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

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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 reiterated 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 1 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 C2H2 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 ify 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 vermiculite (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 TAIL 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 ify-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 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 PHYLIP program (version 4.0b10) (Swoford, 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-32P]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 B1-1 and B1-4 for BOP1, and B2-3 and B2-4 for BOP2. The primers were 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). 18Scompetitors 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, E>0.95 and r²>0.98, where E is the PCR efficiency and r² corresponds to the correlation coefficient obtained with the standard curve. Three replicate assays were performed 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 NcoI (BOP2) and HindIII (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 domain. 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 bop1-3 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 siliqua 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.
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 1. Shoot architecture of bop1 bop2, lfy and lfy bop1 bop2 mutants grown in long days

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

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.

### Table 2. Flower architecture of bop1 bop2 mutants

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

Values are mean±s.e.m.

*First whorl organ with mixed sepal-petal identity.

†Flowers with abaxial gap.
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 Ify, ap1 and ap2 mutants (Dinneny et al.,
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. 5B,D).
Development

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.

**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-12 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
controlled differentiation of tissues (Dinneny et al., 2004). According to this view, the opposing effects of genes like 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. 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 for 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 ASI 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.

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Supplementary material
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