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Rapid Paper

The *ASYMMETRIC LEAVES2* Gene of *Arabidopsis thaliana*, Required for Formation of a Symmetric Flat Leaf Lamina, Encodes a Member of a Novel Family of Proteins Characterized by Cysteine Repeats and a Leucine Zipper

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The *ASYMMETRIC LEAVES2* (*AS2*) gene of *Arabidopsis thaliana* is involved in the establishment of the leaf venation system, which includes the prominent midvein, as well as in the development of a symmetric lamina. The gene product also represses the expression of class 1 *knox* homeobox genes in leaves. We have characterized the *AS2* gene, which appears to encode a novel protein with cysteine repeats (designated the C-motif) and a leucine-zipper-like sequence in the amino-terminal half of the primary sequence. The Arabidopsis genome contains 42 putative genes that potentially encode proteins with conserved amino acid sequences that include the C-motif and the leucine-zipper-like sequence in the amino-terminal half. Thus, the AS2 protein belongs to a novel family of proteins that we have designated the AS2 family. Members of this family except AS2 also have been designated ASLs (AS2-like proteins). Transcripts of *AS2* were detected mainly in adaxial domains of cotyledonary primordia. Green fluorescent protein-fused AS2 was concentrated in plant cell nuclei. Overexpression of AS2 cDNA in transgenic Arabidopsis plants resulted in upwardly curled leaves, which differed markedly from the downwardly curled leaves generated by loss-of-function mutation of *AS2*. Our results suggest that AS2 functions in the transcription of a certain gene(s) in plant nuclei and thereby controls the formation of a symmetric flat leaf lamina and the establishment of a prominent midvein and other patterns of venation.

Key words: *Arabidopsis thaliana* — *ASYMMETRIC LEAVES2* — C-motif — *knox* — Leaf development — Leucine zipper.

Abbreviations: *AS1*; *ASYMMETRIC LEAVES1*, *AS2*; *ASYMMETRIC LEAVES2*, BAC; bacterial artificial chromosome, bp; base pairs, DAPI; 4′,6-diamidino-2-phenylindole, GFP; green fluorescent protein, kbp; kilo base-pairs, *knox*; *knotted*-like homeobox, ORF; open reading frame, SAM; shoot apical meristem.

The nucleotide sequence of the *AS2* cDNA has been submitted to GenBank under accession number AB080802.

Introduction

Leaves of angiosperms, which are relatively flat organs, exhibit remarkable diversity in terms of their shape and complexity. Nonetheless, the basic structure of each leaf can generally be described in terms of three axes: the proximal-distal, medial-lateral and adaxial-abaxial axes (Steeves and Sussex 1989, Waites et al. 1998, Hudson 2000, Byrne et al. 2001). Thus, plants appear to exploit common mechanisms that are responsible for the establishment of these axes during leaf development.

Leaves develop as lateral organs from a shoot apical meristem (SAM). Various mutants have been isolated with alterations in leaf morphology that are related to the development of shape along each of three axes, to adaxial-abaxial identity, and to the overall shapes of leaves. Some genes responsible for the mutant phenotypes have been cloned and characterized (Hake et al. 1989, Conway and Poethig 1997, Hofer et al. 1997, Kim et al. 1998, Berná et al. 1999, Serrano-Cartagena et al. 1999, Timmermans et al. 1999, Tsiantis et al. 1999). *PHANTASTICA* (*PHAN*) of *Antirrhinum majus* (Waites et al. 1998), *PHABULOSA* (*PHB*) and *PHAVOLUTA* (*PHV*) of Arabidopsis (McConnell et al. 2001), which appear to encode myb-like (*PHAN*) and homeobox-containing transcription factors (*PHB* and *PHV*) are involved in adaxial cell fate. *FILAMENTOUS FLOWER* (FIL; Sawa et al. 1999), *YABBY3* (YAB3; Siegfried et al. 1999), *CRABS CLAW* (CRC; Bowman and Smyth 1999) and *KANADI* (KAN; Kerstetter et al. 2001), which also appear to encode transcription factors, are involved in the specification of abaxial cell fate in the leaf lamina. Mutations in these genes convert flat expanded leaves to filamen-

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tous and rod-shaped structures and they also distort adaxial-abaxial identity. Thus, it is likely that the mediolateral development of the leaf lamina might be coupled with the determination by these genes of adaxial-abaxial identity.

With regard to leaf shape along the medial-lateral axis, the leaves of many plant species commonly exhibit obvious but approximate left-right symmetry, with the rachis as the axis (Ogura 1962, Hickey 1973, Hickey 1979, Sinha 1999, Semiarti et al. 2001a), even though exceptions have been reported (Whaley and Whaley 1942, Lieu and Sattler 1976, Dengler 1999). Such symmetry is independent of the complexity of leaf shape (e.g. simple or compound).

To clarify the mechanisms responsible for the development of symmetrical leaves, several groups including our own have taken advantage of the *asymmetric leaves2* (*as2*) and *asymmetric leaves1* (*as1*) mutants of Arabidopsis. The phenotypes of such mutants are very similar in terms of the asymmetric shape of the lamina and a malformed vein system but the abnormalities are not absolutely identical (Byrne et al. 2000, Ori et al. 2000, Semiarti et al. 2001a, Sun et al. 2002). The abnormalities can be summarized as follows. (1) Leaves of *as1* and *as2* mutants have asymmetric lobes and exhibit downward curling that is bilaterally asymmetric. (2) They fail to produce a thick and distinct midvein and the pattern of the secondary veins is asymmetric (Semiarti et al. 2001a, Sun et al. 2002). (3) Leaf sections, cultured in vitro on phytohormone-free medium, regenerate shoots at a higher frequency than sections from wild-type leaves (Semiarti et al. 2001a). (4) Transcripts of class 1 *knox* genes, such as *KNAT1, KNAT2* and *KNAT6*, accumulate in the leaves of the mutants (Semiarti et al. 2001a). (5) Transcripts of *AS1*, which encodes a myb-like transcription factor that is related to the products of *ROUGH SHEATH2* (*RS2*) of maize and *PHAN*, accumulate around vascular tissues in cotyledonary and leaf primordia (Byrne et al. 2000).

The observations summarized above suggest that *AS1* and *AS2* might be involved in the establishment of the entire venation system, which includes the prominent midvein as the structural axis of left-right symmetry of the leaf, as well as in the development of lamina symmetry (Semiarti et al. 2001a). They might also function in maintaining leaf cells in a developmentally determinate state, probably by repressing expression of class 1 *knox* genes. Although the roles of these genes in the establishment of the venation system might be tightly correlated with their roles in maintaining the determinate state of leaf cells, such correlations remain to be investigated. The similarities among abnormalities in *as1*, *as2*, and *as1 as2* double-mutant plants have led to the proposal that *AS1* and *AS2* might somehow interact genetically (Semiarti et al. 2001a, Byrne et al. 2002). To obtain a further insight into the mechanisms whereby *AS1* and *AS2* control lamina formation, it is obviously important to characterize both the *AS2* and the *AS1* genes.

In the present study, we isolated and characterized the *AS2* gene. This gene encodes a putative novel protein that contains the cysteine repeats, designated the C-motif, and a leucine-zipper-like motif. A database search revealed that AS2 belongs to a large family of proteins that we designated the AS2 family. Transcripts of *AS2* were mainly detected in adaxial domains of cotyledonary primordia during embryogenesis. Green fluorescent protein-fused AS2 was localized in plant nuclei even though AS2 does not include an obvious nuclear localization signal. Overexpression of AS2 cDNA resulted in a decrease in the efficiency of generation of transgenic shoots. Transgenic Arabidopsis plants that overexpressed the AS2 cDNA produced upwardly curled leaves and, occasionally, rod-shaped leaves without proper expansion of the lamina. Thus, it appears that AS2 might control the transcription of certain genes in the nucleus, the development of a symmetric flat leaf lamina, and the establishment of leaf venation.

Results

Isolation of the AS2 gene by map-based cloning

The *as2* locus was previously mapped between two derivatives of the *Ac* transposable element known as dAc-I-RS in two transgenic lines of Arabidopsis, #14-22.4.4W3 (T-1200) and #14-68.1.4 (T-1700), which were generated in our laboratory (Machida et al. 1997; Fig. 1A). Each transposable element included a hygromycin-resistance gene. Thus, for map-based cloning, we used these two dAc-I-RS elements as genetic and molecular markers, in addition to other PCR-RFLP markers. As shown in Fig. 1A, the *AS2* locus was finally mapped to a 32-kbp region between two PCR-RFLP markers (Marker 1 and Marker 4) in the BAC clone F5I14. We next performed PCR-RFLP analysis and sequenced this region of DNA from an *as2-1* mutant. We found a 13-bp deletion in the open reading frame (ORF) corresponding to the ORF15 of BAC clone F5I14. In addition, we found that all other available mutant alleles, such as *as2-2*, *as2-4* and *as2-5*, had mutations in this ORF (for details, see Fig. 2A and below). To determine whether a mutation in the ORF15 might be responsible for the *as2* phenotype, we introduced an *Apa*I–*Hpa*I fragment of the wild-type genome (fragment I; 6.16 kb), which included all of ORF15, into *as2-1* plants (Fig. 1B). We also transformed *as2-1* plants with fragment II, which lacked the region upstream of ORF15, and with fragment III, which lacked the upstream region of ORF15 as well as the region from the middle of ORF15 to the 3′ end of fragment I (Fig. 1B). Only fragment I reversed the *as2* phenotype. ORF15 is the only reading frame in fragment I that can encode a protein of more than 3,000 Da. Thus, the results indicate that ORF15 corresponds to the *AS2* gene. The failure of fragment II to complement the *as2* phenotype suggested that the region between *Apa*I and *Bsu*36I of fragment I might be required for the expression of *AS2*.

Characterization of as2 alleles

We isolated a cDNA clone that corresponded to the

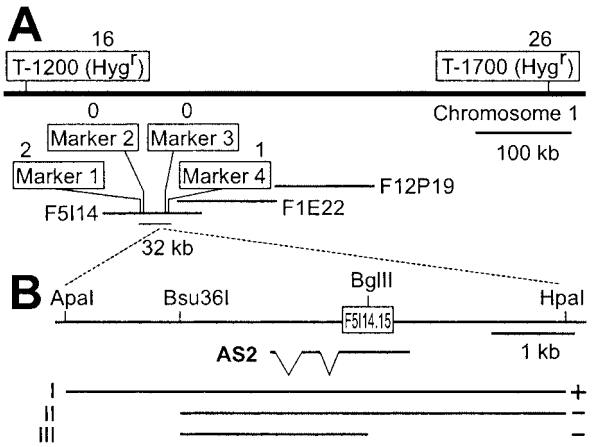

Fig. 1 Structure of the region of the Arabidopsis genome that includes the *AS2* locus, which is located at the lower part of chromosome 1, and the results of complementation analysis with segments of the genome. (A) Map-based cloning of the *AS2* gene. The *AS2* locus was mapped between two markers, Marker 1 and Marker 4 on bacterial artificial chromosome (BAC) clone F5I14. Other BAC clones that overlapped the region of interest are also shown. Numbers above the markers indicate numbers of recombinants between respective markers and the *AS2* locus (in most cases, we examined more than 10,000 chromosomes). T-1200 and T-1700 show the positions of the dAc-I-RS transposable elements with the hygromycin-resistance (Hyg^r) gene from which we started the chromosome walking. Marker 1, Marker 2, Marker 3, and Marker 4 are RFLP markers that we generated for the study. (B) Magnified view of the chromosomal region around *AS2* and the results of complementation analysis. The structure of AS2 cDNA is shown under the genomic structure. Restriction fragments that were used in complementation analysis are indicated by horizontal lines labeled I, II, and III. Symbols + and – on the right indicate positive and negative results, respectively, of complementation tests with the corresponding DNA fragments.

ORF15. Comparison of the nucleotide sequence of the *AS2* gene with that of the cDNA suggested that the mRNA contains two introns in the 5′-untranslated region but no introns in the coding region (Fig. 1B) and, furthermore, that *AS2* encodes a novel protein with 199 amino acid residues (Fig. 2A). The *as2-1* and *as2-2* alleles both had 13-bp deletion at the same position upstream of the coding sequence for the leucine-zipper-like sequence (for details, see below; Fig. 2A, C). This deletion should result in production of a short polypeptide that lacks of most of the carboxyl-terminal (C-terminal) half of AS2 but contains 13 additional amino acid residues (Fig. 2A). In the *as2-4* mutant, there is one bp deletion in the middle of the coding region that corresponded to the C-terminal half of AS2 and this deletion generated a frame-shift mutation (Fig. 2A), which resulted in replacement of the C-terminal 48 amino acid residues of AS2 with the 63 new residues. In the *as2-5* mutant, a single guanine nucleotide was replaced by an adenine nucleotide in the coding region for the amino-terminal (N-terminal) half of the protein, resulting in substitution of the glycine residue at position 46 by a glutamic acid residue (Fig. 2A). This glycine residue is conserved in all members of the AS2 family, as described below. Therefore, this glycine residue is essential for the function of AS2.

Structural characteristics of AS2 and related proteins

The predicted AS2 protein contained a leucine-zipper-like sequence, from residue 81 to residue 109, which included five repetitions of hydrophobic amino acid residues, such as valine, isoleucine and leucine, with six-residue intervals (Fig. 2A). A search of databases revealed that the N-terminal half of AS2 was similar, in terms of amino acid sequence, to the N-terminal halves of a number of putative proteins encoded by hypothetical genes and ESTs of Arabidopsis and other plant species (*Oryza sativa*, *Glycine max*, *Lycopersicon esculentum*, etc.; Fig. 2B). The public databases of sequences of the Arabidopsis genome include at least 41 ORFs that are predicted to encode proteins with N-terminal halves that are related to that of AS2. Of these proteins, several appear to be only distantly related to AS2 (Fig. 2B).

As shown in Fig. 2B, comparative analysis of the putative AS2-like proteins revealed that the $Cx_2Cx_6Cx_3C$ sequence (where x is an unconserved residue; designated the C-motif) was completely conserved in the N-terminal halves of all 41 predicted proteins identified in the database search and AS2 (positions from 10 to 24 in AS2). In addition to the C-motif, the leucine-zipper-like sequence was also strongly conserved in most of the 41 proteins. More than a half of the proteins, including AS2, had additional conserved sequences, as follows: PCAACKFLRRKCxxxCVFAPYFP in and around the C-

Fig. 2 The predicted amino acid sequence of AS2 and comparison of the amino acid sequences of the AS2 domains of AS2 and AS2-like proteins. (A) Predicted amino acid sequence of AS2. The positions of cysteine residues in the C-motif and hydrophobic residues in the leucine-zipper-like sequence are shown by asterisks and dots, respectively. Mutated amino acid residues in various *as2* alleles are indicated in italics above the wild-type sequence. (B) Comparison of the amino acid sequences of the AS2 domains in members of the AS2 family. The sequence from residue 8 to residue 109 of AS2 is aligned with sequences from corresponding regions of members (designated ASL; see text) of the AS2 family. Amino acid residues conserved in more than 20 members are indicated by white characters on a black background. The consensus sequences of the C-motif and hydrophobic residues in the leucine-zipper-like sequence are indicated by asterisks and dots, respectively. The glycine residue that is conserved in all members of the family and mutated in the *as2-5* allele is marked by two asterisks. The members of the Arabidopsis AS2 family can be divided into two classes (class I and class II). Amino acid residues conserved in more than three members of class II are shaded in gray. *O. s.*, *Oryza sativa*; *G. m.*, *Glycine max*; and *L. e.*, *Lycopersicon esculentum*. (C) Domain organization and characteristic features of AS2. The region indicated by a bracket below the box is the AS2 domain. The shaded box and the striped box represent the C-motif and the leucine-zipper-like sequence, respectively. Sites of *as2-1*, *as2-2*, *as2-4,* and *as2-5* mutations are indicated. Numbers below the box indicate positions of amino acid residues.

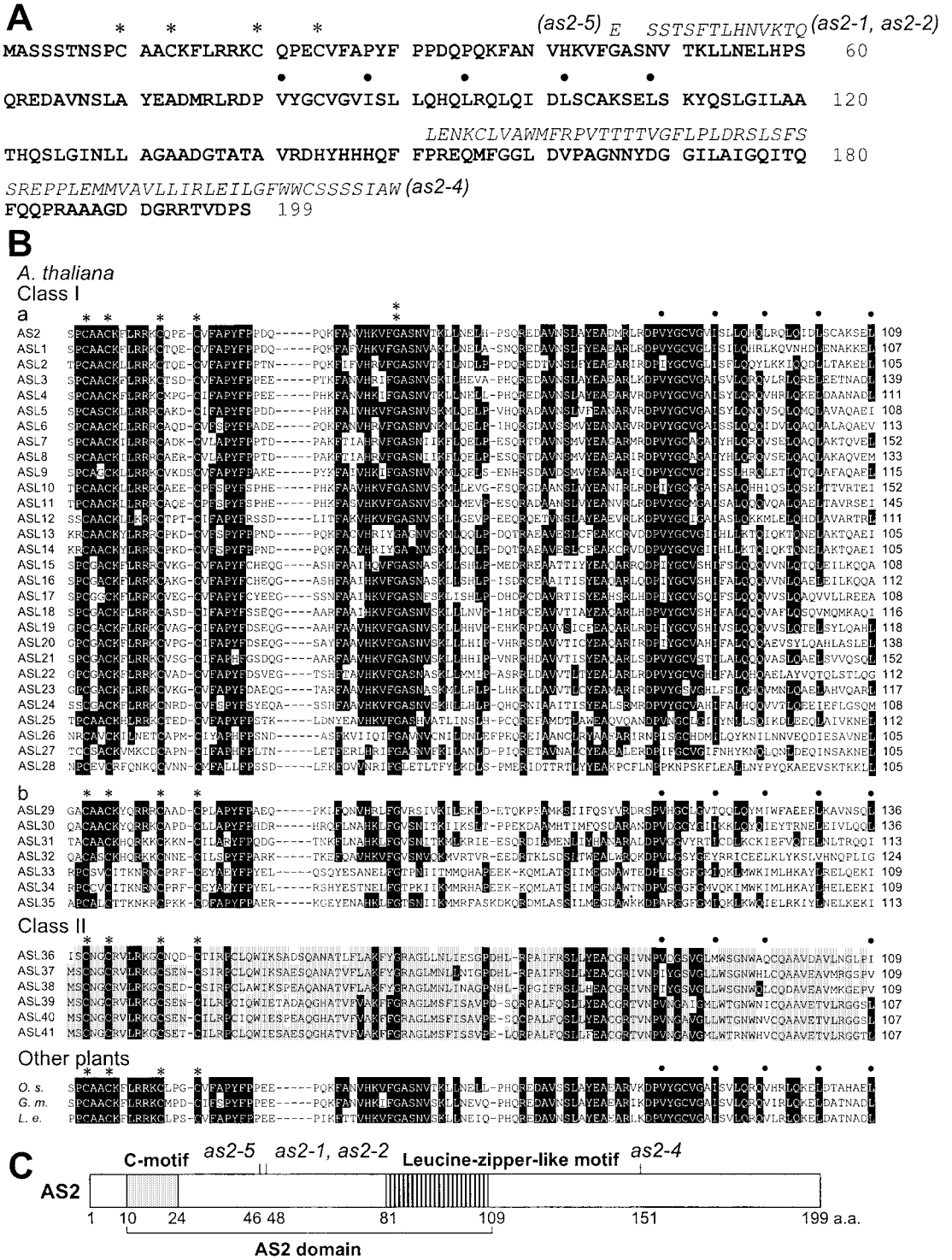

Fig. 2

motif; FAxVHKVFGASNVxKLL between the C-motif and the leucine-zipper-like sequence; and RxxAVxSLxYEAxARxRD-PVYGCVGxISxLQxQL(V or I)xxLQxxLxxxxxxL(V or I) in and around the leucine-zipper-like sequence (Fig. 2B). Despite the strong conservation of amino acid sequence at the N-terminus of AS2, the amino acid sequence of the C-terminal region of AS2 was unlike those of the other AS2-like proteins and other proteins in the database (Fig. 2C).

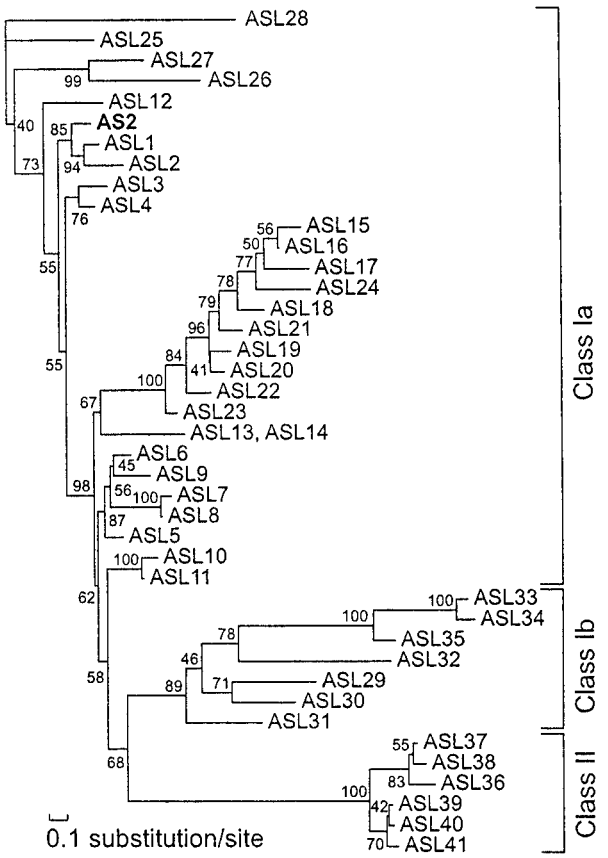

Fig. 3 An unrooted maximum-likelihood tree for 42 members of the AS2 family of proteins from Arabidopsis, as generated by a local rearrangement search. Numbers on branches represent local bootstrap values, which were calculated with the ProtML program. The length of each horizontal branch is proportional to the estimated evolutionary distance. The brackets on the right indicate the classification of members of the AS2 family.

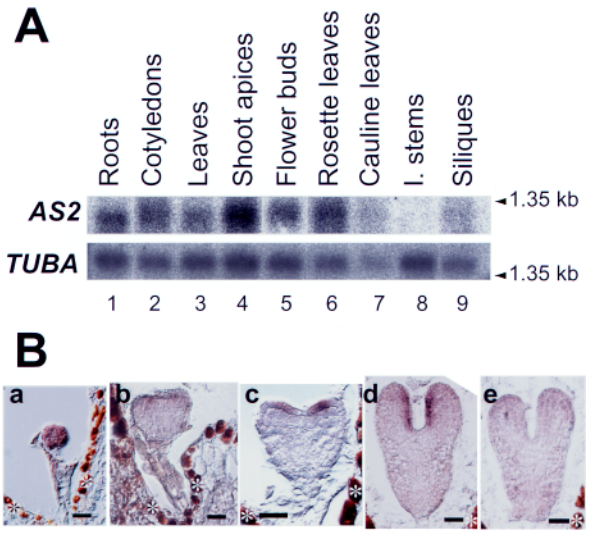

Fig. 4 Sites of accumulation of *AS2* transcripts. (A) Northern blotting analysis of poly(A)$^+$ RNA from various organs of Arabidopsis. Aliquots of 1.0 µg of poly(A)$^+$ RNA were prepared from roots (lane 1), cotyledons (lane 2), leaves (lane 3), and shoot apices (lane 4) of 12-day-old plants; from flower buds (lane 5) of 28-day-old plants; and from rosette leaves (lane 6), cauline leaves (lane 7), inflorescence (I) stems (lane 8), and siliques (lane 9) of 35-day-old plants. The same blot was reprobed with a gene for Éø-tubulin (TUBA) as a control. The numbers on the right indicate the sizes of marker RNA molecules. (B) Detection of *AS2* transcripts by in situ hybridization. (a–d) Patterns of distribution of *AS2* transcripts obtained with an antisense probe. (a) Globular stage; (b) triangular stage; (c) heart-shaped stage; (d) torpedo stage; (e) sense control for panels (a) through (d). Reddish brown coloration in the cell layer beneath the seed coat (indicated by white asterisks) was not specific to AS2 RNA because it was generated by both probes. Scale bars: 20 µm.

In AS2, the N-terminal sequence is characterized by the C-motif and the leucine-zipper-like sequence. We propose that this characteristic region be designated the AS2 domain and that proteins that include this domain be designated members of the AS2 family. According to this designation, the genome of Arabidopsis contains 42 ORFs that potentially encode proteins that belong to the AS2 family. As shown in Fig. 2B, we chose the designation *AS2-like* genes (*ASLs*) for the genes or putative genes for the 41 proteins that resembled AS2 and numbered these genes 1 to 41, respectively. Nucleotide sequences of some cDNAs of these genes have been submitted to the GenBank database and named LOB and LBD by Shuai, B. and Springer, P.S. (2001; Table 2).

The AS2 family consists of at least two major classes of proteins

Members of the AS2 family can be divided into at least two classes, class I and class II (Fig. 2B). Class I consists of AS2 and 35 proteins (from ASL1 through ASL35) that include

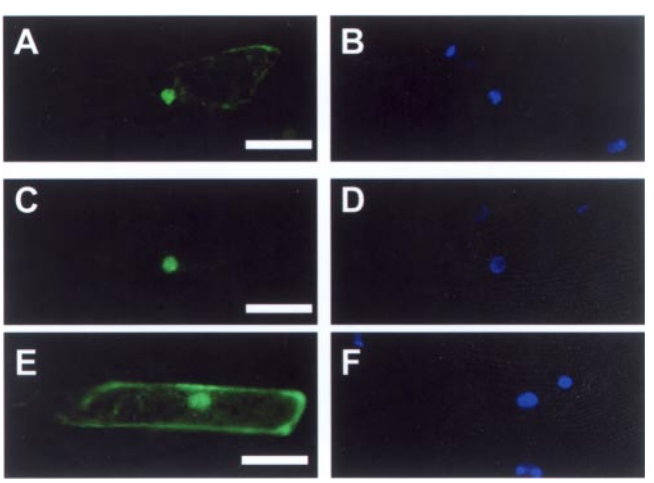

Fig. 5 Subcellular localization of AS2::GFP in onion epidermal cells. Plasmids that carried 35S::AS2::GFP (A, B), 35S::NLS::GFP (C, D) and 35S::GFP (E, F) were introduced by bombardment into onion epidermal cells and introduced tissues were cultured for 16 h at 28°C (see text for details). Fluorescence was monitored under a fluorescence microscope (left panels). Nuclei stained with 1 µg ml^{-1} DAPI are shown in the right panels. Scale bars: 100 µm.

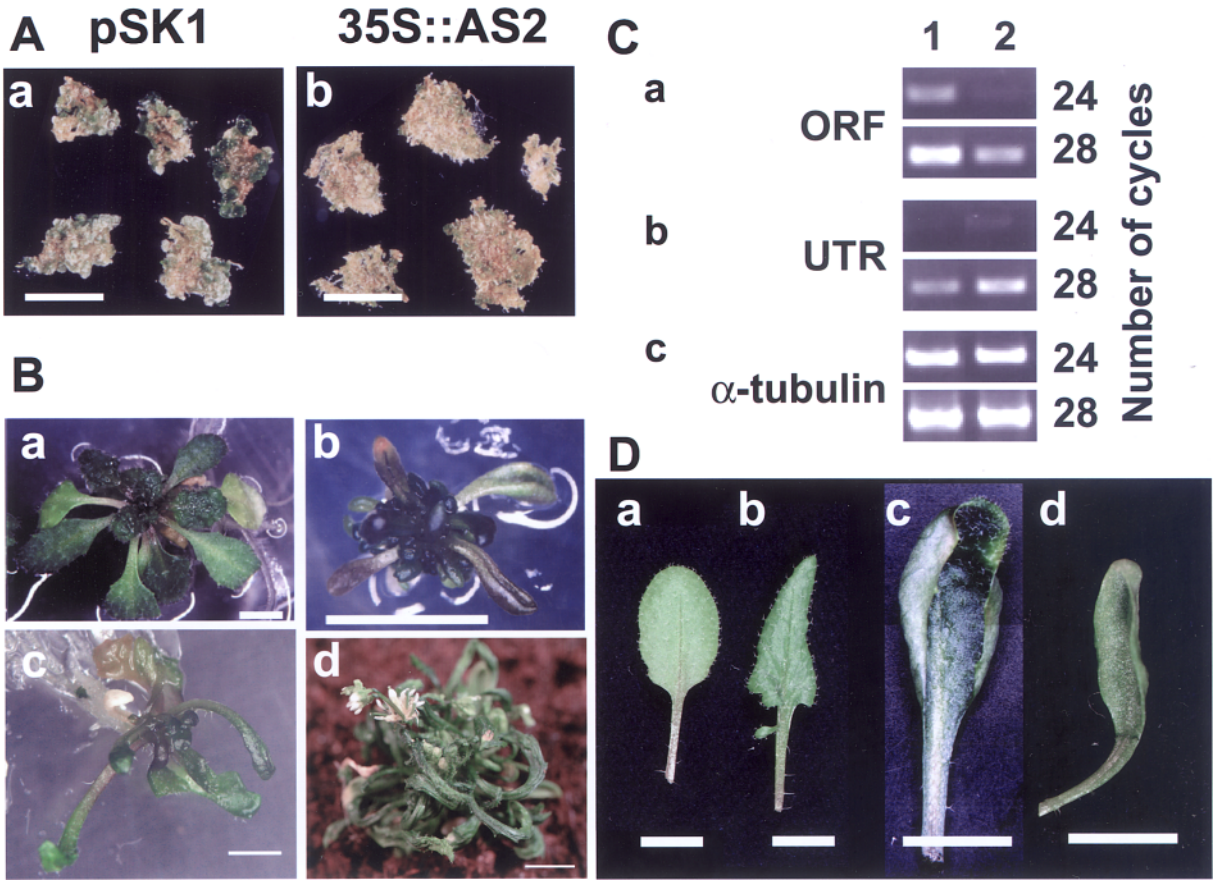

Fig. 6 Phenotypes of transgenic Arabidopsis calli and plants that overexpressed AS2 cDNA and accumulation of transcripts of *AS2* gene(s). (A) Results of transformation of root segments from Col-0 with the empty vector pSK1 (a) and with pSK35S::AS2 (b). Transformed root segments were incubated as described in the text. Photographs were taken 21 days after the start of incubation. Scale bars: 10 mm. (B) Phenotypes of transgenic Arabidopsis plants. (a) A 35-day-old plant transformed with the empty vector pSK1; (b) a 35-day-old plant transformed with pSK35S::AS2; (c) a 25-day-old plant transformed with pSK35S::AS2; (d) the plant in panel c was grown for a further 15 d. Scale bars: 10 mm. (C) Analysis by RT-PCR of transcripts of the *AS2* gene(s) in transgenic and wild-type (Col-0) plants. 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. Lane 1, the transgenic plant shown in panel Bd; Lane 2, wild-type plant (Col-0). Products that were amplified with primers specific for the region of open reading frame of *AS2* transcripts (a; ORF), 5′- and 3′-untranslated regions of the *AS2* transcripts (b; UTR), and transcripts of the gene for α-tubulin (c; α-tubulin) are shown. (D) Leaf phenotypes. The fifth leaves from a typical 35-day-old wild-type Col-0 plant (a) and *as2-1* plant (b) are shown. Typical leaves of transgenic plants that exhibited mild phenotypic changes (c) and severe phenotypic changes (d) are shown. The positions of these latter leaves could not be determined. Scale bars: 5 mm.

a C-motif, a leucine-zipper-like sequence and most of the major conserved residues noted above. Class II consists of six proteins (from ASL36 through ASL41). These proteins include a C-motif and an incomplete leucine-zipper-like sequence, in which the fourth hydrophobic residue is missing, and there is weaker sequence conservation in the AS2 domain. Furthermore, most amino acid residues are conserved among all members of class II, with only a few respective residues being different in each protein in this class.

As shown in Fig. 3, a phylogenetic tree confirmed that class II is only distantly related to class I and suggested that members of class I can be divided further into sub-classes, namely class Ia and class Ib, with weaker sequence conservation but a complete leucine-zipper-like sequence.

Site of expression of the AS2 gene and nuclear localization of the gene product

We examined the sites of accumulation of *AS2* transcripts in wild-type plants. We purified poly(A)$^+$ RNA from roots, cotyledons, leaves, and shoot apices of 12-day-old plants, from flower buds of 28-day-old plants, and from rosette leaves, cauline leaves, influorescence stems and siliques of 35-day-old plants and then subjected the samples of RNA to Northern blot analysis with AS2 cDNA as the probe. We detected *AS2* transcripts in all samples analyzed, with the exception of those from inflorescence stems (Fig. 4A). The level of *AS2* transcripts was highest in the sample from shoot apices that included small developing leaves. These data are consistent with the previous observation that *as2* mutations are evident as

Table 1 Frequency of generation of transformed shoots

Plasmid	Numbers of			
	Masses of roots examined	Green calli	Shoots	Shoots with stems
pSK1	18	76 (4.2) [a]	45 (2.5)	22 (1.2)
pSK1::35S::AS2	21	31 (1.5)	14 (0.67)	4 (0.19)

[a] Numbers in parentheses are average numbers of transformants per mass of roots.

mutant phenotypes in all leaf-like organs, such as cotyledons, rosette leaves, cauline leaves, sepals, and petals, but not in stems (Semiarti et al. 2001a). However, although *AS2* transcripts were detected in roots, the roots of *as2* plants resembled wild-type roots.

We also examined sites of expression of *AS2* during embryogenesis by in situ hybridization with AS2 cDNA as the probe. In embryos of the globular stage, *AS2* transcripts were detected in all cells (Fig. 4Ba). As bulges formed at the upper parts of triangular embryos, *AS2* transcripts accumulated in the uppermost regions of the embryos (Fig. 4Bb). From the heart-shaped stage to the torpedo stage, we detected signals in protoderm cells on the adaxial side of cotyledonary primordia (Fig. 4Bc, Bd). After the walking-stick stage, the accumulation of *AS2* transcripts was detected throughout cotyledonary primordia without any obvious concentration in a specific region (data not shown). After germination, no specific hybridization signals were detected in situ around shoot apical meristems, in leaf primordia and in mature leaves. However, the accumulation of *AS2* transcripts was confirmed by Northern blotting (Fig. 4A).

Nuclear localization of AS2

The predicted AS2 protein does not include an obvious nuclear localization signal (NLS, Fig. 2A). However, to determine whether AS2 is localized in plant cell nuclei, we generated a fusion construct in which AS2 cDNA was fused to the DNA for green fluorescent protein (GFP), with transcription under the control of the 35S promoter of cauliflower mosaic virus (CaMV) (35S::AS2::GFP). The fusion construct was introduced into onion epidermal cells by particle bombardment and fluorescence due to GFP in the cells was monitored. As shown in Fig. 5, fluorescence signals due to AS2-GFP, as well as similar signals due to NLS-GFP, prepared with the NLS of SV40, were detected mainly in nuclei (Fig. 5A, C). When the 35S::GFP construct was introduced into onion cells, signals due to GFP were detected both in the nucleus and the cytoplasm (Fig. 5E). These results suggest that AS2 is a nuclear protein.

Phenotypes of Arabidopsis plants that overproduce AS2

To investigate the function of the *AS2* gene in further detail, we fused AS2 cDNA to the 35S promoter (35S::AS2) and attempted to generate transgenic Arabidopsis plants that overexpressed *AS2*. Our preliminary data demonstrated that it was difficult to generate transformed shoots (Fig. 6A). Therefore, we first determined the approximate efficiency of generation of transformed shoots by a conventional root-transformation procedure. We counted the numbers of transformed green calli, regenerated shoots, and shoots with inflorescence stems per mass of roots used for transformation. Table 1 shows that when 35S::AS2 was introduced into root tissue, the efficiency of formation of green calli was lower than that when empty vector plasmid pSK1 was used for transformation. The efficiencies of regeneration of transgenic shoots and shoots with inflorescent stems were 5- to 10-fold lower (Table 1). These data suggested that overexpression of AS2 cDNA had an inhibitory effect on cell proliferation in green tissues and/or the regeneration of shoots.

We obtained two transgenic lines that overexpressed AS2 cDNA. We confirmed that *AS2* transcripts that were derived from the cDNA were accumulated in one of the transgenic lines that exhibited the severer phenotype (Fig. 6C). One line of transgenic plants had a mildly dwarf phenotype and generated leaves that curled upward (Fig. 6Bb). The other line of transgenic plants, with a severely abnormal phenotype, generated leaves with a very narrow lamina at the early stages of plant growth (Fig. 6Bc, Dc). The leaf lamina failed to expand and generated rod-like leaves (Fig. 6Bd, Dd).

Discussion

Functional domains in the AS2

The *AS2* gene appears to encode a novel protein of 199 amino acid residues that includes a C-motif (defined as $Cx_2Cx_6Cx_3C$) and a leucine-zipper-like sequence in its N-terminal half and an apparently unique sequence in its C-terminal half (Fig. 2A, C). It is generally accepted that a leucine-zipper sequence is involved in protein–protein interactions (Ellenberger et al. 1992) and it seems likely that the leucine-zipper-like sequence of AS2 might also play a role in the association of AS2 with some other protein(s). Such interaction(s) might be essential for actions of AS2.

The C-motif was identified for the first time in this study but it is rather similar to a zinc finger, which generally has the consensus sequence $Cx_{2-3}Cx_nCx_{2-3}C$ (n >12) and which functions in interactions with other macromolecules (Pavletich and Pabo 1991, Wang et al. 1998). It is likely that the C-motif is also involved in association(s) with other macromolecules, such as DNA and proteins, and with AS1, in particular (Semiarti et al. 2001a, Byrne et al. 2002, our unpublished data). The identification and characterization of the molecules with which

Table 2 Members of the AS2 family of proteins in *A. thaliana*

Member	BAC code [a]	Gene code [b]	EST ID [c]	LOB [d]
AS2	F5I14.15	At1g65620	Z33806, Z25656	LBD6
ASL1	MUD21.13	At5g66870		
ASL2	F26B6.31	At2g23660		
ASL3	MGF10.6	At3g27650		LBD25
ASL4	MDC12.5	At5g63090		LOBa
ASL5	T27E13.13	At2g30130		
ASL6	T19E23.11	At1g31320		
ASL7	T17D12.6	At2g28500		
ASL8	F24B9.1	At1g07900		
ASL9	F3O9.33	At1g16530		
ASL10	T9D9.15	At2g30340	AV554524, AV551296, AV538901	LBD13
ASL11	T2P4.18	At2g40470	AI993799, N97300, BE529105	LBD15
ASL12	F9F8.10	At3g11090		
ASL13	MLJ15.5	At3g26660	AV519677, AV565474, AV555285, AV560157, AV556223, AV557695, AV535896, AV564540, AV565846, AV439949, AV561987, AV567074, AV519558	
ASL14	MLJ15.1	At3g26620		
ASL15	MHK10.16	At2g42440		
ASL16	F9D24.100	At3g58190	AV553261, AV544763	LBD29
ASL17	F16D14.15	At2g31310		
ASL18	MHK10.15	At2g42430		LBD16
ASL19	F6N15.4	At4g00220	AI994962, AV563330	LBD30
ASL20	F4L23.41	At2g45420	BE520513, BE521898, BE521897	LBD18
ASL21	F20H23.21	At3g03760		
ASL22	F6N15.25	At4g00210		
ASL23	F4L23.45	At2g45410		
ASL24	K16F4.4	At5g06080		
ASL25	T20E23.110	At3g50510		
ASL26	T12H17.90	At4g22700		
ASL27	MIK22.21	At5g35900		
ASL28	K24A2.3	At3g27940		
ASL29	T23J7.200	At3g47870		
ASL30	MCP4.8	At3g13850		
ASL31	F3N23.18	At1g72980		
ASL32	F9P14.14	At1g06280		
ASL33	T22A15.8	At1g36000		
ASL34	F3P11.11	At2g19510		
ASL35	F6F22.15	At2g19820		
ASL36	T26J14.8	At1g68510	AV559860	LBD42
ASL37	F5A8.2	At1g67100	BE520541, BE520808, BE520344, BE521846, BE524729, BE520809, BE520810	
ASL38	F16B3.18	At3g02550	AV537704, AI996685, AV549715, H76116, AV563797, T42227, AV563144	LBD41
ASL39	K8K14.16	At5g67420	AV525400, AV526154, T41721, AV551431, H36818, F14269, AV550089, T14105, N38449, F14441, AI996949, N65652	LBD37
ASL40	F3A4.20	At3g49940	T76164, Z29130, F13856	
ASL41	F19F18.30	At4g37540	AI994989, R65200	

[a, b] ORF number with BAC clone name and corresponding gene code obtained from TAIR database. [c] EST ID obtained from EMBL database.
[d] Some of ASL proteins have been submitted to the GenBank database as LOB domain (LBD) proteins (see text).

AS2 interacts will be critical to the elucidation of the functions of AS2.

The sequence of the C-terminal half of AS2 is unlike that of any protein in the databases, including known motifs (Fig. 2C). Since the *as2-4* allele had a frame-shift mutation in this region and the phenotype due to this allele is similar to those

due to other *as2* alleles (Semiarti et al. 2001a), it seems that the C-terminal half is essential for the functions of AS2, in addition to the N-terminal half, which contains the newly characterized AS2 domain.

AS2 is a nuclear protein that negatively regulates expression of class 1 knox genes

Both AS1 and AS2 repress the expression of class 1 *knox* homeobox genes (Byrne et al. 2000, Ori et al. 2000, Semiarti et al. 2001a), but it is unclear whether such repression is directly or indirectly attributable to AS1 and AS2. In the present study, we showed that the AS2::GFP fusion protein was localized in the nuclei of plant cells (Fig. 5), as would be expected for a protein that is involved in repression of class 1 *knox* genes in nuclei via interactions with other, as yet unidentified nuclear proteins. The AS1::GFP fusion protein was also localized in nuclei (our unpublished data). This observation is consistent with the hypothesis that AS1 might be one of the molecules with which AS2 interacts in nuclei.

AS2 is involved in the development of a flat leaf lamina

Overexpression of AS2 cDNA induced the upward curling of leaves and cotyledons (Fig. 6). By contrast, loss-of-function mutations in AS2 result in the downward curling of leaves and cotyledons (Semiarti et al. 2001a). Therefore, it seems plausible that AS2 might be involved in suppression of the growth of the adaxial domain of the leaf lamina or in stimulation of the growth of the abaxial domain. Considering the growth-inhibitory effects that we observed upon transformation (Fig. 6B) and the expression of *AS2* in the adaxial domain in the cotyledonary primordia in embryos (Fig. 4B), we can speculate that AS2 might function in suppression of the adaxial domain. It will be of interest to determine whether such suppression is achieved via the inhibition of the proliferation or the expansion of cells.

Whatever the details of the activity of AS2 at the molecular level, the present study of overproducers and previous analysis of *as2* mutant leaves indicate that AS2 is responsible for the expansion of a flat leaf lamina, as well as for leaf symmetry. As described in the Introduction, mutations in genes that are responsible for the adaxial-abaxial fates of leaf domains interfere with the fate determination of leaves and often induce formation of rod-shaped leaves (Waites et al. 1998, Sawa et al. 1999, McConnell et al. 2001). There might be a relationship between *AS2* and these genes. In this context, it is worth noting that AS1 is an Arabidopsis homolog of PHAN, even though it remains to be determined whether the functions of AS1 are analogous to those of PHAN.

Possible functions of ASL proteins

The conservation of amino acid sequence in the N-terminal halves (the AS2 domains) of AS2-like proteins led us to propose that these proteins might form a new family of plant protein, designated as the AS2 family. We named Arabidopsis proteins that are members of this family, with the exception of AS2, ASL (AS2-like) proteins (Fig. 2B). The similarities among amino acid sequences and a phylogenetic tree of AS2 domains of ASLs revealed that ASL proteins could be divided into class I, with sub-classes class Ia and class Ib, and class II (Fig. 2B, 3).

The functions of members of the AS2 family, with the exception of AS2, remain to be identified. AS2 is required for the formation of a symmetric, flat, round leaf lamina and for the establishment of venation patterns that include prominent midveins (this study and Semiarti et al. 2001a). In addition, AS2, acting in conjunction with AS1, has the ability to repress the expression of class 1 *knox* homeobox genes (Ori et al. 2000, Byrne et al. 2000, Semiarti et al. 2001a, Byrne et al. 2002). Since substitution of the conserved glycine residue with the glutamic acid residue in the AS2 domain resulted in generation of mutant phenotypes (Fig. 2), it is clear that this residue as well as the AS2 domain play an important role in the function of AS2. Although some members of class Ia exhibit strong conservation of amino acid sequence of AS2 domains (for example, ASL1 and ASL2), they might have distinct functions from AS2, because the C-terminal region of AS2, which is unique, is required, in addition to the N-terminal AS2 domain for the functions of AS2 (Fig. 2; Semiarti et al. 2001a, our unpublished data). Clearer insight into the roles of these closely related ASLs requires further genetic and molecular experiments.

It was proposed recently that the *LOB* gene (accession number AF447897), which corresponds to the gene for ASL4, is normally expressed at the boundary between meristems and organ primordia but is absent in *as1* and greatly reduced in *as2* (Byrne et al. 2002). The developmental role of the *LOB* gene, however, remains to be determined.

The six ASLs that constitute class II have characteristic structural features that are distinct from class I (Fig. 2B) and their amino acid sequences are very similar to each other. Expression of these genes has been reported (Table 2), suggesting that they are functional genes. Therefore, it seems likely that members of class II might play distinct roles during development in Arabidopsis.

Materials and Methods

Plant strains and growth conditions

A. thaliana ecotype Col-0 (CS1092) and mutants *as2-1* (CS3117) and *as2-2* (CS3118) were obtained from the Arabidopsis Biological Resource Center (Columbus, OH, U.S.A.; ABRC). Our RFLP analysis showed that the background of *as2-1* coincided with the Col-0 ecotype (Semiarti et al. 2001a). L*er*-0 (NW20) and *as2-4* (N463) were obtained from the Nottingham Arabidopsis Stock Center (Nottingham, U.K.; NASC). The *as2-5* allele was isolated from an M2 population of ethylmethanesulfonate-mutagenized L*er*-0 seeds, purchased from Lehle Seeds (Round Rock, TX, U.S.A.; Semiarti et al. 2001a). The transgenic lines #14-22.4.4W3 (T-1200) and #14-68.1.4 (T-1700) were isolated from the (I-RS/dAc-I-RS)#14 line; they carried a trans-

posed dAc-I-RS element (accession no. AB055064) that included a gene for hygromycin phosphotransferase (Machida et al. 1997, Semiarti et al. 2001b). The background of #14-22.4.4W3 (T-1200) and #14-68.1.4 (T-1700) was the L*er* ecotype in both cases. For analysis of plants, seeds were sown on soil. After 2 d at 4°C in darkness, plants were transferred to a regimen of white light at 3,000 lux for 16 h daily at 22°C as described previously (Semiarti et al. 2001a). Ages of plants are given in terms of numbers of days after sowing.

Cloning of the AS2 gene

Meiotic recombination breakpoints were generated and identified near the *AS2* gene by screening for recombination between the *as2-1* mutation and the flanking transposon markers #14-68.1.4 (T-1700) and #14-22.4.4W3 (T-1200). These recombinants, which were shown to be resistant to hygromycin and to have the *as2-1* phenotype, were used to map PCR-RFLP markers relative to the *AS2* gene. The information related to sequences used as PCR-RFLP markers was obtained from the web site of the *Arabidopsis thaliana* Genome Center at the University of Pennsylvania, TAIR web site (http://www.arabidopsis.org/) and the nucleotide sequence of the BAC clone F5I14 (GenBank, accession no. AC001229). PCR-RFLP markers were as follows: for Marker 1, 5′TGTAACTCTTCCGTCCGGTTTG3′ and 5′GCAAAGTCCATAGAGGAGCAAG3′; for Marker 2, 5′TTGGGTTTGCACCGAAACTCAG3′ and 5′AGTGACAGACAGTGACCACAAG3′; for Marker 3, 5′TGGTGGGATGAAACTTTGTGAG3′ and 5′CTCTCTCTTTCTCACTCTTCTC3′; and for Marker 4, 5′AGAAAACTGCTGTCTTCGGGAC3′ and 5′TCCAAAGCACTCTCTAGCTTGG3′ (Fig. 1).

Complementation analysis

To construct the binary plasmids for complementation experiments, the 6.16-kbp *Apa*I–*Hpa*I fragment (fragment I in Fig. 1), the 4.75-kbp *Bsu*36I–*Hpa*I fragment (fragment II in Fig. 1) and the 2.2-kbp *Bsu*36I–*Bgl*II fragment (fragment III in Fig. 1) were inserted separately into the pBI-BAR plasmid, which was a derivative of pBI101 (Jefferson et al. 1987) and contained 1′promoter::bar (Yoshioka et al. 2001). The newly constructed plasmids were introduced into *Agrobacterium tumefaciens* strain GV3101. Whole plants were then transformed by vacuum infiltration, as described elsewhere (Clough and Bent 1998, Galbiati et al. 2000). Transgenic plants were selected on soil that contained 0.01% Basta (AgroEvo, Frankfurt, Germany), which is what genotypes of transgenic plants were analyzed by Southern blotting.

Cloning of cDNA

Poly(A)$^+$ RNA was prepared from 16-day-old plants. For reverse transcription-PCR, first-strand cDNA was generated as described elsewhere (Hamada et al. 2000). Then AS2 cDNA was amplified by PCR with primers ORF15SF (5′GGGTCGACATGGCATCTTCTTCAACAAACTCAC3′) and ORF15NR (5′GGGCGGCCGCTCAAGACGGATCAACAGTACGGC3′). The amplified fragment was then ligated into the *Sal*I/*Not*I site of pBluescript SK(–) (Stratagene, La Jolla, CA, U.S.A.) and its identity was confirmed by nucleotide sequencing. The 5′-end sequence of the cDNA was determined by 5′-RACE with primer ORF15MR (5′TATCTGAAGCTGACGAAGCTGATG3′) and an adapter primer. The 3′-end of the cDNA was determined by 3′-RACE with primer ORF15MF (5′GATCTCAGCTGTGCTAAATCTGAGC3′) and an adapter primer. Nucleotide sequences of mutant alleles (*as2-1*, *as2-2*, *as2-4* and *as2-5*) were determined by amplifying the region of the *as2* gene from the genomic DNA of each mutant. The nucleotide sequence of the *as2* cDNA obtained from the poly(A)$^+$ RNA that has been isolated from each mutant plant was also determined.

Phylogenetic analysis

Arabidopsis genes that resemble *AS2* were obtained from the AGI data set (Proteins from AGI, Total Genome) at TAIR using the TAIR BLAST version 2.0 program (http://arabidopsis.org/Blast/index.html). The *AS2*-like genes of other plant species were obtained from the nr data sets at GenomeNet using the GenomeNet BLAST2 program (http://www.blast.genome.ad.jp/). The sequences of AS2 domains of 42 members of the AS2 family from Arabidopsis were aligned using CLUSTAL W, version 1.8 (Thompson et al. 1994). For construction of the maximum-likelihood (ML) tree, we used a neighbor-joining (NJ) tree as the start tree for a local rearrangement search. We used the NJdist and ProtML programs in the MOLPHY, version 2.3b3, package (http://www.ism.ac.jp/software/ismlib/softother.html#molphy; Adachi and Hasegawa 1996). The NJ tree was obtained with NJdist and the ML tree was obtained with ProtML. The local bootstrap probability of each branch was estimated using the ProtML program (Himi et al. 2001, Sakakibara et al. 2001).

RNA gel blot analysis

For analysis of Col-0 wild-type plants, we used roots, cotyledons, leaves, and shoot apices collected from 12-day-old plants; flower buds collected from 28-day-old plants; and rosette leaves, cauline leaves, influorescence stems, and siliques collected from 35-day-old plants. Aliquots of 1.0 μg of poly(A)$^+$ RNA were isolated and Northern blotting was performed as described previously. Partial cDNA for AS2 was used as the probe. It was amplified by PCR with primer 1 (5′GATCTCAGCTGTGCTAAATC3′) and primer 2 (5′TCAAGACGGATCAACAGTAC3′) and cloned into the *Eco*RV sites of pBluescript SK(–). Its identity was confirmed by nucleotide sequencing. Then a 300-bp fragment, generated by cleavage of the plasmid pAS2c300 with *Eco*RI and *Cla*I extended from codon 151 to codon 200 of the ORF, which has the termination codon, 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. The labeled fragment was used as probe.

In situ hybridization

Details of methods used for fixation of plants, embedding in paraffin and in situ hybridization can be found at http://www.genetics.wisc.edu/CATG/barton/index.html and were described by Nakashima et al. (1998). Sections (thickness, 8 μm) were cut with a microtome (ERMA Inc. Tokyo, Japan). The antisense RNA probe was generated by T3 RNA polymerase after plasmid pAS2c300 has been linearized with *Cla*I. The sense RNA probe was generated by T7 RNA polymerase after linearization of pAS2c300 with *Eco*RI.

Construction of plasmids

The plasmid pSK35S::AS2 was constructed by inserting a fragment of AS2 cDNA (600-bp), which extended from the initiation codon to the termination codon, at *Xba*I and *Not*I sites of binary vector pSK1 (Kojima et al. 1999) downstream of the CaMV 35S promoter. The plasmid p35S::AS2::GFP was constructed by inserting a fragment of AS2 cDNA (597 bp) fragment, which extended from the initiation codon to the 199th amino acid, and three-alanine linker sequence (5′GCAGCTGCC3′) at the *Sal*I and *Nco*I sites of pTH-2 (Chiu et al. 1996). Plasmids that included 35S::NLS::GFP and 35S::GFP were described previously (Nishihama et al. 2001).

Transformation of Arabidopsis

Agrobacterium-mediated transformation of root explants of Arabidopsis was performed as described by Onouchi et al. (1995). Root explants were cocultured with *Agrobacterium* LBA4404/pAL4404 (Hoekema et al. 1984) cells that harbored pSK35S::AS2 or pSK1

(Kojima et al. 1999). Transformants were selected on shoot-induction medium in the presence of 15 mg liter^{-1} hygromycin B (Onouchi et al. 1995). Plasmids pSK35S::AS2 and pSK1 were used to transform *A. tumefaciens* strain GV3101. Whole plants were then transformed by vacuum infiltration. Transgenic plants were selected on MS medium contained 15 mg liter^{-1} hygromycin B and 300 mg liter^{-1} carbenicillin.

Subcellular localization of the AS2::GFP protein

Cells in epidermal layers of onion bulbs were transformed with p35S::AS2::GFP, p35S::NLS::GFP or p35S::GFP, as described previously (von Arnim and Deng 1994). Fluorescent signals were recorded with a fluorescence microscope (Axioplan2; Carl Zeiss, Oberkochen, Germany) equipped with a cooled CCD camera system (Photometrics, Tucson, AZ). Pseudocoloring of the images and measurements of extents of signals from GFP and cell widths were performed with the IPLab software program (Scanalytics, Fairfax, VA). The same cells were stained with 1 μg ml^{-1} 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) and fluorescence was recorded similarly.

Reverse transcription-polymerase chain reaction (RT-PCR)

Flower buds of a transgenic plant and wild type were harvested, 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 as described by Semiarti et al. (2001a). To amplify DNA segments specifically derived from transcripts of the endogenous *AS2* gene, we selected sites for the design of primer sets in 5′- and 3′-untranslated regions of *AS2* transcripts, both of which were absent in the cDNA used for generation of transgenic plants. The primer pairs were as follows: for α-tubulin, pU51 (5′-GGACAAGCTGGGATCCAGG-3′) and pU52 (5′-CGTCTCCACCTTCAGCACC-3′); for untranslated regions of cDNA of *AS2* gene, pU73 (5′-CCCCTCTGAGCAACAGAAGC-3′) and pU74 (5′-CCAAAACCCTAAAATCTCAAGACGG-3′); for the region of the open reading frame of AS2 cDNA, pU328 (5′-GTGTTTGGAGCAAGTAACGT-3′) and pU315 (5′-AAACCTAGGAGACGGATCAACAGTACGGCG-3′). Details of the procedure that we used here will be sent on request.

GenBank accession numbers

The GenBank accession numbers for nucleotide and amino acid sequences reported herein are as follows: AS2 (AB080802); and BACs F5I14 (AC001229), F1E22 (AC007234), and F12P19 (AC009513). Information about members of the AS2 family of proteins from Arabidopsis is summarised in Table 2.

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