Arabidopsis Class I KNOTTED-Like Homeobox Proteins Act Downstream in the IDA-HAE/HSL2 Floral Abscission Signaling Pathway

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Floral organ abscission in Arabidopsis thaliana is regulated by the putative ligand-receptor system comprising the signaling peptide INFLORESCENCE DEFICIENT IN ABSCISSION (IDA) and the two receptor-like kinases HAESA and HAESA-LIKE2. The IDA signaling pathway presumably activates a MITOGEN-ACTIVATED PROTEIN KINASE (MAPK) cascade to induce separation between abscission zone (AZ) cells. Misexpression of IDA effectuates precocious floral abscission and ectopic cell separation in latent AZ cell regions, which suggests that negative regulators are in place to prevent unrestricted and untimely AZ cell separation. Through a screen for mutations that restore floral organ abscission in ida mutants, we identified three new mutant alleles of the KNOTTED-LIKE HOMEBOX gene BREVIPEDICELLUS (BP)/KNOTTED-LIKE FROM ARABIDOPSIS THALIANA1 (KNAT1). Here, we show that bp mutants, in addition to shedding their floral organs prematurely, have phenotypic commonalities with plants misexpressing IDA, such as enlarged AZ cells. We propose that BP/KNAT1 inhibits floral organ cell separation by restricting AZ cell size and number and put forward a model whereby IDA signaling suppresses BP/KNAT1, which in turn allows KNAT2 and KNAT6 to induce floral organ abscission.

INTRODUCTION

Cell separation processes are critical for the development of a plant and play key roles from sculpting the form of the plant to scattering seeds. Abscission, a physiological process that involves programmed changes in cellular adhesion, allows the plant to discard nonfunctional or infected organs. At the cellular level, the presence of an abscission zone (AZ), consisting of small densely cytoplasmic cells at the boundary between organ and plant, is a prerequisite for abscission to take place (McKim et al., 2008). During the subsequent activation of the cell separation process, AZ cells acquire competence to respond to abscission signals and secrete cell wall-modifying and hydrolyzing enzymes that act to degrade the middle lamella between two adjacent cell files (Bleecker and Patterson, 1997; Patterson, 2001; Roberts et al., 2002; Lewis et al., 2006; Stenvik et al., 2006). Shortly before organ shedding, the cells at the proximal side of the AZ expand; however, the relationship between AZ cell enlargement and organ separation is unclear (Patterson, 2001). It has been proposed that the functional role of AZ cell expansion might be to create the tension needed for the final mechanical rupture of the AZ (Sexton and Redshaw, 1981); indeed, the receptor-like kinase (RLK) EVERSHED (EVR) has recently been implicated in regulating the proper timing of floral organ abscission in Arabidopsis thaliana in part by restricting AZ cell size (Leslie et al., 2010).

The correct temporal and spatial regulation of abscission is crucial during plant development. Premature abscission of reproductive organs or immature seeds can compromise reproduction, and unrestricted cell separation can lead to shedding of organs in the proximity of activated AZ cells or interfere with tissue integrity. Studies using Arabidopsis have implicated the involvement of several different genes in the control of floral organ abscission (Aalen, 2011), including a newly identified putative peptide ligand-receptor system (Cho et al., 2008; Stenvik et al., 2008).

Recently, it was discovered that HAESA (HAE) and HAESA-LIKE2 (HSL2), a pair of leucine-rich repeat (LRR)-RLKs, are redundantly required for regulating cell separation during floral organ abscission in Arabidopsis (Cho et al., 2008; Stenvik et al., 2008). hae hsl2 double mutants are phenotypically similar to plants with mutations in INFLORESCENCE DEFICIENT IN ABSCISSION (IDA) (Cho et al., 2008; Stenvik et al., 2008). IDA
encodes a protein with features compatible with it being a peptide ligand (Stenvik et al., 2008) and is essential for the final separation step of floral abscission (Butenko et al., 2003). ida mutant plants are completely deficient in floral organ abscission, and overexpression of the IDA protein leads to premature abscission, an excessively enlarged floral AZ region and ectopic cell separation in vestigial AZs of organs that normally do not abscise in Arabidopsis (Stenvik et al., 2006). In addition, these plants secrete a white substance at organ detachment points consisting mainly of arabinogalactan (AG) (Stenvik et al., 2008). Genetic interaction studies demonstrated that functional receptors are essential for IDA to exert its function (Cho et al., 2008; Stenvik et al., 2008). In addition, genetic studies and in vitro kinase assays showed that the MITOGEN-ACTIVATED PROTEIN KINASE KINASE4 (MKK4) and MKK5 and MAPK3 and MAPK6 act downstream of IDA, HAE, and HSL2 (Cho et al., 2008). Consequently, there seems to be a core signaling pathway regulating cell separation in floral abscission.

A number of mutants have been identified that impediment abscission; the double mutant of the BLADE-ON-PETIOLE1 (BOP1) and BOP2 genes does not differentiate anatomically distinct AZ cells and is consequently completely deficient in abscission (Hepworth et al., 2005; Norberg et al., 2005; McKim et al., 2008). In addition, the recently described mutant reversed (nev) with a mutation in a gene encoding an ADP-riboseylation factor-GTPase-activating protein (Liljegren et al., 2009) has a complete lack of floral abscission. NEV has been implicated in regulating the proper timing of abscission by trafficking key factors required for cell separation and/or for the trafficking of receptor complexes (Liljegren et al., 2009). Interestingly, a suppression screen on nev identified mutations in the LRR-RLKs EVR and SOMATIC EMBRYOGENESIS RECEPTOR KINASE1 (SERK1), which in combination with nev showed precocious floral abscission and enlarged AZ regions reminiscent of those seen in plants overexpressing IDA (Leslie et al., 2010; Lewis et al., 2010). The mechanisms that prevent premature organ separation are largely unknown, but EVR and SERK1 may function as inhibitors of this step in the abscission process. However, both evr and serk1 single mutants have seemingly normal abscission and neither could rescue the abscission defect of ida or hae hsl2, indicating that other factors must be acting as repressors downstream in the IDA-HAE/HSL2 pathway.

To identify additional components of the IDA signaling pathway, we screened progeny of mutagenized ida-1 seeds (Butenko et al., 2003) for normal floral organ abscission. Here, we report the identification and analysis of new alleles of the KNOTTED-like homeobox gene (KNOX), BREVIPEDICELLUS (BP)/KNOTTED-LIKE FROM ARABIDOPSIS THALIANA1 (KNAT1), which suppresses the ida mutation. The bp mutants exhibit similar phenotypes to those observed in plants overexpressing IDA and were also capable of rescuing the abscission defect of hae hsl2. We therefore propose that BP/KNAT1 is important in regulating the correct timing of organ separation by restricting AZ cell size and is a component in the IDA-HAE/HSL2 signaling pathway. We show that two closely related genes in the class I KNOX subfamily, KNAT2 and KNAT6 (Scofield and Murray, 2006; Mukherjee et al., 2009), are likely to act as activators of floral organ separation in close connection with BP/KNAT1. The role of KNAT2 and KNAT6 in the floral abscission process and their genetic relationship to IDA is discussed.

RESULTS

A Suppressor Screen to Identify Components of the IDA Signaling Pathway

Wild-type floral organs abscise shortly after anthesis (Figures 1A and 1B), whereas the perianth and stamens remain attached indefinitely in null alleles of ida (Figures 1C to 1F). We reasoned that it should be possible to identify essential components of the IDA signaling pathway in a screen for suppressor mutants (Figure 1G). After ethyl methanesulfonate mutagenesis, M2 plants generated from mutagenized ida-1 seeds (Butenko et al., 2003) (Figures 1C and 1D) were screened for their ability to undergo floral abscission. Progeny from 13,000 M1 plants were analyzed, and 16 mutant lines were recovered. Three lines partially rescued the abscission defect of ida-1, whereas the remaining thirteen lines showed normal floral abscission. Two of the mutant lines (lines 49 and 595) identified had, in addition to normal organ shedding, the characteristic downward-pointing silique phenotype conferred by mutations in the KNOX gene BP/KNAT1 (Douglas et al., 2002; Venglat et al., 2002) (Figures 1H and 1J; see Supplemental Figure 1A online). An additional mutant line (line 221) portrayed a partial rescue of the ida-1 abscission defect and a less severe bending of the siliques (see Supplemental Figures 1B and 1C online). Crosses between the three mutant lines verified that they were in the same complementation group. Because the phenotypes of lines 49 and 595 were indistinguishable, analysis was performed using line 49.

The F2 progeny of line 49 crossed to C24 wild type segregated for wild type or ida phenotype and plants with a bp-like silique phenotype in a 3:1 ratio (n = 48, segregating 37:11), showing that the mutation in line 49 is fully recessive. Two of the 11 plants with the bp-like silique phenotype were wild-type for the ida alleles (Figure 1L), whereas four were homozygous for the T-DNA insertion of ida-1, confirming that the mutation in line 49 was responsible for the revertant floral phenotype observed.

Mutations in IDA block abscission at the stage when the AZ cells undergo separation; the early steps in the floral abscission process from AZ cell patterning and differentiation to initial organ loosening remain mainly unaffected (Butenko et al., 2003; Stenvik et al., 2006). A stress transducer was used to quantify the force needed to remove petals from the receptacle of the plant, the petal breakstrength (pBS), during the abscission process (Fernandez et al., 2000; Lease et al., 2006). Similar to the wild type, the ida-1 mutant has a high pBS, exceeding 2 g equivalents at positions 2 and 4 (referring to location when counting flowers from anthesis along the inflorescence, according to Bleecker and Patterson [1997]) (Figure 1N). ida-1 shows a gradual decrease in pBS from position 4 to 10, which is indicative of initial cell wall loosening, but the decline is delayed by two positions compared with wild-type flowers (Figure 1N) (Butenko et al., 2003). For ida-1, but not the wild type, there is a gradual increase in pBS from position 10, and flowers at position 20 have pBS similar to those at position 6 (Figure 1N). Line 49 had a pBS profile
Figure 1. Phenotypes of ida and bp Mutants.

(A) and (B) C24 wild-type (WT) plants undergo flower abscission shortly after anthesis, which defines position 1 when counting flowers along the primary inflorescence (arrowhead). At position 7 (left image in [B]) turgid petals and senescent sepals are still attached to the receptacle of the flower. The AZ region at position 12 (arrow) after floral organs have abscised is shown on the right in (B).

(C) to (F) ida-1 (C24; [C] and [D]) and ida-2 (Col; [E] and [F]) retain floral organs indefinitely. Position 7 (left in [D] and [F]) and position 12 (right in [D] and [F]).

(G) Cartoon illustrating suppression screen of ida-1. Revertant mutants portraying normal abscission could either be mutated downstream inhibitors (blunt arrowheads) in the IDA signaling pathway or constitutively active components (arrowheads) of the pathway.

(H) and (I) Line 49 with downward-pointing siliques and abscised floral organs by position 9 (arrowhead). AZ with floral organs attached at position 7 (left in [I]) and abscised at position 12 (right in [I]).

(J) and (K) bp-3 mutant with downward-pointing siliques and abscised floral organs by position 7 (left in [K]) and an enlarged AZ region at position...
similar to that of ida-1 until position 8 (Figure 1N); however, there was no increase in the pBS post position 8, and by position 9, all floral organs had abscised from line 49 (Figure 1).

pBS measurements are supported by anatomical observations in scanning electron micrographs of AZ cells during the course of floral abscission. pBS values above 2 are associated with the presence of broken cells at the fracture plane between the petal and the body of the plant due to high forces keeping the cell walls intact. This corresponds to flowers up to position 4, capable of responding to abscission signals such as ethylene (Butenko et al., 2003) but which still have their cell walls intact. pBS values under 1.5, observed from position 6, reveal a flattened fracture cavity, indicating an initial degradation of the middle lamella between two adjacent AZ cells and an initial cell wall loosening. Measurements below 0.5 are linked to initial rounding of lamella between two adjacent AZ cells and an initial cell wall fracture cavity, indicating an initial degradation of the middle lamella.

Figure 1. (continued).

To substantiate the ability of a mutation in BP/KNAT1 to rescue the abscission phenotype of ida, a Columbia (Col) null-allele deletion mutant of BP/KNAT1, bp-3 (Rim et al., 2009) (see Supplemental Figure 1G online), was crossed to ida-1 and ida-2 and both double mutants portrayed wild-type floral abscission (see Supplemental Figures 2A to 2C online). The observed phenotypes were substantiated by pBS (see Supplemental Figure 2F online), and mature AZs of the double mutants were covered with a white substance similar to that covering AZs of plants overexpressing IDA (see Supplemental Figures 2D and 2E online).

Given the confirmation of the molecular nature of line 49, it will from now on be referred to as bp ida-1 and the single mutant obtained from backcross to C24 as bp-10.

BP/KNAT1 Prevents Premature Floral Organ Abscission

The rescue of organ separation in bp ida-1, bp-3 ida-1, and bp-3 ida-2 flowers suggests an important role for BP/KNAT1 in the developmental control of floral organ abscission. Therefore, we first determined whether BP/KNAT1 was expressed in a temporal and spatial manner compatible with modulating floral abscission using a promoter:GUS (for β-glucuronidase) transgene (BP/KNAT1pro:GUS) according to Ori et al. (2000). GUS expression was first detected in the pedicels of the inflorescence apex and the replum of the gynoecium (see Supplemental Figure 3A online) as shown by Douglas et al. (2002), Alonso-Cantabrana et al. (2007), and Ragni et al. (2008). From flower position one, also defined as stage 13 by Smyth et al. (1990), there was weak GUS staining in the AZ and at the base of the pedicel (see Supplemental Figures 3B and 3C online). This low level of expression was maintained until position 4, from which point there was a strengthened GUS signal that reached a peak at position 8-10, corresponding to the phase at which organs detach, and was
BP/KNAT1 can have a role in both the initial stages of floral abscission, prior to organ dissociation, as well as regulating cell separation.

It has been suggested that BP/KNAT1 could be involved in regulating AZ cell formation (Wang et al., 2006). Loss-of-function bop1 bop2 double mutants lack floral organ abscission due to an inability to specify the anatomy of AZ cells (McKim et al., 2008) (see Supplemental Figure 3D online). We created bop1 bop2 bp-3 and bop1 bop2 bp ida-1 to investigate whether the absence of BP/KNAT1 would have an effect on the abscission phenotype of bop1 bop2 plants. Floral organ abscission was not reestablished in bop1 bop2 bp-3 and bop1 bop2 bp ida-1 plants (see Supplemental Figures 3E and 3F online). Thus, mutations in BP/KNAT1 are not sufficient to initiate AZ formation, suggesting that the reported enlargement of floral AZs (Wang et al., 2006) is dependent on the presence of morphologically distinct AZ cells.

Differences in pBS profiles between mutant and wild-type plants provide information as to where in the abscission process the mutation is exerting its effect (Patterson and Bleecker, 2004). The pBS of bp-3 and bp-10 was compared with that of Col wild-type and C24 wild-type flowers, respectively (n = 15, bars = SD).

To elucidate the role of BP/KNAT1 further, scanning electron microscopy was performed. Petal abscission was carefully observed for the Col wild type and bp-3 by either forceful removal of the petal or natural shedding at different stages of development (Figures 3A to 3C). Loss of BP/KNAT1 in bp-3 mutants affected the petal AZ fracture plane. In contrast with the wild type, which portrayed broken cells at positions 2 and 4, bp-3 had broken cells at position 2 but a smooth fracture plane by position 4, first observed for the wild type at position 6 (Figure 3A). Initial rounding of AZ cells was observed as early as position 5 for bp-3 (as shown for position 6 in Figure 3A), comparable to position 7 in the wild type (Figure 3A). The bp-3 mutant possessed fully rounded cells by position 7, similar to those seen in the wild type at position 8 (Figure 3A). The morphology of petal AZ cells was similar between bp-3 and the wild type at position 12 (Figure 3A).

When comparing scanning electron micrographs of the whole abscission region, it was apparent that at the time of organ separation for bp-3 (position 7), the discrete AZs found at the base of the receptacle in the wild type were less defined in the bp-3 mutant. The filament AZ was larger than that of the wild type and extended closer to the valves of the silique than in the wild type, while the petal and sepal AZs were closer together and extended into the pedicel (Figure 3B). After organ separation (position 8 and 12), the AZ cell expansion was more pronounced, making it difficult to discriminate the petal and sepal AZs from

Figure 2. BP/KNAT1 Involvement in Floral Abscission.
(A) BP/KNAT1::GUS expression during floral organ abscission. Arabic numerals indicate floral positions along the inflorescence. (B) pBS of bp-3 and bp-10 mutants compared with Col wild-type (wt) and C24 wild-type flowers, respectively (n = 15, bars = SD). (C) to (E) Mature siliques stained with the Yariv reagent β-GlcY, resulting in a red precipitate. (F) to (H) Mature siliques stained with the negative control α-GlcY.

then gradually diminished (Figure 2A). A GUS signal could be detected in the AZ as late as position 16, showing that BP/KNAT1 expression was maintained after the floral organs had abscised, indicating that BP/KNAT1 could also play a role after floral organ shedding. The initial augmentation in BP/KNAT1 expression at position 5-6 (Figure 2A) corresponded to the stage at which the pBS was markedly reduced in wild-type petals prior to organ shedding (Figure 1N) and coincides with the onset of IDA::GUS expression (Butenko et al., 2003). Moreover, the BP/KNAT1::GUS profile was similar to the reported expression patterns of HAE::GUS and HSL2::GUS (Jinn et al., 2000; Cho et al., 2008; Leslie et al., 2010). This expression pattern suggests that
Figure 3. Floral AZ Scanning Electron Micrographs of bp-3 and 35S:IDA

(A) Fracture planes of petal AZ (delineated in gray) of the wild type (wt), bp-3, and 35S:IDA at given floral positions. From position 6 onward, it is not possible to distinguish the different AZs for 35S:IDA plants, and consequently the whole AZ region is shown for all positions older than position 6.

(B) Entire AZ region of the wild type and bp-3 at given positions. Note that from position 8 onward in bp-3, it is not possible to distinguish the petal and sepal AZs from each other and the AZ region expands into the pedicel. The extended AZ region is delineated in gray.

(C) Entire AZ region of mature siliques. For bp-3 and 35S:IDA, it is not possible to indicate the different AZs.

f, filament AZ; p, petal AZ; s, sepal AZ. bp-3 displays a larger sepal AZ region on the abaxial side of the pedicel (arrowhead in [B] and [C]). Bars = 30 μm for petal AZ fracture planes and 100 μm for the entire AZ region.
one another (Figure 3B). Interestingly, the normal symmetry seen in wild-type AZs was lost in bp-3 mutants, and the sepal AZs on the abaxial side of the pedicel extended over a larger area (arrowhead in Figures 3B and 3C).

The resemblance between bp-3 and flowers overexpressing IDA, such as the enlargement of the AZ region and the early cell wall loosening (Stenvik et al., 2006; Cho et al., 2008; Leslie et al., 2010), led us to ask the question whether the structural changes in AZ cells occurring during the abscission process would follow a similar progression. Compared with the broken cells observed in bp-3, 35S:IDAI had a flattened fracture cavity at position 2 and plants abscised their floral organs by position 4 where fully rounded AZ cells could be observed (Figure 3A). By comparing the petal AZ cell density of bp-3 and 3SS:IDAI to the wild type (Table 1), we found that both have increased cell size compared with the wild type at the positions where they shed their floral organs. These results, combined with the reduced pBS levels, suggest that cell elongation is important in regulating floral organ abscission and that BP/KNAT1 plays a role in the proper timing of organ shedding by restricting cell expansion. In addition, we compared the height and width of petal AZs of bp-3 and 3SS:IDAI to those of wild-type plants at positions where the cell density between mutants and the wild type were approximately the same (Table 1). For both mutant and transgenic plants, there was a significant increase in size, indicating that the enlarged AZs observed (Figure 3C) are in part also due to an increase in AZ cell number.

A closer look at the AZ region revealed that bp-3 and bp-10 manifested a white substance surrounding the AZ region (Figures 1K and 1M). In 3SS:IDAI plants, rounded AZ cells are gradually covered by a white substance consisting of high amounts of AG (Stenvik et al., 2006). When wild-type AZs are treated with the synthetic chemical reagent β-1-glucosyl Yariv (β-GlcY) (Yariv et al., 1962, 1967), which stains AG proteins (AGPs) red, staining is apparent in AZ cells at position 10, corresponding to the stage of organ separation. It has therefore been suggested that AGP secretion is a normal part of the abscission process (Stenvik et al., 2006). To assess the commonalities between 3SS:IDAI and bp-3 further, mature siliques of bp-3 and bp-10 were treated with β-GlcY and compared with 3SS:IDAI (Figures 2C to 2E). The α-1-glucosyl Yariv does not bind AGPs and was used as a control (Figures 2F to 2H). This confirmed that the white substance covering the AZ region of bp-3 and bp-10 consisted in part of AGPs. Interestingly, a white substance was also observed at the base of bp-3 mutant pedicels, as previously reported for activated vestigial AZs in 3SS:IDAI plants (Stenvik et al., 2006). The similarity between bp-3 and 3SS:IDAI together with the rescue of organ separation in bp ida-1, bp-3 ida-1, and bp-3 ida-2 indicate that BP/KNAT1 and IDA act closely together to regulate floral organ abscission.

**BP/KNAT1 Is an Integral Component of the IDA-HAE/HSL2 Signaling Pathway**

As IDA has been suggested to be the ligand of HAE and HSL2 (Cho et al., 2008; Stenvik et al., 2008) we used genetics to position BP/KNAT1 relative to these genes. We created the triple mutant bp-3 hae hsl2, which showed an almost complete rescue of the floral abscission defect of hae hsl2 (Figures 4A and 4B) with only some stamens attached on few siliques (Figure 4D). ida-1 hae hsl2 plants are phenotypically identical to hae hsl2 and are completely deficient in abscission. However, quadruple mutants of bp ida-1 hae hsl2 (Figure 4C) had a similar but weaker rescue phenotype to that of bp-3 hae hsl2 plants, with a larger proportion of the siliques having unabscised stamens (Figure 4E). The weaker rescue phenotype of bp ida-1 hae hsl2 compared with bp-3 hae hsl2 can be explained if we assume that the bp-10 mutant, obtained from the backcross of bp ida-1 to C24, retains some inhibitory activity lost in the null mutant bp-3. There could additionally be an effect of combining ecotypes. The observed phenotypes were further quantified by pBS measurements (Figure 4H), and the mature AZs of both bp-3 hae hsl2 and bp ida-1 hae hsl2 were covered with a white substance (Figures 4F and 4G).

These results suggest that BP/KNAT1 could be a downstream component of the IDA-HAE/HSL2 signaling pathway acting to inhibit premature organ separation, possibly by regulating genes involved in controlling cell expansion and cell division. We therefore analyzed the expression pattern of BP/KNAT1, KNAT1, KNAT2, and KNAT6 in the AZs of ida-2 and hae hsl2 (see Supplemental Figures 4A and 4B online) to investigate whether IDA, HAE, and HSL2 were regulating BP/KNAT1 at the transcriptional level. No difference in the spatial or temporal distribution of BP/KNAT1 could be observed in the mutant background compared with the wild type (see Supplemental Figure 4C online), and the relative expression levels of BP/KNAT1 in ida and hae hsl2 AZ tissue were unaltered (see Supplemental Figure 4D online). This indicates that BP/KNAT1 could be regulated at the protein, rather than transcriptional, level by the IDA signaling pathway.

**The Inactivation of KNAT2 and KNAT6 Rescues the bp-3 Floral Abscission Phenotype**

BP/KNAT1 is known to restrict the expression of KNAT2 and KNAT6 to promote correct inflorescence growth (Ragni et al., 2008). The inactivation of KNAT6, but not KNAT2, partially rescues the pedicel orientation phenotype of bp; however, a complete rescue of the downward-pointing silique is seen in bp...
Therefore, we assayed the abscission phenotype of bp-3 in single and double mutants of knat2 and knat6. As expected, the downward orientations of siliques in bp-3 was only partially rescued in bp-3 knat6 and fully rescued in bp-3 knat2 knat6 (Figures 5B to 5D). No difference was apparent in the AZ region of bp-3 knat2 and bp-3 knat6 compared with bp-3 (Figures 5E and 5F). In both double mutants, the AZ was enlarged and covered with a white substance. However, pBS measurements suggest that there is a partial rescue of the early cell wall loosening of bp-3 in bp-3 knat6 (see Supplemental Figure 5A online), consistent for what is observed for the downward-pointing siliques phenotype. Compared with each of the double mutants, bp-3 knat2 knat6 triple mutant plants had delayed floral organ separation and the floral

Figure 4. Genetic Interaction of BP/KNAT1, HAE, and HSL2. (A) hae hsl2 mutants with deficiency in floral abscission (B) to (G) bp mutants rescue the abscission defect of hae hsl2. bp-3 hae hsl2 main inflorescence (B), siliques at position 12 (D), and AZ from mature siliques (F). bp ida-1 hae hsl2 main inflorescence (C), siliques at position 12 (E), and AZ from mature siliques (G). (H) pBS of bp-3 hae hsl2 compared with hae hsl2 and Col wild type (wt). pBS by position (n = 15, bars = SD). [See online article for color version of this figure.]

Figure 5. Phenotypes of knat2 and knat6 in bp-3 and 35S:IDA. (A) Phenotype of knat2 knat6 with organs attached at position 15 (arrow). (B) to (H) Genetic interaction phenotypes of knat2 and knat6 with bp-3. bp-3 knat2 (B) and (E), bp-3 knat6 (C) and (F), and bp-3 knat2 knat6 (D), (G), and (H). Inactivation of knat6 but not knat2 partially rescues the siliques phenotype of bp-3 but not the enlarged AZ region. In bp-3 knat2 knat6 triple mutants, siliques display wild-type orientation and a normal-sized AZ region with attached floral organs (arrow in D). (I) and (J) Overexpression phenotypes of 35S:IDA are not apparent in the knat2 knat6 mutant background. Note floral organs attached in 35S:IDA knat2 knat6 (arrow). (K) to (N) Siliques at position 7 have attached floral organs in 35S:IDA knat2 knat6 plants and normal-sized AZs (K) and (M) compared with 35S:IDA (L) and (N). (O) Mature 35S:IDA knat2 knat6 AZ with enlarged AZ region. [See online article for color version of this figure.]
organs remained attached until at least position 15 (Figures 5D and 5G). Although no floral abscission defect has been reported for knat2 knat6 double mutants (Belles-Boix et al., 2006; Ragni et al., 2008), we observed a delay in floral abscission (Figure 5A), which was accentuated with increased light intensity and which could not be distinguished from that in bp-3 knat2 knat6 plants (Figure 5D). The forceful removal of floral organs from bp-3 knat2 knat6 siliques revealed a normal AZ region with no apparent enlargement or secretion of white substance (Figure 5H). The same phenotypes were observed for bp-3 ida-2 knat2 knat6 plants. Thus, the involvement of BP/KNAT1 in regulating the correct timing of floral organ separation is likely dependent on its restrictive action on KNAT2 and KNAT6.

knat2 and knat6 Suppress the 35S:IDA Phenotype

Since we observed that the premature time course of floral abscission in bp-3 was rescued in bp-3 knat2 knat6 triple mutants and that bp-3 was capable of rescuing the abscission defect of ida, we examined the genetic interaction between the double mutant of knat2 knat6 and a transgene overexpressing IDA. If the IDA signaling pathway negatively regulates BP/KNAT1 and the restriction of KNAT2 and KNAT6 by BP/KNAT1 is important for the correct timing of floral abscission, we hypothesized that the untimely organ loss in 35S:IDA plants would be delayed in the knat2 knat6 double mutant background.

In addition to the early floral abscission and enlarged AZ region, plants overexpressing IDA also have ectopic abscission of other organs, reduced stature, small siliques, and early senescence (Stenvik et al., 2006) (Figure 5J). Plants overexpressing IDA in the knat2 knat6 double mutant background showed a wild-type phenotype at the whole-plant level (Figure 5I). The small siliques, which often senesce, on 35S:IDA plants were absent in 35S:IDA knat2 knat6. Moreover, the precocious floral abscission of 35S:IDA plants, where all organs are shed by position 4 (Stenvik et al., 2006), was substantially delayed in 35S:IDA knat2 knat6 plants, and floral organs remained attached to the receptacle of the siliques until at least position 15 (Figures 5K and 5L). The excessive AZ enlargement apparent in siliques of 35S:IDA plants at position 7 (Figures 5L and 5N) was not observed in 35S: IDA knat2 knat6 (Figure 5M). However, as the siliques matured further, it was apparent that the AZ region of 35S:IDA knat2 knat6 plants became larger than that of the wild type (Figure 5O). The relative expression levels of IDA in 35S:IDA knat2 knat6 compared with the wild type verified that the observed phenotypes were not due to silencing of the transgene (see Supplemental Figure 5B online). Taken together, these results show that the phenotypes of 35S:IDA plants are suppressed in the knat2 knat6 mutant background and substantiates the hypothesis that the IDA signaling pathway is involved in the regulation of these genes through BP/KNAT1.

KNAT2 and KNAT6 Are Transcriptionally Regulated by IDA, HAE, and HSL2

Since it has been shown that BP/KNAT1 restricts the expression of KNAT2 and KNAT6 in pedicels (Ragni et al., 2008), we examined the expression patterns of KNAT2 and KNAT6 in ida-1, hae hsl2, and bp mutants during the different stages of floral organ abscission. We first examined KNAT2 and KNAT6 expression in the AZ of flowers along the whole length of the inflorescence using promoter:GUS transgenes (KNAT2pro:GUS and KNAT6pro:GUS) previously described by Ragni et al. (2008). In wild-type flowers, both KNAT2 and KNAT6 were expressed in the AZ region from early floral stages and up to the point of floral organ separation (Figures 6A and 6B). Both had an enhanced expression level at the stage of organ detachment, corresponding to position 10 for KNAT2 in the C24 wild-type background (Figure 6A) and position 6-8 for KNAT6 in the Wassilewskija (Ws) background (Figure 6B). KNAT6 expression was restricted to the sepal, petal, and filament AZs in addition to the dehiscence zone of the siliques, whereas KNAT2 had a broader spatial distribution covering the entire AZ region (Figures 6A and 6B). By contrast, in the ida-1 and hae hsl2 AZs, the GUS expression of KNAT2 and KNAT6 was markedly reduced or totally absent, respectively (Figures 6C and 6D). The presence of KNAT2pro:GUS expression at the base of ida-1 and hae hsl2 floral buds and KNAT6pro:GUS expression in the vasculature of emerged lateral roots (Figures 6C and 6D), as previously reported for wild-type plants (Dean et al., 2004; Ragni et al., 2008), indicates that the expression of these genes in the mutant backgrounds is extenuated only in cells where IDA, HAE, and HSL2 are normally expressed. Thus, during floral abscission, the expression of KNAT2 and KNAT6 in AZ tissue is dependent on the presence of IDA, HAE, and HSL2. Moreover, in the bp mutant background, an elevated level of KNAT2 and KNAT6 expression was observed in the AZ (Figures 6C and 6D), indicating that BP/KNAT1 restricts the expression of KNAT2 and KNAT6 in AZ tissues in a similar way as in the pedicels.

Misexpression of KNAT2 and KNAT6 Rescues the Floral Abscission Defect of ida

To extend further the genetic interaction study between IDA, KNAT2, and KNAT6, plants overexpressing KNAT2 under an inducible cauliflower mosaic virus 35S promoter in the Landsberg erecta ( Ler) wild-type background (Pautot et al., 2001) and a constitutively expressed KNAT6 transgene in Col wild type (Figure 7A) were crossed into the ida-1 mutant background. As expected if KNAT2 and KNAT6 are acting downstream in the IDA signaling pathway and are positive regulators of organ separation, misexpression of either gene in ida-1 restored floral organ abscission (Figures 7B and 7C). Given that high levels of KNAT2 expression leads to homeotic conversions of floral tissue (Pautot et al., 2001), we selected for plants with elevated expression levels of KNAT2 that did not have severe developmental effects (see Supplemental Figure 6A online). pBS measurements for 35S:KNAT2 ida-1 showed a similar reduction compared with ida-1 as that seen in the bp-3 ida-1 double mutants (Figure 7D; see Supplemental Figure 2F online); however, it is possible that the contribution of the Ler genome had an effect in the pBS. In addition, consistent with the pBS measurements showing a partial rescue of the early cell wall loosening of bp-3 in bp-3 knat6 double mutants (see Supplemental Figure 5A online), plants overexpressing KNAT6 have lower pBS at all measurable positions compared with the Col wild type (see Supplemental Figure.
However, the AZ region is morphologically similar to that of the wild type (see Supplemental Figure 6C online). Taken together, these results indicate that BP/KNAT1 acts as a negative regulator and KNAT2 and KNAT6 act as positive regulators of floral organ separation in the IDA signaling cascade.

**DISCUSSION**

**The Role of BP/KNAT1 in Floral Organ Abscission**

In this article, we report on the characterization of a new mutant allele of BP/KNAT1, bp-10, and reveal a role for BP/KNAT1 as a negative regulator of floral organ abscission. BP/KNAT1 belongs to the super-class of TALE (three amino acid loop extension) HD proteins and is known to modulate various aspects of plant development, mainly by regulating cell growth and differentiation (Hamant and Pautot, 2010; Hay and Tsiantis, 2010).

We discovered a role for BP/KNAT1 in abscission via a genetic screen for suppressors of the ida-1 abscission deficiency. AZ cells differentiate early in floral development; however, loss of floral organs cannot be induced prior to anthesis, indicating that shortly after pollination the AZ cells become competent to respond to abscission signals (Patterson, 2001). Ethylene can expedite the abscission process (Van Doorn, 2002), suggesting that the timing of abscission can be modulated. IDA can induce cell separation from the time when AZ cells are able to respond to organ shedding signals, as seen by misexpression of IDA, which effectuates precocious floral abscission (Butenko et al., 2003; Stenvik et al., 2006). As described below, our results indicate that BP/KNAT1 acts as a negative regulator of floral abscission by restricting AZ cell size and number and is likely suppressed by IDA signaling to allow for the progression of abscission.

In wild-type flowers, floral organ separation is preceded by a reduction in pBS that correlates with an initial rounding of AZ cells (Patterson, 2001). Loss of BP/KNAT1, as seen in the bp-3 mutant, causes a substantial reduction in pBS at positions 2 and 4 (Figure 2B) and abscission of floral organs one position earlier than in wild-type inflorescences. In addition, compared with the wild type, the mutant has a significant (P < 0.002, Student’s t test) enlargement of AZ cells at the time of organ separation, also seen for 35S:IDA (Table 1). Although it is not completely clear what role cell expansion plays in the regulation of organ separation (Patterson, 2001), it is apparent that it correlates with the correct timing and the extent of the process. In Arabidopsis, the level of EXP10 transcript, which encodes an expansin originally identified by its involvement in cell elongation (McQueen-Mason et al., 1992), regulates the degree of pedicel separation (Cho and Cosgrove, 2000). The loss of the LRR-RLK EVR shows increased floral AZ cell size and premature floral abscission in the

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**Figure 6.** KNAT2 and KNAT6 Expression in ida-1, hae hsl2, and bp during Floral Abscission.

(A) KNAT2 promoter-driven GUS expression during floral organ abscission in the C24 wild type.

(B) KNAT6 promoter-driven GUS expression during floral organ abscission in the Ws wild type.

(C) KNAT2 promoter-driven GUS expression in ida-2, hae hsl2, and bp-3 AZs for flowers at position 10. GUS activity at the base of wild-type (wt) floral buds was unaltered in the ida-2 and hae hsl2 mutant backgrounds.

(D) KNAT6 promoter-driven GUS expression in ida-2, hae hsl2, and bp-9 (Ragni et al., 2008) AZs. GUS activity in the vasculature of emerging wild-type (wt) lateral roots was unaltered in the ida-2 and hae hsl2 mutant backgrounds.

Arabic numerals indicate floral positions along the inflorescence.
Interestingly, EVR is downregulated in bp mutants (Wang et al., 2006); however, unlike bp ida-1 and bp-3 ida-2 (Figure 1H; see Supplemental Figure 2 online), evr ida-2 mutants have defective abscission (Leslie et al., 2010). Thus, EVR inhibits cell elongation and abscission independently of IDA, but BP/KNAT1 may mediate crosstalk between the IDA-HAE/HSL2 and the EVR pathways (Figure 8). In some plant species, cells in the separation layers undergo cell division prior to abscission (Sexton and Roberts, 1982; Van Doorn and Stead, 1997) and so the increased number of petal AZ cells seen in the bp-3 mutant (Table 1) could also contribute to the early abscission observed, as is the case for mutants of SERK1 in the nev background (Lewis et al., 2010). The protein phosphatase KAPP dephosphorylates HAE and returns the receptor to an off state (Stone et al., 1994). The signal from IDA-HAE/HSL2 is suggested to be transduced via a MAPK cascade that includes MKK4, MKK5, MPK3, and MPK6. Genetic evidence suggests that BP/KNAT1 acts as a negative downstream component of this signaling pathway by restricting cell wall degradation and consequently cell separation and the expression of KNAT2 and KNAT6. Upon activation of the IDA signaling pathway, the BP/KNAT1 restriction of KNAT2 and KNAT6 is elevated, and these transcription factors can act as positive regulators of floral organ separation. In addition, BP/KNAT1 is a positive regulator of EVR, which influences cell elongation by a separate parallel pathway (Leslie et al., 2010).
plants have stronger phenotypes than do bp
apparent only under certain growth conditions and why
35S
and were substantially delayed in abscission compared with
35S
development is observed in
pedicel tissue, suggesting that the transport of the
bp-3
two polygalacturonase-encoding genes ARABIDOPSIS DEHIS-
tation in other proteins in the AZ, such as BEL1-like (BELL) proteins
Bellau et al., 2001). Whereas the defect in pedicel cell differ-
entiation in bp mutants has been attributed in part to the
misregulation of genes involved in lignin biosynthesis (Mele
et al., 2003), it is possible that the cellular defects observed in
bp-3 and bp-10 during abscission affect the transcription of cell
wall remodeling enzymes or genes that act to regulate cell wall
remodeling enzymes. Since polygalacturonases have been shown
to play an important role in cell separation during abscission, it
would be interesting to monitor the expression levels of the
two polygalacturonase-encoding genes ARABIDOPSIS DEHIS-
CENCE ZONE POLYGALACTURONASE2 and QUARTET2 in bp
mutants, as mutations in these genes delay floral abscission
González-Carranza et al., 2007; Ogawa et al., 2009). Since the
functions of half of the TALE protein members remain unknown
Hamant and Pautot, 2010), it will be interesting to investigate if
any have an abscission defect similar to that of bp-3.

The Role of KNAT2 and KNAT6 in Floral Organ Abscission

The inflorescence phenotypic features of the bp mutant correlate with the
misexpression of both KNAT2 and KNAT6. Mutations in
KNAT6 can partially rescue the defects of bp, and normal pedicel
development is observed in bp knat2 knat6 mutants (Ragni et al.,
2008). It has been proposed that BP/KNAT1 could directly
regulate KNAT2 and KNAT6 expression, as motifs nearly identi-
tical to the BP/KNAT1 binding site were found in KNAT2 and
KNAT6 (Ragni et al., 2008); indeed, the expression levels of these
genes is enhanced in floral AZs of bp mutants (Figures 6C and
6D). The triple mutant bp-3 knat2 knat6 and the quadruplet mutant
bp-3 ida-2 knat2 knat6 develop normal-sized AZs (Figures 5D
and 5H) and are delayed in floral abscission (Figure 5G). This shows
that KNAT2 and 6 have antagonistic effects to those of BP/KNAT1
during floral abscission and suggests that KNAT2 and 6 are
positive regulators of cell separation during floral abscission. It is
tempting to speculate that KNAT2 and/or KNAT6 directly regulate
AZ cell size. In part, this appears to be correct, in that plants
overexpressing IDA in the knat2 knat6 mutant background had
smaller AZs compared with 3SS:IDA plants (Figures 5M and 5N)
and were substantially delayed in abscission compared with 3SS:
IDA (Figures 5K and 5L) and the wild type. However, in mature
siliques of 3SS:IDA knat2 knat6, an increased AZ region was
observed (Figure 5O) and 3SS:KNAT6 plants show normal-sized
AZs (see Supplemental Figure 6C online), indicating that additional
factors must be in place to determine AZ cells size. This would also
explain why the defect in floral abscission in knat2 knat6 mutants is
apparent only under certain growth conditions and why 3SS:IDA
plants have stronger phenotypes than do bp mutants.

Integration of the KNOX I Protein Family in the IDA
Signaling Pathway

The isolation and characterization of bp-10 in addition to the
genetic and physiological analysis of bp-3 allow us to expand the
current model for IDA signaling. Our screen was designed to
identify components of IDA signaling as revertants of the ida-1
mutant abscission phenotype. As could be expected for negative
downstream intermediates in this signaling pathway, bp-3 muta-
tions had features in common to 3SS:IDA plants: precocious
cell wall loosening and cell elongation, increased AZ size, early
abscission, and secretion of AGP. Moreover, both bp-3 and bp-
to could rescue the abscission defect of haes2 (Figures 4B and
4C). However, when comparing PBS between bp-3 and Col wild
type or between bp-10 and C24 wild type, it was apparent that
cell wall loosening was more accentuated in bp-3 than in bp-10
(Figure 2B). Moreover, the extent of rescue seen for bp ida-1 and
bp ida-1 haes2 was less than that observed for crosses with
bp-3. This could be due to the molecular nature of bp-10
compared with bp-3. The MEINOX domain, which for KNOX
proteins is divided into two subdomains (KNOX1 and KNOX2)
Bürglin, 1997), is necessary for dimer formation with BELL
proteins (Hay and Tsiantis, 2010) like PENNYWISE, which inter-
acts with BP/KNAT1 and is thought to participate in repression of
KNAT2 and KNAT6 (Smith and Hake, 2003; Ragni et al., 2008;
Hay and Tsiantis, 2010). In bp-10, it is possible that a truncated
protein with the KNOX1 domain, which has been shown to
function as a repressor of transcription (Nagasaki et al., 2001),
could be present. If so, and the truncated protein in part restricts
KNAT2 and KNAT6, this could explain that the bp-10 allele is
weaker than bp-3, which is a deletion mutant.

The findings that expression of KNAT2 and KNAT6 in AZ cells
of ida and haes2 mutants was notably reduced and absent,
respectively (Figures 6C and 6D), in addition to the complete
rescue of the enlarged AZ region of bp-3 by the knat2 knat6
mutant, the reduction of 3SS:IDA phenotypes in knat2 knat6, and
the rescue of ida-1 by overexpression of KNAT2 and 6 allow us to
propose the following signaling pathway (Figure 8): A posttrans-
lationally modified IDA protein (Stenvik et al., 2008) binds the LRR
of either a homo or heterodimer including HAE and/or HSL2 and
induces a MAPK signaling cascade that acts to regulate BP/
KNAT1. Since the transcriptional levels of BP/KNAT1 was unal-
tered in ida and haes2 (see Supplemental Figure 4 online), the
regulation is likely to be at the protein level and could involve the
interaction of BP/KNAT1 with another HD protein. BP/KNAT1
can then have a dual role both in regulating the expression of
EVF and in restricting the expression of KNAT2 and KNAT6, which
in turn will positively regulate the transcription of genes involved
in cell separation.

Future experiments investigating the cellular and subcellular
localization of BP/KNAT1 during wild-type abscission compared
with ida will likely give us information as to how the IDA signaling
pathway is regulating the action of BP/KNAT1. Interestingly in the
postabscission stages, the sepal AZ of bp-3 mutants was not
symmetrical and was larger on the abaxial side of the pedicel,
indicating that at least in sepal AZ cells BP/KNAT1 could act
differentially, similarly to what is seen for pedicel development
Venglat et al., 2002). This opens the possibility that IDA could
induce posttranslational modifications on BP/KNAT1 to regulate its trafficking in AZ cells.

METHODS

Growth Conditions

Arabidopsis thaliana plants were grown on soil in growth chambers at 22°C under long days (16 h day/8 h dark) at a light intensity of 100 μE·m⁻²·s⁻¹. The floral abscission defect of the knat2 knat6 double mutant was monitored at light intensities of 100 μE·m⁻²·s⁻¹ and 250 μE·m⁻²·s⁻¹.

Ethyl Methanesulfonate Mutagenesis and Suppressor Mutant Screen

Approximately 50,000 ida-1 (C24) seeds were treated with 0.3% ethyl methanesulfonate (Sigma-Aldrich) for 16 h at room temperature in the dark. Seeds were then washed twenty times with deionized water, resuspended in 0.01% agar, and transferred to soil. Stratification was done for 1 week at 4°C, and plants were subsequently grown under standard conditions. M1 plants were harvested in pools of 10 plants. The frequency of chlorophyll mutants was 2%, determined by screening 1200 M2 plants.

The mutation points of line 49, line 595, and line 221 were determined by sequencing of their BP/KNAT1 gene region amplified with KNAT1 F and KNAT1 R primers.

Primers

Sequences of all primers used can be found in Supplemental Table 1 online.

Plant Material and Genetic Crosses

Mutant alleles and transgenic plants described previously include ida-1 (C24) (Butenko et al., 2003), ida-2 (Col) (Cho et al., 2008), hae hsl2 (Col) (Cho et al., 2008; Stenvik et al., 2008), bop1 bop2 (Col) (Hepworth et al., 2005; Norberg et al., 2005), 35S:IDA (C24) (Stenvik et al., 2006), knat2 knat6, KNAT2pro:GUS (C24), KNAT6pro:GUS (Ws and Col), KNAT6pro:GUS bp-9 (Ragni et al., 2008), and 35S:KNAT2 (Ler) (Pautot et al., 2001). The transgenic Col line BP:KNAT1pro:GUS (N6141) (Ori et al., 2000) was obtained from the Nottingham Arabidopsis Stock Centre, and bp-3 (Col) (Rim et al., 2009) was kindly provided by Jae-Yeon Kim. The bp-10 allele was generated through the backcross between bp ida-1 and C24. Wild-type plants with subsequent genotyping with ida-1 genotyping primers and GUS64L. Homozygous BP:KNAT1pro:GUS, KNAT2pro:GUS, and KNAT6pro:GUS lines were crossed to ida-1 or ida-2 and hae hsl2. F2 plants with deficiency in abscission were selected for GUS staining. Homozygous KNAT2pro:GUS was crossed to bp-3 and F2 plants with downward-pointing silicles were selected for GUS staining. bp-3 was crossed to ida-1, ida-2, hae hsl2, bop1 bop2, and knat2 knat6, respectively. Double and triple mutants were verified by PCR with the corresponding genotyping primers and Lbb1 (or GUS64L for ida-1). bp ida-1 was crossed to hae hsl2 and bop1 bop2. Quadruple mutants were verified by PCR with the corresponding genotyping primers, Lbb1, and cleaved-amplified polymorphic sequence marker (see Supplemental Table 1 online). knat2 knat6 was crossed to bp3, bp-3 ida-2, and 35S:IDA, respectively, bp-3 knat2, bp-3 knat6, bp-3 knat2 knat6, bp-3 ida-2 knat2, bp-3 ida-2 knat6, bp-3 ida-2 knat2 knat6, and 35S:IDA knat2 knat6 were selected by PCR in the F2 or F3 population with corresponding genotyping primers. 35S:KNAT2 and 35S:KNAT6 were crossed to ida-1, and the transgenes in ida-1 were selected by phenotype, PCR, and fluorescent selection for 35S:KNAT6 (described by Bensmihen et al., 2004).

Generation of Transgenic Plants

For the complementation test, the genomic BP/KNAT1 region was amplified by PCR from BAC F9M13 with the primers attB1KNAT1 and attB2KNAT1 with additional gateway attB sequences at the 5’ ends and introduced into the pDONR/Zeo gateway vector (Invitrogen). The 3.2-kb fragment was thereafter recombined through the entry clone into the pHTW32 vector (Karimi et al., 2002). The construct was transferred to the Agrobacterium tumefaciens strain C58C1 pGV2260, and bp ida-2 plants and C24 wild-type plants were transformed using the A. tumefaciens–mediated floral dip method (Clough and Bent, 1998). Transgenic plants were selected for resistance on medium (Murashige and Skoog, 1962) with 5μg/mL hygromycin. Thirty-six out of 54 transformants showed the characteristic lobed leaf phenotype of 35S:KNAT1 plants.

The KNAT6 cDNA was amplified using primers specific to KNAT6 and cloned into the pCR II TOPO blunt vector (Invitrogen). Subsequently, the primers attB1KNAT6 and attB2KNAT6 were used to amplify KNAT6 and recombine the gene into pDONR201 (Invitrogen). KNAT6 was thereafter introduced by recombination into the binary vector destination alligator 2 vector by Gateway recombination. This vector allows for the selection of transgenic Arabidopsis seeds via GFP expression driven by the A0236 seed-specific promoter by Bensmihen et al. (2004). The new vector called all2KNAT6, harboring the HA-KNAT6 fusion under the double enhanced cauliflower promoter, was introduced into A. tumefaciens strain C58, and C24 wild-type plants were transformed using the A. tumefaciens–mediated floral dip method (Clough and Bent, 1998).

Histochemical GUS Assay and β-GlucY Staining

GUS staining, postfixation, and whole-mount clearing preparations of various plant tissues were performed as described (Grini et al., 2002) and inspected with a Zeiss Axioplan2 imaging microscope equipped with differential interference contrast optics and a cooled Axiocam camera imaging system.

β-GlucY (Biosupplies) was used to test for the presence of AGPs, whereas α-GlucY (Biosupplies) was used as a negative control. Plant tissues were treated as previously described (Stenvik et al., 2006).

Quantitative Real-Time RT-PCR

Total RNA was extracted from rosette leaves and floral AZs (position 4 to 8) using the spectrum plant total RNA kit (Sigma-Aldrich) according to the manufacturer’s recommendations. An optional on-column DNase digestion step was included. cDNA synthesis and real-time RT-PCR were performed as described previously (Grini et al., 2009). Gene amplification was performed using the primers denoted in Supplemental Table 1 online. For all samples, cdNAS were normalized using RCAR1 (At1g01360) and primers CAB F and CAB R.

Breakstrength Measurement

pBS was quantified as the force in gram equivalents required for removal of a petal from a flower (Butenko et al., 2003) and was performed as described by Stenvik et al. (2008). Breakstrength was measured for 15 plants and a minimum of 20 measurements at each position.

Scanning Electron Microscopy

Plant tissues for electron microscopy were fixed and dehydrated as described previously (Butenko et al., 2003) and critical point dried using a Bal-Tec CPD030 critical point dryer. Specimens were mounted on carbon tape and sputter coated with gold palladium using a Cressington Coating System 308R. Samples were viewed at a 5K accelerating voltage on a...
S-4800 field emission scanning electron microscope (Hitachi). AZ measurements were performed using NIH Image software. To determine the cell density of petal AZs, cells that fell completely or partially within a 1600 \( \mu \text{m}^2 \) area in a petal AZ were counted, and a minimum of four flowers were used; for 3SS:IDA flowers older than position 4, it was not possible to distinguish individual petal AZ and an area of 1600 \( \mu \text{m}^2 \) was used; for 35S measurements were performed using NIH Image software. To determine the number of petal AZs, the height and width of petal AZs were measured at positions with similar cell density.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers At1g68765 (IDA), At4g28490 (HAE), At5g65710 (HSL2), At4g08150 (BP/KNAT1), At1g70510 (KNAT2), At1g23380 (KNAT6), At3g657130 (BOP1), and At2g41370 (BOP2).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Phenotypes and Mutation Points of Revertant Lines.

Supplemental Figure 2. Genetic Interaction of BP/KNAT1 and IDA.

Supplemental Figure 3. BP/KNAT1 Expression and Genetic Interaction with bop1 bop2.

Supplemental Figure 4. BP/KNAT1 Expression in ida-2 and hae hsl2.

Supplemental Figure 5. Rescue of Precocious bp-3 Cell Loosening by knat2 and knat6.

Supplemental Figure 6. Contribution of 3SS:KNAT2 and 3SS:KNAT6 to Floral Abscission.

Supplemental Table 1. Primers.

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Arabidopsis Class I KNOTTED-Like Homeobox Proteins Act Downstream in the IDA-HAE/HSL2 Floral Abscission Signaling Pathway

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