The Plant Cell, Vol. 15, 2241–2252, October 2003, www.plantcell.org © 2003 American Society of Plant Biologists

RESEARCH ARTICLES

The Arabidopsis *LATERAL ORGAN BOUNDARIES*–Domain Gene *ASYMMETRIC LEAVES2* Functions in the Repression of *KNOX* Gene Expression and in Adaxial-Abaxial Patterning

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The normal development of lateral organs of the shoot requires the simultaneous repression of meristem-specific genes and the activation of organ-specific genes. *ASYMMETRIC LEAVES2* (*AS2*) is required for the development of normal leaf shape and for the repression of *KNOX* genes in the leaf. *AS2* is a member of the recently identified, plant-specific *LATERAL ORGAN BOUNDARIES* (*LOB*)–domain gene family. Expression of *AS2* at high levels resulted in repression of the *KNOX* homeobox genes *BREVIPEDICELLUS*, *KNAT2*, and *KNAT6* but not of the related *SHOOT MERISTEMLESS* gene. Overexpression of *AS2* also led to a perturbation of normal adaxial-abaxial asymmetry in lateral organs, resulting in the replacement of abaxial cell types with adaxial cell types. These results indicate that *AS2* is sufficient to induce adaxial cell fate and repress *KNOX* gene expression.

INTRODUCTION

The development of leaves and other lateral organs that arise from the shoot apical meristem (SAM) requires the coordinated activities of several distinct morphological processes. Cell division, expansion, and differentiation all contribute to the formation of mature leaves. Lateral organs contain two primary axes of asymmetry, a proximal-distal axis and an adaxial-abaxial axis. These asymmetries are established relatively early during leaf development and are defined relative to the SAM. Surgical experiments designed to examine the relationship between leaf development and the SAM demonstrated that communication between the SAM and leaf primordia is necessary for the establishment of the adaxial-abaxial pattern and for leaf outgrowth (Sussex, 1955; Snow and Snow, 1959). These experiments suggested the existence of a SAM-derived signal that is necessary for the establishment of adaxial-abaxial leaf polarity.

A number of mutations that cause alterations in leaf form have been described. Mutations in the *ASYMMETRIC LEAVES1* (*AS1*) and *AS2* genes result in the formation of rumpled, lobed leaves that curl downward and display vascular pattern defects (Tsukaya and Uchimiya, 1997; Serrano-Cartagena et al., 1999; Byrne et al., 2000; Ori et al., 2000; Semiarti et al., 2001; Sun et al., 2002). *AS1* and *AS2* are required to maintain the repression of the class-1 *KNOX* genes *BREVIPEDICELLUS* (*BP*), *KNAT2*, and *KNAT6* in the leaf. In wild-type plants, class-1 *KNOX* genes are expressed in the SAM and are downregulated at the P0 position before leaf initiation such that their repression predicts the position of the next primordium. In *as1* and *as2* mutants, expression of *BP*, *KNAT2*, and *KNAT6* is expanded into the leaf blade (Byrne et al., 2000; Ori et al., 2000; Semiarti et al., 2001). *KNOX* genes are downregulated appropriately in the P0 position in *as1* and *as2* mutants, however, indicating that *AS1* and *AS2* are not needed for the initial repression of *KNOX* genes in leaf primordia (Ori et al., 2000). Mutations in homologs of *AS1* in *Antirrhinum* (*PHANTASTICA* [*PHAN*]) and maize (*rough sheath2* [*rs2*]) also result in a loss of *KNOX* gene repression in the leaf (Schneeberger et al., 1998; Timmermans et al., 1999; Tsiantis et al., 1999). In addition, *PHAN* is required for the development of proper adaxial-abaxial polarity (Waites and Hudson, 1995; Waites et al., 1998). *phan* mutant leaves are radially symmetric and display abaxial characteristics. Interestingly, mutations in *rs2* and *AS1* do not result in adaxial-abaxial polarity defects (Schneeberger et al., 1998; Ori et al., 2000; Byrne et al., 2002), suggesting that *PHAN* may have a distinct function that is not shared by *rs2* and *AS1*.

Genes that regulate adaxial-abaxial polarity in Arabidopsis include *PHABULOSA* (*PHB*) and *PHAVOLUTA* (*PHV*). Semidominant gain-of-function mutations in *PHB* and *PHV* cause the formation of radially symmetric, adaxialized lateral organs (McConnell and Barton, 1998; McConnell et al., 2001). *PHB* and *PHV* encode class-III homeodomain/Leu zipper transcription factors that are thought to specify adaxial cell fate by preventing the action of abaxial-promoting factors (McConnell et al., 2001; Bowman et al., 2002). Activation of the PHB and PHV proteins is postulated to occur via interaction with a SAM-derived ligand (McConnell et al., 2001). Members of the *YABBY* and *KANADI* gene families specify abaxial cell fate (reviewed by Bowman et al., 2002). Both of these gene families encode presumptive transcription factors that act redundantly, and ectopic expression of individual members of either family promotes abaxial identity

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Article, publication date, and citation information can be found at www.plantcell.org/cgi/doi/10.1105/tpc.014969.

(Bowman and Smyth, 1999; Eshed et al., 1999, 2001; Sawa et al., 1999; Siegfried et al., 1999; Kerstetter et al., 2001). Genetic data indicate that the *KANADI* genes act, at least in part, upstream of *YABBY* genes (Eshed et al., 2001), although the relationship between the two gene families is not completely clear.

AS2 encodes a member of the LATERAL ORGAN BOUNDARIES (LOB) family of plant-specific proteins (Iwakawa et al., 2002). *LOB*, the founding member of this gene family, is thought to play a role in boundary establishment or communication between the meristem and initiating lateral organs (Shuai et al., 2002). *LOB* is expressed at the base of lateral organs and is positively regulated by *AS1* and *AS2* (Byrne et al., 2002; Shuai et al., 2002). Here, we report the effects of the overexpression of *AS2* in wild-type and *as1* mutant backgrounds. We show that the expression of high levels of *AS2* results in repression of the *KNOX* genes *BP*, *KNAT2*, and *KNAT6* but not *SHOOT MERISTEMLESS* (*STM*). In addition, overexpression of *AS2* results in adaxial-abaxial polarity defects, implicating *AS2* in polarity establishment.

RESULTS

AS2 Is Expressed Broadly in Plant Development

To begin to understand the role of *AS2* in plant development, we first examined the expression of *AS2* by reverse transcriptase–mediated (RT) PCR. *AS2* transcripts were detected in most tissues tested (Figure 1). Transcripts were detected in RNA isolated from whole seedlings ranging in age from 2 to 12 days post imbibition. *AS2* transcripts were detected in rosette leaves, where they were more abundant in the petiole than in the blade. RT-PCR products also were detected in cotyledons, cauline leaves, roots, inflorescence stem nodes, open flowers, young floral buds, green siliques, and all four floral organs. *AS2* transcripts were not detected in internodes or pedicels. These data indicate that *AS2* is expressed throughout plant development.

as2 mutants are phenotypically similar to *as1* mutants in many respects. Both mutations have similar effects on leaf morphology, cause ectopic expression of *KNOX* genes, and are epistatic to mutations in *STM* (Tsukaya and Uchimiya, 1997; Serrano-Cartagena et al., 1999; Byrne et al., 2000, 2002; Ori et al., 2000; Semiarti et al., 2001). Thus, *AS1* and *AS2* appear to be components of the same genetic pathway, and it has been proposed that the protein products of these genes might interact directly (Byrne et al., 2002). To compare the *AS1* and *AS2* expression patterns, we examined *AS1* expression by RT-PCR. *AS1* transcripts were detected in all tissues tested, but the overall expression profiles of *AS1* and *AS2* were distinct (Figure 1). For example, *AS1* transcripts were detected in both petioles and blades of rosette leaves and appeared to be slightly more abundant in blade tissue, whereas *AS2* transcripts were more abundant in petioles. *AS1* transcripts also were detected in internode tissue, unlike *AS2*. Only low levels of *AS1* transcripts were detected in the outer three whorls of the flower, whereas a higher level was detected in the carpel. Thus, the domains of expression of *AS1* and *AS2* appear to be largely overlapping but not identical.

AS1 and *AS2* have been implicated in the regulation of *LOB* expression (Byrne et al., 2002), so we also examined the expression profile of *LOB* by RT-PCR (Figure 1). *LOB* was expressed in a more limited pattern than either *AS1* or *AS2*. In contrast to that of *AS1* and *AS2*, *LOB* transcript abundance increased concomitantly with seedling age. *LOB* transcripts were not detected in rosette leaf blades but were detected in petioles and were especially abundant in nodes. *LOB* expression also was detected in buds, open flowers, and pedicels but was absent from individual floral organs. These observations are largely in agreement with the previously reported *LOB* expression pattern, which was based on the *lob*::*ET22* enhancer trap line (containing an enhancer trap insertion in the *LOB* gene) as well as *LOB* promoter:β-glucuronidase (*GUS*) fusions (Shuai et al., 2002).

Phenotypic Effects Resulting from the Overexpression of *AS2*

To investigate the role of *AS2* in plant development, we generated transgenic plants that ubiquitously expressed *AS2* under the control of the *35S* promoter of *Cauliflower mosaic virus*. Of 76 *35S*:*AS2* transgenic seedlings, all formed cotyledons that were narrow and curled upward, toward the adaxial side, in contrast to the flattened cotyledons of wild-type seedlings (Fig-

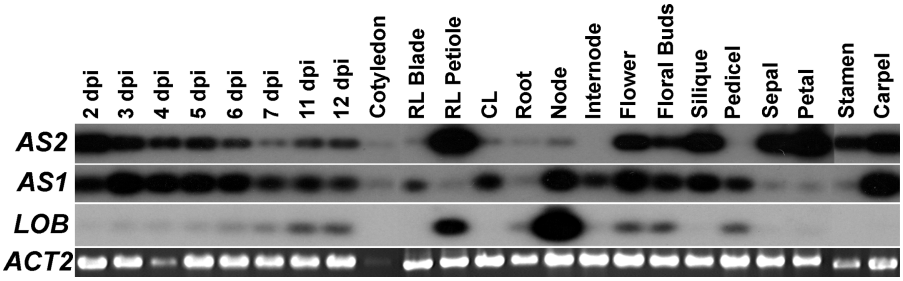

Figure 1. Expression of *AS2*, *AS1*, and *LOB* in Wild-Type Tissues.

RT-PCR was performed on RNA isolated from seedlings at 2, 3, 4, 5, 6, 7, 11, and 12 days post imbibition (dpi), cotyledons, rosette leaf (RL) blades, rosette leaf (RL) petioles, cauline leaves (CL), roots, inflorescence stem nodes, inflorescence stem internodes, open flowers, floral buds, green siliques, flower pedicels, sepals, petals, stamens, and carpels. RT-PCR products were detected by blotting and probing with gene-specific probes after 15 cycles of amplification. *ACTIN2* (*ACT2*) was used as a control.

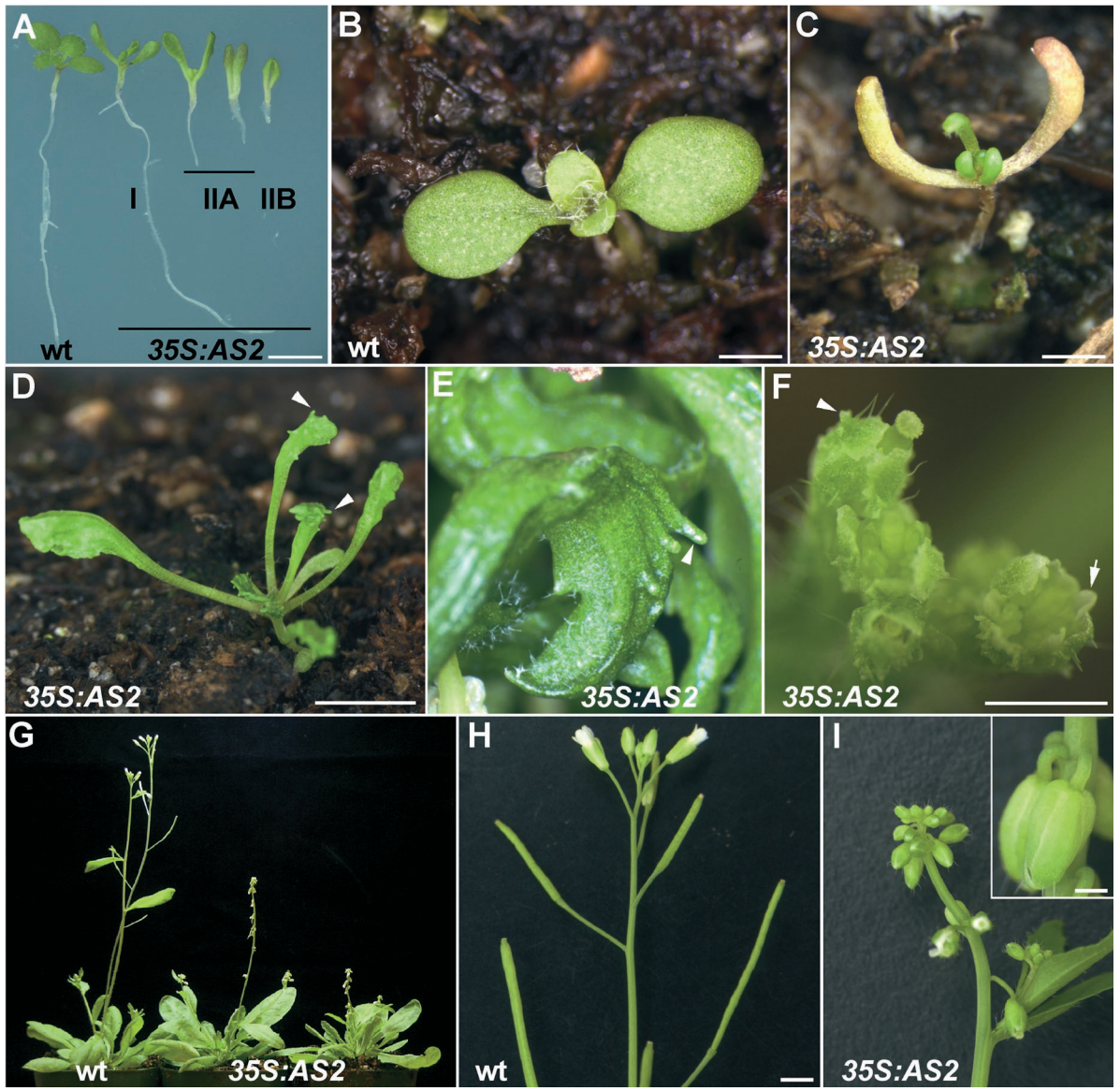

Figure 2. Phenotypes of Transgenic Plants That Overexpress *AS2*.

(A) Phenotypic variability in primary *35S:AS2* Columbia (Col-0) transgenic plants. From left to right: wild-type (wt), *35S:AS2* class-I, class-IIA, class-IIA, and class-IIB 12-day-old transgenic seedlings.
(B) Wild-type (Col-0) 8-day-old seedling.
(C) *35S:AS2* transgenic plant with radial leaves.
(D) *35S:AS2* transgenic plant. The arrowheads indicate outgrowth on the abaxial leaf surface.
(E) *35S:AS2* rosette leaf. The arrowhead shows a finger-like abaxial protrusion.
(F) Inflorescence of a *35S:AS2* plant. The arrowhead shows an abaxial protrusion on a sepal, and the arrow shows an inwardly curled petal.
(G) Wild-type (left) and two different *35S:AS2* plants (right).
(H) Wild-type (Col-0) inflorescence.
(I) *35S:AS2* inflorescence. The inset shows a higher magnification of *35S:AS2* pedicels.
Bars = 3 mm in **(A)**, 1 mm in **(B)**, **(C)**, **(F)**, and **(I)** inset, 5 mm in **(D)**, and 2 mm in **(H)** for **(H)** and **(I)**.

ures 2A to 2C). Among these seedlings, 34 formed a root system similar to that of wild-type seedlings (class I) and 42 produced a primary root that was shorter than that of wild-type seedlings (class II) (Figure 2A). The class-II seedlings could be categorized further into those that initiated lateral roots (class IIA; 12 of 42) and those that did not (class IIB; 30 of 42). Class-IIB seedlings arrested after making only a few leaves that usually failed to expand and occasionally were radial in appearance (Figure 2C). Class-I and class-IIA seedlings did not arrest, and the majority of these plants produced narrow leaves that

were curled upward, twisted, and formed finger-like outgrowths from the abaxial leaf surface (Figures 2D and 2E). Upon flowering, *35S:AS2* plants usually produced abnormal, sterile flowers. Sepals occasionally exhibited protrusions on the abaxial surface, and petals often were curled inward, toward the adaxial side (Figure 2F). The inflorescence stems of *35S:AS2* plants typically did not elongate. Pedicels were shorter than those in the wild type and emerged from the inflorescence stems at abnormal angles (data not shown). Similar phenotypes resulting from the expression of *AS2* from the *35S* promoter have been reported, although they were not analyzed in detail (Iwakawa et al., 2002; Nakazawa et al., 2003).

A small number of *35S:AS2* transgenic plants had relatively normal leaf morphologies, but upon flowering, they had abnormal inflorescence architecture (Figures 2G and 2I). In wild-type plants, pedicels pointed upward (Figures 2G and 2H), but *35S:AS2* plants developed pedicels that pointed downward (Figures 2G and 2I). In addition, internodes between flowers were shortened, and in the most extreme cases, there was no internode elongation between some flowers, so that multiple flowers appeared to be produced at the same node (Figure 2I). The morphological characteristics of the inflorescence resembled those of the *bp* mutant. *BP* encodes the class-1 KNOX homeodomain protein and was previously designated *KNAT1* (Douglas et al., 2002; Venglat et al., 2002).

Expression of *AS2* at High Levels Causes the Repression of *KNOX* Gene Expression

AS2, together with *AS1*, is required to maintain the repression of the *KNOX* genes *BP*, *KNAT2*, and *KNAT6* in Arabidopsis leaves, because transcripts of these genes accumulate in the leaves of *as1* and *as2* mutants (Byrne et al., 2000, 2002; Ori et al., 2000; Semiarti et al., 2001). The observation that the inflorescences of *35S:AS2* plants resembled those of *bp* mutants suggested that *BP* repression is an outcome of *AS2* overexpression. Therefore, we examined the steady state transcript levels of *BP* as well as the related *KNOX* genes *STM*, *KNAT2*, and *KNAT6* in *35S:AS2* plants of varying phenotypic severity using RNA gel blot hybridization and RT-PCR. We initially examined the transcript levels of *BP* in *35S:AS2* plants that had relatively normal vegetative morphology, because they most closely resembled *bp* mutants. *BP* transcript levels were reduced in the inflorescences of these plants (Figure 3A).

We also examined *KNOX* gene expression in the shoots of *35S:AS2* transformants that showed vegetative abnormalities. We examined both class-I seedlings, which formed relatively normal roots and produced narrow leaves with abaxial protrusions, and class-IIB seedlings, which produced very short roots and few leaves. Class-IIB seedlings were predicted to arrest at a later stage. Examination of *AS2* expression in these transgenic plants showed that *AS2* transcript levels correlated with phenotypic severity. Both classes had increased *AS2* expression relative to the wild type, and class-IIB seedlings had higher levels of *AS2* transcripts than class-I seedlings (Figure 3B). Compared with the wild type, a reduction in *KNAT2* and *KNAT6* transcript levels was observed in both classes of transgenic seedlings (Figure 3B). *KNAT2* transcript levels were reduced more dramatically in class-IIB seedlings, whereas the level of *KNAT6* transcripts was

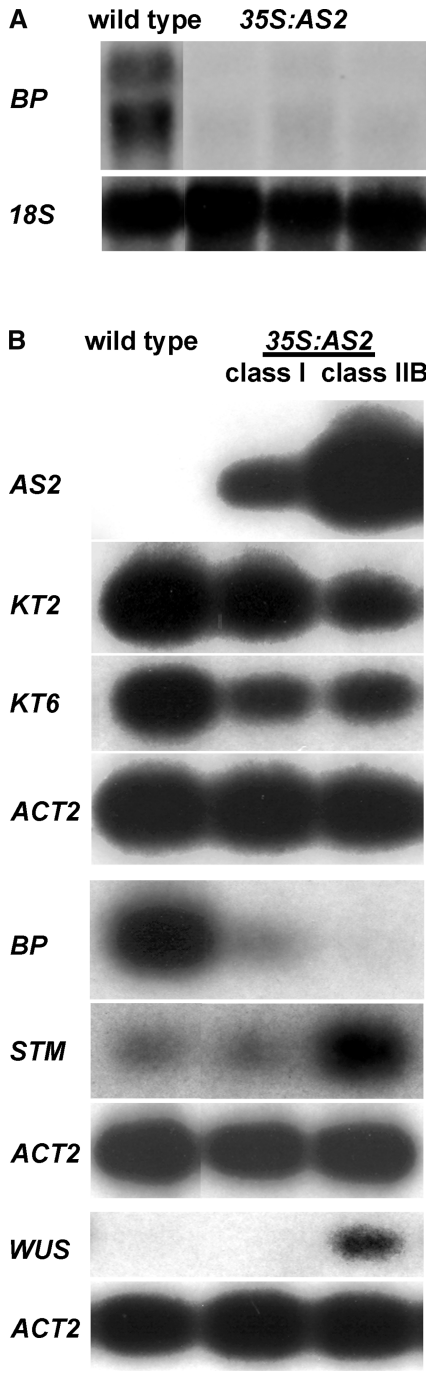

Figure 3. *KNOX* Gene Expression in *35S:AS2* Plants.

(A) RNA gel blots showing *BP* expression in inflorescence tissue of wild-type (left) and three different *35S:AS2* plants. Ten micrograms of total RNA was loaded in each lane. The filter was probed with *BP* cDNA (top gel) or *18S rDNA* as a loading control (bottom gel).

(B) RT-PCR analyses of *AS2*, *KNAT2* (*KT2*), *KNAT6* (*KT6*), *BP*, *STM*, and *WUS* transcripts in wild-type (left), *35S:AS2* class-I (middle), and *35S:AS2* class-IIB (right) seedlings. RT-PCR products were detected by blotting and probing with gene-specific probes after 10 cycles of amplification for *AS2* and 15 cycles of amplification for the other genes. *ACT2* was used as a control with 10 cycles of amplification, and the appropriate control is shown below each experimental set.

similar in both classes. *BP* transcript levels were much more affected, showing a strong reduction in both classes (Figure 3B). *STM* transcript levels were unchanged in class-I seedlings but apparently were increased in class-IIB seedlings (Figure 3B). This apparent upregulation of *STM* likely was attributable to a higher ratio of meristem to leaf tissue in these seedlings. To test this possibility, transcript levels of *WUSCHEL* (*WUS*), a homeodomain gene that is expressed in the central zone of the meristem (Mayer et al., 1998), were examined. Higher levels of *WUS* transcripts also were detected in class-IIB seedlings (Figure 3B), consistent with the presence of more meristem tissue in these seedlings.

To further investigate the decrease in *KNOX* transcript levels in *35S:AS2* transgenic plants, the *35S:AS2* transgene was introduced into plants containing a *GUS* reporter gene driven by either the *BP* promoter (Ori et al., 2000) or the *KNAT2* promoter (Dockx et al., 1995). *35S:AS2* transgenic plants were identified by selection for glufosinate ammonium resistance and stained for GUS activity. In the *BP:GUS* background, all 102 seedlings carrying the *35S:AS2* transgene showed a reduction in staining intensity relative to the wild type. The reduction in GUS staining correlated with phenotypic severity, being reduced most strongly in seedlings with the strongest phenotype (Figures 4A to 4C). By contrast, the majority of class-I *35S:AS2* plants retained high levels of *KNAT2:GUS* expression, similar to the parental line (Figures 4D and 4E). GUS staining intensity was reduced in some class-I seedlings and all class-II individuals (Figures 4F and 4G).

35S:AS2 Plants Exhibit Adaxial-Abaxial Polarity Defects

To further examine the morphological abnormalities of *35S:AS2* plants, they were embedded and sectioned. Transverse sections through cotyledons and leaves of *35S:AS2* plants revealed abnormalities in the organization of internal tissues. A distinct adaxial-abaxial polarity was visible in wild-type cotyledons and leaves. Tightly packed, elongated palisade mesophyll cells were present on the adaxial side, and loosely packed, rounded spongy mesophyll cells were present on the abaxial side (Figures 5A and 5C). This polarity was disrupted in *35S:AS2* cotyledons and leaves. Elongated, densely packed cells that resembled palisade mesophyll were present beneath the abaxial epidermis, and cells on the adaxial side of the leaf resembled spongy mesophyll (Figures 5B, 5D, and 5E). In those leaves that were radial in appearance, a subepidermal ring of palisade cells was observed around the entire leaf (Figure 5F).

Vasculature in *35S:AS2* plants did not develop normally. In wild-type leaves, vascular tissue exhibited adaxial-abaxial polarity: xylem developed on the adaxial side of the leaf, and phloem developed on the abaxial side (Figure 5G). In *35S:AS2* leaves, veins were twisted such that this polarity was variable along the proximal-distal leaf axis. Polarity often was disrupted, resulting in vascular bundles that formed at an abnormal angle relative to the plane of the leaf surface (data not shown). In the most extreme cases, phloem was encircled by xylem (Figure 5H). Occasionally, a strand of xylem developed without any associated phloem (data not shown). The venation pattern of *35S:AS2* seedlings also was different from that of the wild type. The cotyledon midvein frequently failed to develop to the tip, and lateral veins usually did not form. Occasionally, the cotyledon midvein split to form two or more veins (data not shown).

Unlike the internal tissues, polarity defects were apparent only occasionally in the epidermal cells of *35S:AS2* plants. One distinct difference between abaxial and adaxial epidermal sur-

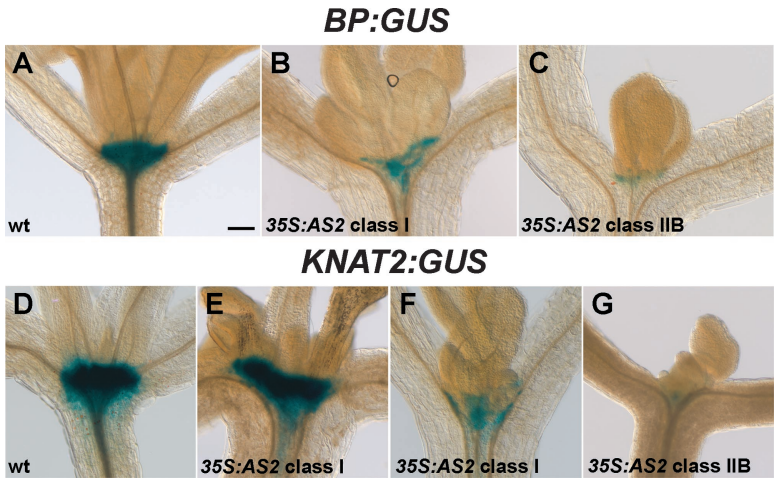

Figure 4. Activity of *BP* and *KNAT2* Promoters in *35S:AS2* Transgenic Plants.

(A) Wild-type (wt) Col-0 seedling carrying a *BP:GUS* construct.
(B) and **(C)** Class-I **(B)** and Class-IIB **(C)** *35S:AS2* transgenic plants carrying a *BP:GUS* construct and showing reduced *GUS* expression.
(D) Wild-type C-24 seedling carrying a *KNAT2:GUS* construct.
(E) Class-I *35S:AS2* transgenic seedling carrying a *KNAT2:GUS* construct and showing similar GUS activity to the wild type.
(F) Class-I *35S:AS2* transgenic seedling carrying a *KNAT2:GUS* construct and showing reduced *GUS* expression.
(G) Class-IIB *35S:AS2* transgenic seeding carrying a *KNAT2:GUS* construct and showing strongly reduced GUS activity.
Bar in **(A)** = 1 mm for **(A)** to **(G)**.

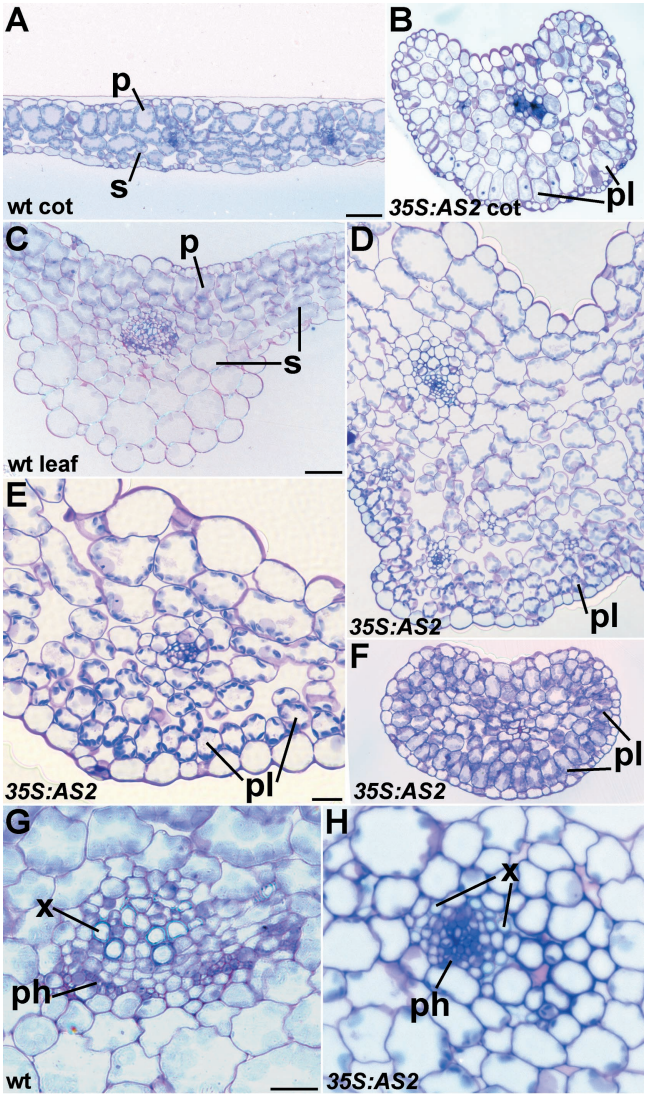

Figure 5. Overexpression of *AS2* Results in Adaxial-Abaxial Polarity Defects.

Cross-section through a wild-type (wt) cotyledon **(A)**, a *35S:AS2* cotyledon **(B)**, a wild-type rosette leaf (**[C]** and **[G]**), and a *35S:AS2* rosette leaf (**[D]** to **[F]** and **[H]**). In each panel, the adaxial leaf surface is at the top of the image. cot, cotyledon; p, palisade mesophyll; ph, phloem; pl, palisade-like mesophyll; s, spongy mesophyll; x, xylem. Bar in **(A)** = 100 μm for **(A)** and **(B)**; bar in **(C)** = 50 μm for **(C)**, **(D)**, and **(F)**; bar in **(E)** = 20 μm; and bar in **(G)** = 20 μm for **(G)** and **(H)**.

faces of wild-type plants is that trichomes are present only on the adaxial leaf surface of the first four to five leaves (Telfer et al., 1997). Consequently, one obvious hallmark of leaf adaxialization is the appearance of trichomes on the abaxial side of early leaves (Kerstetter et al., 2001; McConnell et al., 2001). *35S:AS2* plants were never observed to form trichomes on the abaxial side of early leaves, so by this criterion, the epidermal surface was not adaxialized. In wild-type leaves, there also are differences in the appearance of the puzzle-shaped pavement cells on the abaxial and adaxial surfaces. Pavement cells on the adaxial blade are larger and more uniform in size than those on the abaxial surface (Figures 6A and 6B) (Bowman, 1993). We examined the shape of epidermal cells of *35S:AS2* leaves by scanning electron microscopy. The adaxial surface of *35S:AS2* leaves was not visibly different from that of wild-type leaves (data not shown). The abaxial epidermis of *35S:AS2* leaves appeared to be a mixture of adaxial and abaxial cell types. Patches of epidermis on this side of the leaf resembled the wild-type adaxial epidermis, containing cells that are uniform in size, with an absence of the characteristic large, irregular cells that typify the wild-type abaxial epidermis (Figure 6C). We also observed rare flowers that produced petals with conical cells, characteristic of the adaxial epidermis, on both epidermal surfaces (Figures 6D to 6G).

Relationship Between *AS2* and Polarity Genes

Many of the phenotypes exhibited by *35S:AS2* plants were similar to those caused by mutations in the redundant *KANADI1* (*KAN1*) and *KAN2* genes, which specify abaxial cell identity (Eshed et al., 2001; Kerstetter et al., 2001). To examine possible interactions between *AS2* and the *KANADI* genes, we examined transcript levels of *KAN1* and *KAN2* in *35S:AS2* shoots. *KAN1* and *KAN2* transcripts were detectable in *35S:AS2* shoots, but at reduced levels compared with those in the wild type, and seemed to be reduced more dramatically in the most severe class-IIB seedlings (Figure 7A). Members of the *YABBY* gene family act redundantly to specify abaxial identity, so we also examined transcript levels of the *YABBY* genes *FILAMENTOUS FLOWER* (*FIL*) and *YABBY3* (*YAB3*) in *35S:AS2* shoots. Transcript levels of *FIL* and *YAB3* also were reduced in *35S:AS2* shoots compared with those in the wild type (Figure 7A). By contrast, transcript levels of *PHB*, a gene that regulates adaxial identity, were increased in *35S:AS2* shoots of both classes, with the more severe class-IIB seedlings showing higher levels than class-I seedlings (Figure 7A).

To further investigate the relationship between *AS2* and polarity genes, we examined the expression of *KAN1*, *KAN2*, *FIL*, *YAB3*, and *PHB* in *as2-1* mutants. No significant differences in the transcript levels of these genes were observed in *as2* mutants compared with those in the wild type (Figure 7B). We also examined transcript levels of *AS2* in adaxialized mutant backgrounds. In *kan1 kan2* double mutants, *AS2* transcripts were detected at wild-type levels (Figure 7C). By contrast, *AS2* transcript levels were increased slightly in both *phb-1d* mutants and *fil-8 yab3-2* double mutants (Figure 7C).

AS2 Overexpression in the *as1* Mutant Background

Existing genetic and molecular data indicate that *AS1* and *AS2* function together (Serrano-Cartagena et al., 1999; Ori et al., 2000; Semiarti et al., 2001; Byrne et al., 2002). To determine if the effects of *AS2* overexpression require a functional *AS1* gene, we introduced the *35S:AS2* transgene into an *as1* mutant background. Of 35 *as1* seedlings carrying the *35S:AS2* transgene, 7 resembled *as1* single mutant plants during vegetative growth, whereas the remaining 28 showed additional phenotypes caused by the *AS2* transgene. Those transgenic plants that did not show

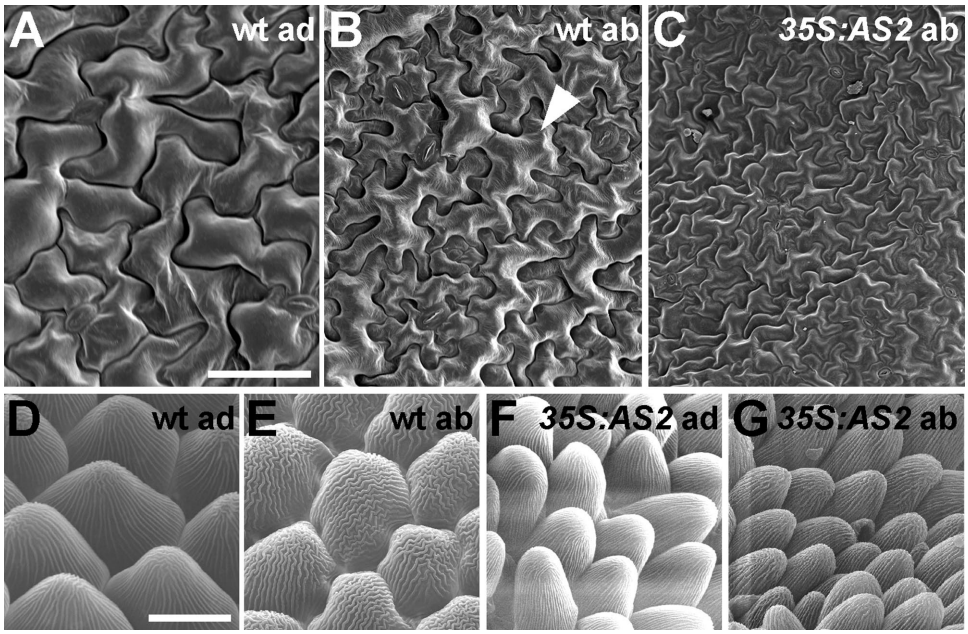

Figure 6. Epidermal Surface of *35S:AS2* Transgenic Plants.

(A) Adaxial epidermal cells of a wild-type (wt) rosette leaf showing uniform cell size.
(B) Abaxial epidermis of a wild-type rosette leaf showing irregular cell size. The arrowhead points to a large, irregular cell.
(C) Abaxial epidermis of a *35S:AS2* rosette leaf resembling a wild-type adaxial epidermis.
(D) Adaxial epidermis of a wild-type petal.
(E) Abaxial epidermis of a wild-type petal.
(F) Adaxial epidermis of a *35S:AS2* petal.
(G) Abaxial epidermis of a *35S:AS2* petal.
Bar in **(A)** = 50 μm for **(A)** to **(C)**; bar in **(D)** = 10 μm for **(D)** to **(G)**.

additional phenotypes were assumed to express low levels of the transgene and were not examined further. Two *as1 35S:AS2* seedlings formed narrow cotyledons that curled upward, similar to the effect of *35S:AS2* in a wild-type background (data not shown). The remaining *as1 35S:AS2* seedlings produced leaves that curled upward at the edges (Figure 8B), in contrast to *as1* mutant leaves, which curled downward (Figure 8A). The upward leaf curling was similar to the effect of the *35S:AS2* transgene in a wild-type background (Figure 2D), although less pronounced. Abaxial protrusions, which were observed commonly in *35S:AS2* transgenic plants in a wild-type background (Figures 2D and 2E), were not formed in the *as1* mutant background (Figure 8B). Upon flowering, most *as1 35S:AS2* plants produced flowers that had short pedicels that emerged from the stem at abnormal angles (Figure 8C). Internodes between flowers were shorter than those in *as1* single mutants, inflorescence stems were thickened, and fertility was reduced in these plants. We detected no change in *BP* transcript levels in these plants (data not shown).

DISCUSSION

AS2 Functions in Adaxial-Abaxial Polarity

Overexpression revealed a role for *AS2* in the establishment of adaxial-abaxial polarity, something that was not apparent from the loss-of-function phenotype. Several aspects of the *35S:AS2* phenotype indicate that *AS2* can promote adaxial identity in lateral organs. The cotyledons and leaves of *35S:AS2* plants were curled upward, toward the adaxial side of the leaf, and had limited blade expansion. Mutations that affect adaxial-abaxial polarity typically show reduced lamina expansion (reviewed by Bowman et al., 2002), consistent with the model that juxtaposition of adaxial and abaxial domains is required for leaf blade outgrowth (Waites and Hudson, 1995). Examination of the internal tissues of *35S:AS2* cotyledons and leaves revealed the presence of cells that resembled palisade mesophyll on the abaxial side. Leaves showing the most extreme phenotype were radial in appearance, and palisade cells ringed the leaf circumference. The abaxial epidermis of leaves and floral organs also was affected in *35S:AS2* plants, often appearing to be a mixture of abaxial and adaxial identities.

The organization of vascular tissues frequently was altered in *35S:AS2* leaves. The orientation of phloem and xylem poles relative to the plane of the leaf surface was abnormal, and some veins appeared to twist as they traveled through the leaf. In some cases, xylem developed on both sides of phloem or developed in the absence of identifiable phloem (data not shown). Vascular patterning also was abnormal in *as1* and *as2* mutant leaves, which may indicate a role for these genes in the establishment of the vascular network (Semiarti et al., 2001; Sun et al., 2002).

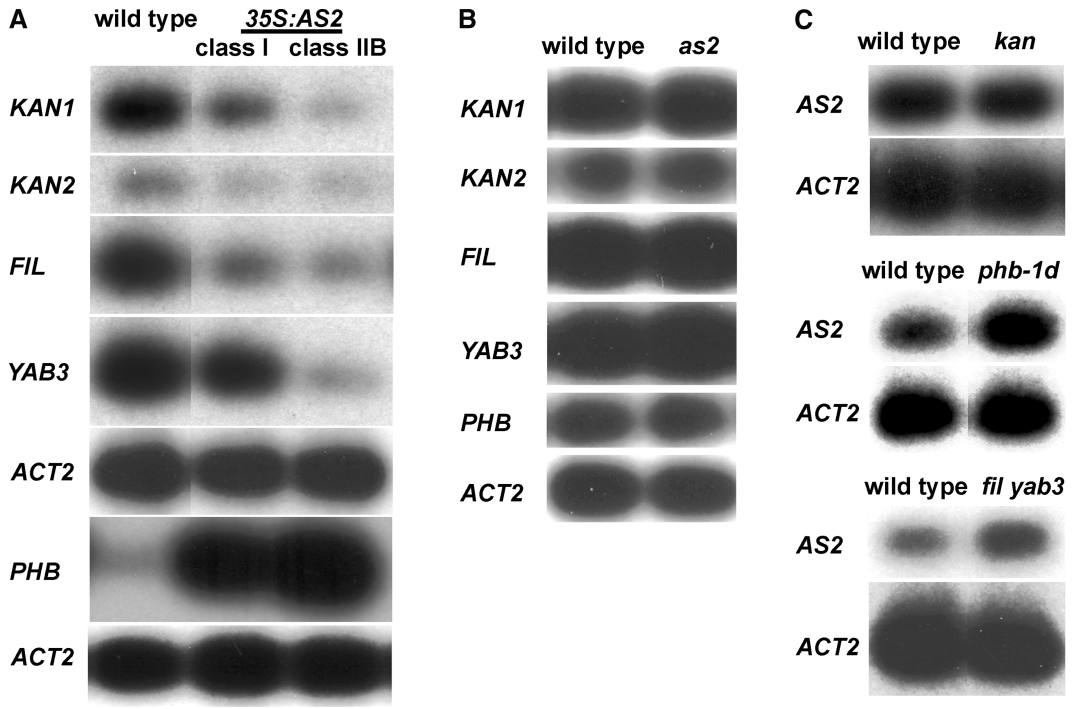

Figure 7. Expression Analyses of Polarity Genes in *35S:AS2* Transgenic Plants and *as2* Mutants and *AS2* in Polarity Mutants.

(A) RT-PCR analyses of *KAN1*, *KAN2*, *FIL*, *YAB3*, and *PHB* transcript levels in 12-day-old shoots of wild-type C-24 and *35S:AS2* transgenic plants.
(B) RT-PCR analyses of *KAN1*, *KAN2*, *FIL*, *YAB3*, and *PHB* expression in 11-day-old shoots of wild-type Landsberg *erecta* and *as2-1* mutant plants.
(C) RT-PCR analyses of *AS2* expression in wild-type L*er* and *kan1 kan2* (*kan*), *phb-1d*, and *fil-8 yab3-2* (*fil yab3*) mutant seedlings.
RT-PCR products were detected by blotting and probing with gene-specific probes after 15 cycles of amplification. Exposure times varied between experiments, and expression levels cannot be compared between experiments. *ACT2* was used as a control with 10 cycles of amplification, and the appropriate control is shown below each experimental set.

Vascular-pattern defects also are associated with the loss of adaxial-abaxial polarity in *phb-1d* mutants and transgenic plants that ectopically express *KAN1* (McConnell and Barton, 1998; Eshed et al., 2001; Kerstetter et al., 2001). These observations may indicate a relationship between vascular patterning and adaxial-abaxial polarity. One possibility is that the formation of a vascular network requires the presence of distinct adaxial and abaxial domains. In this case, overexpression of *AS2* might affect vascular patterning indirectly.

In many respects, *35S:AS2* leaves resembled those of *kan1 kan2* double mutants (Eshed et al., 2001). *KANADI* genes act redundantly to promote abaxial cell fate, and in plants that have lost both *KAN1* and *KAN2* function, adaxial cell types develop in place of abaxial cell types (Eshed et al., 2001). Both *35S:AS2* leaves and *kan1 kan2* mutant leaves develop outgrowths on the abaxial leaf surface. In these leaves, there appears to be an incomplete conversion of abaxial cell types to adaxial cell types, which would result in the development of patches of cells with adaxial identity adjacent to cells with abaxial identity. The outgrowths may represent ectopic margins, which are formed when adaxial and abaxial domains are adjacent to each other (Waites and Hudson, 1995).

The phenotypes caused by overexpression implicate *AS2* in the development of normal adaxial-abaxial polarity in lateral organs. However, *as2* mutants do not show a conspicuous polarity defect, although it has been suggested that the downward curling of *as2* mutant leaves, as well as other aspects of the phenotype, are consistent with abaxialization (Ori et al., 2000). Likewise, *as1* mutants, which exhibit a phenotype nearly identical to that of *as2*, do not display obvious polarity defects, nor do *as1 as2* double mutants (Serrano-Cartagena et al., 1999; Byrne et al., 2000; Ori et al., 2000). This is in contrast to mutations in the *Antirrhinum PHAN* gene, which result in the formation of radially symmetric, abaxialized leaves (Waites and Hudson, 1995; Waites et al., 1998). *PHAN* and *AS1* are related genes, both encoding MYB-domain proteins required for *KNOX* gene repression in the leaf (Waites et al., 1998; Byrne et al., 2000). Therefore, the lack of functional conservation with respect to leaf polarity was initially surprising (Byrne et al., 2000). Our observations implicating *AS2* in the establishment of adaxial-abaxial polarity may help to clarify this issue. *AS2* appears to act redundantly with respect to polarity establishment but not with respect to other aspects of leaf morphogenesis. Functional redundancy is a common theme in genetic pathways that regulate polarity (reviewed by Bowman et al., 2002). The identities of factors that act redundantly with *AS2* are not known, but the closely related *LOB-DOMAIN* (*LBD*) genes are likely candidates. *LBD36*, also known as *ASL1* (Iwakawa et al., 2002), encodes a LOB-domain protein that is closely related to AS2, and overexpression of *LBD36* results in phenotypes similar to those of *35S:AS2* plants, includ-

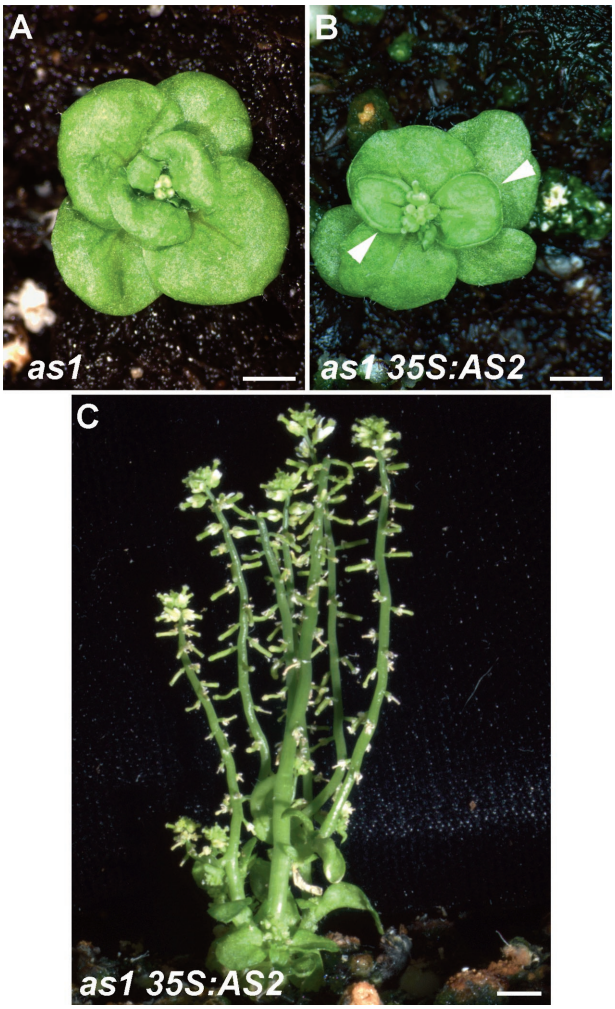

Figure 8. Morphology of *as1 35S:AS2* Transgenic Plants.

(A) 25-day-old *as1* mutant plant.
(B) 25-day-old *as1 35S:AS2* plant. Arrowheads show leaves curling upward at the edges.
(C) *as1 35S:AS2* plant after flowering.
Bars in **(A)** and **(B)** = 2 mm; bar in **(C)** = 3 mm.

ing the repression of *BP* expression (Nakazawa et al., 2003; W.-c. Lin and P.S. Springer, unpublished data).

We do not yet understand the relationship between *AS2* and other polarity genes. Transcript levels of the abaxializing genes *KAN1*, *KAN2*, *FIL*, and *YAB3* were reduced in *35S:AS2* plants, especially in those with the most extreme phenotypes, whereas transcript levels of the adaxializing gene *PHB* were increased in *35S:AS2* plants. The change in expression of these polarity genes might indicate that *AS2* acts upstream of them to positively regulate *PHB* and negatively regulate *KANADI* and *YABBY* genes. However, transcript levels of *PHB*, *KANADI*, and *YABBY* genes were not altered in *as2* mutants, indicating that *AS2* is not required for the regulation of these genes. This finding suggests that the effect of *AS2* overexpression on polarity-gene expression may be indirect, but it also is consistent with *AS2* acting redundantly to control the expression of these genes. *KANADI* and *YABBY* genes are expressed on the abaxial side of lateral organs (Siegfried et al., 1999; Kerstetter et al., 2001), whereas *PHB* is expressed on the adaxial side (McConnell et al., 2001), so a change in the expression of these genes in *35S:AS2* seedlings might simply reflect the presence of adaxialized cell types on the abaxial side of leaves. In this case, *AS2* might define a separate pathway for polarity determination. *AS2* transcripts were upregulated in adaxialized *phb-1d* mutants and *fil yab3* double mutants but appeared to be unaffected in *kan1 kan2* double mutants. These data suggest that *AS2* is positively regulated by *PHB* and negatively regulated by *YABBY* genes, but whether this regulation is direct or indirect is not clear. The relationship between the abaxializing factors *KAN1*, *KAN2*, and the *YABBY* genes also is not clear (reviewed by Bowman et al., 2002).

Interactions between *AS2* and *KNOX* Genes

The *as2* loss-of-function phenotype demonstrates that *AS2* plays an important role, together with *AS1*, in the development of normal leaf morphology. One aspect of this role involves the maintenance of *KNOX* gene repression in the leaf. The *KNOX* genes *BP*, *KNAT2*, and *KNAT6* are misexpressed in *as1* and *as2* mutant leaves (Byrne et al., 2000; Ori et al., 2000; Semiarti et al., 2001). *STM* also appears to be misexpressed late in development in *as1* leaves (Semiarti et al., 2001). However, it is not clear if all aspects of the *as1* and *as2* mutant phenotypes are caused by ectopic *KNOX* gene expression. Individual mutations in *BP*, *KNAT2*, or *STM* do not suppress *as1* or *as2* mutant phenotypes (Byrne et al., 2002), indicating that misexpression of individual *KNOX* genes is not required for the *as1* and *as2* mutant phenotypes.

Several lines of evidence indicate that *AS2* overexpression results in the repression of *KNOX* gene expression. *35S:AS2* plants produced an inflorescence that resembled that of the *bp* mutant, and steady state *BP* transcript levels were decreased in all *35S:AS2* plants examined. Steady state *KNAT2* and *KNAT6* transcript levels also were reduced to a lesser extent in *35S:AS2* transgenic plants, with the largest reduction seen in plants that showed the highest levels of *AS2* expression. These transgenic plants (class IIB) also had extremely short roots and arrested after producing a small number of leaf primordia. This aspect of the phenotype may be attributable to the combined reduction of *BP*, *KNAT2*, and *KNAT6* transcripts.

KNOX genes are downregulated normally in the P0 of *as1* and *as2* mutants (Ori et al., 2000), but this repression is not maintained. Thus, the initial repression of *KNOX* genes must be independent of *AS1* and *AS2*. When *AS2* is overexpressed, it is able to induce the repression of the *KNOX* genes *BP*, *KNAT2*, and *KNAT6* in the meristem. It appears that the transcriptional repression of *KNAT2* and *KNAT6* requires a higher level of *AS2* expression than does the repression of *BP*. In agreement with this observation, *BP* is expressed at higher levels than *KNAT2* and *KNAT6* in *as2* mutant leaves (Byrne et al., 2000; Ori et al., 2000; Semiarti et al., 2001). Together, these data suggest that the mechanism by which *BP* expression is restricted from lateral organs may be distinct from the mechanism of *KNAT2* and *KNAT6* restriction.

AS2 encodes a nuclear protein (Iwakawa et al., 2002) and therefore might directly repress *KNOX* gene transcription. Alternatively, the repression of *KNOX* genes by *AS2* might be indirect. In certain contexts, *KNOX* genes appear to be coexpressed with *AS1* and/or *AS2*, which suggests that the mechanism of repression is indirect. In *fil yab3* double mutants, *KNOX* genes are misexpressed in the leaf, whereas *AS1* expression is unaffected (Kumaran et al., 2002) and *AS2* expression is upregulated (Figure 7C). These findings indicate that *AS1* and *AS2* are not sufficient to repress *KNOX* gene expression in this mutant background. In addition, in tomato, the domain of expression of *Le-PHAN*, an *AS1*/*PHAN*/*rs2* ortholog, overlaps with the expression domain of a class-I *KNOX* gene in the meristem (Koltai and Bird, 2000).

Relationship between *AS2* and *AS1*

Genetic data indicate that *AS1* and *AS2* function together to regulate leaf morphogenesis (Semiarti et al., 2001; Byrne et al., 2002; Iwakawa et al., 2002). Consistent with this idea, the majority of the phenotypes associated with *AS2* overexpression, including leaf polarity defects and *BP* repression, are suppressed in an *as1* mutant background. The expression patterns of *AS1* and *AS2* were not identical in wild-type tissues, because *AS2* was more abundant than *AS1* in some tissues and vice versa. Furthermore, *AS1* is expressed throughout the leaf blade (Byrne et al., 2000), whereas *AS2* expression may be adaxially restricted (Iwakawa et al., 2002). In *35S*:*AS2* plants, *AS1* and *AS2* transcripts may be colocalized in cells that do not normally express both genes. The discrete expression patterns of *AS1* and *AS2* may indicate that, in addition to shared functions, each gene plays a distinct role in plant development. In this regard, it is interesting that transgenic plants overexpressing *AS1* do not resemble *35S*:*AS2* plants (Theodoris et al., 2003). Overexpression of *AS1* resulted in the formation of leaves that were narrower than wild-type leaves and that expressed a *BP*:*GUS* reporter at wild-type levels (Theodoris et al., 2003). Thus, *AS1* appears to function somewhat differently than *AS2*.

AS2 transcripts were detected on the adaxial face of embryonic cotyledons (Iwakawa et al., 2002), consistent with a role in adaxial specification. *AS2* transcripts were more abundant in the leaf petiole than in the blade, suggesting that *AS2* might be expressed predominantly at the base of the leaf. The related *LOB*-domain gene *LOB* also is expressed at the adaxial leaf base, which suggests a similar role for *LOB* in leaf polarity (Shuai et al., 2002). *AS2* positively regulates *LOB* expression (Byrne et al., 2002), which further implicates *LOB* in the regulation of polarity. *LOB* transcript abundance, however, was not affected in *35S*:*AS2* seedlings (data not shown), indicating that *AS2* is not sufficient to induce *LOB* expression. Ectopic expression of *LOB* led to the formation of small leaves that were curled upward but showed no obvious polarity defects (Shuai et al., 2002). Therefore, if *LOB* plays a role in the establishment of adaxial-abaxial polarity, additional factors must be required.

AS2 encodes a LOB-domain protein of unknown function. The LOB domain contains a predicted coiled coil that may function in protein–protein interactions (Shuai et al., 2002). The presence of a coiled-coil domain suggests that AS2 might function via interactions with one or more additional proteins. One candidate interacting protein is AS1, although the nonidentical expression patterns of *AS1* and *AS2* suggest that their functions may overlap only partially.

AS2 and Lateral Organ Development

Overexpression has revealed a role for *AS2* in the regulation of adaxial-abaxial polarity in lateral organs and has provided confirmation for the role of *AS2* in *KNOX* gene repression. The relationship between adaxial-abaxial polarity control and *KNOX* gene repression is not clear, although there does not appear to be a direct connection. *KNOX* genes also are repressed by members of the *YABBY* gene family (Kumaran et al., 2002). *YABBY* genes are expressed early in leaf development and are important regulators of abaxial identity. In *fil yab3* double mutants, which are adaxialized, *KNOX* genes are derepressed (Kumaran et al., 2002). Conversely, in *35S*:*AS2* seedlings, which also are adaxialized, *KNOX* genes are repressed. The opposite effects on *KNOX* gene expression in these different backgrounds suggest that there is no direct relationship between adaxial-abaxial polarity control and *KNOX* gene regulation. Thus, *KNOX* gene repression and adaxial promotion may be two distinct and unrelated functions of *AS2*. Genes that are important for polarity determination also are expressed in the early leaf, and many early leaf genes may function to repress *KNOX* genes. *KNOX* genes negatively regulate gibberellin biosynthesis (Sakamoto et al., 2001; Hay et al., 2002), so an important outcome of *KNOX* gene repression in the leaf is the upregulation of gibberellin signaling pathways, leading to differentiation. *KNOX* genes also are misexpressed in the *blade-on-petiole1* (*bop1*) mutant (Ha et al., 2003). The molecular identity of the *BOP1* gene has not been reported, but it should shed additional light on the important process of *KNOX* gene repression in the leaf.

Many of the factors known to play roles in determining adaxial-abaxial polarity are putative transcription factors that are unique to plants (Siegfried et al., 1999; Eshed et al., 2001; Kerstetter et al., 2001; McConnell et al., 2001). The process of polarity establishment appears to be highly redundant, because most if not all genes that function in this process act redundantly (reviewed by Bowman et al., 2002). *AS2* also appears to act redundantly to regulate organ polarity, a function that was not revealed by loss-of-function mutations. This finding suggests the importance of ectopic expression and overexpression experiments in functional studies. It will be important to investigate the related *LBD* genes for possible overlapping functions with *AS2*.

METHODS

Plant Material, Growth Conditions, and Transformation

Arabidopsis thaliana plants were grown as described previously (Shuai et al., 2002). Binary T-DNA vectors were introduced into *Agrobacterium tumefaciens* strain GV3101 (Koncz and Schell, 1986). Arabidopsis plants were transformed by floral dip (Clough and Bent, 1998). The *35S*:*AS2* transgene was introduced into the wild-type Columbia ecotype or into *as1-1* mutant plants in a Landsberg *erecta* background. The *BP*:*GUS* reporter was in a Columbia background (Ori et al., 2000), and the *KNAT2*:*GUS* reporter was in the C-24 ecotype (Dockx et al., 1995). Transformants were selected on Murashige and Skoog (1962) medium containing 50 μM glufosinate ammonium (Crescent Chemical, Islandia, NY) or on soil by spray-

ing with Finale (1000-fold dilution; AgrEvo Environmental Health, Montvale, NJ). Spraying was initiated at 9 days after germination and was performed every 2 days for 6 days.

Constructs

For the overexpression of *AS2*, an EcoRI-SacI fragment containing the *35S* promoter of *Cauliflower mosaic virus* and a XbaI-HindIII fragment containing the *OCTOPINE SYNTHASE* transcription terminator were excised from SLJ4D4 (Jones et al., 1992) and cloned into binary vector pCAMBIA3300 (www.cambia.org) to generate plasmid pCL0011. The *AS2* coding region was amplified from EST clone VBVYB03 with primers LBD6-F (5′-ATTTCCCCTCTGAGCAACAG-3′) and LBD6-R (5′-AAGACGGATCAACAGTACGG-3′) under the following conditions: denaturation at 94°C for 3 min, followed by 24 cycles of 94°C for 45 s, 56°C for 45 s, and 72°C for 1 min using Ex-Taq Polymerase (Takara, Shiga, Japan) under conditions specified by the manufacturer. The amplified fragment was cloned into the pGEM-T Easy vector (Promega, Madison, WI), sequenced to verify its integrity, and subcloned subsequently into pCL0011 to create the *35S:AS2* construct.

Expression Analyses

Total RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA). RNA gel blot hybridizations were performed as described previously (Martienssen et al., 1989). For reverse transcriptase–mediated PCR studies, cDNA was synthesized from 2 μg of total RNA using an oligo(dT) primer and Superscript Reverse Transcriptase (Invitrogen). One-twentieth volume of each cDNA was used as a template for PCR amplification. The gene-specific primers and PCR conditions were as described below. PCR conditions for *BP* amplification were as follows: denaturation at 94°C for 3 min, followed by 15 cycles of 94°C for 45 s, 64°C for 45 s, and 72°C for 1 min, using primers KNAT1-F (5′-GCTCCACCTGATGTGGTTGA-3′) and KNAT1-R (5′-TGTTGAGGATGTGAATGGGA-3′). PCR conditions for *KNAT2*, *KNAT6*, *STM*, and *WUS* amplification were as follows: denaturation at 94°C for 3 min, followed by 15 cycles of 94°C for 45 s, 57°C for 45 s, and 72°C for 1 min. Gene-specific primers were KNAT2-F (5′-ACCACCGGAGACAATCAAAG-3′) and KNAT2-R (5′-TCCGCTGCTATGTCATCATC-3′) (Byrne et al., 2000); KNAT6-F (5′-TGGCAGACTCGACACCAGTA-3′) and KNAT6-R (5′-CCGGTGAAAATCGTGTCTCT-3′); STM-F (5′-TTAGGGAGCCTCAAGCAAGA-3′) and STM-R (5′-TACAAACTGCATGTCCTCCG-3′); and WUS-5 (5′-GAATCAAACACACATGGAGC-3′) and WUS-3 (5′-AGAGGAAGCGTACGTCGATG-3′).

Primers and amplification conditions for *LOB* and *ACT2* were as described previously (Shuai et al., 2002). *KAN1*, *KAN2*, *FIL*, *YAB3*, and *PHB* cDNAs were amplified with the gene-specific primers KANADI1-5 (5′-ACAACAACGCTTACCGATCA-3′) and KANADI1-R (5′-ATTTCTCGTGCCAATCTGGT-3′); KANADI2-5 (5′-TCATGCCAAGATTCCCAG-3′) and KANADI2-R (5′-TTAGTGAGATCGACCCAGAG-3′); FIL-3 (5′-GCTATGTCCAATGCAACTTT-3′) and FIL-4 (5′-TTCTTGGCAGCAGCACTAAA-3′); YAB3-5 (5′-ACTTCTCATCTACGGACCAG-3′) and YAB3-R (5′-TCAGCCATGAGTCCAAAGTG-3′); and PHB-5 (5′-TGATGGTCCATTCGATGAGC-3′) and PHB-3 (5′-TCTAAACTCACGAGGCCGCA-3′). PCR conditions for these genes were as follows: 94°C for 3 min, followed by 15 cycles of 94°C for 45 s, 55°C for 45 s, and 72°C for 1 min. One-half of each PCR sample was blotted and hybridized as described previously (Springer et al., 1995). Exposure times varied depending on the experiment, and for this reason, expression levels cannot be compared directly between experiments.

Histology and Microscopy

Tissue samples were fixed and processed for scanning electron microscopy as described previously (Shuai et al., 2002). For cross-sections, leaves were fixed in 2.5% (v/v) glutaraldehyde (Electron Microscopy Sciences, Fort Washington, PA) in 0.1 M phosphate buffer, pH 6.8. Tissue was placed under vacuum in fixation solution until it sank and then fixed overnight at 4°C. Leaves were rinsed with phosphate buffer several times and dehydrated through an ethanol series up to 95% ethanol. The ethanol was replaced gradually by historesin (Leica Microsystems, Wetzlar, Germany) through a series of ethanol/historesin mixtures up to 100% historesin. Tissue remained in 100% historesin overnight. All steps before polymerization were performed at 4°C. Resin was polymerized at room temperature. Three-micrometer sections were cut, mounted on glass slides, and stained with 0.5% toluidine blue. *35S:AS2 BP:GUS* and *35S:AS2 KNAT2:GUS* plants were selected, stained for GUS activity, and imaged as described previously (Shuai et al., 2002).

Upon request, materials integral to the findings presented in this publication will be made available in a timely manner to all investigators on similar terms for noncommercial research purposes. To obtain materials, please contact P. Springer, patricia.springer@ucr.edu.

ACKNOWLEDGMENTS

We thank Kathleen Eckard for help with sample embedding and sectioning and Mary Byrne for sharing primer sequences and other unpublished data, *as1* and *as2* mutant seeds, and for helpful discussions. We thank Benjamin Aronson and Elizabeth Bell for critical reading of the manuscript. We thank Sarah Hake for providing *BP:GUS* seeds, Jan Traas for providing *KNAT2:GUS* seeds, John Bowman for providing *kan1 kan2* mutant seeds, and Venkatesan Sundaresan for providing *fil-8 yab3-2* double mutant seeds. *phb-1d* mutant seeds were provided by the ABRC (Columbus, OH). This work was supported National Science Foundation Grant IBN-9875371 to P.S.S.

Received June 27, 2003; accepted August 12, 2003.

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