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AINTEGUMENTA and AINTEGUMENTA-LIKE6 Act Redundantly to Regulate Arabidopsis Floral Growth and Patterning

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An Arabidopsis (Arabidopsis thaliana) flower consists of four types of organs arranged in a stereotypical pattern. This complex floral structure is elaborated from a small number of floral meristem cells partitioned from the shoot apical meristem during reproductive development. The positioning of floral primordia within the periphery of the shoot apical meristem depends on transport of the phytohormone auxin with floral anlagen arising at sites of auxin maxima. An early marker of lateral organ fate is the AP2/ERF-type transcription factor AINTEGUMENTA (ANT), which has been proposed to act downstream of auxin in organogenic growth. Here, I show that the related, AINTEGUMENTA-LIKE6 (AIL6)/PLETHORA3 gene acts redundantly with ANT during flower development. ant ail6 double mutants show defects in floral organ positioning, identity, and growth. These floral defects are correlated with changes in the expression levels and patterns of two floral organ identity genes, APETALA3 and AGAMOUS. ant ail6 flowers also display altered expression of an auxin-responsive reporter, suggesting that auxin accumulation and/or responses are not normal. Furthermore, I show that ANT expression in incipient and young floral primordia depends on auxin transport within the inflorescence meristem. These results show that ANT and AIL6 are important regulators of floral growth and patterning and that they may act downstream of auxin in these processes.

During reproductive development in Arabidopsis (Arabidopsis thaliana), floral meristems are initiated reiteratively from groups of cells within the periphery of the shoot apical meristem. The particular sites at which flowers are initiated correspond to local auxin maxima generated by polar transport of the hormone by efflux carriers such as PINFORMED1 (PIN1; for review, see Fleming, 2007). Two genes closely associated with floral initiation are LEAFY (LFY) and AINTEGUMENTA (ANT), which act in the specification of floral meristem identity and growth, respectively. Both genes are expressed in incipient floral anlagen prior to outgrowth, and their expression patterns are altered in pin1 mutants, which are defective in floral initiation (Weigel et al., 1992; Elliott et al., 1996; Vernoux et al., 2000). The inability of pin1 mutants to initiate flowers can be rescued by the application of auxin paste to the periphery of the shoot apex, demonstrating the necessity and sufficiency of auxin for lateral organ development (Reinhardt et al., 2000).

Once established, floral meristems give rise to floral organ primordia at spatially defined positions within four concentric whorls. In an Arabidopsis flower, four sepal primordia arise in the outer first whorl, four petal primordia arise in the second whorl, six stamen primordia arise in the third whorl, and two carpel primordia arise in the centermost fourth whorl. Four different classes of floral organ identity genes (A, B, C, and E) act in different regions of the flower to specify these different organ identities (for review, see Krizek and Fletcher, 2005). The class A genes APETALA1 (API) and AP2 are active in whorls one and two; the class B genes AP3 and PISTILLATA are active in whorls two and three; the class C gene AGAMOUS (AG) is active in whorls three and four; and the E class, consisting of SEPALLATA1 (SEPI) to SEP4, is active in all four whorls. The region-specific activities of the class A, B, and C genes result primarily from regulation at the level of transcription. LFY encodes a novel transcription factor with similarity to helix-turn-helix proteins (Hames et al., 2008) that is expressed throughout stage 1 and 2 floral meristems (Weigel et al., 1992). The broadly expressed LFY acts in combination with different regionally expressed coregulators to activate the floral organ identity genes in distinct subdomains within the flower (Parcy et al., 1998). In the case of AG activation, LFY acts in combination with the homeodomain protein WUSCHEL (WUS), an important regulator of stem cell identity (Lenhard et al., 2001; Lohmann et al., 2001). AG activity later feeds back to down-regulate WUS expression in the center of the flower, resulting in termination of the floral meristem (Lenhard et al., 2001; Lohmann et al., 2001).
Despite our knowledge about the control of floral organ identity and floral determinacy, other aspects of floral development remain obscure at the mechanistic level. This is especially true of early floral meristem patterning events such as floral organ positioning and primordium outgrowth. These processes are likely to be regulated by auxin because of their analogy with lateral organ positioning and primordium outgrowth from the shoot apical meristem. However, floral meristems are determined, whereas the shoot apical meristem exhibits indeterminate growth. In addition, floral organ primordia arise in a whorled rather than a spiral phyllotaxis, thus requiring the simultaneous generation of multiple auxin maxima within the floral meristem. There is limited information on auxin distribution in early stages of flower development (Benkova et al., 2003; Heisler et al., 2005), and genes that act downstream of auxin in floral meristem patterning have not been identified. In later stages of flower development, auxin accumulates in the tips of developing floral primordia, where it is thought to specify a new growth axis (Benkova et al., 2003).

**ANT** is an important regulator of growth during lateral organ development. *ant* flowers reach a smaller final size, while plants constitutively expressing **ANT** produce flowers that reach a larger final size (Elliott et al., 1996; Klucher et al., 1996; Krizek, 1999; Mizukami and Fischer, 2000). Several pieces of evidence link **ANT** function in organ growth with auxin signaling. **ANT** acts downstream of the novel auxin-inducible gene **ARGOS** (for auxin-regulated gene involved in organ size; Hu et al., 2003). Similar to **ANT**, loss- and gain-of-function **ARGOS** plants display opposite effects on lateral organ growth, and **ANT** activity is required for the increased size of lateral organs in 35S:**ARGOS** plants (Hu et al., 2003). Furthermore, **ANT** expression in maturing organs may be negatively regulated by **AUXIN RÉSPONSE FACTOR2** (ARF2), a repressor of organ growth (Schruff et al., 2005).

**ANT** is a member of the large AP2/ERF transcription factor family, containing a DNA-binding domain that corresponds to two AP2 repeats and the conserved intervening linker region (Nole-Wilson and Krizek, 2000). Seven AINTEGUMENTA-like (**AIL**) /PLETHORA (**PLT**) proteins share high sequence similarity to **ANT** within this DNA-binding domain, but as a group these eight proteins do not share sequence similarity outside of this region (Nole-Wilson et al., 2005). High amino acid similarity is found throughout the sequences of two pairs of **AIL**/**PLT** proteins (**AIL6/**PLT3** and **AIL7**, **PLT1** and **PLT2**; Fig. 1A). **ANT**, **AIL5**, **AIL6/**PLT3**, and **AIL7** show partially overlapping but distinct expression patterns within the inflorescence meristem and developing flowers, suggesting that these genes may function redundantly during flower development (Nole-Wilson et al., 2005). Four **AIL/**PLT** genes (**PLT1**, **PLT2**, **AIL6/**PLT3**, and **BBM**) have been shown to act redundantly in root development (Aida et al., 2004; Galinha et al., 2007).

Here, I show that **AIL6** acts redundantly with **ANT** during shoot development. While **ail6** single mutants display no obvious morphological differences from the wild type, **ant** **ail6** double mutants show defects during both vegetative and reproductive development. **ant** **ail6** flowers exhibit altered positioning of floral organ primordia, loss of floral organ identity, and reduced growth of floral organ primordia. These defects are correlated with altered expression of stem cell and floral regulatory genes as well as the auxin-responsive reporter **AGH3-2:GUS**. **ANT** expression in incipient and young floral primordia is reduced after treatment with the auxin transport inhibitor N-1-naphthylphthalamic acid (NPA), thus suggesting a role for auxin transport in the regulation of **ANT** expression. These results support a model in which **ANT** and **AIL6** promote growth and patterning in flowers downstream of auxin.

**RESULTS**

**ail6** and **ail6** **ail7** Mutants Have a Wild-Type Appearance

To investigate the function of **AIL6** (At5g10510), I identified four T-DNA insertion lines (Fig. 1B; Krysan et al., 1999; Sessions et al., 2002; Alonso et al., 2003; Rosso et al., 2003). The T-DNAs are present in the second intron (WISC 475_E04, **ail6-3**), third intron (SALK_127417, **ail6-1/**plt3-1; SAIL 690_B11, **ail6-2**), and sixth exon (Gabi-Kat 765_G02, **ail6-4**) of **AIL6**. None of these lines display any obvious morphological differences compared with the wild type during either vegetative or reproductive development (Fig. 1, D–G). **AIL6** expression levels were examined in three of these T-DNA lines by in situ hybridization using a probe directed against the second exon of **AIL6** (Fig. 1B). **AIL6** mRNA levels were significantly reduced in **ail6-1**, **ail6-2**, and **ail6-3** inflorescences as compared with Columbia (Col) wild-type inflorescences (Fig. 1, J and K; data not shown). The weak signal detectable in **ail6-2** inflorescences (Fig. 1K) suggests that a partial transcript that includes exon 2 is produced in this allele.

To determine if **AIL6** might act redundantly with the closely related gene **AIL7**, I identified a single T-DNA line carrying an insertion within **AIL7** (SAIL 1167_C10, **ail7-1**; Fig. 1C). The T-DNA is present in the sixth exon of **AIL7**. While partial transcripts have been detected in **ail7-1** plants (J. Mudunkothge and B. Krizek, unpublished data), it is likely that any protein produced in these plants is nonfunctional, as the T-DNA is present within sequences encoding the linker between the two AP2 domains. **ail7-1** plants have a wild-type appearance (Fig. 1H). **ail6-2** was crossed to **ail7-1** to generate the double mutant, which also exhibited no obvious morphological differences compared with wild-type plants (Fig. 1I).

**ant** **ail6** Flowers Display Reduced Petal, Stamen, and Carpel Identities

**AIL6** is more distantly related to **ANT**, but previously published in situ hybridization experiments
showed that these two genes are expressed in partially overlapping domains in inflorescences (Elliott et al., 1996; Nole-Wilson et al., 2005). To investigate whether AIL6 function overlaps with that of ANT, the strong ant-4 allele in the Landsberg erecta (Ler) background (Baker et al., 1997; Nole-Wilson and Krizek, 2006) was crossed to all four ail6 alleles. All of these double mutants displayed a similar enhanced phenotype compared with ant-4 single mutants (Fig. 1, L–O). This double mutant phenotype is not significantly affected by the mixed Col/Ler background (Supplemental Fig. S1). A detailed phenotypic characterization of the ant-4 ail6-2 double mutant is presented here.

ant-4 ail6-2 flowers consist primarily of small green organs; they lack petals and only rarely produce some stamen-like organs (Fig. 1, M–O; Table I). The outermost organs of ant-4 ail6-2 flowers resemble sepals in their overall shape and epidermal cell characteristics (Fig. 2, A–D). Other organs present in ant-4 ail6-2 flowers include filaments, filaments that are swollen or expanded at their distal end (Fig. 2A; Table I), and organs resembling unfused carpel valves (Fig. 2, A, E, and F). Schematic tissue is occasionally present on the margins of these valve-like organs (Fig. 2F), but they completely lack internal tissue normally present within carpels. ant-4 ail6-2 flowers sometimes contain other flat green organs that bear little resemblance to any floral organ found in wild-type flowers (Table I). Short filaments sometimes arise in the very center of ant-4 ail6-2 flowers (Fig. 2B).

Defects in the development of ant ail6 flowers are apparent at early stages. The floral meristem dome is often flatter in the double mutant (Figs. 2J and 3D) and/or consists of fewer cells (Fig. 2I). Fewer floral organ primordia are initiated, and they do not arise in regular positions (Fig. 2, I, J, and L). In both wild-type and ant-4 flowers, four sepal primordia arise in a cross pattern, each arising approximately 90° from the adjacent primordium, with one primordium located in the position adaxial to the inflorescence meristem (Fig. 2, G and H). In ant-4 ail6-2 flowers, a variable number of sepal primordia initiate at random positions within the floral meristem periphery (Fig. 2, I and J). Similarly, more centrally arising floral organ primordia do not exhibit any regular phyllotaxy (Fig. 2, K and L).

AP3 and AG Expression Is Altered in ant ail6 Flowers

Because of the loss of petal, stamen, and carpel identities in ant-4 ail6-2 flowers, I examined the expression of two floral organ identity genes: the class B gene AP3 and the class C gene AG. RNA gel blots showed that both genes are expressed at reduced levels in the double mutant (Fig. 3, A and G), while...
in situ hybridization revealed that the spatial expression patterns are altered. In a stage 3 wild-type flower, AP3 is expressed in cells adjacent to the sepal primordia that will give rise to the second and third whorls of the flower but not in the centermost cells of the floral meristem (Fig. 3B). Fewer cells in ant-4 ail6-2 stage 3

<table>
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<th>Organ Type</th>
<th>ant-4 ail6-2</th>
<th></th>
<th>ant-4</th>
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<tr>
<td></td>
<td>1 to 10</td>
<td>11 to 20</td>
<td>21 to 30</td>
<td>1 to 10</td>
<td>11 to 20</td>
<td>21 to 30</td>
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<tr>
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<td>4.0</td>
<td>3.85</td>
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<td>0.0</td>
<td>0.02</td>
<td>3.33</td>
<td>2.05</td>
<td>1.3</td>
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<tr>
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<td>0.95</td>
<td>0.8</td>
<td>0.18</td>
<td>0.15</td>
<td>0.20</td>
</tr>
<tr>
<td>Swollen filament</td>
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<td>0.2</td>
<td>0.1</td>
<td></td>
<td></td>
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<tr>
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<td>0.3</td>
<td>4.88</td>
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<td>Valve like</td>
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<td>2.22</td>
<td>2.0</td>
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<td>8.9</td>
<td>7.8</td>
<td>14.2</td>
<td>12.3</td>
<td>11.6</td>
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</tbody>
</table>

Table 1. Organs present in ant-4 ail6-2 and ant-4 flowers at positions 1 to 10, 11 to 20, and 21 to 30 on the inflorescence

Organ counts were performed on four plants for each genotype.

Figure 2. ant-4 ail6-2 plants have defects in flower development. A, Mature ant-4 ail6-2 flower with sepals (Se), unfused carpel valve-like structures (Va), and a stamen-like organ (arrow). B, Mature ant-4 ail6-2 flower in which two short filaments (arrows) occupy the center of the flower. C, Abaxial surface of Ler sepal. D, Abaxial surface of ant-4 ail6-2 sepal. E, Epidermal surface of Ler carpel valve. F, Epidermal surface of ant-4 ail6-2 valve-like organ. An arrow points to stigmatic papillae present on the margin of the organ. G, Stage 4 Ler flower. H, Stage 4 ant-4 flower. I and J, Stage 4 ant-4 ail6-2 flowers showing altered patterns of sepal initiation. K, Stage 10 Ler flower in which three sepals have been dissected away. L, ant-4 ail6-2 flower in which inner organ primordia have arisen in random positions. Bars = 400 μm in A, 200 μm in B, 50 μm in C to F and L, and 20 μm in G to K.
flowers, AP3 expression is only observed in a few cells at the adaxial base of the outer whorl organs and/or at the base of more inwardly arising organs (Fig. 3F).

In stage 3 wild-type flowers, AG is expressed in cells of the floral meristem that will develop into the third and fourth whorls of the flower (Fig. 3H). In stage 3 and 4 ant-4 ail6-2 flowers, AG mRNA is reduced and/or absent in the centermost cells of the floral meristem (Fig. 3, I and J). In addition, AG expression in the floral meristem directly abuts the sepal primordia in contrast to wild-type flowers, in which cells adjacent to the sepals do not express AG (Fig. 3, H–J). The extension of AG expression toward the sepals in ant ail6 flowers is consistent with earlier work showing that ANT can act as a second whorl repressor of AG (Krizek et al., 2000). Ectopic AG expression in second whorl cells of ant-4 ail6-2 flowers may be partly responsible for the decreased proliferation of these cells. In older wild-type flowers, AG is expressed in developing stamens and carpels (Fig. 3K). In ant-4 ail6-2 flowers, AG expression is observed in interior organs with valve-like appearances (Fig. 3L).

To determine if the absence of AG mRNA in the center of ant-4 ail6-2 floral meristems might be due to reduced expression of its activators, I examined LFY and WUS expression in the double mutant. LFY mRNA was detected in incipient floral primordia and throughout stage 1 and 2 flowers in both wild-type and ant ail6 flowers (Fig. 4, A–D). Although the signal appeared to be weaker in some ant ail6 flowers, no obvious difference in LFY mRNA levels could be detected by semiquantitative reverse transcription (RT)-PCR (Supplemental Fig. S2). In wild-type stage 3 flowers, LFY expression is strong and uniform throughout the sepal primordia and floral meristem (Fig. 4C). However, in stage 3 ant-4 ail6-2 flowers, LFY expression is found to be patchy (Fig. 4D). In contrast to LFY expression, WUS expression is increased in young ant-4 ail6-2 flowers (Fig. 4, E and F). In addition, WUS expression is detected in a broader domain in the double mutant that includes L1, L2, and L3 cells (Fig. 4, G and H).

**ANT and AIL6 Regulate Floral Meristem Proliferation**

As AG activity is required to down-regulate WUS expression but is missing from the central region of stage 3 ant-4 ail6-2 flowers, WUS expression was examined in older ant-4 ail6-2 flowers. Persistent WUS expression was sometimes observed in the cen-
central region of ant-4 ail6-2 flowers at stages after WUS expression had disappeared from wild-type flowers (Fig. 4, I–L). While persistent expression of WUS in ag flowers results in floral meristem indeterminacy (Lenhard et al., 2001; Lohmann et al., 2001), such indeterminacy was never observed in ant-4 ail6-2 flowers, suggesting that ANT and AIL6 activities are required for floral meristem proliferation.

This possibility was investigated by constructing the ag-1 ant-4 ail6-2 triple mutant. ag-1 ant-4 ail6-2 flowers are determinate and consist primarily of sepalas with a few petal-like organs (Fig. 5, A and B). ag-1 ant-4 ail6-2 flowers typically produce just a few more floral organs than ant-4 ail6-2 flowers (Fig. 5C). In a few ag-1 ant-4 ail6-2 flowers, many more floral organs were produced, but these flowers were never indeterminate (Fig. 5D). Thus, ANT and AIL6 are required for the continued proliferation of the floral meristem in ag mutants. The partial restoration of petal identity in ag-1 ant-4 ail6-2 flowers and the increased number of floral organs could be due to increased proliferation of second whorl cells in the absence of AG.

Figure 4. The expression patterns of LFY and WUS are altered in ant-4 ail6-2 flowers. A, LFY expression in a Ler inflorescence. Arrows point to incipient floral primordia (right) and a stage 1 floral meristem (left). B, LFY expression in an ant-4 ail6-2 inflorescence. Arrows point to incipient primordia (left) and a stage 1 floral meristem (right). C, LFY expression in stage 2 and stage 3 ant-4 ail6-2 flowers. LFY expression is not as uniform in stage 3 ant-4 ail6-2 flowers compared with Ler. D, WUS:GUS inflorescence showing strong GUS staining in the very center of the inflorescence. E, WUS:GUS ant-4 ail6-2 inflorescence showing GUS staining in the center region of a stage 3 wild-type flower. F, WUS mRNA is detected in a broader domain in this stage 3 ant-4 ail6-2 flower. I, The arrow points to WUS expression in a stage 5 wild-type flower. J, WUS is not expressed in a stage 7 wild-type flower. K and L, The arrows point to WUS expression in older ant-4 ail6-2 flowers. Bars = 50 μm in A to D and G to L.

ANT and AIL6 Promote Growth of All Shoot-Derived Organs

ant-4 ail6-2 plants also exhibit defects during vegetative development. The plants are reduced in height, with a bushier appearance due to the outgrowth of additional axillary inflorescences from rosette and cauline leaves (Fig. 6A; Table II). In addition, accessory meristems in cauline leaf axils exhibit enhanced outgrowth in ant-4 ail6-2 plants (Fig. 6B). Both leaves and flowers produced by ant-4 ail6-2 plants are reduced in size compared with those from ant-4 and wild-type plants (Fig. 6, C–F). Rosette leaves of the double mutant plants are thinner than those from ant-4 and wild-type plants (Fig. 6, C–E), with alterations in leaf vein architecture (Supplemental Fig. S3). ant-4 ail6-2 leaves show more severe defects in vein density and complexity than those previously reported for ant single mutants (Supplemental Fig. S3, B and C; Kang et al., 2007).

Epidermal cells of rosette leaves and sepalas were similar in size in both the wild type and ant-4 ail6-2,
suggesting that the reduced size of these organs results primarily from fewer cells (Figs. 2, C and D, and 6, G and H). Epidermal peels of stems at positions midway along the primary inflorescence revealed similar cell lengths in wild-type and ant-4 ail6-2 plants (Fig. 6, I and J). However, cells making up the internodes between successive flowers of ant-4 ail6-2 plants are dramatically reduced in length in ant-4 ail6-2 plants as compared with wild-type plants (Fig. 6, K and L). Thus, the reduced height of ant-4 ail6-2 plants appears to arise primarily from reduced internode length.

**ANT and AIL6 Are Required for Continued Shoot Apical Meristem Function**

Initially, the inflorescence meristems of ant-4 ail6-2 plants have a dome appearance similar to the wild type (Fig. 7, A and B). However, as reproductive development proceeds, ant-4 ail6-2 inflorescence meristems become increasingly sloped, with flowers initiating at positions farther down the sides of the meristem (Fig. 7, C and D). Floral initiation eventually ceases in ant-4 ail6-2 plants, with the inflorescence meristem and surrounding floral primordia aborting growth. While the total number of flowers initiated by ant-4 ail6-2 inflorescence meristems prior to termination is similar to the number produced by wild-type plants prior to senescence (36.2 ± 3.0 in the wild type versus 33.3 ± 4.0 in ant-4 ail6-2), it is less than that produced by sterile mutants such as ant-4 (44.4 ± 8.5).

To gain insight into the basis for these defects in meristem function, I examined the expression of two important regulators of shoot apical meristem activity, WUS and CLAVATA3 (CLV3). WUS expression is confined to a small group of underlying cells in the shoot apical meristem referred to as the organizing center (Fig. 7G), while CLV3 is expressed in the overlying stem cells (Fig. 7E; Mayer et al., 1998; Fletcher et al., 1999). In ant-4 ail6-2 plants, CLV3 expression extends deeper into the inflorescence meristem than in the wild type (Fig. 7F), while WUS expression moves up into L1 and L2 cells within the center of the meristem (Fig. 7H). These changes and the partial overlap of the WUS and CLV3 expression domains suggest that cells within ant-4 ail6-2 inflorescence meristems have altered and/or mixed identities, since they express markers for both stem cell and organizing center fate.

**ant ail6 Flowers Display Altered Expression of the Auxin-Responsive Reporter AGH3-2:GUS**

Because several of the defects observed in ant-4 ail6-2 plants are reminiscent of mutants disrupted in auxin physiology, I examined auxin responses in ant-4 ail6-2 inflorescences using the auxin reporter AGH3-2:GUS. This reporter contains approximately 1.1 kb of 5’ sequence from the Arabidopsis GH3-2 gene (At4g37390; G. Hagen, personal communication). The AGH3-2:GUS reporter shows a similar pattern of expression in inflorescences and flowers as DR5:GFP, with expression in incipient floral primordia (Fig. 8A) and the tips of developing floral organs (Fig. 8, E, G, and I; Benkova et al., 2003; Heisler et al., 2005; Smith et al., 2006). Many AGH3-2:GUS ant-4 ail6-2 inflorescences showed GUS activity in incipient floral primordia similar to that observed in AGH3-2:GUS (Fig. 8B). However, in some AGH3-2:GUS ant-4 ail6-2 inflorescences, GUS staining was observed in a broader domain that extended deeper into the inflorescence meristem (Fig. 8, C and D). Differences in the expression of AGH3-2:GUS in the double mutant were more dramatic in flowers.
ers, GUS activity was not restricted to the tips of developing organs (Fig. 8, F, H, and J). GUS activity was often highest in the central provascular tissue of developing floral organs (Fig. 8H). These differences suggest that auxin responses, distribution, and/or levels are altered in ant-4 ail6-2 flowers. Ant-4 ail6-2 plants are responsive to exogenous auxin, as increased GUS expression was observed in AGH3-2:GUS ant-4 ail6-2 inflorescences treated with 50 μM indole-3-acetic acid (IAA; Fig. 8, K and L).

Auxin Transport Is Necessary for ANT Expression in Incipient Flowers

As ANT and AIL6 expression in incipient floral primordia (Nole-Wilson et al., 2005) correlates with auxin accumulation in these cells (Reinhardt et al., 2003; Heisler et al., 2005; Barbier de Reuille et al., 2006; Smith et al., 2006), I investigated whether auxin might play a role in transcriptional regulation of ANT. No differences in ANT mRNA levels were detected in Ler inflorescences collected 24 h after treatment with 50 μM IAA or 50 μM 2,4-dichlorophenoxyacetic acid as compared with a mock solution (Supplemental Fig. S4, A and B). In addition, no change in the spatial pattern of ANT expression, as assessed using an ANT:GUS reporter line, was observed (Supplemental Fig. S4, C–F). The promoter fragment used in this construct is sufficient to complement ant-4 when fused to the ANT coding sequence (Supplemental Fig. S5).

Because auxin may be necessary but not sufficient for ANT regulation, I treated inflorescences with the auxin transport inhibitor NPA. No difference in ANT mRNA levels was detected in Ler inflorescences treated with 100 μM NPA for 24 h as compared with mock-treated tissue (Fig. 9, A and B). However, ANT expression was reduced in incipient floral primordia and stage 1 and 2 flowers of NPA-treated ANT:GUS inflorescences (Fig. 9, C and D). No obvious changes in ANT expression were observed in older flowers. Sim-
ilar results were obtained by in situ hybridization examining ANT mRNA in Ler inflorescences treated with NPA (Fig. 9, E and F). Thus, auxin transport within the inflorescence meristem is necessary for high levels of ANT expression in the very earliest stages of flower development but not for ANT expression in older flowers. The observed reduction in ANT expression is observed prior to morphological changes in lateral organ initiation that occur as a consequence of NPA treatment (Fig. 9, G and H; Okada et al., 1991; Reinhardt et al., 2000). The inability to detect changes in ANT mRNA levels in the RT-PCR experiment likely results from the very small contribution of cells from early floral primordia to the entire inflorescence harvested for the RT-PCR experiment.

**DISCUSSION**

**ANT and AIL6 Regulate Flower Development**

ANT and AIL6 regulate several aspects of flower development, including floral meristem and organ growth, organ positioning within the floral meristem, and specification of floral organ identity. ant ail6 flowers contain fewer organs per flower than the wild type, as well as organs such as filaments and flat green organs that are not normally found in wild-type flowers. Thus, the absence of petals and stamens likely results from defects in both organ initiation and organ identity specification. Defects in organ initiation may be a consequence of insufficient cells within the floral meristem, while defects in organ identity specification likely result from altered expression of the floral organ identity genes AP3 and AG.

Similar LFY expression in ant ail6 and wild-type flowers of stages 1 and 2 suggests that ANT and AIL6 promote AP3 and AG expression through a LFY-independent pathway (Fig. 10). However, nonuniform LFY expression in stage 3 ant ail6 flowers suggests that ANT and AIL6 may also promote floral organ identity gene expression through the maintenance of LFY expression (Fig. 10). The expanded WUS expression domain in ant ail6 flowers suggests that WUS is not limiting for AG activation. I examined LFY, AP3, and AG regulatory regions (Blazquez et al., 1997; Hill et al., 1998; Tilly et al., 1998; Busch et al., 1999; Deyholos and Sieburth, 2000) for the presence of potential ANT-binding sites (Nole-Wilson and Krizek, 2000). One such site with two mismatches compared with the consensus site was identified in the LFY promoter (21,380 to 21,394 relative to the start codon), while no sequences with three or fewer mismatches were present within 800 bp of AP3 5′ sequence. This suggests that the regulation of AP3 by ANT and AIL6 may be indirect.

The presence of five potential ANT-binding sites, each containing three mismatches, within the AG second intron suggests that ANT and AIL6 may be direct regulators of AG expression. Previous work has shown that ANT acts as a repressor of AG expression, preventing precocious AG expression in flowers (Liu et al., 2000) and acting redundantly with AP2 in repression of AG in the second whorl (Krizek et al., 2000). The data presented here show that ANT and AIL6 act redundantly to negatively regulate AG expression in second whorl cells and to positively regulate AG expression in the centermost cells of the floral

![Figure 7](image)

**Figure 7.** Inflorescence meristems (IM) of ant-4 ail6-2 plants exhibit growth arrest and show altered expression of meristem regulators. A, Scanning electron micrograph of the apex of a 22-d-old Ler plant. B, Scanning electron micrograph of the apex of a 22-d-old ant-4 ail6-2 plant. C, Scanning electron micrograph of the apex of a 30-d-old Ler plant. D, Scanning electron micrograph of the apex of a 30-d-old ant-4 ail6-2 plant. E, CLV3 expression in a Ler inflorescence. F, CLV3 mRNA is detected in a broader domain corresponding to L1, L2, and L3 cells within the inflorescence meristem of ant-4 ail6-2 plants. G, WUS expression in a Ler inflorescence. H, WUS mRNA is detected in the uppermost cells of the inflorescence meristem in ant-4 ail6-2 plants. Bars = 100 μm in A to D and 50 μm in E to H. [See online article for color version of this figure.]
meristem (Fig. 10). Thus, the role of ANT and AIL6 in AG regulation appears to be context dependent and likely involves different coregulators.

ANT and AIL6 Are Required for the Proliferation and Maintenance of a Meristematic Cell Fate

In ag mutants, persistent WUS expression in stage 6 and older flowers results in floral indeterminacy (Lenhard et al., 2001; Lohmann et al., 2001). Additional regulators of floral determinacy have recently been identified and include perianthia (pan) and three enhancers of crabs claw (crc): rebelote (rbl), squint (sqn), and ultrapetala1 (ult1; Prunet et al., 2008; Das et al., 2009; Maier et al., 2009). In crc sqn and crc ult double mutants and in a dominant-negative pan allele, AG expression is reduced in the very center of stage 3 flowers, similar to what is seen in ant ail6 flowers (Prunet et al., 2008; Das et al., 2009). It has been proposed that this region corresponds to an inner fourth whorl subdomain in which meristematic potential can be maintained (Prunet et al., 2008). SQN, ULTI, and PAN promote AG expression in this subdomain, consequently resulting in WUS down-regulation and termination of stem cell fate (Prunet et al., 2008; Das et al., 2009; Maier et al., 2009).

ANT and AIL6 also appear to regulate AG expression in this inner fourth whorl domain, but unlike other genetic backgrounds in which persistent WUS expression results in indeterminacy, ant ail6 flowers are determinate. Furthermore, I have found that ANT and AIL6 activity is required for the indeterminacy of ag flowers. Thus, WUS itself is not sufficient for floral meristem indeterminacy but requires factors such as ANT and AIL6 that promote continued proliferation of stem cells. In the absence of ANT and AIL6, these cells do not proliferate to maintain a stem cell pool from which organ primordia can be initiated.

The eventual termination of ant ail6 inflorescence meristems also supports a role for ANT and AIL6 in...
stem cell proliferation. The basis for shoot apical meristem termination in ant ail6 plants is not clear. While the width of the inflorescence meristem gets progressively smaller during development, it does not appear that all meristematic cells are consumed in the process of lateral organ initiation. Rather, there appears to be an arrest of the shoot apex with the cessation of new floral initiation and growth abortion of young primordia. The expression patterns of CLV3 and WUS are altered in ant ail6 inflorescence meristems, with both genes expressed in a broader domain and some cells within the central part of the meristem expressing both CLV3 and WUS. The broader expression domains of these central zone markers might indicate a loss of peripheral zone identity and consequently an inability to initiate new primordia.

**ANT May Act Downstream of Auxin**

Roles for ANT and AIL6 in auxin regulation of flower development are suggested by the apparent overlap between transient auxin maxima within the inflorescence meristem (Reinhardt et al., 2003; Heisler et al., 2005; Barbier de Reuille et al., 2006; Smith et al., 2006) and the expression of ANT and AIL6 within incipient floral primordia (Nole-Wilson et al., 2005). In addition, the spatial expression pattern of ANT is altered in pin1 mutants, with ANT mRNA detected in a ring around the periphery of the naked shoot apex rather than in distinct groups of cells (Vernoux et al., 2000). This change in ANT expression could be a direct consequence of altered auxin accumulation patterns in pin1 mutants or an indirect consequence related to the inability of pin1 mutants to initiate flowers. Here, I show that ANT expression is reduced in incipient floral primordia and young flowers 24 h after NPA treatment. This change in expression occurs prior to any morphological change in the meristem, suggesting that auxin transport plays a relatively direct role in ANT regulation.

ant ail6 mutants are dwarfed, display altered organ growth, and have reduced amounts of vascular tissue, phenotypes similar to mutants defective in auxin physiology. Treatment of ant ail6 inflorescences with exogenous auxin showed that these plants could re-

![Figure 9](image)

**Figure 9.** ANT expression is responsive to NPA treatment. A and B, Semiquantitative RT-PCR of ANT and ACTIN (internal control) mRNA levels in inflorescences 24 h after mock (A) or NPA (B) treatment. C and D, ANT-GUS inflorescence stained for GUS 24 h after mock (C) or NPA (D) treatment. GUS staining is weaker in the incipient floral primordium (arrow) and stage 2 (st 2) flower of the NPA-treated inflorescence compared with the mock-treated inflorescence. E and F, ANT expression in Ler inflorescences fixed 24 h after mock (E) or NPA (F) treatment. ANT mRNA levels are reduced in the incipient floral primordium (arrow) and stage 2 flower of the NPA-treated inflorescence compared with the mock-treated inflorescence. G and H, Scanning electron micrographs of Ler inflorescences fixed 24 h after mock (G) or NPA (H) treatment. IM, Inflorescence meristem. Bars = 50 μm in C to F and 100 μm in G and H.

![Figure 10](image)

**Figure 10.** Model for AG regulation by ANT and AIL6. In a stage 3 flower, AG expression in the floral meristem (gray) is promoted by the combined action of LFY and WUS. ANT and AIL6 promote AG expression in the centermost part of the floral meristem via a pathway that is likely to be independent of the LFY-WUS pathway. This centermost region is outlined in dark gray. ANT and AIL6 may also indirectly promote AG expression via maintenance of LFY expression (dotted arrow). ANT and AIL6 repress AG expression in second whorl cells (bar). se, Sepal.
spond to auxin, suggesting that at least some primary auxin responses mediated by ARFs are functional in these plants. If ANT and AIL6 do function in auxin signaling, presumably they do so downstream of ARFs. Two potential AuxREs are present within the ANT promoter at positions −1,083 to −1,078 and −2,525 to −2,520 relative to the start codon. No sequences with similarity to AuxRE were identified in AIL6 regulatory regions. Altered expression of the auxin-responsive reporter AGH3-2:GUS in ant ail6 flowers may indicate defects in some auxin responses or alternatively that auxin levels and/or distribution are altered in ant ail6 inflorescences. Future experiments need to be conducted to investigate these possibilities. A role for ANT and AIL6 in mediating auxin distribution would not preclude a possible role for these proteins in signaling downstream of auxin. Feedback between the auxin distribution system and auxin signaling pathways allows the plant to coordinate developmental processes and respond to changing environmental conditions (Leyser, 2006; Benjamins and Scheres, 2008). PLT proteins in the root are thought to regulate developmental patterning in response to auxin as well as auxin distribution (Blilou et al., 2005; Benjamins and Scheres, 2008).

Parallels between AIL/PLT Function in Roots and Shoots

Members of the AIL/PLT gene family play important and somewhat similar roles in roots and shoots. While plt1 and plt2 single mutants show subtle root defects, plt1 plt2 double mutants exhibit reduced root growth, altered cellular patterning in the root tip, and termination of the root apical meristem by 6 to 8 d after germination (Aida et al., 2004). Thus, in both roots and shoots, AIL/PLT genes act redundantly to regulate organ growth, developmental patterning, and meristem maintenance. AIL6/PLT3 also acts redundantly with PLT1 and PLT2 in the root (Galinha et al., 2007), demonstrating that AIL6 is an important regulator of both root and shoot development.

PLT function has been proposed to promote root growth and patterning downstream of auxin. The highest accumulation of PLT1, PLT2, AIL6/PLT3, and BBM proteins in the root occurs in the stem cell niche, which corresponds to a stable auxin maximum (Galinha et al., 2007). PLT1 and PLT2 mRNA levels increase approximately 24 h after treatment of seedlings with auxin, and their expression is largely absent in embryos lacking functional copies of two ARFs (Aida et al., 2004). The work presented here suggests that ANT and AIL6 function downstream of auxin in flowers. Thus, members of the AIL/PLT gene family may represent components of a conserved mechanism by which auxin gradients are converted into growth and patterning outputs within both roots and shoots despite differences in the nature of the auxin maxima in roots and shoots.

MATERIALS AND METHODS

Plant Growth Conditions

Arabidopsis (Arabidopsis thaliana) plants were grown on a soil mixture of either Metro-Mix 360:perlite:vermiculite (5:1:1) or Miracle Gro Moisture Control Potting Mix:perlite:vermiculite (5:1:3) under continuous light or in 16-h days (100–150 μmol m−2 s−1) at a temperature of 22°C.

PCR Genotyping

DNA was isolated from leaves and used in PCR for genotyping (Klimyk et al., 1993). The AIL6 T-DNA alleles were PCR genotyped using a pair of gene-specific primers and a T-DNA primer. ant-4 contains a single T-to-A version that alters the donor splice site of the fourth intron (Nole-Wilson and Krizek, 2006). ant-4 was genotyped by Tetramer-primer ARMS-PCR using TPANT-1 (5′-GATGATCCTTCTTCTTAGTTCATCCGGA-3′), TPANT-2 (5′-TGATCACATGAGGATGTTGTACATA-3′), TPANT-3 (5′-GGGAC-AATGTTTCAGGAGAAAGGTCA-3′), and TPANT-4 (5′-CCGACGATGTCAC-3′); Ye et al., 2001). PCRs of 100 μL contained 100 pmol of TPANT-1 and TPANT-2 and 10 pmol of TPANT-3 and TPANT-4 and consisted of 35 cycles with the following conditions: 1 min at 95°C, 1 min at 55°C, and 1 min at 72°C followed by one cycle of 2 min at 72°C. The allele-specific PCR product for ant-4 is 199 bp, while that for the wild type is 230 bp.

In Situ Hybridization

Inflorescences were fixed, embedded, sectioned, hybridized, and washed as described previously (Krizek, 1999). Digoxigenin-labeled antisense RNA probes (AIL6, AP3, AG, LFY, WUS, and CLV3) were synthesized as described previously (Yanoisky et al., 1990; Jack et al., 1992; Weigel et al., 1992; Mayer et al., 1998; Fletcher et al., 1999; Nole-Wilson et al., 2005).

Scanning Electron Microscopy

Tissue for scanning electron microscopy was fixed, dried, dissected, and coated as described previously (Krizek, 1999). Scanning electron microscopy analyses were performed on either a FEI XL30 ESEM or a FEI Quanta 200 ESEM apparatus.

RNA Isolation and Gel-Blot Analyses

Inflorescences (corresponding to all unopened floral buds) were homogenized in liquid nitrogen, and total RNA was isolated with hot phenol extraction buffer (Verwoerd et al., 1989). Total RNA (10 μg) was separated on a 0.66% formaldehyde, 1% agarose gel, transferred to a Nytran SuperCharge membrane, and hybridized with RNA probes. [32P]UTP-labeled RNA probes were synthesized using the AP3 and AG linearized plasmids described above for in situ hybridization. The blots were scanned on a Molecular Dynamics Storm 860 PhosphorImager.

Semi-quantitative RT-PCR

Total RNA was treated for 4 to 6 h with RQ1 RNase-free DNase (Fisher Scientific) at 37°C. Approximately 5 μg of total RNA was reverse transcribed using the SuperScript III first-strand synthesis system (Invitrogen). PCR conditions were 40 cycles of 30 s at 92°C, 30 s at 50°C, and 2 min at 72°C and one cycle of 5 min at 72°C. Aliquots (5 μL) were removed at five-cycle intervals starting with cycle 20. Actin transcript levels confirmed that equivalent amounts of cDNA were used in the experiment.

Epidermal Peels, Plant Measurements, and Leaf Vascular Staining

The epidermis was removed from a midsection of the inflorescence stem of 5-week-old plants, placed on a drop of water on a glass slide, and stained briefly with toluidene blue. Plants used in height measurements and axillary inflorescence counts were germinated on plates and transplanted into soil at 7 d after germination. Plant height was measured and the number of axillary inflorescences from rosette and cauline leaves was counted when the plants

ANT and AIL6 Act Redundantly in Flower Development

were 6 weeks old. Vascular tissue was examined by fixing the sixth leaf of 25-d-old plants overnight at room temperature in a 3:1 solution of ethanol:acetic acid. The leaves were mounted in 70% ethanol and photographed using a dissecting microscope with illumination from below.

**GUS Staining**

The GUS assays were performed as described previously (Krizek and Meyerowitz, 1996) except that the tissue was incubated in 2 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid for 9 h (sectioned ANT/GUS and AGH3-2/GUS) or 22 h (whole mount WUS/GUS and AGH3-2/GUS) at 37°C. The tissue was either photographed as whole mounts or embedded in paraplast, sectioned, mounted on slides, and observed under bright-field illumination.

**AUXin and NPA Treatment of Inflorescences**

AGH3-2:GUS and AGH3-2 ant-4 ail6-2 inflorescences were treated with 50 μM IAA (in 0.05% methanol and 0.01% Silwet L-77) or a mock solution (0.05% methanol and 0.01% Silwet L-77) using a paintbrush, harvested 6 h after treatment, and stained for GUS. Similar treatments were conducted with 50 μM IAA (in 0.05% methanol and 0.01% Silwet L-77) or a mock solution (0.05% methanol and 0.01% Silwet L-77) and harvested 24 h after treatment. For the NPA experiment, the tissue was treated twice (at 0 and 7 h), as described previously (Niemhauser et al., 2000), with 100 μM NPA (in 0.1% dimethyl sulfoxide and 0.01% Silwet L-77) or a mock solution (0.1% dimethyl sulfoxide and 0.01% Silwet L-77) and collected 24 h after the initial treatment.

**Plasmid Construction**

For construction of the ANT/GUS plasmid, GUS and the 3′ nos terminator sequence were subcloned from pBl121 into pBluescript SK– using BamHI and EcoRI and subsequently into pCGN1547 using BamHI and HindIII. A 6.2-kb region of the ANT sequence 5′ to the start codon was constructed in pBluescript SK– by first subcloning a 5-kb ANT genomic fragment followed by the addition of a 1.2-kb PCR product. The entire 6.2-kb ANT promoter sequence was subsequently subcloned upstream of GUS in pCGN1547 using KpnI. ANT: GUS/pCGN1547 was transformed into Agrobacterium tumefaciens strain ASE by electroporation. Arabidopsis Lr plants were transformed with this Agrobacterium strain by vacuum infiltration (Bechtold et al., 1993). Transformants were selected for kanamycin resistance.

The Arabidopsis Genome Initiative locus numbers for the genes analyzed in this article are as follows: ANT, At5g10510; AIL6, At5g10510; AIL7, At5g65510.

**Supplemental Data**

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

Supplemental Figure S1. ant aild flowers in mixed genetic backgrounds.

Supplemental Figure S2. LFY mRNA levels are not changed in ant-4 aild-2 inflorescences.

Supplemental Figure S3. ant-4 aild-2 leaves have altered leaf vein architecture.

Supplemental Figure S4. ANT expression is not altered after auxin treatment of inflorescences.

Supplemental Figure S5. ANTANT complements ant-4.

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