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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 cauline 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 cauline 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 cauline leaves (Stenvik et al., 2006). Restricting *IDA* expression to the flower is likely to be crucial to prevent abscission 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 petalloid 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 localization 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 adjacent to developing AZs. Thus, *BOP1* and *BOP2* regulate the differentiation of multiple cell types in proximal regions of inflorescence lateral organs.

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 (Butenko 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 pBI101 (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* (L*er*) background. Floral developmental stages were determined according to Smyth et al. (Smyth et al., 1990).

Petal breakstrength

Petal breakstrength is the force in gram equivalents required to remove a petal from the receptacle. Petal 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 cauline 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 heptane and then rinsed with GUS buffer [100 mM NaH_2PO_4 (pH 7.0), 10 mM EDTA, 0.1% Triton X-100, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferricyanide]. 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* (At3g18780) 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 LC480 instrument (Roche) according to the manufacturer's protocol. To ensure that primer combinations did not produce undesired PCR fragments or primer dimers, a SYBER-GREEN qPCR with melting-point analysis was performed using the 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 replicates each. Primers for amplification were: IDA 68 right, 5′-TCAATGAGGAAGAGAGTTAACAAAAG-3′; IDA 68 left, 5′-CTA-AAGGCGTTCCCATTCCT-3′; HAESA 82 right, 5′-GAGAGAGGGAA-TGGAGAGAAGG-3′; HAESA 82 left, 5′-CATGCTCGTCGGACCTTT-3′; ACT2 102 right, 5′-CCGGTACCATTGTCACACAC-3′; and ACT2 102 left, 5′-CGCTCTTTCTTTCCAAGCTC-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

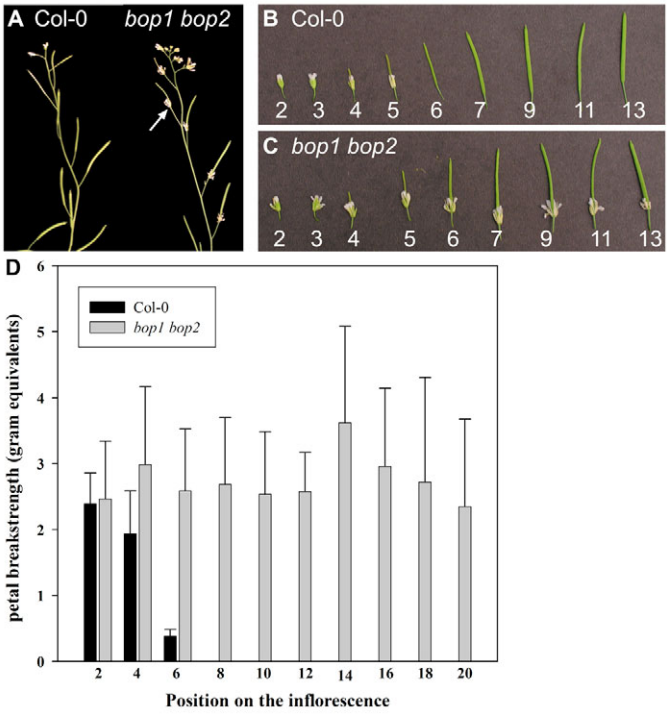

Fig. 1. Abscission phenotype and petal breakstrength of *bop1 bop2*. (**A**) Inflorescences of wild-type *Arabidopsis* plants (Col-0, left) abscise floral organs while *bop1 bop2* plants (right) retain their floral organs. (**B**) Abscission in wild-type flowers occurs between positions 5 and 7. (**C**) Floral organs of *bop1 bop2* never abscise. (**D**) Petal breakstrength measurements of wild-type flowers (black bars) decrease with floral age, while *bop1 bop2* flowers (grey bars) show no change in force over time. Error bars represent s.d. *n*=30 per genotype.

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 silique (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

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

Condition	Line	Sepals yellowing position±s.d.	Floral organs withering position±s.d.	Abscission position±s.d.
Continuous light	Col-0	2.56±0.79	5.40±0.50	6.55±1.09
	bop1bop2	2.59±0.82	6.76±0.83	na

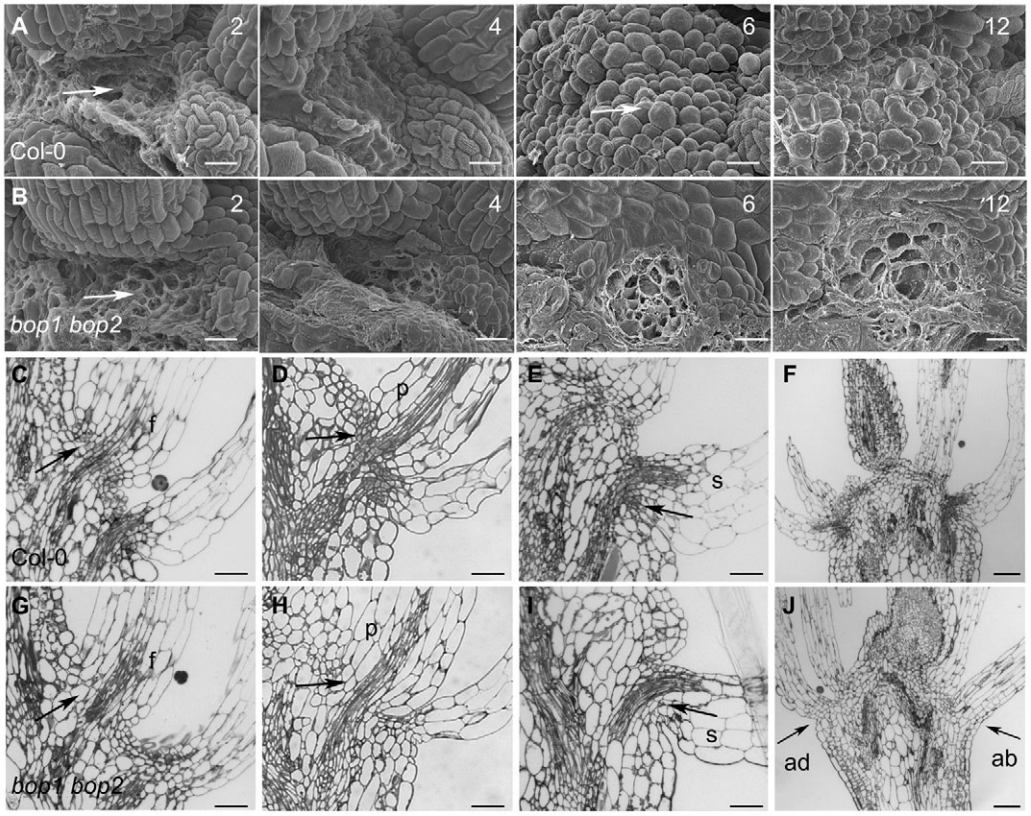

Fig. 2. Morphology of floral AZs in wild type (Col-0) and *bop1 bop2*. (**A**,**B**) Scanning electron micrographs of *Arabidopsis* petal AZs. The fracture plane on the receptacle (arrow) was observed following removal or natural abscission of petals in wild-type (A) and *bop1 bop2* (B) flowers at positions 2, 4, 6 and 12. (A) Wild-type fracture planes demonstrate progression from broken cells (position 2) to rounded AZ cells (position 6) to protective surface cells (position 12). (B) Fracture planes of *bop1 bop2* petals from all positions show broken cells. (C-J) Toluidine Blue-stained sections from flowers at anthesis. (**C-E**) AZ cells at the stamen filament-receptacle (C), petal-receptacle (D) and sepal-receptacle (E) junctions in wild type (arrows). (**F**) Longitudinal section along the adaxial and abaxial faces of wild-type flower. (**G-I**) Receptacle-organ boundaries of stamen filaments (G), petals (H) and sepals (I) of *bop1 bop2* lack cells displaying AZ anatomy (arrows). (**J**) Abaxial petalloid sepal of *bop1 bop2* lacks an organ-receptacle boundary. f, stamen filament; p, petal; s, sepal; ad, adaxial; ab, abaxial. Scale bars: 400 μm in A,B; 35 μm in C-E,G-I; 70 μm in F,J.

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. Interestingly, stipules that flank the wild-type leaf fracture plane (Fig. 3A-C, arrow) and intact wild-type cauline leaves (Fig. 3H, arrowhead) were never observed flanking *bop1 bop2* fracture planes (Fig. 3D-F) or intact leaves (Fig. 3K).

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* receptacle 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.

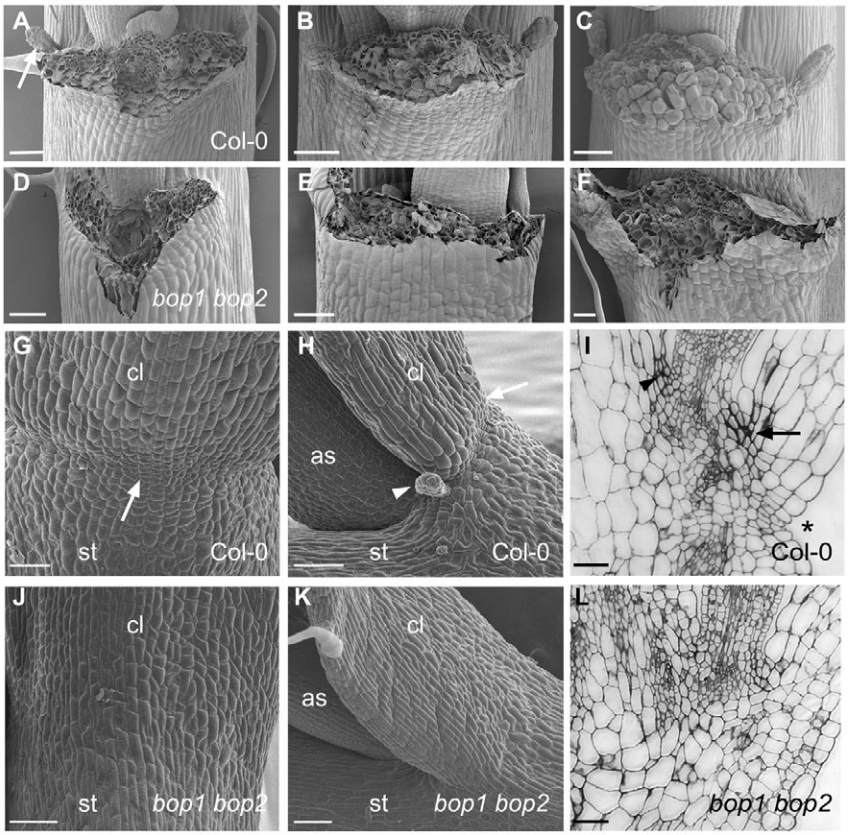

Fig. 3. Morphology of vestigial AZs in wild type (Col-0) and *bop1 bop2*. (A-F) Scanning electron micrographs of stem fracture planes in *Arabidopsis* following removal of cauline leaves. (**A**) Fracture plane in wild type shows ruptured cells following removal of green leaves. (**B**) Removal of 50% yellow leaves reveals a few rounded AZ cells. (**C**) Fracture planes from yellow leaves often show many rounded AZ cells. Stipules always flank the fracture plane (A, arrow). (**D-F**) Corresponding fracture planes of *bop1 bop2* from green (D), 50% yellow (E) and yellow (F) cauline leaves show no development of rounded AZ cells or evidence of flanking stipules. (**G**,**H**) A furrow of narrowed cells (arrows) flanked by stipules (H, arrowhead) is observed between the abaxial surface of the cauline leaf and the stem in wild type. (**I**) Toluidine Blue-stained longitudinal sections show darkened files of cells that mark the vestigial AZ (arrow) at the adaxial boundary between the cauline leaf and stem in wild type and at the branching point with the primary inflorescence stem (arrowhead). The leaf-steam boundary furrow is also apparent (asterisk). (**J**,**K**) *bop1 bop2* cauline leaves lack stipules and leaf-stem boundary furrow. (**L**) No vestigial AZs are visible in *bop1 bop2* sections. as, axillary shoot; cl, cauline leaf; st, primary inflorescence stem. Scale bars: 600 μm in A-H,J,K; 35 μm in I,L.

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

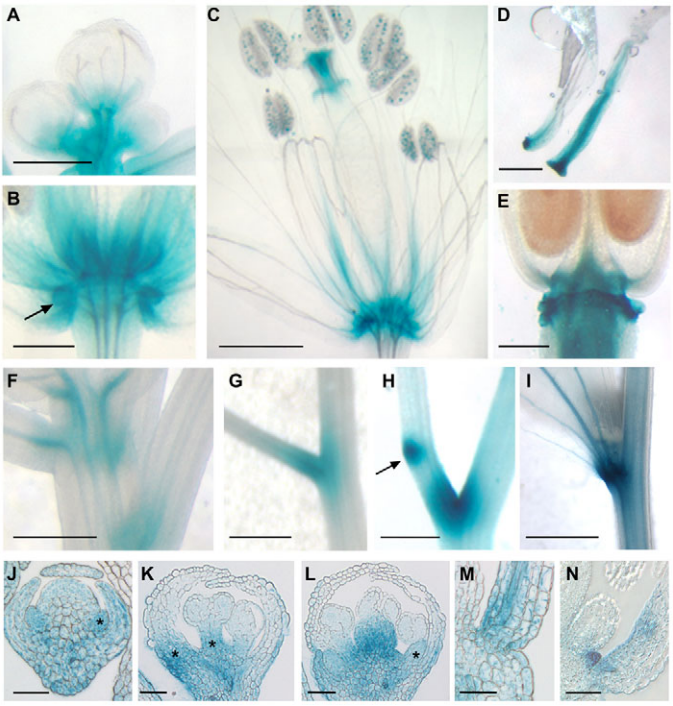

Fig. 4. Expression of *BOP1::GUS*. (A-I) Whole mounts of *BOP1::GUS* (Col-0) transgenic *Arabidopsis* plants stained for GUS activity. (**A**) Expression is restricted to the base of developing organs in stage 9 and 10 flowers. (**B**) Expression at the base of floral organs and in lateral nectaries 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 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.

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 secretary 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 base of the lateral stamen filaments and form large glands

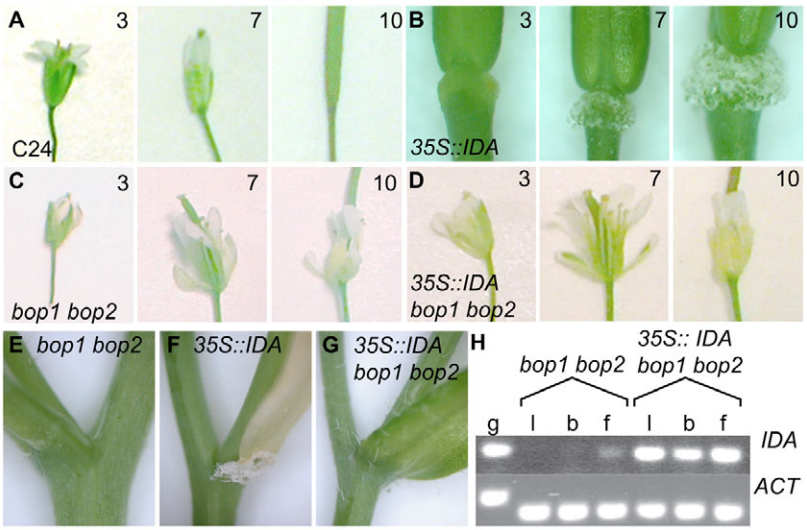

Fig. 5. Suppression of *35S::IDA* phenotype in *bop1 bop2*. (A-D) *Arabidopsis* flowers from positions 3, 7 and 10. (**A**) Wild type (C24) shows floral organ abscission by position 7. (**B**) *35S::IDA* (C24) with premature abscission of floral organs by position 3. (**C**) *bop1 bop2* lacks floral 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). l, leaves; b, buds; f, flowers.

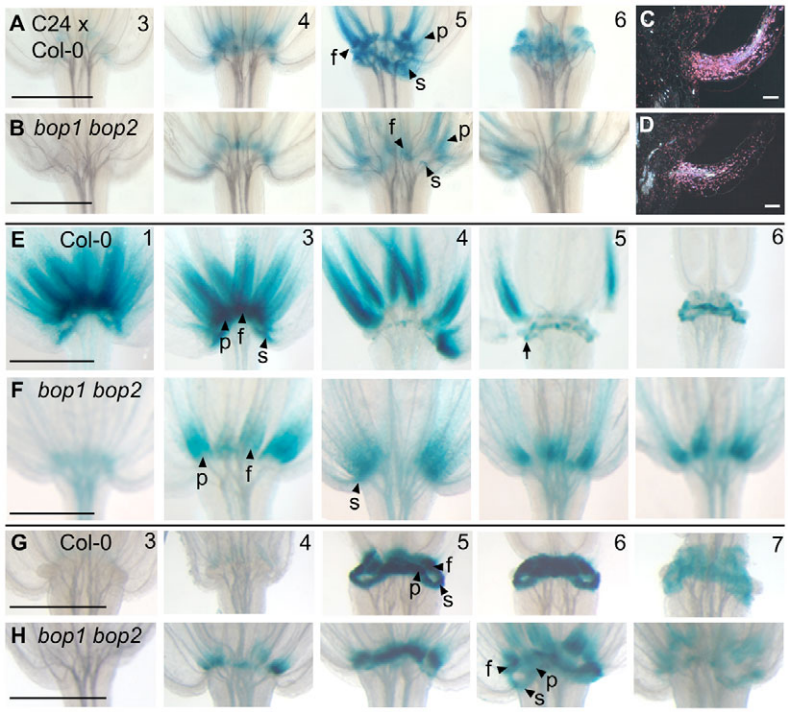

Fig. 6. Expression of *IDA::GUS*, *BAC::GUS* and *CHIT::GUS* in wild type and *bop1 bop2*. *Arabidopsis* floral positions are indicated in the upper right of panels. (**A**) Wild-type (C24×Col-0) flowers at position 3 show very weak *IDA::GUS* activity in the base of floral organs that intensifies throughout position 4 and 5. (**B**) *IDA::GUS* expression in *bop1 bop2* (Col-0×C24) is detected at position 4 and more strongly in position 5. (**C**,**D**) Dark-field microscopy of stained *IDA::GUS* flowers in wild type (C) and *bop1 bop2* show staining throughout the proximal sepal. (**E**,**F**) *BAC::GUS* in wild type (Col-0) or *bop1 bop2* at positions 1, 3-6 stained for GUS activity. (E) *BAC::GUS* plants show strong staining at the base of all floral organs, especially concentrated in the vasculature of position 1 and 3 flowers. Activity persists in position 4 and 5 flowers during abscission and is visible in single AZ cells (arrow). (F) *BAC::GUS bop1 bop2* flowers at position 1 show weak staining at the bases of petals and stamens, and strengthens and enlarges by position 3. Very slight expression is detected on the adaxial side of sepals in position 4. (**G**,**H**) *CHIT::GUS* expression from flowers in wild type (Col-0) or *bop1 bop2* at positions 3-7. (G) *CHIT::GUS* position 3 and 4 flowers demonstrate weak GUS staining, while positions 5 and 6 exhibit very strong AZ staining. (H) *CHIT::GUS bop1 bop2* position 3 and 4 floral organs show weak GUS activity at their base. GUS activity is stronger in position 5. Staining is maintained in position 6 flowers extending further into the base of these floral organs. Arrowheads indicate expression. f, stamen filament; p, petal; s, sepal. Scale bars: 0.5 mm in A,B,E-H; 35 μm in C,D.

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). Nectaries showed strong *BOP1* expression throughout nectary development (Fig. 4B,N).

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×L*er* 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. 7I); 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

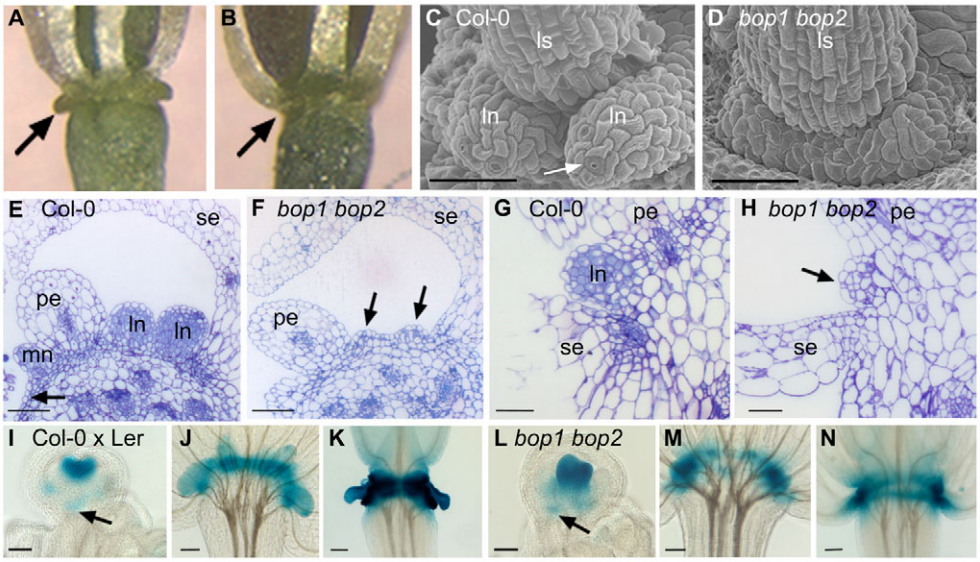

Fig. 7. Nectary morphology and *CRC::GUS* activity in wild type (Col-0) and *bop1 bop2*. (**A**,**B**) Sepals and petals in *Arabidopsis* were removed from wild-type (A) and *bop1 bop2* (B) position 4 flowers. Lateral nectary protrusions are obvious in wild type (arrow) but not in *bop1 bop2*. (**C**) Pair of lateral nectary glands with secretory stomata (arrow) in wild type. (**D**) Lateral nectary glands absent in *bop1 bop2*. (E-H) Toluidine Blue-stained sections from position 3 flowers. (**E**) Wild-type transverse section shows paired lateral nectary glands with distinct epidermal and starch-containing parenchymal regions, a ridge of connecting nectary tissue (arrow) and a medial nectary gland. (**F**) Corresponding *bop1 bop2* cross-section shows two primordia (arrows) in the lateral nectary position. (**G**) A lateral nectary gland visible in longitudinal section of wild type. (**H**) Corresponding longitudinal section of *bop1 bop2* shows a small outgrowth (arrow) in the lateral nectary position. (I-N) *CRC::GUS* plants stained for GUS activity. (I-K) *CRC::GUS* in wild type (Col-0×L*er*). (**I**) Expression in the nectary anlagens of a stage 7/8 flower (arrow). (**J**) Strong nectary gland expression in a mature flower. (**K**) Position 7 flower with GUS staining nectaries and the ring of connecting nectary tissue. (L-N) *CRC::GUS* activity in *bop1 bop2* (Col-0×L*er*). (**L**) Expression in stage 7/8 flowers in the nectary anlagens (arrow). (**M**) Strong expression in the nectary gland region of a mature flower. (**N**) GUS staining in position 7 flowers shows strong staining in the lateral nectary gland region and weaker staining in the medial and connecting nectary tissue regions. ln, lateral nectary; ls, lateral stamen; mn, medial nectary; p, petal; s, sepal. Scale bars: 500 μm in C,D; 70 μm in E-H,J,K,M,N; 35 μm in I,M.

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* phenotype 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

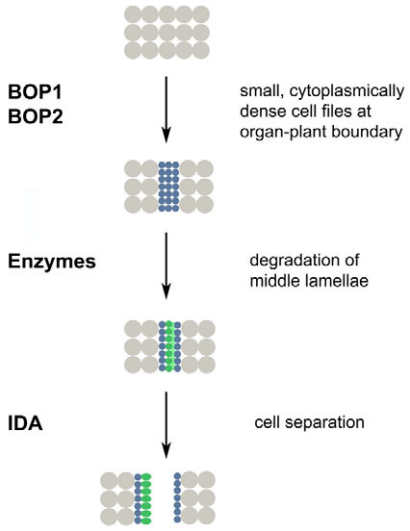

Fig. 8. Model of abscission. *BOP1* and *BOP2* act early to promote the development of AZ specific anatomy of small cytoplasmically dense cell files in *Arabidopsis*. Later in flower development, a suite of enzymes involved in middle lamella degradation are expressed specifically in the AZ although with 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.

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 lamellae degrading enzymes. These enzymes are expressed in the AZ, presumably with specific spatial and temporal profiles, to progressively weaken the middle lamellae. 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 lamellae 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 lamellae 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 anlagens 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 1 Knox gene expression in the shoot (Ha et al., 2003; Ha et al., 2007). Class 1 Knox genes are important to maintain and establish shoot meristem identity and are normally downregulated in incipient lateral organ primordia (Scofield 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.

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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

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