Regulation of floral organ abscission in *Arabidopsis thaliana*

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Abscission is a developmental program that results in the active shedding of infected or nonfunctional organs from a plant body. Here, we establish a signaling pathway that controls abscission in *Arabidopsis thaliana* from ligand, to receptors, to downstream effectors. Loss of function mutations in *Inflorescence Deficient in Abscission* (*IDA*), which encodes a predicted secreted small protein, the receptor-like protein kinases *HAESA* (*HAE*) and *HAESA-like 2* (*HSL2*), the *Mitogen-Activated Protein Kinase Kinase 4* (*MKK4*) and *MKK5*, and a dominant-negative form of *Mitogen-Activated Protein Kinase 6* (*MPK6*) in a *mpk3* mutant background all have abscission-defective phenotypes. Conversely, expression of constitutively active MKKs rescues the abscission-defective phenotype of *hae hsl2* and *ida* plants. Additionally, in *hae hsl2* and *ida* plants, MAP kinase activity is reduced in the receptacle, the part of the stem that holds the floral organs. Plants overexpressing *IDA* in a *hae hsl2* background have abscission defects, indicating *HAE* and *HSL2* are epistatic to *IDA*. Taken together, these results suggest that the sequential action of *IDA*, *HAE* and *HSL2*, and a MAP kinase cascade regulates the programmed separation of cells in the abscission zone.

protein phosphorylation | signal transduction

Abscission is a physiological process that involves the programmed separation of entire organs, such as leaves, petals, flowers, and fruit. Abscission allows plants to discard nonfunctional or infected organs, and promotes dispersion of progeny. At the cellular level, abscission is the hydrolysis of the middle lamella of an anatomically specialized cell layer, the abscission zone (AZ), by cell wall-modifying and hydrolyzing enzymes. Thus, abscission requires both the formation of the AZ early in the development of a plant organ and the subsequent activation of the cell separation response (1–4).

Studies using *Arabidopsis thaliana* have implicated the involvement of several different genes in the control of abscission including potential signal molecules, receptors and other gene products (4). *HAESA* (*HAE*), one of the first *Arabidopsis* receptor-like protein kinases (RLK) identified, is expressed in floral organ AZs and antisense experiments show a reduction in the level of HAE protein is correlated with the degree of defective floral organ abscission. Expression of *HAE* is not altered in *etr1-1* (an ethylene-insensitive mutation), implying an ethylene-independent role in abscission (5). *Inflorescence Deficient in Abscission* (*IDA*) encodes a small protein with an N-terminal signal peptide. Analysis of *ida* mutant plants indicates IDA regulates floral organ abscission through an ethylene insensitive pathway (6). Overexpression of *IDA* results in early abscission and the production of a white substance in the floral AZs. The main components of the white substance are arabinose and galactose (7).

Here, we report that components of a MAPK signaling cascade also have roles in the regulation of abscission. A MAPK cascade is a regulatory module with three protein kinases, a MAP kinase kinase kinases (MKKK) that activates a MAP kinase kinases (MKK), which in turn, activates a MAP kinase (MPK). MAPK cascades play important roles in plant responses to pathogens, hormone responses, and development (8). In addition to demonstrating a role for a MAPK cascade in abscission we also link the MAPK pathway to a putative ligand, IDA, and the two RLKs HAE and HAESA-like 2 (HSL2). Based on the genetic interactions between the genes encoding these proteins we propose a model where the sequential action of IDA, HAE and HSL2, and a MAP kinase cascade regulate floral organ abscission.

Results and Discussion

A growing paradigm in signal transduction pathways features receptor modules that perceive signals and modules such as MAPK cascades that relay and amplify this information to downstream effectors. Because little is known about the signaling modules that regulate abscission in plants, we investigated the potential that MAPK cascades play a role in this process. In *Arabidopsis*, recent evidence shows that *MKK4* and *MKK5* have important and overlapping functions in multiple physiological and developmental processes (9–12). Therefore, we investigated a potential role these genes play in floral organ abscission.

Because loss of function mutant alleles of *Arabidopsis MKK4* and *MKK5* are not available in public depositories, we used RNAi to silence these genes. Single RNAi lines of *MKK4* or *MKK5* did not display mutant phenotypes (10). However, transgenic lines expressing a tandem RNAi transgene of *MKK4* and *MKK5* (*MKK4-MKK5RNAi*) have severe stomata developmental defects, and the majority of transgenic plants are unable to survive beyond the seedling stage (10). Among the transgenic plants that are able to survive, three independent lines were recovered with a ≈50% reduction in the expression of *MKK4* and a ≈80% reduction in *MKK5* expression [see supporting information (SI) Fig. S1], and they display an abscission-defective phenotype (Fig. 1*A*). The *MKK4-MKK5RNAi* transgene appears

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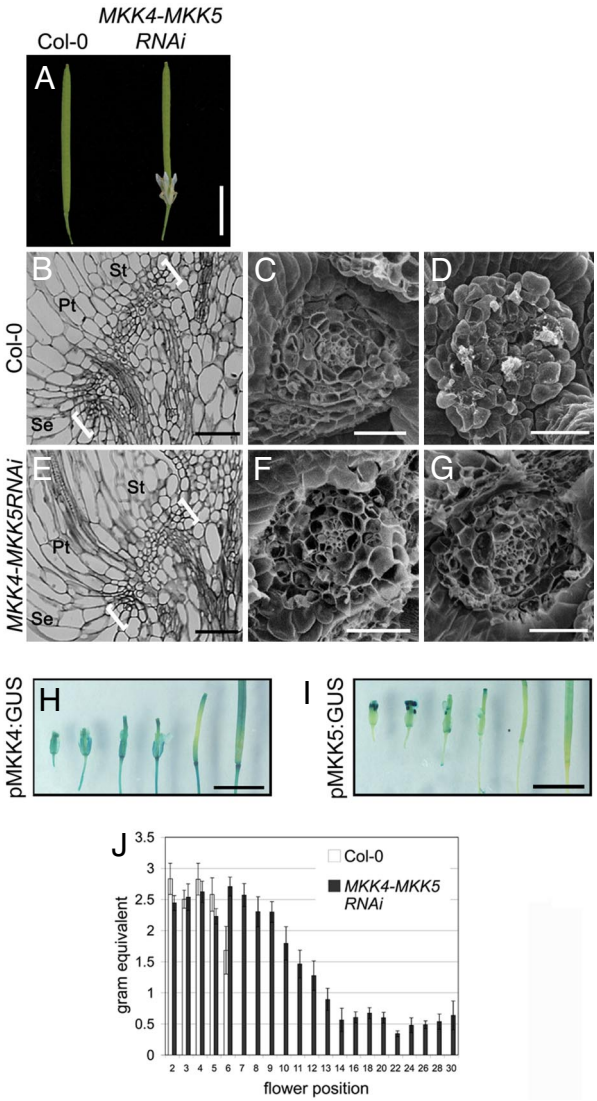

Fig. 1. *MKK4* and *MKK5* regulate floral organ abscission. (*A*) Representative siliques from Col-0 (wild type) and *MKK4-MKK5RNAi* plants at flower position 10. The floral organs of the position 10 flower of the Col-0 have abscised, but the flower from *MKK4-MKK5RNAi* plant displays an abscission defective phenotype. (*B* and *E*) Sepal (Se), petal (Pt) and stamen (St) AZ morphology of Col-0 (*B*) and *MKK4-MKK5RNAi* (*E*) flowers. The AZ (flanked by brackets) consists of the small dense cells at the base of the floral organs. (*C*, *D*, *F*, and *G*) SEM of floral organ AZs from Col-0 (*C* and *D*) and *MKK4-MKK5RNAi* (*F* and *G*) flowers from position 4 (*C* and *F*) and 10 (*D* and *G*). The floral organs were forcibly removed from the Col-0 position 4 (*C*) and from the *MKK4-MKK5RNAi* position 4 (*F*) and 10 (*G*) flowers. The AZ fracture plane created by forcible removal of the floral organs is observed as broken cells. The surface of AZ from Col-0 position 10 flowers (*D*) display the characteristic enlarged and rounded cells of the abscission scar that normally develops during abscission. (*H* and *I*) GUS reporter gene expression of *MKK4* (*H*) and *MKK5* (*I*) in flowers and siliques. (*J*) Petal breakstrength of *MKK4-MKK5RNAi* plants from flowers at different positions. Two petals from a flower were assayed from seven flowers ($n = 14$ per each position). Error bars indicate standard error. The force required to remove the petals could only be measured at positions 2–6 from Col-0 plants. (Scale bars: *A*, *H*, and *I*, 0.5 cm; *B* and *E*, 100 μm; *C*, *D*, *F*, and *G*, 50 μm.)

to selectively silence MKK4 and MKK5 because the expression of the two most closely related *Arabidopsis* MKKs, MKK7 and MKK9, are not significantly altered in *MKK4-MKK5RNAi* plants that have an abscission defective phenotype (Fig. S1). To determine the basis of the phenotype, we examined the morphology of the AZ in flowers at different stages of development. Flower development is commonly described using a standardized nomenclature based on the morphology of the floral organs (13). The floral stages examined in this study include stage 12, petals with long stamens; stage 13, buds open, petals visible, anthesis; stage 14, long anthers extend above stigma; stage 15, stigma extends above long anthers; stage 16, petals and sepals withering; and stage 17, all organs fall from green siliques. In addition, the flower position, which reflects the relative developmental stage along the inflorescence from the top to the bottom, is frequently used in studies of abscission in *Arabidopsis* (1). Position 1 is defined as the youngest flower after anthesis, with older flowers numbered consecutively. With the Col-0 ecotype under our standard growth conditions abscission occurs from flower positions 7 to 9.

AZs are characterized by the presence of small, cytoplasmically dense cells two to six layers deep. AZs from stage 15 *MKK4-MKK5RNAi* flowers, before abscission, appear to be the same as the wild type (Fig. 1 *B* and *E*). Scanning electron microscopy (SEM) is an effective approach to examine the progressive changes during abscission (1). Before cell separation occurs, floral organs can be forcibly removed to create a fracture plane at the AZ (Fig. 1*C*). After separation, the cells of the AZ are enlarged and rounded to form a characteristic abscission scar (1) (Fig. 1*D*). In the *MKK4-MKK5RNAi* flowers, the floral organs do not abscise (Fig. 1*A*) and have to be forcibly removed. The AZ fracture plane (Fig. 1*G*) of older *MKK4-MKK5RNAi* floral organs appears to be identical to what is observed in early flowers that have not completed the cell separation response (Fig. 1 *C* and *F*). Exogenous ethylene, which promotes the cell separation phase of abscission in wild-type plants, does not alter the abscission defective phenotype in the *MKK4-MKK5RNAi* plants (data not shown). If MKK4 and MKK5 have roles in abscission we would expect they would be expressed in the AZ. GUS reporter genes were used to analyze the expression of *MKK4* and *MKK5* in floral organs (Fig. 1 *H* and *I*). Expression of *MKK4* and *MKK5* is detected in the floral organs and the AZs. To quantify the difference in abscission between the wild-type and the *MKK4-MKK5RNAi* plants, we measured the amount of force required to pull the petals from the flower, using a petal breakstrength meter (1, 14) (Fig. 1*J*). Force is required to remove the petals from the *MKK4-MKK5RNAi* plants at all flower positions, which suggest that the normal weakening of the middle lamella of the AZ is disrupted. Based on these observations, we conclude the MKK4 and MKK5 are not required for the formation of the AZ but function subsequently during the cell separation phase of abscission.

The abscission-defective phenotype and the morphology of the AZ in the *MKK4-MKK5RNAi* plants suggest that a MAPK cascade is required for abscission. The *MKK4-MKK5RNAi* phenotype is very similar to the phenotype observed in *ida* plants (6). In addition, the normal AZ morphology and the lack of progressive changes in the AZ are characteristic of both the *MKK4-MKK5RNAi* (Fig. 1) and *ida* flowers (6). These observations suggest IDA and a MAPK cascade involving MKK4/MKK5 function in a signaling pathway that regulates floral organ abscission. The phenotype of antisense *HAE* plants (5) suggests that HAE, a RLK, may also function in this signaling pathway.

To determine whether IDA, HAE, MKK4, and MKK5 function in a common signaling pathway, we first examined the morphology and progressive AZ changes in a *hae* mutant background. Unlike the *HAE* antisense lines, T-DNA insertion loss-of-function *hae* alleles (Fig. S2) do not have an abscission-defective phenotype (Fig. 2*A*). *HAE* belongs to a gene family that includes two paralogs (15) that we have named *HAESA-like 1* (*HSL1*) and *HSL2*. Gene expression data show the expression pattern of *HSL2*, but not *HSL1*, is similar to *HAE*. Developmental expression patterns of *HAE* and *HSL2* were assessed by compiling publicly available microarray data and confirmed by

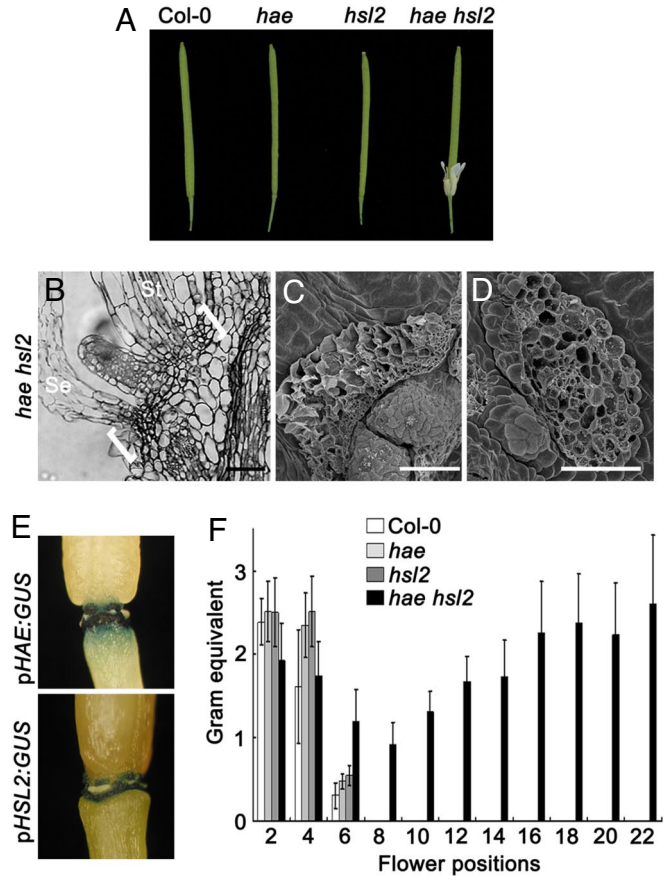

Fig. 2. *HAE* and *HSL2* regulate floral organ abscission. (*A*) Representative siliques from Col-0 (wild type), *hae*, *hsl2*, and *hae hsl2* plants at flower position 10. The floral organs of the position 10 flower of the Col-0, and single mutant *hae* or *hsl2* plants have abscised, but the flower from *hae hsl2* plants display an abscission defective phenotype. (*B*) Sepal (Se) and stamen (St) AZ morphology of a representative *hae hsl2* flower. The AZ (flanked by brackets) consists of the small dense cells at the base of the floral organs. (*C* and *D*) SEM of floral organ AZs from *hae hsl2* flowers from position 4 (*C*) and 10 (*D*). The floral organs were forcibly removed from the *hae hsl2* position 4 and 10 flowers. The AZ fracture plane created by forcible removal of the floral organs is observed as broken cells. (*E*) Reporter gene expression of *HAE* and *HSL2*. Expression analysis using *HAE* or *HSL2* promoter:GUS plants at flower stage 17 demonstrates that *HAE* and *HSL2* expression in flowers is restricted to the AZ. (*F*) Petal breakstrength of Col-0, *hae*, *hsl2*, and *hae hsl2* from flowers at different positions from 15 flowers ($n = 13$ per each position). The error bars indicate standard deviation. The force required to remove the petals could only be measured at positions 2, 4, and 6 from Col-0, *hae*, and *hsl2* plants. (Scale bars: *A*, 0.5 cm; *B*, 100 μm; *C* and *D*, 50 μm.)

GUS reporter gene expression. The compiled data show an increase in the expression of *HAE* and *HSL2* but decreased expression of *HSL1* just before the onset of floral organ abscission (Fig. S3). Plants expressing promoter GUS reporter genes, using the *HAE* or *HSL2* promoter, were examined in stage 17 flowers. GUS expression in the floral organs is restricted in the AZ (Fig. 2*E*).

The expression data and the phenotype of the *hae hsl2* plants suggest that *HAE* and *HSL2* have overlapping functions. Similar to the *hae* single mutant alleles, plants with T-DNA insertion loss-of-function alleles of *HSL2* (Fig. S2) do not show any phenotypic changes (Fig. 2*A*). However, double mutants of *hae* and *hsl2* have a strong abscission defect; the floral organs never abscise (Fig. 2*A*). Like *ida-2* (Fig. S2), a T-DNA insertion allele of *ida* (6) in the Col-0 ecotype, and *MKK4-MKK5RNAi* mutant lines, the *hae hsl2* plants appear to be defective in cell separation. The morphology of the AZ in the *hae hsl2* plants appears normal (Fig. 2*B*), but SEM of the AZ at different stages of development (Fig. 2 *C* and *D*) suggest that the middle lamella remains intact in floral organs that normally would have undergone abscission.

The force required to remove the petals from wild-type, *hae* and *hsl2* single mutants and the *hae hsl2* double mutant was used as a quantitative measure of abscission (Fig. 2*F*). The *hae* and *hsl2* single mutants require approximately the same amount of force as wild type to remove the petals from flowers before abscission. However, force is required to remove the petals from the *hae hsl2* double mutant plants at all flower positions. Exogenous ethylene treatment does not have an effect on the abscission defective phenotype of *hae hsl2* plants (data not shown). Thus, the mutant phenotypes, AZ morphology and absence of cell separation in plants with altered expression of IDA, HAE, HSL2, MKK4, and MKK5 suggests these proteins may function in a common signaling pathway to regulate abscission.

MKK4 and MKK5 have been shown to act through the *Arabidopsis* MAP kinases MPK3 and MPK6 in plant defense responses (8) and in stomatal patterning (10). An in-gel protein kinase assay was used to test the hypothesis that IDA, HAE, and HSL2 modulate the activity of a MAPK cascade. Proteins were isolated from the receptacle, the portion of the pedicel that bears the floral organs and includes the AZ, of wild-type, *hae hsl2*, and *ida*. There is a 40% decrease in the activity of MPK6, a known target MAPK of MKK4 and MKK5 (8), in the mutant lines relative to the wild type (Fig. 3*A* and Fig. S4). This result is consistent with a role for MPK6 in abscission, but the inability to measure MAPK activity specifically in the AZ makes it difficult to determine whether MPK6 is required for abscission using this assay.

Plants that are mutant for either *mpk3* or *mpk6* do not have an abscission defective phenotype and the *mpk3 mpk6* double mutant is embryo lethal (10). Therefore, to further investigate the role of *MPK6* in abscission, we expressed mutant forms of *MPK6* in wild-type and *mpk3* mutant plants. A mutated *MPK6* transgene that contains a Lys to Arg mutation in the protein kinase catalytic domain was constructed. The Lys targeted for mutagenesis is an invariant amino acid present in the protein kinase catalytic domain. This Lys is required for transfer of the phosphate from ATP to the protein substrate. A mutation of this conserved Lys often creates a dominant-negative form of the protein kinase (16). The *MPK6* Lys to Arg ($MPK6^{KR}$) transgene, driven by the *MPK6* promoter, was transformed into wild-type and *mpk3* plants. All of the $MPK6^{KR}$ plants in the wild-type background are normal. However, ≈10% of independently isolated $MPK6^{KR}$ transgenic lines in the *mpk3* background have an abscission defective phenotype (Fig. 3*B*). The morphology of the AZ in the $MPK6^{KR}$ plants appears normal (Fig. 3*C*), but SEM of the AZ at different stages of development (Fig. 3 *D* and *E*) suggests that the cell separation response is defective. Another mutated form of *MPK6* ($MPK6^{AF}$) also confers an abscission defective phenotype in *mpk3* but not the wild-type background (Fig. 3*B*). The conserved Thr and Tyr in the MAPK activation motif ThrXaaTyr, which are sites of phosphorylation by the upstream MKK and are required for MAPK activation (16), were mutated to Ala and Phe in $MPK6^{AF}$. These results support the hypothesis that *MPK6* is a positive regulator of abscission and that MPK3 can substitute for MPK6 in plants lacking a functional *MPK6*. The expression of a GUS reporter gene fused with the promoter of *MPK3* or *MPK6* shows high expression in floral organs and both genes are expressed in the AZs (Fig. 3*F*), which is also consistent with the roles of these genes in the regulation of floral organ abscission in *Arabidopsis*.

To test the hypothesis that the putative ligand and the receptor-like protein kinases are in a common pathway with a

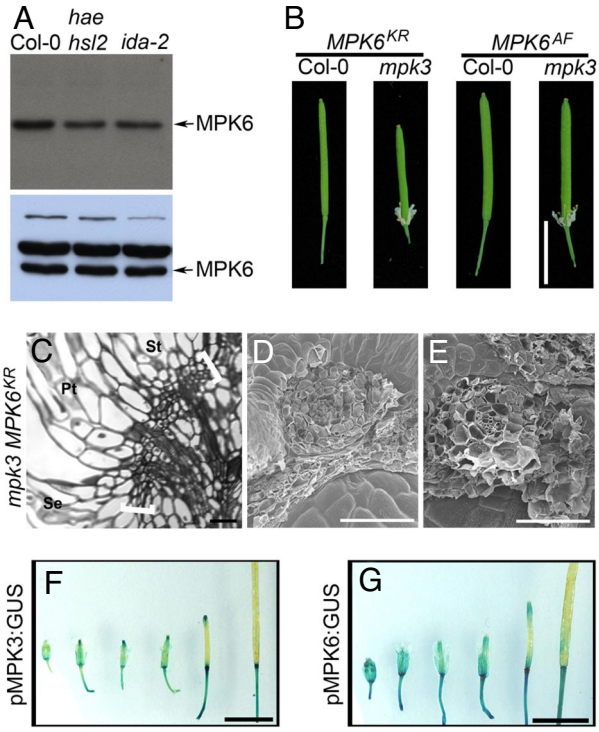

Fig. 3. *MPK6* and *MPK3* are required for floral organ abscission. (*A*) (*Upper*) An in-gel MAP kinase assay of protein extracted from the receptacles of Col-0, *hae hsl2*, and *ida-2* flowers at positions 6–8. (*Lower*) Western blot, using a MPK6 antibody of the same protein samples, showing equivalent amounts of protein were loaded for each sample. (*B*) Representative siliques at flower position 10 of the $MPK6^{KR}$ or $MPK6^{AF}$ transgenes expressed in Col-0 and *mpk3* plants. The abscission defective phenotype is only observed in the transgenic *mpk3* plants. (*C*) Sepal (Se), petal (Pt), and stamen (St) AZ morphology of $MPK6^{KR}$ in *mpk3* flowers. The AZ (flanked by brackets) at the base of the floral organs is normal in the transgenic plants. (*D* and *E*) SEM of the floral organ AZs from $MPK6^{KR}$ in *mpk3* flowers from position 4 (*D*) and 10 (*E*). The floral organs were forcibly removed from the flowers. (*F* and *G*) GUS reporter gene expression of *MPK3* (*F*) and *MPK6* (*G*) in flowers and siliques. Scale bars: *B*, *F*, and *G*, 0.5 cm; *C–E*, 50 μm.)

MAP kinase cascade, *ida-2* and *hae hsl2* plants were transformed with constitutively active forms of *MKK4* or *MKK5* under the control of a steroid-inducible promoter ($GVG\text{-}MKK4^{DD}$ and $GVG\text{-}MKK5^{DD}$) (9). Due to the hypersensitive reaction and cell death induced by high expression of the *MKK*s, the plants were not treated with the steroid inducer. However, the basal level of expression of either transgene is sufficient to rescue MAPK pathway mutant phenotypes (10). Several lines in each mutant background showed a rescued abscission phenotype (Fig. 4). These data suggest that *MKK4* and *MKK5* are epistatic to *IDA*, *HAE* and *HSL2* and support the hypothesis that they are in a common pathway upstream of MKK4 and MKK5.

Although the constitutively active *MKK* transgenes revert both the *ida-2* and *hae hsl2* mutant phenotypes and expression of dominant-negative forms of MPK6 have abscission defective phenotypes, these results do not distinguish between the role of *IDA* and *HAE HSL2* functioning in common or parallel pathways to regulate a MAPK cascade. To test these alternative hypotheses, we reasoned that a gain of function IDA gene would require *HAE* and *HSL2* if they were in a shared pathway but that the gain of function *IDA* phenotype would not be altered if *IDA* and *HAE HSL2* were in parallel pathways. Therefore, we combined *hae hsl2* with overexpression of *IDA*. Similar to the report in ref. 7, overexpression of *IDA* (*IDAOE*)

Fig. 4. IDA and HAE HSL2 regulate abscission via a MAPK signaling cascade. Representative siliques from Col-0, *ida-2*, *hae hsl2*, $GVG\text{-}MKK4^{DD}$ *ida-2* and $GVG\text{-}MKK4^{DD}$ *hae hsl2* plants at flower position 10. The abscission defective phenotype of *ida-2* or *hae hsl2* is rescued by the $GVG\text{-}MKK4^{DD}$ transgene.

in wild type shows a premature abscission phenotype and the production of a white substance in the region of abscission (Fig. 5*A*). However, *IDAOE* in *hae hsl2* has an abscission-defective phenotype, the same as that of *hae hsl2* (Fig. 5*A*). These results show that *hae hsl2* are epistatic to *IDAOE* and support the hypothesis IDA and HAE HSL2 function in a common pathway to regulate abscission.

Multiple gene products, including potential signaling ligands, membrane receptors, protein kinase cascades, regulators of hormone responses, and transcription factors have been implicated in the regulation of abscission in plants (4). We have demonstrated, by several different lines of evidence, that there is a signaling cascade (Fig. 5*B*), from putative ligand (IDA) to receptors (HAE HSL2) to cytoplasmic effectors (MKK4, MKK5, MPK3, and MPK6), which function together to control cell separation during abscission. Additional gene products are also likely to play important roles in abscission and the relationships between them and the signaling pathway outlined here remain to be determined. However, based on the genetic interactions between *IDA*, *HAE*, *HSL2*, *MKK4*, and *MKK5*, it seems that this core signaling cascade is an important regulator of floral abscission.

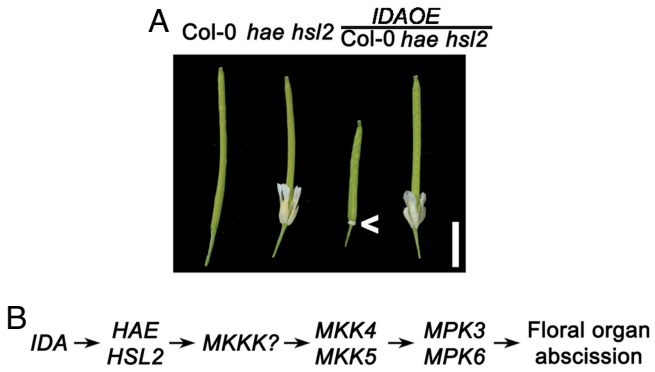

Fig. 5. HAE and HSL2 are epistatic to IDA. (*A*) Representative siliques from Col-0, *hae hsl2*, *IDAOE*, and *IDAOE hae hsl2* plants at flower position 10. The flowers from the *hae hsl2* double mutant plants display an abscission defective phenotype, and overexpression of *IDA* in the Col-0 background shows premature abscission and the production of a white substance in receptacle (indicated by the arrow). The *hae hsl2* double mutant masks the *IDA* overexpression phenotype and maintains the abscission defective phenotype. (Scale bar: 0.5 cm.) (*B*) Schematic diagram of the role of IDA, HAE HSL2, and a MAPK cascade in the regulation of floral organ abscission.

Materials and Methods

Plant Materials and Growth. Columbia ecotype (Col-0) of *Arabidopsis thaliana* was used as wild type and is the background ecotype for all mutants. T-DNA insertion alleles (17) were obtained from the *Arabidopsis* Biological Resources Center (Ohio State University, Columbus, OH) (SALK_105975, SALK_015074, SALK_057117, SALK_030520, and SALK_133209 for *hae-1*, *hae-2*, *hsl2-1*, *hsl2-2*, and *ida-2*, respectively). After identifying homozygous mutant plants, the single *hae-1* and *hsl-12* T-DNA insertion plants were crossed to make *hae hsl2* double mutants, and confirmed by PCR. All of the primers for PCR genotyping are listed in Table S1. Plants were grown at 22°C on a 16 h light:8 h dark cycle.

Cloning and Plant Transformation. Plants were transformed using the floral dip method (18). For the generation of the *MKK4-MKK5* RNAi lines, a transgene identical to that in Wang *et al.* (10) was used. A total of three lines showing an abscission defective phenotype for the *MKK4-MKK5RNAi* transgenics from ≈300 total T1 plants screened were recovered. For the generation of the $MPK6^{KR}$ and $MPK6^{AF}$ constructs, a 2834-bp fragment upstream of ATG start codon of *MPK6* was amplified with MPK6-F and MPK6-B. This upstream sequence was then cloned into pCAMBIA3300 between *Eco*R1 and SmaI. cDNA of MPK6 containing the KR or AF mutations was amplified with cMPK6-F and cMPK6-Nosterminator-B with the NOS terminator incorporated at the 3′. The KR and AF mutations were generated (16). This cMPK6-NOS terminator fragment was then cloned into the SmaI and XbaI sites of pCAMBIA3300 to generate the final construct of *pCAMBIA3300-*P_{MPK6}*:cMPK6-NOSter*. For overexpression constructs of IDA, a genomic fragment of *IDA* was amplified using *Pfu* (Stratagene) and cloned into to the KpnI and SacI sites of pBIB-Hyg (19) under CaMV35S promoter. A total of 60 *IDAOE* Col-0 and 80 *IDAOE hae hsl2* transgenic plants were screened on half-strength Murashige and Skoog salts (½ MS) media with 20 μg/ml hygromycin (Sigma), and their phenotypes scored. Seventeen plants containing the *IDAOE* transgene in Col-0 showed a premature abscission phenotype and the production of a white substance in the receptacle (28.3% of selected plants). All of the *IDAOE hae hsl2* transgenic plants had the abscission defective phenotype (100% of selected plants). Constitutively active *MKK4* (*GVG-*$MKK4^{DD}$) and *MKK5* (*GVG-*$MKK5^{DD}$) (9) in the pTA7002 vector (20) were transformed into *ida-2* and *hae hsl2*, and 90 individual plants each were screened on ½ MS media with 20 μg/ml hygromycin (Sigma). Three plants from *GVG-*$MKK4^{DD}$ in *ida-2* (3.3% of screened plants), three plants from *GVG-*$MKK4^{DD}$ in *hae hsl2* (3.3% of selected plants), and six plants from *GVG-*$MKK5^{DD}$ in *hae hsl2* (6.7% of screened transgenics) showed normal abscission. A different transgene in the pTA7002 vector was transformed into *ida-2* and *hae hsl2*, and 80 individual plants each were screened on ½ MS media with 20 μg/ml hygromycin. None showed normal abscission. The region toward the 5′ end of the start codons (1.6 kb upstream) of *HAE* or *HSL2* was amplified by *Pfu* (Stratagene) and inserted in pBIG-Hyg (19) for GUS constructs. All of the primers for the construction of the transgenes are listed in Table S1.

Expression of *MKK4 and MKK5* by Quantitative Real-Time PCR. The expression of *MKK4* and *MKK5* was assayed using unopened floral buds from Col-0 and *MKK4-MKK5RNAi* plants. RNA was extracted using TRIzol Reagent (Invitrogen) according to the manufacturer's recommendations. One microgram of total RNA was reverse transcribed using an oligo dT primer and the Omniscript RT Kit (Qiagen). For the real-time PCR, Absolute QPCR SYBR green mix (ABgene) was used, and PCR was performed using DNA Engine Opticon 2 (MJ Research). All samples were log-transformed and normalized to an *EF-1-α* control. Primers for the real-time PCR are listed in Table S1.

Histochemical GUS Expression. GUS reporter analyses were performed as reported in ref. 21 with minor modification. Stage 17 (13) flowers from the transgenic plants were harvested and fixed in ice-cold 90% acetone for 1 h at −20 C. The tissues were stained in GUS solution [2 mM potassium ferricyanide, 2 mM potassium ferrocyanide, 10 mM EDTA, 0.2% Triton X-100, 100 μg/ml chloramphenicol, 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-glucuronic acid in 50 mM sodium phosphate buffer (pH 7.0)] for 3 h at 37°C and then cleared in 70% ethanol. GUS expression was observed on a Nikon SMZ-2T microscope.

Petal Breakstrength. The force that is required for pulling a petal from a flower was measured using a petal breakstrength meter (14). Breakstrength from each indicated floral position of Col-0, *hae*, *hsl2*, and *hae hsl2* was analyzed from 15 plants (additional data were collected for the early stage flowers). The maximum and minimum values were considered as outliers. Therefore, a total of 13 petals per position were assayed. In the case of *MKK4-MKK5RNAi* plant, two petals per each flower position from seven plants were analyzed ($n = 14$).

Light Microscopy and Scanning Electron Microscopy. Flowers are numbered from the youngest flowers, where the petals first emerge above the sepals to the oldest, most mature flowers. In Col-0 plants, abscission occurs from flower positions 7 to 9. Flowers from stage 15 (13) of 8-week-old Col-0, *MKK4-MKK5RNAi*, *hae hsl2*, and $MPK6^{KR}$ were embedded in Epon–Spurr's resin (22). Thin sections (2.5 μm) were obtained with a Leica RM2065 microtome equipped with glass knives and stained with in 0.05% toluidine blue. For SEM analysis, flowers from positions 4 and 10 of 8-week-old Col-0, *MKK4-MKK5RNAi*, and *hae hsl2* were collected, fixed in 4% (vol/vol) gluteraldehyde in 0.05 M potassium phosphate buffer (pH 7.4), and then rinsed four times in the buffer. After dehydration in a graded ethanol series, flower samples were critical-point dried in liquid CO_2. Samples were mounted on plates with double-stick tape, and then floral organs were forcibly removed. Samples were sputter coated with Pt, and AZs were viewed at 5 KV on a Hitachi S4700 Field Emission Scanning Electron Microscope or an Amray 1600T Scanning Electron Microscope.

In-Gel Kinase Assay. Protein was isolated as described in ref. 23 from the receptacles of Col-0, *hae hsl2*, and *ida-2*. Fifteen micrograms of protein were separated in 10% SDS/PAGE embedded with myelin basic protein (0.25 mg/ml) as a MAP kinase substrate. H_2O_2-treated leaves (1 mM for 1 h) of Col-0 were used as positive control for MAP kinase activity. The relative amount of myelin basic protein phosphorylation was analyzed using Fuji phosphoimage analyzer and quantified using Multi Gauge software, Version 2.0 (Fuji Film). For the quantity control, the protein was separated in 10% SDS/PAGE, transferred to Immobilon-P transfer membranes (Millipore), and quantified using Anti-AtMPK6 (Sigma).

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