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The *ASYMMETRIC LEAVES2* gene of *Arabidopsis thaliana* regulates formation of a symmetric lamina, establishment of venation and repression of meristem-related homeobox genes in leaves

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SUMMARY

The *asymmetric leaves2* (*as2)* mutant of *Arabidopsis thaliana* generated leaf lobes and leaflet-like structures from the petioles of leaves in a bilaterally asymmetric manner. Both the delayed formation of the primary vein and the asymmetric formation of secondary veins were apparent in leaf primordia of *as2* plants. A distinct midvein, which is the thickest vein and is located in the longitudinal center of the leaf lamina of wild-type plants, was often rudimentary even in mature *as2* leaves. However, several parallel veins of very similar thickness were evident in such leaves. The complexity of venation patterns in all leaf-like organs of *as2* plants was reduced. The malformed veins were visible before the development of asymmetry of the leaf lamina and were maintained in mature *as2* leaves. In vitro culture on phytohormone-free medium of leaf sections from *as2* mutants and from the *asymmetric leaves1* (*as1*) mutant, which has a phenotype similar to that of *as2*, revealed an elevated potential in both cases for regeneration of shoots from leaf cells. Analysis by the reverse transcription-polymerase chain reaction showed that transcripts of the *KNAT1*, *KNAT2* and *KNAT6* (a recently identified member of the class 1 *knox* family) genes accumulated in the leaves of both *as2* and *as1* plants but not of wild type. Transcripts of the *STM* gene also accumulated in *as1* leaves. These findings suggest that, in leaves, the *AS2* and *AS1* genes repress the expression of these homeobox genes, which are thought to maintain the indeterminate cell state in the shoot apical meristem. Taken together, our results suggest that *AS2* and *AS1* might be involved in establishment of a prominent midvein and of networks of other veins as well as in the formation of the symmetric leaf lamina, which might be related to repression of class 1 *knox* homeobox genes in leaves.

Key words: *Arabidopsis thaliana*, *asymmetric leaves1*, *asymmetric leaves2*, *knox* homeobox genes, Leaf morphology, Venation pattern, Midvein, Shoot, Apical meristem

INTRODUCTION

The left-right symmetry of most living things has been of general interest not only to biologists but also to researchers in other fields. In contrast to animals that have developed bodies with left-right symmetry, which is believed to be tightly linked to the capacity for mobility, plants are basically immovable and their overall body shapes exhibit conic symmetry (Gardner, 1990). It is, nonetheless, generally accepted that leaves of many angiosperms exhibit obvious but approximate left-right symmetry with the rachis as the axis (Hickey, 1973; Hickey, 1979; Sinha, 1999), even though exceptions exist, such as *Begonia* spp. (Lieu and Sattler, 1976), *Tropaeolum* (Whaley and Whaley, 1942) and many species of *Urticaceae* (Dengler, 1999). Regardless of the complexity of leaf shape (e.g., a simple leaf or a compound leaf), the two sides of each leaf are nearly mirror images of one another (Ogura, 1962; Hickey, 1973; Hickey, 1979).

Leaves develop from a shoot apical meristem (SAM) along three-dimensional axes (the proximodistal, transverse and adaxial-abaxial axes; Steeves and Sussex, 1989; Waites et al., 1998), as well as with left-right symmetry. Various mutants with altered leaf morphology that is related to abnormal expansion along these axes, altered adaxial and abaxial identity, and the altered overall shape of leaves have been isolated, and some of the genes responsible for the mutant phenotypes have been cloned and characterized (Hake et al., 1989; Conway and Poethig, 1997; Höfer et al., 1997; Waites et al., 1998; Kim et al., 1998; Berná et al., 1999; Serrano-Cartagena et al., 1999; Timmermans et al., 1999; Tsiantis et al., 1999; Sawa et al., 1999; Siegfried et al., 1999). However, our understanding of the way in which the nearly mirror-image

architecture arises during leaf development remains at a descriptive level (see below) and the molecular and genetic basis for this phenomenon remains to be analyzed. Previous studies using *Arabidopsis thaliana* have focused on two aspects of leaf symmetry. It has been demonstrated that the number and the positions of the serrations on the margin of a leaf lamina are bilaterally symmetric (Tsukaya and Uchimiya, 1997). It has also been demonstrated that venation patterns in the leaf lamina of *Arabidopsis* are bilaterally symmetric (Candela et al., 1999). A single primary vein, the midvein, is the thickest vein and is located at the center of the leaf lamina (Hickey, 1973; Hickey, 1979; Kinsman and Pyke, 1998). During the development of the leaf from the SAM, the primary vein grows acropetally in the center of the leaf lamina. The primary vein bifurcates at the tip to form secondary veins that elongate basipetally toward the primary vein. Additional secondary veins differentiate as the lamina expands and they are connected almost symmetrically to each side of the primary vein (Hickey, 1973; Hickey, 1979; Kinsman and Pyke, 1998; Poethig, 1997; Nelson and Dengler, 1997; Candela et al., 1999).

The cells of the SAM resemble stem cells in that they have the capacity for self-regeneration and remain in an undifferentiated state, but the SAM can also generate leaf primordia from its peripheral zone (Steeves and Sussex, 1989; Howell, 1998). The *SHOOT MERISTEMLESS* (*STM*) gene, a member of the family of class 1 *knox* homeobox genes, is required for the development of the SAM, as well as for the maintenance of stem-cell identity throughout the life of the plant (Barton and Poethig, 1993; Long et al., 1996). The *WUS* gene is another type of homeobox gene that also plays an important role in the maintenance of stem-cell identity in the SAM (Laux et al., 1996; Mayer et al., 1998) and it affects heteroblastic leaf development in *Arabidopsis* (Hamada et al., 2000). The expression of the *STM* gene is down-regulated in the presumptive region of initiation of a leaf primordium in the peripheral zone of the SAM, and such down-regulation appears to be crucial for development of the leaf primordium (Long et al., 1996; Lynn et al., 1999; Long and Barton, 2000). The genome of *Arabidopsis* includes other members of the family of class 1 *knotted*-like homeobox (*knox*) genes, *KNAT1* and *KNAT2* (also known as *ATK1*), and the transcripts of these genes are localized primarily in the region around the SAM and the floral meristem, with down-regulation of expression in the presumptive region of initiation of a leaf primordium (Kerstetter and Poethig, 1998). The roles of *KNAT1* and *KNAT2* remain to be established but studies of the ectopic overexpression of these genes in *Arabidopsis* have shown that leaf cells can be converted from the meristematic indeterminate state to the determinate state, and back again, depending on the levels of expression of these genes and, moreover, that their levels of expression are closely related to the extent of leaf serration or formation of lobes (Sinha et al., 1993; Lincoln et al., 1994; Chuck et al., 1996; Serikawa and Zambryski, 1997). Similar observations have been made in other plant species in studies of class 1 *knox* genes (Hareven et al., 1996; Parnis et al., 1997; Nishimura et al., 2000).

As part of our efforts to clarify the mechanisms responsible for the development of symmetrical leaves, we have taken advantage of the *asymmetric leaves2* (*as2*) mutant of *A. thaliana*, which was originally isolated by Rédei (ABRC, Ohio). Another similar mutant, *asymmetric leaves1* (*as1*), was also identified by Rédei and it produced leaves with distorted bilateral symmetry (Rédei and Hirono, 1964; Tsukaya and Uchimiya, 1997). In the present study, we analyzed the phenotype of the *as2* mutant, focusing on patterns of serration, formation of lobes and venation in leaves and leaf-like organs. We found that leaf serration in *as2* was asymmetric, with generation of leaflet-like structures from petioles and malformed entire vein systems. An examination of levels of transcripts of class 1 *knox* genes revealed that *KNAT1* and *KNAT2* mRNAs, which are normally present only in the SAM, accumulated ectopically in leaves of *as2* and *as1* plants, and that *STM* mRNA accumulated in leaves of *as1* plants. In addition, leaves of these mutants exhibited an elevated potential for regeneration of shoots from leaf cells in vitro without exogenous phytohormones, supporting the hypothesis that leaf cells in *as2*, and also in *as1*, have characteristics typical of an enhanced indeterminate state. Thus, it seems possible that a close correlation might exist between lamina formation, the establishment of the venation, including the midvein, and the determinate state of leaf cells.

MATERIALS AND METHODS

Plant strains and growth conditions

Arabidopsis thaliana ecotypes Col-0 (CS1092) and En-2 (CS1138) and mutants *as2-1* (CS3117), *as2-2* (CS3118) and *as1-1* (CS3374) were obtained from the Arabidopsis Biological Resource Center (Columbus, OH, USA; ABRC; Table 1). L*er*-0 (NW20) and *as2-4* (N463) were obtained from the Nottingham Arabidopsis Stock Centre (Nottingham, UK; NASC; Table 1). The *as2-1*, *as2-2* and *as1-1* alleles are X-ray-induced alleles that were isolated from the ER, ER and Col-1 ecotypes, respectively. The mutants are classified as 'Form Mutants' in the G. P. Rédei collection. The *as2-1* and *as1-1* alleles were mapped to chromosomes 1 and 2, respectively (Fabri and Schäffner, 1994; Machida et al., 1997). Although it was reported that the *as2-1* mutant was isolated from the ER ecotype, our analysis of restriction fragment length polymorphism (RFLP) showed that the background coincided with the Col-0 background (data not shown). We outcrossed *as2-1* with Col-0 three times and *as1-1* with Col-0 once and used the progeny for experiments. The *as2-4* (N463) allele was isolated in the En-2 ecotype by Dr Zhuchenko (NASC). Tsukaya and Uchimiya (Tsukaya and Uchimiya, 1997) designated the N463 mutation *as2-2*, while Rédei gave the designation *as2-2* to another allele (ABRC). To avoid confusion, we changed the designation of N463 to *as2-4*. An M2 population of ethylmethanesulfonate-mutagenized L*er*-0 seeds was purchased from Lehle Seeds (Round Rock, TX, USA), and we isolated the *as2-5* allele from this population as line no. E56 (Table 1). The *as2-4* and *as2-5* lines were outcrossed once with En-2 and L*er*-0, respectively. For analyses of phenotypes, seeds were sown on soil, and after 2 days at 4°C in darkness, plants were transferred to a regimen of white light at 3,000 lux for 16 hours and darkness for 8 hours at 22°C. Ages of plants are given in terms of numbers of days after sowing.

Table 1. The various *as2* and *as1* alleles

Allele	Mutagen	Ecotype	Source
as2-1	X rays	ER*	ABRC (CS3117)
as2-2	X rays	ER*	ABRC (CS3118)
as2-4	Unknown	En-2	NASC (N463), Tsukaya and Uchimiya (1997)
as2-5	EMS	L*er*	This article
as1-1	X rays	Col-1	ABRC (CS3374), Rédei and Hirono (1964)

*Our RFLP analysis showed that the background coincided with the Col-0 ecotype.

Histological and vasculature analysis

Plant materials were prepared for sectioning by the procedure described by Nakashima et al. (Nakashima et al., 1998). To determine numbers of branching points (NBPs), leaves from 23-day-old plants were treated as described by Hamada et al. (Hamada et al., 2000).

Scanning electron microscopy

Leaves were pasted on a brass stage and plunged into nitrogen slush at −210°C for 30 seconds for fixation of tissue. The stage was transferred to the chamber of a scanning electron microscope (Philips, Eindhoven, Holland) and each sample was viewed at an operating voltage of 5 kV.

RNA gel blot analysis

Seedlings of *as1-1* and *as2-1* mutants and wild-type (Col-0) were harvested 12 days after sowing. Total RNA from seedlings was isolated and northern blotting was performed as described previously (Banno et al., 1993). To prepare the *KNAT1* probe, a 566 bp fragment, corresponding to the 5′ portion of *KNAT1* cDNA (Lincoln et al., 1994) was generated by *Sal*I and *Xba*I cleavage of a plasmid that contained *KNAT1* cDNA and was labeled with [α-^{32}P]dCTP using a High Prime DNA Labeling Kit (Boehringer Mannheim Biochemica, Mannheim, Germany) according to the manufacturer's instructions.

In situ hybridization

Details of methods used for fixation of plants, embedding in paraffin and in situ hybridization can be found at http://www.wisc.edu/genetics/CATG/barton/index.html and were described previously (Nakashima et al., 1998). Sections (8 μm thick) were cut with a microtome (ERMA Inc., Tokyo, Japan). *KNAT1* cDNA was amplified by PCR with primer 1 (5′GGGTCGACATGGAAGAATACCAGC-ATGACAACAG3′) and primer 2 (5′GGGCGGCCGCTTATGGA-CCGAGACGATAAGG-TCC3′), cloned into the *Sal*I-*Not*I sites of pBluescriptII SK(−) (Stratagene, La Jolla, CA, USA) and its identity was confirmed by nucleotide sequencing. Both antisense and sense probes were prepared from the cloned *KNAT1* cDNA clones.

Reverse transcription-polymerase chain reaction (RT-PCR)

Leaves and shoot apices were harvested 19 days after sowing, and then frozen immediately in liquid nitrogen and stored at –80°C. Poly(A)$^+$ RNA was purified and the first strand of cDNA was synthesized. Sample volumes were normalized for equal amplification of DNA fragments with primers specific for α-tubulin cDNA. Then, PCR was performed under the same conditions except that the primers were specific for cDNAs of homeobox genes. For semi-quantitation of mRNAs, we examined DNA fragments that had been amplified during increasing numbers of cycles during a series of PCRs. Under the conditions that we used, the amount of PCR products increased quantitatively by a factor of two during single reactions for up to 30 cycles. However, the rate of amplification gradually decreased after 30 cycles (data not shown). The products of PCR were analyzed by agarose-gel electrophoresis and sequenced directly using one of the primers used for amplification. To distinguish products amplified from mRNAs from those generated from contaminating genomic DNA, we selected sites for the design of primer sets in two regions that are separated by introns in the cognate genes. The primer pairs were as follows: for α-tubulin, pU51 (5′-GGACAAGCTGGGATCCAGG-3′) and pU52 (5′-CGTCTCCACCTTCAGCACC-3′); for KNAT1, pU13 (5′-ATGGAAGAATACCAGCATGACAAC-3′) and pU28 (5′-GATGATCCCATATTGTCACTCTTCCC-3′); for KNAT2, pU85 (5′-GCGGCGATCACTGATCGTATC-3′) and pU86 (5′-GCGGCG-ATCACTGATCGTATC-3′); for STM, pU87 (5′-CAAAGCATGG-TGGAGGAGATGTG-3′) and pU88 (5′-GGAGAGTGGTT-CCAACAGCAC-3′); for WUS, pU31 (5′-GTGAACAAAAGTCGA-ATCAAACACACATG-3′) and pU34 (5′-GCTAGTTCAGACG-TAGCTCAAGAG-3′); and for KNAT6, pU161 (ATGTACAAT-TTCCATTCGGCCGGTG) and pU162 (TCATTCCTCGGTAA-AGAATGATCCACTAG). Details of the procedure that we used here will be sent on request.

Culture of leaf sections in vitro

Rosette leaves of 19- to 21-day-old plants were halved and incubated on plates of Murashige and Skoog (MS) basic medium (Onouchi et al., 1995) at 22°C under continuous white light.

RESULTS

Prominent leaf lobes, leaflet-like structures and short petioles in *as2* plants

Fig. 1 shows typical leaf phenotypes. In terms of overall shape, the *as2* leaf often had many deep and irregularly split lobes; the leaf lamina was often plump and humped at its base, the leaf surface was wavy and plants had leaflet-like structures on petioles, which were relatively short. We also examined the morphology and growth rates of roots, hypocotyls and inflorescence stems but we found no significant differences among these organs between *as2-1* and wild-type plants (data not shown).

Leaf lobes and leaflet-like structures

The positions of the deep lobes on *as2-1* rosette leaves corresponded to those of the serrations on wild-type rosette leaves, as judged from their positions relative to those of hydathodes on the respective rosette leaves (data not shown). As shown in Table 2, regardless of the age of plants, leaf lobes were usually observed on leaves at positions higher than the second rosette leaf. As the plants matured, the proportion of leaves with lobes at each leaf position increased. However, no lobes were observed on the first and second leaves, namely, the juvenile leaves of *as2-1* plants. Even though they had no lobes, these

Table 2. Positions and proportions of leaves of *as2-1* plants that had lobes and leaflet-like structures, as recorded 23 days, 39 days and 60 days after sowing

	Plants examined		Leaf number						
	Age*	Number	1	2	3	4	5	6	7
Leaf lobe	23	14	0 (0%)	0 (0%)	5 (36%)	6 (43%)	11 (78%)	10 (71%)	12 (85%)
	39	30	0 (0%)	0 (0%)	21 (70%)	23 (76%)	29 (97%)	28 (93%)	29 (97%)
	60	14	0 (0%)	0 (0%)	12 (86%)	13 (93%)	14 (100%)	14 (100%)	14 (100%)
Leaflet-like structure	23	14	0 (0%)	0 (0%)	3 (21%)	1 (7%)	6 (43%)	4 (28%)	6 (43%)
	39	30	0 (0%)	0 (0%)	18 (60%)	13 (43%)	17 (57%)	15 (50%)	23 (77%)
	60	14	0 (0%)	0 (0%)	10 (71%)	11 (79%)	13 (93%)	12 (86%)	13 (93%)

*Age of plants is indicated by days after sowing. Percentages indicate frequencies with which a lobe or leaflet-like structure was observed.

leaves had obvious humps at the base of the leaf lamina (Fig. 1B,C), which also resulted in an asymmetric lamina (Fig. 2B). The juvenile leaves of wild-type plants had no obvious serrations (Poethig, 1997; Hamada et al., 2000). The allelic mutations *as2-2* and *as2-4* also generated leaf lobes at similar frequencies (80-86%), whereas *as2-5* generated them less frequently (20%), indicating that *as2-5* was a weak allele (Table 3).

As *as2-1* plants matured, they frequently produced leaflet-like structures on the petioles of the third leaf upwards (Table 2; Fig. 1E), while younger plants did so less frequently (Fig. 1C). These structures were produced on either side of the petiole but, even as the *as2-1* plants matured, their first and second rosette leaves did not generate such structures (Table 2). As shown in Table 3, the frequency of leaflet-like structures varied among *as2* mutants with different alleles (50%, 60% and 14% for the *as2-1*, *as2-2* and *as2-4* alleles, respectively). No such abnormal structures were found on *as2-5* leaves (Fig. 1M; Table 3). The transheterozygote *as2-1*/*as2-4* produced leaflet-like structures at a low frequency (20%; Table 3; Fig. 1K), resembling the *as2-4* homozygote and indicating that *as2-4* was a weak allele.

Lengths of petioles

Petioles of *as2-1* and *as2-2* leaves were always shorter than those of the wild type (Table 3). There were, however, no significant differences between *as2-1* and the wild type in terms of the length and width of the leaf lamina and the number of palisade mesophyll cells (data not shown).

The presence of a thinner midvein in *as2* leaves: asymmetric development and less efficient connections of secondary veins to the midvein

Patterns of venation

We analyzed the venation in rosette leaves of *as2-1* and wild-type plants by dark-field microscopy. The conditions used allowed tracheary elements with lignin to be visualized (Telfer and Poethig, 1994). In the wild type, there was a single, distinct and maximally thick midvein in the center of each leaf lamina and a number of thinner secondary veins were connected to the midvein (Fig. 2A). The severity of the effect of the *as2* mutation on venation varied among progeny, even when we considered progeny from a particular parental plant with a specific *as2* allele (Fig. 2B-D). In extreme cases, no midvein was obvious, and several veins of similar thickness were evident with a proximodistal orientation (Fig. 2B,C). In less extreme cases, a midvein was present in the center of the leaf lamina but was thinner than the wild-type midvein even when fully matured rosette leaves were compared (Fig. 2A,D). In both mild and extreme cases, several secondary veins failed

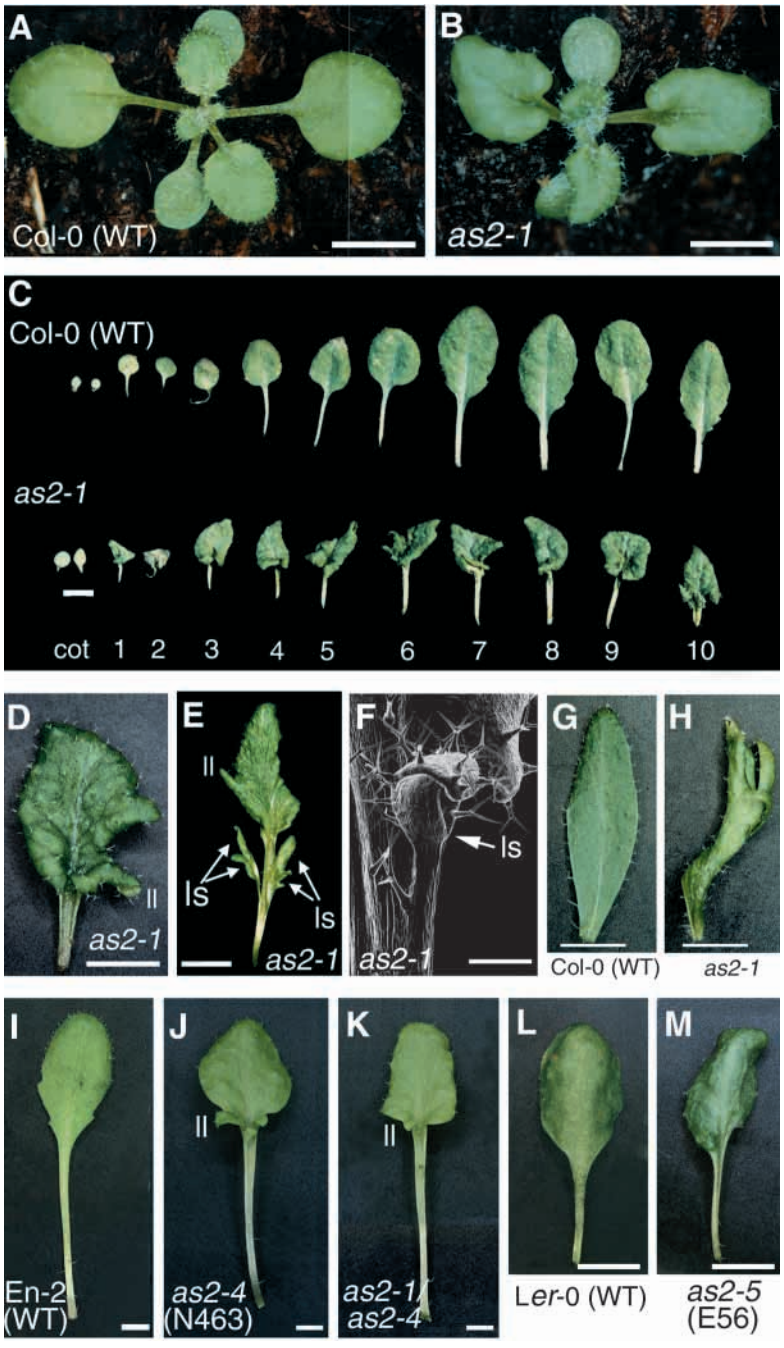


Fig. 1. The typical phenotype of leaves of *as2* mutant plants. (A,B) The overall morphology of wild-type Col-0 and *as2-1* plants. The morphology of each leaf of the wild type is compared to that of *as2-1* in C. Cotyledons and the first to the tenth rosette leaves of a wild-type (upper) and an *as2-1* (lower) plant are shown. (D-F,H) The abnormal phenotype of the *as2-1* mutant. Two types of fifth leaf of *as2-1* plants are shown in D and E. A deep leaf lobe (ll) is shown in D and the arrows in E point to leaflet-like structures (ls) at the petiole. (F) A scanning electron micrograph of the fifth leaf of an *as2-1* plant, at the proximal region of the leaf blade, shows the base of a leaflet-like structure (ls) that is emerging from the petiole. Cauline leaves of Col-0 wild type and *as2-1* are shown in G and H, respectively. (I-M) Phenotypes due to other alleles of *as2* that were seen in the fifth leaves of (I) En-2 wild type (WT), (J) *as2-4* (N463), (K) transheterozygous *as2-1/as2-4*, (L) L*er*-0 wild type (WT) and (M) *as2-5* (E56). The shapes of rosette leaves of *as2-4* (J) and *as2-5* (M) were less severely affected than those of the *as2-1* mutant (C-E; see also text). The photographs were taken 18 days (A,B), 23 days (C,D,F,I-K), 32 days (G,H), 39 days (L,M) and 60 days (E) after sowing. Bars, 5 mm in all panels except F (500 μm).

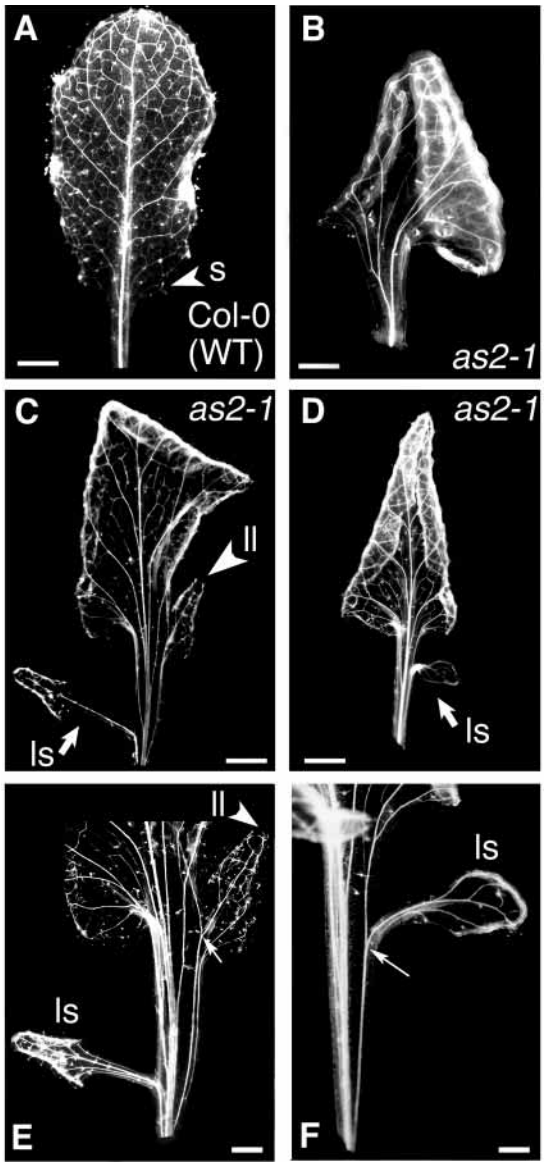


Fig. 2. Patterns of leaf venation in wild-type and *as2* leaves. Dark-field views of cleared rosette leaves of 26-day-old wild-type (A) and *as2-1* (B-F) plants. The fifth leaf of Col-0 wild type (A) and the second (B), fifth (C) and seventh (D) rosette leaves of *as2-1* are shown. Arrowheads in A and C and E indicate a serration(s) and a large leaf lobe (ll) in the wild type and *as2-1*, respectively. Arrows in C and D indicate leaflet-like structures (ls) at the petioles of rosette leaves in *as2-1*. (E,F) Higher magnification views of C and D. Arrows in E and F indicate connections between the central vein of a leaf lobe and leaflet-like structure and the secondary vein of the main rosette leaves. Bars, 1.5 mm.

to connect with the midvein in the leaf lamina and sometimes they ran separately through the petiole (Fig. 2B-F). The venation in each *as2* leaf lamina was bilaterally asymmetrical. Moreover, veins at the leaf margin were often insufficiently interconnected or were disconnected from one another (data not shown). The leaf lobes and leaflet-like structures all had single central veins, which were usually connected to a secondary vein in the basal region of the leaf lamina or the petiole (Fig. 2E,F, arrows). In no cases were the veins from

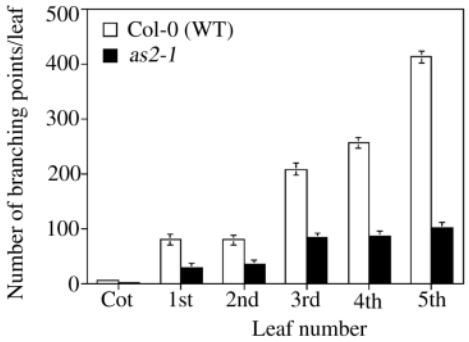


Fig. 3. The effects of the *as2* mutation on the complexity of leaf venation. The numbers of branching points (NBPs) of leaf veins were counted in 23-day old wild-type (WT; open bars) and *as2-1* (filled bars) plants. The results are averages of values obtained from 12 leaves. Error bars indicate s.d.

Table 3. The shapes of leaves and the lengths of petioles of the third leaves of *as2* mutant plants, as recorded 39 days after sowing

Genotype	Number of plants examined	Leaf lobe	Leaflet-like structure	Length of petiole (mm)
+/+ (Col-0)	10	0 (0%)	0 (0%)	14.5±0.76
as2-1	10	7 (70%)	5 (50%)	12.1±0.74
as2-2	10	8 (80%)	6 (60%)	11.8±1.03
+/+ (En-2)	5	0 (0%)	0 (0%)	19.1±1.06
as2-4	7	6 (86%)	1 (14.3%)	19.2±1.52
as2-1/as2-4	5	4 (80%)	1 (20%)	20.2±0.29
+/+ (L*er*-0)	10	0 (0%)	0 (0%)	7.9±0.53
as2-5	10	2 (20%)	0 (0%)	8.2±0.49

Percentages refer to the frequency with which a leaf lobe or leaflet-like structure was noted on the third leaf, relative to the total number of plants examined. For lengths of petioles, means and s.d. are indicated.

these protrusions connected directly to the primary vein of the leaf (Fig. 2E,F), suggesting the limited developmental control of these protruding structures by the midvein.

In addition to abnormalities of the midvein and secondary veins, the patterns of venation in *as2* rosette leaves were less complex than those in wild-type rosette leaves. To quantitate the complexity of leaf venation, we counted the total number of branching points (NBPs; Hamada et al., 2000) in rosette leaves of 23-day-old *as2-1* and wild-type plants. Fig. 3 shows that the NBPs in cotyledons and in the first to fourth rosette leaves of *as2* plants were about 40% of those in the respective wild-type organs. In the fifth rosette leaves and those at higher positions, the NBPs were less than 25% of those in the wild type. The number of NBPs in *as2-1* leaves reached a plateau value in the third to fifth rosette leaves, while those in wild-type leaves increased up to the twelfth rosette leaf (data not shown). Taken together, the data suggest that the *AS2* gene might be involved in the development of the entire vein system in each rosette leaf.

Development of veins

We investigated the earliest stages of vein development in the first rosette leaves of both wild-type and *as2-1* plantlets. Fig. 4A shows the delayed development of the primary vein in *as2*.

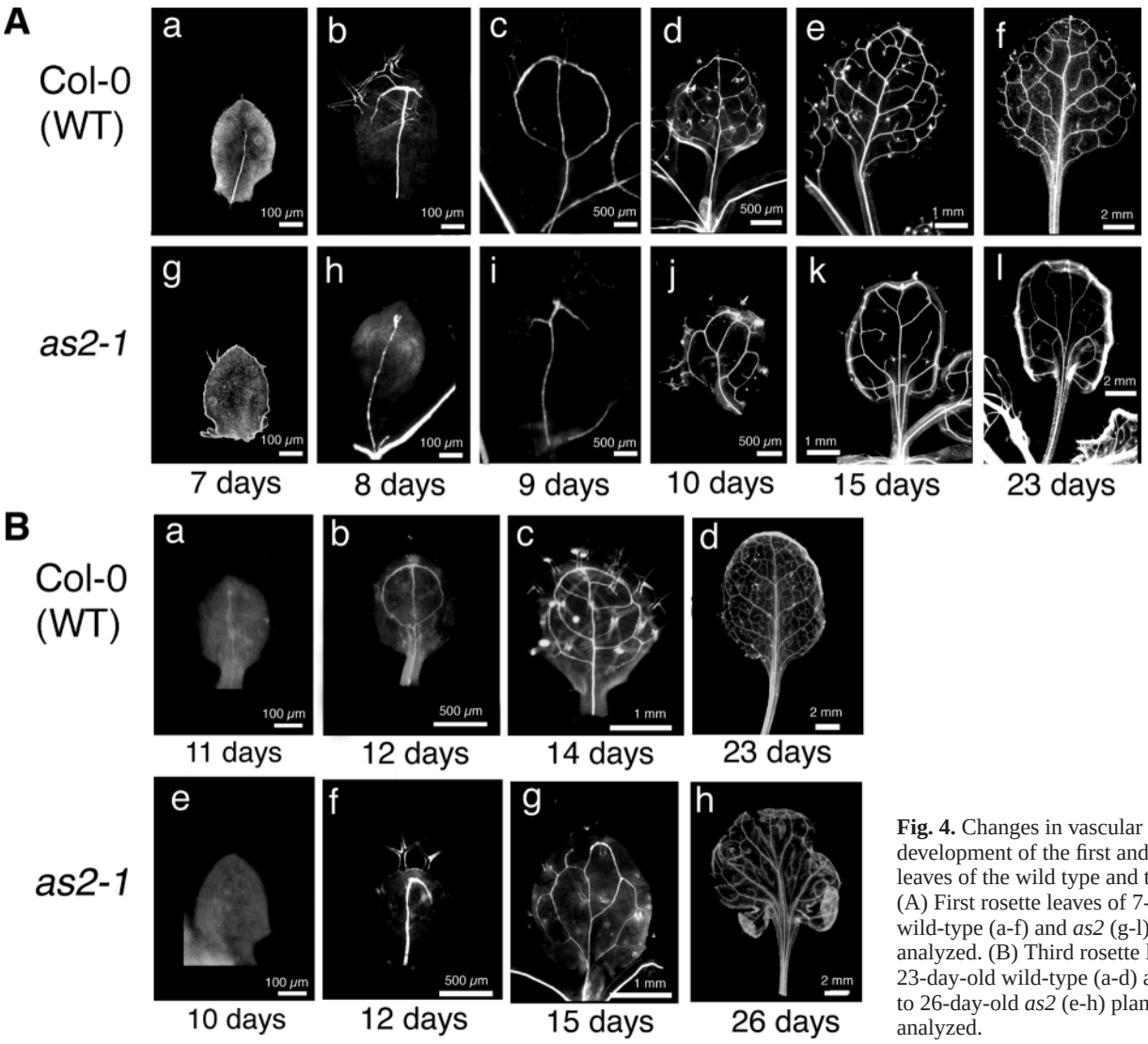


Fig. 4. Changes in vascular patterns during development of the first and third rosette leaves of the wild type and the *as2* mutant. (A) First rosette leaves of 7- to 23-day-old wild-type (a-f) and *as2* (g-l) plants were analyzed. (B) Third rosette leaves of 11- to 23-day-old wild-type (a-d) and those of 10- to 26-day-old *as2* (e-h) plants were analyzed.

In leaf primordia of most wild-type plants 7 days after sowing, primary veins were visible (Fig. 4Aa), but that was not the case in *as2-1* plants, even though the *as2-1* leaf primordia were normal in shape (Fig. 4Ag). The emergence of stipules at the base and trichomes at the top of the leaf lamina also occurred in *as2-1* plants on day 7 (data not shown). On day 8, a primary vein appeared for the first time in the *as2-1* leaf primordium (Fig. 4Ah). In the wild type, the primary vein bifurcated at its distal end on day 8, forming two strands (secondary veins) that extended basipetally (Fig. 4Ab) and connected to middle positions on the primary vein (Fig. 4Ac). By contrast, in almost all the *as2-1* primordia, the primary veins bifurcated irregularly and asymmetrically (Fig. 4Ai). The secondary veins of *as2-1* leaves developed with bilateral asymmetry, approached the primary vein at a more acute angle than in the wild type and, in some cases, did not connect with the primary vein in the leaf lamina (compare Fig. 4Ad,e,f with Aj,k,l). The development of new higher-ordered veins ceased in *as2-1* leaves by day 15 (Fig. 4Ak,l).

We performed similar analysis of vein development in the third rosette leaves of wild-type and *as2-1* plants, which often generated leaf lobes (Table 2). As shown in Fig. 4B, abnormal patterns of veins, similar to those in the first leaf, were seen throughout the development of the third leaf of *as2-1*. At the early stage of the development (Fig. 4Be,f,g), most of the *as2-1* leaf lamina was normal in shape but the secondary veins were being developed in an asymmetric manner. As the leaf developed into the late stage, deep asymmetric lobes were generated (Fig. 4Bh). We observed similar patterns of development of venation and the lamina in almost all other rosette leaves (data not shown).

While the phenotype associated with *as2-1* was apparent in the vein networks of rosette leaves, differentiation of vascular elements, such as xylem and phloem cells, was unaffected (data not shown).

Separate vascular bundles in the petiole

To examine the structural organization of the vascular bundles in *as2-1* rosette leaves, we prepared a series of transverse sections starting from the middle of first rosette leaves and continuing as far as the base of petioles of 15-day-old wild-type and mutant plants. Fig. 5 shows representative sections from both wild-type and *as2-1* rosette leaves. In the wild type, there was a single vascular bundle from the transition region

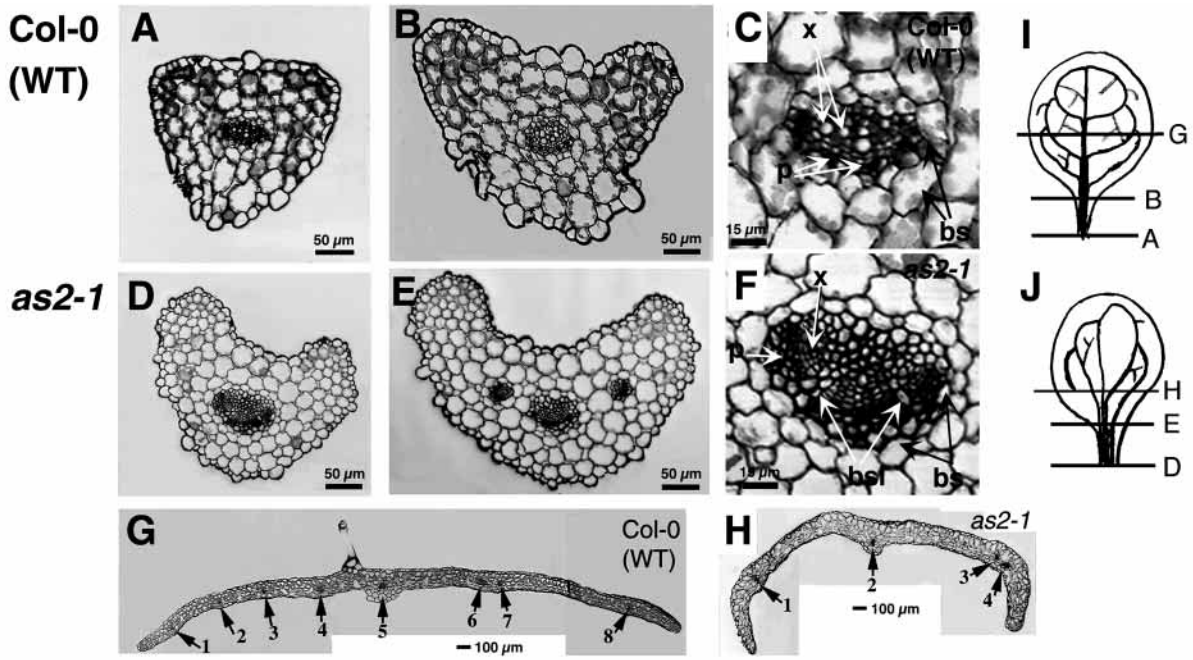


Fig. 5. Structural organization of vascular bundles in the first rosette leaves of the *as2* mutant. Transverse sections of the first rosette leaves of 15-day-old wild type (A-C,G) and *as2* (D-F,H) plants. Transverse sections of petiole near the base (A,D), the leaf blade near the petiole (B,E), and the leaf blade in the middle region (G,H). C and F show higher-magnification views of vascular bundles at the base of the wild-type petiole in A and the *as2* petiole in D, respectively. bs, Bundle sheath cell; bsl, bundle sheath-like cell; x, xylem; p, phloem. Numbers in G and H indicate the numbers of vascular bundles in leaves. I and J are drawings of the wild-type and *as2* first leaves, respectively, that show the positions of sections.

between the leaf lamina and the petiole toward the base of the petiole (Fig. 5A-C). In *as2-1* leaves, there was a wider vascular bundle in the center, with disconnected secondary vascular bundles on both sides of the wider bundle in the transition region (Fig. 5E). Even at the base of the petiole (Fig. 5D,F), the *as2-1* mutant still had a single but wider vascular bundle, which was surrounded by bundle sheath cells (Fig. 5D). Such wider bundles appeared to contain several subvascular arrays, each of which consisted of xylem and phloem and was surrounded by bundle sheath-like cells (Fig. 5F).

In the *as2-1* leaf lamina, the central vascular bundle was thinner than in the wild type but several parallel bundles that were almost equivalent in thickness to the central bundle were observed with a proximodistal orientation (Fig. 5H). By contrast, the wild-type leaf lamina had a single and distinct, central vascular bundle (Fig. 5G). Our analysis of fifth leaves revealed similar organizations of veins (data not shown).

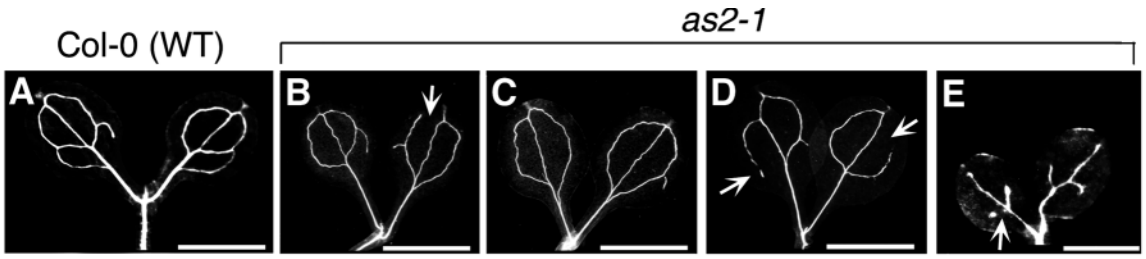
Reduced venation in *as2-1* cotyledons and reproductive leaf-like organs

Although the cotyledons of the *as2-1* mutant did not exhibit severe asymmetry, they were curled, had shorter petioles and bent downwards (data not shown). The complexity of venation in *as2-1* cotyledons was lower than that in the wild type. Fig. 6 shows typical differences between the venation of wild-type and *as2* cotyledons. We classified the complexity of the venation of cotyledons into three types on the basis of the extent of formation of loops due to the joining of primary and secondary veins (Fig. 6F): type I had three or four loops, which corresponded to NBPs of 5-7; type II had one or two loops, which corresponded to NBPs of 2-4; and type III had no loops, corresponding to NBPs of 1 or 2. The results of our analysis are summarized in Fig. 6F. Among wild-type cotyledons, 97% had NBPs of 5-7 and were classified as type I. By contrast, no *as2-1* cotyledons were of type I: 86% of *as2-1* cotyledons were type II and 14% were type III. Thus, secondary veins were less efficiently connected to the primary vein in *as2-1* cotyledons, as was also the case in the foliage leaves.

We also examined the morphology and patterns of venation of cauline leaves and leaf-like reproductive organs. The *as2-1* cauline leaves were severely lobed (Figs 1H, 7D). The relatively narrow sepals and the petals were unusually curled downward, but they were not strongly asymmetric (Fig. 7E,F). Fig. 7 shows typical patterns of venation of cauline leaves, sepals and petals. In all these organs, venation in *as2-1* was less complex than in the wild type (Fig. 7D-F). The *as2-1* sepals and petals always had open loops of veins (Fig. 7E,F) but in most of these organs in the wild type the loops were closed (Fig. 7B,C). These results showed that the development of vein networks was less extensive in the leaf-like organs of *as2-1* plants than in the wild type.

Accumulation in leaves of *as2* and *as1* plants of transcripts of meristem-related homeobox genes in the class 1 *knox* family

The morphology of *as2* and *as1* leaves was similar to that of leaves of transgenic *Arabidopsis* that ectopically express the homeobox gene *KNAT1* (see Introduction). We examined the expression of *KNAT1* by northern blotting of poly(A)$^+$ RNA prepared from 12-day-old wild-type *Arabidopsis* and *as1-1* and *as2-1* mutant plants. Fig. 8A shows that levels of *KNAT1* transcripts in the two mutants were higher than in the wild type.



F

Phenotype	No. of plant examined	Type I (7 6 5)	Type II (4 4 3 2)	Type III (2 1)
Wild type	35	34 (97.2%)	1 (2.8%)	0 (0%)
as2-1	35	0 (0%)	30 (85.7%)	5 (14.3%)

Fig. 6. Venation patterns in *as2* cotyledons. Photographs of cleared cotyledons of (A) wild-type and (B-E) *as2-1* plants are shown. The arrows in B,D,E indicate insufficiently interconnected veins at the margin. Bars, 1.5 mm. (F) Classification of the venation patterns of cotyledons into three types, based on the extents of loops formed by joining of primary and secondary veins. Type I, three or four loops, corresponding to NBPs of 5-7; type II, one or two loops, corresponding to NBPs of 2-4; and type III, no loop, corresponding to NBPs of 1-2. The observations were made using 12-day-old seedlings.

We then attempted to quantify relative levels of *KNAT1* transcripts in separated rosette leaves of wild-type, *as1-1* and *as2-1* plants using RT-PCR. We also examined transcripts of other meristem-related homeobox genes, namely *KNAT2, STM* and *WUS*. Fig. 8B shows representative results. When primers for amplification of *KNAT1* cDNA were used, products of PCR were detected in samples from all the leaves and from shoot apices of *as1* and *as2* mutants but not of wild-type plants. When we used primers for *KNAT2* cDNA, increased levels of products were detected in both *as1* and *as2* (Fig. 8B). Direct sequencing of the products of PCR confirmed that they included sequences of the *KNAT1* and *KNAT2* genes (data not shown). Thus, transcripts of both genes had accumulated in all the leaves and the shoot apices of *as1-1* and *as2-1* plants but only in the shoot apices of wild-type plants. Levels of the *KNAT1* and *KNAT2* transcripts in *as1-1* were slightly higher than those in *as2-1* plants.

The *Arabidopsis* genome contains another *knotted*-like homeobox gene (AC007945). The deduced amino acid sequence of the product is 71% identical to that of the product of the *KNAT2* gene and the gene belongs to the class 1 *knox* family. We designated this gene *KNAT6*. Fig. 8B shows that, in the wild type, transcripts of this gene accumulated in the shoot apices, while very low levels of transcripts were detected in rosette leaves. In the rosette leaves of *as1* and *as2* mutants, levels of transcripts of the *KNAT6* gene increased similarly to those of the *KNAT2* gene (Fig. 8B). The leaves at lower positions tended to accumulate higher levels of the transcripts of these *knox* genes.

Transcripts of the *STM* gene accumulated in the first and second rosette leaves of *as1-1* plants as well as in their shoot apices, although the relative levels were lower than those of the *KNAT1* gene (Fig. 8B). Levels of *STM* transcripts in *as2-1* rosette leaves fluctuated (data not shown) and wild-type leaves did not accumulate any detectable *STM* transcripts. No significant increase in the accumulation of *WUS* transcripts was detected in *as1-1* and *as2-1* rosette leaves (data not shown).

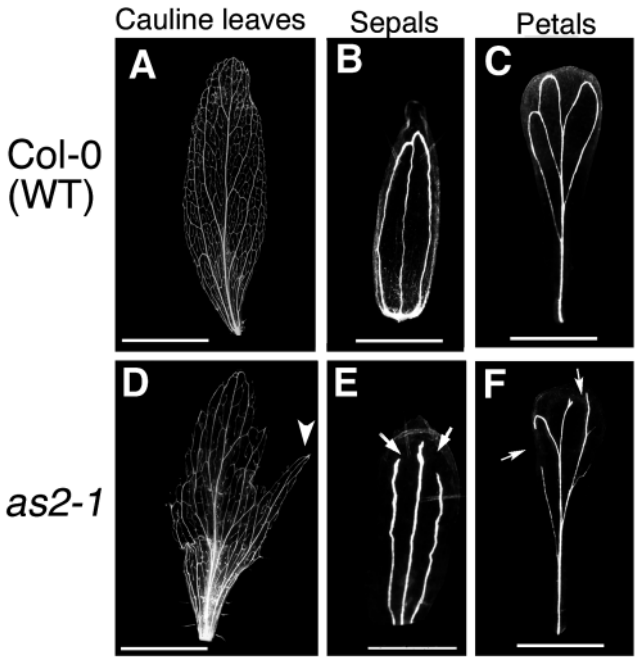


Fig. 7. Venation patterns of *as2* cauline leaves and reproductive leaf-like organs. (A) Wild-type and (D) *as2-1* cauline leaves. The arrowhead in D indicates a deep lobe in the *as2-1* cauline leaf. (E) Sepals and (F) petals of *as2-1* had simpler venation patterns than the wild-type sepals (B) and petals (C). Arrows in E and F indicate some non-connected veins at the margins of the *as2-1* sepal and petal. Bars, 8 mm in A,D; 1 mm in B,C,E,F.

In situ hybridization showed that the pattern of accumulation of *KNAT1* transcripts around vegetative meristems of *as2-1* was similar to that of the wild type (Fig. 8C). In both cases,

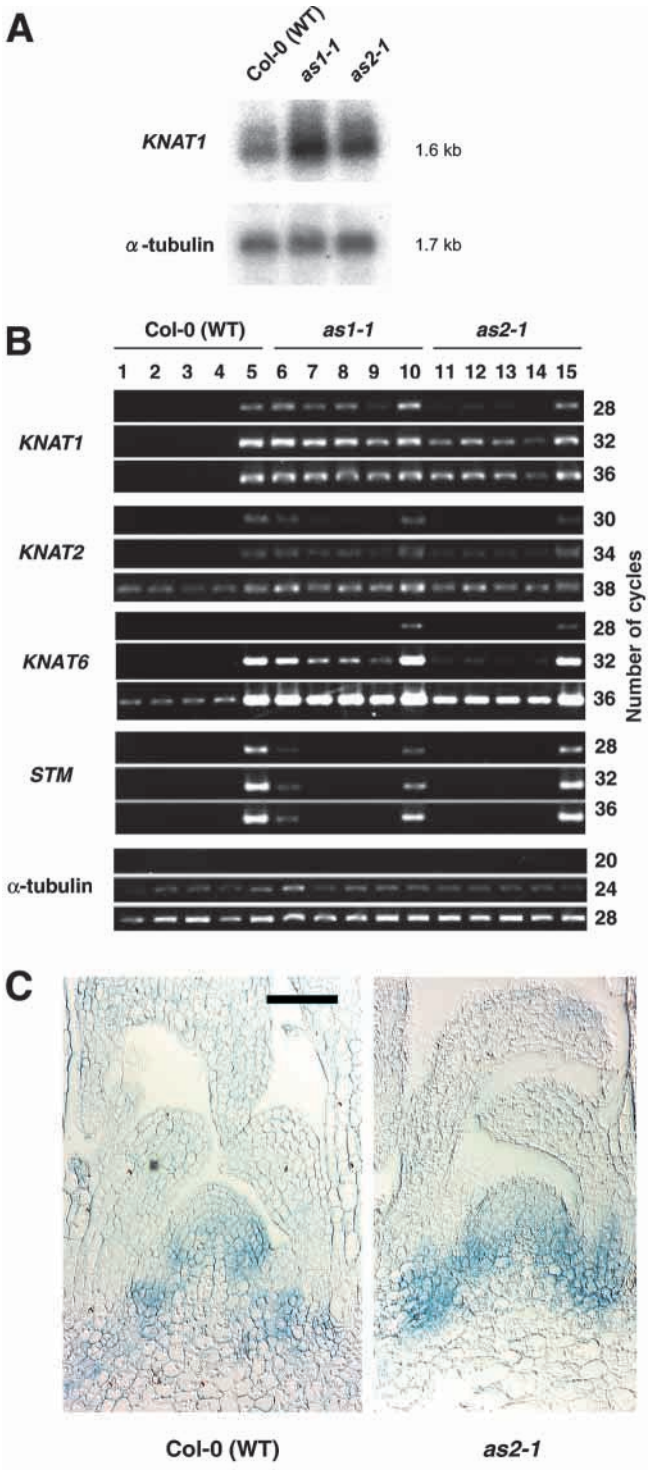


Fig. 8. Accumulation of transcripts of homeobox genes in *as2* and *as1* mutants. (A) A accumulation of *KNAT1* transcripts in wild-type (WT), *as1-1* and *as2-1* seedlings (top). The same blot was reprobed with a gene for α-tubulin (bottom) as a control. (B) Analysis by RT-PCR of transcripts of the *KNAT1*, *KNAT2*, *KNAT6* and *STM* genes in wild-type (WT), *as1-1* and *as2-1* shoot apices and rosette leaves. See Materials and Methods for details of RT-PCR. The number of cycles is indicated at the right of each panel. Amplified DNA fragments were separated by electrophoresis on an agarose gel and visualized by staining with ethidium bromide. Lanes 1, 6 and 11, the first and second rosette leaves; lanes 2, 7 and 12, the third and fourth rosette leaves; lanes 3, 8 and 13, the fifth and sixth rosette leaves that had already expanded; lanes 4, 9 and 14, young leaves that had not yet expanded; lanes 5, 10 and 15, shoot apices. Lanes 1-5, wild type (Col-0); lanes 6-10, *as1-1*; lanes 11-15, *as2-1*. (Bottom panel) Products of control PCR that were amplified with primers specific for transcripts of the gene for α-tubulin. (C) Detection of *KNAT1* transcripts by in situ hybridization. Sections of vegetative meristems of 12-day-old wild-type and *as2-1* mutant plants were probed with a digoxigenin –labeled *KNAT1* antisense probe as described in Materials and Methods. Bar, 50 μm.

Table 4. Generation of shoots from leaf sections of *as1* and *as2* mutants after incubation for 23 to 27 days on MS plates

Genotype	No. of leaf sections examined	No. of leaf sections producing shoots	No. of leaf sections producing roots
WT (Col-0)	271	0 (0.0%)	33 (12.3%)
as1-1	252	8 (3.2%)	3 (1.2%)
as2-1	281	7 (2.5%)	2 (0.7%)
as2-4	56	1 (1.8%)	2 (3.4%)

Percentages indicate the frequency with which shoots or roots were observed relative to the total number of sections examined.

transcripts were detected in the peripheral zones and basal regions of leaf primordia but not in the central zones, presumptive leaf initials and small leaf primordia. The pattern of accumulation of *KNAT1* transcripts in the *as1-1* meristem was also similar to that in the wild type (data not shown). Thus, mutations in the *AS1* and *AS2* genes did not affect transcription of *KNAT1* in meristems. By contrast, we detected relatively strong hybridization signals at the bases of *as2-1* leaf primordia. Furthermore, we occasionally detected weak but significant hybridization signals in some *as2-1* leaf primordia and such signals were not detected in the wild type.

The enhanced ability of *as1* and *as2* leaves to develop autonomous shoots in vitro

Overexpression of the *KNAT1* gene results in the formation of ectopic shoots on rosette leaves of some transformants of *Arabidopsis* with an extreme phenotype (Chuck et al., 1996). However, no ectopic shoots appeared on rosette leaves of *as1-1*, *as2-1* and *as2-4* mutant plants. We examined whether *as1-1*, *as2-1* and *as2-4* mutant rosette leaves could regenerate shoots during culture in vitro. We prepared sections of rosette leaves from mutant and wild-type Col-0 plants and incubated them on MS medium without exogenous phytohormones. Table 4 shows that shoots were regenerated from 2-3% of leaf sections from *as1-1*, *as2-1* and *as2-4* plants, but not from those of wild-type plants. The shoots appeared on adaxial surfaces at the base of petioles of rosette leaves (Fig. 9A,B) and at the sinus of leaf lobes (Fig. 9C). Although small calli were produced from the cut edges of mutant leaves, no shoots formed from these calli. However, such shoots appeared to be generated directly from leaf cells. These results suggested that *as1-1*, *as2-1* and *as2-4* rosette leaves had a higher potential for regeneration of shoots in vitro than did wild-type leaves. When wild-type leaf sections were incubated under the same conditions in vitro, roots were sometimes regenerated from the

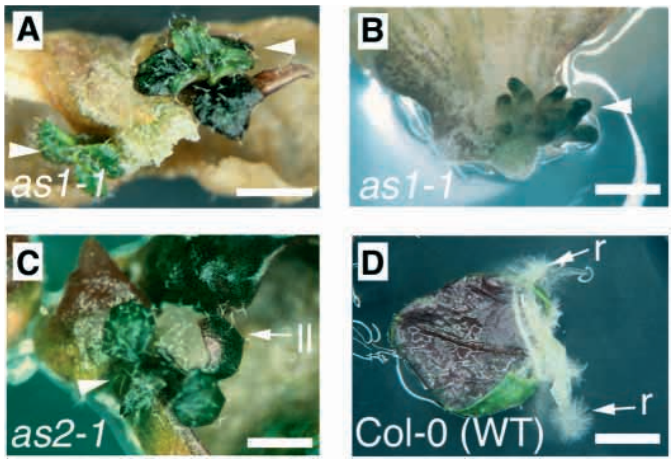


Fig. 9. The regeneration of shoots on *as1* and *as2* leaves in vitro. Sections of the sixth leaf of an *as1-1* plant were photographed after incubation for 13 days (A) and 16 days (B) on MS basic medium. Each section produced a shoot near the base of the petiole. A section of the fifth leaf of an *as2-1* plant was photographed after incubation for 16 days on MS basic medium (C). It had produced a shoot at the sinus of a leaf lobe. The arrowheads in (A-C) indicate shoots and the arrow in (C) indicates a leaf lobe (ll). (D) A section of the third leaf of a wild-type (Col-0) plant was photographed after incubation for 14 days on MS basic medium; it had produced roots (r; arrows) at its margin. Bars, 3 mm.

small calli that were produced close to the margins of the sections (Table 4; Fig. 9D). However, roots were rarely produced from leaf sections from *as1-1*, *as2-1* and *as2-4* plants (Table 4). Thus, these mutations might reduce the rooting potential of leaf cells.

DISCUSSION

Results in the present study suggest that *AS2* is involved in the establishment of the entire vein system including the prominent midvein, which is the structural axis of left-right symmetry in the leaf, as well as in the development of lamina symmetry. *AS2* also appears to function in maintaining leaf cells in a developmentally determinate state and in repressing the expression of class 1 *knox* genes. These morphological, physiological and molecular phenomena might be related to each other but possible relationships remain to be investigated.

Distorted expansion of the leaf lamina and the asymmetric leaf lobes of *as2* plants

Distortions of the lamina, such as curlings and vein abnormalities, were commonly observed in all the leaf-like organs of *as2* mutants. The formation of asymmetric lobes was not, however, seen in all these organs. The cotyledons, first and second leaves, sepals and petals of *as2-1* plants had no obvious lobes (Table 2; Fig. 7). Cauline leaves (Fig. 7D) and rosette leaves at positions above the second rosette leaf (Table 2) in *as2-1* plants had obvious lobes. In the wild type, the corresponding rosette leaves have obvious serrations, while other leaf-like organs are relatively simple and lack serration. For generation of obvious leaf lobes, the *as2* mutation might require some additional function(s), which might be related to the potential of leaves to develop serration.

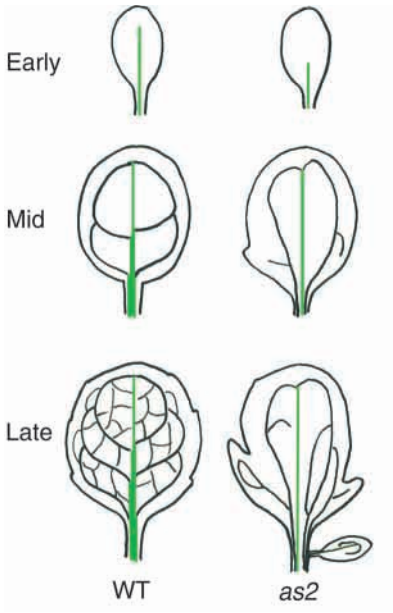


Fig. 10. Schematic representation of the development of veins in wild-type and *as2* leaves. Green lines indicate midveins. See text for details.

The *AS2* gene is required for the establishment of the vein system

As depicted schematically in Fig. 10, the developmental patterns of three distinct veins in *as2* leaves were abnormal. First, the *as2* mutation delayed the formation of the primary vein at the early stage. This vein, however, did develop in the center of the leaf primordium in *as2* plants, indicating that the position of the rachis was still correctly determined in the mutant leaf. Second, the secondary veins branched asymmetrically in *as2* leaf primordia; they elongated basipetally, rarely joining the primary vein but running through the petiole in parallel with the midvein at the mid stage of leaf development. Thus, even mature *as2* leaves failed to develop a thickened and prominent midvein. Third, the complexity of venation of higher-order veins was also reduced at the late stage. These results suggest that the *AS2* gene might be involved in the establishment of the prominent midvein and the network of lateral veins.

The shape of the lamina in *as2-1* leaf primordia in which the primary vein had not been observed yet was normal. In these primordia, asymmetric and abnormal venation was found. Thus, lamina formation seems to be independent of vein formation at the early stage. However, in the third leaf of *as2-1*, conspicuous lamina lobes and leaflet-like structures asymmetrically appeared at the late stage of leaf development when both abnormal midvein and secondary veins were already almost fully developed. It is, therefore, likely that *AS2* might suppress the formation of abnormal lobes in the lamina and might support the establishment of the vein system, including the midvein in the mature leaves. Relationships between formation of the lamina and that of veins remain to be clarified, and characterization of the *AS2* gene might provide clues to this relationship.

The combination of asymmetric lobes and venations is unique to *as2* and *as1* mutations

Mutations at other loci, such as *LOPPED 1* (*LOP1*), *VAN2* (also *3, 4, 5, 6* and *7*), *SCARFACE* (*SFC*), *MONOPTEROS* (*MP*) and *CVP1* (*CVP2*) also affect patterns of veins in rosette leaves (Carland and McHale, 1996; Koizumi et al., 2000; Deyholos et al., 2000; Przemerck et al., 1996; Carland et al., 1999). The *lop1*, *van5* and *van7* (*emb30-7*), *sfc* and *mp* mutants also have defects, in terms of thickness and shape, in midveins, as well as in lateral veins of leaves, while *cvp1* and *cvp2* have defects primarily in lateral veins.

Among the mutations mentioned above, *lop1* is associated with the severest disruption in development of the midvein, which include bifurcation and failure to produce the normal series of lateral veins (Carland and McHale, 1996). The *lop1* leaf lamina is asymmetric but there are no leaf lobes. Thus, *LOP1* appears to function differently from *AS2* although the *lop1* mutation affects both lamina symmetry and midvein formation. The *van5* and *emb30-7* mutations produce midveins that are thicker than the wild-type midvein (Koizumi et al., 2000). These mutations also cause alterations in overall morphology of rosette leaf lamina, but this phenotype is again different from that of *as2* which has the thinner midvein. The lamina of *sfc* and *mp* leaves, which have thinner midveins and fragmented lateral veins, appears to be symmetric (Deyholos et al., 2000; Przemerck et al., 1996). Phenotypes of these mutants suggest that vein disruption might not be sufficient to generate asymmetric lobes in laminae. The formation of an asymmetric leaf lamina appears to be uniquely associated with *as2* and *as1* mutations.

The presence in *as2* leaves of multiple secondary veins, unconnected to the primary vein, is reminiscent of the multiple strands in the center of the leaf lamina and petiole in the *pin1* mutant (Mattsson et al., 1999), in which the capacity for polar transport of auxin is reduced (Okada et al., 1991). The vascular pattern in *as2* leaves was similar only to the proximal portion of *pin1* leaves. Thus, the state of cells in the proximal portion of *as2* leaves might be similar to that in *pin1* leaves. However, the polar transport of auxin in *as2* plants was not different from that in wild-type plants (our unpublished data).

Relationship between ectopic expression of class 1 *knox* genes in leaves and the phenotype of *as2* leaves

The *as1-1* and *as2-1* mutations both caused the accumulation of transcripts of the *KNAT1*, *KNAT2* and *KNAT6* genes in rosette leaves (Fig. 8). Very recently, other groups have also reported that *KNAT1* and *KNAT2* transcripts accumulate in the rosette leaves of *as1* (Byrne et al., 2000) and *as2* (Ori et al., 2000) mutants. Abnormalities in *as2* leaves might be related to the ectopic expression of these homeobox genes. In an earlier study, overexpression of *KNAT1* revealed that the *KNAT* genes are responsible for the formation of leaf lobes, the enlargement of secondary veins and the regeneration of ectopic shoots on leaves, and, furthermore, that a relationship exists between the extent of development of leaf lobes and the efficiency of regeneration of ectopic shoots on leaves (Chuck et al., 1996). Thus, the similar morphological changes that we observed in *as2* in the present study might be explained by the ectopic expression of these homeobox genes in the mutant leaves (Fig. 8). The *STM* gene was also expressed ectopically in the lower leaves of *as1-1* plants (Fig. 8), and the *STM* gene might contribute to the mutant phenotype of *as1-1* leaves. Since these homeobox genes belong to the class 1 *knox* family and no other member is known in *Arabidopsis*, expression of all the members of this family is probably regulated by a similar mechanism involving AS1 and AS2.

Hareven et al. (1996) reported that *Tkn2*, a tomato homologue of *kn1*, is expressed in leaf primordia as well as the SAM of wild-type tomato plants which produce compound leaves consisting of many leaflets. They propose that such expression in leaf primordia is related to the formation of the compound leaves in tomato. The overall shape of a tomato leaf is, however, roughly symmetrical. Formation of asymmetric lamina lobes and leaflet-like structures in *as2* plants might require ectopic expression of the multiple members of the class 1 *knox* family and/ or by functions of other unknown factors.

Repression by products of the *AS2* and *AS1* genes of expression of the *KNAT1*, *KNAT2* and *KNAT6* genes in leaves

The *AS1* and *AS2* genes apparently repress the expression of the *KNAT1*, *KNAT2* and *KNAT6* genes by inducing reductions in the levels of their transcripts, and at least three plausible models can be proposed for the mode of action of wild-type *AS1* and *AS2* genes. These genes might repress the rates of transcription of these homeobox genes; they might be involved in the degradation of the transcripts; or one of them might be involved in the former process and either might be involved in the latter. It remains unknown whether such repression is directly or indirectly attributable to the encoded AS1 and AS2 proteins. The *as1-1* and *as2-1* mutations affected levels of transcription of the *STM* gene differently. Low levels of *STM* transcripts accumulated in the first and second leaves of *as1-1* plants (Fig. 8). The accumulation of *STM* transcripts in *as2-1* leaves was not reproducible (our unpublished data). Thus, *AS1* represses the expression of *STM* in leaves at least to some extent, but the role of *AS2* is still unclear.

With regard to repression of class 1 *knox* genes, the *PHANTASTICA* (*PHAN*) gene of *Antirrhinum majus* and the *ROUGH SHEATH2* (*RS2*) gene of *Zea mays*, which encode plant homologues of the myb protein (Waites et al., 1998), have been shown to have such a repressive function (Schneeberger et al., 1998; Timmerman et al., 1999; Tsiantis et al., 1999). Recently, the *AS1* gene was shown to encode a domain that is similar to the myb repeat (Byrne et al., 2000). This result suggests that a similar mechanism is involved in regulation of the class 1 *knox* gene family in *Arabidopsis*.

The enhanced indeterminate state of leaf cells from *as1* and *as2* plants

Culture of sections of *Arabidopsis* leaves in vitro revealed that the ability to regenerate shoots from *as1* and *as2* leaves was higher and the ability to regenerate roots was lower than in wild-type leaves (Table 4 and Fig. 9). Therefore, it seems likely that the cells in the growing leaves of the mutant plants might have an enhanced tendency to shift at random to a more indeterminate state, which might resemble that of cells in the SAM. Since ectopic overexpression of the *KNAT1* gene in *Arabidopsis* plants induces shoot meristems on leaves (Chuck et al., 1996), such an indeterminate state of leaf cells in these

mutants might be due to the ectopic expression of the *KNAT1*, *KNAT2* and *KNAT6* genes in these leaves (Fig. 8).

For the formation of a bilaterally symmetric leaf, the division and expansion of cells must be coordinated on both sides of the midvein of the leaf lamina. The random regeneration of meristematic cells in mutant leaves might interfere with the necessary coordinated cell division and expansion, with eventual formation of asymmetric leaves with irregular curls, lobes and leaflet-like structures. Therefore, it might be critical for leaf cells to maintain a determinate state if leaves with bilateral symmetry are to be generated.

As described above, the establishment of the vein system, including the midvein, appears to require the wild-type *AS2* gene. It will be of interest to examine whether a relationship exists between establishment of the vein system and the maintenance of the determinate state of leaf cells. There are at least three plausible mechanisms by which *AS2* might regulate these two phenomena. *AS2* might regulate these events independently. Alternatively, it might act first to induce the determinate state of leaf cells, which in turn might establish the vein system, or *vice versa*. These possibilities should be investigated in future by functional analysis of the corresponding genes.

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REFERENCES

Banno, H., Hirano, K., Nakamura, T., Irie, K., Nomoto, S., Matsumoto, K. and Machida, Y. (1993). *NPK1*, a tobacco gene that encodes a protein with a domain homologous to yeast BCK1, STE11, and Byr2 protein kinases. *Mol. Cell Biol.* **13,** 4745-4752.

Barton, M. K. and Poethig, R. S. (1993). Formation of the shoot apical meristem in *Arabidopsis thaliana*: an analysis of development in the wild type and in the *shoot meristemless* mutant. *Development* **119,** 823-831.

Berná, G., Robles, P. and Micol, J. L. (1999). A mutational analysis of leaf morphogenesis in *Arabidopsis thaliana*. *Genetics* **152,** 729-742.

Byrne, M., Barley, R., Curtis, M., Arroyo, J. M., Dunham, M., Hudson, A and Martienssen, R. (2000). *Asymmetric leaves* mediates leaf patterning and stem cell function in *Arabidopsis*. *Nature* **408,** 967-971.

Candela, H., Martinez-Laborda, A. and Micol, J. L. (1999). Venation pattern formation in *Arabidopsis thaliana* vegetative leaves. *Dev. Biol.* **205,** 205-216.

Carland, F. M. and McHale, N. A. (1996). *LOP1*: a gene involved in auxin transport and vascular patterning in *Arabidopsis*. *Development* **122,** 1811-1819.

Carland, F. M., Berg, B. L., FitzGerald, J. N., Jinamrnphongs, S., Nelson, T. and Keith, B. (1999). Genetic regulation of vascular tissue patterning in *Arabidopsis*. *Plant Cell* **11,** 2123-2137.

Chuck, G., Lincoln, C. and Hake, S. (1996). *KNAT1* induces lobed leaves with ectopic meristems when overexpressed in *Arabidopsis*. *Plant Cell* **8,** 1277-1289.

Conway, L. J. and Poethig, R. S. (1997). Mutations of *Arabidopsis thaliana* that transform leaves into cotyledons. *Proc. Natl. Acad. Sci. USA* **94,** 10209-10214.

Dengler, N. G. (1999). Anisophylly and dorsiventral shoot symmetry. *Int. J. Plant Sci*. **160** (6 Suppl.): S67-S80.

Deyholos, M. K, Cordner, G., Beebe, D. and Sieburth, L. E. (2000). The *SCARFACE* gene is required for cotyledon and leaf vein patterning. *Development* **127,** 3205-3213.

Fabri, O. C. and Schäffner, A. R. (1994). An *Arabidopsis thaliana* RFLP mapping set to localize mutations to chromosomal regions. *Plant J.* **5,** 149-156.

Gardner, M. (1990). *The New Ambidextrous Universe: Symmetry and Asymmetry from Mirror Reflections to Superstrings.* 3rd rev. edition, pp. 53-63. New York: W. H. Freeman and Company.

Hake, S., Vollbrecht, E. and Freeling, M. (1989). Cloning *Knotted*, the dominant morphological mutant in maize using *Ds2* as a transposon tag. *EMBO J.* **8,** 15-22.

Hamada, S., Onouchi, H., Tanaka, H., Kudo, M., Liu, Y. –G., Shibata, D., Machida, C. and Machida, Y. (2000). Mutations in the *WUSCHEL* gene of *Arabidopsis thaliana* result in the development of shoots without juvenile leaves. *Plant J.* **24**, 91-101.

Hareven, D., Gutfinger, T., Parnis, A., Eshed, Y. and Lifschitz, E. (1996). The making of a compound leaf: Genetic manipulations of leaf architecture in tomato. *Cell* **84,** 735-744.

Hickey, L. J. (1973). Classification of the architecture of dicotyledonous leaves. *Am. J. Bot.* **60**, 17-33.

Hickey, L. J. (1979). A revised classification of the architecture of dicotyledonous leaves. In *Anatomy of the Dicotyledons* (ed. C. R. Metcalfe and L. Chalk), pp. 25-39. New York: Oxford University Press.

Höfler, J. Turner, L., Hellens, R., Ambrose, M., Matthews, P., Michael, A. and Ellis, N. (1997). *UNIFOLIATA* regulates leaf and flower morphogenesis in pea. *Curr. Biol*. **7,** 581-587.

Howell, S. (1998). *Molecular Genetics of Plant Development.* Cambridge, UK: Cambridge University Press.

Kerstetter, R. A. and Poethig, R. S. (1998). The specification of leaf identity during shoot development. *Annu. Rev. Cell Dev. Biol.* **14,** 373-398.

Kim, G.-T., Tsukaya, H. and Uchimiya, H. (1998). The *ROTUNDIFOLIA3* gene of *Arabidopsis thaliana* encodes a new member of the cytochrome P-450 family that is required for the regulated polar elongation of leaf cells. *Genes Dev.* **12,** 2381-2391.

Kinsman, E. A. and Pyke, K. A. (1998). Bundle sheath cells and cell-specific plastid development in *Arabidopsis* leaves. *Development* **125,** 1815-1822.

Koizumi, K., Sugiyama, M. and Fukuda, H. (2000). A series of novel mutants of *Arabidopsis thaliana* that are defective in the formation of continuous vascular network: calling the auxin signal flow canalization hypothesis into question. *Development* **127,** 3197-3204.

Laux, T., Mayer, K. F. X., Berger, J. and Jürgens, G. (1996). The *WUSCHEL* gene is required for shoot and floral meristem integrity in *Arabidopsis*. *Development* **122,** 87-96.

Lieu, S. M. and Sattler, R. (1976). Leaf development in *Begonia hispida* var. *cucullifera* with special reference to vascular organization. *Can. J. Bot.* **54,** 2108-2121.

Lincoln, C., Long, J., Yamaguchi, J., Serikawa, K. and Hake, S. (1994). A *knotted1-like* homeobox gene in *Arabidopsis* is expressed in the vegetative meristem and dramatically alters leaf morphology when overexpressed in transgenic plants. *Plant Cell* **6,** 1859-1876.

Long, J. A., Moan, E. I., Medford, J. I. and Barton, M. K. (1996). A member of the KNOTTED class of homeodomain proteins encoded by the *STM* gene of *Arabidopsis*. *Nature* **379,** 66-69.

Long, J. A. and Barton, M. K. (2000). Initiation of axillary and floral meristems in *Arabidopsis*. *Dev. Biol.* **218,** 341-353.

Lynn, K., Fernandez, A., Aida, M., Sedbrook, J., Tasaka, M., Masson, P. and Barton, M. K. (1999). The *PINHEAD/ZWILLE* gene acts pleiotropically in *Arabidopsis* development and has overlapping functions with the *ARGONAUTE1* gene. *Development* **126,** 469-481.

Machida, C., Onouchi, H., Koizumi, J., Hamada, S., Semiarti, E., Torikai, S. and Machida, Y. (1997). Characterization of the transposition pattern of the *Ac* transposable element in *Arabidopsis thaliana* using endonuclease I-*Sce*I. *Proc. Natl. Acad. Sci. USA* **94,** 8675-8680.

Mattsson, J., Sung, Z. R. and Berleth, T. (1999). Responses of plant vascular systems to auxin transport inhibition. *Development* **126,** 2979-2991.

Mayer, K. F. X., Schoof, H., Haecker, A., Lenhard, M., Jürgens, G. and Laux, T. (1998) Role of *WUSCHEL* in regulating stem cell fate in the *Arabidopsis* shoot meristem. *Cell* **95,** 805-815.

Nakashima, M., Hirano, K., Nakashima, S., Banno, H., Nishihama, R. and Machida, Y. (1998). The expression pattern of the gene for *NPK1* protein kinase related to mitogen-activated protein kinase kinase kinase

(MAPKKK) in a tobacco plant: correlation with cell proliferation. *Plant Cell Physiol*. **39,** 690-700.

Nelson, T. and Dengler, N. (1997). Leaf vascular pattern formation. *Plant Cell* **9**, 1121-1135

Nishimura, A., Tamaoki, M., Sakamoto, T. and Matsuoka, M. (2000). Over-expression of tobacco *knotted*-1-type class1 homeobox genes alters various leaf morphology. *Plant Cell Physiol.* **41,** 583-590.

Ogura, Y. (1962). *Plant Anatomy and Morphology*. pp. 102-134, Tokyo, Japan: Youkendo, Inc.

Okada, K., Ueda, J., Komaki, M. K., Bell, C. J. and Shimura, Y. (1991). Requirement of the auxin polar transport system in early stages of *Arabidopsis* floral bud formation. *Plant Cell* **3**, 677-684.

Onouchi, H., Nishihama, R., Kudo, M., Machida, Y. and Machida, C. (1995). Visualization of site-specific recombination catalyzed by a recombinase from *Zygosaccharomyces rouxii* in *Arabidopsis thaliana*. *Mol. Gen. Genet*. **247,** 653-660.

Ori, N., Eshed, Y., Chuck, G., Bowman, J. L. and Hake, S. (2000). Mechanisms that control *knox* gene expression in the *Arabidopsis* shoot. *Development* **127**, 5523-5532.

Parnis, A., Cohen, O., Gutfinger, T., Hareven, D., Zamir, D. and Lifschitz, E. (1997). The dominant developmental mutants of tomato, *Mouse-ear* and *Curl*, are associated with distinct modes of abnormal transcriptional regulation of a *Knotted* gene. *Plant Cell* **9,** 2143-2158.

Poethig, R. S. (1997). Leaf morphogenesis in flowering plants. *Plant Cell* **9**, 1077-1087.

Przemeck, G. K., Mattsson, J., Hardtke, C. S., Sung, Z. R. and Berleth, T. (1996). Studies on the role of the *Arabidopsis* gene *MONOPTEROS* in vascular development and plant cell axialization. *Planta* **200,** 229-237.

Rédei, G. P. and Hirono, Y. (1964). Linkage studies. *Arabidopsis Inf. Serv*. **1,** 9.

Sawa, S., Watanabe, K., Goto, K., Liu, Y. G., Shibata, D., Kanaya, E., Morita, E. H. and Okada, K. (1999). *FILAMENTOUS FLOWER*, a meristem and organ identity gene of *Arabidopsis*, encodes a protein with a zinc finger and HMG-related domains. *Genes Dev.* **13,** 1079-1088.

Schneeberger, R., Tsiantis, M., Freeling, M. and Langdale, J. A. (1998). The *ROUGH SHEATH2* gene negatively regulates homeobox gene expression during maize leaf development. *Development* **125**, 2857-2865.

Serikawa, K. A. and Zambryski, P. C. (1997). Domain exchanges between KNAT3 and KNAT1 suggest specificity of the kn1-like homeodomains requires sequences outside of the third helix and N-terminal arm of the homeodomain. *Plant J.* **4,** 863-869.

Serrano-Cartagena, J., Robles, P., Ponce, M. R. and Micol, J. L. (1999). Genetic analysis of leaf form mutants from the *Arabidopsis* Information Service collection. *Mol. Gen. Genet.* **261,** 725-739.

Siegfried, K. R., Eshed, Y., Baum, S. F., Otsuga, D., Drews, G. N. and Bowman, J. L. (1999). Members of the *YABBY* gene family specify abaxial cell fate in *Arabidopsis*. *Development* **126**, 4117-4128.

Sinha, N. (1999). Leaf development in angiosperms. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **50,** 419-446.

Sinha, N. R., Williams, R. E. and Hake, S. (1993). Overexpression of the maize homeobox gene, *KNOTTED-1*, causes a switch from determinate to indeterminate cell fates. *Genes Dev.* **7,** 787-795.

Steeves, T. A. and Sussex, I. M. (1989) Patterns in Plant Development. Cambridge: Cambridge University Press.

Telfer, A. and Poethig, R.S. (1994). Leaf Development in *Arabidopsis*. In *Arabidopsis* (ed. E. M. Meyerowitz and C. R. Somerville), pp. 379-401. New York: Cold Spring Harbor Laboratory Press.

Timmermans, M. C. P., Hudson, A., Becraft, P. W. and Nelson, T. (1999). ROUGH SHEATH2: a Myb protein that represses *knox* homeobox genes in maize lateral organ primordia. *Science* **284,** 151-153.

Tsiantis, M., Schneeberger, R., Golz, J. F., Freeling, M. and Langdale, J. A. (1999). The maize *rough sheath2* gene and leaf development programs in monocot and dicot plants. *Science* **284,** 154-156.

Tsukaya, H. and Uchimiya, H. (1997). Genetic analyses of the formation of the serrated margin of leaf blades in *Arabidopsis*: combination of a mutational analysis of leaf morphogenesis with the characterization of a specific marker gene expressed in hydathodes and stipules. *Mol. Gen. Genet.* **256,** 231-238.

Waites, R., Selvadurai, H. R. N., Oliver, I. R. and Hudson, A. (1998). The *PHANTASTICA* gene encodes a MYB transcription factor involved in growth and dorsoventrality of lateral organs in *Antirrhinum*. *Cell* **93,** 779-789.

Whaley, W. G. and Whaley, C. Y. (1942). A developmental analysis of inherited leaf patterns in *Tropaeolum*. *Am. J. Bot.* **29**, 105-194.