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EMBRYONIC FLOWER2, a Novel Polycomb Group Protein Homolog, Mediates Shoot Development and Flowering in Arabidopsis

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In higher plants, developmental phase changes are regulated by a complex gene network. Loss-of-function mutations in the *EMBRYONIC FLOWER* genes (*EMF1* and *EMF2*) cause Arabidopsis to flower directly, bypassing vegetative shoot growth. This phenotype suggests that the *EMF* genes play a major role in repression of the reproductive program. Positional cloning of *EMF2* revealed that it encodes a zinc finger protein similar to FERTILIZATION-INDEPENDENT SEED2 and VERNALIZATION2 of Arabidopsis. These genes are characterized as structural homologs of *Suppressor of zeste 12* [*Su(z)12*], a novel Polycomb group gene currently identified in Drosophila. In situ hybridization studies have demonstrated that *EMF2* RNA is found in developing embryos, in both the vegetative and the reproductive shoot meristems, and in lateral organ primordia. Transgenic suppression of *EMF2* produced a spectrum of early-flowering phenotypes, including *emf2* mutant–like phenotype. This result confirms the role of *EMF2* in phase transitions by repressing reproductive development.

INTRODUCTION

Postembryonic plant development undergoes phase changes (Poethig, 1990). Each phase is characterized by lateral organs with distinct morphological features that are produced by the shoot apical meristem (SAM). The major phase change involves the transition from vegetative to reproductive development (floral transition), which results in flowering. In Arabidopsis, the floral transition occurs when the vegetative SAM converts to the inflorescence SAM. During reproductive development, the main inflorescence shoot produces lateral buds that develop into additional inflorescences or flowers.

The floral transition is regulated by endogenous factors such as plant hormones and environmental factors such as photoperiod and temperature (Martínez-Zapater et al., 1994; Koornneef et al., 1998). These developmental regulations are mediated by a complex network of flowering time genes (Levy and Dean, 1998; Simpson et al., 1999; Blázquez, 2000). *APETALA1* (*AP1*) and *LEAFY* (*LFY*), which encode transcription factors, are necessary for flower initiation (Mandel et al., 1992; Weigel et al., 1992). They accelerate the floral transition when ectopically expressed under the constitutive 35S promoter of *Cauliflower mosaic virus* (Mandel and Yanofsky, 1995; Weigel and Nilsson, 1995). Gibberellin and long days promote flowering in part through the upregulation of *LFY* (Simon et al., 1996; Blázquez et al., 1998), suggesting that *LFY* integrates environmental and endogenous signals. Other flowering time genes, such as *TERMINAL FLOWER1* (*TFL1*) and *FLOWERING LOCUS T* (*FT*), which encode similar phosphatidylethanolamine binding protein homologs, act antagonistically in flowering time regulation. *FT* promotes and *TFL1* delays flowering (Ratcliffe et al., 1998; Kardailsky et al., 1999; Kobayashi et al., 1999). Plants that overexpress both *FT* and *LFY* flower immediately after germination, indicating that these two genes are sufficient to bypass vegetative shoot development (Kardailsky et al., 1999; Kobayashi et al., 1999). In Arabidopsis, long-day photoperiods promote flowering. *CONSTANS* (*CO*) is a major mediator of the photoperiod-dependent regulation pathway that encodes the mammalian GATA factor–like zinc finger protein (Putterill et al., 1995). Evidence indicates that *CO* promotes flowering through the upregulation of *LFY*, *FT* (Simon et al., 1996; Kobayashi et al., 1999), and another flowering time gene, *AGAMOUS-LIKE 20* (Borner et al., 2000; Lee et al., 2000; Samach et al., 2000). However, winter annual ecotypes of

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Arabidopsis are vernalization sensitive with respect to flowering. In these ecotypes, active alleles of *FRIGIDA* (*FRI*) and *FLOWERING LOCUS C* (*FLC*) repress flowering through the negative regulation of *FT* and other flowering time genes. Vernalization promotes flowering by suppressing FLC activity in these ecotypes (Sheldon et al., 2000). Molecular cloning of *FRI* and *FLC* revealed that they encode a novel protein with a coiled-coil domain and a MADS box protein, respectively (Michaels and Amasino, 1999; Sheldon et al., 1999; Johanson et al., 2000). Several other genes, including *VERNALIZATION1* (*VRN1*), *VRN2*, and *VRN3*, were identified as vernalization signal mediators from their mutant phenotypes (Chandler et al., 1996).

The *EMBRYONIC FLOWER* (*EMF*) genes also are involved in shoot development and phase transitions in Arabidopsis. Loss-of-function *emf* mutants (*emf1* and *emf2*) skip rosette growth, producing small inflorescences whose lateral buds produce only flowers and no additional inflorescences (Sung et al., 1992). Genetic analysis using double mutations demonstrated that the *EMF* genes are epistatic to flowering time genes such as *CO*, *AP1*, *FT*, and *FWA* with respect to the rosette-to-inflorescence transition (Chen et al., 1997; Haung and Yang, 1998). These phenotypes suggest that the *EMF* genes play major roles in the repression of reproductive development. Moreover, the *emf* mutants express *AP1* precociously and *AGAMOUS* ectopically in young seedlings, suggesting negative regulation of these genes by *EMF* genes (Chen et al., 1997).

Here, we report the molecular cloning of *EMF2* and its functional analysis. *EMF2* encodes a novel zinc finger protein that is related to regulatory proteins in Arabidopsis and animals. *EMF2* transcripts were detected throughout the life cycle of Arabidopsis, especially in actively proliferating tissues. Altering *EMF2* expression by transgenic approaches caused phenotypic changes in flowering time and shoot morphogenesis, supporting the idea that EMF2 activity regulates phase transitions and shoot development. The possible molecular function of *EMF2* in Arabidopsis development also is discussed based on functional similarity to *EMF2*-related genes.

RESULTS

Map-Based Cloning of *EMF2*

We isolated a new *emf2* mutant, *emf2-9*. Like other *emf2* mutants (Yang et al., 1995), *emf2-9* displays the characteristic features of the weak *emf1* mutant (Sung et al., 1992), namely, petioleless cotyledons and leaves, abbreviated inflorescence bearing terminal flowers, and impairment of petal development. Using *emf2-9*, we cloned *EMF2* using a map-based strategy (Figure 1). The *EMF2* locus was mapped between the restriction fragment length polymorphism markers *mi2* (Lister and Dean, 1995) and *Ds 389-14* (Smith et al., 1996) on chromosome 5. The DNA contig covering this 5.7-centimorgan region was established with DNA clones from the CEPH, INRA, and CNRS (CIC)-yeast artificial chromosome library (Creusot et al., 1995), the P1 library (Liu et al., 1995), and the transformation-competent artificial chromosome (TAC) library (Liu et al., 1999). Fine mapping with cleaved amplified polymorphic sequence (CAPS) markers (Konieczny and Ausubel, 1993; Glazebrook et al., 1998) narrowed the locus to ~80 kb. The TAC clones, which cover the 80-kb region, were tested to rescue the *emf2* mutant phenotype by Agrobacterium-mediated root transformation (Akama et al., 1992). As a result, three complementary clones, 11K22, 22K2, and 19A20, were identified. Using a 50-kb fragment common to these three clones as a probe, we isolated six independent cDNA clones from a library that was made from various tissues and developmental stages (Newman et al., 1994). In addition, one open reading frame (ORF) was predicted from the genomic sequence. To identify the gene complementing the *emf2* mutation, a series of deletion subclones covering the 50-kb region was constructed and introduced into the mutants. Consequently, the

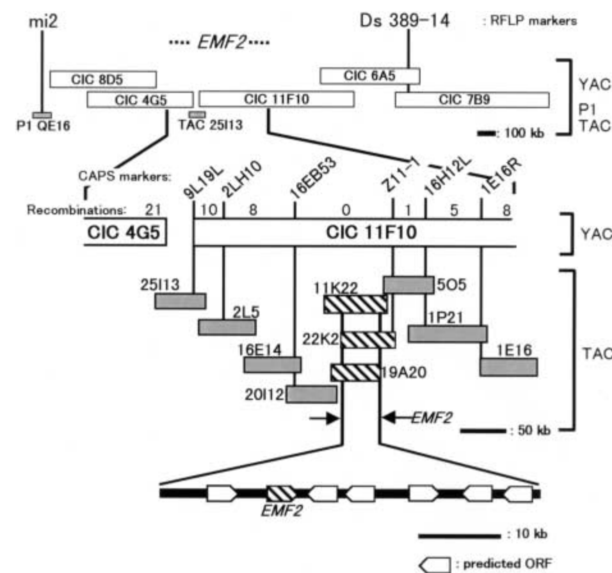

Figure 1. Map-Based Cloning of the *EMF2* Locus.

Scheme of the physical map of the *EMF2* locus. CEPH, INRA, and CNRS (CIC)-yeast artificial chromosome (YAC) clones are shown as open bars, and TAC and P1 clones are shown as gray or striped bars. The three striped TAC clones can complement the *emf* mutation. The positions of restriction fragment length polymorphism (RFLP) and CAPS markers are represented by vertical lines. Recombination numbers in the 2000 tested chromosomes are indicated between each pair of CAPS markers. Predicted ORFs are mapped in the region common to the three complementary TAC clones.

region corresponding to the cDNA, named 58-1, was identified as responsible for the complementation. A nearly full-length clone of the cDNA was obtained through rescreening of the library. Comparison of genomic (GenBank accession number AB053262) and cDNA sequences revealed that the ORF consists of 21 exons spanning 5813 bp. Molecular lesions of four *emf2* alleles were mapped in the ORF (Figure 2A). Thus, we concluded that the ORF is *EMF2*.

EMF2 Encodes a Novel Polycomb Group Protein Homolog

The predicted *EMF2* ORF encodes a novel protein with 631 amino acid residues whose molecular mass is estimated to be 71.7 kD (Figure 2B). The EMF2 protein has a single C_2H_2 zinc finger motif as well as putative nuclear localization signals. The C-terminal region of the protein is characterized by an acidic cluster and a tryptophan/methionine-rich sequence. We named this region the acidic-W/M domain. Molecular lesions in the two *emf2* alleles (*emf2-3* and *emf2-9*) were mapped to this domain (Figure 2A); thus, the domain is crucial for EMF2 function.

Through a survey of Arabidopsis genome databases, at least three *EMF2*-related genes were detected: *FIS2* (GenBank accession number AF096096), *VRN2* (GenBank accession number AX03288), and one hypothetical ORF. *VRN2*, which is involved in the vernalization-dependent promotion of flowering, was isolated and characterized recently (A.R. Gendall, personal communication). The *VRN2* gene encodes a protein containing a C_2H_2 zinc finger and an acidic-W/M domain, showing 67 and 76% identity with corresponding domains of EMF2, respectively, although it lacks the sequence corresponding to six exons in the N-terminal half of EMF2. The second gene, *FIS2*, was characterized originally by its loss-of-function mutation phenotype of partial seed development in the absence of fertilization (Luo et al., 1999). FIS2 also is similar to EMF2 in the zinc finger and the acidic-W/M domain, showing 55 and 49% identity, respectively, but it differs from EMF2 in the unique repetitive sequences that constitute a putative protein–protein interaction domain (Figure 3) (Luo et al., 1999). The third gene, a hypothetical ORF, was tentatively termed *EMF2-LIKE 1* (*EML1*; GenBank accession number AB053265). It seems to be a pseudogene because the transcripts were not detected by reverse transcriptase–mediated polymerase chain reaction (RT-PCR) using the total RNA from seedlings as templates (data not shown). This gene can potentially encode an acidic-W/M domain, showing 54% identity with EMF2, but it lacks zinc fingers. The exon-intron organization is conserved among these *EMF2*-related genes, suggesting that they have evolved from the same ancestral sequence (data not shown).

Interestingly, these EMF2-related proteins show homology with a newly identified Polycomb group (PcG) protein, Su(z)12, of Drosophila (Figure 3) (Birve et al., 2001). Mutations of the *Su(z)12* gene cause typical phenotypes of PcG mutants, such as homeotic transformations and misexpression of homeobox genes, in the developmental process of Drosophila. Until now, three classes of PcG gene homologs have been reported in Arabidopsis. Those are *CURLY LEAF* (*CLF*) (Goodrich et al., 1997), *FIS1/MEDEA* (*MEA*) (Grossniklaus et al., 1998; Luo et al., 1999), and *FIS3/FERTILIZATION-INDEPENDENT ENDOSPERM* (*FIE*) (Ohad et al., 1999). Thus, *EMF2*, *VRN2*, and *FIS2* constitute the fourth class of PcG gene homologs in Arabidopsis. A cDNA clone, KIAA1060 (Nagase et al., 1995), should be the human homolog of *Su(z)12*, because these proteins are conserved over the entire sequence. The Arabidopsis and animal genes share three conserved domains, the N-terminal basic domains, zinc finger domains, and acidic-W/M domains, except that *FIS2* lacks the N-terminal basic domain (Figure 3).

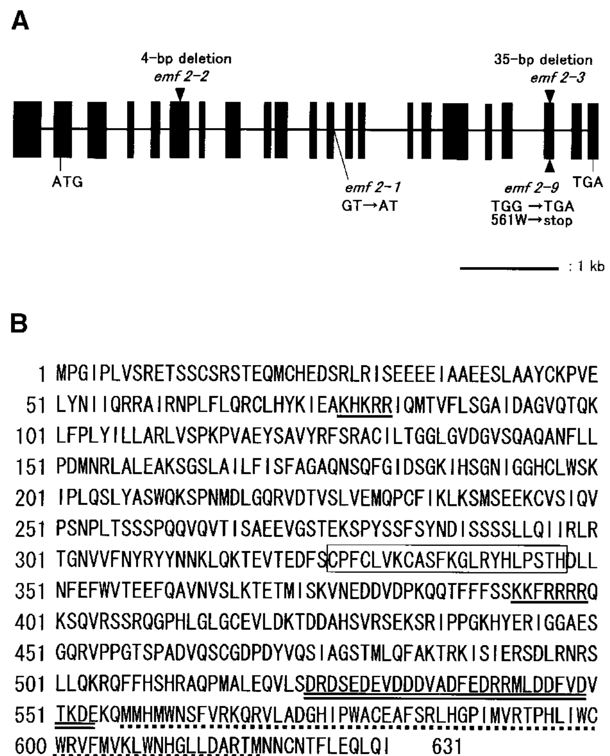

Figure 2. Structure of the *EMF2* Gene.

(A) Scheme of the *EMF2* gene. Solid boxes and lines represent exons and introns, respectively. Mutations of four *emf2* alleles are indicated at the corresponding sites.

(B) Deduced amino acid sequence of the EMF2 protein. A C_2H_2-type zinc finger motif is boxed. Two putative nuclear localization signals and an acidic cluster are underlined with single solid lines and a double solid line, respectively. The sequence marked with a dotted line is rich in tryptophan and methionine.

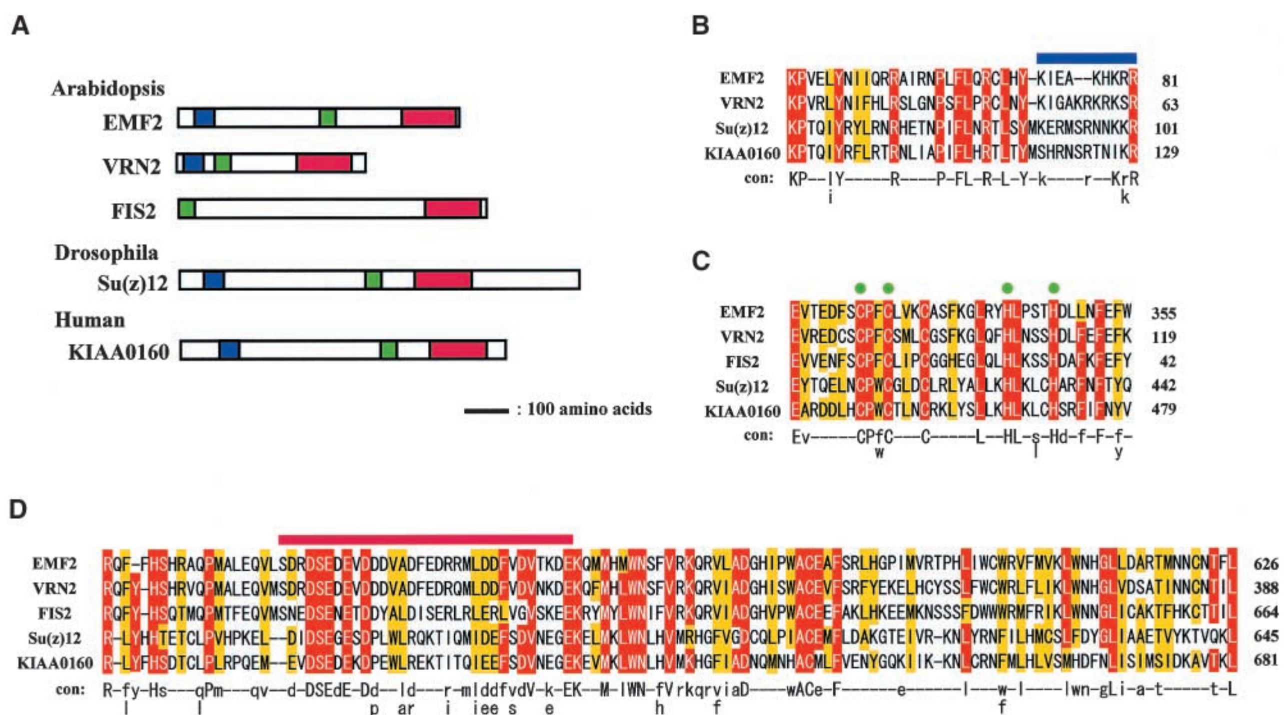

Figure 3. Structural Similarity in the EMF2/Su(z)12 Protein Family.

(A) Schematic comparison of EMF2, VRN2, FIS2, Su(z)12, and KIAA1610 protein structures. Conserved N-terminal basic domains, C_2H_2-type zinc finger domains, and C-terminal acidic-W/M domains are colored blue, green, and pink, respectively.

(B) to **(D)** Sequence alignment of the proteins. Identical amino acids and similar amino acids are boxed in red and yellow, respectively. con, consensus sequence.

(B) Sequence alignment in N-terminal basic domains. Basic amino acid clusters are indicated by a blue bar.

(C) Sequence alignment in zinc finger regions. Conserved cysteine and histidine residues involved in zinc finger formation are indicated with green circles.

(D) Sequence alignment in acidic-W/M domains. Acidic amino acid clusters are indicated by a pink bar.

Expression of *EMF2* in Wild-Type Arabidopsis

It has been hypothesized that the *EMF* genes play a major role in repressing floral transition and that a decrease in EMF activity leads to flowering. To determine whether the transcriptional regulation of *EMF2* is involved in floral transition, semiquantitative RT-PCR analysis was performed at different stages of growth. The time course of RNA accumulation in seedlings showed that the *EMF2* transcript level did not change significantly during vegetative and reproductive development, whereas the *FT* transcript level was upregulated during flower initiation (Figure 4). Thus, transcriptional regulation of *EMF2* appeared not to be involved in the floral transition.

We further studied the spatial expression pattern of *EMF2* by in situ RNA hybridization. The results demonstrated that *EMF2* is expressed throughout the life cycle of Arabidopsis with preferential localization in actively proliferating tissues. In early seed development, *EMF2* mRNA was observed in the developing embryos and endosperm. *EMF2* mRNA was detected in the entire embryos as early as the globular stage. When embryos were about to mature, *EMF2* mRNA decreased to an undetectable level, tending to remain for a longer period in the embryonic SAMs (Figures 5A to 5D). In endosperm, *EMF2* mRNA was observed in cytoplasm around the free nuclei in syncytium and in early cellularized endosperm (Figure 5B), but it was reduced to an undetectable level during later endosperm development. After germination, *EMF2* mRNA was again detected in the SAMs, leaf primordia, and young leaves (Figure 5E). In the reproductive shoots, transcripts were detected in both the inflorescence and the floral meristems (Figure 5F). At later stages of flower development, *EMF2* mRNA appeared to accumulate in floral organ primordia (Figure 5F). In coflorescences, the transcripts were detected in SAMs and lateral organs, as seen in the main

shoots (Figure 5G). In roots, higher levels of the transcripts were detected in root tips (Figure 5H).

Suppression of *EMF2* in Transgenic Plants

To confirm that EMF2 activity regulates flowering, we generated transgenic Arabidopsis plants expressing antisense or sense *EMF2* under the control of the 35S promoter of *Cauliflower mosaic virus*. For the antisense experiment, the construct containing 2280 bp of the entire cDNA in the antisense orientation was introduced into wild-type plants. Among 69 T1 transformants, 46 plants (67%) exhibited early flowering phenotypes with formation of terminal flowers, whereas 18 plants (26%) showed the wild-type–like phenotype under long-day (LD) conditions. Five plants (7%) had other morphological abnormalities. A gradient of the early-flowering phenotype was observed, which can be grouped into two categories: *emf2*–like (Figure 6A) and *terminal flower1* (*tfl1*)–like (Figures 6B to 6D). In *emf2*-like plants, the main and lateral shoots are terminated by infertile flowers. They have petiolated cotyledons but formed two to six small sessile leaves instead of rosette leaves. On the other hand, *tfl1*-like plants had petiolated rosette leaves and cotyledons and retained some fertility. Under LD conditions, these plants produced six to seven rosette leaves before flowering, the same as wild-type plants, but they are distinguished by terminal flowers and curled leaves (Figure 6C), like *clf* mutants (Goodrich et al., 1997). *emf2*-like plants accumulated higher levels of the antisense *EMF2* RNA compared with those of the *tfl1*-like plants (Figure 6E, *EMF2*−), although the two categories were not significantly different with respect to accumulation levels of the endogenous *EMF2* (Figure 6E, *EMF2*+). This finding suggests that the phenotypic difference may result from varying the amounts of the antisense RNA, which directs the suppression of endogenous EMF2 activity. These results indicate that the suppression of *EMF2* affects flowering time by converting inflorescence to floral meristems prematurely, as in 35S::*LFY* plants (Weigel and Nilsson, 1995) or *tfl1* mutants (Shannon and Meeks-Wagner, 1991), and that further suppression causes the plants to bypass the rosette phase, as in *emf2* mutants.

In the case of sense transgenic plants, 138 plants among 292 T1 transformants (47%) showed the early-flowering/terminal flower phenotype like the antisense plants (data not shown), whereas 148 (51%) were wild type–like and six (2%) were other mutants. Unexpectedly, no late-flowering phenotype was observed in the sense transformants. Enhancement of only *EMF2* expression might be insufficient to repress the reproductive program in Arabidopsis development. Alternatively, ectopic expression of *EMF2* might cause suppression of the endogenous gene function in a cosuppression mechanism.

The T2 generation of the antisense transformants was cultivated under LD or short-day (SD) conditions (Table 1). The *tfl1*-like phenotype was not affected by daylength (Table 1). Some wild-type–like lines segregated the early-flowering phenotype only under SD conditions (Table 1). They bolted after developing 10 to 12 rosette leaves, in contrast to 26.7 leaves for wild-type plants. They produced no terminal flowers and were completely fertile. This early-flowering phenotype under SD conditions appears to be mediated by moderate suppression of *EMF2*, suggesting that the LD signal might promote flowering partly by suppressing the endogenous *EMF2* in wild-type Arabidopsis.

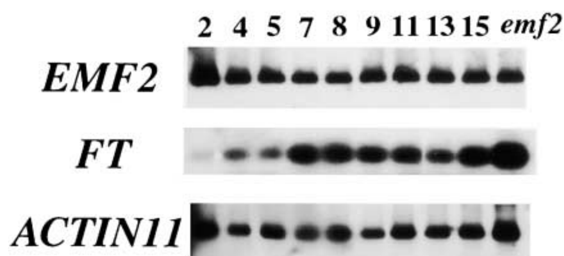

Figure 4. Expression Time-Course of *EMF2* during Arabidopsis Growth.

Wild-type Columbia plants were cultivated in vitro under LD conditions (see Methods), and seedlings were harvested at 2, 4, 5, 7, 8, 9, 11, 13, and 15 days after sowing. Semiquantitative RT-PCR was performed using total RNA as a template for *EMF2*, *FT*, and *ACTIN11* RNA amplifications. PCR products were detected by DNA gel blot analysis. Numbers indicate days of cultivation before harvesting. Floral buds are visible after 10 to 14 days of cultivation in this condition. *emf2*, *emf2-3* plants harvested after 14 days of cultivation.

DISCUSSION

Structural and Functional Homology between *EMF2* and PcG Genes

EMF2, together with *FIS2* and *VRN2*, shows significant homology with *Su(z)12*, a newly identified class of PcG gene from Drosophila (A.R. Gendall, personal communication; Birve et al., 2001). In Drosophila, PcG proteins repress certain sets of target genes and maintain the repression state through cell division (Elgin, 1996; Pirrotta, 1997). The loss-of-function mutations of the *EMF* genes skip a large part of vegetative growth and flower precociously. This phenotype suggests that the *EMF* genes could repress the reproductive program, which is likely to be a default program of Arabidopsis development. Similarly, the *FIS* genes (*FIS1*/*MEA*, *FIS2*, and *FIS3*/*FIE*) are thought to repress seed development

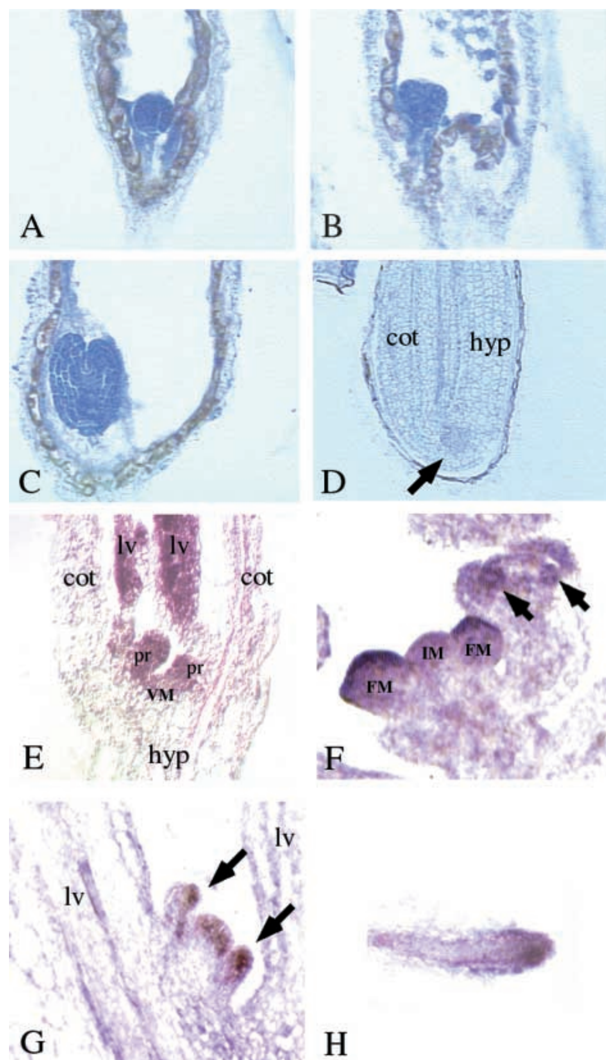

Figure 5. In Situ Localization of *EMF2* mRNA in Wild-Type Arabidopsis.

(A) to **(D)** EMF2 mRNA signals are detected as dark blue areas with bright-field optics. Longitudinal sections of developing embryos are shown.
(A) Globular stage.
(B) Early heart stage.
(C) Early torpedo stage.
(D) Maturing stage. Arrow indicates the embryonic shoot apex, which showed slightly higher signals than other parts of the embryo. cot, cotyledon; hyp, hypocotyl.
(E) to **(H)** *EMF2* mRNA signals are detected as purple areas with differential interference contrast optics. Longitudinal sections of shoot and root apical meristems are shown.
(E) Shoot apex of a 6-day-old seedling. Stronger signals are detected at the meristem (VM), leaf primordia (pr), and young leaves (lv).
(F) Inflorescence apex. Stronger signals are detected on the inflorescence meristem (IM), floral meristem (FM), and floral organ primordia (arrows).
(G) Coflorescence shoot emerging from the axil of a rosette leaf. Stronger signals are detected on the meristem and leaf primordia. Arrows indicate two leaf primordia emerging from the meristem.
(H) Longitudinal section of a root tip. Stronger signals are detected at the meristem and the provascular tissue.

until fertilization occurs (Grossniklaus et al., 1998; Luo et al., 1999; Ohad et al., 1999). As a result of the discovery of Su(z)12, all of the *FIS* genes can be characterized as PcG gene homologs. *EMF2* and the *FIS* genes appear to share structural and functional homology, although the EMF1 protein has no similarity to previously characterized proteins (Aubert et al., 2001).

CLF, another PcG gene homolog in Arabidopsis, functions as a repressor of the floral homeotic genes *AGAMOUS* (*AG*) and *PISTILLATA* (*PI*) in vegetative tissue (Goodrich et al., 1997). *clf* mutants display early-flowering and curled leaf phenotypes, which also are observed in antisense *EMF2* transgenic plants (Figure 6C). Moreover, a previous study showed precocious expression of *AP1* and ectopic expression of *AG* in *emf2* mutants (Chen et al., 1997). From these facts, it can be hypothesized that *EMF2* and *CLF* act in the same pathway of phase transitions by regulating proper expression of the floral homeotic genes.

In animals, PcG proteins form large protein complexes (Tie et al., 1998, 2001; Shao et al., 1999) and act to remodel chromatin structures, altering the accessibility of DNA to factors required for transcription. In plants, protein–protein interactions were examined among the *FIS* genes, and only the MEA–FIE interaction was detected by the yeast two-hybrid system (Luo et al., 2000; Yadegari et al., 2000). Protein interaction between *CLF* and *EMF2* is intriguing. The role of the Arabidopsis PcG homologs in chromatin remodeling remains to be investigated, which should lead to further understanding of the molecular mechanisms of plant development and the evolutionary correlation of developmental regulation between the animal and plant kingdoms.

Involvement of *EMF2* in Shoot Development and Flowering

It has been hypothesized that the *EMF* genes repress reproductive development by delaying the vegetative-to-inflorescence (V/IF) and inflorescence-to-flower (IF/F) transitions. The early-flowering/terminal flower phenotypes of the transgenic plants harboring the antisense *EMF2* support this hypothesis. *emf2*-like and *tfl1*-like phenotypes demonstrate the role of *EMF2* in the repression of the V/IF and IF/F transitions, whereas early flowering under SD conditions suggests *EMF2*-mediated, photoperiod-dependent regulation

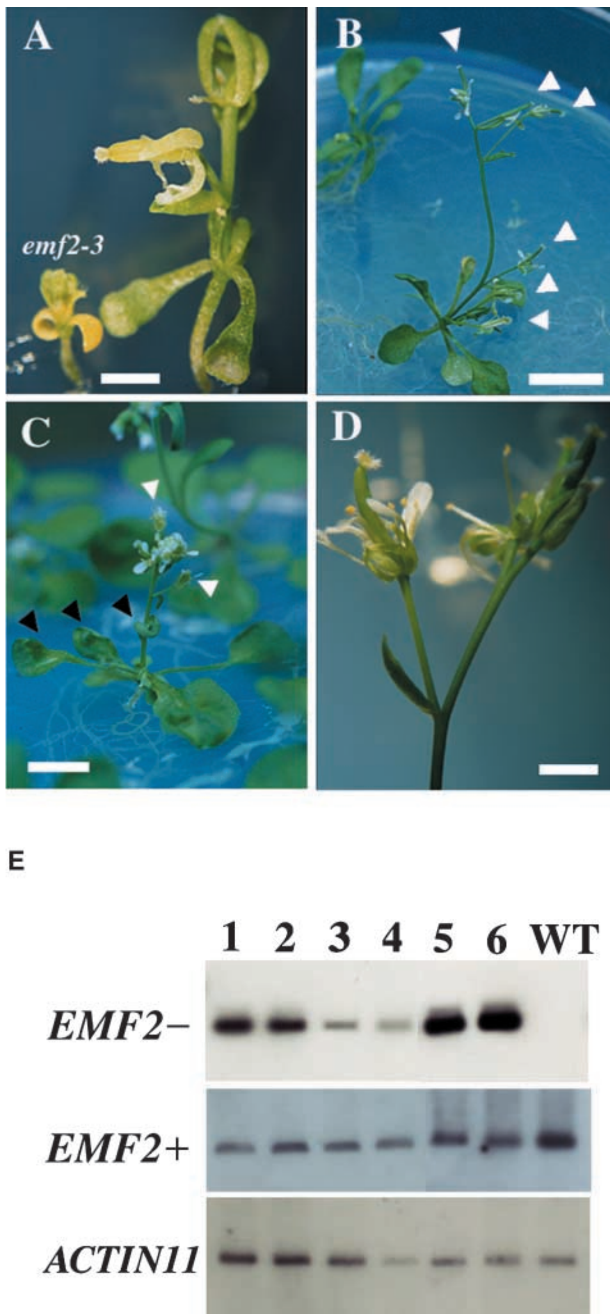

Figure 6. Phenotypes and RNA Levels of Antisense *EMF2* Transgenic Plants.

(A) An *emf2*-like plant. Both the main and lateral shoots develop into terminal flowers. An *emf2-3* plant is shown for comparison of size. Bar = 2 mm.

(B) A typical *tfl1*-like plant. Both the main and all lateral shoots develop into terminal flowers (white arrowheads). Bar = 1 cm.

(C) The *clf*-like phenotype of a *tfl1*-like plant. Rosette and cauline leaves are curled (black arrowheads). Both the main and all lateral shoots develop into terminal flowers (white arrowheads). Bar = 5 mm.

(D) Close-up view of terminal flowers of a *tfl1*-like plant. There is no visible structural abnormality. Bar = 2 mm.

(E) Antisense (*EMF2*−) and endogenous (*EMF2*+) RNA levels in transgenic plants. Semiquantitative RT-PCR analysis was performed using total RNA as a template. *ACTIN11* RNA was amplified as a control. Four independent *tfl1*-like plants (lanes 1 to 4) and two *emf2*-like plants (lanes 5 and 6) were tested. WT, wild type, ecotype Columbia.

of the V/IF transition. However, our findings indicate that transcriptional regulation of endogenous *EMF2* is not likely to direct the phase transitions. The possibility of other regulatory mechanisms, such as modification of the protein, remains to be investigated. The expression pattern of *EMF1*, another *EMF* gene, is similar to that of *EMF2* (Aubert et al., 2001). Like the 35S::sense *EMF2* transgenic plants, ectopic 35S::sense *EMF1* transgenic plants do not exhibit late-flowering phenotypes (Aubert et al., 2001). These findings indicate that each *EMF* gene is strictly required but not sufficient for repression of the transitions. The two genes should act in the same pathway, but their molecular interaction remains to be determined. A previous study suggested that the *EMF* genes interact with *LFY* and *AP1* genes in a negative, reciprocal manner (Chen et al., 1997). Thus, modification of EMF activity by *LFY* and *AP1* may contribute to the phase transitions. *EMF2* shows structural similarity to PcG genes, which regulate the expression of homeotic genes that interact reciprocally with Trithorax group (TrxG) genes. An important question is whether a regulatory system similar to the PcG/TrxG system in animals exists in plants.

In inflorescence development, the *TFL1* gene interacts with *AP1*/*LFY* genes in a reciprocal, negative manner to specify meristem identity (Liljegren et al., 1999). Arabidopsis shoot development is thought to be a highly integrated process that is controlled by a common mechanism, named the controller of phase switch (COPS) (Schultz and Haughn, 1993). In *tfl1* mutants, all phases are shortened, whereas ectopic expression of *TFL1* causes the extension of all phases (Ratcliffe et al., 1998). These observations support the COPS hypothesis and suggest that *TFL1* affects COPS activity. The phenotype of *emf* mutants also can be interpreted by compressing all of the developmental phases, and the *EMF2* transcripts accumulate in all phases that we analyzed. Thus, it can be speculated that *EMF2* may be involved in COPS by acting in a manner similar to *TFL1*. Otherwise, EMF2 activity, possibly along with EMF1 activity, may be required for *TFL1* activity. The phenotypic similarity between *tfl1* and *tfl1-like* transgenic plants supports this speculation. In the reproductive phase, *EMF2* mRNA was detected throughout the inflorescence and floral meristems (Figure 5G), whereas the *TFL1* mRNA accumulated specifically in a group of cells just below the apical dome of inflorescence meristems (Bradley et al., 1997). Further investigations of

Table 1. Flowering Times of the Antisense *EMF2* Transgenic Plants

Line, Photoperiod[a]	Ratio of Hygromycin-Resistant Plants	Number of Rosette Leaves[b]	*n*
WTL-1, LD	0.75	6.6 ± 0.6	21
WTL-1, SD	0.75	11.6 ± 1.6	38
WTL-2, LD	1.00	6.4 ± 1.0	25
WTL-2, SD	1.00	11.5 ± 1.3	23
TFL-1, LD	N. d.[c]	6.8 ± 1.2	14
TFL-1, SD	N. d.[c]	6.0 ± 0.8	42
Co-0, LD	0	6.5 ± 1.0	23
Co-0, SD	0	26.7 ± 3.5	7

[a] WTL and TFL represent wild-type–like and *terminal flower1*–like transgenic lines, respectively. Co-0 represents wild-type ecotype Columbia plants.
[b] Average leaf numbers ±SD at the time of bolting. Leaf numbers of hygromycin-resistant plants and *tfl1*-like plants were counted for the WTL and TFL lines, respectively.
[c] N. d., not determined.

gene interaction between *EMF1*/*EMF2* and *TFL1* will help explain this diverse expression pattern. In addition to these investigations, discovery of the genes regulated directly by *EMF2* should provide further insights into the gene network that controls Arabidopsis shoot development.

METHODS

Plant Material and Mutant Screening

All *Arabidopsis thaliana* plants were grown under white light at 24°C. The photoperiod was 16 hr of light and 8 hr of dark for long-day (LD) conditions and 8 hr of light and 16 hr of dark for short-day (SD) conditions. Plants were planted on rock fiber blocks and supplied Hyponex (1:1000 dilution; Hyponex, Inc., Tokyo, Japan) as liquid nutrient. For in vitro culture, 0.8% agar plates containing half-strength B5 salts (Sigma), 1% sugar, and Hyponex diluted 1:2000 (pH adjusted to 5.8) were used. M2 seed of ethyl methanesulfonate–mutagenized Landsberg ecotype were purchased from Lehle Seed (Round Rock, TX). Approximately 5000 M2 seed from each of 10 parental groups, totaling 50,000 seed, were sowed on the agar medium and screened for *emf2*-like seedlings. Five thousand M3 lines were established from the parental group in which the *emf2*-like mutant was obtained. Consequently, the line segregating the mutant was isolated. We confirmed the allelism between this line and *emf2-3* by reciprocal crossing. The line was identified as the ninth allele of *emf2* (*emf2-9*).

Map-Based Cloning

Genetic and physical mapping of *emf2-9* was conducted as described by Schmidt and Dean (1992). All genetic complementation analysis was performed using the root transformation method (Akama et al., 1992). *Agrobacterium tumefaciens* strain MP90 was transformed with transformation-competent artificial chromosome (TAC) plasmids (Liu et al., 1999), and then root sections from *emf2-9* plants were infected with the transformed bacteria. Complementation was judged from regeneration of the normal inflorescence stalks from transformed calli.

In Situ RNA Hybridization

Wild-type Columbia plants were grown under LD conditions. Shoots, flowers, roots, and siliques at different developmental stages were harvested and fixed as described (Chen et al., 1997). Methods for digoxigenin labeling of RNA probes, slide preparation, and in situ hybridization were as described on the World Wide Web (http://www.arabidopsis.org/cshl-course/5-in_situ.html; http://www.wisc. edu/genetics/CATG/barton/protocols.html). For synthesis of the *EMF2*-specific probe, the 441-bp PstI fragment of *EMF2* cDNA, the region deleted in *VRN2*, was subcloned into pBluescript KS+ and transcribed in vitro with digoxigenin-UTP using T3 and T7 RNA polymerase for the antisense and sense probes, respectively.

Semiquantitative Reverse Transcriptase–Mediated Polymerase Chain Reaction

Total RNA was extracted from seedlings of wild-type, mutant, or transgenic plants using RNeasy (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Reverse transcription–mediated polymerase chain reaction (RT-PCR) was performed using the mRNA Selective PCR kit (TaKaRa; Kyoto, Japan) according to the manufacturer's instructions using 0.1 μg of total RNA as starting material. PCR was stopped in the exponential phase of amplification, and the products were detected by DNA gel blot analysis. Primers used to amplify the endogenous *EMF2* mRNA were 5′-GGCAAG-ACTCGTTTCTCCTAAGC-3′ (EMF-F) and 5′-GCAACAAGGAAGAGG-AAGGATGT-3′ (EMF-R), designed for amplification of the *EMF2*-specific region deleted in *VRN2*. For amplification of *FT* mRNA, previously described primers (Kobayashi et al., 1999) were used. As a control reaction, the Arabidopsis *ACTIN11* gene was amplified using primers 5′-ATGGCAGATGGTGAAGACATTCAG-3′ and 5′-GAAGCA-CTTCCTGTGGACTATTGATG-3′. For amplification of the antisense *EMF2* RNA, 5′-GTTTGAACGATCGGGGAAATTC-3′, the sequence upstream of the polyadenylation signals in the nopaline synthase terminator, was used in combination with EMF-R or other *EMF2*-specific primers to check for reproducibility. For amplification of

endogenous *EMF2* mRNA from the antisense transformants, DNase I–treated RNA samples were reverse transcribed by ReverTraAce (Toyobo, Osaka, Japan) using the EMF-R primer. PCR was performed successively using KOD Dash polymerase (Toyobo) with the following program: 94°C for 3 min followed by 18 cycles of 94°C for 30 sec, 60°C for 10 sec, and 74°C for 1 min.

Generation of Transgenic Arabidopsis

The antisense *EMF2* construct was generated as follows. The cDNA clone was cut with SalI and NotI and blunted with the Klenow fragment. The cDNA region was separated by agarose gel electrophoresis and ligated with XbaI-digested and blunted KH2 vector, the pBI121-based binary plasmid with a hygromycin selection marker, courtesy of Dr. Kenzo Nakamura (Nagoya University, Nagoya, Japan). The clones containing the antisense-oriented *EMF2* coding sequence were selected by enzymatic digestion of their plasmids. The antisense constructs were introduced into Agrobacterium strain MP90 by electroporation. Wild-type Columbia plants were infected with the bacteria harboring the constructs by means of the vacuum infiltration method (Bechtold and Pelletier, 1998). T1 transformants were selected on the agar medium containing 30 mg/L hygromycin B.

Accession Number

The GenBank accession number for the *EMF2* cDNA clone is AB053171.

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