The Plant Cell, Vol. 13, 1865–1875, August 2001, www.plantcell.org © 2001 American Society of Plant Biologists

EMF1, A Novel Protein Involved in the Control of Shoot Architecture and Flowering in Arabidopsis

Dominique Aubert,[a,1,2] Lingjing Chen,[a,1] Yong-Hwan Moon,[a,1] David Martin,[a,3] Linda A. Castle,[a,4] Chang-Hsien Yang,[b] and Z. Renee Sung[a,5]

[a] Department of Plant and Microbial Biology, University of California, Berkeley, California 94720
[b] Graduate Institute of Agricultural Biotechnology, National Chung Hsing University, Taichung, Taiwan 40227, Republic of China

Shoot architecture and flowering time in angiosperms depend on the balanced expression of a large number of flowering time and flower meristem identity genes. Loss-of-function mutations in the Arabidopsis *EMBRYONIC FLOWER* (*EMF*) genes cause Arabidopsis to eliminate rosette shoot growth and transform the apical meristem from indeterminate to determinate growth by producing a single terminal flower on all nodes. We have identified the *EMF1* gene by positional cloning. The deduced polypeptide has no homology with any protein of known function except a putative protein in the rice genome with which EMF1 shares common motifs that include nuclear localization signals, P-loop, and LXXLL elements. Alteration of *EMF1* expression in transgenic plants caused progressive changes in flowering time, shoot determinacy, and inflorescence architecture. EMF1 and its related sequence may belong to a new class of proteins that function as transcriptional regulators of phase transition during shoot development.

INTRODUCTION

Higher plants undergo successive developmental phase changes that are regulated by factors extrinsic and intrinsic to the shoot apical meristem (Poethig, 1990). In Arabidopsis, the main shoot development begins with the rosette/vegetative phase, that is, production of a rosette shoot comprising leaves with long petioles but no internode elongation. The transition from the vegetative to the reproductive phase results in the production of the inflorescence shoot, which displays elongated internodes with nodes that produce coflorescence shoots in the axils of cauline leaves and flowers without subtending cauline leaves. Eventually, the main shoot apical meristem ceases growth but does not form a flower.

Environmental and endogenous cues control the length of each developmental phase (Levy and Dean, 1998). In Arabidopsis, ~40 genes are involved in regulating the transition to flowering (for a recent review, see Blazquez, 2000). Impairments in early- or late-flowering genes, such as *CONSTANS* (*CO*), *EARLY FLOWERING3* (*ELF3*), and *FLOWERING LOCUS T* (*FT*), hasten or delay the transition from rosette to inflorescence development (Koornneef et al., 1991; Zagotta et al., 1992). Mutations in floral meristem identity genes, such as *APETALA1* (*AP1*) and *LEAFY* (*LFY*), lengthen the inflorescence and delay the initiation of flower development (Schultz and Haughn, 1991; Huala and Sussex, 1992; Weigel et al., 1992).

The *TERMINAL FLOWER1* (*TFL1*) gene and the two *EMBRYONIC FLOWER* (*EMF*) genes, *EMF1* and *EMF2*, are involved in delaying both the vegetative to reproductive transition and flower initiation in Arabidopsis (Shannon and Meeks-Wagner, 1991; Sung et al., 1992; Schultz and Haughn, 1993; Yang et al., 1995). Loss-of-function mutations in *TFL1* shorten both rosette and inflorescence development (Alvarez et al., 1992; Bradley et al., 1997). Loss-of-function *emf* mutants display more dramatic phase-reduction phenotypes: there is no rosette shoot development; only a reduced inflorescence with several flowers lacking petals is produced.

Mutants of the *TFL1* and *EMF1* genes display another similar phenotype, the conversion of the inflorescence apex from indeterminate to determinate growth by production of a terminal flower (Alvarez et al., 1992; Chen et al., 1997). The *TFL1* ortholog in Antirrhinum, *CENTRORADIALIS* (*CEN*), also specifies shoot indeterminacy, which led Bradley et al. (1996) and Amaya et al. (1999) to propose that a common

[1] These authors contributed equally to this work.
[2] Current address: Plant Biology and Biogeochemistry Department, Risoe National Laboratory, P.O. Box 49, DK-4000 Roskilde, Denmark.
[3] Current address: Department of Plant Sciences, University of Oxford, South Parks Road, Oxford OX1 3RB, UK.
[4] Current address: Maxygen, Inc., 515 Galveston Drive, Redwood City, CA 94063.
[5] To whom correspondence should be addressed. E-mail zrsung@nature.berkeley.edu; fax 510-642-4995.

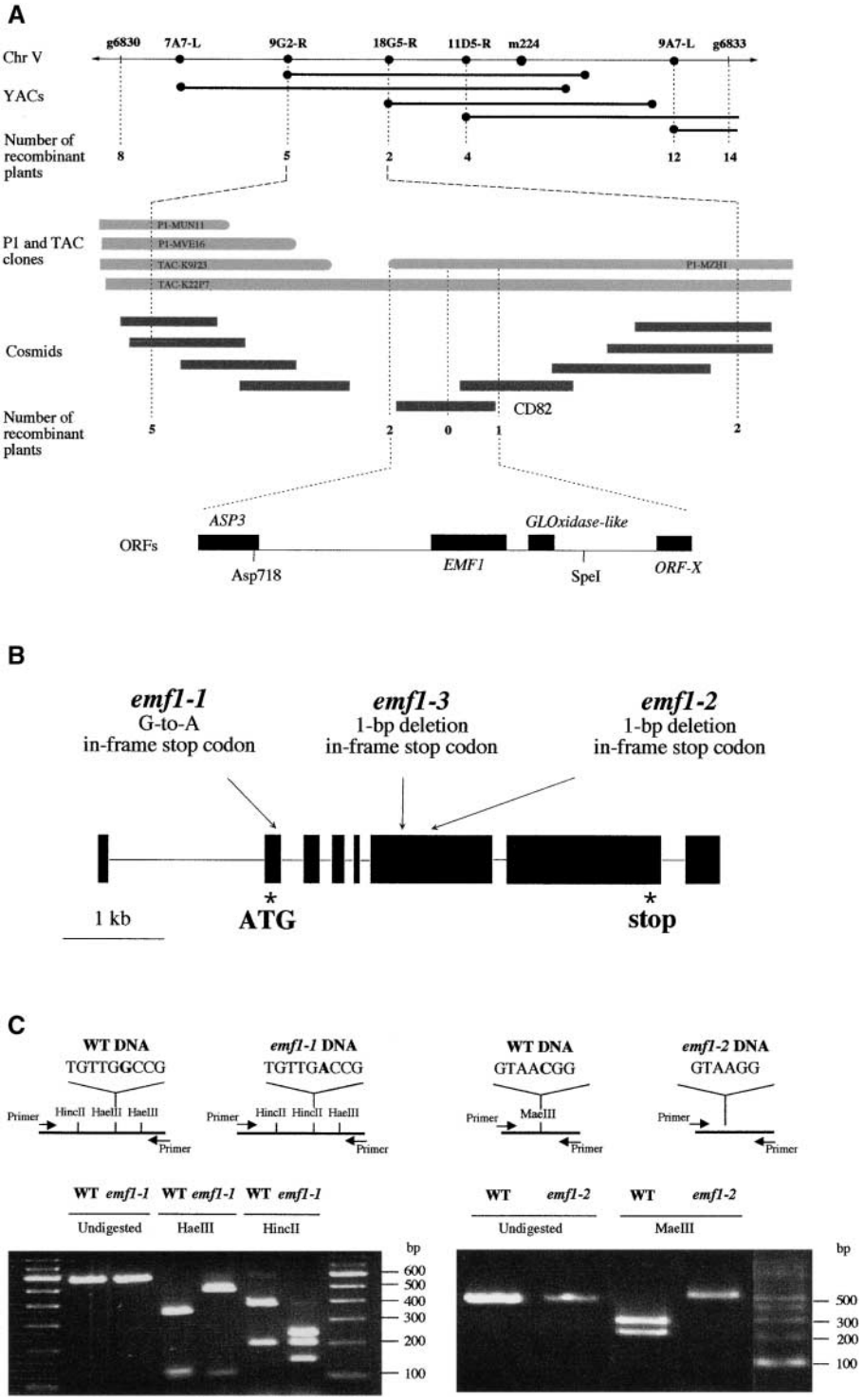

Figure 1. Map-Based Cloning and Gene Structure of *EMF1*.

(A) Summary of the physical and genetic positions of *EMF1* on chromosome (Chr) V. The top horizontal line represents the markers used to fine map the *EMF1* locus between 9G2-R and 18G5-R on chromosome V. Values indicate the number of recombinant plants identified between the *EMF1* locus and a given RFLP marker (circles). The bottom horizontal line shows the organization of the four genes deduced from the sequence of the CD82 clone: *ORF-X* (a putative ORF), the *GLOxidase-like* gene, *EMF1*, and *ASP3* (*aspartate aminotransferase3*).

mechanism underlies indeterminacy in Antirrhinum and Arabidopsis. Shoot determinacy affects many aspects of inflorescence architecture. For example, development of a terminal flower could cause a reduction in apical dominance, activating the emergence of a secondary inflorescence and drastically changing the shoot architecture. The *EMF1* and *TFL1* genes may play an important role in specifying the two major inflorescence types in angiosperms, determinate (cymose) and indeterminate (racemose) (Cronquist, 1988).

To enable investigation of the molecular function of *EMF1*, we cloned the *EMF1* gene by positional cloning. It encodes a new class of regulatory proteins also found in rice, a monocot. By manipulating EMF1 activity in transgenic plants, we confirmed that EMF1 activity controls shoot indeterminacy and flowering time. The possible function of EMF1 and its role in regulating rosette and inflorescence development are discussed.

Table 1. Complementation of *emf1* Mutant Alleles by the SpeI–Asp718 Genomic DNA

		Number of T2 plants[b]				
T1 Line	Mutant Allele[a]	Km^R WT	Km^S WT	Km^R Mutant	Km^S Mutant	Total
1-4	*emf1-1*	44	10	0	2	56
3-12	*emf1-1*	45	15	0	5	65
3-11	*emf1-1*	47	0	0	9	56
3-10	*emf1-1*	51	3	0	0	54
Total		187	28	0	16	231
1-2	*emf1-2*	107	32	0	11	150
2-2	*emf1-2*	134	0	0	8	142
7-2	*emf1-2*	110	0	0	36	146
8-2	*emf1-2*	128	0	0	18	146
9-1	*emf1-2*	81	11	0	8	100
9-2	*emf1-2*	141	45	0	14	200
Total		701	88	0	95	884

[a] Mutant alleles of T1 transgenic plants were confirmed by the allele-specific RFLP or by the occurrence of Km^S *emf1* mutants in T2 populations.

[b] Km^R, kanamycin-resistant; Km^S, kanamycin-sensitive; WT, wild type.

RESULTS

Positional Cloning of the *EMF1* Locus

The *EMF1* locus has been mapped to the upper part of chromosome V (Yang et al., 1995). Figure 1A diagrams the positional cloning experiments. By using restriction fragment length polymorphism (RFLP) markers and a segregating population representing ~1200 meiotic events, *EMF1* was fine mapped between markers 9G2-R and 18G5-R, which were derived from the ends of yeast artificial chromosome (YAC) clones included in a large contig covering that region. P1 and transformation-competent bacterial artificial chromosome (TAC) clones were anchored to these markers, and a contig was assembled based on information available at www.kazusa.or.jp/kaos/kazusa/chr5/pmap/P1_map_3.html. The 9G2-R and 18G5-R ends were used as probes to initiate a chromosome walk. Cosmid clones were isolated from binary cosmid libraries. Internal fragments of cosmid or P1 clones were converted into RFLP markers and used to delimit the *EMF1* locus on cosmid clone CD82.

Sequence analysis of the 17,341-bp CD82 clone and sequence data from bacterial artificial chromosome clone F15N18 revealed the presence of four open reading frames (ORFs) on cosmid clone CD82 (Figure 1A). RFLP mapping localized the *EMF1* locus to a fragment common to P1 clone MZH1, TAC K22P7, and cosmid CD82 (Figure 1A). We subcloned a SpeI–Asp718 fragment from the genomic DNA of TAC K22P7 and transformed *emf1-1* and *emf1-2* segregating plants. T2 seed from T1 lines carrying the *emf1* alleles were sown on kanamycin (Km) medium, and Km resistance and the *emf* mutant phenotype were scored in progeny of four *emf1-1* T1 lines totaling 231 plants and six *emf1-2* T1 lines totaling 884 plants (Table 1). The absence of any Km-resistant *emf* mutants showed that this region contained a functional *EMF1* gene that complements the *emf1* phenotypes. The complementing fragment has two ORFs (Figure 1A). One ORF has sequence homology with L-gulono-lactone oxidase (GLOxidase) and encodes a GLOxidase-like protein (Koshizaka et al., 1988). The second ORF has homology with two expressed sequence tags (ESTs) from Arabidopsis encoding putative polypeptides of unknown

Figure 1. (continued).

(B) Structure of the *EMF1* gene and positions of the three mutations in the mutant alleles, *emf1-1*, *emf1-2*, and *emf1-3*. Black boxes indicate exons, and lines between the boxes indicate introns.

(C) Allele-specific RFLPs created by the *emf1-1* and *emf1-2* mutations. The positions of primers and enzyme restriction sites for each genotype, wild type (WT) and mutants (*emf1-1* and *emf1-2*), are depicted in the schemes. Below, the left and right panels show the allele-specific RFLP analysis associated with the *emf1-1* and *emf1-2* alleles, respectively. Molecular mass markers are identified to the right of each gel.

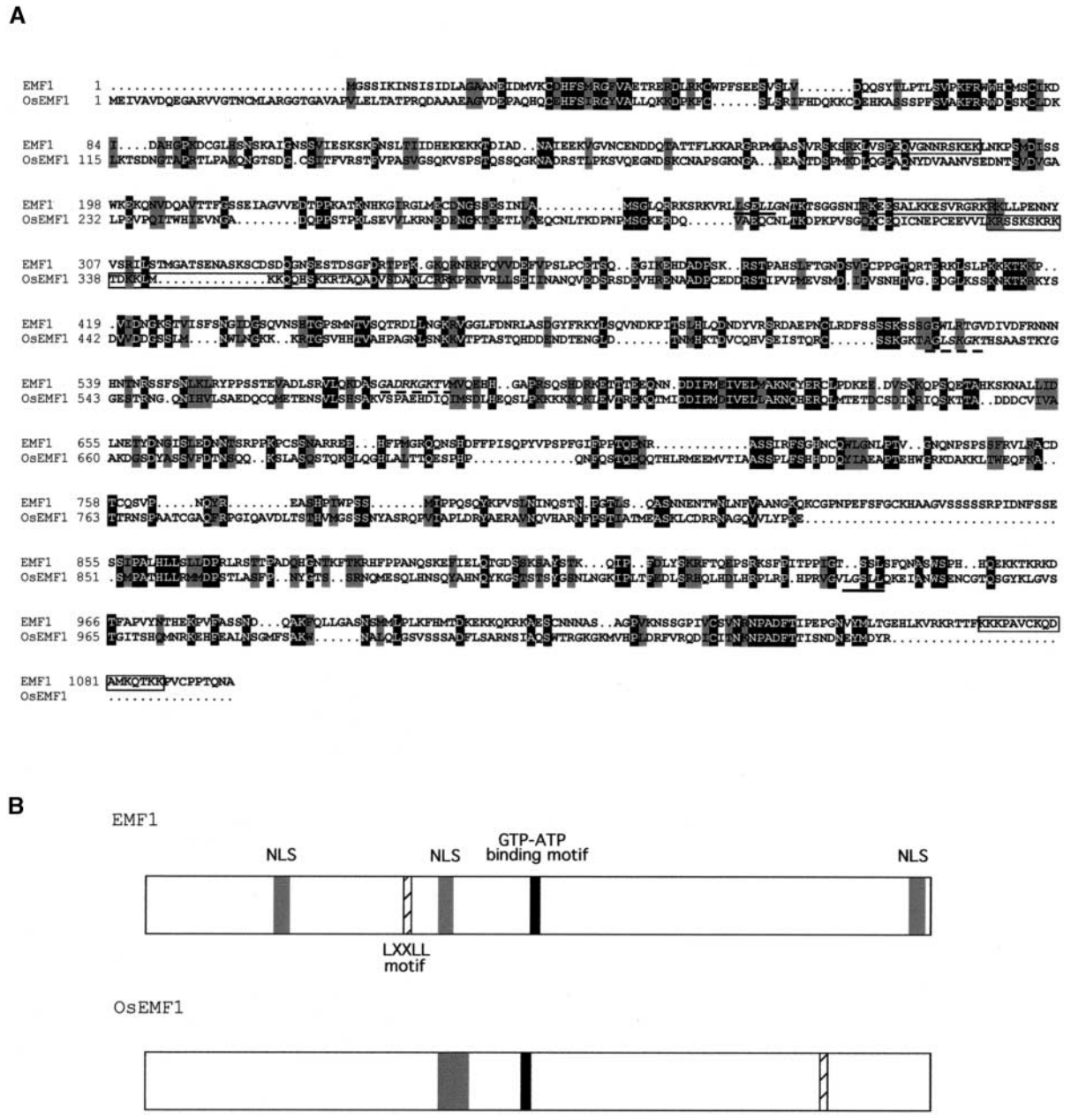

Figure 2. Alignment between Predicted EMF1 and OsEMF1 Amino Acid Sequences and Protein Structures.

(A) Alignment between predicted EMF1 and OsEMF1 amino acid sequences. Identical amino acid residues are shaded in black, and similar amino acid residues are shaded in gray. Dots denote gaps introduced by the alignment program. Boxed sequences indicate putative nuclear localization signals (NLSs). In Arabidopsis, each box includes one possible NLS, whereas in rice, the box includes four possible overlapping NLSs. The solid underlines mark the LXXLL motif. The dashed underlines mark the GTP-ATP binding motif (P-loop motif).

(B) Predicted EMF1 and OsEMF1 protein structures. Gray boxes, NLSs; black boxes, GTP-ATP binding motif (P-loop motif); hatched boxes, LXXLL motif.

function and a hypothetical protein from the rice genomic sequencing project.

We sequenced these two candidate genes from genomic DNA isolated from the *emf1* mutants and corresponding wild-type plants. All three *emf1* mutant alleles (*emf1-1*, *emf1-2*, and *emf1-3*) have a single base pair mutation in the second ORF: each mutation creates a stop codon interrupting the predicted ORF (Figure 1B). The *emf1-1* and *emf1-2* mutations generate allele-specific RFLPs that were detected using a polymerase chain reaction (PCR)-based method derived from the cleaved amplified polymorphic sequence marker (Konieczny and Ausubel, 1993; Michaels and Amasino, 1998). A G-to-A change in *emf1-1* and a deleted C in *emf1-2* changed a HaeIII restriction site to HincII in *emf1-1* and deleted a MaeIII site in *emf1-2*. The PCR products obtained from wild type, *emf1-1*, and *emf1-2* and digested with the relevant restriction enzymes had the predicted fragment length polymorphisms (Figure 1C). Based on the complementation experiment and the presence of mutations in all three mutant alleles, we concluded that we had identified the *EMF1* gene.

EMF1 and *OsEMF1*: A Novel Class of Putative Transcriptional Regulators

Using reverse transcription (RT)-PCR, we generated a 3.8-kb cDNA (see Methods) that is likely the full-length *EMF1* cDNA because the first ATG initiating a 1096–amino acid polypeptide is preceded by one or more stop codons in all frames. This cDNA detected a single low-abundance transcript of ~4 kb on poly(A)$^+$ RNA gel blots (see below). Sequence comparison between Arabidopsis genomic DNA and this cDNA product revealed seven introns (Figure 1B), all of which display the consensus border sequence GT/AG (Hanley and Schuler, 1988). The first and last introns are located in the untranslated transcribed regions; the other five introns are located within the coding region (Figure 1B). The *EMF1* gene encodes a predicted 121.7-kD protein (Figure 2A) with similarity to two ESTs from Arabidopsis and a rice sequence, as mentioned above. The two EST clones are identical to the cDNA clone amplified by RT-PCR. Analysis of the size of the two ESTs showed that they are partial cDNA clones. Furthermore, analysis of the Arabidopsis genome sequence did not reveal any other sequence closely related to the *EMF1* gene. Thus, we conclude that the *EMF1* gene is a single-copy gene in the Arabidopsis genome.

To better characterize the rice *EMF1* homolog (*OsEMF1*), we isolated the corresponding cDNA clone by the rapid amplification of cDNA ends technique (see Methods). The *OsEMF1* cDNA of 3896 nucleotides predicts a 1057–amino acid polypeptide (estimated molecular mass, 116.4 kD) that is 328 amino acids shorter than the predicted protein in BAA94774.1. The organization of introns and exons predicted at the 5′ end in BAA94774.1 was not confirmed by the sequence of the *OsEMF1* cDNA (Figure 2A). The *OsEMF1* cDNA likely includes a complete ORF because several stop codons are found in all three possible reading frames upstream of the first ATG initiating the 1057–amino acid polypeptide. The Arabidopsis and rice predicted protein sequences display 37% similarity and 20% identity over their entire lengths.

Neither *EMF1* nor *OsEMF1* displays significant homology with proteins of known function from any organism. Nevertheless, several domains could be identified in the predicted EMF1 and OsEMF1 polypeptides (Figure 2B), including nuclear localization signals (Raikhel, 1992), phosphorylation sites, an ATP/GTP binding motif (P-loop) (Walker et al., 1982), and an LXXLL motif. The LXXLL motif has been demonstrated to mediate the binding of steroid receptor coactivator complexes to a nuclear receptor (Heery et al., 1997; Torchia et al., 1997). In plants, it has been identified in the RGA and GAI proteins, two putative transcriptional regulators in the gibberellic acid signal transduction pathway (Peng et al., 1997; Silverstone et al., 1998). A PSI-BLAST homology search (Altschul et al., 1997) revealed a region of the EMF1 protein between amino acids 901 and 1034 that displays similarity (identity, 23%; positive, 37%) with two members of a nuclear receptor gene family. This gene family comprises one of the most abundant groups of transcriptional regulators in mammals, with members involved in various developmental processes (Sluder et al., 1999). Furthermore, the EMF1 protein displays homopolymeric stretches of serine residues, as do the two putative transcriptional regulators RGA and GAI (Silverstone et al., 1998). The identification of these motifs indicates that EMF1 and OsEMF1 could represent a new class of molecules that could function as transcriptional regulators during shoot development in higher plants.

Ubiquitous Expression of *EMF1*

To investigate the molecular mechanism of *EMF1*-regulated shoot development, we studied the spatial and temporal expression of *EMF1*. RNA gel blot analysis found *EMF1* mRNA in all organs examined: roots, rosette leaves, stems, cauline leaves, and flower clusters (Figure 3A). Using the *glyceraldehyde 3-phosphate dehydrogenase c* (*GAPc*) gene as a loading control, we found that *EMF1* was expressed in all vegetative organs and was more abundant (~20 to 30%) in flower clusters, which contain the inflorescence meristem, many flower meristems, and flowers of all stages. Thus, *EMF1* RNA appeared to be expressed constitutively. Figure 3B shows that *EMF1* RNA levels remained constant throughout the development of wild-type Arabidopsis plants grown under short-day conditions. Although *EMF1* RNA expression did not decrease during Arabidopsis development, as proposed previously (Chen et al., 1997), EMF1 protein activity may be modulated during development by protein modification via phosphorylation, nuclear localization, or other means.

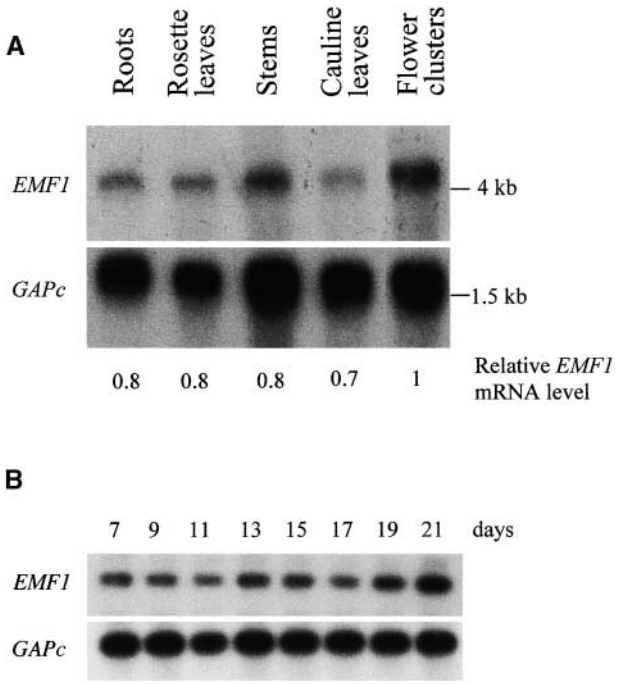

Figure 3. Expression of *EMF1* RNA in Wild-Type Arabidopsis.

(A) Autoradiographic determination of the relative amount of *EMF1* RNA from a blot containing 1 μg of poly(A)$^+$ RNA isolated from different tissues. The RNA gel blot was hybridized with an *EMF1* radioactive probe and, after stripping, with a *GAPc* probe as a loading control. The numbers below the gels indicate the relative amounts of *EMF1* RNA after standardization using the *GAPc* signal as a reference. Tissue samples were from roots of 2-week-old plants on agar plates; rosette leaves of 3- to 4-week-old plants in soil under short-day conditions; stems, cauline leaves, and flower clusters from an inflorescence shoot apex with developing buds and open flowers.

(B) An autoradiograph from semiquantitative RT-PCR analysis of the *EMF1* level in wild-type plants grown under short-day conditions. Total RNAs were isolated from seedlings at the times indicated. RT-PCR products were amplified with *EMF1* primers and *GAPc* primers and hybridized with an *EMF1* probe (top) or a *GAPc* probe (bottom).

Modulation of the *EMF1* Level Alters Flowering Time and Shoot Determinacy

To study the function of *EMF1*, we attempted to decrease *EMF1* expression in wild-type plants. Three constructs containing the *EMF1* coding sequence extending 0.6, 2.4, and 3.3 kb from the translation initiation codon in the antisense orientation under the control of the 35S cauliflower mosaic virus promoter (35S) (see Methods) were introduced into wild-type Arabidopsis (Bechtold et al., 1993). The 2226 T1 transgenic plants carrying the three different antisense constructs displayed a spectrum of *emf1*-like, early-flowering, and wild-type–like phenotypes (Figures 4A and 4B). The *emf1*-like plants were sterile, whereas the early-flowering plants were fertile and could grow in soil. The proportion of the three phenotypic categories observed varied among the constructs (Table 2). The two longer antisense constructs (2.4 and 3.3 kb) gave higher proportions of *emf1*-like transgenic plants and lower proportions of early-flowering plants than the shortest construct. The *emf1*-like transgenic plants, like *emf1* mutants (Figure 4C), lacked rosette leaves and flowered at 14 to 16 days after sowing. Early-flowering transgenic plants produced two to eight normal petiolated rosette leaves and flowered at 16 to 20 days after sowing (Figure 4A); in the same growth conditions, wild-type–like plants produced 10 to 13 rosette leaves and flowered at ~25 days after sowing. The endogenous *EMF1* transcript levels of the early-flowering and *emf1*-like antisense plants were decreased greatly relative to those of wild-type–like antisense plants and wild-type plants (Figure 4E).

All of the *emf1*-like and early-flowering antisense plants made the shift from indeterminate to determinate growth by producing terminal flowers (Figures 4A and 4B). Additionally, some early-flowering plants showed a sympodial branching phenotype during shoot development, a phenotype that is seen in nature (Foster and Gifford, 1974) but that is never observed in wild-type Arabidopsis. We also found evidence of the sensitivity of flower organ differentiation to *EMF1* level. Some *emf1*-like and early-flowering antisense plants with three or four rosette leaves produced stigmatic papillae and ovule-like structures on stamens or sepals (Figure 4D).

To study the effect of ectopic *EMF1* expression on shoot development, we generated 35S::sense *EMF1* transgenic plants (see Methods). Approximately 400 T1 plants and 3000 T2 plants were analyzed. The sense transgenic plants displayed the same flowering time phenotypes as the antisense transgenic plants: *emf1*-like, early-flowering, and wild-type–like plants. None of the 35S::sense *EMF1* transgenic plants were late flowering. In the *emf1*-like sense transgenic plants, no *EMF1* RNA was detected by RT-PCR and RNA gel blot analyses (data not shown). Thus, the *emf1*-like sense transgenic plant phenotypes are best explained by the occurrence of post-transcriptional gene silencing in response to the level of overexpression of *EMF1* RNA (Hamilton and Baulcombe, 1999). Transgenic plants that were verified to overexpress *EMF1* RNA by RNA gel blot analysis had wild-type–like phenotypes (data not shown).

Table 2. Phenotypes of 35S::Antisense *EMF1* T1 Transgenic Plants

Length of Antisense cDNA (kb)	Percent of *emf1*-Like Plants	Percent of Early-Flowering Plants	Percent of WT[a]-Like Plants	No. of Transgenic Plants Analyzed[b]
0.6	49.3	28.7	22.0	608
2.4	64.6	4.2	31.2	1025
3.3	63.9	6.8	29.3	593

[a] WT, wild-type.

[b] Analyzed at 25 to 30 days after germination.

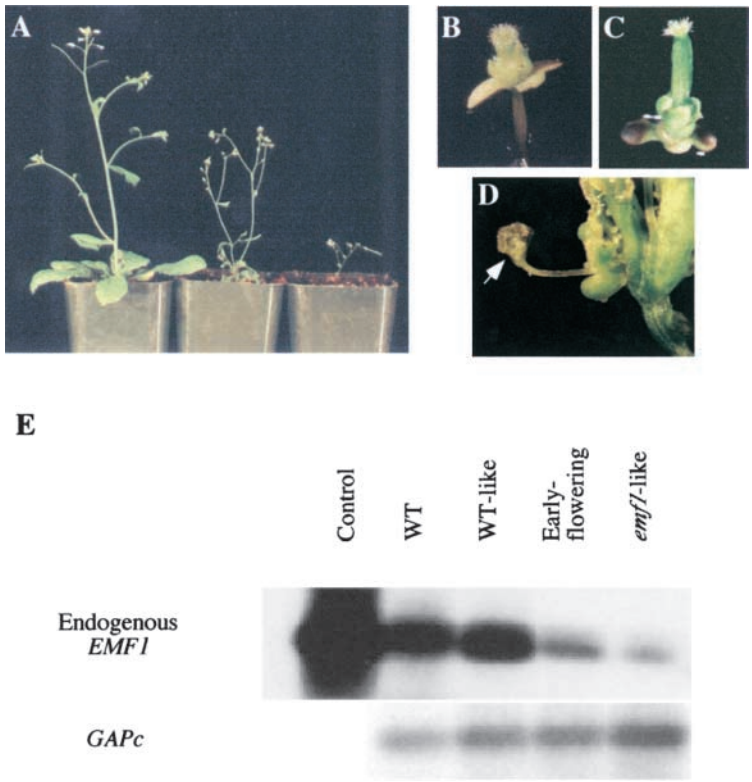

Figure 4. Phenotypes and *EMF1* mRNA Levels of 35S::Antisense *EMF1* Transgenic Plants.

Phenotypes of antisense transgenic plants and *emf1-1* mutants are shown in **(A)** to **(D)**.
(A) Thirty-four-day-old plants grown under long-day conditions. A wild-type–like plant is shown on the left, and two early-flowering antisense transgenic plants are shown on the right.
(B) A 25-day-old *emf1-1*–like transgenic plant grown under short-day conditions.
(C) A 25-day-old *emf1-1* mutant grown under short-day conditions.
(D) A flower of a 32-day-old early-flowering transgenic plant with ovule-like structures on stamens (arrow) or sepals.
(E) Semiquantitative RT-PCR analysis of endogenous *EMF1* levels in 25-day-old Columbia wild-type (WT) plants and antisense transgenic plants grown under short-day conditions. Total RNA in the wild-type plants, wild-type–like transgenic plants, and early-flowering transgenic plants was isolated from rosette/cauline leaves, and total RNA in *emf1*-like transgenic plants was isolated from seedlings. Control *EMF1* fragments were amplified using *EMF1* cDNA as a template. Shown are autoradiographs of RT-PCR products of endogenous *EMF1* mRNA (top) and *GAPc* mRNA (bottom).

DISCUSSION

Cloning of *EMF1* revealed that it encodes a novel protein that might function as a transcriptional regulator. The ubiquitous presence of *EMF1* RNA in Arabidopsis tissues indicates limited transcriptional regulation; however, the activity of the EMF1 protein may be modulated during development by protein phosphorylation, nuclear localization, or other means. *EMF1* affects diverse developmental processes. The phenotypes of the transgenic plants indicate that *EMF1* regulates flowering time, shoot determinacy, and floral organ identity. These various *EMF1* functions could be explained by involvement in multiple pathways via interaction with different partners or by varying EMF1 activities in different developmental stages. Below, we analyze the phenotypes of *EMF1* transgenic plants in an attempt to elucidate the function of the *EMF1* gene.

The Role of the *EMF1* Gene in Regulating Flowering (Bolting) Time

Flowering time is known to be regulated by photoperiod, vernalization, nutrients, and hormones (Blazquez, 2000). The

relationship between the proteins involved in these flowering pathways and the EMF1 protein remains to be characterized. *EMF1* loss-of-function mutants form no petiolated or rosette leaves; rather, they develop an inflorescence shoot directly from the embryo. This observation raised the possibility that *EMF1* specifies rosette leaf development and influences flowering time indirectly. The early-flowering phenotype would be a pleiotropic effect of defective leaf development. However, transgenic plants with an early-flowering phenotype caused by suppression of *EMF1* activity formed normal rosette leaves with long petioles before flowering, although there were fewer such leaves (Figure 4). This observation indicates that *EMF1* activity affects the number of rosette leaves produced before flowering but not leaf size or morphology.

Antisense *EMF1* transgenes caused wild-type plants to flower early, supporting the notion that *EMF1* suppresses flowering. Furthermore, the reduction of *EMF1* expression in the antisense transgenic plants correlated with flowering time (Figure 4E). This result suggests that a decrease in *EMF1* expression is required for flowering in wild-type plants. However, results from RT-PCR analysis showed that the level of *EMF1* expression did not decrease during development (Figure 3), suggesting that post-translational regulation of *EMF1* may be required in wild-type plant development.

Ectopic expression of *EMF1* in sense transgenic plants produced no late-flowering plants. On the contrary, we observed a gradient of early-flowering phenotypes, from extremely early, as in an *emf1* mutant, to moderately early, as in a *tfl1* mutant. Because these phenotypes were also observed in antisense transgenic plants, we postulate that they may result from gene silencing (Hamilton and Baulcombe, 1999). The normal phenotype of the transgenic plants that actually overexpressed *EMF1* suggests that overexpression of *EMF1* alone cannot delay flowering in wild-type plants. If other factors are required for a functional EMF1 complex, overexpression of these genes as well as *EMF1* may be required for a delayed-flowering phenotype.

Role of *EMF1* in Inflorescence Development

Based on mutant analysis, a role of *EMF1* in controlling Arabidopsis shoot meristem identity was proposed (Chen et al., 1997). The early-flowering transgenic plants with a determinate inflorescence and normal leaves demonstrated that this role was played by EMF1. The inflorescence development might be regulated by the level of EMF1 activity, as shown by the various levels of *EMF1* RNA found in the antisense transgenic plants with terminal flowers or determinate inflorescences.

Previous studies have suggested that both the *EMF1* and *TFL1* genes interact with the *LFY* and *AP1* genes in a reciprocal or mutual negative regulation manner (Chen et al., 1997; Liljegren et al., 1999). The production of a terminal flower was correlated with the ectopic expression of *AP1* and *LFY* on the *tfl1* inflorescence meristem (Weigel et al., 1992; Bowman et al., 1993; Gustafson-Brown et al., 1994; Bradley et al., 1997). In *emf1* mutants, the *AP1* promoter was activated prematurely in the shoot apical meristem (Chen et al., 1997). Plants constitutively expressing *LFY* and *AP1* produced a phenotype similar to that of the *tfl1* mutant (Mandel and Yanofsky, 1995; Weigel and Nilsson, 1995). Interestingly, alteration in *EMF1* expression also produced the *tfl1* phenotype, as seen in antisense transgenic plants; this finding suggests that, in contrast to *AP1* and *LFY*, which are negative regulators of *TFL1*, EMF1 activities may be required by *TFL1*. Furthermore, modification in the overexpression of *AP1* or *LFY* generated new phenotypes, such as an umbel-like shoot in 35S::*AP1 lfy* plants; this phenotype could be enhanced greatly under short-day conditions (Liljegren et al., 1999).

Modification of EMF1 activities in transgenic plants also produced such a phenotype under short-day conditions (data not shown). In a *35S*::*LFY 35S*::*TFL1* population, some carpelloid structures that developed secondary flowers terminated a shoot (Ratcliffe et al., 1999). This feature also was seen in some of our transgenic plants (data not shown). It is unclear whether the similar inflorescence structures produced by alteration of AP1, LFY, TFL1, and EMF1 activities ultimately were mediated by EMF1 activities or by a common downstream gene regulated by a combination of these genes' activities. The molecular basis of these phenotypes awaits further study. Nevertheless, these results show that *EMF1* mediates the same process regulated by *TFL1*, *AP1*, and *LFY* during inflorescence development and that *EMF1* is one of the key regulators of a network of genes that regulate global shoot architecture.

METHODS

Plant Material and Growth Conditions

Surface-sterilized *Arabidopsis thaliana* seed were stratified for 3 days in the cold and germinated on agar plates containing half-strength Murashige and Skoog (1962) salts and 15 g/L sucrose. Seedlings were grown under short-day conditions (8 hr of light/16 hr of dark) for 10 days and then transferred to soil and grown under long-day conditions (16 hr of light/8 hr of dark) except as indicated. The *emf1* mutants or *emf1*-like transgenic plants were grown on agar plates continuously under short-day conditions.

Identification of the Arabidopsis and Rice *EMF1* cDNAs

The longest Arabidopsis *EMF1* cDNA was identified from cDNA prepared by reverse transcription–polymerase chain reaction (RT-PCR) using 1 μg of total RNA from 4-day-old seedlings (AMLV-RT; Promega, Madison, WI). One-fifth of the reaction was amplified using a pfu Taq polymerase (Stratagene, La Jolla, CA). The PCR products were purified by agarose gel electrophoresis. DNA was isolated from

the gel using a gel extraction kit (Qiagen, Valencia, CA) and cloned into a blunt end vector (pCR-Blunt; Invitrogen, Carlsbad, CA). Comparison of amplified sequences with genomic sequences revealed the intron positions. The sequence of the 3′ end primer was 5′-CCCTCTCTTTGTATCCCTC-3′. Several primers were designed from the genomic sequence at the putative 5′ end of the *EMF1* transcript. The primer 5′-ATCGAGCTCGAATCTCGC-3′, situated 1033 bp upstream of a putative start codon that initiates the longest open reading frame (ORF), gave the longest RT-PCR product (~3.8 kb).

To clone the *OsEMF1* cDNA, we isolated total RNA from 7-day-old rice (*Oryza sativa* cv Nipponbare) seedlings using the RNeasy plant mini kit (Qiagen). Using 1 μg of total RNA and the SMART RACE (rapid amplification of cDNA ends) cDNA amplification kit (Clontech, Palo Alto, CA), we synthesized first-strand cDNAs and amplified cDNAs corresponding to the *OsEMF1* transcript. The amplified PCR fragments were cloned into the pCR2.1-TOPO vector (Invitrogen) and sequenced.

Constructs and Plant Transformation

For the complementation experiment, an SpeI–Asp718 genomic fragment of the transformation-competent bacterial artificial chromosome K22P7 was cloned into the binary vector pPZP211 (Hajdukiewicz et al., 1994) and transformed into *emf1-1* and *emf1-2* segregating plants. Seed of T1 transgenic plants were sown on kanamycin (Km) Murashige and Skoog medium, and Km resistance and the *emf1* mutant phenotypes were scored. T1 lines with *emf1* mutant alleles were identified by allele-specific restriction fragment length polymorphism (RFLP) or by the occurrence of Km-sensitive *emf1* mutants in T2 populations.

The antisense constructs were made by inserting ORF fragments extending 0.6 and 2.4 kb from the initiation codon into the Asp718 site of pGA1535 and inserting a 3.3-kb fragment into the XbaI–Asp718 site of pGA1535 in the antisense orientation. For the sense construct, a *uidA* gene in pBI121 was replaced with the fragment containing the longest cDNA of *EMF1* in the sense orientation.

Constructs were transformed into *Agrobacterium tumefaciens* strain GV3101 (pMP90) and then into Arabidopsis plants as recommended by Bechtold et al. (1993). Seed from the transformed plants were selected with 50 mg/L Km.

RT-PCR Experiments

The amount of *EMF1* transcript was determined by semiquantitative RT-PCR. The RT conditions were as described for the cloning of *EMF1* cDNA. The PCR amplification was performed in a final volume of 12.5 μL using a Promega Taq polymerase according to the recommendations of the supplier. Amplification was verified as being in the exponential phase: 15 to 20 cycles for the *EMF1* gene, 10 to 15 cycles for the *GAPc* gene. The data shown are representative of the tissues or time points in at least three independent RT-PCR experiments.

Poly(A)$^+$ RNA Isolation and Hybridization

Total RNA was isolated according to the protocol established by Logemann et al. (1987). Poly(A)$^+$ RNA was then purified using the Oligotex mRNA kit (Qiagen). RNA and DNA gel blots were analyzed as described (Sambrook et al., 1989). The hybridization was performed with Church buffer (Church and Gilbert, 1984). After hybridization, membranes were washed at 65°C in 2 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate) and 0.1% SDS, then in 1 × SSC and 0.1% SDS, and finally in 0.1 × SSC and 0.1% SDS for 20 min each.

GenBank Accession Numbers

The GenBank accession numbers are as follows: bacterial artificial chromosome clone F15N18, AL163815; ESTs from Arabidopsis encoding putative polypeptides, N96450 and Z46543; hypothetical protein from the rice genomic sequencing project, BAA94774-1; *EMF1* gene, AF319968; *OSEMF1* cDNA of 3896 nucleotides, AF326768.

ACKNOWLEDGMENTS

We are grateful to T. Ito and K. Shinozaki (Institute of Physical and Chemical Research, RIKEN, Tsukuba, Japan) for sharing unpublished information and for their efforts in the search for the *EMF1* cDNA clones, to S. Tabata and S. Sato (Kazusa DNA Research Institute, Kisarazu, Chiba, Japan) for sharing their data before publication, and to Denise Schichnes and Steven Ruzin (Biological Imaging Facility, College of Natural Resources, University of California) for assistance with image processing. We also thank W. Lukowitz (Carnegie Institution of Washington, Department of Plant Biology, Stanford, CA), G. An (POSTECH, Pohang, Korea), and P. Maliga (Waksman Institute, Rutgers University, New Brunswick, NJ) for providing aliquots of binary cosmid library and binary vectors, and the Arabidopsis Biological Resource Center at the Ohio State University (Columbus) for providing the cDNA library and clones. This work was supported by U.S. Department of Agriculture Grant 99-35301-7984, by funds from the Norvatis Agricultural Discovery Institute to Z.R.S., and by a Korean Government fellowship (KOSEF) to Y.-H.M.

Received March 6, 2001; accepted May 29, 2001.

REFERENCES

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D.J. (1997). Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. Nucleic Acids Res. **25,** 3389–3402.

Alvarez, J., Guli, C.L., Yu, X.-H., and Smyth, D.R. (1992). *terminal flower*: A gene affecting inflorescence development in *Arabidopsis thaliana*. Plant J. **2,** 103–116.

Amaya, I., Ratcliffe, O.J., and Bradley, D.J. (1999). Expression of *CENTRORADIALIS* (*CEN*) and *CEN*-like genes in tobacco reveals a conserved mechanism controlling phase change in diverse species. Plant Cell **11,** 1405–1418.

Bechtold, N., Ellis, J., and Pelletier, G. (1993). *In planta Agrobacterium*-mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. C. R. Acad. Sci. Paris Life Sci. **316,** 1194–1199.

Blazquez, M. (2000). Flower development pathways. J. Cell Sci. **113,** 3547–3548.

Bowman, J.L., Alvarez, J., Weigel, D., Meyerowitz, E.M., and Smyth, D.R. (1993). Control of flower development in *Arabidopsis thaliana* by *APETALA1* and interacting genes. Development **119,** 721–743.

Bradley, D., Carpenter, R., Copsey, L., Vincent, C., Rothstein, S., and Coen, E. (1996). Control of inflorescence architecture in Antirrhinum. Nature **379,** 791–797.

Bradley, D., Ratcliffe, O., Vincent, C., Carpenter, R., and Coen, E. (1997). Inflorescence commitment and architecture in Arabidopsis. Science **275,** 80–83.

Chen, L., Cheng, J.C., Castle, L., and Sung, Z.R. (1997). *EMF* genes regulate Arabidopsis inflorescence development. Plant Cell **9,** 2011–2024.

Church, G.M., and Gilbert, W. (1984). Genomic sequencing. Proc. Natl. Acad. Sci. USA **81,** 1991–1995.

Cronquist, A. (1988). The Evolution and Classification of Flowering Plants, 2nd ed. (Bronx, NY: New York Botanical Garden).

Foster, A.S., and Gifford, E.M., Jr. (1974). Comparative Morphology of Vascular Plants. (San Francisco: W.H. Freeman).

Gustafson-Brown, C., Savidge, B., and Yanofsky, M.F. (1994). Regulation of the floral homeotic gene *APETALA1*. Cell **76,** 131–143.

Hajdukiewicz, P., Svab, Z., and Maliga, P. (1994). The small, versatile *pPZP* family of *Agrobacterium* binary vectors for plant transformation. Plant Mol. Biol. **25,** 989–994.

Hamilton, A.J., and Baulcombe, D.C. (1999). A species of small antisense RNA in posttranscriptional gene silencing in plants. Science **286,** 950–952.

Hanley, B.A., and Schuler, M.A. (1988). Plant intron sequences: Evidence for distinct groups of introns. Nucleic Acids Res. **16,** 7159–7176.

Heery, D.M., Kalkhoven, E., Hoare, S., and Parker, M.G. (1997). A signature motif in transcriptional co-activators mediates binding to nuclear receptors. Nature **387,** 733–736.

Huala, E., and Sussex, I.M. (1992). *LEAFY* interacts with floral homeotic genes to regulate floral development. Plant Cell **4,** 901–913.

Konieczny, A., and Ausubel, F.M. (1993). A procedure for mapping Arabidopsis mutations using co-dominant ecotype-specific PCR-based markers. Plant J. **4,** 403–410.

Koornneef, M., Hanhart, C.J., and Van der Veen, J.H. (1991). A genetic and physiological analysis of late flowering mutants in *Arabidopsis thaliana*. Mol. Gen. Genet. **229,** 57–66.

Koshizaka, T., Nishikimi, M., Ozawa, T., and Yagi, K. (1988). Isolation and sequence analysis of a complementary DNA encoding rat liver L-gulono-gamma-lactone oxidase, a key enzyme for L-ascorbic acid biosynthesis. J. Biol. Chem. **263,** 1619–1621.

Levy, Y.Y., and Dean, C. (1998). The transition to flowering. Plant Cell **10,** 1973–1989.

Liljegren, S.J., Gustafson-Brown, C., Pinyopich, A., Ditta, G., and Yanofsky, M.F. (1999). Interactions among *APETALA1*, *LEAFY*, and *TERMINAL FLOWER1* specify meristem fate. Plant Cell **11,** 1007–1018.

Logemann, J., Schell, J., and Willmitzer, L. (1987). Improved method for the isolation of RNA from plant tissues. Anal. Biochem. **163,** 16–20.

Mandel, M.A., and Yanofsky, M.F. (1995). A gene triggering flower formation in Arabidopsis. Nature **377,** 522–524.

Michaels, S.D., and Amasino, R.M. (1998). A robust method for detecting single-nucleotide changes as polymorphic markers by PCR. Plant J. **14,** 381–385.

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue culture. Physiol. Plant. **15,** 473–497.

Peng, J., Carol, P., Richards, D.E., King, K.E., Cowling, R.J., Murphy, G.P., and Harberd, N.P. (1997). The Arabidopsis *GAI* gene defines a signaling pathway that negatively regulates gibberellin responses. Genes Dev. **11,** 3194–3205.

Poethig, R.S. (1990). Phase change and the regulation of shoot morphogenesis in plants. Science **250,** 923–930.

Raikhel, N. (1992). Nuclear targeting in plants. Plant Physiol. **100,** 1627–1632.

Ratcliffe, O.J., Bradley, D.J., and Coen, E.S. (1999). Separation of shoot and floral identity in Arabidopsis. Development **126,** 1109–1120.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, 2nd ed. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Schultz, E.A., and Haughn, G.W. (1991). *LEAFY*, a homeotic gene that regulates inflorescence development in Arabidopsis. Plant Cell **3,** 771–781.

Schultz, E.A., and Haughn, G.W. (1993). Genetic analysis of the floral initiation process (FLIP) in Arabidopsis. Development **119,** 745–765.

Shannon, S., and Meeks-Wagner, D.R. (1991). Genetic interactions that regulate inflorescence development in Arabidopsis. Plant Cell **3,** 877–892.

Silverstone, A.L., Ciampaglio, C.N., and Sun, T.-p. (1998). The Arabidopsis *RGA* gene encodes a transcriptional regulator repressing the gibberellin signal transduction pathway. Plant Cell **10,** 155–169.

Sluder, A.E., Mathews, S.W., Hough, D., Yin, V.P., and Maina, C.V. (1999). The nuclear receptor superfamily has undergone extensive proliferation and diversification in nematodes. Genome Res. **9,** 103–120.

Sung, Z.R., Belachew, A., Shunong, B., and Bertrand-Garcia, R. (1992). *EMF*, an Arabidopsis gene required for vegetative shoot development. Science **258,** 1645–1647.

Torchia, J., Rose, D.W., Inostroza, J., Kamei, Y., Westin, S., Glass, C.K., and Rosenfeld, M.G. (1997). The transcriptional coactivator p/CIP binds CBP and mediates nuclear-receptor function. Nature **387,** 677–684.

Walker, J.E., Saraste, M., Runswick, M.J., and Gay, N.J. (1982). Distantly related sequences in the α- and β-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. EMBO J. **1,** 945–951.

Weigel, D., and Nilsson, O. (1995). A developmental switch sufficient for flower initiation in diverse plants. Nature **377,** 495–500.

Weigel, D., Alvarez, J., Smyth, D.R., Yanofsky, M.F., and Meyerowitz, E.M. (1992). *LEAFY* controls floral meristem identity in Arabidopsis. Cell **69,** 843–859.

Yang, C.H., Chen, L.J., and Sung, Z.R. (1995). Genetic regulation of shoot development in Arabidopsis: Role of the *EMF* genes. Dev. Biol. **169,** 421–435.

Zagotta, M.T., Shannon, S., Jacobs, C., and Meeks-Wagner, R. (1992). Early flowering mutants of *Arabidopsis thaliana*. Aust. J. Plant Physiol. **19,** 411–418.