Chromatin-Dependent Repression of the Arabidopsis Floral Integrator Genes Involves Plant Specific PHD-Containing Proteins

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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...
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 (HDAS) 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
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 dwarfishsm 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, Ler, 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, Ler, 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 Ler (L), shl-2 (s), and ebs (e) plants grown both under LD and SD. Error bars show SD.

(F) Rosette leaves of Ler 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.

[See online article for color version of this figure.]
Table 1. Flowering Time (Average ± se) of shl and ebs Double Mutants

<table>
<thead>
<tr>
<th>Mutant</th>
<th>No. of Leaves LD</th>
<th>No. of Leaves SD</th>
</tr>
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<tbody>
<tr>
<td>Ler</td>
<td>11.2 ± 1.0</td>
<td>37.8 ± 0.6</td>
</tr>
<tr>
<td>shl-2</td>
<td>9.3 ± 0.7</td>
<td>26.6 ± 0.7</td>
</tr>
<tr>
<td>ebs</td>
<td>8.4 ± 0.5</td>
<td>22.4 ± 0.8</td>
</tr>
<tr>
<td>ebs shl-2</td>
<td>4.9 ± 0.8</td>
<td>13.1 ± 1.0</td>
</tr>
<tr>
<td>co-2</td>
<td>26.7 ± 0.9</td>
<td>35.4 ± 1.7</td>
</tr>
<tr>
<td>shl-2 co-2</td>
<td>20.4 ± 0.4</td>
<td>n.d.</td>
</tr>
<tr>
<td>gi-3</td>
<td>37.6 ± 1.7</td>
<td>46.0 ± 1.3</td>
</tr>
<tr>
<td>shl-2 gi-3</td>
<td>30.4 ± 1.5</td>
<td>35.5 ± 0.8</td>
</tr>
<tr>
<td>fve-1</td>
<td>23.3 ± 1.4</td>
<td>68.5 ± 1.7</td>
</tr>
<tr>
<td>shl-2 fve-1</td>
<td>17.7 ± 0.4</td>
<td>62.4 ± 1.6</td>
</tr>
<tr>
<td>fca-1</td>
<td>24.4 ± 0.4</td>
<td>60.6 ± 2.0</td>
</tr>
<tr>
<td>shl-2 fca-1</td>
<td>22.2 ± 1.6</td>
<td>60.8 ± 2.0</td>
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<tr>
<td>ga-2</td>
<td>20.4 ± 0.9</td>
<td>61.4 ± 1.9</td>
</tr>
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<td>13.5 ± 0.4</td>
<td>42.3 ± 2.0</td>
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<td>n.f.</td>
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<tr>
<td>shl-2 ga1-3</td>
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<td>n.f.</td>
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<tr>
<td>clf-16</td>
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<td>10.2 ± 0.7</td>
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<td>9.9 ± 0.8</td>
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<tr>
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<td>62.2 ± 0.4</td>
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<tr>
<td>ft-1</td>
<td>18.6 ± 1.6</td>
<td>43.3 ± 1.2</td>
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<td>38.0 ± 0.9</td>
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<tr>
<td>soc1-1</td>
<td>16.6 ± 0.8</td>
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<tr>
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<td>64.6 ± 0.9</td>
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<tr>
<td>Col</td>
<td>16.4 ± 1.1</td>
<td>68.7 ± 1.2</td>
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<td>ebs (Col)</td>
<td>12.0 ± 1.7</td>
<td>22.8 ± 2.5</td>
</tr>
<tr>
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<tr>
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<td>66.6 ± 2.2</td>
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<td>shl-2 (Col)</td>
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<td>56.7 ± 6.4</td>
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<tr>
<td>tt-2</td>
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<tr>
<td>shl-2 tt-2</td>
<td>10.2 ± 0.5</td>
<td>13.2 ± 0.8</td>
</tr>
<tr>
<td>ebs tt-2</td>
<td>8.2 ± 0.5</td>
<td>10.6 ± 0.6</td>
</tr>
<tr>
<td>ebs shl-2 ft</td>
<td>13.0 ± 1.7</td>
<td>31.4 ± 3.4</td>
</tr>
<tr>
<td>ebs shl-2 soc1-1</td>
<td>12.1 ± 1.4</td>
<td>33.4 ± 2.4</td>
</tr>
<tr>
<td>Muc-SHL shl-2</td>
<td>10.3 ± 0.4</td>
<td>33.8 ± 1.4</td>
</tr>
<tr>
<td>Muc-EBS ebs</td>
<td>10.4 ± 0.5</td>
<td>34.7 ± 1.2</td>
</tr>
</tbody>
</table>

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 ACIDS (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 (Píñeiro 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; Píñeiro 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 (Píñeiro 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
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 3.** SHL Is Required to Repress the Expression of the Floral Integrator Gene SOC1.

(A) RT-PCR showing expression of FT in Ler (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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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.

[See online article for color version of this figure.]
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 H3K4me3 (Wysocka et al., 2006) (Figure 8B). 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, H3K36me2, H3K36me1, 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.
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 HDAC6**

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**.

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**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.
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 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
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 JMJD4/14 (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-Anoro 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 H3K4me2/3 forms. 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 PHVA, 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 ATUM4 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 H3K9Ac marks 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 (Ler) 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), ftz-2 (Jarson et al., 1998), atx1-2 (Pien et al., 2008), atx7-1 and atx7-2 (Tamada et al., 2009), elf7-2 (He et al., 2004), elf6-4 (Jeong et al., 2009), and axel-1-5 (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 Ler 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) (EBSpro:GUS and SHLpro: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., 2011). 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 SHLthF (5′-AACAGACGACTTCTCTGTG-3′) and SHLthR (5′-TTCACGCTATGCTTCT-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 (Pitreiro 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 QuickChange 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 from Upstate Biotechnology. PCR was used to amplify four different fragments of the FT gene and three different fragments of the SOC1 gene (Bouvieret 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 pSHL-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 pEBS-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: AI-EBL1 (XP_002868884.1), AI-EBL2 (XP_002872686.1), Th-EBL (BAJ33950.1), Br-EBL1 (EM:DK63381), Br-EBL2 (EM:OY20949), Os-EBL (BAC79935.1), Pt-EBL (XP_002360545.1), Vv-EBL (OJB38025.3), 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 (AT4G52420), MAFI1 (AT1G77080), MAF2 (AT5G65050), MAF3 (AT5G65060), MAF4 (AT5G65070), MAF5 (AT5G65080), SHL (At4g39100), SOC1 (AT2G45660), TSF (AT4G20370), UBC10 (AT4G05320), At-ING1 (At3g24010), At-ING2 (At1g54390), Hs-ING2 (AAQ13674.1), Hs-BPTF (NP_872579.2), and GSE33270 (arrays of sh1 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 sh1 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 sh1-2 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 sh1 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


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Chromatin-Dependent Repression of the Arabidopsis Floral Integrator Genes Involves Plant Specific PHD-Containing Proteins
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