The Plant Cell, Vol. 20: 277–291, February 2008, www.plantcell.org

EMBRYONIC FLOWER1 Participates in Polycomb Group–Mediated *AG* Gene Silencing in *Arabidopsis* [W]

Myriam Calonje,[1] Rosario Sanchez, Lingjing Chen, and Z. Renee Sung[2]

Department of Plant and Microbial Biology, University of California, Berkeley, California 94720

Polycomb group (PcG)–mediated gene silencing is a common developmental strategy used to maintain stably inherited repression of target genes and involves different protein complexes known as Polycomb-repressive complexes (PRCs). In animals, the two best-characterized PcG complexes are PRC1 and PRC2. In this report, we demonstrate that the plant-specific protein EMBRYONIC FLOWER1 (EMF1) functions in maintaining the repression of the flower homeotic gene *AGAMOUS* (*AG*) during vegetative development in *Arabidopsis thaliana* by acting in concert with the EMF2 complex, a putative equivalent of *Drosophila melanogaster* PRC2. We show that *AG* regulatory sequences are required for its ectopic expression in both *emf1* and *emf2* mutants and that EMF2 is required for trimethylation of histone 3 lysine 27 on the *AG* chromatin. We found that EMF1 interacts directly with *AG* and that this interaction depends on the presence of EMF2. Together with the finding of EMF1 interference with transcription in vitro, these results suggest that EMF1 enables transcriptional repression of *AG* after the action of the putative EMF2 complex. Our data indicate that EMF1 plays a PRC1-like role in the PcG-mediated floral repression mechanism.

INTRODUCTION

Plants undergo phase changes from vegetative to reproductive development (Schultz and Haughn, 1991). Extended vegetative growth allows plants to accumulate sufficient reserves needed for energy-intensive flowering and seed production (Boss et al., 2004). In *Arabidopsis thaliana*, vegetative development results from repression of flowering (Sung et al., 2003). This is evidenced by the elimination of vegetative rosette development in mutants impaired in epigenetic floral repressors (Moon et al., 2003; Schubert et al., 2005) or in transgenic plants that constitutively express floral inducers (Kardailsky et al., 1999; Honma and Goto, 2000). The epigenetic mechanism required for maintaining vegetative development involves the putative EMBRYONIC FLOWER2 (EMF2) complex that comprises four core proteins: EMF2 (Yoshida et al., 2001), CURLY LEAF (CLF) (Goodrich et al., 1997), FERTILIZATION INDEPENDENT ENDOSPERM (FIE) (Kinoshita et al., 2001), and MULTICOPY SUPPRESSOR OF IRA1 (MSI1) (Hennig et al., 2003). Plants impaired in members of the putative EMF2 complex display pleiotropic phenotypes, including early flowering, curly leaves, terminal flower, and abnormal flower organs, caused by the widespread misexpression of multiple flower homeotic genes (Hennig et al., 2003; Moon et al., 2003; Chanvivattana et al., 2004; Katz et al., 2004).

In addition to the predicted EMF2 complex, EMF1 is also required for floral repression during vegetative development (Aubert et al., 2001). EMF1 is a plant-specific protein that does not display significant homology with proteins of known function from organisms other than plants. Except for the nuclear localization signals, a putative ATP/GTP binding motif (P-loop) and a putative LXXLL motif that is present in many transcription factors and cofactors and mediates interactions that can activate or repress transcription, it does not contain any recognizable domain (Aubert et al., 2001). Like *EMF2*, *EMF1* mRNA is ubiquitous and constitutively expressed (Aubert et al., 2001). Phenotypic and gene expression analyses suggest that EMF1 functions in the same pathway as EMF2 (Moon et al., 2003), but its role in the repression process is not understood nor is its relationship to the putative EMF2 complex.

Loss-of-function *emf1* or *emf2* mutants skip vegetative development and produce flowers upon germination (Sung et al., 1992), suggesting that both genes are required for vegetative development. Both *emf1* and *emf2* mutant seedlings are characterized by short hypocotyl, sessile, and oval-shaped cotyledons. Weak *emf1* (*emf1-1*) and most *emf2* mutants are morphologically similar; they germinate into a short inflorescence with a terminal flower and a few axillary flowers that lack petals and are sterile, bypassing rosette growth. Strong *emf1* mutants, such as *emf1-2*, germinate into a terminal pistil, lacking other floral organs (Yang et al., 1995; Chen et al., 1997). Despite the pleiotropic phenotypes observed in *emf* mutants, suppression of *EMF1* or *EMF2* genes in transgenic plants results in, besides *emf* phenotypes, early flowering plants with normal lateral organs (Aubert et al., 2001; Yoshida et al., 2001), indicating that EMF proteins are principally involved in floral repression.

In *Drosophila melanogaster*, the Polycomb group (PcG)–mediated repression of the target genes is controlled by the cooperation of at least three PcG complexes: Polycomb

[1] Current address: Heidelberg Institute for Plant Science, Biodiversity and Plant Systematics, Heidelberg University, 69120 Heidelberg, Germany.

[2] Address correspondence to zrsung@nature.berkeley.edu.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Z. Renee Sung (zrsung@nature.berkeley.edu).

[W] Online version contains Web-only data.

www.plantcell.org/cgi/doi/10.1105/tpc.106.049957

repressive complex 2 (PRC2), PRC1, and Pleiohomeotic repressive complex (PhoRC). PRC2 labels the targets by adding histone 3 lysine 27 (H3-K27) methyl marks (Cao et al., 2002; Czermin et al., 2002; Kuzmichev et al., 2002; Muller et al., 2002), and PRC1 mediates chromatin compaction and inhibition of the transcription machinery, playing an essential role in one of its components, Posterior sex combs (Psc) that can recapitulate the in vitro inhibition activities of the entire complex (Dellino et al., 2004; Francis et al., 2004; King et al., 2005). These protein complexes are proposed to be recruited to the target genes via a DNA binding protein of the third PcG complex, the PhoRC (Klymenko et al., 2006), which may also participate in the gene silencing mechanism (Muller and Kassis, 2006).

In *Arabidopsis*, three PRC2 equivalents have been proposed based on subunit composition and protein function; however, homologs of PhoRC and PRC1 components have not been identified. Thus, it is unclear how plant PcGs recognize and maintain the transcriptional repression of the target genes. The predicted *Arabidopsis* EMF2 complex is equivalent to *Drosophila* PRC2 in terms of subunit composition and biochemical function (Chanvivattana et al., 2004; Lindroth et al., 2004; Schubert et al., 2005, 2006). There are two other predicted *Arabidopsis* PRC2 equivalents, the FERTILIZATION INDEPENDENT SEED2 complex and the VERNALIZATION2 (VRN2) complex that are involved in seed development and in the vernalization response, respectively (Kohler et al., 2003; Chanvivattana et al., 2004; Wood et al., 2006). Recently, it has been shown that another protein, VERNALIZATION INSENSITIVE3 (VIN3), copurifies with the core components of the VRN2 complex (VRN2, FIE, CLF and SWINGER) (Wood et al., 2006). VIN3 contains a plant homeodomain (PHD) and a fibronectin 3 (FNIII) domain (Sung and Amasino, 2004). The protein is implicated in *FLOWERING LOCUS C* (*FLC*) histone 3 deacetylation that is proposed to occur prior to the VRN2 complex-mediated histone methylation (Wood et al., 2006).

In contrast with PRC2, homologs of *Drosophila* PhoRC and PRC1 have not been identified in plants; thus, it is unclear how the PRC2 is recruited to the target gene and how the gene is silenced after histone methylation. However, a recent report (Zhang et al., 2007) shows that LIKE HETEROCHROMATIN PROTEIN1 (LHP1; also called TERMINAL FLOWER2 [TFL2]) is functionally similar to Pc, a subunit of PRC1 that recognizes and binds the H3H27-trimethyl(me3) marks created by PRC2. Sequence homology suggests that *Arabidopsis* LHP1 encodes a HETEROCHROMATIN1 (HP1) homolog (Gaudin et al., 2001; Kotake et al., 2003). Based on animal and fungal HP1 homolog function and observations that LHP1 can bind H3K9-methylated peptides in vitro, it has been proposed that LHP1 might be involved in heterochromatic gene silencing in plants (Jackson et al., 2002). *Drosophila* Pc shares homology in the chromodomain and in the mechanism of action with HP1. Despite these similarities, HP1 and Pc have distinct biological functions, regulating different components of the genome (de Wit et al., 2007). Zhang et al. (2007) showed that *Arabidopsis* LHP1 binds genomic regions associated with H3K27me3, probably facilitated in part by the direct interaction between its chromodomain and H3K27me3, thus acting as a Pc equivalent.

Here, we demonstrate that EMF1 participates in the PcG-mediated gene silencing by acting downstream of EMF2 to effect transcriptional repression of the flower homeotic gene *AGAMOUS* (*AG*). Our data show that, despite the lack of homology at the protein level, EMF1 plays a PRC1-like role and could have a function in part analogous to *Drosophila* Psc.

RESULTS

emf1 Is Epistatic to *emf2*

To test whether EMF1 and EMF2 act in the same pathway, we generated double mutants. Previous results showed that *emf1emf2* double mutants resembled either parent if one of the parents used is the weak *emf1-1*, which is phenotypically indistinguishable from *emf2* mutants (e.g., *emf2-1* and *emf2-6*) (Sung et al., 2003). Double mutants made by crossing strong *emf1-2* and *emf2-1* were also generated (Yang et al., 1995). A small fraction of the segregating progeny developed severe phenotypes (i.e., callus), suggesting that the callus-generating individuals could be the double mutants (Yang et al., 1995). However, the genotypes of the putative double mutants could not be investigated because neither *EMF* gene was cloned at the time.

To investigate the phenotype of *emf1emf2* double mutants, now that both *EMF* genes have been cloned (Aubert et al., 2001; Yoshida et al., 2001), we generated again the *emf1-2 emf2-1* double mutants and performed PCR-based genotypic analysis. In the segregating population, only three phenotypes were detected: wild type, *emf1-2*, and *emf2-1* at a ratio of 865 wild type-like:299 *emf1-2*-like:249 *emf2-1*-like ($n = 1413$). Figures 1A to 1D show the different phenotypes of the *emf* mutants and wild-type plants 11 d after germination (DAG). The *emf1-2* mutant shows semi-open, small petioleless cotyledons, extremely short hypocotyls, and no leaf primordia. The *emf1-1* and *emf2-1* mutants show open, small petioleless, oval-shaped cotyledons and short hypocotyl and leaf primordia, whereas the wild-type seedling at 11 DAG shows open, round cotyledon blade with long petiole, the first two true leaves, leaf primordia, and long hypocotyl.

Although some *emf1-2*–like plants produced callus upon culture for more than 1 month, we found that the control *emf1-2* single mutant would also generate callus. The *emf1-2* and *emf2-1* mutations generate allele-specific restriction fragment length polymorphisms (RFLPs) derived from the cleaved amplified polymorphic sequences. The *emf1-2* mutation eliminates one of the *Mae*III sites present in the wild-type allele, and *emf2-1* generates an *Hpy*188III site that is not present in the wild-type allele (see Methods and Figure 1F).

To test the presence of the *emf1-2* allele, we amplified the region containing the mutation site for *emf1-2* in the *EMF1* locus from genomic DNAs extracted from the segregating progeny. All *emf1-2*–like plants in the segregating population displayed the restriction pattern with one *Mae*III site eliminated in the *EMF1* locus as did control *emf1-2* mutants, but not *emf2-1* or the wild type (Figure 1F, left panel). To test for the presence of the *emf2-1* allele, we amplified the region containing the *emf2-1* mutation site in the *EMF2* locus. Figure 1F (right panel) showed that the control single *emf2-1* mutant presented the restriction pattern expected for the generation of an *Hpy*188III site, but neither the single *emf1-2* mutant or wild-type seedlings did. This pattern was also observed in 1/7 *emf1-2*–like plants from the segregating population.

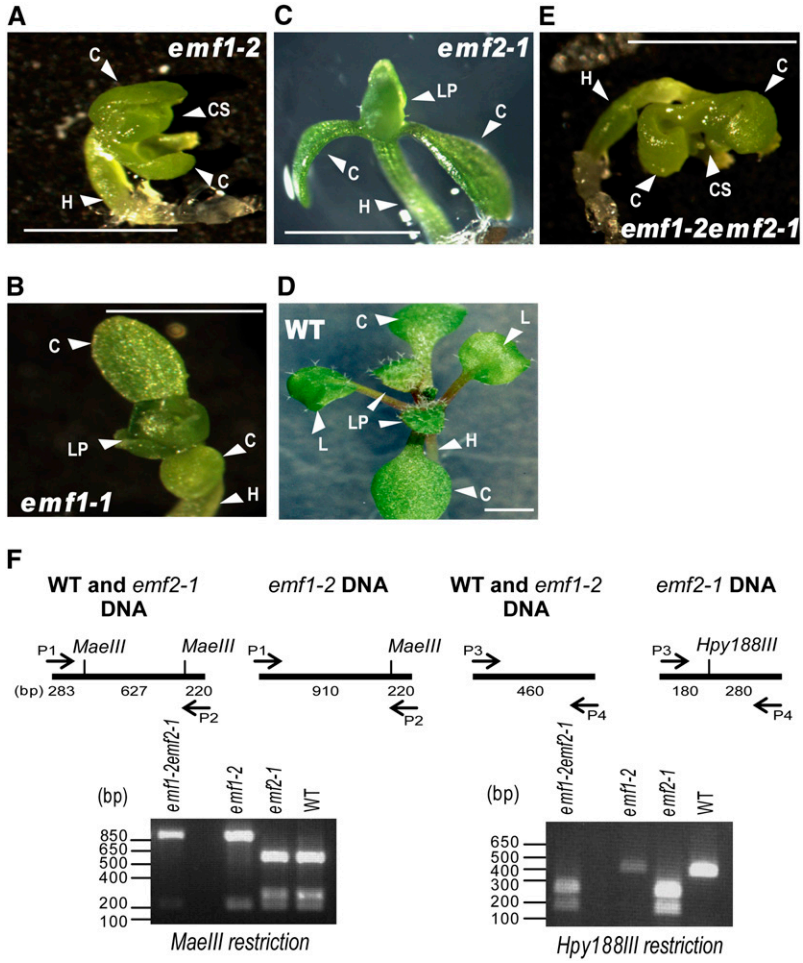

Figure 1. *emf* Mutant Phenotypes.

(A) to **(E)** Phenotypic comparison among *emf1-2* **(A)**, *emf1-1* **(B)**, and *emf2-1* **(C)** mutants, a wild-type plant **(D)**, and *emf1-2 emf2-1* double mutant **(E)** at 11 DAG. C, cotyledon; LP, leaf primordia; CS, carpeloid structures; H, hypocotyl; L, leaf. Bars = 2 mm.

(F) Allele-specific RFLPs created by the *emf1-2* and *emf2-1* mutations. Top: the PCR fragments, the primers (P1, P2, P3, and P4 arrows), the restriction enzyme sites, and the predicted length (bp) of the fragments for each genotype (wild type, *emf1-2*, and *emf2-1*) are illustrated. Bottom: the left and right panels show the allele-specific RFLP analysis using restriction with *Mae*III and *Hpy*188III, respectively. DNA size markers (bp) are identified at the left of each gel.

Thus, the *emf1-2*–like plants that display the RFLP of both parents are the double mutants (Figure 1E). Since the double mutant resembles the *emf1-2* parent, it would suggest that *emf1* is epistatic to *emf2*. Alternatively, it could be argued that the double mutant may display a novel phenotype, which was masked by the severe *emf1-2* phenotype. However, double mutant between weak *emf1-1* and *emf2* mutants did not show a novel phenotype or an additive effect (Yang et al., 1995). Taken together, our molecular and phenotypic analyses indicate that *emf1* is epistatic to *emf2* and that the two genes act in the same pathway.

EMF1 Interacts with MSI1 in Vitro

We next investigated whether EMF1 is another member of the putative EMF2 complex by studying its interaction with the core components of the complex EMF2, CLF, FIE, and MSI1 (Chanvivattana et al., 2004). No interaction between EMF1 and EMF2 or CLF has been detected (Y.-H. Moon and Z.R. Sung, unpublished data). Glutathione *S*-transferase (GST) pull-down experiments, in which either a GST-FIE or GST-MSI1 fusion protein was incubated with hemagglutinin (HA)–tagged EMF1 and collected using Glutathione-Sepharose beads (Figure 2A), showed that EMF1-HA could effectively bind GST-MSI1 but not GST-FIE or GST alone (Figure 2B). In the reciprocal experiment, we used anti-HA antibody to coimmunoprecipitate GST-MSI1 from *Escherichia coli* extract. GST-MSI1 protein was detected (by an anti-GST antibody) only when EMF1-HA was present in the extract (Figure 2C), indicating that GST-MSI1 was bound to EMF1-HA. To determine which part of EMF1 protein was implicated in the interaction with MSI1, we generated various

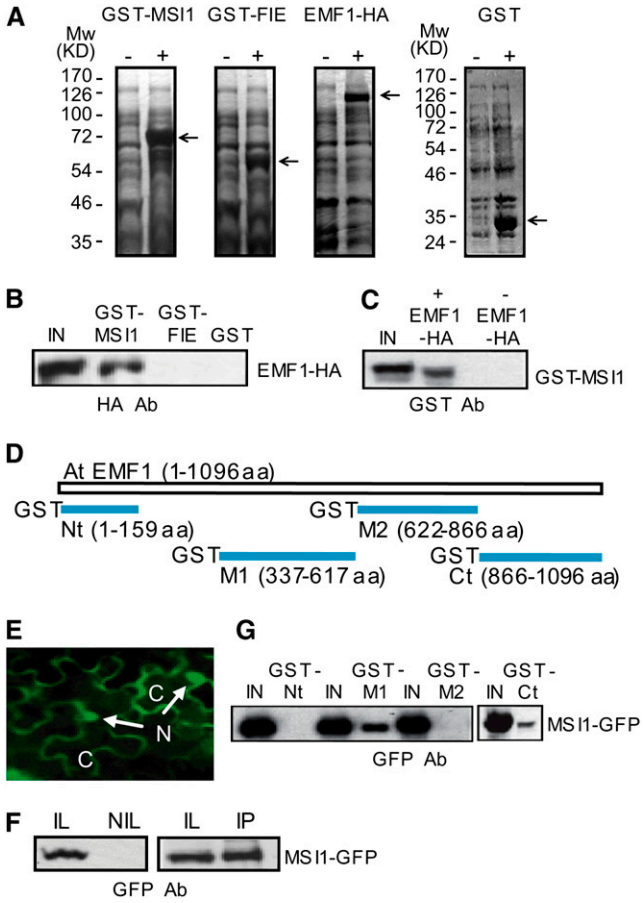

Figure 2. EMF1 Interacts with MSI1.

(A) Coomassie blue stain of the SDS-PAGE gels loaded with GST-MSI1, GST-FIE, EMF1-HA, and GST *E. coli* extracts before (−) and after (+) induction with isopropyl-D-thiogalactopiranoside. Arrows indicate the induced proteins.

(B) For GST pull-down assays, an equal volume of either GST-MSI1, GST-FIE, or GST bacterial extract was combined with EMF1-HA extract. Proteins were recovered using Glutathione-Sepharose beads and detected by protein gel blotting using an anti-HA antibody (HA Ab). IN corresponds to 10% of the EMF1-HA extract used in the GST pull downs.

(C) EMF1-HA extract (+EMF1-HA) or noninduced *E. coli* extract (−EMF1-HA) was mixed with GST-MSI1 extract and immunoprecipitated with an anti-HA antibody. Precipitated proteins were detected by protein gel blotting using an anti-GST antibody (GST Ab). IN corresponds to 10% of the GST-MSI1 extract used in the immunoprecipitation.

(D) Diagram depicts EMF1 protein fragments (Nt, M1, M2, or Ct) expressed as GST fusion proteins. aa, amino acids.

(E) MSI1-GFP protein transiently expressed in *N. benthamiana* leaves showing a nuclear (N) and a cytoplasmic (C) localization probably due to its high expression level.

(F) Detection of MSI1-GFP in infiltrated tobacco leaf (IL) compared with noninfiltrated tobacco leaf (NIL) and immunoprecipitation (IP) of MSI1-GFP from IL using GFP antibody.

(G) Mapping of the interaction between EMF1 and MSI1-GFP. Antibodies (Ab) used for the protein gel blots are indicated in each panel.

GST-fused EMF1 fragments (Figure 2D). The different EMF1 fragments were designed based on the conserved regions among EMF1 and its homologs in rice (*Oryza sativa*) and *Lotus japonica* (see Supplemental Figure 1 online). We found that M1 and Ct fragments could pull down MSI1 fused to the green fluorescent protein (MSI1-GFP) transiently expressed in *Nicotiana benthamiana* leaves (Figures 2E to 2G), indicating that these regions are required for the interaction with MSI1. The Ct fragment contains a highly conserved region among EMF1 homologs (see Supplemental Figure 1 online) that could be implicated in the interaction.

EMF1- and EMF2-Mediated *AG* Repression

Plants impaired in core members of the predicted EMF2 complex or in EMF1 display precocious and ectopic expression of the flower homeotic genes (e.g., *AG*, *APETALA3* [*AP3*], and *PISTILLATA* [*PI*]) (Hennig et al., 2003; Moon et al., 2003; Chanvivattana et al., 2004; Katz et al., 2004), indicating the involvement of EMF proteins in their repression. CLF and EMF2 proteins require *cis*-acting sequences located in the region spanning from the promoter to the 2nd intron of *AG* for *AG* repression (Chanvivattana et al., 2004). To determine whether these sequences are required for EMF1-mediated *AG* repression as well, we crossed three transgenic plants with the β-*glucuronidase* (*GUS*) reporter gene expressed under the control of different regulatory regions of the *AG* gene (*AG:GUS*) (Busch et al., 1999; Deyholos et al., 2003) to plants containing *emf1* or *emf2* alleles and analyzed the GUS activity in the *emf* seedlings harboring the transgene (Figure 3). pMD200 contains the 5′ region spanning from the promoter to the end of the 2nd intron, KB9R contains the 2nd intron, and pMD222 construct is pMD200 with a large deletion in the 2nd intron (Busch et al., 1999; Deyholos et al., 2003).

While pMD200 and KB9R constructs confer a carpel and stamen GUS staining pattern during flower development in wild-type plants, pMD222 confers just a carpel GUS staining pattern (Busch et al., 1999; Deyholos et al., 2003). Wild-type seedlings containing these three constructs do not show GUS activity before flowering under these assay conditions (Figures 3A to 3C). *emf1-2* and *emf2-6* mutants containing pMD200 displayed precocious and strong ectopic expression of the reporter gene in hypocotyl, cotyledons, and shoot apex of 4- and 8-d-old plants, and GUS staining was reduced to the shoot apex in 14-d-old seedlings (Figure 3A). By such time, the shoot apex of both mutants has developed into carpel primordium, and cotyledons of *emf1-2* mutants have become carpelloid (Chen et al., 1997).

By contrast, GUS activity in *emf1-2* and *emf2-6* mutants carrying KB9R or pMD222 (Figures 3B and 3C, respectively) did not display ectopic expression. Instead, GUS activity appeared only in the shoot apices that have differentiated into carpel primordium. In 4-d-old *emf1-2*, the radicle has emerged from the seed, but shoot apex and cotyledons are still enclosed within the seed coat; however, the shoot apex has already differentiated into carpel primordium (Yang et al., 1995). Consequently, there is GUS activity in the shoot apex in 4- and 8-d-old *emf1-2* mutants harboring the KB9R and pMD222 constructs. In *emf2-6* mutants, the carpel primordium appears in 8- to 14-d-old seedlings. Therefore, the GUS staining is detectable in 8 and

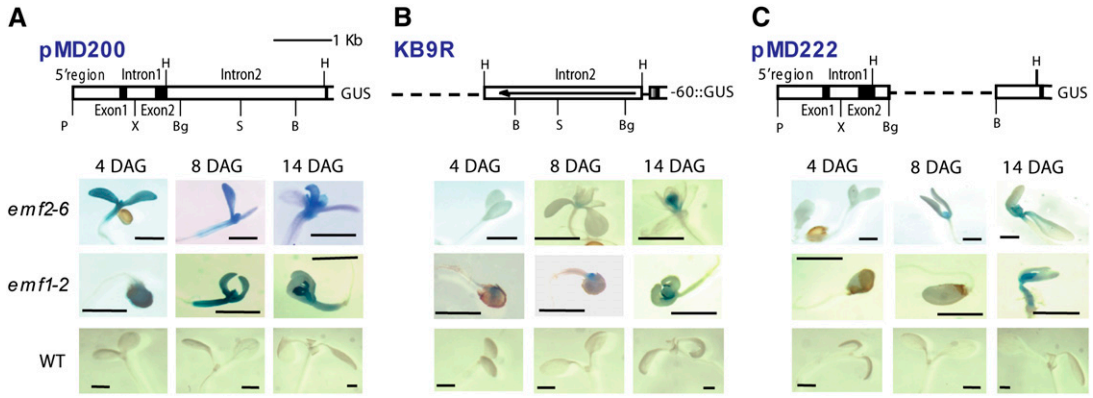

Figure 3. GUS Staining Patterns in Wild-Type, *emf2-6*, and *emf1-2* Mutant Plants Carrying *AG:GUS* Reporter Gene Constructs.

The structures of the pMD200 **(A)**, KB9R **(B)**, and pMD222 **(C)** constructs are depicted in the top of the corresponding panels. Exons and introns are indicated by black boxes and open boxes, respectively. Dashed lines in **(B)** and **(C)** indicate a deletion in the pMD200 construct. Arrow indicates the 5′-to-3′ orientation of the fragment in **(B)**. Where arrows are omitted, the fragments are in their normal 5′-to-3′ orientation. -60, minimal *35S* promoter; P, *Pst*I; X, *Xba*I; H, *Hin*dIII; Bg, *Bgl*II; S, *Spe*I; B, *Bam*HI. Pictures in the bottom set of panels show the GUS activity in 4, 8, and 14 DAG wild-type plants and *emf1-2* and *emf2-6* mutants carrying pMD200, KB9R, or pMD222 construct, respectively. Bars = 2mm.

14 but not 4-d-old *emf2-6*. Together, these data revealed that the 5′ region spanning from the promoter to the end of the 2nd intron is required for *AG* ectopic expression in *emf1* and *emf2* mutants. These results indicate that EMF1 and EMF2 proteins interact with the same regulatory DNA region when mediating *AG* repression in vegetative tissues; however, since this is a large region, it is not clear whether the two proteins overlap in their site of interaction.

Constitutive Expression of EMF1 Protein

EMF1 RNA is constitutively expressed throughout *Arabidopsis* development (Aubert et al., 2001). To study the expression pattern of EMF1 protein, we tagged *EMF1* cDNA with three repeats of a *FLAG* sequence and expressed it under the control the of *EMF1* promoter (see Methods). The *ProEMF1:EMF1cDNA-FLAG* construct was introduced into plants harboring the *emf1-2* allele. This construct rescued the *emf1-2* mutant phenotype. The *emf1-2/EMF1-FLAG* transgenic plants or rescued mutants (RMs) displayed the wild-type phenotype (Figure 4A), indicating that this construct was functional in vivo. The RM genotype was confirmed by PCR-based genotyping and RFLP (see Methods and Figure 4A). Protein gel blots probed with monoclonal anti-FLAG antibody detected EMF1-FLAG protein in nuclear extracts from RM but not from wild-type plants that do not harbor the construct (Figure 4B, left panel).

To study the temporal expression of EMF1 protein, we extracted the nuclear proteins from wild-type or RM plants grown on agar plates samples at either 4, 14, or 21 DAG and from various organs of plants grown in soil for protein gel blot analysis. The antibody detected EMF1-FLAG in the RM in all stages of *Arabidopsis* grown on agar plates as well as in rosette leaves, flower buds, stems, and open flowers (Figure 4B, left panel). The highest amount of EMF1-FLAG was found in flower buds. These results show that like *EMF1* RNA, EMF1 protein is ubiquitously present throughout plant life.

AG Is a Direct Target of EMF1

To investigate whether EMF1 can interact directly with *AG*, *AP3*, and *PI* loci, we performed chromatin immunoprecipitation (ChIP) experiments. For this purpose, we used the RM plants to isolate chromatin associated with the EMF1-FLAG protein. Chromatin from 7-d-old RM seedlings was immunoprecipitated with anti-FLAG antibody followed by quantitative real-time (qRT-PCR) analysis using primers specific for the *AP3*, *PI*, and *AG* promoters and the *AG* 2nd intron, which is known to contain *cis*-regulatory sequences of the *AG* gene (Deyholos et al., 2003) (Figure 4C). Since *AP3* promoter is sufficient to confer ectopic expression of *AP3* in *emf2-10* and *clf* mutants (Chanvivattana et al., 2004) and no additional information is available about intragenic regulatory sequences required for *PI* repression, we tested the promoter region of *AP3* and *PI*.

We also analyzed regions located downstream of *AG* after the At4G18970 gene (*down-AG*) and in the *ACTIN* gene, which are not expected to be targets of EMF1. We found that EMF1 was associated with sites located in the promoter and 2nd intron of *AG* but was not bound to the *AG* downstream region or to the *ACTIN* gene (Figure 4D), indicating that EMF1 interacts in vivo with specific regions of *AG*. EMF1 could also be associated with the *AP3* and *PI* promoter sites due to the higher percentage of these fragments obtained in the immunoprecipitation samples compared with the mock samples (Figure 4D). However, further investigation of EMF1 interaction with other regions is necessary to confirm the binding of EMF1 to the *AP3* and *PI* promoters.

EMF1 Requires EMF2 to Interact with the Target Genes

EMF1 interaction with MSI1 suggests its association with the putative EMF2 complex, either in a stable or transient manner, and that they target the same genes. EMF1 could either recruit the putative EMF2 complex to the target sites or act after EMF2 complex-mediated histone methylation (Schubert et al., 2006). If

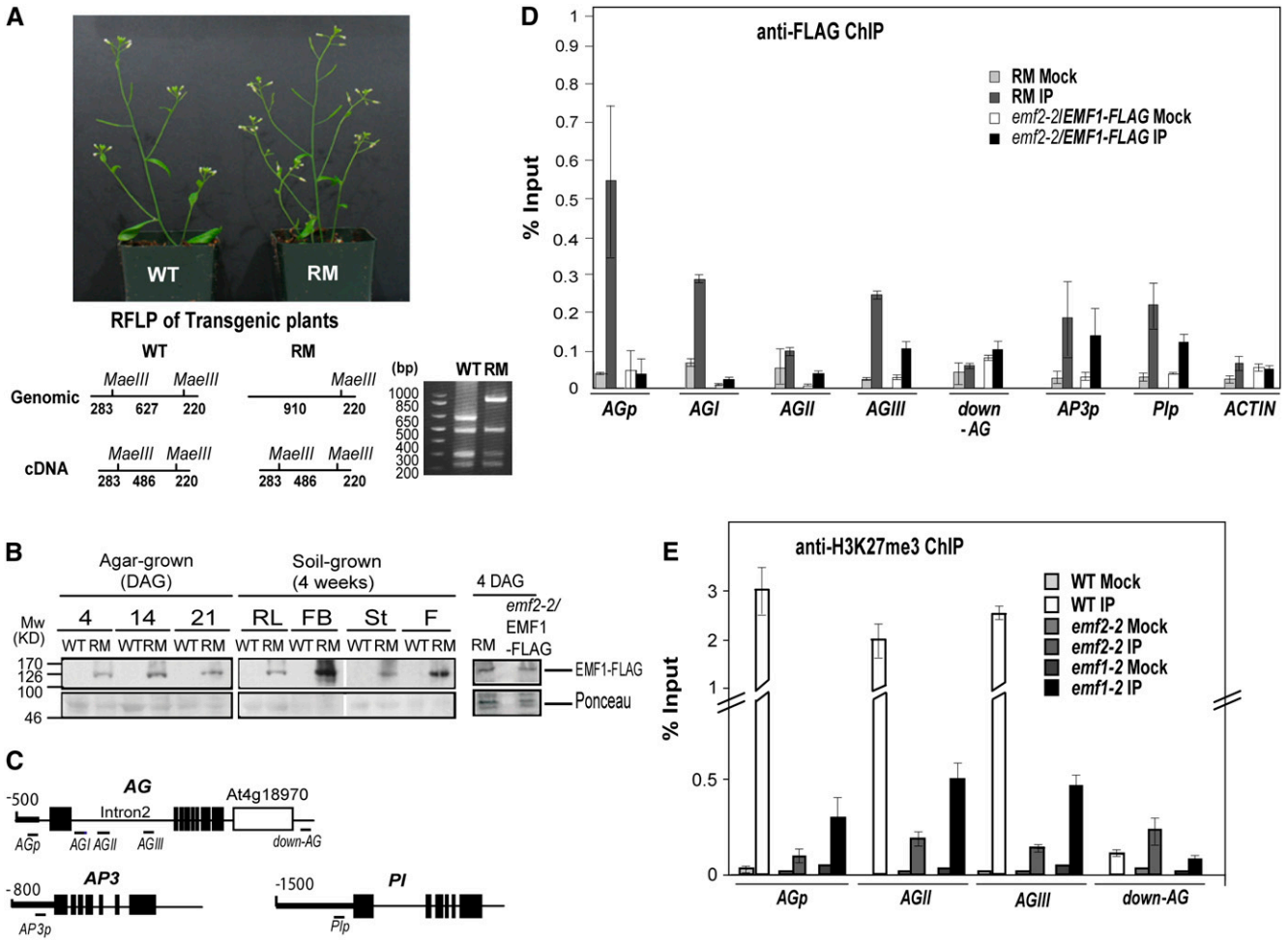

Figure 4. Expression of EMF1 Protein, Its Association with the Target Genes, and the Histone Methylation Pattern of the *AG* Gene.

(A) Phenotype (top) and genotype (bottom) analysis of wild-type and RM plants. Allele-specific RFLPs created by the *emf1-2* mutation (bottom panel). The PCR fragments, the restriction enzyme sites, and the predicted genomic and cDNA restriction fragments for each genotype are illustrated at the bottom left and middle. The RFLP analysis is shown at the bottom right.

(B) Nuclear extracts from wild-type plants, RM, and *emf2-2/EMF1-FLAG* transgenic plants were subjected to protein gel blot analysis using monoclonal anti-FLAG antibody. Nuclear extracts were prepared from 4-, 14-, and 21-d-old seedlings grown on agar plates and from different plant organs: rosette leaves (RL), flower buds (FB), stems (St), and open flowers (F) from plants grown in soil. Ponceau red staining (bottom line) showing a 50-kD protein as a loading control.

(C) Schematic representation of *AP3*, *PI*, and *AG* loci and *AG* 3′ flanking region. For *AP3*, *PI*, and *AG*, the exon/intron structure (black boxes/black lines, respectively), and for the *AG* downstream gene, the transcribed regions (white boxes) are depicted. The promoter regions of *AP3*, *PI*, and *AG* are also indicated (thick lines). The regions amplified by qRT-PCR are depicted as horizontal lines.

(D) ChIP results expressed as percentage of input showing the association of EMF1 with the promoter of the target genes and *AG* 2nd intron. *AG* downstream region (*down-AG*) and *ACTIN* amplifications were used as negative controls.

(E) ChIP results showing the *AG* H3-K27me3 pattern in wild-type seedlings, *emf1-2*, and *emf2-2* mutants. In all the ChIP experiments the average IP from two chromatin samples is expressed on graphs as percentage of corresponding input DNA with error bars representing the standard deviations. Mock represents the chromatin immunoprecipitation without the Ab, and IP represents the chromatin immunoprecipitation with the Ab.

EMF1 recruits the EMF2 complex to the target genes, it could function like a DNA binding protein that recognizes specific DNA sequences on the target genes. To test this possibility, we investigated whether EMF1 binds DNA by probing a protein gel blot with DNA. In this assay, different *E. coli* extracts were resolved by SDS-PAGE, transferred to a membrane, renatured, and finally incubated with a labeled *AG* promoter DNA probe. This study revealed that, while EMF1 can bind DNA (Figure 5A, left panel), this activity is not sequence specific, as we observed interaction with a wide range of unrelated DNA, such as different DNA fragments obtained after digestion of a pUC18 plasmid using different restriction enzymes. Shift assays using different fragments of EMF1 protein (Figure 5B) identified two regions with DNA binding activity, M1 and Ct (Figures 5D and 5F). We also

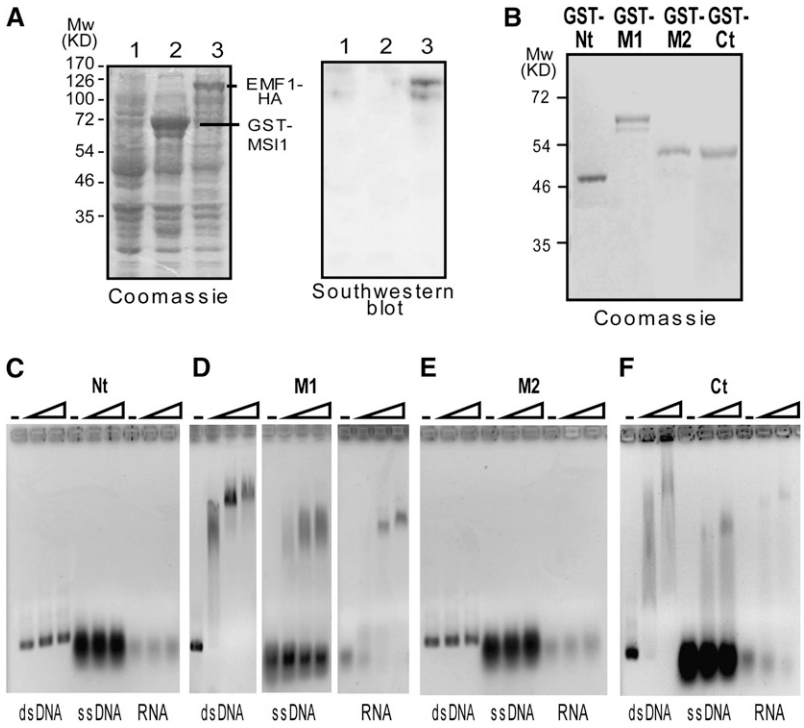

Figure 5. DNA and RNA Binding Activity of EMF1.

(A) Protein gel blots probed with DNA. Noninduced *E. coli* extract (lane 1) and *E. coli* expressing GST-MSI1 extract (lane 2), used as controls, and *E. coli* expressing EMF1-HA extracts (lane 3) were resolved by SDS-PAGE, transferred to an Immobilon-P membrane, renatured, and finally incubated with a radiolabeled DNA probe.

(B) Purified GST fusion proteins of EMF1 fragments: GST-Nt, GST-M1, GST-M2, and GST-Ct (see Figure 2D for constructs).

(C) to **(F)** Different concentrations of EMF1 protein fragments (0, 20, and 40 nM of GST-Nt, GST-M2, and GST-Ct and 0, 20, 40, and 60 nM of GST-M1) were incubated with a 250-bp NOS terminator DNA PCR fragment (double-stranded DNA [dsDNA]), a 100-base oligonucleotide (single-stranded DNA [ssDNA]), or a 100-nucleotide RNA transcript (RNA) for 30 min at 4°C. Samples were loaded in a 1% agarose gel and stained with ethidium bromide.

found that these fragments bind RNA and single-strand DNA in a non-sequence-specific fashion (Figures 5D and 5F). These results indicate that EMF1 is not involved in the sequence-specific recruitment of EMF2 complex to the target sites.

Next, we tested whether EMF1 interaction with the target genes depends on the presence of EMF2. To do this, we introduced the *EMF1-FLAG* transgene into the *emf2* mutant background by crossing RM homozygous plants with *emf2-2* heterozygous plants. Protein gel blots probed with monoclonal anti-FLAG antibody detected EMF1-FLAG protein in nuclear extracts from *emf2-2* mutants containing the *EMF1-FLAG* transgene (Figure 4B, right panel). Chromatin from 7-d-old *emf2-2* mutants containing the *EMF1-FLAG* transgene (*emf2-2*/*EMF1-FLAG*) was immunoprecipitated and analyzed as described above. Interestingly, we found that EMF1 failed to bind to *AG* sites in the *emf2-2* background (Figure 4D), demonstrating the requirement of EMF2 for EMF1 target interaction and indicating that EMF1 acts downstream of EMF2. The requirement of EMF2 for EMF1 target interaction in *AP3* and *PI* promoter sites is less clear, since there is not a significant decrease of EMF1 binding to these sites in the *emf2-2* background compared with the RM background. Additional experiments testing other sites in *AP3* and *PI* regions are required to determine whether EMF1 binding of these genes depends on the presence of EMF2.

To determine whether EMF1 is required for histone methylation, chromatin from 2 g (fresh tissue) of 7-d-old wild-type seedlings, *emf2-2* mutants, or *emf1-2* mutants was immunoprecipitated with anti-trimethyl H3-K27 antibody followed by qRT-PCR amplification using primers specific to several *AG* regulatory regions (*AGp*, *AGII*, and *AGIII*) or to the *AG* downstream region (Figure 4C). We found H3-K27 trimethyl marks (H3-K27me3) over all regions of the *AG* locus that we tested, as has been reported (Schubert et al., 2006) (Figure 4E). The presence of H3-K27me3 throughout the gene has also been reported for *Drosophila* PcG target genes (Kahn et al., 2006; Papp and Muller, 2006). As expected, the H3-K27me3 marks were dramatically reduced in *emf2-2* mutants; however, we also detected a significant, but lesser, decrease of H3-K27me3 marks in *emf1-2* mutants (Figure 4E). These data could indicate that EMF1 is also required for histone methylation or for maintaining a stable histone methylation pattern of the target gene. On the other hand, it could be a consequence of the derepression of the *AG* gene in *emf1-2* mutants. Nevertheless, these data indicate EMF1 and EMF2 acting at the same *AG* sites.

EMF1 Interfered with Transcription in Vitro

The fact that EMF1 requires the presence of EMF2 to interact with the target genes raises the possibility that EMF1 could have a PRC1-like function, converting the methyl marks imparted by PRC2 into stable gene silencing (Wang et al., 2004; Zhang et al., 2004b). The *Drosophila* PRC1 core complex inhibits chromatin remodeling and transcription in vitro (Francis et al., 2001). Remarkably, isolated Psc, a component of *Drosophila* PRC1, has the same inhibitory activities as the entire core complex (Francis et al., 2001; King et al., 2005). Psc can inhibit in vitro transcription by RNA polymerase II and T7 RNA polymerase from chromatin or naked DNA templates (King et al., 2002). Hence, we investigated the ability of EMF1 to inhibit in vitro transcription by RNA polymerase II and T7 RNA polymerase from DNA fragments containing the *cytomegalovirus* (*CMV*) immediate early gene promoter and the *T7* promoter, respectively.

For this assay, we used the four GST-fused EMF1 protein fragments described before (Figure 2C). It was not possible to use the purified EMF1 full-length protein because the fusion protein was insoluble. Purified EMF1 protein fragments were incubated with the naked DNA template before the transcription reactions. We found that EMF1 interfered with transcription by RNA polymerase II (Figure 6A) and T7 RNA polymerase in vitro (Figure 6B). In both cases, the presence of M1, M2, and Ct EMF1 fragments in the reaction led to a significant reduction in the transcript levels, whereas the presence of Nt had no effect in either transcription process. Previous DNA and RNA shift assay experiments using these protein fragments showed that M1 and Ct can bind DNA and RNA in a non-sequence-specific fashion and that none of the protein fragments displayed DNase or RNase activity under these conditions (Figures 5C and 5D).

In addition, there are no signs of RNA degradation evidenced by a smear in the gel after electrophoresis, indicating that the reduced transcript level is not a consequence of transcript degradation. Separating T7 RNA polymerase reaction products by native agarose gel (Figure 6C) allows visualization of protein-nucleic acid complex formation. We found that M1 and Ct bind the nucleic acids present in the reaction, possibly due to their DNA/RNA binding ability. This suggests that the M1 and Ct protein fragments interfered with transcription by complexing with the DNA template and the RNA transcript. However, M2 did not show DNA or RNA binding activity, suggesting that it probably reduced transcription by directly interfering with the polymerase.

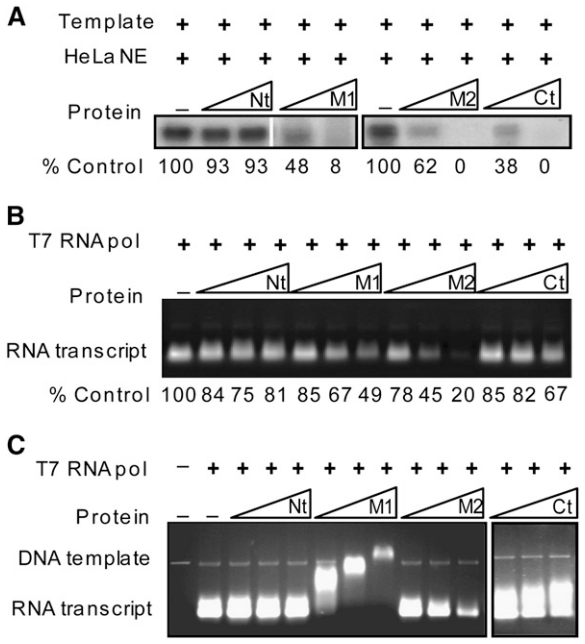

Figure 6. Inhibition of in Vitro Transcription by EMF1.

(A) For the RNA polymerase II transcription assays, a restriction fragment containing the *CMV* immediate early gene promoter was transcribed in vitro, and the radiolabeled runoff transcript was resolved on a 6% denaturing polyacrylamide gel. Reaction mixtures contained 100 ng of DNA template, 0, 20, or 40 nM of the different EMF1 protein fragments (see Figure 2D), and HeLa nuclear extract (NE). Quantitations of the transcript are relative to the "−" protein lane.

(B) T7 RNA polymerase transcription reactions contained 100 ng of linearized pGEM DNA template, 0, 20, 40, or 60 nM of EMF1 protein fragment, and T7 RNA polymerase. Products were visualized on denaturing 1% agarose gels. Quantitations of the transcript are relative to the "−" protein lane.

(C) T7 RNA polymerase assays following the same conditions as in **(B)** but visualizing the products in a native 1% agarose gel. All these experiments were reproduced at least three times obtaining in all repetitions a similar amount of transcript relative to the control.

EMF1 Localizes in the Nucleus in a Speckle-Like Pattern

Animal PRC1 proteins accumulate in characteristic nuclear foci, termed PcG bodies (Zhang et al., 2004a; Hernandez-Munoz et al., 2005), which are proposed to be concentrated areas of transcriptional repression, possibly containing multiple PcG complexes (Grimaud et al., 2006). Using an EMF1-GFP fusion protein transiently expressed in *N. benthamiana* leaves, we found that EMF1-GFP localized exclusively to the nucleus and was dispersed in a speckle-like pattern (Figure 7B) similar to that of the animal PcG proteins. To determine which part of EMF1 is responsible for this subnuclear distribution pattern, we made three chimeric GFP-tagged constructs using different fragments of the protein (Figure 7A). Each of these fragments contains at least one predicted nuclear localization signal and different highly conserved regions among EMF1 homologs (see Supplemental Figure 1 online). E_{1-336}-GFP signal was concentrated in one spot within the nucleus (Figure 7C). $E_{866-1096}$-GFP fusion protein was targeted mainly to the nucleolus and to a few nuclear bodies (Figure 7E). $E_{337-1096}$-GFP fusion protein localization was indistinguishable from that of the intact protein, indicating that the region between amino acids 337 and 866 is responsible for the EMF1 subnuclear pattern (Figure 7D). The characteristic nuclear localization of the different fragments was observed in all the nuclei of the infiltrated tobacco leaves, indicating a consistent pattern of the fragments when expressed under the control of the cauliflower mosaic virus *35S* promoter. Further studies using the EMF1 promoter will be required to confirm these results.

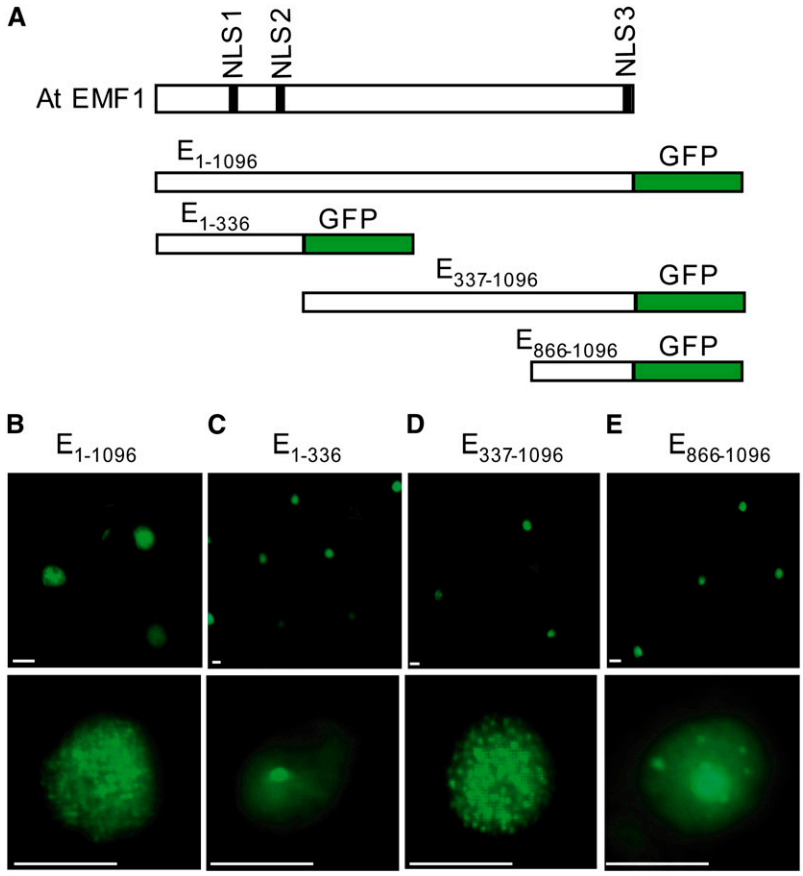

Figure 7. Subcellular Localization of EMF1-GFP Fusion Proteins in *N. benthamiana* Leaves.

(A) Schematic representations of EMF1 and truncated EMF1 proteins fused to GFP.
(B) to **(E)** Nuclear localization of EMF1 full-length and protein fragments. Top and bottom panels show images of nuclei containing each construct using a lower magnification (top panels) or higher magnification (bottom panels) to demonstrate that all the nuclei displayed the specific expression pattern. All these experiments were reproduced at least three times. NLS, nuclear localization signal. Bars = 10 μm.

DISCUSSION

EMF1 Functions in the Same PcG-Mediated Silencing Mechanism as EMF2

Previous phenotypic and gene expression analyses suggested a cooperation of EMF1 and EMF2 in the repression of the flower homeotic genes during vegetative development (Chen et al., 1997; Moon et al., 2003); however, the role of EMF1 in the process was not understood. Genetic and molecular evidences presented here show that EMF1 indeed functions in concert with the putative EMF2 complex in mediating *AG* gene silencing. Epistasis analysis showed that EMF1 and EMF2 function in the same pathway. The members of the predicted EMF2 complex (EMF2, CLF, MSI1, and FIE) are expressed constitutively, indicating that the complex is required throughout development to maintain the epigenetic control of gene expression (Kinoshita et al., 2001; Hennig et al., 2003; Katz et al., 2004). Previous results showed that, like *EMF2* (Yoshida et al., 2001), *EMF1* RNA is constitutively expressed in *Arabidopsis* (Aubert et al., 2001). However, *EMF1* RNA could be posttranscriptionally regulated, and the EMF1 protein levels could be different from the RNA levels. Our finding that EMF1 protein was present in all organs analyzed is consistent with the fact that PcG proteins function in multiple stages of development to maintain cell identity.

In addition, we found that EMF1 interacts with a member of the predicted EMF2 complex, MSI1. EMF1 interaction with MSI1 could indicate that EMF1 is a component of the putative EMF2 complex. Alternatively, this interaction could be transiently established and not related to the EMF2 complex activity. In *Drosophila*, there are three PcG complexes with different functions implicated in gene silencing: PhoRC, PRC2, and PRC1. The three PcG complexes colocalize at the polycomb response elements (PREs), which consist of specific DNA regulatory elements present in the target genes required for the repression, and transient interactions between members of different complexes have been reported (Poux et al., 2001; Wang and Brock, 2003; Wang et al., 2004). Thus, it is possible that EMF1 interacts with the EMF2 complex while performing another function in the gene silencing mechanism.

Since MSI1 was also reported to function together with FASCIATA1 (FAS1) and FAS2 in the *Arabidopsis* chromatin assembly factor 1 (CAF-1) complex (Kaya et al., 2001), it may be argued that EMF1 functions as a member of the CAF-1 complex via its interaction with MSI1. However, this scenario is not likely because *fas1* and *fas2* single or *fas1 fas2* double mutants do not display the *emf1* phenotype or gene expression pattern, while *MSI1* cosuppressed plants and plants impaired in any other member of the putative EMF2 complex do (Leyser and Furner, 1992; Kaya et al., 2001; Hennig et al., 2003; Moon et al., 2003; Chanvivattana et al., 2004; Katz et al., 2004). Hence, EMF1 interacts with the MSI1 that is involved in the PcG-mediated silencing mechanism.

EMF1 and the Putative EMF2 Complex Both Require the Promoter and Intragenic Regions to Mediate *AG* Repression in Vegetative Tissues

Consistent with the two *EMF* genes acting in the same pathway, we found that the region of the *AG* promoter to the end of the 2nd intron is needed to confer ectopic expression of *AG* in *emf1* and *emf2* mutants. Interestingly, the VRN2 complex-mediated *FLC* repression requires *FLC* promoter and intragenic sequences (Sheldon et al., 2002; Bastow et al., 2004; Sung and Amasino, 2004; Sung et al., 2006). In *Drosophila*, PcG proteins act on target genes by association with the PREs (Ringrose and Paro, 2004). Each target gene is normally controlled by more than one PRE, and one of these PREs is located in the promoter region and others may be located intragenically (Ringrose and Paro, 2004).

Neither the 2nd intron nor its upstream sequences alone confers ectopic expression of *AG* in *emf* mutants or wild-type seedlings. Although the 2nd intron is sufficient to confer flower-specific *AG* expression, it is insufficient to confer ectopic expression in *emf* mutants. The same result was observed when the construct containing the 2nd intron of *AG* was introduced into *clf* or *emf2-10* mutant backgrounds (Chanvivattana et al., 2004). Only the construct containing the promoter and the intragenic regions (1st intron, 1st exon, and 2nd intron) showed ectopic GUS staining in these mutants (Sieburth and Meyerowitz, 1997; Chanvivattana et al., 2004).

If EMF1 and EMF2 require the region spanning from the promoter to the 2nd intron to mediate the repression of *AG*, we should expect an ectopic expression of the reporter gene in mutant and wild-type plants containing the constructs with the incomplete region; however, we did not observe ectopic expression of GUS in these plants. It is possible that the regulatory region required for the repression is also required for the transcriptional activation. In *Drosophila*, the trithorax group (TrxG) proteins act antagonistically to the PcG proteins, maintaining the transcriptional activation of the homeotic genes (Ringrose and Paro, 2004). The PREs have a dual function as PcG and TrxG response elements. Thus, if the regulatory elements recognized by the repressors/activators are not present, the activation could not take place. In *Arabidopsis*, several TrxG protein homologs have been identified. Interestingly, *ARABIDOPSIS* TRITHORAX1 (ATX1), the homolog of *Drosophila* trithorax, functions as an activator of homeotic genes, including *AG*, *PI*, and *AP3* (Alvarez-Venegas et al., 2003). A recent report showed that simultaneous loss of ATX1 and CLF restored *AG* repression and normalized leaf phenotypes (Saleh et al., 2007).

EMF1 Plays a PRC1-Like Role

Our findings suggest that EMF1 plays a role in the PcG-mediated silencing mechanism. In *Drosophila*, the recruitment of the PcG protein complex to the target genes implicates the PhoRC, which contains Pleiohomeotic (Pho), a DNA sequence-specific binding protein, and the histone-hypomethylated binding protein *Scm*-related gene containing four mbt domains (Sfmbt). Sfmbt contains a conserved protein-interacting sterile α motif domain, which was first identified in the *Drosophila* PcG gene *Sex comb on midleg* (*Scm*), and four malignant brain tumor (mbt) domains implicated in the methyl histone binding activity (Klymenko et al., 2006; Muller and Kassis, 2006). Several lines of evidence indicate that in *Drosophila* PRC1 and PRC2 are targeted to the PREs through interactions with Pho and probably other DNA binding proteins and not through interactions with nucleosomes or by covalent histone modifications (Mohd-Sarip et al., 2002, 2005; Muller and Kassis, 2006). Homologs of these DNA binding proteins have not been detected in *Arabidopsis*. It is possible that, in plants, the recruitment of the PcG proteins involves plant-specific proteins, but the fact that EMF1 binds DNA and RNA nonspecifically and acts downstream rather than upstream of EMF2 indicate that it cannot be involved in the target recognition.

In *Drosophila*, although PRC1 by itself is unable to target PRE sequences, some of its subunits have nonspecific DNA binding activity (Francis et al., 2001). Interestingly, PRC1, after recruitment by Pho to the PREs, facilitates the DNA binding activity of Pho to naked PRE DNA templates and extends the footprinting pattern of Pho, suggesting additional protein-DNA contacts (Mohd-Sarip et al., 2005). Recent reports showed that the core of *Drosophila* PREs is depleted of nucleosomes (Kahn et al., 2006; Papp and Muller, 2006), indicating that the direct interaction of the PcG complexes with PRE DNA plays an important role in the silencing mechanism. However, recent work showed that H3K27me3 regions in *Arabidopsis* do not colocalize with nucleosome-depleted regions of significant length (Zhang et al., 2007).

Nevertheless, although nucleosomal DNA presents a barrier for proteins to contact the DNA, it is known that nucleosomes spontaneously undergo conformational fluctuations in which a stretch of their DNA transiently lifts off the histone surface (Li et al., 2005). Therefore, EMF1 DNA binding ability could assist in the stabilization of the PcG complex through, albeit transient, protein-DNA contact. In addition, PcG proteins maintain the status of their target genes through mitosis. In mitotic cells, DNA of inactive genes is temporarily naked during S phase (or DNA synthesis), and the epigenetic marks need to be established on newly synthesized daughter strands after S phase. The DNA binding properties of EMF1 could help in reestablishing the PcG complex on the target genes to maintain the silenced state. Interestingly, the nonspecific DNA binding property is also found in other PcG proteins, including plant PcG proteins such as VRN1 (Levy et al., 2002).

Finally, the ability of EMF1 to interfere with in vitro transcription, as does the component of *Drosophila* PRC1, Psc (Francis et al., 2001; King et al., 2005), led us to the conclusion that EMF1 performs a PRC1-like function in *Arabidopsis*. The in vitro inhibitory activity of Psc is as efficient as that of the core complex. The

Psc N-terminal region contains a RING finger domain implicated in the PRC1-mediated mono-ubiquitination of histone 2A at Lys-119 (Buchwald et al., 2006; Li et al., 2006); however, the inhibitory activities of Psc reside in its long C-terminal region that has no recognizable structural motifs and no obvious homology to other proteins (King et al., 2005). EMF1 does not display significant homology with proteins of known function either (Aubert et al., 2001). Nevertheless, despite the lack of sequence homology between EMF1 and Psc, they have similar transcriptional inhibitory properties.

Drosophila PRC1 induces nucleosomal array compaction in vitro (Francis et al., 2004), and interaction between PRC1 and promoter factors, such as TATA binding protein (TBP) and TBP-associated proteins, has been reported (Breiling et al., 2001; Saurin et al., 2001). Moreover, TBP, TBP-associated proteins, and RNA polymerase II have been found at the promoter of the repressed target genes (Dellino et al., 2004; Papp and Muller, 2006). These observations suggest that PRC1 repression is mediated by a combined mechanism of chromatin compaction and direct inhibition of the transcription machinery (Dellino et al., 2004; Francis et al., 2004). Some of the components of *Drosophila* PRC1 (e.g., Psc and polyhomeotic) can interact in vitro with DNA (Francis et al., 2001) and with RNA (Zhang et al., 2004a). This property could help the complex to mediate chromatin compaction and transcription inhibition. Like these PcG proteins, EMF1 may represses transcription by a mechanism that involves DNA/RNA binding and inhibition of the transcription machinery.

In summary, our results indicate that EMF1 plays a PRC1-like role in the PcG-mediated gene silencing mechanism; however, it is not clear if it functions as a member of EMF2 complex or as a member of a PRC1 equivalent. In the former case, *Arabidopsis* PRC2 would have expanded its role to include transcriptional repression activity. In the latter case, it would require the identification of the other PRC1 components in *Arabidopsis*. In either case, the discovery of the molecular mechanism of EMF1-mediating flower homeotic gene silencing opens a new avenue in plant PcG investigation and brings us closer to the elucidation of the epigenetic mechanism involved in floral repression.

METHODS

Plant Material and Growth Conditions

Surface-sterilized *Arabidopsis thaliana* (cultivar Columbia) seeds were plated on agar plates containing half-strength salts and vitamins, 1.5% sucrose, and 0.8% agar (Murashige and Skoog, 1962). The plates were placed for 2 d at 4°C, and then seedlings were grown under short-day conditions (8 h of light/16 h of dark) at 21°C. Fifteen-day-old seedlings grown under short-day conditions were transferred to soil (potting compost and vermiculite 2:1) and grown under long-day conditions (16 h of light/8 h of dark) at 21°C in the greenhouse. *Nicotiana benthamiana* plants were grown in 10-cm pots. Seeds were sown on a mix of 70% soil with vermiculite and 30% sea sand and were irrigated from below. Plants were grown in the greenhouse under long-day conditions at 27°C.

In Vitro Pull Down, Coimmunoprecipitation Assays, and Purification of Recombinant Protein

cDNA encoding *EMF1* tagged with *HA* (5′-TATCCATATGACGTCCCAGACTCTGCC-3′) was cloned into pET19b (Novagen), cDNA encoding *EMF1* fragments (*MSI1* or *FIE*) was cloned into pGEX-4T-3 (Amersham Biosciences), and these were transformed into *Escherichia coli* strain BL21. Cells expressing GST fusion proteins or EMF1-HA protein were collected by centrifugation, resuspended in 1 mL of extraction buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 μM $ZnSO_4$, 0.1% Triton X-100, 10% glycerol, and 1 mM phenylmethylsulfonyl fluoride [PMSF]), and sonicated. The extracts were centrifuged for 20 min at 14,000 rpm at 4°C. An equal volume of GST fusion protein extract was mixed with EMF1-HA extract. For pull-down assays, 30 μL of Glutathione-Sepharose beads (Amersham Biosciences) were added to the mix and incubated for 2 h at 4°C. The beads were then washed three times with extraction buffer. For coimmunoprecipitation assays, 20 μL of HA matrix (Amersham Biosciences) were added to the mix. Incubation and washes were as in the pull-down assay. Standard procedures were used for protein gel blotting. Antibodies used are as follows: anti-GST-HRP conjugate (Amersham Biosciences) polyclonal antibody (1:10,000); anti-HA-peroxidase (Roche) monoclonal antibody (1:500).

Recombinant GST fusion proteins were purified following the recommendations of the Glutathione-Sepharose manufacturer (Amersham Biosciences) using purification buffer (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 1 mM DTT, 0.1% Triton X-100, 10% glycerol, and 1 mM PMSF) as binding and washing buffer too. Protein elution was performed using elution buffer (20 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 25% glycerol).

Genetic Crosses, GUS Activity Assays, and Transgenic Plants

emf1-2 and *emf2-1* heterozygous plants were crossed. The F2 segregating progeny was subjected to PCR-based genotyping. Genomic DNAs extracted from the segregating progeny were used to amplify the region containing the mutation site for *emf1-2* and *emf2-1* in the *EMF1* and *EMF2* loci, respectively. The primers used were as follows: P1 (5′-GTGGGTTTGACAGAACTCCA-3′) and P2 (5′-GTCCTCAAGTGAGATCCCGT-3′) for *EMF1* and P3 (5′-AACAACAAATTGCAGAAGACTGAAG-3′) and P4 (5′-CTTGGATATCATTGTCTCAGTCTTG-3′) for *EMF2*. The *emf1-1* and *emf2-1* mutations generate allele-specific RFLPs (Figure 1F). In *emf1-2*, a deletion of a C base in position 5898 bp in *EMF1* genomic DNA results in the deletion of a *Mae*III site. In *emf2-1*, substitution of a G-to-A base in position 2994 bp in *EMF2* genomic DNA generates an *Hpy*188III site. *Mae*III digestion would generate three fragments for the wild type and *emf2-1* (i.e., 283, 627, and 220 bp) and two fragments for *emf1-2* (i.e., 910 and 220 bp). *Hpy*188III would not cut the *emf1-2* or the wild type's 460-bp fragment but would cut it into two fragments, 180 and 280 bp, in *emf2-1*.

emf1-2 and *emf2-6* heterozygous plants were crossed with lines homozygous for the *AG:GUS* transgenes. F1 plants were selfed. F2 plants homozygous or heterozygous for *AG:GUS* transgenes and heterozygous for *emf1-2* or *emf2-6* were grown to generate F3 plants. GUS activity in transgenic plants was assayed as described before (Moon et al., 2003).

The 7-kb *EMF1* promoter region, the 3′ three *FLAG* (5′-GACTACAAAGACCATGACGGTGATTATAAAGATCATGATATCGATTACAAGGATGACGATGACAAG-3′)-tagged *EMF1* cDNA, which was generated using a PCR-based epitope tagging method, and the *NOS* terminator were cloned in this order into the binary vector pPZP221 (Hajdukiewicz et al., 1994). The construct was introduced into *Agrobacterium tumefaciens* strain GV3101 by the freeze-thaw method (Chen et al., 1994) and subsequently transformed into *emf1-2* heterozygous plants by the floral-dip method (Clough and Bent, 1998). The *emf1-2/EMF1-FLAG* transgenic plants or rescued *emf1-2* mutants (RM) were confirmed by PCR-based genotyping. Genomic DNAs extracted from kanamycin resistant (Km^r) wild-type–like plants were used to amplify the region that contains the *emf1-2* mutation in the *EMF1* locus with the P1/P2 pair of primers as

described above. The resulting fragments were digested with *Mae*III. The primers amplify a fragment in the *EMF1* genomic and in the inserted *EMF1* cDNA sequences. The *EMF1* cDNA fragment can be distinguished from the genomic one because it does not contain a 141-bp intron. The *EMF1* cDNA fragment contributes 283, 486, and 220 bp bands in all Km^r plants. RM showed the restriction pattern characteristic for the *emf1-2* mutants (220- and 910-bp fragments) and the fragments resulting from the digestion of the cDNA, whereas wild-type transgenic plants showed the restriction pattern for the wild-type allele (283-, 627-, and 220-bp fragments) together with the bands from the cDNA.

Homozygous RM plants were crossed with *emf2-2* heterozygous plants and then selfed to introduce the *EMF1-FLAG* transgene into the homozygous *emf2* mutants (*emf2-2*/*EMF1-FLAG* transgenic plants).

Nuclei from different ages and tissues of *Arabidopsis* wild-type and transgenic plants were obtained essentially as described (Bowler et al., 2004). Nuclei were then resuspended in SDS-PAGE 1× loading buffer, heated 5 min at 95°C, and analyzed by SDS-PAGE and protein gel blots to detect EMF1-FLAG protein using anti-FLAG M2 (Sigma-Aldrich).

Transient Expression

PCR-amplified fragments of *EMF1* cDNA or *MSI1* cDNA were cloned as *Nco*I/*Spe*I fragments into a modified pCAMBIA vector (pCAMBIA 130N) that contains a double cauliflower mosaic virus *35S* promoter followed by the *TEVL* (translational enhancer from tobacco etch virus) sequence and the enhanced GFP (*eGFP*) coding sequence (Cormack et al., 1996). The fragments were placed in frame downstream of the *TEVL* and before the *eGFP*. *A. tumefaciens* strain GV3101 harboring the appropriate plasmid was grown at 30°C in L-broth (10 g tryptone, 5 g yeast extract, 5 g NaCl, and 15 g agar in 1 liter of water) plates supplemented with 50 μg/mL kanamycin and 25 μg/mL gentamycin. Bacteria were collected and resuspended in infiltration medium (5 mM MES, pH 5.6, 10 mM $MgCl_2$, and 162.5 μM acetosyringone). Two- to four-week-old *N. benthamiana* leaves were coinfiltrated with a bacteria suspension containing two *A. tumefaciens* strains harboring the desired construct and the suppressor of transgene silencing p19 (pGD-p19) construct, respectively, at OD_{600} of 0.6. Protein expression was examined 72 h following the injection in a Zeiss Axiophot epifluorescence microscope equipped with a 5 MPix Q Imaging Micropublisher low-light, cooled CCD color digital camera.

To extract soluble proteins, leaves were batch frozen in liquid N_2 and ground into powder using a mortar and pestle. Leaf powder was subsequently resuspended in extraction buffer (previously described). To remove insoluble material, the resulting extract was centrifuged at 16,000 rpm for 15 min at 4°C. The supernatant was collected and used for protein gel blot analysis, coimmunoprecipitation, or pull-down assay. Antibodies used are as follows: GFP (FL) (Santa Cruz Biotechnology) polyclonal antibody (1:500) for protein gel blot and GFP full-length A. v. (BD Living Colors) polyclonal antibody for immunoprecipitation.

ChIP

ChIP was performed on chromatin isolated from 2 g of 7-d-old seedlings using monoclonal anti-FLAG M2 (Sigma-Aldrich) or anti-trimethyl H3-K27 (Upstate) antibody. Buffers and procedures were essentially as described in a published protocol (Bowler et al., 2004).

The precipitated DNA was dissolved in 100 μL of TE buffer (10 mM Tris-HCl, pH 7.5, and 1 mM EDTA) of which 4 μL was used per PCR reaction. An aliquot of the original input chromatin was processed in parallel with the immunoprecipitated samples to determine efficiency of the immunoprecipitation reaction. Immunoprecipitated DNA was analyzed by qRT-PCR (Thermocycler ABI 7000) using SYBR Green and standard settings (Applied Biosystems). PCR was performed in duplicate, and serial dilutions of purified input DNA were measured together with the immunoprecipitated DNA samples. Quantification was determined by applying the 2^{-Ct} formula (SuperArray ChIP-qPRC user manual; Bioscience Corporation). Average immunoprecipitates from chromatin isolated independently are expressed on graphs as percentage of corresponding input DNA with error bars representing the standard deviations. Selection of optimal primers and data analysis was performed essentially as described (SuperArray ChIP-qPRC user manual; Bioscience Corporation). The following primers were used to amplify *Arabidopsis* genomic DNA: *AGp*, 5′-CTGCATCTCATGAGTGATTGCCCA-3′ and 5′-TGGTGGGTAGTTCTTGTGTGGTCT-3′; *AGI*, 5′-ATGCTGAAGTCGCACTCATCGTCT-3′ and 5′-GAGCACGAGAAGAAGAAGAAACCTG-3′; *AGII*, 5′-GGGAGAGAGTAAGGAAGGACTATGAGGT-3′ and 5′-GACACAGACATTAACAACAATGGAGGATGG-3′; *AGIII*, 5′-AGAGTTTGGTCTGCCTTCTACGATCC-3′ and 5′-GATGTCTGAACTCTCAACCTTCTCG-3′; *down-AG*, 5′-TTGCTAGTGTGCCAAGGGATGAGA-3′ and 5′-TTCCCAGGACACAGGACACATGAT-3′; *AP3p*, 5′-AAAGCCAACCAAATCCACCTGCAC-3′ and 5′-GGAGCTCCGTTAGCTTCTACTTTG-3′; *Plp*, 5′-CCATGACTGTGCCCTCAAGAAAGT-3′ and 5′-GAAGAACAAGAAGAGGAGCATTGG-3′; *ACTIN2*, 5′-CACTTGCACCAAGCAGCATGAAGA-3′ and 5′-AATGGAACCACCGATCCAGACACT-3′.

DNA–Protein Interaction

E. coli protein extracts were denatured at 95°C for 5 min prior to loading onto 10% SDS-PAGE, and,following electrophoresis, proteins were electrotransferred onto an Immobilon-P membrane (Millipore). Proteins were renatured in renaturing buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 M $ZnSO_4$, 1 mM $MgCl_2$, 0.1% Nonidet P-40, 10% glycerol, and 5% [w/v] nonfat milk) overnight at 4°C and incubated with the labeled DNA probe for 1 h in binding buffer (renaturing buffer without nonfat milk). The membrane was then washed three times for 10 min in the same buffer and exposed to x-ray film (Kodak BioMax M_R films).

AG regulatory region DNA fragments and unrelated DNA fragments were obtained by PCR using a 5′-specific primer and a 3′ primer containing an *Eco*RI site. After digestion, the DNA fragments were end-labeled with ^{32}P by incorporating [α-^{32}P]ATP during a 3′ fill-in reaction using DNA polymerase Klenow fragment (New England Biolabs).

Electrophoresis Shift Assay

Different concentrations of GST fusion proteins (GST plus EMF1 protein fragments) were incubated with a 250-bp DNA fragment, a 120-base oligonucleotide, or a 100 nucleotide RNA transcript (obtained by in vitro transcription) using a Riboprobe transcription system (Promega) and a linearized pBlueScript KS vector as template in 20 μL of binding buffer (10 mM Tris-HCl, pH 7.9, 50 mM NaCl, 10 mM $MgCl_2$, 1 μM $ZnSO_4$, 1 mM DTT, and 1% BSA) for 30 min at 4°C. Samples were then loaded in a 1% agarose gel and electrophoresed at 100 V in 1× TAE buffer (40 mM Tris-HCl, pH 8, 20 mM acetic acid, and 1 mM EDTA) at 4°C. The gel was soaked in 1× TAE buffer containing 1 μg/mL ethidium bromide for 15 min and then visualized in a UV transilluminator.

In Vitro RNA Polymerase II and T7 RNA Polymerase Transcription

RNA polymerase II transcription assays were performed using a HeLaScribe Nuclear Extract in vitro transcription system (Promega). As a template, we used a linearized 1.2-kb DNA fragment containing the *CMV* immediate early gene promoter provided with the system as a positive control DNA. Reaction mixtures (25 μL) contained 100 ng of DNA template, 0, 20, or 40 nM of the different EMF1 protein fragments, and HeLa nuclear extract. To maintain the same conditions in the different reactions, the protein concentration was adjusted using the same buffer in which the protein is kept in the stock solution. T7 RNA polymerase

transcription assays were performed following the standard protocol (Riboprobe transcription system; Promega). The DNA template was generated by cloning a 500-bp DNA fragment into pGEM-T vector (Promega) and further linearized by digestion with *Nde*I. T7 RNA polymerase transcription reactions (20 μL) contained 100 ng of linearized pGEM DNA template, 0, 20, 40, or 60 nM of EMF1 protein fragment, and T7 RNA polymerase. To maintain the same conditions in the different reactions, the protein concentration was adjusted as described before. Fragment intensity was quantified using ImageQuant (version 5.2) software (Molecular Dynamics).

Accession Numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative database under the following accession numbers: *EMF1*, At5g11530; *EMF2*, At5g51230; *MSI1*, At5g58230; *FIE*, At3g20740; *AG*, At4g18960; *AP3*, At3g54340; *PI*, At5g20240. Germplasm information for the mutants is as follows: *emf1-1*, CS16235; *emf1-2*, CS16236; *emf2-1*, CS16238; *emf2-6*, CS16243; *emf2-2*, CS16239. Other sequence data are as follows: *Oryza sativa* accession number NC 008394 (gene Os01g0229300); *Lotus japonicus*, AG247130.

Supplemental Data

The following material is available in the online version of this article.

Supplemental Figure 1. Sequence Alignment among EMF1 Protein Homologs.

ACKNOWLEDGMENTS

We thank L. Sieburth (University of Utah, Salt Lake City, UT) and D. Weigel (Max Planck Institute, Tubingen, Germany) for pMD200, KB9R, pMD222, and pMD228 transgenic lines, H. Nakayama (University of California, Berkeley, CA) for pCAMBIA 130N vector, A. Jackson (University of California, Berkeley, CA) for the pGD-p19 construct, Y.H. Moon (Pusan National University, Busan, Korea) for *EMF1* promoter and the *EMF1* cDNA, S. Luan and D. Lisch (University of California, Berkeley, CA) for helpful suggestions of the manuscript, O. Phan and E. Abbott (University of California, Berkeley, CA) for crossing *AG:GUS* constructs into *emf* mutants and GUS assays, T. Ha Tran, A. Meyer, and C. Heng (University of California, Berkeley, CA) for their technical support, K. Lertpiriyapong (University of California, Berkeley, CA) for stimulating discussion, O. Puig and T. Kutzkey from R. Tjian's laboratory (University of California, Berkeley, CA) for assisting with the transcription assays, and J. Alfonso (Heidelberg University, Germany) and A. Kaul (Heidelberg University, Germany) for helping us with the qRT-PCR assays. This work is supported by National Science Foundation Grant IBN 0236399 and USDA Grant 03-35301-13244 to Z.R.S. R.S. was supported by a postdoctoral fellowship from the Spanish Ministry of Education and Science.

Received December 22, 2006; revised January 7, 2008; accepted January 31, 2008; published February 15, 2008.

REFERENCES

Alvarez-Venegas, R., Pien, S., Sadder, M., Witmer, X., Grossniklaus, U., and Avramova, Z. (2003). ATX-1, an Arabidopsis homolog of trithorax, activates flower homeotic genes. Curr. Biol. **13:** 627–637.

Aubert, D., Chen, L., Moon, Y.H., Martin, D., Castle, L.A., Yang, C.H., and Sung, Z.R. (2001). EMF1, a novel protein involved in the control of shoot architecture and flowering in Arabidopsis. Plant Cell **13:** 1865–1875.

Bastow, R., Mylne, J.S., Lister, C., Lippman, Z., Martienssen, R.A., and Dean, C. (2004). Vernalization requires epigenetic silencing of FLC by histone methylation. Nature **427:** 164–167.

Boss, P.K., Bastow, R.M., Mylne, J.S., and Dean, C. (2004). Multiple pathways in the decision to flower: Enabling, promoting, and resetting. Plant Cell **16**(Suppl): S18–S31.

Bowler, C., Benvenuto, G., Laflamme, P., Molino, D., Probst, A.V., Tariq, M., and Paszkowski, J. (2004). Chromatin techniques for plant cells. Plant J. **39:** 776–789.

Breiling, A., Turner, B.M., Bianchi, M.E., and Orlando, V. (2001). General transcription factors bind promoters repressed by Polycomb group proteins. Nature **412:** 651–655.

Buchwald, G., van der Stoop, P., Weichenrieder, O., Perrakis, A., van Lohuizen, M., and Sixma, T.K. (2006). Structure and E3-ligase activity of the Ring-Ring complex of polycomb proteins Bmi1 and Ring1b. EMBO J. **25:** 2465–2474.

Busch, M.A., Bomblies, K., and Weigel, D. (1999). Activation of a floral homeotic gene in Arabidopsis. Science **285:** 585–587.

Cao, R., Wang, L., Wang, H., Xia, L., Erdjument-Bromage, H., Tempst, P., Jones, R.S., and Zhang, Y. (2002). Role of histone H3 lysine 27 methylation in Polycomb-group silencing. Science **298:** 1039–1043.

Chanvivattana, Y., Bishopp, A., Schubert, D., Stock, C., Moon, Y.H., Sung, Z.R., and Goodrich, J. (2004). Interaction of Polycomb-group proteins controlling flowering in Arabidopsis. Development **131:** 5263–5276.

Chen, H., Nelson, R.S., and Sherwood, J.L. (1994). Enhanced recovery of transformants of *Agrobacterium tumefaciens* after freeze-thaw transformation and drug selection. Biotechniques **16:** 664–668, 670.

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

Clough, S.J., and Bent, A.F. (1998). Floral dip: A simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana.* Plant J. **16:** 735–743.

Cormack, B.P., Valdivia, R.H., and Falkow, S. (1996). FACS optimized mutants of the green fluorescent protein (GFP). Gene **173:** 33–38.

Czermin, B., Melfi, R., McCabe, D., Seitz, V., Imhof, A., and Pirrotta, V. (2002). Drosophila enhancer of Zeste/ESC complexes have a histone H3 methyltransferase activity that marks chromosomal Polycomb sites. Cell **111:** 185–196.

Dellino, G.I., Schwartz, Y.B., Farkas, G., McCabe, D., Elgin, S.C., and Pirrotta, V. (2004). Polycomb silencing blocks transcription initiation. Mol. Cell **13:** 887–893.

de Wit, E., Greil, F., and van Steensel, B. (2007). High-resolution mapping reveals links of HP1 with active and inactive chromatin components. PLoS Genet. **3:** e38.

Deyholos, M.K., Cavaness, G.F., Hall, B., King, E., Punwani, J., Van Norman, J., and Sieburth, L.E. (2003). VARICOSE, a WD-domain protein, is required for leaf blade development. Development **130:** 6577–6588.

Francis, N.J., Kingston, R.E., and Woodcock, C.L. (2004). Chromatin compaction by a polycomb group protein complex. Science **306:** 1574–1577.

Francis, N.J., Saurin, A.J., Shao, Z., and Kingston, R.E. (2001). Reconstitution of a functional core polycomb repressive complex. Mol. Cell **8:** 545–556.

Gaudin, V., Libault, M., Pouteau, S., Juul, T., Zhao, G., Lefebvre, D., and Grandjean, O. (2001). Mutations in LIKE HETEROCHROMATIN PROTEIN 1 affects flowering time and plant architecture in Arabidopsis. Development **128:** 4847–4858.

Goodrich, J., Puangsomlee, P., Martin, M., Long, D., Meyerowitz, E.M., and Coupland, G. (1997). A Polycomb-group gene regulates homeotic gene expression in Arabidopsis. Nature **386:** 44–51.

Grimaud, C., Bantignies, F., Pal-Bhadra, M., Ghana, P., Bhadra, U., and Cavalli, G. (2006). RNAi components are required for nuclear clustering of Polycomb group response elements. Cell **124:** 957–971.

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

Hennig, L., Taranto, P., Walser, M., Schonrock, N., and Gruissem, W. (2003). Arabidopsis MSI1 is required for epigenetic maintenance of reproductive development. Development **130:** 2555–2565.

Hernandez-Munoz, I., Lund, A.H., van der Stoop, P., Boutsma, E., Muijrers, I., Verhoeven, E., Nusinow, D.A., Panning, B., Marahrens, Y., and van Lohuizen, M. (2005). Stable X chromosome inactivation involves the PRC1 Polycomb complex and requires histone MACROH2A1 and the CULLIN3/SPOP ubiquitin E3 ligase. Proc. Natl. Acad. Sci. USA **102:** 7635–7640.

Honma, T., and Goto, K. (2000). The Arabidopsis floral homeotic gene PISTILLATA is regulated by discrete cis-elements responsive to induction and maintenance signals. Development **127:** 2021–2030.

Jackson, J.P., Lindroth, A.M., Cao, X., and Jacobsen, S.E. (2002). Control of CpNpG DNA methylation by KRYPTONITE histone H3 methyltransferase. Nature **416:** 556–560.

Kahn, T.G., Schwartz, Y.B., Dellino, G.I., and Pirrotta, V. (2006). Polycomb complexes and the propagation of the methylation mark at the *Drosophila Ubx* gene. J. Biol. Chem. **281:** 29064–29075.

Kardailsky, I., Shukla, V.K., Ahn, J.H., Dagenais, N., Christensen, S.K., Nguyen, J.T., Chory, J., Harrison, M.J., and Weigel, D. (1999). Activation tagging of the floral inducer FT. Science **286:** 1962–1965.

Katz, A., Oliva, M., Mosquna, A., Hakim, O., and Ohad, N. (2004). FIE and CURLY LEAF polycomb proteins interact in the regulation of homeobox gene expression during sporophyte development. Plant J. **37:** 707–719.

Kaya, H., Shibahara, K.I., Taoka, K.I., Iwabuchi, M., Stillman, B., and Araki, T. (2001). FASCIATA genes for chromatin assembly factor-1 in arabidopsis maintain the cellular organization of apical meristems. Cell **104:** 131–142.

King, I.F., Emmons, R.B., Francis, N.J., Wild, B., Muller, J., Kingston, R.E., and Wu, C.T. (2005). Analysis of a polycomb group protein defines regions that link repressive activity on nucleosomal templates to in vivo function. Mol. Cell. Biol. **25:** 6578–6591.

King, I.F., Francis, N.J., and Kingston, R.E. (2002). Native and recombinant polycomb group complexes establish a selective block to template accessibility to repress transcription in vitro. Mol. Cell. Biol. **22:** 7919–7928.

Kinoshita, T., Harada, J.J., Goldberg, R.B., and Fischer, R.L. (2001). Polycomb repression of flowering during early plant development. Proc. Natl. Acad. Sci. USA **98:** 14156–14161.

Klymenko, T., Papp, B., Fischle, W., Kocher, T., Schelder, M., Fritsch, C., Wild, B., Wilm, M., and Muller, J. (2006). A Polycomb group protein complex with sequence-specific DNA-binding and selective methyl-lysine-binding activities. Genes Dev. **20:** 1110–1122.

Kohler, C., Hennig, L., Bouveret, R., Gheyselinck, J., Grossniklaus, U., and Gruissem, W. (2003). Arabidopsis MSI1 is a component of the MEA/FIE Polycomb group complex and required for seed development. EMBO J. **22:** 4804–4814.

Kotake, T., Takada, S., Nakahiqashi, K., Ohto, M., and Goto, K. (2003). Arabidopsis TERMINAL FLOWER 2 gene encodes a heterochromatin protein 1 homolog and represses both FLOWERING LOCUS T to regulate flowering time and several floral homeotic genes. Plant Cell Physiol. **44:** 555–564.

Kuzmichev, A., Nishioka, K., Erdjument-Bromage, H., Tempst, P., and Reinberg, D. (2002). Histone methyltransferase activity associated with a human multiprotein complex containing the Enhancer of Zeste protein. Genes Dev. **16:** 2893–2905.

Levy, Y.Y., Mesnage, S., Mylne, J.S., Gendall, A.R., and Dean, C. (2002). Multiple roles of Arabidopsis VRN1 in vernalization and flowering time control. Science **297:** 243–246.

Leyser, H.M.O., and Furner, I.J. (1992). Characterisation of three shoot apical meristem mutants of *Arabidopsis thaliana.* Development **116:** 397–403.

Li, G., Levitus, M., Bustamante, C., and Widom, J. (2005). Rapid spontaneous accessibility of nucleosomal DNA. Nat. Struct. Mol. Biol. **12:** 46–53.

Li, Z., Cao, R., Wang, M., Myers, M.P., Zhang, Y., and Xu, R.M. (2006). Structure of a Bmi-1-Ring1B polycomb group ubiquitin ligase complex. J. Biol. Chem. **281:** 20643–20649.

Lindroth, A.M., et al. (2004). Dual histone H3 methylation marks at lysines 9 and 27 required for interaction with CHROMOMETHYLASE3. EMBO J. **23:** 4286–4296.

Mohd-Sarip, A., Cleard, F., Mishra, R.K., Karch, F., and Verrijzer, C.P. (2005). Synergistic recognition of an epigenetic DNA element by Pleiohomeotic and a Polycomb core complex. Genes Dev. **19:** 1755–1760.

Mohd-Sarip, A., Venturini, F., Chalkley, G.E., and Verrijzer, C.P. (2002). Pleiohomeotic can link polycomb to DNA and mediate transcriptional repression. Mol. Cell. Biol. **22:** 7473–7483.

Moon, Y.H., Chen, L., Pan, R.L., Chang, H.S., Zhu, T., Maffeo, D.M., and Sung, Z.R. (2003). EMF genes maintain vegetative development by repressing the flower program in Arabidopsis. Plant Cell **15:** 681–693.

Muller, J., Hart, C.M., Francis, N.J., Vargas, M.L., Sengupta, A., Wild, B., Miller, E.L., O'Connor, M.B., Kingston, R.E., and Simon, J.A. (2002). Histone methyltransferase activity of a Drosophila Polycomb group repressor complex. Cell **111:** 197–208.

Muller, J., and Kassis, J.A. (2006). Polycomb response elements and targeting of Polycomb group proteins in Drosophila. Curr. Opin. Genet. Dev. **16:** 476–484.

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

Papp, B., and Muller, J. (2006). Histone trimethylation and maintenance of transcriptional ON and OFF states by trxG and PcG proteins. Genes Dev. **20:** 2041–2054.

Poux, S., McCabe, D., and Pirrotta, V. (2001). Recruitment of components of Polycomb group chromatin complexes in Drosophila. Development **128:** 75–85.

Ringrose, L., and Paro, R. (2004). Epigenetic regulation of cellular memory by the Polycomb and Trithorax group proteins. Annu. Rev. Genet. **38:** 413–443.

Saleh, A., Al-Abdallat, A., Ndamukong, I., Alvarez-Venegas, R., and Avramova, Z. (2007). The Arabidopsis homologs of trithorax (ATX1) and enhancer of zeste (CLF) establish 'bivalent chromatin marks' at the silent AGAMOUS locus. Nucleic Acids Res. **35:** 6290–6296.

Saurin, A.J., Shao, Z., Erdjument-Bromage, H., Tempst, P., and Kingston, R.E. (2001). A Drosophila Polycomb group complex includes Zeste and dTAFII proteins. Nature **412:** 655–660.

Schubert, D., Clarenz, O., and Goodrich, J. (2005). Epigenetic control of plant development by Polycomb-group proteins. Curr. Opin. Plant Biol. **8:** 553–561.

Schubert, D., Primavesi, L., Bishopp, A., Roberts, G., Doonan, J., Jenuwein, T., and Goodrich, J. (2006). Silencing by plant Polycomb-group genes requires dispersed trimethylation of histone H3 at lysine 27. EMBO J. **25:** 4638–4649.

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

Sheldon, C.C., Conn, A.B., Dennis, E.S., and Peacock, W.J. (2002). Different regulatory regions are required for the vernalization-induced repression of FLOWERING LOCUS C and for the epigenetic maintenance of repression. Plant Cell **14:** 2527–2537.

Sieburth, L.E., and Meyerowitz, E.M. (1997). Molecular dissection of the AGAMOUS control region shows that cis elements for spatial regulation are located intragenically. Plant Cell **9:** 355–365.

Sung, S., and Amasino, R.M. (2004). Vernalization in Arabidopsis thaliana is mediated by the PHD finger protein VIN3. Nature **427:** 159–164.

Sung, S., He, Y., Eshoo, T.W., Tamada, Y., Johnson, L., Nakahigashi, K., Goto, K., Jacobsen, S.E., and Amasino, R.M. (2006). Epigenetic maintenance of the vernalized state in Arabidopsis thaliana requires LIKE HETEROCHROMATIN PROTEIN 1. Nat. Genet. **38:** 706–710.

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

Sung, Z.R., Chen, L., Moon, Y.H., and Lertpiriyapong, K. (2003). Mechanisms of floral repression in Arabidopsis. Curr. Opin. Plant Biol. **6:** 29–35.

Sung, Z.R., Chen, L., Moon, Y.H., and Yoshida, N. (2003). Molecular mechanism of shoot determinacy and flowering in Arabidopsis. HortScience **38:** 1325–1327.

Wang, L., Brown, J.L., Cao, R., Zhang, Y., Kassis, J.A., and Jones, R.S. (2004). Hierarchical recruitment of polycomb group silencing complexes. Mol. Cell **14:** 637–646.

Wang, Y.J., and Brock, H.W. (2003). Polyhomeotic stably associates with molecular chaperones Hsc4 and Droj2 in Drosophila Kc1 cells. Dev. Biol. **262:** 350–360.

Wood, C.C., Robertson, M., Tanner, G., Peacock, W.J., Dennis, E.S., and Helliwell, C.A. (2006). The *Arabidopsis thaliana* vernalization response requires a polycomb-like protein complex that also includes VERNALIZATION INSENSITIVE 3. Proc. Natl. Acad. Sci. USA **103:** 14631–14636.

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

Yoshida, N., Yanai, Y., Chen, L., Kato, Y., Hiratsuka, J., Miwa, T., Sung, Z.R., and Takahashi, S. (2001). EMBRYONIC FLOWER2, a novel polycomb group protein homolog, mediates shoot development and flowering in Arabidopsis. Plant Cell **13:** 2471–2481.

Zhang, H., Christoforou, A., Aravind, L., Emmons, S.W., van den Heuvel, S., and Haber, D.A. (2004a). The *C. elegans* Polycomb gene SOP-2 encodes an RNA binding protein. Mol. Cell **14:** 841–847.

Zhang, Y., Cao, R., Wang, L., and Jones, R.S. (2004b). Mechanism of Polycomb group gene silencing. Cold Spring Harb. Symp. Quant. Biol. **69:** 309–317.

Zhang, X., Germann, S., Blus, B.J., Khorasanizadeh, S., Gaudin, V., and Jacobsen, S.E. (2007). The Arabidopsis LHP1 protein colocalizes with histone H3 Lys27 trimethylation. Nat. Struct. Mol. Biol. **14:** 869–871.