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RESEARCH ARTICLES

# Chromatin-Dependent Repression of the *Arabidopsis* Floral Integrator Genes Involves Plant Specific PHD-Containing Proteins[C][W]

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**The interplay among histone modifications modulates the expression of master regulatory genes in development. Chromatin effector proteins bind histone modifications and translate the epigenetic status into gene expression patterns that control development. Here, we show that two *Arabidopsis thaliana* paralogs encoding plant-specific proteins with a plant homeodomain (PHD) motif, *SHORT LIFE* (*SHL*) and *EARLY BOLTING IN SHORT DAYS* (*EBS*), function in the chromatin-mediated repression of floral initiation and play independent roles in the control of genes regulating flowering. Previous results showed that repression of the floral integrator *FLOWERING LOCUS T* (*FT*) requires *EBS*. We establish that *SHL* is necessary to negatively regulate the expression of *SUPPRESSOR OF OVEREXPRESSION OF CO1* (*SOC1*), another floral integrator. SHL and EBS recognize di- and trimethylated histone H3 at lysine 4 and bind regulatory regions of *SOC1* and *FT*, respectively. These PHD proteins maintain an inactive chromatin conformation in *SOC1* and *FT* by preventing high levels of H3 acetylation, bind HISTONE DEACETYLASE6, and play a central role in regulating flowering time. SHL and EBS are widely conserved in plants but are absent in other eukaryotes, suggesting that the regulatory module mediated by these proteins could represent a distinct mechanism for gene expression control in plants.**

## INTRODUCTION

Chromatin remodeling processes play an essential role in the control of gene expression patterns that direct cell differentiation and development in eukaryotic organisms. A number of protein complexes are known to mediate the deposition of histone marks in the chromatin of underlying genes. In addition to these "writer" activities, "reader" proteins that recognize specific histone modifications are necessary to recruit chromatin remodeling complexes and transcription factors to target loci and modulate their transcriptional status. In this way, downstream effectors can translate histone modifications into patterns of gene expression that in turn drive developmental transitions (Suganuma and Workman, 2011).

Chromatin remodeling factors are widely conserved in different eukaryotic organisms, and a large number of proteins previously known to function in chromatin reorganization in animals have been identified in plants. However, plant development shows distinct features such as the continuous postembryonic differentiation of organs and the ability to adapt developmental transitions to environmental cues. These differences strongly argue for the existence of plant specific chromatin remodeling mechanisms accounting for the higher degree of plasticity characteristic of plant development (Jarillo et al., 2009).

The timing of the floral transition is a critical developmental switch for the reproductive success of plant species. In *Arabidopsis thaliana*, a number of genetic pathways, including the photoperiod, the vernalization, the autonomous, and the gibberellin pathways, function in the induction of flowering (Fornara et al., 2010; Srikanth and Schmid, 2011; Andrés and Coupland, 2012; Song et al., 2013b). The balance between these floral promoting pathways and floral repressors finely controls the expression of a few floral integrator genes such as *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR OF OVEREXPRESSION OF CO1* (*SOC1*) that trigger floral initiation when both environmental and developmental signals are appropriate (Fornara et al., 2010; Jarillo and Piñeiro, 2011). The dynamics of chromatin organization is crucial in the transcriptional regulation of flowering, and a large number of chromatin remodeling activities control the expression of flowering genes that participate both in the induction and the repression of flowering in *Arabidopsis* (He, 2012). One example of this is the regulation of the floral repressor *FLOWERING LOCUS C* (*FLC*), which has become a paradigm of epigenetic control of gene expression in plants. Several chromatin modifying activities act on *FLC* chromatin to modulate the activation and silencing of this

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locus during *Arabidopsis* development and also in response to vernalization (Kim et al., 2009; Crevillén and Dean, 2011; Song et al., 2013a). Some of these activities rely on protein complexes that are homologous to those present in other eukaryotic organisms, but some other appear to be specific to plants (Crevillén and Dean, 2011). Besides *FLC*, the expression of other flowering time genes is also under the influence of chromatin remodeling processes (Jarillo and Piñeiro, 2011). Recent studies have revealed that chromatin-mediated repression of the floral integrator *FT* is essential for proper control of flowering time in *Arabidopsis* (Jiang et al., 2008; Jeong et al., 2009; Adrian et al., 2010; del Olmo et al., 2010; Yang et al., 2010; He, 2012). In particular, the activity of histone deacetylases (HDACs) of the Reduced Potassium Dependency-3 (RPD3) type is necessary to establish the appropriate level of *FT* expression and prevent premature flowering in response to inductive photoperiods (Gu et al., 2013).

EARLY BOLTING IN SHORT DAYS (EBS) is an *Arabidopsis* protein related to chromatin remodeling factors, and EBS is necessary to repress *FT* expression and is also involved in the control of other developmental processes such as floral organ determination and seed dormancy (Gómez-Mena et al., 2001; Piñeiro et al., 2003). EBS bears a BAH (bromo adjacent homology) motif and a PHD (plant homeodomain) Zn finger and is part of a widely conserved family of plant-specific transcriptional regulators with no counterparts in other eukaryotic organisms. Here, we show that an *Arabidopsis* paralog of *EBS*, *SHORT LIFE* (*SHL*), is also involved in the repression of flowering. However, *SHL* has a distinct function in the regulation of flowering and is required to repress *SOC1* expression. Moreover, we reveal that the PHD domains present in EBS and SHL act as effectors of di- and trimethylated Lysine 4 in histone H3 (H3K4me2/3). In yeast and animals, the binding of PHD-containing proteins to these histone modifications triggers local changes in the levels of histone acetylation or methylation, playing a pivotal role in the activation and silencing of gene expression in eukaryotic organisms (Becker, 2006; Mellor, 2006; Sanchez and Zhou, 2011). In *Arabidopsis*, the binding of the PHD-containing protein ORIGIN OF REPLICATION COMPLEX1 (ORC1) correlates with increased levels of active histone modifications in the chromatin of target genes and with transcriptional activation (de la Paz Sanchez and Gutierrez, 2009). In contrast, PHD-containing proteins of the ALFIN-like family of *Arabidopsis* also recognize H3K4me3 and interact with POLYCOMB REPRESSIVE COMPLEX1 (PRC1) proteins to mediate the transition from the H3K4me3-associated active status to an inactive transcriptional state associated with H3K27me3 of seed genes during germination (Molitor et al., 2014). Here, we show that SHL and EBS act as repressors of the floral integrator genes *SOC1* and *FT*, respectively. Both transcriptional regulators bind discrete genomic regions of these floral integrators and are required to maintain an inactive chromatin configuration by preventing high levels of H3 acetylation in their regulatory regions. Furthermore, these PHD-containing proteins bind HISTONE DEACETYLASE6 (HDA6) and play a central role in the chromatin-mediated repression of flowering in *Arabidopsis*. Our results indicate that *SHL* and *EBS* are also involved in the control of other developmental processes and biological responses, suggesting that the regulatory mechanism mediated by these chromatin effector proteins has a more general role in the modulation of plant development. Furthermore, since these PHD-containing factors are characteristic of the plant kingdom, we propose that they could represent a fundamental difference in gene expression control strategies between plants and other eukaryotic organisms.

## RESULTS

### *Arabidopsis* SHL Is a Plant-Specific Protein Related to Chromatin Remodeling Factors and Involved in the Repression of Flowering

SHL is a nuclear protein with a BAH domain and a PHD Zn finger (Figure 1A) (Müssig et al., 2000). Both motifs are frequently found in chromatin remodeling factors involved in the control of gene expression (Sampath et al., 2009; Armache et al., 2011; Sanchez and Zhou, 2011). Based on overexpression and antisense approaches, *SHL* was proposed to be required for proper development and fertility of *Arabidopsis* plants (Müssig et al., 2000) and to participate in the regulation of *AGAMOUS-LIKE* (*AGL*) genes such as *AGL20*/*SOC1* and *AGL9*/*SEPALLATA3* (*SEP3*) (Müssig and Altmann, 2003). SHL shares high similarity with EBS (Figure 1A), an *Arabidopsis* protein involved in the repression of *FT* and in the control of other developmental processes (Gómez-Mena et al., 2001; Piñeiro et al., 2003). Homologs of EBS and SHL are highly conserved among plant species but not found in other eukaryotic organisms. The regions corresponding to the BAH and PHD domains are particularly conserved, while the C termini of these proteins are more divergent both within species and in different taxa (Figure 1A). Like *EBS*, *SHL* is ubiquitously expressed, and both transcripts are present from very early stages of germination to inflorescence development (Figures 1B and 1C) (Piñeiro et al., 2003). In addition, the levels of *SHL* expression remain constant throughout the day and after different days of vegetative growth (Supplemental Figure 1). This pattern of expression is consistent with a putative role for *SHL* in the control of different developmental processes.

To further investigate the role of *SHL* in the control of plant development and particularly of flowering time, we obtained two insertion alleles of this gene (Figure 2A). Expression analyses of both alleles showed that *shl-1* could generate a truncated version of the SHL protein that might retain some activity. In contrast, *shl-2* was likely a null allele since no *SHL* transcript could be detected in this mutant line (Figures 2B and 2C). A phenotypic analysis of both *shl* alleles revealed that the flowering time of *shl-1* was indistinguishable from the Columbia (Col) wild-type plants (Figure 2I, Table 1; Supplemental Figure 2A). In contrast, *shl-2* mutant plants showed an acceleration of flowering that was more conspicuous under short-day (SD) conditions (Figure 2D, Table 1), indicating that, similarly to *EBS*, *SHL* also has a role in the repression of flowering in *Arabidopsis*. As previously shown for *ebs* mutants, this reduced duration of vegetative growth in *shl-2* mutants was associated with a shorter adult vegetative phase (Figure 2E). Besides the early flowering phenotype, *shl-2* plants also displayed other developmental defects including smaller leaves and siliques (Figure 2). Moreover, rosette leaves of the *shl-2* mutant senesce prematurely (Figure 2F), an alteration that is absent in *ebs* mutants. These developmental defects were rescued when a gene construct

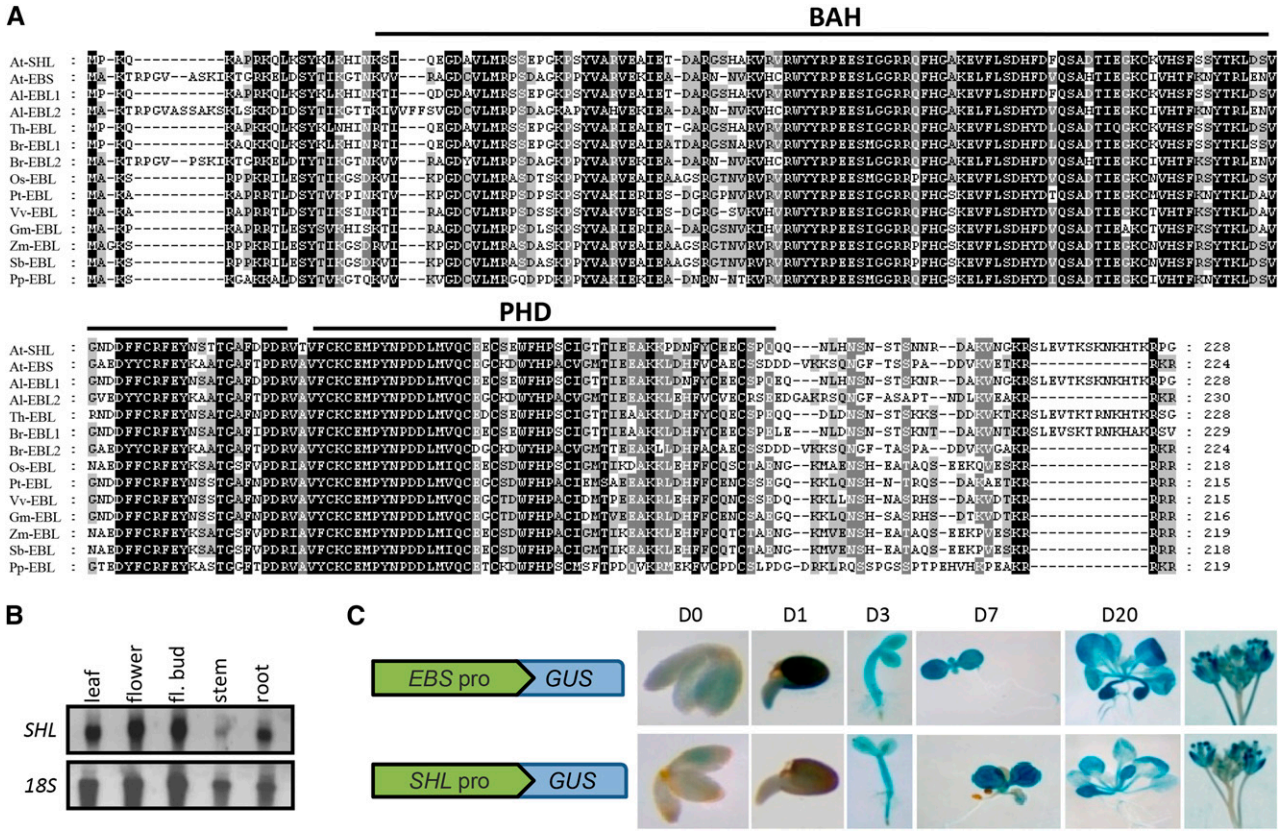


**Figure 1.** SHL Belongs to a Plant-Specific Family of Transcriptional Regulators and Is Ubiquitously Expressed in *Arabidopsis*.

**(A)** Alignment of plant proteins showing the conservation of the BAH and PHD domains. The sequences shown correspond to members of the EBS-like family found in *A. thaliana* (At), *Arabidopsis lyrata* (Al), *Thellungiella halophila* (Th), *Brassica* sp (Br), *Oryza sativa* (Os), *Populus trichocarpa* (Pt), *Vitis vinifera* (Vv), *Glycine max* (Gm), *Zea mays* (Zm), *Sorghum bicolor* (Sb), and *Physcomitrella patens* (Pp). Black, dark-gray, and light-gray boxes represent conservation percentages of 100, 80, and 60, respectively.

**(B)** Expression of *SHL* in different *Arabidopsis* organs as shown by RNA gel blot.

**(C)** Tissue expression pattern of *EBS* and *SHL* revealed by histochemical GUS staining of *EBS*pro:*GUS* and *SHL*pro:*GUS* plants. Staining was performed at different times after seed imbibition (days 0, 1, 3, 7, and 20) or in inflorescences. *SHL*pro and *EBS*pro promoter fragments are sufficient to complement the defects present in *shl* and *ebs* mutants, respectively, when fused to translational fusions containing a C-Myc epitope and the corresponding wild-type cDNA (Table 1).

containing a translational fusion of a c-Myc epitope with the *SHL* cDNA under the control of the *SHL* promoter (SHLpro:Myc-SHL) was introduced into *shl-2* mutant plants (Figure 2G, Table 1), confirming that the loss of *SHL* function is responsible for the phenotypes observed in this mutant.

To investigate a possible functional redundancy between both loci, we generated double mutants combining *ebs* with *shl-1* and *shl-2*. As shown in Figure 2H, the double mutant *shl-2 ebs* displays an extremely early flowering phenotype (Supplemental Figure 2; Table 1). In addition, these plants show extreme dwarfism and severely reduced fertility (Figures 2H and 2J). Interestingly, despite the absence of flowering time defects in the *shl-1* allele, when combined with *ebs* the double mutant *shl-1 ebs* is again extremely early flowering, dwarf, and almost sterile, similarly to *shl-2 ebs* (Figures 2I and 2J, Table 1; Supplemental Figure 2). Moreover, the defects in flowers and fruits of the *shl* mutants are dramatically enhanced in both double mutants, *shl-1 ebs* and *shl-2 ebs* (Figure 2J). Altogether, these observations suggest that *EBS* can partially compensate for the loss of *SHL* function in the repression of flowering and other developmental processes. This partial redundancy could explain the lack of phenotypic alterations in the *shl-1* allele in which a partially functional SHL protein and a partially redundant EBS protein could result in active chromatin remodeling complexes. In contrast, the complete knockout of *SHL* function cannot be fully compensated by *EBS* resulting in the developmental defects described above for the *shl-2* allele.

### *SHL* Is Required for the Repression of the Floral Integrator *SOC1*

The early flowering displayed by *ebs* mutants and knockout alleles of *SHL* indicates that these loci are not fully redundant in the repression of flowering; therefore, *SHL* must have independent roles from *EBS* in the regulation of this developmental process. For that reason, we investigated the effect of the *shl-2*

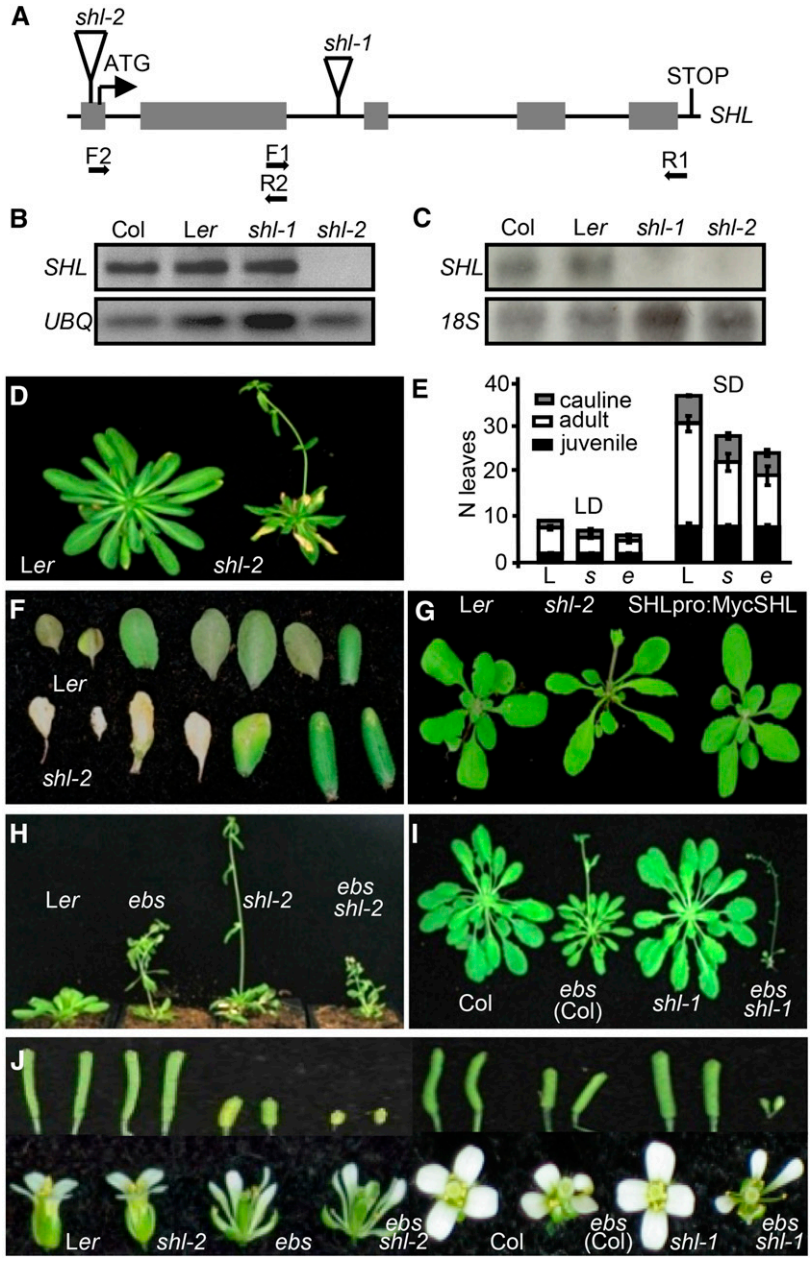


**Figure 2.** Knockout Mutations Affecting *SHL* Cause Early Flowering.

**(A)** Schematic representation of *SHL* genomic region. *shl-1* and *shl-2* insertion sites on the genomic sequence of *SHL* are marked by inverted triangles. Gray boxes represent exons and black lines, introns.

**(B)** Expression of the 5′ end of the *SHL* transcript in Col, L*er*, *shl-1*, and *shl-2* plants. RT-PCR assays were performed on plants grown for 18 d under SD.

**(C)** Expression of the 3′ end of the *SHL* mRNA in Col, L*er*, *shl-1*, and *shl-2* plants. RNA gel blot from plants grown for 15 d under SD. 18S RNA is used as loading control.

**(D)** Flowering time phenotype of the *shl-2* mutant under SD conditions.

**(E)** Number of juvenile, adult, and cauline leaves of L*er* (L), *shl-2* (*s*), and *ebs* (*e*) plants grown both under LD and SD. Error bars show SD.

**(F)** Rosette leaves of L*er* and *shl-2* grown under LD, displaying the premature senescence in the *shl* mutant.

**(G)** Complementation of the *shl-2* mutant with a SHLpro:Myc-SHL construct.

**(H)** and **(I)** Flowering time phenotype of the double mutants *shl-2 ebs* **(H)** and *shl-1 ebs* (Col) **(I)**; wild-type plants and single mutants are shown for comparison.

**(J)** Siliques and flowers of the double mutants *shl-2 ebs* and *shl-1 ebs* (Col). Plants of the same age are shown in each panel.

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**Table 1.** Flowering Time (Average ± SE) of *shl* and *ebs* Double Mutants

| Mutant | No. of Leaves LD | No. of Leaves SD |
|---|---|---|
| L*er* | 11.2 ± 1.0 | 37.8 ± 0.6 |
| *shl-2* | 9.3 ± 0.7 | 26.6 ± 0.7 |
| *ebs* | 8.4 ± 0.5 | 22.4 ± 0.8 |
| *ebs shl-2* | 4.9 ± 0.8 | 13.1 ± 1.0 |
| *co-2* | 26.7 ± 0.9 | 35.4 ± 1.7 |
| *shl-2 co-2* | 20.4 ± 0.4 | n.d. |
| *gi-3* | 37.6 ± 1.7 | 46.0 ± 1.3 |
| *shl-2 gi-3* | 30.4 ± 1.5 | 35.5 ± 0.8 |
| *fve-1* | 23.3 ± 1.4 | 68.5 ± 1.7 |
| *shl-2 fve-1* | 17.7 ± 0.4 | 62.4 ± 1.6 |
| *fca-1* | 24.4 ± 1.4 | 65.7 ± 2.2 |
| *shl-2 fca-1* | 22.2 ± 1.6 | 60.6 ± 2.0 |
| *ga2-1* | 20.4 ± 0.9 | 61.1 ± 1.9 |
| *shl-2 ga2-1* | 13.5 ± 0.4 | 42.3 ± 2.0 |
| *ga1-3* | 17.5 ± 1.4 | n.f. |
| *shl-2 ga1-3* | 15.2 ± 0.8 | n.f. |
| *clf-16* | 7.5 ± 0.5 | 10.2 ± 0.7 |
| *shl-2 clf-16* | 7.5 ± 0.6 | 9.9 ± 0.8 |
| *ebs clf-16* | 3.0 ± 0.0 | 6.2 ± 0.4 |
| *ft-1* | 18.6 ± 1.6 | 43.3 ± 1.2 |
| *shl-2 ft-1* | 14.3 ± 1.2 | 38.0 ± 0.9 |
| *soc1-1* | 16.6 ± 0.8 | 65.6 ± 1.2 |
| *shl-2 soc1-1* | 15.8 ± 1.4 | 64.6 ± 0.9 |
| Col | 16.4 ± 1.1 | 68.7 ± 1.2 |
| *ebs* (Col) | 12.0 ± 1.7 | 22.8 ± 2.5 |
| *ebs shl-1* | 5.4 ± 1.8 | 18.6 ± 1.7 |
| *shl-1* | 16.2 ± 0.9 | 66.6 ± 2.2 |
| *shl-2* (Col) | 14.5 ± 1.5 | 56.7 ± 6.4 |
| *tfl-2* | 11.5 ± 0.6 | 17.0 ± 0.8 |
| *shl-2 tfl-2* | 10.2 ± 0.5 | 13.2 ± 0.8 |
| *ebs tfl-2* | 8.2 ± 0.5 | 10.6 ± 0.6 |
| *ebs shl-2 ft* | 13.0 ± 1.7 | 31.4 ± 3.4 |
| *ebs shl-2 soc1-1* | 12.1 ± 1.4 | 33.4 ± 2.4 |
| Myc-*SHL shl-2* | 10.3 ± 0.4 | 33.8 ± 1.4 |
| Myc-EBS *ebs* | 10.4 ± 0.5 | 34.7 ± 1.2 |

n.d., not determined; n.f., these plants did not flower.

mutation on the expression of floral integrators and other flowering time genes. Accordingly with previous observations, *ebs* mutations cause a premature upregulation of *FT* (Figure 3A; Supplemental Figure 3A). In contrast, the expression of this floral integrator is not affected in *shl-2* mutants at different time points of the day (Figure 3A) or at different days after germination (Supplemental Figure 3A). The expression of the *FT-LIKE* gene *TWIN SISTER OF FT* (*TSF*) is not altered in *ebs* or *shl* mutants (Supplemental Figure 3B). However, the expression of another floral integrator, *SOC1*, is upregulated in *shl-2* (Figure 3B), indicating that *SHL* is necessary for the repression of *SOC1*. Furthermore, the expression of other genes ascribed to different pathways that control flowering in *Arabidopsis* such as *CONSTANS* (*CO*), *FVE*, *GIBBERELLIC ACID5* (*GA5*) or *FLC* and the *FLC-LIKE* genes *MADS AFFECTING FLOWERING1-5* (*MAF1-5*) is not affected in *shl-2* mutants (Supplemental Figures 3C to 3G) nor in *ebs* plants, as previously described (Piñeiro et al., 2003).

To further understand the interaction of *SHL* with pathways controlling the initiation of flowering in *Arabidopsis*, we performed a thorough genetic analysis combining *shl-2* with mutations affecting the floral integrators and representative genes of these floral inductive pathways. The early flowering phenotype of *ebs* is fully suppressed by mutations in *FT* but not in *SOC1* (Gómez-Mena et al., 2001; Piñeiro et al., 2003). In contrast to *ebs*, the premature flowering of *shl-2* mutants is only partially suppressed in *ft* mutant background, but loss of function of *SOC1* completely eliminates the early flowering of *shl-2* plants under both long-day (LD) and SD conditions (Figures 3C to 3F, Table 1). These results indicate that *SOC1* is required for the early flowering phenotype of *shl-2*. Double mutants combining *shl-2* with representative mutations of the photoperiod, the autonomous and the GA pathways did not reveal any genetic interaction consistent with a role for *SHL* in the control of flowering through the modulation of these inductive pathways (Figure 4, Table 1). Only mutations in the autonomous pathway gene *FCA*, but not *FVE*, suppress the early flowering phenotype of *shl* mutants (Figures 4D and 4E). These observations suggest that *SHL* does not interact with the autonomous pathway, although we cannot rule out a specific interaction with *FCA*. Alternatively, the effect of *shl* mutations on flowering time could be masked by the increased expression levels of the strong floral repressor *FLC* that are present in mutants affecting genes in the autonomous pathway. This interpretation is consistent with the observation that *FLC* expression levels are not altered by mutations in *SHL* (Supplemental Figures 3F and 3G). Together with the expression analysis, these results indicate that *SHL* has a distinct role from *EBS* in the repression of flowering in *Arabidopsis*. While *EBS* is required to prevent premature activation of *FT* (Piñeiro et al., 2003), *SHL* is necessary to negatively regulate the expression of *SOC1* independently of other floral pathways that control this floral integrator gene.

Since SHL and EBS are necessary to repress *SOC1* and *FT* expression, respectively, we reasoned that mutations affecting one of these two floral integrator genes should not suppress the early flowering phenotype of the double mutant *shl-2 ebs*. Indeed, both *shl-2 ebs soc1* and *shl-2 ebs ft* triple mutants display an intermediate flowering time phenotype between that of the double mutant *shl-2 ebs* and each of the late flowering mutants *ft* or *soc1* (Figures 5A and 5B, Table 1). Moreover, the expression of both *FT* and *SOC1*, but not other flowering time genes tested such as *CO* or *FLC*, is increased in *shl-2 ebs* plants (Figures 5C and 5D), confirming that both floral repressors are required for the precise control of master genes of flowering initiation.

### Transcriptomic Analysis Confirms Distinct Roles of *SHL* and *EBS* in the Regulation of Gene Expression

*SHL* and *EBS* act on the repression of two different floral integrator genes, *SOC1* and *FT*. This observation suggests target specificity for these repressors of flowering in the control of gene expression. To investigate the level of functional divergence between *SHL* and *EBS* in relation to their role in transcriptional control, we performed transcriptomic profiling of both *shl* and *ebs* mutants. We found 218 genes with an altered level of expression in the *shl* mutant and 178 genes in *ebs* (fold change ± 2, P value < 0.05) (Supplemental Data Set 1). The genes differentially expressed in both mutants are enriched in upregulated loci (66 and 80% in *shl* and *ebs*, respectively), in agreement with a possible repressor function for

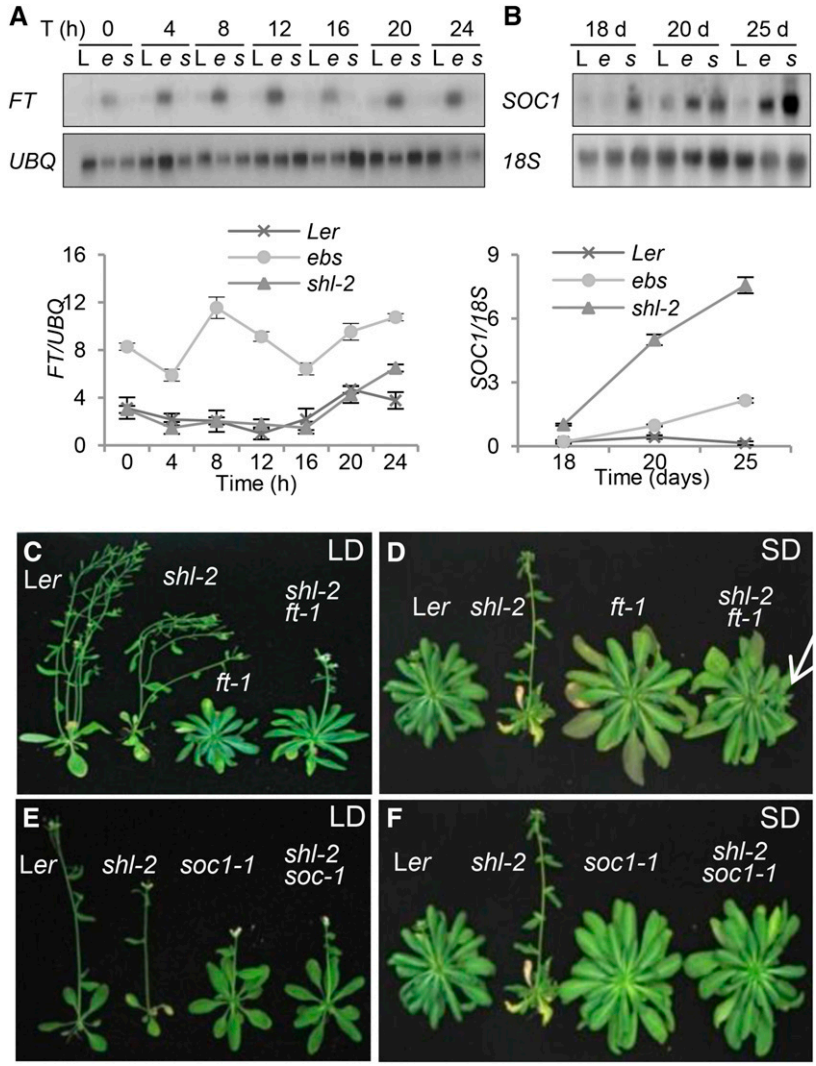


**Figure 3.** *SHL* Is Required to Repress the Expression of the Floral Integrator Gene *SOC1*.

**(A)** RT-PCR showing expression of *FT* in L*er* (L) and in *shl-2* (*s*) and *ebs* (*e*) mutant plants grown for 18 d under SD; samples were taken every 4 h. Error bars show SD.

**(B)** RNA gel blot showing expression of *SOC1* in *shl-2* and *ebs* mutant plants grown for 18, 20, and 25 d under SD.

**(C)** and **(D)** Additive flowering time phenotype of double mutants *shl-2 ft* under LD **(C)** and SD **(D)**; a white arrow marks the tip of the inflorescence.

**(E)** and **(F)** Suppression of the early flowering phenotype of the *shl-2* mutant by *soc1* mutations under LD **(E)** and SD **(F)**. Plants of the same age are shown in each panel.

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SHL and EBS in the control of gene expression. Moreover, consistent with a role for both *SHL* and *EBS* in the transcriptional regulation of development and other biological processes, a Gene Ontology analysis demonstrated that transcription factors are the most overrepresented molecular function category among the genes misregulated in *shl* or *ebs* mutants (Figure 6A). More transcription factors are downregulated in the *shl* mutant (56%), while this proportion is reversed in the *ebs* mutant (25%), but in both cases several families of transcription factors including homeodomains, MADS box proteins, bHLH, WRKY, MYB, AP2-like, and others are represented in the genes with altered level of expression (Supplemental Data Set 1). Some of these transcriptional regulators are known to play central roles in the control of different biological processes in *Arabidopsis*, such as light responses, diverse aspects of development (flowering and flower development or root architecture), and responses to stress, indicating that the function of *SHL* and *EBS* is not restricted to the control of flowering time (Figure 6B). These results are consistent with the pleiotropic phenotypic alterations observed in *shl*, *ebs*, and *shl ebs* mutants and suggest a role for these two homologous proteins as master regulators of gene expression programs.

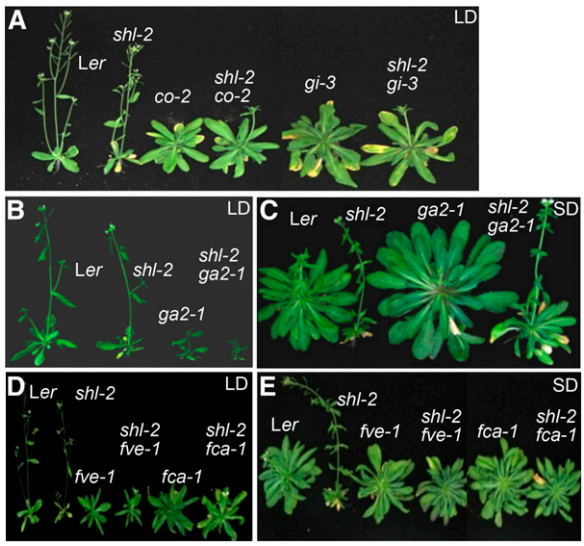


**Figure 4.** *SHL* Acts Independently of the Photoperiod, Gibberellin, and Autonomous Pathways in the Control of Flowering Time.

**(A)** Additive flowering time phenotype of double mutants *shl co-2* and *shl-2 gi-3* (photoperiod pathway) under LD.
**(B)** and **(C)** Flowering time phenotype of *shl ga2-1* (gibberellin-dependent pathway) plants under LD **(B)** and SD **(C)**.
**(D)** and **(E)** Flowering time phenotype of double mutants *shl fve-1* and *shl-2 fca-1* (autonomous pathway) under LD **(D)** and SD **(E)**. Plants of the same age are shown in each panel.
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In contrast to transcription factors that are overrepresented in both *shl* and *ebs*, other functional categories representing the molecular function of misregulated genes are significantly enriched in only one of the mutants but not in the other (Figure 6A). Moreover, despite the overlapping in some functional categories, the genes with altered expression in the two mutants differ significantly, and only 48% of the genes derepressed in the *shl* mutant are also upregulated in *ebs*. Furthermore, only 22% of genes downregulated in *shl* overlap with *ebs* (Figure 6C). These observations are consistent with our molecular and genetic analyses of the *shl* and *ebs* mutants and confirm that, although some degree of overlapping exists, *SHL* and *EBS* play distinct roles in the control of gene expression.

### *SHL* and *EBS* Genetically Interact with Genes Encoding Chromatin Remodeling Factors That Regulate Flowering Time

The presence of a BAH domain and a PHD Zn finger in SHL and EBS suggests that these proteins might be involved in the control of gene expression by modulating the organization of chromatin. In order to unveil functional links between the genes encoding these plant-specific proteins and other chromatin remodeling factors also involved in the repression of flowering, we analyzed their genetic interaction with *CURLY LEAF* (*CLF*) and *LIKE-HETEROCHROMATIN PROTEIN1/TERMINAL FLOWER2* (*LHP1/TFL2*). *CLF* encodes the PRC2 subunit that catalyzes the trimethylation of Lys (K) 27 in histone H3 (H3K27me3) (Schubert et al., 2006), a chromatin mark associated with transcriptional inactivation, while LHP1/TFL2 is a PRC1 protein involved in binding H3K27me3 (Turck et al., 2007; Zhang et al., 2007). Both loci are involved in the repression of flowering by inhibiting the expression of *FT* (Jarillo and Piñeiro, 2011). The double mutant combination of *ebs* with *clf* displayed an extreme early flowering phenotype and a dramatic upregulation of the floral integrators, particularly *FT*, measured at early stages of development (10 d after sowing in SD) when flowering initiation has not taken place in this extremely early flowering double mutant (Figures 7A and 7E, Table 1). Consistent with previous observations, upregulation of *FT* in the *ebs* mutant is not observed at this early stage of vegetative development, although it is clearly detected after 2 weeks of growth (Piñeiro et al., 2003). Both *EBS* and *SHL* are required to delay flowering during the adult but not the juvenile phase of vegetative development (Figure 2E), suggesting that the repression activity of these transcriptional regulators on the floral integrators is required several days after germination. The described synergistic interaction between *EBS* and *CLF* suggests that these loci are functionally related in the control of flowering time. In contrast, *CLF* is epistatic to *SHL* since *shl* mutations do not cause any additional acceleration of flowering in the absence of an active *CLF* gene, suggesting that *CLF* may act downstream of *SHL* in a pathway that controls flowering time (Figure 7B, Table 1). Moreover, the expression of *FT* is similar in the double mutant *shl clf* and in the *clf* mutant, while *SOC1* is expressed at slightly higher levels in the double mutant as

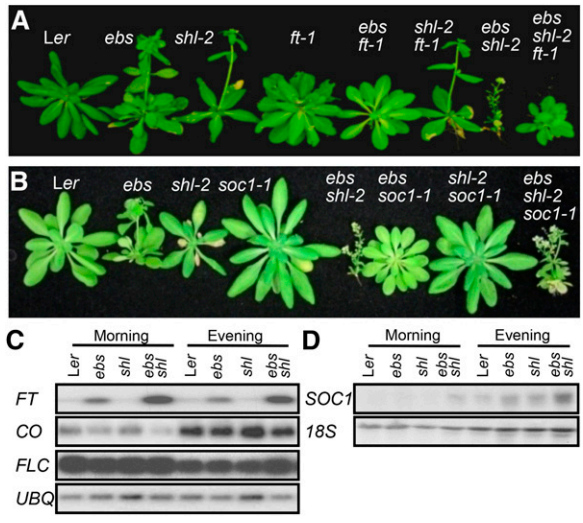


**Figure 5.** Mutations in *FT* or *SOC1* Cannot Suppress the Early Flowering of *shl-2 ebs* Double Mutants.

**(A)** and **(B)** Flowering time phenotype of triple mutants *shl ebs ft* **(A)** and *shl ebs soc1* **(B)** under SD. Plants of the same age are shown in **(A)** and **(B)**.
**(C)** and **(D)** Expression of *FT*, *CO*, and *FLC* **(C)** shown by RT-PCR and *SOC1* **(D)** shown by RNA gel blot in *shl-2 ebs* double mutants after 10 d of growth under SD. Samples were taken early in development to ensure that the transcript levels of the floral integrator genes were measured prior to the initiation of flowering in the double mutant.
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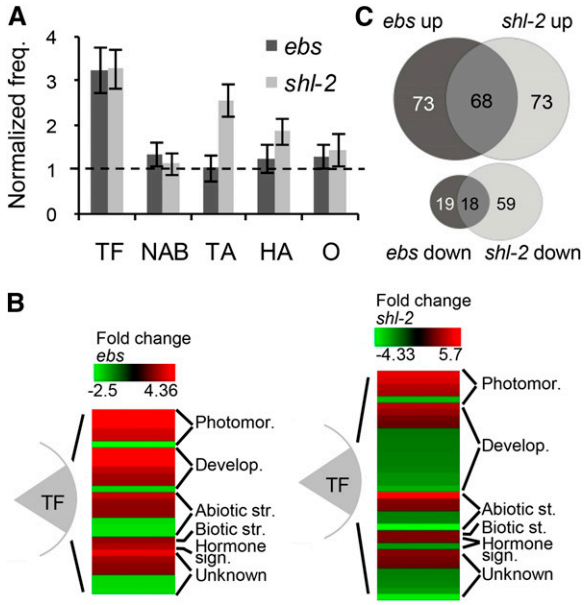


**Figure 6.** Transcriptional Profiling of *shl-2* and *ebs* Mutants Demonstrates Distinct Roles for Both Loci in the Control of Gene Expression.

**(A)** Normalized frequency of functional categories among the genes misregulated in *shl* and *ebs* mutants, attending to their molecular function (classification of BAR, Bio Array Resource for Plant Biology, University of Toronto, Canada). The normalized frequency, as defined in BAR (http://bar.utoronto.ca/welcome.htm), represents the frequency of genes in each category in the input set normalized relative to the frequency in the reference set that includes all genes in the *Arabidopsis* database (25 K) $((N\_in\_Class_{input\_set}/N\_Classified_{input\_set})/(N\_in\_Class_{reference\_set\ 25\ K}/N\_Classified_{reference\_set\ 25\ K}))$. Only overrepresented categories (normalized frequency > 1) are shown. TF, transcription factor; NAB, nucleic acid binding; TA, transferase activity; HA, hydrolase activity; O, other. Error bars show SD.

**(B)** Transcription factors showing altered levels of expression in *shl-2* and *ebs* mutants based on TAIR classification.

**(C)** Venn diagrams showing the number of genes misregulated in the *ebs* mutant (dark gray), in the *shl* mutant (lightest gray), or in both (intermediate gray).

compared with the single mutant *clf* (Figure 7E). This moderate increase in the expression of the floral integrators in the double mutant *shl clf* is in contrast with the marked increase in the expression of *FT* and *SOC1* observed in the double mutant *ebs clf*. The distinct genetic interactions observed between *shl* and *ebs* with *clf* is again consistent with *EBS* and *SHL* playing independent roles in the regulation of flowering.

We also analyzed the flowering phenotype of double mutants combining *shl* or *ebs* with *lhp1/tfl2*. These double mutants also displayed an extreme acceleration of flowering, especially under SD conditions, together with drastic alterations of plant development (Figures 7C and 7D, Table 1). Consistent with the enhancement of the early flowering phenotype observed in the double mutant combinations, *shl-2 tfl2-1* plants showed increased expression of both floral integrators *FT* and *SOC1* (Figure 7F). However, while *SOC1* expression is very similar in the *ebs tfl2* plants and in the *tfl2* mutant, the expression of *FT* is increased in this double mutant suggesting that *FT* could mediate the acceleration of flowering observed in these plants (Figure 7G). These observations suggest that both *EBS* and *SHL* interact synergistically with *LHP1/TFL2* in the repression of flowering in *Arabidopsis* and are consistent with a role for these two PHD-containing proteins in the chromatin-mediated modulation of flowering.

## SHL and EBS Function as Chromatin Effectors That Mediate the Repression of Master Genes Involved in the Control of Flowering

A number of PHD fingers have been shown to act as readers of covalent modifications present in the N-terminal tail of histone H3 and, particularly, the methylation state of Lys (K) 4 in H3. These PHD domains act as effectors that translate the epigenome into patterns of gene expression by recruiting chromatin regulators and transcription factors to target loci (Sanchez and Zhou, 2011; Molitor et al., 2014). The PHD domains present in SHL and EBS (S-PHD and E-PHD) contain several conserved residues that are important for the aromatic cage that mediates the recognition of H3K4me3 in different PHD-containing proteins both in animals (Shi et al., 2006; Wysocka et al., 2006) and plants (Lee et al., 2009) (Figure 8A). In addition, the predicted 3D model of the EBS and SHL PHD motifs fits the structure of PHD domains such as that in the BPTF protein (bromodomain PHD-finger transcription factor), the largest subunit of the NuRF (nucleosome remodeling factor) complex in animals, which was shown to bind H3K4me3 (Wysocka et al., 2006) (Figure 8B). For those reasons, we decided to investigate the ability of the PHD present in EBS and SHL to bind the N-terminal tail of histone H3 bearing different levels of K4 methylation. In vitro binding assays of E-PHD and S-PHD to H3 peptides carrying a different number of methyl groups at K4 (0 to 3, levels of H3K4 methylation found in vivo) showed that both motifs can preferentially recognize H3K4me2/3 (Figure 8C). Site-directed mutagenesis of the W170 residue in E-PHD and W163 in S-PHD (both substituted by A) completely abolished this binding. The position of these W residues corresponds to a highly conserved W that is essential in the aromatic cage that recognizes H3K4me3 in a number of PHD-containing proteins (Figure 8A) (Sanchez and Zhou, 2011). Furthermore, both E-PHD and S-PHD can pull down H3K4me3 from *Arabidopsis* histone extracts (Figure 8D). In contrast, other versions of histone H3, methylated in different lysine residues of the N terminus (H3K36me3, H3K9me2, and H3K27me3) also important for the regulation of gene expression, are not recognized by any of the assayed polypeptides (Figure 8D), suggesting the specificity of the observed binding. Again, a W/A residue substitution in the aromatic cage of the PHDs completely eliminates the binding to H3K4me3 (Figure 8D). Furthermore, 91 and 97% of the genes misregulated in the *ebs* and *shl* mutants, respectively, carry H3K4me2/3, according to genome-wide maps of these marks in *Arabidopsis* (Roudier et al., 2011). From these results, we concluded that the PHD domains in SHL and EBS are responsible for the binding of these proteins to the histone mark H3K4me2/3 and that the function of both floral repressors may be mediated by their roles as chromatin effectors.

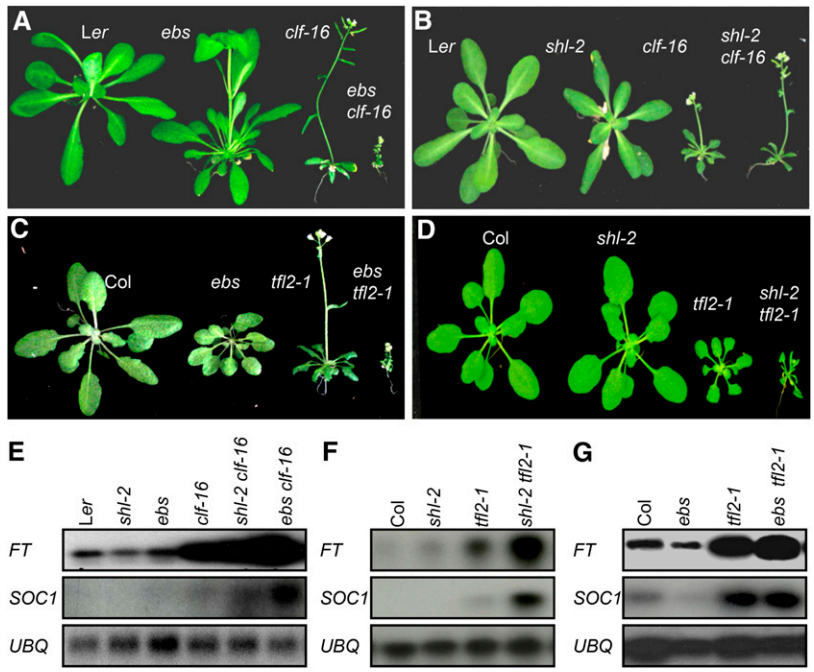


**Figure 7.** Genetic Interaction of *SHL* and *EBS* with Genes Encoding the PcG-Related Chromatin Remodeling Factors CLF and LHP1/TFL2.

**(A)** to **(D)** Flowering time phenotype under SD of the double mutants *ebs clf-16* **(A)**, *shl-2 clf-16* **(B)**, *ebs tfl2-1* **(C)**, and *shl-2 tfl2-1* **(D)**. Plants of the same age are shown in each panel; wild-type plants and single mutants are shown for comparison.

**(E)** Expression of the floral integrator genes *FT* and *SOC1* in double mutants combining *shl* or *ebs* with *clf-16*.

**(F)** RT-PCR showing expression of the floral integrator genes *FT* and *SOC1* in the double mutant *shl tfl2-1*.

**(G)** RT-PCR showing expression of the floral integrator genes *FT* and *SOC1* in the double mutant *ebs tfl2-1*. In **(E)** to **(G)**, samples were taken early in development (10 d after sowing) to ensure that the transcript levels of the floral integrator genes were measured prior to the initiation of flowering in the double mutants.

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In animals, the recognition of H3K4me3 by PHD-containing proteins results in the recruitment of protein complexes that modulate the expression of underlying genes (Sanchez and Zhou, 2011). For instance, BPTF and INHIBITOR OF GROWTH2 (ING2) associate with chromatin-modifying complexes that direct histone acetyl-transferase or HDAC complexes to target loci to mediate their activation or repression (Becker, 2006). To assess the relevance of SHL and EBS proteins in the chromatin-mediated regulation of gene expression, we analyzed changes in the chromatin acetylation of loci whose expression is affected by the *shl* and *ebs* mutations, namely, *SOC1* and *FT*, respectively. We performed chromatin immunoprecipitation (ChIP) experiments on different genomic regions of the *SOC1* gene in the *shl-2* mutant. These regions were previously shown to be important for the chromatin-mediated regulation of *SOC1* (Bouveret et al., 2006). For these assays, we used an antibody against a histone mark associated with transcriptional activation such as H3K9K14Ac. As shown in Figures 9A and 9B, the upregulation of *SOC1* observed in *shl-2* mutants correlates with higher levels of H3K9K14 acetylation in the genomic region of the *SOC1* locus. In addition, our ChIP experiments demonstrate that the SHL protein can bind regulatory regions corresponding to the second intron of *SOC1* (Figures 9A and 9C). Similarly, *ebs* mutations cause increased levels of H3K9K14Ac throughout the *FT* gene (Figures 9D and 9E). Moreover, a functional C-Myc-EBS protein binds in vivo one of the *FT* regions identified in the *ebs* mutant for their increased levels of H3 acetylation (Figures 9D and 9F). These higher levels of H3 acetylation are consistent with the derepression of *FT* observed in *ebs* mutants (Piñeiro et al., 2003). Furthermore, our results show that SHL does not bind the regulatory regions of *FT* where EBS is found, and vice versa, EBS does not bind the *SOC1* locus (Supplemental Figure 4). These observations led us to conclude that the floral repressors SHL and EBS directly regulate the expression of the floral integrator genes *SOC1* and *FT*, respectively, and are required for low levels of histone H3 acetylation in the regulatory regions of these master genes of flowering.

### SHL and EBS Bind HDA6

All together, the data presented suggest that *SHL*/*EBS* could mediate the crosstalk between H3K4me2/3 and histone H3 acetylation in the chromatin of the floral integrators. To further test this hypothesis, we decided to analyze the existence of genetic interactions between these two genes encoding PHD-containing proteins and different genes responsible for the modulation of those histone marks. For that, we built double mutants combining *ebs* or *shl* with plants defective in histone methyltransferases,

such as *ARABIDOPSIS TRITHORAX1* (*ATX1*) and *ARABIDOPSIS TRITHORAX-RELATED7* (*ATXR7*), which mediate trimethylation of H3K4 and are involved in the repression of flowering controlling the expression of *FLC* and probably other flowering time genes (He, 2012). Double mutants *ebs atx1-2*, *ebs atxr7-1*, and *ebs atxr7-2* displayed an additive acceleration of flowering as compared with the single mutants both under LD and SD (Supplemental Figures 5A, 5B, 5E, and 5F). Similarly, double mutants combining *ebs* with mutations affecting the *Arabidopsis* PAF1-C, thought to act as a docking platform for histone methyltransferase complexes during transcriptional activation (He, 2012), such as *early flowering7* (*elf7*) also result in an additive decrease of flowering time under both photoperiodic conditions assayed (Supplemental Figures 5C, 5E, and 5F). In contrast, the double mutants *shl atx1-2*, *shl atxr7-1*, and *shl atxr7-2* showed no further acceleration of flowering as compared with each single mutant, suggesting that *SHL* and *ATX1*/*ATXR7* could act in the same genetic pathway to control flowering time (Supplemental Figures 5E and 5F). This result is consistent with a functional link between *SHL* and genes modulating H3K4me2/3 levels. Furthermore, these observations reinforce the notion that *EBS* and *SHL* play independent roles in the control of flowering time in *Arabidopsis*.

Additional analyses revealed genetic interactions of *EBS* and *SHL* with *Arabidopsis* genes encoding histone H3K4 demethylases, such as *ELF6* and *JUMONJI 4/14* (*JMJ4*/*JMJ14*) (He, 2012). JMJ4/14 is required to repress the expression of floral integrator genes like *FT*,

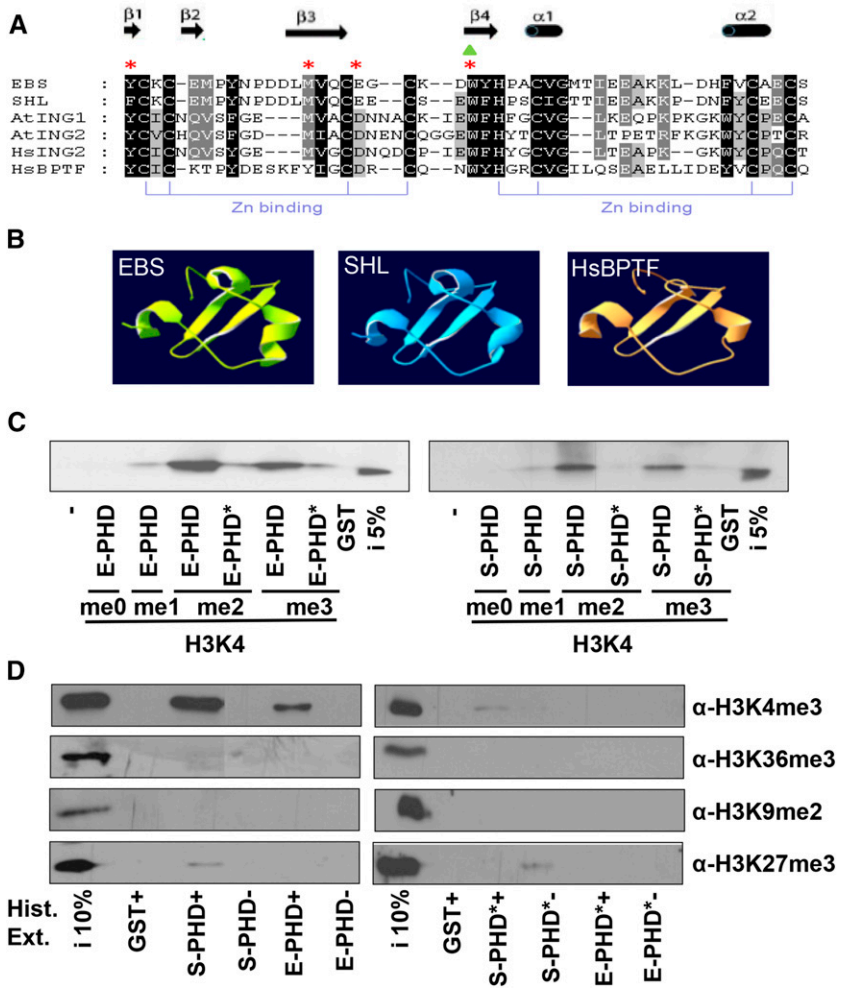


**Figure 8.** The PHD Domains in SHL and EBS Are Responsible for the Binding of These Proteins to H3K4me2/3 Residues.

**(A)** Alignment of the PHD domains in SHL and EBS with those of other PHD-containing proteins known to bind H3K4me3. Important residues for H3K4me3 recognition are marked (red asterisk). The green triangle corresponds to the highly conserved W residue mutated in **(C)** and **(D)**.

**(B)** Predicted three-dimensional structure of the SHL and EBS PHDs, based on Swiss-PdbViewer v4.01.

**(C)** Binding assays of wild-type and mutated (asterisk) versions of the SHL and EBS PHDs with unmodified, mono-, di-, or trimethylated-K4 H3 peptides. In mutated PHDs (asterisk), W163 in SHL and W170 in EBS were replaced by A. "i 5%" corresponds to input.

**(D)** Protein gel blots (with antibodies against H3K4me3, H3K36me3, H3K9me2, or H3K27me3) of modified histones pulled down from *Arabidopsis* histone extracts using wild-type or mutated (asterisk) versions of the SHL and EBS PHDs.

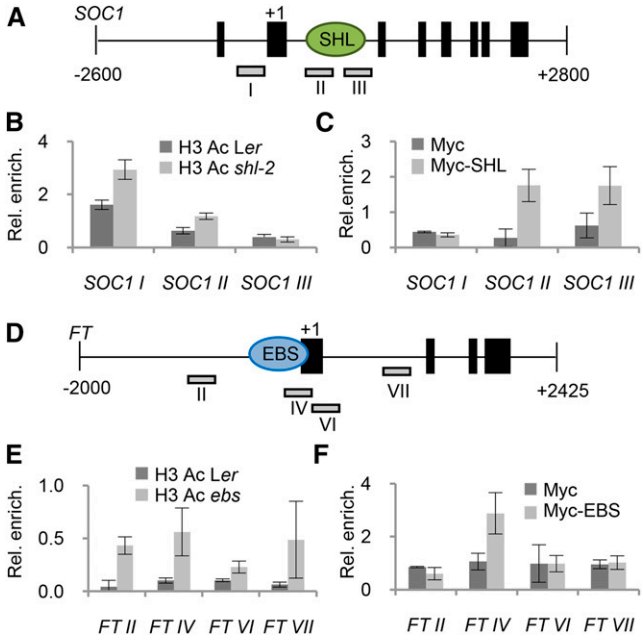


**Figure 9.** SHL and EBS Bind Discrete Genomic Regions of *SOC1* and *FT*, Respectively, and Are Required to Maintain Low Levels of H3Ac in These Floral Integrator Genes.

**(A)** Schematic representation of the *SOC1* locus showing the location of the quantitative PCR amplicons used in ChIP assays (gray boxes). Black boxes represent exons.

**(B)** Discrete regions of *SOC1* are enriched in H3K9K14Ac in the *shl-2* mutant. Relative increase of this histone mark, as shown by ChIP, in the mutant is shown in comparison with the wild type.

**(C)** Binding of Myc-SHL protein to the regions of *SOC1* hyperacetylated in the *shl-2* mutant. Myc-SHL corresponds to *shl-2* mutant plants containing the complementing fusion SHLpro:Myc-SHL described in Figure 2G. Myc denotes transgenic plants expressing the Myc epitope not fused to any *Arabidopsis* gene.

**(D)** *FT* genomic region showing the location of the quantitative PCR amplicons used in ChIP assays (gray boxes). Black boxes represent exons.

**(E)** Discrete regions of *FT* are enriched in H3K9K14Ac in the *ebs* mutant. Relative increase of this histone mark in the mutant, as shown by ChIP, is shown in comparison with the wild type.

**(F)** Binding of Myc-EBS protein to the regions of *FT* hyperacetylated in the *ebs* mutant. Myc-EBS corresponds to *ebs* mutant plants containing the complementing fusion EBSpro:Myc-EBS, while Myc is the same as in **(C)**. Plant material used in the ChIP experiments described in **(B)**, **(C)**, **(E)**, and **(F)** was harvested at Zeitgeber time 8 after 18 d of growth under SD conditions.

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*SOC1*, and *LFY* (Lu et al., 2010), and both ELF6 and JMJ4/14 have been proposed to mediate H3K4 demethylation of the *FT* locus to modulate flowering time (Jeong et al., 2009; Yang et al., 2010). Consistent with a functional relationship of EBS and H3K4me2/3 levels in the *FT* chromatin, *EBS* interacts genetically with *ELF6* in the control of flowering initiation, and when combined with *ebs*, *elf6* mutations do not cause a further acceleration of flowering in the double mutant plants (Supplemental Figures 5D to 5F). In contrast, *SHL* appears to act additively with *ELF6* in the control of flowering time under both LD and SD conditions (Supplemental Figures 5E and 5F). In addition, *SHL* interacts genetically with *JMJ4* at least under SDs (Supplemental Figures 5E and 5F).

Further to the above discussed genetic interactions with genes involved in H3K4 methylation and demethylation, we also assessed a possible interaction of *EBS* with HDACs that mediate histone H3 deacetylation and are known to be required for the control of flowering time, such as *HDA6* and *HDA19* (Berr et al., 2011; Kim et al., 2012). Double mutants *ebs hda19* turned out to be severely impaired in flower development, resulting in sterile plants that prevented the flowering time analysis. However, the flowering time analyses of the double mutant combining *ebs* with the *hda6* mutant allele *axe1-5* revealed that *ebs* fully suppresses the late flowering phenotype of *axe1-5* under LD and drastically accelerates flowering of the double mutant under SD (Figures 10A and 10B; Supplemental Figure 6A). This observation, together with the high levels of histone acetylation measured in the chromatin of *FT* in the *ebs* mutant, led us to investigate a possible physical interaction between EBS and HDA6. Different experimental approaches allowed us to reveal the binding of EBS and this RPD3/Class I histone deacetylase both in in vitro pull-down assays (Figure 10C) and in planta bimolecular fluorescence complementation (BiFC) and coimmunoprecipitation (co-IP) experiments (Figures 10D and 10E), suggesting that this class of HDACs could be involved in the repression mechanism mediated by EBS. In order to extend these observations to SHL, we performed BiFC experiments that demonstrated the in vivo binding of this PHD-containing protein and HDA6 (Supplemental Figure 7), suggesting that this Class I HDAC could also participate together with SHL in the control of gene expression. In contrast to *ebs axe1-5*, the double mutant *shl axe1-5* displayed an intermediate flowering time phenotype under LD as compared with the parental single mutants (Supplemental Figure 6B), confirming again that *EBS* and *SHL* interact differentially with the genetic pathways that regulate the initiation of flowering.

All together, the data presented are consistent with the idea that the function of both floral repressors, SHL and EBS, likely contributes to maintain the chromatin of *SOC1* and *FT* in an inactive conformation. This transcriptional repression appears to implicate the activity of HDAC complexes and is required to prevent the premature initiation of flowering, ensuring that the floral transition takes place at the appropriate time (Figure 10F).

## DISCUSSION

Histone modifications are key elements in the transcriptional regulation of developmental master genes (Jarillo et al., 2009). However, these histone marks are not directly responsible for changes in the levels of expression of underlying genes. Instead, a variety of effector proteins are required to specifically recognize histone modifications and translate them into gene expression patterns. Therefore, functional domains present in these "reader" proteins play a central role in mediating the functional consequences of histone marks on transcriptional regulation. Despite the relevance of these mechanisms for proper control of plant development, the molecular basis of the process is far from understood. Here, we show that SHL and EBS, two homologous *Arabidopsis* proteins involved in the repression of flowering and

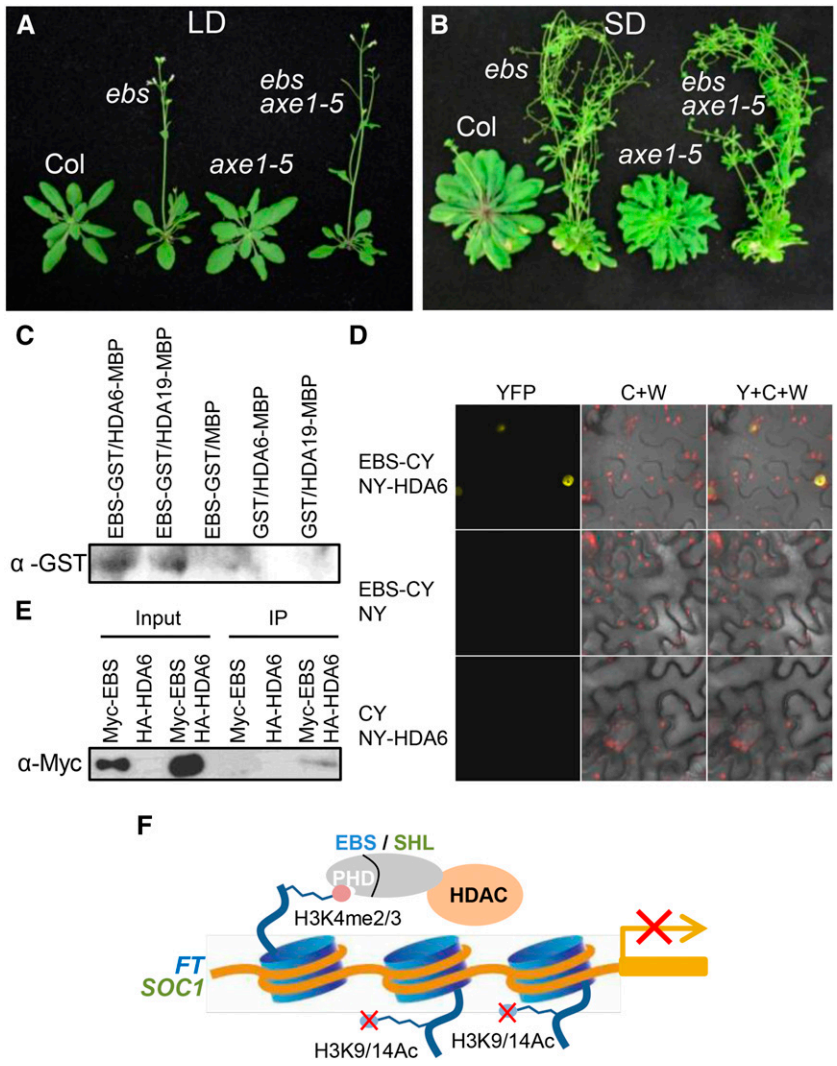


**Figure 10.** EBS Interacts with HDACs.

**(A)** and **(B)** Flowering time phenotype of the double mutant *ebs axe1-5* under LD **(A)** and SD **(B)** conditions.
**(C)** In vitro pull-down assays showing the interaction of the EBS protein, fused to GST, with HDA6 and 19, fused to maltose binding protein.
**(D)** BiFC assays showing the in vivo interaction between EBS and HDA6 (top panel). The EBS and HDA6 coding sequences were fused to the C-YFP (CY) and N-YFP (NY), respectively, and coexpressed in *Nicotiana benthamiana* cells. YFP signal (YFP), chlorophyll autofluorescent plus bright field (C+W), and overlay (Y+C+W) microscopy images are shown. Fluorescence is not observed when EBS fused to the C-terminal moiety of YFP is infiltrated with only the N terminus of YFP (middle panel) or when HDA6 fused to the N terminus of YFP is infiltrated together with the C terminus of the YFP.
**(E)** In planta interaction between EBS and HDA6 fused to Myc and HA epitopes, respectively. Samples were immunoprecipitated with HA antibody and the immunoblot was probed with anti-Myc antibody.
**(F)** Working model for the hypothetical repression mechanism of the floral integrator genes mediated by SHL and EBS, showing their interaction with HDACs.

the regulation of different developmental processes, function in the chromatin-mediated control of gene expression. Both proteins appear to specifically recognize H3K4me2/3 marks, are required to maintain low levels of H3K9K14Ac in the chromatin of their target loci, and interact in planta with the RPD3/Class I HDAC HDA6, preventing the premature activation of the floral integrator genes. Interestingly, SHL and EBS homologs are widely conserved from mosses to higher plants (Figure 1A), but not in yeast or animals, indicating that the repression mechanism mediated by these H3K4me2/3 effector proteins could represent a regulatory module that operates specifically in the plant kingdom and contributes to unique features, such as postembryonic organogenesis and developmental plasticity, that characterize plant development.

*SHL* and *EBS* show partial genetic redundancy in the control of flowering time (Figure 2). However, both the genetic and the molecular analyses demonstrate that these two loci have independent functions in the repression of flowering and act on

different targets. In contrast to *EBS*, *SHL* is necessary to negatively regulate the expression of the floral integrator *SOC1* (Figure 3). This observation is consistent with transcriptomic profiling experiments showing that alterations in the levels of expression of *SHL* result in moderate changes in the levels of *SOC1* expression (Müssig and Altmann, 2003). Our genome-wide expression analyses of *shl* and *ebs* mutants reveal that transcription factors are overrepresented among the genes misregulated in both mutants (Figure 6). These transcription factors participate in the regulation of different developmental processes, including reproductive development. This observation is consistent with the pleiotropic alterations observed in these mutants and fits well with a role for these PHD-containing proteins in the control of diverse aspects of development and other biological responses. Furthermore, the genes misregulated in the *shl* and *ebs* mutants differ significantly (Figure 6), suggesting that both paralogs have evolved to control different target genes. The amino acid sequence of EBS and SHL are very similar, except for the C terminus, suggesting that this region could mediate interactions with a different set of proteins that could be responsible for the specificity for different regulatory regions. Additional work will be needed to establish the role of the C-terminal domain in target gene discrimination.

Mutant plants defective in the repression mechanisms mediated by SHL or EBS and CLF or LHP1/TFL2 display an extreme acceleration of flowering and a severe deregulation of the floral integrator genes *FT* and *SOC1* during early stages of plant development (Figure 7). Our results are consistent with the hypothesis that a relaxed chromatin conformation caused by loss of function of these floral repressors is likely responsible for the observed upregulation of the floral promoter genes and the premature induction of the floral transition, supporting a role for SHL and EBS in the modulation of chromatin organization. Moreover, misregulation of other target genes is probably responsible for the pleiotropic developmental alterations observed in these double mutants. Further genetic analyses showed that *SHL* genetically interacts with genes encoding chromatin remodeling factors involved in the modulation of H3K4 methylation levels, such as *ATX1/ATXR7* and *JMJ4/14* (Supplemental Figure 5), while *EBS* displayed genetic interactions with *ELF6* and *HDA6* (Figure 10; Supplemental Figures 5D and 6A), supporting again the involvement of *SHL* and *EBS* in chromatin remodeling processes. Consistent with this interpretation, our data show that the PHD domains present in SHL and EBS are responsible for the ability of these proteins to bind H3K4me2/3 (Figure 8). A number of animal proteins containing PHDs have been shown to control gene expression by recognizing H3K4me3 and recruiting chromatin remodeling complexes that can activate or repress the transcription of underlying genes. The BPTF subunit of the NURF chromatin remodeling complex is recruited to the promoters of target genes where it acts to activate transcription. However, binding of the PHD-containing protein ING2 to H3K4me3 recruits HDAC complexes that promote the acquisition of an inactive chromatin conformation and repression of transcription (Becker, 2006). The *Drosophila* PHD-containing protein UpSET is also required to restrict chromatin accessibility by directly binding an Rpd3 HDAC complex (Rincon-Arano et al., 2012). In plants, the PHD-containing protein ORC1 has been shown to bind H3K4me3 and activate transcription of several target genes (de la Paz Sanchez and Gutierrez, 2009). In contrast, the ALFIN-like PHD-containing proteins also bind H3K4me3 and mediate the switch from an active chromatin conformation of seed genes to an inactive H3K27me3-associated state during germination by interacting with PRC1 proteins (Molitor et al., 2014). Other PHD proteins, such as VERNALIZATION INSENSITIVE3 (VIN3) and VIN3-LIKEs, are required to repress the expression of *FLC* in response to vernalization (Kim et al., 2009), although their histone binding properties remain to be elucidated. Although it is possible that SHL and EBS may be recruited to regulatory regions of target loci independently of H3K4me2/3, the results obtained in this work are consistent with a role for these PHD-containing proteins in the recognition of H3K4me2/3 and the repression of gene expression. Our data show that both *shl* and *ebs* mutants display increased accumulation of H3K9K14Ac in regulatory regions of target genes (Figure 9). We cannot rule out at this stage that high levels of acetylation in *shl* and *ebs* mutants are a consequence of increased transcription activity, but our observations together with the detected interactions between these PHD-containing proteins and HDA6 (Figure 10; Supplemental Figure 7) are consistent with the notion that both transcriptional regulators are required to maintain low levels of H3 acetylation in the chromatin of regulatory regions of their target loci by recruiting HDACs. HDA6 was shown to be involved in the transcriptional regulation of *FLC* through the interaction with the histone demethylase FLOWERING LOCUS D (FLD) and the HDAC-associated protein FVE (He, 2012). However, genetic analyses demonstrated that part of the flowering time phenotype observed in *HDA6* mutant plants *axe1-5* was independent of this floral repressor gene, suggesting that this Class I HDAC is regulating additional loci involved in the control of flowering initiation (Yu et al., 2011). In addition, other HDACs could play at least partially redundant roles with *HDA6* in the regulation of flowering and other developmental processes, hampering the progress in understanding the function of these chromatin remodeling proteins in modulating developmental gene expression control. The data presented in this work open a new window to better understand the transcriptional regulation of developmental genes in plants, although further studies will be necessary to fully clarify the chromatin remodeling complexes that mediate the repression mechanism in which these two PHD-containing proteins are involved.

The PHDs in SHL and EBS bind preferentially H3K4me2/3 marks (Figure 8). This result is similar to previous observations obtained with the Pygopus (Pygo) protein (Fiedler et al., 2008). The presence of D residues in the aromatic cage that is formed by the PHD motif has been evoked as the possible explanation for the ability of the Pygo protein to recognize both H3K4me2/3 forms. Consistent with this interpretation, a Y/E substitution in the aromatic cage of the PHD-containing protein BPTF increases the affinity of this transcription factor for H3K4me2 (Li et al., 2007). Two acidic residues are present in the predicted aromatic cage of SHL and EBS and could provide the basis for the recognition of H3K4me2/3 by both PHD-containing proteins. Additional analyses will be necessary to confirm this hypothesis and to establish the functional relevance of this binding specificity in the mechanism of transcriptional regulation mediated by these PHD proteins.

Histone acetylation is frequently present in the regulatory regions and the 5′ end of genes and has been shown to be

involved in transcriptional initiation and elongation (Choi and Howe, 2009). In *Arabidopsis*, the histone acetylation status has been shown to influence the regulation of some genes such as *FLC*, *TIMING OF CAB EXPRESSION1*, and *PHYA*, where crosstalk between H3K9/14Ac and H3K4me3 appears to be important for changes in the light-dependent transcriptional status of the locus (Perales and Más, 2007; Jang et al., 2011; He, 2012). The chromatin conformation of *FT* is also correlated with the expression of this locus (Adrian et al., 2010). In fact, the levels of H3K9K14Ac are increased in transcribed and regulatory regions of the *FT* locus in plants overexpressing *CO*, an upstream regulator of this floral integrator that activates *FT* expression in response to LD (Andrés and Coupland, 2012). Moreover, *FT* expression is associated with a depletion of the H3K27me3 effector protein LHP1, although activation of this floral integrator can take place without a decrease in the levels of the H3K27me3 mark (Adrian et al., 2010). However, histone acetylation changes in the chromatin of *FT* do not appear to be a prerequisite for the activation of this locus, and it has been suggested that the chromatin-mediated repression of this floral integrator provides a means for the fine-tuning of the transcription of this gene rather than an on/off switch (Adrian et al., 2010). In fact, recent results have demonstrated that a histone deacetylation mechanism is necessary for the photoperiodic control of *FT* expression and that core components of HDAC complexes are required for this regulatory mechanism in *Arabidopsis* (Gu et al., 2013). Chromatin remodeling processes are also important for the regulation of *SOC1*, and changes in the levels of H3K4me2/3 and H3K9Ac marks have been shown to correlate with the transcriptional status of this floral integrator (Bouveret et al., 2006; Adrian et al., 2009).

Our results reveal that SHL and EBS can bind genomic regions of *SOC1* and *FT*, respectively. In *SOC1*, these regions are important for the transcriptional regulation mediated by MSI1 (Bouveret et al., 2006), while EBS binds sequences near the initiation codon of *FT* where the transcription factor TEMPRANILLO1 and the H3K4 demethylases ELF6 and AtJMJ4 also bind to repress *FT* expression (Castillejo and Pelaz, 2008; Jeong et al., 2009). Remarkably, both EBS and SHL are necessary to modulate the levels of H3 acetylation in the chromatin of these master genes of flowering and both recognize H3K4me2/3, chromatin marks that are also essential for proper control of gene expression, suggesting that the crosstalk between H3K4me2/3 and H3K9K14Ac is a key element in the precise control of flowering time, as previously discussed for the FLD-HDA6-FVE regulation of *FLC* (He, 2012). Therefore, the SHL and EBS proteins appear to be part of a regulatory mechanism for the chromatin-mediated repression of central genes in the control of flowering time. The transcriptomic analysis and the phenotype of double mutants indicate that *SHL* and *EBS* are also involved in the regulation of other aspects of plant development besides flowering time, suggesting that these plant-specific chromatin effectors may function as master regulators of a number of gene expression programs. Therefore, the modulation of the chromatin status in the genomic regions of the floral integrators *SOC1* and *FT* mediated by SHL and EBS, respectively, may represent a model for the regulation of target genes involved in a variety of developmental processes. In a chromatin context where both active and inactive histone marks are present, the function of these plant-specific proteins may be crucial in finely modulating the expression of the floral integrators and other genes playing pivotal roles in plant developmental programs.

## METHODS

### Plant Material and Growth Conditions

*Arabidopsis thaliana* mutant seed stocks used were in Landsberg *erecta* (L*er*) and Col genetic backgrounds and were obtained from public stock centers and personal donations. Monogenic mutants were described previously: *fve-1*, *fca-1*, *ft-1*, *co-2*, and *gi-3* (Koornneef et al., 1991), GA-deficient *ga1-3* and *ga2-1* (Koornneef and van der Veen, 1980), *soc1-1* (Samach et al., 2000), *ebs* (Piñeiro et al., 2003), *tfl2-1* (Larsson et al., 1998), *atx1-2* (Pien et al., 2008), *atxr7-1* and *atxr7-2* (Tamada et al., 2009), *elf7-2* (He et al., 2004), *elf6-4* (Jeong et al., 2009), and *axe1-5* (Yu et al., 2011). *clf-16* was previously isolated in our laboratory. The *shl-1* allele, in Col background, corresponds to line SALK_053996, and *shl-2* allele, in L*er* background, corresponds to line GT442, obtained from Cold Spring Harbor Laboratory. Molecular markers used for the genotyping of double mutants are detailed in Supplemental Table 1.

The transcriptional fusions of the *SHL* and *EBS* promoters to β-glucuronidase (GUS) (*EBS*pro:*GUS* and *SHL*pro:*GUS*; 2 and 1 kb, respectively) were transformed into Col plants. For the generation of the EBSpro:Myc-EBS construct, the chimeric Myc-EBS cDNA, previously in pGWB18, was cloned into the EBSpro:GUS plasmid by substituting the *GUS* gene by the Myc-EBS cassette. To generate the SHLpro:Myc-SHL construct in the pGreen0229 plasmid, the Myc-SHL cassette in pGWB18 was cloned downstream of the *SHL* promoter. The primers used are listed in the Supplemental Table 1. *Agrobacterium tumefaciens* (AGL0)-mediated transformation of *Arabidopsis* plants was performed using the floral dip method (Clough and Bent, 1998). Transformant plants were selected on germination medium (Murashige and Skoog medium with 1% sucrose) (Murashige and Skoog, 1962) with appropriate antibiotics.

### Phenotypic Characterizations and Genetic Analyses

Flowering time, measured as total leaf number, and the duration of vegetative developmental phases were scored as previously described in ASL-Ibercex and Aralab walk-in growth chambers (Lázaro et al., 2008). Double mutants were isolated from selfed F2 progenies derived from crosses of *shl-2* or *ebs* with different flowering time mutants. To generate double mutants of *shl-2* or *ebs* with flowering time mutants in Col background, *shl-2* or *ebs* was previously introgressed. The molecular markers used for selection are listed in Supplemental Table 1.

### Expression Analyses

Isolation of total RNA from seedlings and cDNA synthesis were performed according to previously described procedures (Lázaro et al., 2008). Total RNA was extracted from whole seedlings grown under SD for the times indicated. For *SHL*, *CO*, *FLC*, *MAF1-5*, *GA5*, *FT*, and *TSF* genes, we performed RT-PCR followed by radioactive detection according to described procedures (Lázaro et al., 2008) (the primers used are described in Supplemental Table 1). *UBIQUITIN10* (*UBQ10*) was used as a loading control in these experiments and was amplified for 25 cycles. *SHL*, *FT*, *TSF*, *GA5*, *FLC*, and *MAF1-5* were amplified for 30 cycles. *CO* was amplified for 28 cycles. *SHL* expression was analyzed by RNA gel blot, using as a probe a specific fragment from the 3′ end of the cDNA generated by PCR using the primers SHLnthF (5′-AAACGACGACTTCTTCTGTCG-3′) and SHLnthR (5′-TGAGAAACCA CCATACGCTATAC-3′). *SHL* expression was also monitored by RT-PCR (Supplemental Table 1). *FVE*, *FLC*, and *SOC1* expression was analyzed by RNA gel blot. *FVE* expression was detected using the complete *FVE* cDNA (1.9 kb) as a probe. *FLC* and *SOC1*/*AGL20* were

detected as described (Piñeiro et al., 2003). All experiments were repeated at least three times with independent samples.

Transcriptomic analyses were performed on ATH1 arrays using RNA from 18-d-old seedlings grown in SD and harvested at Zeitgeber time 8. Three independent biological replicates were hybridized. We used The Bio-Array Resource for Plant Biology (http://bar.utoronto.ca/welcome.htm), the resources of Genevestigator (https://www.genevestigator.com/gv/), as well as Venny (http://bioinfogp.cnb.csic.es/tools/venny/index.html) software for microarray data analysis. Obtained raw data have been submitted to the Gene Expression Omnibus public repository with reference GSE33270.

### Histochemical GUS Assays

GUS staining was performed as previously described (Lázaro et al., 2008).

### Protein Modeling

Three-dimensional models for the EBS-PHD and SHL-PHD domains were generated using the SWISS-PROT/TrEMBL tool (http://swissmodel.expasy.org/) described by Schwede et al. (2003).

### Protein Expression and Protein Interaction Assays

The *EBS* and *SHL* complete cDNAs together with the fragments corresponding to the PHD domains of both proteins (E-PHD and S-PHD, respectively) were fused to glutathione *S*-transferase (GST) by cloning them into the pGEX 2T vector compatible with the Gateway system and expressed in *Escherichia coli* BL21 Rosetta. The primers used are listed in Supplemental Table 1. To generate the EBS-GST and SHL-GST mutated versions, the QuikChange site-directed mutagenesis kit (Stratagene) was used. Nuclear extracts enriched in histones were prepared from MM2d *Arabidopsis* suspension-cultured cells according to described procedures (de la Paz Sanchez and Gutierrez, 2009).

For histone pull-down assays, EBS-GST, SHL-GST, E-PHD-GST, S-PHD-GST, and the mutated versions of the proteins bound to glutathione sepharose beads (Upstate) were incubated with histone extracts according to described procedures (de la Paz Sanchez and Gutierrez, 2009). Pulled down histones were analyzed by immunoblot. For binding assays to the N-terminal tail of H3 methylated at K4, we used biotinylated histone peptides H3K4me0 (12-357), H3K4me1 (12-563), H3K4me2 (12-460), and H3K4me3 (12-564) from Upstate. The proteins E-PHD-GST, S-PHD-GST, and the corresponding mutated versions bound to glutathione sepharose beads were incubated with 0.5 μg of each peptide according to described procedures (de la Paz Sanchez and Gutierrez, 2009). Bound peptides to different versions of the PHDs were detected with horseradish peroxidase conjugated to Streptavidin after transfer of proteins to Immobilon (Millipore) membranes. For EBS-HDAC pull-down assays, EBS-GST protein was incubated with maltose binding protein fusions to HDA6 and 19 in the same conditions described above for binding assays to histone peptides. Pulled down proteins were visualized with anti-GST antibodies.

For co-IP assays, the different coding sequences were cloned into Gateway destination vectors pGWB18 (C-Myc fusions with EBS and SHL) and pEARLEYGATE201 (HA fusion to HDA6). *Agrobacterium* strain AGL0 carrying the different constructs was used to infiltrate *Nicotiana benthamiana* leaves. Co-IP was performed as previously described (Yu et al., 2011) using anti-HA High Affinity antibody (Roche). Proteins were visualized by immunoblot using an anti-Myc antibody (Millipore).

Coding sequences of the different proteins were cloned into the Gateway binary destination vectors pNXGW (nYFP-) and pXCGW (-cCFP) for BiFC assays. EBS and SHL were tagged with cCFP, and HDA6 was tagged with nYFP at either the N or C terminus, respectively. Assays were performed as previously described (Yuan et al., 2013). The BiFC constructs were introduced in *N. benthamiana* leaves by agroinfiltration. YFP-derived fluorescence was analyzed by laser scanning microscopy using a Leica TCS SP8 confocal microscope.

### ChIP Assays

After chromatin isolation according to previously described methods (Lázaro et al., 2008), the H3 acetylated fractions were immunoprecipitated using specific antibodies to acetylated K9 and K14 (ref. 06-599 from Upstate Biotechnology). PCR was used to amplify four different fragments of the *FT* gene and three different fragments of the *SOC1* gene (Bouveret et al., 2006) (the primers used are described in Supplemental Table 1). All PCR reactions and quantification of the amplified DNA were done as described (Lázaro et al., 2008). We conducted three repeats of each experiment from independent biological replicates. *ACTIN2* was used as an internal control for the ChIP analyses.

Binding of SHL to the *SOC1* gene was analyzed by ChIP experiments with p*SHL*:Myc-SHL plants; quantitative PCR was performed on the immunoprecipitates obtained with anti-Myc antibodies with the three primer sets used for ChIP experiments performed with anti-H3 modifications (Supplemental Table 1). Error bars correspond to SD of the mean of at least three quantitative PCR replicates. To establish the binding of EBS to the *FT* gene, quantitative PCR was performed on the immunoprecipitates obtained with anti-Myc antibodies from p*EBS*:Myc-EBS plants by using the four primer sets used for ChIP experiments performed with anti-H3 modifications (Supplemental Table 1). Error bars correspond to SD of the mean of at least three quantitative PCR replicates.

### Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL under the following accession numbers: Al-EBL1 (XP_002868884.1), Al-EBL2 (XP_002872686.1), Th-EBL (BAJ33950.1), Br-EBL1 (EM:DK463881), Br-EBL2 (EM:DY020946), Os-EBL (BAC79935.1), Pt-EBL (XP_002305450.1), Vv-EBL (CBI38025.3), Gm-EBL (ACU15947.1), Zm-EBL (NP_001151899.1), Sb-EBL (XP_002459456.1), Pp-EBL (XP_001781596.1), *CO* (AT5G15840), *EBS* (At4g22140), *FLC* (At5g10140), *FT* (AT1G65480), *FVE* (AT2G19520), *GA5* (AT4G25420), *MAF1* (AT1G77080), *MAF2* (AT5G65050), *MAF3* (AT5G65060), *MAF4* (AT5G65070), *MAF5* (AT5G65080), *SHL* (At4g39100), *SOC1* (AT2G45660), *TSF* (AT4G20370), *UBQ10* (AT4G05320), At-ING1 (At3g24010), At-ING2 (At1g54390), Hs-ING2 (AAQ13674.1), Hs-BPTF (NP_872579.2), and GSE33270 (arrays of *shl* and *ebs* mutants).

### Supplemental Data

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

**Supplemental Figure 1.** *SHL* Is Expressed at Constant Levels along the Daily Cycle and at Different Times of Development.

**Supplemental Figure 2.** Flowering Time Phenotype of the Double Mutants *shl ebs* Grown under LD.

**Supplemental Figure 3.** The Expression of the Floral Integrator *FT* and Representative Genes from the Different Pathways Controlling Flowering Time Is Independent of *SHL*.

**Supplemental Figure 4.** EBS Binds Genomic Regions of *FT* but Not of *SOC1*, While SHL Binds the *SOC1* Locus but Not *FT*.

**Supplemental Figure 5.** Flowering Time Phenotype of the Double Mutants Combining *ebs* and *shl-2* with Mutations in Genes Encoding Chromatin Remodeling Factors Related with the Levels of H3K4me3.

**Supplemental Figure 6.** Flowering Time Quantification of the Double Mutants Combining *ebs* and *shl* with *axe1-5*.

**Supplemental Figure 7.** SHL Interacts in Vivo with HDA6.

**Supplemental Table 1.** Primers Used in This Work.

**Supplemental Data Set 1.** Transcriptomic Profiling of *shl* and *ebs* Mutants.


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## AUTHOR CONTRIBUTIONS

J.A.J. and M.P. designed research. L.L.-G., A.M., L.N.-D., and R.B. performed research, J.M.M.-Z., J.A.J., M.P., and L.L.-G. analyzed data. J.M.M.-Z., J.A.J., and M.P. wrote the article.

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## REFERENCES


**Adrian, J., Torti, S., and Turck, F.** (2009). From decision to commitment: the molecular memory of flowering. Mol. Plant **2:** 628–642.

**Adrian, J., Farrona, S., Reimer, J.J., Albani, M.C., Coupland, G., and Turck, F.** (2010). cis-Regulatory elements and chromatin state coordinately control temporal and spatial expression of *FLOWERING LOCUS T* in Arabidopsis. Plant Cell **22:** 1425–1440.

**Andrés, F., and Coupland, G.** (2012). The genetic basis of flowering responses to seasonal cues. Nat. Rev. Genet. **13:** 627–639.

**Armache, K.J., Garlick, J.D., Canzio, D., Narlikar, G.J., and Kingston, R.E.** (2011). Structural basis of silencing: Sir3 BAH domain in complex with a nucleosome at 3.0 Å resolution. Science **334:** 977–982.

**Becker, P.B.** (2006). Gene regulation: a finger on the mark. Nature **442:** 31–32.

**Berr, A., Shafiq, S., and Shen, W.H.** (2011). Histone modifications in transcriptional activation during plant development. Biochim. Biophys. Acta **1809:** 567–576.

**Bouveret, R., Schönrock, N., Gruissem, W., and Hennig, L.** (2006). Regulation of flowering time by Arabidopsis MSI1. Development **133:** 1693–1702.

**Castillejo, C., and Pelaz, S.** (2008). The balance between CONSTANS and TEMPRANILLO activities determines *FT* expression to trigger flowering. Curr. Biol. **18:** 1338–1343.

**Choi, J.K., and Howe, L.J.** (2009). Histone acetylation: truth of consequences? Biochem. Cell Biol. **87:** 139–150.

**Clough, S.J., and Bent, A.F.** (1998). Floral dip: a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*. Plant J. **16:** 735–743.

**Crevillén, P., and Dean, C.** (2011). Regulation of the floral repressor gene *FLC*: the complexity of transcription in a chromatin context. Curr. Opin. Plant Biol. **14:** 38–44.

**de la Paz Sanchez, M., and Gutierrez, C.** (2009). Arabidopsis ORC1 is a PHD-containing H3K4me3 effector that regulates transcription. Proc. Natl. Acad. Sci. USA **106:** 2065–2070.

**del Olmo, I., López-González, L., Martín-Trillo, M.M., Martínez-Zapater, J.M., Piñeiro, M., and Jarillo, J.A.** (2010). *EARLY IN SHORT DAYS 7* (*ESD7*) encodes the catalytic subunit of DNA polymerase epsilon and is required for flowering repression through a mechanism involving epigenetic gene silencing. Plant J. **61:** 623–636.

**Fiedler, M., Sánchez-Barrena, M.J., Nekrasov, M., Mieszczanek, J., Rybin, V., Müller, J., Evans, P., and Bienz, M.** (2008). Decoding of methylated histone H3 tail by the Pygo-BCL9 Wnt signaling complex. Mol. Cell **30:** 507–518.

**Fornara, F., de Montaigu, A., and Coupland, G.** (2010). SnapShot: Control of flowering in Arabidopsis. Cell **141:** 550–550.

**Gómez-Mena, C., Piñeiro, M., Franco-Zorrilla, J.M., Salinas, J., Coupland, G., and Martínez-Zapater, J.M.** (2001). *early bolting in short days*: an Arabidopsis mutation that causes early flowering and partially suppresses the floral phenotype of leafy. Plant Cell **13:** 1011–1024.

**Gu, X., Wang, Y., and He, Y.** (2013). Photoperiodic regulation of flowering time through periodic histone deacetylation of the florigen gene FT. PLoS Biol. **11:** e1001649.

**He, Y.** (2012). Chromatin regulation of flowering. Trends Plant Sci. **17:** 556–562.

**He, Y., Doyle, M.R., and Amasino, R.M.** (2004). PAF1-complex-mediated histone methylation of *FLOWERING LOCUS C* chromatin is required for the vernalization-responsive, winter-annual habit in Arabidopsis. Genes Dev. **18:** 2774–2784.

**Jang, I.-C., Chung, P.J., Hemmes, H., Jung, C., and Chua, N.-H.** (2011). Rapid and reversible light-mediated chromatin modifications of Arabidopsis *phytochrome A* locus. Plant Cell **23:** 459–470.

**Jarillo, J.A., and Piñeiro, M.** (2011). Timing is everything in plant development. The central role of floral repressors. Plant Sci. **181:** 364–378.

**Jarillo, J.A., Piñeiro, M., Cubas, P., and Martínez-Zapater, J.M.** (2009). Chromatin remodeling in plant development. Int. J. Dev. Biol. **53:** 1581–1596.

**Jeong, J.H., Song, H.R., Ko, J.H., Jeong, Y.M., Kwon, Y.E., Seol, J. H., Amasino, R.M., Noh, B., and Noh, Y.S.** (2009). Repression of *FLOWERING LOCUS T* chromatin by functionally redundant histone H3 lysine 4 demethylases in Arabidopsis. PLoS ONE **4:** e8033.

**Jiang, D., Wang, Y., Wang, Y., and He, Y.** (2008). Repression of *FLOWERING LOCUS C* and *FLOWERING LOCUS T* by the Arabidopsis Polycomb repressive complex 2 components. PLoS ONE **3:** e3404.

**Kim, D.-H., Doyle, M.R., Sung, S., and Amasino, R.M.** (2009). Vernalization: winter and the timing of flowering in plants. Annu. Rev. Cell Dev. Biol. **25:** 277–299.

**Kim, J.M., To, T.K., and Seki, M.** (2012). An epigenetic integrator: new insights into genome regulation, environmental stress responses and developmental controls by histone deacetylase 6. Plant Cell Physiol. **53:** 794–800.

**Koornneef, M., and van der Veen, J.H.** (1980). Induction and analysis of gibberellin sensitive mutants in *Arabidopsis thaliana* (L.) heynh. Theor. Appl. Genet. **58:** 257–263.

**Koornneef, M., Hanhart, C.J., and van der Veen, J.H.** (1991). A genetic and physiological analysis of late flowering mutants in *Arabidopsis thaliana*. Mol. Gen. Genet. **229:** 57–66.

**Larsson, A.S., Landberg, K., and Meeks-Wagner, D.R.** (1998). The *TERMINAL FLOWER2* (*TFL2*) gene controls the reproductive transition and meristem identity in *Arabidopsis thaliana*. Genetics **149:** 597–605.

**Lázaro, A., Gómez-Zambrano, A., López-González, L., Piñeiro, M., and Jarillo, J.A.** (2008). Mutations in the Arabidopsis *SWC6* gene, encoding a component of the SWR1 chromatin remodelling complex, accelerate flowering time and alter leaf and flower development. J. Exp. Bot. **59:** 653–666.

**Lee, W.Y., Lee, D., Chung, W.I., and Kwon, C.S.** (2009). Arabidopsis ING and Alfin1-like protein families localize to the nucleus and bind to H3K4me3/2 via plant homeodomain fingers. Plant J. **58:** 511–524.

**Li, H., Fischle, W., Wang, W., Duncan, E.M., Liang, L., Murakami-Ishibe, S., Allis, C.D., and Patel, D.J.** (2007). Structural basis for lower lysine methylation state-specific readout by MBT repeats of L3MBTL1 and an engineered PHD finger. Mol. Cell **28:** 677–691.

**Lu, F., Cui, X., Zhang, S., Liu, C., and Cao, X.** (2010). JMJ14 is an H3K4 demethylase regulating flowering time in Arabidopsis. Cell Res. **20:** 387–390.

**Mellor, J.** (2006). It takes a PHD to read the histone code. Cell **126:** 22–24.

**Molitor, A.M., Bu, Z., Yu, Y., and Shen, W.H.** (2014). Arabidopsis AL PHD-PRC1 complexes promote seed germination through H3K4me3-to-H3K27me3 chromatin state switch in repression of seed developmental genes. PLoS Genet. **10:** e1004091.

**Murashige, T., and Skoog, F.** (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant. **15:** 473–497.

**Müssig, C., and Altmann, T.** (2003). Changes in gene expression in response to altered *SHL* transcript levels. Plant Mol. Biol. **53:** 805–820.

**Müssig, C., Kauschmann, A., Clouse, S.D., and Altmann, T.** (2000). The Arabidopsis PHD-finger protein SHL is required for proper development and fertility. Mol. Gen. Genet. **264:** 363–370.

**Perales, M., and Más, P.** (2007). A functional link between rhythmic changes in chromatin structure and the Arabidopsis biological clock. Plant Cell **19:** 2111–2123.

**Pien, S., Fleury, D., Mylne, J.S., Crevillen, P., Inzé, D., Avramova, Z., Dean, C., and Grossniklaus, U.** (2008). ARABIDOPSIS TRITHORAX1 dynamically regulates *FLOWERING LOCUS C* activation via histone 3 lysine 4 trimethylation. Plant Cell **20:** 580–588.

**Piñeiro, M., Gómez-Mena, C., Schaffer, R., Martínez-Zapater, J.M., and Coupland, G.** (2003). EARLY BOLTING IN SHORT DAYS is related to chromatin remodeling factors and regulates flowering in Arabidopsis by repressing *FT*. Plant Cell **15:** 1552–1562.

**Rincon-Arano, H., Halow, J., Delrow, J.J., Parkhurst, S.M., and Groudine, M.** (2012). UpSET recruits HDAC complexes and restricts chromatin accessibility and acetylation at promoter regions. Cell **151:** 1214–1228.

**Roudier, F., et al.** (2011). Integrative epigenomic mapping defines four main chromatin states in Arabidopsis. EMBO J. **30:** 1928–1938.

**Samach, A., Onouchi, H., Gold, S.E., Ditta, G.S., Schwarz-Sommer, Z., Yanofsky, M.F., and Coupland, G.** (2000). Distinct roles of CONSTANS target genes in reproductive development of Arabidopsis. Science **288:** 1613–1616.

**Sampath, V., Yuan, P., Wang, I.X., Prugar, E., van Leeuwen, F., and Sternglanz, R.** (2009). Mutational analysis of the Sir3 BAH domain reveals multiple points of interaction with nucleosomes. Mol. Cell. Biol. **29:** 2532–2545.

**Sanchez, R., and Zhou, M.-M.** (2011). The PHD finger: a versatile epigenome reader. Trends Biochem. Sci. **36:** 364–372.

**Schubert, D., Primavesi, L., Bishopp, A., Roberts, G., Doonan, J., Jenuwein, T., and Goodrich, J.** (2006). Silencing by plant Polycomb-group genes requires dispersed trimethylation of histone H3 at lysine 27. EMBO J. **25:** 4638–4649.

**Schwede, T., Kopp, J., Guex, N., and Peitsch, M.C.** (2003). SWISS-MODEL: An automated protein homology-modeling server. Nucleic Acids Res. **31:** 3381–3385.

**Shi, X., et al.** (2006). ING2 PHD domain links histone H3 lysine 4 methylation to active gene repression. Nature **442:** 96–99.

**Song, J., Irwin, J., and Dean, C.** (2013a). Remembering the prolonged cold of winter. Curr. Biol. **23:** R807–R811.

**Song, Y.H., Ito, S., and Imaizumi, T.** (2013b). Flowering time regulation: photoperiod- and temperature-sensing in leaves. Trends Plant Sci. **18:** 575–583.

**Srikanth, A., and Schmid, M.** (2011). Regulation of flowering time: all roads lead to Rome. Cell. Mol. Life Sci. **68:** 2013–2037.

**Suganuma, T., and Workman, J.L.** (2011). Signals and combinatorial functions of histone modifications. Annu. Rev. Biochem. **80:** 473–499.

**Tamada, Y., Yun, J.Y., Woo, S.C., and Amasino, R.M.** (2009). ARABIDOPSIS TRITHORAX-RELATED7 is required for methylation of lysine 4 of histone H3 and for transcriptional activation of FLOWERING LOCUS C. Plant Cell **21:** 3257–3269.

**Turck, F., Roudier, F., Farrona, S., Martin-Magniette, M.L., Guillaume, E., Buisine, N., Gagnot, S., Martienssen, R.A., Coupland, G., and Colot, V.** (2007). Arabidopsis TFL2/LHP1 specifically associates with genes marked by trimethylation of histone H3 lysine 27. PLoS Genet. **3:** e86.

**Wysocka, J., Swigut, T., Xiao, H., Milne, T.A., Kwon, S.Y., Landry, J., Kauer, M., Tackett, A.J., Chait, B.T., Badenhorst, P., Wu, C., and Allis, C.D.** (2006). A PHD finger of NURF couples histone H3 lysine 4 trimethylation with chromatin remodelling. Nature **442:** 86–90.

**Yang, W., Jiang, D., Jiang, J., and He, Y.** (2010). A plant-specific histone H3 lysine 4 demethylase represses the floral transition in Arabidopsis. Plant J. **62:** 663–673.

**Yu, C.-W., Liu, X., Luo, M., Chen, C., Lin, X., Tian, G., Lu, Q., Cui, Y., and Wu, K.** (2011). HISTONE DEACETYLASE6 interacts with FLOWERING LOCUS D and regulates flowering in Arabidopsis. Plant Physiol. **156:** 173–184.

**Yuan, L., Gu, R., Xuan, Y., Smith-Valle, E., Loqué, D., Frommer, W.B., and von Wirén, N.** (2013). Allosteric regulation of transport activity by heterotrimerization of Arabidopsis ammonium transporter complexes in vivo. Plant Cell **25:** 974–984.

**Zhang, X., Germann, S., Blus, B.J., Khorasanizadeh, S., Gaudin, V., and Jacobsen, S.E.** (2007). The Arabidopsis LHP1 protein colocalizes with histone H3 Lys27 trimethylation. Nat. Struct. Mol. Biol. **14:** 869–871.