The EPIP Peptide of INFLORESCENCE DEFICIENT IN ABSCISSION Is Sufficient to Induce Abscission in Arabidopsis through the Receptor-Like Kinases HAESA and HAESA-LIKE2

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ABSCISSION is sufficient to induce abscission in Arabidopsis through the receptor-like kinases HAESA and HAESA-LIKE2.

In Arabidopsis thaliana, the final step of floral organ abscission is regulated by INFLORESCENCE DEFICIENT IN ABSCISSION (IDA): ida mutants fail to abscise floral organs, and plants overexpressing IDA display earlier abscission. We show that five IDA-LIKE (IDL) genes are expressed in different tissues, but plants overexpressing these genes have phenotypes similar to IDA-overexpressing plants, suggesting functional redundancy. IDA/IDL proteins have N-terminal signal peptides and a C-terminal conserved motif (extended PIP [EPIP]) at the C terminus (EPIP-C). IDA can, similar to CLAVATA3, be processed by an activity from cauliflower meristems. The EPIP-C of IDA and IDL1 replaced IDA function in vivo, when the signal peptide was present. In addition, synthetic IDA and IDL1 EPIP peptides rescued ida and induced early floral abscission in wild-type flowers. The EPIP-C of the other IDL proteins could partially substitute for IDA function. Similarly to ida, a double mutant between the receptor-like kinases (RLKs) HAESA (HAE) and HAESA-LIKE2 (HSL2) displays nonabscising flowers. Neither overexpression of IDA nor synthetic EPIP or EPIP-C peptides could rescue the hae hsl2 abscission deficiency. We propose that IDA and the IDL proteins constitute a family of putative ligands that act through RLKs to regulate different events during plant development.

INTRODUCTION

The cell wall provides plant cells with strength and rigidity but also constrains the activity and autonomy of individual cells. Most cells remain attached to adjacent cells throughout their life cycle, but in some situations, it is crucial for the plant to break that attachment. Coordinated breakdown of cell-to-cell adhesion plays a key role throughout the life cycle of plants, and separation events may be triggered by environmental, hormonal, or developmental signals (Roberts et al., 2002; Lewis et al., 2006). Cell separation, cell wall loosening, and cell wall degradation are critical aspects of processes like cell elongation, sloughing of cells at the root tip, formation of vascular tissue, stomata, and hydathodes, root emergence, and organ shedding (Roberts et al., 2002). Although the biochemical mechanisms that lead to loss of adhesion between cells of specific organs probably are similar, the signals that induce these changes are likely to be different so that such developmental events only take place at the right time and at the right location.

Plants may shed entire organs for many reasons: if they no longer serve a function, for dispersal or propagation, to aid pollination, or as a defense mechanism. Due to the diverse life styles of plants and their adaptations to different environments, different species have evolved distinct patterns of organ loss (Sexton and Roberts, 1982). For example, Arabidopsis thaliana displays abscission only of floral organs and seeds (Patterson, 2001). A prerequisite for abscission to take place is the presence of an abscission zone (AZ), consisting of small densely cytoplasmic cells that can respond to abscission signals (Sexton and Roberts, 1982; Patterson, 2001; Stenvik et al., 2006). A prerequisite for abscission to take place is the presence of an abscission zone (AZ), consisting of small densely cytoplasmic cells that can respond to abscission signals (Sexton and Roberts, 1982; Patterson, 2001; Stenvik et al., 2006). In Arabidopsis, the formation of the AZ is dependent on BLADE-ON-PETIOLE1 (BOP1) and BOP2 (McKim et al., 2008), while INFLORESCENCE DEFICIENT IN ABSCISSION (IDA) is essential for the final separation step of abscission (Butenko et al., 2003). ida mutant plants are deficient in abscission, and overexpression of the IDA protein leads to premature floral organ abscission and ectopic cell separation in vestigial AZs of organs that normally do not abscise in Arabidopsis (Stenvik et al., 2006).

The IDA protein has an N-terminal signal sequence that can direct proteins through the secretory pathway (Butenko et al., 2003; Aalen et al., 2006), suggesting that it is involved in cell-to-cell communication. One way in which plant cells can perceive and respond to external signals is through the interaction of extracellular peptides with plasma membrane–bound receptor-like kinases (RLKs). Since the first identification of a plant RLK in maize (Zea mays; Walker and Zhang, 1990), >400 genes with
receptor configurations have been classified in Arabidopsis (Shiu and Bleecker, 2001b). However, only a handful of these have been assigned a function and in even fewer cases has it been possible to establish a genetic interaction between receptor and peptide encoding genes (reviewed in Torii, 2004). We have proposed that IDA could function as a signaling peptide, possibly interacting with the Leu-rich repeat (LRR) RLK HAESA (HAE). HAE is expressed in the floral organ AZs and has been implicated in regulating floral abscission (Jinn et al., 2000; Butenko et al., 2003; Torii, 2004).

There are two HAESA-LIKE (HSL) genes in the LRR XI RLK subfamily that are closely related to HAE and share similar extracellular LRR and cytoplasmic Ser/Thr protein kinase domains (Shiu and Bleecker, 2001b). IDA also belongs to a multi-gene family with five additional IDA-LIKE (IDL) Arabidopsis genes. The IDL genes are differentially expressed and encode small proteins (<100 amino acids), all with predicted N-terminal secretion signals (Butenko et al., 2003). The proteins encoded by IDL genes in Arabidopsis and other plant species share a conserved 12–amino acid PIP motif near their C termini (Butenko et al., 2003).

To explore the biological function of the IDL genes and their possible similarities in function, we investigated both their expression patterns by promoter-reporter gene constructs and their overexpression phenotypes. Furthermore, to dissect the functional contribution of the different regions of IDA and the IDL proteins, especially the conserved C-terminal region, the \textit{ida} mutant was complemented with several gene and domain swap constructs. In addition, \textit{ida} plants were exposed to synthetic peptides containing the conserved motifs of IDA and IDL1. Several IDA deletion constructs were used to investigate the level of complementation when expressed in the \textit{ida} mutant.

To further unravel the contribution of HAE in the regulation of floral organ abscission and to investigate the genetic interaction between IDA and HAE, T-DNA mutant plants for HAE and HSL2 were investigated for floral abscission defects. The \textit{hae hsl2} double mutant turned out to have an abscission phenotype very similar to that of \textit{ida}. We therefore investigated whether IDA function was dependent on the HAE and HSL2 genes by introducing \textit{35S:IDA} into the \textit{hae hsl2} mutant background. The significance of the different parts of IDA and IDL proteins on protein function and how these may relate to HAE and HSL2 are discussed.

RESULTS

\textbf{Promoter:GUS Constructs Uncovered Distinct Expression Patterns for the IDL Genes}

To elucidate the temporal and spatial activity of the IDL genes in more detail, \textit{IDL} promoter fragments were cloned in front of the \textit{β-glucuronidase (GUS)} gene in a T-DNA vector that was transformed into C24 wild-type plants (see Methods). An in-depth analysis of reporter gene expression in root tissues and above-ground organs was conducted at different stages of development on T1 \textit{IDL:GUS}-transformed Arabidopsis plants and their T2 siblings.

The GUS expression patterns were consistent with the previous RT-PCR data (Butenko et al., 2003) and confirmed that the \textit{IDL} genes are expressed in a wide range of tissues, including sites where cell separation occurs (Roberts et al., 2002) (Figure 1). The \textit{IDL1} promoter was primarily active in roots, driving expression in the two outermost cell layers of the columella root cap (Figure 1A). The promoters of \textit{IDL2}, \textit{IDL3}, and \textit{IDL4} all directed GUS expression at the base of the pedicel and in floral organ AZs (Figures 1B and 1C). Contrary to IDA, where the GUS expression is at its strongest at position 5 (referring to location when counting flower and silique positions from the top of the inflorescence, according to Bleecker and Patterson [1997]) (Butenko et al., 2003), only a weak GUS signal is seen at position 5 for \textit{IDL2}, \textit{IDL3}, and \textit{IDL4} (see Supplemental Figure 1 online). In the C24 ecotype, wild-type floral organs are shed at position 10. The intensity of \textit{IDL2:GUS}, \textit{IDL3:GUS}, and \textit{IDL4:GUS} shows a peak at position 10 through 12, position 15, and position 20 through 23, respectively (see Supplemental Figure 1). Thereafter, the GUS activity decreases. For \textit{IDL2}, the GUS activity was observed until position 18, and for \textit{IDL3} and \textit{IDL4}, through position 27. \textit{IDL3:GUS} and \textit{IDL4:GUS} expression was first present in regions above and below the actual AZs of the sepal, petal, and filaments and only at very late stages localized to these cells (see Supplemental Figure 1). \textit{IDL2:GUS} and \textit{IDL4:GUS} were in addition expressed in the funicle AZ (Figure 1D), while \textit{IDL2:GUS}, \textit{IDL3:GUS}, \textit{IDL4:GUS}, and \textit{IDL5:GUS} expression was detected in vascular tissue (Figure 1E). Cross sections of cotyledons revealed no spatial differences between the expression patterns of these genes in the vascular bundles (see

Figure 1. GUS Expression under the Control of the IDL Promoters.  
(A) \textit{IDL1:GUS} expression in the columella root cap and in cells that are shed from the root.  
(B) to (D) Expression at the base of the pedicel, in the floral organ AZ, and in the funicle AZ, here represented by \textit{IDL2:GUS}.  
(E) \textit{IDL3:GUS} expression in vascular tissue of a young seedling. Expression in vascular tissue was also observed for \textit{IDL2:GUS}, \textit{IDL4:GUS}, and \textit{IDL5:GUS}.  
(F) \textit{IDL4:GUS} expression in guard cells.  
(G) GUS activity was seen in hydathodes both for \textit{IDL4:GUS} and \textit{IDL5:GUS}, here shown for \textit{IDL5:GUS}.  

1806 The Plant Cell
Supplemental Figure 2 online). IDL4:GUS was also expressed in guard cells of young seedlings (Figure 1F), whereas both IDL4:GUS and IDL5:GUS showed expression in hydathodes (Figure 1G).

Overexpression of IDL Genes Mimics Overexpression of IDA

To elucidate IDL gene function, constructs for overexpression of all the IDL genes were made, using the strong constitutive cauliflower mosaic virus 35S promoter (see Methods), and primary transformants were investigated for abnormal phenotypes.

The AZ of 35S:IDA plants, characterized by premature floral organ abscission, shows an increased number of rounded AZ cells secreting a white substance shown to be rich in arabino-galactan (AG) (Stenvik et al., 2006). Overexpression of all IDL genes resulted in a similar abscission phenotype. However, the severity of the phenotype varied; 35S:IDL1 plants usually had the strongest resemblance to 35S:IDA and 35S:IDL5 the weakest (Figure 2; see Supplemental Figures 3A to 3C online). Compared with the wild type (Figure 2A) and similar to 35S:IDA plants (Figure 2B), the 35S:IDL lines showed premature abscission of floral organs and had shed their floral organs by position 7 (Figures 2C and 2D). After abscission, AZs with an increase in the number of rounded AZs cells and secretion of AG were found in all lines (Figures 2F to 2H; see Supplemental Figures 3A to 3C) but not found in wild-type AZs (Figure 2E). As for plants overexpressing IDA (Stenvik et al., 2006), siliques, flowers, and cauline leaves were in some cases abscised, and AZ-like cells covered with AG were also seen at these abscission sites (see Supplemental Figures 3D to 3F online). These results indicate some degree of functional redundancy.

**Figure 2. Phenotype of Plants Overexpressing IDL Genes.**

(A) and (E) Wild-type Columbia (Col) floral organ AZs at positions 7 and 20 on the inflorescence. 

(B) and (F) 35S:IDA AZs at positions 7 and 20 showing premature abscission of floral organs and enlarged rounded AZ cells. 

(C) and (G) 35S:IDL1 AZs at positions 7 and 20.

(D) and (H) 35S:IDL5 AZs at positions 7 and 20.

A Double Mutant between hae and hsl2 Displays No Floral Organ Abscission

To further investigate the role of HAE in abscission, a line (SALK 021905) (Alonso et al., 2003) with a T-DNA inserted in the sequence encoding the 10th LRR (see Supplemental Figure 4 online) was analyzed. Unlike the HAE antisense lines, which were reported to have delayed floral organ abscission (Jinn et al., 2000), all homozygous plants for the T-DNA insertion (hae mutants) were indistinguishable from wild-type plants (data not shown).

HAE is closely related to the two genes HSL1 (At1g28440) and HSL2 (At5g65710) (Shiu and Bleecker, 2001b). HSL1 and HSL2 share 58 and 45% overall identity, respectively, to HAE (see Supplemental Figure 4 online). The gene expression pattern of HSL2 in flower development and during stamen abscission (Schmid et al., 2005; Cai and Laahbrook, 2008) is similar to HAE, with low transcript levels prior to anthesis followed by an increase shortly before the onset of abscission (see Supplemental Figure 5 online). HSL1, on the other hand, shows a different profile, with levels decreasing shortly before the onset of abscission.

The congruent expression profile of HAE and HSL2 suggests that these two genes may be functionally redundant. A homozygous T-DNA insertion line for HSL2 (SALK 030520), with an insertion in the predicted transmembrane domain coding sequence (see Supplemental Figure 4 online) depicting normal abscission (data not shown), was crossed to the hae T-DNA mutant. Similar to the ida mutant, hae hsl2 double mutant plants were completely deficient in floral organ abscission, retaining their petals, sepals, and filaments indefinitely (Figure 3A). RT-PCR performed on cDNA from AZ tissue of flowers from positions 4 to 8 failed to detect any HAE or HSL2 transcripts (Figure 3B). This combined with the position of the T-DNA insertions suggests that the insertions have resulted in null alleles for both genes.

**Figure 3. Abscission by IDA and IDL Works through RLKs 1807**

IDA is Dependent on the Presence of HAE and HSL2 to Function

A quantitative determination of hae hsl2 floral organ abscission was obtained by measuring the force needed to remove the petals from the receptacle of the plant (i.e., petal break strength [pBS]) (Fernandez et al., 2000; Lease et al., 2006). The double mutant had high pBS at positions 2 and 4, exceeding 2-gram equivalents, following a substantial decrease by position 8, which compared with wild-type flowers is a delay by two positions (Figure 3C). This pBS profile was also recorded for ida (Figure 3C) and is indicative of cell wall loosening (Butenko et al., 2003). For both hae hsl2 and ida but not the wild type, the drop in pBS is followed by an increase, where flowers at position 20 have pBS similar to those at position 6 (Figure 3C).

To explore the possibility that the IDA, HAE, and HSL2 genes could be acting in the same genetic pathway, a single locus 35S:IDA line overexpressing IDA (Figure 3A) was crossed to hae hsl2. An F2 population of plants was genotyped for the presence of homozygote T-DNA insertions in HAE and HSL2 and for the inclusion of the 35S:IDA sequence. All homozygous hae hsl2...
plants containing the 35S:IDA construct had the hae hsl2 phenotype and none of the observed 35S:IDA features, although they overexpressed IDA (Figures 3A, 3D, and 3F). However, plants that expressed the 35S:IDA transgene, but were wild-type or heterozygous for the T-DNA insertions in HAE and HSL2, displayed all the characteristic features of 35S:IDA plants (Figures 3A, 3E, and 3F). These results show that hae hsl2 is epistatic to 35S:IDA and substantiates the hypothesis that IDA, HAE, and HSL2 act in a common pathway.

The N-terminal Signal Peptide Is Necessary for IDA/IDL Function

Green fluorescent protein fusions of IDA and IDL3 or the IDA signal peptide (SP) coupled to green fluorescent protein are exported to the apoplastic space (Butenko et al., 2003; Aalen et al., 2006). This supports the idea that the SPs function to ensure proper localization of these proteins, as to enable a potential receptor interaction. To test this suggestion, plants harboring constructs designed to overexpress IDA or IDL lacking the SP, 35S:IDAΔSP or 35S:IDL1ΔSP, respectively, were investigated. When these plants were compared with the wild type and 35S:IDA, they portrayed none of the phenotypes observed in 35S:IDA or 35S:IDL1 plants, here shown for 35S:IDAΔSP (see Supplemental Figure 6 online). These results indicate that the proteins fail to function properly when lacking the signal peptide.

IDL Coding Regions Partially Rescue the ida Mutation

To study the degree of functional redundancy, we investigated whether the IDL genes could rescue the ida mutant when the IDA upstream and downstream cis-regulatory elements (Butenko et al., 2003) were used to drive IDL expression (see Methods; Figure 4A). ida mutant plants were transformed with the resulting IDA:IDL gene swap constructs using the full-length IDA gene as a control (Butenko et al., 2003).

A majority of the IDA:IDL1 transgenic lines showed rescue of ida in primary transformants (Table 1), and all progeny of these plants also showed abscission of floral organs. The seeds of primary transformants that did not show a C24 wild-type phenotype were pooled, and plants germinated from the pool were

**Figure 3.** hae hsl2 Is Epistatic to 35S:IDA.

(A) Position 12 siliques from wild-type Col, the hae hsl2 double mutant, the ida mutant, the 35S:IDA single locus homozygous line, and a hae hsl2 35S:IDA mutant plant.

(B) RT-PCR performed on cDNA from AZ tissue of wild-type and hae hsl2 flowers (positions 4 to 8) using primers spanning the single intron of both HAE and HSL2, generating products of 120 and 1214 bp, respectively, from wild-type AZ cDNA, but not from hae hsl2 AZ cDNA. Genomic DNA controls (G) for HAE and HSL2 primers gave bands of 192 and 1301 bp, respectively. ACTIN2-7, giving a fragment of 255 bp with primers on each side of intron 2, was used as positive control.

(C) pBS (i.e., the force required to remove petals from the flower) measured from positions 2 to 20 along the inflorescence of 15 wild-type (Col and C24), ida, and hae hsl2 plants. Standard deviations are shown as thin lines at the top of the columns.

(D) hae hsl2 35S:IDA plant. Notice the abscission-deficient hae hsl2 phenotype.

(E) HAE HSL2 35S:IDA plant with the 35S:IDA phenotype. Notice the early abscission and short siliques.

(F) RT-PCR performed on cDNA from rosette leaf tissue of wild-type, hae hsl2, 35S:IDA, hae hsl2 35S:IDA, and a hae hsl2 plant without 35S:IDA from a segregating F2 population of a hae hsl2 35S:IDA cross. IDA primers amplified a fragment of 237 bp. ACTIN2-7 was used as positive control.
analyzed by pBS. The majority of these plants also rescued the ida phenotype (Table 1). The remaining plants showed a partial rescue, in that the force needed to remove the petals approached nil at several positions in the pBS profile (Figure 4B). Evidently, IDL1 is capable of replacing the activity of IDA when expressed in the cell types that normally express IDA.

Full rescue of the ida mutant phenotype was not observed for any of the transgenic lines transformed with the IDA:IDL2, IDA:IDL3, IDA:IDL4, or IDA:IDL5 constructs. However, compared with ida, the pBS profiles (Figure 4B) demonstrated a significant reduction in the average force needed for petal removal at positions 12, 14, and 16, according to a Student’s t test (P < 0.05; see Supplemental Table 1 online), suggesting some ability for these IDL proteins to substitute for IDA.

The Nature of the Variable Region Is Not Crucial for IDA Function

Except for the C-terminal PIP motif, Pro-58 to Asn-69 in IDA (Figure 4C), very little amino acid similarity between IDA and the various IDL proteins can be identified (Butenko et al., 2003; see Supplemental Figure 7 online). Based on the full-length protein sequences, IDL1 is most closely related to IDA (Figure 4D). However, some of the eight amino acids N-terminal of the PIP motif are conserved between IDA and the five Arabidopsis IDL proteins (Figure 4C). These eight amino acids together with the 12 amino acids of the PIP motif are from here on referred to as the extended PIP domain (EPIP). The EPIP plus the remaining residues at the C terminus is referred to as EPIP-C (Figure 4C).

(A) Schematic presentation of various IDA:IDL constructs with IDA upstream (P_IDA) and downstream (T_IDA) cis-elements driving the expression. In the IDA:IDL gene swap constructs, the coding sequence of IDA has been exchanged with the coding sequence of IDL genes. In the IDA:IDL-IDIA domain swap constructs, the IDA sequence encoding the signal peptide (SP) and variable region (V) has been exchanged with the corresponding IDL sequences. In the IDA:IDIA-IDL domain swap constructs, the sequence encoding the EPIP-C of IDA has been exchanged with the corresponding IDL sequences.

(B) pBS measured from positions 2 to 20 along the primary inflorescence of wild-type C24, the ida mutant, and the IDA:IDIA-IDL EPIP-C swap constructs (cf. [A]) for which full rescue of the ida mutant phenotype was not seen. Note reduced pBS compared with ida (i.e., partial rescue) at positions 12, 14, and 16.

(C) Alignment of the C terminus of IDA and the five Arabidopsis IDLs. IDL1 is most similar to IDA and best-functioning. The middle cluster is the IDLs that can partially substitute for IDA, and at the bottom, IDL5, which cannot substitute for IDA, is shown. Amino acids in the EPIP-Cs of IDL identical to the IDA sequence are shaded; an asterisk indicates the EPIP residues common to IDA and IDL1 but not all the other IDLs.

(D) Relationship between IDA and the IDL proteins. The phylogenetic tree was constructed using maximum likelihood analysis after alignment of the full-length protein sequences (see Supplemental Data Set 1 and Supplemental Figure 7 online). Figures indicate bootstrap values in percentages.

(E) pBS for wild-type C24, the ida mutant, and the IDA:IDIA-IDL EPIP-C swap constructs (cf. [A]) for which full rescue of the ida mutant phenotype was not seen. Note partial rescue (reduced pBS compared with ida) for several constructs at positions 8 to 20.
The most diverged regions of the IDA and IDL proteins are the SPs and the variable regions (VARs) (Butenko et al., 2003; see Supplemental Figure 7 online). To investigate the possibility that these regions influence the ability of the IDL proteins to rescue the ida mutation, IDA:IDL\textsuperscript{SP-VAR-IDA\textsuperscript{EPIP-C}} constructs were generated. We replaced the C terminus of the IDL proteins, from EPIP to the end, with the corresponding EPIP-C region of IDA (see Methods). These constructs, from now on referred to as IDA:IDL\textsuperscript{VAR-IDAEPIP-C} (Figure 4A), were used to investigate whether IDA\textsuperscript{EPIP-C} could restore function to the IDL proteins with a limited ability to substitute for IDA in planta.

When transformed into ida, all hybrid genes provided a complete rescue in multiple independent transgenic plants (Table 1); 95.2% of the IDA:IDL1-IDA and 97.5% of IDA:IDL5-IDA plants and ~70% of the primary transformants of IDA:IDL2-IDA, IDA:IDL3-IDA, and IDA:IDL4-IDA rescued ida. Seeds of primary transformants not rescuing ida were pooled for each of the three latter constructs, and a high proportion of IDA:IDL2-IDA, IDA:IDL3-IDA, and IDA:IDL4-IDA plants did rescue ida in the second generation (Table 1). Even though a varying rescue ability was observed, the high percentage of plants with wild-type phenotype indicate that the IDA EPIP-C domain is capable of restoring function to the less functional IDL2, IDL3, IDL4, and IDL5 genes.

### Table 1. Number of Transgenic Plants Showing a Wild-Type Abscission Phenotype (Rescue of ida) or Showing Partial/No Rescue of the ida Mutant

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wt ph t, wild-type abscission phenotype; p/n r, partial/no rescue.
a T2 plants of pooled T1 seeds from transformants not showing a wild-type abscission phenotype.

The most diverged regions of the IDA and IDL proteins are the SPs and the variable regions (VARs) (Butenko et al., 2003; see Supplemental Figure 7 online). To investigate the possibility that these regions influence the ability of the IDL proteins to rescue the ida mutation, IDA:IDL\textsuperscript{SP-VAR-IDA\textsuperscript{EPIP-C}} constructs were generated. We replaced the C terminus of the IDL proteins, from EPIP to the end, with the corresponding EPIP-C region of IDA (see Methods). These constructs, from now on referred to as IDA:IDL\textsuperscript{VAR-IDAEPIP-C} (Figure 4A), were used to investigate whether IDA\textsuperscript{EPIP-C} could restore function to the IDL proteins with a limited ability to substitute for IDA in planta.

When transformed into ida, all hybrid genes provided a complete rescue in multiple independent transgenic plants (Table 1); 95.2% of the IDA:IDL1-IDA and 97.5% of IDA:IDL5-IDA plants and ~70% of the primary transformants of IDA:IDL2-IDA, IDA:IDL3-IDA, and IDA:IDL4-IDA rescued ida. Seeds of primary transformants not rescuing ida were pooled for each of the three latter constructs, and a high proportion of IDA:IDL2-IDA, IDA:IDL3-IDA, and IDA:IDL4-IDA plants did rescue ida in the second generation (Table 1). Even though a varying rescue ability was observed, the high percentage of plants with wild-type phenotype indicate that the IDA EPIP-C domain is capable of restoring function to the less functional IDL2, IDL3, IDL4, and IDL5 genes.

### The IDA Variable Region Can Assist the Less Functional IDL EPIP-C Domains

Both the gene swap experiment and the variable region domain swap experiment suggested that EPIP-C contained the functional domain of IDA and the IDL proteins. However, we could not rule out that the variable region of IDA could play a role in providing a correct context for the less optimal IDL EPIP-Cs. To investigate this, a new domain swap experiment was performed. The EPIP-C of IDA was exchanged with the IDL EPIP-Cs, resulting in IDA:IDAEPIP\textsuperscript{SP-VAR,IDA\textsuperscript{EPIP-C}} constructs, hereafter called IDA:IDAEPIP-C (see Methods; Figure 4A). For IDL1, the primary transformants showed a complete rescue of the ida phenotype, whereas for IDL5, no abscission was seen and the pBS profile resembled that of ida (Figure 4E). Also for IDL2, IDL3, and IDL4, no abscission was observed. However, the pBS profile did not resemble the profile of ida but was significantly reduced (Student’s t test, P < 0.01; see Supplemental Table 1 online). These results indicate that the IDL suboptimal EPIP-Cs (Figure 4C) can be functionally supported by the IDA variable region.

### An Extended PIP Motif Is Sufficient for Rescue of the ida Mutation

To further delineate the parts of the IDA gene needed for IDA function, different IDA deletion constructs were made (see Methods; Figure 5A). In the IDA\textsuperscript{EPIP-C} construct, the C-terminal part of IDA from the EPIP motif to the end was removed. No phenotypic restoration was observed in ida plants carrying this construct (Table 1). The pBS of IDA\textsuperscript{EPIP-C} transgenic plants was measured (Figure 5B), but no significant difference was observed when comparing to the pBS profile of ida for positions 10, 12, 16, 18, and 20 by Student’s t test P < 0.05 (see Supplemental Table 1 online).

Therefore, we wanted to investigate the function of the C terminus in more detail (Figure 5A). In the IDA\textsuperscript{VAR} construct, the coding sequence downstream of the EPIP motif was deleted, while in the IDA\textsuperscript{VAR} construct, the variable region between the SP and the EPIP motif was removed. Restoration of the wild-type phenotype was observed in all ida plants carrying constructs with the EPIP motif, both for IDA\textsuperscript{VAR} and IDA\textsuperscript{SC-end}. The pBS of IDA\textsuperscript{VAR} transgenic plants confirmed that this construct was capable of complementing the ida mutation (Figure 5B). Thus, neither the variable region nor the amino acids downstream of the EPIP motif are needed for IDA function.

### A Synthetic EPIP Peptide Can Induce Abscission

To substantiate whether EPIP was sufficient for inducing abscission, we examined the effect of synthetic peptides. Wild-type C24 and ida mutant flowers at anthesis were placed on MS2 control plates (see Methods) either containing or not containing 10 μM of EPIP or EPIP-C peptides (Figure 4C). In plates containing IDA EPIP-C or EPIP, wild-type flowers abscised earlier than on control plates (Figures 6A and 6C; see Supplemental Figure 8 online). To investigate the possibility that EPIP could restore function to the IDL proteins with a limited ability to substitute for IDA in planta, we examined the effect of synthetic peptides.
The effect of the synthetic peptides on the flowers tested (n = 90) retained their floral organs, substantiating that these RLKs are needed for IDA/IDL peptide signaling.

**IDA Is Processed in Vitro by CLE Proteolytic Activity**

Studies of CLAVATA3 (CLV3) and related proteins have shown both sequence conservation and functional sufficiency of the C-terminally located CLE domain (Jun et al., 2008); moreover, strong evidence both in vitro and in vivo of proteolytic processing to release the active CLE peptide has been observed (Kondo et al., 2006; Ni and Clark, 2006). Given the parallel evidence of both conservation and functional importance of the IDA and IDL EPIP domain, we hypothesized that a peptide might be proteolytically released from the IDA protein. As a first approach to investigate whether IDA can be processed, a glutathione S-transferase (GST)-tagged version of the protein lacking the signal peptide (Stenvik et al., 2006), GST-IDAΔSP, was expressed in *Escherichia coli*. The fusion protein was incubated with cauliflower extracts in in vitro reactions previously shown to process the CLE domain from CLV3 (Ni and Clark, 2006). Upon detection by protein gel blots, mobility shifts consistent with a distinct C-terminal processing of GST-IDAΔSP and GST-mCLV3 (Ni and Clark, 2006) were observed (Figure 7A). To determine if IDA processing was catalyzed by the same activity as CLV3, competition experiments were performed. Excess His-mCLV3, included in GST-IDAΔSP processing reactions, partially inhibited processing of GST-IDAΔSP, while excess BSA had no effect (Figure 7B). This suggests the presence of a single or related processing activity capable of generating peptides from the C terminus of both CLV3 and IDA.

Interestingly, there is some similarity between the PIP motif and the active mature CLV3 peptide, MCLV3 (Kondo et al., 2006), as well as the residues preceding these peptides (Figure 7C). MCLV3 and PIP have a common core, PS[G,A]P, surrounded by small residues, and two amphoteric residues at the end, while hydrophobic residues flank each side of the charged E or K preceding the CLE/PIP motifs.

**DISCUSSION**

**IDA and IDL Genes Are Expressed at Sites Where Cell Separation Occurs**

One of the aims of this work has been to elucidate the function of the IDL genes, which, in contrast with IDA, were identified using bioinformatics tools (Butenko et al., 2003). The promoter-GUS transgenic plants showed that IDL2, IDL3, and IDL4 are expressed in floral AZs (Figure 1B; see Supplemental Figure 1 online), suggesting that these genes are the results of gene duplication events of an ancient abscission-related gene. The most closely related genes, IDL2 (At5g64667) and IDL3 (At5g09805) (Figure 4D), are in fact found in two duplicated regions on chromosome 5, encompassing the genes At5g03600-65640 and At5g08570-10570 (cf. http://wolfe.gen.tcd.ie/athal/dup). The expression of IDL genes in the AZ is apparently not
A Functional Part of the IDA and IDL Proteins Is Found in the EPIP

The overexpression of the IDL genes resulted in phenotypes similar to the overexpression of IDA itself (Figure 2; see Supplemental Figure 3 online). Therefore, one could have expected that all IDL proteins would be capable of complementing the ida mutant when expressed under the control of the IDA promoter. However, this was only the case for IDL1, which has the highest overall sequence similarity to IDA (Figures 4C and 4D).

The failure of the other IDL proteins to rescue the mutant phenotype was not due to the low sequence similarity in the N terminus and variable regions, as all the IDA:IDL-IDA constructs were functional. The gene deletion analysis substantiated that the variable region of IDA was not required for IDA function (Figure 5). All constructs containing the EPIP motif rescued the ida mutant. This suggests that the active parts of these IDL proteins, most likely found within the EPIPs, are not sufficiently similar to IDA to fully substitute for IDA function (cf. Figure 4C).

Involvement in floral organ abscission may not be the normal and/or major function of the IDL genes, as the IDL:GUS expression was seen in a range of tissues (Figure 1). The expression of IDL2 and IDL3 at the base of the pedicel (Figure 1B) could be a remainder of an ancient expression pattern. Pedicel abscission occurs naturally in a number of plant species, although not Arabidopsis, which has a vestigial AZ at the pedicel junction (Stenvik et al., 2006). In plant species displaying such abscission, it can be hypothesized that an IDA or IDL ortholog induces the pedicel separation event. Sloughing (i.e., detachment of the root-cap from the remaining root), release of the seed body from the funiculus, and disruption of adhesion at the junction between two adjacent cells during the formation of stomata are also cell–cell separation processes (Stevens and Martin, 1978; Zhao and Sack, 1999; Roberts et al., 2002; del Campillo et al., 2004). During the maturation of continuous tubes of tracheary elements, the middle lamella between two neighboring tracheary elements is digested completely by cell wall–degrading enzymes (Fukuda, 2004). Interestingly, IDL genes are also expressed at these sites (Figures 1A and 1D to 1F; see Supplemental Figure 2 online). The investigation of future IDL knockout mutants may reveal whether the IDL proteins, similar to IDA, represent signals that trigger the cell separation processes at these sites.

Figure 6. Effects of Synthetic EPIP-C and EPIP Peptides on Abscission.

(A) Percentage of abscised wild-type flowers (n = 30) after exposure to 10 μM EPIP IDA peptide (black) compared with control flowers (n = 30) without peptide in the medium (control, in green).

(B) Percentage of abscised ida flowers (n = 30) after exposure to 10 μM EPIP IDA peptide (black) compared with ida flowers (n = 30) without peptide in the medium (control, in green).

For (A) and (B), standard deviations based on three independent experiments are shown.

(C) Floral AZ and pedicel AZ of wild-type flowers exposed to EPIP-C IDA or EPIP IDL1. Note secretion of white substance on floral AZ cells exposed to peptides and rupturing of the epidermal layer on pedicel AZ (arrow). The control had no peptide in the medium.
ida mutant, but the construct that lacked the EPIP domain did not. Thus, in the presence of the SP, the EPIP of IDA is sufficient to trigger cell separation at floral organ AZs. Further proof of the functionality of the IDA EPIP was that the synthetic EPIP and EPIP-C peptides could induce floral organ abscission in the ida mutant and earlier abscission in wild-type flowers (Figure 6).

The fact that IDL1 was able to replace IDA function suggested that the functional domain of the IDL proteins was also contained in the EPIP domain. This was substantiated for IDL1 as its synthetic EPIP functioned as efficiently as the synthetic IDA EPIP in the EPIP domain. This was substantiated for IDL1 as its functional domain of the IDL proteins was also contained in the EPIP domain. This was substantiated for IDL1 as its synthetic EPIP functioned as efficiently as the synthetic IDA EPIP (Figure 6C). The functionality of the IDL EPIPs was also tested with the IDA:IDA-IDL constructs. Somewhat to our surprise, the IDA:IDA-IDL2, IDA:IDA-IDL3, and IDA:IDA-IDL4 constructs were more efficient in rescuing the ida mutation than the IDA:IDL gene swap constructs (with the same genes). This implies that the variable region of IDA positively supports the IDL EPIP-C functionality.

For CLV3, it has been suggested that the variable domain could play a role in allowing translation/translocation of the CLE domain into the lumen of the endoplasmic reticulum (Ni and Clark, 2006). However, our construct lacking the variable region of IDA was observed to rescue the ida mutant phenotype, and the variable region of IDA can therefore rather be suggested to function to facilitate correct protein processing.

### IDA and IDL Proteins Are Suggested to Be Processed

Several members of the CLE family can rescue the CLV3 loss-of-function phenotype (Ni and Clark, 2006). The CLE domain of CLV3 and the CLE proteins is the functional peptide released from the precursor protein (Kondo et al., 2006; Ni and Clark, 2006), and an active peptide could similarly be released from the IDA or IDL precursor. Although no obvious cleavage recognition site has been found in IDA, the protein can be processed, as demonstrated using extracts from cauliflower meristem (Figure 7A). However, predicted cleavage sites are not present in several peptide ligands, such as phytosulfokine, systemin, and CLV3, that clearly are processed in planta (Yang et al., 1999; Pearce et al., 2001; Matsubayashi, 2003; Fiers et al., 2006; Kondo et al., 2006). Sequence similarity of the CLE and EPIP domains and competition of CLV3 for IDA processing suggest that CLV3 and IDA share a common or similar processing mechanism (Figures 7B and 7C). The cauliflower processing results for CLV3 are consistent with the CLV3 peptide isolated in vivo (Kondo et al., 2006; Ni and Clark, 2006). The presence of a processing activity in cauliflower meristem that is functionally identical to an activity in the Arabidopsis shoot meristem may seem likely. At this point, however, we cannot conclude whether the cauliflower processing activity, although capable of processing IDA in vitro, reflects the situation in Arabidopsis floral organ AZs. Further in vivo studies may delineate the shortest peptide necessary for biological function and the potential release of this from an IDA proprotein.

### IDA and IDL Represent a Family of Putative Ligands Likely to Interact with RLKs

All the IDL proteins are capable of initiating the final step of cell separation of floral organs when being ectopically expressed. This suggests that the proteins function through common mechanisms. In analogy to CLV3, which is exported to the apoplastic space and binds the CLV1 receptor (Rojo et al., 2002; Ogawa et al., 2008), IDA and the IDL proteins may act as ligands that interact with plasma membrane–bound receptors. This implies that the signal peptides of IDA and the IDL proteins are crucial for proper function. In agreement with this hypothesis, 35S:IDA\(\text{∆}\)SP and 35S:IDL\(\text{∆}\)SP plants showed none of the phenotypes observed in 35S:IDA and 35S:IDL\(\text{∆}\) plants (see Supplemental Figure 6 online).

We have earlier proposed that IDA could be the ligand of the LRR-RLK HAE (Jinn et al., 2000; Butenko et al., 2003). IDA and HAE have overlapping expression patterns in the flower, and HAE is expressed at the base of the pedicel, where ectopic expression of IDA can induce abscission (Jinn et al., 2000; Butenko et al., 2006; Stenvik et al., 2006). As the hae hsl2 double mutant shows a strong defect in floral organ abscission (Figures 3A and 3D), it is likely that the HAE antisense RNA that resulted in delayed abscission (Jinn et al., 2000) also silenced the endogenous expression of HSL2. In contrast with the bop1 bop2 double mutant that is defect in the formation of the AZ and therefore cannot abscise (Hepworth et al., 2005; McKim et al., 2008), the pBS profiles of the hae hsl2 mutant is very similar to that of ida mutant plants. The pBS profiles of both ida and hae hsl2 show an initial decrease, suggesting the onset of cell wall
loosening, followed by an increase in pBS as the actual cell separation is not taking place (Figure 3C). This makes these RLKs likely candidates for interaction with IDA and IDL peptides. The need to knock out both RLK genes to get an aberrant phenotype suggests that IDA/IDL can signal through both HAE and HSL2 (i.e., HAE and HSL2 have redundant functions). However, from our results we cannot say which of the two (or both) receptors normally relay the IDA signal. As both have a kinase domain (see Supplemental Figure 3 online), this scenario may differ from that demonstrated for CLV1 and CLV2, where these receptors have been proposed to function as heterodimers and only CLV1 has a kinase domain (Jeong et al., 1999).

Also consistent with the hypothesis that HAE/IDL2 act as receptors for IDA, we have shown that the floral abscission defect of hae hsl2 was maintained in the presence of 3SS:IDA and that none of the observed 3SS:IDA phenotypic traits appeared in the hae hsl2 mutant background (Figures 3A and 3D). That hae hsl2 is epistatic to 3SS:IDA provides genetic evidence for the notion that IDA, HAE, and HSL2 act in the same pathway. This is further substantiated by the fact that the synthetic IDA EPIP was unable to induce abscission in hae hsl2 flowers.

The simplest interpretation of the common overexpression phenotype seen for all the 3SS:IDL lines, consistent with our ligand-receptor hypothesis, is that the IDL peptides can interact with each other or a common receptor. However, when expressed using the IDA promoter, only ID1 showed full rescue of the ida mutant phenotype. This suggests that the functions of IDL2, IDL3, IDL4, and IDL5 are not completely redundant with IDA but can provide limited function when present in a low dosage, which potentially can be overcome by a huge increase in concentration when driven by the 3SS promoter.

When overexpressed, the putative IDL ligands may exert their effects both through an IDA receptor and perhaps through other similar receptors. The expression of the IDL genes in diverse tissues other than the floral organ AZs suggests that their normal function differs from that of IDA. Other receptors similar to HAE with IDL-compatible expression patterns may be postulated as signaling partners for the IDL proteins. However, the Arabidopsis family of LRR-RLKs consists of >400 members (Shiu and Bleecker, 2001a). Finding how and which of the many newly identified putative ligands encoded in the Arabidopsis genome (Lease et al., 2006; Hanada et al., 2007) interact with these receptors will surely provide insights into the regulation of many important developmental processes.

METHODS

Primers

The sequences of all primers used can be found in Supplemental Table 2 online.

GUS constructs

The five IDL:GUS constructs were made using Gateway technology. The promoter fragments, which included 1555, 1864, 1908, 1980, and 2020 bp upstream of the ATG of IDL1, IDL2, IDL3, IDL4, and IDL5, respectively, were amplified using primers R-AtIDL1 promoter GW, R-AtIDL2 promoter GW, R-AtIDL3 promoter GW, R-AtIDL4 promoter GW, R-AtIDL5 promoter GW, L-AtIDL1 promoter GW, L-AtIDL2 promoter GW, L-AtIDL3 promoter GW, L-AtIDL4 promoter GW, and L-AtIDL5 promoter GW, which all have additional Gateway att sequences at the 5' ends. The PCR products were introduced into pDONR201 (Invitrogen) and thereafter recombined into the pFPZP211GAWI vector (Butenko et al., 2003), generating pFPZP IDL1:GUS, pFPZP IDL2:GUS, pFPZP IDL3:GUS, pFPZP IDL4:GUS, and pFPZP IDL5:GUS.

IDL and IDA 35S Overexpression Constructs

For overexpression of the five IDL genes and IDA (Stenvik et al., 2006), and IDA and IDL1 without signal sequences, the Gateway destination vector pK7WG2, based on the backbone of the pFPZP200 vector (Karimi et al., 2002), was used. The att-flanked PCR products were amplified using the primers IDL1 attB1, IDL1 minus signal attB1, IDL1 stop attB2, IDL2 attB1, IDL2 stop attB2, IDL3 attB1, IDL3 stop attB2, IDL4 attB1, IDL4 stop attB2, IDL5 attB1, IDL5 stop attB2, IDA minus signal attB1, and IDA stop attB2. The PCR products were recombined into the pDONR/zeo (Invitrogen) and thereafter into the destination vector pK7WG2, generating pK7IDL12, pK7IDL1minus signal2, pK7IDL22, pK7IDL32, pK7IDL42, pK7IDL52, and pK7IDAnminus signal2.

Identification of Homologous Lines and Genetic Crosses

HAE SALK line (021905) was genotyped with LPHAE and RPHAE and Lba1. HSL2 SALK line (030520) was genotyped with LPHSL2 and RPHSL2 and Lba1. PCR products obtained with RPHAE, RPHSL2, and Lba1 were cloned into pCR2.1-TOPO vector (Invitrogen) and sequenced to obtain the flanking T-DNA sequence for both genes. Homozygous T-DNA lines for both genes were reciprocally crossed, and 54 F2 plants from each cross were investigated for aberrant phenotypes. As for the double homozygote SALK lines (SALK 105975, HAE: SALK 057117, HSL2; and SALK 030520, HSL2; SALK 015074, HAE) (http://www.mcb.arizona.edu/tax/2010), one and two F2 siblings from each cross, respectively, showed a severe defect in floral organ abscission. The plants deficient in abscission and three plants with normal abscission were genotyped with LPHAE, RPHAE, LPHSL2, RP HSL2, and Lba1 primers. Only plants with a defect in abscission were homozygous for both T-DNA insertions. One double homozygous plant was crossed to a single locus homozygous 3SS:IDA line. F2 plants were genotyped with LPHAE, RPHAE, LPHSL2, RP HSL2, and Lba1 primers, and the presence of the 3SS:IDA construct was verified by PCR with primers IDAF (Stenvik et al., 2006) and NptII.

IDL Gene Swap Constructs

For IDA cis-elements, a 303-bp fragment downstream of the IDA stop codon was amplified from C24 plants with primers IDA T term Smal and complements pipp L. This fragment, hereafter termed the IDA terminator, was cloned into TOPO pcr2.1 vector, generating pcr2.1.IDA-T. The fragment covering 1482 bp upstream of the IDA start codon was amplified from C24 DNA with primers IDA prom BarnHI and IDA prom MultSmal. This fragment, defined as the IDA promoter region, was inserted into pcr2.1.IDA-T using the BarnHI restriction site of TOPO pcr2.1 and IDA term Smals’s Mult restriction site to generate pcr2.1.IDA-P-T. The IDL gene sequences were amplified using IDLX sense Mulx and IDLX antisense Mul primers. The amplified IDLX sequences were ligated into pcr2.1.IDA-P-T using the Mult site, resulting in pcr2.1.IDA-P-IDLX-T constructs, referred to as IDA:IDLX.

Variable Swap Constructs

A fragment covering the IDA EPIP domain from Phe-50 and the C-terminal IDA cis-elements was amplified with primers IDA F C-term Mult and complements pipp L. This fragment, defined as the IDA EPIP domain and
RT-PCR. Wild-type (C24 and Col) and transgenic IDA terminator, was cloned into TOPO pcr2.1 vector, generating pcr2.1IDA-
IDAesp -T. The IDA promoter region (amplified and generated as de-
scribed earlier) was inserted into pcr2.1DA-IDAesp -T, resulting in
pcr2.1IDA- P-IDAesp -T. IDA and IDL sense MuI and IDA and IDL variable
muI restriction site, resulting in pcr2.1IDA- P-SIDLX-VIDLX-
IDAesp -T, referred to as IDA/IDLX-IDA.

EPIP Motif Swap Constructs
The IDA promoter and the first 147 bp of the IDA gene, ending at
Phe-50, was amplified from C24 DNA using the primer IDA prom BamHI
and the primer IDA 67224R Smal MuI. This fragment was inserted into
pcr2.1IDA-T, using the pcr2.1 Smal site and the primer’s BamHI site to
generate a pcr2.1IDA-P-S-V-T.

The sequences encoding IDA and IDL EPIPs were amplified from C24
genomic DNA, starting at the conserved Phe-50 in the IDA sequence, and
at Val or Phe in the IDL genes and ending at the stop codon of the gene in
question with primers IDA and IDLX F C-terminal MuI, and IDA and IDLX
antisense MuI. The IDA and IDL EPIP sequences were then ligated into
pcr2.1IDA-P-T by the attached restriction sites (MuI), integrated after the
IDA variable region to generate pcr2.1IDA-P-IDLXesp -T, referred to as
IDA/IDA-IDLX.

IDA Deletion Constructs
A fragment covering the downstream IDA cis-element and the conserved
EPIP region, from the conserved Phe-50, was amplified using the primers
pip aa50 MuI and complement pip L. The fragment was cloned into
TOPO pcr2.1 vector, generating pcr2.1IDA-IDAesp -T. A fragment
covering the promoter and the signal sequence of IDA was amplified
using IDA prom BamHI and IDA signal reverse MuI and ligated in front of
the IDA eip and the IDA downstream cis-elements in the pcr2.1IDA-
IDAesp50 -T, generating pcr2.1IDA-P-SIDAesp50 -T, IDA1LID. In addition,
constructs containing the IDA promoter, the C-terminal cis-elements, and
different parts of the IDA coding sequence were generated: the first in
which a fragment containing the IDA promoter and the open reading
frame from the start codon to Asn-69 was amplified using IDA prom
BamHI and PIP down reverse with stop Smal and then ligated into the
pcr2.1IDA-T, creating pcr2.1IDA-P-S-V-P-T, IDA1Ac-vpr. The second
construct covers a fragment with the IDA promoter and the IDA open
reading frame from the start codon to lie-49.

This fragment was amplified with IDA prom BamHI and pip upper
reverse m/stop Smal and was ligated into pcr2.1IDA-A-T, resulting in
pcr2.1IDA-P-S-V-T, IDA2EPIP-C.

Plant Material and Generation of Transgenic Plants
All the final sequences were amplified from the 20 different pcr2.1
constructs using the Gateway primers pip U attB1 and pip complement
L attB2. The att-flanked PCR products were subcloned into pDONR/zeo
using Gateway cloning technology (Invitrogen) and then recombined into
the destination vector pgSVW1704. The pgSVW1704 constructs were
transferred to the Agrobacterium tumefaciens strain C5881 pGVC2260,
and ida (ecotype C24) plants were transformed using the A. tumefaciens-
mediated floral dip method (Clough and Bent, 1998). Transformants were
selected on MS (Murashige and Skoog, 1962) medium with 50 μg/mL
kanamycin and 20 μg/mL hygromycin. The overexpression and GUS
constructs were transformed into Col and C24 ecotypes, respectively,
and selected on medium with 50 μg/mL kanamycin. Elevated IDL
expression in the different organs of the 3SS:IDL plants was verified by
RT-PCR. Wild-type (C24 and Col) and transgenic Arabidopsis plants were
cultivated in growth chambers at 22°C for 8 h of dark and 16 h of light
(100 μE m-2 s-1).

Peptide Assays
Wild-type (C24), ida (C24), and hae hs2 (Col) flowers (n = 30) at anthesis
were excised from primary inflorescences, and the pedicel was rinsed
b Briefly in 70% ethanol followed by distilled water before being pushed
Into MS2 (Murashige and Skoog, 1962) plates. Two plates were used for
each line, one containing 10 μM of peptide and the other containing the same
volume of chromatography water (Merck). The plates were divided into
three sections with each section containing one of either the wild type,
ida, or hae hs2. The plates were placed in growth chambers at 22°C for 8
h of dark and 16 h of light (100 μE m-2 s-1), and the scoring of floral
abscission was done as described by Gonzalez-Carranza et al. (2007).
Experiments were repeated three independent times. All peptides were
synthesized by MIMOTOPES, IDA EPIP, and IDA EPIP-C with a purity of
>90% and IDL1 EPIP with a purity of >70%.

Histochemical Analysis
Tissues for thin sections were fixed in 0.3% (v/v) glutaraldehyde (Electron
Microscopy Sciences) in 50 mM KPO4 buffer, pH 7.2, rinsed four times
with potassium phosphate buffer before incubated in staining buffer
(50 mM KPO4 buffer, pH 7.2, 2 mM potassium ferricyanide, 2 mM
potassium ferricyanide, 0.1% Triton X-100, and 2 mM X-Gluc [X-GLUC
Direct]) for 12 h at 37°C. After several washes with 50 mM KPO4 buffer,
the tissue was fixed at 4°C ON with 4% (v/v) glutaraldehyde in the same
buffer. A graded ethanol series to 100% ethanol was used to dehydrate
the samples. The tissue was then embedded in LR-white (Fluka), and
1- and 2-μm sections were cut and viewed unstained using both light field
and dark field. GUS staining, postfixation, and whole-mount clearing
preparations of various plant tissues were performed as described (Grini
et al., 2002). All tissues were inspected with a Zeiss Axioplax2 imaging
microscope equipped with differential interference contrast optics and a
cooled Axiocam camera imaging system.

RT-PCR
Total RNA was extracted from different tissues using the RNeasy plant
mini kit (Qiagen) according to the manufacturer’s recommendations. An
optional on-column DNase digestion step was included. First-strand
cDNA synthesis with SuperScript III reverse transcriptase (Invitrogen)
was performed as described by the manual. Experiments in which the reverse
transcriptase was omitted were used as negative controls. ACTIN2-7
primers designed to amplify a fragment spanning intron 2 were used to
control the quality and amount of first-strand cDNA. The following gene-
specific primers were used for PCR amplification from the cDNA: for
IDA, IDA F and IDA R (Stenvik et al., 2006); for IDL, IDL1-5’- and
IDL1-3’; for IDL2, IDL2-5’ and IDL2-3’; for IDL3, IDL3-5’ and
IDL3-3’; for IDL4, IDL4 R and IDL4 L; for IDL5, IDL5 R and IDL5 L; for
HAE, HAE F and HAE R; for
HSL2, HSL2 F and HSL2 R; and for ACTIN2-7, ACTIN2-7 sense and
ACTIN2-7 antisense. The PCR was run for 30 cycles.

Break Strength Measurement
pBS was quantified as the force in gram equivalents required for removal
of a petal from a flower (Butenko et al., 2003). pBS was measured by an
apparatus built after the description in Lease et al. (2006), with a few
modifications. Instead of using a petal gripper and an aluminum strip, an
ultralight miniature clamp was suspended, using lightweight nylon thread,
from a load transducer IAD Instruments ML7050/D, range 0 to 50 g). This
is to facilitate pBS measurements where the force required to remove the
petal approached values of 0.2 g equivalents. The output from the
transducer is amplified by a differential amplifier (Linear Technology LT1013). The signal is digitized by a microcontroller’s 10-bit AD converter (Microchip 12F675). A second microcontroller (Microchip 16F676) was used to handle the user interface push buttons and indicator LEDs. The transducer’s output is repeatedly sampled at a rate of 21 ksps. After memorizing the greatest value, the microcontroller converts the 10-bit value to a 4-byte ASCII packet that is transmitted to a PC at baud rate of 9600 by software-implemented UART using a MAX 202 communication chip.

Proteolytic Processing of GST-IDA3JSP in Cauliflower Extracts

Protein extracts from cauliflower (*Brassica oleracea*) meristem were prepared, and proteolytic processing assays were performed as described (Ni and Clark, 2006). In brief, *Escherichia coli*-purified GST-tagged IDA and CLV3 (without their signal peptides) were incubated with cauliflower protein extracts or buffer alone (50 mM HEPES, 10 mM EDTA, and 0.1% Triton X-100) for 2 h at room temperature with rotation. The processing assays of GST-IDA3JSP were performed in the presence or absence of 20-fold excessive His-m-CLV3 or BSA as competitor substrates. SDS loading buffers were added to the samples, and samples were boiled at 100 °C for 8 min before analysis. Approximately 20 ng of each of the GST-tagged proteins was loaded onto a 15% Ready Gel Tris-HCl gel (Bio-Rad Laboratories). Chicken anti-GST antibodies (1:5000) (kindly provided by Ken Cadigan) and horseradish peroxidase–conjugated rabbit anti-chicken secondary antibodies (1:5000) (Promega) were used for protein gel blot analysis. SuperSignal West Pico Chemiluminescent Substrate (34080; Pierce) was used to visualize HRP activity.

Phylogenetic Analysis

Alignment of the full-length protein sequences (see Supplemental Data Set 1 online) was done using the ClustalW2 program (http://www.ebi.ac.uk/Tools/clustalw2/) followed by manual adjustment using GeneDoc (http://www.psc.edu/biomed/genedoc/). The evolutionary model for the protein alignment was chosen on the basis of AIC criterion implemented in Treefinder under four gamma rate categories (Jobb et al., 2004). The best fitting evolutionary model for the alignment was estimated to be the LG model. Maximum likelihood analysis was performed with Treefinder (Jobb et al., 2004). The optimal topology (highest negative log likelihood) was obtained from 100 separate heuristic searches from random starting trees, while bootstrap analyses were performed by 1000 pseudoreplicates with the same evolutionary model as the initial search (LG with four gamma rate categories).

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers AT1G68765 (IDA), AT3g25655 (IDL1), AT5G64667 (IDL2), AT5G09805 (IDL3), AT3G18715 (IDL4), AT1G76952 (IDL5), AT4G28490 (HAE), and AT5G65710 (HSL2).

Supplemental Data

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

**Supplemental Figure 1.** IDL::GUS Expression in the Abscission Zone.

**Supplemental Figure 2.** IDL::GUS Expression in Vascular Tissue.

**Supplemental Figure 3.** Phenotypes 3SS:IDL Plants.

**Supplemental Figure 4.** Alignment of HAE, HSL1, and HSL2.

**Supplemental Figure 5.** Expression Profiles of HAE, HSL1, and HSL2 during Flower Development.

**Supplemental Figure 6.** The Significance of the N-Terminal Signal Peptide for IDA Function.

**Supplemental Figure 7.** Alignment of IDA and the IDL Full-Length Proteins.

**Supplemental Figure 8.** The Synthetic Peptides EPIP-C of IDA and EPIP of IDL induce Abscission Similar to EPIP of IDA.

**Supplemental Table 1.** Petal Break Strength Measurements.

**Supplemental Table 2.** Primers.

**Supplemental Data Set 1.** Text File Corresponding to Alignment Used to Generate Figure 4D.

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The EPIP Peptide of INFLORESCENCE DEFICIENT IN ABSCISSION Is Sufficient to Induce Abscission in Arabidopsis through the Receptor-Like Kinases HAESA and HAESA-LIKE2

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