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Floral organ abscission is regulated by a positive feedback loop

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Abscission is the process by which plants shed unwanted organs, either as part of a natural developmental program or in response to environmental stimuli. Studies in *Arabidopsis thaliana* have elucidated a number of the genetic components that regulate abscission of floral organs, including a pair of related receptor-like protein kinases, *HAESA* and *HAESA-like 2* (*HAE/HSL2*) that regulate a MAP kinase cascade that is required for abscission. *HAE* is transcriptionally up-regulated in the floral abscission zone just before cell separation. Here, we identify AGAMOUS-like 15 (AGL15; a MADS-domain transcription factor) as a putative regulator of *HAE* expression. Overexpression of *AGL15* results in decreased expression of *HAE* as well as a delayed abscission phenotype. Chromatin immunoprecipitation experiments indicate that AGL15 binds the *HAE* promoter in floral receptacles. AGL15 is then differentially phosphorylated through development in floral receptacles in a MITOGEN-ACTIVATED PROTEIN KINASE KINASE 4/5-dependent manner. MAP kinase phosphorylation of AGL15 is necessary for full *HAE* expression, thus completing a positive feedback loop controlling *HAE* expression. Together, the network components in this positive feedback loop constitute an emergent property that regulates the large dynamic range of gene expression (27-fold increase in *HAE*) observed in flowers when the abscission program is initiated. This study helps define the mechanisms and regulatory networks involved in a receptor-mediated signaling pathway that controls floral organ abscission.

transcriptional regulation | transcription factor | protein phosphorylation | signal transduction | abscission

Abscission is the process that plants use to shed unwanted organs. Various plants can abscise leaves, fruits, and flowers. One of the most noticeable abscission events occurs when deciduous trees and shrubs shed their leaves in the fall. Plants can abscise organs as part of a developmental program or in an inducible manner in response to stimuli like abiotic or biotic stress. For example, tomatoes can abscise leaves and flowers in response to drought or insect feeding (1–3). In order for abscission to occur, a layer of small and cytoplasmically dense cells, known as an abscission zone, must be laid down during development at the boundary between the organ to be abscised and the body of the plant (4).

Arabidopsis thaliana has been used to elucidate a number of the molecular and genetic components that regulate abscission of floral organs. In *Arabidopsis*, floral organs abscise shortly after pollination, which corresponds to stage-16 flowers or approximately floral position 4–6, where anthesis is defined as position 1 and older flowers are defined as increasing numerical positions (Fig. 1 *A* and *B*) (5). A number of genetic components regulating this abscission process have been described thoroughly in recent reviews (6, 7). In brief, a pair of related receptor-like protein kinases, *HAESA* and *HAESA-like 2* (*HAE/HSL2*), are required for floral abscission (Fig. 1*C*) and are thought to be triggered by a peptide derived from INFLORESCENCE DEFICIENT IN ABSCISSION (IDA) (8, 9). A mitogen-activated protein kinase (MAPK) cascade consisting of MITOGEN-ACTIVATED PROTEIN KINASE KINASE 4/5 (MKK4/5) and MPK3/6 is positioned genetically downstream from *HAE/HSL2* (8). Mutations in the transcription factor *BREVIPEDICELLUS* (a knotted1-like homeobox transcription factor) suppress the abscission defect of the *hae hsl2* double mutant (10). Additionally, *nevershed* (*nev*; an ARF–GAP domain protein) plants do not abscise, whereas secondary mutations in *evershed* [*evr*; a leucine-rich repeat (LRR) receptor-like protein kinase], *somatic embryogenesis receptor kinase1* (a LRR receptor-like protein kinase), and *cast away* (a receptor-like cytoplasmic protein kinase) suppress the abscission defect of *nev* (11–14). *HAE* is transcriptionally up-regulated in the floral abscission zone just before cell separation (Fig. 1 *D–F*) (15, 16). The initial goal of this study was to extend the known floral abscission pathway by identifying upstream components that regulate *HAE* expression. However, the course of experimentation resulted in uncovering previously unidentified mechanisms and integrates known regulatory networks that control the floral abscission pathway. There is a large dynamic range of *HAE* expression through the process of abscission because *HAE* progressively increases 27-fold from stage-12 to -15 abscission zones (Fig. 1*F*) (16). This study also helps explain the large dynamic range of gene expression that is observed in the process of abscission.

Here, we report that AGAMOUS-like 15 (AGL15) is a putative regulator of *HAE* expression. Overexpression of *AGL15* results in decreased expression of *HAE* in stage-15 floral receptacles, as well as a delayed abscission phenotype (17). Chromatin immunoprecipitation (ChIP) experiments indicate that AGL15 binds the *HAE* promoter in floral receptacles, where it is also phosphorylated by a MAPK cascade. Overall, this work defines a positive feedback loop controlling *HAE* expression.

Significance

Dramatic changes in gene expression occur in abscission zones immediately prior to abscission. This study helps explain the mechanisms and emergent properties of a regulatory network that controls abscission. Two important connections are made that help explain the observed dynamic range of abscission gene expression. First, it connects a MAP kinase cascade that positively regulates abscission to a MADS-domain transcription factor, AGAMOUS-like 15 (AGL15). Second, it connects AGL15 to the expression of a receptor kinase known to control abscission. All together, the findings define a positive feedback loop that provides exponential signal amplification that starts with a receptor, goes through a MAP kinase cascade and a transcription factor, and then returns to the receptor.

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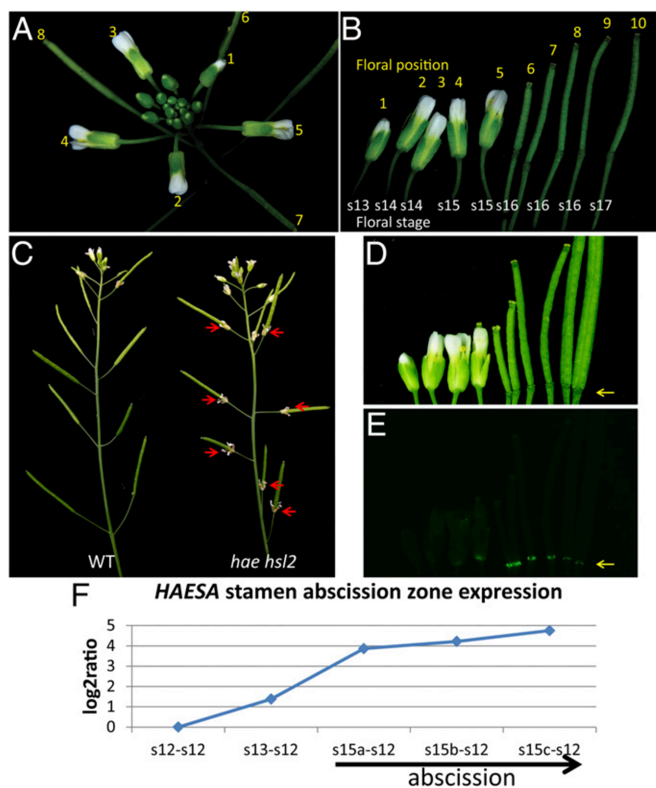

Fig. 1. HAE is expressed in abscission zones and is required along with HSL2 for floral organ abscission. (*A*) WT inflorescence photographed from above with floral position numbered. (*B*) Same flowers from inflorescence in *A* arranged in floral position order with both floral stage and position indicated. (*C*) *hae hsl2* double mutant does not abscise its floral organs. Arrows indicate abnormally attached floral organs. (*D* and *E*) White light photograph (*D*) and photograph (*E*) of YFP fluorescence of *HAE-promoter-HAE-YFP* plants expressing HAE–YFP specifically in abscission zones of flowers that are about to abscise and after abscission has occurred. The arrows indicate the position of the abscission zone. (*F*) *HAE* transcript expression is induced shortly before abscission in stamen abscission zones. Floral stages of stamen abscission zones are indicated in which s15a, s15b, and s15c are progressively older subdivisions of stage 15 flowers (16).

Results

Promoters of Genes Coexpressed with *HAE* Have Overrepresented AGL15 and WRKY Binding Sites. *HAE* expression increases in stamen abscission zones from stage-12 to late-stage-15 flowers (Fig. 1*F*) (16). This increase of HAE expression is largely confined to the abscission zones (Fig. 1 *D* and *E*). We sought to understand this abscission-related transcriptional event by identifying components that control *HAE* transcription. Analysis of the promoters of the top 100 genes coexpressed with *HAE* (Pearson's correlation, $r > 0.85$ across 28 conditions in which *HAE* expression changes) revealed an overrepresentation of several transcription factor families (Table 1 and Datasets S1 and S2). The *HAE* promoter has at least one of all of the *cis*-elements listed in Table 1 and has five AGL15 binding sites and five WRKY binding sites (W boxes) (Fig. 2*A*; refs. 18 and 19). Previously, it was shown that AGL15 overexpression in *Arabidopsis* (ecotype Wassilewskija/WS) delays floral abscission (17). AGL15 binds a CArG motif with a longer A/T-rich core than other MADS-domain proteins (18). AGL15 has been characterized in great detail (17, 18, 20–22), especially in embryos and the regulation of flowering time, making hypothesis generation easier than for less-studied transcription factors. Moreover, *AGL15* mRNA is detectable in stamen abscission zones and is differentially expressed through the process of abscission (Fig. 2*B*) (16). We thus hypothesized that AGL15 may be a repressor that keeps *HAE* expression low before abscission, and we designed experiments to test this hypothesis.

Strong Overexpression of AGL15 Leads to Delay of Abscission and Decreased *HAE* Expression in Floral Receptacles. We made Col-0 (ecotype Columbia) plants that express AGL15 tagged with a double hemagglutinin (HA) tag at the C terminus to determine whether AGL15 could alter *HAE* expression and also bind the *HAE* promoter. The phenotypes of 43 T_1 transgenic plants were scored. Two independent transgenic plants showed a delayed floral organ abscission and senescence phenotype similar to that previously described for AGL15 overexpressors in the WS background (Fig. 3*A*) (17). The remaining transgenic plants were indistinguishable from wild-type (WT) plants and looked similar to the line-1 flowers shown in Fig. 3*A*. Western blot analyses on flowers from the two plants that showed a delayed abscission phenotype as well as from four plants that had a WT-like appearance showed that the abscission-delayed phenotype is correlated with strong overexpression of AGL15 protein in flowers (Fig. 3*B* and Fig. S1). Floral receptacles from stage-15 flowers were isolated from AGL15 overexpressors with the abscission-delayed phenotype (line 2) and from WT flowers to test whether *HAE* transcript accumulation was altered. *HAE* expression was statistically decreased fivefold in AGL15 overexpressors (Fig. 3*C*). This finding suggests that AGL15 may be a negative regulator of *HAE* expression. Stage-11 (two floral positions before anthesis) and stage-15 floral receptacles were collected from WT and from T-DNA insertion lines *agl15-4*, *agl18-2*, and *agl15-4 agl18-2* and analyzed for *HAE* transcript accumulation. *AGL18* is *AGL15's* closest relative and has been shown to have overlapping functions with *AGL15*, including the ability to delay abscission when overexpressed (21, 23). No statistically significant differential expression was observed between WT and single or double mutants (Fig. 3*D*). These loss-of-function experiments suggest that if AGL15 is a repressor of *HAE* expression, then there are either additional redundant repressors or multiple levels of control over *HAE* expression. Although the *agl15-4 agl18-2* double-mutant plant did not have statistically different *HAE* expression, it did have a statistically earlier abscission phenotype than WT plants (Fig. 3*E* and Fig. S2). Qualitatively *agl15-4 agl18-2*

Table 1. Overrepresented transcription factor binding sites in promoters of *HAE* coexpressed genes

Cis-element name	Consensus sequence	*P* value	Promoters with the motif (of 100)	Total sites in 100 promoters
AGL15 binding site	CW_8G	$<10^{-10}$	91	472
W-box promoter motif	TTGACY	$<10^{-10}$	95	291
Myc *cis*-element of EDR1	CATGTG	$<10^{-10}$	81	135
AtMYC2 binding site in RD22	CACATG	$<10^{-10}$	81	135
ARF binding site motif	TGTCTC	$<10^{-6}$	67	112
GA-responsive element	TAACAAR	$<10^{-5}$	80	175
MYB4 binding site motif	AMCWAMC	$<10^{-4}$	90	301

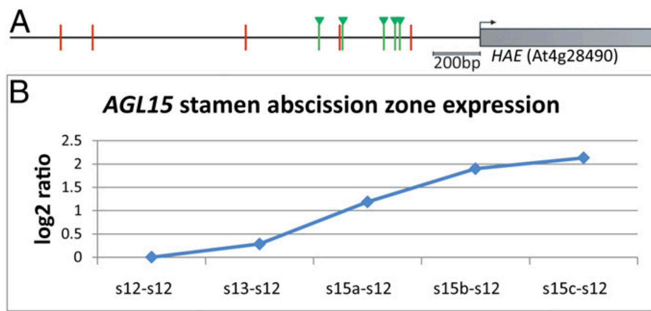

Fig. 2. *AGL15* is transcriptionally increased through the process of abscission and is predicted to bind the *HAE* promoter. (*A*) The *HAE* promoter has five AGL15 binding sites (red) and five W boxes (green). (*B*) *AGL15* is transcriptionally up-regulated immediately before abscission in stamen abscission zones (16).

double mutants look very similar to WT plants; however, their quantitatively early abscission was robustly reproducible (Fig. S2), lending additional support for AGL15 as a negative regulator of abscission.

AGL15 Directly Interacts with the *HAE* Promoter in Floral Receptacles. We next asked whether AGL15 directly controls *HAE* expression by binding to the *HAE* promoter. Results of ChIP-quantitative PCR (qPCR) performed on stage-15 floral receptacles from plants that overexpress AGL15 show that AGL15 interacts directly with the *HAE* promoter (Fig. 4*A*). To determine whether AGL15 could interact with the *HAE* promoter at natural protein levels, we made a binary vector with the *AGL15* native promoter and the *AGL15* genomic coding sequence followed by a double HA tag. This construct was used to make transgenic plants in the *agl15-4* background (*AGL15* null) (18, 23). Several transgenic lines were chosen for future use that had WT appearance, similar *AGL15* transcript accumulation as in WT plants, and detectable AGL15-HA on Western blots in flowers. AGL15 native promoter lines had much less or no detectable protein in rosette leaves than in flowers (Fig. S3). ChIP-qPCR performed on floral receptacles from AGL15 native promoter plants show that AGL15 interacts with the *HAE* promoter at native protein levels (Fig. 4*B*). AGL15 binding to the *HAE* promoter can be detected in floral receptacles isolated before anthesis (stage 10/11) and after pollination (stage 15) (Fig. 4*B*). ChIP performed on empty vector plants did not produce *HAE* promoter enrichment, whereas an independent transgenic line did produce enrichment (Fig. 4*B*). Together, these results suggest that AGL15 directly regulates *HAE* expression through physical interaction with the promoter of *HAE*, but differential binding at different floral stages may not be part of the regulatory mechanism.

AGL15 Is Differentially Phosphorylated Through the Process of Abscission and Requires a MAPK Cascade to Be Fully Phosphorylated. While running Western blots of flowers and floral receptacles from *35S-AGL15* plants, we observed that AGL15 ran as two bands. Previous studies have shown that AGL15 is phosphorylated by MPK6 in vitro on a protein microarray and, to a lesser extent, by MPK3 (24). We reasoned that one of the two bands might be a phosphorylated version of AGL15. To test this AGL15 phosphorylation hypothesis, we prepared protein extract from floral receptacles and split it into two halves; one half was treated with alkaline phosphatase. The sample treated with alkaline phosphatase showed only a single band of AGL15 (Fig. 5*A*), which supports the hypothesis that AGL15 has a phosphorylated isoform in floral receptacles.

We next explored the possibility that AGL15 might be differentially phosphorylated through the process of abscission. Protein extracts isolated from a developmental series of floral receptacles from AGL15 native promoter plants were analyzed on 25 μM Phos-tag gels. Phos-tag SDS/PAGE gels have immobilized metal ions (manganese or zinc) that provide phosphate affinity for mobility shift detection of phosphorylated proteins. Fig. 5*B* shows that AGL15 is differentially phosphorylated across a developmental series. Floral receptacles three positions before anthesis (stage 10) have predominantly nonphosphorylated AGL15. As floral buds mature, AGL15 can be separated clearly into three bands. Running the same extracts on identical gels, minus the 25 μM Phos-tag, results in single AGL15 bands among all floral stages (Fig. 5*C*). A MAPK cascade consisting of MKK4/5 and MPK3/6 has been shown to regulate floral abscission in *Arabidopsis* (8). To determine whether this MAPK cascade is responsible for AGL15 phosphorylation in floral receptacles, we crossed native promoter AGL15 plants to *MKK4/5* tandem RNA interference (RNAi) plants. The resulting F_1 plants had a HA-tagged AGL15 that could be followed on Western blots and an abscission-delayed phenotype (although not as strong of a phenotype as

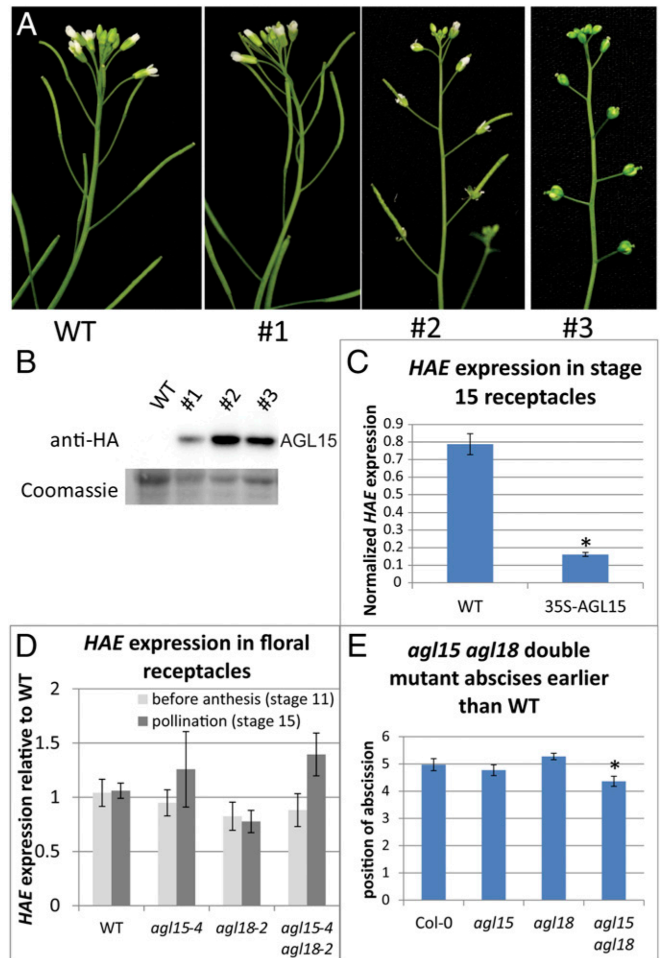

Fig. 3. Overexpression of AGL15 results in reduced *HAE* expression in floral receptacles. (*A* and *B*) Strong overexpression of AGL15–double-HA tag (AGL15-DHA) results in Col-0 plants that do not abscise normally (lines 2 and 3), whereas medium overexpression results in plants that resemble WT Col-0 (line 1). Rubisco is shown as a loading control. (*C*) *HAE* expression in stage-15 receptacles. *HAE* expression was reduced fivefold in plants that strongly overexpress AGL15. *$P <$ 0.005. $n = 3$; SEM error bars are shown. (*D*) *HAE* expression in floral receptacles. Single or double *agl15*, *agl18* mutants do not have altered *HAE* expression ($n =$ 5). (*E*) *agl15 agl18* double mutants abscise statistically earlier than WT, whereas single mutants do not. *$P < 0.05$ (*t* test). $n = 12$; SEM error bars are shown.

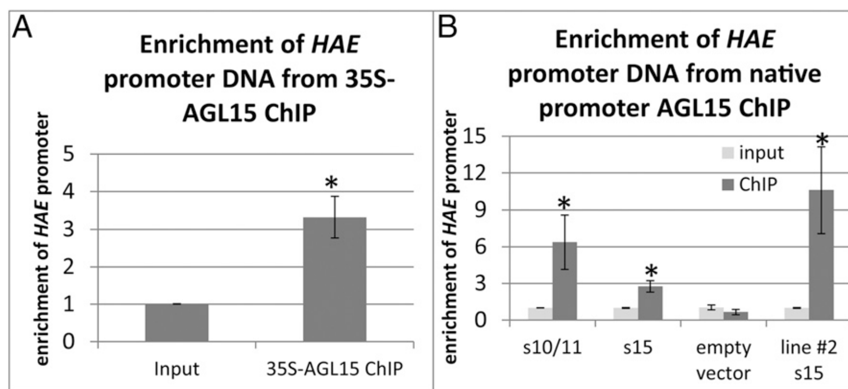

Fig. 4. AGL15 binds to the *HAE* promoter in floral receptacles. (*A*) Enrichment of *HAE* promoter DNA from 35S–AGL15 ChIP. ChIP on s15 receptacles from 35S–AGL15-DHA indicate that AGL15 binds the *HAE* promoter in vivo. $^{*}P = 0.03$ (*t* test). $n = 3$; SEM error bars are shown. (*B*) Enrichment of *HAE* promoter DNA from native promoter AGL15 ChIP. ChIP on floral receptacles of AGL15p:AGL15-DHA plants indicate AGL15 can interact with the *HAE* promoter in floral receptacles at native expression levels. $^{*}P < 0.05$ (*t* test). $n = 4$; SEM error bars are shown.

homozygous *MKK4/5 RNAi* plants, which have completely blocked abscission) (Fig. 5 *F–H*). The plants with reduced MKK4/5 have greatly reduced phosphorylation of one of the three phosphorylation isoforms (labeled AGL15 B) (Fig. 5*D*). AGL15 runs as a single band on an identical gel without Phos-tag (Fig. 5*E*). This experiment suggests that full AGL15 phosphorylation requires the MKK4/5 cascade.

***MKK4/5* Are Required for Full *HAE* Expression Before Abscission.** AGL15 appears to regulate *HAE* expression, and AGL15 is itself likely phosphorylated by the MAPK cascade consisting of MKK4/5 and MPK3/6. Previously, this MAPK cascade was positioned genetically downstream of HAE (8). One interpretation of these two results is that AGL15 could be both upstream of *HAE* and downstream of the MAPK cascade. Thus, we hypothesized that there may be a loop controlling *HAE* expression. To test this hypothesis, *HAE* expression was analyzed in stage-15 floral receptacles of *MKK4/5 RNAi* plants (homozygous RNAi; Fig. 5*G*). We found that *HAE* expression is reduced 3.5-fold in *MKK4/5 RNAi* plants compared with WT plants (Fig. 6*A*). The *hae-3 hsl2-3* double-mutant plant does not abscise, but has nearly identical *HAE* transcript accumulation as WT, indicating that not all mutants that do not abscise have decreased *HAE* expression (25). Reduced *HAE* expression in *MKK4/5 RNAi* plants suggests both that MAPK phosphorylation is necessary for *HAE* expression and that a positive feedback loop exists that controls *HAE* expression. This positive feedback loop could serve to amplify the expression of *HAE* and other genes necessary for abscission. Further support for this positive feedback loop is the observation that transient overexpression of an active form of MKK4 increases *HAE* expression twofold (Fig. 6*B*) (26).

Here we show that the specific expression pattern of *HAE* is regulated by both AGL15 and the MAPK cascade consisting of MKK4/5. We also show that posttranslational regulation of AGL15 occurs, in part, downstream of the MAPK cascade. Thus, this work describes both a transcription factor that directly regulates *HAE* expression in floral receptacles and how the transcription is itself directly regulated by MAPK phosphorylation in the floral abscission pathway. Based on these findings, we propose that the linear description of the core floral organ abscission pathway (8) be modified to include a positive feedback loop (Fig. 6*C*). MAPK cascades serve to amplify input signals (activation of one MAPKKK can result in activation of many downstream MAPKs). A positive feedback loop that connects components downstream of MPK3/6 with expression of the receptor-like protein kinase HAE, which is upstream of the MAPK cascade, would allow for both further signal amplification and more points of control. Multiple means of signal amplification could account for the large dynamic range of expression that is observed for *HAE* and other genes that function in abscission.

Discussion

To date, much of the work to elucidate the molecular processes that control floral abscission in *Arabidopsis* has focused on genetic approaches. Currently, a number of genes have been described that are necessary for abscission. Additionally, a number of genes have been described that, when overexpressed, delay abscission. Finally, secondary mutations that suppress abscission defects of several mutants have also been characterized (6, 7, 10, 12–14, 17, 21, 27). A limited number of biochemical approaches have been used to improve our understanding of abscission, largely due to the small size of *Arabidopsis* flowers and abscission zones. The exceptions are genome-scale transcript profiling experiments that describe both the transcriptional changes that occur through the process of abscission and transcriptional differences between the *hae hsl2* double mutant and WT (16, 25). Here we describe a transcription factor, AGL15, that binds the *HAE* promoter in floral receptacles and regulates *HAE* expression. AGL15 is differentially phosphorylated in floral receptacles through the process of abscission in a MKK4/5-dependent manner. All together, these results support a model involving a positive feedback loop that regulates *HAE* expression and the process of abscission. This work extends our understanding of a core abscission regulatory pathway from receptor to transcription factor and helps explain the observed dynamic range of abscission gene expression. This study defines mechanisms and regulatory networks controlling abscission.

Specifically, we show that the transcription factor AGL15 is a direct target of MAPK phosphorylation in floral organ abscission and that AGL15 directly binds the *HAE* promoter and regulates *HAE* expression. In general, organisms have sophisticated levels of control to regulate important biological processes. The positive feedback loop we describe would allow a lot of fine control over abscission gene expression and a large dynamic range of expression due to the loop's ability to amplify the initial signal. *HAE* expression in stamen abscission zones illustrates the large dynamic range of expression that is possible. From stage-12 flowers to the end of stage-15 flowers, HAE expression increases 27-fold (Fig. 1*F*) (16). AGL15 may not be the only transcription factor that regulates *HAE*. The lack of major changes in *HAE* expression in *agl15*, *agl18*, or *agl15 agl18* single/double mutants suggest that additional factors may be needed to activate *HAE* expression. Although we focus on AGL15's regulation of *HAE* because HAE is a well-studied positive regulator of abscission, AGL15 likely regulates other known components of abscission as well. For example, *EVR* is coexpressed with *HAE* (Dataset S2), and AGL15 has been shown to bind the *EVR* and *HAE* promoter in somatic embryos (ChIP-Chip experiment) (20). It is attractive to speculate whether *HSL2* may also be regulated by AGL15. *HSL2* has four AGL15 binding sites in the 2,000 base pairs upstream from its transcription start, which suggests that AGL15 may be able to regulate both *HAE* and *HSL2*. However, *HSL2* is not a *HAE*-coexpressed gene (Dataset S2).

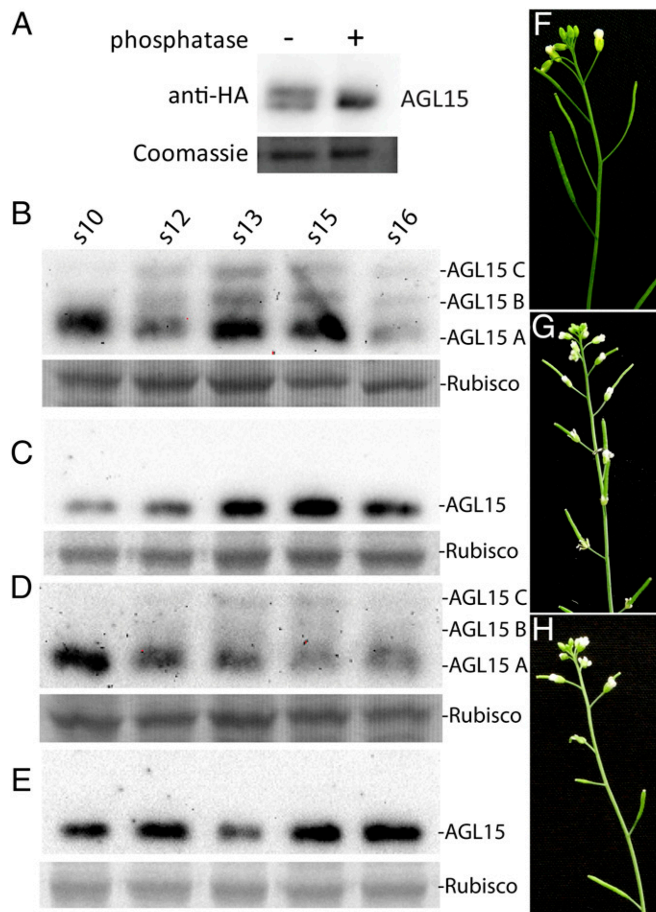

Fig. 5. AGL15 is differentially phosphorylated in floral receptacles, and AGL15 phosphorylation requires the MKK4/5 cascade. (*A*) AGL15 in floral receptacles produced by the 35S promoter runs as two bands on a 12% gel. Calf intestinal phosphatase treatment converts the two bands into one band. Rubisco is shown as a loading control. (*B*) AGL15 produced by its own promoter runs as a single band in receptacles from young unopened flowers (stage 10) and runs as three bands in floral receptacles from stage-12 to -16 flowers on 10% SDS/PAGE gels containing 25 μM Phos-tag. The fast-migrating AGL15 isoform is arbitrarily labeled AGL15 A, and the two slower-migrating species are labeled AGL15 B and C. (*C*) Same protein extracts as in *B* run on a 10% SDS/PAGE gel without Phos-tag produce only a single band for AGL15. (*D*) F_1 plants from a cross between AGL15p:AGL15-DHA x *MKK4/5 RNAi* (both dominant) have less phosphorylated AGL15 in floral receptacles than AGL15p:AGL15-DHA plants on 10% SDS/PAGE gels with 25 μM Phos-tag. In particular, AGL15 isoform B is greatly reduced. (*E*) Same protein extracts in *D* run on 10% SDS/PAGE gels without Phos-tag. (*F*) AGL15p:AGL15-DHA plants cannot be differentiated from WT Col-0 plants. (*G*) *MKK4/5 RNAi* plants do not abscise their floral organs (8). (*H*) AGL15p:AGL15-DHA x *MKK4/5 RNAi* F_1 plants have delayed abscission, but are not completely defective in abscission. The plants abscise after stage-16 flowers, whereas WT Col-0 abscises after stage-15 flowers. The above Western blots were repeated at least three times with the same result.

Additionally, in data from somatic embryos, *HSL2* transcript is not repressed by *35S–AGL15* or bound by AGL15 in global ChIP experiments (20). ChIP-sequencing experiments with AGL15 and floral receptacles may help provide a global view of AGL15 targets. The model presented (Fig. 6*C*) has AGL15 drawn as a transcriptional repressor that is inactivated by MAPK phosphorylation. A slight variation on this model could be that, rather than MAPK phosphorylation inactivating AGL15, it might change AGL15 from a repressor to an activator. AGL15 protein actually peaks in stage-15 receptacles (Fig. 5*C*), suggesting that it has some role to play immediately before abscission. Currently, we lack strong evidence for AGL15 being switched to an activator by phosphorylation.

It also seems unlikely that AGL15 is the only transcription factor that is a target for MAPK phosphorylation in floral abscission. AGL18 can also be phosphorylated by MPK3 in vitro (24). AGL18 has one putative MAPK phosphorylation site (SP or TP), whereas AGL15 has two. Experimentation will be necessary to determine whether AGL18 can substitute for AGL15 in regulation of *HAE*. At present, another MADS-domain protein, FOREVER YOUNG FLOWER (FYF/AGL42), also delays floral abscission when overexpressed. FYF is proposed to regulate *IDA*, but not *HAE* (27). FYF does not have any putative MAPK phosphorylation sites, so it is unlikely to be regulated by MAPK phosphorylation. It is well established that *AGL15* and *AGL18* regulate the transition from vegetative growth to flowering and that AGL15 binds the key flowering time regulator, *FLOWERING LOCUS T* in vivo (21, 22). It will be interesting to see whether MPK3/6 also regulates AGL15 by phosphorylation in the flowering time pathway. In general, *Arabidopsis* floral organ abscission is an excellent system for studying MAPK signaling. With the addition of AGL15 into the mix, only stomata development and shoot apical meristem development have a similar number of positioned components tied to MAPK signaling in *Arabidopsis* development (28–30). In the stomata development pathway, MPK3/6 are proposed to phosphorylate and regulate the basic helix–loop–helix

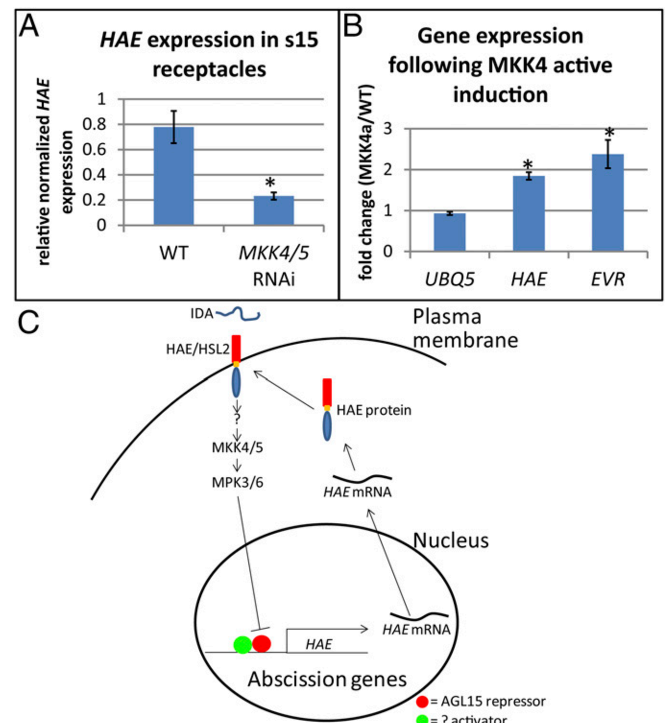

Fig. 6. MKK4/5 positively regulates *HAE* expression. (*A*) *HAE* expression in stage-15 receptacles. *HAE* expression is reduced 3.5-fold in stage-15 floral receptacles from *MKK4/5 RNAi* plants. $^*P < 0.05$ (statistical significance, *t* test; $n = 5$; SEM error bars are shown). (*B*) Gene expression following MKK4 active induction. *HAE* expression is increased twofold in seedlings that transiently express MKK4 active under the control of the dexamethasone-inducible system. The *HAE* coexpressed gene and player in abscission, *EVR*, is also increased (26). $^*P < 0.05$ (statistical significance, *t* test; SEM error bars are shown). (*C*) Proposed core positive feedback loop controlling abscission. IDA peptides trigger HAE/HSL2 to activate a MAPK cascade consisting of MKK4/5 and MPK3/6, which then phosphorylates AGL15 that binds the *HAE* promoter. MPK3/6 phosphorylation relieves AGL15 repression of *HAE* expression, leading to production of *HAE* transcript and later HAE protein.

transcription factor SPEECHLESS (28, 29). The *35S–AGL15* plants have wrinkly leaves with a slightly different epidermis (17). It might be interesting to investigate whether AGL15 plays some role in stomata/epidermis patterning, perhaps via MPK3/6. In summary, *Arabidopsis* floral organ abscission is a robust genetic, biochemical, and phenotypic system to study a developmental process. Moreover, findings in this system have broad translational ability, because abscission is an agriculturally important trait (31, 32).

Materials and Methods

Plant Material, Growth Conditions, Constructs, and Transgenics. The Columbia ecotype of *Arabidopsis* was used as a WT (Col-0; ABRC stock no. CS70000). Plants were grown in Promix BX (Premier Tech Horticulture) at 22 °C, 16-h light/8-h dark, 100–150 $\mu E \cdot m^{-2} \cdot s^{-1}$, and 40–70% humidity. T-DNA mutants were ordered from the *Arabidopsis* Biological Resources Center at Ohio State University. *agl15-4* (CS16480/salk_076234C) and *agl18-2* (CS16482/salk_144022C) (23) were crossed to make *agl15-4 agl18-2* and confirmed by PCR. Plants were planted in a randomized complete block experimental design for data collection. Details for making constructs and transgenic plants are provided in *SI Materials and Methods*.

Promoter Overrepresentation Analysis. Details for promoter overrepresentation analysis are provided in *SI Materials and Methods*.

qRT-PCR. Details for gene expression analysis and qRT-PCR are provided in *SI Materials and Methods*.

Position of Abscission Measurements. Five weeks after planting the flowers from the tallest and second tallest inflorescence of each plant were gently touched to find the first flower that abscised. The position was counted from anthesis (or stage 13) being position 1. Paired *t* test analysis was performed to determine statistical significance.

Analysis of AGL15 Protein Accumulation. Details for analysis of AGL15 protein accumulation are provided in *SI Materials and Methods*.

Separation of AGL15 Phospho-Isoforms and Phosphatase Treatment. Details for separation of AGL15 phospho-isoforms and phosphatase treatment are provided in *SI Materials and Methods*.

ChIP. Tissue (10 floral receptacles) was fixed in PBS with 1% formaldehyde on ice for 10 min and then quenched with 0.125 M glycine for 10 min. Samples were washed three times with PBS. Crude nuclei were isolated by powdering tissue in liquid N_2 and resuspending in PBS supplemented with 0.5% Triton X-100 and 10 mM $MgCl_2$. The extract was pasted through a 70-μm nylon mesh. The nuclei were washed once in the same buffer followed by a wash with PBS (pH 7.4) supplemented with 2 mM $MgCl_2$. Nuclei were lysed for 5 min at room temperature by adding 40 μL of PBS supplemented with 9 mM EDTA, 0.5% sarkosyl, and Complete Protease Inhibitor (Roche). The lysate was diluted with 260 μL of PBS with 9 mM EDTA, 0.5% Triton X-100, 1× Complete, and 1 μg/μL BSA (sonication buffer). The lysate was sonicated three times for 10 s at 20% power and then cleared by centrifugation. A total of 40 μL was saved as an input. The cleared lysate was added to a tube that had 5 μL of anti-HA matrix (Roche; clone 3F10), 5 μL of 10 μg/μL salmon sperm DNA, and 5 μL of sonication buffer and was rocked at 4 °C overnight. The beads were washed four times with PBS with 0.1% Triton X-100 and 10 mM EDTA and once with TE buffer. DNA was eluted/de-cross-linked by adding 200 μL of 1% SDS, 10 mM EDTA, and 50 μg/mL Proteinase K and incubating at 65 °C for 30 min followed by 70 °C for 1.5 h. The input sample was de-cross-linked by adding 2 μL of 20% (wt/vol) SDS and 0.1 μL of RNaseA to the 40-μL input sample and incubated at 70 °C for 2 h. Both the ChIP sample and the input sample were phenol:chloroform:isoamyl alcohol (25:24:1)-extracted and precipitated. Enrichment relative to input was determined by qPCR (described above) with UBQ5 as a reference.

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