EMF Genes Regulate Arabidopsis Inflorescence Development

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Mutations in EMBRYONIC FLOWER (EMF) genes EMF1 and EMF2 abolish rosette development, and the mutants produce either a much reduced inflorescence or a transformed flower. These mutant characteristics suggest a repressive effect of EMF activities on reproductive development. To investigate the role of EMF genes in regulating reproductive development, we studied the relationship between EMF genes and the genes regulating inflorescence and flower development. We found that APETALA1 and AGAMOUS promoters were activated in germinating emf seedlings, suggesting that these genes may normally be suppressed in wild-type seedlings in which EMF activities are high. The phenotype of double mutants combining emf1-2 and apetala1, apetala2, leafy1, apetala1 cauliflower, and terminal flower1 showed that emf1-2 is epistatic in all cases, suggesting that EMF genes act downstream from these genes in mediating the inflorescence-to-flower transition. Constitutive expression of LEAFY in weak emf1, but not emf2, mutants increased the severity of the emf phenotype, indicating an inhibition of EMF activity by LEAFY, as was deduced from double mutant analysis. These results suggest that a mechanism involving a reciprocal negative regulation between the EMF genes and the floral genes regulates Arabidopsis inflorescence development.

INTRODUCTION

Arabidopsis primary shoots undergo a series of developmental phase changes as they mature and age (Schultz and Haughn, 1993). Their development can be broadly categorized into three major phases based on node morphologies: first, the rosette or vegetative phase, with nodes closely compressed and bearing a petiolated leaf and an axillary bud; second, the early-inflorescence phase, with nodes separated by internode elongation and bearing a sessile leaf and a corymb; and third, the late-inflorescence phase, with nodes bearing solitary flowers. Thus, two major phase transitions are involved in Arabidopsis main shoot development: the transition from rosette to early inflorescence when the rosette begins to bolt and the transition from early to late inflorescence (or from inflorescence to flower) when the primary shoot switches from producing cauline leaves and corymb-like inflorescences to flowers (Schultz and Haughn, 1993). Ultimately, the primary shoot meristem becomes senescent and ceases producing flowers from its flanks (Shannon and Meeks-Wagner, 1991).

The transition from rosette to early inflorescence is considered to be the vegetative-to-reproductive transition. It is regulated by many flowering-time genes, that is, floral repression and floral promotion genes (or early- and late-flowering genes, respectively) (Koornneef et al., 1991; Zagotta et al., 1992). Loss-of-function mutations in floral repression genes, such as EARLY FLOWER 1 (ELF1), cause early flowering, whereas mutations in floral promotion genes, such as CONSTANS (CO), delay transition from the rosette-to-inflorescence stage (Koornneef et al., 1991). In addition, two EMBRYONIC FLOWER (EMF) genes, EMF1 and EMF2, are proposed to be involved in this process as floral repressors, suppressing the onset of reproductive development (Sung et al., 1992; Martinez-Zapater et al., 1994; Castle and Sung, 1995; Yang et al., 1995). Based on this floral repressor concept, vegetatively growing plants must decrease EMF1 and EMF2 activities to initiate reproductive growth. It has been proposed that the floral repression genes maintain whereas floral promotion genes inhibit EMF1 and EMF2 activities. A balance of these gene actions would cause a gradual decline in EMF activities and determine the time of vegetative-to-reproductive transition (Castle and Sung, 1995; Yang et al., 1995).

The transition from inflorescence to flower is regulated by flower meristem identity genes, such as LEAFY (LFY), APETALA1 (AP1), AP2, and CAULIFLOWER (CAL) (Irish and Sussex, 1990; Mandel et al., 1992; Bowman et al., 1993; Jofuku et al., 1994). Mutants with defective LFY, AP1, AP2, or AP1 CAL genes are impaired in flower initiation; thus, inflorescence-like or flowerlike shoots, instead of flowers, initiate peripherally from the apical meristem during the late-inflorescence phase. In addition to these genes, the TERMINAL FLOWER1 (TFL1) gene is reported to negatively regulate meristem identity gene function in inflorescence development. Both the primary shoot and the lateral shoots in tfl1
mutants terminate in a flower, reflecting a precocious inflorescence-to-flower transition (Alvarez et al., 1992). Molecular data have shown that the LFY gene is ectopically expressed in the entire apical meristem of tf1 primary and lateral shoots, which is consistent with the tf1 phenotype (Bradley et al., 1997). Thus, TFL1 functions to maintain inflorescence development. Mutants impaired in EMF1 or EMF2 produce a reduced inflorescence and a terminal flower, indicating a role for the EMF genes in delaying the inflorescence-to-flower transition.

The development of Arabidopsis floral organs also depends on normal EMF gene function. As in ap1 and ap2 mutants, weak emf mutants, such as emf1-1 and all of the emf2 mutants, lack petals (Yang et al., 1995). The strong emf mutant, emf1-2, is impaired in the development of all floral organs: only carpelloid organs form (Yang et al., 1995). The effects of emf mutations on inflorescence and flower development suggest that EMF1 and EMF2 continue to function during reproductive development.

To understand the role of EMF1 and EMF2 during reproductive development, we (1) further characterized the emf mutant phenotypes, including that of a new allele emf1-3, (2) examined the expression of AP1 and AGAMOUS (AG) in emf mutants, (3) constructed and analyzed double mutants combining emf and mutants defective in inflorescence-to-flower transition, and (4) generated and analyzed emf plants harboring a chimeric construct of the cauliflower mosaic virus 35S promoter fused to a LFY gene (35S::LFY) (Weigel and Nilsson, 1995). Our results show that EMF gene activities regulate inflorescence development through their interaction with floral genes. To explain the role of EMF activity in regulating multiple phase transitions, we propose that during rosette and early-inflorescence development, the floral genes, such as AP1 and AG, are suppressed by high EMF gene activities, which decline as the plant develops. In late-inflorescence development, the low level of EMF activities allows the expression of floral genes, which subsequently repress EMF activities. Our findings indicate a reciprocal negative regulatory relationship between the EMF genes and some of the floral genes during inflorescence development.

**RESULTS**

**Transition from the Inflorescence-to-Flower Phase Is Altered in emf Mutants**

**Reproductive Development in emf Mutants with Weak Phenotypes**

emf mutants germinated as seedlings lacked petiolated rosette leaves, indicating the possible loss of vegetative growth. Regarding the expression of rosette and early-inflorescence development, the floral genes, as was the case with all emf mutants, emf1-3 had sessile cotyledons, lacked rosette leaves, had a short hypocotyl, and had normal roots. Like emf1-2, (Sung et al., 1992; Bai and Sung, 1995; Yang et al., 1995). Besides the defect in rosette leaf development, emf mutants were also abnormal in reproductive development. emf1-1 and the six emf2 mutants had similar phenotypes, as shown in Figures 1A, 1B, 1G, and 2F. Here, we describe the characteristics of reproductive development in emf mutants with weak phenotypes, using emf1-1 as an example.

Homozygous emf1-1 seedlings grown under short- or long-day conditions produced a short stem, ~1 to 5 mm in height, with approximately five or six sessile leaves on it. The length of the leaves varied from 0.3 to 1.5 mm. As for the wild-type plants shown in Figure 2A, all of the leaves were flanked by tiny stipules at their base (Figure 2B).

During early-inflorescence development, emf1-1 plants often produced one to three solitary flowers in the axils of upper cauline leaves (Figure 2F), whereas in wild-type plants, instead of solitary flowers, cofloreresences were produced. The ability to form flower buds decreased basipetally. Seldom did a flower develop in the axil of lower leaves before the plant became senescent. During late-inflorescence development, emf1-1 produced one to four flowers that were not subtended by cauline leaves. In contrast to the wild type, the inflorescence of emf1-1 terminated in a flower (Figures 1A and 2F) or sometimes a fused floral cluster (Figure 1B).

Flower development in emf1-1 was also affected, as indicated by the apparent apetalous phenotype (Figure 1A). Both stamens and pistils were sterile. In the homozygous mutant population, some plants did not blossom. Their flower buds turned yellow and ceased to enlarge at early developmental stages as a result of early senescence (Bai and Sung, 1995). emf2 plants showed the same phenotypes as emf1-1 (Figure 1G). The accelerated floral formation in leaf axils and shoot apex showed that, like tf1 mutants grown in long-day conditions (Shannon and Meeks-Wanger, 1991), weak emf mutants are affected in the early- to late-inflorescence transition even under short-day conditions.

**Characteristics of the Strong emf Mutant Phenotype**

emf1-2 displayed strong mutant phenotypes. No emf2 mutants with strong phenotypes as emf1-2 were found. Bhatt et al. (1996) described an emf-like (leafless) mutation from a transposon mutagenesis experiment using a modified autonomous Activator (Ac) element. Our complementation result showed that it was allelic to emf1 mutants (data not shown). Thus, it was named emf1-3. The phenotype of emf1-3 (Figures 1E, 1F, 2D, and 2E) was similar to that of emf1-2 (Figures 1C and 1D) (Yang et al., 1995). Like emf1-2, emf1-3 seedlings required more time to germinate than did emf1-1 or emf2 mutants. As was the case with all emf mutants, emf1-3 had sessile cotyledons, lacked rosette leaves, had a short hypocotyl, and had normal roots. Like emf1-2,
stigmatic papillae developed at the base of cotyledons and all sessile leaf primordia, indicating the carpelloid nature of the primordia. Additional characteristics of the two strong emf alleles are described below.

Upon germination, cotyledons of the mutants underwent reproductive differentiation. Stigmatic papillae appeared at the lower margin of the cotyledons as early as 4 days after germination. Ovulelike structures might develop from the adaxial surface at the base of the cotyledons. Those ovulilike structures sometimes developed into tubular structures, with a clear junction connecting the integument and the elongated nucellus (Figure 2C).

Phenotypic variations existed among emf1-2 and emf1-3 plants. Three types of shoot were observed: (1) a much-reduced inflorescence (Figures 1E and 2D), (2) a transformed flower (Figure 1D), and (3) a pistil (Figures 1C and 1F). In the first type, the shoot was composed of several conspicuous carpelloid structures that sometimes surrounded a transformed terminal flower. The carpelloid structures were sessile and developed stigmatic papillae cells on the top and ovulilike structures on the edges. The ovulilike structures sometimes developed into a tubular structure, with the differentiation of stigmatic papillae cells at the tips. In the axis of the carpelloid structures, one small, poorly developed and highly carpelloid flower occasionally developed. In addition, the carpelloid feature, no trichomes formed on the organs and no stipules flanked their bases (Figure 2C). The transformed terminal flower was composed of one whorl of carpelloid sepals, one whorl of reduced stamens, which sometimes became carpelloid, and a pistil that was often an open carpel. Pedicels were not always present below the terminal flowers.

In the second type, the shoot consisted of a single transformed flower with one whorl of carpelloid sepals, one whorl of reduced stamen, and one prominent pistil (Figure 1D). The carpelloid sepals developed stigmatic papillae cells at their tips. In the third type, the shoot was composed of only one pistil: no other organs formed between the pistili and cotyledons (Figures 1C and 2E). Variations in the pistil phenotype included carpel development inside the pistil, which could burst out of the top of the pistil and form a stack of carpelloid structures (Figure 1F). These features confirmed the absence of vegetative growth and the acceleration of flower development in emf mutants.

The absence of stipules and trichomes on emf1-2 and emf1-3 lateral organs is consistent with their carpelloid nature and origin as floral organs. Stipules are the tiny organs that flank the base of wild-type rosette and cauline leaves, but they are absent from that of the floral organs (Bowman, 1994; Medford et al., 1994). In mutants that display floral reversion, for example, ap1-1, ap2-1, and ify, stipules develop at the base of "sepalas" as these sepals revert to cauline leaflike structure (Smyth et al., 1990; Bowman et al., 1991; Huala and Sussex, 1992). Thus, the absence of stipules indicates floral organ identity. Similarly, trichomes are not found on stamens and pistils of wild-type plants.

**AP1 and AG Genes Are Precociously Expressed in emf Mutants**

emf mutants showed acceleration in the inflorescence-to-flower transition. The carpelloid lateral organs of emf strong mutants suggested that EMF activities suppress the expression of floral genes in the early stages of shoot development. To investigate this possibility, we examined the expression of AP1 and AG in emf mutants.

**AP1::GUS Expression in emf Mutants**

In wild-type Arabidopsis, AP1 RNA is first detected in young floral primordia as soon as they are visible on the flanks of the inflorescence meristem (Mandel et al., 1992). As flower development progresses, AP1 gene expression is localized to the developing sepals and petals (Mandel et al., 1992). To study the temporal and spatial expression of AP1 in emf mutants, we produced transgenic emf1-1, emf1-2, and emf2-3 plants harboring a chimeric construct of the AP1 promoter fused to a β-glucuronidase gene (AP1::GUS).

In emf1-1 and emf2-3 transgenic plants, AP1::GUS activity became detectable as early as 5 days after germination. Both shoot meristem and leaf primordia were GUS-positive, as shown in Figure 3A. As the leaves expanded, GUS activity was lost in the developing leaves. However, the subsequent leaf primordia were again GUS-positive (Figures 3B and 3F). In contrast, the parental AP1::GUS seedlings were GUS-negative during this time (Figure 3E). During flower formation, unlike the expression pattern in wild-type plants, AP1 expression in the mutants was not limited to the perianth whorl (emf mutants lack petals) but was seen in the stamen and pistil primordia as well (Figure 3G).

In emf1-2, AP1::GUS activity was first detected in 4-day-old seedlings (Figure 3C). In addition to the shoot apex, the hypocotyl and cotyledons were also GUS-positive (Figure 3C). With the development of the lateral organs, GUS activity disappeared in the hypocotyl and cotyledons of 10-day-old emf1-2 seedlings. However, the emerging shoot apical tissues were GUS-positive (Figure 3D). When the lateral organs differentiated into carpelloid structures, GUS activity was lost at their tips where the stigmatic tissue developed. Eclectic AP1 expression in both weak and strong emf mutants suggested that AP1 expression normally may be suppressed by high levels of EMF1 and EMF2 activities. The temporal difference in AP1 expression between the strong and weak emf1 mutants is consistent with the notion of EMF decline during shoot development (Yang et al., 1995). The amount of EMF1 activity in emf1-1 cotyledons is probably higher than that in leaf primordia, so AP1 expression is inhibited.

**AG Gene Expression in emf Mutants**

AG ectopic expression was found in the carpelloid sepals in ap2 mutants (Bowman et al., 1991; Weigel and Meyerowitz,
Figure 1. *emf* Single and Double Mutants and Transgenic Plants.

(A) A 25-day-old *emf1-1* seedling showing that the primary shoot terminated in an apetalous flower.

(B) A fused flower on shoot apex of a 22-day-old *emf1-1* plant.

(C) A 25-day-old *emf1-2* plant with a pistil developed upon germination and stigmatic tissue (arrows) developed at the base of the cotyledons.

(D) A 25-day-old *emf1-2* plant with a pistil surrounded by carpelloid structures and reduced stamens. The arrow indicates a cotyledon.

(E) A 25-day-old *emf1-3* plant showing carpelloid structures. The arrow indicates the stigmatic tissue developed at the base of cotyledons.
To investigate the molecular basis of the emf mutant phenotype, we examined AG expression in emf mutants by using in situ RNA hybridization. In wild-type plants, AG transcripts were first detectable in the floral meristem after the emergence of sepal primordia. As organogenesis progressed, AG gene expression was restricted to developing stamens and carpels, as shown in Figures 4A and 4B. Later, AG transcripts decreased in the ovary wall and remained in the ovules. In emfl-1 and emf2-3 plants grown under short-day conditions, flower buds appeared 10 days after germination. AG was expressed in the developing stamen and pistil as in wild-type plants. But in general, the signals were weaker than that in wild-type flowers (Figures 4I and 4J). No expression of AG was detected in cotyledons, hypocotyls, stems, and sessile leaves.

In emfl-2, there were no detectable AG transcripts in the shoot apical meristem of 5-day-old plants (Figures 4C and 4D). AG transcripts became detectable after the primordia of the carpelloid structures were formed. With the differentiation of the primordia into carpelloid structures, the transcription of AG continued (Figures 4E and 4F). The region of cotyledon that would develop papillae or ovulelike protrusions also showed AG expression (Figures 4G and 4H). As the plants aged, AG expression subsided from the carpelloid structures. The temporal pattern of AG expression in the carpelloid structures of emfl-2 was similar to that in wild-type pistils (Drews et al., 1991). The precocious and ectopic AG expression in the lateral organs was consistent with their carpelloid features and suggests negative regulation of AG by EMF directly or indirectly. Expression of both AP1 and AG was affected in emf mutation, suggesting that the wild-type level of EMF activity in early developmental stages suppressed multiple aspects of the floral program.

**Relationship between EMF and Genes Regulating the Inflorescence-to-Flower Transition**

As Arabidopsis undergoes transition from early- to late-inflorescence development, the shoot produces flowers instead of another shoot, the coflorescence. As a result, solitary flowers form during the late-inflorescence stage. The levels of LFY, AP1, AP2, and CAL RNA are increased at this time to promote flower initiation (Mandel et al., 1992; Weigel et al., 1992; Okamuro et al., 1993; Yanofsky, 1995). In contrast, EMF gene activities seem to retard flower initiation.

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**Figure 1.** (continued)

- **(F)** A 25-day-old emfl-3 plant. Arrows indicate the primary pistil, which is split at the base because of the emergence of a secondary pistil.
- **(G)** A 25-day-old emf2-3 plant showing a terminal flower and two solitary flowers in the axils of cauline leaves (arrows).
- **(H)** A 22-day-old emfl-1 ap2-1 seedling. Sepals of the terminal flower (arrow) were similar to that of emfl-1 but not of ap2-1.
- **(I)** A 30-day-old emfl-1 ap2-1 plant. Arrows point to the sepals that were severely lobed and tipped with stigmatic tissue.
- **(J)** An upper cauline leaf of a 30-day-old emfl-1 ap2-1 plant. Arrows point to the stigmatic tissue developed at the tips of the lobes.
- **(K)** A sepal of an emfl-1 ap2-1 flower. Arrows point to the stigmatic tissue developed at the tips of the lobes.
- **(L)** A 30-day-old emfl-1 ap2-1 plant with a solitary flower developed from the axil of a lower sessile leaf. The sepals were not bractlike, as with ap2-1.
- **(M)** A 22-day-old emfl-1 ap1-1 plant showing a flower (arrow) terminating the inflorescence. No secondary flower developed in the axils of the sepals.
- **(N)** A 30-day-old emfl-1 ap1-1 plant showing solitary flowers in the axils of cauline leaves and no secondary flowers developed in the axils of sepals. Arrows indicate the cauline leaves.
- **(O)** A 30-day-old emf2-3 tfl-1 plant showing that only carpelloid structures developed after germination.
- **(P)** A 22-day-old emf2-3 ap1-1 plant showing that only one pistil developed after germination, as was the case for emfl-2 shown in (C).
- **(Q)** A 22-day-old emfl-1 ap2-1 plant. A flowerlike shoot developed after germination. The central pistil of the flowerlike shoot was surrounded by reduced stamens and carpelloid structures, similar to that of emfl-1-2 shown in (O).
- **(R)** Top view of a 30-day-old emfl-2 ap2-1 plant. The lower lateral carpelloid organs (arrow) became repeatedly lobed.
- **(S)** A lateral carpelloid organ of the emfl-2 ap2-1 plant. The carpelloid structure was cut off from the plant and entirely stained with toluidine blue O (0.05% in benzoate buffer).
- **(T)** A cotyledon of a 30-day-old emfl-2 ap2-1 plant. The arrow points to the lobed basal edge.
- **(U)** A 22-day-old emfl-2 ap1-1 cal plant. Only carpelloid structures were developed after germination.
- **(V)** A 25-day-old 35S::LFY emfl-1 plant differentiating stigmatic tissues on the sessile leaves but not cotyledons. At upper right, a side view of one of the carpelloid structures is shown. Arrows point to the ovules borne on the edges of the carpelloid structure. The ovule on the right developed stigmatic papillae cells at the tip (arrow).
- **(W)** A 25-day-old 35S::LFY emf2-3 plant with two cotyledons, one sessile leaf (arrow), and several carpelloid organs.
- **(X)** A 25-day-old 35S::LFY emf1-1 plant with 16 reduced stamens and a central pistil. The arrow and box at bottom right indicate the first reduced stamen produced after germination.
- **(Y)** A 25-day-old 35S::LFY emf2-3 plant. All of the sessile leaves were like those of emf2-3 single mutants and were not transformed. The ovary with a long gynophore below was shorter and thicker than that of emf2-3 plants.

C, cotyledon; Ca, carpelloid sepal; St, reduced stamen. Bars = 0.5 mm.
To study the relationship between EMF and these genes in regulating inflorescence and flower development, we performed double mutant analysis.

**Double Mutants with Weak emf Alleles**

In this study, three weak phenotype mutants, emf1-1, emf2-2, and emf2-3, were crossed with ap1-1, ap1-1 cal, and ap2-1 plants, and double mutants were obtained as described in Methods.
In general, double mutants resembled the emf parent, as indicated in Figures 1H, 1M, 1N, 5, and Table 1. There was little difference between double mutants constructed with emf1-1 and with emf2-2 or emf2-3. Like the emf parents, the double mutant seedlings displayed oval-shaped, sessile cotyledons and short hypocotyls. The inflorescence of double mutants also displayed the emf phenotype in producing solitary flowers in the leaf axils and terminal flowers (Figures 1L and 1N). Unlike the ap1-1 parent plants (Irish and Sussex, 1990; Bowman et al., 1993), no secondary flowers developed inside of the flowers of the emf ap1-1 double mutants, and no extra coflorescences without subtending cauline leaves were found on the emf ap1-1 inflorescences. Unlike the ap2-1 parent (Bowman, 1992), no massive proliferation of meristems was found to replace the flower of emf1-1 cal or emf2-2 ap1-1 cal mutants. Unlike ap2-1 parents (Bowman et al., 1991), no bractlike sepals were found on the emf1-1 ap2-1 or emf2-3 ap2-1 double mutants. The effect of ap1-1 and cal in floral reversion may be seen only in the form of internode elongation between sepals in some flowers of emf1-1 ap1-1 and emf1-1 ap1-1 cal double mutants. These cases were found in axillary flowers in the early-inflorescence phase. No internode elongation was found between sepals of emf2-2 ap1-1 cal and emf2-3 ap1-1 flowers. The floral organs of double mutants were generally similar to that of emf parents. For example, both stamens and pistils were sterile. However, the role of AP1 and AP2 in specifying sepal identity was visible in sepals, which became carpelloid in some flowers (Table 1).

In the population of emf1-1 ap2-1 double mutants, there was a fraction, <5%, of the plants (Figure 1I) that produced an inflorescence twice the length of the emf parent and cauline leaves, which were larger than average emf leaves. Sepals on this 5% of double mutant flowers were severely carpelloid. Their margins lobed repeatedly, and filamentous structures sometimes differentiated from them (Figure 1K). Both lobes and filamentous structures developed stigmatic papillae at their tips. No petals formed on the flowers. Stamens also became carpelloid. The pistil frequently developed as an open carpelloid structure with many small carpelloid ovules. In this small population of plants, the ap2-1 mutation caused sepals and cauline leaves (Figure 1J) of weak emf mutants to become more carpelloid. The reason for the appearance of the extreme phenotype in the emf1-1 ap2-1 population is not clear. The appearance of this phenotype may be due to the multiple roles AP2 plays in development and the weak allele used in the experiment.

**Double Mutants with emf1-2**

To further investigate emf epistasis, double mutants combining a strong emf mutant, emf1-2, and ify1-1, ap1-1, ap1-1 cal, ap2-1, or tfl1-1 were constructed as described in Methods. The double mutants of emf1-2 ify1-1, emf1-2 ap1-1, emf1-2 ap1-1 cal, emf1-2 ap2-1, and emf1-2 tfl1-1 were
similar to the emf1-2 parent (Figures 10 to 1R, 1U, 5, and Table 1). Like emf1-2 mutants, all double mutants lacked rosette growth. They developed several carpelloid structures, with or without a transformed terminal flower, and then ceased shoot development. Thus, emf1-2 was epistatic to ify-1, ap1-1, ap2-1, and tfll-1 with respect to flower initiation, suggesting that EMF1 acted downstream from these meristem identity genes and was regulated by them in mediating the transition from inflorescence to flower development.

With respect to the lateral organs, a small fraction, ~8%, of the emf1-2 ap1-1 double mutants had larger carpelloid organs than that of emf1-2. But this did not have a profound impact on overall shoot morphology of the double mutants. However, enlarged carpelloid structures occurred in ~70% of the emf1-2 ap2-1 double mutants. Because of unrestrained marginal growth, the carpelloid structures, especially the basal ones, were enlarged and lobed (Figures 1R and 1S). All of these structures developed stigmatic papillae at their tips, causing the shoot to look like a fuzzy green ball. The cotyledons were affected too. Lobes with stigmatic papillae at the tips were produced at the base of the cotyledons (Figure 1T). These results are similar to those of double mutants with the weak emf mutants in that the ap2-1 mutation had a stronger impact than other mutations on the carpelloidy of emf lateral organs. This might be explained by the more ubiquitous role of AP2 that extends beyond the regulation of flower initiation or by its cadastral role in defining the AG expression domain (Jofuku et al., 1994; Weigel and Meyerowitz, 1994).

**LFY Negatively Regulates EMF1 Activity**

To seek further evidence supporting the notion of negative regulation of EMF by floral genes, we introduced a chimeric construct of a LFY gene fused to the 35S cauliflower mosaic virus promoter (35S::LFY) into emf1-1, emf1-2, and emf2-3 mutants, as described in Methods, and analyzed the impact of 35S::LFY on the emf phenotype. Transgenic 35S::LFY plants flowered earlier than did wild-type plants under short-
day conditions. Their coflorescences were converted to solitary flowers, and primary shoots terminated in terminal flowers (Weigel and Nilsson, 1995).

**emf1 Mutants Harboring 35S::LFY Constructs**

35S::LFY emf1-1 plants germinated like emf1-1 parents. The seedlings displayed oval-shaped and sessile cotyledons and short hypocotyls. However, the lateral organs produced from the embryonic shoot meristem developed differently from those of the emf1-1 parents. In contrast to emf1-1 sessile leaves on which trichomes became visible 6 days after germination, the lateral organs of 35S::LFY emf1-1 plants did not develop trichomes (Figures 1V to 1X). By 20 days after germination, some of the transgenic plants developed stigmatic tissue on the tips and ovules or ovuliform-like structures at the lower edges of the lateral organs, resulting in lateral carpelloid structures similar to that of emf1-2 (Figure 1V). Some plants produced one or more glabrous sessile leaves and then carpelloid structures (Figure 1W). Other plants produced two to 16 staminoid structures followed by a short, thick, pistil-like structure (Figure 1X). These phenotypes showed that 35S::LFY emf1-1 plants had a stronger tendency toward reproductive growth than did emf1-1 plants after germination. More significantly, they produced carpelloid organs. The carpelloid nature of 35S::LFY emf1-1 lateral organs is a characteristic similar to the strong emf mutants. On the other hand, the 35S::LFY emf1-1 plants differed from emf1-2 plants in that no stigmatic papillae developed at the base of the cotyledons (Figures 1V to 1X).

The production of staminoid and carpelloid organs on the primary shoot after germination had not been seen in any other single or double emf mutants. Promotion of stamen formation in 35S::LFY emf1-1 plants was consistent with one of the LFY functions deduced from the ify phenotype: ify "flowers" always lack stamens (Huala and Sussex, 1992). Like emf single mutants, all stamens and pistils were sterile in the transgenic plants.

In contrast to emf1-1, constitutive expression of LFY had little effect on the phenotype of emf1-2. The shoot structures of 35S::LFY emf1-2 were similar to those of emf1-2.

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<td>38</td>
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</table>

³Parent 1 plants have wild-type-like seedling and rosette phenotypes, but their reproductive development is altered. ap1-1 is characterized by the development of secondary flowers, coflorescences without subtending cauline leaves, and bractlike sepals, with the meridian pair being separated from the lateral pair at late floral development; ap1-1 cal is characterized by the proliferation of floral meristems; ap2-1 by the development of bractlike sepals, stigmatic tissue on tips of sepals, and secondary flowers; ify-1 by the development of coflorescence-like and flowerlike shoots; and tf1-1 by the development of terminal flowers and solitary flowers in leaf axils.

²The inflorescence trait of emf mutants include short hypocotyls and oval-shaped and petiole-less cotyledons, with a lack of petiolated rosette leaves.

²The inflorescence trait of emf mutants include short hypocotyls and oval-shaped and petiole-less cotyledons, with a lack of petiolated rosette leaves.

²The lateral organ trait of emf mutants means that the cauline leaves and sepals are similar to those of emf1-1, emf2-2, or emf2-3, or that the carpelloid structures are similar to those of emf1-2; both means that the sepals became carpelloid or separated by an internode (specific in emf1-1 ap1-1 and emf1-1 ap1-1 cal); novel means that the sepals lobed repeatedly and cauline leaves developed stigmatic papillae at the tips (specific in emf1-1 ap2-1) or that the carpelloid structures became lobed and enlarged (in emf1-2 ap2-1).

²Because of the sterility of ify-1 plants, double mutants segregated from plants heterozygous for both ify-1 and emf1-2. In total, 156 emf1-2-like plants were observed, with 39 theoretically expected to be double mutants.
single mutants. After germination, carpelloid lateral organs were produced in 35S::LFY emf1-2 plants. No staminoid structures were observed. In short, ectopic LFY expression in young emf1-1 seedlings increased the severity of the emf phenotype. The conversion of emf1-1 to an emf1-2-like phenotype by 35S::LFY suggested negative regulation of EMF1 by LFY.

emf2-3 Mutants Harboring 35S::LFY Constructs

Because emf2 single and double mutants shared shoot phenotypes similar to emf1-1, we might expect constitutive LFY expression to alter the emf2-3 phenotype as it did in emf1-1. However, we found that the introduction of 35S::LFY had limited effect on the emf2-3 phenotype. The shoot phenotype of 35S::LFY emf2-3 was quite different from that of 35S::LFY emf1-1. Upon germination, the shoot apical meristem produced normal sessile leaves that were similar to those in emf2-3. No carpelloid or staminoid transformation occurred. After producing the same number of sessile leaves as in emf2-3, plants produced a terminal flower (Figure 1Y). The effect of constitutive expression of LFY seemed only to be the shortening and thickening of the ovary (Figure 1Y). Although EMF1 and EMF2 play similar roles in rosette and inflorescence development, the differences in phenotype between 35S::LFY emf2-3 and 35S::LFY emf1-1 suggested that EMF1 plays a stronger repressive role than EMF2 in flower initiation and floral organ differentiation. This is consistent with the fact that two-thirds of the known emf1 alleles are strong, but no strong emf2 allele was found among the six emf2 mutants.

DISCUSSION

The Interaction of EMF with AG and AP1

AP1 and AG have been classified as both flower meristem identity and flower organ identity genes (Weigel, 1995; Yanofsky, 1995). Because emf mutants are also affected in floral meristem and floral organ development, we analyzed the relationship between EMF and these genes. Absence of EMF1 expression is sufficient for AP1 and AG ectopic expression, suggesting that EMF1 negatively regulates these two genes. However, the residual EMF activities in a germinating emf1-1 seedling is enough to cause precocious expression of AP1 but not of AG. Spatial pattern of AP1 and AG expression also differs in emf1-2 mutants, indicating that the regulatory mechanisms vary and that perhaps the genetic interaction between EMF1 and AG may be indirect and involve other genes.

Our previous study showed that an ag mutation rescued petal formation in emf1-1 but not in emf1-2 mutants (Yang et al., 1995). To explain this phenomenon, we have proposed that the weak emf mutations caused ectopic AG expression, which, based on the ABC model (Bowman et al., 1991; Coen and Meyerowitz, 1991; Weigel and Meyerowitz, 1994), would suppress AP1 and AP2, resulting in the loss of petals. To explain the inability of the ag mutation to rescue petal formation in emf1-2, we proposed a requirement of minimal EMF1 activity for AP1 expression (Yang et al., 1995). However, our findings, that is, that AG RNA was not detected in the outer whorl of emf1-1 mutant flowers and that the AP1::GUS activity was detected in emf1-1 sepal cells, do not support these explanations. Owing to the loss of vegetative and early inflorescence phases in emf mutants, there may be a disordered expression of genes involved in the flower program, resulting in the loss of petals in weak and the loss of most of the floral organs in strong emf mutants. Therefore, EMF1 activity may be required by other gene activities specifying petal formation; or it may specify the extent of floral competence, and the ectopic AG and AP1 expression may only be indicators of the expression of late floral program (Weigel, 1995).

The EMF Genes Encode Floral Repressors That Specify the Vegetative State of Arabidopsis Shoot Development

A genetic model for the control of vegetative-to-reproductive transition has been proposed (Martínez-Zapater et al., 1994; Weigel, 1995). The main scheme of the model is that flowering is a default state and is negatively regulated by floral repressors (Martínez-Zapater et al., 1994; Weigel, 1995). The hypothesis assumes that vegetative development is maintained as a result of the suppression of reproductive development. The EMF gene products are regarded as the floral repressors because weak emf mutants produce an inflorescence directly after germination (Sung et al., 1992). In this study, the characteristics of emf1 strong mutants lend further support to this hypothesis. The severe emf1 alleles cause the shoot to shift further into the reproductive state than do weak alleles, as evidenced by several distinct floral characteristics, including lack of stipules (Figure 2C) and trichomes on lateral organs, carpelloid of lateral organs (Figure 1E), direct development of a single flower or pistil (Figures 1B, 1C, and 1F), and precocious expression of floral genes (Figures 3C, 4F, and 4H). Both morphological and molecular data showed that floral programs proceeded in emf seedlings with little or no vegetative phase, indicating that EMF activity suppresses the reproductive transition in wild-type seedlings and rosettes.

To flower, juvenile plants must acquire floral competence first (McDaniel et al., 1992). Because LFY is the first one to express among the flower meristem identity genes and it is expressed before floral commitment (Bradley et al., 1997), LFY may potentially encode the floral competence factor. However, evidence suggests that it is not directly responsible for floral competence (Weigel and Nilsson, 1995). There-
fore, a pathway, the floral competence factor, parallel to the flower meristem identity genes was proposed to regulate the floral target genes. It is hypothesized that the floral competence factor causes the floral target genes to undergo a change from a completely repressed to a partially derepressed state, which then can be completely derepressed by high LFY activity (Weigel and Nilsson, 1995). The notion of floral repressor is compatible with the suppression of floral competence. In this respect, EMF products can be viewed as specifying the level of floral competence, which must be abated to a level to enable the partial derepression of floral target genes for LFY to initiate flower development.

In the absence of LFY, as in ify and ifly ap1 plants, continued increase of floral competence would still occur, resulting in floral target gene expression and carpelloid organ formation.

**EMF Gene Products Maintain Inflorescence Development**

Like the tfl1-1 mutant, the primary shoot meristems of emf weak mutants develop terminal flowers, and the auxiliary shoot meristems develop solitary flowers in leaf axis. Loss of EMF or TFL1 activities switches the meristem from inflorescence to flower development. EMF and TFL1 seem to have similar effects on maintenance of inflorescence growth. However, major differences exist between TFL1 and EMF. EMF is and TFL1 is not required for the photoperiodic response of Arabidopsis flowering (Sung et al., 1992; Schultz and Haughn, 1993; Martinez-Zapater et al., 1994). emf mutants lack and tfl1 plants possess petiolated rosette leaves. Both ify-1 and ap1 can lessen the tfl1 phenotype, resulting in a coflorescence-like shoot rather than a solitary flower in the leaf axis of double mutants (Schultz and Haughn, 1993). This implies no absolute requirement of TFL1 activity for maintaining inflorescence development (Schultz and Haughn, 1993). In contrast, neither ify-1 nor ap1 could lessen the strong emf phenotype, suggesting that EMF activities are indispensable for inflorescence development. Epistasis of emf1-2 to tfl1-1 (Figures 1O and 5) suggests that EMF activities are required for TFL1 function and that TFL1’s function in inflorescence development may be mediated via its positive regulation of EMF.

**Decline of EMF Activity Is Responsible for the Appearance of Floral Character**

Many observations indicate the existence of a gradient of “floral character” along the Arabidopsis inflorescence axis. For example, the coflorescence-like shoots of ify plants become more flowerlike as plants develop (Schultz and Haughn, 1991, 1993; Weigel et al., 1992). Ectopic expression of PisTILLATA and AP3 can only cause the upper cauline leaves to be petaloid, whereas the lower leaves remain untransformed (Krizek and Meyerowitz, 1996). The gradient of floral character can also be seen on the shoots of other annual plants, such as tobacco (Tran Than Van, 1973). The common features seen in different plants suggest that the mechanism controlling plant shoot maturation may be a conserved one in angiosperms. This gradient effect may be interpreted as resulting from an increasing amount of floral activators or a decreasing amount of floral repressors during inflorescence development (Krizek and Meyerowitz, 1998).

Our study shows that the decline of floral repressor responsible for the vegetative-to-reproductive transition is also responsible for increasing the floral character during inflorescence development. For example, the differences in weak and strong emf1 phenotypes suggest that the extent of floral character corresponds with the EMF level. Ectopic expression of LFY alone cannot change the vegetative fate of the lateral organs (Weigel and Nilsson, 1995). This can be explained by the relatively high level of EMF activity in the shoot. When EMF is reduced, as in emf1-1 mutants, the development of lateral organ primordia is more easily shifted into the floral program by constitutive expression of LFY, as shown by the cluster of carpelloid and staminoid structures on the 35S::LFY emf1-1 plants (Figure 1X).

**Reciprocal Negative Regulation between EMF and Floral Genes**

Epistasis of emf to floral repression and floral promotion mutations suggests that these floral genes act by modulating EMF activity to cause vegetative-to-inflorescence transition (Yang et al., 1995). Likewise, epistasis of emf1-2 to ify-1, ap1-1, ap2-1, and ap1-1 cal suggests that EMF acts downstream from those floral genes in mediating the inflorescence-to-flower transition. The phenotype of 35S::LFY emf1-1 plants confirms the regulation of EMF1 activities by LFY. On the other hand, EMF appears to suppress floral genes, as evidenced by the precocious expression of AP1 and AG in emf seedlings. Therefore, there seems to be a reciprocal negative interaction between EMF and floral genes in controlling the development of Arabidopsis shoots from inflorescence to flower phase. This kind of interaction may seem somewhat paradoxical, but it is consistent with the controllers of phase switching (COPS) hypothesis, which places EMF in the center of the COPS activity (Schultz and Haughn, 1993).

The COPS hypothesis holds that a high level of COPS activity suppresses reproductive development, allowing vegetative growth. If COPS activities continue to decline throughout the life span, the plant can progress from the rosette to inflorescence and to the flower phase. The reciprocal negative regulation between EMF and the floral genes provides a plausible mechanism for this hypothesis. During rosette growth, high EMF activity suppresses floral genes. EMF decline, mediated by the flowering-time genes, allows the activation of floral genes, which in turn suppress EMF activity,
resulting in the sequential activation of other floral genes and the gradual decline of EMF activity during inflorescence and flower development (Castle and Sung, 1995; Yang et al., 1995).

No single floral gene activity can suppress EMF completely, as evidenced by the phenotype of 35S::LYF emf1-1, which is more severe than that of emf1-1 but not as severe as that of emf1-2 (Figures 1V to 1X). Thus, in addition to LFY, other genes may also suppress EMF activity. This is compatible with the fact that ectopic expression of one or two floral genes can shorten the rosette phase but the plant cannot simply skip to floral development, as seen in 35S::AP1, 35S::LFY, 35S::AP3 35S::PISTILLATA, and 35S::AG transgenic plants (Mandel and Yanofsky, 1995; Weigel and Nilsson, 1995; Krizek and Meyerowitz, 1996; Mizukami and Ma, 1997). A possible working model for the EMF regulation of shoot development is proposed (Castle and Sung, 1995; Yang et al., 1995). Figure 6 summarizes the relationship between EMF and flower meristem identity genes in regulating inflorescence-to-flower development. EMF activities continue to decline in this process, allowing the initiation of floral program. The higher EMF activities in the earlier stage prohibit floral program by directly or indirectly suppressing the activities of floral genes. And in the later stage, the increasing activities of flower meristem identity genes promote the further decline of EMF activities, allowing the late floral program to be activated. To confirm the gradual decline of EMF activities, it is necessary to measure EMF activity directly during Arabidopsis shoot development. To do this, we are now cloning the EMF genes.

METHODS

Genetic Materials and Growth Conditions

emf Alleles

Nine emf mutations, three in the EMF1 gene (emf1-1, emf1-2, and emf1-3) and six in the EMF2 gene (emf2-1, emf2-2, emf2-3, emf2-4, emf2-5, and emf2-6), have been characterized previously (Yang et al., 1995; Bhatt et al., 1996). All alleles are in the Columbia background, except for emf1-3, emf2-5, and emf2-6, which are in the Landsberg erecta background. The six emf2 mutants have phenotypes similar to that of emf1-1.

emf1-2 and emf1-3 have the most severe phenotype and are distinct from the other mutants. Based on the phenotypes, the emf1-1 and six emf2 alleles are considered weak alleles, whereas emf1-1-2 and emf1-3 are strong alleles. The emf1-3 mutant was provided by C. Dean (John Innes Center, Norwich, UK). Although it was derived from an Ac transposon tagging experiment, the mutation is not linked to the Ac (Bhatt et al., 1996).

Seeds from plants heterozygous for emf were surface sterilized, germinated, and grown on Murashige and Skoog medium (Murashige and Skoog, 1962) as described (Yang et al., 1995). Seedlings on agar plates were grown in a growth chamber (Conviron, Winnipeg, Manitoba, Canada), which was maintained under short-day (8 hr of light and 16 hr of dark) conditions at 21°C. The light intensity of the growth chamber was 150 μE. After 10 days, plants showing normal rosette leaves were removed, leaving only homozygous emf plants on plates for examination and analysis.

Mutants Affected in Inflorescence and Flower Development and Transgenic Plants

Seeds carrying ap1-1, ap2-1, and ap1-1 cal-1 mutations were obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus, OH). The lfy-7 strain was obtained from E. Huala (University of California at Berkeley, Berkeley, CA), and the tf1-1 strain was from R. Meeks-Wagner (University of Oregon, Eugene, OR).

Transgenic plants harboring a chimeric construct of the AP1 promoter fused to a β-glucuronidase gene (AP1::GUS) were obtained from M. Yanofsky (University of California at San Diego, La Jolla, CA). Transgenic 35S::LFY plants were obtained from D. Weigel (Saik Institute for Biological Studies, La Jolla, CA).

Seeds were germinated on agar plates, as described previously (Yang et al., 1995), and transplanted to soil after 10 days. After 1 week under short-day conditions, the plants were moved to long-day conditions (16 hr of light and 8 hr of dark) in the greenhouse.

Genetic Crosses

Three weak emf alleles (emf1-1, emf2-2, and emf2-3) and a strong allele (emf1-2) were chosen for genetic crosses. Eleven double mutants were constructed and are listed in Table 1. Heterozygous emf lines were crossed with plants homozygous for ap1-1, ap1-1 cal, ap2-1, or tf1-1. F1 plants were selfed. F2 plants homozygous for ap1-1, ap1-1 cal, ap2-1, and tf1-1 and heterozygous for emf were grown to generate F3 seed that segregated 25% of the double mutants. For
generation of emf1-2 lfy-1 double mutants, plants heterozygous for emf1-2 and lfy-1 were crossed. F1 plants that segregated both lfy-1 and emf1-2 in the F2 generation were used for production of double mutants. In these lines, the F2 population segregated one-sixteenth to avoid the interference of erecta in double mutant analysis. For Landsberg background that carries the erecta mutation, only F1 section

To generate AP1::GUS homozygous plants, plants showing GUS activity in whorl 1 and whorl 2 of a flower were selfed. The plants that showed GUS activity in all offspring were selected as homozygous parental plants. To cross 35S::LFY and AP1::GUS into the three emf backgrounds, heterozygous emf lines were crossed with lines homozygous for 35S::LFY or AP1::GUS and heterozygous for emf were grown to generate F1 plants, of which one-fourth were the desired strains.

Light and Scanning Electron Microscopy

For light microscopy, samples were fixed in FAA (10% formaldehyde, 50% ethanol, and 5% acetic acid) for 1 day. The samples were dehydrated and embedded in paraffin. Sections were cut 10 mm thick and stained with toluidine blue O (0.05% in benzcooler). Finished specimens were examined and photographed with a microscope (Axioptot; Carl Zeiss, Inc., Heidelberg, Germany) under bright-field illumination.

For scanning electron microscopy, samples were fixed in 4% formaldehyde, 50% ethanol, and 5% acetic acid overnight and dehydrated in a graded ethanol series. Specimens were critical point dried in liquid CO2. The dried materials were mounted and coated with palladium in a sputtercoater (Technics Hummerr I; Polaron, Inc., Watford, UK). Specimens were examined and photographed with a microscope (model ISI-DS-130; Topcon Technologies, Inc., Paramus, NJ) with an accelerating voltage of 10 kV.

In Situ Hybridization

In situ hybridization was performed mainly according to the method of Drews et al. (1991). Two probes were used in these experiments. The AG antisense probe was synthesized with T7 RNA polymerase from a Hindlll-digested pCIT565 template and contained nucleotides 240 to 977 of the AG sequence published in Yanofsky et al. (1990). Two probes were used in these experiments.

ACKNOWLEDGMENTS

We thank Drs. Robert Wilson, Jim Mattsson, Dominique Aubert, Dave Martin, and Regina McClinton for critical reading of the manuscript and for suggestions. We also thank Dr. Caroline Dean for providing emf1-3; Dr. Eva Huala for lfy-1; Dr. Ry Meeks-Wagner for tf1-1; Dr. Martin Yanofsky for AP1::GUS plants; Dr. Detlef Weigel for 35S::LFY plants; the Arabidopsis Biological Resource Center (Ohio State University) for ap1-1, ap2-1, and ap1-1 cal; Dr. Hong Ma for plasmid pCIT565; and Dr. Allen Sessions for help with scanning electron microscopy. This work was supported by U.S. Department of Agriculture Competitive Grant No. 93-37301-8704 to L. Chen. In part by Chinese National Science Foundation Grant No. 39570046 to L. Shen.

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EMF genes regulate Arabidopsis inflorescence development.
L Chen, J C Cheng, L Castle and Z R Sung
DOI 10.1105/tpc.9.11.2011

This information is current as of July 8, 2017