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AINTEGUMENTA-like (*AIL*) genes are expressed in young tissues and may specify meristematic or division-competent states

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Abstract

Although several members of the AP2/ERF family of transcription factors are important developmental regulators in plants, many genes in this large protein family remain uncharacterized. Here, we present a phylogenetic analysis of the 18 genes that make up the AP2 subgroup of this family. We report expression analyses of seven *Arabidopsis* genes most closely related to the floral development gene *AINTEGUMENTA* (*ANT*) and show that all *AINTEGUMENTA*-like (*AIL*) genes are transcribed in multiple tissues during development. They are expressed primarily in young actively dividing tissues of a plant and not in mature leaves or stems. The spatial distribution of *AIL5*, *AIL6*, and *AIL7* mRNA in inflorescences was characterized by *in situ* hybridization. Each of these genes is expressed in a spatially and temporally distinct pattern within inflorescence meristems and flowers. Ectopic expression of *AIL5* resulted in a larger floral organ phenotype, similar to that resulting from ectopic expression of *ANT*. Our results are consistent with *AIL* genes having roles in specification of meristematic or division-competent states.

Introduction

AP2/ERF proteins make up one of the largest transcription factor families in *Arabidopsis* (Riechmann *et al*., 2000). Members of this family contain either one or two copies of a DNA-binding domain called the AP2 domain (Riechmann and Meyerowitz, 1998) and can be broadly divided into three subfamilies. Members of the AP2 subfamily have two AP2 domains, members of the ERF subfamily have a single AP2 domain, and members of the RAV subfamily contain one AP2 domain and a second DNA binding domain (the B3 domain). ERF-type proteins constitute 125 of the total 146 AP2/ERF proteins, while the AP2 and RAV subfamilies consist of 15 and 6 members, respectively (Riechmann *et al*., 2000; Sakuma *et al*., 2002; Cernac and Benning, 2004). A more detailed phylogenetic analysis of the family divided these proteins into five groups: the AP2 subfamily, the RAV subfamily, the DREB subfamily, the ERF subfamily, and others (Sakuma *et al*., 2002). Three of the genes in the 'others' subfamily contain a single AP2 domain but more closely resemble members of the AP2 subfamily (Sakuma *et al*., 2002; Schmid *et al*., 2003).

Biological functions have only been determined for a fraction of these proteins. Data obtained so far for members of this protein family in *Arabidopsis,* petunia, maize, rice, and tobacco suggests that members of the AP2 subfamily play roles in development (Riechmann and Meyerowitz, 1998) while members of the ERF subfamily can play roles in development (Wilson *et al*., 1996; van der Graaff *et al*., 2000; Banno *et al*., 2001; Chuck *et al*., 2002; Kirch *et al*., 2003; Komatsu *et al*., 2003) or responses to biotic and abiotic stresses (Riechmann and Meyerowitz, 1998). While the AP2/ERF family of transcription factors is unique to plants, proteins containing homologs of the

AP2 domain have been identified recently in cyanobacteria, ciliates, and viruses (Magnani *et al.*, 2004). These AP2-domain containing proteins are predicted to function as HNH endonucleases, a class of mobile endonuclease genes. HNH endonucleases are encoded within mobile intervening sequences and make double stranded breaks in DNA that allow the movement of their host sequence (reviewed in Chevalier and Stoddard, 2001).

Several members of the *Arabidopsis* AP2 subfamily have been characterized genetically. *APETALA2* (*AP2*), one of the founding members of the family, is a class A floral homeotic gene that specifies sepal and petal identity in floral whorls one and two (Bowman *et al.*, 1989, 1991; Kunst *et al.*, 1989). In addition, AP2 acts as a negative regulator of the class C floral homeotic gene, *AGAMOU*S (*AG*) (Drews *et al.*, 1991). Mutations in *AP2* result in homeotic transformations of organ identity in the first and second whorls due to ectopic *AG* expression (Bowman *et al.*, 1991). Two genes related to *AP2* have also been characterized recently. *TOE1* and *TOE2* are repressors of flowering that have overlapping functions (Aukerman and Sakai, 2003). Two of the genes containing a single AP2 domain but more closely resembling members of the AP2 subfamily, *SCHLAFMÜTZE* (*SMZ*) and *SCHNARCHZAPFEN* (*SNZ*), may also act as repressors of flowering as misexpression of either gene confers a late-flowering phenotype (Schmid *et al.*, 2003).

AINTEGUMENTA (*ANT*), another member of the AP2 subfamily, is required for integument initiation and promotion of growth within developing floral organs (Elliott *et al.*, 1996; Klucher *et al.*, 1996). Mutations in *ANT* cause a reduction in the number and size of floral organs (Elliott *et al.*, 1996; Klucher *et al.*, 1996), while ectopic expression of *ANT* results in the production of larger floral organs (Krizek, 1999; Mizukami and Fischer, 2000). In addition, *ANT* acts redundantly with *AP2* in repression of *AG* in second whorl cells (Krizek *et al.*, 2000). Recently, two other members of the AP2 subfamily, *PLETHORA1* (*PLT1*) and *PLETHORA2* (*PLT2*) were found to be required for specification and maintenance of stem cells within the root apical meristem (Aida *et al.*, 2004). A role in embryo development has been implicated for another member of the AP2 subfamily, *BABY BOOM* (*AtBBM*). Although the effects of mutations in *AtBBM* have not been reported, ectopic expression of *Brassica napus BBM* results in the formation of somatic embryos on seedlings (Boutilier *et al.*, 2002).

We are interested in determining whether previously uncharacterized members of the AP2 subfamily play roles in flower development. We are focusing initial studies on those genes most similar in sequence to *ANT*, which we have designated *AINTEGUMENTA*-like (*AIL*) genes. To begin to determine their functions, we have examined the expression of the *AIL* genes in different plant tissues by real time reverse transcription PCR (RT-PCR). We find that all of the *AIL* genes are expressed in multiple tissues with significantly higher expression levels in young dividing tissues (seedlings, roots, inflorescences, and siliques) as compared with mature tissues (rosette leaves and stems). In situ hybridization experiments reveal that *AIL5*, *AIL6*, and *AIL7* have distinct patterns of expression within inflorescence tissues. Ectopic expression of *AIL5* can alter organ size suggesting that this gene may play a role in organ growth control. Our initial studies combined with recent work by others, suggests that *AIL* genes play important developmental roles in different tissues of a plant and at different stages in the life cycle of a plant.

Materials and methods

Bioinformatics tools

Database searches were performed using TAIR BLAST (http://www.arabidopsis.org/Blast/). Amino acid sequences were aligned using Clustal X. The neighbor-joining AP2 subfamily tree was generated with PAUP* 4.0 (Sinauer Associates, Sunderland, MA) based on amino acid alignments of the AP2 repeat region of each protein.

Plant materials and growth conditions

Arabidopsis plants were grown in a 4:1:1 mix of Fafard 4P mix:perlite:vermiculite at 22 °C in continuous light.

RNA extraction and cDNA preparation

Seedlings and roots were collected from 10 to 12 day-old L*er* plants grown on Murashige and

Skoog (MS) medium under continuous light at 22 °C. For root collection, all aerial portions were removed. Stems, rosette leaves, inflorescences, and siliques were dissected from L*er* plants grown in the soil and light conditions described above. Mature rosette leaves were collected from plants of a variety of ages. Cauline leaves were removed from stem sections. Inflorescences included flowers at all developmental stages. Green siliques were harvested after all other floral organs had fallen off. Tissue was homogenized in liquid nitrogen and total RNA was extracted (Verwoerd *et al*., 1989). The RNA was treated with RQ1 RNase-free DNase (Fisher Scientific, Pittsburgh, PA) for at least 3 h at 37 °C according to the manufacturer's instructions. Approximately 2 μg of total RNA was reverse transcribed using the Superscript First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA).

Real time RT-PCR

Real time RT-PCR was performed with gene-specific oligonucleotides (Table 1) that amplified products between 75 and 131 bp. UBQ-1 (5′-TGGTGGTATGCAGATTTTCG-3′) and UBQ-2 (5′-GGCTTTCACGTTATCAATGG-3′) were used to amplify *UBQ10* for normalization. Real time RT-PCR reactions were performed on an iCycler (BioRad, Hercules, CA) using iQ SYBR green Supermix (BioRad, Hercules, CA) or Brilliant SYBR green QPCR Master Mix (Stratagene, La Jolla, CA). Melt curve analyses were performed at the end of each experiment to check the specificity of the reaction. The experiments were performed at least twice using biological tissue replicates. The software calculated concentrations based on a standard curve generated using known molar concentrations of a plasmid containing a fragment of the appropriate *AIL* gene. The data was standardized by dividing the expression level of each *AIL* gene by the expression level of polyubiquitin mRNA (*UBQ10*) for that particular tissue.

Table 1. Primers used in real time RT-PCR assay.

Gene	Forward/reverse primers
ANT	5′-GCGAAGGAACAACAACAGCATT-3′
	5′-CCACCTTCCACAACAGCATT-3′
AIL1	5′-CGCTGTAACCAATTTCGATATA-3′
	5′-AGCTGGTGGGAGAATGTTTG-3′
AtBBM	5′-GGTAGATACGAGGCACATTTATG-3′
	5′-AGTGCGGCTAAATCGTAAGC-3′
PLT1	5′-ACGAAAACCAATCCAACCAC-3′
	5′-ATTGGACGCTAGGCATCAAG-3′
PLT2	5′-GAGGTTCCAAAAGTGGCTGA-3′
	5′-CGTTGGTTTGATGAATGTCG-3′
AIL5	5′-CTACTCCGGTGGACACTCGT-3′
	5′-CGTTCTTCTTCGGAGTAGGC-3′
AIL6	5′-CGGTTCAGAGGTCGATGACT-3′
	5′-AGATCCACCGTGAAACCCTA-3′
AIL7	5′-TTTCCTCGGTGATTCCTTTG-3′
	5′-TGACGTGGATCGTAGAATGG-3′

In situ *hybridization*

Inflorescences were fixed, embedded, sectioned, hybridized, and washed as described previously (Krizek, 1999). Sequences outside of the AP2 repeat region were cloned into pCRScript (Stratagene) and used to generate gene specific digoxygenin-labeled probes. DNA blots confirmed the specificity of the probes. Two different *AIL5* probes were used in these experiments and gave the same results. A 542 bp *AIL5* sequence 5′ of the AP2 repeats was PCR amplified from L*er* inflorescence cDNA using AIL5-1 (5′-TCTTCTTCTAGCTATGATTCTTC T-3′) and AIL5-2 (5′-CGGAGTAGGCGAAGC CTC-3′). This fragment was cloned into pCRScript to generate pAIL5(1/2). A sense *AIL5* probe was synthesized using T7 RNA polymerase and pAIL5(1/2) linearized with *Not*I. An antisense *AIL5* probe was generated using T3 RNA polymerase and pAIL5(1/2) linearized with *Asp*718. The second *AIL5* probe corresponded to a 456 bp sequence at the 3′ end of the gene and was PCR amplified from the *AIL5* cDNA using AIL5-3 (5′-GTGAA-TACTCATTCCAACCCAAAAACC G-3′) and AIL5-4 (5′-CGTCCCTCACGTT CAATGTGGCA-3′). This fragment was cloned into pCRScript to generated pAIL5(3/4). A sense *AIL5* probe was synthesized using T7 RNA polymerase and pAIL5(3/4) linearized with *Not*I. An antisense *AIL5* probe was synthesized using T3 RNA polymerase and pAIL5(3/4) linearized with *Bam*HI. A 545 bp fragment of *AIL6* corresponding to a sequence 5′ to the AP2 repeats was PCR amplified from genomic DNA using AIL6-4 (5′-TAGCAGCAGCAGCTTCAATG-3′) and AIL6-3 (5′-CAACAGCCTTCTCCTTCT-3′). This fragment was cloned into pCRScript in both directions

to generate pAIL6AS and pAIL6S. An antisense *AIL6* probe was made using T7 RNA polymerase and pAIL6AS linearized with *Not* I. A sense *AIL6* probe was made using T7 RNA polymerase and pAIL6S linearized with *Not*I. A 535 bp fragment of *AIL7* corresponding to a sequence 5′ to the AP2 repeats was PCR amplified from genomic DNA using AIL7–4 (5′-GAATCTCGAGGGATCCA TGGCGGATTCAACAACC-3′) and AIL7–6 (5′-CGGGTGACTCCACGATAAAT-3′). This fragment was cloned into pCRScript to generate pAIL7. An antisense *AIL7* probe was made using T3 RNA polymerase and pAIL7 linearized with *Bam*HI. A sense *AIL7* probe was made using T7 RNA polymerase and pAIL7 linearized with *Not* I.

Generation of 35S::AIL5IR and 35S::AIL5 plants

For the *35S::AIL5IR* construct, a 574 bp fragment of *AIL5* was PCR amplified with AIL5-5 (5′-ATTAGGTACCATCGATCGGAGTAGGCGAAG CCTC-3′) and AIL5-6 (5′-CGTTCTCGAGGGA TCCTCTTCTTCTAGCTATGATTCTTCT-3′) primers and cloned into pHannibal in the sense and antisense directions (Wesley, *et al*., 2001). The entire inverted repeat construct including the *35S* promoter was digested from pHannibal and subcloned into pART27 as a *Not*I fragment. For the *35S::AIL5* construct, the *AIL5* cDNA was subcloned from pBluescript SK$^-$ into pART7 using *Eco*RI/*Kpn*I. *35S::AIL5* was subsequently subcloned from pART7 into pMLBART as a *Not*I fragment. Both the *35S::AIL5IR*/pART27 and *35S::AIL5*/pMLBart plasmids were transformed into *Agrobacterium* ASE by electroporation. *Arabidopsis* L*er* plants were then transformed with these *Agrobacterium* strains by vacuum infiltration (Bechtold *et al*., 1993). Transformants were selected for kanamycin or Basta resistance.

ESEM

ESEM was performed on a XL30 at an accelerating voltage of 5 kV. Two petals from the oldest non-senescing flower on an inflorescence were removed from 30 day-old plants. The petals were immediately placed adaxial side up inside the chamber. A total of eight petals were examined for L*er* and *35S::AIL5* lines 16 and 20. Images of cells near the tip of the petal were recorded at 1000 × magnification. These images were then opened in Adobe Photoshop (San Jose, CA) and the number of whole cells within the borders of the image were counted and recorded.

Results

AIL genes in Arabidopsis

The relationships among the 15 members of the AP2 subfamily and the three 'other' proteins containing a single AP2 domain (SMZ, SNZ, and At2g41710) are shown in Figure 1. SMZ, SNZ, and At2g41710 share homology with members of the AP2 subfamily in the first AP2 domain and the linker region. All other members of the AP2 subfamily share homology through both AP2 domains and the intervening linker region. Seven of the predicted genes are most similar in sequence to ANT and have been designated *AIL*s for *AINTEGUMENTA*-like. The *AIL* group includes *AtBBM*/*AIL2* (At5g17430) and two genes, *PLETHORA1* (*PLT1*)/*AIL3* (At3g20840) and *PLETHORA2* (*PLT2*)/*AIL4* (At1g51190), recently found to be required for specification and maintenance of stem cells within the root meristem (Aida *et al*., 2004). The remaining members of the family: *AIL1* (At1g72570), *AIL5* (At5g57390), *AIL6* (At5g10510), and *AIL7* (At5g65510) have not been characterized. *TOE3* (At5g67180), *TOE1* (At2g28550), *TOE*2 (At5g60120), *SMZ* (At3g54990), and *SNZ* (At2g39250) closely resemble AP2 (At4g36920) while At1g16060, At1g79700, At2g41710, and *WRINKLED1* (*WRI1*) (At3g54320 and At3g54330) form a distinct third group within the AP2 subfamily. *WRI1* is a recently cloned gene involved in the regulation of seed storage metabolism (Cernac and Benning, 2004).

An alignment of the AP2 repeat region for the 15 proteins with two AP2 domains is shown in Figure 2. The predicted protein sequences correspond to cDNAs publicly available (*ANT*, *AIL1*, *AtBBM*, *PLT1*, *PLT2*, *AIL5*, *AIL6*, *AP2*, *TOE1*, *TOE*2, *TOE3*, At1g16060, and At1g79700) or isolated by us (*AIL7*). Two alternative transcripts have been reported for At1g79700. For the alignment, we have used the predicted protein sequence corresponding to the longer transcript. Within the AP2 repeat region, these 15 proteins share 25% sequence identity. The sequence identity is significantly greater when considering just members of the AIL group (70% identity) (Figure 2). While the only region shared by all

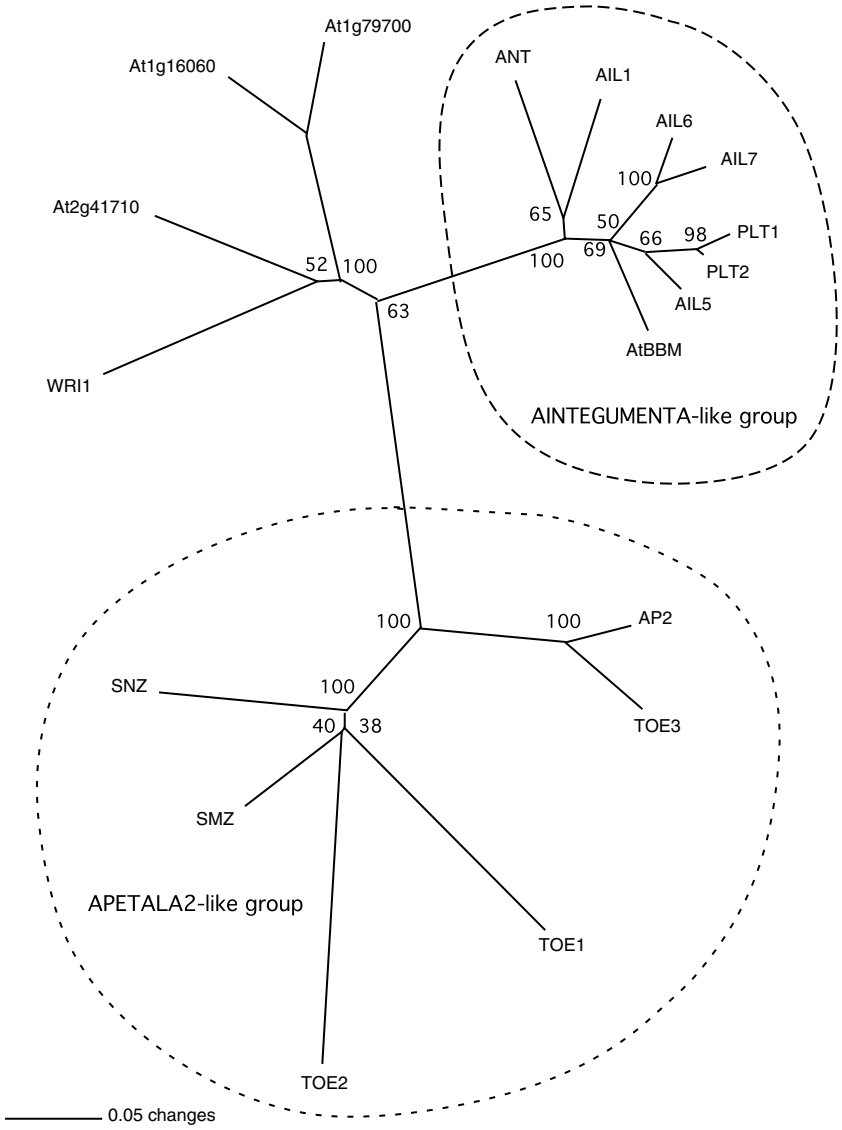

Figure 1. Phylogenetic relationships between members of the AP2 subgroup based on the sequence of the two AP2 repeats and linker region. Three genes (SMZ, SNZ, and At1g41710) that contain a single AP2 repeat are also included. This neighbor joining (NJ) tree was generated using PAUP* 4.0. The bootstrap values for 1000 replicates are indicated. Those proteins most closely related to ANT are members of the AINTEGUMENTA-like (AIL) group. Those proteins most closely related to AP2 are members of the APETALA2-like group. Similar trees were obtained if either the first AP2 repeat or second AP2 repeat alone was used to construct the tree.

members of the AP2 subfamily is the AP2 repeat region, pairs of genes share similarity throughout their sequence. For example, *PLT1* and *PLT2* are 75% identical and 86% similar throughout their entire sequence. *AIL6* and *AIL7* are 62% identical and 74% similar throughout their sequence.

Besides the AP2 domain DNA-binding region, members of the AP2 subfamily contain other regions characteristic of transcriptional regulators. Putative nuclear localization signals and Ser-rich, Gln-rich, or Asn-rich regions suggestive of transcriptional activation domains are present within several of the AILs (Table 2) and other members of the AP2 subfamily. It has been reported earlier that several AP2-like proteins share three additional motifs outside of the AP2 domains (Vahala *et al.*, 2001; Shigyo and Ito, 2004). Two of these three motifs are present within all members

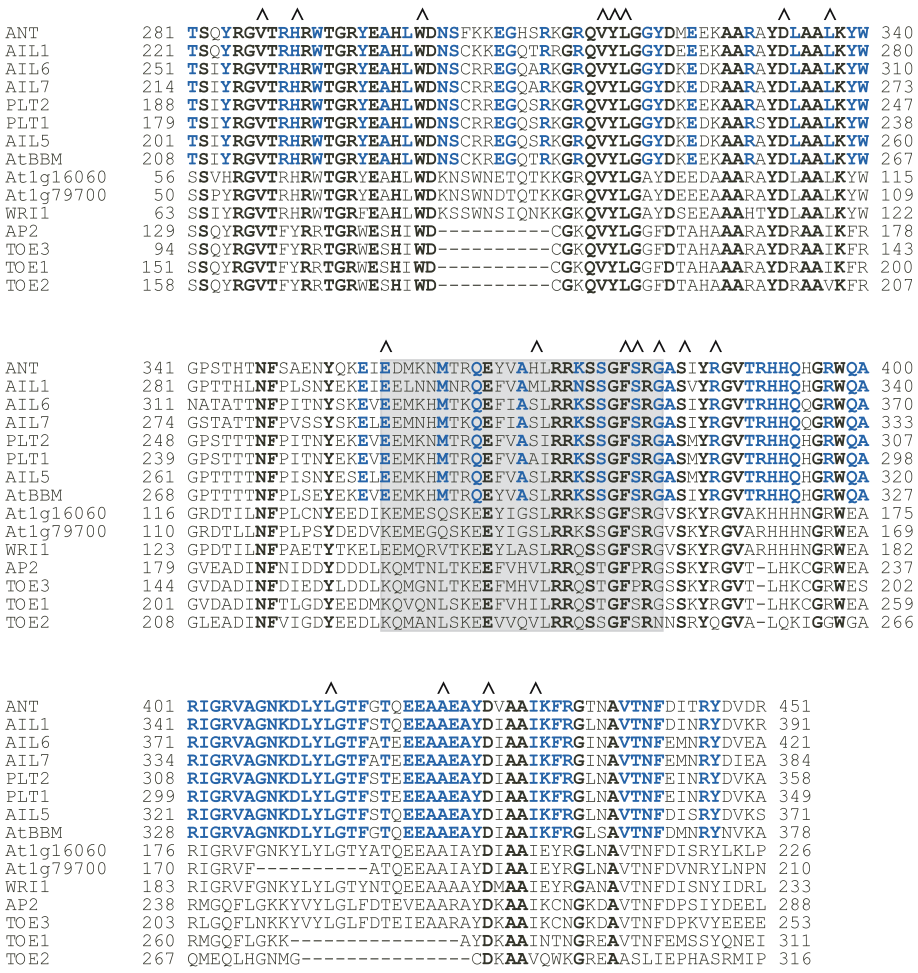

Figure 2. Sequence alignment of the AP2 repeat region for the 15 members with two AP2 domains. Numbers indicate the amino acid positions in the context of the entire protein. Amino acids that are identical in all members of the AP2 subfamily are indicated in bold. Additional amino acids that are identical in all AIL proteins are indicated in blue. The linker region is highlighted in gray. ∧ denote positions important for DNA binding by ANT (Krizek, 2003).

of the AP2-like group shown here. The third of these conserved motifs corresponds to a binding site for the microRNA, miRNA172 (Park *et al.*, 2002). This miRNA regulates the expression of AP2 (and most likely TOE1, TOE2, TOE3, SMZ, and SNZ) at the translational level (Aukerman and Sakai, 2003; Chen, 2004). None of these motifs are present in AILs, WRI1, At1g16060, At1g79700, or At2g41710.

Expression analysis of AILs by real time RT-PCR

As a starting point in the determination of function for the *AIL* family members, their expression was examined in different plant tissues by real time RT-PCR. All of the *AIL* genes showed expression in multiple plant tissues, with higher levels of expression in young tissues (seedlings, roots, inflorescences, and siliques) as compared with older tissues (mature rosette leaves and stems) (Figure 3). *ANT* is expressed at higher levels in most tissues compared to other members of the *AIL* group. All *AIL*s are expressed in seedlings although the level of *PLT2* mRNA was very low. All *AIL* genes are expressed in roots with *ANT*, *AtBBM, PLT1, PLT2, AIL5,* and *AIL6* expressed at higher levels than *AIL1* and *AIL7*. Our root expression data for *PLT1* and *PLT2* are consistent with the recent finding that these genes play a role in root development (Aida *et al.*, 2004). *ANT,*

Table 2. Ser, Gln, and Asn rich regions in AIL proteins.

Protein	Ser rich	Gln rich	Asn rich
AIL2		aa537–546	aa145–180
PLT1		aa451–474	
AIL5	aa8–88		aa37–49
AIL6		aa456–528	aa187–219

AIL1, AIL5, AIL6, and *AIL7* are expressed in inflorescences. The expression levels of *PLT1* and *PLT*2 were extremely low in inflorescence tissue. All *AIL*s were expressed in siliques.

Our examination of *ANT* expression by real time RT-PCR produced results that are in agreement with previous reports of *ANT* expression in both vegetative and reproductive tissues including roots, seedlings, floral buds, flowers, and siliques (Elliott *et al.*, 1996; Klucher *et al.*, 1996). However, we also observed low expression in mature rosette leaves, which was not previously detected by RNA gel blot analysis (Klucher *et al.*, 1996).

Expression analysis of AILs by in situ *hybridization*

Because of our interest in flower development, we have examined the spatial pattern of expression of the four *AIL* genes that are expressed at significant levels in inflorescences.

AIL1

We were not able to detect a specific signal with our *AIL1* antisense probe.

AIL5

AIL5 mRNA was detected at low levels in the inflorescence meristem and throughout young floral meristems (Figure 4A). In stage 3 flowers, *AIL5* is expressed in sepal primordia and the floral meristem (Figure 4C). *AIL5* continues to be expressed in sepal primordia until stage 6. At this time, *AIL5* mRNA is detected in petal, stamen, and carpel primordia (Figure 4D). Later, *AIL5* mRNA becomes restricted to internal regions of stamens and carpels, specifically sporogenous tissue in the stamens and the medial ridge of the carpel (Figure 4E and F). *AIL5* continues to be expressed in petals, with higher expression in the petal margins compared to the central part of the organ (Figure 4E). At later stages of development, *AIL5* expression became restricted to petal epidermal cells and was maintained there at low levels through stage 12 of flower development (Figure 4G). *AIL5* was expressed in tissues that develop from the medial ridge including the placenta and ovule primordia (Figure 4H and P). *AIL5* expression was strongest in the base of newly initiated ovule primordia (Figure 4H). At later stages in ovule development, *AIL5* was localized to the central region of the ovule including the distal part of the funiculus and the outer integument (Figure 4I). *AIL5* expression decreased in the integuments once they surrounded the nucellus but continued to be expressed in the distal part of the funiculus. No signal was detected in *AIL5* sense controls (Figure 4B). To further examine *AIL5* expression in the inflorescence meristem, serial transverse sections were examined. *AIL5* is expressed at low levels throughout the inflorescence meristem with higher expression in young floral meristems that have just initiated from the inflorescence meristem (Figure 4J–M).

AIL6

AIL6 mRNA was detected in inflorescence meristems, throughout young floral meristems (stages 1 and 2), and in procambial cells in the stem (Figure 5A). In stage 3 floral meristems, *AIL6* expression was highest in cells in the central dome of the floral meristem and lower in sepal primordia (Figure 5A and C). *AIL6* expression continued to decrease in sepal primordia and was no longer present by stage 6 (Figure 5D). During stage 6, *AIL6* mRNA was observed in second, third and fourth whorl organ primordia (Figure 5D and data not shown). By stage 7, only occasional patches of *AIL6* mRNA were observed in stamens (Figure 5E). *AIL6* expression was visible in petals upon inception and continuing until stage 9 (Figure 5E). After stage 9, no *AIL6* transcript was detected in any floral organs. No signal was detected in *AIL6* sense controls (Figure 5B).

To further investigate the pattern of *AIL6* expression in inflorescence meristems, transverse sections were used for *in situ* hybridization (Figure 5F–J). *AIL6* is expressed in the topmost cells of the inflorescence meristem with the strongest expression in circular regions around the periphery of the meristem and lower expression in the central region (Figure 5F). In subepidermal cells, *AIL6* is

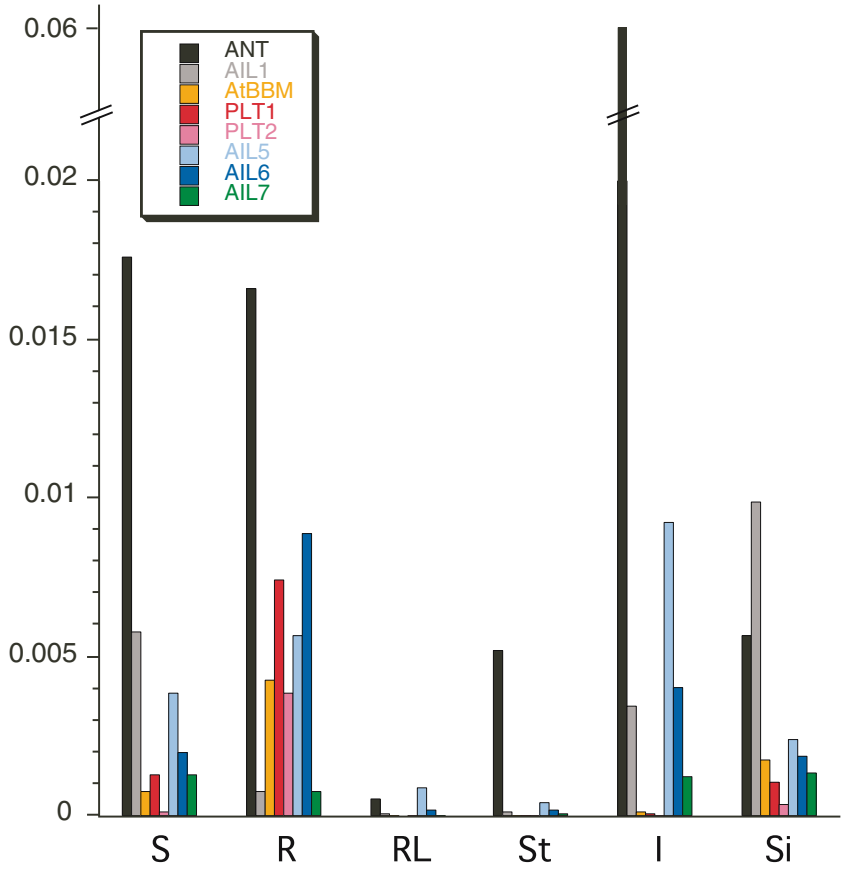

Figure 3. Relative expression profiles of the *AIL* genes as determined by real time RT-PCR. Ratios of the absolute value of *AIL* gene expression divided by the absolute value of *UBQ10* expression were calculated for each experiment. The average value from 2 to 4 experiments is shown here. Because of this ratio calculation, standard deviations are not displayed. RNA from seedlings (S), roots (R), rosette leaves (RL), stems (St), inflorescences (I), and siliques (Si) was examined for each gene.

expressed in small groups of cells within the meristem (Figure 5G–J). These spots of *AIL6* expression presumably correspond to floral anlagen and newly initiated floral meristems based on their spatial arrangement within the inflorescence meristem.

AIL7

AIL7 is expressed in the central region of inflorescence and floral meristems (Figure 6A). *AIL7* mRNA was not detected in stage 1 floral meristems but reappears in stage 2 floral meristems (Figure 6C). After initially being expressed throughout stage 2 floral meristems, *AIL7* become restricted to a more central region by the end of stage 2 of flower development (Figure 6C). During stage 3 and 4 of flower development, *AIL7* mRNA is expressed in the innermost cells of the dome of the floral meristem (Figure 6A). This expression pattern is more spatially restricted than that of *AIL6*, which is expressed in all floral meristem cells between the sepal primordia in a stage 3 flower (compare Figure 6C with Figure 5A and C). *AIL7* is expressed at low levels in the central region of a stage 5 flower (Figure 6D) and is not detected in stage 6 or stage 7 flowers. *AIL7* expression reappears in the developing stamen locules during stage 8 and sporogenous cells within the locules of stage 9 flowers (Figure 6E, F). Within carpel primordia, *AIL7* mRNA is detected at low levels in the placenta derived from the medial ridge during stage 9 (Figure 6E, F) and in young ovule primordia of stage 9 and 10 flowers (Figure 6G).

To further investigate the pattern of *AIL7* expression in inflorescence meristems, transverse sections of L*er* inflorescences were used for *in situ*

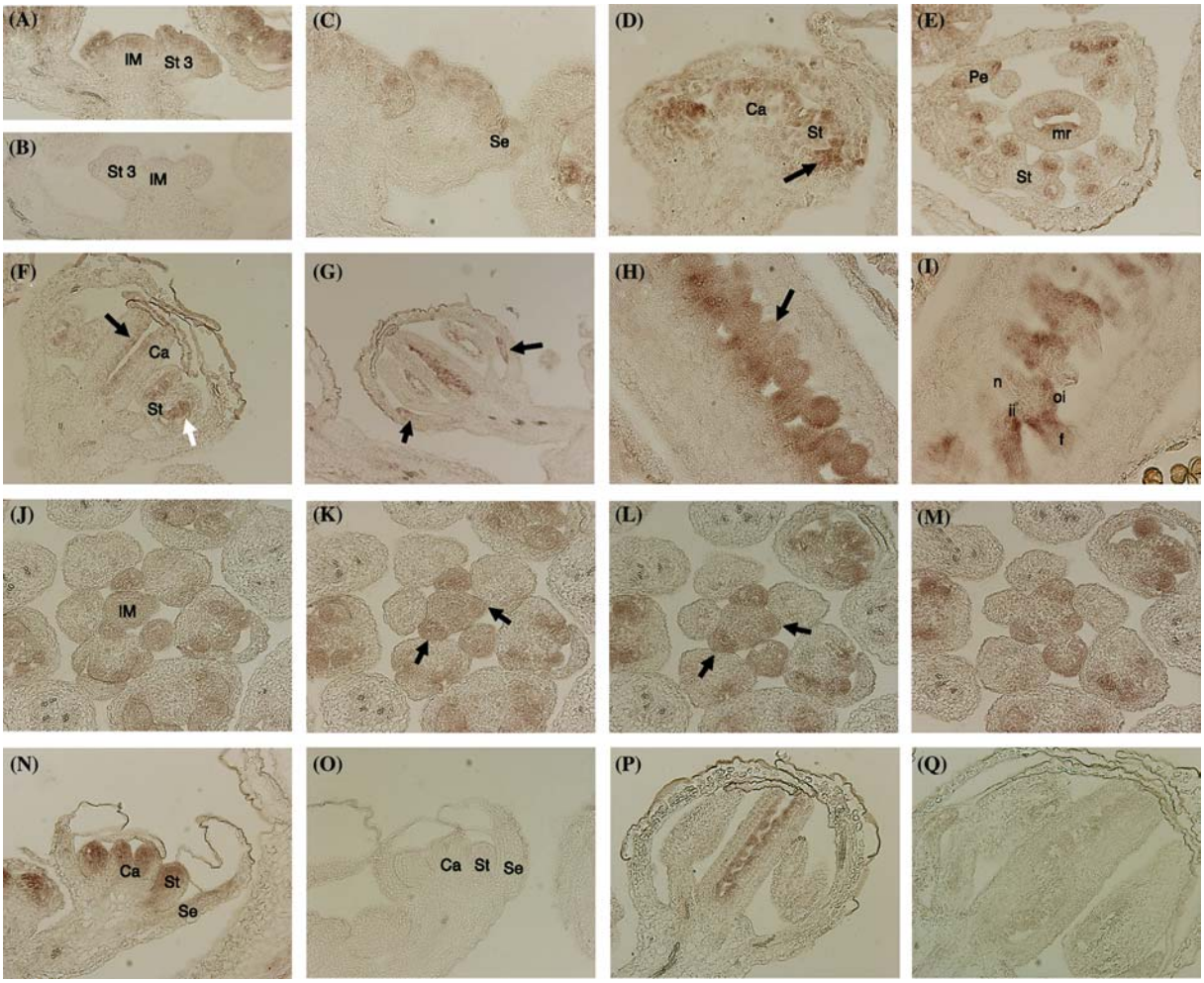

Figure 4. *In situ* hybridization of *AIL5* in L*er* inflorescences. A, C–Q were probed with an *AIL5* antisense probe. B was probed with an *AIL5* sense probe. (A) *AIL5* is expressed at low levels in the inflorescence meristem and throughout stages 2 and 3 floral meristems. (B) Tissue section probed with an *AIL5* sense probe showed no signal. (C) *AIL5* is expressed at low levels in the sepal primordia and floral meristem of a stage 3 flower. (D) *AIL5* is expressed in petal, stamen, and carpel primordia of a stage 6 flower. Arrow points to a petal primordia. (E) Transverse section of an approximately stage 8 flower. *AIL5* mRNA is detected in the petals, sporogenous tissue of the stamens, and the medial ridge of the carpel. *AIL5* mRNA is localized to the edges of some developing petals. (F) Longitudinal section showing *AIL5* expression in the stamens (white arrow) and medial ridge of the carpel (black arrow). (G) Longitudinal section of a stage 12 carpel showing *AIL5* expression in ovules, stamens, and the epidermis of petals (arrows). (H) A longitudinal section of a stage 10 carpel showing *AIL5* expression in ovules. *AIL5* expression is strongest at the base of the ovule primordia and weaker at the tip (one primordia is indicated with an arrow). (I) *AIL5* is expressed in the outer integument and the distal region of the funiculus. (J–M) Serial transverse sections through an inflorescence meristem. (J) *AIL5* is expressed at low levels throughout the topmost cells of an inflorescence meristem. (K–M). *AIL5* expression is higher in flower meristems that have just initiated from the inflorescence meristem (arrows). (N) *AIL5* expression in a L*er* stage 7 flower. (O) *AIL5* expression in a *35S::AIL5IR* stage 7 flower. The L*er* and *35S::AIL5IR* tissues shown in N and O were hybridized side by side on the same slide. (P) *AIL5* expression in a L*er* stage 10 flower. (Q) *AIL5* expression in a *35S::AIL5IR* stage 12 flower. The L*er* and *35S::AIL5IR* tissues shown in P and Q were hybridized side by side on the same slide. Abbreviations: IM, inflorescence meristem, St 3, stage 3 flower; Se, sepal; Pe, petal; St, stamen; Ca, carpel; mr, medial ridge; n, nucellus; f, funiculus; oi, outer integument; ii, inner integument.

hybridization (Figure 6H–L). *AIL7* mRNA was detected in the central region but not the more peripheral regions of the inflorescence meristem (Figure 6H–J). Circular regions free of *AIL7* expression are visible around the edges of the inflorescence meristem (Figure 6I). These regions

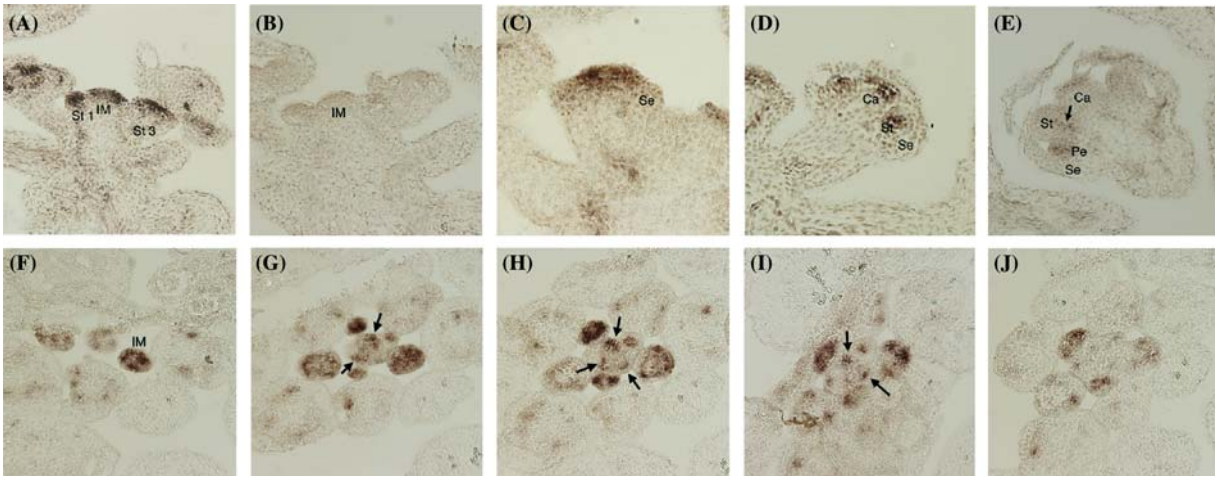

Figure 5. *In situ* hybridization of *AIL6* in L*er* inflorescences. A, C–J were probed with an *AIL6* antisense probe. B was probed with an *AIL6* sense probe. (A) *AIL6* is expressed in inflorescence meristems and young floral meristems (stages 1 and 3 flowers are shown here). (B) No signal is detected in tissue hybridized with an *AIL6* sense probe. (C) *AIL6* is expressed most strongly in the center of a stage 3 floral meristem. (D) *AIL6* is expressed in the stamen and carpel primordia of a stage 6 flower. (E) *AIL6* mRNA is expressed throughout the petal primordia and small domains within stamen primordia (arrow) of a stage 7 flower. (F–J) Serial transverse sections through an inflorescence meristem. (F). *AIL6* is expressed in the topmost cells of an inflorescence meristem with weaker expression in the centermost cells and stronger expression in cells on the periphery. (G–J). *AIL6* is expressed in patches corresponding to young flower anlagen (arrows). Abbreviations: IM, inflorescence meristem; St 1, stage 1 flower; St 3, stage 3 flower; Se, sepal; Pe, petal; St, stamen; Ca, carpel.

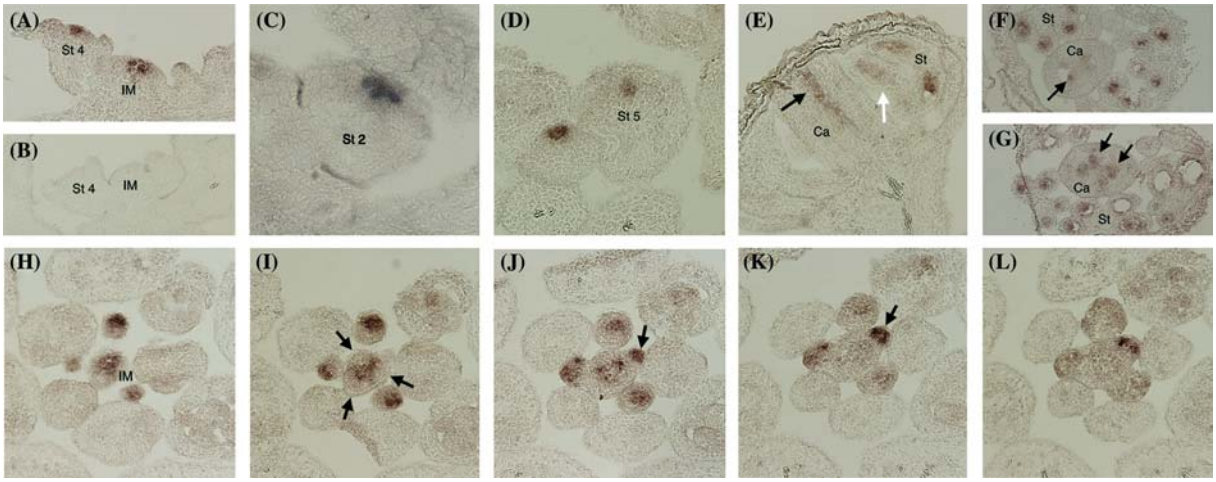

Figure 6. *In situ* hybridization of *AIL7* in L*er* inflorescences. A, C–L were probed with an *AIL7* antisense probe. B was probed with an *AIL7* sense probe. (A) *AIL7* is expressed in the central region of the inflorescence meristem and the stage 4 floral meristem. (B) No signal is detected in tissue hybridized with an *AIL7* sense probe. (C) *AIL7* mRNA is detected in the centermost cells of a late stage 2 flower. (D) *AIL7* mRNA is present at relatively lower expression levels in the center of a stage 5 flower. (E) *AIL7* mRNA is detected in a stage 9 flower in sporogenous cells of stamens (white arrow) and the placenta of the carpel (black arrow). (F) Transverse section showing *AIL7* mRNA in the placenta (arrow) and in the stamen locules. (G) Transverse section showing *AIL7* mRNA in young ovule primordia (arrows). (H–L) Serial transverse sections through an inflorescence meristem. (H) *AIL7* is expressed in the central region of an inflorescence meristem. (I) *AIL7* mRNA is detected in the center of the inflorescence meristem but not in peripheral regions of the meristem corresponding to newly initiated stage 1 floral meristems and floral anlagen (arrows). (J–L) *AIL7* mRNA is detected in a young stage 2 floral meristem that has been initiated by the inflorescence meristem (arrow). Abbreviations: IM, inflorescence meristem; St 4, stage 4 flower; St 5, stage 5 flower; Se, sepal; St, stamen; Ca, carpel.

appear to correspond to floral anlagen and stage 1 floral meristems. Although *AIL7* expression is downregulated prior to flower initiation, *AIL7* expression returns at approximately stage 2 of flower development (Figure 6J, K). The expression patterns of *AIL5*, *AIL6*, and *AIL7* during stages 1-12 of flower development are summarized in Figure 7.

Identification of AIL *insertional alleles*

To address the function of *AIL* genes in *Arabidopsis*, we have identified lines containing T-DNA insertions in *AIL1*, *AIL5*, *AIL6*, and *AIL7* (Sessions *et al.*, 2002; Alonso *et al.*, 2003). SALK_003704 contains a T-DNA in the second exon of *AIL1*, SALK_067199 contains a T-DNA in the first intron of *AIL5*, SALK_021823 contains a T-DNA in the eighth exon of *AIL6*, and SAIL 1167_C10 contains a T-DNA in the sixth exon of *AIL7*. Plants homozygous for any one of these insertions do not display developmental defects. However, it is not clear whether any of these insertions corresponds to a null allele. *AIL5* mRNA was detected in inflorescence tissue of SALK_067199, *AIL6* mRNA was detected in inflorescence tissue of SALK_021823, and *AIL7* mRNA was detected in inflorescence tissue of SAIL 1167_C10 (data not shown). To investigate the consequences of reduced *AIL5* expression, we generated transgenic plants containing an inverted repeat *AIL5* construct. These *35S*::*AIL5IR* lines do not accumulate *AIL5* mRNA (Figure 4N–Q) yet have a wild-type phenotype.

Our *in situ* hybridization experiments indicate that many tissues within an inflorescence express multiple *AIL* genes. The expression patterns of *AIL5*, *AIL6,* and *AIL7* overlap with each other and with that of *ANT* in the inflorescence meristem, young floral meristems, and floral organ

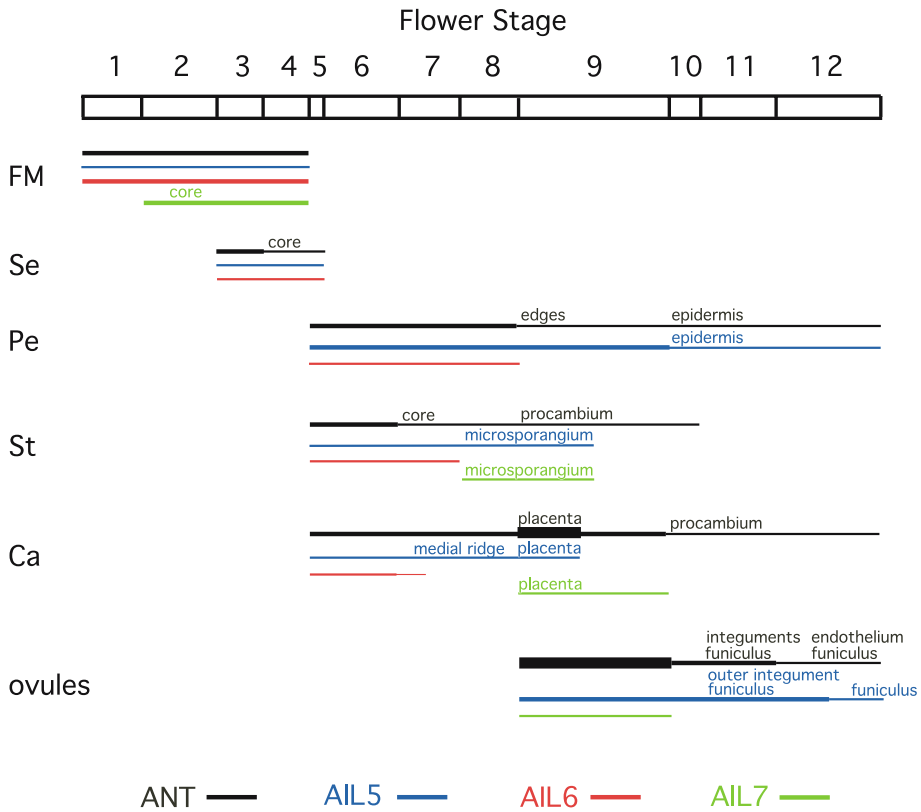

Figure 7. Summary of the expression patterns of *ANT*, *AIL5*, *AIL6*, and *AIL7* in developing flowers. The stages of flower development and their durations are as described earlier (Smyth *et al.*, 1990). The relative thickness of the lines indicates the levels of expression. Restriction of expression to particular cell types is as indicated above the lines. The *ANT* expression data is from (Elliott *et al.*, 1996) with some modifications. Abbreviations: FM, floral meristem; Se, Sepal; Pe, Petal; St, Stamen; Ca, carpel.

primordia (Figure 7). Thus it is possible that the absence of a phenotype in these lines may be due to genetic redundancy. We are currently investigating this possibility.

Ectopic expression of AIL5 *increases floral organ size*

An additional approach to determining the function of *AIL* genes is ectopic expression. This approach can provide information on gene function in cases where there is functional redundancy (Zhang, 2003). The *AIL5* cDNA was placed behind the *35S* cauliflower mosaic virus promoter and transformed into wild-type *Arabidopsis* plants. Seven out of 10 independent lines showed increased growth of floral organs but not vegetative organs (Figure 8A, B). Two of these seven lines (lines 16 and 20) exhibited a more dramatic increase in organ size similar to that resulting from ectopic expression of *ANT* (Krizek, 1999; Mizukami and Fischer, 2000). Petal length was increased approximately 20% in *35S::AIL5* line 20 compared to L*er* (3.5 mm ± 0.3 vs. 2.9 mm ± 0.4). Cells were approximately the same size in petals of L*er*, *35S::AIL5* line 16, and *35S::AIL5* line 20 suggesting that the increased size of *35S::AIL5* petals is primarily due to increases in cell number (Figure 8C, D).

In addition, *35S::AIL5* plants exhibit reduced seed set that is apparently due to a reduction in pollen production and transfer to the stigma. Manual pollination using pollen from wild-type plants resulted in normal seed set. The decreased fertility of *35S::AIL5* plants appears to be correlated with the severity of the organ size phenotype, as transgenic lines with the largest floral organs produce the fewest seeds. *AIL5* transcript levels were examined in five *35S::AIL5* lines by real time RT-PCR. Expression of *AIL5* was dramatically increased in three lines with larger floral organs (lines 5, 16, and 20) but was only slightly increased in two lines (lines 6 and 7) that had a wild-type appearance. *AIL5* mRNA levels were not strictly correlated with phenotypic severity as lines 5 and 16 had similar *AIL5* mRNA levels but line 5 exhibited only a slight increase in floral organ size.

Discussion

AIL *genes are expressed in different plant tissues*

By real time RT-PCR and *in situ* hybridization, we have shown that members of the *AIL* gene family exhibit a rather broad pattern of expression in both vegetative and reproductive tissues. Similar to *ANT,* expression of *AIL* genes is higher in young tissues and very low or absent in mature leaves and stems (Elliott *et al*., 1996; Klucher *et al*., 1996). Typically, expression levels were at least 100 fold lower than that of *UBQ10*. In almost all of the tissues tested, *AIL* genes are expressed at lower levels than *ANT*. This is also seen in

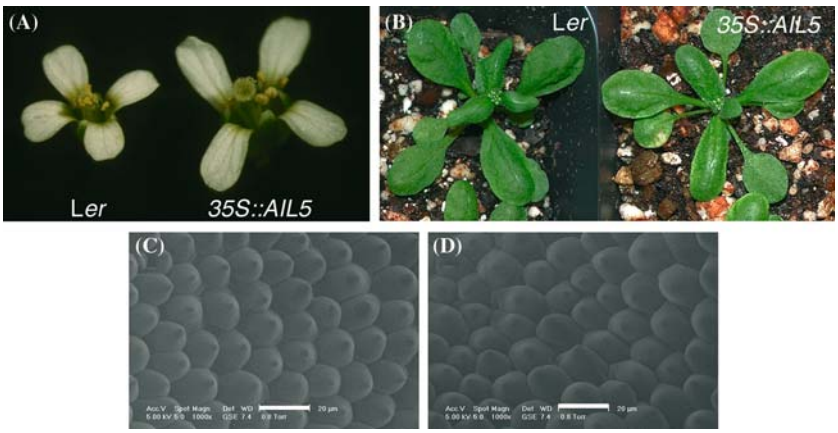

Figure 8. Phenotype of *35S::AIL5* plants. (A) L*er* and *35S::AIL5* flowers. (B) L*er* and *35S::AIL5* rosettes. (C) Scanning electron micrograph of cells on the adaxial surface on a L*er* petal. (D) Scanning electron micrograph of cells on the adaxial surface on a *35S::AIL5* petal. Size bars correspond to 20 μM.

publicly available expression data from MPSS (http://mpss.udel.edu/at/) and AtGenExpress (http://www.weigelworld.org/resources/microarray/AtGenExpress/).

Multiple *AIL* genes are expressed in the majority of tissues tested, suggesting that genetic redundancy may exist among members of this gene family. This has been shown recently for *PLT1* and *PLT2* (Aida *et al*., 2004) and will need to be investigated for other *AIL* genes. Additional *in situ* hybridization experiments will be necessary to determine the extent of *AIL* gene expression overlap at the cellular level in other tissues of the plant such as seedlings and roots. Different *AIL* genes do function in different tissues and at different developmental stages within the plant life cycle. For the members previously characterized, *AtBBM* likely functions during embryogenesis (Boutilier *et al*., 2002), *ANT* functions during flower development (Elliott *et al*., 1996; Klucher *et al*., 1996), and both *PLT* genes function in roots (Aida *et al*., 2004).

AIL *genes have distinct spatial patterns of expression in reproductive tissues*

The expression patterns of *ANT*, *AIL5*, *AIL6,* and *AIL7* are complex with each of these genes being expressed in multiple reproductive tissues and exhibiting spatial variation in these tissues during development (Figure 7). Of the three new *AIL* genes characterized, *AIL5* has the most similar expression pattern to *ANT*.

AIL5, *AIL6*, and *AIL7* are all expressed in the inflorescence meristem but in distinct patterns. *AIL7* is expressed in regions of the inflorescence meristem that will remain meristematic. *AIL6* is expressed in a complementary group of cells, those that will leave the inflorescence meristem to become a flower. *AIL5* is expressed at low levels throughout the inflorescence meristem with higher levels in newly initiated floral meristems. *ANT*, *AIL5*, and *AIL6* are all expressed in stage 1 floral meristems (Elliott *et al*., 1996). *AIL7* expression reappears in stage 2 floral meristems. As floral organs are initiated from the meristem, first *AIL7* and later *AIL6* expression disappears in these primordia. *AIL5* expression becomes restricted to petals and certain reproductive tissues. *ANT* expression is maintained as organ primordia arise and generally remains on during stages 3–8 (depending on the organ type) (Elliott *et al*., 1996). *ANT* expression then becomes more restricted to particular regions within developing petals, stamens, and carpels (Elliott *et al*., 1996).

AIL5 is expressed within a meristematic-like tissue, the medial ridge. The medial ridge arises from cells on the adaxial side of the gynoecium cylinder. This group of proliferative cells gives rise to the septum, placenta, ovules, transmitting tract and the adaxial region of the style (reviewed in Bowman *et al*., 1999). *ANT*, *AIL5,* and *AIL7* are all expressed in the placenta and subsequently in ovule primordia that are initiated from the placenta. *ANT* and *AIL5* continue to be expressed in developing ovules while *AIL7* is downregulated once the primordia have initiated and start to elongate. Both *ANT* and *AIL5* are initially expressed in the basal half of ovule primordia. At later stages of ovule development, *ANT* is expressed in both developing integuments while *AIL5* is expressed in the outer integument (Elliott *et al*., 1996). Both genes are expressed in the distal part of the funiculus. *ANT* and *AIL5* are downregulated as the integuments grow to surround the nucellus with *AIL5* expression persisting longer than that of *ANT*. *ANT* expression becomes localized to the inner wall of the inner integument that differentiates into the endothelium (Elliott *et al*., 1996).

AIL *genes may specify a meristem or growth competent state*

AIL gene expression is associated with actively growing and developing tissues within a plant. Results from expression analyses, loss of function (*ant*, *ptl1 ptl2*) alleles, and gain of function (*35S::ANT*, *35S::BBM*, *35S::AIL5*) studies suggest that genes within this family act to specify meristematic and/or developmentally young tissue states. Within the MPSS data set, six *AIL* genes (*AtBBM*, *PLT1*, *PLT2*, *AIL5*, *AIL6*, and *AIL7*) exhibit their strongest expression in callus tissue. This is consistent with these genes specifying an undifferentiated fate.

In meristems, some *AIL* genes may function to suppress differentiation. All cells within the root apical meristem of *ptl1 ptl2* double mutants undergo differentiation (Aida *et al*., 2004), indicating that these genes promote an undifferentiated state and are required to maintain a root meristem.

Based on its expression pattern, *AIL7* may play a similar role in shoot meristems. Several aspects of the *AIL7* expression pattern are reminiscent of meristem genes like *CLAVATA1* (*CLV1*) and *CLAVATA3* (*CLV3*) (Clark *et al*., 1997; Fletcher *et al*., 1999). All three genes are expressed in the center of an inflorescence meristem and are not expressed on the flanks of the inflorescence meristem or in stage 1 flowers. In addition, the expression of all three genes reappears in stage 2 floral meristems but is downregulated as floral organ primordia are initiated.

AIL6 expression is an early marker of cells within the inflorescence meristem that will embark on a pathway toward differentiation and is maintained in these cells as they give rise to a floral meristem. *AIL6* may act to reprogram cells as they switch from an indeterminate to determinate fate. During floral organ development, *ANT* and *AIL5* may act to maintain immature organ primordia in a growth-competent state by promotion of cell division. Continued expression of *ANT* and *AIL5* within developing organs results in prolonged growth of organs (Krizek, 1999; Mizukami and Fischer, 2000).

Brassica napus BBM (*BnBBM1*) appears to promote an embryogenic state and repress the transition to a more mature developmental phase. *35S::BnBBM1* and *UBI::BnBBM1* plants reiteratively form somatic embryo and cotyledon-like organs on the surface of cotyledons (Boutilier *et al*., 2002). The production of lobed leaves and ectopic shoots on the leaf surface of these plants as well as plants misexpressing *PLT1* (M. Aida and B. Scheres, personal communication) suggests that these genes are sufficient to confer a meristematic fate, at least in certain contexts. Because of the similarity of these phenotypes to those resulting from ectopic expression of class I *KNOX* genes (Chuck *et al*., 1996; Lincoln *et al*., 1994), it is possible that *KNOX* genes get activated in the leaves of *PLT1* and *BBM* overexpressing plants.

AIL5 *might function in organ growth control*

Ectopic expression of *AIL5* in wild type plants produces larger floral organs. This phenotype is similar to that seen upon ectopic expression of *ANT* (Krizek, 1999; Mizukami and Fischer, 2000) and suggests that *AIL5*, like *ANT*, might promote growth within developing floral organs. The similar overexpression phenotypes also suggest that AIL5 regulates many of the same genes as ANT. It is possible that all of the AIL proteins possess similar DNA binding specificities as the predicted proteins have identical amino acids in all but one of the positions that have been shown to be important for the DNA binding ability of ANT (Krizek, 2003) (Figure 2). Only one of these positions, corresponding to the 14th amino acid within the linker region, is variable between AIL proteins. Whether a single amino acid difference is sufficient to mediate distinct DNA binding specificities by AIL proteins is not known.

AIL5 is expressed in many of the same tissues as *ANT* yet it cannot complement an *ant* loss of function mutant. One possible reason is the significantly lower expression levels of *AIL5* as compared with *ANT*. It will be interesting to investigate the potential of *AIL5* to complement *ant* when expressed under the control of the *ANT* promoter. Further defining the function of *AIL5* and the other *AIL*s may require the construction of several double and triple mutant combinations as multiple *AIL* genes are expressed in all floral tissues (Figure 7). Characterization of such plants should provide a clearer picture of the role of *AIL* genes in specification of meristematic and growth competent states.

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