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EAF1 Regulates Vegetative-Phase Change and Flowering Time in Arabidopsis[1]

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We have identified a new locus that regulates vegetative phase change and flowering time in Arabidopsis. An early-flowering mutant, *eaf1* (*early flowering 1*) was isolated and characterized. *eaf1* plants flowered earlier than the wild type under either short-day or long-day conditions, and showed a reduction in the juvenile and adult vegetative phases. When grown under short-day conditions, *eaf1* plants were slightly pale green and had elongated petioles, phenotypes that are observed in mutants altered in either phytochrome or the gibberellin (GA) response. *eaf1* seed showed increased resistance to the GA biosynthesis inhibitor paclobutrazol, suggesting that GA metabolism and/or response had been altered. Comparison of *eaf1* to other early-flowering mutants revealed that *eaf1* shifts to the adult phase early and flowers early, similarly to the *phyB* (*phytochrome B*) and *spy* (*spindly*) mutants. *eaf1* maps to chromosome 2, but defines a locus distinct from *phyB*, *clf* (*curly leaf*), and *elf3* (*early-flowering 3*). These results demonstrate that *eaf1* defines a new locus involved in an autonomous pathway and may affect GA regulation of flowering.

The transition from vegetative to reproductive development is of vital importance for the survival of virtually all flowering plants. The vegetative phase of Arabidopsis can be subdivided into juvenile and adult phases that can be distinguished by physiological and morphological markers (Martinez-Zapater et al., 1995; Chien and Sussex, 1996; Telfer et al., 1997). Juvenile leaves are small and round in shape, with adaxial trichomes and no abaxial trichomes (Telfer et al., 1997). As development continues, emerging adult leaves are more elongated and lanceolate in shape, and trichomes begin to appear on the abaxial surfaces (Chien and Sussex, 1996; Telfer et al., 1997). Application of the hormone GA_3 will induce abaxial trichomes on leaves where they are not normally present (Chien and Sussex, 1996; Telfer et al., 1997), although the earliest arising leaves do not respond to this induction. At the appropriate stage in development and in response to specific cues, the shoot apical meristem becomes reprogrammed and generates a reproductive shoot that carries either an inflorescence or a single flower. Daylength is a key regulator of flowering in many plant species, and in Arabidopsis flowering is hastened under LD conditions (Koornneef et al., 1998). Vernalization accelerates flowering in some Arabidopsis ecotypes, and the shoot meristem itself is thought to be the site of perception of the vernalization signal (Dennis et al., 1996; Wilson and Dean, 1996).

Genetic studies with Arabidopsis and pea have identified a large number of genes that regulate flowering (Reid et al., 1996; Koornneef et al., 1998). In Arabidopsis at least two flowering pathways are thought to exist, a photoperiod-sensitive pathway and an autonomous pathway. GA may function as part of the autonomous pathway or could define a third pathway. Each of the flowering pathways includes both activator and repressor genes, and epistasis tests and physiological experiments have led to the idea that these pathways function in parallel. In Arabidopsis several genes that serve as promoters of flowering have been recently cloned, and the sequences of the proteins encoded as well as their expression patterns have suggested possible functions for these genes. For example, the genes *CO* (Putterill et al., 1995) and *LD* (Lee et al., 1994) both appear to encode transcription factors, whereas *FCA* possibly affects RNA metabolism (Macknight et al., 1997), suggesting that a cascade of gene activation events likely controls flowering.

Genes that function to repress the transition to adult and/or reproductive phases have been identified in Arabidopsis (Koornneef et al., 1998). *elf3* (*early-flowering 3*) (Hicks et al., 1996; Zagotta et al., 1996) and *lhy* (*long hypocotyl)* (Schaffer et al., 1998) are photoperiod-insensitive mutants that show alterations in circadian clock function and appear to be involved in the repression of flowering in the LD pathway. The *HST* (*HASTY*) gene is thought to promote a juvenile pattern of development, and loss-of-function mutations in this gene result in early transition to the adult phase and early flowering (Telfer and Poethig, 1998). Arabidopsis mutants defective in phytochrome synthesis (Reed et al., 1994; Koornneef et al., 1995) or phytochrome function (Ahmad and Cashmore, 1996) flower early, indicating that this light receptor is involved in repression of floral initiation. At least some of the effects of phytochrome may be mediated by GA, because recent studies (Reed et al., 1996) show that the hypocotyl tissue of *phyB* (*phytochrome B*) mutant seedlings is more responsive to exogenous GA than wild-type seedlings. The *tfl* (*terminal flower*)

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Abbreviations: LD, long-day; SD, short-day.

mutant flowers early and produces a determinate inflorescence, often generating only one or a few terminal flowers.

Application of GA accelerates the onset of the adult phase, and induces early flowering and the production of larger, slightly pale-green leaves in wild-type Arabidopsis (Jacobsen and Olszewski, 1993; M. Honma, unpublished data). The GA-deficient mutant *ga1* of Arabidopsis flowers later when grown in LD and does not flower in SD conditions, indicating that GA is required for the photoperiod-insensitive (autonomous) flowering pathway (Wilson et al., 1992). GA levels and response to the hormone are sensitive to changes in photoperiod (Pharis et al., 1987; Zeevaart and Gage, 1993), suggesting a role for GA in photoperiodic induction of flowering. Suppressors of the *ga1* mutant have been identified and shown to suppress the late-flowering phenotype of *ga1*. These suppressors known as *spy* (*spindly*) (Jacobsen and Olszewski, 1993) and *rga* (*repressor of ga1-3*) (Silverstone et al., 1997) are thought to have partially activated the GA response. *spy* flowers early, and *spy* mutant seed show increased resistance to the GA biosynthesis inhibitor paclobutrazol. The double mutant *rga ga1* flowers earlier than *ga1* alone. The phenotypes of *spy* and *rga* suggest that activating the GA response can cause earlier flowering.

We have identified a new gene, *eaf1* (*early flowering 1*), that appears to function in the autonomous pathway to repress the transition from juvenile to adult development. *eaf1* mutant plants exhibit truncated juvenile and adult phases, resulting in early flowering. *eaf1* mutant plants exhibit phenotypes similar to the GA response mutants *spy* and *rga*, and *eaf1* seed show increased resistance to paclobutrazol. Our results are consistent with the notion that the *eaf1* mutant is altered in either GA biosynthesis or response to the hormone, and that this change is responsible for the alteration in flowering time.

MATERIALS AND METHODS

Arabidopsis Seed Stocks

Seed stocks were obtained from the Arabidopsis Biological Resource Center at Ohio State University, Columbus, or from individual researchers (Drs. D.R. Meeks-Wagner, J. Reed, G. Coupland, T.-p. Sun, and R. Amasino). Ac^{st} and *Ds* transgenic lines of ecotype Nossen used to generate the *eaf1* mutant line were described previously (Honma et al., 1993). The 35S-Ac^{st} and rbcS-Ac^{st} lines express the *Ac* transposase under the control of either the cauliflower mosaic virus 35S or the Arabidopsis rbcS-1A promoters. The Ds^{ALS} construct carries the Arabidopsis acetolactate synthase gene that encodes resistance to the herbicide chlorsulfuron. The Ds^{ALS} element resides within the untranslated leader of a kanamycin resistance gene that serves as marker for excision of *Ds*. The *eaf1* mutant was originally identified in a mutant screen of a population carrying transposed *Ds* elements (Honma et al., 1993; M. Honma, unpublished data). The *eaf1* mutant line contained three transposed *Ds* insertions: *Ds-1*, *Ds-2*, and *Ds-3*. *Ds-1* and *Ds-2* are tightly linked to the *eaf1* mutation, and most of the characterization described in this paper was done using a line that carried both of these insertions.

Introgression of the *eaf1* mutation into the Landsberg background was accomplished by crossing *eaf1* plants carrying *Ds-1* and *Ds-2* to wild-type Landsberg plants. Backcross progeny were screened using chlorsulfuron to select for the presence of either *Ds-1* or *Ds-2*, both of which are linked to the *eaf1* mutation. These chlorsulfuron-resistant plants were then backcrossed four more times to the Landsberg parent. No selection for the early-flowering trait was done during the introgression, to prevent selective maintenance of additional loci in the Nossen ecotype that may condition earlier flowering. F_1 plants from the fifth backcross were self-fertilized and early-flowering F_2 progeny were identified.

Growth Conditions

Plants were grown either under sterile conditions on germination medium (Valvekens et al., 1988) supplemented with appropriate antibiotics, as described previously (Honma et al., 1993), or in soil under LD (16 h of light/8 h of dark) or SD (8 h of light/16 h of dark) conditions at 18°C to 20°C during the dark period and at 20°C to 24°C during the light period. For most of the flowering-time experiments, seeds were either allowed to imbibe in water at 4°C for 4 d (in the dark or dim light) before transfer to soil or planted directly in moist soil and cold treated. After the cold treatment, seeds were transferred to growth rooms. Light intensity was 440 $\mu E\ m^{-2}\ s^{-1}$ for SD and 240 $\mu E\ m^{-2}\ s^{-1}$ for LD conditions. Lighting was supplied by a 3:1 mixture of cool-white:Wide Spectrum bulbs (General Electric). Plants were grown individually in divided flats (Hummert, St. Louis, MO) at a density of 60 to 96 plants/1290 cm^2 flat. In the screen used to isolate the *eaf1* mutant, seedlings were grown for 2 weeks on germination medium plates, followed by transfer to soil at a density of 150 plants/1290 cm^2 flat. Chlorsulfuron was a gift from DuPont.

In the initial experiment to determine linkage of the *Ds* elements to the early-flowering phenotype, seedlings were first grown on germination medium plates for 10 to 14 d before transfer to soil. Plants were grown in a growth chamber (Conviron, Winnipeg, Manitoba, Canada) with a mixture of cool-white and incandescent bulbs. The light intensity was approximately 120 $\mu E\ m^{-2}\ s^{-1}$ and the temperature was maintained at 22°C. In the GA experiment sterilized seeds were plated on germination medium plates with or without 10^{-5} M GA_3 and allowed to imbibe at 4°C for 2 d before growth under SD (220 μmol photons $m^{-2}\ s^{-1}$) conditions in a growth chamber (model CU32L, Percival Scientific, Boone, IA). After 11 d of growth the seedlings were transferred to soil and grown under SD conditions, 440 $\mu E\ m^{-2}\ s^{-1}$. GA_3 (100 μL of 10^{-5} M) was applied weekly to the base of each plant and continued until the plants had flowered.

For vernalization treatment, seeds were allowed to imbibe by planting in moist soil; they were grown at 4°C for 8 weeks under low-intensity SD conditions (19 $\mu E\ m^{-2}\ s^{-1}$) before transfer to standard SD conditions (440 $\mu E\ m^{-2}\ s^{-1}$,

22°C). Untreated control seeds were allowed to imbibe for 4 d at 4°C and transferred to the SD growth rooms on the same day as the vernalized plants.

Morphological Analysis

Days to flowering was scored as the length of time between germination and visible appearance of the first floral bud. The number of rosette leaves was counted weekly, and cauline leaves were counted after seed set. Juvenile-stage leaves were those true leaves present in the rosette that lacked abaxial trichomes. The appearance of abaxial trichomes was monitored using a stereomicroscope.

Hypocotyl elongation in response to red light was measured as described previously by Nagatani et al. (1993).

Allelism Tests

eaf1 was crossed to the early-flowering mutants *clf* (curly leaf), *elf3*, and *hy3*, and F_1 and F_2 plants were scored for days to flowering and leaf number under SD conditions. *clf* and *phyB*(*hy3*) are alleles in the Landsberg ecotype and *elf3* is in the Columbia ecotype. The control crosses *eaf1* × Landsberg, *eaf1* × Columbia, and Nossen × *clf*, *elf3*, or *phyB*(*hy3*) were included. Twenty to fifty plants were scored in each experiment.

Molecular Analysis

DNA was isolated from leaf tissue using a modification of the method described by Dellaporta et al. (1983). Southern blotting was by reverse capillary transfer as described previously (Ausubel et al., 1988) and Southern hybridizations were carried out according to the method of Church and Gilbert (1984). DNA fragments used as probes were a 1.5-kb fragment from the 5′ end of *Ac* and the *Ds-2* genomic flanking sequence (PCR product). These probes were generated by random-prime labeling (Feinberg and Vogelstein, 1983).

Genetic Mapping of *Ds-2* Insertion and the *eaf1* Mutation

Inverse PCR was used to isolate genomic DNA sequences flanking the *Ds-2* insertion (Healy et al., 1993). Mapping of this genomic sequence was done using recombinant inbred lines as described previously by Osborne et al. (1995).

Wild-type Nossen, Columbia, and Landsberg ecotypes were compared using restriction fragment-length polymorphism analysis to determine if the region carrying *eaf1* in Nossen showed polymorphisms with respect to the Columbia or Landsberg ecotypes. Southern analysis with restriction fragment-length polymorphism clones that map to the middle of chromosome 2 (Lister and Dean, 1993) showed that the *eaf1* region in Nossen appears to be polymorphic compared with the same region in Columbia (data not shown). Thus, the Nossen × Columbia cross was the most likely to yield polymorphisms that could be used for mapping. A mapping population was constructed by crossing the *eaf1* mutant (Nossen background) carrying *Ds-1* and *Ds-2* to Columbia wild type, and the F_1 plants were self-fertilized to generate F_2 siblings. Tissue was collected for molecular analysis from 596 early-flowering plants that arose from a population of approximately 2500 F_2 plants. The position of the *eaf1* mutation was determined using cleaved-amplified polymorphic sequence markers positioned on chromosome 2 (Lister and Dean, 1993) (http://genome-www.stanford.edu/Arabidopsis/ww/Aug98RImaps/index.html). Progeny from plants that showed recombination events between the *eaf1* mutation and the marker tested were scored for their flowering phenotype to confirm that they were homozygous for the *eaf1* mutation.

Germination Assays

Paclobutrazol resistance was determined as described previously (Jacobsen and Olszewski, 1993) with minor modifications. For each paclobutrazol treatment, 120 seed of each line were sterilized by treatment with 0.1% Triton. After the seeds were rinsed with water, they were washed with the respective paclobutrazol solutions and allowed to imbibe in the same solutions for 4 d at 4°C. Seeds were suspended in a small volume of 0.1% agarose, plated on four stacked filter paper circles in small Petri dishes, and allowed to dry (30 seeds/plate). Paclobutrazol (1.5 mL) in 0.01% Tween was applied to the filter paper and the Petri dishes were sealed with parafilm and incubated at 22°C for 7 d under a LD photoperiod. Germination was scored under a stereomicroscope as emergence of the radicle. Paclobutrazol was a gift from Zeneca (Wilmington, DE).

RESULTS

Isolation of an Early-Flowering Mutant, *eaf1*

A mutant that flowered earlier than the wild type was identified in a *Ds*-mutagenized population of plants of the Nossen ecotype grown under LD conditions. This early-flowering mutant was designated *eaf1*. The *eaf1* mutant was characterized with regard to flowering time, appearance, and overall growth and development. Under LD conditions the mutant plants flowered 2 d before the wild type and generated three fewer leaves (Table I). Under SD conditions the early-flowering phenotype became much more extreme, and *eaf1* flowered 20 d earlier with 27 fewer leaves than the wild type. The *eaf1* mutant flowered earlier under LD than under SD conditions, indicating that it remains responsive to changes in the photoperiod. This is in contrast to the phenotype of other flowering-time mutants, *elf3*, *co* (*constans)*, and *gi* (*gigantea*), that flower at the same time under either SD or LD conditions and appear to be photoperiod insensitive.

Vernalization accelerates flowering in some Arabidopsis ecotypes (Napp-Zinn, 1985), and it is possible that *eaf1* has an activated vernalization response. *eaf1* and Nossen wild-type plants were tested for a response to vernalization. Seedlings were vernalized under SD conditions and scored for flowering time. As shown in Table II, both Nossen wild type and *eaf1* respond to vernalization, flowering earlier with fewer rosette leaves. This result suggests that re-

Table I. *Days to flowering and rosette leaf no. of wild-type Nossen and the eaf1 mutant in response to photoperiod*

Days to flowering was measured as the no. of days from germination to appearance of the floral bud. Leaf no. is the no. of rosette leaves produced before flowering. Each value represents the mean ± 2 SE. Unless otherwise noted, plants from within each group were significantly different from the wild-type (WT) controls ($P < 0.05$, Student's *t* test).

Genotype	Growth Condition	Days to Flowering	Leaf No.	*n*
WT (Nossen)	LD	21.2 ± 0.5	9.0 ± 0.3	66
eaf1	LD	19.3 ± 0.3	5.8 ± 0.1	66
WT (Nossen)	SD	59.2 ± 1.0	43.5 ± 1.0	63
eaf1	SD	38.9 ± 0.5	16.8 ± 0.3	69

sponse to vernalization has not been altered in *eaf1* or that this response has not been saturated.

eaf1 Is Recessive and Not Allelic to Other Early-Flowering Mutants

Genetic analysis showed that *eaf1* is a recessive mutation. Homozygous *eaf1* plants were crossed to wild-type Nossen, and the resulting *eaf1*/+ F_1 plants from two independent crosses (a and b) were scored for flowering (Table III). Plants that were heterozygous for *eaf1* flowered similarly to wild-type control plants, showing no statistical difference in terms of days to flowering. Although the *eaf1*/+ plants flowered at the same time as wild-type plants, the numbers of rosette leaves appeared to be slightly reduced. One possibility is that the heterozygous plants have a slightly reduced rate of leaf initiation, and if so, this would indicate that the *eaf1* mutation is not completely recessive for this phenotype. Two F_1 plants were self-fertilized, and the F_2 progeny were scored for flowering phenotype under SD conditions. The two F_2 populations segregated mutant: wild-type plants in the ratios of 1:3, indicating that *eaf1* is recessive and that the early-flowering phenotype was due to a mutation at a single locus (data not shown).

The original *eaf1* mutant line carried three *Ds* insertions, *Ds-1*, *Ds-2*, and *Ds-3*. Backcross of *eaf1* to wild-type Nossen generated a set of lines carrying different combinations of the *Ds* insertions. Lines that were hemizygous for *Ds-1* and *Ds-2* or *Ds-2* and *Ds-3* produced 25% mutant progeny. Plants carrying only *Ds-3* flowered similarly to the wild type. These results indicated that *Ds-2* is most closely linked to the *eaf1* mutation, with *Ds-1* and *Ds-3* not responsible for causing the early-flowering phenotype. The *Ds-1* insertion is approximately 2 centimorgans from *eaf1*, with the *Ds-3* insertion loosely linked on the same chromosome (data not shown). The genomic sequence flanking *Ds-2* was isolated by inverse PCR (see "Materials and Methods") and used in Southern hybridization experiments (data not shown). Analysis of 50 early-flowering F_2 progeny showed that all were homozygous for the *Ds-2* insertion, indicating that *eaf1* was <1 centimorgan from *Ds-2*. Two lines hemizygous for *Ds-2* alone have been identified; when self-fertilized, both lines produced mutant:wild-type progeny in the ratio of 1:3 (18 mutant:60 wild type and 20 mutant:59 wild type), confirming that *Ds-1* and *Ds-3* were not responsible for causing the early-flowering phenotype and that the *eaf1* mutation was tightly linked to the *Ds-2* insertion. However, meiotic mapping experiments using cleaved-amplified polymorphic sequence markers have more precisely localized the *Ds-2* insertion site to 0.35 ± 0.2 centimorgans away from the *eaf1* mutation; thus *Ds-2* is tightly linked but not inserted into the *eaf1* gene (W. Jin and M. Honma, unpublished data). Moreover, sequence analysis of the genomic region flanking *Ds-2* indicates that the element is not inserted within an open reading frame (W. Jin and M. Honma, unpublished data).

Assignment of an initial map position to the *eaf1* mutation was determined with the aid of the *Ds-2* insertion, which lies 0.35 centimorgans away. The genomic sequence flanking *Ds-2* was isolated by inverse PCR (see "Materials and Methods") and this genomic sequence was mapped using a recombinant inbred population (Lister and Dean, 1993). The *Ds-2* insertion resides on chromosome 2, near mi238. A mapping population was generated (as described in "Materials and Methods") by crossing *eaf1* to wild-type Columbia plants, and the early-flowering phenotype was mapped using cleaved-amplified polymorphic sequence markers located in the middle of chromosome 2 (Lister and

Table II. *Days to flowering and rosette leaf no. of wild-type Nossen and the eaf1 mutant in response to vernalization*

Days to flowering was measured as the no. of days from germination to appearance of the floral bud. Leaf no. is the no. of rosette leaves produced before flowering. Each value represents the mean ± 2 SE. Unless otherwise noted, plants from within each group were significantly different from the wild-type (WT) controls ($P < 0.05$, Student's *t* test). vern, Vernalization.

Genotype	Growth Condition	Days to Flowering	Leaf No.	*n*
WT (Nossen)	SD	54.8 ± 1.0	41.9 ± 1.6	53
eaf1	SD	41.0 ± 0.6	20.2 ± 0.3	54
WT (Nossen)	vern + SD	36.2 ± 1.4	39.9 ± 1.4	63
eaf1	vern + SD	26.1 ± 1.9	15.4 ± 0.3	63

Table III. *Days to flowering and rosette leaf no. of the eaf1 mutant in the Nossen background*

Days to flowering was measured as the no. of days from germination to appearance of the floral bud. Leaf no. is the no. of rosette leaves produced before flowering. Each value represents the mean ± 2 SE. Unless otherwise noted, plants from within each group were significantly different from the wild-type (WT) controls ($P < 0.05$, Student's *t* test).

Genotype	Growth Condition	Days to Flowering	Leaf No.	*n*
WT (Nossen)	SD	64.0 ± 3.1	49.6 ± 3.5	24
eaf1	SD	39.9 ± 6.0	20.3 ± 1.2	11
WT × *eaf1* F_1 a	SD	70.0 ± 2.8	44.0 ± 1.0	12
WT × *eaf1* F_1 b	SD	65.0 ± 2.4[a]	39.0 ± 3.4	8

[a] Not significantly different from wild-type control ($P > 0.05$, Student's *t* test).

Dean, 1993) (http://genome-www.stanford.edu/Arabidopsis/ww/Aug98RImaps/index.html). Our results placed the *eaf1* mutation between mi139 and mi238. The early-flowering mutations *phyB*(*hy3*), *clf*, and *elf3* map within this region, but at locations different from *eaf1* (Fig. 1). *eaf1* lies 1.9 centimorgans south of *phyB* (*hy3*) and is >1.7 centimorgans north of *clf*. *elf3* is 6.6 centimorgans south of *eaf1*, which is close to the marker GPA1 (Zagotta et al., 1996; K.A. Hicks, T.M. Albertson, and D.R. Meeks-Wagner, personal communication). To confirm that *eaf1* was not allelic to *phyB* (*hy3*), *clf*, or *elf3*, complementation tests (described in "Materials and Methods") were done. *eaf1* (in Nossen) was crossed to *phyB* (*hy3*) (in Landsberg), *clf* (in Landsberg), *elf3* (in Columbia), wild-type Landsberg, or wild-type Columbia. *phyB*(*hy3*), *clf*, and *elf3* were each crossed to wild-type Nossen. All F_1 plants flowered at the same time, and after self-fertilization they produced both early-flowering and wild-type F_2 progeny (data not shown). These results demonstrate that *eaf1* is not allelic to *phyB*(*hy3*), *clf*, and *elf3*. Therefore, *eaf1* defines a new locus on chromosome 2 that affects flowering time, in addition to the previously known *phyB*(*hy3*), *clf*, and *elf3* loci.

eaf1 Regulates Vegetative-Phase Transition

eaf1 mutant and wild-type plants were analyzed for the appearance of abaxial trichomes, a marker associated with the shift from the juvenile to the adult phase (Chien and Sussex, 1996; Telfer et al., 1997). The first rosette leaf bearing abaxial trichomes is counted as the first adult leaf. The number of juvenile, adult, and reproductive (cauline) leaves was determined, and the results are presented in Figure 2. Abaxial trichomes first appeared on leaf 8 in

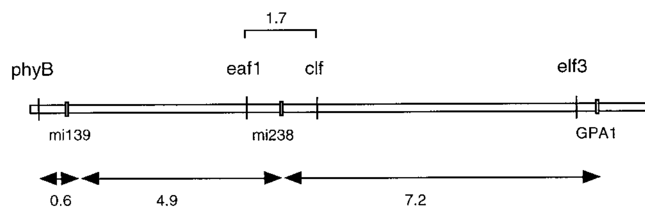

Figure 1. Map location of *eaf1* and other early-flowering mutants on chromosome 2. Distance shown with a bracket indicates number of centimorgans between the marker and the early-flowering phenotype. Distances shown with arrows were derived from the recombinant inbred lines (Lister and Dean, 1995).

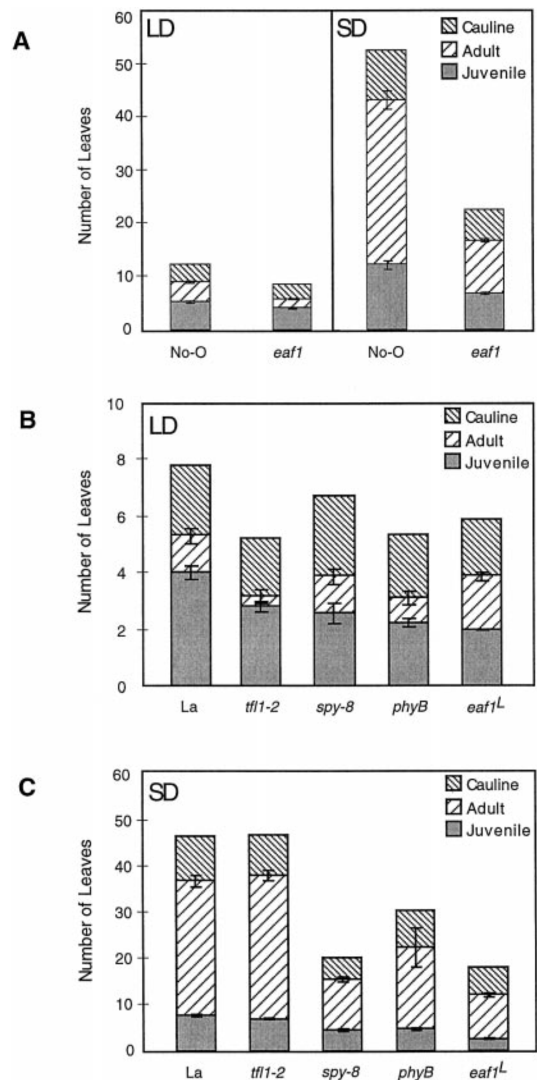

Figure 2. Effect of daylength on number and type of leaves produced in wild-type Nossen and *eaf1* mutant plants (A) grown under LD or SD conditions. Wild-type Landsberg and *tfl*, *spy*, *phyB*(*hy3*), and *eaf1* mutant plants all in the Landsberg background grown under LD (B) or SD (C) conditions. Twenty to fifty plants of each line were tested and error bars represent ±2 SE.

Figure 3. Wild-type and *eaf1* mutant plants (Landsberg ecotype) grown under SD conditions for approximately 8 weeks. *eaf1* plants had flowered 3 weeks before this photograph was taken and wild-type plants had not yet flowered.

mutant plants as compared with leaf 13 in wild-type plants grown under SD conditions. Under LD conditions abaxial trichomes appeared on leaf 5 in mutant plants and leaf 6 in wild-type plants. These results show that the juvenile phase has been shortened in the mutant plants when grown in either SD or LD conditions. The adult phase in the mutant was also affected, because only nine adult leaves with abaxial trichomes were produced when grown under SD, as compared with 30 adult leaves in the wild type.

eaf1 Mutant Plants Resemble Phytochrome and GA Signal Transduction Mutants

eaf1 mutant plants had elongated petioles, were lighter green compared with wild-type plants (Fig. 3), and produced hypocotyls that were approximately 20% longer than the wild type (data not shown). The elongated petiole and pale-green phenotype has also been observed with other early-flowering mutants, *phyB*, *spy*, and *elf3* (Jacobsen and Olszewski, 1993; Reed et al., 1993; Zagotta et al., 1996) and the late-flowering mutant *lhy* (Schaffer et al., 1998). In addition, *elf3* produces long hypocotyls when grown under SD conditions (Zagotta et al., 1992), and *phyB* and *lhy* mutant seedlings produce long hypocotyls when grown under LD conditions (Koornneef et al., 1980; Schaffer et al., 1998).

Although we had previously shown that *eaf1* was not allelic to *phyB*, it is still possible that *eaf1* is defective in some other phytochrome or phytochrome-related process. *eaf1* mutant and Nossen wild-type seedlings were tested for inhibition of hypocotyl elongation in response to red light, a phytochrome-mediated response. Both wild-type and *eaf1* mutant seedlings responded by producing shorter hypocotyls at higher fluences of red light, indicating that the *eaf1* mutation was not affected in this aspect of phytochrome function. (M. Honma and J. Reed, unpublished data).

GA has long been known to be involved in flowering, and application of GA accelerates the transition to the adult phase and flowering in Arabidopsis (Jacobsen and Olszewski, 1993). Some of the phenotypic characteristics of *eaf1* mutant plants, such as early flowering, pale color, and elongated petioles, are similar to GA-treated plants or mutants altered in GA response (Jacobsen and Olszewski, 1993). Therefore, GA levels might be elevated or GA response may have been increased in the *eaf1* mutant. To test whether *eaf1* is able to respond to exogenous GA, an experiment was performed by applying GA_3 and measuring flowering time. If the GA signal transduction pathway has been activated such that its response is saturated, then increased levels of exogenous GA will not have an effect. Our results showed that *eaf1* was still able to respond to applied GA, and treated plants exhibited more elongated petioles, were paler green (data not shown), and were earlier flowering (Table IV). This suggests that, even if GA synthesis or signaling has been activated, the response to the hormone had not been saturated.

Quantitative measurements of response to GA or GA inhibitors on soil-grown plants are extremely difficult. To address the question of whether *eaf1* is altered in some aspect of GA function, seed germination in response to paclobutrazol was examined. Germination requires GA, and paclobutrazol interferes with GA biosynthesis such that germination of wild-type seeds is inhibited. Germination of wild-type Nossen and *eaf1* mutant seeds was measured in the presence of varying levels of paclobutrazol (Fig. 4). *eaf1* showed increased resistance to paclobutrazol compared with wild-type Nossen. Between 0 and 3 μM paclobutrazol, both the wild type and *eaf1* showed similar high levels of germination. Germination of wild-type Nossen dropped to below 75% on 10 μM paclobutrazol, whereas *eaf1* germination remained at 95%. Increasing lev-

Table IV. *Days to flowering and rosette leaf no. of wild-type Nossen and the eaf1 mutant in response to GA*

Days to flowering was measured as the no. of days from germination to appearance of the floral bud. Leaf no. is the no. of rosette leaves produced before flowering. Each value represents the mean ± 2 SE. Unless otherwise noted, plants from within each group were significantly different from the wild-type controls ($P < 0.05$, Student's *t* test). ND, Not determined.

Genotype	Growth Condition	Days to Flowering	Leaf No.	*n*
WT (Nossen)	SD	60.1 ± 1.0	ND	47
eaf1	SD	37.2 ± 0.7	ND	41
WT (Nossen)	GA + SD	53.9 ± 1.8	ND	40
eaf1	GA + SD	33.8 ± 1.7	ND	32

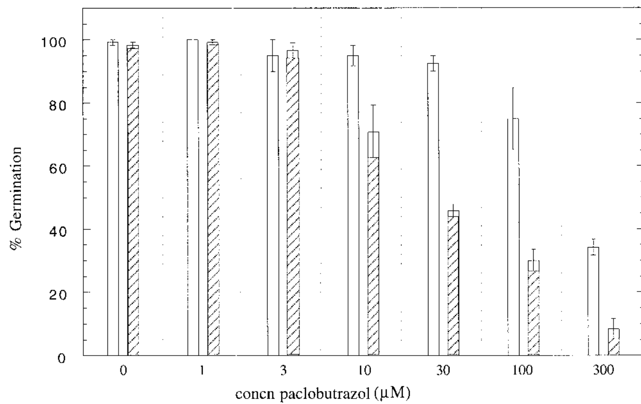

Figure 4. Response of *eaf1* to paclobutrazol. Percentage germination of Nossen wild-type and *eaf1* mutant seed in the presence of exogenous paclobutrazol. Open bars represent *eaf1*; striped bars represent wild type. Error bars represent ±1 SE.

els of paclobutrazol showed a decreasing level of germination for the wild type, with only 15% germination at 300 μM. In contrast, germination of *eaf1* seed remained high at 100 μM, and even at 300 μM paclobutrazol, 35% of the seed still germinated. Thus, the *eaf1* mutant shows increased resistance to paclobutrazol, similarly to the *spy* mutant (Jacobsen and Olszewski, 1993). This resistance could be the result of elevated levels of GA, such that higher levels of paclobutrazol are required to have an effect, or the result of an altered response to GA. The pale pigmentation, early-flowering, elongated petiole, and paclobutrazol-resistant phenotypes of *eaf1* mutant plants are all consistent with the notion that GA levels or responses have been altered.

Comparison of *eaf1* and Other Early-Flowering Mutants

To determine whether all early-flowering mutants show premature appearance of abaxial trichomes, flowering time and abaxial trichome formation were examined in a variety of early-flowering mutants grown under different photoperiods (Table V; Fig. 2). *eaf1* was compared with the early-flowering mutants *phyB*(*hy3*), *spy*, and *tfl*, of which alleles exist in the Landsberg ecotype. Because wild-type Landsberg and Nossen differ in flowering time and other characteristics, the *eaf1* mutation was introgressed into the Landsberg background for this comparison. Plants of each mutant line were grown in either LD or SD conditions, and the appearance of abaxial trichomes and time of floral bud emergence were noted. Two experiments were done and similar results were obtained.

One set of experiments is shown in Table V and Figure 2. In the Landsberg background, *eaf1* exhibited a shorter juvenile phase and flowered early in LD conditions. *spy* also produced fewer juvenile leaves and both *eaf1* and *spy* produced the normal number of adult leaves. *phyB*(*hy3*) and *tfl* had slightly shortened juvenile phases, had markedly reduced adult phases, and flowered early. The phenotype of *tfl1-2* in the LD condition is similar to what has been previously reported for Columbia alleles of *tfl* (Telfer and Poethig, 1998). When grown under SD conditions, *tfl* appeared to be very similar to wild-type Landsberg. The reason for no observable phenotype in the SD condition is not clear, but alleles of *tfl* in the Landsberg ecotype have been previously reported to show a milder phenotype than those in the Columbia ecotype (Alvarez et al., 1992). Perhaps the light intensity, light quality, or temperatures used in our experiments were unable to reveal such a phenotype. *phyB*(*hy3*) flowered early under SD conditions, but the length of the adult phase appeared to be less affected than either *spy* or *eaf1*. When grown under the LD condition, the adult phase of *phyB*(*hy3*) appeared to have been shortened compared with the wild type. *spy* and *eaf1* behaved very similarly when grown in SD conditions, with reduced juvenile and greatly reduced adult phases and early flowering. Thus, by comparison, *eaf1* is similar phenotypically to *spy* under both photoperiod regimes tested.

DISCUSSION

The *EAF1* gene that controls vegetative-phase change and flowering time has been identified by mutational anal-

Table V. *Days to flowering and rosette leaf no. of wild-type Landsberg and various early-flowering mutants*

Days to flowering was measured as the no. of days from germination to appearance of the floral bud. Leaf no. is the no. of rosette leaves produced before flowering. Each value represents the mean ± 2 SE. Unless otherwise noted, plants from within each group were significantly different from the wild-type controls ($P < 0.05$, Student's *t* test).

Genotype	Growth Condition	Days to Flowering	Leaf No.	*n*
WT (Landsberg)	LD	19.5 ± 0.5	5.3 ± 0.2	40
tfl1-2	LD	15.9 ± 0.4	3.2 ± 0.1	40
spy-8	LD	17.5 ± 0.4	4.0 ± 0	7
phyB(hy3)	LD	16.7 ± 0.5	3.1 ± 0.1	38
eaf1^L	LD	15.7 ± 0.4	3.9 ± 0.1	23
WT (Landsberg)	SD	61.3 ± 1.3	36.5 ± 1.3	28
tfl1-2	SD	62.3 ± 1.5[a]	37.3 ± 1.0[a]	25
spy-8	SD	36.6 ± 0.6	14.8 ± 0.5	22
phyB(hy3)	SD	55.2 ± 2.5	23.4 ± 2.7	9
eaf1^L	SD	31.6 ± 0.3	12.0 ± 0.3	29

[a] Not significantly different from wild-type controls ($P > 0.05$, Student's *t* test).

ysis and shown to reside on chromosome 2. Both the juvenile and adult phases of *eaf1* mutant plants are shortened, resulting in an early transition to reproductive development. *eaf1* appears primarily to affect the length of the adult phase, with a less dramatic alteration of the juvenile phase. The *eaf1* allele behaves as a recessive mutation, and if this mutation is due to loss-of-function of the *eaf1* gene, then the *EAF1* gene product may function to repress flowering by delaying adult development. Alternatively, *eaf1* could be a recessive neomorph, in which case the wild-type product may not normally function to regulate flowering. Additional alleles of the *eaf1* gene will provide important information regarding the role of *EAF1* in control of flowering.

Seed of *eaf1* show increased resistance to paclobutrazol compared with Nossen wild type, a phenotype also seen with the *spy* mutant. Increased resistance to paclobutrazol suggests that *eaf1* is involved in regulation of GA levels or response to the hormone. An increase in bioactive GAs could be the result of increased biosynthesis, decreased catabolism or inactivation, or loss of feedback regulation on the biosynthetic pathway (Chiang et al., 1995). Elongation of the inflorescence stem (bolt) after flowering is a GA-regulated process, and paclobutrazol treatment will inhibit elongation. Preliminary results indicate that *eaf1* plants are more resistant to paclobutrazol than wild-type Nossen in terms of bolt elongation, demonstrating that early developmental stages such as germination and late stages such as bolting are both altered in *eaf1*. These results support the idea that alteration in GA metabolism or signaling in *eaf1* is responsible for the early-flowering phenotype. Levels of paclobutrazol resistance observed with the Nossen wild type are higher than has been seen previously with wild-type seeds of Landsberg or Columbia ecotypes (D. Scott and M. Honma, unpublished data). Thus, the Nossen ecotype may produce more bioactive GA than other ecotypes or have an altered response to the hormone. Plants of the Nossen ecotype have leaves that are paler green with longer petioles than Landsberg or Columbia plants, phenotypes consistent with increased GA levels or GA signaling. Identification of loci that differ between Nossen and Landsberg responsible for resistance to paclobutrazol is currently in progress.

GA is known to promote vegetative-phase change and flowering in a variety of plants, including Arabidopsis (Chien and Sussex, 1996; Telfer et al., 1997). The *SPY*, *RGA*, and *GAI* genes are negative regulators of GA signaling (Jacobsen and Olszewski, 1993; Peng et al., 1997; Silverstone et al., 1997), and loss-of-function alleles exhibit phenotypes indicating that these genes act downstream of GA biosynthesis. The *spy* mutant of Arabidopsis, which is altered in response to GA, exhibits phenotypic modifications similar to GA-treated plants and is able to suppress most of the *ga1* phenotypic changes, including reduced germination. Although *eaf1* does possess some phenotypic alterations in common with *spy*, it does not show increased height or reduced fertility. However, because only one allele of *eaf1* is currently available, other mutant alleles may have more severe phenotypes or *eaf1* may regulate a different subset of GA-controlled functions than *spy*. Both *rga* and *spy* are able to suppress the late-flowering defect of *ga1* and accelerate the production of adult leaves (Silverstone et al., 1997). It is thought that *rga* functions downstream of GA biosynthesis, in a pathway independent of *spy* (Silverstone et al., 1997). Preliminary characterization of an *eaf1 ga1* double-mutant line suggests that *eaf1* is not able to suppress the germination defect of *ga1*, similar to *rga* and *gai* mutations (H. Ledford and M. Honma, unpublished data). If *eaf1* is also involved in GA response, this mutation will likely be able to suppress the late-flowering alteration of *ga1*. Thus, we would expect that *ga1 eaf1* would flower earlier than *ga1* under LD conditions. *GAI* also functions as a negative regulator of GA response, and GA can release this repression (Peng et al., 1997). Recently, *RGA* and *GAI* have been shown to encode proteins with similar amino acid sequences, suggesting that they may have redundant functions in GA signaling (Peng et al., 1997; Silverstone et al., 1998).

The role of GAs in promotion of flowering could be the consequence of early transition to the adult phase, which then hastens transition to reproductive development. Alternatively, regulation of transition to the adult phase might be independent of transition to the reproductive phase, but with components in common, one of which may be GAs. GAs could contribute to generate a signal that promotes phase transition or may function in making the meristem more competent to respond to such factors. Thus, GAs may act by causing developmental changes that eventually result in early flowering, rather than acting directly as a floral inducer. Recently it was shown that expression of the floral meristem identity gene *LEAFY* is regulated in response to GA (Blázquez et al., 1998). Construction of double-mutant lines altered in *eaf1* and the GA and phytochrome genes will allow study of genetic interactions between these genes. Such experiments are in progress and will indicate whether *eaf1* functions within the GA or photoperiod pathways.

Flowering-time mutants can be grouped into classes based on duration of juvenile and adult phases. To compare *eaf1* with other early-flowering mutants, it was introgressed into the Landsberg background and the duration of juvenile and adult phases and flowering time of all genotypes compared. *eaf1* appears to be most similar to the *spy* mutant, which shows reduced juvenile and adult phases. In contrast, the *hst* gene (Telfer and Poethig, 1998) appears to have a primary role during the juvenile phase. *hst* mutants exhibit a shortened juvenile phase and a normal-length adult phase, flower earlier than wild-type plants, and appear to be pleiotropic (Telfer and Poethig, 1998). When grown under LD conditions, we find that *tfl* mutant plants have a slightly shorter juvenile phase, a greatly shortened adult phase, and flower early, as has been previously reported (Shannon and Meeks-Wagner, 1991). The existence of mutants that primarily affect one phase but not the other would suggest that flowering and phase transition are separate processes, which may share common regulatory factors. *eaf1* defines a new locus in Arabidopsis that represses the shift to the adult phase. Future studies of *eaf1* in conjunction with GA response and flowering-time genes will further our understanding of the complex interactions

that control vegetative-phase change and reproductive development.

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