The BLADE-ON-PETIOLE genes are essential for abscission zone formation in Arabidopsis

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The Arabidopsis BLADE-ON-PETIOLE 1 (BOP1) and BOP2 genes encode redundant transcription factors that promote morphological asymmetry during leaf and floral development. Loss-of-function bop1 bop2 mutants display a range of developmental defects, including a loss of floral organ abscission. Abscission occurs along specialised cell files, called abscission zones (AZs) that develop at the junction between the leaving organ and main plant body. We have characterized the bop1 bop2 abscission phenotype to determine how BOP1 and BOP2 contribute to the known abscission developmental framework. Histological analysis and petal breakstrength measurements of bop1 bop2 flowers show no differentiation of floral AZs. Furthermore, vestigial cauleine leaf AZs are also undifferentiated in bop1 bop2 mutants, suggesting that BOP proteins are essential to establish AZ cells in different tissues. In support of this hypothesis, BOP1/BOP2 activity is required for both premature floral organ abscission and the ectopic abscission of cauleine leaves promoted by the INFLORESCENCE DEFICIENT IN ABSCISSION (IDA) gene under the control of the constitutive CaMV 35S promoter. Expression of several abscission-related marker genes, including IDA, is relatively unperturbed in bop1 bop2 mutants, indicating that these AZ genes respond to positional cues that are independent of BOP1/BOP2 activity. We also show that BOP1 and BOP2 promote growth of nectary glands, which normally develop at the receptacle adjacent to developing AZs. Taken together, these data suggest that BOP1/BOP2 activity is required for multiple cell differentiation events in the proximal regions of inflorescence lateral organs.

KEY WORDS: Abscission, BLADE-ON-PETIOLE, Flower development, INFLORESCENCE DEFICIENT IN ABSCISSION

INTRODUCTION

Plants alter their body plan in response to developmental and environmental cues by the addition of lateral organs, such as leaves and flowers. However, plant form is also shaped by the removal of these organs. Organs may be actively shed by the plant in a developmentally regulated process known as abscission (Lewis et al., 2006; Patterson, 2001; Roberts et al., 2002). Separation of organs from the plant body occurs at anatomically distinct cell files called abscission zones (AZs). Differentiated AZ cells are small, isodiametric and cytoplasmically dense compared with surrounding cells, and are responsive to signals promoting abscission. These signals induce a directed, enzymatic dissolution of the middle lamellae between AZ cell walls, resulting in a loss of adhesion between the organ and plant body (Addicott, 1982; Sexton and Roberts, 1982). Many enzymes have been suggested to be involved in the separation process, including cellulases, polygalacturonidases (PGs) and expansins (Roberts et al., 2002). After the organ is shed, the cells exposed at the AZ on the plant body differentiate to form a protective surface (Patterson, 2001).

Arabidopsis petals, stamens and sepals develop an abscission zone four to six cell layers thick, where the bases of the organs meet the receptacle (Bleecker and Patterson, 1997). Following fertilization, these floral organs senesce and abscise. Various genes are specifically expressed at floral organ AZs in Arabidopsis. For example, the promoter from an Arabidopsis abscission-related PG (PGAZAT) gene was able to drive floral organ AZ-specific expression of β-glucuronidase (GUS) during abscission (Gonzalez-Carranza et al., 2002). Furthermore, GUS constructs driven by the soybean (Glycine max) chitinase (CHIT) promoter or the bean (Phaseolus vulgaris) abscission cellulase (BAC) promoter are also upregulated in abscission zones during floral organ abscission (Bleecker and Patterson, 1997; Butenko et al., 2006; Chen and Bleecker, 1995; Patterson and Bleecker, 2004).

In several plant species, ethylene promotes abscission, whereas auxin inhibits it (Roberts et al., 2002). Although considerably delayed, floral organ abscission occurs in ethylene-insensitive mutants of Arabidopsis, suggesting that ethylene signalling is important for the timing of abscission but is not essential for it to occur (Patterson and Bleecker, 2004). Numerous loci are proposed to modulate floral organ abscission in Arabidopsis, including HAESA (Jinn et al., 2000), AGAMOUS-LIKE 15 (Fernandez et al., 2000), the DELAYED ABSCISSION loci (Patterson and Bleecker, 2004), genes for the actin-related proteins ARP4 and ARP7 (Kandasamy et al., 2005b; Kandasamy et al., 2005a), several AUXIN RESPONSE FACTOR genes (Ellis et al., 2005; Okushima et al., 2005), INFLORESCENCE DEFICIENT IN ABSCISSION (IDA) (Butenko et al., 2003), and HAWAIIAN SKIRT (Gonzalez-Carranza et al., 2007). Disruption of the expression of these genes results in delayed floral organ abscission, with the exception of ida plants, which retain floral organs indefinitely (Butenko et al., 2003). Floral organs of ida develop AZs; however, the middle lamellae only partially dissolves. IDA encodes a putative secreted peptide ligand suggested to act late in abscission to promote final middle lamellae dissolution (Butenko et al., 2003). Interestingly, ectopic and overexpression of IDA in 35S::IDA transgenic plants lead to precocious floral organ abscission and ectopic abscission at the

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vestigial AZs of pedicels and cauleine leaves (Stenvik et al., 2006). Restricting IDA expression to the flower is likely to be crucial to prevent abscession at vestigial AZs.

Two recently identified Arabidopsis genes encoding redundant regulators of leaf and flower patterning, BLADE-ON-PETIOLE 1 (BOP1) and BOP2 (Ha et al., 2003; Ha et al., 2004; Ha et al., 2007; Hepworth et al., 2005; Norberg et al., 2005), were also shown to be required for floral organ abscission (Hepworth et al., 2005; Norberg et al., 2005). Loss-of-function bop1 bop2 mutants develop leafy projections from petioles and form floral bracts. In addition, flowers of bop1 bop2 develop two petaloid sepals instead of a single abaxial sepal. Furthermore, as in ida mutants, bop1 bop2 mutant floral organs fail to abscise. However, the molecular and anatomical basis of this defect has not been studied and it is unclear how the BOP1 and BOP2 genes function in the abscission process. BOP1 and BOP2 are part of the NPR1 (NON-EXPRESSOR OF PR1) protein family, which is characterised by a series of conserved cysteines and two protein–protein interaction domains. NPR1 is a positive regulator of systemic acquired resistance (SAR), a plant immune response induced following a local infection. During SAR, accumulation of salicylic acid causes a reductive shift in the cellular redox balance, prompting NPR1 to preferentially localise to the nucleus where it interacts with the TGACG sequence-specific binding transcription factors (TGAs) to activate the transcription of PATHOGENESIS RELATED (PR) genes (Dong, 2004). Several aspects of the BOP1/BOP2 signalling mechanism are conserved with NPR1, including localisation to both the cytoplasm and nucleus, and interaction with the TGA PERIANTHIA, which acts in the same genetic pathway to control perianth patterning in flowers (Hepworth et al., 2005). It is unknown whether BOP1/BOP2 activity is controlled by redox-control of nuclear localization and TGA interaction, as is the case for NPR1.

This study examines the contribution of BOP1 and BOP2 to abscission zone development. We show that BOP1 and BOP2 are required for abscission in both wild-type and 35S::IDA plants, as well as for all aspects of AZ-related anatomy in both functional and vestigial AZs. These data suggest that BOP1 and BOP2 promote formation of the specialized AZ anatomy necessary for abscission. Interestingly, the expression of abscission-related genes was relatively unperturbed in bop1 bop2 mutants, indicating that activation of AZ-specific gene expression is independent of AZ anatomy. In addition, our analysis determined that BOP1 and BOP2 promote nectary gland formation, which occurs at the receptacle and is controlled by redox-control of nuclear localization and TGA interaction, as is the case for NPR1.

MATERIALS AND METHODS
Plant material and growth conditions
Seedlings were germinated onto Arabidopsis minimal medium (Haughn and Somerville, 1986) and plants were grown in continuous light at 20°C as described (Dean et al., 2007). Wild type was the Columbia-0 (Col-0) ecotype, unless otherwise noted. Plants for 35S::IDA experiments were grown at 22°C for 8 hours dark/16 hours light and wild type was the C24 ecotype. T-DNA mutants bop1-3 (Col-0) and bop2-1 (Col-0) alleles have been described previously (Hepworth et al., 2005). T-DNA/ida mutant (C24) and IDA::GUS (C24) lines have been described previously (Butenkov et al., 2003). The 35S::IDA construct described by Stenvik et al. (Stenvik et al., 2006) was transformed into bop1 bop2 plants by floral dip (Clough and Bent, 1998). Analysis was conducted on the T2 generation. bop1 bop2 ida (Col-0×C24) triple mutants were generated by crossing. BOP1::GUS plants (Col-0) contain transcriptional fusions generated by fusing ~4 kb upstream of the putative BOP1 start codon to the β-glucuronidase (GUS) reporter gene in the binary vector pBl101 (Jefferson et al., 1987). CHIT::GUS (Chen and Bleecker, 1995) and BAC::GUS (Koehler et al., 1996) marker lines have been introgressed into Col-0 and were provided by Dr Sara Patterson (University of Wisconsin, Madison, WA). CRABS CLAW (CRC)::GUS transgenic plants (Baum et al., 2001) and crc-1 mutants (Bowman and Smyth, 1999) were provided by Dr John Bowman (Monash University, Melbourne, Australia) and are both in the Landsberg erecta (Ler) background. Floral developmental stages were determined according to Smyth et al. (Smyth et al., 1990).

Petals breakstrength
Petals breakstrength is the force in gram equivalents required to remove a petal from the receptacle. Petals breakstrength was measured by the apparatus described by Lease et al. (Lease et al., 2006).

Microscopy and histology
For scanning electron microscopy of floral organ and vestigial AZs, sepals, petals and cauleine leaves were removed prior to fixation. Following critical-point drying, tissues were mounted onto steel stubs, coated with gold palladium and observed using a Hitachi VP-4600 scanning electron microscope (Tokyo, Japan). For detection of GUS activity, tissue was fixed in 90% acetone, treated with hexane and then rinsed with GUS buffer [100 mM Na2HPO4 (pH 7.0), 10 mM EDTA, 0.1% Triton X-100, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide]. Samples were incubated with GUS buffer supplemented with 0.05% X-gluc (5-bromo-4-chloro-3-indolyl β-D-glucuronide cyclohexylamine) salt (Rose Scientific, Alberta, Canada) at 37°C for 2 hours for CRC::GUS and IDA::GUS plants, or overnight for BOP1::GUS, CHIT::GUS, GLUC::GUS plants. Tissues were cleared overnight in 70% ethanol, then cleared and mounted in chloral hydrate:water:glycerol (8:1:2). Tissues for sectioning were embedded in standard Spurr’s resin. BOP1::GUS and IDA::GUS samples were stained for GUS activity before embedding. For nectary and AZ analyses, sections were stained in Toluidine Blue.

Reverse transcriptase-mediated expression analyses
Total RNA from green rosette leaves, mature flowers and floral buds was isolated as described by Stenvik et al. (Stenvik et al., 2006). First-strand cDNA synthesis with SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA) was performed in a total volume of 20 µl, using 1350 ng of total RNA as template, and incubated at 50°C for 60 minutes. Reverse transcription was omitted in negative controls. The open reading frame sequence of ACTIN2 (At13g18780) was used as a positive internal control. Primers used for ACTIN2 and IDA have been described by Stenvik et al. (Stenvik et al., 2006).

Real-time quantitative RT-PCR (qPCR)
Total RNA for qPCR was isolated from position 5-7 floral organ AZs. The reaction was performed on a LightCycler 480 SYBR Green I Master Kit (Roche). Probe-based qPCR with these primers was performed using Universal Probe Library (UPL) hydrolysis probes (Roche). UPL probes 68 (IDA), 82 (HAESA) and 102 (ACTIN2), and the LightCycler 480 Probes Master Kit (Roche). All samples and reference controls were performed in two biological replicates and with two technical duplicates each. Primers for amplification were: IDA 68 left 5'-TCAATGAGGAGAGATCTCAAAAG-3', IDA 68 right 5'-CTAAAGGCGTTCCTCACTTF-3'; ACTIN2 82 left 5'-CATGGCTGTCGGACCTTT-3'; ACTIN2 82 right 5'-CCCGTACCATGGACACAC-3'; and ACT2 102 left 5'-CGCTCCTTCTTCTCAAGCTC-3'.

RESULTS
Loss of abscission in bop1 bop2 mutants is independent of senescence
Loss of floral organ abscission is a striking phenotype of bop1 bop2 plants (Fig. 1A). In wild type, sepals, petals and stamens normally abscise shortly after anthesis (flower opening). The convention used
to stage abscission is to label the youngest flower with visible white petals as position 1 (Bleecker and Patterson, 1997). Flowers further down the inflorescence are numbered consecutively. Wild-type (Col-0) flowers shed sepals, petals and stamens at position 6.55±1.09 (Fig. 1B, Table 1), whereas bop1 bop2 plants retained all floral organs indefinitely (Fig. 1C).

Under growing conditions of continuous light, wild-type floral organs began to senesce before abscission. Sepals began to yellow starting at the tips around position 2-3, and sepals, petals and stamens showed signs of withering by position 5-6 (Table 1). Floral organs of bop1 bop2 mutants showed a similar progression (Table 1), and became completely senesced while remaining attached to the base of the elongating siliques (Fig. 1B), suggesting that the senescence of floral organs is unimpeded in bop1 bop2 mutants and does not lead to the observed loss of abscission.

**BOP1 and BOP2 are necessary for the formation of floral AZ and vestigial AZ anatomy**

The abscission defect of bop1 bop2 was further characterized using a petal breakstrength meter (Lease et al., 2006), which measures the amount of force required to remove a petal from the receptacle (Craker and Abeles, 1969). Owing to the progressive degradation of the middle lamellae in the AZ, breakstrength force decreased as the organs approach abscission (Fig. 1D). Dramatically, bop1 bop2 showed no decrease in breakstrength at any position, suggesting that cell-wall adhesion at the organ-receptacle boundary does not weaken in bop1 bop2 (Fig. 1D).

We used scanning electron microscopy (SEM) to examine the organ-receptacle boundary of bop1 bop2. Petals were removed at the receptacle if not yet abscised from wild-type and bop1 bop2 flowers at positions 2, 4, 6 and 12. The petal fracture surface of wild-type and bop1 bop2 position 2 flowers was composed of cells that ruptured upon petal removal (Fig. 2A,B, arrows). Owing to weakening of the middle lamellae, petal removal at position 4 (Fig. 2A) did not cause cell rupture but revealed a smooth fracture cavity that is typical of wild-type AZ cells (Bleecker and Patterson, 1997). At position 6, petals readily abscised to reveal a mature AZ characterised by spherical elongated cells (arrow). By position 12, the exposed plant body had differentiated into a protective surface. By contrast, the fracture surface of bop1 bop2 showed no evidence of AZ activation; cells ruptured during petal removal from all positions (Fig. 2B).

To determine more precisely the differences in cellular morphology between bop1 bop2 and wild type, longitudinal sections of flowers at anthesis were examined. Cytoplasmically dense AZ cells were visible in the stamen filament-receptacle, petal-receptacle and sepal-receptacle boundaries of wild type (Fig. 2C-E, arrows), but such a layer never was observed in bop1 bop2 (Fig. 2G-I, arrows). Interestingly, the abaxial petalloid sepals of bop1 bop2 did not form an obvious junction with the floral receptacle – the cell layers were fused (Fig. 2J, arrow). In summary, analyses via petal breakstrength, SEM and light microscopy strongly suggest that cytologically distinct and active floral AZs do not form in bop1 bop2.

Vestigial AZs develop in Arabidopsis at branching points and at the base of pedicels and cauline leaves (Stenvik et al., 2006). Given the absence of floral AZs in bop1 bop2, we investigated whether bop1 bop2 also lacks vestigial AZs. To this end, SEM was performed on the vestigial AZs exposed after removal of cauline leaves. Fully expanded cauline leaves were removed from the stem at three maturation phases based on leaf colour: green, yellowing (50%) and yellow. The exposed fracture plane on wild type and bop1 bop2 stems following removal of green cauline leaves showed breakage of cells along the surface (Fig. 3A,D). Removal of yellowing cauline leaves from wild-type stems revealed enlarged rounded cells at the edges of the attachment point, indicative of an AZ (Fig. 3B), and when fully yellow, revealed rounded cells, although the vasculature cells remain broken (Fig. 3C). Corresponding surfaces of bop1 bop2 lacked any signs of these enlarged AZ cells (Fig. 3E,F). The bases of attached green cauline leaves were also examined. Abaxial surfaces of wild-type leaves at the stem showed a furrow of narrowed cells (Fig. 3G,H, arrows) flanked by stipules (Fig. 3H, arrowhead). This boundary furrow corresponded to the lower cleavage site formed when wild-type leaves are removed. Abaxial surfaces of bop1 bop2 cauline leaves lacked an obvious boundary furrow (Fig. 3J,K). Sections through expanded green wild-type cauline leaves revealed the narrowed cell layers of the leaf-stem boundary furrow (Fig. 3I, asterisk). Wild-type

![Image](https://example.com/image.png)

**Table 1. Characterisation of floral organ abscission in wild type and bop1 bop2 (n=70)**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Line</th>
<th>Sepals yellowing position±s.d.</th>
<th>Floral organs withering position±s.d.</th>
<th>Abscission position±s.d.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Continuous light</td>
<td>Col-0</td>
<td>2.56±0.79</td>
<td>5.40±0.50</td>
<td>6.55±1.09</td>
</tr>
<tr>
<td></td>
<td>bop1 bop2</td>
<td>2.59±0.82</td>
<td>6.76±0.83</td>
<td>na</td>
</tr>
</tbody>
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sections also showed darkened vestigial AZ cells at the adaxial leaf-stem boundary (Fig. 3I, arrow) and between the primary and axillary stem branching point (Fig. 3I, arrowhead). Wild-type vestigial AZs were readily apparent in serial sections through the sides of the leaf-stem boundary, but were less obvious through the vasculature (see Fig. S1 in supplementary material). However, neither cauline leaf nor branching point vestigial AZs were present in any position for expanded green bop1 bop2 cauline leaves; cells at the junction remained large and vacuolated (Fig. 3L). In summary, these data indicate that BOP1 and BOP2 are essential for vestigial AZ formation.

BOP1 expression analyses

In situ hybridization has shown BOP1 and BOP2 mRNAs at the base of developing floral organs and in the AZ of mature flowers (Ha et al., 2004; Hepworth et al., 2005; Norberg et al., 2005). We examined the expression pattern in more detail using a GUS reporter gene under the control of the BOP1 promoter. BOP1::GUS was expressed at the base of developing floral organs in stage 9/10 (Fig. 4A), stage 12 (Fig. 4B) and stage 14 (Fig. 4C) flowers, agreeing with previous results (Norberg et al., 2005). In addition, bases of recently shed organs had GUS activity (Fig. 4D). BOP1::GUS receptor expression was detected for a prolonged period following abscission (Fig. 4E). The adaxial regions of young pedicel-stem junctions showed GUS activity (Fig. 4F) that spreads around the entire boundary in older pedicels (Fig. 4G). BOP1::GUS expression exhibited similar patterns in bop1 bop2 as in wild type (data not shown), showing that BOP1 expression does not depend on functional BOP proteins. Interestingly, BOP1 was ectopically expressed in the bop1 bop2 pedicel where the ectopic bract develops (Fig. 4H, compare with 4G). In addition, expression of BOP1::GUS was detected at the bases of cauline leaves (Fig. 4I), as previously reported (Norberg et al., 2005).

To further characterise BOP1 expression, BOP1::GUS stained tissue was sectioned. Corresponding to in situ hybridisation results of BOP2 (Hepworth et al., 2005), BOP1::GUS was expressed in early floral organ primordia (Fig. 4J). As the stamens become stalked, GUS activity was basally restricted (Fig. 4K,L), while the petal primordia, which develop later, displayed diffuse staining (Fig. 4L). The petal expression also became restricted to the petal base as petals grew out (data not shown). Mature flowers showed strong staining through the AZ (Fig. 4M) agreeing with BOP2 in situ hybridisation (Hepworth et al., 2005; Norberg et al., 2005). In summary, patterns of BOP1::GUS expression correlate with a putative role in abscission zone development.
Suppression of ida and 35S::IDA phenotypes by bop1 bop2

The ida mutant shows a complete lack of abscission; however, unlike bop1 bop2, ida flowers differentiate floral organ AZs and have reduced petal breakstrength (Butenko et al., 2003). BOP1 and BOP2 are essential for anatomical differentiation of the AZ, suggesting that these roles are fulfilled prior to IDA function. To test this hypothesis, triple mutants were constructed and examined. The triple mutant bop1 bop2 ida did not differentiate AZs or show decreases in petal breakstrength (see Fig. S2 in the supplementary material), demonstrating that bop1 bop2 is epistatic to ida.

Absence of abscission in bop1 bop2 is presumed to occur because of a lack of proper AZ anatomy. We tested whether overexpression of IDA by the CaMV 35S promoter in bop1 bop2 plants can circumvent this requirement. Compared with wild type (C24) (Fig. 5A), precocious floral organ abscission was a striking phenotype of 35S::IDA plants (Fig. 5B), as previously described (Stenvik et al., 2006). These plants also exhibited activation of vestigial cauline AZs (Fig. 5F) and ectopic abscission at pedicels and branching points (Stenvik et al., 2006). Similar to bop1 bop2 plants (Fig. 5C), 35S::IDA bop1 bop2 plants showed no floral organ abscission (Fig. 5D), indicating that bop1 bop2 is completely epistatic to 35S::IDA. Furthermore, ectopic abscission at vestigial AZs in pedicels, branching points (data not shown) and cauline leaves (Fig. 5F) was lost in 35S::IDA bop1 bop2 plants, which resembled bop1 bop2 (Fig. 5E). Both C24 and Col-0 backgrounds demonstrate these phenotypes when transformed with 35S::IDA, suggesting that the lack of 35S::IDA phenotype in 35S::IDA bop1 bop2 is not due to the Col-0 ecotype. In addition, the 35S::IDA construct was not transcriptionally silenced in bop1 bop2 (Fig. 5H).

Thus, earlier and ectopic expression of IDA is not sufficient to promote abscission in bop1 bop2 because of an absence of proper AZ architecture.

Abscission-related gene expression in bop1 bop2

We were interested in determining whether BOP1 and BOP2 regulate expression of genes encoding putative signalling components, such as IDA, and/or enzymes normally transcribed in AZs. RT-PCR results indicated that IDA, HAESA and HAWAIIAN SKIRT had the same expression levels in bop1 bop2 and wild-type flowers (results not shown). Whereas mutation in HAWAIIAN SKIRT leads to delayed abscission and fusion of sepal margins that precludes shedding (Gonzalez-Carranza et al., 2007), downregulation of IDA or HAESA results in abscission defects only (Jinn et al., 2000; Butenko et al., 2003). The RT-PCR results were confirmed by quantitative PCR for IDA and HAESA; the expression level in wild-type (Col-0) and bop1 bop2 floral AZs did not differ as the relative expression was very close to 1: 0.94 for IDA and 0.95 for HAESA (see Fig. S3 in supplementary material). To further examine IDA regulation in bop1 bop2, IDA::GUS plants (C24) were crossed into bop1 bop2. Wild-type IDA::GUS expression was examined in Col-0×C24 background to compare with bop1 bop2 IDA::GUS plants generated by crossing. IDA::GUS was expressed shortly following anthesis in bop1 bop2 flowers and persisted until after the stage when abscission would normally occur (Fig. 6B) – a temporal profile akin to that observed in wild type (Fig. 6A). Spatially, IDA::GUS expression was present at the base of the floral organs in bop1 bop2, similar to wild type (Fig. 6C,D).

Two reporter constructs driven by promoters from genes encoding enzymes that are specifically upregulated in AZs, BAC::GUS and CHIT::GUS, were also examined. As observed previously in wild type (Fig. 6D), these constructs were not transcriptionally silenced in bop1 bop2 (Fig. 6B).
type (Butenko et al., 2006). BAC::GUS expression appeared early (before anthesis) and throughout the AZ by position 1 (Fig. 6E). BAC::GUS was expressed also in the proximal petal and stamen filaments. Although this temporal pattern was retained in bop1 bop2 (Fig. 6F), BAC::GUS expression was reduced in intensity, restricted to the bases of the petals and stamens and absent from single sepal AZ cells, as seen in the wild type (Fig. 6E, arrow).

Consistent with published results (Patterson and Bleecker, 2004; Butenko et al., 2006), CHIT::GUS was expressed in the floral organ AZ cells starting at position 4 and increased to maximal intensity, coincident with abscission at position 6, after which expression weakens (Fig. 6G). Despite the lack of anatomical AZ structure in bop1 bop2, temporal expression of CHIT::GUS was maintained, albeit with lower intensity (Fig. 6H). Often CHIT::GUS activity was detected earlier in bop1 bop2 but this was not consistent. CHIT::GUS expression also occurred in the proximal floral organs in the bop1 bop2 background (Fig. 6H). These data suggest that activation signals for BAC::GUS and CHIT::GUS are operating in bop1 bop2 regardless of a lack of AZ morphology. Interestingly, ida also shows normal temporal and spatial expression of both these reporters in AZ cells (Butenko et al., 2006), suggesting that signals activating their expression also function independently of IDA.

**BOP1 and BOP2 are necessary for nectary development**

Nectaries are secretory organs that develop from receptacle tissue after maturation of floral organs at stage 9 (Smyth et al., 1990), appearing as outgrowths at the base stamen filaments and connected to one another via a ring of nectary tissue that encircles the receptacle (Davis et al., 1994). Lateral nectaries surround the receptacle (asterisk) and are operating in bop1 bop2 (Fig. 6H). Young pedicel-stem junctions have an abaxial GUS signal that spreads around the attachment point in older pedicels (Fig. 6G). BOP1::GUS bop1 bop2 plant with GUS activity at the base of the ectopic bract (arrow). I Cauline leaves show GUS activity at their base. J-N Sections of stained BOP1::GUS flowers. (J) Stage 5/6 flower showing GUS activity in stamen primordia (asterisk) and outgrowing sepals. (K) Staining of a stage 7/8 flower is restricted to the base of stalked stamens (asterisk) and outgrowing sepals. (L) Section through the same stage 7/8 flower showing staining throughout the petal primordia (asterisk). (M,N) Mature flowers with GUS activity through the AZ (M) and nectaries (N). Scale bars: 250 μm in A,B,D-F; 0.5 mm in C; 1 mm in G-I; 35 μm in J-N.

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**Fig. 4. Expression of BOP1::GUS.** (A-I) Whole mounts of BOP1::GUS (Col-0) transgenic Arabidopsis plants stained for GUS activity. (A) Expression is restricted to the base of developing organs in stage 9 and 10 flowers. (B) Expression at the base of floral organs and in lateral nectaries of a stage 12 flower (arrow). (C) GUS activity at the base of floral organs, sepal vasculature, style and pollen of stage 14 flowers. (D) Newly abscised petal and stamen show expression in their AZs and filaments. (E) Position 9 siliques show AZ expression in the receptacle AZ. (F) Young pedicel-stem junctions have an abaxial GUS signal that spreads around the attachment point in older pedicels (G). (H) BOP1::GUS bop1 bop2 plant with GUS activity at the base of the ectopic bract (arrow). I Cauline leaves show GUS activity at their base. (J-N) Sections of stained BOP1::GUS flowers. (J) Stage 5/6 flower showing GUS activity in stamen primordia (asterisk) and outgrowing sepals. (K) Staining of a stage 7/8 flower is restricted to the base of stalked stamens (asterisk) and outgrowing sepals. (L) Section through the same stage 7/8 flower showing staining throughout the petal primordia (asterisk). (M,N) Mature flowers with GUS activity through the AZ (M) and nectaries (N). Scale bars: 250 μm in A,B,D-F; 0.5 mm in C; 1 mm in G-I; 35 μm in J-N.

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**Fig. 5. Suppression of 35S::IDA phenotype in bop1 bop2.** (A-D) Arabidopsis flowers from positions 3, 7 and 10. (A) Wild type (C24) shows floral organ abscission by position 7. (B) 35S::IDA (C24) with premature abscission of floral organs by position 3. (C) bop1 bop2 lacks floral organ abscission. (D) 35S::IDA bop1 bop2 (Col-0) showing no floral organ abscission. (E) Cauline leaves do not abscise in bop1 bop2. (F) Cauline leaves of 35S::IDA show ectopic abscission. (G) 35S::IDA bop1 bop2 cauline leaves resemble bop1 bop2. (H) RT-PCR from cDNA derived from rosette leaves, buds and flowers from bop1 bop2 and 35S::IDA bop1 bop2. Upper panel shows RT-PCR with IDA primers. IDA is expressed in bop1 bop2 flowers only. 35S::IDA bop1 bop2 plants show expression in rosette leaves, buds and flowers. Lower panel, RT-PCR of ACTIN2-7 (ACT) as a positive control. Lane 1 is a genomic PCR control (g), 1, leaves; b, buds; f, flowers.
subtending the abaxial side. Medial nectaries are smaller and develop at the abaxial base of medial stamens. As the glands mature, they develop modified stomata designed for secretion and the cuticle becomes heavily reticulated (Baum et al., 2001).

During our AZ analyses, we noticed that bop1 bop2 flowers did not show obvious nectary glands; analysis of the F2 progeny of a bop1 bop2 × wild type cross demonstrated this phenotype co-segregated with other bop1 bop2 phenotypes (data not shown). Wild-type position 4 flowers with sepals and petals removed showed paired lateral nectary glands protruding at the base of the lateral stamens (Fig. 7A). By contrast, bases of bop1 bop2 lateral stamens lacked large nectary outgrowths (Fig. 7B). Wild-type lateral nectaries had characteristic deeply reticulated cuticle and associated secretory stomata (Fig. 7C), while in bop1 bop2, the area where lateral nectaries normally develop had slightly bulging areas of weak striation lacking secretory stomata (Fig. 7D).

From the onset of nectary development, two distinct nectary cell types exist: an outer epidermal layer and an inner starch granule-containing parenchymal tissue (Baum et al., 2001), as seen in transverse and longitudinal sections (Fig. 8E,G); a ridge of connecting nectary tissue also is present (Fig. 8E, arrow). The corresponding section of bop1 bop2 lacks distinct epidermal and parenchymal cells but shows slight bulges where the paired lateral glands would normally arise (Fig. 8F,H, arrows). Thus, although some residual cell division may occur, bop1 bop2 lacks differentiation of most nectary tissue characteristics.

**Genetic interaction between CRABS CLAW and BOP1 and BOP2**

CRABS CLAW (CRC) is a key gene regulating nectary development in Arabidopsis and encodes a putative zinc-finger transcription factor containing a YABBY domain (Bowman and Smyth, 1999; Siegfried et al., 1999). While bop1 bop2 flowers retain residual bulging reminiscent of nectary glands, crc mutants show a complete loss of nectary development (Bowman and Smyth, 1999). To determine whether loss of nectary growth observed in bop1 bop2 was due to misregulation of CRC expression, CRC::GUS expression was examined in bop1 bop2. Wild-type CRC::GUS expression was examined in Col-0 × Ler background to compare with bop1 bop2 CRC::GUS plants generating by crossing. As described by Baum et al. (Baum et al., 2001), CRC::GUS is expressed in stage 7/8 flowers in the nectary gland anlagen (Fig. 71); expression then expands throughout the connecting nectary tissue between the glands and is maintained after abscission of floral organs (Fig. 7J,K). CRC::GUS is also expressed in stage 7/8 bop1 bop2 flowers (Fig. 7L), suggesting that BOP1 and BOP2 are...
not necessary for CRC expression in the nectary anlagen. As bop1 bop2 flowers mature, receptacle regions expressing CRC::GUS expand into lateral and medial nectary regions and in connecting areas although at reduced levels compared with wild type (Fig. 7M,N).

Like BOP1 and BOP2, CRC regulates other aspects of floral development. To determine whether these genes overlap in other functions, bop1 bop2 crc-1 triple mutants were constructed and examined. Nectary development was completely abolished in the triple mutants (data not shown) showing that the severe nectary phenotype of crc-1 is epistatic to that of bop1 bop2.

**DISCUSSION**

**BOP1 and BOP2 act early to specify AZ anatomy**

Abscission occurs in the AZ positioned at the junction between a lateral organ and the main plant body. The AZ is characterized by a unique anatomy and although it is thought to be essential for abscission, this has not been explicitly demonstrated in Arabidopsis as previously described mutants retain the development of an AZ. We show that two redundant NPR1-like homologues, BOP1 and BOP2, are expressed in the AZ and that lack of BOP1/2 proteins results in a complete absence of abscission. Moreover, the absolute loss of floral organ abscission in bop1 bop2 is uniquely correlated with an absence of cellular anatomy typical of the AZ, suggesting that the AZ anatomy is necessary for abscission and that BOP1 and BOP2 initiate differentiation of the AZ. However, we cannot rule out the possibility that BOP1 and BOP2 control other downstream events also necessary for abscission.

Several lines of evidence implicate that the specification by BOP1 and BOP2 of abscission zone cells as the earliest known step necessary for abscission. First, BOP1 and BOP2 genes are transcribed in early floral organ primordia, and resolve to a region corresponding to the future AZ prior to other known abscission-related genes. Second, appearance of AZ anatomy, which is absent in bop1 bop2, is the earliest identified event associated with AZ development. Third, the bop1 bop2 genotype is epistatic to that of both ida and 35S::IDA, suggesting that the BOP1 and BOP2 act upstream of the only other gene known to be absolutely required for abscission.

BOP1 and BOP2 are expressed earlier than IDA, raising the possibility that BOP1 and BOP2 are positive regulators of IDA expression. However, we have shown that IDA is expressed similarly to wild type in bop1 bop2. It is possible that BOP proteins regulate IDA activity post-transcriptionally but given the requirement of BOP1 and BOP2 for AZ-specific anatomy, we favour a model where BOP1/2 function early to specify the AZ cell type and IDA acts relatively independently to finalize the cell separation process. Significantly, as is the case for IDA (Butenko et al., 2006), BOP1 and BOP2 are not required for the correct temporal transcription of known abscission-related genes tested here, including one encoding a cell wall hydrolytic enzyme.

A model of the known essential players in abscission is presented in Fig. 8. We propose that BOP1 and BOP2 act early to specify AZ-unique anatomy. The characteristics of this anatomy that make it crucial for abscission remain to be identified but could include cell shape and/or cell wall structure amenable to middle lamellae.
digestion. Upstream factors that regulate initiation of abscission act through both ethylene-dependent and ethylene-independent pathways that converge to activate the expression of abscission-related genes, including middle lamella degrading enzymes. These enzymes are expressed in the AZ, presumably with specific spatial and temporal profiles, to progressively weaken the middle lamella. This expression is driven independently from BOP1/2-mediated differentiation of the AZ. As yet, none of these enzymes has individually been shown as essential for abscission. Finally, IDA is necessary for abscission and is expressed in the AZ just prior to abscission in response to an unknown signal. Given that partial enzymatic dissolution of the middle lamella occurs in ida mutants, IDA must act downstream from the initiation of abscission. Expression of IDA alone is insufficient for abscission as premature abscission of 35S::IDA plants occurs only at flower positions with differentiated AZ (Stenvik et al., 2006), and is dependent on BOP1 and BOP2-mediated differentiation of the AZ. The specific role of IDA and its putative receptor are unknown but must be involved in the final essential steps of the cell separation process.

BOP1 and BOP2 also specify vestigial AZ anatomy

Although Arabidopsis leaves do not abscise, ectopic abscission induced by 35S::IDA has suggested that vestigial AZs develop at the bases of cauline leaves, branching points and at the base of pedicels (Stenvik et al., 2006). Several lines of evidence presented here strongly support this hypothesis. First, cauline leaf AZs have characteristic AZ anatomy and a boundary furrow that demonstrates progressive degradation of middle lamella with age similar to floral AZs. Second, the characteristic anatomy, boundary furrow and 35S::IDA-induced abscission at these putative vestigial AZs are dependent on BOP activity. Finally, vestigial AZs develop on the adaxial side of the leaf base, corresponding well to BOP1 and BOP2 expression, as shown here and in other studies (Ha et al., 2004; Norberg et al., 2005). Taken together, these data suggest that vestigial AZs do develop and that their anatomy is regulated by BOP1 and BOP2.

Nectary formation and the role of BOP1 and BOP2

Nectaries are not entirely absent in bop1 bop2, but rather are greatly reduced in size and do not differentiate key nectary features such as parenchymal and secretory tissue, and modified stomata. Our analysis suggests that bop1 bop2 mutants retain CRC::GUS activity in both the nectary anlagen and the bulges that later develop, indicating that the lack of nectary outgrowth is not due to a loss in CRC expression. Similar to CRC, BOP is expressed very early in nectary development and may be controlling other downstream elements in conjunction with CRC.

Formation of the third whorl, although not the presence of stamens in this whorl, is essential for nectary formation (Baum et al., 2001). Third whorls in bop1 bop2 often develop an extra medial stamen between the other two medial stamens and the adjacent petal on the abaxial side (Hepworth et al., 2005). Given this additional growth, the nectary phenotype in bop1 bop2 may be a secondary effect of ectopic growth around the stamen attachment areas.

The role of BOP1 and BOP2 in plant development

Previous research on BOP1 and BOP2 has suggested a role in defining the identity of the proximal regions of lateral organs (Ha et al., 2003; Hepworth et al., 2005; Norberg et al., 2005). The receptacle may be thought of as a proximal feature of a flower, just as the petiole is a proximal area of a leaf. Therefore, the absence of AZs and reduction in nectaries in bop1 bop2 suggests that BOP1 and BOP2 regulate multiple differentiation events in the proximal flower. Prior work has demonstrated that BOP1 and BOP2 may repress class I Knox gene expression in the shoot (Ha et al., 2003; Ha et al., 2007). Class I Knox genes are important to maintain and establish shoot meristem identity and are normally downregulated in incipient lateral organ primordia (Scotsfield and Murray, 2006). Temporal and/or spatial misregulation of class I Knox genes in developing AZs may contribute to defects in AZ differentiation.

Although AZs may be thought of as defining the organ-plant body junction, cauline leaves in bop1 bop2 do not display gross malformations where they meet the stem. Furthermore, expression of floral organ AZ markers, such as IDA and HAESA, persists in bop1 bop2, suggesting the positional information is intact. Thus, bop1 bop2 plants lack some but not all of the features of the organ-plant body junction, suggesting that other factors are responsible for overall boundary patterning, such as CUP-SHAPED COTYLEDONS (Aida and Tasaka, 2006), while the BOP1 and BOP2 genes are later effectors of specific aspects of the organ-plant body interface.

Fig. 8. Model of abscission. BOP1 and BOP2 act early to promote the development of AZ specific anatomy of small cytoplasmically dense cell files in Arabidopsis. Later in flower development, a suite of enzymes involved in middle lamella degradation are expressed specifically in the AZ although with differing temporal patterns. Transcription of these enzymes is independent of BOP-driven formation of AZ anatomy. Abscission occurs following the expression of IDA in the AZ, which promotes cell separation. Expression of IDA is also driven independently from BOP1/2 activity.

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Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/135/8/1537/DC1


