Polycomb repression of flowering during early plant development

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Contributed by Robert B. Goldberg, September 25, 2001

All plants flower late in their life cycle. For example, in *Arabidopsis*, the shoot undergoes a transition and produces reproductive flowers after the adult phase of vegetative growth. Much is known about genetic and environmental processes that control flowering time in mature plants. However, little is understood about the mechanisms that prevent plants from flowering much earlier during embryo and seedling development. *Arabidopsis embryonic flower* (*emf1* and *emf2*) mutants flower soon after germination, suggesting that a floral repression mechanism is established in wild-type plants that prevents flowering until maturity. Here, we show that polycomb group proteins play a central role in repressing flowering early in the plant life cycle. We found that mutations in the *Fertilization Independent Endosperm* (*FIE*) polycomb gene caused the seedling shoot to produce flower-like structures and organs. Flower-like structures were also generated from the hypocotyl and root, organs not associated with reproduction. Expression of floral induction and homeotic genes was derepressed in mutant embryos and seedlings. These results suggest that FIE-mediated polycomb complexes are an essential component of a floral repression mechanism established early during plant development.

Plants flower late in their life cycle, after embryogenesis, seedling development, and a period of vegetative growth (1). For example, during the *Arabidopsis* life cycle, double fertilization results in the formation of an embryo and endosperm, an organ that nourishes the embryo. The embryo develops within the seed and forms an axis, cotyledons, and shoot and root apical meristems (2). When the seed germinates, the seedling has two cotyledon leaves of embryonic origin. Subsequently, the shoot apical meristem generates a rosette of vegetative leaves. After a period of vegetative growth, in response to genetic and environmental signals, the shoot apical meristem of the adult plant undergoes a dramatic transition, and the inflorescence is initiated by internode elongation and the production of sessile cauline leaves and secondary inflorescences. Finally, in the late inflorescence phase, nodes give rise to solitary flowers comprised of whorls of sepals, petals, stamens, and carpels arranged in a crucifer phyllotaxis typical of *Arabidopsis* flowers (3).

A great deal is understood about the genes and environmental signals (e.g., day length, light quality, temperature) that control the transition to flowering in the mature *Arabidopsis* plant (4–6). This knowledge is due, in part, to the isolation and analysis of mutations in genes that accelerate or delay flowering time during the adult stage of *Arabidopsis* development after the formation of vegetative rosette leaves. By contrast, much less is known about processes that prevent the embryo or the seedling from initiating floral development. Seedlings with mutations in the *Embryonic Flower* (*EMF1* and *EMF2*) genes skip the vegetative phase of growth and immediately initiate inflorescence development (7, 8). Thus, *EMF* genes are required for normal vegetative shoot development. It is possible that *EMF* genes represent part of a floral repression mechanism, established early in plant development, that prevents flowering until later in the vegetative stage of plant development (3, 7, 9). Recently, *EMF1* was shown to encode a polypeptide that has no homology with any protein of known function (10). Thus, little is known about the molecular mechanisms responsible for early floral repression in the *Arabidopsis* seedling.

Arabidopsis Fertilization Independent Endosperm (FIE) is a Trp-Asp dipeptide (WD)-motif polycomb protein related to *Drosophila* Extra Sex Combs (ESC) and mouse Embryonic Ectoderm Development (EED) (11). The maternal *FIE* allele is required for proper development of the embryo and endosperm. Loss-of-function *fie* mutations cause precocious endosperm formation before fertilization and prolonged endosperm nuclear proliferation after fertilization, indicating that FIE functions as a suppressor of endosperm development (12–14). However, because embryos with a maternal null *fie* allele abort, it has not been possible to generate and examine the postembryonic phenotypes of homozygous *fie* mutant plants. Thus, the extent that FIE-mediated polycomb complexes might regulate postembryonic development is not known. We have addressed this issue by using a modified *FIE* transgene that specifically provides FIE protein for seed viability, revealing new functions for polycomb proteins during plant development. Here, we show that the shoot apical meristem of *fie* mutant seedlings skips vegetative development and produces flowers and floral organs. Floral-like shoots were also observed on the seedling hypocotyl and root. Moreover, *fie* mutant embryos and seedlings activate the expression of floral induction genes. These results indicate that FIE-mediated polycomb complexes constitute a floral repression mechanism that is established very early during plant development.

Materials and Methods

Plant Materials. Seedlings were grown on hormone-free agar plates [0.5× MS salts (GIBCO/BRL)], 2% sucrose, Gamborg's B5 vitamins (GIBCO/BRL), and 0.8% agar (Difco). Plants were grown on soil under long day conditions (16 h light). *LFY*::*GUS* (*LFY* promoter ligated to *GUS* cDNA) seed was provided by the *Arabidopsis* Biological Resource Center (CS6297). *AG*::*GUS* (*AG* promoter ligated to *GUS* cDNA; KB9) and *AP3*::*GUS* (*AP3* promoter ligated to *GUS* cDNA; 890-7) seed were provided by D. Weigel and T. Jack.

Transgenic Plants. To construct a FIE-green fluorescent protein (GFP) fusion protein, the *FIE* cDNA was amplified with primers *FIE-Sal* (5′-ATGTCGACGAGAGTCAGACAGAGAG-AGAG-3′) and *FIE-Nco*I (5′-CACCATGGCTCCGCCACCTC-

Abbreviations: FIE, Fertilization Independent Endosperm; GFP, Green Fluorescent Protein; *pFIE*::*FIE-GFP*, *FIE* promoter ligated to *FIE* and *GFP* cDNA sequences; *pCaMV*::*FIE-GFP*, cauliflower mosaic virus promoter ligated to *FIE* and *GFP* cDNA sequences; GUS, β-glucuronidase; *LFY*::*GUS*, *LFY* promoter ligated to *GUS* cDNA; *AG*::*GUS*, *AG* promoter ligated to *GUS* cDNA; *AP3*::*GUS*, *AP3* promoter ligated to *GUS* cDNA; EMF, Embryonic Flower.

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 www.pnas.org/cgi/doi/10.1073/pnas.241507798

CGCCACCCTTGGTAATCACGTCCCAGCG-3′), digested with *Sal*I and *Nco*I, and inserted into the *CaMV35S-sGFPS65T-Nos* vector (15) to obtain the *CaMV35S*::*FIE-sGFPS65T-Nos* plasmid. The *GFP* gene used in these experiments lacks subcellular localization sequences. To have transcription of the *FIE-GFP* fusion gene under the control of a *FIE* promoter, 1,639 base pairs of *FIE* 5′-flanking sequences were amplified with primers *FIE-Sph* (5′-TTCCTATAAGAGGCATGCGAGGAAGCGAGCAAGTACACA-3′) and *FIE-SalRV* (5′-TCTGACTCTCGTCGACTAATCTAAGCTCACAAGTCTCTCA-3′), digested with *Sal*I and Sph, and inserted into the *CaMV35S*::*FIE-sGFPS65T-Nos* plasmid to create the *CaMV35S*::*pFIE*::*FIE-sGFPS65T-Nos* plasmid. This plasmid was digested with *Pst*I and *Hin*dIII to liberate the *pFIE*::*FIE-GFP* (*FIE* promoter ligated to *FIE* and *GFP* cDNA sequences) transgene that was then inserted into pBI101.1 (16), replacing the β-glucuronidase (GUS) reporter, to create plasmid *pBI(pFIE*::*FIE-GFP)* that was introduced into Agrobacterium GV3101. *Arabidopsis* plants (i.e., Columbia-0 ecotype) were transformed as described (11). Transgenic plants were crossed with heterozygous *fie-1*/*FIE* plants, and F_1 progeny were self-pollinated to generate plants heterozygous for *fie-1*/*FIE* and homozygous for *pFIE*::*FIE-GFP*. Expression of *pFIE*::*FIE-GFP* was highly restricted compared with previously reported constructs (17), and this result may be due, at least in part, to the process of creating a *Sal*I site in the *FIE* cDNA that changed nucleotides −38 to −36 (translation start site is position 1) in the 5′-untranslated region from GTG to CGA.

Plant Genotypes. To amplify endogenous *FIE* gene sequences we used primers *579dXba* (5′-CATTACTGCCATTGGTGTATCTCTTATTATCTA-3′) and *48S4* (5′-CACTGTTGACGTCAATGACTCGG-3′). Because the *579dXBa* primer is located in the first intron of the *FIE* gene, it does not amplify any sequences associated with the *pFIE*::*FIE-GFP* transgene. We distinguished *fie-1* and wild-type *FIE* alleles by digesting the amplified products with *Xba*I restriction endonuclease followed by agarose gel electrophoresis. The PCR amplified product from the wild-type *FIE* allele is digested, whereas the *fie-1* allele is not. To amplify *pFIE*::*FIE-GFP* transgene sequences, we used a primer in the *FIE* cDNA region, *FIE-RTf* (5′-CTGTAATCAGGCAAACAGCC-3′) and a primer in the *GFP* cDNA region, *GFP274r* (5′-GCATGGCGGACTTGAAGA-3′). PCR reactions were performed as described (17). Seedlings with the *pFIE*::*FIE-GFP* transgene were identified by growth on agar plates with 50 μg/ml kanamycin.

Gene Expression. RNA (0.2 μg) was converted to cDNA as described (18). Amplification of cDNA by PCR involved incubation at 94°C for 2 min, followed by 30 cycles except when indicated at 94°C for 30 s, 55°C for 30 s, and 72°C for 10 s. Gene-specific primers were as follows: *AP1* (AP1500f 5′-GATGATATAAGAACATCGAACATTTGCCA-3′ and AP1991r 5′-GATGATATAAGAACATCGAACATTTGCCA-3′), *LFY* (LFY4042f 5′-GCTAAAGACCGTGGCGAA-3′ and LFY5371r 5′-GCATCCACCACGTCCAGA-3′), *AG* (AG5523f 5′-GTTGATTTGCATAACGATAACCAGA-3′ and AG6116r 5′-TTCACTGATACAACATTCATGGGAT-3′), *PI* (PI1500f 5′-CACGCCATTGAACATGGCCT-3′ and PI2020r 5′-TCGATGATCAATCGATGACCAA-3′), actin (ACT.conf 5′-GATTTGGCATCACACTTTCTACAATG-3′ and ACT.conr 5′-GTTCCACCACTGAGCACAATG-3′), *PK* (PKf 5′-CTTCACCACATGGGTCACA-3′ and PKr2 5′-CTAAACCGGAAGGAATGGA-3′), *APG* (APGf 5′-CTTGTGTCTCTGGTTGATCA-3′ and APGr2 5′-CTCTGTGTTTGCTTGGAGGA-3′), *FIE* (FIE-RTr 5′-TCAAGGTCTCAGGGAGTAGCA-3′ and FIE-RTf), and *FIE-GFP* (FIE-RTf and GFP274r).

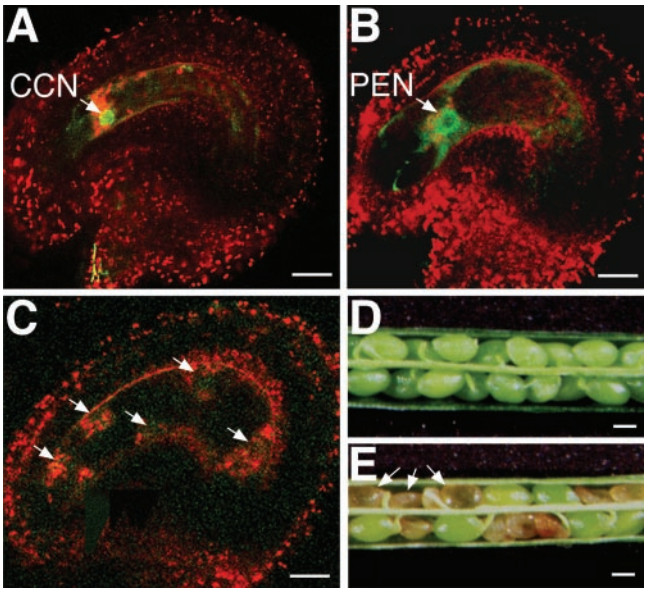

Fig. 1. Expression of a *pFIE*::*FIE-GFP* transgene rescues viability of seeds with a maternal mutant *fie-1* allele. In *A*–*C*, stage 12 flowers (11) from homozygous *pFIE*::*FIE-GFP* transgenic plants were emasculated and manually self-pollinated. Siliques were harvested at the indicated time and dissected, and isolated ovules were mounted on slides. GFP fluorescence was visualized by confocal laser microscopy. Excitation wave-length was 488 nm, GFP emission was 500 to 530 nm, and chlorophyll florescence was above 650 nm. GFP and chlorophyll florescences were converted to green and red, respectively. (*A*) Mature ovule before fertilization. (*B*) Seed 6 h after pollination. (*C*) Seed with an eight-nuclei endosperm 24 h after pollination. Arrows point to endosperm nuclei. (*D*) Silique that is heterozygous *fie-1*/*FIE* and homozygous for the *pFIE*::*FIE-GFP* transgene. (*E*) Heterozygous *fie-1*/*FIE* silique. Arrows point to aborted seeds. In *A*–C, bars = 20 μm. In *D* and *E*, bars = 0.4 mm. CCN, central cell nucleus; PEN, primary endosperm nucleus.

Results

Rescue of *fie* Seed Viability by the *pFIE*::*FIE-GFP* Transgene. To understand FIE polycomb function, and to visualize the pattern of recombinant FIE protein accumulation, we generated *Arabidopsis* lines bearing a transgene, *pFIE*::*FIE-GFP*, with 1,639 bp of *FIE* 5′-flanking sequences (*pFIE*), a modified *FIE* 5′-untranslated region, a sequence that encodes the full-length FIE protein (*FIE*), a linker sequence encoding six glycine amino acids, and then a sequence encoding a green fluorescent protein (*GFP*; ref. 15). GFP fluorescence was detected in the progenitor of the endosperm, the central cell nucleus, before (Fig. 1*A*) and after (Fig. 1*B*) fertilization. By the eight-nuclei endosperm stage, GFP fluorescence was no longer detectable (Fig. 1*C*), and could not be detected at any later stage of plant development (data not shown). Nor could *FIE-GFP* RNA be detected in *pFIE*::*FIE-GFP* transgenic seedlings when the endogenous *FIE* gene is expressed (Fig. 2). Thus, expression of the *pFIE*::*FIE-GFP* transgene in the very early stages of seed development, observed in multiple independently transformed lines, was highly restricted when compared with the pattern of *FIE* RNA accumulation in the ovule, female gametophyte, embryo, endosperm, seedling (Fig. 2), leaf, stem, root, and flower (11, 19). Although *pFIE*::*FIE-GFP* transgene expression did not recapitulate the wild-type pattern of *FIE* RNA accumulation, perhaps because of a requirement for additional regulatory sequences and/or the sensitivity of GFP fluorescence detection, it was able to complement the *fie* seed abortion phenotype. That is, self-pollinated heterozygous *fie-1*/*FIE* plants that are homozygous for the *pFIE*::*FIE-GFP* transgene displayed siliques with no seed abortion (Fig. 1*D*). When germinated, we

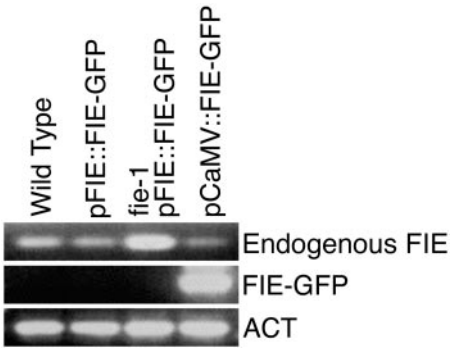

Fig. 2. Expression of the endogenous *FIE* gene, *pFIE*::*FIE-GFP*, and *CaMV*::*FIE-GFP* transgenes in seedlings. Lane designations refer to seedlings used for RNA isolation: pFIE::FIE-GFP, homozygous for the wild-type *FIE* allele and the *pFIE*::*FIE-GFP* transgene; fie-1 pFIE::FIE-GFP, homozygous for the mutant *fie-1* allele with at least one copy of the *pFIE*:: *FIE-GFP* transgene; pCaMV::FIE-GFP, homozygous for the wild-type *FIE* allele and the *pCaMV*::*FIE-GFP* transgene. Total RNA from 7-day seedlings was isolated (18) and was amplified by reverse transcription–PCR as described in *Materials and Methods*, except that 40 cycles were used. ACT, actin RNA; Endogenous FIE, RNA from the endogenous *FIE* or *fie-1* allele as indicated in the lane designation.

observed seedlings, all homozygous for the *pFIE*::*FIE-GFP* transgene, with Mendelian 1:2:1 segregation (20:28:12, $\chi^2 = 2.6$, $P = 0.35$) of *FIE*/*FIE*:*fie-1*/*FIE*:*fie-1*/*fie-1* genotypes. In contrast, siliques from control self-pollinated *fie-1*/*FIE* plants contain 50% nonviable seeds (Fig. 1*E*) and when germinated the seedlings segregate 1:1 for *FIE*/*FIE*:*fie-1*/*FIE* genotypes. Thus, expression of the *pFIE*::*FIE-GFP* transgene in the central cell and early endosperm rescues embryo and seed abortion associated with inheritance of a maternal mutant *fie-1* allele. This result shows that the FIE-GFP fusion protein is active and suggests that *fie* embryo and seed abortion may be due primarily to a defect in endosperm development.

Mutant *fie* Seedlings Produce Flowers. Plants homozygous for the *pFIE*::*FIE-GFP* transgene that were either homozygous for the wild-type *FIE* allele, or heterozygous *fie-1*/*FIE*, developed normally (Fig. 3*A* and data not shown). Thus, in the presence of a wild-type *FIE* allele, the *pFIE*::*FIE-GFP* transgene caused no detectable alteration in plant development. However, none of the seedlings tested that were homozygous for the *fie-1* allele and the *pFIE*::*FIE-GFP* transgene developed normally. These seedlings were smaller (Fig. 3*B*) and did not produce normal rosettes. In some cases, highly disorganized structures emerged from the shoot apical meristem (Fig. 3*C*). Seedlings often produced sessile leaves (Fig. 3*D*) with trichomes (Fig. 3 *J* and *K*) that resembled cauline leaves. We also observed white petal-like organs lacking trichomes (Fig. 3*E*). In some cases, floral buds emerged that were surrounded by sessile leaves (Fig. 3 *F* and *K*). Within the floral buds, outer whorl sepals could be distinguished by the appearance of unbranched trichomes on their abaxial surfaces (Fig. 3*K*) and by their highly elongated cells (Fig. 3*L*). Inner whorl organs whose distinctive shapes resembled immature stamens and carpels were also observed (Fig. 3*L*). Finally, floral organs within flower buds often displayed proper floral crucifer phyllotaxy (Fig. 3 *F* and *L*). The mutant phenotypes described above were observed in multiple independently isolated transgenic lines (data not shown) as well as in seedlings homozygous for the null *fie-1* allele and for a *FIEp*::*FIE* transgene that produces FIE protein without a GFP moiety (data not shown). Thus, the mutant phenotypes were not due to any effect of GFP on the structure or cellular location of FIE. Taken together, these results show that the transition from vegetative to inflorescence development has prematurely occurred in the shoot

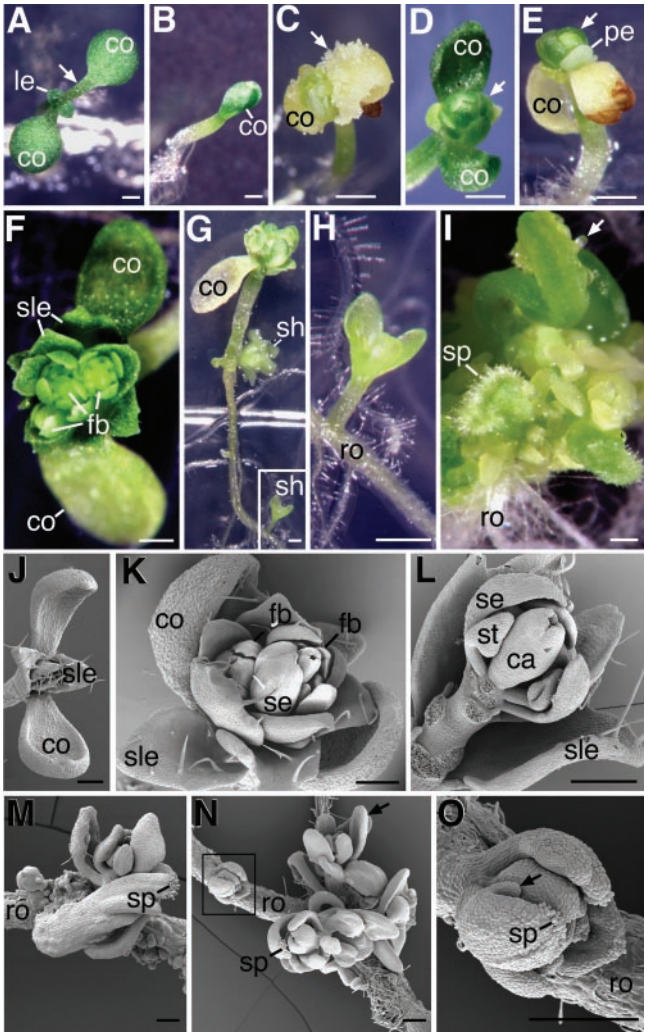

Fig. 3. Early flowering phenotypes. Seedlings were analyzed by light microscopy (*A–I*) or scanning electron microscopy (*J–O*). (*A*) Control seedling homozygous for the wild-type *FIE* allele and the *pFIE*::*FIE-GFP* transgene 6 days after germination. Arrow points to cotyledon petiole. (*B–O*) Seedlings homozygous for the null *fie-1* allele and the transgene, *pFIE*::*FIE-GFP*. (*B*) Mutant seedling 4 days after germination. (*C*) Three-week mutant seedling. Arrow points to region of disorganized growth. (*D*) Ten-day mutant seedling. Arrow indicates sessile leaf. (*E*) Three-week mutant seedling. Arrow indicates sessile leaf. (*F*) Three-week mutant seedling. (*G*) Four-week mutant seedling. The boxed region is magnified in *H* showing the shoot emerging from the root. (*I*) A portion of a root from a 1-week seedling was excised and cultured for 3 weeks on hormone-free agar media. Arrow points to an ovule-like primordium. (*J*) One-week mutant seedling. (*K*) Three-week mutant seedling. (*L*) Three-week mutant seedling. Cotyledons, sessile leaves, secondary flower buds, sepals, petals, and stamens have been removed to reveal sepal, stamen, and carpel organs in a single flower bud. (*M*) Root of a 4-week intact seedling. (*N*) Intact root of a 4-week seedling. Arrow points to organs arranged in a floral crucifer phyllotaxy. A second shoot in the boxed region magnified in *O* has an organ with stigmatic papillae and an ovule-like primordium (arrow). ca, carpel; co, cotyledon; fb, flower bud; le, vegetative rosette leaf; pe, petal; ro, root; se, sepal; sh, shoot; sle, sessile leaf; sp, stigmatic papillae; st, stamen. Bars in *A–I* = 0.5 mm. Bars in *J–O* = 0.25 mm.

apical meristem of seedlings homozygous for the null *fie-1* allele and the *pFIE*::*FIE-GFP* transgene. This result suggests that one of the functions of the *FIE* gene is to repress the transition to flowering in the seedling shoot apical meristem.

Production of shoots and flower-like organs was not limited to the shoot apical meristem in seedlings homozygous for the *fie-1* allele and the *pFIE*::*FIE-GFP* transgene. Shoots frequently

emerged from the hypocotyl and roots of intact seedlings (Fig. 3 *G* and *H*). Carpel-shaped organs with stigmatic papillae at their tips were present in such adventitious shoots (Fig. 2*M*). In addition, organs tipped with stigmatic papillae and with structures resembling ovule primordia on their edges were observed (Fig. 3 *N* and *O*). In some cases, shoots with organs arranged in a floral crucifer phyllotaxy were observed (Fig. 3*N*). When roots were cultured in hormone-free media, many additional organs were produced with stigmatic papillae and structures resembling ovule primordia (Fig. 3*I*). These results show that ectopic shoots and organs resembling flowers were produced in seedlings homozygous for the *fie-1* allele and the *pFIE*::*FIE-GFP* transgene. This result suggests that another function of the wild-type *FIE* allele is to repress the formation of shoots and floral organs outside of the seedling shoot apical meristem region.

Rescue of *fie* Vegetative Development by the *pCaMV*::*FIE-GFP* Transgene. The mutant phenotypes described above were observed in transgenic *pFIE*::*FIE-GFP* seedlings that were also homozygous for the null *fie-1* allele, and were never observed when a wild-type *FIE* allele was present. Thus, the *pFIE*::*FIE-GFP* transgenic allele is recessive to the wild-type *FIE* allele. This fact, along with the restriction of *pFIE*::*FIE-GFP* gene expression to very early seed development (Fig. 1 *A–C*), suggests that loss of FIE activity is responsible for early flowering and ectopic shoot formation in seedlings that are homozygous for the null *fie-1* allele and have a *pFIE*::*FIE-GFP* transgene. From this result, we reasoned that expanding the period of FIE activity during embryogenesis and seedling development with a different transgene might result in plants that develop more normally. To test this hypothesis, we generated *pCaMV*::*FIE-GFP* transgenic plants where transcription of *FIE-GFP* is under the control of the cauliflower mosaic virus (*pCaMV*) promoter (20). In *pCaMV*::*FIE-GFP* lines, we detected a broad pattern of GFP fluorescence throughout embryo development, including the later stages, that diminished in the germinating seedling (data not shown). Whereas *pFIE*::*FIE-GFP* gene expression was not detected in seedlings, *FIE-GFP* RNA encoded by the *pCaMV*::*FIE-GFP* gene was present (Fig. 2). By genetic crosses, we obtained plants that were homozygous for *fie-1*, *pFIE*::*FIE-GFP*, and *pCaMV*::*FIE-GFP*. These plants did not display any of the mutant phenotypes shown in Fig. 3 and could not be distinguished from wild-type plants. They produced rosette leaves, followed by inflorescences, fertile flowers, and siliques with viable seed (data not shown). Thus, we were able to adjust the level of FIE activity with the two transgenes to recapitulate the wild-type flowering time phenotype. These results verify that early flowering and ectopic shoot and floral organ formation is due to a critical lack of FIE activity in seedlings homozygous for *fie-1* and the *pFIE*::*FIE-GFP* transgene.

Expression of Floral Induction and Homeotic Genes in *fie* Embryos and Seedlings. To understand the molecular basis for the early flowering phenotypes, we measured the expression of meristem and floral organ identity genes in mutant and control seedlings. *LEAFY* (*LFY*) and *APETALA1* (*AP1*) encode transcription factors that promote floral meristem identity in *Arabidopsis* and, with the aid of other factors, activate transcription of floral meristem and organ identity genes such as *AGAMOUS* (*AG*), *APETALA3* (*AP3*), and *PISTILLATA* (*PI*) (21). As shown in Fig. 4*A*, *LFY* RNA was present at a low level, and *AP1* RNA was not detected in wild-type 7-day and 14-day seedlings. The same result was observed in 7- and 14-day seedlings containing the *pFIE*::*FIE-GFP* transgene. However, in seedlings that were homozygous *fie-1* with a *pFIE*::*FIE-GFP* transgene, *LFY* RNA concentration was significantly elevated in 7-day seedlings, and *AP1* RNA concentration was increased in both 7-day and 14-day

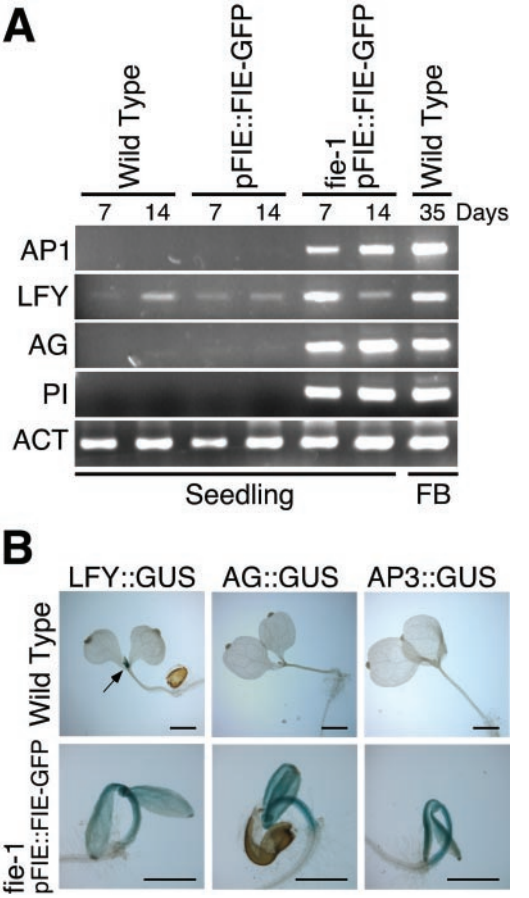

Fig. 4. Analysis of expression of floral genes in seedlings. (*A*) RNA analysis. Lane designations refer to seedlings used for RNA isolation: pFIE::FIE-GFP, homozygous for the wild-type *FIE* allele and the *pFIE*::*FIE-GFP* transgene; fie-1 pFIE::FIE-GFP, homozygous for the mutant *fie-1* allele with at least one copy of the *pFIE*::*FIE-GFP* transgene. Total RNA from seedlings was isolated at the indicated day after germination, or from stage 1 to stage 12 (11) floral buds (FB) and was amplified by reverse transcription–PCR as described in *Materials and Methods*. (*B*) Analysis of floral promoter activity. β-glucuronidase enzyme activity was measured as described (16). Arrow points to GUS stained shoot apical meristem region in the *LFY*::*GUS* seedling. fie-1 pFIE::FIE-GFP, seedlings that are homozygous for the mutant *fie-1* allele with at least one copy of the *pFIE*::*FIE-GFP* and indicated reporter transgene. Bars = 1 mm. ACT, actin RNA.

seedlings. No effect on the expression of other genes that accelerate flowering time, *CONSTANS* (*CO*; ref. 22) or *FLOWERING LOCUS T* (*FT*; refs. 23 and 24), was observed (data not shown). Because ectopic expression of either *LFY* or *AP1* is sufficient to convert the normally indeterminate shoot apex to a floral meristem that forms a terminal flower (25, 26), it is likely that aspects of the *fie* early flowering phenotype are due to their ectopic expression. Floral organ identity gene expression was also affected by the level of FIE activity. Whereas *AG* and *PI* RNAs were not detected in control wild-type seedlings, or control seedlings with the *pFIE*::*FIE-GFP* transgene, both RNAs accumulated in homozygous *fie-1*, *pFIE*::*FIE-GFP* seedlings (Fig. 4*A*). Taken together, these results suggest that FIE-mediated polycomb complexes, either directly or indirectly, repress expression of both floral meristem identity genes and floral organ identity genes during seedling development.

To investigate the spatial regulation of gene transcription by FIE-mediated polycomb complexes, we determined the activity of *LFY*, *AG*, and *AP3* promoters ligated to a *β-glucuronidase* (*GUS*) reporter gene (16) in transgenic seedlings. As shown in

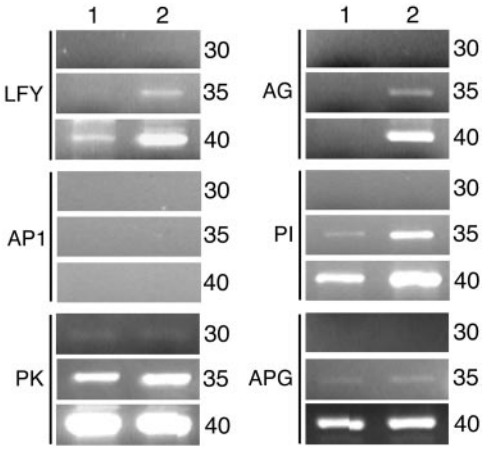

Fig. 5. Analysis of expression of floral genes in embryos. Total RNA was isolated from walking stick stage embryos dissected from either self-pollinated wild-type siliques (lane 1) or from self-pollinated siliques that were heterozygous *fie-1/FIE* and homozygous for the *pFIE*::*FIE-GFP* transgene (lane 2). Twenty-five percent of the embryos used for RNA isolation in lane 2 were predicted to be homozygous for *fie-1* and for the *pFIE*::*FIE-GFP* transgene. As controls, we measured the expression of two genes that flank the *AG* gene in the *Arabidopsis* genome: PK (protein kinase-like protein; CAB78897.1) and APG (proline rich protein; CAB78899.1). Cycles, number of PCR cycles.

Fig. 4*B*, *LFY*::*GUS* transcription was restricted to the wild-type seedling shoot apical meristem, whereas there was no detectable transcription of the *AG*::*GUS* or *AP3*::*GUS* transgenes in wild-type seedlings. In contrast, homozygous *fie-1* seedlings with the *pFIE*::*FIE-GFP* transgene showed elevated *LFY*::*GUS*, *AG*::*GUS*, and *AP3*::*GUS* transcription in the shoot apical meristem, cotyledon, and hypocotyl regions. These results show that FIE-mediated polycomb complexes, either directly or indirectly, repress transcription of floral meristem identity and floral organ identity genes in the aerial seedling.

When does repression of flowering and floral-promoting gene transcription begin? One possibility is that aspects of floral repression initiate early in plant development during embryogenesis. To test this hypothesis, embryo RNA was isolated from self-pollinated plants heterozygous for *fie-1/FIE* and homozygous for the *pFIE*::*FIE-GFP* transgene (Fig. 5, lane 2). Twenty-five percent of these embryos are homozygous for *fie-1* and the *pFIE*::*FIE-GFP* transgene. Control wild-type embryo RNA was also isolated (Fig. 5, lane 1). Semiquantitative reverse transcription–PCR analysis indicated that the level of *LFY*, *AG*, and *PI* RNA was elevated in the population that included homozygous *fie-1*, *pFIE*::*FIE-GFP* embryos. In this experiment, we could not detect *AP1* embryo RNA in either embryo population, perhaps because a factor not regulated by *FIE* is required for *AP1* expression in the embryo. Finally, expression of control genes *PK* and *APG* was the same in both embryo populations. These results suggest that FIE-mediated polycomb complexes function during embryogenesis, either directly or indirectly, to repress the expression of specific floral meristem and organ identity genes.

Discussion

Polycomb proteins represent an evolutionarily highly conserved cellular memory system that maintains transcriptional repression of target genes (27). For example, during *Drosophila* embryogenesis, gap proteins repress homeotic gene transcription outside their appropriate expression domain. However, gap proteins are only transiently expressed, and it is the formation of polycomb complexes that ensure that the repressed state is maintained over many cell divisions. Failure to maintain repression because of mutations in polycomb genes can result in dramatic homeotic transformations of organ identity. Here, we show that the plant FIE polycomb protein, either directly or indirectly, represses homeotic gene transcription (e.g., *LFY*, *AP1*, *AG*, *AP3*, and *PI*) outside their appropriate temporal and spatial domains during embryo and seedling development (Figs. 4 and 5). Diminished FIE activity because of loss-of-function mutations in the *FIE* gene results in premature flowering by the seedling shoot apical meristem and inappropriate formation of shoots and flower-like structures along the root and hypocotyl (Fig. 3). Previously, we showed that the FIE polycomb protein prevents premature formation of an endosperm before fertilization (11). Taken together, these results demonstrate that, similar to animal systems (27), plant polycomb proteins function to repress inappropriate spatial and temporal programs of gene transcription and development very early in the plant life cycle.

Multiple genes have been shown to repress flowering during *Arabidopsis* development. Loss-of-function mutations in the cloned genes *CLF, EBS, HY1, PHYB, ELF3, HY2, SPY*, and *TFL* all result in accelerated flowering. However, these mutant plants flower only after the production of embryonic cotyledon leaves and multiple vegetative rosette leaves by the shoot apical meristem (28–33). By contrast, *fie* (Fig. 3) and *emf* (7, 8) mutant seedlings can skip the normal adult vegetative phase and flower. Thus, *FIE* and *EMF* genes function uniquely during the earliest stages of plant development to repress flowering.

WD motif polycomb proteins related to FIE in mammals and *Drosophila* function by assembling polycomb protein complexes (27). The WD motifs form surface loops that are used as scaffolds for the generation of protein complexes that include additional polycomb proteins (e.g., SET-domain polycombs) and histone deacetylase. These complexes remodel chromatin and repress gene transcription (27). With regard to its endosperm repression function, FIE is likely to be associated with a complex that includes the FIS2 zinc finger protein and the MEA SET-domain polycomb protein. Evidence for complex formation among these three proteins includes the similar mutant phenotypes of loss-of-function mutations in the *FIE*, *MEA*, and *FIS2* genes, and experiments which show that FIE and MEA directly interact (17, 19, 34). Analogous to the *Drosophila* ESC polycomb, FIE may be recruited to specific chromatin sites by a zinc finger protein such as FIS2. However, in contrast to what is observed for *fie* seedlings, homozygous *mea* and *fis2* mutant seedlings do not display early flowering phenotypes (14, 35), strongly suggesting that FIE represses flowering by associating with other protein partners. To repress flowering, it is possible that FIE interacts with additional SET-domain polycomb group proteins encoded in the *Arabidopsis* genome (36). One candidate is the SET-domain polycomb protein Curly Leaf (CLF), because mature *clf* mutant plants display somewhat early flowering and ectopic *AG* and *AP3* expression in vegetative leaves (28). However, the fact that *clf* mutant seedlings do not flower suggests that FIE may interact with other SET-domain polycomb proteins that function to repress flowering during the early phases of plant development. FIE may also interact, directly or indirectly, with proteins encoded by *EMF* genes. This hypothesis is supported by the fact that *emf1* and *emf2* mutant seedlings bear a striking resemblance to *fie* seedlings and display derepressed *AP1* and *AG* promoter activity (8, 37). FIE, encoded by a single-copy gene in the *Arabidopsis* genome (11), therefore might form complexes with distinct transcription factors and polycomb proteins in the embryo and seedling to repress transcription of floral induction and homeotic genes.

In animals, the formation of polycomb complexes ensures that the repressed state is mitotically stable and maintained over many cell divisions, perhaps over the lifetime of the organism. However, the repression of development by plant polycomb proteins, at least in some cases, appears to be reversible during the plant life cycle. For example, repression of endosperm

development ends immediately after fertilization of the central cell, and flowering at the shoot apical meristem, initially repressed in the embryo and seedling, initiates when the plant reaches the appropriate stage of development. Recently, it has been shown in *Drosophila* that there is a discontinuity in polycomb silencing of a transgene between embryo and subsequent larval stages, suggesting that differences exist between the maintenance properties of polycomb complexes at different stages of development (38). It is possible that modulation of polycomb repression occurs during plant development as well.

A model has been proposed whereby flowering initiates when floral repression has decreased in response to genetic (i.e., autonomous and gibberellin pathways) and environmental (i.e., vernalization and photoperiod pathways) signals (3, 7–9). An alternative model states that the genetic and environmental promotion pathways are directly integrated at the promoters of the floral meristem identity genes and do not directly interact with a floral repression mechanism (4). These models are not mutually exclusive and it is possible that aspects of both contribute to the control of flowering. Results from this study reveal that, early during the plant life cycle, FIE-mediated polycomb protein complexes prevent flowering in both the shoot apical meristem and along the hypocotyl and root of the seedling. It is not known how the effect of polycomb repression is diminished in the shoot apical meristem where flowering occurs later in plant development. One possibility is that genetic and environmental promotion pathways produce transcriptional activators that can function in the presence of polycomb complexes. Alternatively, polycomb complex activity may diminish in the shoot apical meristem during plant development. This result might be due to reduced polycomb gene expression, modification of a key polycomb protein, failure to maintain the polycomb complex during mitosis, and/or antagonistic interactions between polycomb proteins and components of the genetic and environmental promotion pathways. Additional experiments are needed to determine whether there is a relationship between polycomb-mediated embryonic and seedling floral repression and the activation of floral promotion pathways later in plant development.

This research was supported by U.S. Department of Agriculture (USDA; 2000-01539), Ceres, Inc. (B970602), and Binational Agricultural Research and Development (USDA; IS-3158-99C) grants to R.L.F. and by a fellowship from the Japan Society for the Promotion of Science to T.K.

1. Walbot, V. (1985) *Trends Genet.* **1,** 165–169.
2. Goldberg, R. B., de Paiva, G. & Yadegari, R. (1994) *Science* **266,** 605–614.
3. Haughn, G. W., Schultz, E. A. & Martinez-Zapater, J. M. (1995) *Can. J. Bot.* **73,** 959–981.
4. Araki, T. (2001) *Curr. Opin. Plant Biol.* **4,** 63–68.
5. Koornneef, M., Alonso-Blanco, C., Peeters, A. J. M. & Soppe, W. (1998) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **49,** 345–370.
6. Levy, Y. Y. & Dean, C. (1998) *Plant Cell* **10,** 1973–1998.
7. Sung, Z. R., Belachew, A., Shunong, B. & Bertrand-Garcia, R. (1992) *Science* **258,** 1645–1647.
8. Yang, C.-H., Chen, L.-J. & Sung, Z. R. (1995) *Dev. Biol.* **169,** 421–435.
9. Weigel, D. (1995) *Annu. Rev. Genet.* **29,** 19–39.
10. Aubert, D., Chen, L., Moon, Y.-H., Martin, D., Castle, L. A., Yang, C.-H. & Sung, Z. R. (2001) *Plant Cell* **13,** 1865–1875.
11. Ohad, N., Yadegari, R., Margossian, L., Hannon, M., Michaeli, D., Harada, J. J., Goldberg, R. B. & Fischer, R. L. (1999) *Plant Cell* **11,** 407–415.
12. Vinkenoog, R., Spielman, M., Adams, S., Fischer, R. L., Dickinson, H. G. & Scott, R. J. (2000) *Plant Cell* **12,** 2271–2282.
13. Ohad, N., Margossian, L., Hsu, Y.-C., Williams, C., Repetti, P. & Fischer, R. L. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 5319–5324.
14. Chaudhury, A. M., Ming, L., Miller, C., Craig, S., Dennis, E. S. & Peacock, W. J. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 4223–4228.
15. Niwa, Y., Hirano, T., Yoshimoto, K., Shimizu, M. & Kobayashi, H. (1999) *Plant J.* **18,** 455–463.
16. Jefferson, R. A., Kavanagh, T. A. & Bevan, M. V. (1987) *EMBO J.* **6,** 3901–3907.
17. Yadegari, R., Kinoshita, T., Lotan, O., Cohen, G., Katz, A., Choi, Y., Katz, A., Nakashima, K., Harada, J. J., Goldberg, R. B., *et al*. (2000) *Plant Cell* **12,** 2367–2381.
18. Kinoshita, T., Yadegari, R., Harada, J. J., Goldberg, R. B. & Fischer, R. L. (1999) *Plant Cell* **11,** 1945–1952.
19. Spillane, C., MacDougall, C., Stock, C., Kohler, C., Vielle-Calzada, J.-P., Nunes, S. M., Grossniklaus, U. & Goodrich, J. (2000) *Curr. Biol.* **10,** 1535–1538.
20. Rogers, S. G., Klee, H. J., Horsch, R. B. & Fraley, R. T. (1987) *Methods Enzymol.* **153,** 253–277.
21. Parcy, G., Nilsson, O., Busch, M. A., Weigel, I. L. & Weigel, D. (1998) *Nature (London)* **395,** 561–566.
22. Putterill, J., Robson, R., Lee, K., Simon, R. & Coupland, G. (1995) *Cell Mol. Life Sci.* **80,** 847–857.
23. Kobayashi, Y., Kaya, H., Goto, K., Iwabuchi, M. & Araki, T. (1999) *Science* **286,** 1960–1962.
24. Kardailsky, I., Shukla, V. K., Ahn, J. H., Dagenail, N., Christensen, S. K., Nguyen, J. T., Chory, J., Harrison, M. J. & Weigel, D. (1999) *Science* **286,** 1962–1965.
25. Weigel, D. & Nilsson, O. (1995) *Nature (London)* **377,** 495–500.
26. Mandel, M. A. & Yanofsky, M. F. (1995) *Nature (London)* **377,** 522–524.
27. Francis, N. J. & Kingston, R. E. (2001) *Nat. Rev. Mol. Cell Biol.* **2,** 409–421.
28. Goodrich, J., Puangsomlee, P., Martin, M., Long, D., Meyerowitz, E. M. & Coupland, G. (1997) *Nature (London)* **386,** 44–51.
29. Goto, N., Kumagai, T. & Koornneef, M. (1991) *Physiol. Plant.* **83,** 209–215.
30. Zagotta, M. T., Hicks, K. A., Jacobs, C. I., Young, J. C., Hangarter, R. P. & Meeks-Wagner, R. (1996) *Plant J.* **10,** 691–702.
31. Jacobsen, S. E. & Olszewski, N. E. (1993) *Plant Cell* **5,** 887–896.
32. Shannon, S. & Meeks-Wagner, R. (1991) *Plant Cell* **3,** 877–892.
33. Gomez-Mena, C., Pineiro, M., Franco-Zorrilla, J. M., Salinas, J., Coupland, G. & Martinez-Zapater, J. M. (2001) *Plant Cell* **13,** 1011–1024.
34. Luo, M., Bilodeau, P., Koltunow, A., Dennis, E. S., Peacock, W. J. & Chaudhury, A. M. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 296–301.
35. Kiyosue, T., Ohad, N., Yadegari, R., Hannon, M., Dinneny, J., Wells, D., Katz, A., Margossian, L., Harada, J., Goldberg, R. B. *et al*. (1999) *Proc. Natl. Acad. Sci. USA* **96,** 4186–4191.
36. The *Arabidopsis* Genome Initiative. (2000) *Nature (London)* **408,** 796–815.
37. Chen, L., Cheng, J.-C., Castle, L. & Sung, Z. R. (1997) *Plant Cell* **9,** 2011–2024.
38. Poux, S., McCabe, D. & Pirrotta, V. (2001) *Development (Cambridge, U.K.)* **128,** 75–85.