

early bolting in short days: An Arabidopsis Mutation That Causes Early Flowering and Partially Suppresses the Floral Phenotype of *leafy*

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The time of flowering in *Arabidopsis* is controlled by multiple endogenous and environmental signals. Some of these signals promote the onset of flowering, whereas others repress it. We describe here the isolation and characterization of two allelic mutations that cause early flowering and define a new locus, *EARLY BOLTING IN SHORT DAYS (EBS)*. Acceleration of flowering time in the *ebs* mutants is especially conspicuous under short-day photoperiods and results from a reduction of the adult vegetative phase of the plants. In addition to the early flowering phenotype, *ebs* mutants show a reduction in seed dormancy, plant size, and fertility. Double mutant analysis with gibberellin-deficient mutants indicates that both the early-flowering and the precocious-germination phenotypes require gibberellin biosynthesis. Analysis of the genetic interactions among *ebs* and several mutations causing late flowering shows that the *ft* mutant phenotype is epistatic over the early flowering of *ebs* mutants, suggesting that the precocious flowering of *ebs* requires the *FT* gene product. Finally, the *ebs* mutation causes an increase in the level of expression of the floral homeotic genes *APETALA3 (AP3)*, *PISTILLATA (PI)*, and *AGAMOUS (AG)* and partially rescues the mutant floral phenotype of *leafy-6 (lfy-6)* mutants. These results suggest that *EBS* participates as a negative regulator in developmental processes such as germination, flowering induction, and flower organ specification.

INTRODUCTION

The reproductive success of plants depends on initiation of flowering occurring under the most favorable conditions. Plants have developed mechanisms to sense environmental conditions as well as their developmental and nutritional status and to integrate this information to regulate their flowering time. Flowering in *Arabidopsis* is promoted by low nonfreezing temperatures (vernalization) and long photoperiods, and delayed by short photoperiods (Koornneef et al., 1998). Before the induction of flowering, *Arabidopsis* plants grow vegetatively as rosettes that result from the repetitive production of leaves from lateral primordia initiated at the flanks of the apical meristem. Two developmental phases, juvenile and adult, have been distinguished during rosette

growth on the basis of leaf morphology, trichome distribution, and acquisition of meristem competence to flower (Telfer et al., 1997). As a result of floral induction, leaf production is inhibited, lateral primordia develop into flowers, and the main stem elongates to give rise to an inflorescence. Analyses of *Arabidopsis* mutants have allowed the identification of many genes involved in the regulation of flowering time. Physiological, genetic, and molecular analyses of flowering time mutants have shown that flowering is promoted or inhibited by several pathways, some of which are dependent on the environment (reviewed by Koornneef et al., 1998; Levy and Dean, 1998; Piñeiro and Coupland, 1998; Simpson et al., 1999).

Three floral-promotion pathways have been proposed in *Arabidopsis*: the long-day (LD) pathway, the autonomous pathway, and a gibberellin-dependent pathway. Genes in the LD pathway, such as *FHA*, *CONSTANS (CO)*, *GIGANTEA (GI)*, *LATE ELONGATED HYPOCOTYL*, *FWA*, and *FT*, have been identified by mutations that delay flowering specifically under LD (Koornneef et al., 1998). Among them, the differential interactions of *ft* and *fwa* with mutations that affect

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flower meristem identity suggest that *FT* and *FWA* participate in the final steps of the LD pathway (Ruiz-García et al., 1997; Nilsson et al., 1998; Kardailsky et al., 1999; Kobayashi et al., 1999). The autonomous pathway has been defined on the basis of mutants delayed in flowering under both LD and short days (SD), which are responsive to vernalization, and include genes such as *FCA*, *FPA*, *FVE*, *FY*, and *LUMINIDEPENDENS* (Koornneef et al., 1998). Finally, flowering of Arabidopsis under noninductive SD conditions is absolutely dependent on gibberellin biosynthesis. This is demonstrated by the inability to flower under SD of strong gibberellic acid (GA)-deficient mutants such as *ga1-3* (Wilson et al., 1992) and the early SD flowering phenotype of *spindly* (*spy*) mutants, which have a constitutively activated GA signal transduction pathway (Jacobsen and Olszewski, 1993).

The repression of flowering in Arabidopsis has not been analyzed so intensively, and only a few genetic interactions among late- and early-flowering mutants have been described (reviewed by Hicks et al., 1996a; Koornneef et al., 1998; Levy and Dean, 1998). Some of the genes involved in repression of the floral transition act independently of environmental factors. Among them, *HASTY* is required early in development to regulate the competence to flower of the shoot apical meristem (Telfer and Poethig, 1998). Once plants have reached the adult vegetative phase and are competent to flower, several regulatory systems prevent floral initiation until the appropriate developmental stage is reached and inductive environmental conditions are present. Genes such as *TERMINAL FLOWER 1* seem to function to repress reproductive development irrespective of photoperiodic conditions, allowing the plant to reach further vegetative development before flowering (Alvarez et al., 1992). Under noninductive photoperiods (SD), flowering inhibition depends largely on genes involved in light perception (phytochrome B and related phytochromes) and signal transduction (Hicks et al., 1996a). Mutants defective in phytochromes, such as *long hypocotyl 1* (*hy1*), *hy2*, and *hy3* (=phytochromeB) (Koornneef et al., 1980), or in phytochrome signal transduction, such as *phytochrome-signaling early-flowering 1* (*pef1*), *pef2*, and *pef3* (Ahmad and Cashmore, 1996), show reduced sensitivity to photoperiodic inhibition of flowering. Furthermore, *early flowering 3* shows almost complete photoperiod insensitivity (Hicks et al., 1996b). Finally, in vernalization-requiring genotypes, flowering repression under regular growing temperatures is provided by dominant alleles at loci such as *FRIGIDA* (Levy and Dean, 1998) and *FLOWERING LOCUS C* (*FLC*; Michaels and Amasino, 1999; Sheldon et al., 1999). The abundance of the *FLC* transcript seems to be negatively controlled by both vernalization and the activity of the autonomous flowering promotion pathway (Michaels and Amasino, 1999; Sheldon et al., 1999).

Ultimately, the promotion and repression pathways regulate the initiation of flowering by modulating the expression of floral meristem identity genes such as *LEAFY* (*LFY*) and *APETALA1* (*AP1*) (Simon et al., 1996; Kardailsky et al., 1999;

Kobayashi et al., 1999; Blázquez and Weigel, 2000). Mutations in any of these genes produce flowers with shootlike characteristics, supporting their role in the specification of floral fate (Irish and Sussex, 1990; Schultz and Haughn, 1991). Furthermore, *LFY* has been identified as an upstream regulator of *AP1*, *APETALA3* (*AP3*), and *AGAMOUS* (*AG*), which are responsible for the A, B, and C functions, respectively, in the specification of flower organ identity (Parcy et al., 1998; Busch et al., 1999; Wagner et al., 1999).

In a screening for early-flowering mutants under SD, we have identified two allelic mutations that define a new locus of Arabidopsis, *EARLY BOLTING IN SHORT DAYS* (*EBS*), that is involved in flowering repression. These mutants show a regular juvenile phase but bolt early once the adult vegetative phase has been reached. Moreover, *ebs* mutants show additional phenotypic defects, including reduced dormancy of the seed. The construction and characterization of double mutants show that both the early-flowering and germination phenotypes of *ebs* mutants require gibberellin biosynthesis, whereas the early-flowering phenotype also requires *FT* function. Moreover, a partial rescue of petal and stamen development was observed in the *ebs-1 lfy-6* double mutant. We discuss the role of *EBS* in flowering repression and other developmental processes.

RESULTS

Isolation of Mutant Alleles of the *EBS* Locus

Early-flowering mutants in the Landsberg *erecta* (*Ler*) background were selected under SD from one ethyl methane-sulfonate (EMS)-mutagenized M2 population and from progeny families derived from self-fertilizing plants carrying mobilized *Dissociation* (*Ds*) elements (Long et al., 1997). Two mutants, one derived from each population, showed a similar early-flowering phenotype and were studied further. In both mutants, a recessive mutation at a single locus was responsible for an early-bolting phenotype, particularly under SD but also under LD. Phenotypic similarities observed in both mutant plants suggested that the mutations might be allelic, and this was confirmed by a complementation test. The locus was named *EBS* for *EARLY BOLTING IN SHORT DAYS*, and the isolated alleles were named *ebs-1* and *ebs-2* for the EMS- and transposon-induced alleles, respectively (this locus has been designated *SPEEDY* in previous reviews [Levy and Dean, 1998; Simpson et al., 1999]). Both alleles were backcrossed to *Ler* twice before further analyses.

Plants homozygous for the *ebs-1* allele were crossed to Columbia to determine the map position of the mutation relative to molecular markers. *ebs-1* was located on the lower arm of chromosome 4, specifically at 2.29 ± 0.46 centimorgan (cM) south from marker g3883 and 3.08 ± 0.02 cM north from RPS2. No other early-flowering mutation had

been mapped to this location, indicating that *EBS* could be a new locus regulating flowering time. The *ebs-2* line carried a single *Ds* element, and genetic analysis showed that the *Ds* was tightly linked to the mutation (<0.4 cM). However, molecular analyses of the transposon-induced allele indicated that the *Ds* insertion was associated with a chromosomal rearrangement, probably a deletion or an inversion (see Methods). This precluded the direct identification of the *EBS* gene but suggested that *ebs-2* probably is a null allele.

Mutations at the *EBS* Locus Cause Early Flowering and Have Pleiotropic Effects on Shoot, Leaf, and Flower Development

Plants homozygous for each of the mutant alleles were grown under inductive (LD) and noninductive (SD) photoperiods and compared with *Ler* wild-type plants (Figures 1A and 1B). Under LD, mutant plants flowered slightly earlier

than did the wild type (20 versus 25 days) and with fewer leaves (eight versus nine). However, the early-flowering phenotype was much more conspicuous under SD. Under short photoperiods, both mutant alleles flowered after 35 days with 18 to 20 leaves, whereas wild-type plants took more than 50 days to flower and produced more than 30 leaves. Thus, the *ebs* mutations cause premature flowering under both LD and SD, although the mutant alleles retain a photoperiodic response. To determine whether the *ebs* mutations shortened a specific developmental phase or all of the phases, we analyzed the presence of trichomes in the abaxial surface of the leaves, which has been used as a criterion to distinguish juvenile and adult rosette leaves (Telfer et al., 1997). As shown in Figure 1B, the *ebs* mutations seemed to shorten specifically the adult vegetative phase, because fewer leaves of this class were produced in both mutant plants under LD or SD. This effect was particularly conspicuous when plants were grown under SD; under this condition, both mutant alleles produced ~ 10 adult leaves fewer

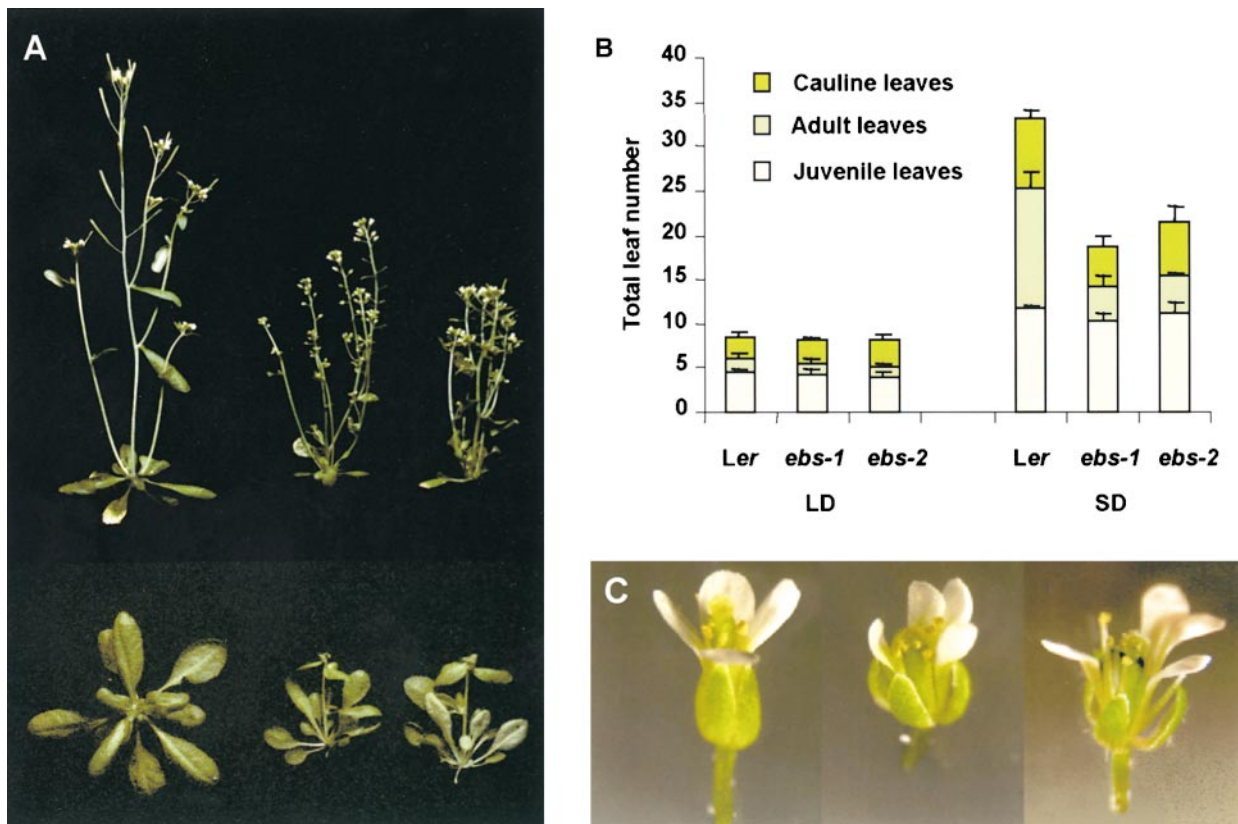


Figure 1. Phenotype of *ebs* Mutants.

(A) *Ler* (left), *ebs-1* (middle), and *ebs-2* (right) 5-week-old plants grown under LD (top) or SD (bottom).

(B) Average number of juvenile, adult, or cauline leaves for *Ler* and *ebs* mutants grown under LD or SD photoperiods. Bars indicate \pm SE.

(C) Flowers from *Ler* (left), *ebs-1* (middle), and *ebs-2* (right).

than did the wild type. The fact that both mutations caused a very similar phenotype indicates that the EMS allele *ebs-1* is as strong as the presumed null allele.

Mutant plants also showed other pleiotropic effects in shoot development. Mutant leaves were generally narrower and smaller than were wild-type leaves. The stems, inflorescences, and flower pedicels were shorter, and the plants showed a semidwarf phenotype (Figure 1A). Mutant flowers were smaller and slightly asymmetric. Flower organs also showed some developmental defects: petals were generally narrower, stamens produced a reduced amount of pollen, and carpels often showed fusion abnormalities (Figure 1C).

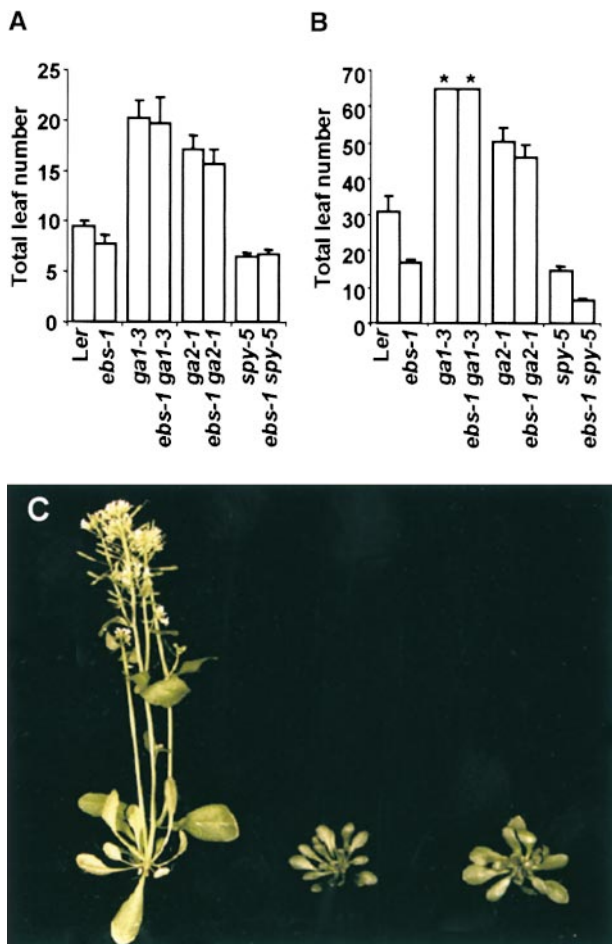


Figure 2. Effect of the *ebs* Mutation on Total Leaf Number of *ga1-3*, *ga2-1*, and *spy-5* Mutants.

(A) Total leaf number under LD.

(B) Total leaf number under SD. Asterisks indicate that plants were unable to flower after 3 months of growth under SD. During this time, they produced 65 leaves.

(C) Phenotype of *ebs-1* (left), *ebs-1 ga1-3* (middle), and *ga1-3* (right) 5-week-old plants grown under LD.

Error bars in (A) and (B) indicate \pm SE.

Occasionally, mutant plants grown under higher-intensity illumination formed terminal flowers (data not shown).

Early Flowering of *ebs* Mutants under SD Requires Gibberellin Biosynthesis

Flowering under short photoperiods in *Arabidopsis* is strongly dependent on gibberellin biosynthesis (Wilson et al., 1992). Because *ebs* mutants are early flowering under SD, we tested whether this phenotype was dependent on gibberellin biosynthesis or signal transduction. Double mutants carrying *ebs-1* and mutations affecting GA biosynthesis (*ga1-3*, *ga2-1*) or response (*spy-5*) were constructed. Both *ga1-3* and *ga2-1* mutations affect early steps in GA biosynthesis (Sun and Kamiya, 1994; Yamaguchi et al., 1998) and delay flowering under LD and SD (Wilson et al., 1992). Furthermore, the *ga1-3* mutation completely prevents flowering under SD (Wilson et al., 1992). The double mutants *ebs-1 ga1-3* and *ebs-1 ga2-1* showed the same flowering time phenotype as the single GA-deficient mutants under both LD and SD (Figures 2A and 2B). In both cases, the phenotype of these double mutants was very similar to the phenotype of the single GA-deficient mutants (Figure 2C). The GA deficiency mutations, therefore, are epistatic over the *ebs* phenotype, indicating that GA biosynthesis is required for early flowering of *ebs* mutants.

The *spy* mutation alters GA signal transduction, producing a constitutive GA response even in the absence of gibberellins (Jacobsen and Olszewski, 1993). The flowering time phenotype of *spy* mutants is similar to that of *ebs*: they flower earlier than does the wild type under both LD and SD, but they are sensitive to photoperiod, showing a delay in flowering time under SD. The double mutant *ebs-1 spy-5* was similar to *spy-5* in flowering time and slightly earlier than *ebs* under LD (Figure 2A). Furthermore, this double mutant was daylength insensitive, flowering with the same number of leaves under both LD and SD (Figures 2A and 2B). These mutations, therefore, have an additive effect on flowering under SD.

ebs Mutant Seed Shows Reduced Dormancy

The phenotype of the *ebs-1 ga1-3* double mutant demonstrated that GA biosynthesis is required for the early-flowering phenotype of *ebs* mutants, suggesting that *ebs* might affect flowering time by enhancing GA biosynthesis or response. GA also is required for germination (Koornneef and van der Veen, 1980); therefore, we tested whether *ebs* had an effect on germination. Seeds of *Ler*, *ebs-1*, and *ebs-2* were stored for different periods of time after harvest, and their ability to germinate was scored 14 days after sowing. In contrast to the wild type, *ebs-1* and *ebs-2* mutant seeds showed almost no dormancy response, and a much higher percentage of mutant than wild-type seeds germinated when sown immediately after harvest (Figure 3A). These freshly

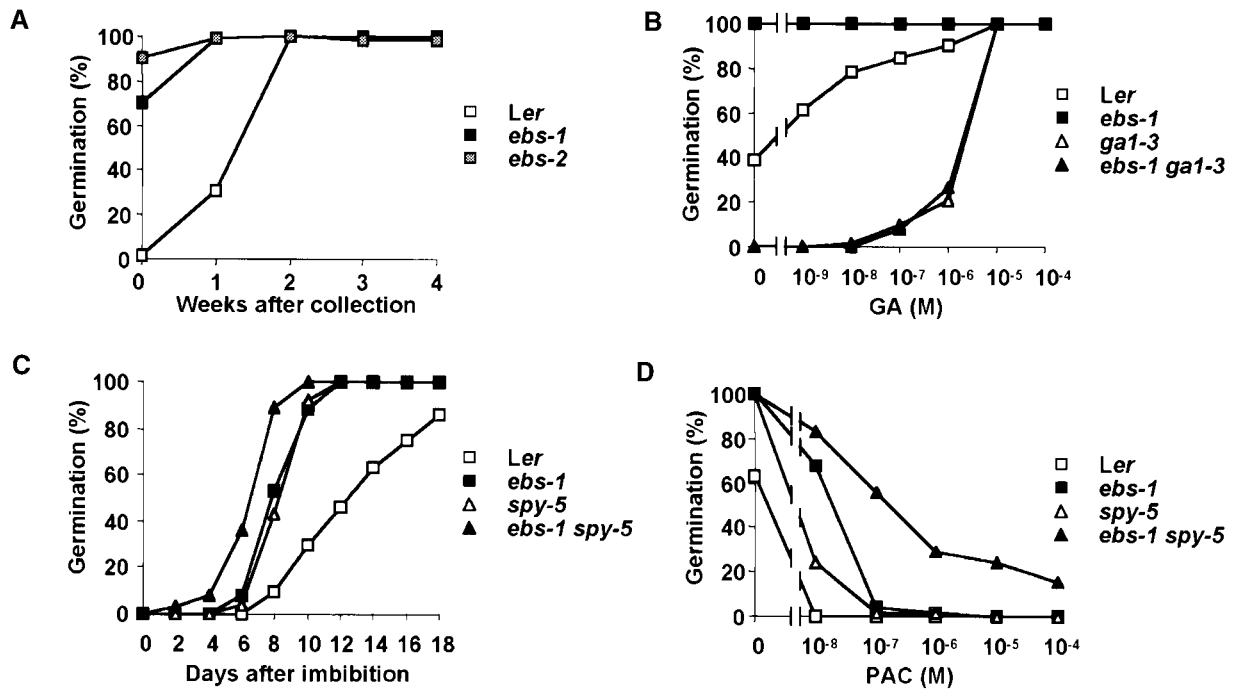


Figure 3. Reduction in the Dormancy of *ebs* Mutant Seeds and Its Effect on the Germination of Double Mutants with *ga1-3* and *spy-5*.

Germination was scored after 2 weeks of incubation except in (C), where germination was scored as indicated.

(A) Germination of *Ler*, *ebs-1*, and *ebs-2* seeds after different weeks of storage.

(B) Germination rates of *Ler*, *ebs-1*, *ga1-3*, and *ebs-1 ga1-3* freshly harvested seeds in the presence of different GA concentrations.

(C) Time course of germination of *Ler*, *ebs-1*, *spy5*, and *ebs-1 spy-5* freshly harvested seeds.

(D) Germination rates of *Ler*, *ebs-1*, *spy5*, and *ebs-1 spy-5* freshly harvested seeds in the presence of different concentrations of PAC.

harvested mutant seeds also were able to germinate in complete darkness, a condition that prevents germination of fresh wild-type seeds (data not shown).

Mutations affecting GA biosynthesis, such as *ga1-3*, can completely abolish the ability of seeds to germinate unless GA is added exogenously (Koornneef and van der Veen, 1980). Because the early flowering phenotype of *ebs* mutants requires GA, we tested whether the reduced seed dormancy of these mutants also requires GA. Freshly harvested seeds of *Ler* wild type, *ebs-1*, *ga1-3*, and the double mutant *ebs-1 ga1-3* were sown with increasing concentrations of GA, and germination was scored after 14 days. As shown in Figure 3B, seeds of the double mutant *ebs-1 ga1-3* germinated only in the presence of concentrations of GA very similar to those required for the germination of *ga1-3*. Therefore, as observed for the early-flowering phenotype, the premature germination of *ebs* mutants also requires GA biosynthesis.

Phenotypic analysis of the double mutant *ebs-1 spy-5* showed that both mutations act additively in the control of flowering time in SD. Because both the *ebs* and *spy* mutations show reduced dormancy and increased resistance to paclobutrazol (PAC), an inhibitor of GA biosynthesis, we

tested the effect of combining both mutations on the germination of freshly harvested seeds and on the resistance of these seeds to PAC. Seeds of the double mutant *ebs-1 spy-5* showed a further reduction in dormancy and started germinating earlier than any of the single mutants (Figure 3C). Furthermore, the double mutant also showed increased resistance to PAC compared with either single mutant (Figure 3D), suggesting that the *ebs* and *spy* mutations also have additive effects in reducing dormancy.

FT Is Required for the Early Flowering of *ebs* Mutants

To test the interaction between *EBS* and the pathways proposed to promote flowering in Arabidopsis, we made double mutants carrying *ebs* and mutations in representative genes for each of the pathways that promote flowering. Double mutants in which *ebs* was combined with mutations that affect the autonomous pathway (*fve-2* or *fpa-1*) showed an intermediate phenotype measured as the total number of leaves produced before flowering when grown under LD or SD (Figures 4A and 4B). The time of bolting was also intermediate in these double mutants (data not shown). All of the

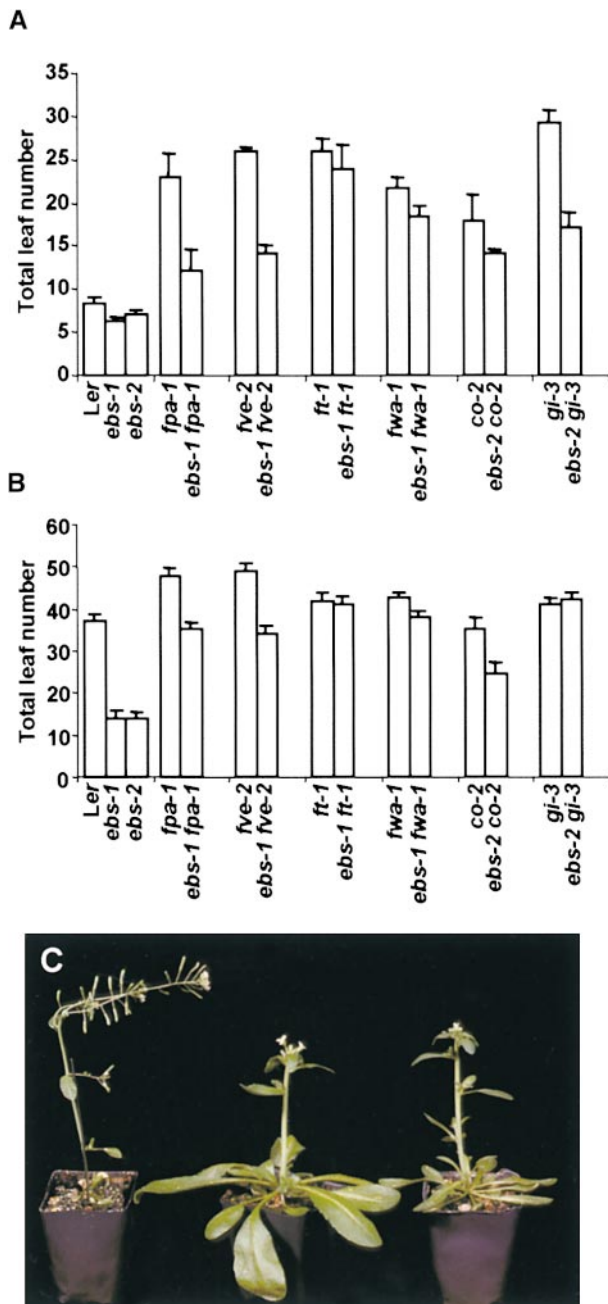


Figure 4. Effect of the *ebs* Mutation on Total Leaf Number of Late-Flowering Mutants Affecting the LD Pathway and the Autonomous Pathway.

(A) Total leaf number under LD.

(B) Total leaf number under SD.

(C) Phenotype of *ebs-1* (left), *ft-1* (middle), and *ebs-1 ft-1* (right) 5-week-old plants grown under LD.

Error bars in (A) and (B) indicate \pm SE.

pleiotropic effects caused by the *ebs* mutations on the morphology of the plant, such as the reduced elongation of inflorescence stems and the smaller size of leaves and flowers, also were present in these double mutants (data not shown). These results demonstrate a lack of interaction between these mutations and suggest that *EBS* acts in a pathway that is parallel to the pathway represented by the genes *FVE* and *FPA*.

Double mutants carrying *ebs* and different mutations affecting the LD pathway showed flowering time phenotypes that differed depending on the late-flowering mutant used. The double mutants *ebs-2 co-2* and *ebs-2 gi-3* showed an intermediate flowering time under LD (Figure 4A). Under SD, the double mutant *ebs-2 co-2* also exhibited an intermediate phenotype in terms of both total number of leaves (Figure 4B) and bolting time (data not shown). However, the double mutant *ebs-2 gi-3* bolted 2 weeks earlier than did the late flowering parent, although with a number of rosette and cauline leaves similar to that of *gi-3* (*ebs-2 gi-3*, 30.0 ± 1.2 rosette and 12.4 ± 0.7 cauline leaves; *gi-3*, 31.2 ± 1.7 rosette and 10.0 ± 0.5 cauline leaves; Figure 4B). These results indicate that under SD, the rate of leaf production is higher in *ebs-2 gi-3* than in the *gi-3* parent. Moreover, the double mutant *ebs-2 gi-3* formed a large number of cofillorescences not subtended by leaves (19.7 on average) before the development of the first flower, suggesting a delay in the establishment of floral meristem identity. These results, together with a number of other pleiotropic effects displayed by *gi* mutants, such as elongated hypocotyls and higher starch levels (Araki and Komeda, 1993; Eimert et al., 1995), suggest a unique role for *GI* in the LD promotion pathway. In conclusion, the phenotypes of the double mutants with *co* and *gi* indicate a lack of interaction between *EBS* and *CO* and *GI* loci in the regulation of bolting time. However, the phenotypic differences observed between these double mutants support the existence of different roles for *CO* and *GI* within the LD pathway and an SD requirement of the *GI* function for the initiation of flowers in *ebs* mutants.

Within the LD pathway, *ft* and *fwa* behave differently from the other mutants when combined with the flower meristem identity mutation *lfy*, and they may participate in later steps of this pathway (Ruiz-García et al., 1997; Nilsson et al., 1998). Consequently, we analyzed the possible interaction between *ebs* and these two mutations. The *ebs-1 ft-1* and *ebs-1 fwa-1* double mutants showed a different phenotype than did the other combinations of *ebs* with late-flowering mutants. When grown under both LD and SD, the *ebs-1 ft-1* and *ebs-1 fwa-1* double mutants showed a late-flowering phenotype similar to that of each single late parent, measured either as number of leaves or time of bolting, indicating that the *ft* and *fwa* mutant phenotypes are epistatic to *ebs* with respect to flowering time (Figures 4A and 4B). These double mutants also showed the pleiotropic phenotypes caused by the *ebs* mutation (Figure 4C). These results indicate that the early-flowering phenotype of *ebs* mutants requires the *FT* function. Considering the dominant nature of

the *fwa-1* mutation, this result could be interpreted as *FWA* having a negative effect on the induction of flowering downstream of the *EBS* function. Exactly the same results were obtained when these double mutants were generated with the *ebs-2* allele (data not shown).

***ebs* Partially Corrects the *lfy* Phenotype**

FT seems to be required together with *LFY* in the determination of flower meristem identity (Ruiz-García et al., 1997; Nilsson et al., 1998). Because *FT* was required for the early flowering of *ebs* mutants, we tested whether *LFY* also was required for the early-flowering phenotype of *ebs*. A double mutant carrying *ebs-1* and the strong mutant allele *lfy-6* was constructed. The *lfy-6* mutant is slightly delayed in flowering time under LD with respect to wild-type plants; however, the double mutant *ebs-1 lfy-6* flowered at the same time and with a similar number of rosette leaves as did the *ebs* mutants (*ebs-1*, 5.7; *lfy-6*, 8.7; *ebs-1 lfy-6*, 5.5, on average) and showed the characteristic *ebs* phenotype in rosettes and inflorescences. The absence of the *LFY* function prevents the development of petals and stamens in the flowerlike structures produced by *lfy-6* (Weigel et al., 1992).

Interestingly, *ebs-1 lfy-6* formed a variable number of petaloid and staminoid organs with variable degrees of differentiation as well as mosaic organs intermediate between petals and stamens (Figure 5). This partial rescue of the floral organ phenotype of *lfy-6* mutants was observed in all *ebs-1 lfy-6* double mutant plants, under both LD and SD. To quantify the phenomenon, 102 flowers from 10 LD-grown *ebs-1 lfy-6* double mutant plants were tested for the presence of floral organs normally absent in *lfy-6* flowerlike structures. Petals or petaloid organs were present in 90% of the *ebs-1 lfy-6* flowerlike structures analyzed. These structures contained an average of 2.8 ± 1.8 petals or petaloid organs. The occurrence of stamens or staminoid organs was less frequent: up to 25% of the flowerlike structures analyzed showed an average of 1.2 ± 0.6 stamens or staminoid organs. However, pollen was observed only occasionally in the flowers of the *ebs-1 lfy-6* double mutant. These transformations were observed only rarely in the *lfy-6* progenitor, in which less than 1% of flowers contained petaloid structures and no staminoid structures were observed in 10 plants tested (Figure 5A). In conclusion, the early-flowering *ebs* phenotype does not require *LFY* function, and *ebs* mutations are able to partially rescue the specification of floral organ identity in the second and third whorls of *lfy-6* flowers.

The overexpression of *AP3* under the control of the 35S cauliflower mosaic virus (CaMV) promoter can partially rescue the development of petals and stamens in flowers of the *lfy-6* mutant (Jack et al., 1994). Because *ebs-1 lfy-6* mutants are able to develop second and third whorl organs in older flowers, *AP3* and/or *PI* expression could be enhanced by *ebs* mutations. To test this hypothesis, we performed RNA gel blot experiments using total RNA from reproductive api-

ces and leaves of wild-type *Ler* plants and *ebs* mutants. As shown in Figure 6A, *AP3* and *PI* mRNA were found at higher levels in the apices of *ebs* mutants than in those of wild-type plants, whereas neither transcript was detected in leaves. The level of *AG* mRNA, which also is involved in the specification of third whorl organs, also was higher in apices of *ebs* mutants than in apices of *Ler* wild-type plants (Figure 6A). Furthermore, the expression of *AP3* and *PI* was enhanced in the double mutant *ebs-1 lfy-6* compared with *lfy-6* (Figure 6B). In contrast, the level of *AG* mRNA was not altered significantly in the *ebs-1 lfy-6* double mutant compared with the *lfy-6* single mutant, indicating a differential effect of the *ebs* mutation on the regulation of *AP3/PI* and *AG* in the *lfy-6* mutant background. The observed increase in the expression of *AP3* and *PI* caused by the *ebs* mutation seems to be enough to promote the development of petals and stamens in the absence of *LFY* product, suggesting a role for *EBS* in the regulation of *AP3* and *PI* expression.



Figure 5. Partial Rescue of the *lfy-6* Floral Phenotype by the *ebs* Mutation.

- (A) Inflorescence of a *lfy-6* mutant plant grown under LD.
 (B) Inflorescence of an *ebs-1 lfy-6* double mutant plant grown under LD.
 (C) Inflorescence of an *ebs-1 lfy-6* double mutant plant grown under SD.
 (D) Flower from an SD-grown *ebs-1 lfy-6* plant showing the partial rescue of petals and stamens.

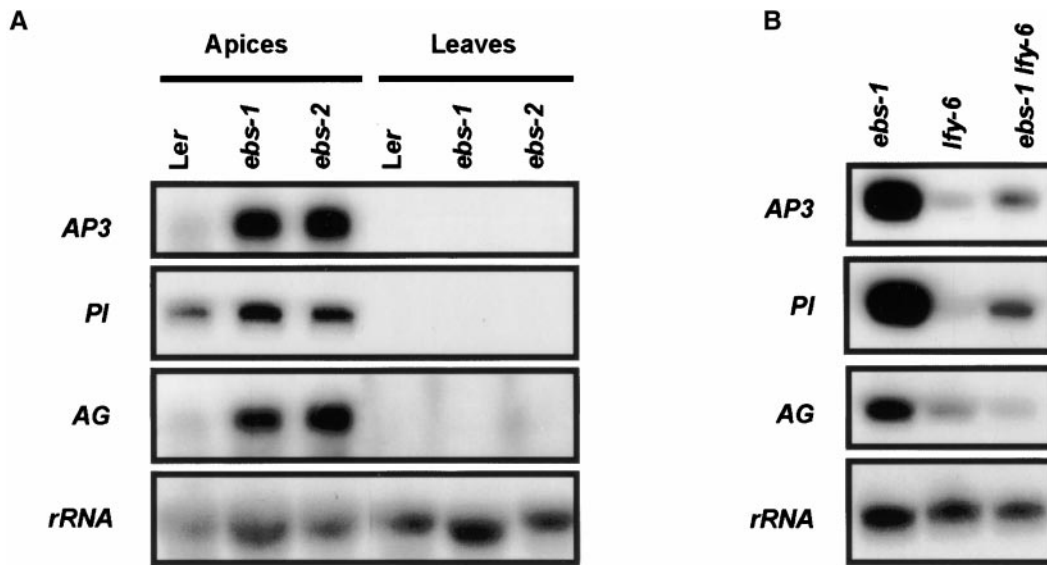


Figure 6. Expression of *AP3*, *PI*, and *AG* in *ebs-1* and the *ebs-1 lfy-6* Double Mutant.

Total RNA was isolated from reproductive apices or leaves of LD-grown plants, and 20 μ g was loaded in each lane. Blots were probed with radiolabeled *AP3*, *PI*, and *AG* cDNAs and then reprobbed with rDNA as a loading control.

(A) Steady state levels of *AP3*, *PI*, and *AG* mRNA in apices and leaves of *Ler* and *ebs* mutants.

(B) Steady state levels of *AP3*, *PI*, and *AG* mRNA in apices of *ebs-1*, *lfy-6*, and *ebs-1 lfy-6* plants.

The lack of homeotic transformations in the organs of *ebs* flowers suggests that *AP3* and *AG* messages are not expressed ectopically during flower development. To test this possibility, we performed RNA in situ hybridization with *AP3* and *AG* probes on flower meristems and flowers of *Ler* and *ebs-1* plants. As shown in Figure 7, the temporal and spatial patterns of *AP3* and *AG* expression were very similar in wild-type and *ebs* mutant plants. *AP3* mRNA was first detected in early stage 3 flowers, localized in whorls 2 and 3, in both *Ler* and *ebs* (Figures 7A and 7B). Later in flower development (stages 6 and 9), *AP3* transcript was detected on developing stamens and petals, with a similar pattern in *Ler* (Figures 7E and 7I) and *ebs* (Figures 7F and 7J). A greater hybridization signal was always detected on floral meristems of the *ebs* mutant, consistent with the results obtained by RNA gel blot hybridization. The pattern of expression of *AG* also was the same in *Ler* and *ebs*: during stages 3 and 5, *AG* mRNA was restricted to the central region of the floral meristem (*Ler*, Figures 7C and 7G; *ebs*, Figures 7D and 7H). Later in development, during stage 9, *AG* mRNA was located in developing carpels and stamens in both *Ler* (Figure 7K) and *ebs* (Figure 7L).

To determine whether the partial rescue of petal and stamen development in the *ebs-1 lfy-6* double mutant could be caused by the observed increase in *AP3* expression, we performed RNA in situ hybridizations on inflorescences of *lfy-6* and *ebs-1 lfy-6* plants. As expected, *AP3* expression was reduced markedly in *lfy-6* plants compared with wild-

type plants, although with low frequency a weak hybridization signal could be observed in the axils of the bracts and on the basal region of the flowerlike structures of *lfy-6* mutants (Figure 7M). When the inflorescences of the *ebs-1 lfy-6* double mutant were analyzed, the localization of the *AP3* transcript was similar to that observed in the *lfy-6* single mutant, although the hybridization signal was higher and detected more frequently in the axils of the bracts (Figure 7N). The pattern of expression of *AG* was similar in the inflorescences of *lfy-6* and *ebs-1 lfy-6* (Figures 7O and 7P). In conclusion, the observed increase in *AP3* and *AG* expression in the *ebs* mutant background is not the result of ectopic expression of these homeotic genes, suggesting a role for *EBS* in their regulation in those cells in which they are normally expressed.

DISCUSSION

EBS, a New Locus Required for the Repression of Flowering in Arabidopsis

The early-flowering phenotype of *ebs* mutants and the map position of *EBS* indicate that it is a new locus involved in the regulation of flowering time. Two aspects of the mutant phenotypes strongly support a role for *EBS* as a flowering repressor under noninductive photoperiods. First, the re-

duction in flowering time caused by the *ebs* mutations is not the result of a general acceleration of the development of the plant; rather, it results specifically from a reduction in the duration of the adult vegetative phase (Figure 1). During this phase, the apical meristem is already competent to initiate reproductive development once the environmental conditions are adequate (Telfer et al., 1997). This observation places the role of the *EBS* locus in the negative regulation of flowering time once the shoot apical meristem is competent to flower. Second, the epistatic relationships revealed by the late-flowering phenotype of the *ebs ft* double mutant suggest a specific role for *EBS* in the repression of *FT*, a gene required (along with *LFY*) to promote the initiation of flowering in Arabidopsis (Ruiz-García et al., 1997).

FT has been shown to induce flowering under SD when it is expressed constitutively from a CaMV 35S promoter (Kardailsky et al., 1999; Kobayashi et al., 1999). The pleiotropic phenotype shown by *ebs* mutants, which include reduced seed dormancy, reduced plant size, altered flower morphology, and reduced fertility, suggests the involvement of *EBS* in other developmental processes in addition to the repression of flowering. However, we cannot completely exclude the possibility that *EBS* might have a very early role in development that could affect later developmental stages. Most of the early-flowering mutants already characterized in Arabidopsis also show pleiotropic defects (Koornneef et al., 1998). On the one hand, early-flowering mutants such as *elongated* (Halliday et al., 1996), *early flowering 1* (Scott et al., 1999), and *early flowering in short days* (Soppe et al., 1999) are affected in either dormancy or plant size. On the other hand, mutants defective in light or gibberellin response also can show an early-flowering phenotype. This is the case with mutants defective in phytochrome biosynthesis, such as *hy1* (Parks and Quail, 1991) and *phyB* (Somers et al., 1991), or in signal transduction, such as *pef* (Ahmad and Cashmore, 1996), *constitutively photomorphogenic 1* (Deng and Quail, 1992), and *de-etiolated 1* (Pepper et al., 1994), which show early-flowering phenotypes together with alterations in organ and plant size and chlorophyll content. Some of the mutants altered in GA-mediated signal transduction, such as *spy* (Jacobsen and Olszewski, 1993), also show early flowering together with reduced dormancy and increased elongation of the plant.

GA-deficient mutants are impaired in germination and flowering under noninductive photoperiods (Koornneef and van der Veen, 1980; Wilson et al., 1992). The reduced dormancy of *ebs* mutants and their early bolting under SD would suggest that *EBS* could act as a repressor of GA biosynthesis or could participate as a negative regulator in GA-mediated signal transduction. However, several aspects of the mutant and double mutant phenotypes are not consistent with these hypotheses. The gibberellin requirement shown by *ebs* mutants for both early flowering and premature germination is not in agreement with *EBS* negatively regulating GA-mediated signal transduction, because a GA-independent phenotype like that observed in *spy* mutants

(Jacobsen and Olszewski, 1993) would have been expected. On the other hand, the semidwarf phenotype shown by *ebs* mutants is not consistent with the phenotype of Arabidopsis transgenic plants that overproduce GA (Hedden and Phillips, 2000) or the phenocopies obtained in Arabidopsis by exogenous GA treatments (Chandler and Dean, 1994). Thus, the phenotypes of *ebs* and the double mutants *ebs-1 ga1-3* and *ebs-1 ga2-1* do not support a role for *EBS* in the regulation of GA biosynthesis or signaling pathways. However, *EBS* could participate as a repressor in at least two developmental processes (germination and flowering under SD) that also are regulated by gibberellins.

EBS* Mediates the Repression of Flowering through *FT

Analyses of double mutants carrying *ebs* and late-flowering mutations indicate a specific interaction of *EBS* with *FT* and *FWA* genes, which have been shown to act downstream of other genes participating in the photoperiod-dependent pathway (Kardailsky et al., 1999; Kobayashi et al., 1999). These results suggest that *EBS* could act as a direct or indirect repressor of *FT* expression under noninductive photoperiods. This is consistent with recent reports demonstrating that the overexpression of *FT* is enough to promote early flowering under SD (Kardailsky et al., 1999; Kobayashi et al., 1999). Furthermore, considering the dominant nature of the *fwa* mutations, the late-flowering phenotype displayed by the double mutant *ebs-1 fwa-1* indicates that *FWA* could act as a repressor downstream of *EBS*. This hypothesis is in agreement with the late-flowering phenotype of *fwa 35S::FT* plants, which suggests that *FWA* represses flowering downstream of *FT* (Kardailsky et al., 1999; Kobayashi et al., 1999). Two reports have suggested a transcriptional regulation of *FT* by *CO*. First, there is a direct correlation between *CO* expression and the *FT* transcript level (Kobayashi et al., 1999; Samach et al., 2000). Second, *ft* mutations partially suppress the early-flowering phenotype of transgenic Arabidopsis plants expressing a 35S::*CO* construct (Onouchi et al., 2000). However, the intermediate-flowering time phenotype of *ebs-2 co-2* suggests that *EBS* and *CO* might regulate flowering through *FT* independently.

The gibberellin requirement for flowering under noninductive conditions in Arabidopsis is clearly established (Wilson et al., 1992), and these hormones have been proposed to function in regulating *LFY* expression (Blázquez et al., 1998; Blázquez and Weigel, 2000). We have shown here that GA biosynthesis is absolutely required for the early-flowering phenotype of *ebs* mutants under SD and that this early-flowering phenotype does not require the *LFY* function. These observations, together with the *FT* requirement for the early flowering of *ebs*, suggest that GA also could have a regulatory role on either *FT* or *CO*, or downstream of them, a hypothesis that can be tested in future experiments.

Thus, *EBS* could participate in the repression of flowering under SD through the repression of *FT*. Upon removal of

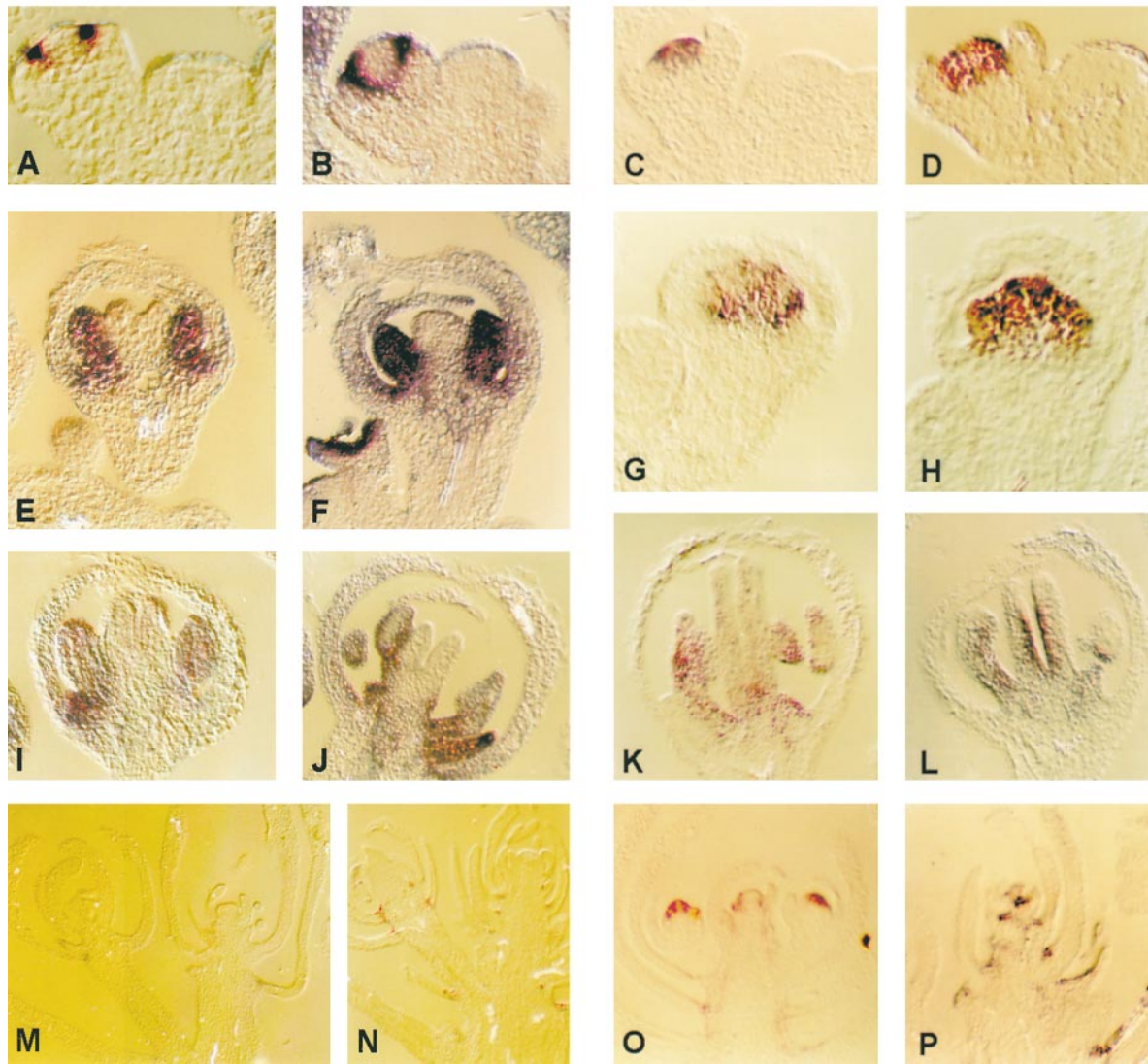


Figure 7. Pattern of Expression of *AP3* and *AG* in Floral Meristems of *ebs* and Inflorescences of the *ebs-1 lfy-6* Double Mutant.

The expression of *AP3* and *AG* was analyzed by in situ hybridization on longitudinal sections of apical buds from plants grown under LD.

- (A) *Ler* stage 3 flower probed with *AP3*.
 (B) *ebs-1* stage 3 flower probed with *AP3*.
 (C) *Ler* stage 3 flower probed with *AG*.
 (D) *ebs-1* stage 3 flower probed with *AG*.
 (E) *Ler* stage 6 flower probed with *AP3*.
 (F) *ebs-1* stage 6 flower probed with *AP3*.
 (G) *Ler* stage 5 flower probed with *AG*.
 (H) *ebs-1* stage 5 flower probed with *AG*.
 (I) *Ler* stage 9 flower probed with *AP3*.
 (J) *ebs-1* stage 9 flower probed with *AP3*.
 (K) *Ler* stage 9 flower probed with *AG*.
 (L) *ebs-1* stage 9 flower probed with *AG*.
 (M) *lfy-6* mutant inflorescence probed with *AP3*.
 (N) *ebs-1 lfy-6* double mutant inflorescence probed with *AP3*.
 (O) *lfy-6* mutant inflorescence probed with *AG*.
 (P) *ebs-1 lfy-6* double mutant inflorescence probed with *AG*.

EBS repression, probably as a consequence of inductive photoperiods, *FT* activation still would be dependent on gibberellins and *CO* transcriptional activation. This model of interaction could be extended to other developmental processes such as germination, in which *EBS*-mediated repression and GA-mediated activation could act on common targets with additional specific transcriptional activators. This hypothesis is consistent with the additive phenotype shown by the double mutant *ebs-1 spy-5* in terms of both germination and flowering initiation under noninductive conditions. Furthermore, the lack of a photoperiodic response of these double mutants suggests that SD inhibition of flower induction depends largely on both *EBS*-mediated flowering repression and GA-mediated flowering activation. The roles of photoreceptors and photoperiod in the regulation of these two pathways remain to be elucidated.

***EBS* Involvement in the Regulation of Floral Organ Identity Genes**

Partial rescue of the differentiation of petals and stamens in the *ebs-1 lfy-6* double mutant suggests increased activation of *AP3* and *PI* in the *ebs* background. In fact, higher steady state transcript levels for *AP3*, *PI*, and *AG* were confirmed by RNA gel blot experiments in apices of the *ebs* mutants. Contrary to what has been reported for mutants such as *clf* (Goodrich et al., 1997), in situ hybridization experiments indicate that both *AG* and *AP3* are not expressed ectopically in the *ebs* mutants. These results suggest a direct or indirect repression effect of *EBS* on *AG* and *AP3* expression, independent of the positional regulation provided by additional factors. The expression of *AP3* and *PI* in the *ebs-1 lfy-6* background suggests that these genes can be activated in the absence of LFY product. However, the enhancement of *AG* expression in *ebs* mutants might be dependent on LFY, because the levels of *AG* transcript are similar in *lfy-6* and the *ebs-1 lfy-6* double mutant. Because the *ft* mutation is epistatic over the *ebs* mutation for the initiation of flowering, it is possible that the *EBS* effect on *AP3*, *PI*, and *AG* expression could be mediated by *FT*. Alternately, *EBS* could participate in the repression of those genes independently of *FT*. Our genetic analysis does not discriminate between these two hypotheses. However, the fact that 35S::*FT* does not correct the *lfy-6* flower phenotype (D. Weigel, personal communication) supports the second possibility. Additional experiments are under way to elucidate the *FT* requirement in *AP3*, *PI*, and *AG* overexpression. Whether gibberellins also are required for complete activation of these homeotic genes remains to be shown.

In conclusion, we have identified a new locus of Arabidopsis whose product participates as a negative regulator in several developmental processes during the life cycle of the plant, from germination to flower development. We also show that in at least two of these processes, which are positively regulated by gibberellins, biosynthesis of these hor-

mones is required for the observation of the *ebs* mutant phenotype. Finally, in two of these processes, flower induction and flower development, we have identified genes such as *FT*, *AP3*, *PI*, and *AG* that could be direct or indirect targets of the *EBS* function. Confirmation of these hypotheses and further research on the molecular role of the *EBS* gene product require its molecular cloning and characterization, a task that is currently under way.

METHODS

Plant Material

The *Arabidopsis thaliana* mutant lines used in this work are all in the ecotype Landsberg and carry the *erecta* mutation. Seed stocks were obtained from the Arabidopsis Biological Resource Center of Ohio State University (Columbus) or the Nottingham Arabidopsis Centre (UK). The monogenic mutants *ve-2*, *fpa-1*, *ft-1*, *fwa-1*, *co-2*, and *gi-3* were described by Koornneef et al. (1991), *ga1-3* and *ga2-1* were described by Koornneef and van der Veen (1980), *spy-5* was described by Jacobsen and Olszewski (1993), and *lfy-6* was described by Weigel et al. (1992). Because *ga1-3* and *ga2-1* mutants require gibberellin treatment for germination, seed carrying these mutations were incubated with 100 μ M gibberellic acid (GA) during 2 days in darkness and were rinsed thoroughly with water before sowing.

The *ebs-1* mutant allele was isolated from ethyl methanesulfonate (EMS)-mutagenized seed, whereas the *ebs-2* mutant allele was identified in a mutant screen of a population carrying *Ds* elements. The *ebs-2* mutant line contains a single transposed *Ds* insertion that is tightly linked to the *ebs* mutation. In an attempt to identify the *Ds* insertion site on *ebs-2*, an inverse polymerase chain reaction fragment on the 3' end of the *Ds* element was generated and sequenced. However, the flanking sequence predicted to be adjacent to the 5' end of the transposon was not present in *ebs-2*, suggesting a chromosomal rearrangement generated upon insertion of the *Ds*. We confirmed that both mutations were allelic by their failure to complement the early flowering phenotype in F1 plants derived from crosses between them. In addition, all plants from the F2 generation exhibited early-flowering and a similar pleiotropic phenotype when grown under short day (SD) photoperiods.

Growth Conditions

Plants were grown in plastic pots containing a mixture of substrate and vermiculite (3:1). Controlled environmental conditions were provided in growth chambers at 18°C and 80% RH. Plants were illuminated with cool-white fluorescent lights. Long day (LD) conditions consisted of 16 hr of light followed by 8 hr of darkness; SD conditions consisted of 8 hr of light followed by 16 hr of darkness. For germination experiments, sterilized seed were sown aseptically in 9-cm Petri dishes on 0.8% (w/v) agar containing Murashige and Skoog (1962) mineral salts supplemented with 1% sucrose. Germination tests were performed subsequently under the LD conditions used for plant growth or in total darkness. For GA or paclobutrazol (PAC) sensitivity tests, sterilized seed were sown on plates in the presence of the GA or PAC concentrations indicated in Figures 4C and 4D, respectively. Unless mentioned otherwise, germination was scored after 2 weeks of incubation.

Flowering Time Analysis

The total number of leaves was recorded as an adequate measurement of flowering time. Total leaf number was scored as the number of leaves in the rosette (excluding cotyledons) plus the number of leaves in the inflorescence at the time of opening of the first flower. The appearance of abaxial trichomes was monitored using a magnifying glass. Adult and juvenile leaves were scored independently. Rosette leaves lacking abaxial trichomes were considered juvenile leaves (Telfer et al., 1997).

Genetic Analysis

The *ebs-1* mutation was mapped relative to cleaved-amplified polymorphic sequence (Bell and Ecker, 1994) and simple sequence length polymorphism molecular markers (Konieczny and Ausubel, 1993). Recombination fractions were used to calculate the map distances using the Kosambi mapping function (Kosambi, 1944). Double mutants were constructed by crossing the monogenic *ebs-1* or *ebs-2* mutant with lines carrying the mutations *fve-2*, *fpa-1*, *ft-1*, *fwa-1*, *co-2*, *gi-3*, *ga1-3*, *ga2-1*, *spy-5*, and *lfy-6*. Double mutants were isolated from selfed F2 progeny that showed the *ebs* phenotype and that segregated for the second mutation. The genotypes of double mutants were confirmed by crosses with the monogenic parental lines.

Expression Analysis

Total RNA was isolated from both leaves and apices using the FastRNA Kit-GREEN (BIO101; Vista, California) according to the instructions of the manufacturer. Total RNA was electrophoresed in 1.5% formaldehyde agarose gels (Sambrook et al., 1989) and transferred to Hybond N⁺ membranes (Amersham). The *AP3* probe was an EcoRI-BglII fragment from pD793 plasmid that contains the cDNA of the *AP3* gene (Jack et al. 1992). The *PI* probe was a NsiI-XhoI fragment from plasmid pNX that contains the cDNA of the *PI* gene (Goto and Meyerowitz, 1994). The *AG* probe was a Scal-EcoRI fragment from the pCIT565 plasmid that contains the cDNA of the *AG* gene (Yanofsky et al., 1990). As a loading control, we used a 300-bp fragment of the cauliflower 18S rDNA gene. For in situ hybridization, apical buds were fixed and embedded by standard methods. In situ hybridization was performed essentially as described by Huijser et al. (1992). Immunological detection was performed according to the DIG Nucleic Acid Detection kit (Boehringer Mannheim). Antisense probes for the *AP3* messenger were made using T7 RNA polymerase and pD793 plasmid linearized with BglII, which cuts once, 216 bp from the 5' end of the cDNA, just past the 3' end of the MADS box (Jack et al. 1992). For the *AG* probe, plasmid pCIT565 was linearized with HindIII, and labeled RNA was made using SP6 polymerase (Drews et al., 1991).

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