Development 130, 4097-4107

doi:10.1242/dev.00622

Novel *as1* and *as2* defects in leaf adaxial-abaxial polarity reveal the requirement for *ASYMMETRIC LEAVES1* and *2* and *ERECTA* functions in specifying leaf adaxial identity

Lin Xu[1,2], Yi Xu[1], Aiwu Dong[3], Yue Sun[4], Limin Pi[1], Yuquan Xu[2] and Hai Huang[1,*]

[1]National Laboratory of Plant Molecular Genetics, Shanghai Institute of Plant Physiology and Ecology, Shanghai Institute for Biological Sciences, Chinese Academy of Sciences, 300 Fenglin Road, Shanghai 200032, China
[2]College of Life Science and Biotechnology, Shanghai Jiao Tong University, 1954 Hua Shan Road, Shanghai 200030, China
[3]College of Life Sciences, Fudan University, 220 Han Dan Road, Shanghai 200433, China
[4]College of Life Sciences, East China Normal University, 3663 North Zhongshan Road, Shanghai 200062, China
*Author for correspondence (e-mail: hhuang@iris.sipp.ac.cn)

Accepted 21 May 2003

SUMMARY

The shoot apical meristem (SAM) of seed plants is the site at which lateral organs are formed. Once organ primordia initiate from the SAM, they establish polarity along the adaxial-abaxial, proximodistal and mediolateral axes. Among these three axes, the adaxial-abaxial polarity is of primary importance in leaf patterning. In leaf development, once the adaxial-abaxial axis is established within leaf primordia, it provides cues for proper lamina growth and asymmetric development. It was reported previously that the *Arabidopsis ASYMMETRIC LEAVES1* (*AS1*) and *ASYMMETRIC LEAVES2* (*AS2*) genes are two key regulators of leaf polarity. In this work, we demonstrate a new function of the *AS1* and *AS2* genes in the establishment of adaxial-abaxial polarity by analyzing *as1* and *as2* alleles in the Landsberg *erecta* (L*er*) genetic background. We provide genetic evidence that the *Arabidopsis ERECTA* (*ER*) gene is involved in the *AS1-AS2* pathway to promote leaf adaxial fate. In addition, we show that AS1 and AS2 bind to each other, suggesting that AS1 and AS2 may form a complex that regulates the establishment of leaf polarity. We also report the effects on leaf polarity of overexpression of the *AS1* or *AS2* genes under the control of the cauliflower mosaic virus (CAMV) 35S promoter. Although plants with *as1* and *as2* mutations have very similar phenotypes, *35S::AS1*/L*er* and *35S::AS2*/L*er* transgenic plants showed dramatically different morphologies. A possible model of the *AS1*, *AS2* and *ER* action in leaf polarity formation is discussed.

Key words: Adaxial-abaxial axis, Arabidopsis thaliana, ASYMMETRIC LEAVES1, ASYMMETRIC LEAVES2, ERECTA, Polarity formation

INTRODUCTION

The establishment of polarity is a fundamental theme in leaf development. Generally, leaf primordia initiate from the peripheral zone of shoot apical meristem (SAM), and establish their adaxial-abaxial, proximodistal and mediolateral axes. Genetic studies of leaf axis formation have uncovered a number of mutants that exhibit abnormalities in leaf polarity (Talbert et al., 1995; Waites and Hudson, 1995; McConnell and Barton, 1998; Schneeberger et al., 1998; Berna et al., 1999; Clarke et al., 1999; Serrano-Cartagena et al., 1999; Kerstetter et al., 2001; Semiarti et al., 2001; Ha et al., 2003). Phenotypic and molecular genetic analyses of some of these mutants have led to the identification of genes that play important roles in the leaf polarity establishment (for a review, see Bowman et al., 2002).

In *Arabidopsis*, the *PHABULOSA* (*PHB*) and *PHAVOLUTA* (*PHV*) genes, together with a closely related gene, *REVOLUTA* (*REV*), encode members of a homeodomain/leucine-zipper (HD-ZIP) family of proteins. Semi-dominant gain-of-function mutations in either *PHB* or *PHV* result in the transformation of abaxial leaf tissues into adaxial ones (McConnell and Barton, 1998; McConnell et al., 2001). Phenotypes in loss-of-function *rev* mutants could be interpreted as a partial loss of adaxial identity (Talbert et al., 1995; Otsuga et al., 2001). It was suggested that these genes are required for promoting the adaxial cell fate in lateral organs (McConnell and Barton, 1998; McConnell et al., 2001). In addition, *YABBY* and *KANADI* (*KAN*) genes are expressed in the abaxial face of lateral organs and specify the abaxial cell identity in *Arabidopsis* (Chen et al., 1999; Sawa et al., 1999a; Sawa et al., 1999b; Eshed et al., 1999; Eshed et al., 2001; Kerstetter et al., 2001). Members of the *YABBY* and *KAN* gene families are candidate abaxial-promoting factors because mutations in these genes cause abnormality in the specification of the abaxial fate (Siegfried et al., 1999; Eshed et al., 2001; Kerstetter et al., 2001; Bowman et al., 2002).

Other key regulators of leaf polarity include a group of

functional homologs: *PHANTASTICA* (*PHAN*) in *Antirrhinum*, *ROUGH SHEATH2* (*RS2*) in maize and *ASYMMETRIC LEAVES1* (*AS1*) in *Arabidopsis* (Waites and Hudson, 1995; Schneeberger et al., 1998; Serrano-Cartagena et al., 1999). *PHAN*, *RS2* and *AS1* all encode MYB-domain containing putative transcription factors, with a high degree of sequence similarity among them (Waites et al., 1998; Timmermans et al., 1999; Tsiantis et al., 1999; Byrne et al., 2000; Sun et al., 2002). In situ hybridization and immunolocalization experiments demonstrated that transcripts or proteins of members in the class 1 *KNOX* (knotted-like homeobox) gene family are ectopically accumulated in leaves of *phan*, *rs2* and *as1* mutants (Waites et al., 1998; Timmermans et al., 1999; Tsiantis et al., 1999; Byrne et al., 2000). These results suggest that PHAN, RS2 and AS1 act to down-regulate *KNOX* genes in leaf initials, or these genes might initiate a process by which *KNOX* gene expression is epigenetically repressed.

Furthermore, mutations in the *Arabidopsis AS2* gene, another important gene in leaf development, cause very similar phenotypes to those of *as1* mutants (Serrano-Cartagena et al., 1999; Ori et al., 2000; Sun et al., 2000; Semiarti et al., 2001). In addition, *as2* mutants show increased accumulation of *KNOX* transcripts in leaves (Semiarti et al., 2001), similar to that in *as1* mutants (Byrne et al., 2000). It was proposed that the *AS1* and *AS2* genes function in the same regulatory pathway (Serrano-Cartagena et al., 1999; Byrne et al., 2002; Xu et al., 2002). *AS2* has been cloned recently and the gene encodes a protein with a leucine-zipper motif (Iwakawa et al., 2002; Xu et al., 2002). *AS2* is expressed in almost all of the above ground portion of the wild-type plant except the stem (Iwakawa et al., 2002; Xu et al., 2002).

Although the isolation and characterization of the *AS1* and *AS2* genes have provided important insights into the mechanisms that control the establishment of polarity during leaf development, they also raised further questions. First, what is the molecular basis for AS1 and AS2 action? Do they form a complex if they function in the same regulatory pathway? Second, do *AS1* and *AS2* also regulate leaf polarity in the adaxial-abaxial axis, in addition to their roles in proximodistality and mediolaterality in leaves (Byrne et al., 2000; Tsiantis, 2001)? Finally, are there any other genes required for leaf polarity formation in the *AS1* and *AS2* regulatory pathways?

To address these questions, we previously isolated and characterized new *as1* and *as2* alleles in the Landsberg *erecta* (L*er*) genetic background (Sun et al., 2000; Sun et al., 2002; Xu et al., 2002). Unlike other *as1* and *as2* alleles in the Columbia, ER and En-D backgrounds, the alleles in the L*er* background showed a novel leaf phenotype: in some rosette leaves the petiole is attached to the under surface of the leaf lamina. We referred to this structure as a lotus-leaf. Here, we further characterize the lotus-leaf defects and demonstrate that the primary *AS1* and *AS2* functions in the establishment of leaf polarity are the regulation of adaxial-abaxial axis. We also provide evidence that *ER* function acts in the *AS1-AS2* pathway to regulate polarity formation during leaf development. We report a physical interaction between AS1 and AS2 proteins in vitro and in yeast. Based on these results as well as the phenotypes of *35S::AS1* and *35S::AS2* transgenic plants, we propose a model of *AS1*, *AS2* and *ER* actions in leaf polarity formation.

MATERIALS AND METHODS

Plant material and growth conditions

Seeds of mutant *brevipedicellus* (*bp*), *as1-1*, *as2-1* and wild-type Landsberg (Lan, with the wild-type allele for the *ERECTA* gene) were obtained from the *Arabidopsis* Biological Resource Center (ABRC). The *as1-101* and *as2-101* mutants are in the L*er* genetic background and have been previously described (Sun et al., 2000; Sun et al., 2002; Xu et al., 2002). Plants were grown on soil according to our previous conditions (Chen et al., 2000).

Yeast two-hybrid assay

The cDNA fragments encoding the entire AS1 and AS2 predicted proteins were amplified using polymerase chain reaction (PCR) and cloned into the *Nde*I and *Bam*HI restriction sites of the MATCHMAKER two-hybrid vectors pGADT7 and pGBKT7 (Clontech, USA), to generate pGADT7-AS1, pGBKT7-AS1, pGADT7-AS2 and pGBKT7-AS2, respectively. The PCR primers were as follows: 5′-gccatATGAAAGAGCGTCAACGTTGG-3′ and 5′-gtggatccTTAT CAGGGGCGGTCTAATCTG-3′ (for AS1), and 5′-gccatATGGCATCTTCTTCAACAAAC-3′ and 5′-gtggatccTTAT-CAAGACGGATCAACAGTAC-3′ (for AS2). In each of the above primer sequences, the lowercase letters represent additional nucleotides to introduce restriction sites. All PCR fragments were verified by sequencing.

Construct combinations pGADT7-AS1/pGBKT7-AS2 and pGADT7-AS2/pGBKT7-AS1 were co-transformed into the yeast strain AH109, and transformants were selected for growth on media lacking tryptophan and leucine. The interaction between the AS1 and AS2 proteins was tested by growth of the transformants on media lacking histidine and adenine, indicating expression of the reporter genes *HIS3* and *ADE2*. Analysis of the relative β-galactosidase activity was as described in the Yeast Handbook (Clontech, USA).

Enzyme-linked immunosorbent assay

For synthesis and purification of recombinant AS1 and AS2 proteins, cDNAs containing the entire coding regions of these two proteins were amplified by PCR. The amplified *AS1* cDNA was cloned into the vector pET-14b (Novagen, USA) by using the *Nde*I and *Bam*HI sites to yield His-AS1, and the AS2 cDNA was cloned into the vector pGEX-4T1 (Pharmacia, USA) by using the *Bam*HI and *Sal*I sites to result in GST-AS2. The PCR primers for the *AS1* amplification were 5′-gccatATGAAAGAGCGTCAACGTTGG-3′ and 5′-gtggatccTT-ATCAGGGGCGGTCTAATCTG-3′, and those for the *AS2* amplification were 5′-caggatccATGGCATCTTCTTCAACAAAC-3′ and 5′-cagtcgacTTATCAAGACGGATCAACAGTAC-3′. In each of above sequences the lowercase letters represent additional nucleotides to introduce restriction sites. All constructs were verified by sequencing. Production and purification of His-AS1 and GST-AS2 fusion proteins were according to the manufactures' recommended protocols (Novagen and Pharmacia, USA). The resultant proteins were analyzed by SDS-PAGE before enzyme-linked immunosorbent assay (ELISA) experiments. ELISA was performed by coating wells of microtiter plates (Nunc., USA) with the GST-AS2, followed by addition of the His-AS1 at different concentrations to the coated wells. The retained His-AS1 was determined by incubation with a primary antibody against the His tag (Sigma, USA), at 4°C overnight. Then the second antibody, a POD-conjugated anti-mouse antibody (Sigma, USA), and the substrate 3,3′,5,5′-tetramethylbenzidine (TMB) were added. The reaction was examined by recording the absorbance at 655 nm, using a 450 Microplate Reader (Bio-Rad, USA).

Reverse transcription-polymerase chain reaction

For reverse transcription-polymerase chain reaction (RT-PCR), total RNA was extracted as described previously (Huang et al., 1995). After treatment with DNase (Promega, USA), complementary DNA was

synthesized using a reverse transcription kit (Promega, USA). PCR reactions were performed with *KNAT1* gene-specific primers (5′-TGTCAGAGTCCCATTCAC-3′ and 5′-GCAACGAGAGGTTGT-TATT-3′), which span the exon3/exon5 region. PCR products were examined by separating on a 1.0% agarose gel.

Construction of transgenic plants

The overexpression construct *35S::AS1* was constructed previously (Sun et al., 2002). For overexpression of the *AS2* gene, a 0.6 kb genomic DNA containing the entire *AS2* coding region was PCR-amplified from the L*er* plants and sequenced. This DNA fragment was then inserted into a binary T-DNA vector pMON530 (Monsanto, USA), downstream of a 35S promoter. The constructs *35S::AS1* and *35S::AS2* were introduced into the L*er*, *as1-101* and *as2-101* plants by *Agrobacterium*-mediated transformation. Ten *35S::AS1*/L*er*, thirty-two *35S::AS2*/L*er*, five *35S::AS1*/*as2-101* and fifteen *35S::AS2*/*as1-101* transgenic lines were obtained. Gene overexpression was verified by RT-PCR from the *35S::AS1*/L*er* and the *35S::AS2*/L*er* transgenic lines that were used for phenotypic analysis in this work (data not shown), and primers used in the PCR experiment were described previously (Xu et al., 2002). *35S::AS1*/*as2-101* and *35S::AS2*/*as1-101* transgenic lines were verified by PCR using a forward *35S* primer (5′-GCTCCTAC-AAATGCCATCA-3′) and reverse primers (5′-ttgaattcCATTACA-AGTTACAAC-3′ for the *AS1* and 5′-GTTTTCTCATCACCAAGCG-3′ for the *AS2*). Phenotypes of the transgenic lines were consistent among progeny from each transformation.

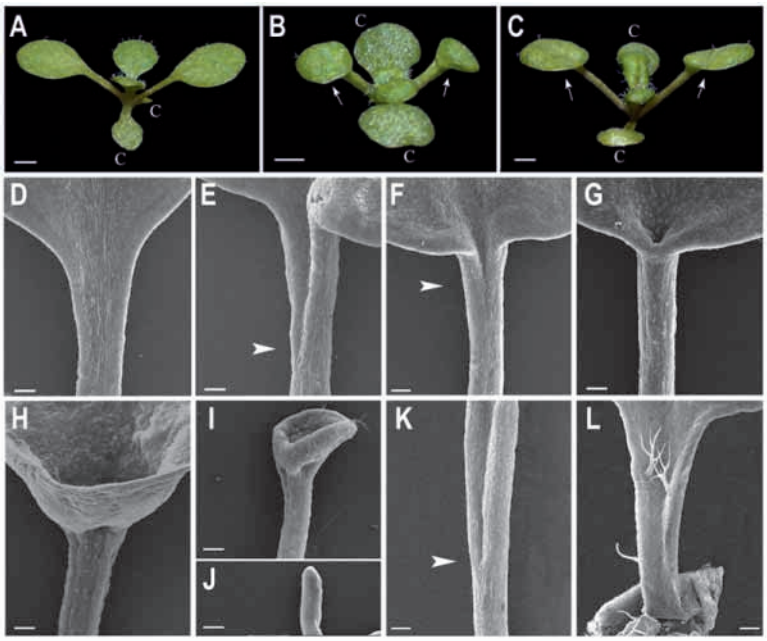

Fig. 1. The lotus-leaf structure of *as1-101* and *as2-101* mutants in the L*er* genetic background. (A) L*er*; (B) *as1-101* and (C) *as2-101* seedlings. Note that first pairs of rosette leaves in the *as1* and *as2* mutants often show the lotus-leaf structure (arrows). c, cotyledon. (D-L) Each panel shows one of the first pairs of rosette leaves, all of similar ages, and photos were taken from an adaxial view. (D) A L*er* rosette leaf with an asymmetric petiole in the adaxial-abaxial axis. (E,F) *as2-101* rosette leaves showing petioles with radially symmetric portions that vary in length (arrowheads). Note that the petiole portions below arrowheads are radially symmetric. (G-I) *as2-101* rosette leaves with a varying degree of severity of the lotus-leaf structure. (J) A needle-like structure of an *as2-101* seedling. (K) An *as2-1* rosette leaf with the radially symmetric portion in the petiole (arrowhead). (L) An *as1-1* petiole. Scale bars:1 mm (A-C); 0.2 mm (D-L).

Histology and microscopy

Fresh leaves and whole seedlings of wild-type and mutant plants were examined using a SZH10 dissecting microscope (Olympus, Japan), and photos were taken using a Nikon E995 digital camera (Nikon, Japan). Preparation of thin section specimens and scanning electron microscopy (SEM) were as described previously (Chen et al., 2000), using the first pair of rosette leaves.

RESULTS

Lotus-leaves in *as1* and *as2* mutants reveal defects in the adaxial-abaxial polarity

Previous characterizations of the *as1* mutants showed that the mutant pattern reflects a change in proximodistal and mediolateral patterning of the leaf, but not in the adaxial-abaxial axis (Byrne et al., 2000). Our more recent results demonstrated that *as1* and *as2* mutants in the L*er* genetic background displayed a novel leaf structure: in some rosette leaves the petiole is attached to the abaxial surface of the leaf lamina, showing a lotus-leaf structure (Sun et al., 2002; Xu et al., 2002). This structure might suggest a defect in the adaxial-abaxial axis in leaves. To understand better the *AS1* and *AS2* functions, we characterized this lotus-leaf structure extensively. Since *as1* and *as2* mutants have very similar overall phenotypes, we focused our phenotypic analyses mainly on the *as2-101* mutant, except where otherwise noted. In comparison with wild-type plants (Fig. 1A), all *as1* and *as2* alleles that we have obtained in the L*er* ecotype produced the lotus-leaf structure (Fig. 1B,C, arrows), and this type of organs usually appears among the first two rosette leaves. In our growth conditions, 15-30% (depending on individual alleles) of all first two rosette leaves in *as1* and *as2* seedlings, were lotus leaves.

Petioles of the L*er* plants have an asymmetric adaxial-abaxial axis with a flat and a slightly wider adaxial side (Fig. 1D). In the *as2-101* mutant, however, each petiole showed a radially symmetric proximal portion, the length of which varied in a continuous series, depending upon leaf positions and leaf ages. Some leaves showed a radially symmetric portion at the very proximal end (Fig. 1E), while in others it was more distal (Fig. 1F). If the radially symmetric tissue reached high enough to affect the region where leaf lamina grew, the lotus-leaf was formed (Fig. 1G,H). The higher the radial portion ended, the smaller the whole leaf structure became. If the radially symmetric portion extended extremely distally, lamina development would be severely affected, resulting in either leaves with a very small lamina (Fig. 1I) or even needle-like organs without any lamina growth (Fig. 1J). We also analyzed petioles of *as1-1* and *as2-1* plants, these are the previously identified alleles that are in the mixed Col/L*er* and ER genetic backgrounds, respectively. Although lotus-leaves appeared in *as2-1* at a very low frequency (Xu et al., 2002), all first pairs of rosette leaves that we have analyzed contained a radially symmetric portion in the petiole (Fig. 1K). *as1-1* exhibited a less severe petiole phenotype, having

Table 1. Effects of *ER* gene on frequency of the lotus-leaves

	as2 mutant phenotype*	Total leaves†	Lotus-leaves	Frequency
as2-101 × Lan	267 Lan-like plants	534	6	1.1%
	88 L*er*-like plants	176	34	19.3%

*Plants with *as2* phenotypes were identified among the F_2 population from a cross between *as2-101* in the L*er* genetic background and Lan with a normal *ER* gene.

†Total numbers of the first-pair rosette leaves were analyzed.

neither lotus-leaves nor radially symmetric petioles. However, margins of the *as1-1* petioles curled upwards (Fig. 1L), resembling the portion distal to the radially symmetric one in petioles in *as1-101* (Sun et al., 2002), *as2-101* (Fig. 1E,F) and *as2-1* (Fig. 1K). These results indicate that the *as1* and *as2* single mutants, regardless of genetic backgrounds, exhibited defects in leaf adaxial-abaxial axis.

ER function is involved in the leaf polarity formation

The *as1* and *as2* mutants were identified and first characterized several decades ago (Redei, 1965). However, the lotus-leaf phenotype was not reported from previous analyses of the *as1* and *as2* alleles (Redei, 1965; Serrano-Cartagena et al., 1999; Byrne et al., 2000; Ori et al., 2000; Semiarti et al., 2001). Previously, we reported the observation of lotus-leaves in newly isolated *as1* alleles in the L*er* background (Sun et al., 2002). We also compared *as2* alleles in different genetic backgrounds, and found that only those in the L*er* background produced lotus-leaves at relatively high frequencies (Xu et al., 2002). These results indicate that the lotus-leaf phenotype is likely to be sensitive to the genetic background. L*er* carries a mutated *ER* gene. To determine whether the lotus-leaf morphology is associated with the *er* mutation, we crossed *as2-101* (L*er*) with a wild-type Landsberg *ER* (Lan) plant. Since the *er* mutation causes distinctive morphologies from those of the *ER* (Lan), it is easy to score the F_2 *as2-101 er* and *as2-101 ER* plants for the lotus-leaf phenotype. Our data showed that the *as2-101 er* plants had a much higher frequency of lotus-leaves than that in the *as2-101 ER* plants (Table 1), indicating that *ER* function is indeed involved in the leaf polarity establishment.

Aberrant adaxial cell identity in *as2* petioles

To identify abnormalities of adaxial-abaxial polarity in *as2* leaves at the cellular level, we analyzed cell patterns by transverse sectioning of *as2* petioles. In a L*er* rosette leaf, adaxial and abaxial surfaces of a petiole can be recognized by the pattern of their epidermal cells (Fig. 2A). The adaxial epidermal cells of a mature petiole are usually large and irregularly shaped, whereas the abaxial epidermal cells are relatively small. Between adaxial and abaxial epidermis, there are small and dense epidermal cells of the petiole margins. The adaxial and abaxial asymmetry of petioles was lost completely in lotus-leaf petioles (Fig. 2B) and the radially symmetric portion of non-lotus-leaf petioles (data not shown) in the *as2-101* mutant. The overall epidermal characters of the radial petiole resembled those of abaxial epidermis in the wild type (for comparison, see Fig. 2A). Moreover, sub-epidermal cells in the lotus-leaf petioles seemed abnormal in shapes compared

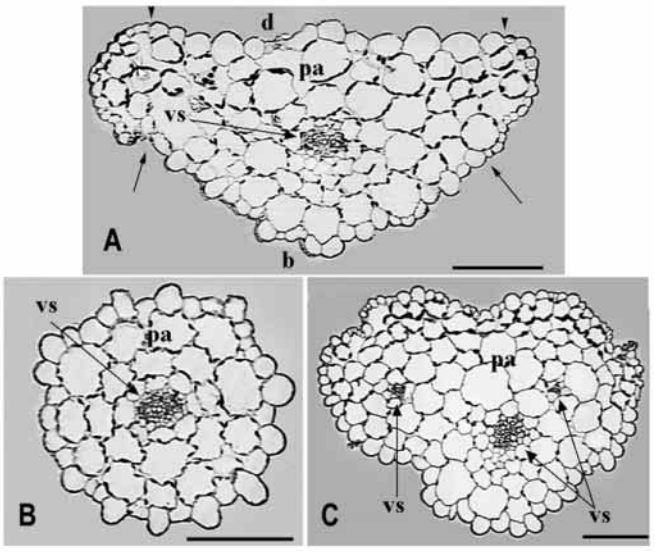

Fig. 2. Transverse sections showing the effects of the *as2* mutation on petiole anatomy. (A) L*er* petiole. (B) Lotus-leaf petiole of *as2-101*. (C) Non-lotus-leaf petiole of *as2-101*. Note that three vascular bundles were seen in the mutant. d, adaxial epidermis between two arrowheads; b, abaxial epidermis between two small arrows; pa, parenchyma; vs, vascular bundle. The small and dense epidermal cells between the arrowheads and small arrow are the petiole margin epidermis in the L*er* plant. Scale bars: 0.1 mm.

with those in the L*er* plant: most cells in L*er* petioles were irregularly shaped (Fig. 2A) while cells in the lotus-leaf petiole were arranged in an orderly fashion (Fig. 2B). In the asymmetric portion of the non-lotus-leaf petioles of the *as2-101* mutant, epidermal cells on the adaxial side were also aberrant as shown in Fig. 2C. In addition to the abaxial epidermal cells, cells similar to the margin epidermis seemed to occupy the adaxial positions. The phenotypic analysis of the lotus-leaf petiole indicates that the *AS2* gene plays an important role in the formation of leaf adaxial-abaxial polarity.

Overexpression of *AS1* and *AS2* results in dramatically different plant morphologies

To further investigate *AS1* and *AS2* functions in the leaf polarity formation, we fused *AS1* and *AS2* cDNAs to the 'constitutive' CAMV 35S promoter and introduced the constructs into L*er* and the corresponding *as1* or *as2* mutant plants, respectively. We analyzed the first pair of rosette leaves, and found that *35S::AS1*/L*er* and *35S::AS2*/L*er* transgenic plants displayed dramatically different phenotypes, although the overall phenotypes of *as1* and *as2* mutants are very similar. L*er* and *as2-101* plants carrying *35S::AS2* had narrow leaves with laminae curled upwards (Fig. 3A,B). In comparison, L*er* plants containing *35S::AS1* displayed a reduced plant stature with normally shaped leaves (Fig. 3C). We also analyzed lamina epidermis that was located midway up the length of the lamina and midway between the margin and the midvein of L*er* and *35S::AS1*/L*er* plants by SEM. For *35S::AS2*/L*er* plants, we analyzed the central portion of the laminae, as the adaxial epidermis in this region could be viewed in a curled leaf. The L*er* adaxial epidermis of leaves was characterized by an undulating surface composed of uniformly sized cells with a

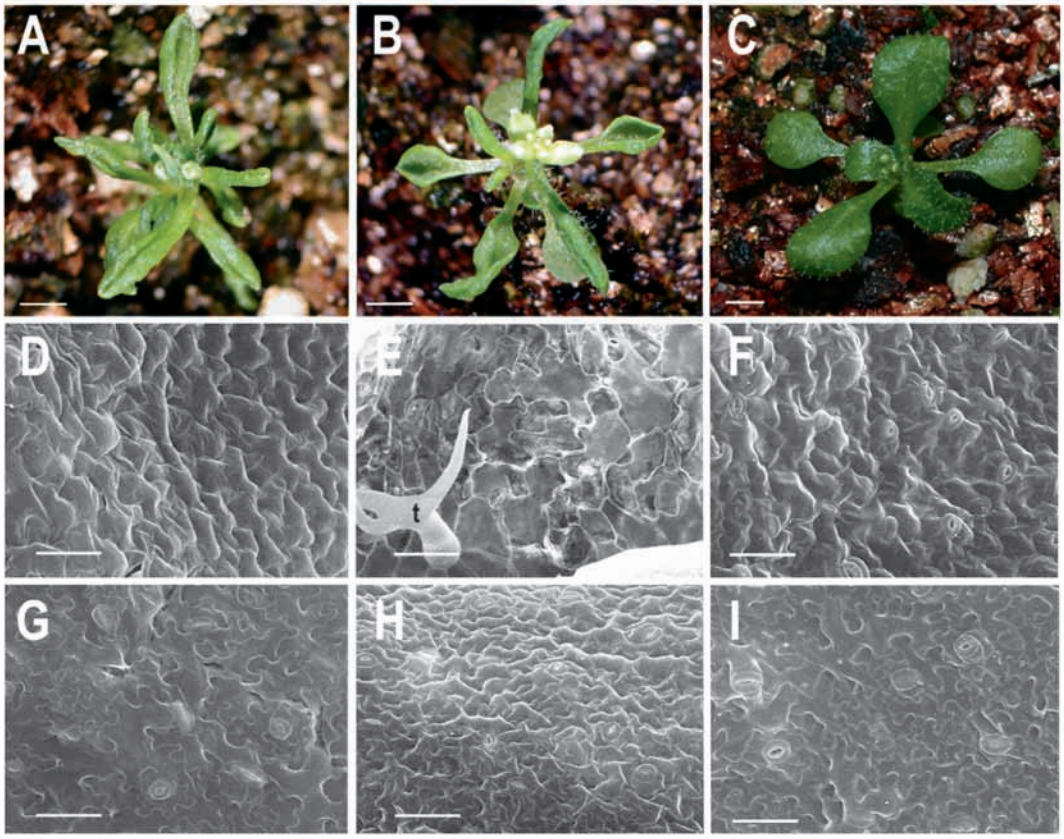

Fig. 3. Phenotypes of *35S::AS1* and *35S::AS2* transgenic plants. (A) Seedling carrying *35S::AS2* in the L*er* background. (B) A plant containing *35S::AS2* in the *as2-101* mutant background. (C) Plant carrying the *35S::AS1* in the L*er* background showing dark green leaves. (D-F) Adaxial lamina epidermis of (D) L*er*, (E) *35S::AS2*/L*er* and (F) *35S::AS1*/L*er*. Note that since leaves of *35S::AS2*/L*er* seedlings are curled upwards, only the central portion of the adaxial surface is visible. (G-I) Abaxial lamina epidermis of (G) L*er*, (H) *35S::AS2* and (I) *35S::AS1*. First pairs of rosette leaves were used for the SEM images. t, three-branched trichome. Scale bars: 1 mm (A-C); 0.05 mm (D-I).

low density of stomata (Fig. 3D). In contrast, the L*er* abaxial epidermis was characterized by a flat surface and a high density of stomata with jigsaw-puzzle-shaped cells (Fig. 3G).

On the adaxial side of laminae, epidermal patterns in L*er* and the *35S::AS1*/L*er* transgenic plants were similar (Fig. 3D,F), although the *35S::AS1* lamina contained more stomata (Fig. 3F). Abaxial epidermal cells in L*er* (Fig. 3G) and the *35S::AS1*/L*er* transgenic plants (Fig. 3I) were also similar in shape. In comparison, the identity of adaxial and abaxial epidermal cells on laminae of the *35S::AS2*/L*er* transgenic plants was altered dramatically. Abaxial-like epidermal cells appeared on part of the adaxial side of laminae of the first pair of rosette leaves (Fig. 3E), whereas the abaxial side was almost entirely covered in cells with adaxial features (Fig. 3H). The other rosette leaves also displayed adaxial-abaxial transformation, albeit weaker: only ectopic patches of adaxial and abaxial epidermal cells appeared on the abaxial and adaxial sides, respectively (data not shown). These results further support the hypothesis that the *AS2* function is required for the adaxial-abaxial polarity in leaves.

To examine *AS1* and *AS2* functions in leaf polarity along the proximodistal axis, we further analyzed adaxial epidermal identity in the *as1*, *as2* and *35S::AS2*/L*er* leaves. Fig. 4A,B shows the adaxial epidermis in the proximal part of a L*er* lamina. There were two distinctive cell types: elongated cells of the midvein and the relatively uniform epidermal cells that covered most of the lamina. The equivalent region of the *as2* leaf epidermis (Fig. 4D,E) contained only one type of cell that was long and narrow in shape. These cells resembled the epidermal cells on the margin of L*er* petiole (Fig. 4C, arrowhead), and were very similar to the epidermal cells on the adaxial side of the *as2* petiole (Fig. 4F), consistent with the results from transverse sections (Fig. 2). Epidermal patterns in the *as1* mutant were very similar to those in the *as2* mutant (data not shown). In comparison, *35S::AS2*/L*er* petioles contained the uniformly shaped epidermal cells (white arrowhead) and elongated midvein-like cells (black arrowhead, Fig. 4G,H). This type of cell is usually positioned in the more distal region in the L*er* lamina. In the more proximal portion of the petiole, epidermal cells were mosaic with a mixture of adaxial- and abaxial-type cells (Fig. 4I). This abnormal proximodistal differentiation, however, was not seen in the *35S::AS1*/L*er* plants (data not shown). These results indicate that the *AS1* and *AS2* functions are also required for promotion of cell fate along the proximodistal axis.

Interestingly, *35S::AS2*/L*er* transgenic plants also produced needle-like leaves amongst the first appearing rosette leaves, similar to those in *as2-101* mutant in terms of whole organ structure and size (Fig. 1J; Fig. 5A,E). However, the epidermal cells of these structures are markedly different in the *as2-101* mutant and *35S::AS2*/L*er* transgenic plants (Fig. 5A,E). Epidermal cells on most of the *as2-101* needle-like structure were long and narrow (Fig. 5B), similar to those on the petiole margin in the L*er* plant (Fig. 4C). However, the epidermal cells on *35S::AS2*/L*er* needle-like leaves looked more similar to the lamina adaxial epidermal cells (Fig. 5F). In more distal regions of the *as2-101* needle-like leaves, the long and narrow epidermal cells were partially developed into lamina abaxial cells (Fig. 5C, arrowheads), or even completely abaxialized (Fig. 5D). However, in the equivalent region in *35S::AS2*/L*er*

needle-like leaves, the surface was undulating with dense stomata, reflecting a trend to being expanded into lamina (Fig. 5G), or with three-branch trichomes (Fig. 5H). This type of trichome is usually associated with the adaxial surface of the wild-type laminae in the early-appearing rosette leaves. Therefore, the needle-like structure in *35S::AS2*/L*er* seedlings was an adaxialized organ. This needle-like structure was not observed in any of the *35S::AS1*/L*er* transgenic plants. Phenotypic analysis of the needle-like organs of the *as2* mutant and *35S::AS2* transgenic plants supports the hypothesis that *AS2* function is required for adaxial cell differentiation.

Ectopic expression of *AS2* suppresses *KNAT1* in floral inflorescence

AS1 and *AS2* down-regulate *KNAT1*, an *Arabidopsis* class I *KNOTTED1*-like gene, in leaves (Byrne et al., 2000; Semiarti et al., 2001). To determine whether the *AS1* or *AS2* gene is sufficient for this down-regulation, we analyzed inflorescence phenotypes and *KNAT1* expressions in inflorescence of *35S::AS1*/L*er* and *35S::AS2*/L*er* transgenic plants. The *Arabidopsis brevipedicellus* (*bp*) mutant carries a *knat1* mutation, which causes altered inflorescence architecture, with reduced internode and pedicel lengths, bending at the nodes, and downward-oriented flowers and siliques (Douglas et al., 2002; Venglat et al., 2002). Therefore, if *KNAT1* expression is suppressed in the transgenic plants, we expected to see *bp*-like phenotypes. In comparison to the L*er* (Fig. 6A), *as1* (Fig. 6B) and *as2* (Fig. 6D) plants, the *35S::AS2*/L*er* transgenic plants exhibited downward-pointing siliques (Fig. 6E) with very short pedicels, very similar to those in the *bp* mutant (Fig. 6F). Plants overexpressing *AS1* did not show such an inflorescence phenotype (Fig. 6C). RT-PCR results showed that *KNAT1* expression was dramatically reduced in the inflorescence in the *35S::AS2*/L*er* transgenic plants, but was not affected in plants that carried *35S::AS1*/L*er* (Fig. 6G). This result suggests that although either *AS1* or *AS2* is required for the down-regulation of *KNAT1* in leaves (Byrne et al., 2000; Semiarti et al., 2001), the amount or location of AS2 may be more crucial for the down-regulation in inflorescence.

AS1 and AS2 can interact physically

The *Arabidopsis as1* and *as2* mutants display very similar phenotypes, which led to the hypotheses that the AS1 and AS2 function in the same regulatory pathway (Serrano-Cartagena et al., 1999) or that they may interact with each other (Byrne et al., 2002; Xu et al., 2002). To gain more direct evidence that *AS1* and *AS2* function together in the leaf polarity formation, we constructed *35S::AS1*/*as2-101* and *35S::AS2*/*as1-101* transgenic plants. Phenotypes including leaf shapes (Fig. 7A,B) and epidermal patterns (data not shown) of *35S::AS1/as2-101* and *35S::AS2/as1-101* plants resembled those of the *as2* and *as1* single mutants, respectively. The curled rosette leaves (Fig. 3A) and the downward-pointing flowers (Fig. 7C) in the *35S::AS2/*L*er* plants were not seen in *35S::AS2/as1-101* plants (Fig. 7A,D). Again, the increased stomata on the adaxial side in the *35S::AS1*/L*er* leaves (Fig.

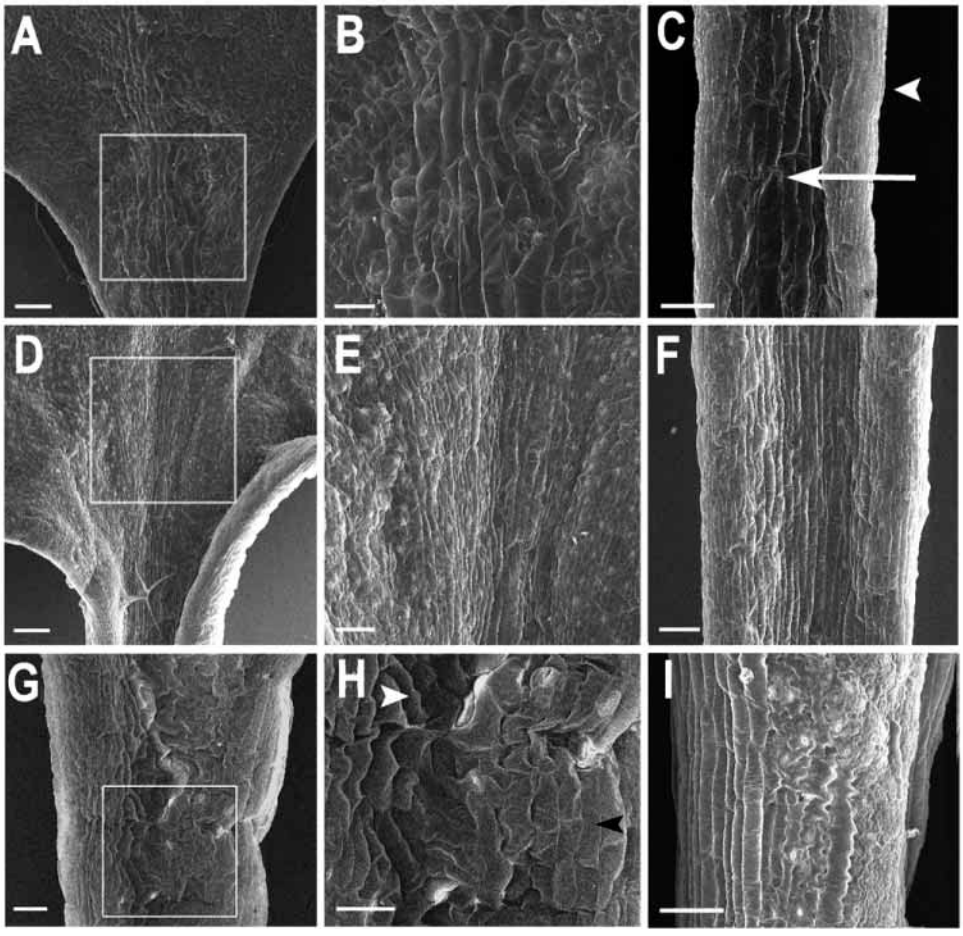

Fig. 4. SEM of epidermal cells of L*er*, *as2-101* and *35S::AS2*/L*er* leaves. (A) Adaxial surface of the proximal part of a L*er* lamina. (B) The boxed region of A showing a close-up of two types of epidermal cells. (C) Adaxial epidermal cells on a L*er* petiole. Arrowhead, margin cells; arrow, adaxial cells. (D) The proximal part of the adaxial surface of an *as2-101* lamina. (E) The boxed region of D showing a close-up of the long and narrow epidermal cells. (F) Adaxial epidermal cells on an *as2-101* petiole, showing that all epidermal cells on the petiole are long and narrow. Note that these cells show a very similar pattern to those found on the proximal part of the *as2-101* leaf lamina in D, and are also similar to petiole margin epidermal cells of L*er*. (G) Epidermal cells of an adaxial *35S::AS2* petiole. (H) The boxed region of G showing the uniformly shaped adaxial epidermis (white arrowhead) and the thick midvein-like epidermis (black arrowhead). (I) Abaxial side of a *35S::AS2* petiole with mosaic adaxial and abaxial patches. Scale bars: 0.2 mm (A,D); 0.1 mm (B,C,E-G,I); 0.05 mm (H).

3F) were not observed in the *35S::AS1/as2-101* transgenic plants (data not shown). These results indicate that the *AS1* function in the regulation of leaf polarity formation needs the presence of the *AS2* function, and vice versa. Flower phenotypes of *35S::AS1/as2-101* and *35S::AS2/as1-101* plants differed from those of the corresponding *as2-101* and *as1-101* mutants. Briefly, *as2-101* (Xu et al., 2002) and *35S::AS1/as2-101* (data not shown) had similar flower shapes, but fertility in *35S::AS1/as2-101* flowers was reduced. Phenotypes of *as1-101* (Sun et al., 2002) and *35S::AS2/as1-101* flowers differed markedly. Flowers in the *35S::AS2/as1-101* plants were all sterile with shortened sepals, petals and stamens. The aberrant flower phenotypes in *35S::AS1/as2-101* and *35S::AS2/as1-101* transgenic plants indicate that AS1 and AS2 have separate functions in flower development in addition to their regulations in the leaf polarity formation.

To test the possible physical interaction between AS1 and AS2 proteins, we first carried out a yeast two-hybrid assay. Yeast cells that coexpressed the AS1 bait and the AS2 prey fusion proteins, and the AS2 bait and the AS1 prey fusion proteins both showed clear β-galactosidase activity (Fig. 8A). However, cells coexpressing AS1 or AS2 together with a vector-only control were β-galactosidase negative. A parallel yeast two-hybrid experiment showed that coexpression of AS1 and AS2 also promoted the expression of *HIS3* and *ADE2* reporter genes, allowing cells to grow on media lacking tryptophan, leucine, histidine and adenine (Fig. 8B). These results demonstrate that AS1 and AS2 bind each other in yeast cells. To further confirm the physical interaction between AS1 and AS2 proteins, we performed ELISA experiments using purified His-AS1 and GST-AS2 (Fig. 8C). Our results showed that the increased absorbance could be recorded only in the presence of both AS1 and AS2 proteins (Fig. 8D), indicating these two proteins can indeed associate physically.

DISCUSSION

AS1 and *AS2* in leaf adaxial-abaxial polarity

The *as1* and *as2* mutants in the L*er* genetic background show a higher frequency of lotus-leaf structure. In the most severe case, a needle-like organ forms in place of some rosette leaves. This structure is very similar to that in the *phan* mutant in *Antirrhinum* (Waites and Hudson, 1995). A *phan* allele, *phan-607*, grown at 17°C produced almost exclusively needle-like leaves. Epidermal cells on these radialized leaves in the *phan* mutant are long and narrow, resembling those on the wild-type abaxial epidermis of leaves (Waites and Hudson, 1995). Epidermal cells on needle-like leaves of the *as1* and *as2* mutants are similar to those of the *phan* mutants, suggesting

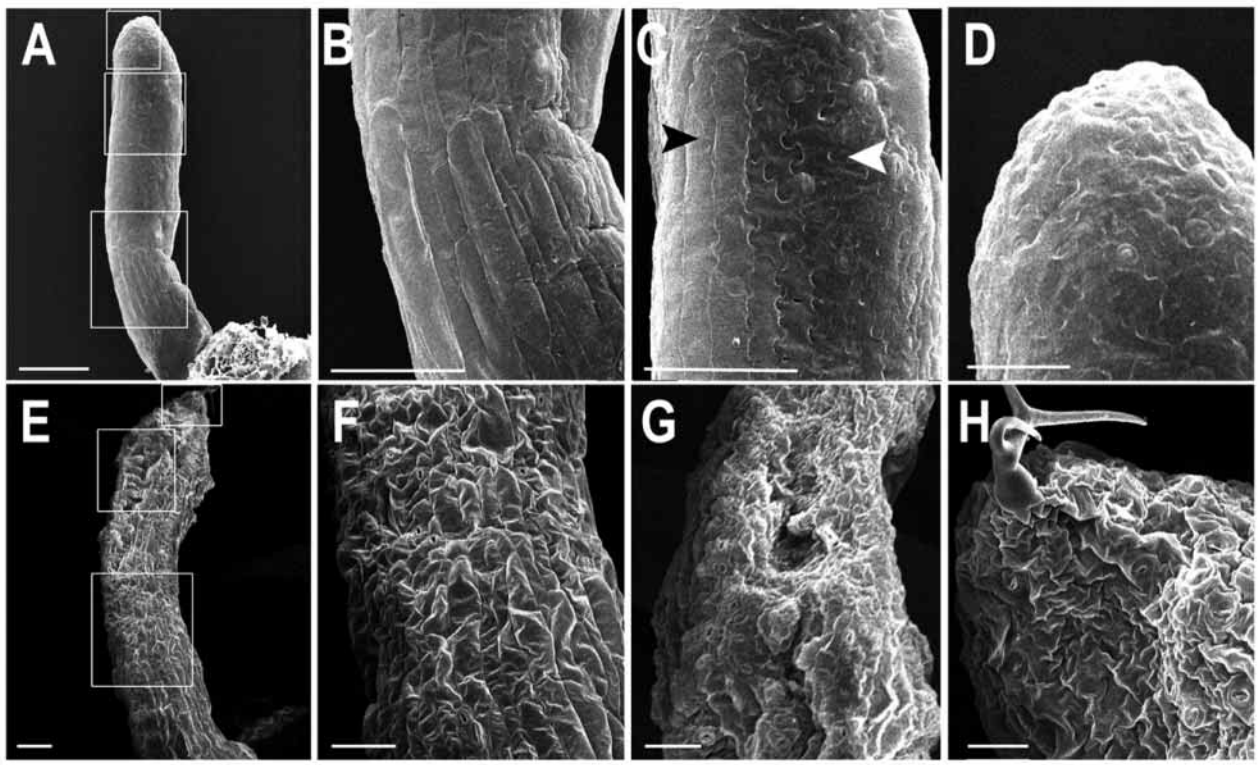

Fig. 5. SEM of needle-like leaves in *as2-101* and *35S::AS2*//L*er* plants. (A) A first *as2-101* rosette leaf with needle-like structure. (B-D) The bottom, middle and top boxed areas of A, respectively. (B) The epidermal cells of the proximal region of this structure mimic those of the petiole abaxial cells in L*er* plants, and are also similar to those of *phan-607* needle-like leaves (Waites and Hudson, 1995). (C) In the more distal portion, cells differentiate into abaxial epidermis (white arrowhead) with some intermediate cells between the jigsaw-shaped abaxial epidermal cells and the petiole abaxial cells in the L*er* (black arrowhead). (D) At the tip of the needle-like organ, all cells are abaxilized. (E) A first appearing *35S::AS2*//L*er* leaf with needle-like structure. (F-H) The bottom, middle and top boxed areas of E, respectively. Note that the epidermal pattern of this structure is similar to that of the needle-like leaves in the *phb-1d* mutant (McConnell and Barton, 1998). Scale bars: 0.2 mm (A,E); 0.1 mm (B,C,F,G); 0.05 mm (D,H).

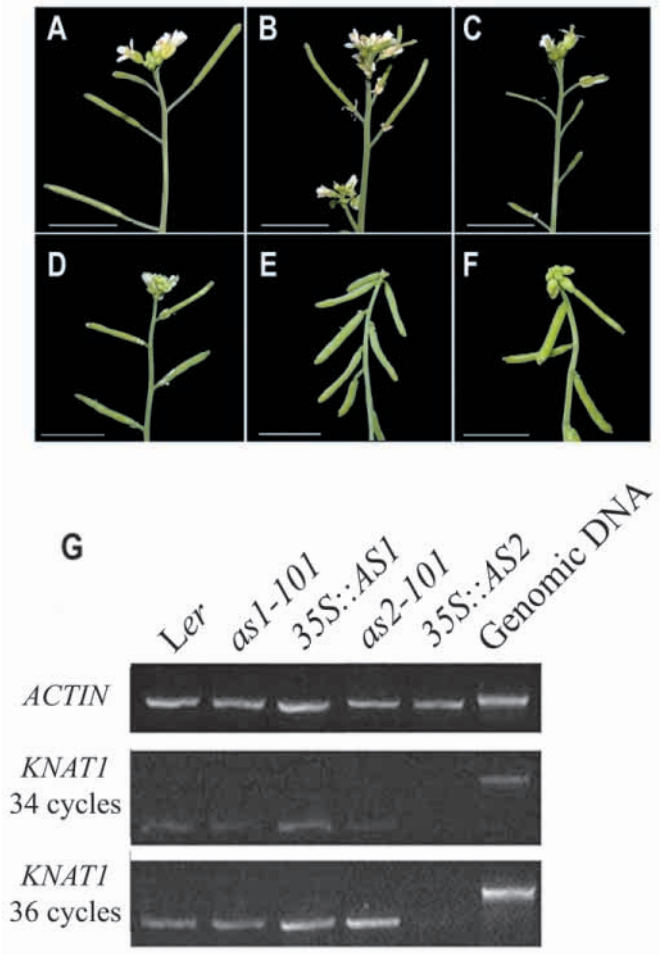

Fig. 6. Overexpression of *AS2* suppresses *KNAT1* expression. (A-F) Same-stage inflorescences. (A) L*er*, (B) *as1-101*, (C) *35S::AS1*/L*er*, (D) *as2-101*, (E) *35S::AS2*/L*er*, (F) *bp*. Note that *35S::AS2* transgenic plants and *bp* mutant plants exhibit similar altered inflorescence architecture, with downward-pointing siliques. (G) RT-PCR. RNA was extracted from the primary inflorescence, and the amplified DNA fragments were separated by electrophoresis on an agarose gel and visualized by staining with ethidium bromide. Scale bars: 1 mm (A-F).

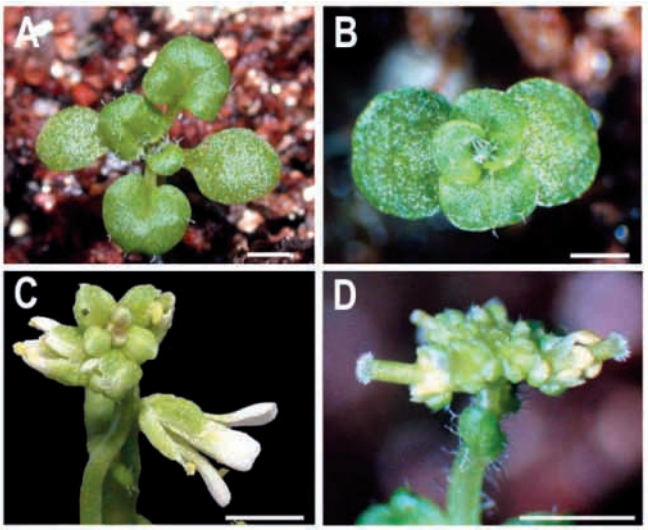

Fig. 7. Phenotypes of *35S::AS1/as2* and *35S::AS2/as1* transgenic plants. (A) *35S::AS1/as2-101* seedling that is similar to the *as2* mutant. (B) *35S::AS2/as1-101* seedling that resembles the *as1* mutant. Note, the curled leaves in *35S::AS2/Ler* were not seen in the *35S::AS2/as1-101* plants. (C) *35S::AS2*/L*er* inflorescence with a downward-pointing flower. (D) *35S::AS2/as1-101* inflorescence. Flowers are not downward-pointing. Scale bars: 1 mm.

that the needle-like leaves in *as1* and *as2* are also abaxialized organs. Although petioles in the less severe *as1* and *as2* leaves can grow asymmetrically, cell specialization at the adaxial surface is aberrant. All these results plus the fact that *AS2* is preferentially expressed adaxially in cotyledons in the embryonic stages (Iwakawa et al., 2002) strongly suggest that *AS2* is an adaxial-promoting factor in leaves.

Of the three axes of leaves, establishment of adaxial-abaxial polarity is the primary and most essential process for leaf development. It was previously proposed that the establishment of adaxial-abaxial polarity is a requirement for proper lamina growth (Sussex, 1954; Sussex, 1955). *as1* and *as2* mutant plants and *35S::AS2*/L*er* transgenic plants all have needle-like leaves, however, the features of these organs are totally different. The needle-like structure in *as1* and *as2* is due to a reduction in adaxial differentiation, whereas that in the *35S::AS2*/L*er* transgenic plants shows only the adaxial epidermis. Moreover, the *Arabidopsis* semidominant mutant *phb-1d* also has needle-like structures, which were thought to be adaxialized organs (McConnell and Barton, 1998; McConnell et al., 2001). The epidermal pattern of needle-like leaves in *35S::AS2*/L*er* transgenic plants is very similar to that of needle-like organs in *phb-1d*, indicating the needle-like leaves in *35S::AS2*/L*er* transgenic plants may also be adaxialized organs. Needle-like leaves cannot develop further to form laminae, regardless of their adaxialized or abaxialized nature. This is consistent with the proposal of Sussex that proper establishment of adaxial-abaxial polarity is required for lamina development (Sussex, 1954; Sussex, 1955).

Interestingly, the lotus-leaf in *as1* and *as2* mutants is also very similar to the trumpet-shaped leaves in the *phb-1d* mutant (McConnell and Barton, 1998). However, the inside and outside cell identities in lotus-leaves and trumpet-shaped leaves is reversed (data not shown) (McConnell and Barton, 1998). Cells with adaxial identity are on the inside surface of the *as1/as2* lotus-leaf, while such cells are on the outside surface of the *phb-1d* trumpet-shaped leaf. The analysis of leaf phenotypes in *35S::AS2*/L*er* transgenic plants, especially needle-like structures in the *as2* mutants and the *35S::AS2*/L*er* transgenic plants further supports the hypothesis that the primary function of *AS2* is related to the promotion of the adaxial cell differentiation. Since AS1 associates with AS2, and the *as1* mutant also showed lotus-leaves and needle-like leaves (Sun et al., 2002) (data not shown), it is possible that *AS1* also functions as an adaxial promoting factor in leaf polarity formation. Our recent results using RT-PCR showed that expression of the *PHB* gene was enhanced in the *35S::AS1*/L*er* and *35S::AS2*/L*er* transgenic plants, and expression of the *FILAMENTOUS FLOWER* (*FIL*), a member in the *YABBY* family, was promoted in the *as1-101* and *as2-101* mutant plants (L.X., H. Li and H.H., unpublished). These results suggest that the *AS1* and *AS2*

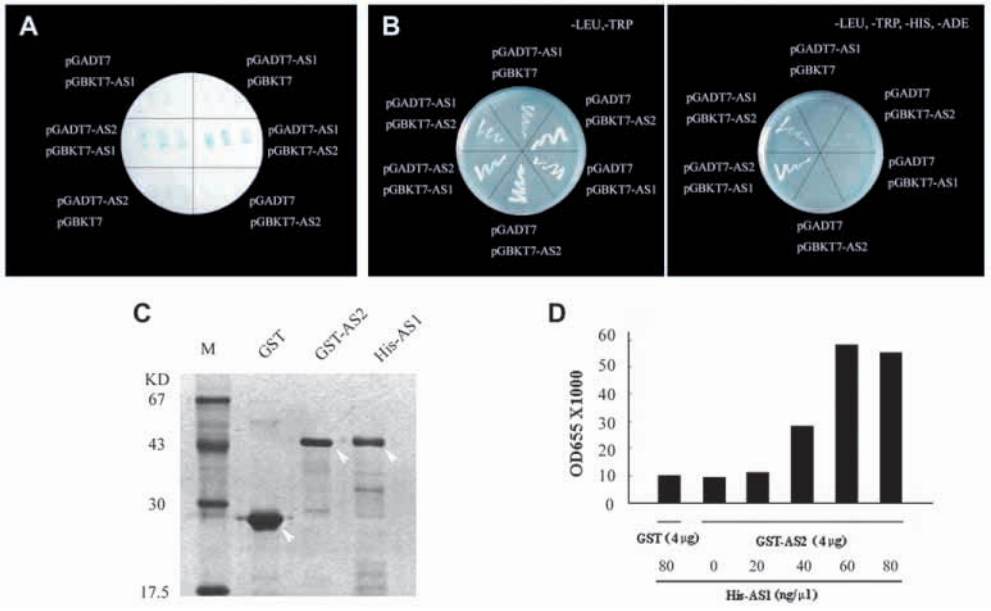

Fig. 8. AS1 and AS2 interaction in yeast and in ELISA. (A,B) Yeast two-hybrid analyses by co-expression of AS1 and AS2, showing that the interaction of AS1 and AS2 turns on the reporter genes *lacZ* (A) and *HIS3* and *ADE2* activities (B). (C) Recombinant purified proteins His-AS1 and GST-AS2 were analyzed by SDS-PAGE and stained by Coomassie Blue R-250. Note, sizes of the recombinant His-AS1 and GST-AS2 proteins agreed with their predicted molecular masses. M, molecular mass marker (in kDa); GST, glutathione *S*-transferase control; GST-AS2, GST tag and AS2 fusion protein; His-AS1, His tag and AS1 fusion protein. (D) ELISA was performed showing protein-protein interaction between the His-AS1 and GST-AS2.

are genetically upstream to the *PHB* and *FIL* genes in the regulation of leaf polarity.

ER function in the *AS1-AS2* pathway for leaf polarity

We previously demonstrated that lotus-leaves appeared at a much higher frequency in *as1* and *as2* mutants in the L*er* background than that in any other genetic backgrounds analyzed (Sun et al., 2002; Xu et al., 2002). Although genomes from different *Arabidopsis* ecotypes contain polymorphisms, a major difference between L*er* and other *Arabidopsis* strains is that L*er* carries a mutated form of the *ER* gene. This mutation confers plants with a compact inflorescence, blunt fruits, and short petioles (Torii et al., 1996). In this work, we provide direct evidence that the higher frequency of lotus-leaves in *as2* mutant was caused by the *er* mutation. Therefore, both AS1 and AS2 (possibly the AS1-AS2complex), as well as ER contribute to the leaf polarity formation. The *ER* gene encodes a receptor protein kinase with extracellular leucine-rich repeats (Torii et al., 1996). It is widely expected that ER regulates signaling in plant development.

The *Arabidopsis bp* mutant carries mutated *KNAT1* and *ER* genes. It was proposed that *ER* functions redundantly with *KNAT1* to regulate plant architecture and stem differentiation (Douglas et al., 2002; Venglat et al., 2002). Although *AS1-AS2* and *ER* also seem to be redundant in the promotion of the adaxial cell fate, similar to the *KNAT1* and *ER* pair in the *bp* mutant, we hypothesize that *AS1-AS2* and *ER* may play different roles in the establishment of leaf polarity. First, we have observed that *as2* mutations in genetic backgrounds other than L*er* also showed lotus-leaves, although at much lower frequencies (Xu et al., 2002). In addition, petioles of the first pair of rosette leaves in all *as2* alleles, regardless of genetic backgrounds, contain a radially symmetric portion. Although petioles of *as1-1* plants in the mixed Col/L*er* background did not show even the radially symmetric portion, petioles in *as1-1* and plants with the other *as1* and *as2* alleles all reflect a same defect. These observations indicate that the *AS1* and *AS2* functions, but not the *ER* function, are primarily necessary for the normal adaxial-abaxial polarity in leaves. Second, the length of the radially symmetric portion in *as1 er* (data not shown) and *as2 er* was highly variable: from fully expanded leaves to needle-like leaves. Nevertheless, *as2 ER* showed very few lotus-leaves and less variable portions of radially symmetric petioles, and *as1 ER* did not contained any radially symmetric position. These observations suggest that the *ER* function may reduce the sensitivity of plant cells to yet unknown internal or environmental signals for leaf development.

Function of the AS1-AS2 complex

Arabidopsis as1 and *as2* mutants have very similar leaf morphology (Redei, 1965; Serrano-Cartagena et al., 1999; Sun et al., 2000). Both mutants also show misexpression of the class 1 *KNOX* genes (Byrne et al., 2000; Ori et al., 2000; Semiarti et al., 2001; Byrne et al., 2002) and suppression of the *LATERAL ORGAN BOUNDARIES* (*LOB*) gene (Shuai et al., 2002). All these suggest that these two genes function in the same regulatory pathway. In this work, we provided direct genetic evidence showing a requirement of the *AS1* and *AS2* functions together in the leaf development: *35S::AS1/as2* and *35S::AS2/as1* transgenic plants demonstrated only the *as1*- or *as2*-like leaf phenotypes, which are markedly different from those in the corresponding *35S::AS1*/L*er* and *35S::AS2*/L*er* plants. To explore the underlying molecular mechanisms of *AS1* and *AS2* actions in leaf development, we previously examined *AS1* expression in the *as2* mutant and *AS2* expression in the *as1* mutant to determine if these two genes are regulated by each other. There were no obvious changes in either *AS1* or *AS2* transcripts when one gene was expressed in the other mutant background (Xu et al., 2002), suggesting that the direct transcriptional regulation of one by the other is not likely.

In this work, we tested the possibility of protein-protein interactions between AS1 and AS2. We showed that AS1 and AS2 can indeed associate together both in vitro and in yeast cells. These results suggest that AS1 and AS2 may form a

complex to regulate their downstream genes during leaf development.

This regulatory model is similar to that with products of floral organ identity genes *APETALA3* (*AP3*) and *PISTILATA* (*PI*) in *Arabidopsis.* AP3 and PI can associate to form a complex, and mutation in either *AP3* or *PI* results in very similar floral phenotypes (Jack et al., 1992; Goto and Meyerowitz, 1994).

Although *as1* and *as2* have comparable defects in leaf development, transgenic plants carrying *35S::AS1*/L*er* and *35S::AS2*/L*er* exhibited dramatically different phenotypes, not only morphologically but also at the molecular level, such as the suppression of *KNAT1* expression. One possibility is that the AS1 and AS2 proteins are not present at similar levels in wild-type plants. The AS1 protein may be more abundant than AS2, such that the increase of AS2 dosage results in the formation of more functional AS1-AS2 complexes. Another possibility is that the different phenotypes are caused by the endogenous gene expression pattern. *AS1* is expressed throughout the leaf, a pattern similar to that of the *35S*-driven gene expression in leaves. The same expression pattern of the *AS1* gene may not generate altered phenotypes. *AS2* is expressed only adaxially as reported in the embryonic cotyledons (Iwakawa et al., 2002), and therefore ectopic *AS2* expression under the control of the *35S* promoter may cause dramatic phenotypic changes.

It is known that both *AS1* and *AS2* are needed to down-regulate class 1 *KNOX* genes, because loss-of-function mutations in *AS1* and *AS2* result in the ectopic expression of *KNOX* genes in leaves (Byrne et al., 2000; Semiarti et al., 2001). Based on the analyses of *35S::AS1*/L*er* and *35S::AS2*/L*er* transgenic plants, only the *AS2* ectopic overexpression suppressed *KNAT1* expression in the inflorescence and generated *bp*-like phenotypes. This phenomenon can also be accounted for by the less abundant AS2 dosage and (or) the strict AS2 distribution in wild-type inflorescence failing to form enough complexes to suppress *KNAT1*, although the AS1 appears in the same-stage inflorescence (Byrne et al., 2000).

A proposed genetic model for *AS1*, *AS2* and *ER* actions in leaves

Based on the AS1 and AS2 expression patterns (Byrne et al., 2000; Iwakawa et al., 2002) and the results in this work, we propose a model of *AS1*, *AS2* and *ER* actions in the establishment of leaf polarity (Fig. 9). AS1 and AS2 can bind each other (evidence from the yeast two-hybrid assays and the in vitro protein binding experiment). The AS1-AS2 complex may efficiently suppress *KNAT1* expression in leaves (*KNAT1* was expressed ectopically in the *as1* and *as2* leaves, but was repressed in wild-type leaves). The AS1-AS2 complex can efficiently promote adaxial leaf identity (the *as1* and *as2* single mutants both showed defective epidermal cells on the adaxial surface, indicating that the independent *AS1* and *AS2* functions cannot normally promote the adaxial identity; and evidence also from *35S::AS2*/*as1-101* plants as they failed to reproduce the phenotypes of *35S::AS2*/L*er* plants, which had adaxialized leaves). The *ER* function is required for promoting adaxial-abaxial polarity formation in the AS1-AS2 regulatory pathway (*as1 ER* and *as2 ER* plants show much weaker adaxial-abaxial defects of leaves than *as1 er* and *as2 er* plants, respectively),

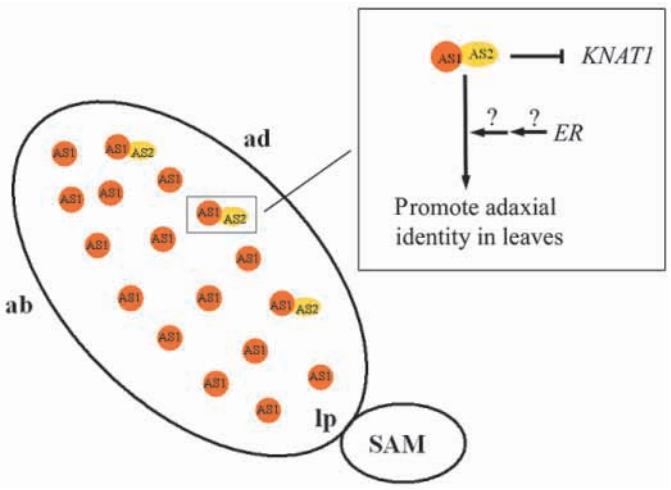

Fig. 9. A genetic model for *AS1*, *AS2* and *ER* actions in establishment of the leaf adaxial-abaxial polarity. lp, leaf primodium; ad, adaxial side; and ab, abaxial side.

however the exact involvement of *ER* action in this pathway remain to be elucidated.

We noted that the strongest phenotypes in the *as1* and *as2* mutant plants and the *35S::AS2*/L*er* transgenic plants all appear in the earliest rosette leaves (the first pair of rosette leaves). It is possible that there might be some other regulators in *Arabidopsis* to promote leaf adaxial identity in addition to AS1/AS2 and PHB/PHV. These participants may have partially overlapping action domains with *AS1*/*AS2* and *PHB/PHV* but play major roles in promoting leaf adaxial identity only in the late appearing leaves. Identification and characterization of new genes and elucidation of the regulatory network for all these genes will refine our views of axis formation in plants, and therefore provide new insights into the leaf development.

The authors would like to thank the Ohio State University *Arabidopsis* Stock Center for providing *as1-1*, *as2-1*, *bp* and Lan seeds, J. Mao and Y. Dou for SEM, X. Gao for thin sectioning, and W. Shen, X. Chen and H. Ma for helpful discussions and comments on the manuscript. This work was supported by grants from the Chinese Administration of Science and Technology (863 and 973), the Chinese National Scientific Foundation and the Shanghai Scientific Committee, to H.H.

REFERENCES

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

Bowman, J. L., Eshed, Y. and Baum, S. F. (2002). Establishment of polarity in angiosperm lateral organs. *Trends Genet* **18**, 134-141.

Byrne, M. E., Barley, R., Curtis, M., Arroyo, J. M., Dunham, M., Hudson, A. and Martienssen, R. A. (2000). *Asymmetric leaves1* mediates leaf patterning and stem cell function in *Arabidopsis*. *Nature* **408**, 967-971.

Byrne, M. E., Simorowski, J. and Martienssen, R. A. (2002). *ASYMMETRIC LEAVES1* reveals *knox* gene redundancy in *Arabidopsis*. *Development* **129**, 1957-1965.

Chen, C., Wang, S. and Huang, H. (2000). *LEUNIG* has multiple functions in gynoecium development in *Arabidopsis*. *Genesis* **26**, 42-54.

Chen, Q., Atkinson, A., Otsuga, D., Christensen, T., Reynolds, L. and Drews, G. N. (1999). The *Arabidopsis FILAMENTOUS FLOWER* gene is required for flower formation. *Development* **126**, 2715-2726.

Clarke, J. H., Tack, D., Findlay, K., Montagu, M. V. and Lijsebettens, M.

V. (1999). The *SERRATE* locus controls the formation of the early juvenile leaves and phase length in *Arabidopsis*. *Plant J.* **20**, 493-501.

Douglas, S. J., Chuck, G., Dangler, R. E., Pelecanda, L. and Riggs, C. D. (2002). *KNAT1* and *ERECTA* regulate inflorescence architecture in Arabidopsis. *Plant Cell* **14**, 547-558.

Eshed, Y., Baum, S. F. and Bowman, J. L. (1999). Distinct mechanisms promote polarity establishment in carpels of *Arabidopsis*. *Cell* **99**, 199-209.

Eshed, Y., Baum, S. F., Perea, L. V. and Bowman, J. L. (2001). Establishment of polarity in lateral organs of plants. *Curr. Biol.* **11**, 1251-1260.

Goto, K. and Meyerowitz, E. M. (1994). Function and regulation of the *Arabidopsis* floral homeotic gene *PISTILLATA*. *Genes Dev.* **8**, 1548-1560.

Ha, C. M., Kim, G., Kim, B. C., Jun, J. H., Soh, M. S., Ueno, Y., Machida, Y., Tsukaya, H. and Nam, H. G. (2003). The *BLADE-ON-PETIOLE1* gene controls leaf pattern formation through the modulation of meristematic activity in *Arabidopsis*. *Development* **130**, 161-172.

Huang, H., Tudor, M., Weiss, C. A., Hu, Y. and Ma, H. (1995). The *Arabidopsis* MADS-box gene *AGL3* is widely expressed and encodes a sequence-specific DNA-binding protein. *Plant Mol. Biol.* **28**, 549-567.

Iwakawa, H., Ueno, Y., Semiarti, E., Onouchi, H., Kojima, S., Tsukaya, H., Hasebe, M., Soma, T., Ikezaki, M., Machida, C. and Machida, Y. (2002). The *ASYMMETRIC LEAVES2* gene of *Arabidopsis thaliana*, required for formation of a symmetric flat lamina, encodes a member of a novel family of proteins characterized by cystein repeats and a leucine zipper. *Plant Cell Physiol.* **43**, 467-478.

Jack, T., Brockman, L. L. and Meyerowitz, E. M. (1992). The homeotic gene *APETALA3* of *Arabidopsis thaliana* encodes a MADS box and is expressed in petals and stamens. *Cell* **68**, 683-697.

Kerstetter, R. A., Bollman, K., Taylor, R. A., Bomblies, K. and Poethlg, R. S. (2001). *KANADI* regulates organ polarity in *Arabidopsis*. *Nature* **411**, 706-709.

McConnell, J. R. and Barton, M. K. (1998). Leaf polarity and meristem formation in *Arabidopsis*. *Development* **125**, 2935-2942.

McConnell, J. R., Emery, J., Eshed, Y., Bao, N., Bowman, J. and Barton, M. K. (2001). Role of *PHABULOSA* and *PHAVOLUTA* in determining radial patterning in shoots. *Nature* **411**, 709-713.

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

Otsuga, D., DeGuzman, B., Prigge, M. J., Drews, G. N. and Clark, S. E. (2001). *REVOLUTA* regulates meristem initiation at lateral positions. *Plant J.* **25**, 223-236.

Redei, G. P. (1965). Non-mendelian megagametogenesis in *Arabidopsis*. *Genetics* **51**, 857-872.

Sawa, S., Ito, T., Shimura, Y. and Okada, K. (1999a). *FILAMENTOUS FLOWER* controls the formation and development of Arabidopsis inflorescences and floral meristems. *Plant Cell* **11**, 69-86.

Sawa, S., Watanabe, K., Goto, K., Kanaya, E., Morita, E. H. and Okada, K. (1999b). *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.

Semiarti, E., Ueno, Y., Tsukaya, H., Iwakawa, H., Machida, C. and Machida, Y. (2001). 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. *Development* **128**, 1771-1783.

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.

Shuai, B., Reynaga-pena, C. G. and Springer, P. S. (2002). The *LATERAL ORGAN BOUNDARIES* gene defines a novel, plant specific gene family. *Plant Physiol.* **129**, 747-761.

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

Sun, Y., Zhang, W., Li, F., Guo, Y., Liu, T. and Huang, H. (2000). Identification and genetic mapping of four novel genes that regulate leaf development in *Arabidopsis*. *Cell Res.* **10**, 325-335.

Sun, Y., Zhou, Q., Zhang, W., Fu, Y. and Huang, H. (2002). *ASYMMETRIC LEAVES1*, an *Arabidopsis* gene that is involved in the control of cell differentiation in leaves. *Planta* **214**, 694-702.

Sussex, I. M. (1954). Experiments on the cause of dorsoventrality in leaves. *Nature* **174**, 351-352.

Sussex, I. M. (1955). Morphogenesis in *Solanum tuberosum* L: Experiment investigation of leaf dorsoventrality and orientation in the juvenile shoot. *Phytomorphology* **5**, 286-300.

Talbert, P., Adler, H. T., Parks, D. W. and Comai, L. (1995). The *REVOLUTA* gene is necessary for apical meristem development and for limiting cell divisions in the leaves and stems of *Arabidopsis thaliana*. *Development* **121**, 2723-2735.

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.

Torii, K. U., Mitsukawa, N., Oosumi, T., Matsuura, Y., Yokoyama, R., Whittier, R. F. and Komeda, Y. (1996). The Arabidopsis *ERECTA* gene encodes a putative receptor protein kinase with extracellular leucine-rich repeats. *Plant Cell* **8**, 735-746.

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.

Tsiantis, M. (2001). Control of shoot cell fate: beyond homeoboxes. *Plant Cell* **13**, 733-736.

Venglat, S. P., Dumonceaux, T., Rozwadowski, K., Parnell, L., Babic, V., Keller, W., Martienssen, R., Selvaraj, G. and Datla, R. (2002). The homeobox gene *BREVIPEDICELLUS* is a key regulator of inflorescence architecture in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA* **99**, 4730-4735.

Waites, R. and Hudson, A. (1995). *phantastica*: a gene required for dorsoventrality of leaves in *Antirrhinum majus*. *Development* **121**, 2143-2154.

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.

Xu, Y., Sun, Y., Liang, W. and Huang, H. (2002). The *Arabidopsis AS2* gene encoding a predicted leucine-zipper protein is required for the leaf polarity formation. *Acta Bot. Sin.* **44**, 1194-1202.