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The *BLADE-ON-PETIOLE 1* gene controls leaf pattern formation through the modulation of meristematic activity in *Arabidopsis*

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SUMMARY

The plant leaf provides an ideal system to study the mechanisms of organ formation and morphogenesis. The key factors that control leaf morphogenesis include the timing, location and extent of meristematic activity during cell division and differentiation. We identified an *Arabidopsis* mutant in which the regulation of meristematic activities in leaves was aberrant. The recessive mutant allele *blade-on-petiole1-1* (*bop1-1*) produced ectopic, lobed blades along the adaxial side of petioles of the cotyledon and rosette leaves. The ectopic organ, which has some of the characteristics of rosette leaf blades with formation of trichomes in a dorsoventrally dependent manner, was generated by prolonged and clustered cell division in the mutant petioles. Ectopic, lobed blades were also formed on the proximal part of cauline leaves that lacked a petiole. Thus, *BOP1* regulates the meristematic activity of leaf cells in a proximodistally dependent manner. Manifestation of the phenotypes in the mutant leaves was dependent on the leaf position. Thus, *BOP1* controls leaf morphogenesis through control of the ectopic meristematic activity but within the context of the leaf proximodistality, dorsoventrality and heteroblasty. *BOP1* appears to regulate meristematic activity in organs other than leaves, since the mutation also causes some ectopic outgrowths on stem surfaces and at the base of floral organs. Three class I *knox* genes, i.e., *KNAT1, KNAT2 and KNAT6,* were expressed aberrantly in the leaves of the *bop1-1* mutant. Furthermore, the *bop1-1* mutation showed some synergistic effect in double mutants with *as1-1* or *as2-2* mutation that is known to be defective in the regulation of meristematic activity and class I *knox* gene expression in leaves. The *bop1-1* mutation also showed a synergistic effect with the *stm-1* mutation, a strong mutant allele of a class I *knox* gene, *STM*. We, thus, suggest that *BOP1* promotes or maintains a developmentally determinate state in leaf cells through the regulation of class I *knox* genes.

Key words: *Arabidopsis thaliana*, Meristem, Differentiation, Leaf morphogenesis, Class I *knox* genes, *BOP1*

INTRODUCTION

The leaf organs of higher plants appear in succession around the periphery of the shoot apical meristem (SAM), which has the ability to self-perpetuate (Steeves and Sussex, 1989). The SAM can be divided into the central, peripheral and rib zones. The central zone contains a core of stem cells. The rib zone lies beneath the central zone and contributes to the differentiation of the vasculature and interior stem structures. The peripheral zone is located on the flanks of the SAM and provides founder cells that direct cell division and differentiation in both spatial and temporal manners to produce leaf primordia with three primary developmental axes: the proximodistal, dorsoventral and mediolateral axes (Steeves and Sussex, 1989; Tsukaya, 1998; Waites et al., 1998). The leaf organ is formed from the leaf primordium through a series of cell divisions, differentiation and expansion processes that are also controlled both spatially and temporally. Therefore, the intricate and precise mechanisms that regulate the timing, location and extent of cell division, differentiation and expansion contribute to leaf morphogenesis.

Arabidopis provides an excellent tool to genetically dissect developmental processes of the leaf organ owing to its simple and stable pattern formation. Each *Arabidopsis* leaf can be divided into a proximal petiole and a distal blade region. A key factor that contributes to the control of leaf morphogenesis in *Arabidopsis* is the spatial and temporal regulation of meristematic activity, which involves cellular differentiation. Class I *knox* genes are believed to play crucial roles in the specification of leaf primordia and in the control of leaf

patterning through regulation of the meristematic activities of leaf cells. Class I *knox* genes include *KNAT1* (for *KNOTTED*-like from *Arabidopsis thaliana*), *KNAT2*, *KNAT6* and *SHOOTMERISTEMLESS* (*STM*) in *Arabidopsis* (Lincoln et al., 1994; Long et al., 1996; Semiarti et al., 2001), *knotted1* and *ROUGH SHEATH1* in maize (Becraft and Freeling, 1994; Jackson et al., 1994; Schneeberger et al., 1995), and *Nicotiana tabacum Homeobox 15* in tobacco (Tamaoki et al., 1997). These genes are strongly expressed in the SAM but not in the incipient young primordia (Jackson et al., 1994; Long et al., 1996; Tamaoki et al., 1997; Nishimura et al., 1999; Sentoku et al., 1999). Suppression of class I *knox* gene expression in primordia and mature leaf organs is critical for the determination of leaf cell fate. Aberrant expression of class I *knox* genes triggers multiple abnormal developmental responses, including altered leaf development and, in extreme cases, formation of ectopic meristems (Matsuoka et al., 1993; Sinha et al., 1993; Lincoln et al., 1994; Schneeberger et al., 1995; Chuck et al., 1996; Hareven et al., 1996; Tamaoki et al., 1997; Sentoku et al., 2000). Furthermore, mutations of the *Arabidopsis* gene *ASYMMETRIC LEAVES1* (*AS1*) and *AS2*, which produce lobed rosette leaves with leaflet-like organs on their petioles, result in aberrant expression of class I *knox* genes (Byrne et al., 2000; Ori et al., 2000; Semiarti et al., 2001). Abnormal expression of class I *knox* genes in leaf cells due to a lack of negative regulators, such as *AS1* and *AS2*, leads to the aberrant regulation of meristematic activity and ectopic growths. *AS1* encodes a Myb protein domain and is a homologue of both the *ROUGH SHEATH2* (*RS2*) of maize and the *PHANTASTICA* (*PHAN*) of *Antirrhinum* (Waites et al., 1998; Timmermans et al., 1999; Tsiantis et al., 1999; Byrne et al., 2000). *AS2* encodes a novel protein with cysteine repeats (C-motif) and a leucine-zipper-like sequence (Iwakawa et al., 2002). In addition, *LEAFY PETIOLE* is thought to be involved in controlling cell division in the marginal meristem of leaves (van der Graaff et al., 2000).

In spite of considerable efforts to reveal the genetic and molecular mechanisms underlying cell fate determination in leaves, our understanding is still largely limited (Tsukaya, 2002a; Tsukaya, 2002b; Tsukaya, 2002c). In this report, we describe the *bop1-1* mutation that induces abnormal expression of class I *knox* genes. The mutation was originally identified by the phenotype of vigorous ectopic outgrowths along leaf petioles. Through characterization of this mutation, we suggest that *BOP1* has a critical role in regulating meristematic activity in leaves by modulating expression of class I *knox* genes.

MATERIALS AND METHODS

Plant strains and growth conditions

The *Arabidopsis thaliana* Landsberg *erecta* (L*er*; CS20) ecotype and the *as1-1* (CS3774), *as2-2* (CS3118), *stm-1* (CS8154) and *bp-1* (CS30) mutants were obtained from the *Arabidopsis* Biological Resource Center (ABRC, Ohio State University, USA). The *KNAT1::GUS* transgenic plants (Col Background) were kindly provided Sarah Hake. For the phenotypic analyses, the seeds were imbibed at 4°C in darkness for 3 days, and then sown on a mixture of vermiculite, peat moss and perlite (1:1:1, v/v) in a plant growth room (KOENCON, Seoul, Korea) with 16 hours of light and 8 hours of darkness daily, and with a day/night temperature cycle of 22°C/18°C.

Isolation of mutants

Approximately 40,000 seeds of the ecotype L*er* were mutagenised by soaking in a 0.33% solution of ethylmethane sulfonate (EMS) in 100 mM phosphate buffer (pH 7.0) for 8 hours, as described previously (Oh et al., 1996). The M_1 plants from the mutagenised seeds were grouped into 8 subpopulations and the progeny of self-fertilised M_1 plants from each group was harvested separately. A single M_2 plant that exhibited the *bop1-1* phenotype was identified from more than 200,000 M_2 plants. A backcross was performed using *bop1-1* mutant pollen and L*er* gynoecia. Progenies of a single plant that were derived from four backcrosses to L*er* were used for genetic and phenotypic analyses.

Mapping of the *BOP1* gene

The *bop1-1* mutation was initially mapped using the cleaved amplified polymorphic sequence (CAPS) markers (Konieczny and Ausubel, 1993). The *bop1-1* mutant was crossed with the Columbia (Col) ecotype. DNA for mapping studies was prepared from 60 individual F_2 progenies with the mutant phenotype. In addition, we generated the *bop1-1 tt5-1* double mutant and examined the segregation ratio of the mutant phenotypes in 261 F_2 progenies that were derived from a cross between the double mutant and Col. The map distance was estimated using the Kosambi mapping function (Koorneef and Stam, 1992).

Analysis of vasculature

The vein patterns in cotyledons and rosette leaves of plants were examined as described previously (Hamada et al., 2000; Jun et al., 2002). Photographs were taken under dark-field microscopy.

Histological analysis

Plant materials for histological analysis were fixed overnight at room temperature in a solution of 45% ethanol, 2.5% glacial acetic acid and 2.5% formaldehyde (v/v). The samples were then dehydrated by sequential 30-minute incubations in 50%, 60%, 70%, 80%, 90%, 95% and 99.5% (v/v) ethanol, followed by two incubations of 1 hour each in 100% (v/v) ethanol. The dehydrated samples were set in Technovit 7100 resin (Heraeus Kulzer, Wehrheim/Ts., Germany) at room temperature, once in 50% (v/v) resin and twice in 100% resin. Serial 2-μm thick sections of the plant tissues were cut with a rotary microtome (MICROM International, Walldorf, Germany), and stained with 0.5% Methylene Blue and 0.5% borate for 30 seconds.

Scanning electron microscopy

The samples used for scanning electron microscopy were prepared in the same way as those for the histological analysis. After 100% ethanol treatment, the pretreated samples were soaked overnight in a solution of amyl acetate (Aldrich). The material was then critical point dried in liquid CO_2, coated with gold and palladium at 10-20 nm thickness, and examined at an acceleration voltage of 10-20 kV using a scanning electron microscope (Model 1420; LEO Electron Microscopy Ltd., Cambridge, England).

Reverse transcription polymerase chain reaction (RT-PCR) analysis

RNA was prepared from 10-day-old plants grown on Gamborg's B-5 Medium (GIBCOBRL) using TRI REAGENT (MCA). The cDNA molecules were synthesised from total cellular RNA using 1 μg of oligo(dT) primer and a first-strand cDNA Synthesis Kit (Roche). The PCR conditions for each cycle were as follows: 0.5 minutes at 94°C, 0.5 minutes at 55°C, and 1.5 minutes at 72°C. PCR was performed over 31 cycles for the *KNAT1*, *KNAT2* and *KNAT6* cDNAs and 26 cycles for the β-tubulin cDNA. The primers used for amplification of the β-tubulin cDNA (Kim et al., 1998), *KNAT1*, *KNAT2* and *KNAT6* cDNAs (Semiarti et al., 2001) were as described previously. The amplification of β-tubulin cDNA was used for normalization of the RT-PCR (Kim et al., 1998).

β-Glucuronidase (GUS) activity

Plants were grown on Gamborg's B-5 Medium (GibcoBRL) in a culture room under the long day condition. Histochemical staining of the GUS activity was performed as described earlier (Jun et al., 2002).

Analysis of double mutants

We reciprocally crossed *bop1-1* mutant plants with *as1-1*, *as2-2*, *stm-1* and *bp-1* mutants. The double mutants were identified as novel or additive phenotypes in the F_2 generation. The frequencies at which these plants arose were compatible with a Mendelian segregation ratio of 1:16 for two genetically unlinked recessive loci.

RESULTS

Isolation of the *bop1* mutant

Identification of the *bop1-1* mutation was based on its pronounced phenotype, i.e., the presence of ectopic organ outgrowths along the leaf petioles of rosette leaves (Fig. 1A,B). The ectopic outgrowths were observed, along with extensive lobe formation, on both sides of the petiole midvein. This phenotype was observed in all of the rosette leaves. The development of ectopic organs was also observed on the petioles and on the basal regions of cotyledons (Fig. 1C) and cauline leaves (Fig. 1D), respectively.

The mutant plants exhibited pleiotropic phenotypes other than leaf patterning. In the cotyledons and the first two leaves of the mutant, an extended, adventitious, petiole-like organ grew out from the base of the original petiole (Fig. 1B,E,F, arrowheads; see Fig. 3 for the nature of these ectopic organs). The petiole region of the early rosette leaves of mutants frequently fused with the basal part of the petiole of a cotyledon or adjacent leaf (Fig. 1G). The leaves of the mutant exhibited extended longevity (Fig. 1H). The leaves of a 37-day-old mutant were still green and viable, whereas wild-type leaves had already turned yellow. The vegetative growth of mutant plants was extended compared to that of wild-type plants (Table 1). The average number of rosette and cauline leaves formed on the mutant plant was slightly lower than that of the wild-type plant (Table 1). Mutant flowers were often slightly distorted and had more variable numbers of sepals and petals, as evidenced by a larger standard error in these experiments (Fig. 1I, Table 2). The mutant flower also contained a slightly reduced number of stamens.

Table 1. Decreases in numbers of leaves and delayed bolting in *bop1-1* mutant plants

Genotype	Rosette leaves	Cauline leaves	Days to bolting
L*er*	6.10±0.43 (*n*=110)	1.90±0.32 (*n*=96)	15.48±0.74 (*n*=101)
bop1-1	4.85±0.40 (*n*=110)	1.13±0.69 (*n*=90)	19.29±0.56 (*n*=96)

Values are means±s.e.m.
n, number of plants analyzed.

Table 2. Number of floral organs

Genotype	Sepals	Petals	Stamens	Carpels
L*er*	4.01±0.09	4.01±0.09	5.89±0.31	2±0
bop1-1	3.81±0.40	3.80±0.42	5.38±0.54	2±0

Values are means±s.e.m.
115 samples were counted for each whorl.

The F_1 offspring from crosses between mutant and wild-type plants had the wild-type phenotype. The F_2 offspring from these plants segregated at a Mendelian segregation ratio of 3:1 (123 wild type: 45 mutant; χ^2=0.28, P>0.5) for the ectopic blade phenotype, demonstrating that the mutant phenotype was caused by a single recessive nuclear mutation. We named the mutation *blade-on-petiole 1* (*bop1*). The genetic locus of the *bop1* mutation was mapped to the lower arm of chromosome 3 at approximately 7.49 cM below the *AFC* CAPS locus and 7.28 cM below the *TT5* locus.

Electron microscopy of mutant leaves

The leaf phenotypes of the mutant suggested that the mutation played a critical role in leaf development and patterning. Therefore, we initially focused our analyses on leaf

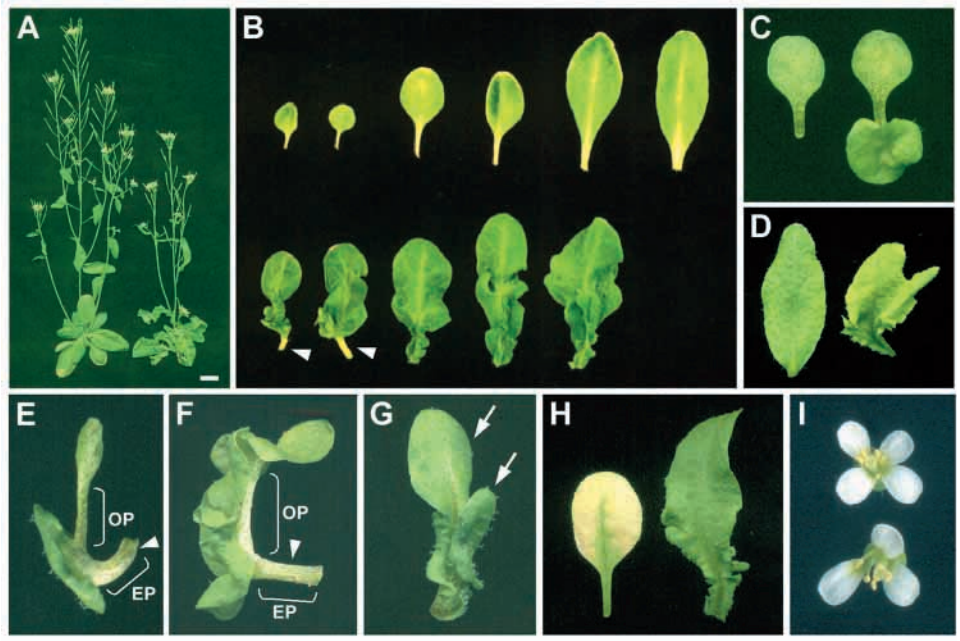

Fig. 1. Phenotypes of *bop1-1* mutant plants. (A) Gross morphologies of a 40-day-old wild-type (left) and a *bop1-1* mutant (right) plant. (B) Morphology of each rosette leaf of the wild-type (upper) compared with that of *bop1-1* mutant leaves (lower). The arrowheads in B indicate extended petiole regions. (C) Morphology of *bop1-1* (right) and wild-type (left) cotyledons of 25-day-old plants. (D) Mature cauline leaf from a wild type (left) and a *bop1-1* mutant (right) plant. (E,F) Cotyledon (E), and the first rosette leaf (F) of a 30-day-old *bop1-1* plant showing the original petiole region (OP) and the extended petiole region (EP; arrowheads). (G) Fused rosette leaves from a *bop1-1* mutant. Arrows indicate two fused leaves. (H) Third leaves from a 37-day-old wild-type (left) and a *bop1-1* mutant (right) plant. (I) Flowers from the wild-type (upper) and the *bop1-1* mutant (lower). Bar in A, 10 mm.

development. When examined by scanning electron microscopy, the proximal parts of the cotyledons and the first leaf of wild-type plants had smooth surfaces without protrusions (Fig. 2A,C, respectively). In contrast, the adaxial side of the basal regions of the petioles of *bop1-1* cotyledons showed several outgrowths (Fig. 2B, arrowheads). Ectopic organogenesis was also observed on the first rosette leaf of the mutant (Fig. 2D). The ectopic organs (Fig. 2D, arrows) that formed on the adaxial side of the rosette leaf resembled leaf blades with flattened structures. These ectopic outgrowths appeared to possess dorsoventrality, with differential growth between the two surfaces of the blade-like structures, and trichome development on only one of the two surfaces. The abaxial side of the first rosette leaf of *bop1-1* also developed ectopic outgrowths (Fig. 2F). However, the shapes of these ectopic outgrowths differed from those on the adaxial side. They never developed into blade-like structures, but remained as small, unidentifiable outgrowths. The development of ectopic outgrowths was much more pronounced on the third rosette leaf of a mutant than on the first rosette leaf (Fig. 2G,H). Importantly, numerous trichomes and leaf primordia were found in the proximal region of the leaf (Fig. 2H). Cauline leaves of wild-type plants appeared as smooth surfaces in the proximal region (Fig. 2I). In contrast, ectopic outgrowths on blade-like structures were observed at the base of the cauline leaves in the mutant (Fig. 2J). In addition, many filamentous outgrowths developed in the mutant on the abaxial and proximal sides of the cauline leaves (Fig. 2J).

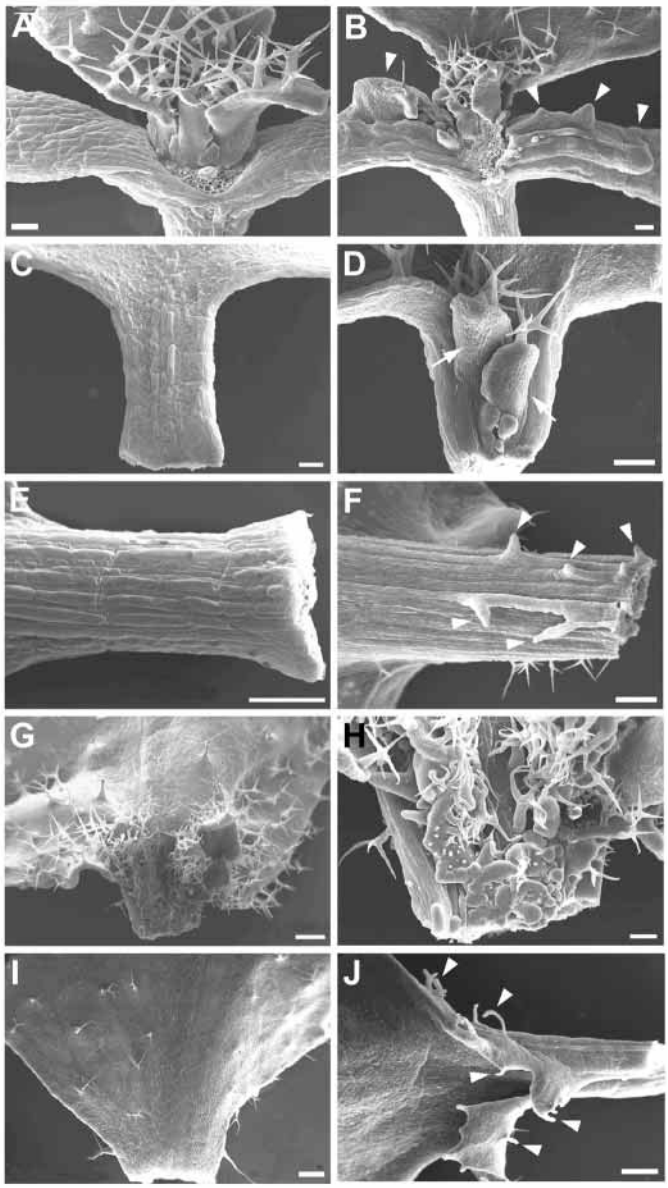

Fig. 2. Ectopic organ outgrowths on *bop1-1* mutant plants. (A,C,E,I) Wild type. (B,D,F-H,J) *bop1-1* mutant. (A,B) Ten-day-old seedlings. One leaf has been detached. Ectopic outgrowths are seen on the petioles of mutant cotyledons (B, arrowheads). (C,D) Detached first leaves of 10-day-old wild-type (C) and *bop1-1* (D) seedlings. Adaxial side views. Arrows in D indicate ectopic outgrowths at the base of the petiole of a mutant rosette leaf. (E,F) Detached first leaves of 15-day-old wild-type (E) and mutant (F) seedlings. Abaxial side views. Note the ectopic outgrowths on the petiole of the mutant (F, arrowheads). (G,H) Detached third leaves of a 23-day-old mutant plant. Note the development of numerous trichomes and leaf primordia on the proximal region of the mutant leaf (H). (I,J) Cauline leaves of a mature wild-type (I) and mutant (J) plant. The arrowheads in J represent ectopic outgrowths on mutant plants. Bars, 100 μm (A-D,H), and 300 μm in the remaining panels.

Cellular phenotypes of the ectopic organs on the petiole

The effect of the *bop1-1* mutation was also examined at the cellular level. On both cotyledons and rosette leaves of 17-day-old mutant plants the ectopic organs were clearly seen on the adaxial surfaces (Fig. 3B,D, respectively). The cellular development of the ectopic outgrowths on the adaxial side resembled that of the leaf blade. Indeed, the ectopic outgrowths had flattened structures that resembled those of leaf blades. Methylene Blue staining revealed clear differences in cellular arrangements between the dorsal and ventral sides of the ectopic blade-like outgrowths. In addition, trichome development occurred only on the adaxial side of the ectopic outgrowth that formed on rosette leaves. The formation of secondary outgrowths on the primary ectopic outgrowths was observed frequently (Fig. 3D, arrows). Interestingly, we noticed that the development of ectopic outgrowths was associated with the formation of a new structure that resembled a vascular system (Fig. 3B,D, arrowheads).

The wild-type petiole has a single, round vascular system in its centre (Fig. 3A,C,E). In contrast, the vascular system of the mutant leaves had a flattened shape (Fig. 3B,D,F). The phloem- and xylem-like tissues were located on the abaxial and adaxial sides of the leaf, respectively (Fig. 3F), which is the arrangement in wild-type petioles (Fig. 3E). The flattened structure appeared to be due to the parallel development of many vascular bundles.

The newly formed, ectopic, adventitious petioles at the base of the original petioles of the cotyledons and the first two leaves of *bop1-1* (Fig. 1B,E,F, arrowheads) had features that were intermediate between stems and petioles. First, the vascular system of the ectopic organs had a eustelic arrangement of vascular bundles (Fig. 3G,H), which is typical for stem bundles (see Fig. 7C,D for comparison). Furthermore, a pith-like structure was observed in the region surrounding the central vascular system (Fig. 3G,H), which is another feature of stems (see Fig. 7C,D for comparison). In cross section the ectopic

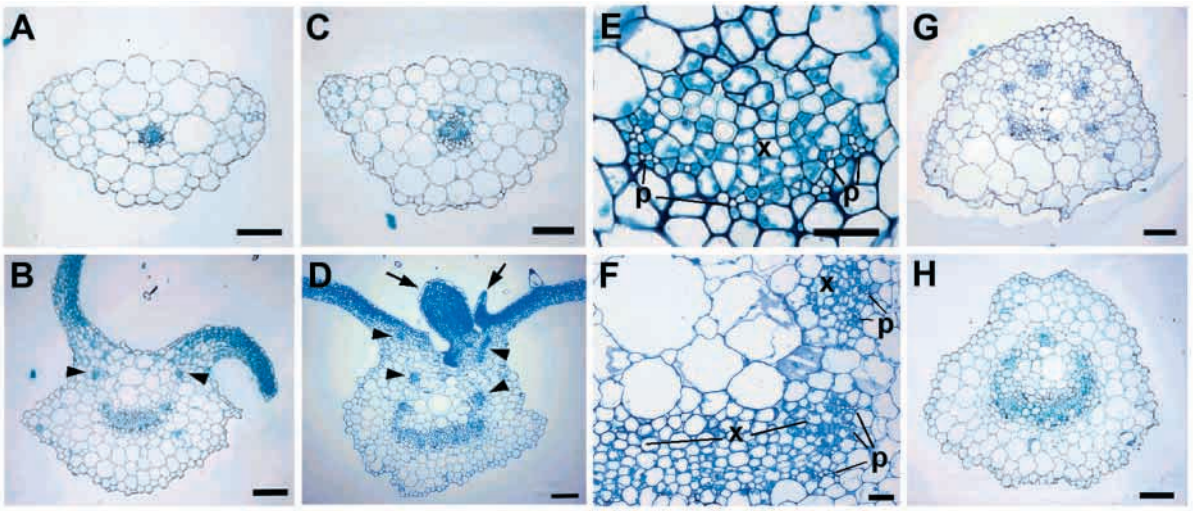

Fig. 3. Histological analysis of *bop1-1* mutant plants. (A,C,E) Wild-type leaf petioles. (B,D,F) *bop1-1* mutant leaf petioles. The adaxial surface is at the top. (A,B) Petioles of cotyledon. (C,D) Petioles of rosette leaf. E and F are magnified views of C and D, respectively. Note that ectopic outgrowths develop on the vasculature (B,D, arrowheads). Secondary outgrowths are formed on the primary ectopic outgrowths (D, arrows). (G,H) Extended petioles. The adaxial surface is at the top. (G) Extended cotyledonary petiole. (H) Extended petiole of first rosette leaf. P, phloem cells; X, xylem cells. Bars, 100 μm in (A-D,G,H), and 20 μm in (E,F).

organs were rounder than the wild-type petioles but not as round as the wild-type stem. In addition, the surface was not as smooth as the wild-type stem (Fig. 3G,H; see Fig. 7C for comparison). We conclude that although the ectopic organ grows from the base of the original petiole, it is not a simple petiole.

Vein patterning in the ectopic blades of the mutant

The electron microscopic and histological analyses indicated that the ectopic organs of *bop1-1* plants had some of the characteristics of true blades. To confirm this, we examined the venation of wild-type and *bop1-1* leaves. The wild-type cotyledons had a very simple pattern of veins, with one central midvein (primary vein) and three or four lateral veins that branched from the midvein (Fig. 4A), as reported previously (Sieburth, 1999; Jun et al., 2002). The wild-type rosette leaves had a central midvein (primary vein), six to eight secondary veins that branched from the midvein, and numerous tertiary and quaternary veins that branched from the secondary and tertiary veins, respectively (Fig. 4B) (Nelson and Dengler, 1997; Sieburth, 1999). The cotyledons of *bop1-1* plants had a typical venation pattern of wild-type cotyledons in the distal regions where no ectopic organogenesis was observed (Fig. 4C). The ectopic organ that formed on the proximal part of the mutant cotyledon developed a reticulated venation pattern that resembled that of wild-type leaf blades. However, the venation pattern was intermediate between those of cotyledons and rosette leaves of the wild type in terms of complexity: the ectopic organ in the mutant cotyledons had more complex venation than the wild-type cotyledon and less complex venation than wild-type rosette leaves. The ectopic organs that formed on the rosette leaves of the *bop1-1* plants also developed extensively reticulated venation systems (Fig. 4D), which were similar to those seen in the blades of wild-type rosette leaves (Fig. 4B). However, unlike the wild-type leaves, the midvein systems in the proximal region of the mutant rosette leaves revealed an extensive vasculature with numerous parallel vein elements. The secondary lateral veins branched from the ectopic midveins to the ectopic outgrowths on the *bop1-1* leaves.

These results show that ectopic outgrowths formed along the petioles of *bop1-1* mutants have the features of leaf blades in that they develop the higher order vascular system. The results also show that the *bop1-1* mutation severely affects vein development in the proximal region of the midvein. This observation is consistent with the histological analysis of the petiole region of the mutant leaves (see above, Fig. 3B,D,F).

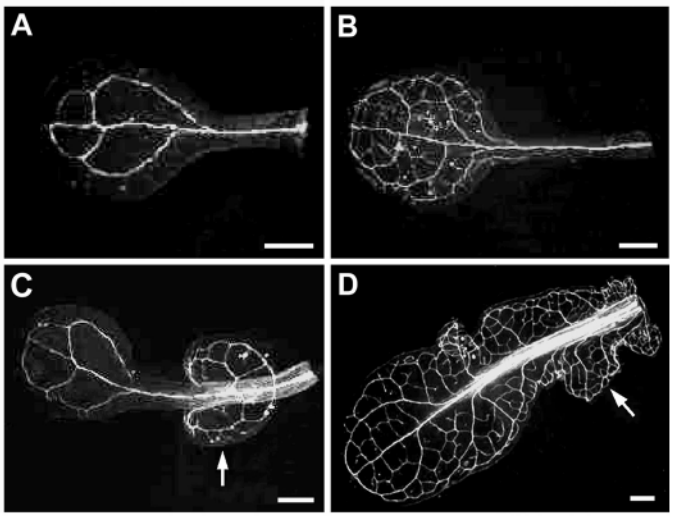

Fig. 4. Leaf venation pattern in *bop1-1* mutant plants. (A,C) Cotyledons of 24-day-old wild type (A) and *bop1-1* mutant (C). (B,D) First rosette leaves of 16-day-old wild type (B) and *bop1-1* mutant (D). Note that the reticulate patterns of venation are distinct in the regions of ectopic outgrowth on petioles of cotyledons and rosette leaves (C,D, arrows). Bars, 1 mm.

Ectopic meristematic activity in the *bop1* mutant

To identify the origin of the abnormal features described above, the lesions in the pattern and timing of expression of the mutant phenotypes were examined in early seedlings of the mutant. In 2-day-old seedlings, young and relatively undifferentiated cells with distinct nuclei were visible in both wild-type and *bop1-1* cotyledons, particularly on the adaxial sides of cotyledons (Fig. 5A,B). At this time, there were no noticeable differences in cellular differentiation between the wild type and the mutant. In addition, scanning electron microscopic examination of the 2-day-old *bop1-1* mutant cotyledons did not reveal structural differences on the surface of the cotyledon (data not shown). The first indication of the *bop1-1* phenotype was recognized in 3-day-old seedlings (Fig. 5D,F). At this stage, most of the cells in the petiole of wild-type cotyledon had already differentiated (Fig. 5C,E). In contrast, the petiole of mutant cotyledon had clusters of young cells on the adaxial side (Fig. 5D,F). The differentiation status of these clustered cells was evident from the large nucleus and a lack of large vacuoles, which indicated that the cells were relatively undifferentiated. This feature was also observed in longitudinal sections (Fig. 5H). The mutant cotyledons had more undifferentiated cells along the longitudinal axis than the wild-type cotyledons. The *bop1-1* cotyledonary cells near the rosette leaf primordia had large nuclei and were stained more strongly than the surrounding cells (Fig. 5F,H, arrowheads). At 6 days of age, the differences between wild-type and mutant seedlings became more evident (Fig. 5I,J). The wild-type cells had more extensive differentiation and a more developed vascular system than the mutant cells. The clusters of young cells in the mutant, however, continued to divide and subsequently produced ectopic outgrowths (Fig. 5J, arrowheads).

Taken together, the results show that the mutant petiole cells have ectopic meristematic activities that lead to the production of ectopic organs.

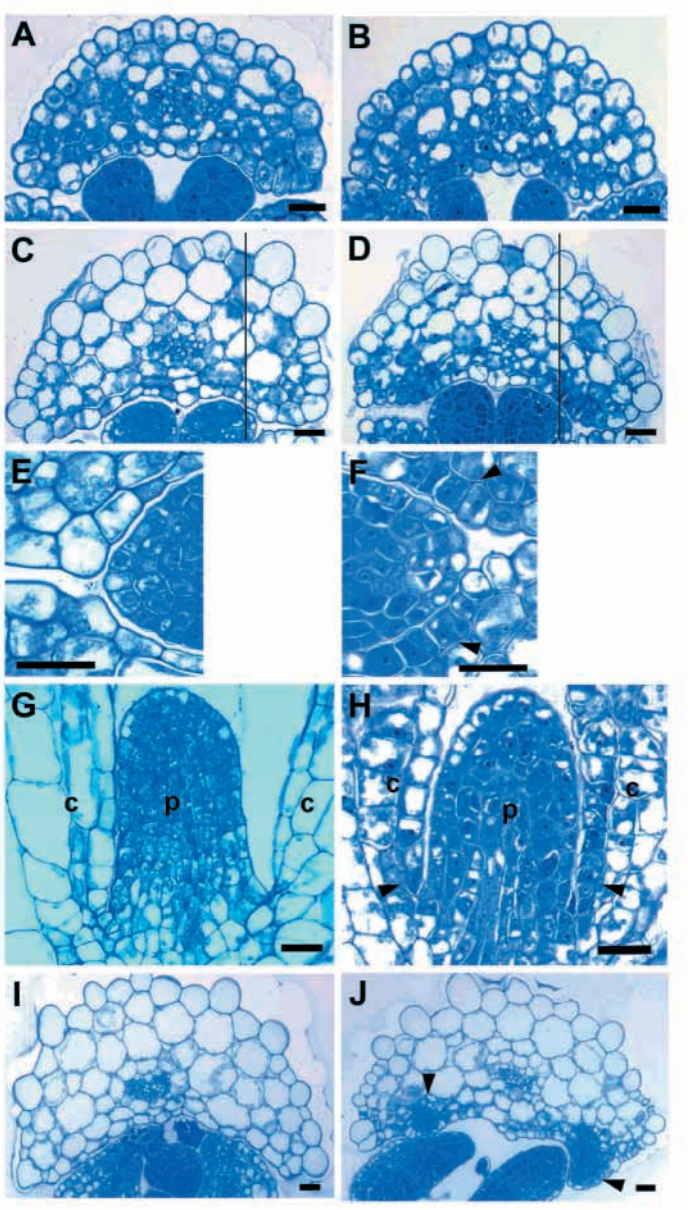

Fig. 5. Origins of the defects in the *bop1-1* mutant. (A-F,I,J) Cross sections and (G,H) longitudinal sections of the shoot apex. (A,B) Two-day-old wild-type (A) and mutant (B) seedlings. (C-H) Three-day-old wild-type (C,E,G) and mutant (D,F,H) seedlings. E and F are magnified views of C and D, respectively. Note that ectopic meristematic regions are visible in the *bop1-1* mutant (F,H, arrowheads). The black lines in C and D indicate the planes of the sections shown in G and H, respectively. (I,J) Six-day-old wild-type (I) and mutant (J) seedlings. Ectopic outgrowths are distinct on the adaxial side of the cotyledonary petiole (J, arrowheads). c, cotyledons; p, leaf primordia. Bars, 20 μm.

Ectopic organs are also formed on flowers and stems

We investigated whether ectopic organs were formed on other parts of the mutant plants. Initially, we examined the floral organs, which are modified leaves. *Arabidopsis* has complete flowers, with four concentric whorls of floral organs, i.e., four sepals, four petals, six stamens and two fused carpels (Fig. 6A) (Smyth et al., 1990). In contrast to wild-type petals, filamentous organs of an unidentified nature developed at the base of mutant sepals (Fig. 6B,C). The development of these ectopic organs was more extensive in certain flowers (Fig. 6D, arrowheads). The petals of *bop1-1* plants had some ectopic outgrowths in their proximal regions (Fig. 6F), in contrast to the smooth surfaces of wild-type petals (Fig. 6E). However, these outgrowths did not develop into the extensive outgrowths such as the ones observed in the mutant leaves.

Ectopic organ development was also found in the stem. The surface of the mutant stem, unlike the smooth surface of the wild-type stem (Fig. 7A), was rough in certain places along the longitudinal direction, and ectopic protrusions were evident in the areas of roughness (Fig. 7B). When the stems were sectioned, the eustelic bundle vasculatures were distinctively observed in the wild-type and mutant stems (Fig. 7C and D, respectively), although the number of vascular bundles was lower in the mutant stem. Furthermore, a few small ectopic outgrowths were observed on the outer surfaces (Fig. 7D,F), which was consistent with the results from electron microscopy. However, the ectopic growths on the stems were not as extensive as those on the leaves. The cellular morphology of the ectopic outgrowths on *bop1-1* stems indicated that they were probably formed from outgrowths of cortical and/or epidermal cells. Trichomes often developed on

these ectopic outgrowths (Fig. 7D, arrows), which indicated that the outer cells of the ectopic outgrowths resembled wild-type epidermal cells in some aspects.

Ectopic expression of knox genes in *bop1-1* leaves

The most pronounced phenotypic abnormalities observed in the *bop1-1* mutant were the lobes on rosette and cauline leaves. These abnormalities are typical of *Arabidopsis* plants that ectopically express class I *knox* genes (Lincoln et al., 1994; Chuck et al., 1996; Ori et al., 2000; Semiarti et al., 2001; Iwakawa et al., 2002). Therefore, we examined the expression patterns of class I *knox* genes using RNA from 10-day-old seedlings. In the wild-type plants, the *KNAT1*, *KNAT2*, and *KNAT6* transcript levels in cotyledons and leaves were low (*KNAT2* and *KNAT6*) or undetectable (*KNAT1*) (Fig. 8A). This result was consistent with earlier reports (Lincoln et al., 1994; Dockx et al., 1995; Byrne et al., 2000; Semiarti et al., 2001). The expression levels of these genes in the cotyledons and rosette leaves of *bop1-1* seedlings were significantly higher than those in wild-type seedlings, except in the case of the *KNAT2* that showed only a slightly increased expression in the mutant rosette leaf. The expression levels of all of these genes were higher in the SAM than in leaves, as reported previously (Lincoln et al., 1994; Dockx et al., 1995; Byrne et al., 2000; Semiarti et al., 2001), but there were no notable differences between the wild-type and mutant SAM regions in the expression of these genes. *STM* transcription was not detectable in our experiment in the wild-type or mutant cotyledons and rosette leaves (data not shown). To complement the above results, we analysed transgenic plants that expressed the β-glucuronidase (GUS) reporter gene under the control of the *KNAT1* promoter. In wild-type plants, expression of *KNAT1::GUS* was observed in the shoot meristem and the upper part of hypocotyl (Fig. 8B), as reported previously (Ori et al., 2000). In wild-type cotyledons and leaves, weak GUS expression was only detected at the basal tip (Fig. 8C,D, arrows). In *bop1-1* plants, *KNAT1::GUS* was expressed in a pattern similar to that in wild-type plants, but in more expanded regions (Fig. 8E). Examination of the expression pattern in single leaves of *bop1-1* showed that, unlike in wild-type leaves, *KNAT1::GUS* was detected in the petioles of cotyledons and rosette leaves (Fig. 8F,G), suggesting that *KNAT1::GUS* was misexpressed in these regions in the *bop1-1*. Thus, at least for the *KNAT1* gene, the promoter activity observed through expression of the GUS reporter gene is consistent with the result of the RT-PCR analysis.

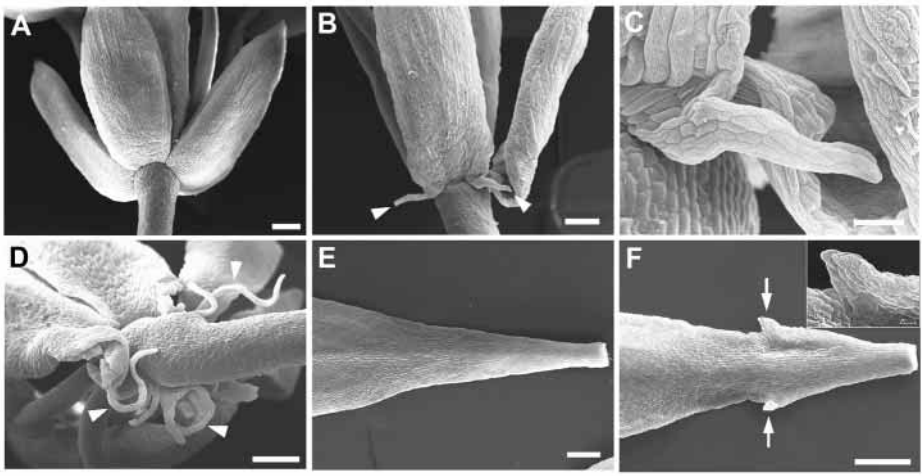

Fig. 6. Development of *bop1-1* flowers. (A) Mature wild-type flower. (B,D) Mature *bop1-1* mutant flowers. (C) Magnified view of B. Note the filamentous organs on the mutant flowers (B,D, arrowheads). (E) Wild-type petal. (F) *bop1-1* mutant petal. The inset shows a magnified view of the ectopic outgrowths (arrows). Bars, 200 μm in (A,B,D-F), and 50 μm in (C).

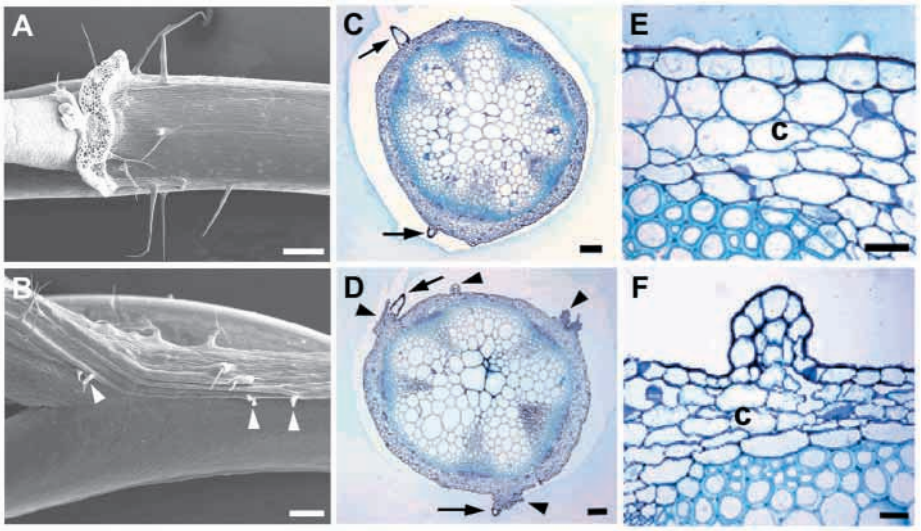

Fig. 7. Development of ectopic outgrowths on the stems of the *bop1-1* mutant. (A,C) Stem of mature wild-type plant. (B,D) Stem of mature *bop1-1* mutant plant. E and F are magnified views of C and D, respectively. The arrows in C and D indicate developing trichomes and the arrowheads in B and D indicate regions of ectopic outgrowths in the mutant. c, cortex. Bars, 300 μm in (A,B), 100 μm in (C,D) and 20 μm in (E,F).

Genetic interaction of *bop1-1* with other shoot mutants

Our results suggested that the *bop1-1* mutant has a lesion in controlling expression of class I *knox* genes. Several mutants are also known to have defects in the regulation of class I *knox* genes in *Arabidopsis* (Byrne et al., 2000; Ori et al., 2000; Semiarti et al., 2001; Iwakawa et al., 2002).

AS1 and *AS2* promote or maintain the differentiated state of leaf cells through negative regulation of class I *knox* genes (Byrne et al., 2000; Ori et al., 2000; Semiarti et al., 2001). A

mutation in either *AS1* or *AS2* produces rumpled and lobed rosette leaves with leaflet-like organs on their petioles (Fig. 9A,B,E,F) (Tsukaya and Uchimiya, 1997; Ori et al., 2000; Semiarti et al., 2001).

The expression pattern of class I *knox* genes and the leaf phenotype of the *bop1-1* mutant suggests that *BOP1* plays a role similar to that of *AS1* and *AS2* in leaf development. Therefore, we examined the genetic interactions between *BOP1* and *AS1* and *AS2* by constructing double mutants. None of the *bop1-1 as1-1* and *bop1-1 as2-2* double mutants showed a simple epistatic relationship. Instead, the *bop1-1 as1-1* and *bop1-1 as2-2* double mutants produced numerous leaflet-like structures along the fasciated leaf petioles (Fig. 9C,D,G,H). On the primary leaflet outgrowths of the double mutant, we observed smaller second- and third-degree leaflet outgrowths that made supercompound leaves. This phenomenon can be viewed as an enhancement of each mutant phenotype.

The other class I *knox* gene is *STM*. *STM* functions in the formation and maintenance of the SAM in *Arabidopsis* (Barton and Poethig, 1993; Endrizzi et al., 1996; Long et al., 1996). The *stm-1* mutant, a strong mutant allele, fails to establish a SAM: cells at the site of the presumptive SAM undergo terminal differentiation (Barton and Poethig, 1993). As a result, *stm-1* plants lack all the organs that normally develop from the SAM. Instead, some adventitious, rescued leaves differentiate ectopically from a region below the fused cotyledons (Fig. 9I). The rescued leaves in *stm-1* show a nearly wild-type appearance but with abnormal phyllotaxy. However, in the *bop1-1 stm-1* double mutant, rescued leaves did not fully expand and were lobed (Fig. 9J). The whole plants showed severely fasciated morphology with a rough surface and many leaflet-like structures. These phenotypes, thus, appear more severe than those expected from a simple addition of the two mutant phenotypes.

BREVIPEDICELLUS (*BP*) encodes the homeodomain protein KNAT1 (Douglas et al., 2002; Venglat et al., 2002). A loss-of-function mutation in *BP* (*KANT1*) is characterized by compact floral internodes, short pedicles and downward-pointing siliques (Fig. 9K). The vegetative rosette leaves of the *bop1-1 bp-1* double mutant exhibited a lobed morphology characteristic for *bop1-1*. The reproductive shoot of the double mutant produced a compact inflorescence meristem, short pedicles and downward-oriented siliques, which are characteristic for *bp-1* (Fig. 9L). The phenotypes of the double mutant, thus, appear additive.

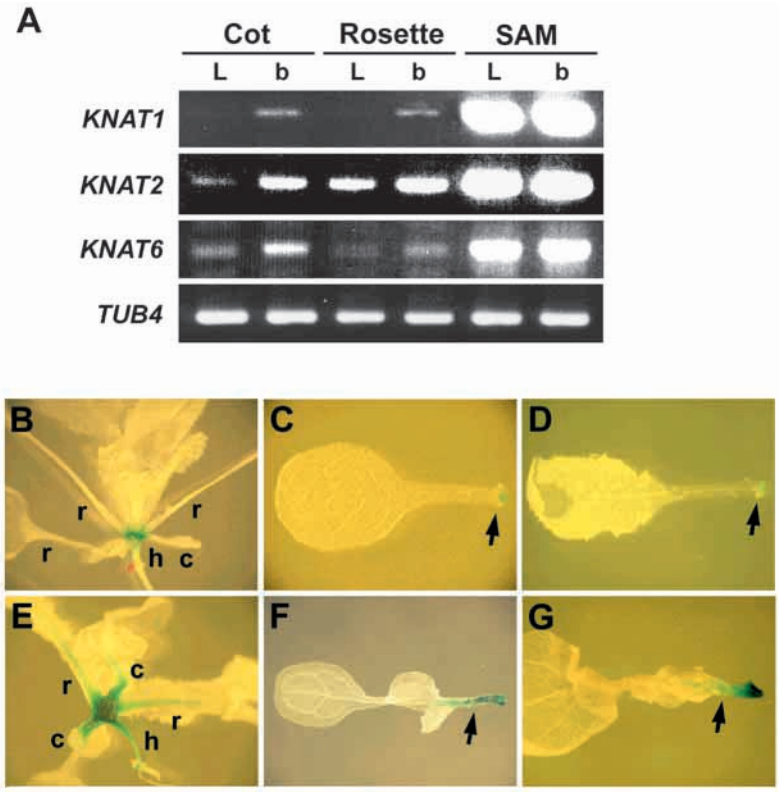

Fig. 8. Analysis of class I *knox* gene expression in the *bop1-1* mutant. (A) Analysis by RT-PCR of transcripts of the *KNAT1*, *KNAT2* and *KNAT6* genes. Cot, cotyledon (including ectopic outgrowths in the case of the *bop1-1* mutant); Rosette, rosette leaves (including ectopic outgrowths in the case of the *bop1-1* mutant); SAM, shoot apical meristem region. L, L*er*; b, *bop1-1*. The β-tubulin (TUB4) gene was amplified as a positive control. (B) A 23-day-old wild-type seedling. (C) A detached cotyledon from wild-type plant. (D) A detached leaf from wild-type plant. Arrows in C and D represent the weak GUS-expressing region at the petiole base. (E) A 23-day-old *bop1-1* mutant seedling. Note the strong expression of GUS on the hypocotyls and the abaxial side of leaf petioles. (F) A detached cotyledon from *bop1-1* plant. (G) A detached leaf from *bop1-1* plant. Note the strong expression of GUS on the basal region of petioles (F,G, arrows). (C,D,F,G) Adaxial side views. r, rosette leaves; c, cotyledons; h, hypocotyls.

DISCUSSION

BOP1 is involved in determining or maintaining the fate of petiole cells

Arabidopsis has a typical simple leaf, which consists of a petiole and a blade. The developmental control of petioles is presumed to be important in the effective capture of light energy by ensuring that the leaf blades do not overlap. In order to produce this leaf shape, the cells on the proximal side of the leaf differentiate into petioles without producing blades or other organs. The most pronounced phenotype of the *bop1-1* mutant was the ectopic development of outgrowths along the leaf petioles. This ectopic structure resembled the leaf blade in many aspects, since it had a blade-like shape with flattened growth pattern, dorsoventral growth differences on the adaxial and abaxial sides of the flattened outgrowths, and trichomes on the adaxial side. Furthermore, the ectopic structures possessed an extensive vasculature with a pattern similar to that of wild-type blades. The *bop1-1* mutation also caused ectopic development of a structure with intermediate features of petiole and stem on the base of the original petiole of the cotyledons and the first two rosette leaves.

These observations show that, following mutation of the *BOP1* gene, the petiole cells do not undergo correct developmental specification and can be diverted towards other developmental fates. Thus, *BOP1* may function to determine or maintain the fate of petiole cells.

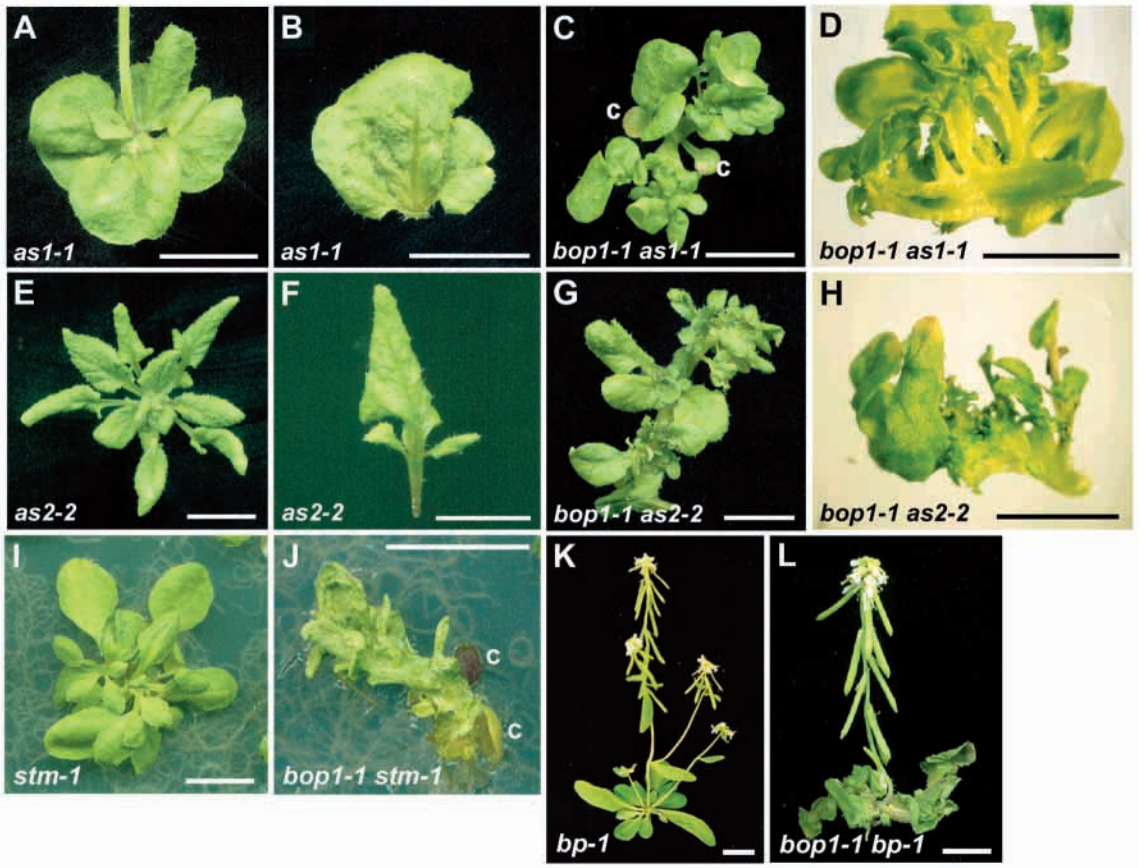

Fig. 9. Phenotypes of double mutants. (A-H) Whole plant and detached rosette leaves of (A,B) *as1-1*; (C,D) *bop1-1 as1-1* double mutant; (E,F) *as2-2*; (G,H) *bop1-1 as2-2* double mutant. (I) A rescued *stm-1* seedling plant. (J) A rescued *bop1-1 stm-1* seedling plant. (K) A *bp-1* seedling plant. (L) A *bop1-1 bp-1* seedling plant. c, cotyledon. The photographs were taken 25 days (A-H), 30 days (I,J) and 38 days (K,L) after planting. Bars, 10 mm.

BOP1 regulates the meristematic activity of leaf cells in a proximodistal manner

The *bop1-1* mutant plants developed extensive ectopic outgrowths in the petiole region as a result of defective cellular differentiation. Normally, petiole cells undergo cellular determination upon growth. However, in the mutant plants, some cells escaped determination and remained as clusters of young cells. These young cells continued dividing and produced ectopic outgrowths. Thus, the wild-type *BOP1* gene is involved in the control of meristematic activity in the petiole region. However, the meristematic cells in the mutant petiole did not develop into a new shoot meristem. Instead, a leaflet-like structure appeared in the early developmental stages of the mutant cotyledons. This result appears to be consistent with previous reports that the formation of leaf primordia can be uncoupled from shoot meristem formation (Jackson et al., 1994; Long et al., 1996; Tamaoki et al., 1997; Nishimura et al., 1999; Sentoku et al., 1999).

Moreover, the ectopic outgrowth of leaf blades was not limited to the petiole regions of cotyledons and rosette leaves. Ectopic outgrowth of blades was also observed in cauline leaves that lacked the petiole. This means that *BOP1* is involved in mechanisms other than petiole cell differentiation. Indeed, the effect of the *bop1-1* mutation was seen in all leaves, including cotyledons, rosettes and cauline leaves. A common feature found among all of these leaves in the mutant was that the proximal region produced ectopic blades. Thus, the *bop1-1* defect in leaves may be explained in terms of the disruption of cellular differentiation along the proximodistal axis. In *Arabidopsis*, the leaves mature basipetally, from the tip to the base of the leaf (Pyke et al., 1991; Donnelly et al., 1999). Thus, cells of the proximal petiole region differentiate later and have a developmental competence that is different from that of distal blade cells. The combination of a less differentiated proximal region and *bop1-1* mutation may have caused the mutant leaf phenotype to produce ectopic blades in the petioles of the cotyledons and rosette leaves and in the proximal regions of the cauline leaves. Developmental lesions were even observed in petals. Although the ectopic outgrowths in petals were not extensive, they appeared primarily in the proximal regions. Therefore, we speculate that *BOP1* controls meristematic activity in a proximodistal manner in a broad range of leaf types.

BOP1 functions within a specific developmental context

The meristematic cells that developed on the petiole of the mutant had limited developmental fates and were not totipotent. The ectopic outgrowths that developed on the adaxial side of the petiole were leaf blades. The base of the original petiole in the mutant cotyledon and first two rosette

leaves had some stem features, particularly with respect to the morphology of the vascular system. Other structures, such as a new shoot or inflorescence, did not develop.

Furthermore, the ectopic organs on the abaxial side of the mutant petiole had different characteristics than those on the adaxial side. While the outgrowths on the adaxial side of the petiole developed mainly into blades, those that formed on the abaxial side did not develop beyond small unidentifiable protrusions. This observation implies that the dorsoventrality of the petiole region of the mutant was maintained. This phenomenon was also observed in the ectopic blades that were generated on the adaxial side of the mutant petioles. The ectopic blades maintained dorsoventrality, as evidenced by trichome formation on the adaxial side only. The ectopic vascular system of the petiole also showed dorsoventrality, such that the xylem cells were oriented towards the adaxial side and the phloem cells were oriented towards the abaxial side. Thus, *BOP1* appears to be minimally, if at all, involved in the control of dorsoventrality.

There were also some position-dependent differences in the developmental defects seen in the leaves. A clear example of this was the extended petiole-like region that occurred exclusively in the cotyledons and the first two leaves. The degree of ectopic organ formation also differed depending on the leaf position, which may be due to the heteroblastic nature of leaf development. In most plant species, including *Arabidopsis*, the leaves that form early in shoot development (juvenile leaves) are morphologically and physiologically different from the leaves that appear later (adult leaves) (Telfer et al., 1997; Kerstetter and Poethig, 1998; Tsukaya et al., 2000). Thus, the production processes for juvenile and adult leaves are modulated by different developmental programs (Kerstetter and Poethig, 1998). The extended petiole-like outgrowth of the *bop1-1* mutant may have been caused by a combination of prolonged and ectopic meristematic activity that is unique to the developmental program of juvenile leaves.

As mentioned above, the mutation did not completely disrupt the developmental fate of the leaf cells, although it remains possible that this is a characteristic of this specific allele. Thus, although *BOP1* regulates the meristematic activity of leaf cells, it functions within a given developmental context that encompasses dorsoventrality, proximodistality and heteroblasty. In other words, *BOP1* controls the developmental fate of leaf cells in association with other factors that are involved in leaf development.

Ectopic leaf blade formation on the petiole, which was the most prominent phenotype observed for the *bop1-1* mutant, may be explained as follows. The mutation caused a disturbance in cellular specification along the proximodistal axis. The mutant petiole (essentially the proximal part of the leaf) displayed prolonged meristematic activity while escaping the differentiation program. Thus, these meristematic cells produced leaf blades by a developmental program that was specified in the leaf.

BOP1 functions in locations other than leaf organs

The mutant phenotypes of the *bop1-1* plants was apparent in organs other than leaves, such as stems and floral organs. Ectopic outgrowths were observed in the proximal parts of petals and ectopic filamentous organs developed at the base of the sepals. Ectopic outgrowths were also observed on the surface of the stem. The morphology of these ectopic outgrowths did not resemble that of any wild-type organ, and they appeared to be simply unidentifiable overgrowths of cells. Thus, *BOP1* also functions in the developmental control of organs other than leaves. The defects in these other organs are probably due to the ectopic meristematic activities of the mutant cells, as is the case in leaves.

Interestingly, the number of floral organs in mature flowers was different in *bop1-1* plants than in wild-type plants. The morphological defects in *bop1-1* mutant flowers mainly appeared in the organs of the first and second whorl. Therefore, *BOP1* may have a role in the development of floral organs in addition to controlling the meristematic activities of lateral organs and stems.

A possible mechanism for *BOP1* function

Our results suggest that *BOP1* is crucial for cellular differentiation by controlling meristematic activity in various organs and during certain developmental stages. How does *BOP1* control this process?

The *bop1-1* mutation led to a wide range of defects that were reminiscent of transgenic *Arabidopsis* lines that overexpress class I *knox* genes (Lincoln et al., 1994; Chuck et al., 1996; Pautot et al., 2001). Expression of the three class I *knox* genes, *KNAT1*, *KNAT2* and *KNAT6*, was increased in the cotyledons and rosette leaves of the mutant, in accordance with the phenotype. Thus, *BOP1* is involved in controlling the expression of class I *knox* genes in these organs. Plants with defective expression of class I *knox* genes exhibit developmental abnormalities along the proximodistal axis, such as displacement of the proximal features of leaves (stipule, sheath and petiole) to more distal tissues (blades) (Becraft and Freeling, 1994; Jackson et al., 1994; Lincoln et al., 1994; Schneeberger et al., 1995; Chuck et al., 1996; Tamaoki et al., 1997; Ori et al., 2000). These features are similar to those of the *bop1-1* mutant. In fact it would seem that the distal lamina of the leaf is now displaced to more proximal regions. This leads us to suggest that the ectopic outgrowths in *bop1-1* mutants are attributable to the failure of *BOP1* to perform spatial and temporal regulation of class I *knox* genes. This proposition is supported by the synergistic genetic interaction of the *bop1-1* mutation with the mutations in other *knox* gene regulators, *AS1* and *AS2*.

The *bop1* mutation was found to be recessive in terms of the ectopic outgrowth phenotype. Thus, the mutant phenotype is probably the result of a functionally defective wild-type protein. In this regard, *BOP1* may negatively regulate the expression of class I *knox* genes and the resulting meristematic activity of leaf cells. However, molecular analysis of the allele is needed to confirm this proposition.

Although *BP* is misexpressed in the *bop1-1* mutant, the analysis of the *bop1-1 bp-1* double mutant showed a rather simple additive genetic interaction. This suggests that misexpression of *BP* may be neither responsible nor sufficient to induce the *bop1-1* phenotypes. One possible explanation would be that the lobed-leaf phenotypes of the *bop1-1* mutant are caused by misexpression of class I *knox* genes other than *BP* or by combined misexpression of three class I *knox* genes, *KNAT1*, *KNAT2* and *KNAT6*, as has been suggested for the *as1* and *as2* mutatns (Byrne et al., 2002). In this regard, it is notable

that the *bop1-1 stm-1* double mutant shows some degree of synergistic mutant phenotype.

The morphology of the leaf organs and the misregulation of class I *knox* genes in the *as1* and *as2* mutants were similar to those found in the *bop1-1* mutant. This indicates that these genes may function in related pathways that control leaf morphogenesis and regulate class I *knox* gene expression. Furthermore, the double mutants (*bop1-1 as1-1* and *bop1-1 as2-2*) showed synergistic mutant phenotypes. Although it is difficult to precisely define genetic relationships based on the morphological phenotypes of the double mutants, it is clear that they interact both genetically and functionally to control leaf development. An interesting hypothesis is that both combinations of *BOP1* and *AS1* and/or *BOP1* and *AS2* control the expression of class I *knox* genes in a synergistic manner and thereby control leaf development.

The *bop1* mutants had some leaf phenotypes that were not observed in the *as1* or *as2* mutants. Unlike the case of *bop1*, no lobed structures were observed on the cotyledons or the first two leaves of *as1* and *as2* mutant plants at early stages of development (Tsukaya and Uchimiya, 1997; Ori et al., 2000; Semiarti et al., 2001). The occurrence of petiole-like outgrowths at the base of cotyledons and the first two leaves was also not observed in the *as1* and *as2* mutants. In addition, *as1 se* and *as2 se* double mutants had phenotypes that were very similar to those of *KNAT1* overexpressers (Ori et al., 2000). In contrast, the *bop1* mutation did not appear to interact with the *se* mutation (data not shown). Thus, *BOP1* may have functions that are distinct from those of *AS1* and *AS2* as well as the functions that are shared among them in controlling leaf development.

The known regulators of class I *knox* genes in higher plants include the *RS2* of *Zea mays*, the *AS1* gene of *Arabidopsis* and *PHAN* gene of *Antirrhinum majus* (Schneeberger et al., 1998; Byrne et al., 2000; Ori et al., 2000; Semiarti et al., 2001; Waites et al., 1998; Timmermans et al., 1999; Tsiantis et al., 1999; Byrne et al., 2000). The lesion in the *rs2* and *as1* mutants is characterized by alterations in their proximodistal identity (Schneeberger et al., 1995; Timmermans et al., 1999; Tsiantis et al., 1999; Ori et al., 2000). The *as1* mutants also exhibit some defects in the dorsoventral identity (Ori et al., 2000). The *phan* mutation can be explained by defects in proximodistal and dorsoventral character (Waites and Hudson, 1995; Waites et al., 1998). In addition to these genes, *KANADY* (*KAN*) also plays a major role in the promotion of abaxial cell fates (Kerstetter et al., 2001; Eshed et al., 2001). In *kan1 kan2* double mutants, ectopic outgrowths develop on their abaxial side. Although *bop1-1* mutants developed some ectopic outgrowths in the abaxial side of the leaf, mostly in the proximal region, we suggest that the effect of the *bop1-1* mutation on dosoventrality is minimal, if any. This is supported by the normal cellular and vascular arrangement and by trichome formation only on the adaxial side in ectopic young leaves.

The cotyledons of the *bop1-1* embryos did not exhibit any distinct morphological defects prior to germination. Instead, the mutant cotyledons produced ectopic blades on their petioles only a few days after germination, at which stage cell expansion and differentiation were taking place in the wild-type petioles (Tsukaya et al., 1994). In addition, a clear difference in cellular morphology, i.e., the appearance of meristematic clusters of cells at the bases of cotyledonary petioles, was observed in 3-day-old seedlings of *bop1-1*. Although we cannot completely rule out the possibility that the gene functions at an earlier stage, it appears that the major effect of *BOP1* is on post-embryonic development.

The data presented here strongly suggest that *BOP1* is a key component in the differentiation of cellular states and in the specification of spatially and temporally regulated developmental patterns in plant leaves. Molecular analyses of the nature and function of the gene product should provide further insights into this important phenomenon.

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REFERENCES

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

Becraft, P. W. and Freeling, M. (1994). Genetic analysis of *Rough sheath1* developmental mutants of maize. *Genetics* **136**, 295-311.

Byrne, M. E., Barley, R., Curtis, M., Arroyo, J. M., Dunham, M., Hudson, A and Martienssen, R. A. (2000). *Asymmetric leaves* 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.

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

Dockx, J., Quaedvlieg, N., Keultjes, G., Kock, P., Weisbeek, P. and Smeekens, S. (1995). The homeobox gene *ATK1* of *Arabidopsis thaliana* is expressed in the shoot apex of the seedling and in flowers and inflorescence stems of mature plants. *Plant Mol. Biol.* **28**, 723-737.

Donnelly, P. M., Bonetta, D., Tsukaya, H., Dengler, R. E. and Dengler, N. G. (1999). Cell cycling and cell enlargement in developing leaves of *Arabidopsis*. *Dev. Biol*. **215**, 407-419.

Douglas, S. J., Chuck, G., Denger, R. E., Pelecanda, L. and Riggs, C. D. (2002). *KNAT1* and *ERECTA* regulate inflorescence architecture in Arabidopsis. *Plant Cell* **14**, 1-13.

Endrizzi, K., Moussian, B., Haecker, A., Levin, J. Z. and Laux, T. (1996). The *SHOOT MERISTEMLESS* gene is required for maintenance of undifferentiated cells in *Arabidopsis* shoot and floral meristems and acts at a different regulatory level than the meristem genes *WUSCHEL* and *ZWILLE*. *Plant J.* **10**, 101-113.

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

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

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

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 leaf lamina, encodes a member of a novel family of proteins characterized by cysteine repeats and a leucine zipper. *Plant Cell Physiol*. **43**, 467-478.

Jackson, D., Veit, B. and Hake, S. (1994). Expression of maize *KNOTTED1* related homeobox genes in the shoot apical meristem predicts patterns of morphogenesis in the vegetative shoot. *Development* **120**, 405-413.

Jun, J. H., Ha, C. M. and Nam, H. G. (2002). Involvement of the *VEP1* Gene in vascular strand development in *Arabidopsis thaliana*. *Plant Cell Physiol*. **43**, 323-330.

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

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

Kim, G.-T., Tsukaya, H. and Uchimiya, H. (1998). The *ROTUNDIFOLIA3* gene of *Arabidopsis thaliana* encodes a new member of the cytochrome P450 family that is required for the regulated polar elongation of leaf cells. *Genes Dev.* **12**, 2381-2391.

Konieczny, A. and Ausubel, F. M. (1993). A procedure for mapping *Arabidopsis* mutations using codominant ecotype-specific PCR-based markers. *Plant J.* **4**, 403-410.

Koornneef, M. and Stam, P. (1992). Genetic Analysis. In *Methods in Arabidopsis Research* (ed. C. Koncz, N.-H. Chua and J. Schell), pp. 85-99. Singapore: World Scientific.

Lincoln, C., Long, J., Yamaguchi, J., Serikawa, K. and Hake, S. (1994). A *Knotted1*-like homeobox gene in *Arabidopsis* is expressed in the vegetative meristem and dramatically alters leaf morphology when overexpressed in transgenic plants. *Plant Cell* **6**, 1859-1876.

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

Matsuoka, M., Ichikawa, H., Saito, A., Tamda, Y., Fujimura, T. and Kano Murakami, Y. (1993). Expression of a rice homeobox gene causes altered morphology of transgenic plants. *Plant Cell* **5**, 1039-1048.

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

Nishimura, A., Tamaoki, M., Sato, Y. and Matsuoka, M. (1999). The expression of tobacco *knotted1*-type class 1 homeobox genes correspond to regions predicted by the cytohistological zonation model. *Plant J.* **18**, 337-347.

Oh, S. A., Lee, S. Y., Chung, I. K., Lee, C. H. and Nam, H. G. (1996). A senescence-associated gene of *Arabidopsis thaliana* is distinctively regulated during natural and artificially induced leaf senescence. *Plant Mol. Biol*. **30**, 739-754.

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

Pautot, V., Dockx, J., Hamant, O., Kronenberger, J., Grandjean, O., Jublot, D. and Traas, J. (2001). KNAT2: evidence for a link between *knotted*-like genes and carpel development. *Plant Cell* **13**, 1719-1734.

Pyke, K. A., Marrison, J. L. and Leech, R. M. (1991). Temporal and spatial development of the cells of the expanding first leaf of *Arabidopsis thaliana* (L.) Heynh. *J. Exp. Bot.* **42**, 1407-1416.

Schneeberger, R. G., Becraft, P. W., Hake, S. and Freeling, M. (1995). Ectopic expression of the *knox* homeo box gene *rough sheath1* alters cell fate in the maize leaf. *Genes Dev*. **9**, 2292-2304.

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.

Sentoku, N., Sato, Y., Kurata, N., Ito, Y., Kitano, H. and Matsuoka, M. (1999). Regional expression of the rice *kn1*-type homeobox gene family during embryo, shoot, and flower development. *Plant Cell* **11**, 1651-1664. in monocot and dicot plants. *Science* **284**, 154-156.

Sentoku, N., Sato, Y. and Matsuoka, M. (2000). Overexpression of rice *OSH* genes induces ectopic shoots on leaf sheaths of transgenic rice plants. *Dev. Biol.* **220**, 358-364.

Sieburth, L. E. (1999). Auxin is required for leaf vein pattern in *Arabidopsis*. *Plant Physiol*. **121**,1179-1190.

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

Smyth, D. R., Bowman, J. L. and Meyerowitz, E. M. (1990). Early flower development in *Arabidopsis. Plant Cell* **2**, 755-767.

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

Tamaoki, M., Kusaba, S., Kano-Murakami, Y. and Matsuoka, M. (1997). Ectopic expression of a tobacco homeobox gene, *NTH15*, dramatically alters leaf morphology and hormone levels in transgenic tobacco. *Plant Cell Physiol.* **38**, 917-927.

Telfer, A., Bollman, K. M. and Poethig, R. S. (1997). Phase change and the regulation of trichome distribution in *Arabidopsis thaliana*. *Development* **124**, 645-654.

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

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

Tsukaya, H., Tsuge, T. and Uchimiya, H. (1994). The cotyledon: a superior system for studies of leaf development. *Planta* **195**, 309-312.

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

Tsukaya, H. (1998). Genetics evidence for polarities that regulate leaf morphogernesis. *J. Plant Res.* **111**, 113-119.

Tsukaya H., Shoda K., Kim G. T. and Uchimiya H. (2000). Heteroblasty in *Arabidopsis thaliana* (L.) Heynh. *Planta* **210**, 536-542.

Tsukaya, H. (2002a). Interpretation of mutants in leaf morphology: genetic evidence for a compensatory system in leaf morphogenesis that provides a new link between Cell and Organismal theory. *Int. Rev. Cytol*. **217**, 1-39.

Tsukaya, H. (2002b). The leaf index: heteroblasty, natural variation, and the genetic control of polar processes of leaf expansion. *Plant Cell Physiol*. **43**, 372-378.

Tsukaya, H. (2002c). *Leaf Development* In *The* Arabidopsis *Book* (ed. C. R. Somerville and E. M. Meyerowitz) American Society of Plant Biologists (http://www.aspb.org/downloads/arabidopsis/tsukayafinal.pdf).

van der Graaff, E., Dulk-Ras, A. D., Hooykaas, P. J. and Keller, B. (2000). Activation tagging of the *LEAFY PETIOLE* gene affects leaf petiole development in *Arabidopsis thaliana*. *Development* **127**, 4971-4980.

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 inforescence 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 *Antirrhinu*m. *Cell* **93**, 779-789.