EMF Genes Maintain Vegetative Development by Repressing the Flower Program in Arabidopsis[W]

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The *EMBRYONIC FLOWER* (*EMF*) genes *EMF1* and *EMF2* are required to maintain vegetative development and repress flower development. *EMF1* encodes a putative transcriptional regulator, and *EMF2* encodes a Polycomb group protein homolog. We examined expression profiles of *emf* mutants using GeneChip technology. The high degree of overlap in expression changes from the wild type among the *emf1* and *emf2* mutants was consistent with the functional similarity between the two genes. Expression profiles of *emf* seedlings before flower development were similar to that of Arabidopsis flowers, indicating the commitment of germinating *emf* seedlings to the reproductive fate. The germinating *emf* seedlings ectopically expressed flower organ genes, suggesting that vegetative development in wild-type plants results from *EMF* repression of the flower program, directly or indirectly. In addition, the seed development program is derepressed in the *emf1* mutants. Gene expression analysis showed no clear regulation of *CONSTANS* (*CO*), *FLOWERING LOCUS T* (*FT*), *LEAFY* (*LFY*), and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* by *EMF1*. Consistent with epistasis results that *co*, *lfy*, or *ft* cannot rescue rosette development in *emf* mutants, these data show that the mechanism of *EMF*-mediated repression of flower organ genes is independent of these flowering genes. Based on these findings, a new mechanism of EMF-mediated floral repression is proposed.

INTRODUCTION

Higher plants must flower to undergo sexual reproduction. To increase flower and progeny production, plants delay flowering until they have built up body size through vegetative growth. Flowering time can be finely regulated by floral inducers and floral repressors, as indicated by the isolation of late- and early-flowering mutants (Koornneef et al., 1998; Araki, 2001). The floral induction pathway in Arabidopsis has been under intense investigation, and multiple floral signal transduction pathways have been identified (Blazquez, 2000; Mouradov et al., 2002; Simpson and Dean, 2002). For example, daylength is perceived by phytochromes and cryptochromes and transduced via the late-flowering genes *GIGANTEA* (*GI*) and *CONSTANS* (*CO*) (Koornneef et al., 1991) to genes such as *FLOWERING LOCUS T* (*FT*) and *SUPPRESSOR OF OVEREXPRESSION OF CON-STANS1* (*SOC1*) (*AGAMOUS-LIKE20* [*AGL20*]) (Samach et al., 2000), which activate the flower meristem identity gene *APETALA1* (*AP1*). The vernalization signal is mediated by *FLOWERING LOCUS C* (*FLC*) (Michaels and Amasino, 1999), which represses flowering by negatively regulating the expression of the floral promotion genes *SOC1* and *FT* (Samach et al., 2000; Hepworth et al., 2002). *LEAFY* (*LFY*) is a key link between the flowering signals and flower development. The large increase in *LFY* RNA level in the flower meristem activates the expression of the downstream flower organ identity genes *AP1*, *AP3*, *PISTILLATA* (*PI*), and *AGAMOUS* (*AG*) to initiate flower organ development (Krizek and Meyerowitz, 1996; Busch et al., 1999; Wagner et al., 1999; Blazquez and Weigel, 2000).

Less is known about the mechanism that prevents flower development. Approximately a dozen early-flowering genes have been reported in Arabidopsis (Zagotta et al., 1992; Sung et al., 2003b). Some of these genes encode proteins that sense light or daylength, such as *EARLY FLOWERING3* (*ELF3*) and *ELF4* (Hicks et al., 2001; Doyle et al., 2002). Other genes, such as *FLC*, *TERMINAL FLOWER2* (*TFL2*), and *EARLY BOLTING IN SHORT DAYS* (*EBS*) (Larsson et al., 1998; Michaels and Amasino, 1999; Gomez-Mena et al., 2001), inhibit the action of a key floral integrator, *FT* (Blazquez et al., 2001). The *TFL1* gene encodes a possible membrane-associated protein that can bind hydrophobic ligands, and it acts antagonistically to *LFY* and *AP1* and vice versa (Bradley et al., 1997; Liljegren et al., 1999).

The *EMF* genes are required for vegetative development. Plants defective in the *EMF1* or *EMF2* gene skip vegetative growth and flower upon germination (Sung et al., 1992). Four *emf1* mutants and nine *emf2* mutants have been identified (Sung et al., 2003a). The weak *emf1* mutant *emf1-1* and all nine *emf2* mutants germinate directly into a small inflorescence that produces a few sterile flowers. In the strong *emf1* mutants, all lateral organs, including the cotyledons, differentiate into carpelloid structures (Chen et al., 1997). *EMF1* encodes a novel protein of 121 kD, which is predicted to be a transcriptional

regulator (Aubert et al., 2001). *EMF2* encodes a 71-kD Polycomb group (PcG) protein containing a zinc finger motif and a VEFS domain conserved among other PcG proteins, such as VERNALIZATION2 (Gendall et al., 2001), FERTILIZATION-INDEPENDENT SEED2, and Suppressor of zeste12 (Birve et al., 2001; Yoshida et al., 2001).

The *EMF* genes have been shown to regulate flowering time because modulation of EMF activities generates transgenic plants that flower at different times (Aubert et al., 2001; Yoshida et al., 2001). Consistent with previous findings based on genetic studies (Chen et al., 1997), transgenic studies confirm the role of the *EMF* genes in regulating inflorescence development. Some of the novel inflorescence architecture seen in transgenic plants with modulated EMF activities resembles that of transgenic plants with altered flower meristem identity gene activities (Liljegren et al., 1999). Both the nature of the EMF proteins and the phenotypes of the transgenic plants support the notion that the *EMF*-mediated floral repression mechanism interacts with the floral induction mechanism mediated by the late-flowering and floral meristem identity genes. However, the early-flowering phenotype of *emf* mutants cannot be rescued by any flowering time, flower meristem, or flower organ identity mutants studied (Yang et al., 1995; Chen et al., 1997; Haung and Yang, 1998), indicating that these genes do not act downstream from EMF to mediate flowering time. Thus, the nature of the EMF-mediated floral repression is not well understood.

To gain insights into the mechanism of *EMF*-regulated floral repression, we used Affymetrix GeneChip technology to examine the expression profiles of *emf* mutants. Mutant expression profiles were compared with wild-type developmental stage–specific expression profiles. The expression patterns of the flower organ identity genes and some flowering genes were verified by reverse transcription (RT) PCR analysis. An EMF-mediated repression mechanism based on chromatin remodeling is proposed to explain the global repression of the flower and seed programs during vegetative development.

RESULTS

Global Gene Expression Patterns in *emf* Mutants

GeneChip technology (Zhu et al., 2001) was used to profile global gene expression patterns in *emf1-1*, *emf1-2*, *emf2-1*, and the wild type. Total RNA was isolated from whole plants grown on agar plates (Figure 1) under short-day (SD) conditions as described in Methods. Each GeneChip contains ∼8900 probe sets representing 8300 unique Arabidopsis genes (Zhu and Wang, 2000; Zhu et al., 2001). Each probe set includes 16 probe pairs of perfect match probes and mismatch probes for each gene. The expression level of each gene was determined by the difference in the intensity of the hybridization signal between perfect match and mismatch probes (Zhu et al., 2001). Duplicate experiments for 15-day-old wild-type seedlings showed a very high correlation coefficient (0.997) among all 8735 probe sets, excluding Affymetrix GeneChip controls, demonstrating the reproducibility of the experiments (data not shown).
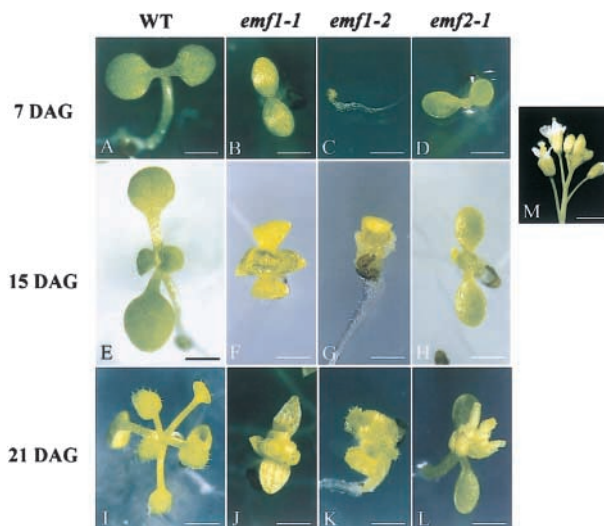
Figure 1. Phenotypes of Wild-Type and *emf* Mutants.

Seven-day-old wild-type (WT) Columbia ecotype **(A)**, *emf1-1* **(B)**, *emf1-2* **(C)**, and *emf2-1* **(D)**, 15-day-old wild-type Columbia **(E)**, *emf1-1* **(F)**, *emf1-2* **(G)**, and *emf2-1* **(H)**, and 21-day-old wild-type Columbia **(I)**, *emf1-1* **(J)**, *emf1-2* **(K)**, and *emf2-1* **(L)**. A Columbia ecotype flower cluster is shown in **(M)**. Bars = 1 mm in **(A)** to **(D)** and **(F)** to **(H)**, 1.4 mm in **(E)**, 1.5 mm in **(I)**, 1.1 mm in **(J)** to **(L)**, and 3.8 mm in **(M)**.

A High Percentage of Overlap in Gene Expression Change among the Three emf Mutants

To compare the gene expression profiles in mutants of the weak and strong phenotypes and of the two different *EMF* genes, we chose three mutants: two weak mutants (*emf1-1* and *emf2-1*) and a strong mutant (*emf1-2*). Based on preliminary analysis, a hybridization signal of <50 was considered background on this set of GeneChips (Zhu et al., 2001); only genes with hybridization signals of >50 in at least one of the four plant types (wild type and the three mutants) were included for further analysis. From the 7-, 15-, and 21-day-old samples, 3872, 3659, and 3820 genes corresponding to 4213, 4009, and 4222 probe sets, respectively, met our stringency criterion (Table 1).

Gene expression changes were determined by the ratio of the hybridization signal of the mutant to the wild type. Genes showing a greater than twofold difference in hybridization signals between the *emf* mutant and wild-type samples were identified as genes upregulated or downregulated in the mutant (Table 1). Of the 3872 genes identified in the 7-day-old samples, 1287 (33.3%), 1448 (37.4%), and 892 (23.0%) showed differential expression in *emf1-1*, *emf1-2*, and *emf2-1*, respectively. The 21-day-old samples showed greater differences between the mutants and the wild type; of 3820 genes identified, 1878 (49.1%), 2298 (60.2%), and 1744 (45.7%) showed differential expression in *emf1-1*, *emf1-2*, and *emf2-1*, respectively (Table 1).

We then determined whether these genes were upregulated or downregulated similarly across the three mutants and found a high percentage of overlap in the genes that showed expres-

Table 1. Changes in Gene Expression in *emf* Mutants

Mutant	7 DAG (3872)[a]			15 DAG (3659)			21 DAG (3820)		
	Up[b]	Down	Total	Up	Down	Total	Up	Down	Total
emf1-1	831 (21.5)	456 (11.8)	1287 (33.3)	448 (12.2)	459 (12.5)	907 (24.7)	830 (21.7)	1048 (27.4)	1878 (49.1)
emf1-2	847 (21.9)	601 (15.5)	1448 (37.4)	575 (15.7)	659 (18.0)	1234 (33.7)	981 (25.7)	1317 (34.5)	2298 (60.2)
emf2-1	484 (12.5)	408 (10.5)	892 (23.0)	–	–	–	732 (19.2)	1012 (26.5)	1744 (45.7)

[a] Number of genes showing intensity of >50 in at least one of the four samples, the wild type, and the three mutants.
[b] Number of genes (percentage) upregulated or downregulated more than twofold in the mutant(s).

sion changes. Among the 3872 genes selected from the 7-day-old plants, 1214 were upregulated and 765 were downregulated in at least one of the 7-day-old *emf* mutants. A total of 262 of 1214 genes (21.6%) were upregulated and 241 of 765 genes (31.5%) were downregulated in all three mutants (Figures 2A and 2B). Higher overlap in expression change was observed among the three mutants at 21 days after germination (DAG). Among the 3820 genes selected, 478 of 1246 (38.4%) were upregulated and 819 of 1476 (55.5%) were downregulated in all three 21-day-old mutants (Figures 2C and 2D). The high percentage of genes with similar expression changes in *emf1* and *emf2* mutants indicates that these mutations affect similar gene programs and that the two *EMF* genes are related functionally.

Gene Expression Pattern Is More Similar between the Two *emf1* Mutants Than between the Two Weak Mutants

The percentage of genes that showed similar expression change was compared among the three mutants. First, we found that the expression profiles of the two *emf1* mutants were very similar. At 7 DAG, 44.6% of the genes were upregulated similarly and 50.1% of the genes were downregulated similarly (Figures 2A and 2B). In the 21-day-old samples, similar upregulation and downregulation were observed in more genes, 50.2 and 64.5%, respectively (Figures 2C and 2D), even though the two *emf1* mutants had developed into distinctly different forms at 21 days (Figure 1). Second, we found that the overlap in expression profiles between the two weak mutants (*emf2-1* and *emf1-1*) was less than that between the two *emf1* mutants. In 7-day-old plants, 31.1 and 37.1% of the genes were upregulated and downregulated similarly in the two weak mutants (Figures 2A and 2B). In 21-day-old plants, 45.6 and 57.7% of the genes were upregulated and downregulated similarly (Figures 2C and 2D). Despite the phenotypic similarity between *emf1-1* and *emf2-1*, which are morphologically distinct from *emf1-2* (Figure 1), a greater molecular difference was found between *emf2-1* and *emf1-1* than between the two *emf1* mutants. These results suggest differences in the gene programs regulated by the two *EMF* genes.

A High Degree of Overlap in Gene Expression Change between *emf* Seedlings and Wild-Type Floral Buds

Because *emf* mutants are expected to be committed to a reproductive fate (Sung et al., 1992), we compared the expres-

sion patterns of the mutants and wild-type floral buds and found a high degree of overlap. For example, 193 of 262 (73.7%) upregulated genes in all three *emf* mutants at 7 DAG also were upregulated in wild-type floral buds relative to wild-type seedlings. A total of 384 of 478 (80.3%) of the genes upregulated in all three *emf* mutants at 21 DAG were upregulated in the floral buds. An even higher percentage of overlap was observed among the downregulated genes. Ninety and 83.4% of the downregulated genes in the 7- and 21-day-old *emf* samples also were downregulated in wild-type floral buds (data not shown). Because 7-day-old *emf* mutants have not produced
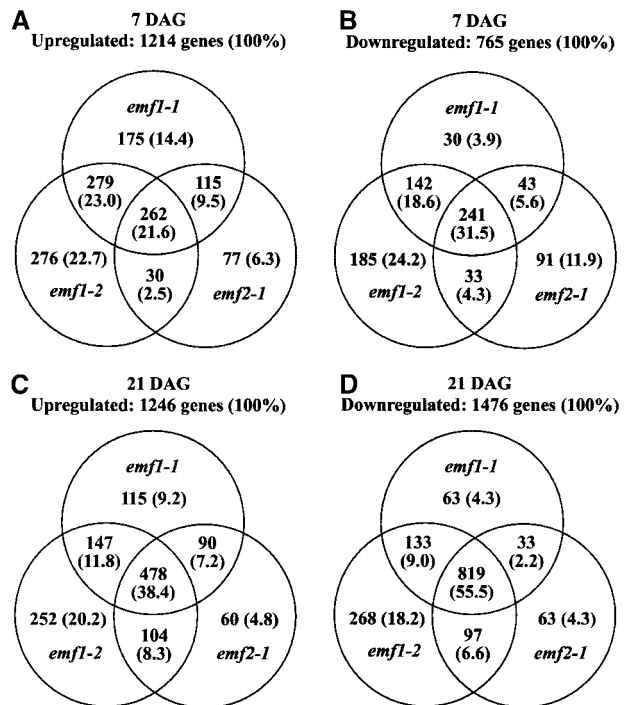
Figure 2. Genes Upregulated or Downregulated in 7- and 21-Day-Old *emf1-1*, *emf1-2*, and *emf2-1* Mutants Relative to the Wild Type.

From the GeneChip data, 3872 and 3820 genes with a hybridization signal of >50 in 7- and 21-day-old plants, respectively, were selected and analyzed. Numbers and percentages (in parentheses) of genes upregulated at 7 **(A)** and 21 **(C)** DAG and downregulated at 7 **(B)** and 21 **(D)** DAG in a mutant with greater than twofold difference are shown.

floral buds or floral organ primordia (Bai and Sung, 1995), activation of the flower program appears to have occurred before flower development in the *emf* mutants.

Functional Classification of the Genes Upregulated and Downregulated in *emf* Mutants

Functionally related genes were categorized to investigate the possible function of the *EMF* genes (Table 2). The gene names were based on the improved annotations for probe sets of the Affymetrix GeneChip (http://wwwbiology.ucsd.edu/labs/schroeder/downloads.html and http://genetics.mgh.harvard.edu/sheenweb/search_affy.html). Flowering genes, including flowering time, flower meristem identity, and flower organ identity genes, and seed maturation genes, including late embryogenesis–abundant (LEA) genes and seed storage protein genes, were upregulated in *emf* mutants. However, indoleacetic acid (IAA)–inducible and IAA transport genes, expansin genes, and genes related to photosynthesis light reaction were downregulated in *emf* mutants (Table 2).

Flowering Genes, Particularly the Flower Organ Genes, Are Upregulated in emf Mutants

Twenty-one of 35 flowering genes investigated showed an increase in transcript level in at least one of the three mutants (Tables 2 and 3). Flower organ–specific genes, including the flower homeotic or MADS box genes, such as *AP1*, *AP3*, *PI*, *AG*, *SHATTERPROOF1* (*SHP1*) (*AGL1*), *SEPALLATA2* (*SEP2*) (*AGL4*), *SEP3* (*AGL9*), *AGL11*, *CRABS CLAW*, and *f-AtMBP*,

showed ectopic expression in all three *emf* mutants (Tables 2 and 3). These genes are expressed ectopically as early as 7 DAG, and the transcript levels of many of these genes continued to increase during the 3-week growth period (Table 3, Figure 3), probably because of the increase in *FT*, *LFY*, or *SOC1* RNA in the last 2 weeks (Table 3).

The expression pattern of the flowering time genes and floral pathway integrators was examined. *FT* transcripts tended to increase in the three mutants relative to the wild type by 21 DAG, and *GI* transcripts were high in all samples. However, *CO*, *LFY*, and *SOC1* levels were very low on the GeneChip in all four samples (Table 3; see also supplemental data online).

The Seed Maturation Program Is Upregulated in emf1 Mutants

Twenty-eight of 32 seed maturation genes, including the LEA genes and the seed storage protein genes, were upregulated in *emf* mutants by 21 DAG (Tables 2 and 4). During the 3-week period, the numbers and RNA levels of upregulated seed maturation genes increased (Tables 2 and 4). The strong mutant, *emf1-2*, had the highest transcript level at the earliest time point, whereas the *emf2-1* mutant showed the least change from the wild type. At 7 DAG, 16 of the 32 seed maturation genes were upregulated in *emf1-2* mutants, 3 were upregulated in *emf1-1*, and none was upregulated in *emf2-1* (Table 2). At 21 DAG, 20 and 27 seed maturation genes were upregulated in the two *emf1* mutants, whereas only 6 were upregulated in *emf2-1*, with much lower expression levels than in the two *emf1* mutants (Tables 2 and 4).

Table 2. Number of Genes Involved in Different Functional Groups Upregulated or Downregulated at Least Twofold in *emf* Mutants

Category	Total No. of Genes Investigated	Change in Gene Expression	*emf1-1*			*emf1-2*			*emf2-1*		Names of Representative Upregulated or Downregulated Genes[b]
			7[a]	15	21	7	15	21	7	21	
Flowering	35	Up	9	13	16	12	10	16	6	18	See Table 3
		Down	0	1	0	0	0	0	0	0	See Table 3
Seed maturation	32	Up	3	15	20	16	13	27	0	6	See Table 4
		Down	0	0	0	0	1	0	0	0	See Table 4
Photoreceptors	12	Up	2	2	7	4	0	6	1	4	PHYC/D, PHY-associated protein 2, PHYA suppressor, COP9
		Down	4	2	2	4	3	2	4	2	PHYA, PHY-associated protein 3, NPH1, flavin-type blue-light photoreceptor
Photosynthesis light reaction	29	Up	0	0	0	0	0	0	0	1	–
		Down	29	4	19	29	26	23	1	13	CAB, PSI subunit precursors, PSII OEC
Auxin-related	25	Up	1	0	2	1	0	2	0	3	IAA12/21
		Down	7	8	8	7	10	5	9	8	IAA2/3/7/17/19, PIN3/4/7
Gibberellin-related	13	Up	0	0	2	0	1	3	0	0	GA 20 oxidase, GA-regulated gene
		Down	3	1	2	3	2	2	1	1	GASA4, GAST1-like
Ethylene-related	9	Up	0	3	3	1	5	4	0	3	EREBF4/5, ACC synthase
		Down	2	1	2	3	1	2	2	3	ACC oxidase
Expansin	5	Up	0	1	1	0	0	0	0	0	–
		Down	4	2	3	3	3	4	4	4	AtEXP1/6, putative expansins

[a] Data shown are days after germination.
[b] See supplemental data online.

Table 3. Expression Levels of Flowering Genes Upregulated or Downregulated in *emf* Mutants

Gene[a]	Probe Set	Protein ID[b]	Wild Type			*emf1-1*			*emf1-2*			*emf2-1*		Floral Bud
			7[c]	15	21	7	15	21	7	15	21	7	21	
GI	17580	CAB56039	779	759	676	554	370	596	477	654	1535	541	1565	523
FT	16113	BAA77838	−1	16	−4	6	173	146	4	12	58	1	202	28
SOC1	14123	AAC06175	0	12	20	3	17	35	0	10	50	−1	120	22
FLF	17501	AAD21249	10	5	20	31	33	10	54	12	48	32	31	12
AGL8	12876	AAA97403	3	8	2	5	209	372	6	11	22	−1	113	265
CAL	19456	AAC67513	3	0	8	3	29	97	42	46	105	6	91	44
AP1	12882	CAA78909	−11	−13	−14	11	172	104	105	8	75	−7	37	319
PI	16648	BAA87000	0	12	8	247	294	564	482	840	1320	819	1222	625
AP3	19527	AAD51900	6	5	3	410	318	149	561	521	209	247	127	216
AG	12864	CAA37642	6	5	3	280	240	134	388	264	163	224	169	232
SEP2 (AGL4)	12873	AAF02125	−9	−9	−16	128	380	628	278	403	1164	79	808	314
SEP3 (AGL9)	16614	AAC00586	−4	50	17	235	504	547	362	286	583	262	782	630
SHP1 (AGL1)	12865	AAA32730	1	12	5	129	61	345	159	51	597	47	370	179
SHP2 (AGL5)	12874	AAA32735	−3	5	3	24	23	61	23	80	107	3	22	14
CRABS CLAW	17538	AAD30526	63	47	80	168	661	1462	1162	1394	3214	126	708	669
SPT	19740	CAB16798	13	20	12	28	33	52	60	97	116	5	67	25
AGL6	12875	AAC06173	16	14	26	27	24	22	19	49	343	18	296	169
AGL11	17131	AAC49080	9	12	8	112	108	168	370	391	1225	84	96	104
BELL1	16162	AAB05099	13	48	19	42	36	41	49	71	45	32	78	31
ANR1	19609	AAD25638	3	−1	−3	10	60	216	50	44	232	44	129	0
f-AtMBP	20227	BAA82151	5	19	−3	96	401	1047	193	16	48	21	1228	1834

[a] Genes selected based on intensity level of >50 in at least one of the four samples, the wild type and the three *emf* mutants.

[b] Protein accession number in GenBank.

[c] Data shown are days after germination.

The temporal expression patterns of the seed maturation genes in the *emf* mutants are plotted in Figure 3, which demonstrates the continuous increase in the transcript levels in the *emf1* mutants during the 3-week period. This result indicates that despite their ectopic expression, the expression of the seed maturation genes in *emf1* plants still was developmentally controlled. Moreover, the progressive derepression of these seed-specific genes during *emf1* development shows that their ectopic expression does not result from the residual expression of the seed maturation program after germination. Because strong *emf1-2* mutants do not have carpelloid organs at 7 DAG and none of the *emf1* mutants can produce seeds, seed-specific gene expression (Gaubier et al., 1993; Conceicao Ada and Krebbers, 1994) in these mutants does not result from seed development. Unlike the *emf1* mutants, the seed maturation genes did not show obvious upregulation in the *emf2-1* mutants, suggesting functional differences between *EMF1* and *EMF2* genes in the repression of the seed maturation genes.

Other Functional Categories of Genes That Show Differential Expression in the Mutants

Table 2 shows that auxin-inducible genes (*IAA2*, *IAA3*, *IAA7*, *IAA17*, and *IAA19*) and auxin transport genes (*PIN3*, *PIN4*, and *PIN7*) were downregulated in all three *emf* mutants. Compared with 7-, 15-, and 21-day-old wild-type genes, the auxin-inducible genes and auxin transport genes also were downregulated in floral buds (see supplemental data online). Although the role of these genes in floral buds is not clear, they serve as molecular markers confirming the floral state of the *emf* mutants.

Photosynthesis light reaction–related genes were downregulated in all three *emf* mutants (Table 2; see also supplemental data online). However, differences were detected between the two *emf1* mutants and the *emf2-1* mutant. For example, at 7 DAG, all 29 genes investigated were downregulated in both *emf1-1* and *emf1-2*, but only one gene was slightly downregulated in *emf2-1* (Table 2; see also supplemental data online). This finding again shows a functional difference between the *EMF1* and *EMF2* genes.

### RT-PCR Results Confirm the Ectopic Expression of Flower Organ Identity Genes

To confirm the GeneChip results on the ectopic expression of flower organ identity genes in *emf* mutants, we analyzed the temporal expression patterns of the four flower organ identity genes (*AP1*, *AP3*, *PI*, and *AG*) in the mutants and the wild type grown under SD conditions by RT-PCR. Flower clusters, consisting of inflorescence meristem and floral buds of various stages, were used as a positive control. Relative amounts of the flower organ identity gene transcripts were standardized against *glyceraldehyde 3-phosphate dehydrogenase c* (*GAPc*) transcripts; the ratio of the hybridization intensity of the flower organ identity gene to that of *GAPc* was determined as described in Methods.

As expected, the four flower organ identity genes were highly

Flowering genes
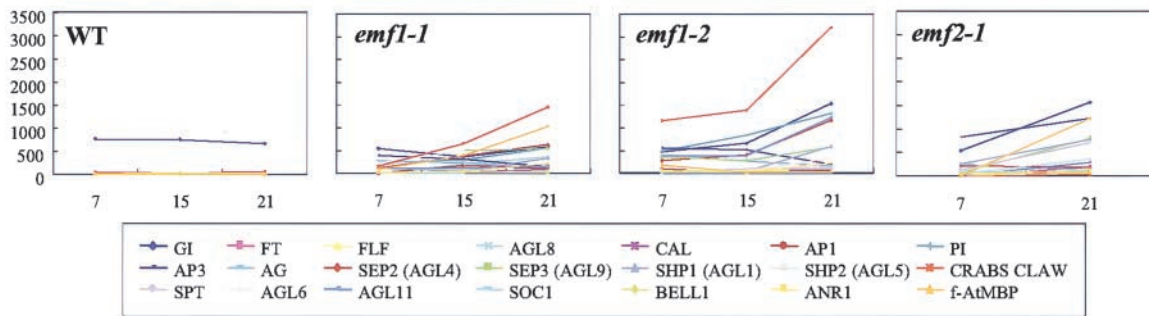
Seed maturation genes
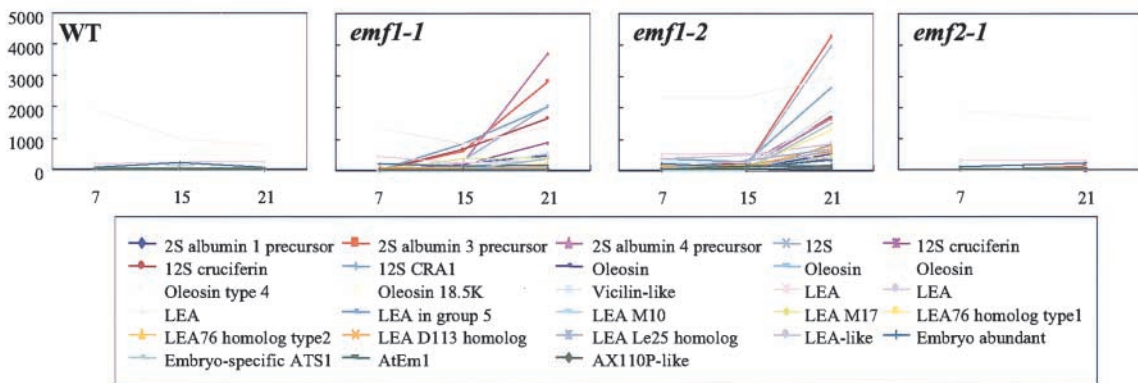
Figure 3. Temporal Expression Patterns of the Flowering Genes and Seed Maturation Genes in the Wild Type and *emf* Mutants.

Horizontal and vertical axes represent days after germination and intensities of the hybridization signal, respectively. See Tables 3 and 4 for the intensity of each gene's hybridization signal. WT, wild type.

expressed in the wild-type flower clusters but not in wild-type seedlings or rosette shoots, except for the low levels of *PI* and *AG* transcripts in 21-day-old samples (Figure 4). On the other hand, all four genes were expressed ectopically in the three mutants. The *AP1* and *PI* RNA levels were higher in all three mutants than in the wild type as early as 4 DAG. The ectopic expression of *AP3* and *AG* was detected as early as 4 DAG in the two weak mutants and at 7 DAG in the strong mutant (Figure 4). The ratio of hybridization intensities showed a temporal increase in the transcript levels after the initial ectopic expression in the mutants. The RT-PCR results were consistent with the GeneChip results in that the flower organ identity genes were expressed ectopically in *emf* mutants and there was a progressive increase in transcript levels during mutant development.

The two weak mutants have an inflorescence consisting of a few floral buds and several sessile leaves at 14 DAG and open flowers at 21 DAG (Chen et al., 1997) (Figure 1). Although the strong *emf1* mutants do not seem to have an inflorescence and lack most floral organs, its lateral organs became carpelloid at

21 DAG (Figures 1G and 1K). Thus, the expression of the flower organ identity genes in 2- and 3-week-old *emf* mutants could result from flower and flower organ development. However, because 4- and 7-day-old *emf* mutants do not contain flower organ primordia, expression of the flower organ identity genes in the first week precedes, and thus does not result from, flower development.

Temporal Expression Patterns of *CO*, *FT*, *LFY*, and *SOC1* in *emf* Mutants

During wild-type development, the expression of flower organ identity genes is activated by the floral pathway integrators *FT*, *SOC1*, and *LFY* (Mouradov et al., 2002; Simpson and Dean, 2002). To confirm the GeneChip results on *FT* and to investigate whether upregulation of the flower organ identity genes is caused by the upregulation of floral pathway integrators, we studied the expression of *LFY*, *FT*, *SOC1*, and a late-flowering gene, *CO*, by RT-PCR. RNA levels of these genes in 4-, 7-, 14-, and 21-day-old wild-type, *emf1-1*, *emf1-2*, and *emf2-1* plants

Table 4. Expression Levels of Seed Maturation Genes, Including Seed Storage Protein and LEA Genes, Upregulated or Downregulated in *emf* Mutants

Gene[a]	Probe Set	Protein ID[b]	Wild Type 7[c]	15	21	emf1-1 7	15	21	emf1-2 7	15	21	emf2-1 7	21	Floral Bud
2S albumin 1 precursor	15983	CAB38844	−11	49	−3	−5	242	483	12	12	806	−14	6	−9
2S albumin 3 precursor	13194	CAB38846	−10	92	−8	−7	617	2832	161	140	4259	−6	127	−8
2S albumin 4 precursor	15984	AAA32746	−3	28	8	1	244	3723	20	92	1660	3	30	0
12S	13195	CAA32493	−1	70	−2	−2	295	2052	170	90	3967	−1	85	−2
12S cruciferin	13200	AAM13004	−25	−16	−31	−4	176	868	22	21	367	−5	−17	−3
12S cruciferin	16025	AAB17379	−17	80	−14	−18	662	1658	103	228	1722	−29	11	−9
12S CRA1	16425	AAA32777	−2	139	−2	2	849	2024	362	266	2637	14	53	13
Oleosin	18714	CAA44225	1	−2	−24	−11	12	118	19	29	531	−2	−3	−28
Oleosin	19003	AAC42242	−3	−2	1	6	42	45	74	105	323	0	8	46
Oleosin	16575	AAA87295	6	68	7	7	258	509	287	231	1804	16	21	13
Oleosin type 4	18991	CAA63011	3	24	−12	5	105	336	31	24	645	10	1	19
Oleosin 18.5K	20412	CAB36756	65	102	69	44	293	986	159	128	1780	59	76	49
Vicilin-like	20535	AAD21484	4	11	10	1	47	558	76	41	1897	4	22	9
LEA	16023	AAC23428	185	235	250	443	247	325	505	560	382	307	301	249
LEA	20004	AAC61808	−2	0	1	20	103	41	367	484	853	13	9	9
LEA	19181	AAM64340	1901	976	780	1362	840	1381	2305	2356	2948	1924	1622	427
LEA in group 5	15597	BAA11016	1	3	7	2	−1	2	2	−33	174	1	−1	0
LEA M10	19355	AAC78544	−1	−2	3	3	7	38	−3	−1	63	−1	1	0
LEA M17	16896	AAC78545	−3	−8	−9	3	363	412	85	30	464	−10	26	−6
LEA76 homolog type 1	19918	CAA63006	−6	−4	−5	3	81	217	177	113	1287	2	−1	−3
LEA76 homolog type 2	20641	CAA63012	0	17	1	50	108	93	135	85	790	22	23	31
LEA D113 homolog	19152	CAA63008	3	7	−6	90	57	146	133	181	602	44	25	16
LEA Le25 homolog	14439	CAA61676	4	5	6	1	41	75	81	298	697	7	8	−6
LEA-like	18872	CAA10352	−3	2	2	5	19	66	38	57	685	3	1	1
Embryo abundant	14083	AAC78535	55	214	56	223	140	163	216	92	323	110	232	18
Embryo-specific ATS1	20681	CAB36520	4	4	−3	1	16	363	6	11	1496	0	12	2
AtEm1	17282	CAA77509	−1	−1	1	0	16	17	41	144	155	0	−1	−3
AX110P-like	20025	AAD39613	29	24	27	31	22	31	45	31	71	29	61	22

[a] Genes selected based on intensity level of >50 in at least one of the four samples, the wild type and the three *emf* mutants.
[b] Protein accession number in GenBank.
[c] Data shown are days after germination.

grown under SD conditions were investigated (Figure 5). RNA extracted from wild-type flower clusters consisting of inflorescence meristems and floral buds was used as a positive control. As expected, these genes were highly expressed in the flower clusters (Figure 5). In general, there was a temporal increase in transcript levels of the four genes in both mutants and the wild type. The temporal patterns of *FT* expression detected among the four plants were consistent with those determined by the GeneChip analysis.

CO and *FT* RNA levels appeared to be higher in the two weak mutants than in the wild type as early as 4 DAG and definitely by 7 DAG. But these two genes did not show precocious expression in the strong mutants (Figure 5). Similar data were obtained with samples grown under long-day conditions (data not shown). *SOC1* RNA levels were comparable between the two weak mutants and the wild type, whereas they were lower in the strong mutant. *LFY* RNA levels did not show upregulation in any of the three mutants relative to that in the wild type in the first week after germination. By the second week, the two weak mutants contained more *LFY* RNA than the wild type but the strong mutant did not (Figure 5).

In summary, the weak mutants expressed *CO*, *FT*, and *LFY* at an earlier time than the wild type. However, the strong *emf1* mutant, *emf1-2*, did not show upregulation in any of the genes investigated, suggesting that precocious expression of these four genes is not responsible for the ectopic expression of the flower organ identity genes in *emf1* mutants. Because there is no strong *emf2* mutant available for study, it is difficult to determine the roles of *CO* and *FT* in the ectopic expression of the flower organ identity genes in the *emf2* mutant.

Temporal and Spatial *LFY::β-Glucuronidase* Expression Pattern in *emf* Mutants

It has been reported that a high level of *LFY* expression activates the transcription of flower organ identity genes in wild-type plants (Parcy et al., 1998). Because *LFY* expression is restricted spatially to the shoot apex, an in situ assay would more accurately depict the temporal and spatial patterns of *LFY* expression than GeneChip or RT-PCR assays. We reported previously the ectopic *AP1*::β-glucuronidase (*GUS*) expression in *emf* mutants (Chen et al., 1997). In this study, we asked
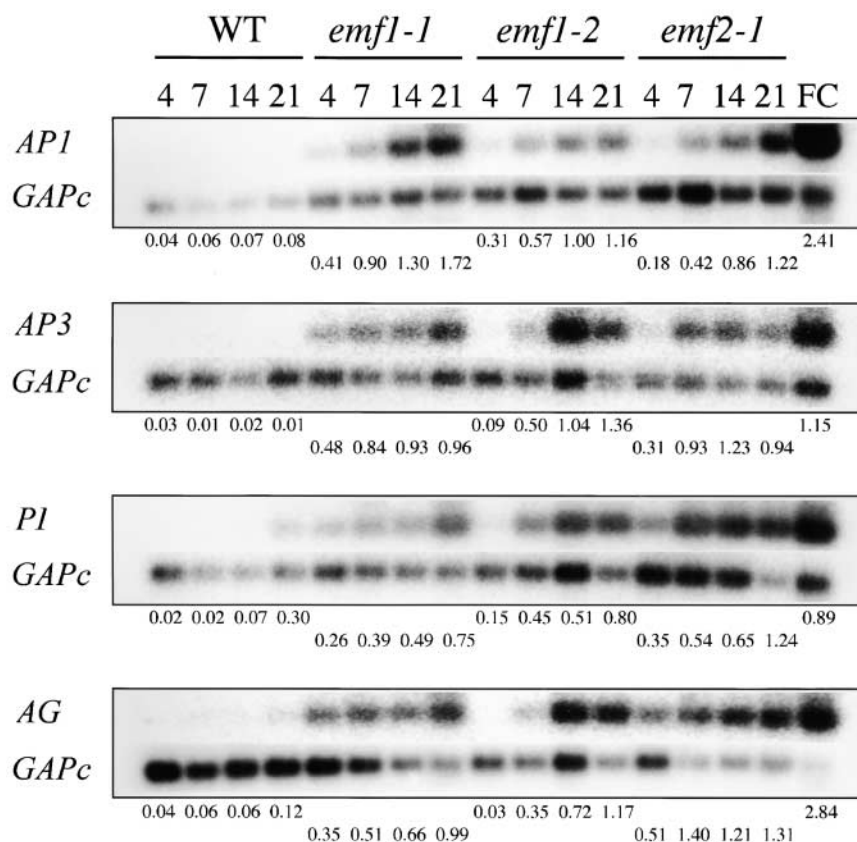
Figure 4. Expression of *AP1*, *AP3*, *PI*, and *AG* in *emf* Mutants.

Autoradiograph of semiquantitative RT-PCR analysis of *AP1*, *AP3*, *PI*, *AG*, and *GAPc* RNA levels in 4-, 7-, 14-, and 21-day-old wild-type (WT) Columbia, *emf1-1*, *emf1-2*, and *emf2-1* plants grown under SD conditions. *GAPc* was used as a RT-PCR control. RT-PCR products amplified were hybridized with a probe of each gene. The relative amount of each gene after standardization using the *GAPc* signal as a reference is presented below each gel. FC, flower clusters consisting of inflorescence meristems and floral buds.

whether ectopic *LFY*::*GUS* expression in *emf* mutants could be responsible for the ectopic *AP1* expression.

LFY::*GUS* activity was weak but detectable at the shoot apex of 4-day-old wild-type plants, as reported previously (Bai et al., 2000), and the activity was detected readily at the shoot apex of 7- and 14-day-old wild-type plants (Figures 6A and 6B). In *emf1-1* and *emf1-2*, the *LFY*::*GUS* activity pattern was similar to that of the wild type, except that the activity in 14-day-old *emf1-2* began to disappear, showing GUS activity on patches of carpelloid tissues in only a few plants (Figures 6C to 6F). At 4 and 7 DAG, no ectopic GUS activity was detected in *emf1* mutants compared with the wild type, and at all ages investigated, no GUS activity was found outside of the mutant shoot apex.

For comparison with the *LFY*::*GUS* result, we performed the *AP1*::*GUS* assay in parallel with the *LFY*::*GUS* assay. Precocious expression of *AP1*::*GUS* activity in *emf* seedlings was detected at 4, 7, and 14 DAG, as reported previously (Chen et al., 1997; Chou et al., 2001). *emf1-2* mutants showed strong *AP1*::*GUS* activity in tissues other than the shoot apex, that is,

in cotyledons and hypocotyls at 7 DAG (Figure 6G), and in the basal region of cotyledons containing carpelloid structures at 14 DAG (Figure 6H). The fact that *LFY*::*GUS* activity was confined strictly to the shoot apical region, and was never present in the cotyledons and hypocotyls, indicates that the ectopic *AP1* expression in *emf1-2* is not activated by *LFY*.

DISCUSSION

To study the molecular mechanism of EMF-mediated floral repression, we compared gene expression patterns in the *emf* mutants and the wild type. Our findings show that both *EMF* genes repress the flower program. There are differences in the gene expression patterns among the three *emf* mutants: some may be gene specific and others may reflect different developmental states of the mutants.

The transcript levels of *FT*, *AP1*, *AP3*, *PI*, and *AG* determined by GeneChip and RT-PCR analysis show similar trends and lead to similar conclusions (Table 3, Figures 4 and 5). The ec-

topic expression of the flower organ identity genes in *emf1* seedlings in the GeneChip and RT-PCR results confirms the increased *AG* RNA and *AP1::GUS* activities in the mutants reported previously (Chen et al., 1997).

### *EMF* Genes Maintain Vegetative Development by Repressing the Flower Program

The high degree of overlap in upregulated and downregulated genes among the three *emf* mutants suggests that the *EMF* genes regulate similar gene programs. Although 7-day-old *emf* mutants have the same organs as the wild-type seedlings (i.e., cotyledon, root, and hypocotyl), they show a similar gene expression pattern as the floral buds (Figure 1, Tables 1 and 3). Of particular interest are the flower organ identity genes (*AP1*, *AP3*, *PI*, and *AG*) and other flower organ–specific genes (*CRABS CLAW*, *SEP2*, *SEP3*, and *AGL11*), which are upregulated in all three *emf* mutants at all time points investigated (Table 3, Figure 3). Because 7-day-old *emf* mutants do not have flower organ primordia, the expression of these genes is not a result of flower development but rather a consequence of the lack of *EMF* re-

pression. In wild-type plants, rosette shoots develop as a result of *EMF* repression of the flower program. In fact, constitutive activation of the flowering program can generate an *emf*-like phenotype (Kardailsky et al., 1999; Simpson and Dean, 2002). These phenomena demonstrate that vegetative development could result from the repression of reproductive development.

### The Three Mutants Are Committed to Different Reproductive States

The *emf* mutants skip the vegetative shoot and germinate as reproductive shoots. The distinct morphological features of the strong and weak mutants must result from developmental differences, which are reflected by the differential expression patterns of the developmentally regulated genes.

The inflorescence state of the two weak mutants is reflected by their precocious expression of *CO*, *FT*, and *LFY* (Table 3, Figure 5). Similar results were reported by other investigators. For example, 14-day-old *emf2-3* mutants under long days contain more *FT* transcripts than the wild type (Yoshida et al.,
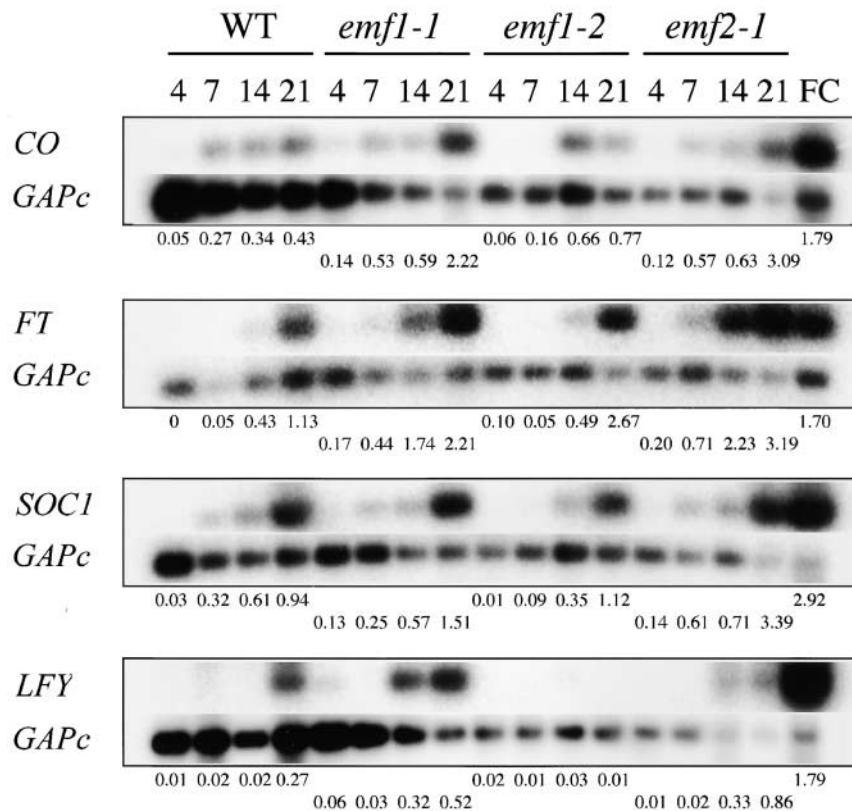
Figure 5. Expression of *CO, FT, SOC1,* and *LFY* in *emf* Mutants.

Autoradiograph of semiquantitative RT-PCR analysis of *CO*, *FT*, *SOC1*, *LFY*, and *GAPc* RNA levels in 4-, 7-, 14-, and 21-day-old wild-type (WT) Columbia, *emf1-1*, *emf1-2*, and *emf2-1* plants grown under SD conditions. *GAPc* was used as a RT-PCR control. RT-PCR products amplified were hybridized with a probe of each gene. The relative amount of each gene after standardization using the *GAPc* signal as a reference is presented below each gel. FC, flower clusters consisting of inflorescence meristems and floral buds.
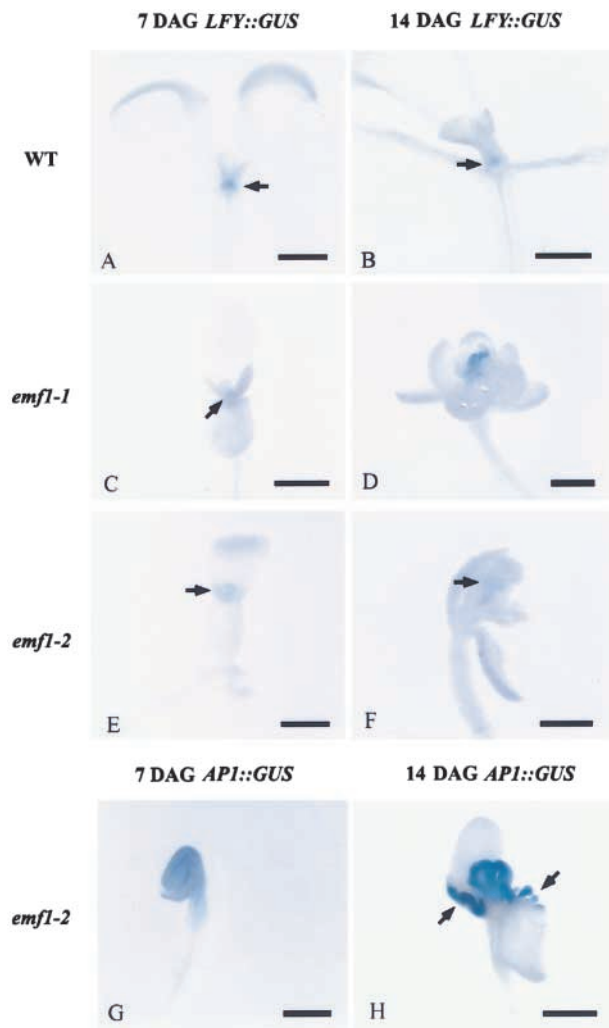
Figure 6. Histochemical Localization of GUS Activity in 7- and 14-Day-Old Wild-Type and *emf1* Seedlings Harboring the *LFY*::*GUS* or *AP1*::*GUS* Transgene.

GUS activity is indicated by blue color. Bars = 0.5 mm.

(A) *LFY*::*GUS* wild type (WT) at 7 DAG, showing GUS activity at the shoot tip (arrow).

(B) *LFY*::*GUS* wild type at 14 DAG, showing GUS activity at the meristematic region of the shoot tip (arrow).

(C) *LFY*::*GUS emf1-1* at 7 DAG, showing GUS activity at the shoot tip (arrow).

(D) *LFY*::*GUS emf1-1* at 14 DAG, showing GUS activity at the shoot tip.

(E) *LFY*::*GUS emf1-2* at 7 DAG, showing GUS activity at the shoot tip (arrow).

(F) *LFY*::*GUS emf1-2* at 14 DAG, showing GUS activity in a patch of the carpelloid tissue (arrow).

(G) *AP1*::*GUS emf1-2* at 7 DAG, showing GUS activity in the shoot tip, cotyledons, and hypocotyl.

(H) *AP1*::*GUS emf1-2* at 14 DAG, showing GUS activity in the carpelloid structure and the papillae tissue developed at the base of the cotyledons (arrows).

2001), and 10-day-old *emf1-1* and *emf2-1* mutants contain more *LFY* transcripts than the wild type (Chou et al., 2001). On the other hand, the strong mutant, *emf1-2*, showed no clear increase in *CO*, *FT*, *SOC1*, and *LFY* transcript levels relative to the wild type (Figure 5), consistent with the notion of the absence of an inflorescence stage in *emf1-2*.

The seed maturation gene expression pattern also indicates developmental differences among the three *emf* mutants (Table 4, Figure 3). Upregulation of seed maturation genes, including LEA and seed storage protein genes, was observed in 7-day-old *emf1-2* plants but not as early in the weak mutants, *emf1-1* and *emf2-1* (Table 4).

Consistent with the mutant phenotypes, the differential gene expression pattern—normal or reduced expression of the floral pathway integrator genes in *emf1-2*, yet stronger expression of flower organ and seed maturation genes than in the two weak mutants—indicates that, at germination, *emf1-2* resides at a more advanced reproductive state than the two weak mutants.

Possible Mechanism of EMF-Mediated Floral Repression

The *EMF* genes may regulate flowering time by modulating the activity of key floral pathway integrators, such as *FT*, *LFY*, and *SOC1* (Mouradov et al., 2002; Simpson and Dean, 2002), as do the other floral repressors *FLC* and *EBS* (Blazquez et al., 2001; Gomez-Mena et al., 2001; Hepworth et al., 2002). However, genetic analysis shows that this is not the case. *ft* and *lfy* cannot delay flowering time in *emf* mutants, as do the other flowering time and flower organ identity mutants tested (Yang et al., 1995; Chen et al., 1997; Haung and Yang, 1998). Although *ft* can lengthen the inflorescence phase, it cannot rescue rosette development in *emf* mutants (Haung and Yang, 1998). Gene expression analysis based on temporal and spatial expression pattern is in agreement with genetic data in that *LFY*, *FT*, or *SOC1* does not mediate *EMF1* action on flowering time (Figures 5 and 6). Although *FT* is upregulated as early as the flower organ identity genes in the 4-day-old weak mutants, the absence of precocious expression of *FT* in the strong *emf1* mutant and the inability of *ft* to rescue rosette development in both *emf1* and *emf2* mutants preclude the possibility of *FT*-mediated *EMF* action on flowering time. Thus, precocious *FT* expression probably is attributable to the inflorescence state of the weak *emf* seedling.

The predicted amino acid sequence suggests that EMF1 is a transcriptional regulator and that EMF2 has a C_2H_2 zinc finger domain. These two proteins could be involved in transcriptional regulation, resulting in the global repression of reproductive gene programs during vegetative development. Because *EMF2* encodes a PcG protein homolog, it may be involved in transcriptional regulation at the chromatin level. In *Drosophila*, PcG proteins form protein complexes that restrict the access of transcriptional activators to the target genes (Lewin, 2000; Tie et al., 2001). The discovery that several other early-flowering genes encode chromatin-related proteins (Goodrich et al., 1997; Gaudin et al., 2001) suggests that these proteins may be involved in remodeling the chromatin, thereby restricting the access of the floral promoters (e.g., *LFY*) to the flower organ

identity genes during vegetative development. As promoters increase in concentration in the reproductive shoot, they gain access to the target genes and potentially can activate gene transcription via the recruitment of chromatin-remodeling proteins (Wagner and Meyerowitz, 2002). The progressive increase in the expression of flower organ–specific genes in the *emf* mutants suggests that in the absence of *EMF* repression, the expression of the flower organ genes is subject to regulation by the floral promoters. Hence, we propose that to control flowering time, the floral promoters and EMF repressors converge on the same target genes, the flower organ–specific genes. Recently it was demonstrated that FERTILIZATION-INDEPENDENT ENDOSPERM, a PcG protein, is involved in floral repression as well as the repression of endosperm development (Kinoshita et al., 2001). It is possible that these proteins display overlapping functions through participation in common protein complexes that modulate chromatin structure.

METHODS

Plant Materials and Growth Conditions

Surface-sterilized *Arabidopsis thaliana* seeds were plated on agar plates containing half-strength Murashige and Skoog (1962) salts and vitamins, 1.5% Suc, and 0.8% agar. The plates were placed for 2 days in the cold, and then seedlings were grown under short-day conditions (8 h of light/16 h of dark) at 21°C. For plants grown on soil, 10- to 12-day-old normal plants grown under short-day conditions were transferred to soil and grown under long-day conditions (16 h of light/8 h of dark) at 21°C in the greenhouse.

Semiquantitative Reverse Transcription–PCR and Hybridization

Total RNA was isolated using RNAWIZ (Ambion, Austin, TX) from whole seedlings harvested between 11 AM and 4 PM. Using 2 μg of total RNA pretreated with RNase Free DNase I (Amersham Pharmacia Biotech, Piscataway, NJ) and reverse transcriptase from *Moloney murine leukemia virus* (Promega, Madison, WI), cDNA was synthesized. PCR amplification was performed using 2 μL of 50 μL of RT reaction and Promega Taq DNA polymerase according to the recommendations of the supplier. Twenty-eight to 30 cycles were used for *CO* and *LFY*, 26 to 28 cycles were used for *FT* and *SOC1*, 25 to 27 cycles were used for *AP1*, 24 to 26 cycles were used for *AP3* and *AG*, 22 to 24 cycles were used for *PI*, and 21 to 23 cycles were used for *GAPc*. The number of PCR cycles chosen was shown to be in the linear range of the reaction in a separate experiment using different amounts of cDNA template. The control, *GAPc*, was amplified in the same tube as each gene studied. Primers used for the PCR reactions were as follows: 5′-GAACAAATTGAGCAGCTCAAG-3′ and 5′-GCAGCTTTAGAGTTTTGTTAC-3′ for *SOC1*; 5′-TGAAGGACG-AGGAGCTTGAAG-3′ and 5′-CATCTTTCCTTGACCTGCGTC-3′ for *LFY*; 5′-TTGAACGCTATGAGAGGTAC-3′ and 5′-TTTTCCCTCTCCTTGATC-TG-3′ for *AP1*; 5′-AGGAGATCGTAGATCTGTAC-3′ and 5′-TCCTCCATT-GTCTACTAGTC-3′ for *AP3*; 5′-ATCTTGGTGCTATGTTGGAC-3′ and 5′-CTCATCATCATTCCTCTTGC-3′ for *PI*; and 5′-GGTACAAGAAGGCAA-TATCG-3′ and 5′-TTGTTCCTCTCATTTTCAGC-3′ for *AG*. *FT* primers described by Kardailsky et al. (1999), *CO* primers described by Putterill et al. (1995), and *GAPc* primers described by Aubert et al. (2001) were used. The amplified fragments were separated on an agarose gel, blotted onto a membrane, and hybridized with radiolabeled probes. Radioactive signals were detected using a Typhoon 8600 imager (Molecular

Dynamics, Sunnyvale, CA) and quantified using ImageQuant (version 5.2) software (Molecular Dynamics). The data shown are representative of the time points in at least two independent RT-PCR experiments.

GeneChip Experiments

For total RNA isolation, 800 to 1500 whole plants for each *emf1-1*, *emf1-2*, and *emf2-1* mutant sample were pooled from different experiments to eliminate much of the variation in gene expression patterns caused by subtle differences in environmental conditions and among individuals. All samples were harvested between 11 AM and 4 PM. Total RNA was isolated using RNeasy columns (Qiagen, Valencia, CA) according to the manufacturer's protocols. cDNA synthesis, biotinylated cRNA probe synthesis, and hybridization were performed as described by Tepperman et al. (2001) and Zhu et al. (2001). The probe array was scanned twice, and the intensities were averaged with a Hewlett-Packard GeneArray Scanner. GeneChip Suite 4.0 (Affymetrix, Santa Clara, CA) was used for image analysis. The average intensity of all probe sets was used for normalization and scaled to 100 in the absolute analysis for each probe array.

Genetic Crosses and β-Glucuronidase Activity Assays of the *LFY*::β-*Glucuronidase* and *AP1*::β-*Glucuronidase* Transgenic Plants

To introduce *LFY*::β-*glucuronidase* (*GUS*) into *emf1* mutants, heterozygous *emf1* plants were crossed with a line homozygous for *LFY*::*GUS*, which was kindly supplied by Detlef Weigel (Salk Institute, San Diego, CA). F1 plants were selfed. F2 plants homozygous or heterozygous for *LFY*::*GUS* and heterozygous for *emf1* were grown to generate F3 plants, of which one-fourth or three-sixteenths were the desired strains. The *AP1*::*GUS emf1* lines were described by Chen et al. (1997).

Four- to 14-day-old seedlings of *LFY*::*GUS emf1-1*, *LFY*::*GUS emf1-2*, *AP1*::*GUS emf1-1*, and *AP1*::*GUS emf1-2* plants were prepared for GUS assays. GUS activity in these plants was detected histochemically using a protocol adapted from Jefferson et al. (1987) with slight modifications. Briefly, the tissue was incubated in 2 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid in 50 mM phosphate buffer, pH 7.0, containing 0.5 mM $K_3Fe(CN)_6$ and 0.5 mM $K_4Fe(CN)_6$ for 16 h at 37°C, rinsed with 50 mM phosphate buffer, fixed and cleared with ethanol (95%):acetic acid (9:1, v/v) for 2 to 4 h at room temperature, and observed and photographed using a Zeiss Stemi SV 11 stereomicroscope equipped with a DEI 450 digital video camera (Optronics, Goleta, CA). Images were captured using NIH Image software as modified by Scion Images (version 1.62c). Captured images were converted from 72 to 300 dots per inch with Adobe Photoshop version 5.5 (Adobe Systems, Mountain View, CA). All subsequent image manipulation and figure preparation were performed with Adobe Photoshop.

Upon request, all novel materials described in this article will be made available in a timely manner for noncommercial research purposes.

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