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Epigenetic control of juvenile-to-adult phase transition by the Arabidopsis SAGA-like complex

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Accession number: The sequences of genes cited in this article can be obtained from the TAIR database (www.arabidopsis.org) with the following accession numbers: *HAG1* (At3g54610), *HAG2* (At5g56740), *HAG3* (At5g50320), *HAM1* (At5g64610), *HAM2* (At5g09740), *HAC1* (At1g79000), *HAC5* (At3g12980), *HAF1* (At1g32750), *HAF2* (At3g19040), *ADA2a* (At3g07740), *PRZ1* (At4g16420), *SPL2* (At5g43270), *SPL3* (At2g33810), *SPL4* (At1g53160), *SPL5* (At3g15270), *SPL7* (At5g18830), *SPL8* (At1g02065), *SPL9* (At2g42200), *SPL10* (At1g27370), *SPL11* (At1g27360), *SPL13* (At5g50570), *SPL14* (At1g20980), *SPL15* (At3g57920), *AP1* (At1g69120), *CAB2* (At1g29920), *Mir156A* (At2g25095), *Mir156B* (At4g30972), *Mir156C* (At4g31877), *Mir156H* (At5g55835), *FT* (At1g65480), *SOC1* (At2g45660), *LFY* (At5g61850), *FUL* (At5g60910), *AGL42* (At5g62165), *TRY* (At5g53200), and *TCL1* (At2g30432).

SUMMARY

During growth and development, plants undergo a series of phase transitions from the juvenile-to-adult vegetative phase to the reproductive phase. In Arabidopsis, vegetative phase transitions and flowering are regulated by SQUAMOSA PROMOTER BINDING PROTEIN-LIKE (SPL) factors. *SPL* mRNAs are post-transcriptionally regulated by miR156 in an age-dependent manner; however, the role of other mechanisms in this process is not known. In this study, we demonstrate that the HAG1/GCN5- and PRZ1/ADA2b-containing SAGA-like histone acetyltransferase (HAT) complex directly controls the transcription of *SPLs* and determines the time for juvenile-to-adult phase transition. Thus, epigenetic control by the SAGA-like HAT complex determines the transcriptional output of *SPLs*, which might be a prerequisite for the subsequent post-transcriptional regulation by miR156. Importantly, this epigenetic control mechanism is also crucial for miR156-independent induction of *SPLs* and acceleration of phase transition by light and photoperiod or during post-embryonic growth.

Keywords: HAG1, histone acetylation, phase transition, flowering, *SPLs*, *Arabidopsis thaliana*.

INTRODUCTION

Higher plants pass through a series of morphologically and physiologically distinct developmental phases. Vegetative phase transition from a juvenile to an adult phase in Arabidopsis and maize is controlled by a master regulatory module made of conserved microRNAs of the miR156 family and their target transcription factors, SQUAMOSA PROMOTER BINDING PROTEIN LIKEs (SPLs). In Arabidopsis, miR156 levels, which are highest in seedlings, gradually decrease with age, resulting in a gradual increase in the transcript levels of target *SPLs* (Wu and Poethig, 2006; Wang *et al.*, 2009; Wu *et al.*, 2009). Besides vegetative phase transition, *SPLs* are also known to be critical factors for the transition from the vegetative to the reproductive phase especially in non-inductive short days (SD; Wang *et al.*, 2009). Although miR156 appears to be a major regulator of SPL expression during the developmental time course, recent studies have provided evidence that the expression or activity of SPLs is also regulated in a miR156-indpendent manner in response to environmental cues or the phytohormone gibberellin (Wang *et al.*, 2009; Jung *et al.*, 2012; Yu *et al.*, 2012).

General Control Nonrepressed 5 (GCN5) is a catalytic component of histone acetyltransferase (HAT) complexes that are structurally conserved in yeast, flies, and mammals, e.g., the Spt–Ada–Gcn5–acetyltransferase (SAGA) complex. Generally, transcriptional activators recruit the SAGA complex to target loci. This leads to loss of chromatin compaction due to acetylating histones, thereby facilitating transcription initiation (Sterner *et al.*, 1999; Nagy and Tora, 2007). In addition, the SAGA complex has also been shown to enhance progression of RNA polymerase II (PolII) during transcription elongation (Govind *et al.*, 2007; Sansó *et al.*, 2011).

In Arabidopsis, mutant studies of HAG1 (the Arabidopsis homolog of GCN5) and ADA2b/PROPORZ 1 (hereafter,

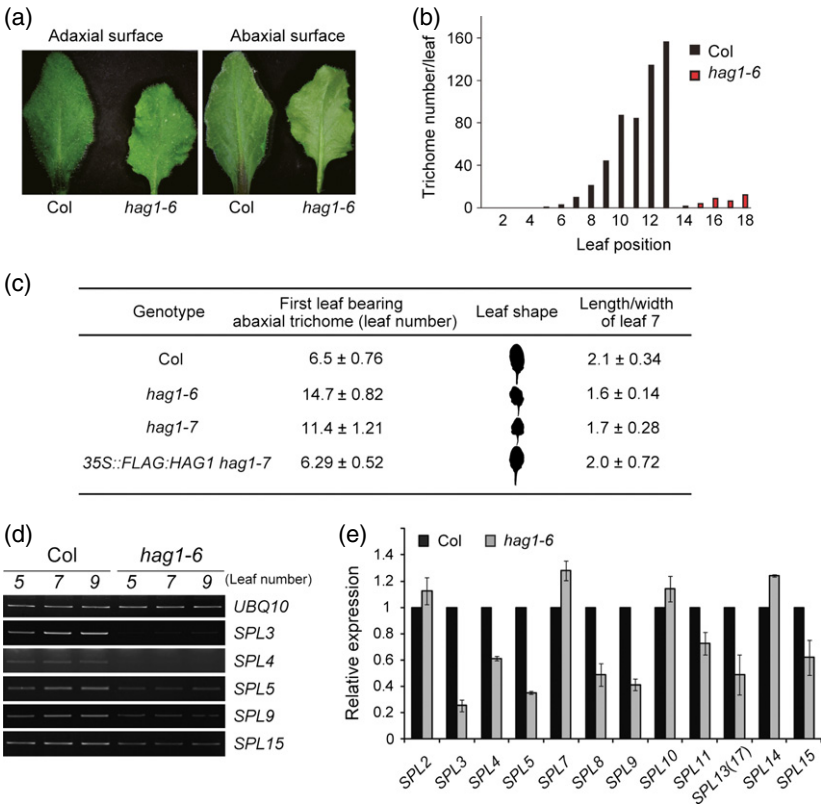


Figure 1. *HAG1* is required for the juvenile-to-adult phase transition.
(a) Adaxial and abaxial surfaces of the seventh rosette leaves of wild type (WT) and *hag1-6*. Col: Columbia-0 WT.
(b) Trichome numbers on the abaxial surface of WT (black bars) and *hag1-6* (red bars) leaves at indicated positions.
(c) The leaf shape and the position of the first leaf with abaxial trichome in WT, *hag1-6*, *hag1-7*, and *35S::FLAG:HAG1 hag1-7*. Values are the averages of 20 plants per genotype, and error bars represent standard deviation (SD).
(d) Reverse transcription followed by polymerase change reaction (RT-PCR) analyses of several *SPL* transcripts in WT and *hag1-6* at different developmental stages. RNA was isolated from the above ground tissues in plants with five, seven, or nine visible leaves. *Ubiquitin 10* (*UBQ10*) is an expression control.
(e) Transcript levels of multiple *SPL* genes in WT and *hag1-6* at the seven-leaf stage as determined by RT followed by quantitative real-time PCR (RT-qPCR). *SPL13*(*17*) indicates duplicate *SPL13* and *SPL17* genes (Guo *et al.*, 2008). WT levels were set to 1 after normalization to *UBQ10*. Values are the means ± standard error (SE) of three biological replicates.

PRZ1; an Arabidopsis homolog of ADA2) have shown that the Arabidopsis SAGA-like complex plays a critical role in development and responses to environmental cues or endogenous hormones (Bertrand *et al.*, 2003; Sieberer *et al.*, 2003; Vlachonasios *et al.*, 2003; Benhamed *et al.*, 2006, 2008; Cohen *et al.*, 2009; Kornet and Scheres, 2009; Anzola *et al.*, 2010; Servet *et al.*, 2010). A microarray study revealed that ~5% of the genes tested were affected by *hag1* and *prz1* mutations (Vlachonasios *et al.*, 2003), suggesting a limited target range of HAG1/PRZ1 in Arabidopsis. Although the effect of HAG1 and PRZ1 in gene expression has been addressed in these studies, their regulatory pathways, direct targets, and molecular mechanisms have yet to be fully elucidated.

Here, we show that HAG1 and PRZ1 play an essential role in the control of *SPL* genes for successful juvenile-to-adult phase transition. HAG1 and PRZ1 establish or maintain high histone acetylation levels at *SPLs* throughout vegetative development, and this allows for high transcriptional output of *SPLs* that is necessary for miR156-mediated fine-tuning. We further demonstrate that HAG1-mediated histone acetylation is crucial for miR156-independent induction of *SPLs* by environmental cues that accelerate phase transitions. Germination-specific targeting of HAG1 to *SPL3* is also required for miR156-independent induction and establishment of early *SPL3* expression in vegetative tissues.

RESULTS

Mutations in *HAG1* strongly delay juvenile-to-adult phase transition in Arabidopsis

In our attempts to characterize the phenotypes of *HAG1* loss-of-function mutants, we noticed that the *hag1-6* mutants (Long *et al.*, 2006) had severely lower trichome numbers on the abaxial, but not on the adaxial, leaf surface compared with that in wild type (WT) (Figure 1a). Quantitative analysis of the abaxial trichome numbers of leaves at various positions indicated that not only were the number of trichomes formed far less, but the developmental onset of trichome production was also severely delayed in *hag1-6* compared with those in WT (Figure 1b). Delay in abaxial trichome initiation was also observed in another allele of *HAG1*, *hag1-7* (Figure 1c), and the overexpression of either FLAG-tagged (*35S::FLAG:HAG1*) or yellow fluorescence protein (YFP)- and HA-tagged (*35S::HAG1:YFP:HA*) HAG1 fully rescued the phenotypes of *hag1-7* or *hag1-6*, respectively (Figures 1c and S1a–c). Furthermore, the seventh leaves of both *hag1-6* and *hag1-7* were smaller and rounder as measured by length-to-width ratio, and their petioles were also more elongated than those in WT (Figure 1c). The morphological characteristics observed in *hag1* are considered juvenile vegetative traits (Telfer *et al.*, 1997; Wu *et al.*, 2009), indicating that juvenile-to-adult vegetative phase transition is delayed in *hag1* mutants.

Transcript levels of a group of *SPL*-family genes are reduced in *hag1* mutants

Because the timing of the juvenile-to-adult phase transition is known to be controlled by several members of the SPL-family of transcription factors (e.g., SPL3, SPL4, SPL5, SPL9, SPL15), we investigated whether delayed vegetative phase transition of *hag1* is related to altered expression of *SPL* genes. The transcript levels of *SPL3*, *SPL4*, *SPL5*, *SPL9* and *SPL15* which were higher in older plants than in younger plants irrespective of genotype (Figures 1d and 2d), were greatly reduced in *hag1-6* compared with WT at all developmental stages examined (Figures 1d and 2d,e). Several other *SPLs* were also down-regulated in *hag1* mutants (Figures 1e and S1d). Among these, *SPL3*, *SPL4*, *SPL5*, *SPL9*, *SPL11*, *SPL13(17)*, *SPL15* but not *SPL8* transcripts contain predicted miR156-binding sites (Rhoades *et al.*, 2002), suggesting that the regulation of *SPLs* by HAG1 might be independent of miR156. Furthermore, the levels of some predicted miR156-target transcripts including *SPL2* and *SPL10* were not affected by *hag1-6* (Figure 1e). In accordance with the rescue of the phase-transition phenotypes, the transcript levels of all *SPLs* down-regulated in *hag1* were restored back to WT levels by the introduction of *35S::FLAG:HAG1* (Figure S1d).

Mutations in *HAG1* result in delayed flowering and reduced expression of *SPL*-target genes

SPLs are believed to directly regulate several MADS-box genes associated with reproductive transition and determination of floral meristem identity in an FT/FD-independent manner (Wang *et al.*, 2009; Yamaguchi *et al.*, 2009). Correspondingly, *HAG1* mutations resulted in decreased levels of *LEAFY* (*LFY*), *FRUITFULL* (*FUL*), *APETALA 1* (*AP1*), *AGAMOUS-LIKE 42* (*AGL42*), and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1* (*SOC1*) transcripts, but not of *FT* transcript, and delayed flowering (Figures 3c and S1e). Consistent with the fact that SPLs are critical factors in an endogenous flowering pathway, flowering delay in *hag1* was more pronounced in non-inductive SD (Figure S1f). Expression of *TRY* and *TCL1*, SPL9 targets without flowering-related function (Yu *et al.*, 2010), was also severely reduced in *hag1-6* (Figure 3c).

miR156-independent transcriptional control of *SPL3* and *SPL9* by HAG1

To test if the reduced expression of *SPLs* in *hag1* was a result of increased miR156 expression, we compared the primary transcript levels of several *MIR156* genes and the level of mature miR156 in the WT and *hag1-6.* The primary transcripts levels of *MIR156A* and *MIR156B* were reduced, whereas those of *MIR156C* and *MIR156H* were slightly increased in *hag1-6* (Figure 2a). The level of mature miR156 was ~1.35-fold higher in *hag1-6* than in WT (Figure 2b).

Because the extent of the increase in miR156 appeared insufficient to account for the strong down-regulation of *SPLs* and the severe delay in vegetative phase transition in *hag1*, we further analyzed *HAG1* function in plants expressing a miR156-resistant *SPL9* under the *SPL9* promoter (*pSPL9::rSPL9;* Wang *et al.*, 2008). The strong acceleration of abaxial trichome production by *pSPL9::rSPL9* was greatly delayed by the *hag1-6* mutation (Figure 2c). The floral promotion effect of *pSPL9::rSPL9* was also less effective in the *hag1-6* mutant background (Figure S2a). Consistently, a large reduction in the transcript levels of *rSPL9* as well as endogenous *SPL9* was observed in *hag1-6* at all developmental stages examined (Figures 2d and S2b), demonstrating that the effects of *HAG1* on *SPL9* is miR156 independent. Expression of SPL-target genes was also reduced in *pSPL9::rSPL9 hag1-6* compared to *pSPL9::rSPL9* (Figure S2c).

Like *SPL9*, *HAG1* control of *SPL3* expression was independent of miR156. Both *pSPL3::SPL3:GUS* and *pSPL3::rSPL3:GUS*, expressing miR156-sensitve and -resistant *SPL3* tagged with β-*glucuronidase* (*GUS*) under the *SPL3* promoter, respectively (Yang *et al.*, 2011), showed lower GUS expression in *hag1-6* than in WT (Figures 2e and S2d). We then studied the effect of a *hag1* loss-of-function mutation on PolII activity and found that PolII occupancies in the promoter and transcribed regions of *SPL3* and *SPL9* were severely reduced in *hag1-6* than in WT (Figure 2f,g). Taken together, these results indicate that HAG1 affects the transcriptional activity of *SPLs* independently of the miR156 pathway.

Histone acetylation at the *SPL3* and *SPL9* loci is controlled by the HAG1- and PRZ1-containing HAT complex

Next, we investigated whether *SPL3* and *SPL9* are the direct targets of HAG1. For this, we used *35S::HAG1:YFP:HA hag1-6* plants (Figure S1) and performed ChIP assays using an anti-HA antibody. Enrichment of the HAG1:YFP:HA protein was observed in the promoters and transcribed regions of *SPL3* and *SPL9*, but not at the negative control locus *CHS* (Benhamed *et al.*, 2006; Figure 3a). Thus, *SPL3* and *SPL9* are likely the direct targets of HAG1. HAG1:YFP:HA was also enriched at *KRP7*, a PRZ1 target (Anzola *et al.*, 2010), supporting the view that HAG1 functions together with ADA2 homologs (ADA2a and PRZ1) in the SAGA-like complex (Mao *et al.*, 2006) as in other organisms.

To determine whether the relationship between HAG1 and ADA2 is also observed in the regulation of phase transition, we examined the abaxial trichome phenotypes of *ada2a-3* and *prz1-1* mutant leaves at various positions. The *prz1-1*, but not *ada2a-3* mutation, resulted in a severe delay in the onset of abaxial trichome production (Figure 3b). Moreover, the transcript levels of several *SPLs* and *SPL3* and *SPL9* -target genes, which were reduced in *hag1* (Figure 1e), were also substantially decreased in *prz1-1* (Figures 3c and S3a), indicating that the HAG1/PRZ1-containing

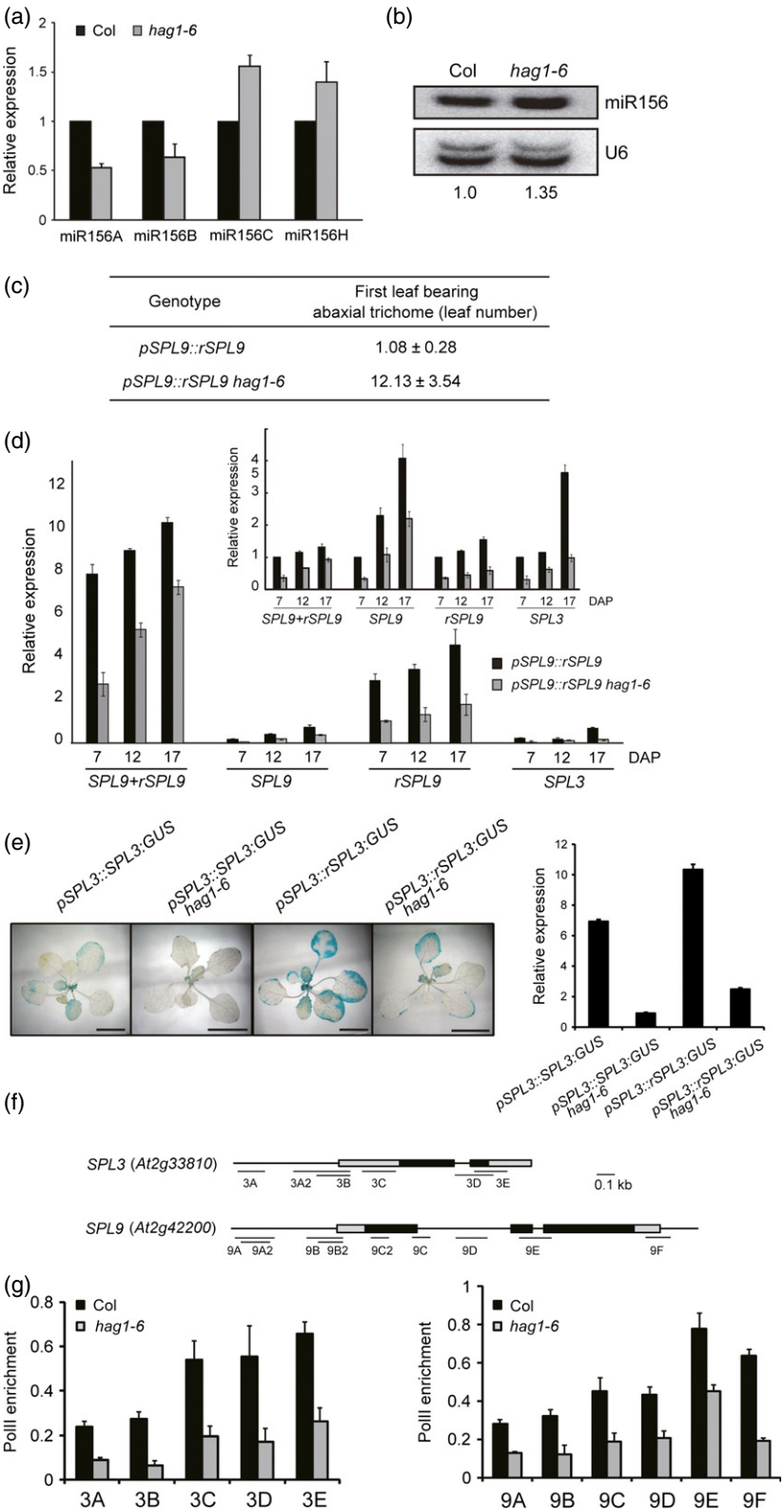
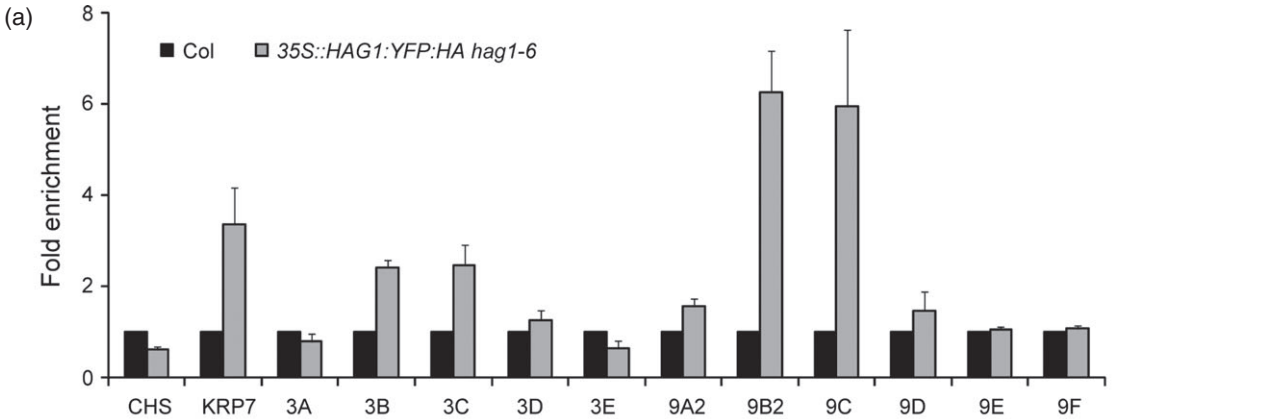


Figure 2. miR156-independent transcriptional control of *SPLs* by HAG1.
(a) Primary transcript levels of miR156 genes in WT and *hag1-6*. WT levels were set to 1 after normalization with *UBQ10*. Values are the means ± standard error (SE) of three biological replicates.
(b) Northern blot analysis of miR156. Numbers indicate fold change relative to WT.
(c) Abaxial trichome phenotypes of *pSPL9::rSPL9* and *pSPL9::rSPL9 hag1-6* plants. Leaf numbers were scored as in Figure 1(c).
(d) Transcript levels of *SPL9*, *rSPL9*, total *SPL9* (*SPL9* + *rSPL9*), and *SPL3* after normalization to *UBQ10* in *pSPL9::rSPL9* and *pSPL9::rSPL9 hag1-6* plants at indicated days after planting (DAP). Values are the means ± SE of three biological replicates. The inset shows relative values to WT at 7 DAP.
(e) Histochemical GUS staining of transgenic plants harboring *pSPL3::SPL3:GUS* or *pSPL3::rSPL3:GUS* in WT or *hag1-6* backgrounds (left). Scale bars: 1 cm. Quantitative analysis of *GUS*-fused transcript levels by RT-qPCR (right). Normalization was to *UBQ10*. Shown are the means ± SE of three biological replicates.
(f) Schematic representation of the *SPL3* and *SPL9* loci. Regions tested in chromatin immunoprecipitation followed by qPCR (ChIP-qPCR) are depicted. Black boxes represent exons, and gray boxes indicate untranslated regions. Intergenic regions or introns are marked with lines.
(g) PolII enrichment at *SPL3* (left) and *SPL9* (right) in WT and *hag1-6* as determined by ChIP-qPCR. The means ± SE of three biological replicates as relative values to *Actin2/7* after normalization to input DNA are shown.

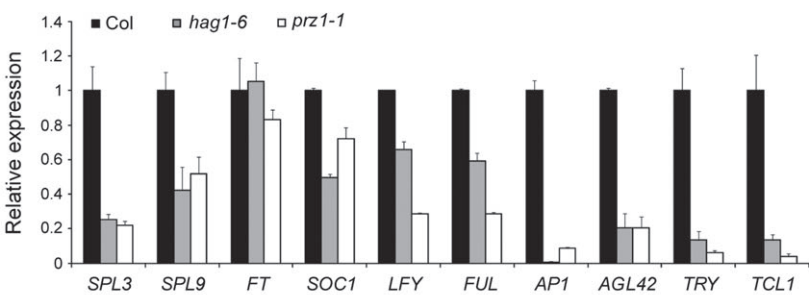
SAGA-like complex plays a role in the transcriptional control of *SPLs* and subsequent developmental phase transitions.

We then investigated whether the HAG1/PRZ1-containing SAGA-like complex affects histone acetylation at *SPL3* and *SPL9*. ChIP assays using anti-histone H3 (H3) and



(b)

Genotype	First leaf bearing abaxial trichome (leaf number)
Col	6.77 ± 0.8
hag1-6	12.89 ± 1.3
prz1-1	12.2 ± 1.2
Col	6.11 ± 0.6
ada2a-3	5.28 ± 0.6



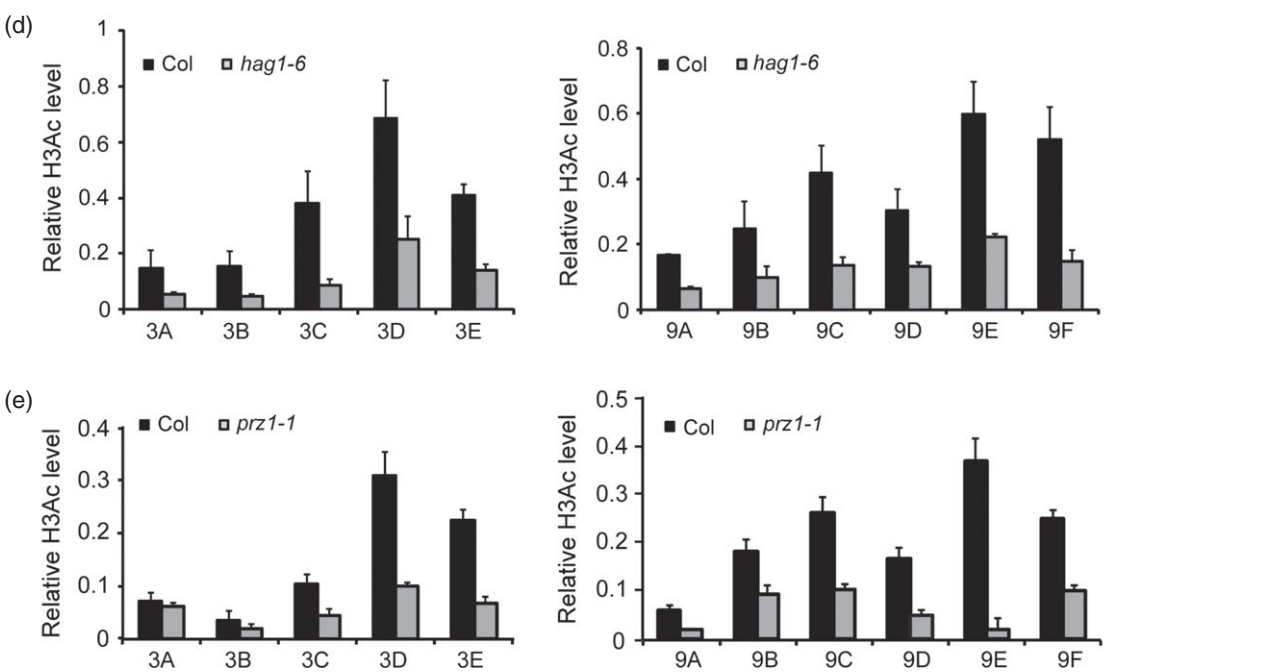


Figure 3. Control of *SPL* transcription by the HAG1/PRZ1-containing HAT complex.
(a) Direct association of HAG1:YFP:HA with *SPL3* and *SPL9* chromatin as determined by ChIP-qPCR using an anti-HA antibody. Regions tested are as depicted in Figure 2(a, d, e, f). Values are the means ± standard error (SE) of three biological replicates obtained after normalization to input DNA.
(b) Abaxial trichome phenotypes of WT, *hag1-6*, *prz1-1*, and *ada2a-3*. Leaf numbers were scored as in Figure 1(c).
(c) Transcript levels of *SPL3*, *SPL9*, and their downstream target genes in WT, *hag1-6*, and *prz1-1* at the seven-leaf stage as determined by RT-qPCR. Error bars represent SE of three biological replicates.
(d, e) H3Ac levels within *SPL3* (left) and *SPL9* (right) chromatin from WT and *hag1-6* (d) or WT and *pzr1-1* (e). Values are the means ± SE of three biological replicates as relative values to *Actin2/7* after normalization to input DNA. Plant samples were harvested at the seven-leaf stage.

anti-acetylated histone H3 (H3Ac) antibodies showed that disruption of *HAG1* and *PRZ1* reduced H3Ac to similar levels without affecting total H3 levels in the promoters and transcribed regions of *SPL3* and *SPL9* (Figures 3d,e and S3b). As HAG1 was previously reported to target H3K9, H3K14, and H3K27 for acetylation (Benhamed *et al.*, 2006), we then tested H3K9Ac, H3K14Ac, and H3K27Ac levels for limited regions of the *SPL3* and *SPL9* loci. In line with the previous report, all the three acetylation levels were reduced by the *hag1-6* mutation (Figure S4). These results, together with the finding that *SPL3* and *SPL9* are direct targets of HAG1, indicate that the SAGA-like complex directly controls *SPL* transcription through histone acetylation.

HAG1 plays a major role in juvenile-to-adult phase transition and *SPL* activation among Arabidopsis HATs

To determine whether the role of HAG1 in *SPL* regulation and phase transitions is shared by other HATs, we examined the abaxial trichome emergence in the leaves of single or double mutants of several HATs (Figure S3c and Data S1; Pandey *et al.*, 2002). The timing of abaxial trichome emergence was not significantly altered in the plants lacking the GNAT-family HAT2 (*hag2-2*) or two MYST-family HATs, HAM1 and HAM2 (*ham1-1 ham2-1*). On the other hand, it was moderately delayed in *elo3-1*, *haf1-2 haf2-5*, and *hac1-2 hac5-2* mutants, which lacked the Elongator HAT ELO3/HAG3, two TAF250-family HATs (HAF1 and HAF2), and two CBP-family HATs, HAC1 and HAC5, respectively. Consistent with delayed trichome emergence, the transcript levels of *SPL3*, *SPL4*, *SPL5*, *SPL9 and SPL15* were reduced in *elo3-1*, *haf1-2 haf2-5*, and *hac1-2 hac5-2* compared with those in WT (Figure S3d–f). However, although small decreases were observed in parts of the transcribed regions of *SPL3* and *SPL9* in *hac1-2 hac5-2*, H3Ac levels at *SPL3* and *SPL9* in these mutants were not as substantially altered as in *hag1* or *prz1* (Figure S3g,i,j). Furthermore, at *SPL5*, where the transcript levels were most severely reduced, H3Ac levels were not affected at all by the *hac1-2 hac5-2* mutations (Figure S3g), indicating a lack of correlation between transcription and histone acetylation. Finally enrichment of HAC1:HA fusion protein was not observed in the *SPL3* and *SPL9* chromatin (Figure S3h). Thus, the role of HACs, HAFs, and ELO3 in *SPL* regulation might be either indirect or minor in contrast to that of the HAG1/ PRZ1 complex.

HAG1-mediated H3Ac is important for miR156-independent light and photoperiodic induction of *SPL9*

Photoperiodic induction of *SPL3* and *SPL9* is known to be a miR156-independent process (Wang *et al.*, 2009; Jung *et al.*, 2012). By studying *rSPL9* expression under light-shifting conditions, we found that *SPL9* is also induced by light in a miR156-independent manner (Figure 4a). Light induction of *SPL9* as well as its previously known photoperiodic induction was substantially reduced by the *hag1-6* mutation (Figures 4b and S5a). This led us to investigate whether the effects of light and photoperiod involve changes in histone acetylation that is mediated by HAG1. These environmental cues clearly increased H3Ac levels at *SPL9* (Figures 4c and S5b). Notably, H3Ac levels at *SPL9* in *hag1-6* were lower than those in WT under dark conditions and increased in response to light, but only to WT levels in the dark (Figure 4c). This indicates that HAG1 plays a role

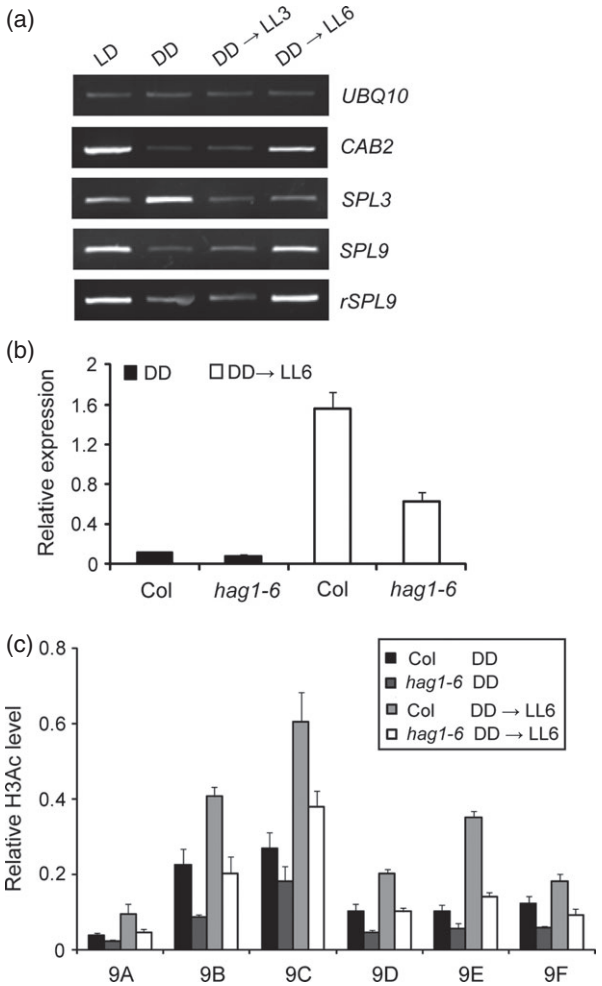


Figure 4. Light induction of *SPL9* is controlled by a miR156-independent epigenetic mechanism.
(a) RT-PCR analysis of *SPL3* and *SPL9* mRNA expression in *pSPL9::rSPL9* seedlings during the dark-to-light transition. Eight-day-old seedlings grown in long days were either kept in long days (LD) or transferred to darkness for 5 days without (DD) or with subsequent transfer to white light for 3 (DD → LL3) or 6 h (h; DD → LL6).
(b) RT-qPCR analysis of light-induced *SPL9* expression in WT and *hag1-6*. Two-week-old seedlings grown in LD were used for DD or DD → LL6 treatment as described in (a). Normalization was to *UBQ10*, and shown are the means ± standard error (SE) of three biological replicates.
(c) ChIP-qPCR analysis of H3Ac levels at *SPL9* in WT and *hag1-6* before and after light exposure. Plant samples were prepared as in (b). Regions tested are as depicted in Figure 2(f). Values are the means ± SE of three biological replicates as relative values to *Actin2/7* after normalization to input DNA.

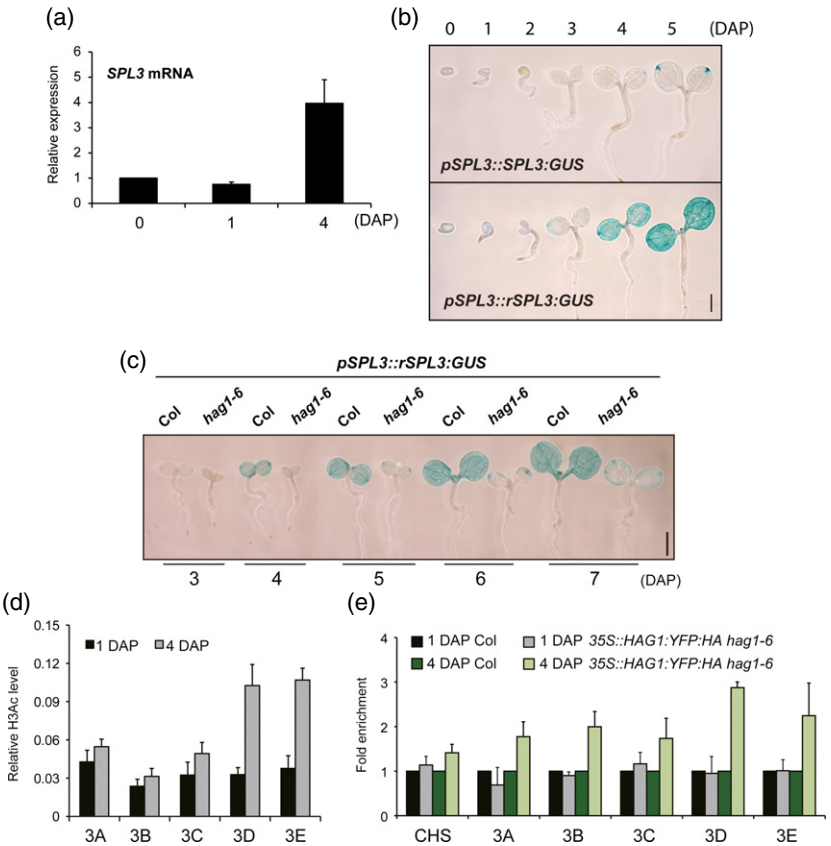


Figure 5. Transcriptional activation of *SPL3* by the germination-dependent targeting of HAG1.
(a) RT-qPCR analysis of *SPL3* mRNA expression during germination. Seeds were plated on Murashige and Skoog (MS) agar medium with 1% sucrose and grown for the indicated days in long day (LD) conditions
(b) Histochemical GUS staining of transgenic plants harboring *pSPL3::SPL3:GUS* or *pSPL3::rSPL3:GUS.* Plants were grown for the indicated days in LD conditions. Scale bars: 1 mm (b, c).
(c) Histochemical GUS staining of transgenic plants harboring *pSPL3::rSPL3:GUS* in WT or *hag1-6* backgrounds. Plants were grown for the indicated days in LD conditions.
(d) ChIP-qPCR analysis of H3Ac levels at the *SPL3* locus in germinating seeds. Seed samples were prepared as in (a). Regions tested are as depicted in Figure 2(d–f). The means ± standard error (SE) of three biological replicates as relative values to *UBQ11* after normalization to input DNA are shown.
(e) Targeting of HAG1:YFP:HA into *SPL3* chromatin in germinating seeds as determined by ChIP-qPCR using anti-HA antibody. Seed samples were prepared as in (a). Values are the means ± SE of three biological replicates obtained after normalization to input DNA.

in maintaining basal histone acetylation and establishing additional histone acetylation in response to light signals at *SPL9*, which induce efficient *SPL9* transcription. Reduced yet detectable increase in H3Ac levels at *SPL9* induced by light in *hag1-6* suggests that HATs other than HAG1 might also be involved in this process. Alternatively, a decrease in histone deacetylase activity might be accompanied with increased HAG1 activity.

HAG1-mediated H3Ac is important for miR156-independent induction of *SPL3* during germination

We were curious about how initial expressional status of *SPLs* is established during post-embryonic growth. We challenged this question using *SPL3* as a test model and noticed that *SPL3* mRNA is substantially increased in germinating seeds (Figure 5a). Although the expression level was different, the induction pattern *per se* during the course of germination between the miR156-sensitive *pSPL3::SPL3:GUS* and the miR156-resistant *pSPL3::rSPL3:GUS* was similar (Figure 5b), indicating that the inductive process is regulated by other mechanism independent of miR156. Thus, the *SPL3* induction during seed germination was not likely due to the release from miR156 inhibition but rather might be contributed by the onset of transcriptional activation. We investigated whether histone acetylation mediated by HAG1 is required for this transcriptional activation of *SPL3* during germination. Gradual increase of GUS activity encoded by *pSPL3::rSPL3:GUS* during germination and early-seedling growth was substantially dampened by the *hag1-6* mutation (Figure 5c). Moreover, the level of H3Ac at the *SPL3* loci was clearly higher in samples 4 days after planting (DAP) compared with 1 DAP samples (Figure 5d). Finally, the enrichment of HAG1:YFP:HA fusion protein within *SPL3* chromatin was observed in four DAP but not in one DAP samples (Figure 5e). Taken together, these results indicate that the targeting of HAG1-mediated histone acetylation activity is crucial for initial establishment of *SPL3* expression during post-embryonic growth.

DISCUSSION

The miR156-SPL module acts as a quantitative developmental clock that orchestrates phase transitions in Arabidopsis (Pulido and Laufs, 2010). However, accumulating evidence has shown that Arabidopsis is equipped with additional layers of regulation for SPLs. In this report, we provide evidence that HAG1 and PRZ1, Arabidopsis homologs of GCN5 and ADA2 of the SAGA complex, provide a miR156-independent transcriptional control mechanism for a group of *SPLs* through histone acetylation. Highly acetylated *SPL* chromatin seems to allow increased engagement and processivity of PolII. Although GCN5-mediated H3Ac in

transcribed regions was reported to facilitate PolII progression by promoting nucleosome eviction in yeast (Govind *et al.*, 2007; Sansó *et al.*, 2011), HAG1/PRZ1-mediated H3Ac in the coding regions of *SPL3* and *SPL9* appears not to induce nucleosome eviction as evidenced by the comparable total H3 levels in WT and *hag1-6.*

HAG1/PRZ1-dependent histone acetylation may act as an important gear to attain the appropriate levels of *SPL* expression required for legitimate developmental phase transitions. The consistently high levels of *rSPL3* and *rSPL9* transcripts observed throughout development indicate that *SPL3* and *SPL9* transcription is facilitated by an active HAG1/PRZ1-containing complex regardless of plant age. Such robust transcription of *SPL*s may be a prerequisite for miR156-dependent post-transcriptional regulation; i.e., the HAG1/PRZ1-mediated transcriptional mechanism might be required for sufficient supply of *SPL* transcripts such that the miR156-mediated post-transcriptional mechanism can gradually tune the final output of *SPL* transcripts in accordance with age (Figure S6).

Another important aspect to the HAG1/PRZ1-mediated transcription of *SPLs* might be in conveying environmental and developmental inductive cues to control phase transition. Although phase transitions are primarily related with developmental cues, plants are also able to modulate phase-transition programs depending on environmental conditions to gain higher reproductive success. Our study shows that environmental changes such as shifts from dark-to-light or photoperiodic change from SD to long days (LD) can be transduced into the regulation of epigenetic status and *SPL* transcription. Moreover, we found that the initial induction of *SPL3* during post-embryonic growth is regulated by the specific targeting of HAG1-mediated histone acetylation activity. Low levels of H3Ac at *SPL* loci in non-inductive conditions or developmental stages may determine the basal expression levels of *SPLs.* Upon exposure to inducing signals (such as light, photoperiod, and seed germination), HAT activity and H3Ac levels increase at *SPLs* and result in enhanced transcriptional competence. Although other, yet unrevealed, mechanisms and factors may also be required for conveying the environmental cues that lead to activation of *SPL* transcription, our study indicates that the HAG1/PRZ1-containing complex and histone acetylation are critical components of this process.

EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

Plants were grown under 100 μmol m^{-2} sec^{-1} cool white fluorescent lights at 22°C. Unless otherwise noted, plants were grown in LD with a 16 h light and 8 h dark photoperiod (16L/8D). SD conditions included an 8L/16D. *pSPL9::rSPL9* and *pSPL3::SPL3:GUS/ pSPL3::rSPL3:GUS* were provided by Detlef Weigel (Max Planck Institute for Developmental Biology, Tübingen, Germany) and Scott Poethig (University of Pennsylvania, Philadelphia, PA, USA), respectively. Leaf-shape analysis and abaxial trichome scoring were performed as previously described (Wu *et al.*, 2009). Flowering time was measured as described (Han *et al.*, 2007). Details of the *HAT* mutant alleles used in this study are provided in Data S1.

Constructs and plant transformation

Construction details of *35S::FLAG:HAG1*, *35::HAG1:YFP:HA* and *HAC1:HA* are described in Data S1. *35S::FLAG:HAG1* or *35S:: HAG1:YFP:HA* was transformed into heterozygous *hag1-7* or *hag1-6* plants and *HAC1:HA* into WT Col respectively, via *Agrobacterium*-mediated transformation using floral dip method (Clough and Bent, 1998). Homozygous transgenic *35S::FLAG:HAG1 hag1-7* and *35::HAG1:YFP:HA hag1-6* plants were selected in the subsequent generations through genotyping.

RT-PCR or RT-qPCR analysis

Total RNA was isolated from plants as previously described (Suzuki *et al.*, 2004; Han *et al.*, 2007). Details of the RT-PCR and RT-qPCR analyses are provided in Data S1.

ChIP assay

ChIP was performed as previously described (Johnson *et al.*, 2002; Han *et al.*, 2007; Kaufmann *et al.*, 2010) with minor modifications. Details are provided in Data S1.

Northern blot analysis

In total, 50 μg of RNA was separated on a 15% polyacrylamide gel containing 8 M urea, transferred to a Hybond™-NX membrane (Amersham, www.gelifesciences.com), and fixed by ultraviolet (UV) light cross-linking. Blots were then hybridized with a miR156-complementary oligonucleotide (5′-GTGCTCACTC TCTTCTGTCA-3′) labeled with γ-^{32}P-ATP. The same blot was also analyzed with an oligonucleotide probe (5′-AGGGGCCATGCTAATCTTCTC-3′) specific to *U6* snRNA, which was used as a loading control. Images were quantified using MOLECULAR IMAGING Software (Kodak, www.kodak.com/go/molecular) and normalized to *U6* expression.

Histochemical GUS assay

Plants at similar developmental stages were fixed with 90% acetone for 20 min on ice. GUS staining was performed as described (Han *et al.*, 2007). The GUS expression pattern was observed with a STEM2000 stereomicroscope (Carl Zeiss, www.zeiss.com).

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AUTHOR CONTRIBUTIONS

J.-Y.K., Y.-S.N., and B.N. designed research; J.-Y.K. and J.E.O. performed experiments; Y.-S.N. and B.N. contributed new reagents or analytic tools; J.-Y.K., J.E.O., Y.-S.N., and B.N. analyzed data; and J.-Y.K., Y.-S.N., and B.N. wrote the paper.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. *hag1* mutant phenotypes and their complementation by *HAG1* overexpression.

Figure S2. Regulation of miR156-resistant *SPL3* and *SPL9* by HAG1.

Figure S3. Role of other HATs in vegetative phase transition.

Figure S4. H3K9Ac, H3K14Ac, H3K27Ac levels at the *SPL3* and *SPL9* loci in WT and *hag1-6.*

Figure S5. Transcriptional control of the photoperiodic induction of *SPL3* and *SPL9.*

Figure S6. Schematic model for the regulation of *SPL3* and *SPL9* by the HAG1/PRZ1 containing SAGA-like complex.

Data S1. Experimental procedures.

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