997, 2001; Rose et al., 2002). Similarly, cluster 1 transcripts include multiple peroxidases including *PER21* (At2g37130), *PER30* (At3g21770), and *PER33* and/or *PER34* (At3g49120 and/or At3g49110). Peroxidases, like XTHs, may loosen or stiffen cell walls in different cellular contexts (Passardi et al., 2005). In roots, PER34 increases root length (Passardi et al., 2005), presumably via cell wall loosening.

Up-Regulated Cluster 2 Transcripts Encode Proteins with Roles in Pectin Modification, Hormone Synthesis and Degradation, Receptor Binding, and Signal Transduction

Cluster 2 transcript levels rise throughout all stages assayed whereas cluster 1 mRNA abundance stabilizes after stage 15a (Fig. 2). It is possible that gene expression at later stages in cluster 2 contributes to processes necessary for final stages of organ detachment. Cluster 2 is enriched in transcripts with likely roles in cell wall structural modification (Table II). Regulated cell wall genes encode two glycosyl hydrolases with structural similarities to endo-β-1,3-glucanases (At4g18340) and endo-β-1,4-glucanases (At2g32990). However, most up-regulated cell wall genes encode pectolytic enzymes, including three pectin methylesterases (PMEs; At2g45220, At2g47550, and At4g02330), two polygalacturonases (At2g43890 and At3g07970), one pectate lyase-like protein (At3g27400), and a member of the invertase/PME inhibitor (PMEI) family (At3g47380). PMEs deesterify pectin to establish structurally and functionally distinct pectin classes (Ridley et al., 2001; Willats et al., 2001) and interact cooperatively with polygalacturonase (PG) to regulate homogalacturonan disassembly (Lashbrook, 2005). PMEIs regulate PMEs at the transcriptional and/or posttranslational levels.

Pectate lyases, like PG, prefer homogalacturonan substrate de-esterified by PMEs, suggesting that cluster 2 transcripts have the potential to act cooperatively. The preponderance of pectin-modifying proteins is consistent with presumptive roles for pectin disassembly in reducing intercellular adhesion between AZ cells (Roberts et al., 2002). González-Carranza et al. (2002) reported that the promoter of abscission-related polygalacturonase *PGAZAT* (At2g41850) directed GUS expression to the bases of all floral organs after natural abscission was initiated. At2g41850 was not a member of our AZ$_{551}$ population restricted by *P*-value. However, evaluation of its expression elsewhere in our data revealed low probe set signal intensities between stages 12 and 15b and increased expression at stage 15c (data not shown). These results may be consistent with the results of González-Carranza et al. (2002); *PGAZAT* gene expression initiated upon natural abscission would be expected to follow stage 15c, where the first signs of completed cell separation appear in Figure 1B.

IDA (At1g68765) encodes a 77-amino-acid ligand secreted into the extracellular matrix (Butenko et al., 2003). *ida* knockout mutants possess AZs of apparent normal structure but fail to abscise floral organs (Butenko et al., 2003). Like antisensed *HAE* plants (Jinn et al., 2000), *ida* mutants are ethylene sensitive (Butenko et al., 2003). Lines overexpressing *IDA* exhibit ectopic abscission of nonfloral organs along with normal organ shed (Stenvik et al., 2006). These data initially suggested that IDA was a component of an ethylene-independent pathway (Butenko et al., 2003), although subsequent studies showed ethylene might affect spatial distribution of *IDA* gene expression (Butenko et al., 2006). In fact, stimulation of IDA signaling in floral AZs may occur in a more complex hormonal background. Controllers of AZ hormone response may include ERF086 (At5g18560) and BRS1 (At4g30610), a suppressor of the brassinosteroid receptor BRI1. Up-regulated transcripts of hormone biosynthesis include anthranilate synthase (At5g05730), which encodes the enzyme catalyzing the first committed step of Trp biosynthesis and hence potential auxin production. GA 2-OXIDASE2 expression (At1g30040; Supplemental Fig. S1) leads to GA catabolism via 2-β-hydroxylation (Thomas et al., 1999). *OPR1* (At1g76680) and/or *OPR2* (At1g76690), homologs of the *OPR3* jasmonate (JA) synthesis gene, are also up-regulated. The ATH1 GeneChip cannot distinguish between *OPR1* and *OPR2*, but promoter-GUS studies suggest that up-regulated AZ activity is *OPR1* (Biesgen and Weiler, 1999). OPR1 and OPR2 are considered unrelated to JA production (Schaller et al., 1998) because they act on steroisomeric forms of OPDA substrate unsuitable for JA biosynthesis (Schaller and Weiler, 1997; Schaller et al., 2000).

Likely Functions of Cluster 3 and 4 Genes Overlap with Those of Clusters 1 and 2

Multiple up-regulated Myb factors complement those of other clusters, including *MYB2, 17, 37, 49,*

68, 74, 116, and a Myb-like factor (Supplemental Table S2). MYB17 and MYB68 are regulated by nitrogen and sugar status (Kranz et al., 1998). Nutrient recycling precedes abscission, and transporter activity in AZ_{551} and cluster 4 exceeds that found in the whole genome (Tables I and II). Transporters that might remove nutrients from senescing or detaching organs are exemplified by cluster 3's sugar alcohol permease homolog, AtPLT5 (At3g18830). Strong up-regulation of *AtPLT5* in floral AZs is consistent with previous reports suggesting a role in the reuptake of sugars released by polysaccharide degrading enzymes (Reinders et al., 2005).

Multiple regulated genes in clusters 3 and 4 control hormone signal transduction and cell wall structure. They include *ERF043* (At4g32800), *ERF092* (At3g23240), and *ERF113* (At5g13330). Expression of *ERF092*, formerly termed *ERF1*, is induced by JA and ethylene in defense gene systems (Lorenzo et al., 2003). A potential hormone-regulated target during abscission is ethylene-responsive protein 33-like (At1g05710). Modulation of ethylene sensitivity is accompanied by activation of auxin-responsive proteins (At3g60690 and At5g35735). Up-regulation of a BRI1 receptor homolog (At3g13380) is consistent with the up-regulation in cluster 1 of the BRS1 component of early BR signaling (Li et al., 2001). Like *HAE*, *BRI1* encodes a Leu-rich repeat RLK (Friedrichsen et al., 2000) and its induction in AZ_{551} suggests that RLK signaling is central to cell separation control. Expression data for two additional RLKs in cluster 4 are listed in Supplemental Table S2.

GO cellular component analysis suggests that the cell wall is an overrepresented site of cluster 3 gene expression (Table II). Cell wall modifying protein candidates (Supplemental Table S2) are AtXTH12 (At5g57530) and AtEXT4 (At1g76930). Another XTH, *XTH7* (At4g37800) is up-regulated in cluster 4 (Supplemental Table S2). As noted earlier, XTHs can strengthen or loosen cell walls (Thompson and Fry, 2001; Rose et al., 2002). Extensins are insoluble proteins that increase cell wall strength. Merkouropoulos and Shirsat (2003) detected the expression of *AtEXT4* within floral organ AZs. *AtEXT4* overexpression increases the thickness of Arabidopsis inflorescences (Roberts and Shirsat, 2006). A plethora of regulated cluster 3 peroxidases (Supplemental Tables S1 and S2) may also contribute to cell wall modifications. The inclusion of potential cell wall strengthening proteins in cluster 3 could suggest a role for wall reinforcement during net disassembly.

Potential Functions of Down-Regulated Gene Clusters

Selected Myb Factors and Regulators of Hormone Response and Cell Wall Structure Are Down-Regulated in Clusters 5 and 6

Positive regulation of cell wall extensibility is counterbalanced by reduced expression of other genes. Several cellulose synthase (CESA) and synthase-like (CSLA) genes are down-regulated in cluster 5 (Sup-

plemental Table S2). Some members of the CSLA gene family encode β-1,4-mannan and glucomannan synthases (Liepman et al., 2005). Hamann et al. (2004) postulated that individual CSLAs might exert tissue-specific roles given their low expression in whole organs; *AtCSLA10*, a cluster 5 gene, exhibited enhanced expression in flowers. Transcripts for expansin-like protein AtEXLA2 and expansin AtEXPA8 (At4g38400 and At2g40610) decline in AZs at stages 12 and 13, respectively. Expansins regulate cell wall extensibility via pH-dependent, nonenzymic means; this may involve interfering with hydrogen bonding of hemicelluloses and cellulose to convert wall tension to polymer creep (Cosgrove et al., 2002). Our data would not suggest an obvious role for AtEXPA8 or AtEXLA2 in regulating cell separation during stage 15. However, elevated EXP or EXP-like expression between stages 12 and 13 could facilitate subsequent entry of pectolytic enzymes into the cell wall matrix. Maximal EXP/EXP-like expression between stages 12 and 13 is accompanied by previously mentioned up-regulation of multiple genes encoding pectolytic enzymes. Reduced expression of expansin genes thereafter is accompanied by a reduction in mRNA abundance for a number of PG, PME, and PMEI genes (Supplemental Table S2, clusters 5 and 6), as well as *XTH4* (At2g06850).

GO categorization assigns cluster 6 transcripts to the nucleus more often than it assigns all transcripts represented on the ATH1 GeneChip (Table II). Just as multiple Myb genes were up-regulated elsewhere, many Mybs are down-regulated in cluster 6 (Supplemental Table S2). These include *AtMYB6, 21, 39*, and *57*. *MYB21* is expressed in flower buds (Kranz et al., 1998) and in stamens where it is JA responsive (Mandaokar et al., 2006). *MYB6*, first characterized by Li and Parish (1995) is weakly responsive to ethylene, ABA, and IAA (Kranz et al., 1998). Other transcription factors in this cluster (Supplemental Table S1) include AGAMOUS (At4g18960) and an AGAMOUS-activated regulator of GA biosynthesis (At4g32980). Supporting a role for enhanced GA response postpollination is down-regulation of the *RGL1* (Supplemental Fig. S1) and *RGL2* negative regulators of GA response. Both of these DELLA proteins normally restrain cellular expansion. It would be interesting to determine if derepression of RGL targets potentiates AZ cell expansion.

Cluster 7 Contains Multiple Down-Regulated Genes of JA Biosynthesis

Cluster 7 transcripts exhibit steep declines in abundance after stage 12 (Fig. 2). A hallmark of such gene expression is the coordinate down-regulation of JA biosynthesis. JA synthesis requires sequential action of lipoxygenase (LOX), allene oxide synthase (AOS), allene oxide cyclase (AOC), and OPR3 (Delker et al., 2006). JA can be converted to volatile methyl JA (MeJA) by JA carboxyl methyltransferase (At1g19640; Seo et al., 2001). Down-regulation of at least one enzyme from each of these steps occurs prior to

abscission. Down-regulated genes in AZ_{551} include *LOX3* and *LOX3*-like lipoxygenases (At1g17420 and At1g72520), *AOS* (At5g42650), and *AOC1* (At3g25760). Transcripts for *OPR3* (At2g06050) and JA carboxyl methyltransferase (At1g19640) were not represented in AZ_{551} restricted by *P*-value. High JA biosynthetic gene expression at stage 12 likely attests to requirements for JA in anther filament elongation, anther dehiscence, and pollen viability (Sanders et al., 2000; Stintzi and Browse, 2000).

Down-regulation of JA biosynthesis was unexpected in that both JA and MeJA promote abscission when applied externally (Ueda et al., 1996; Miyamoto et al., 1997; Hartmond et al., 2000). In citrus (*Citrus sinensis*), external application of MeJA or the structural and functional JA analog coronatine likely induces abscission by stimulating levels of ethylene (Hartmond et al., 2000; Burns et al., 2003). In bean (*Phaseolus vulgaris*), no ethylene production was evident following JA or MeJA-induced abscission (Ueda et al., 1996) although JA significantly promotes senescence (Ueda and Kato, 1982; Ueda et al., 1991). In Arabidopsis, exogenous JA also elicits premature senescence (He et al., 2002). Senescence may promote abscission by lowering auxin and/or inducing ethylene. Our results in Arabidopsis do not suggest an obvious direct role for JA or MeJA biosynthesis in controlling cell separation. Of note, reduction of JA levels after stage 13 is accompanied by apparent loss of JA responsiveness. Coronatine-responsive protein (At1g19670) is one down-regulated example. If JAs positively modulate abscission it is likely that they act indirectly or, like *HAE*, act very early in the abscission pathway. However, constitutive expression of the sole allene oxide synthase gene in Arabidopsis did not lead to precocious floral organ abscission in studies of Kubigsteltig and Weiler (2003). There, floral parts of the *cas* AOS overexpresser persisted along much of the inflorescence. Wild-type Arabidopsis plants typically retain flowers only at uppermost positions.

Cluster 8 Gene Expression Declines Postpollination But Is Restored to Near-Original Levels Prior to Cell Separation

Cluster 8 contains only 25 transcripts, and thus represents less than 5% of AZ_{551}. mRNAs decline in abundance between stages 12 to 15a but are up-regulated to near-original levels by the time of cell separation (Fig. 2). GO analysis reveals cluster transporter activities are overrepresented relative to all ATH1 GeneChip transcripts (Table II). Electron transport proteins represent a subset of transporters; others shuttle one or more ions (e.g. H+, Na+, K+, and phosphate) or nucleotides (e.g. GTP), or nucleotide sugars (e.g. UDP-Gal). Resumption of cluster 8 transcript accumulation before cell separation may be necessary for energy generation, transmembrane trafficking, and/or maintenance of ionic homeostasis. The production of UDP-Xyl by the UXS3 isoform of UDP-GlcUA decarboxylase (At5g59290) could conceivably regulate cell wall synthesis late in abscission to temper cell wall disassembly. UDP-GlcUA decarboxylases generate substrate for making cell wall material and regulate biosynthetic enzymes through feedback mechanisms (Harper and Bar-Peled, 2002).

Functional Analyses Reveal Likely Participation of AtZFP2, a Zinc Finger Protein, in Processes Affecting Abscission Capacity

Figure 3 showed that AZ_{551} contains multiple expressed genes shown by previous reverse and forward genetic strategies to be associated with the abscission process. Their presence imparted confidence that AZ_{551} would contain other transcripts of unknown and potentially novel roles in cell separation. We hypothesized that likely sources of novel abscission genes would include the disproportionately represented GO functional categories for cell wall modifying proteins, extracellular regulators, and transcription factors (Table I). A subset of AZ_{551} transcripts was considered for potential functional analyses; one such target was *AtZFP2* (At5g57520).

Zinc finger proteins regulate many developmental and stress responses (Takatsuji, 1998, 1999). At least 33 Arabidopsis ZFPs contain only one zinc finger domain (Englbrecht et al., 2004). Twenty-eight members of this so-called C1-l1 class of zinc finger proteins share a conserved QALGGH sequence within a putative DNA-contacting surface and a C-terminal Leu-rich sequence (Tague and Goodman, 1995; Takatsuji, 1998, 1999; Englbrecht et al., 2004). However, sequence alignments show low similarity elsewhere. Thus, ZFPs may carry out both distinct and overlapping functions. *AtZFP2* is a single copy, single zinc finger domain gene (Tague and Goodman, 1995). Our transcriptional profiling revealed that *AtZFP2* was up-regulated in cluster 3 of AZ_{551} (Supplemental Table S1). Promoter-GUS assays revealed enhanced *AtZFP2::GUS* transgene expression in open flowers (Fig. 4A) and in floral AZ scars after organ shed (Fig. 4B). Reporter gene expression persisted in AZ scars late into silique maturation (Fig. 4C). The presence of strong expression in AZs validated *AtZFP2* transcript accumulation patterns in microarrays and suggested a potential contribution of *AtZFP2* to abscission. However, the *AtZFP2* gene product appears to participate in non-AZ localized processes as well (Fig. 4, D–I). Promoter:GUS assays show that sites of *AtZFP2::GUS* transgene expression include stamens and carpels (Fig. 4, D and E), cotyledons and major veins of rosette leaves (Fig. 4F), trichomes of inflorescence leaves (Fig. 4, G and H), and stems (Fig. 4, G and I).

As the first step toward testing *AtZFP2*'s potential role in floral organ abscission, we constitutively expressed a 35S::*AtZFP2* transgene in Arabidopsis. Ninety-five hygromycin-resistant T1 plant lines were transplanted; almost half of these lines subsequently showed distinct phenotypes relative to wild-type controls. Q-PCR analysis of transgenic flowers was used to assess the combined level of 35S::*AtZFP2* and

Figure 4. Reporter gene expression in plants harboring *AtZFP2* promoter-*GUS* gene fusion. A, Promoter-*GUS* expression in transgenic inflorescences. B, AZ scars following floral organ detachment. C, AZ scars later in silique development. D, Stamen. E, Carpel. F, Two-week-old seedling. G, Inflorescence. H, Magnified view of leaf surface of G. I, Magnified view of stem surface of G. Scale bars, 1,000 μm.
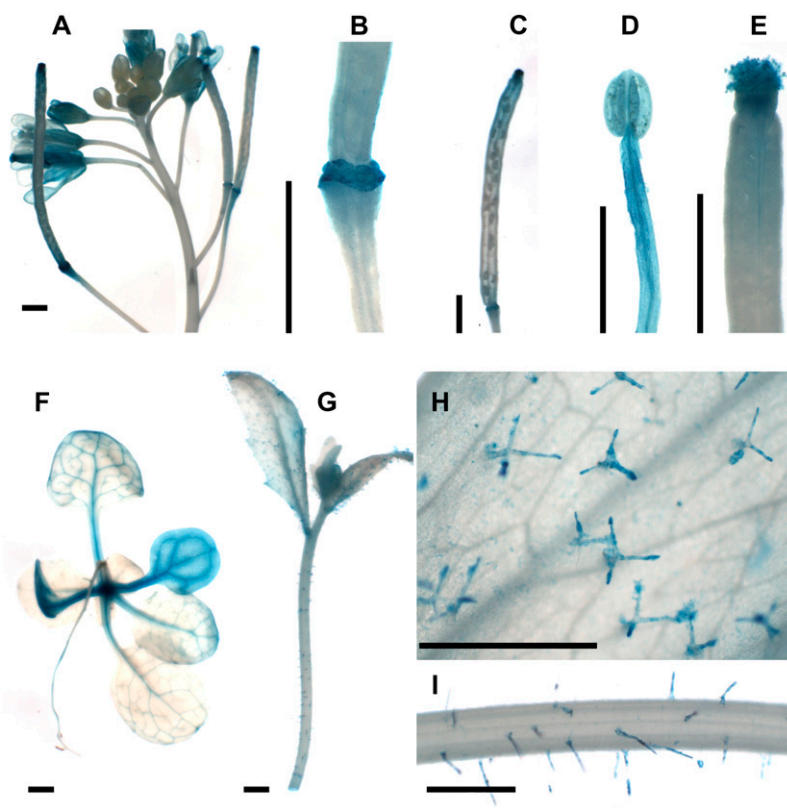
endogenous *AtZFP2* (Supplemental Fig. S2A) and the *35S::AtZFP2* transgene level alone (Supplemental Fig. S2B). Data in Supplemental Figure S2 show that transgenic flowers exhibit significant increases in total *AtZFP2* transcript level relative to wild-type controls.

To analyze potential *AtZFP2* functions, transgenic phenotypes were visually classified into three types: mild, strong, and severe. In all types, stamen filaments appeared shorter than in wild-type plants (data not shown) and plants were sterile. Transgenic lines were characterized in the T1 generation due to sterility. Other phenotypic changes observed in transgenic flowers are depicted in Supplemental Figure S3. Floral phenotypes included curving of carpels, greening of petals, and increases in trichome number (Fig. S3). Of special interest, transgenic lines were defective in abscission, with abscission delays accentuated in plants exhibiting strong and severe pleiotropic phenotypes. In Figure 5, floral organ retention is compared between wild-type plants (Fig. 5A) and transgenic lines with mild (Fig. 5B), strong (Fig. 5, C and E), and severe phenotypes (Fig. 5D). Because transgenic flowers do not open and have other defects, staging them using standard morphological features was not feasible. Instead, abscission was monitored as a function of inflorescence position. In wild-type plants, the flower stage at which white petals are first visible has been defined as position 1 and developmentally older flowers have increased position numbers (Patterson and Bleecker, 2004). In Figure 1A, position 1 in wild-type

plants would correspond to stage 13: anthesis. *AtZFP2* transgenic flowers that fail to open cannot, by definition, undergo anthesis. Therefore, we noted the number of floral buds present when abscission first occurred, not including the tight cluster of young flowers at the top of the inflorescence. Wild-type plants usually abscise parts 2 to 3 d after anthesis and, at that time, retain 4 to 6 intact flowers at uppermost positions (Fig. 5A). In contrast, abscission in transgenic *35S::AtZFP2* plants with mild phenotypes is delayed by several positions (Fig. 5B). In strong phenotype transgenic plants, organ retention is very prolonged (Fig. 5, C and E). Over 40 closed buds are present on the strong inflorescence depicted in Figure 5E. The extremely slow inflorescence elongation rates of severe phenotype plants like that in Figure 4D limited the number of flowers on the inflorescence and all flower parts remained green for 3 to 4 weeks.

Figure 5, F to I, depict reproductive organs from the inflorescences shown in A to D, respectively. About 30 d postpollination, wild-type floral organs are long gone and the silique has elongated, browned, and initiated dehiscence (Fig. 5F). At this time, all floral organs have also detached from plants with mild *35S::AtZFP2* phenotypes and mature siliques have initiated dehiscence (Fig. 5G). In contrast, retention of multiple floral organs is marked in plants with strong and severe transgene phenotypes (Fig. 5, H and I, respectively).

In wild-type *Arabidopsis*, developmental abscission follows pollination and fertilization. We wished to es-
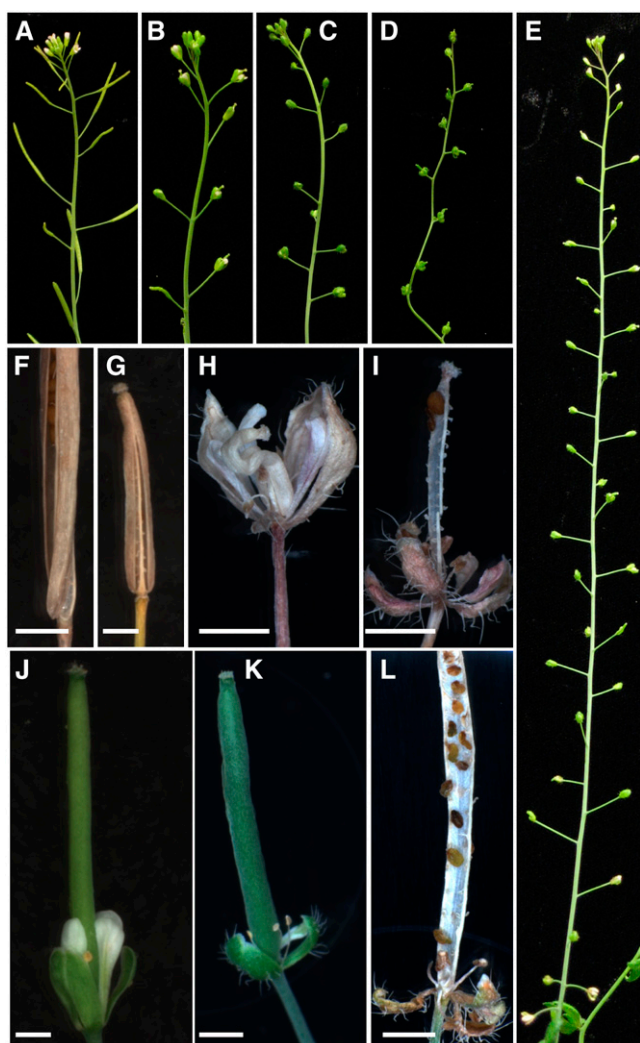
Figure 5. Floral organ abscission in wild-type and *35S::AtZFP2* plants. A, Inflorescence morphology in wild-type plants. B, Transgenic line with mild phenotype. C and E, Transgenic lines with strong phenotypes. D, Transgenic line with severe phenotype. F to I, Reproductive structures from plants in A to D, respectively. J to L, Floral organ abscission responses after cross-pollination with wild-type pollen grains. J, Mild transgenic line 4 d postpollination. K, Strong transgenic line 12 d postpollination. L, Severe transgenic line 32 d postpollination. Scale bars, 1 mm.

tablish whether *35S::AtZFP2* abscission delays could be due to impairment of these functions, particularly because the short stamen filaments of transgenic plants (data not shown) would be expected to limit effective pollination. Pollen grains from wild-type flowers were used to pollinate *35S::AtZFP2* stigma; results are shown in Figure 5, J to L. Four days after pollination, the mild phenotype plant in Figure 5J has elongated siliques but still retains floral parts. Floral parts are retained even longer in strong phenotype plants (Fig. 5K; 12 d postpollination) and severe phenotype plants (Fig. 5L; 32 d postpollination). Collectively, these experiments show that pollination cannot rescue abscission delays or blockages in *35S::AtZFP2* transgenic plants.

The increased floral organ retention seen in Figure 5 has several possible causes, including a failure to differentiate AZs or a failure to separate AZ cells once differentiated. In theory, such failures could occur in all AZs of the floral whorl or just a subset. For example, failure to differentiate or separate only the AZs of sepals would entrap petals and stamens and lead to unobservable abscission. To distinguish between these possibilities, SEM was used to visualize the parental faces of AZ fracture planes after manual removal of wild-type and transgenic floral parts (Fig. 6). When organs were removed from flowers at position 3 of wild-type plants or transgenic plants with mild, strong, or severe phenotypes, cells at bases of petals, sepals, and stamens were torn. Tearing is thought to reflect damage to cells that were tightly linked to their neighbors via cell wall connections at the time of organ removal, i.e. were nonabscised. At position 5, cells at the bases of all wild-type floral organs are intact, suggesting that middle lamellar connections to adjacent cells were at least partially dissolved due to initiation of stamen, sepal, and petal abscission. In Figure 6, arrowheads depict the position at which intact stamen AZ cells first appear. In *35S::AtZFP2* transgenic lines with mild pleiotropic phenotypes, the first intact AZ cells for stamen AZs are observed at position 9. Thus, completion of stamen AZ cell separation in mild phenotype lines is delayed relative to wild-type stamen AZs by 2 to 3 d. Notably, sepal AZs in mutants with both strong and severe phenotypes do not exhibit features indicative of cell separation over all positions assayed—up to 30 positions in all. These data reveal that overexpression of *AtZFP2* results in asynchronous abscission of different floral organs, behavior not seen in wild-type organs. It also suggests that some abscission-competent stamens and petals could be entrapped within a whorl of abscission-incompetent sepals at certain inflorescence positions in *35S::AtZFP2* transgenic lines. We conclude that the native *AtZFP2* gene product might participate in processes that initiate and/or coordinate cell separation in floral organ AZs.

Structural features of AtZFP2 suggest that this transcription factor may serve as an active repressor in processes affecting organ shed. *AtZFP2* encodes a TFIIIA-type zinc finger protein containing the carboxy-terminal amino acid sequence DLSLRL. Hiratsu et al. (2004) showed that the DLSLRL domain of AtZFP2 had a moderate level of repression activity, similar to that observed in known repressors containing an ERF-associated amphiphilic repression (EAR) domain (Hiratsu et al., 2004). Adamczyk et al. (2007) discuss similar domains found in the MADS-box proteins AGL15 and AGL18 and observe their similarity to JOINTLESS-like MADS proteins (Johansen et al., 2002; Becker and Theißen, 2003). The mechanism whereby modulation of repressor activity could alter abscission capacity is uncertain but it is clear that pedicel abscission is blocked in *jointless* mutants (Butler, 1936) and floral organ abscission is delayed in plants overexpressing *AGL15* (Fernandez et al., 2000), *AGL18* (Adamczyk

Figure 6. SEMs of wild-type and *35S::AtZFP2* floral organ AZ fracture planes. Organs abscised naturally or were manually removed at designated positions along plant inflorescences. Arrowheads designate stamen AZs at the first position where intact cells were observed after organ removal.
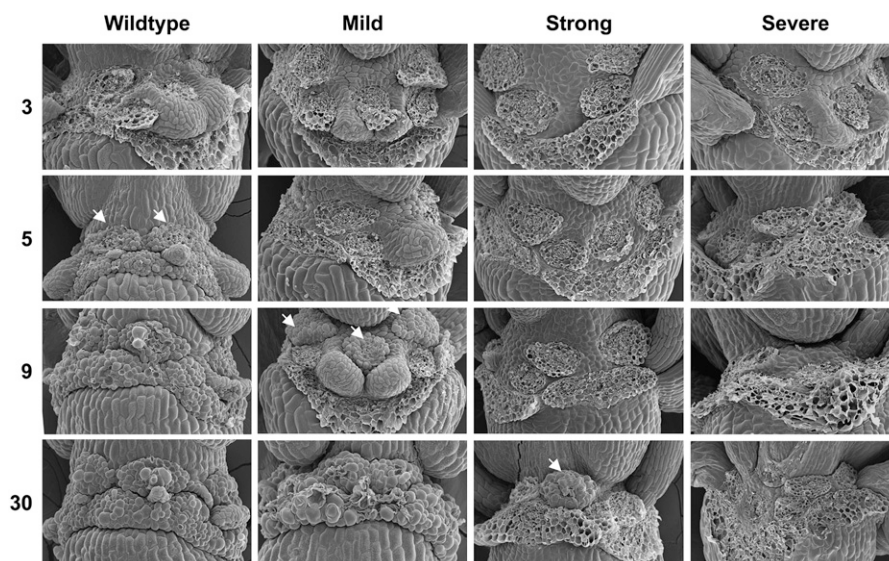
et al., 2007), and *AtZFP2*. It is possible that repression activity indirectly affects abscission competence via the slowing of certain developmental transitions seen in all of these abscission-impaired transgenic systems (Szymkowiak and Irish, 1999; Fernandez et al., 2000; Adamczyk et al., 2007).

CONCLUSION

We have characterized a 551-member slice of the AZ transcriptome whose expression is significantly regulated prior to developmental stamen shed. Supplemental Figure S4 summarizes floral stage intervals at which transcription of different gene classes is up- or down-regulated by at least 2-fold. Quantitative data corresponding to Supplemental Figure S4 is in Supplemental Table S2. Some expressed genes have been shown by others to be associated with the abscission and/or dehiscence processes, suggesting that the AZ_{551} population may also represent a valuable source of new cell separation determinants. This hypothesis was confirmed via functional analyses showing an influence of one up-regulated AZ_{551} gene, *AtZFP2*, on abscission. Abscission of *35S::AtZFP2* floral organs was asynchronous and delayed; in plants with severe pleiotropic phenotypes abscission did not occur over time periods of 2 to 3 weeks. Interestingly, it appears that *35S::AtZFP2* expression does not similarly inhibit cell separation during dehiscence because transgenic silique splitting was observed in Figure 5, G and I.

Comparison of AZ_{551} to the sequenced Arabidopsis genome reveals that cell wall proteins are disproportionately represented in AZs. Transcripts for PGs, pectate lyases, PMEs, and PMEIs are especially well represented, not surprising given the known roles of pectin-modifying enzymes in reducing adhesion between contiguous AZ cells. Our data set would actually be expected to underestimate the potential contribu-

tions of pectin modification to abscission control because the size of AZ_{551} was limited by F-testing to transcripts with *P*-values less than 0.0001. Indeed, additional pectin-related proteins exhibiting modulated accumulation prior to abscission are represented by signals lying just outside that window of statistical significance. These transcripts include PMEs and PGs not listed in Supplemental Tables S1 and S2. Interestingly, the significant up-regulation reported in Supplemental Table S2 for one PG (At3g07970) before abscission was at odds with prior reports that this transcript was not expressed in Arabidopsis AZs (González-Carranza et al., 2007a). We therefore investigated whether the At3g07970 probe set might be cross-hybridizing to another structurally similar PG present in the stamen AZ sample. BLAST analysis defined one structural relative of this PG as abscission-related *PGAZAT*/At2g41850 (González-Carranza et al., 2002). However, our expression profiles for At3g07970 are markedly different from those of *PGAZAT* (data not shown) and do not support the presence of substantial cross-hybridization. A second close structural relative of the At3g07970 PG is *ADPG1*/At3g57510 (Sander et al., 2001). The promoter of this Arabidopsis homolog of the oilseed rape (*Brassica napus*) polygalacturonase *RDPG1* was previously shown to direct GUS expression to floral AZs of transgenic Arabidopsis (Sander et al., 2001). Our expression profiles for At3g07970 also significantly differ from those for *RDPG1* (data not shown). At present, the reason for discrepancies between our data and that of González-Carranza et al. (2007a) is unclear.

Pectin modification is most prevalent at stage 13 whereas multiple regulators of the hemicellulosic-cellulosic structure are turned on at stage 12 (Supplemental Fig. S4). It is possible that modifications of the hemicellulose-cellulose structure by XTHs, expansins, peroxidases, and/or extensins before or at stage 13 facilitate entry of pectolytic proteins to their substrates as occurs in ripening fruit (Lashbrook, 2005). Alterna-

tively, cell wall changes prior to stage 13 could include reinforcement activities of some XTHs, peroxidases, or extensins. Although net cell wall modification during abscission results in disassembly, wall stiffening early in the abscission pathway would be a way to temper the rate of pectin and hemicellulose disassembly. Given the large size of gene families for cell wall modifying proteins (The Arabidopsis Genome Initiative, 2000), our data should help prioritize gene family members for future functional analyses to answer such questions.

The *HAE* mRNA component of the ethylene-independent abscission route accumulates significantly between stages 12 and 13, whereas the earliest signs of cell separation occur late in stage 15. Ethylene signaling components with the same pattern of mRNA accumulation include two *ERFs* and an EIN3-binding protein component of the SCF complex used for ubiquitination-mediated degradation (Potuschak et al., 2003). Thus, ethylene-dependent and -independent pathways seem to be initiated at similar times. Potential convergence of ethylene-dependent and -independent routes may link cell separation regulators (Butenko et al., 2006). The activation of both receptor kinase and ethylene-regulated pathways so close to the time of floral pollination emphasizes that developmental competence to respond to abscission cues is realized before cellular rounding or other visible manifestations of cell separation.

Early induction of abscission pathways presents both opportunities and challenges to researchers interested in understanding abscission control. Opportunities include the potential to identify novel, early regulators suitable for use in plant improvement. Crops including soybean and cotton exhibit high rates of precocious shed prior to fruit set, limiting yield potential (Wiebold et al., 1981; Guinn, 1982). Others like citrus resist detachment at harvest and require chemical harvesting aids that may bring undesirable side effects (Kender et al., 2001). Our studies should help identify gene targets whose modification may reduce or promote shedding of commercially important organs. However, a key challenge will be devising methods to select targets that preferentially control abscission rather than multiple plant processes such as were observed with *AtZFP2*. For example, distinguishing genes controlling abscission from those co-regulating unrelated processes like anther dehiscence, pollination, and/or floral senescence will be important. Of course, it is possible that the primary abscission cue that initiates ethylene-independent and/or -dependent abscission pathways may be derived from these or other events.

A second challenge will be to determine if current abscission models should incorporate hormones beyond ethylene and auxin. Our studies reveal significant changes in the synthesis, perception, and transport of many hormones in AZs prior to organ shed. These include enhancement of GA catabolism, derepression of GA signal transduction, down-regulation of JA biosynthesis, enhanced auxin conjugation and efflux,

and increased expression of BR-related genes including the BRI1 receptor (Supplemental Fig. S4; Supplemental Table S2). Some hormonal changes are likely to contribute to processes other than abscission or may influence abscission via ethylene and/or auxin. Certainly, complex modulation of hormone status in stamen AZs suggests the potential for significant cross-talk between abscission pathways. We are presently assessing the scope of possible hormone communication in detaching organs via the analysis of an expanded population of highly regulated AZ genes.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia grown in a growth chamber under daily cycles of 16-h light and 8-h darkness at 21°C were fertilized with Peters EXCEL (Scotts). Light intensity was approximately 100 to 130 μmol m^{-2} s^{-1}. Flowers were harvested when plants were approximately 6 weeks old and bore a total of 12 to 14 open flowers and siliques. Flowers were collected at the same daily time (4–5 PM) to reduce circadian effects. Floral stages were assigned according to descriptions of Smyth et al. (1990). Flowers at stage 12 ("petals level with long stamens"), 13 ("petal visible, anthesis"), and early stage 15 ("stigma extends above long anthers"; S15a) were collected from the primary inflorescence of the same plant. Flowers of the same stage from the same plant batch were pooled as one independent sample. Three separately grown plant batches formed three biological replications. Flowers at stage S15c were approximately 24 h older than those at stage S15a. Flowers one position above S15c flowers were termed stage S15b. Flowers at stages 15b and 15c were harvested from different plant batches 6 months later. Flowers were fixed, processed, and paraffin-embedded according to Cai and Lashbrook (2006).

LCM, RNA Extraction, aRNA Preparation, and Microarray Hybridization

Protocols of Cai and Lashbrook (2006) were used for longitudinal paraffin sectioning, LCM, and RNA isolation, amplification, and biotin labeling. Paraffin sections were mounted onto adhesive-coated slides (Cai and Lashbrook, 2006) with a paraffin tape transfer system (Instrumedics). Prior to LCM, tape was carefully removed from slides submerged in xylenes using forceps. AZ microdissection using the Pix-Cell II LCM system employed CapSure Macro LCM caps (Arcturus Engineering). RNA was isolated from microdissected cells with the PicoPure RNA isolation kit (Arcturus Engineering). Biotin-labeled aRNA was generated with the BioArray RNA amplification and labeling system (Enzo Life Sciences). ATH1 GeneChip (Affymetrix) hybridizations and scanning at the Affymetrix GeneChip Facility of Iowa Sate University employed Affymetrix protocols.

Data Analysis

Genes that changed significantly over the five developmental stages were selected by applying linear mixed model analysis (Wolfinger et al., 2001; Nettleton, 2006) with the SAS mixed model procedure. Stage was considered as a fixed effect and plant batch as a random effect. Natural log transformed MAS 5.0 signal intensities were used as response variables. False discovery rate was calculated as per Storey and Tibshirani (2003); 551 genes with $P \leq 0.0001$ are listed in Supplemental Table S1. Annotation of each microarray element was accomplished with the "microarray elements search and download" program on The Arabidopsis Information Resource Web site (www.arabidopsis.org). Functional categorization of AZ$_{551}$ using GO analytical tools (Berardini et al., 2004) permitted comparisons between predicted functions of AZ transcripts and transcripts within the Arabidopsis genome. Fisher's exact test established statistical significance of overrepresented functions (Fisher, 1922).

Cluster Analysis

AZ$_{551}$ transcripts were clustered using R software (http://www.R-project.org). The mean normalized signal intensity for each probe set was calculated

from three biological replications. A data matrix was constructed with rows corresponding to genes and columns representing means of each gene at each stage. Signal intensities of each gene were standardized so that each gene (row) would have a mean of zero and an SD of 1. Eight clusters were chosen by the gap statistic (Tibshirani et al., 2001). The K-medoid clustering method was used to assign membership of each probe set (Kaufman and Rousseeuw, 1990).

AtZFP2 Cloning and Transformation

Full-length *AtZFP2* cDNA was reverse transcribed (RETROscript; Ambion) from flower RNA and amplified with Platinum Taq DNA Polymerase High Fidelity (Invitrogen). Primers used were: forward 5′-CACCATGGACTAC-CAGCCAAACACATC-3′; reverse 5′-TTAGAGCCTTAAGGATAAGTCAAG-3′.

For promoter-GUS construction, a 5′ upstream region of 3,000 bp preceding the 5′ untranslated region of *AtZFP2* was amplified from genomic DNA. Primers used were: forward 5′CACCCATTTTCCCTATTGGTTGACGTC-3′; reverse 5′-AGAGAAGTGTGTTTGAAGAGTTTGG-3′.

Transformation constructs were obtained with Gateway technology. Cloning blunt-ended PCR product into pENTR/D-TOPO vector (Invitrogen) generated an entry clone. Pvu1 enzyme digestion destroyed kanamycin resistance of the entry construct. An LR recombination reaction was performed using Gateway LR clonase II enzyme mix (Invitrogen). This transferred AtZFP2 and the promoter from the entry construct into Gateway Destination vectors PMDC32 and PMDC164, respectively (Curtis and Grossniklaus, 2003). Vectors were introduced into *Agrobacterium tumefaciens* (strain GV3101) by freeze and thaw (Holsters et al., 1978). Arabidopsis was transformed by floral dip (Clough and Bent, 1998) and transgenic plants selected on Murashige and Skoog-hygromycin plates (50 mg/L).

Q-PCR

For validation of gene expression pattern obtained from microarray studies, Q-PCR employed gene-specific primers provided by the QuantiTect primer assay (QIAGEN). The QuantiTect Reverse transcription kit (QIAGEN) generated cDNA using RNA isolated from laser-captured stamen AZs. Reactions in a Mx3000P instrument (Stratagene) used the QuantiTect SYBR-Green PCR kit (QIAGEN); 18S rRNA was amplified in parallel with each target gene as an internal control. A relative amount of Q-PCR product was represented by $-\Delta\Delta Ct$ according to described methods (Livak and Schmittgen, 2001; Ehlting et al., 2005). Basically, threshold detection cycles (Ct) were normalized using corresponding 18S rRNA Ct values to generate ΔCt values. ΔCt values for each gene were compared to the highest ΔCt value among 15 samples obtained for that gene to generate $-\Delta\Delta Ct$ values. Q-PCR was replicated three times and results were shown in plots parallel with normalized signal intensities from microarray data.

For examinations of the transcript level of *35S::AtZFP2* and the combined level of *35S::AtZFP2* and endogenous *AtZFP2* in transgenic plants, flower RNA was reverse transcribed. The primer set for amplifying the transgene only is: forward 5′-TGGTGGTCATCAAAACGCTCAT-3′ and reverse 5′-ATT-GCCAAATGTTTGAACGA-3′. The reverse primer recognizes the nopaline synthase terminator of the transgene. The QuantiTect primer assay, At_ZFP2-1-SG (QIAGEN), was then used to collectively PCR amplify 35S::AtZP2 and endogenous *AtZFP2*. Relative expression was calculated according to Livak and Schmittgen (2001) with 18S rRNA as the internal control and wild-type expression normalized to 1. Three biological replications were conducted for wild-type flowers; eight biological replications were conducted for *35S::AtZFP2* flowers.

Histochemical Analysis of GUS Activity

Gus staining used 5-bromo-4-chloro-3-indolyl-b-D-GlcUA substrate (Jefferson et al., 1987). Tissues from T2 plants were incubated at 37°C overnight in 0.1 M phosphate buffer containing 10 mM EDTA, 0.5 mM K-ferricyanide, 0.5 mM K-ferrocyanide, 1.0 mM X-glucuronide, and 0.1% Triton X-100. Tissues were cleared in 70% ethanol and viewed under a stereomicroscope.

SEM

Flowers were fixed in formaldehyde-acetic acid (45% ethanol, 2.5% acetic acid, and 2.5% formalin) overnight, dehydrated in an ethanol series, and critical-point dried (DCP-1; Denton Vacuum). Samples were mounted on aluminum stubs, silver painted, sputter-coated (Denton Vacuum) with Au/Pd, and viewed under a SEM (JSM-5800LV; JEOL) at 10 kV.

Supplemental Data

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

Supplemental Figure S1. Validation of microarray results using Q-PCR.

Supplemental Figure S2. Q-PCR measurement of transcripts representing combined *35S::AtZFP2*/endogenous *AtZFP2* and *35S::AtZFP2* only in flowers of wild-type and *35S::AtZFP2* plants.

Supplemental Figure S3. Phenotypes of wild-type and 35S::AtZFP2 floral organs.

Supplemental Figure S4. Developmental timing of accumulation of selected AZ_{551} transcripts.

Supplemental Table S1. AZ_{551}, the 551 most significantly regulated genes of the stamen AZ transcriptome.

Supplemental Table S2. Fold changes of selected AZ_{551} transcripts within floral stage intervals.

Note Added in Proof

Up-regulated expression of the At3g07970 polygalacturonase in floral organ AZs prior to abscission was previously reported by Kim and Patterson (**Kim J, Patterson SE** [2006] Expression divergence and functional redundancy of polygalacturonases in floral organ abscission. Plant Signal Behav **1:** 281–283).

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