

EARLY BOLTING IN SHORT DAYS Is Related to Chromatin Remodeling Factors and Regulates Flowering in Arabidopsis by Repressing *FT*

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The timing of flowering initiation depends on the balanced expression of a complex network of genes that are regulated by both endogenous and environmental factors. We showed previously that mutations at the *EARLY BOLTING IN SHORT DAYS (EBS)* locus of Arabidopsis result in an acceleration of flowering, especially in noninductive photoperiods (short days), and other phenotypic anomalies. We have identified the *EBS* gene and demonstrate that it encodes a nuclear protein that contains a bromodomain homology domain and a plant homeodomain Zn finger. Both types of motif are thought to mediate protein–protein interactions and occur in transcriptional regulators involved in chromatin remodeling, suggesting that *EBS* is part of a transcriptional repressor complex that modulates chromatin structure and is required to repress the initiation of flowering in short days. Overexpression of *EBS* has phenotypic effects similar to those of recessive *ebs* mutations, suggesting that both might disrupt the formation of protein complexes that contain *EBS*. Analysis of the expression of flowering-time genes in *ebs* mutants and in *EBS*-overexpressing plants indicates that *EBS* participates in the regulation of flowering time by specifically repressing the expression of *FT*, a key gene in the integration of floral promotion pathways in Arabidopsis.

INTRODUCTION

During the embryogenesis and organogenesis of all organisms, groups of genes are specifically activated or silenced to establish the spatial and temporal patterns of expression that allow proper development. In higher plants, the production of organs continues during postembryonic growth, and changes in gene expression initiate progressive phases of development that are characterized by the identity of the lateral primordia produced on the flanks of the shoot apical meristem. Initially, vegetative organs are formed, but after the floral transition, the shoot apical meristem initiates the production of inflorescences and flowers. The time of the initiation of flowering is crucial for the reproductive success of plants; therefore, they have developed mechanisms to integrate both environmental and endogenous cues to regulate flowering time precisely.

In Arabidopsis, the floral transition is promoted by exposure to low temperatures (vernalization) and growth under long-day

(LD) conditions; by contrast, growth under short-day (SD) conditions delays the initiation of flowering. As a result of physiological, genetic, and molecular analyses of Arabidopsis mutants altered in flowering time, the existence of four floral promotion pathways was proposed in Arabidopsis (for reviews, see Mouradov et al., 2002; Simpson and Dean, 2002). The LD and vernalization pathways promote flowering in response to photoperiod and low temperature, respectively, whereas the autonomous and gibberellin (GA) pathways act independently of environmental signals, although the latter is required most strongly under SD conditions (Wilson et al., 1992). Recently, significant progress was made in understanding how these floral promotion pathways are integrated at the molecular level to regulate the time of flowering (Mouradov et al., 2002; Simpson and Dean, 2002). The pathways that regulate vernalization requirement and response converge on *FLOWERING LOCUS C (FLC)*, a gene that encodes a MADS-box transcription factor that represses flowering. The analysis of *FLC* expression revealed that both the vernalization (in vernalization-responsive accessions) and autonomous pathways promote flowering by repressing *FLC* expression (Michaels and Amasino, 1999; Sheldon et al., 1999). *FLC*, in turn, appears to repress the expression of *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1 (SOC1)* (Lee et al., 2000), a gene that encodes another MADS-box protein, and *FT* (Samach et al., 2000), which encodes a protein with similarity to RAF kinase inhibitor proteins (Kardailsky et al., 1999; Kobayashi et al., 1999). However, the expression of *SOC1* and *FT* is not regulated exclusively

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by *FLC*, because both genes also are direct targets of *CONSTANS* (*CO*), a gene that mediates the flowering response to LD conditions (Suárez-López et al., 2001). These observations suggest that *FT* and *SOC1* integrate signals from the LD pathway (mediated by *CO*) and from the autonomous and vernalization pathways, which promote flowering by relieving the repression of *FT* and *SOC1* that is mediated by *FLC* (Michaels and Amasino, 2001; Hepworth et al., 2002).

Besides these pathways that promote flowering, Arabidopsis mutants that exhibit early flowering have revealed the existence of genes involved in the repression of flowering. Some of these repressors act independently of environmental factors and are required to inhibit flowering during the initial stages of development. For example, *EMBRYONIC FLOWER1* (*EMF1*) and *EMF2* act as strong repressors of flowering, as suggested by the extremely early flowering of *emf* mutants (Chen et al., 1997). *EMF2* encodes a homolog of the *Drosophila* Polycomb Group (PcG) protein Su(z)12, whereas *EMF1* encodes a putative transcription factor (Aubert et al., 2001; Yoshida et al., 2001). *CURLY LEAF* (*CLF*), which also encodes a PcG protein, acts by preventing the expression of the floral meristem identity gene *AGAMOUS* (*AG*) during vegetative growth, so that *clf* mutations result in the ectopic expression of *AG* and premature flowering (Goodrich et al., 1997).

We have shown previously that *early bolting in short days* (*ebs*) mutations result in an acceleration of flowering, especially under noninductive SD conditions. In addition to early flowering, *ebs* mutants show a reduction in seed dormancy and an increase in the level of expression of the floral organ identity genes *APETALA3* (*AP3*), *AG*, and *PISTILATA* that can partially rescue the floral phenotype of *leafy-6* (*lfy-6*) mutant plants. These observations suggested that *EBS* participates as a repressor in several developmental processes, such as germination, induction of flowering, and expression of floral homeotic genes. Genetic analyses demonstrated that both the precocious germination and the early flowering of *ebs* mutants require GA biosynthesis and that FT function also is required for the premature flowering of *ebs* mutants (Gómez-Mena et al., 2001).

We have identified the *EBS* gene and shown that it encodes a nuclear protein with a bromo-adjacent homology (BAH) domain and a plant homeodomain (PHD) Zn finger. These motifs are thought to mediate protein-protein interactions and are found in transcriptional regulators and chromatin-remodeling factors in other organisms, suggesting that *EBS* is part of a transcriptional repressor complex involved in the modulation of chromatin structure. Analysis of the expression of key genes in the regulation of flowering time in *ebs* mutants indicates that *EBS* participates in the regulation of floral induction specifically repressing the expression of *FT*, a crucial gene in the integration of signals from different pathways that control flowering time in Arabidopsis.

RESULTS

Identification of the *EBS* Gene

Mutations at the *EBS* locus cause early flowering in Arabidopsis, especially under SD conditions, indicating that its product is involved in the repression of flowering. Previously, we de-

scribed the isolation of two mutant alleles of the *EBS* locus. The mutation present in the ethyl methanesulfonate-induced allele, *ebs-1*, was mapped initially to the bottom arm of chromosome IV, between markers g3883 and RPS2. Preliminary analysis of the transposon-induced allele, *ebs-2*, suggested that it contained a chromosomal rearrangement (Gómez-Mena et al., 2001). Detailed molecular analysis of the mutation present in *ebs-2* confirmed that a deletion is present in this mutant (see Methods). This deletion spans the region between JGB9 and RJS (~150 kb), which is included completely in the g3883-RPS2 interval. Subsequently, to identify the *EBS* locus, the mapping population generated with the *ebs-1* allele (904 mutant plants derived from the F2 population of the cross *ebs-1* × Columbia) was used to locate the gene within the deletion present in *ebs-2*. The gene was mapped to a region of ~25 kb that contained four predicted open reading frames (ORFs), At4g22130 to At4g22160 (Figure 1A). Sequencing of these predicted ORFs in *ebs-1* and Landsberg *erecta* (*Ler*) allowed the identification of the mutation present in this mutant allele. At4g22140 carried a point mutation in *ebs-1* (C to T), which caused an amino acid substitution (Pro-41 to Leu) in the predicted protein (Figure 1B).

To confirm that this ORF was *EBS*, a genomic fragment containing only At4g22140 was introduced into *ebs-1* mutant plants, generating 30 independent transformant lines (TnORF2); in the progeny of these transformant plants, all individuals carrying the T-DNA (1008 of 1292 plants analyzed) showed a wild-type phenotype in LD conditions. Because the flowering-time phenotype of *ebs* mutants is more conspicuous in noninductive photoperiods, we also scored the number of leaves at flowering of several T2 lines under SD conditions. All of these lines displayed a flowering time close or identical to that of wild-type plants (Figures 1C and 1D); therefore, on the basis of these complementation experiments, we concluded that At4g22140 is *EBS*.

EBS Is a Member of a Family of Putative Plant Chromatin-Remodeling Factors

Two ESTs corresponding to *EBS* were identified (F3C6T7A and 135G1T7). Sequencing of the cDNA clones revealed that the *EBS* gene contains five exons and encodes a protein of 224 amino acids (Figure 1B). The deduced amino acid sequence for the *EBS* protein contains a BAH domain, a PHD Zn finger, and a nuclear localization signal. Among plant proteins, *EBS* is highly similar (54% identity) to the barley ES43 protein (Speelman and Salamini, 1995), to a rice predicted protein (64% identity), and to two Arabidopsis proteins: a predicted protein (At4g04260; 70% identity) and SHORT LIFE (57% identity) (Müssig et al., 2000) (Figure 1E). The regions corresponding to the BAH and PHD domains are highly conserved in all five proteins, whereas the N and C termini are more divergent. In addition, ESTs from other plant species, such as cotton, tomato, wheat, and sorghum, also display a high level of identity to the *EBS* cDNA, suggesting the existence of a family of this type of putative transcriptional regulator in higher plants. No proteins with the same modular architecture as *EBS* have been found in the animal genomes sequenced to date.

BAH domains appear to mediate protein-protein interactions in protein complexes involved in the silencing of regions of chro-

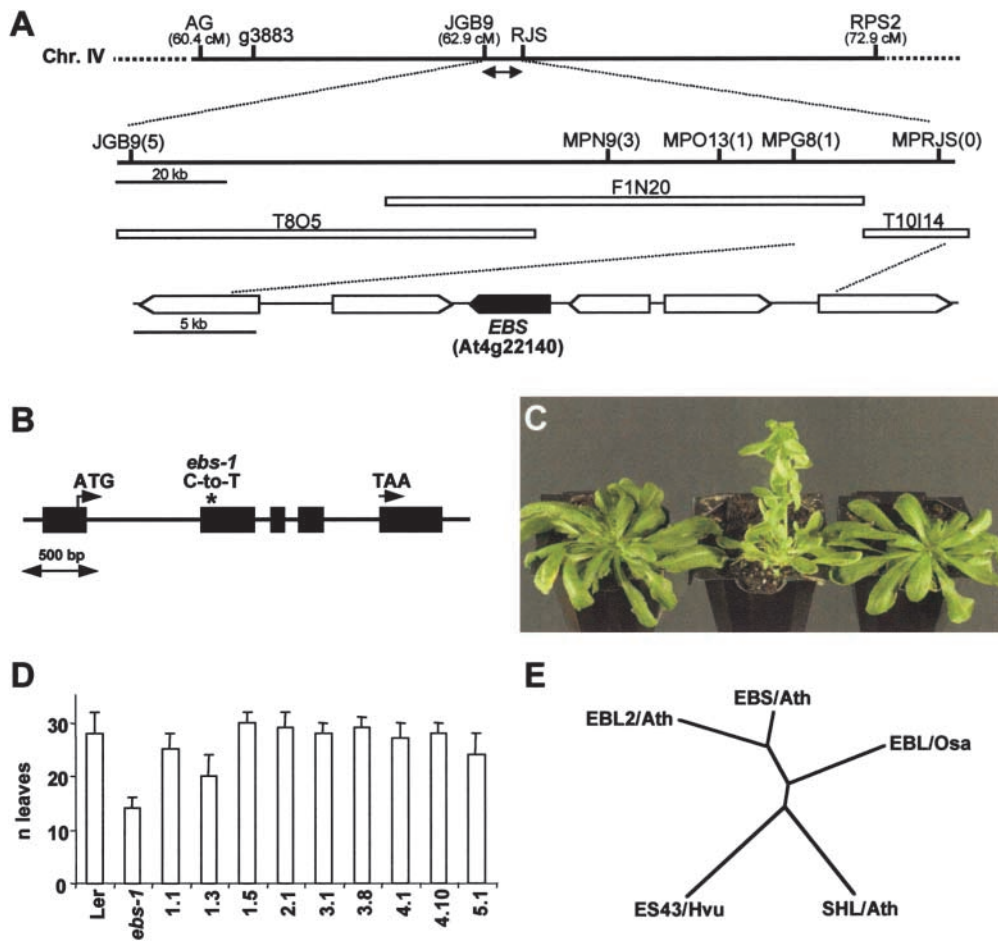


Figure 1. Identification of the *EBS* Gene and Phylogenetic Relationships among EBS-Like Proteins.

(A) Map-based cloning of *EBS*. The genetic interval, molecular markers, and BAC clones in the *EBS* region are shown. The number of recombinant events between molecular markers within the JGB9-RJS region, deleted in *ebs-2* (double-headed arrow), and *EBS* are given in parentheses. cM, centimorgan.

(B) Scheme of the *EBS* gene showing the position of the mutation present in *ebs-1* (asterisk). Exons are shown as black boxes. The positions of the start and stop codons are indicated by arrows.

(C) Complementation of the *ebs* mutant. From left to right: *Ler*, *ebs-1*, and TnORF2 transgenic line 1.1 containing the complementing genomic fragment. Plants were grown for 10 weeks under SD conditions. Both *Ler* and TnORF2 are shown at the time of bolting initiation.

(D) Flowering time, measured as the number of leaves produced before flowering, of *Ler*, *ebs-1*, and several TnORF2 transformant lines containing the complementing genomic fragment. Plants were grown under SD conditions. Error bars indicate standard errors.

(E) Phylogenetic diagram showing the relatedness of full-length *EBS* and *EBS*-like proteins from different species. *EBS*/Ath is At4g22140, *EBL2*/Ath is At4g04260, and *SHL*/Ath is At4g39100 from *Arabidopsis* (Müssig et al., 2000); *ES43*/Hvu (Speulman and Salamini, 1995) and *EBL*/Osa are barley and rice homologs, respectively. CLUSTAL W (www.ebi.ac.uk/clustalw) and TreeView (<http://genetics.stanford.edu/~alok/treeview>) were used to generate the dendrogram.

matin (Callebaut et al., 1999; Goodwin and Nicholas, 2001). The BAH motif was identified first in the chicken POLYBROMO1 (PB1) protein and also is found in the yeast Rsc1 and Rsc2 (REMODELING STRUCTURE OF CHROMATIN 1 and 2) proteins, which are orthologs of the vertebrate PB proteins and are involved in gene transcriptional regulation (Goodwin and Nicholas, 2001). BAH domains also occur in eukaryotic DNA methyltransferases, in ORIGIN OF REPLICATION COMPLEX1 (Orc1) proteins of several species, and in the yeast-silencing factor Sir3 as well as other transcriptional regulators (Callebaut et al., 1999). Methyltransferases modify cytosine residues in the

DNA, and this DNA methylation is associated with transcriptional repression, perhaps by promoting an inactive chromatin conformation (Li et al., 2002). Yeast Orc1 and Sir3 proteins are required to maintain transcriptional silencing by recruiting other silencing factors and compacting regions of chromatin. These observations led to the proposal that the BAH motif plays a role in transcriptional repression through chromatin remodeling (Callebaut et al., 1999).

As with the BAH motif, PHD domains seem to mediate protein-protein interactions and also are present in transcriptional regulators involved in chromatin remodeling (Aasland et al., 1995).

PHD fingers are found in the *Drosophila* NUCLEOSOME REMODELING FACTOR, in the human KAP1 corepressor, which was shown to act by recruiting chromatin-remodeling activities such as histone deacetylase complexes (Schultz et al., 2001), and in Mi-2 proteins, which are involved in transcriptional repression by interacting with the NuRD type of histone deacetylase complex; one Mi-2 protein, corresponding to the protein PICKLE, was isolated in Arabidopsis and shown to be required for the repression of embryonic differentiation genes during postembryonic development (Li et al., 2002). The *Drosophila* Polycomb-like protein also contains a PHD domain and is involved in the transcriptional repression of homeotic gene expression during development by altering chromatin structure. The presence of both BAH and PHD motifs suggests that EBS participates in a protein complex involved in the transcriptional regulation of gene expression through the modulation of chromatin structure.

A nuclear localization signal is predicted in the C terminus of the EBS protein. To determine whether EBS is a nuclear protein, a green fluorescent protein (GFP)–EBS fusion protein under the control of the 35S promoter (35S:GFP-EBS) was expressed transiently in epidermal onion cells. In cells that transiently express the fusion protein (GFP-EBS), GFP activity appeared restricted to the nuclei, whereas in cells that express the 35S:GFP construct, GFP activity was detectable in both the nuclei and the cytoplasm (Figure 2). Therefore, the nuclear localization signal present in the EBS protein can drive GFP to the nucleus in onion epidermal cells. The presence of a functional nuclear localization signal in EBS is consistent with its proposed role in the transcriptional regulation of gene expression.

EBS Is Expressed Ubiquitously

The expression of *EBS* was examined by both RNA gel blot analysis and in situ hybridization. *EBS* transcript was as abun-

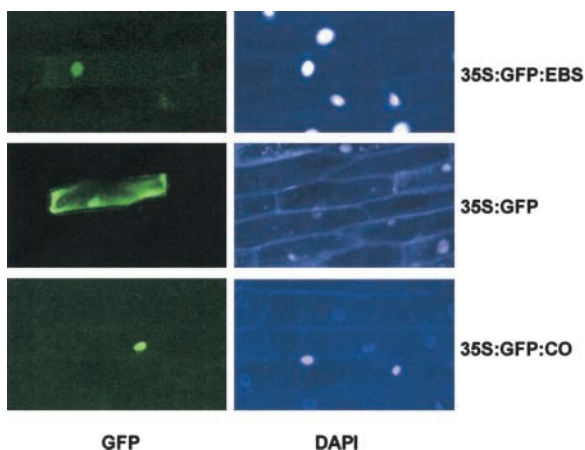


Figure 2. Nuclear Localization of the EBS Protein.

At left, GFP activity in onion epidermal cells bombarded with 35S:GFP-EBS (top), 35S:GFP (middle), and 35S:GFP-CO (bottom) as a positive control for nuclear localization (Robson et al., 2001). At right, the same groups of cells stained with 4',6-diamidino-2-phenylindole (DAPI).

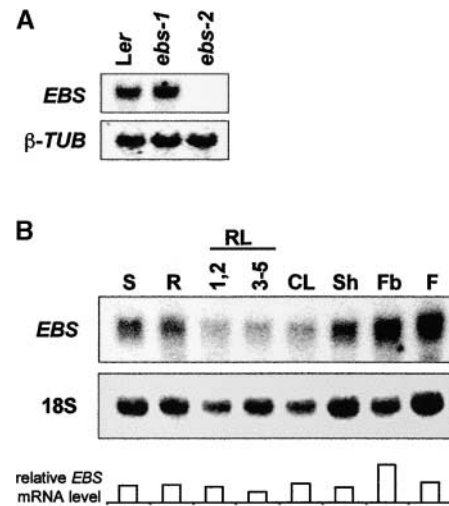


Figure 3. Expression of the *EBS* Gene.

(A) RNA gel blot showing the expression of *EBS* in 7-day-old seedlings of *Ler*, *ebs-1*, and *ebs-2* grown under LD conditions. β -*TUBULIN*.

(B) Expression of *EBS* in different organs: S, 7-day-old seedlings; R, roots; RL, rosette leaves (1,2 and 3-5 indicate leaves 1 and 2 and 3 to 5, respectively); CL, cauline leaves; Sh, shoot; Fb, floral buds; F, flowers. The relative abundance of *EBS* transcript is given for comparison.

dant in the *ebs-1* mutant as in wild-type plants, whereas, as expected, it was absent in the *ebs-2* deletion allele (Figure 3A), which also demonstrates the specificity of the probe. *EBS* transcript was present at similar levels in all adult organs except for floral buds, in which it appeared to be slightly higher (Figure 3B). The level of *EBS* mRNA remained constant during the day, indicating that the transcription of *EBS* is not subject to circadian or light-dark regulation (data not shown). Similarly, *EBS* mRNA abundance was not sensitive to photoperiod and remained at similar levels throughout development under both LD and SD conditions (data not shown).

To investigate in detail possible expression patterns of *EBS* within organs, we performed in situ hybridization experiments (Figure 4). This analysis revealed that *EBS* transcript was present throughout the shoot apical meristem and young primordia during both vegetative and reproductive development. The relative levels of *EBS* mRNA seemed to be higher in young inflorescence and floral primordia, particularly in outer cell layers, as well as in floral organ primordia within the developing flower (Figures 4A to 4C). As expected, no hybridization signal was observed on sections of the *ebs-2* mutant allele (Figure 4D). The expression pattern of *EBS* was consistent with the observed pleiotropic effects of *ebs* mutations and the proposed involvement of the EBS product in the regulation of flowering time and other aspects of plant development (Gómez-Mena et al., 2001).

Overexpression of *EBS* Causes Early Flowering

To investigate the effect of increasing the abundance of the *EBS* transcript, we generated 28 transgenic lines expressing *EBS* under the control of the 35S promoter (35S:*EBS*). Approximately

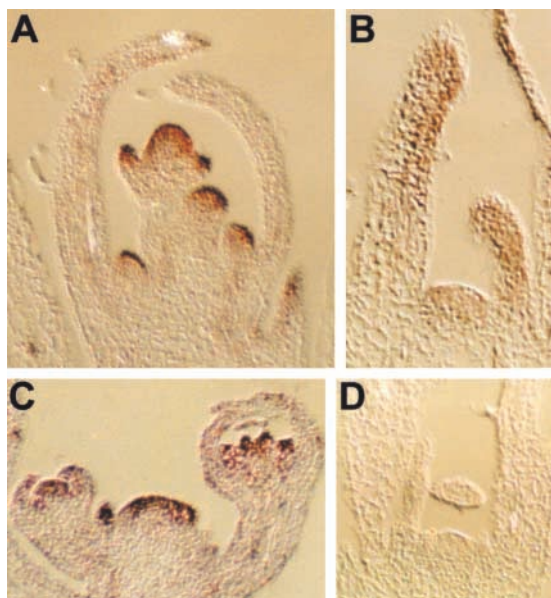


Figure 4. Expression Patterns of *EBS* at Different Developmental Stages.

In situ hybridization was performed on sections prepared from *Ler* seedlings grown for 14 days under LD conditions (A), *Ler* seedlings grown for 14 days under SD conditions (B), inflorescences of *Ler* plants with flower primordia at different developmental stages (C), and *ebs-2* seedlings grown for 14 days under SD conditions (D).

60% of these overexpressor lines showed similar phenotypes to the loss-of-function *ebs* mutants, namely, early flowering, a dwarf phenotype, and reduced fertility (Figure 5A). Further characterization of some of these lines revealed that those containing higher levels of *EBS* transcript were earlier flowering and showed a more extreme dwarf phenotype (Figures 5A to 5C). The high abundance of the *EBS* mRNA in these plants suggested that gene silencing is unlikely to explain the similarity in the phenotype of overexpressor and loss-of-function mutants. As with *ebs* mutants (Gómez-Mena et al., 2001), the effect of *EBS* overexpression was more conspicuous under SD conditions. The presence of the BAH and PHD motifs suggests that *EBS* could be part of a protein complex involved in the modulation of chromatin structure. The accumulation of *EBS* protein in 35S:*EBS* lines could result in an impairment of the formation of the putative *EBS* protein complex, perhaps by sequestering components of the complex into inactive associations, resulting in the deregulation of gene expression similar to that caused by loss-of-function mutations. To further explore this hypothesis, we generated transformant lines overexpressing either the mutant version of the *EBS* gene present in the *ebs-1* mutant (35S:*ebs*) or a truncated protein containing the PHD domain and the nuclear localization signal (35S:M-PHD). 35S:*ebs* plants showed a phenotype similar to that of 35S:*EBS* plants (data not shown), suggesting that the mutant version of the protein retains the ability to impair the proper formation of the *EBS* complex, perhaps by binding to other proteins normally present in the complex. On the other hand, 35S:M-PHD plants also showed an

early-flowering phenotype and dwarfism, although in this case, both phenotypes were less severe than in plants that overexpressed any of the full-length proteins (data not shown). Again, this result suggests that PHD domain overexpression can interfere with the organization or regulation of the complex containing *EBS*.

***EBS* Regulates Flowering Time through the Specific Repression of *FT* Expression**

The sequence of the *EBS* protein suggests that it could be part of a transcriptional repressor complex involved in the modulation of chromatin structure. Because *ebs* mutations accelerate flowering, especially under SD conditions, *EBS* could be involved in the repression of genes that promote the transition to flowering in noninductive photoperiods. With respect to flowering time, the *ft* mutation is epistatic to *ebs*, indicating that *FT* is required

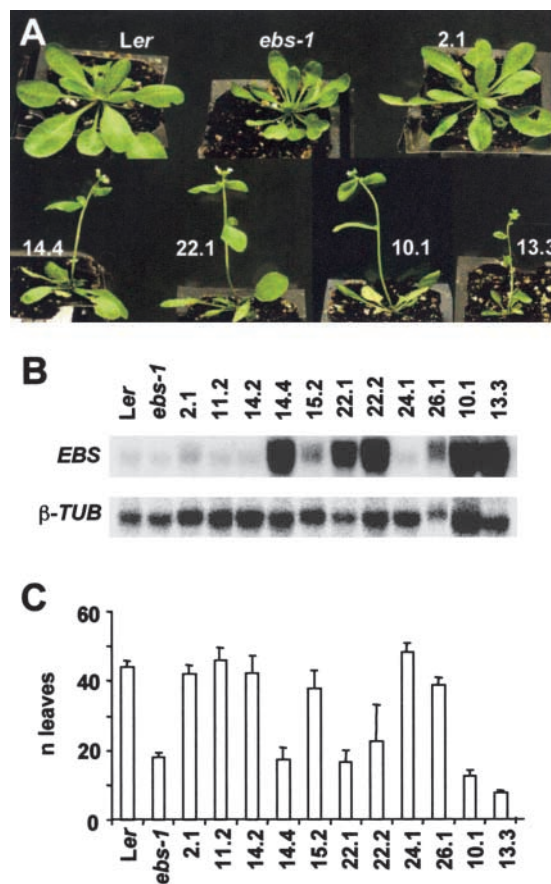


Figure 5. Effect of *EBS* Overexpression on Flowering Time.

(A) Flowering time phenotypes of several transgenic lines overexpressing *EBS* (35S:*EBS*) compared with those of *Ler* and *ebs-1*.

(B) Accumulation of *EBS* transcript in different 35S:*EBS* lines. β -*TUB*, β -*TUBULIN*.

(C) Flowering time of 35S:*EBS* lines expressed as the number of leaves produced before flowering.

All plants were grown under SD conditions.

for the early flowering of *ebs* mutants (Gómez-Mena et al., 2001). *FT* expression is promoted by growth under LD conditions through the activity of *CO*. Under these conditions, *FT* mRNA cycles with a daily pattern that appears to follow that of *CO* expression (Suárez-López et al., 2001). Because the overexpression of *FT* causes early flowering (Kardailsky et al., 1999; Kobayashi et al., 1999), we tested whether the acceleration of flowering observed in *ebs* mutants is caused by the upregulation or premature induction of *FT*. The early flowering of *ebs* mutants is especially clear in SD conditions; therefore, we compared the oscillations in *FT* expression at different times of a 24-h cycle in *Ler* and *ebs-1* plants grown under SD conditions. As shown in Figure 6A, *FT* expression in *ebs* mutants was higher than that in wild-type plants for most of the 24-h period. By contrast, the abundance of *CO* mRNA was not affected by the *ebs* mutation (Figure 6A). This increase in *FT* expression in *ebs* mutants was further confirmed by collecting samples at dawn after different periods of growth in SD conditions. Again, the level of *FT* expression in *ebs* mutants appeared to increase prematurely compared with the level in *Ler*, and no significant differences in *CO* expression were detected between *Ler* and *ebs-1* (Figure 6B). These results suggest that EBS represses *FT* expression under SD conditions independently of *CO*. Consistent with this conclusion, the abundance of *FT* mRNA abundance was similar in *ebs co* double mutant and *ebs* mutant plants, and in both cases, it was higher than that in wild-type plants (Figure 6C).

As mentioned above, the overexpression of *EBS* causes early flowering, and as with *ebs* mutations, this phenotype is more conspicuous under SD conditions. To determine whether this acceleration of flowering observed in 35S:*EBS* plants also required *FT*, we analyzed the effect of *ft* mutations on the flowering time of 35S:*EBS* plants. If the accumulation of the EBS product causes a deregulation of *FT* expression that leads to early flowering in 35S:*EBS* lines, then in the absence of *FT* functional product, the flowering time of these transformants should be delayed. The results obtained show that 35S:*EBS ft* plants grown under SD conditions flowered with a similar number of leaves as *ft* mutants (Figures 6D and 6E), despite the high levels of EBS mRNA present in 35S:*EBS ft* plants (Figure 6F), indicating that the early-flowering phenotype of 35S:*EBS* requires *FT*. Furthermore, the overexpression of *EBS* also causes a premature increase of *FT* expression under SD conditions (Figure 6G), which can explain the observed early-flowering phenotype of 35S:*EBS* plants. Therefore, the early flowering caused by *EBS* overexpression appears to be mediated by the effect of EBS on the regulation of *FT* expression.

In addition to *FT*, two other genes, *SOC1* and *FLC*, integrate signals from different pathways to regulate flowering time in *Arabidopsis* (Mouradov et al., 2002; Simpson and Dean, 2002). To determine whether the EBS protein also is involved in their regulation, we monitored the expression of these genes around the time of *FT* induction in SD-grown *ebs* mutants. As shown in Figure 6H, the *ebs* mutation did not significantly alter the expression of *FLC* in the *Ler* or *fve-1* background, in which the level of *FLC* transcript is higher. Similarly, no significant differences in the expression of *SOC1* were detected between *Ler* and *ebs* plants (Figure 6I), indicating that the EBS product is not involved in the transcriptional regulation of either *FLC* or

SOC1. Phenotypic analysis of the double mutant *ebs-1 soc1* provided further indication that *EBS* and *SOC1* act in independent pathways. The flowering time of the double mutant, measured as the number of leaves produced before flowering, was intermediate between both parental lines (*soc1*, 57.4 ± 1.6 ; *ebs-1*, 12.8 ± 2.3 ; *ebs-1 soc1*, 22.4 ± 2.3), demonstrating the lack of interaction between *EBS* and *SOC1*.

The flowering of *Arabidopsis* under SD conditions is strongly dependent on GA (Wilson et al., 1992). GA biosynthesis also is required for the early flowering of *ebs* mutants, because mutations in GA biosynthetic enzymes eliminate the acceleration of flowering observed in *ebs* mutants (Gómez-Mena et al., 2001). *GA5* encodes a key enzyme in the regulation of the GA biosynthetic pathway (20-oxidase), and changes in the level of GAs feed back on the level of expression of *GA5* (Hedden and Phillips, 2000). Therefore, we examined the effect of the *ebs* mutation on *GA5* expression under SD conditions. As shown in Figure 6I, there was no significant difference in the expression of *GA5* between *Ler* and *ebs*. These results are consistent with previous genetic data (Gómez-Mena et al., 2001), which suggested that *EBS* is not involved in the regulation of GA biosynthesis but regulates developmental processes also regulated by GAs, such as flowering under SD conditions. Together, the results obtained from the analysis of the expression of key genes in the regulation of flowering time suggest that EBS acts to delay flowering under SD conditions by repressing specifically the expression of *FT*, and this repression appears to be independent of the activity of other genes, such as *CO* or *FLC*, that are known to participate in the regulation of *FT* expression.

Mutations in *EBS* can partially rescue the floral phenotype of *lfy* mutants, and both petals and stamens, which normally are absent in *lfy-6* mutants, differentiate in the double mutant *ebs lfy-6* (Gómez-Mena et al., 2001) (Figure 7A). *FT* appears to be required, together with *LFY*, in the determination of floral meristem identity (Ruiz-García et al., 1997) (Figure 7B). Because the effect of EBS on flowering time is mediated specifically by *FT*, it is possible that the effect of *EBS* on the determination of floral organ identity also requires *FT*. To test this hypothesis, we generated the triple mutant *ebs-1 ft lfy-6*. As shown in Figures 7C and 7D, the development of petals and stamens was suppressed completely in the triple mutant plants, suggesting that *FT* is required for the specification of these floral organs in the *ebs lfy-6* background. This observation suggests that *FT* mediates the effect of EBS on different aspects of reproductive development, such as the transition to flowering and the specification of floral organ identity.

DISCUSSION

EBS Belongs to a Family of Plant Proteins with Features Characteristic of Chromatin-Interacting Factors

EBS, a gene involved in the repression of the floral transition and other developmental processes, such as germination and floral organ specification (Gómez-Mena et al., 2001), encodes a nuclear protein that contains a BAH and a PHD domain. Both motifs are thought to be involved in the establishment of protein-protein interactions and are present in several transcrip-

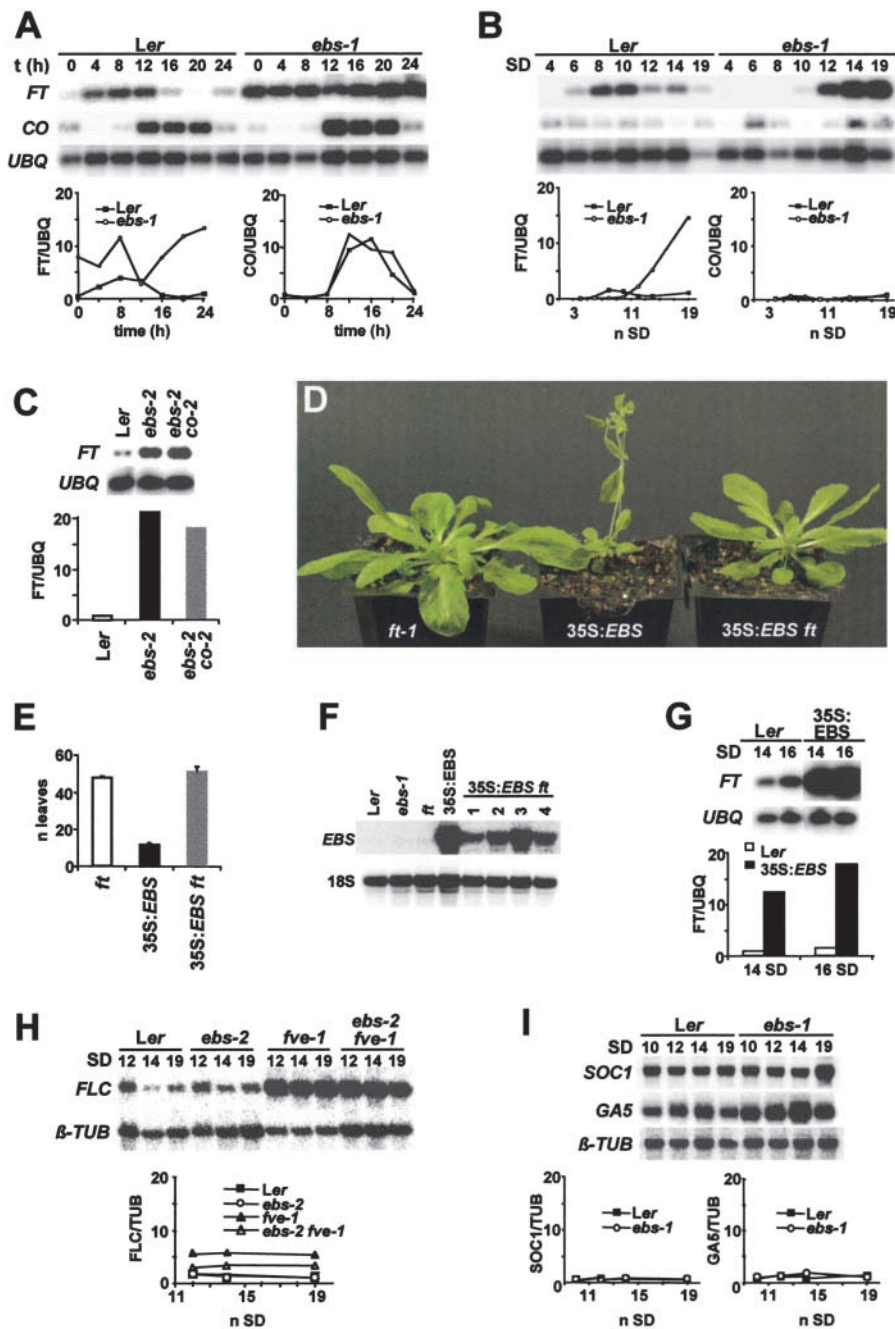


Figure 6. Effect of *ebs* Mutations and *EBS* Overexpression on the Expression of Genes Involved in the Control of Flowering Time in Arabidopsis.

(A) *FT* and *CO* expression in *Ler* and *ebs-1* mutant plants during a 24-h cycle. Seedlings were grown for 14 short days, and RNA was isolated from tissue collected at the times indicated after dawn. *UBQ*, *UBIQUITIN*.

(B) *FT* and *CO* expression in *Ler* and *ebs-1* plants collected at different days after sowing under SD conditions.

(C) *FT* expression in *Ler*, *ebs-2*, and *ebs-2 co-2* plants after 16 days of growth under SD conditions.

(D) Effect of the *ft* mutation on the flowering time of *35S:EBS* plants.

(E) Flowering time of *ft*, *35S:EBS*, and *35S:EBS ft* lines expressed as the number of leaves produced before flowering.

(F) Level of accumulation of *EBS* transcript in different *35S:EBS ft* lines.

(G) *FT* expression in *35S:EBS* lines compared with *Ler* wild type after 14 and 16 days of growth under SD conditions.

(H) *FLC* expression in *ebs-2* in both *Ler* and *fve-1* mutant background at different days after sowing under SD conditions. β -*TUB*, β -*TUBULIN*.

(I) *SOC1* and *GA5* expression in *Ler* and *ebs-1* mutant plants at different days after sowing under SD conditions.

All values are expressed relative to *Ler* at 24 h (A), day 19 (B), (H), and (I), day 16 (C), or day 14 (G) after normalization using *UBIQUITIN* (A) to (C) and (G) or β -*TUBULIN* (H) and (I). Unless indicated otherwise, samples for RNA isolation were collected immediately after dawn. All experiments were repeated at least twice.

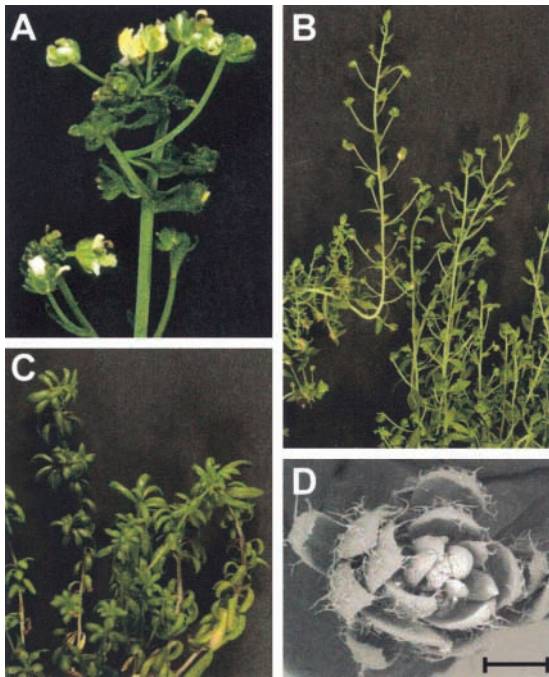


Figure 7. Inflorescence Phenotype of the *ebs-1 ft lfy-6* Triple Mutant.

The partial rescue of the floral phenotype of *lfy-6* observed in the *ebs-1 lfy-6* double mutant is suppressed in the presence of *ft* mutations.

(A) The floral phenotype of the double mutant *ebs-1 lfy-6* displaying the development of petals and stamens (described previously by Gómez-Mena et al. [2001]) is shown for comparison.

(B) Inflorescence of the *lfy-6 ft* double mutant.

(C) Inflorescence of the triple mutant *ebs-1 lfy-6 ft*.

(D) Scanning electron micrograph showing the absence of petals and stamens in the triple mutant. Bar = 500 μm .

tional regulators that act through chromatin remodeling (Aasland et al., 1995; Callebaut et al., 1999). Therefore, the presence of both BAH and PHD motifs suggests that EBS is part of a nuclear protein complex involved in the chromatin-mediated regulation of gene expression.

A small family of plant proteins share the same modular architecture as EBS—that is, they are made up of a BAH and a PHD domain together with a nuclear localization signal. The first protein of this type to be identified was ES43 from barley (Speulman and Salamini, 1995). Two EBS homologs have been identified in the *Arabidopsis* genome, SHL (SHORT LIFE) and EBL2 (EBS-LIKE2). Like EBS, SHL is a nuclear protein, and based on the phenotype of transgenic lines with altered levels of expression, it was proposed to be required for proper plant growth and development (Müssig et al., 2000). Observations made in our laboratory indicate that loss-of-function insertion alleles of the *SHL* gene show no visible effect on flowering time; however, when this mutation was combined with *ebs-1*, the double mutant flowered much earlier than *ebs-1* alone (J. Jarillo, personal communication), suggesting that SHL is partially redundant with EBS.

A number of chromatin-remodeling factors are conserved functionally in animals and plants. However, some of the plant

proteins contain different combinations of motifs than their animal counterparts (Wagner, 2003) or belong to novel classes of chromatin-modifying proteins not found in animals (Bartee et al., 2001). Proteins with the same modular architecture as EBS are present only in plants, although the BAH and PHD domains are closely associated in a number of other animal and yeast proteins that also contain other motifs often involved in epigenetic mechanisms of gene transcriptional regulation (such as SET domains) or DNA binding motifs (such as AT hooks) (Callebaut et al., 1999). The functional significance of this association remains unknown but raises the intriguing question of whether those modules present in BAH/PHD-containing proteins from other organisms are provided by different proteins in plants.

EBS Regulates Flowering by Repressing *FT* Expression

FT plays a crucial role in integrating the information from different pathways that regulate flowering time in *Arabidopsis* (Mouradov et al., 2002; Simpson and Dean, 2002). *FT* promotes the initiation of flowering, and its overexpression is sufficient to accelerate flowering under noninductive SD conditions (Kardailsky, et al., 1999; Kobayashi et al., 1999). We have shown previously that the early-flowering phenotype of *ebs* mutants requires *FT* (Gómez-Mena et al., 2001). Here, we present evidence demonstrating that *FT* expression is upregulated in *ebs* mutants grown under SD conditions and therefore that EBS is required to directly or indirectly repress *FT* expression in noninductive photoperiods. This regulation is specific for *FT*, because the expression of other genes, such as *CO* and *FLC*, that are known to regulate *FT* (Samach et al., 2000; Hepworth et al., 2002) is not affected in *ebs* mutants. This is consistent with previous conclusions from the analysis of double mutants that combine *ebs* with late-flowering mutants, which indicated that *EBS* acts independently of *CO* and the autonomous pathway to regulate flowering time (Gómez-Mena et al., 2001). Our data also indicate that *EBS* and *SOC1* do not interact within the same pathway to regulate the floral transition.

In addition to the requirement for *FT*, the early-flowering phenotype of *ebs* mutants under SD conditions also showed an absolute requirement for GA biosynthesis. However, the levels of expression of *GA5* under SD conditions were not affected by *ebs* mutations, confirming previous genetic analyses that indicated that the phenotype of *ebs* mutants is not likely to be caused by alterations in GA levels (Gómez-Mena et al., 2001). Because EBS is required to repress *FT* expression but not GA biosynthesis under SD conditions, the requirement of both *FT* and GAs for the early flowering of *ebs* mutants suggests that GAs play a regulatory role in *FT* expression or downstream of it to promote the floral transition under SD conditions. GA-deficient mutants are expected to suppress the flowering of *35S:FT* transgenic plants if GAs are required downstream of *FT* but not if GAs regulate *FT* expression. Constitutive overexpression of *FT* can promote flowering under SD conditions in the *ga1-3* mutant background (Blázquez et al., 2002), supporting a model in which GAs are involved in the regulation of *FT* expression under SD conditions. This and our observation that the *ga1* mutation suppresses the early-flowering phenotype of *ebs* under SD conditions are consistent with the proposal that GA is required for

the increased expression of *FT* observed in the *ebs* mutant. This could be tested by directly analyzing *FT* expression in *ebs ga1* double mutants, but to date, poor germination of these double mutants has prevented us from performing this experiment.

Other phenotypic alterations caused by *ebs* mutations during reproductive development, such as the partial recovery of petal and stamen development in the *lfy* background (Gómez-Mena et al., 2001), also appear to be mediated by *FT*, because *ft* mutations completely eliminate the rescue of the *lfy* floral phenotype observed in *ebs lfy* double mutants. This may be attributable to a reduction in *AP1* expression, because *ft* mutations were shown previously to reduce *AP1* mRNA levels when combined with *lfy* (Ruiz-García et al., 1997). In conclusion, both genetic and molecular data support a role for *EBS* in the control of reproductive development by specifically repressing *FT* expression.

The Molecular Mechanism of EBS-Mediated Repression

EBS is required to repress *FT* expression during vegetative growth in noninductive photoperiods; later in development, this repression must be relieved to allow the onset of reproductive development. The *CO* protein is required to promote *FT* expression under LD conditions, and under these conditions, *CO* may overcome the repression mediated by *EBS*. Under SD conditions, however, *FT* activation probably occurs independently of *CO*, and consistent with this possibility, the peak in *FT* expression under SD conditions occurred before that in *CO*. Transcriptional repression of *EBS* is unlikely to be responsible for the relief of repression of *FT*, because *EBS* transcript was present at similar levels in all organs analyzed and in different photoperiod regimes; therefore, other regulatory mechanisms, such as protein modifications or changes in protein-protein interactions, could be invoked to explain this repression release.

The nature of the *EBS* protein suggests that it is part of a protein complex involved in the control of gene expression through changes in chromatin structure. A number of recent studies have shown that proteins involved in chromatin remodeling, such as plant PcG protein homologs, play an essential role in repressing the expression of key regulatory genes required for the onset of different developmental processes in *Arabidopsis*, including the reproductive program (Wagner, 2003). PcG proteins such as CLF, EMF2, and FERTILIZATION-INDEPENDENT ENDOSPERM (FIE) appear to repress the expression of floral homeotic genes during vegetative growth to prevent precocious flowering (Goodrich et al., 1997; Kinoshita et al., 2001; Yoshida et al., 2001); VERNALIZATION2, another PcG protein, is required to maintain the repression of the key floral repressor FLC in response to vernalization (Gendall et al., 2001). Other types of chromatin-remodeling factors also are involved in the regulation of different aspects of reproductive development; the SWI/SNF ATPase homolog SPLAYED is a repressor of the floral transition proposed to regulate LFY activity (Wagner and Meyerowitz, 2002). Mutations in the LIKE HETEROCHROMATIN PROTEIN1 (LHP1) gene, a homolog of *Drosophila* HP1, cause early flowering, which correlates with precocious *CO* expression (Gaudin et al., 2001) and is dependent on FT. As in *ebs*, *FT* is up-regulated in *lhp1/tfl2* mutants, suggesting that LHP1/TFL2 may

be required to repress *FT* during vegetative development (K. Goto, personal communication) (Wagner, 2003).

The mechanism of repression of PcG proteins and other chromatin-remodeling factors remains largely unknown in plants, although, as in animals, they are likely to form large protein complexes responsible for altering the accessibility of DNA to the transcriptional machinery (Simon and Tamkun, 2002). The domains present in *EBS* suggest that it could be part of a multiprotein repressor complex. The observed early-flowering phenotype of 35S:*EBS* plants is consistent with this view. Despite the fact that *EBS* is required for the repression of flowering, 35S:*EBS* plants do not flower late, suggesting that *EBS* is necessary but not sufficient for the repression of floral initiation and that other components probably are required for the repressor activity of *EBS*. Similar results were obtained in the case of the PcG protein EMF2, because both *emf2* mutants and 35S:*EMF2* transgenic plants displayed early flowering (Yoshida et al., 2001). The precocious flowering of 35S:*EBS* plants could be explained by the disruption caused in the formation or function of the *EBS* complex by the hyperaccumulation of one of the members of the protein complex.

Together, our data suggest that *EBS* likely is part of a protein-repressor complex involved in the control of gene expression. The ubiquitous pattern of expression of *EBS* and the pleiotropic phenotype observed in *ebs* mutants suggest that the *EBS* protein complex participates in the regulation of different stages of plant development, including flowering time. Further research will be required to increase our understanding of the composition, organization, and regulation of the *EBS* complex and its role in plant development as well as to identify the target genes of the complex.

METHODS

Plant Material and Growth Conditions

Arabidopsis thaliana mutant lines used in this work are in the *Ler* background and were obtained from the ABRC (Ohio State University, Columbus) and the Nottingham Arabidopsis Centre (UK). The late-flowering mutants *ft-1*, *fve-1*, and *co-2* were described previously (Koornneef et al., 1991). The *ebs-1* and *ebs-2* mutant alleles were isolated as described previously (Gómez-Mena et al., 2001). Seeds were stratified for 4 to 7 days before sowing on soil containing a mixture of substrate and vermiculite (3:1). Plants were grown under controlled environmental conditions (18°C and 80% RH) and illuminated with cool-white fluorescent lights for either 16 h followed by 8 h of darkness (long days) or 8 h followed by 16 h of darkness (short days).

Molecular Characterization of the *ebs-2* Allele and Map-Based Cloning

A chromosomal rearrangement was present in the *Dissociation* (*Ds*)-induced *ebs-2* allele (Gómez-Mena et al., 2001). To determine the nature of the mutation present in *ebs-2*, we generated a cosmid library of this mutant using the Gigapack II XL kit (Stratagene). The isolation of several clones containing *Ds* and the DNA flanking the mobile element on both ends confirmed that a deletion was present in *ebs-2*. This deletion spans the genomic region between RJS (the inverse PCR fragment isolated from the 3' end of *Ds*) and JGB9 (the launching site of *Ds*) (Long et al., 1997).

To finely map the *ews-1* mutation within the interval deleted in *ews-2*, we generated cleaved amplified polymorphic sequence (Bell and Ecker, 1994) and simple sequence length polymorphism (Konieczny and Ausubel, 1993) markers between JGB9 and RJS: MPRJS, a DNA fragment generated by primers RTMP11 (5'-TGGACTGCTTTTCA-CGGTTTTTC-3') and RTMP10 (5'-GGAGCCAGCTCCGTAGG-3'), cleaved with *Bsa*I; MPO13, a fragment generated by primers MP502.9 (5'-CAAAGCAATGAAATAGTTGCC-3') and MP502.10 (5'-AGCGCC-GATCCCACC-3'), cleaved with *Mun*I; MPG8, a fragment generated by primers MP502.11 (5'-GCATTAGAAATCTCTCCTGC-3') and MP502.12 (5'-GCTCCGCGAGTGAC-3'), cleaved with *Av*all; and MPN9, a fragment generated by primers MP502.3 (5'-CAGCATTCTCCATCCCCG-3') and MP502.4 (5'-GTGTTGAAGCTATTGCCTGTC-3').

Plasmid Construction

Standard molecular biology procedures were used for plasmid construction. To make the fusion protein GFP-EBS for nuclear localization experiments, we used vector pAVA393 derived from pAVA321 (von Arnim et al., 1998). To insert EBS cDNA in frame with GFP, we used primers 5'-AAAACCAGATCTTCCATGGCG-3' (which creates a *B*glII site upstream of the EBS ATG), and 5'-AGAACTCTAGATTACCTTTTCTGG-3' (which creates an *X*baI site after the stop codon).

To make the 35S:EBS construct, EBS cDNA was inserted into pJIT60 vector, carrying two copies of the 35S promoter. Subsequently, a *Sac*I-*X*hoI fragment containing the promoter and the EBS cDNA was moved into the binary vector pGREEN(O229) (Hellens et al., 2000). p35S:*ews* was constructed to generate a PCR fragment with primer OXF (5'-AAACCCTGTCGACCATGGC-3'), which creates a *S*alI site upstream of the EBS ATG, and OXR (5'-CCCAAGAATTCGCGATTAC-3'), generating an *E*coRI site after the stop. To construct p35S:M-PHD, a PCR fragment was generated with primers OXPHDF (5'-CCTGGTCGACTTGCTATG-TACTG-3') and OXR. OXPHDF creates a Met codon by replacing the first base of the Val-147 codon (GTG) and introduces a *S*alI site upstream of it. Both PCR fragments were cloned into pJIT60 and moved into pGREEN(O229) as described above for the p35S:EBS construct.

Plant Transformation and Transient Expression Assays

Arabidopsis plants were transformed using the floral-dip method (Clough and Bent, 1998). The *Agrobacterium tumefaciens* strain used was C58C1 pGV3101 pMP90. Transformant plants were selected on soil by spraying seedlings with BASTA. Transient expression assays in onion epidermal cells have been described (Varagona et al., 1992). DNA-coated particles were bombarded onto onion epidermal peels using a Biolistic PDS-1000/He System (Bio-Rad). After bombardment, onion cell layers were incubated overnight to allow for GFP expression. To visualize nuclei, the onion peels were immersed in 0.1% (v/v) 4',6-diamidino-2-phenylindole for 5 min. Subsequently, samples were examined by epifluorescence microscopy (Leica DMR, Wetzlar, Germany).

Expression Analyses

RNA was extracted from seedlings at the times indicated in the figures according to procedures described previously (Logemann et al., 1987). For RNA gel blot analysis, 10 μ g of RNA was loaded onto 1.2% agarose denaturing formaldehyde gels and transferred onto Hybond NX nylon membranes (Amersham). The EBS probe was a 270-bp HindIII-Ddel fragment from EBS cDNA. The β -TUBULIN and the 18S rRNA probes have been described (Onouchi et al., 2000; Samach et al., 2000). The *SOC1* probe was from pAGL20-3 (Samach et al., 2000), and the *FLC* probe was a cDNA fragment lacking the MADS-box domain (Michaels and Amasino, 1999). The *GA5* probe was a PCR fragment (Peng et al.,

1997). For low-abundance mRNAs, such as *FT* and *CO*, we performed reverse transcriptase-mediated PCR according to described procedures (Blázquez and Weigel, 1999). In situ hybridization experiments were performed as described by Gómez-Mena et al. (2001). EBS probes (+ and -) were generated using T7 polymerase and plasmid pCD124 containing the coding region of EBS cDNA cloned into pBS with inverted orientations.

Upon request, all novel materials described in this article will be made available in a timely manner for noncommercial research purposes.

Accession Numbers

The accession numbers for the sequences mentioned are as follows: EST F3C6T7A, N96619; EST 135G1T7, T46081; ES43 barley protein, X77575; and rice EBS-like protein, BAC24958.

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EARLY BOLTING IN SHORT DAYS Is Related to Chromatin Remodeling Factors and Regulates Flowering in Arabidopsis by Repressing *FT*

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