Arabidopsis Genes *AS1*, *AS2*, and *JAG* Negatively Regulate Boundary-Specifying Genes to Promote Sepal and Petal Development[1][W]

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Boundary formation is crucial for organ development in multicellular eukaryotes. In higher plants, boundaries that separate the organ primordia from their surroundings have relatively low rates of cell proliferation. This cellular feature is regulated by the actions of certain boundary-specifying genes, whose ectopic expression in organs can cause inhibition of organ growth. Here, we show that the *Arabidopsis thaliana ASYMMETRIC LEAVES1* and *2* (*AS1* and *AS2*) and *JAGGED* (*JAG*) genes function in the sepal and petal primordia to repress boundary-specifying genes for normal development of the organs. Loss-of-function *as1 jag* and *as2 jag* double mutants produced extremely tiny sepals and petals. Analysis of a cell-cycle marker *HISTONE4* revealed that cell division in sepal primordia of the double mutant was inhibited. Moreover, these abnormal sepals and petals exhibited ectopic overexpression of the boundary-specifying genes *PETAL LOSS* (*PTL*) and *CUP-SHAPED COTYLEDONS1* and *2* (*CUC1* and *CUC2*). Loss of *PTL* or *CUC1* and *CUC2* functions in the *as1 jag* background could partially rescue the tiny sepal and petal phenotypes, supporting the model that the tiny sepal/petal phenotypes are caused, at least in part, by ectopic expression of boundary-specifying genes. Together, our data reveal a previously unrecognized fundamental regulation by which *AS1*, *AS2*, and *JAG* act to define sepal and petal from their boundaries.

In higher plants, formation of organ primordia requires creation of boundaries that separate an organ primordium from surrounding tissues. Morphological boundaries between the meristem and organs are termed M-O boundaries, whereas those between adjacent organs are called O-O boundaries (Aida and Tasaka, 2006). It was noted that the rate of cell proliferation was decreased in the boundary tissues (Hussey, 1971), within which DNA synthesis and expression of cell-cycle-related genes both became undetectable (Breuil-Broyer et al., 2004). In Arabidopsis (*Arabidopsis thaliana*), several genes are known to be involved in boundary specification, including *CUP-SHAPED COTYLEDONS1* and *2* (*CUC1* and *CUC2*) for both M-O and O-O boundaries and *PETAL LOSS* (*PTL*) and *RABBIT EARS* (*RBE*) for O-O boundaries (Aida et al., 1997; Griffith et al., 1999; Takeda et al., 2003; Brewer et al., 2004; Aida and Tasaka, 2006; Krizek et al., 2006). *CUC1*, *CUC2*, and an additional *CUC* gene, *CUC3*, encode members in the NAC family of putative transcription factors. Loss-of-function mutations in any two of these three *CUC* genes resulted in plants with loss of embryonic shoot apical meristem (SAM) and fused cotyledons (Aida et al., 1997; Vroemen et al., 2003; Hibara et al., 2006).

Previous studies have shown that these boundary-specifying genes all affect flower development. The flower contains different types of organs, forming concentric whorls within a flower. In Arabidopsis, the wild-type flower consists of four whorls: four sepals in the first whorl, four petals in the second whorl, six stamens in the third whorl, and two fused carpels in the fourth whorl (Bowman et al., 1989). *CUC1*and *CUC2* transcripts were detected in boundaries between each type of floral organ during flower development (Ishida et al., 2000; Takada et al., 2001). *PTL* encodes a plant-specific trihelix transcription factor. In addition to its functions for sepal and petal architecture, *PTL* is also important in boundary formation to separate sepals (Brewer et al., 2004). Flowers of loss-of-function *ptl* mutant plants showed fusions of some adjacent sepals, whereas *ptl cuc1* and *ptl cuc2* double-mutant plants both exhibited the more severe sepal-fusion phenotype than *ptl* (Brewer et al., 2004). *RBE* encodes a zinc-finger transcription factor, playing a role in the regulation of petal development (Takeda et al., 2003). In addition,

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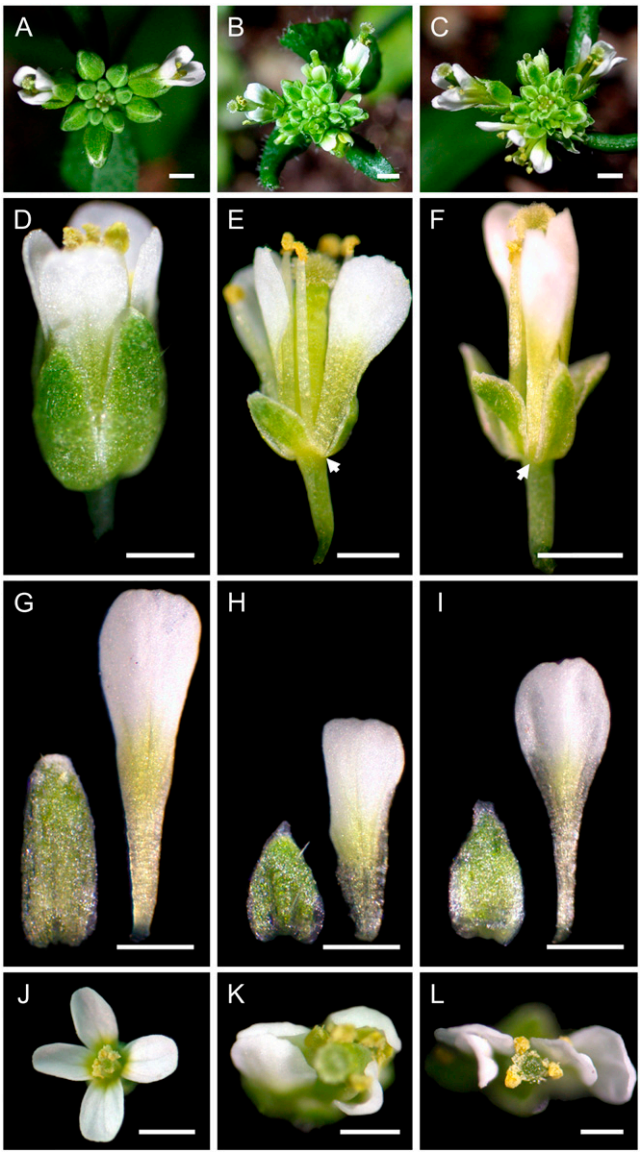

Figure 1. *AS1* and *AS2* are required for sepal and petal sizes. A to C, Inflorescences of wild-type L*er* (A), *as1-101* (B), and *as2-101* (C). D to F, Floral phenotypes of wild-type L*er* (D), *as1-101* (E), and *as2-101* (F). Arrows indicate an open space between two adjacent sepals. G to I, Sepal and petal sizes of wild-type (G), *as1-101* (H), and *as2-101* (I) flowers. J to L, Top view of flowers from wild-type L*er* (J), *as1-101* (K), and *as2-101* (L). In wild-type plants, the concave adaxial surface of petals usually faces inward, but in *as1-101* and *as2-101* mutant flowers it faced other directions. Bars = 1 mm (A–L).

RBE is also required for boundary formation between adjacent sepals (Krizek et al., 2006). Although these genes are important in boundary establishment, little is known about their regulation during floral organ development. Here, we show that *AS1*, *AS2*, and *JAG* are critical in repressing *PTL*, *CUC1*, and *CUC2* in sepal and petal primordia for their normal growth.

as1 and *as2* mutants have overall similar phenotypes with both abnormal leaves and floral organs (Ori et al., 2000; Sun et al., 2000, 2002; Semiarti et al., 2001; Byrne et al., 2002). *AS1* encodes an R2-R3 MYB-domain protein (Byrne et al., 2000), whereas *AS2* encodes a plant-specific lateral organ boundary (LOB) domain-containing transcription factor (Iwakawa et al., 2002) that associates with AS1 (Xu et al., 2003). In leaves, *AS1* and *AS2* are known to be required for repression of meristematic genes and for leaf adaxial-abaxial polarity establishment, whereas their roles in flower development are largely unknown. *JAG* encodes a C_2H_2 transcription factor and promotes morphogenesis in multiple types of lateral organs (Dinneny et al., 2004; Ohno et al., 2004). However, targets regulated by *JAG* during flower development still remain elusive. In this article, we present evidence that *AS1*/*AS2* and *JAG* act in parallel to repress boundary-specifying genes in sepals and petals to define these organs from their boundaries.

RESULTS

AS1 and *AS2* Play Important Roles in Sepal and Petal Growth

Flowers of *as1* and *as2* exhibit several defects (Ori et al., 2000; Byrne et al., 2002; Sun et al., 2002). We provide a more detailed description of their floral phenotypes here. Compared with the inflorescence of wild-type Landsberg *erecta* (L*er*) plants (Fig. 1A), those from *as1-101* (Fig. 1B) and *as2-101* (Fig. 1C) produced floral buds that opened prematurely, consistent with previously reported *as1-1* and *as2-2* phenotypes (Ori et al., 2000; Byrne et al., 2002). In comparison to the size of sepals and petals in wild-type plants (Fig. 1, D and G), those from *as1-101* (Fig. 1, E and H) and *as2-101* (Fig. 1, F and I) were notably reduced, resulting in an open space between adjacent sepals (arrows). This sepal phenotype appeared more severe than those observed in previously reported *as1-1* and *as2-2* flowers (Ori et al., 2000; Byrne et al., 2002). In addition, orientation of petals within the mutant flowers was abnormal. In wild-type plants, the concave adaxial surface of petals usually faces inward (Fig. 1J), but in *as1-101* (Fig. 1K) and *as2-101* (Fig. 1L) it faced other directions instead. Because petals of *as1* and *as2* mutants do not twist (Fig. 1, H and I), this aberrant petal phenotype may result from misorientation of the petal primordium. Whereas sizes of sepals and petals were reduced in *as1* and *as2* flowers, stamens and carpels of mutants appeared relatively normal, indicating that *AS1* and *AS2* are important for sepal and petal growth.

Previous studies showed that *AS1* is expressed throughout primordia of all floral organs (Byrne et al., 2000), whereas the *AS2* expression pattern in the Arabidopsis flower was not reported. We thus examined *AS2* expression patterns by in situ hybridization. *AS2* was expressed in the central part of the inflorescence meristem, highly concentrated in the L1 and L2 layers of cells, but not in the floral meristem (Fig. 2A). Interestingly, *AS2* exhibited a polar expression pattern in

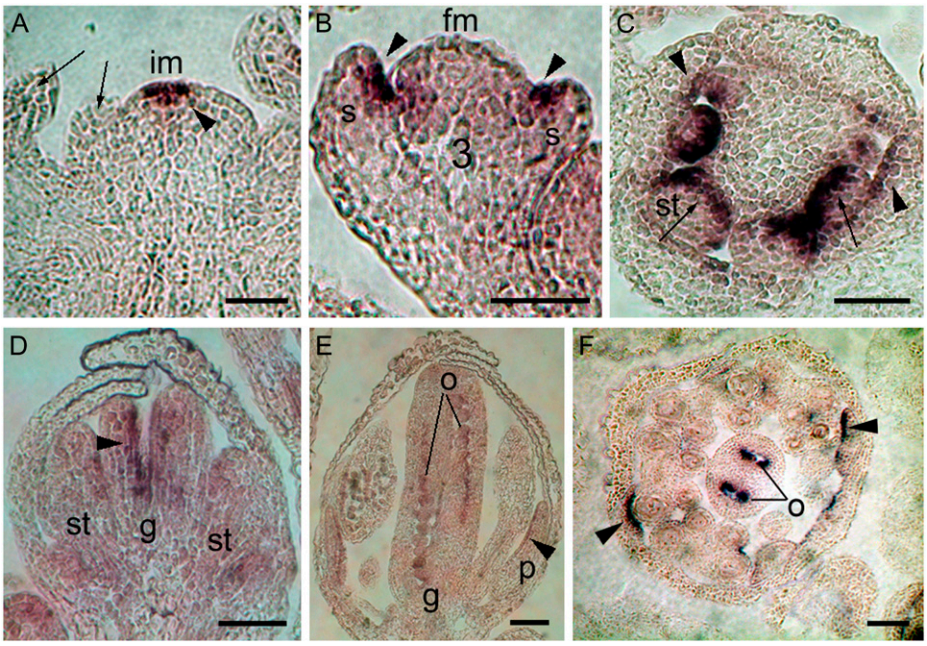

Figure 2. In situ hybridization showing *AS2* expression patterns in wild-type flowers. A, *AS2* transcripts were detected in the L1 and L2 layers in the central part of the inflorescence meristem (arrowhead), whereas they were absent in floral meristem (arrows). B and C, *AS2* was expressed in the adaxial side of sepals (B and C, arrowheads) and stamens (C, arrows). The number shows flower stage. D, In gynoecia, *AS2* mRNA was detected more abundantly in the adaxial side (arrowhead), but was also found in other gynoecial parts. E and F, In nearly mature flowers, *AS2* transcripts were found in ovules and the adaxial side of petals (arrowheads). im, Inflorescence meristem; fm, floral meristem; s, sepal; p, petal; st, stamen; g, gynoecium; and o, ovule. Bars = 20 μm (A–F).

floral organs. *AS2* hybridization signals were detected in the adaxial side of sepals in stage 3 flowers (Fig. 2B). In stamens, *AS2* transcripts were initially concentrated in the adaxial side of stage 6 flowers (Fig. 2C) and then became detectable from other parts of the stamens in stage 8 flowers (Fig. 2D). In the gynoecium, *AS2* was expressed more abundantly in the adaxial side (Fig. 2D). In stage 9 to 11 flowers, *AS2* signals appeared in the ovules (Fig. 2E) and the adaxial side of petals (Fig. 2, E and F, arrowheads). The *AS2* expression pattern suggests that the function of the AS1 and AS2 complex is likely regulated spatially and temporally during flower development.

as1 jag and *as2 jag* Flowers Have Dramatically Reduced Sepal and Petal Sizes

To investigate how *AS1* and *AS2* function in flower development and to explore possible functional redundancy between *AS1* and *AS2* and other flower genes, we constructed double mutants with *as1-101* or *as2-101* and one of several floral mutants. *as1-101 jag-2* and *as2-101 jag-2* double mutants were dramatically affected in sepal and petal morphologies compared with those in wild-type (Fig. 1D), *as1-101* (Fig. 1E), *as2-101* (Fig. 1F), and *jag-2* flowers (Fig. 3A; Ohno et al., 2004). All *as1-101 jag-2* and *as2-101 jag-2* flowers produced very tiny structures in the outer two whorls (Fig. 3, B and C, arrows). We examined more than 500 flowers from each of the two double mutants and the phenotype was very consistent (without any exception). Although stamens and carpels of the double mutants appeared morphologically normal, fertility of the double-mutant flowers was severely reduced due to both male and female defects (data not shown).

To determine the earliest time when the double-mutant phenotypes occur, we analyzed floral phenotypes by using scanning electron microscopy (SEM). Because phenotypes of *as1-101 jag-2* and *as2-101 jag-2* double mutants were similar, we describe here only the *as1-101 jag-2* phenotypes. SEM revealed no difference in the shape or size of the inflorescence meristem and young flowers before stage 5 between wild type (Fig. 3D) and *as1-101 jag-2* (Fig. 3E). At stage 5, sepals of wild-type flowers extended their growth and two opposite ones overlap to cover the interior floral organs (Fig. 3D). However, sepal growth in *as1-101 jag-2* was arrested at and after this stage (Fig. 3E). During subsequent flower development, wild-type sepals and petals grew along with the inner whorl organs (Fig. 3F). In contrast, growth of the *as1-101 jag-2* sepals and petals was severely affected after stage 5, even though stamens and carpels in the same mutant flower continued to grow (Fig. 3G).

Most organ primordia in the first and second whorls of *as1-101 jag-2* flowers developed to filamentous structures (Fig. 3H). Occasionally, a few filament-like structures became thicker and were capped with stigmatic papillae (Fig. 3I). To learn more about the nature of the filamentous organs in *as1-101 jag-2* mutant flowers, we examined their surface characteristics using SEM. For the wild-type sepal, the relatively uniformly sized epidermal cells from the distal part have cuticular ridges on the surface (Fig. 3J). Similarly, some *as1-101 jag-2* filaments in the first whorl also had cell surfaces with cuticular ridges (Fig. 3K), although they were varied in cell size. In the *jag* single-mutant plant, surface cell characteristics of the distal part of petals resembled those of the proximal part of wild-type petals (Dinneny et al., 2004, 2006; Ohno et al.,

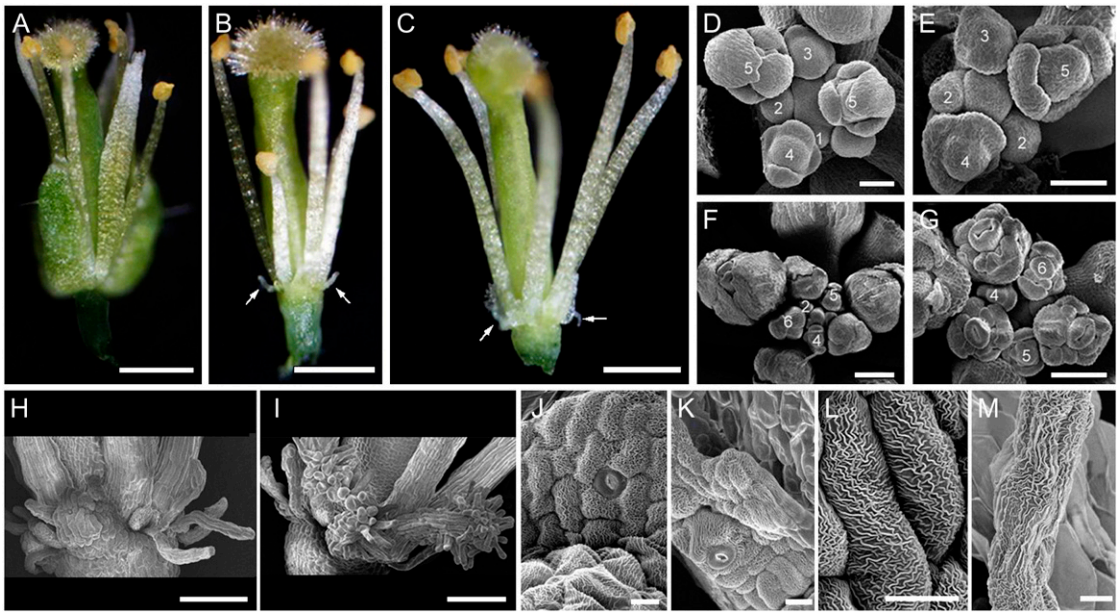

Figure 3. *AS1*, *AS2*, and *JAG* regulate sepal and petal development. A to C, Floral phenotypes of *jag-2* (A), *as1-101 jag-2* (B), and *as2-101 jag-2* (C). Sepals and petals in the double-mutant flowers were transformed to very small filamentous structures. D and E, Early flower development in wild-type (D) and *as1-101 jag-2* (E), showing that morphology of *as1-101 jag-2* flowers before stage 5 was nearly normal. F and G, Flowers of wild type (F) and *as1-101 jag-2* (G) became different after stage 5, with perianth growth in *as1-101 jag-2* being arrested. H and I, In the outer two whorls of the *as1-101 jag-2* flowers, only filamentous structures were seen (H, arrowheads) and, occasionally, some of these filaments capped with stigmatic papillae (I, arrow). J to M, Analyses of the abaxial side of sepals and petals revealed that the filamentous structures in the double mutants have the sepal or petal characteristics. J, Cells on the top part of the wild-type sepal. K, Cells on the top part of some double-mutant filaments, which showed similar cell surfaces to those of the wild-type sepal. L, Cells on the proximal part of the wild-type petal. M, Cells on the top part of some other double-mutant filaments, which were similar to the wild-type cells in L. Numbers indicate flower stages. Bars = 1 mm (A–C), 50 μm (D and E), 100 μm (F–I), and 10 μm (J–L).

2004). Interestingly, cell surface characteristics of the proximal part of wild-type petals (Fig. 3L) and those from tips of the *as1-101 jag-2* filaments in the second whorl (Fig. 3M) were also similar.

To investigate whether the reduced sepal size involved a decrease in cell division, we analyzed the expression of a specific cell division marker, *HISTONE4* (*H4*), whose expression represents cell cycle activity in developing organs (Krizek, 1999). Compared to wild-type stage 6 flowers (Fig. 4A), numbers of cells expressing *H4* were reduced in both *as1-101* and *jag-2* sepals (Fig. 4, B and C). Furthermore, cells with *H4* expression in the *as1-101 jag-2* sepals were even more decreased (Fig. 4D). These phenotypes were consistent in stage 5 and 6 flowers (Table I). These results indicate that the cell division defect in the double mutant is likely to be an important factor affecting sepal size.

Ectopic Expression of Boundary-Specifying Genes in *as1-101 jag-2* Flowers

It was reported previously that transgenic lines with ectopic expression of *PTL*, driven by *AP3* promoters, produced arrested floral organ primordia and filamentous floral organs instead of petals and stamens (Brewer et al., 2004). However, it is unknown which genes normally regulate *PTL* expression. Phenotypes of the *as1-101 jag-2* double mutant suggested that the double mutant might ectopically express *PTL* and AS1 and JAG might normally repress *PTL* expression. To test these hypotheses, we analyzed *PTL* expression by in situ hybridization, first examining transverse sections. *PTL* expression patterns in wild-type (Fig. 5A) and *as1-101 jag-2* (Fig. 5B) flowers before stage 5 were similar, with hybridization signals detected between adjacent sepals. In stage 7 or 8 flowers, *PTL* signals were detected only in sepal margins of wild-type flowers (Fig. 5C), but were strong in the interior of the *as1-101 jag-2* sepals. Interestingly, *PTL* transcripts were generally found to be concentrated in the adaxial side of the sepal (Fig. 5D).

Because *PTL* has a boundary-specifying function that is required for sepal separation during flower development (Griffith et al., 1999; Brewer et al., 2004), we decided to test whether other boundary genes, including *CUC1*, *CUC2*, and *RBE*, are also affected in the *as1-101 jag-2* sepals. Compared with the wild-type flowers (Fig. 5, E and G), *as1-101 jag-2* mutant flowers exhibited ectopic expression of *CUC1* (Fig. 5F) and *CUC2* (Fig. 5H) in the sepal. Ectopic *CUC1* expression was found predominantly in the adaxial side of sepals (Fig. 5F) and *CUC2* hybridization signals were detected

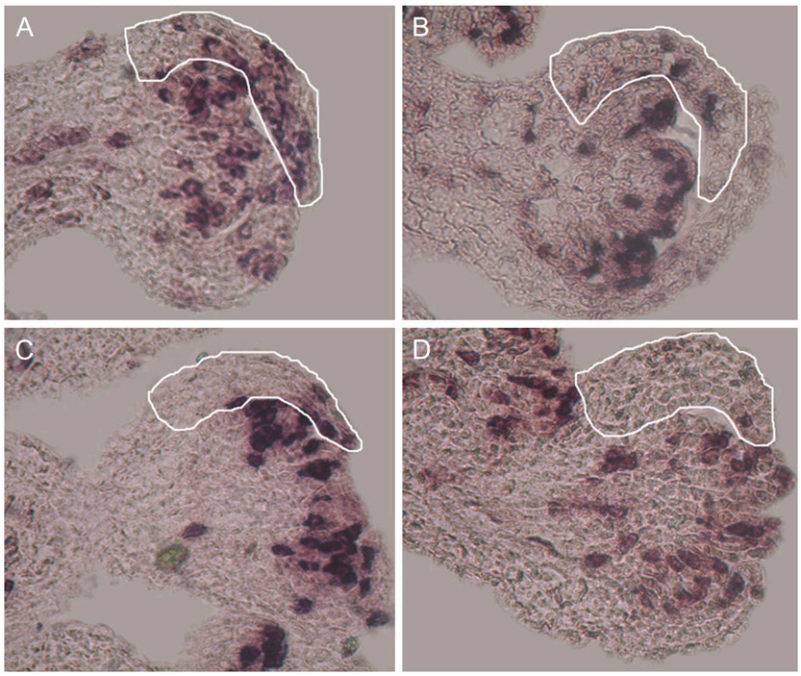

Figure 4. *AS1* and *JAG* promote cell division in sepals. A to D, In situ hybridization to examine *H4* expression in stage 6 sepals of wild-type (A), *as1-101* (B), *jag-2* (C), and *as1-101 jag-2* (D) flowers. Sepals are highlighted by white frames.

throughout the sepal (Fig. 5H). In contrast, *RBE* expression in floral organs could be detected only in flowers before stage 5, with similar patterns between wild-type (Fig. 5I) and *as1-101 jag-2* (Fig. 5J) flowers.

To confirm that *PTL*, *CUC1*, and *CUC2* are abnormally expressed in sepals of the double mutant and to examine whether their expressions are also altered in petals, we further analyzed longitudinal sections of flowers. Compared with wild-type plants (Fig. 5, K, M, and O), the *as1-101 jag-2* double mutant clearly had overexpression of *PTL*, *CUC1*, and *CUC2* in the sepal (Fig. 5, L, N, and P). In addition, these three genes were only weakly expressed in wild-type petal primordia (Fig. 5, K, M, and O), but were more robustly expressed in petals of double-mutant flowers (Fig. 5, L, N, and P), with *CUC2* showing the highest level (Fig. 5P). We also analyzed *PTL*, *CUC1*, and *CUC2* expression in the *as1-101* and *jag-2* flowers. However, the difference in gene expression between wild type and single mutants was not evident (Supplemental Fig. S1). These results support the hypothesis that AS1, AS2, and JAG are negative regulators of the expression of the boundary-specifying genes *PTL*, *CUC1*, and *CUC2* during normal sepal and petal growth.

Partial Restoration of Sepals and Petals in *as1 jag ptl* and *as1 jag cuc1 cuc2*

To further test whether ectopic expression of *PTL*, *CUC1*, and *CUC2* was responsible for reduced growth of the *as1-101 jag-2* sepals and petals, we constructed the *as1 jag ptl* triple mutant and the *as1 jag cuc1 cuc2* quadruple mutant. It was reported that the L*er* background has a dominant modifier that boosts the number of petals in the *ptl* flower (Griffith et al., 1999). To avoid the L*er* background, we used *as1-1* (Columbia-0 [Col-0]), *jag-1* (Wassilewskija [Ws]), and *ptl-1* (Col-0) to construct the *as1-1 jag-1 ptl-1* triple mutant. Compared to the wild-type Col-0 flower (Fig. 6A), *as1-1* (Fig. 6B) and *jag-1* (Fig. 6C) produced flowers with defects similar to those in the *as1-101* (Fig. 1E) and *jag-2* (Fig. 3A) mutants, respectively, with reduced sizes of sepals and petals. Similarly, the overall flower architecture of *as1-1 jag-1* (Fig. 6D) resembled that of *as1-101 jag-2*

Table I. *Cell numbers in Ler, as1-101, jag-2, and as1-101 jag-2 sepals that expressed H4*[a]

Genotype	L*er*	*as1-101*	*jag-2*	*as1-101 jag-2*
Stage 5 flowers ($n = 4$)[b]	9.0 ± 0.8	6.8 ± 1.3	4.3 ± 1.0	2.3 ± 1.0
Stage 6 flowers ($n = 10$)[b]	10.5 ± 2.2	6.6 ± 1.6	4.6 ± 1.4	2.2 ± 0.8

[a]Values are mean ± SE. A total of four and 10 sepals from stage 5 and 6 wild-type and mutant flowers, respectively, were analyzed. Whole inflorescences with small floral buds were longitudinally sectioned. *H4*-expressing cells in medial-longitudinal sections of sepal primordia of about stage 5 and 6 flowers were counted. An average was determined from the respective stage 5 and 6 *H4*-expressing cells. [b]$P < 0.05$ for both stage 5 and 6 groups.

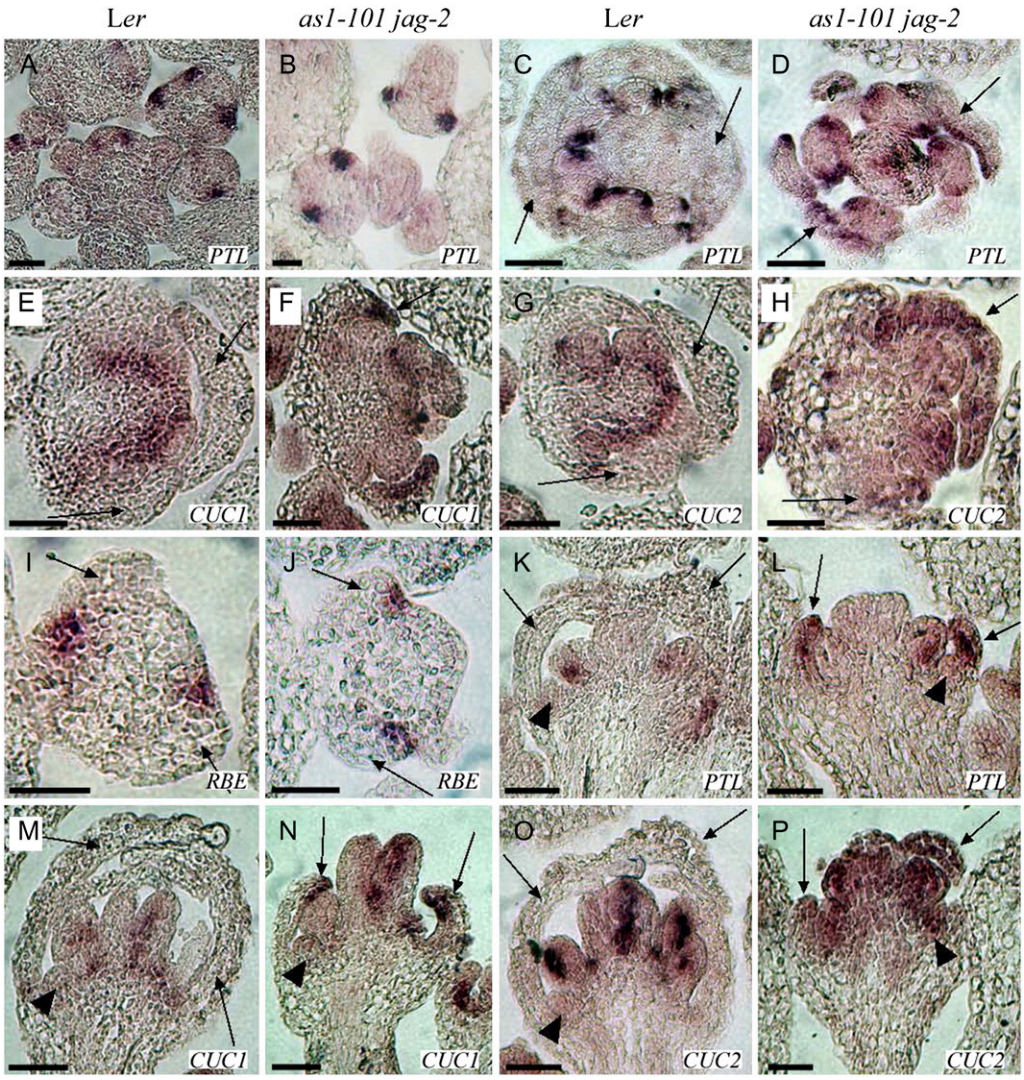

Figure 5. Altered expression of *PTL*, *CUC1*, and *CUC2* in *as1-101 jag-2* flowers. A to J, Transverse sections. A and B, Expression of *PTL* in early wild-type (A) and *as1-101 jag-2* (B) flowers, showing no obvious differences in expression patterns. C and D, *PTL* expression in wild-type (C) and *as1-101 jag-2* (D) flowers, with *PTL* ectopically expressed in the adaxial side of *as1-101 jag-2* sepals. E and F, *CUC1* expression in wild-type (E) and *as1-101 jag-2* (F) flowers. G and H, *CUC2* expression in wild-type (G) and *as1-101 jag-2* (H). Note that *CUC1* and *CUC2* were both ectopically expressed in sepals of the *as1-101 jag-2* flowers. I and J, *RBE* expression in wild-type (I) and *as1-101 jag-2* (J) flowers. There was no obvious difference in the *RBE* expression pattern between wild-type and *as1-101 jag-2* flowers before stage 5. K to P, Longitudinal sections. K and L, *PTL* expression in wild-type (K) and *as1-101 jag-2* (L) flowers. M and N, *CUC1* expression in wild-type (M) and *as1-101 jag-2* (N) flowers. O and P, *CUC2* expression in wild-type (O) and *as1-101 jag-2* (P) flowers. Arrows show sepals and arrowheads indicate petals. Specimens in C to J and K to P were from about stage 7 or 8 flowers. Note that sense probes of *PTL*, *CUC1*, *CUC2*, and *RBE* did not result in hybridization signals. Bars = 20 μm (A–P).

(Fig. 3B), with all flowers only producing tiny sepals and petals. The major defects of *ptl-1* single-mutant flowers were in the second whorl, with either reduced petal numbers or variable petal orientations (Fig. 6E; Griffith et al., 1999). Flowers of *as1-1 ptl-1* and *jag-1 ptl-1* double mutants showed additive phenotypes of the single mutants. For example, the aberrant petal orientation in *as1-1 ptl1-1* became more severe, with some petals facing outward (Fig. 6F, arrow). In addition, sepals in *jag-1 ptl-1* were often narrow (Fig. 6G). In contrast, a few partially expanded sepals and petals, which showed an expanded structure, were noted in early-appearing flowers of *as1-1 jag-1 ptl-1* (Fig. 6H; 7.9 $\pm$ 1.0, first 10 flowers of 10 independent triple-mutant plants), although the frequency of such partially rescued flowers was reduced in later flowers.

cuc1 cuc2 double-mutant plants lack the embryonic SAM and produce fused cotyledons (Aida et al., 1997). However, the *as1* mutation can rescue the *cuc1-1 cuc2-1* phenotypes, resulting in partially separated cotyledons

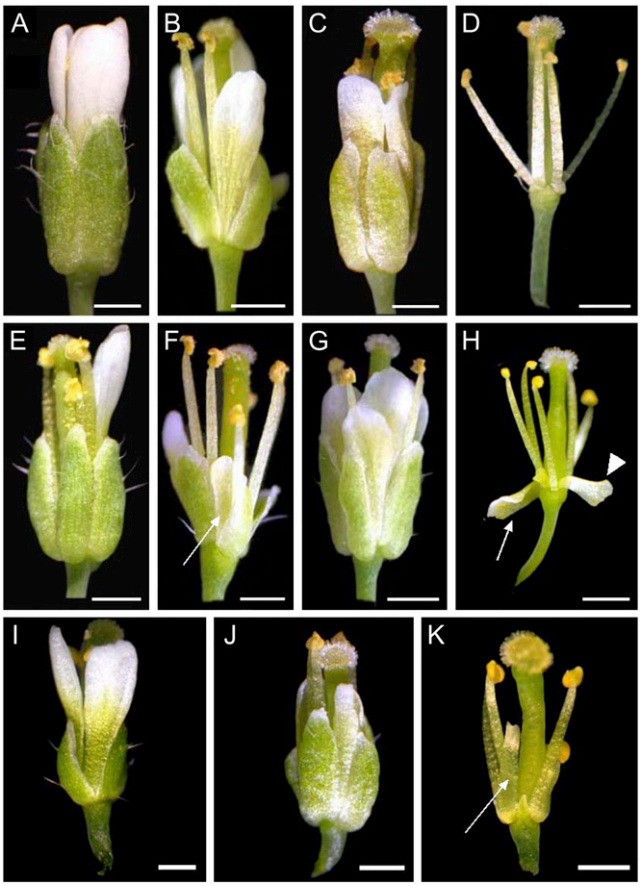

Figure 6. *ptl* mutation or *cuc1 cuc2* mutations partially rescue the *as1 jag* phenotypes. A to D, Floral phenotypes of wild type Col-0 (A), *as1-1* (B), *jag-1* (C), and *as1-1 jag-1* (D). Note that *as1-1 jag-1* and *as1-101 jag-2* have similar floral phenotypes with very small outer whorl organs (for comparison, see Fig. 2B). E to H, Floral phenotypes of *ptl-1* (E), *as1-1 ptl-1* (F), *jag-1 ptl-1* (G), and *as1-1 jag-1 ptl-1* (H). Note that the aberrant petal orientation in *as1-1 ptl1-1* (F) became more severe, with some petals facing outward (arrow), and the triple-mutant flowers (H) showed some partially expanded sepals (arrow) and petals (arrowhead). I to K, Floral phenotypes of *as1-101 cuc1-1 cuc2-1* (I), *jag-2 cuc1-1 cuc2-1* (J), and *as1-101 jag-2 cuc1-1 cuc2-1* (K). Note that the quadruple-mutant flower showed some partially rescued sepals (arrow) and all plants were grown under the same conditions. Bars = 1 mm (A–K).

(Hibara et al., 2003), suggesting that the *as1* defect might allow other genes to be expressed and partially substitute for the *CUC1* and *CUC2* function. Under our conditions, a few *as1-101 cuc1-1 cuc2-1* plants even produced flowers (Fig. 6I). Notably, *jag-2 cuc1-1 cuc2-1* plants also had less severe phenotypes compared with the *cuc1-1 cuc2-1* double mutant, sometimes producing flowers (Fig. 6J). Different from the *as1-101 cuc1-1 cuc2-1* and *jag-2 cuc1-1 cuc2-1* triple mutants, all *as1-101 jag-2 cuc1-1 cuc2-1* quadruple mutant plants were able to flower. Compared with the *as1-101 jag-2* double mutant (Fig. 3B), increased growth of sepals that had a flat structure was observed in early-appearing flowers of *as1-101 jag-2 cuc1-1 cuc2-1* (Fig. 6K; 5.5 ± 1.3, first 10 flowers of 10 independent quadruple-mutant plants). These observations suggest that the ectopically expressed boundary-specifying genes *PTL* and *CUC1/CUC2* are responsible for the sepal and petal defects in the *as1 jag* and *as2 jag* flowers.

DISCUSSION

In this study, we found that *as1 jag* and *as2 jag* double mutants had dramatically reduced growth of the floral organ sepals and petals. We further showed that *as1 jag* double-mutant flowers exhibited ectopic expression of the boundary-specifying genes *PTL*, *CUC1*, and *CUC2*. This is consistent with the previous findings that transgenic plants with ectopic expression of *PTL* or microRNA164-resistant *CUC1* and *CUC2* repressed sepal growth (Brewer et al., 2004; Mallory et al., 2004; Baker et al., 2005; Sieber et al., 2007). Our finding that *as1 jag ptl* and *as1 jag cuc1 cuc2* both had weaker phenotypes than *as1 jag* indicates that the reduced floral organ growth is at least in part due to the ectopic expression of the *PTL*, *CUC1*, and *CUC2* genes. All these results suggest that *AS1*, *AS2*, and *JAG* support normal sepal and petal growth by restricting the expression domain of boundary-specifying genes. Interestingly, although the *CUC1* and *CUC2* genes are known to be required for stamen separation and *AS1*, *AS2*, and *JAG* are also expressed in the inner two whorl organs, stamens and carpels in *as1 jag* and *as2 jag* were less affected. We propose that the reproductive organs stamen and carpel may differ from the sepal and petal; they might use different mechanisms in repressing boundary genes or have more redundant repressors during organ separations. We also tested whether *RBE* is a target of *AS1/AS2* and *JAG* by construction of the *as1 jag rbe* triple mutant. However, *as1 jag rbe* did not show the restored sepal and petal (data not shown), indicating that *RBE* may not be a target of *AS1*, *AS2*, and *JAG*.

AS2 belongs to the *LOB* gene family. It was previously reported that the *LOB* gene, the founding member in the family, is expressed in boundaries of all lateral organs and at the base of lateral roots (Shuai et al., 2002), implying that *LOB* may be potentially involved in boundary formation. Although the *lob* mutant does not show obvious phenotypic abnormalities, further studies of functional redundancy in the *LOB* family would be of great interest because these may uncover new regulatory mechanisms of the family.

Organ primordia in the outer two whorls of *as1-101 jag-2* flowers became filamentous structures and a few of these structures showed some carpel features, capped with stigmatic papillae. We propose that this phenotype may be caused by ectopic *PTL* expression. This phenotype resembles that of some transgenic plants that ectopically expressed *PTL*, driven by an *APETALA1* (*AP1*) promoter (Brewer et al., 2004), whose expression is known to be limited in sepals and petals in stage 3 to 5 flowers (Mandel et al., 1992). This phenotype also suggests that *as1 jag* and *as2 jag* mutations not only

affect expression of *PTL*, *CUC1*, and *CUC2*, but also many other genes, either directly or indirectly. This is consistent with the observation that *as1 jag ptl* and *as1 jag cuc1 cuc2* mutants both only partially rescued the *as1 jag* phenotypes.

Lateral organ development is generally considered to be along three axes: the proximo-distal, mediolateral, and adaxial-abaxial axes (Hudson, 2000). *AS1* and *AS2* are important regulators of leaf adaxial-abaxial polarity. Recent results revealed that *JAG* was also involved in leaf adaxial-abaxial polarity formation because double-mutant plants with loss of functions in *JAG* and its homolog *NUBBIN* showed the leaf adaxial-abaxial polarity defect (Dinneny et al., 2006). Because floral organs are thought to be modified leaves, it is possible that AS1 and AS2 also affect the adaxial-abaxial polarity in sepals and petals during their growth. *AS2* is predominantly expressed in the adaxial sides of young floral organs, similar to its expression pattern in cotyledons (Iwakawa et al., 2002) and overexpression of *AS2* could result in a polarity defect of petal epidermal cells (Lin et al., 2003). AS1 and AS2 are known to be capable of forming a protein complex (Xu et al., 2003) and *as1 jag* and *as2 jag* had similar floral defects, indicating that the AS1 and AS2 complex acts in the adaxial domain of floral organs, although *AS1* is known to be expressed throughout each type of floral organ (Byrne et al., 2000). In addition, *PTL* and *CUC* transcripts were accumulated in the adaxial side of the *as1 jag* sepals, further supporting the idea that AS1/AS2 act in polarity in regulating their targets for normal sepal and petal development.

AS1, *AS2*, and *JAG* play roles in both boundary-specifying gene repression and adaxial-abaxial polarity establishment. On the other hand, boundary-specifying genes, such as *CUC3*, could also affect establishment of leaf adaxial-abaxial polarity (Hibara et al., 2006). Sepal and petal phenotypes and the ectopic expression of boundary-specifying genes in the *as1 jag* and *as2 jag* flowers imply that the adaxial-abaxial polarity and the boundary specification and lateral growth might be functionally related. We noticed that many *as1* and *as2* flowers contain petals with incorrect orientation, whereas the role of *AS1* and *AS2* in controlling petal orientation is not yet clear. We hypothesize that, at the early petal developmental stages, the correct petal orientation might depend at least in part on a balance of adaxial and abaxial cell growth. Therefore, the aberrant petal orientation in the *as1* and *as2* mutants could be caused by loss of normal adaxial-abaxial polarity in the petal primordium. It should be of interest to examine this model in the future.

The AS1 and AS2 protein complex is a critical repressor because it is known to repress important regulatory genes, such as *BREVIPEDICELLUS*, *KNAT2*, *KNAT6*, *FILAMENTOUS FLOWER* (*FIL*), *KANADI*s (Byrne et al., 2000; Semiarti et al., 2001; Lin et al., 2003), and the microRNA genes *MIR165/166* (Li et al., 2005; Ueno et al., 2007). Recent studies have demonstrated that AS1, AS2, and histone deacetylases function in the same genetic pathway to regulate leaf polarity (Ueno et al., 2007), and AS1 is able to bind to a putative chromatin-remodeling factor (Phelps-Durr et al., 2005). These results suggest that the AS1 and AS2 complex may be involved in epigenetic regulation. The JAG protein also appears to have a repressor function (Dinneny et al., 2004; Ohno et al., 2004), whereas how JAG represses its downstream targets is unknown. Recent studies have shown that *CUC1*and *CUC2* expression is regulated by chromatin-remodeling factors (Kwon et al., 2006), indicating that these two genes might be modulated by an epigenetic mechanism. On the other hand, it is not clear yet whether AS1, AS2, and JAG regulation of the boundary target genes is direct or indirect through other genes. In future studies, it will be important to investigate how AS1, AS2, and JAG repress their targets at the molecular level.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

For the Arabidopsis (*Arabidopsis thaliana*) mutants used in this study, *as1-1* and *ptl-1* were in the Col-0, *jag-1* in the Ws, and others in the L*er* backgrounds. *as1-101* and *as2-101* were generated as described previously (Sun et al., 2000, 2002; Xu et al., 2002, 2003). Seeds of *jag-1* and *jag-2* were kindly provided by D. Weigel (Max Plank Institute for Developmental Biology) and C.K. Ohno (California Institute of Technology), respectively. Seeds of *as1-1*, *ptl-1*, *cuc1-1*, and *cuc2-1* were obtained from the Arabidopsis Biological Resource Center (Ohio State University). Plants were grown according to our previous conditions (Xu et al., 2003).

Constructions of Double, Triple, and Quadruple Mutants

Homozygous *jag-2* plants were crossed to homozygous *as1-101* and *as2-101*, respectively. F_1 progeny of those crosses were all phenotypically normal. For the *jag-2* and *as1-101* cross, double mutants were recognized among the F_2 plants as those with novel phenotypes of flowers, with F_2 segregation of 59 normal, 18 *as1-101*, 22 *jag-2*, and seven with novel phenotypes. *AS2* and *JAG* loci are known to be linked, and a total of 490 F_2 plants from the *jag-2* and *as2-101* cross showed a distribution of 268 normal, 115 *as2-101*, 105 *jag-2*, and two plants that had similar novel phenotypes to those from the *as1-101* and *jag-2* cross. These phenotypically novel plants from both crosses were considered to be *as1-101 jag-2* and *as2-101 jag-2* double mutants, respectively, and were further verified by PCR and sequence analysis. To construct the *as1-1 jag-1 ptl-1* triple mutant, homozygous *ptl-1* and *as1-1 jag-1* plants were crossed. In the F_2 progeny, seeds from eight individual plants that had *ptl-1*-like phenotypes were harvested. One F_3 population contained 239 *ptl-1*-like plants, 79 and 73 plants with additive *as1-1* and *ptl-1*, and *jag-1* and *ptl-1* phenotypes, respectively, and 23 plants with novel phenotypes. Plants with additive phenotypes were considered to be the *as1-1 ptl-1* and *jag-1 ptl-1* double mutants, respectively, and plants with novel phenotypes were considered to be the *as1-1 jag-1 ptl-1* triple mutant. One plant from each of these putative double or triple mutants was genotyped either by PCR (for the *jag-1* locus) or PCR and DNA sequencing (for the *as1-1* and *ptl-1* loci) for verification. To obtain *as1-101 jag-2 cuc1-1 cuc2-1*, pollen from *cuc1-1/+ cuc2-1/cuc2-1* plants was used to pollinate *as1-101 jag-2* plants. Because the insertional *cuc2-1* mutation could be genotyped by PCR, among F_2 progeny, a number of individual plants with *as1-101* or *jag-2* phenotypes and with the homozygous *cuc2-1* mutation were identified. In the F_3 generation, plants with consistent novel phenotypes appeared among some *as1-101 cuc2-1* and *jag-2 cuc2-1* groups. These novel phenotypic plants were considered to be the putative quadruple mutants, and one of these plants was genotyped by PCR and sequencing for verification. In addition, plants with phenotypes similar to those of previously reported *as1 cuc1 cuc2* and plants with both *jag* and *cuc1 cuc2* phenotypes were also genotyped to confirm that they are *as1 cuc1 cuc2* and *jag cuc1 cuc2* triple mutants.

In Situ Hybridization

In situ hybridizations were performed according to previous methods (Drews et al., 1991; Long and Barton, 1998). The *H4*, *RBE*, and *CUC1* probes were made according to the previously reported sequences (Takada et al., 2001; Dinneny et al., 2004; Krizek et al., 2006). *PTL* and *CUC2* probes were made from constructs containing cDNA fragments, derived from reverse transcription-PCR using the following primers: for *PTL*, 5′-GAGGATGGAAGCTAGGGATG-3′ and 5′-GAGCCTCAAACCATAACTC-3′; and for *CUC2*, 5′-TGGGATGAAGAAGACTCTTG-3′ and 5′-GGAAAAGGGTCAAAGTCAAACC-3′. These cDNA fragments were subcloned into pBluescript SK− for probe preparation. *AS2* probes were made according to a previous method (Iwakawa et al., 2002).

Microscopy

Fresh tissue from wild-type and mutant plants was examined using a SZH10 dissecting microscope (Olympus). SEM was performed as previously described (Chen et al., 2000).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers NM129319 (Arabidopsis *AS1* cDNA), NM105235 (Arabidopsis *AS2*), and NM105519 (Arabidopsis *JAG*).

Supplemental Data

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

Supplemental Figure S1. Expression patterns of *PTL*, *CUC1*, and *CUC2* in *as1-101* and *jag-2* single-mutant flowers.

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