The *BLADE ON PETIOLE* genes act redundantly to control the growth and development of lateral organs

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Accepted 23 February 2005

Development 132, 2203-2213
Published by The Company of Biologists 2005
doi:10.1242/dev.01815

Summary

Developmental processes in multicellular organisms involve an intricate balance between mechanisms that promote cell division activity and growth, and others that promote cell differentiation. Leaf development in *Arabidopsis thaliana* is controlled by genes like *BLADE ON PETIOLE1* (*BOP1*), which prevent the development of ectopic meristematic activity that leads to the formation of new organs, and *JAGGED* (*JAG*), which control the proximodistal development of the leaf by regulating cell-division activity. We have isolated and characterized the *BOP1* gene together with a functionally redundant close homolog that we name *BOP2*. The *BOP* genes are members of a gene family containing ankyrin repeats and a BTB/POZ domain, suggesting a role in protein-protein interaction. We show that the *BOP* genes are expressed in the proximal parts of plant lateral organs where they repress the transcription not only of class 1 *knox* genes but also of *JAG*. We also show that the *BOP* genes are acting together with the flower meristem identity gene *LEAFY* in the suppression of bract formation. These findings show that the *BOP* genes are important regulators of the growth and development of lateral organs.

Key words: *Arabidopsis*, Leaf development, Flower organ abscission, *BLADE ON PETIOLE1*, *JAGGED*, *knox* genes, *LEAFY*

Introduction

In plants, in contrast to in animals, the vast majority of growth and development takes place after embryogenesis. Furthermore, plant growth and development are dependent not only on internal cues, but also on signals from the environment to a much greater extent than are animal growth and development. This means that a plant can adapt its growth and development in accordance with shifting environmental conditions. All above ground parts of a plant are ultimately derived from the activity of the shoot apical meristem (SAM). Through a reiterative process of organogenesis, the SAM produces primordia on its flanks that develop into lateral organs: first leaves and shoots, and later flowers. In *Arabidopsis thaliana* flowers are not subtended by leaves ('bracts'), so the shift from vegetative to reproductive development involves the simultaneous action of two tightly connected processes: promotion of floral primordium identity and suppression of leaf primordium identity. Both of these processes are controlled by the flower meristem-identity gene *LEAFY* (*LFY*). Instead of developing flowers, a *lfy* mutant plant develops shoot-like structures, or structures that are intermediate between shoots and flowers, subtended by bracts (Huala and Sussex, 1992; Schultz and Haughn, 1991; Weigel et al., 1992). Although we have deep insights into the way *LFY* induces flower meristem-identity, the mechanism whereby *LFY* suppresses the leaf development program is not known.

The identity of the Arabidopsis SAM is controlled by class I *knox* genes including *BREVIPEDICELLUS* (*BP*), *KNOTTED*-like from *Arabidopsis thaliana2* (*KNAT2*), *KNAT6* and *SHOOTMERISTEMLESS* (*STM*) (Reiser et al., 2000). In order to promote normal leaf development, the expression of these genes needs to be tightly suppressed in the incipient leaf primordium and in the developing leaf. This suppression is partly attributable to the action of genes like *ASYMMETRIC LEAVES1* (*AS1*), *AS2* and *BLADE ON PETIOLE1* (*BOP1*) (Byrne et al., 2000; Ha et al., 2004; Ha et al., 2003; Ori et al., 2000; Semiarti et al., 2001).

Loss-of-function mutations in *AS1* and *AS2* lead to ectopic *knox*-gene expression in the leaf, which is associated with the formation of lobed rosette leaves with ectopic leaf-like organs on their petioles (Byrne et al., 2000; Ori et al., 2000; Semiarti et al., 2001). This phenotype is also seen, although much weaker, in a *bop1* null mutant (Ha et al., 2004). BOP1 has recently been shown to belong to a family of proteins containing BTB/POZ domains and ankyrin repeats that have not previously been associated with the regulation of plant development (Ha et al., 2004). Ectopic leaf formation is also caused by strong constitutive expression of *BP* (previously *KNAT1*) from the Cauliflower Mosaic Virus *35S* promoter (Chuck et al., 1996; Lincoln et al., 1994), suggesting that the *as1*, *as2* and *bop1* mutant phenotypes are caused, at least partly, by the ectopic *knox*-gene expression. However, the originally described *bop1-1* mutant also displays another leaf development phenotype that is not seen in *as1*, *as2* or *bop1* null mutants. It develops extensive growth of the proximal parts of the leaf lamina, leading to enlarged leaves without petioles (Ha

et al., 2003). It has been suggested that the strong *bop1-1* mutant phenotype is caused by a dominant-negative interaction between the mutant allele and the wild-type allele, which may interfere with the normal function of other proteins in the leaf morphogenesis pathway (Ha et al., 2004). It has also been suggested, but not shown, that the very weak phenotype of the *bop1* null mutant may be attributed to functional redundancy with a similar gene (Ha et al., 2004).

Recently, several genes have been identified that control the balance between cell division and cell differentiation in the proximal versus distal parts of the leaf. The *JAGGED* (*JAG*) gene encodes a transcription factor with a C_2H_2 zinc finger domain (Dinneny et al., 2004; Ohno et al., 2004). In *jag* mutants, the growth of distal parts of leaves, sepals, petals and stamens is suppressed, leading to these organs being smaller than wild type, with serrated margins (Dinneny et al., 2004; Ohno et al., 2004). *JAG* is expressed in the distal parts of leaves and petals, and appears to have a role in the maintenance of cell-division activity. *JAG* expression is necessary for the development of bracts in *lfy* mutants, as well as for the development of bract-like organs in *ap1* and *ap2* mutant backgrounds (Dinneny et al., 2004; Ohno et al., 2004). Interestingly, ectopic *JAG* expression in a wild-type background leads to the production of bracts and to ectopic growth of the proximal parts of the leaf, a phenotype that is very similar to that of *bop1-1* mutants, suggesting that these genes may interact functionally (Dinneny et al., 2004; Ha et al., 2003; Ohno et al., 2004).

We have cloned and characterized the *Arabidopsis BOP1* gene as well as a functionally redundant closely related gene that we call *BOP2*. Through analysis of double mutants, we show that the *BOP* genes have a previously uncharacterized role in the suppression of bract formation and that this suppression is achieved through a strong synergistic interaction with the flower meristem-identity gene *LFY*. We also show that the *BOP* genes are expressed in proximal parts of plant organs in a region that is non-overlapping with that of *JAG* expression, and that *bop1 bop2* mutants display ectopic *JAG* expression in regions corresponding to the regions of wild-type *BOP* expression. Taken together, our data show that BOP1 and BOP2 are important repressors of both *knox* gene and *JAG* expression in the developing leaf, and that the coordination of *LFY*, *BOP* and *JAG* expression is important for the balance between cell-division activity and differentiation sculpting the architecture of the leaf and the development of lateral organs.

Materials and methods

Plant material and growth conditions

All plants were grown on soil mixed with vermaculite (1:1) under long days (16 hours light) or short days (9 hours light) with a temperature of 23°C.

Wild type was Columbia (Col-0). The *bop2-1* and *bop2-2* mutants were identified after screening of the Salk T-DNA insertion lines (Alonso et al., 2003), and seed was obtained from the Nottingham Arabidopsis Stock Center (NASC). The seed stock numbers were N533520 (*bop2-1*) and N575879 (*bop2-2*). The *bop1-5* was identified after screening of the Syngenta SAIL T-DNA insertion lines (Sessions et al., 2002) as line 14.c02. The *bop1-6D* mutation was identified after screening of activation tagged lines transformed with pSK1015 (Weigel et al., 2000). Seeds from *jag-1* and *jag-5D* (Dinneny et al., 2004) were kindly provided by José Dinneny and Detlef Weigel. The *lfy-12* mutant is a null mutant in Col-0.

Cloning of *BOP1* and *BOP2* cDNA

Plasmid rescue of *bop1-6D* genomic DNA was performed as described previously (Weigel et al., 2000). 5′ and 3′ RACE (rapid amplification of cDNA ends) of *BOP1* and *BOP2* cDNA was performed using the SMART RACE cDNA amplification kit (CLONTECH) according to the manufacturer's instructions. Full-length cDNA was generated using gene-specific primers B1-1 and B1-2 for *BOP1*, and B2-1 and B2-2 for *BOP2* (see Table S1 in the supplementary material for oligonucleotide sequences).

Protein alignment and phylogenetic analysis

Protein sequences were aligned using the Clustal W program (Thompson et al., 1994) followed by a phylogenetical analysis using the PAUP* program (version 4.0b10) (Swofford, 2003).

Construction of transgenic lines

The *35S::BOP1* and *35S::BOP2* vectors were constructed by placing the full-length cDNA sequences from *BOP1* and *BOP2* downstream of the Cauliflower Mosaic Virus (CaMV) *35S* promoter in the binary vector pPCV702 (Walden et al., 1990). The *BOP1::GUS* and *BOP2::GUS* vectors were constructed by placing 2 kb of the genomic region 5′ of the *BOP1* and *BOP2* translational start sites upstream of the reporter gene *uidA* (*GUS*) in the binary vector pPCV812 (Walden et al., 1990). The *BOP1* promoter region was amplified using the gene specific primers B1p-1 and B1p-2, whereas the *BOP2* promoter was amplified with B2p-1 and B2p-2. Transgenic *Arabidopsis* lines were generated by the floral dipping method (Clough and Bent, 1998).

Northern blot

RNA was extracted from 9-day-old *bop1-6D* and Col-0 wild-type plants using a Qiagen RNA plant minikit (Qiagen). Total RNA (10 μg) was run on a 0.8% formaldehyde gel and blotted on a Hybond-N+ membrane (Amersham Biosciences) as described (Sambrook et al., 1989). The membrane was probed with [α^{32}P]dATP labelled DNA from a 500 bp fragment from exon 1 of the *BOP1* gene, and washed as described (Church and Gilbert, 1984).

RT-PCR

Total RNA was isolated from 11- and 25-day-old Col-0 wild-type plants using the RNAqueous kit (Ambion). cDNA synthesis was performed using the SuperScript II Reverse Transcriptase (Invitrogen) according to the manufacturer's instructions. The primers used were B1-3 and B1-4 for *BOP1*, and B2-3 and B2-4 for *BOP2*. The primers are flanking the intron of both *BOP1* and *BOP2* in order to selectively amplify the respective cDNA. The PCR program used was 94°C for 3 minutes, then 94°C for 15 seconds, 57°C for 30 seconds and 72°C for 30 seconds for 29-31 cycles (as indicated in Fig. S2 in supplementary material), followed by 72°C for 10 minutes. An *18S* ribosomal RNA fragment was amplified as a control using the QuantumRNA Universal 18S kit according to the manufacturer's instructions (Ambion). *18S* competimers in a ratio of seven to three were added to equalize the expression of the target gene with that of the *18S* control.

Real-time RT-PCR

RNA was extracted from 8- and 11-day-old Col-0 wild-type and *bop1-5 bop2-2* double mutant plants grown in long days. Leaf 1 and 2 were sampled together with the apical part of the shoot carrying leaves smaller than 1 mm. Poly(dT) cDNA synthesis was performed using the iScript cDNA Synthesis Kit (BIO-RAD) according to the manufacturer's instructions. Quantification was performed on an iCycler iQ real-time PCR detection system (BIO-RAD) using the BIO-RAD iQ SYBR Green Supermix. PCR was carried out in 96-well optical reaction plates heated to 95°C for 3 minutes, followed by 45

cycles of 10 seconds at 95°C and 30 seconds at 54°C, followed by a melting curve analysis from 54°C to 95°C with 0.5°C per step to verify that quantification was not caused by primer self-amplification but by a pure and common PCR product. For each quantification conditions were, 1>E>0.95 and r^2>0.98, where E is the PCR efficiency and r^2 corresponds to the correlation coefficient obtained with the standard curve. Three replicate assays were preformed with independently isolated RNA and each sample was loaded in triplicates. Results were normalized to the expression of *18S* ribosomal RNA, then to the value of the wild-type control. The primers used to detect *JAG* were J-1 and J-2, whereas *JGL* was detected using JG-1 and JG-2.

In situ hybridization

In situ hybridization was performed on 10 μm thick sections as described previously (Jackson, 1992). Templates for the digoxigenin-labelled RNA probes were generated by amplifying gene-specific sequences using the primers B2-5 and B2-6 for the *BOP2* probe, and J-3 and J-4 for the *JAG* probe. The *BOP2* probe spans the end of exon 2, which is divergent between *BOP1* and *BOP2*. The products were ligated into the vector pGEM-T easy (Promega), linearized using *Nco*I (*BOP2*) and *Hin*dIII (*JAG*); ligation was followed by in vitro transcription using SP6 (*BOP2*) and T7 (*JAG*) polymerase to generate antisense probes. As a control, in situ hybridization using the *BOP2* probe on sections from the *bop2-2* mutant and the *JAG* probe on sections from the *jag-1* mutant was performed. None of these hybridizations resulted in a detectable signal (results not shown).

Analysis of GUS activity

For analysis of GUS activity in *BOP1::GUS* and *BOP2::GUS* plants, plants were harvested and tissue samples were subjected to histochemical staining of the *GUS* activity as described (Weigel and Glazebrook, 2002). Samples for histological analysis were fixed in 50% ethanol, 40% LR-WHITE (TAAB Laboratories) and 10% PEG 400 for 20 minutes at room temperature. The samples were transferred to 90% LR-WHITE with 10% PEG 400, put into a capsule and baked overnight at 65°C. Sections (10 μm) were mounted in glycerol before microscopy.

Results

Identification of *BOP1* and *BOP2*

Three T-DNA insertion alleles in and around *BOP1* have been described (Ha et al., 2004). We identified a fourth T-DNA insertion allele, called *bop1-5*, in the ecotype Columbia (Col-0) (Fig. 1A). We also identified two new T-DNA insertion alleles in the paralogous gene At2g41370, which we named *bop2-1* and *bop2-2* (Fig. 1A). Two of the isolated mutant alleles, *bop1-5* and *bop2-2*, resulted in the loss of detectable transcription as determined by RT-PCR (Fig. 1A), suggesting that these alleles are null alleles.

The *BOP1* and *BOP2* genes encode two very similar proteins (81% amino acid identity) containing putative ankyrin repeats (Sedgwick and Smerdon, 1999) and a BTB/POZ

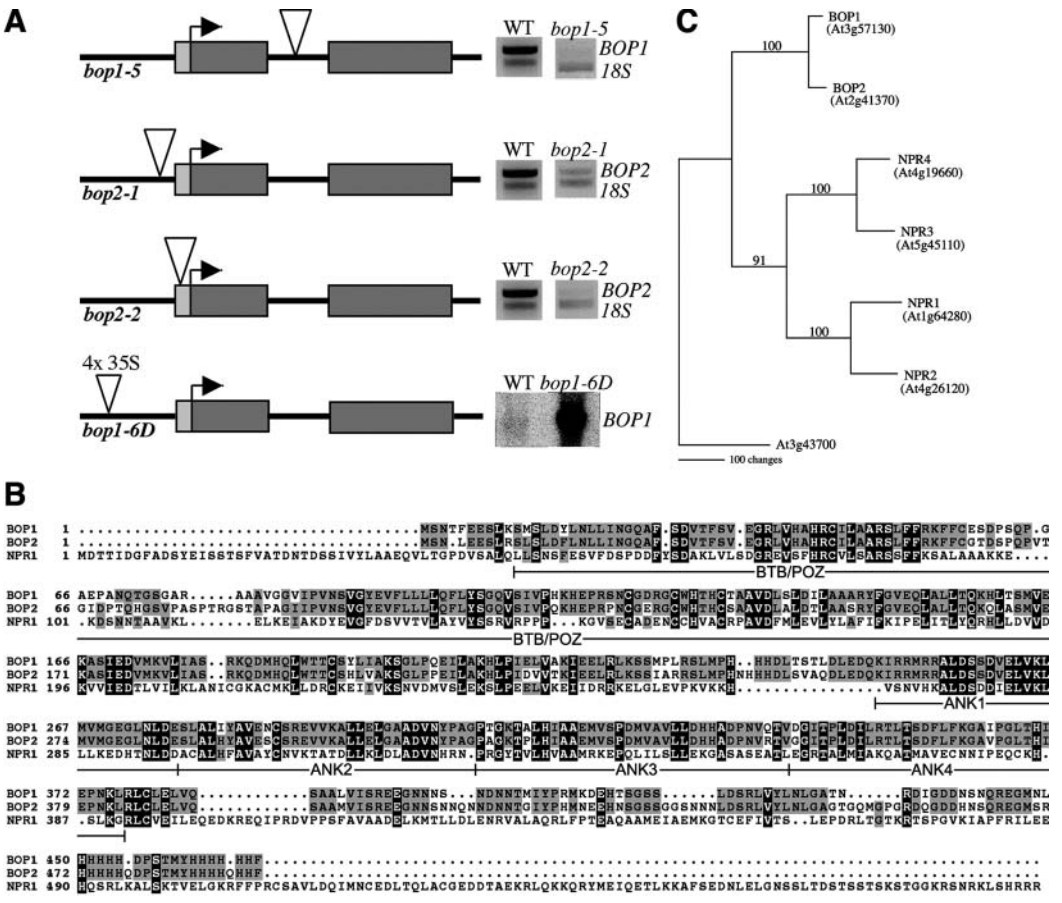

Fig. 1. Molecular characterization of *BOP1* and *BOP2*. (A) Genomic structure (left) and *BOP1* and *BOP2* expression in the various mutant alleles compared with wild type (right). Dark-shaded boxes are exons, light-shaded boxes are 5′ untranslated regions. Black arrows show translational start sites. Triangles mark the T-DNA insertion sites in the various mutant alleles. 4×35S indicates the four 35S enhancers present in the activation-tagging T-DNA. The expression levels of *BOP1* and *BOP2* in the mutants and wild type were quantified by RT-PCR with *18S* ribosomal RNA used as control. The *BOP1* expression in *bop1-6D* was detected with northern blot. (B) Alignment of predicted amino-acid sequences of BOP1, BOP2 and NPR1, with BTB/POZ domains and ankyrin-repeats (ANK) indicated. Identical residues between all three proteins are shaded in black, residues that are identical in at least two sequences are shaded in grey. (C) Phylogenetic interrelationship of all *Arabidopsis* proteins predicted to contain BTB/POZ domains followed by ANK repeats. The At3g43700 gene contains only the BTB/POZ domain and has been used as an outgroup. Bootstrap support values are indicated.

(Broad-Complex, Tramtrack, and Brick-a-brac/Pox virus and Zinc finger) domain (Collins et al., 2001), suggesting that they have a role in protein-protein interaction (Fig. 1B). A homology search and phylogenetic analysis revealed four other proteins containing BTB/POZ-domains followed by ankyrin repeats in the *Arabidopsis* genome (Fig. 1B,C). These proteins include the transcription factor NONEXPRESSOR OF PR GENES1 (NPR1) (Cao et al., 1997; Ryals et al., 1997), which is a regulator of salicylic acid signaling and systemic acquired resistance, and the NPR1-like proteins NPR2-4 (Liu et al., 2005). NPR1 and the NPR1-like proteins group together but are clearly separated from the well-supported monophyletic group containing BOP1 and BOP2 (Fig. 1C).

Defects caused by loss of *BOP1* and *BOP2* function

The *BOP1* and *BOP2* genes display a high level of functional redundancy. The *bop2-2* mutant plants have no discernible mutant phenotypes (not shown), whereas the *bop1-5* loss-of-function mutant plants display a very weak mutant phenotype that can only be detected under growth in short-day conditions. In short days, all *bop1-5* mutants form a few ectopic leaves on the rosette leaves (Fig. 2A), a phenotype that is also seen, but more weakly and in only 2-3% of the plants, in the previously described *bop1-3* and *bop1-4* mutants (Ha et al., 2004). By contrast, the *bop1 bop2* double mutants display severe developmental defects that are very similar to all previously described *bop1-1* mutant phenotypes (Fig. 2). The double mutant combinations *bop1-5 bop2-1* and *bop1-3 bop2-2* both show the same, but slightly weaker, mutant phenotype as the *bop1-5 bop2-2* double mutant (data not shown). The *bop1 bop2* double mutant has a retarded growth compared with wild type (Fig. 2B), but eventually reaches the same overall height. The most dramatic developmental effect is on leaf development, where *bop1 bop2* leaves display extensive lobe formation and ectopic growth of the leaf lamina, producing larger leaves without petioles. This is true for all leaves, but is especially evident for leaves 1 and 2 (Fig. 2C). In wild-type plants these leaves stop growing at a much earlier stage than the other rosette leaves and are consequently much smaller. In *bop1 bop2* plants the proximal parts of the leaves continue growing

Fig. 2. Phenotypes of *bop1* and *bop1 bop2* mutants. (A) Ectopic leaf formed from the petiole of a *bop1-5* mutant plant grown in short days. (B) 24-day-old Col-0 wild-type plant (left) and *bop1-5 bop2-2* double mutant plant (right). (C) Growth and development of leaf 1 and 2 from Col-0 wild type (left) and *bop1-5 bop2-2* (right) from day 7 to day 22 after germination. Scale bar: 1 cm. (D,E) Inflorescences of Col-0 wild type (D) and a *bop1-5 bop2-2* double mutant (E). Arrowheads point to bracts subtending the flowers. (F,G) Flowers of Col-0 wild type (F) and a *bop1-5 bop2-2* double mutant (G). Arrowheads point to stipules growing from the base of the bract. Inset in G is a magnification of the proximal part of the bract. Scale bars: 1 mm. (H) Phenotypes of plants grown in short days. From left to right: Col-0 wild type (2 months old), *bop1-5 bop2-2* double mutant (2 months old) and *bop1-5 bop2-2* double mutant (5 months old). (I-K) Floral organ abscission in wild type (I) and *bop1-5 bop2-2* double mutant (J,K). While wild type abscise their flower organs at an early stage (I), *bop1-5 bop2-2* double mutants never abscise their flower organs (J,K). Scale bars: 1 cm. (K) Dry silique from a *bop1-5 bop2-2* double mutant plant after dehiscence of seeds. The large arrowhead indicates the dry floral organs that are still attached. The small arrowhead (b) points to a bract that is delayed in senescence compared with the sepals.

Table 1. Shoot architecture of *bop1 bop2*, *lfy* and *lfy bop1 bop2* mutants grown in long days

	Col-0 (*n*=20)	*bop1-5 bop2-2* (*n*=16)	*lfy-12* (*n*=18)	*lfy-12 bop1-5 bop2-2* (*n*=15)
Rosette leaves	10.5±0.2	7.1±0.2	11.9±0.2	7.9±0.2
Second order inflorescence*	2.2±0.1	3.5±0.1	17.7±1.0	17.9±0.6
Flowers without bracts†	31.4±1.6	15.3±0.5	12.2±1.6	0.0±0.0
Flowers with bracts†	0.0±0.0	10.6±0.8	13.4±1.7	15.1±1.1
Bracts without flowers‡	0.0±0.0	0.0±0.0	0.0±0.0	7.8±0.7

Values are mean±s.e.m.
*With cauline leaves.
†Flowers of *lfy-12* and *lfy-12 bop1-5 bop2-2* plants are defined as flower-like structures with no apparent elongation between leaves and floral organs.
‡Only a leaf develops, no development of the axillary meristem.

throughout development and, therefore, reach gigantic proportions compared with leaves 1 and 2 in wild-type plants (Fig. 2C). As previously shown for the *bop1-1* mutant, leaves of the *bop1 bop2* double mutants frequently develop ectopic organs along the petioles and midveins (results not shown).

Ectopic growth of bracts in *bop1 bop2* mutants

Defects also occur in flower development in *bop1 bop2*. In contrast to wild type, mutant flowers are frequently subtended by bract-like organs developing ectopically on the inflorescence (Fig. 2D-G, Table 1). That these leaf-like organs are indeed bracts is confirmed by the fact that they are elongate, flanked by stipules (which are dramatically elongated compared with wild-type counterparts; Fig. 2G), they lack a petiole, their tips are pointed and they are significantly delayed in senescence compared with sepals (Fig. 2G,K). These traits are all typical for bracts (Bowman et al., 1993; Irish and Sussex, 1990). Late-developing bracts are sometimes tipped with stigmatic papillae (not shown). Sectioning through the inflorescence meristem also shows that the flower develops from the axil of the bract (Fig. 6D). The bracts are often displaced to a more distal position on the pedicel (Fig. 2E), this could be interpreted as an ectopic growth of the proximal part of the pedicel, similar to the ectopic growth of proximal petioles described for the *bop1-1* mutant and also seen in *bop1 bop2* (Fig. 2C) (Ha et al., 2003). This interpretation is supported by the fact that the *BOP* genes are specifically expressed at the base of the pedicels (Fig. 4I).

The *BOP* genes affect flower development

The mutant flowers have an open structure, in which the abaxial sepals are missing, irrespective of whether the flowers are subtended by bracts or not (Fig. 2G, Fig. S1 in supplementary material). Three sepals and four petals grow tightly together on the adaxial side of the flower, while organs with a mixed sepal/petal identity flank the gap created by the missing sepal (Table 2, Fig. S1 in supplementary material). One explanation for this phenotype is that the development of the bract has interfered with the normal development of the flower and that the *BOP*-genes might have a role in controlling the distribution of meristem cells between the bract and the flower primordium. The hypothesis that the *BOP* genes have a role in controlling floral initiation is further supported by the fact that flowering is delayed in *bop1 bop2* mutants grown under short-day conditions, with 56.7±5.1 days to the first visible floral bud for Col-0 wild type (mean±s.e.m., *n*=14) compared with 121.8±7.7 days for *bop1 bop2* (mean±s.e.m., *n*=14; Fig. 2H). However, this delay is to a large extent caused by a slower leaf initiation rate in *bop1 bop2* compared with wild type, with *bop1 bop2* forming only 0.78±0.05 leaves per day (mean±s.e.m., *n*=14), whereas the wild type forms 1.67±0.04 leaves per day (mean±s.e.m., *n*=14). Compared with wild type, *bop1 bop2* mutants form around 15 more leaves until flowering, a number which is comparable to that of *lfy* mutants grown in short days (see Table S2 in supplementary material). Under these conditions, plant senescence is also dramatically delayed and *bop1 bop2* mutants can continue to grow for at least 10 months before senescing, compared with 4 months for wild-type plants in our growth conditions (results not shown). Also, *bop1 bop2* mutants never abscise their flower organs (Fig. 2I-K), showing that *BOP*-gene expression is necessary for this process.

Ectopic *BOP1* and *BOP2* expression has severe developmental effects

We first identified the *BOP1* gene when the *bop1-6D* mutant, which contains a single activation-tagging T-DNA 1 kb upstream of *BOP1* was characterized (Fig. 1A). *bop1-6D* expressed *BOP1* much more strongly than wild type did (Fig. 1A). The *bop1-6D* mutant displays a range of mutant traits that are seen in all plants, including severely stunted growth (Fig. 3A) and the development of multiple leaves and flowers from the same node (Fig. 3B), indicating that ectopic *BOP1* expression has strong effects on meristem function. Interestingly, many aspects of the *bop1-6D* phenotype resemble the phenotype of double mutants with mutations in the class I *knox* gene *BP* and the *BELL1*-like homeodomain gene *BELLRINGER* (*BLR*) [also known as *PENNYWISE*

Table 2. Flower architecture of *bop1 bop2* mutants

	Sepals	Sepal/petals*	Petals	Stamens	Open flowers†	Bracts
Col-0 (*n*=64)	4.0±0	0.0±0.0	4.0±0	5.9±0.1	0.0±0.0	0.0±0.0
bop1-5 bop2-2 (*n*=379)	3.3±0.1	1.4±0.1	3.8±0.1	5.8±0.1	83%	38%

Values are mean±s.e.m.
*First whorl organ with mixed sepal-petal identity.
†Flowers with abaxial gap.

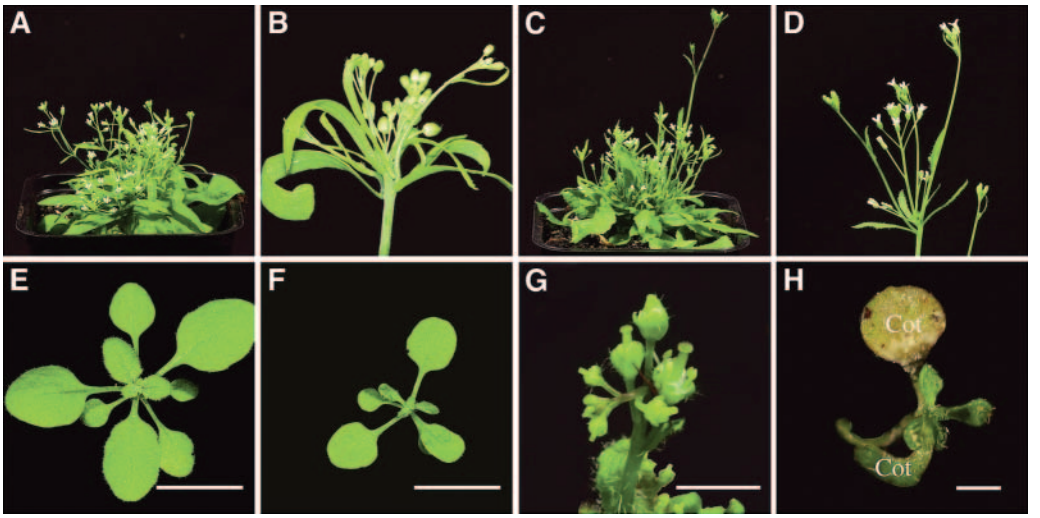

Fig. 3. Phenotypes of *bop1-6D*, *35S::BOP1* and *35S::BOP2* plants. (A) *bop1-6D* mutant plant. (B) *bop1-6D* inflorescence. (C) *35S::BOP2* plant (D) *35S::BOP2* inflorescence. Note the clusters of paraclades and flowers formed from the same node and the similarity between the *bop1-6D* and *35S::BOP2* phenotypes. (E) 16-day-old wild-type plant. Scale bar: 1 cm. (F) 16-day-old *35S::BOP1* plant. Note the reduction in leaf size. Scale bar: 1 cm. (G) *35S::BOP1* inflorescence carrying very small flowers with reduced organ size. Scale bar: 2 mm. (H) Extreme phenotype of a 30-day-old *35S::BOP2* plant. Cot, cotyledon. Scale bar: 1 mm.

(*PNY*)] (Byrne et al., 2003; Smith and Hake, 2003). *bp blr/pny* double mutants are bushy and form clusters of paraclades and flowers from the same node, phenotypes also seen in *bop1-6D* (Fig. 3B) (Byrne et al., 2003; Smith and Hake, 2003). This suggests that ectopic *BOP1* expression may suppress *knox* gene activity.

We also analyzed the effects of *BOP* gene overexpression by making constructs where the expression of the *BOP1* and *BOP2* cDNAs are under the control of the Cauliflower Mosaic Virus *35S* promoter. Transgenic plants expressing *35S::BOP1* or *35S::BOP2* displayed very similar phenotypes that included many of the mutant phenotypes seen in the *bop1-6D* mutant (Fig. 3A-D), seven out of 25 transgenic *35S::BOP1* lines (20%) and seven out of 33 transgenic *35S::BOP2* lines (21%) completely recapitulated the *bop1-6D* mutant phenotypes (Fig. 3C,D). A weaker phenotype, where plants initiated leaves and flowers in a normal fashion but the leaves were reduced in size (Fig. 3E,F), could be seen in 10 out of 25 transgenic *35S::BOP1* lines (40%) and in 19 out of 33 transgenic *35S::BOP2* lines (58%). A few transgenic lines of both *35S::BOP1* and *35S::BOP2* displayed an even more pronounced effect on the growth of shoots, leaves and flowers, leading to the development of severely dwarfed plants with very small leaves and flowers (Fig. 3G,H), and with a reduced meristem size (results not shown). These findings confirm that *BOP1* and *BOP2* are functionally equivalent and that *BOP* expression can repress the growth of leaves and flowers as well as affecting the function of the shoot and flower meristems.

Expression patterns of *BOP1* and *BOP2*

BOP1 and *BOP2* transcripts were detected in all plant organs tested, although at various levels (see Fig. S2 in supplementary material). *BOP1* is not as strongly expressed as *BOP2*, but can be detected in all the tissues displaying *BOP2* expression. This is not unexpected given that the two genes display almost complete functional redundancy.

A more detailed picture of *BOP1* and *BOP2* expression was obtained by in situ localization of *BOP1* and *BOP2* mRNA. *BOP1* has previously been shown to be expressed in leaf and flower primordia, and at the base of developing leaves, sepals and petals (Ha et al., 2004). In our analysis *BOP1* and *BOP2* displayed very similar expression patterns (*BOP1* expression is not shown here), but the *BOP2* signal was always stronger. In vegetative shoot apical meristems weak expression of *BOP2* can be detected in incipient leaf primordia (Fig. 4A). Later in development, *BOP2* expression is restricted to the base of the developing leaf (Fig. 4A). In inflorescence meristems, *BOP2* is expressed at stronger levels at the sites of the incipient floral primordia (Fig. 4B). The expression then disappears in the young flower primordia. At later stages the expression reappears, but is confined to the proximal parts of the developing floral organs (Fig. 4B).

To further analyze the expression patterns of *BOP1* and *BOP2*, transgenic plants expressing promoter fusions to the reporter gene β-glucuronidase (GUS; *uidA*) were analyzed. GUS expression corresponded well with the patterns found in the in situ localization analysis, showing that the *BOP* genes are expressed in the proximal margins of young developing leaves and along the midveins (Fig. 4C-E). At later stages, *BOP* expression is confined to the base of the petioles (Fig. 4F,G) and the proximal parts of the floral organs (Fig. 4H). At even later stages strong *BOP* expression can be seen at the base of the floral organs in an area overlapping the floral organ abscission zone (Fig. 4I). There is also expression at the base of the pedicels (Fig. 4I).

BOP1 and *BOP2* regulate the expression of *JAGGED* and *JAGGED-LIKE*

It has previously been shown that expression of the putative transcription factor *JAG* is necessary for the proper development of distal parts of leaves and petals, as well as for bract formation in the *lfy, ap1* and *ap2* mutants (Dinneny et al.,

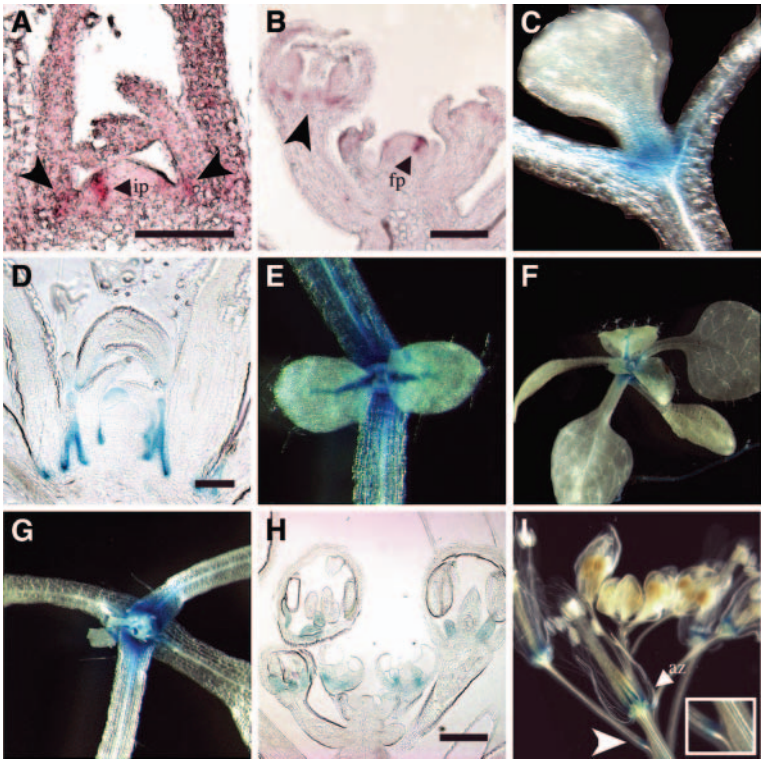

Fig. 4. Expression of *BOP1* and *BOP2*. (A,B) *BOP2* expression in Col-0 wild type as determined by in situ hybridization. (A) Vegetative shoot apex from an 11-day-old plant. *BOP2* is expressed in the incipient leaf primordia (ip) and in the proximal regions of emerging leaves (arrowheads). Scale bar: 100 µm. (B) *BOP2* expression in the inflorescence apex. Expression can be seen in the incipient flower primordium (fp) and in the proximal parts of developing flower organs (arrowhead). Scale bar: 100 µm. (C-I) Expression of *BOP1::GUS*. (C-E) 5-day-old seedling. (C) *BOP1* expression can be found in the proximal parts of leaf 1 and 2 and at the base of the cotyledons. (D) Histological section showing *BOP1* expression in the proximal margins of leaf primordia. (E) *BOP1* is expressed along the midveins of leaf 1 and 2, and along the cotyledon petioles. (F,G) 10-day-old seedling. *BOP1* is expressed at the base of the petioles. In G, young leaves have been removed for clarity. (H) Section of an inflorescence apex showing *BOP1* expression in proximal parts of developing flower organs. Scale bar: 100 µm. (I) Inflorescence showing *BOP1* expression overlapping the flower organ abscission zone (az) and at the base of the pedicels (arrowhead). The base of the indicated pedicel has been magnified (inset) to show the *BOP1* expression.

2004; Ohno et al., 2004). Furthermore, ectopic *JAG* expression is sufficient to induce growth of proximal parts of leaves, and leads to bract formation in the wild type (Dinneny et al., 2004; Ohno et al., 2004). These bracts are tipped with stigmatic papillae in late development stages, and *JAG* has been shown to suppress floral meristem identity (Dinneny et al., 2004). Because all of these phenotypes are also seen in *bop1 bop2* mutants, we decided to investigate how *BOP1* and *BOP2* affect the expression of *JAG*. However, because *JAG* has a close homolog in *Arabidopsis* called *JAGGED-LIKE* (*JGL*) and it has been speculated that *JAG* and *JGL* might be at least partially functionally redundant (Dinneny et al., 2004; Ohno et al., 2004), we also analyzed the expression of *JGL*.

In 11-day-old leaves 1 and 2 of *bop1 bop2* mutants, and in shoot apices, *JAG* and *JGL* expression was dramatically increased when compared with wild type (Fig. 5A,C). As it has been shown previously that ectopic expression of *JAG* is sufficient to promote leaf growth (Dinneny et al., 2004; Ohno et al., 2004), this suggests that the increased growth of leaves 1 and 2 in *bop1 bop2* mutants could at least partially be caused by the increased *JAG* expression, and that BOP1 and BOP2 function as repressors of *JAG* and *JGL* transcription. This hypothesis was further corroborated by analyzing the expression of *JAG* and *JGL* in *bop1-6D* plants. In leaves 1 and 2, as well as in shoot apices, *JAG* and *JGL* expression was decreased in *bop1-6D* when compared with wild type (Fig.

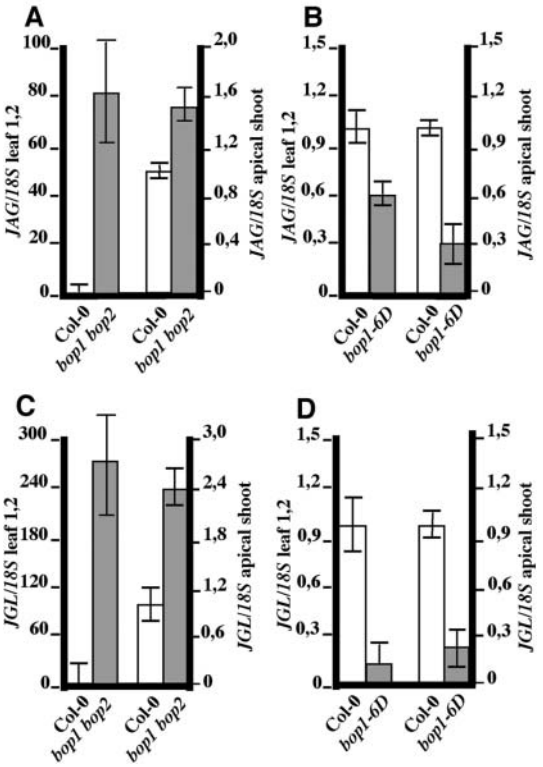

Fig. 5. The *BOP* genes repress the expression of *JAG* and *JGL*. Gene expression was analyzed using quantitative RT-PCR. Results were normalized to the expression of *18S* ribosomal RNA, then to the value of the wild-type control that was arbitrarily set to 1. Bars represent standard deviation of the mean for three separate biological replicates. (A,C) Relative expression levels of *JAG* (A) and *JGL* (C) in leaf 1 and 2 (left) and apical shoots (right) of 11-day-old Col-0 wild-type and *bop1-5 bop2-2* mutant plants. (B,D) Relative expression levels of *JAG* (B) and *JGL* (D), in leaf 1 and 2 (left) and apical shoots (right) of 8-day-old Col-0 wild-type and *bop1-6D* mutant plants.

5B,D). In situ localization of *JAG* mRNA in wild-type and *bop1 bop2* mutant plants showed that *JAG* and *BOP* display non-overlapping expression patterns in the leaves and flowers of wild-type plants (Fig. 6A,B). Whereas *JAG* is expressed in the distal parts (Fig. 6A), the *BOP* genes are expressed in the proximal part of the leaves and flowers (Fig. 6B). Interestingly, the *bop1 bop2* double mutants display ectopic *JAG* expression in the areas of wild-type *BOP* expression (Fig. 6C), confirming that *BOP* expression represses *JAG*, and that the balance between *BOP* and *JAG* expression is an important determinant of leaf architecture. By contrast, *BOP1* and *BOP2* expression was not significantly altered in either the *jag-1* loss-of-function mutant or the *jag-5D* activation-tagged mutant, as determined by RT-PCR (data not shown). Furthermore, although *JAG* is not expressed in wild-type incipient flower primordia, it is strongly expressed in the bract primordia that form in *bop1 bop2* double mutants (Fig. 6D). This suggests that the formation of bracts in *bop1 bop2* mutants is at least partially caused by upregulation of *JAG* expression in the cryptic bract and that the *BOP* genes contribute to suppression of *Arabidopsis* bracts by repressing *JAG*.

To test the importance of *JAG* expression for the *bop1 bop2* mutant phenotype, we analyzed the phenotype of *bop1 bop2 jag* triple mutant plants. As both *JAG* and *JGL* were shown to be upregulated in the *bop1 bop2* mutant and it has been suggested that *JAG* and *JGL* might be functionally redundant (Dinneny et al., 2004; Ohno et al., 2004), we hypothesized that the loss of *JAG* expression might not be sufficient to suppress the *bop1 bop2* mutant phenotype. Indeed, *bop1 bop2 jag* plants display an almost identical phenotype to *bop1 bop2* plants, including bract formation (Fig. 7A). The only difference can be found in the flowers of *bop1 bop2 jag*, which are similar to *jag* mutant flowers in the sense that they develop sepals and petals that are narrower and shorter than in *bop1 bop2*. This shows that *JAG* expression is not necessary for manifestation of the *bop1 bop2* mutant phenotype.

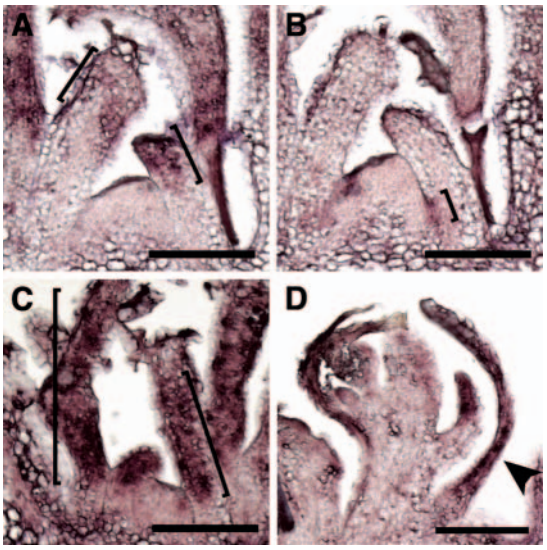

Fig. 6. Ectopic expression of *JAG* in *bop* mutants. *JAG* (A,C,D) and *BOP2 (*B*)* expression, as determined by in situ hybridization. (A-C) Vegetative shoot apex from 11-day-old wild-type (A,B) and *bop1-5 bop2-2* (C) plants. (A) *JAG* is expressed in the distal parts of leaf primordia (brackets). (B) *BOP2* is expressed in the proximal parts of leaf primordia (bracket). (C) In *bop1-5 bop2-2*, *JAG* is ectopically expressed in the whole leaf primordium (brackets). (D) Section through a *bop1-5 bop2-2* flower. *JAG* shows a uniform expression in the bract (arrowhead). Scale bars: 100 μm.

BOP1 and *BOP2* interacts with *LFY* to suppress bract formation

As it has previously been shown that *LFY* and *AP1* suppress outgrowth of bracts through the repression of *JAG* expression (Dinneny et al., 2004; Ohno et al., 2004), and as our data suggest that *BOP1* and *BOP2* are also acting upstream of *JAG*/*JGL*, we decided to test the genetic interaction between the *BOP* genes and *LFY* in the suppression of bract formation. As can be seen from Table 1, *bop1 bop2* mutants are very similar to the *lfy* null mutant *lfy-12*, in the sense that they produce approximately the same number of flowers or flower-like structures that are subtended by bracts. These bracts are in general relatively small in size (Fig. 2E, Fig. 7B), although *bop1 bop2* occasionally develop larger bracts (Fig. 2G). Surprisingly, all flower-like structures of *bop1 bop2 lfy* triple mutant plants are subtended by well-developed bracts (Table 1, Fig. 7C), which are all much larger than the average *bop1 bop2* or *lfy* bracts (Fig. 7D). Furthermore, *bop1 bop2 lfy* mutant plants display a new mutant phenotype not seen in either parent; after formation of the flower-like structures subtended by bracts, the inflorescence meristems form leaves with no apparent development of the associated axillary meristem (Table 1). We interpret this as the formation of bracts where the associated ‘flower’ meristem fails to develop. This shows that there is a strong synergistic interaction between the *BOP* genes and *LFY* in the suppression of bract formation, and that late in development this interaction is important for the development of the axillary meristem.

Discussion

It has been proposed that the shape of organs and the architecture of shoots in plants may be, in part, the result of

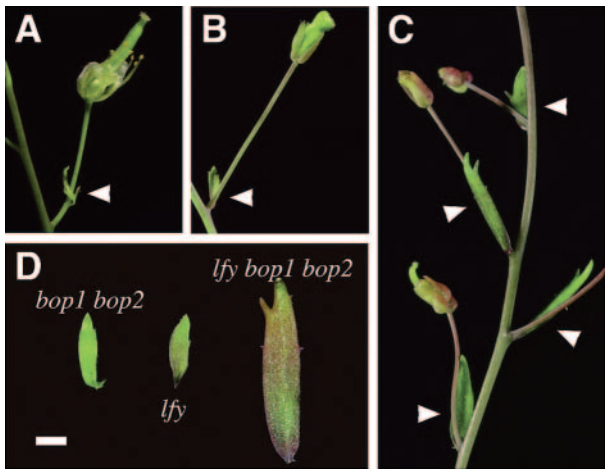

Fig. 7. The *BOP* genes and *LFY* cooperate in the suppression of bract formation. (A) *bop1-5 bop2-2 jag-1* flower with bract (arrowhead). (B) *lfy-12* flower-like structure with bract (arrowhead). (C) *bop1-5 bop2-2 lfy-12* flower-like structures with bracts (arrowheads). (D) Comparison of typical bracts from, from left to right: *bop1-5 bop2-2*, *lfy-12* and *bop1-5 bop2-2 lfy-12* mutants. Scale bar: 1 mm.

controlled differentiation of tissues (Dinneny et al., 2004). According to this view, the opposing effects of genes like *CINCINNATA* (*CIN*), which promotes cell-cycle arrest during the development of leaf blades in *Anthirrinum* (Nath et al., 2003), and *JAG*, which suppresses cell-cycle arrest (Dinneny et al., 2004; Ohno et al., 2004), help sculpt the development of plant organs. However, although the activity of genes like *CIN* and *JAG* might contribute to the control of cell division activity, it is equally important to prevent the formation of new ectopic meristem activity on the leaf. For this, genes like *AS1* and *AS2* have already been shown to be important, as they suppress the expression of the class I *knox* genes in the leaf, which would otherwise have the capacity to induce ectopic meristematic activity, leading to lobed leaves and growth of organs from petioles (Byrne et al., 2000; Ori et al., 2000; Semiarti et al., 2001). Our results, together with recently published data (Ha et al., 2003; Ha et al., 2004), suggest that the genes *BOP1* and *BOP2* are involved in controlling both of these processes by repressing not only class I *knox* genes but also *JAG* and *JGL*. We propose that aspects of the *bop1 bop2* mutant phenotype are caused by a combination of ectopic *knox*-gene and *JAG/JGL* expression. The ectopic *knox*-gene expression could be the cause of ectopic organ formation, while ectopic *JAG/JGL* expression could contribute to the ectopic growth of the leaf lamina and bract formation. These findings show that the *BOP* genes have important roles in the network of genes controlling leaf initiation and growth.

The regulation of flower organ abscission

Here we show that the *bop1-5 bop2-2* mutants display additional mutant traits to those previously described for the *bop1-1* mutant (Ha et al., 2003). These include the suppressed abscission of flower organs (Fig. 2I-K), bract formation (Fig. 2D-G, Table 1) and delayed flower initiation under short-day conditions (Fig. 2H). The flower organ abscission phenotype correlates to strong expression of the *BOP* genes in the presumed flower organ abscission zone (Fig. 4I). Interestingly, this expression overlaps with that of the *INFLORESCENCE DEFICIENT IN ABSCISSION* (*IDA*) gene, and *ida* mutants, just like *bop1 bop2*, never shed their flower organs (Butenko et al., 2003). As ectopic expression of neither *JAG* nor *knox* genes has been reported to cause suppression of floral organ abscission, this phenotype might reflect a *BOP*-specific function. IDA belongs to a family of small proteins encoding putative receptor ligands. It will be interesting to determine whether the BOP proteins and IDA physically interact.

Suppression of bract formation

Our data reveals that there is a strong functional cooperation between the *BOP* genes and *LFY* in the suppression of *Arabidopsis* bract formation. The *BOP* genes are already expressed at weak levels in the incipient leaf primordia and at considerably higher levels in the incipient floral/bract primordia (Fig. 4A,B), although at this point we cannot determine whether *BOP* expression is specifically localized to the cryptic bract. The expression pattern is consistent with a role in bract suppression and is very similar to that of *LFY* (Blazquez et al., 1997). The cooperation between the *BOP* genes and *LFY* also provides an explanation to the floral initiation defect seen in *bop1 bop2* mutants grown under short-day conditions. Floral initiation in *Arabidopsis* requires the simultaneous action of two tightly connected developmental processes: suppression of leaf development and activation of flower development. We show here that *bop1-5 bop2-2* mutants are late flowering under short-day conditions and form more leaves than wild-type plants before the first flower is initiated (Fig. 2H, Table S2 in supplementary material). Under short-day conditions *LFY* expression is lower than under long-day conditions (Blazquez et al., 1998). It is possible that, in a *bop1 bop2* mutant grown under short days, the ability of *LFY* to suppress the leaf development program is severely reduced. The development of the leaf could affect the ability of *LFY* to promote the development of the floral primordium, leading to the production of more leaves. Later in development, *LFY* might be able to induce floral meristem identity, although the associated leaf still develops into a bract. That the *LFY-BOP* cooperation is important also for the development of the floral meristem can be deduced from the fact that the inflorescences of *bop1 bop2 lfy* mutants, in contrast to *lfy-12* or *bop1 bop2* mutants, revert to forming leaves with no growth of axillary meristems after forming flower-like structures (Table 1). In this context it is also interesting to note that ectopic expression of *JAG* can suppress flower meristem identity and cause a *lfy* mutant-like phenotype (Dinneny et al., 2004; Ohno et al., 2004) very similar to that of *bop1 bop2* in short days.

The *BOP* genes and *JAG/JGL*

We show here that BOP1 and BOP2 are repressors of *JAG* and *JGL* transcription, as in *bop1 bop2* mutants *JAG* and *JGL* both display strong ectopic expression. Because many aspects of the *bop1 bop2* mutant phenotype are very similar to the phenotype of *35S::JAG* overexpressors, including bract formation, enhanced outgrowth of stipules, ectopic leaf lamina formation and suppression of flower meristem identity (see above) (see also Dinneny et al., 2004; Ohno et al., 2004), it seemed likely that these aspects of the *bop1 bop2* phenotype could be explained by the ectopic expression of *JAG*. It has also been shown that *JAG* expression is necessary for the outgrowth of bracts in *lfy* and *ap1* mutants (Dinneny et al., 2004; Ohno et al., 2004). However, we show here that in a *bop1 bop2 jag* triple mutant, *JAG* expression is no longer necessary for the outgrowth of bracts, and there is no suppression of the *bop1 bop2* mutant phenotype. Obviously, the need for *JAG* expression in the developing bract has been replaced by another factor regulated by the *BOP* genes. We propose that the simplest explanation to this result is the fact that the *BOP* genes repress the expression of both *JAG* and the very similar gene *JGL* (Fig. 5), and that *JGL* can functionally replace *JAG* when overexpressed. This hypothesis could be tested in a *bop1 bop2 jag jgl* quadruple mutant, but that analysis will have to await the characterization of a *jgl* mutant.

Molecular function of the *BOP* genes

The BOP proteins are predicted to contain BTB/POZ domains and ankyrin repeats, suggesting a role in protein-protein interaction (Fig. 1). The BTB/POZ domain is thought to provide a scaffold for the organization of higher-order structures, such as the cytoskeleton, chromatin, and ubiquitin ligase substrate complexes (Ahmad et al., 1998; Geyer et al., 2003; Kobayashi et al., 2000). Interestingly, the BTB/POZ domain-containing gene *PLZF* has been shown to mediate

transcriptional repression by recruiting histone deacetylase complexes (Lin et al., 1998), providing an interesting parallel to the transcriptional repression activity of the *BOP* genes. Furthermore, the BTB/POZ domain has been shown to interact with elements of the basal transcriptional machinery suggesting that this domain can perform many different functions in transcriptional complexes (Pointud et al., 2001). The only previously characterized proteins containing both BTB/POZ domains and ankyrin repeats are the transcription factors NPR1 and NPR4, which have been shown to interact differentially with members of the TGA family of basic-domain/Leucine zipper (bZIP) transcription factors (reviewed by Dong, 2004; Liu et al., 2005). Although most of the TGA family members have been implicated in the regulation of glutathione *S*-transferase and pathogenesis-related (PR) genes (reviewed by Dong, 2004), one TGA factor gene, *PERIANTHIA* (*PAN*), has been shown to be involved in the restriction of organ initiation from the flower meristem (Chuang et al., 1999). The PAN protein is localized in both floral and vegetative tissues, and it has been suggested that PAN exerts its action through interaction with spatially and/or quantitatively regulated factors that might heterodimerize with PAN. It will be interesting to investigate whether the BOP proteins interact with PAN or other members of the TGA factor family.

In conclusion, we show here that the *BOP* genes affect leaf growth and development by influencing two different processes. First, together with *AS1* and *AS2*, the formation of ectopic meristem activity on the leaf is prevented, most likely through their mutual repression of *knox* gene activity in the leaf. Secondly, probably through repression of *JAG/JGL* and through a strong cooperation with the flower meristem-identity gene *LFY*, the development of proximal parts of the leaf and the development of the bracts are suppressed. As the BOP proteins contain domains indicative of a role in protein-protein interaction it will be very interesting to investigate whether the BOP proteins interact with any of the known proteins affecting *knox* gene regulation or the regulation of cell-cycle activity in the leaf. Elucidating such phenomena should significantly advance our understanding of how the network of regulators affecting leaf initiation and growth interact in order to sculpt the development of the leaf as well as other lateral organs.

We thank Hong Gil Nam, Chan Man Ha, José Dinneny and Detlef Weigel for providing mutant seeds, and Miguel Blázquez and Eugenio Gomez Minguet for initial help with in situ hybridizations. We also thank Catherine Bellini, Alan Marchant and François Parcy for comments and suggestions on the manuscript. This work was supported by an 'INGVAR' grant from the Swedish Foundation for Strategic Research to O.N.

Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/132/9/2203/DC1

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