Epigenetic regulation of gene programs by EMF1 and EMF2

in Arabidopsis

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The *EMBRYONIC FLOWER (EMF)* genes are required to maintain vegetative development in *Arabidopsis*. Loss-of-function *emf* mutants skip the vegetative phase, flower upon germination, and display pleiotropic phenotypes. *EMF1* encodes a putative transcriptional regulator, while *EMF2* encodes a Polycomb group (PcG) protein. PcG proteins form protein complexes that maintain gene silencing via histone modification. They are known to function as master regulators repressing multiple gene programs. Both EMF1 and EMF2 participate in PcG-mediated silencing of the flower homeotic genes, *AGAMOUS (AG)*, *PISTILLATA (PI)* and *APETALLA3 (AP3)*. Full-genome expression pattern analysis of *emf* mutants showed that both EMF proteins regulate additional gene programs including photosynthesis, seed development, hormone, stress and cold signaling. Chromatin Immunoprecipitation (ChIP) was carried out to investigate whether EMF regulates these genes directly. It was determined that EMF1 and EMF2 interact with genes encoding the transcription factors, *ABSCISIC ACID 3 (ABI3)*, *LONG VEGETATIVE PHASE 1 (LOV1)* and *FLOWERING LOCUS C (FLC)*, which control seed development, stress and cold signaling, and flowering, respectively. Our results suggest that the two EMFs repress the regulatory genes of individual gene programs to effectively silence the genetic pathways necessary for vegetative development and stress response. A model of the regulatory network mediated by EMF is proposed.
INTRODUCTION

Epigenetics entails stable gene expression changes not caused by changes in DNA sequence (Vaillant and Paszkowski, 2008). The major mechanisms underlying epigenetic changes include histone tail methylation (or acetylation), DNA methylation and non-coding RNA. The Polycomb group (PcG) proteins maintain repression of homeotic genes during *Drosophila* embryogenesis via histone methylation (Schwartz and Pirrotta, 2008). These PcG proteins form multiprotein complexes, Polycomb Repressive Complex1 (PRC1) and PRC2. PRC2 contains Enhancer of Zeste (E(z), the methyltransferase), Suppressor of Zeste 12 (Su(z)12) and Extra Sex Combs (ESC). PRC2 methylates histone3 at Lysine27 (H3K27) PRC1 binds to the methylated histone protein to maintain the repressed state (Lund et al., 2004). PRC2 components are conserved in plants and animals. In *Arabidopsis*, MEDEA (MEA), CURLY LEAF (CLF) and SWINGER (SWN) are E(z) homologs and FERTILIZATION INDEPENDENT ENDOSPERM (FIE) and MULTICOPY SUPPRESSOR OF IRA1 (MSI1) are homologs of ESC and p55, respectively (Grossniklaus et al., 1998; Goodrich et al., 1997; Chanvivattana et al., 2004; Kinoshita et al., 2001; Henning et al., 2003). EMBRYONIC FLOWER2 (EMF2), FERTILIZATION INDEPENDENT SEED2 (FIS2) and VERNALIZATION2 (VRN2) are Su(z)12 homologs (Yoshida et al., 2001; Gendall et al., 2001; Chaudhury et al., 1997). Although little is known about PRC1 in plants, a recent study showed that, despite lack of sequence similarity, EMF1 is functionally similar to an animal PRC1 protein (Calonje et al., 2008).

Molecular genetic analysis found that the three *Arabidopsis* PRC2s regulate diverse functions (Sung et al., 2003; Chanvivattana et al., 2004). The PRC2 composed of MEA, FIE, FIS2 and MSI1 represses *PHERES1* (*PHE1*) gene expression during seed development (Kohler et al., 2003b; Makarevich et al., 2006). The complex composed of CLF/SWN, FIE, VRN2 and MSI1 represses *FLOWERING LOCUS C* (*FLC*), mediating the vernalization response (Chanvivattana et al., 2004; Wood et al., 2006; De Lucia et al., 2008). FLC is a floral repressor that suppresses the floral activators, *FLOWERING LOCUS T* (*FT*) and *SUPRESSOR OF OVEREXPRESSION OF CO1* (*SOC1*) (Michaels et al., 2005). FLC repression would activate *FT* and *SOC1*, leading to flowering. The third complex, composed of CLF/SWN, FIE, EMF2 and MSI1, represses the flower *MADS-
box genes, e.g., *AGAMOUS (AG)*, enabling vegetative growth (Calonje and Sung 2006; Schubert et al., 2006; Yang et al., 1995; Chen et al., 1997; Goodrich et al., 1997).

Previous gene expression analysis using an 8000 *Arabidopsis* gene GeneChip array (8K GeneChip) identified eight categories of genetic pathways modulated by EMF activities (Moon et al., 2003). Here we report a genome-wide analysis of gene expression in *emf* mutants that identified additional pathways regulated by the two EMF genes, the abscisic acid (ABA), stress, cold and heat signaling pathways. We employed Chromatin Immunoprecipitation (ChIP) to determine whether EMF regulates these pathways directly. Analysis showed that EMF1 and EMF2 are recruited to the transcription factor genes of several genetic pathways. A model of EMF regulation of seed maturation, stress/cold signaling, and flowering is presented.

**RESULTS**

**Genome-wide gene expression pattern in *emf1* and *emf2* mutants**

Previous 8K GeneChip analyses showed that EMF1 and EMF2 negatively regulate the flower organ identity and seed maturation genes in *Arabidopsis* (Moon et al., 2003). To study genome-wide regulation of EMF, we performed a global gene expression analysis using a custom designed Affymetrix GeneChip containing probe sets representing 25,996 unique *Arabidopsis* genes (26K GeneChip, Zhu, 2003).

The expression level of each gene was determined by the difference in hybridization signal intensity measured by perfect match and mismatch probes (Zhu, 2003). Gene expression patterns of 7- and 15-day old *emf* mutants (*emf1-1, emf1-2* and *emf2-1*) were compared to that of wild-type (WT) at the same age to identify genes showing expression change. Expression patterns of genes in WT flower buds and flowers were also examined to investigate the causal relationship of the gene expression change to the *emf* mutation or its reproductive state.

The high-density *Arabidopsis* oligonucleotide arrays were designed based on the unigene set assembled at the time. The expression level of each gene was determined by hybridization signal intensity measured by perfect match probes using the MOID
algorithm (Zhou et al. 2002). Despite some differences in probe sets, a high correlation coefficient (0.85) between the two arrays among 7,582 commonly presented genes was reported (Zhu, 2003). This supports the strategy of cross-comparison for commonly presented genes between the previous analysis (Moon et al., 2003) and the present study. The present microarray study is a quick survey of the transcriptome to identify additional putative components of EMF regulated pathways. The previous study (Moon et al., 2003) showed that a simple, stringent cutoff can effectively produce a global reference profile of the EMF regulated transcriptome. Transcript changes of key genes in these pathways were confirmed by RT-PCR (see below).

Genes with hybridization signal >50 in at least one of the four lines (WT and the three \(emf\) alleles) were selected for further analysis. Of the 25,996 genes analyzed, 11,447 from 7-day old and 10,204 from 15-day old samples satisfied this criterion (Table I). Fold change of gene expression was determined by the hybridization signal ratio between the WT and the mutants. Genes showing more than a two-fold change in the hybridization signal were designated up-regulated if those signals were higher in the mutants and down-regulated if the signals were lower in the mutants as described in Table I. For example, in the 7-day old \(emf1-1\) sample, 3,336 (29.1%) genes were up-regulated and 1,033 (9%) down-regulated; in total, 38% of genes changed expression. All three mutants showed extensive changes from the WT gene expression pattern. In general, more genes were up-regulated than down-regulated in the mutants. We compared gene expression patterns among the three \(emf\) mutants to determine whether the same genes were similarly up- or down-regulated. Among the 11,447 genes selected from the 7-day old samples, 3,957 genes were up-regulated and 1,629 down-regulated in at least one of the three \(emf\) alleles (Figure 1A and 1B). A total of 1,654 of the 3,957 genes (41.8%) were up-regulated and 303 of the 1,629 genes (18.6%) down-regulated in all three mutants. The overlap in up-regulated genes between the two \(emf1\) alleles, \(emf1-1\) and \(emf1-2\), was higher than between the two mutants of similar phenotypes, \(emf1-2\) and \(emf2-1\). These similar phenotype mutants are both weak alleles compared to \(emf1-2\). The overlap between the two \(emf1\) alleles for the 7-day old samples was 69.9% (2,773 of 3,957) in up-regulated (Figure 1A), and 48.6% (803 of 1,629) in down-regulated genes (Figure 1B). The overlaps between \(emf1\) and \(emf2\) were fewer, 44.2% (\(emf1-2\) vs. \(emf2-1\), Figure 1A)
and 47.3% (emf1-1 vs. emf2-1) for up-regulated genes (Figure 1A). Similar results were found for the down-regulated genes.

In 15-day old samples, among 10,204 selected genes, 2,522 were up-regulated and 1,909 down-regulated in at least one of the three emf alleles (Figure 1C and 1D). 556 of the 2,522 genes (22%) were up-regulated and 457 of the 1,909 genes (23.9%) down-regulated in all three emf alleles. Again, there was a higher percentage of overlap between the two emf1 alleles than between emf2-1 and emf1-1.

In summary, our 8K and 26K GeneChip results were similar in that the emf mutants display major gene expression pattern changes from WT at the same number of days after germination. Our results also confirmed that, despite the fact that emf1-1 is morphologically more similar to emf2-1 than to emf1-2, its gene expression pattern is more similar to that of emf1-2. That the gene expression patterns correlate with alleles rather than phenotype, suggests possible EMF1 and/or EMF2-specific gene programs.

**Functional analysis of EMF regulated genes**

Genes showing expression change in emf mutants were grouped into 15 functional categories including the eight previously described, i.e., Flowering, Seed maturation, Photoreceptors, Photosynthesis, Auxin, Gibberellin (GA), Ethylene and Expansin (Moon *et al.*, 2003; Table II). In Moon *et al.*, (2003), the Flowering category included both flowering time genes and flower organ identity genes, which are separated into two different categories for the 26K GeneChip data analysis. As 26K GeneChip data showed expression changes in many more genes, we created additional categories to cover these genes and their homologs: ABA synthesis and signaling, Stress, Cold, Heat and Transcription Factors. The Histone category is also included because histone proteins are major components of the chromatin and their modifications by PcG proteins affect gene activity. Genes with hybridization signal >50 in at least one of the eight RNA samples were grouped into functional categories based on annotation of the probe set in the 26K GeneChip and their relations to biological functions, processes, and cellular locations (Supplemental data S1 and S2).

**Flower organ and flowering time genes**
Consistent with the 8K GeneChip data analysis, the 26K GeneChip data showed increased expression of most flower organ genes that include the flower MADS-box or organ identity genes, namely **AG, PI, APETALLA1 (AP1), AP3, SEPALLATA (SEP) 1, 2,** and **3,** in the *emf* mutants. Table II shows that all but one of 23 flower organ genes examined were up-regulated in the *emf* mutants. (Table II, Supplemental data S1-A).

Most flower MADS-box genes are also up-regulated in WT flowers, consistent with the notion that *emf* mutants are in the reproductive state. However, no flower organ primordia have yet developed in the 7-day old *emf* mutants, suggesting that up-regulation of the MADS-box genes results from the loss of EMF activity, rather than as a consequence of flower organ development.

In contrast to the flower organ genes, some genes in the Flowering Time category were up-regulated and others down-regulated (Table II, supplemental data S1-B). In addition, flowering time genes did not show corresponding expression changes from WT seedlings between WT flower and *emf* mutants. For example, **GIGANTEA (GI)** and **FLC** were up-regulated in *emf* mutants, but not in WT flowers (Table III, supplemental data S1-B). Hence, their expression change in *emf* mutants is not related to the reproductive state of the *emf* mutants.

Differential gene expression of major flowering time genes was observed in the *emfl* and *emf2* mutants. For example, both 8K and 26K GeneChip data as well as RT-PCR analysis showed that **FT** is up-regulated in *emf2* mutants, but not in *emfl* mutants (Table III, Figure 2, Yoshida *et al.*, 2001; Moon *et al.*, 2003). The 26K GeneChip data showed up-regulation of **GI** in *emf* seedlings (Table III) that is confirmed by RT-PCR analysis in 7-day old *emf* mutants (Figure 2). As **GI** and **FT** are activators of **CONSTANS (CO)** and **SOC1,** respectively (Kim *et al.*, 2008), we investigated **CO** and **SOC1** RNA levels (Table III). Unexpectedly, **CO** and **SOC1** were not up-regulated in *emf* seedlings. As transcript levels were low, we carried out RT-PCR analysis to confirm expression levels, and found no significant differences between WT and *emf* mutants (Figure 2). These results suggest additional factors controlling **CO** and **SOC1** expression. Several negative regulators of **CO** and **SOC1** were investigated. Interestingly, the transcript level of **LONG VEGETATIVE PHASE 1 (LOV1),** a known **CO** repressor (Yoo *et al.*, 2007), and **FLC,** a known **SOC1** repressor, were increased in all three *emf* mutants on the 26K
GeneChip array (Table III). RT-PCR results confirmed increased LOV1 and FLC expression in the emf mutants (Figure 2). Since CO activates FT, it is curious that the FT transcript is elevated in emf2-1 while the CO level remained constant. To understand this, we examined the expression patterns of FT repressors such as the TARGET OF EAT 1 (TOE1) and TEMPRANILLO (TEM) genes (Jung et al., 2007; Castillejo and Pelaz, 2008). Table III shows that TOE1, TEM1, TEM2 and RELATED TO ABI3/VP 1 (RAV1) transcripts levels were all reduced in emf mutants. It, however, is not clear if the down-regulation of these genes is related to the lack of EMF repressors or to the reproductive state, as these genes were also down-regulated in WT flowers.

Genes involved in seed development

The 32 seed maturation genes investigated in the 8K GeneChip analysis were all up-regulated in the 2 emf1 mutants, but not in emf2-1 (Moon et al., 2003). We examined 120 genes involved in seed development in the 26K GeneChip data set (Supplemental data S1-C). Many were up-regulated in emf1 or emf2 mutants, but some did not change or showed reduced expression (Table II). Of the 120 Seed category genes examined, 61 were up-regulated in at least one of the 3 emf mutants at 7 day after germination (DAG), and 56 at 15 DAG. Figure 3 shows that 26.3% were up-regulated in all three emf mutants at 7 DAG and 26.7% at 15 DAG. The two emf1 allele samples have more genes with overlapping expression pattern than the emf1 and emf2 mutant samples. There is only 31.2 and 30.2% overlap between emf1-1 and emf2-1, but 37.7 and 55.2% overlap between the 2 emf1 mutants at 7 and 15 DAG, respectively (Figure 3). For example, the AtEm1, ABI3, and 4 Arabidopsis 2S Albumin 3 (At2S3) genes were up-regulated in the two emf1 mutants but not in emf2-1 (Supplemental data S1-C).

ABI3, a master regulator of the seed maturation program that activates these seed storage protein genes (Krebbers et al., 1988), has low transcript level, thus may be precluded GeneChip detection as a gene changing expression in the emf mutants, Thus, we performed RT-PCR analysis that showed transcripts of ABI3, as well as At2S3 and AtEm1, are increased in emf1-1 and emf1-2, but not in emf2-1 (Figure 4). The differential expression pattern of ABI3 in the mutants is likely to account for the expression pattern of the downstream seed storage protein genes.
Genes involved in hormone synthesis and signaling

In the Auxin and Gibberellin (GA) categories, the 26K GeneChip data showed a similar number of genes up- and down-regulated in all three emf mutants (Table II and Supplemental data S1-D and S1-E).

In the Ethylene category, more genes were up-regulated than down-regulated in 7-day old mutants, but more genes were down-regulated in 15-day old emf mutants (Table II). This is in part a result of age-dependent expression changes in WT, where transcript levels of many genes were increased from 7- to 15-day old. Many genes in the mutants also showed increased expression from 7- to 15-day, but to a lesser extent than genes in WT (Supplemental data S1-F). Therefore, down-regulation in 15-day-old mutants is not necessarily a direct result of the loss-of-EMF function, rather may result from developmental change in WT. However, the results from 7-day old samples do show an up-regulation of gene expression in emf mutants, indicating that EMF negatively regulates genes in the Ethylene category.

In contrast, almost all of the ABA-related genes investigated were up-regulated in emf mutants (Table II, Supplemental data S1-G). There is no obvious age-dependent change in gene expression in WT samples. Thus, ABA signaling is clearly negatively impacted by EMF. EMF regulation is consistent with ABA’s role in repressing seed maturation genes after germination.

Genes involved in cold, stress and heat signaling

As ABA also regulates stress gene expression (Zhang et al., 2008; Kempa et al., 2009), we examined the expression of stress-related genes, i.e., genes in the Cold, Stress and Heat categories. Many were up-regulated in emf mutants (Table II, Supplemental data S1-H, I and J).

In the Cold category, more genes were up- than down-regulated in the 3 emf mutants compared to WT in both the 7- and 15-day old samples (Table II). C-REPEAT BINDING FACTOR 1 (CBF1) encodes the primary cold response regulator that binds to cis-elements in the promoter of the COLD REGULATED (COR) genes to activate their
expression (Chinnusamy et al., 2007). The 26K GeneChip data showed up-regulation of
COR15A and CBF1 in emf mutants (Table IV). RT-PCR analysis also showed a dramatic
increase of transcript levels of CBF1, COR15A and KIN1 in all three emf mutants (Figure
4). Since LOV1, which activates KIN1 and COR15A (Yoo et al., 2007), was up-regulated
in all three emf mutants at 7 DAG (Table III and Figure 2), it is possible that increased
LOV1 and CBF1 in emf synergistically activate downstream genes KIN1 and COR15A in
cold signaling. KIN1 is also regulated by stresses, such as dehydration and osmotic
pressure (Wang et al., 1995).

In the Stress category, which represents salt tolerance, dehydration-induced and
cation efflux family protein/metal tolerance (Park et al., 2009; Supplemental data S1-I),
transcript levels of many genes were distinctly elevated in 7-day, but not in 15-day, old
mutants (Table II). This is because WT showed an age-dependent increase in transcript
level from 7- to 15-day (Supplemental data 1-I). As a result, it is difficult to determine
whether the mutation affected transcription for 15-day old samples. However, the up-
regulation of stress genes in all 7-day samples of all 3 mutants is consistent with EMF
repression of stress genes.

In the Heat category, most WT genes showed an age-dependent increase in
transcript level that is not consistently observed in the 3 mutants (Supplemental data 1-J).
This has caused an apparent down-regulation of Heat genes in the 15-day-old emf mutant
samples. In the 7-day old samples, nearly 100% of Heat category genes examined were
up-regulated in all 3 emf mutants (Table II). Thus, like the Ethylene and Stress categories,
the 7-day old samples showed negative regulation of heat genes by EMF. Most genes in
the Heat category encode Heat Shock Proteins (HSPs) (Supplemental data S1-J), e.g.,
HSP70, which is involved in a variety of cellular processes (Su and Li, 2008). Our data
suggest EMF regulation of diverse cellular processes via the modulation of the heat shock
genexpression.

Genes in other categories
The Photosynthesis category genes investigated in the 8K array displayed the same
expression pattern on the 26K array. All but one of 54 photosynthesis genes examined on
the 26K array were down-regulated in one of the three mutants (Table II). The RNA level
of 31 of the 54 genes was similar in WT seedling (7-day old and 15-day old) and flower (Supplemental data S1-K). However, the expression of these 31 genes was significantly down-regulated in emf mutants. For example, the transcript level of At2g47450, a chlorophyll a/b binding protein gene, was similar between WT seedlings and flowers, but was reduced by at least 3 fold in emf mutants. This result suggests that the expression change in photosynthesis genes is caused by the lack of EMF proteins, not the reproductive state of the emf mutants.

In contrast, in the Photoreceptor category, most genes examined were up-regulated in the emf mutant samples (Table II, Supplemental data S1-L).

In the Histone category, H1, H2A, H2B, H3 and H4, most genes in WT showed age-dependent decreased expression from 7- to 15-day old plants, while many in emf mutants showed age-dependent increased expression (Supplemental data S1-M). This opposite expression pattern resulted in the up-regulation of all histone genes in 15-day old mutants that cannot be attributed directly to the emf mutation. The comparative data of 7-day old samples do not show an apparent expression change resulting from the emf mutations.

In the Expansin category, despite the age-dependent decreased expression in WT from 7- to 15 days (Supplemental data S1-N), there is nevertheless an apparent down-regulation of expansin genes in emf mutants relative to WT, most notably in the emfl-2 samples (Table II). These findings suggest that EMF normally promotes the expression of expansin genes.

The Transcription Factor category (Qu and Zhu, 2006), which includes homeo-domain, zinc finger, WRKY, SET domain, MYB, MADS, AP2, bHLH, etc., (Supplemental data S1-O), is by far the largest category of genes showing expression change in emf mutants (Table II). More genes are up- than down-regulated in 5 of the 6 mutant samples. In the 2 emfl mutants, more than 30% of the genes were up-regulated and less than 10% of the genes down-regulated in 7-day old emf mutants. In 15-day old mutants, only 15% of the genes were up-regulated. EMF regulation of transcription factor genes is consistent with the PcG’s role of master regulator of gene programs (Schwartz et al., 2006; Tolhuis et al., 2006).
Target genes that interact directly with EMF1 and EMF2

Functional grouping of the genes that showed expression change in emf mutants indicated EMF regulation of multiple pathways. However, it is not clear whether genes showing expression change are directly or indirectly regulated by the two EMFs. Using protein tagging and ChIP technologies, we showed that AG, AP3 and PI are direct targets of the EMF1 protein (Calonje et al., 2008). EMF1 proteins are recruited to the promoter region of these genes and also to the second intron of AG. To investigate whether EMF2 also interacts with these genes directly, we employed the same strategy to tag EMF2 and study EMF2 interaction with potential target genes.

EMF2 tagging and protein expression pattern

EMF2 cDNA was tagged with three repeats of a FLAG sequence and expressed under the control of the EMF2 promoter (Figure 5A). The EMF2promoter::EMF2 cDNA::3XFLAG construct was introduced into an emf2-1 mutant background as described in Materials and Methods. It rescued the mutant: transgenic plants grew like WT (Figure 5B). Nuclear proteins were extracted from WT and E2-3F transgenic plants. EMF2-FLAG proteins were detected on western blots in 7- and 14-day old seedlings and in three different organs, rosette leaf, inflorescence apex, and silique of plants grown in soil (Figure 5C). This result is similar to the EMF1 protein expression pattern (Calonje et al., 2008). Finding EMF2 in all organs examined is consistent with constitutive EMF2 gene expression (Yoshida et al., 2001).

EMF1 and EMF2 recruitment to target genes

To determine whether the EMF1 target genes recruit EMF2 proteins, chromatin from E2-3F transgenic seedlings was immunoprecipitated by anti-FLAG antibody as described (Calonje et al., 2008). Primers from the regulatory region of a flower organ identity gene, PI, were able to amplify DNA in the immunoprecipitated chromatin, indicating that EMF2 was recruited to the PI promoter region (Figure 6).

To investigate whether the two EMF proteins directly regulate pathways other than those involved in flower organ development, we performed ChIP analysis on genes that encode transcription factors involved in seed development, stress, cold signaling and
flowering, *ABI3*, *LOV1*, *FLC* and *FT*, respectively. *PI* serves as a positive control of EMF target genes in the ChIP experiments. *PHERES 1* (*PHE1*) is a *MADS*-box gene expressed in seed. *PHE1* gene expression is regulated by FIS2-, not the EMF2-, containing PRC2 (Kohler et al., 2003a). ChIP results showed that *PHE1* promoter sequence did not recruit EMF1 or EMF2 proteins (Figure 6). Thus *PHE1* serves as a negative control in experiments aimed at identifying EMF target genes.

Using primers amplifying a promoter region of *FT* (*FT-P* in Figure 6), we found that the *FT* promoter sequences did not recruit EMF1 or EMF2, suggesting that *FT* is not a target gene of EMF. However, using primers from the second exon of *FT*, Jiang et al., (2008) showed that *FT* chromatin is marked with K27me3 and bound to CLF. To examine possible EMF interaction with the second exon of *FT*, we tested the same primer sequences reported by Jiang et al. (2008; *FT-I* in Figure 6). Neither EMF1 nor EMF2 was bound by *FT-I*. Thus, *FT* is not likely to be controlled by EMF directly. *FT* up-regulation in *emf2* mutants maybe a secondary effect. For example, *FT* may be negatively controlled by *TOE1* and *TEM*, which, in turn, are regulated by EMF.

Both EMF1 and EMF2 were recruited to *ABI3*, *LOV1* and *FLC* genes (Figure 6, Supplemental data S3). Recruitment of EMF proteins to these genes is consistent with EMF repression of the master regulatory genes of seed development, cold signaling, and *FLC*-mediated flowering pathway during vegetative development.

**DISCUSSION**

*emf* mutants are characterized by their prominent precocious flowering phenotype (Sung et al., 1992; Bai and Sung, 1995); however, the mutation is pleiotropic, consistent with expression changes in genes in multiple pathways (Moon et al., 2003). The present genome-wide GeneChip study confirms and extends the previous findings by identifying additional gene programs coordinately regulated by EMF1 and EMF2, notably, stress-related gene programs.

The full-genome analysis identified 15 gene programs regulated by the 2 EMF genes. In most functional categories, i.e., Flower organ, Flowering time, Photosynthesis,
Photoreceptor, Seed, Auxin, GA, ABA, and Cold, gene expression levels remain relatively unchanged in 7- and 15-day old WT seedlings, enabling the investigation of the effect of emf mutation on gene expression. WT transcript levels changed from 7- to 15-day in several of the functional categories. For example, genes in the Ethylene, Stress and Heat categories had higher transcript levels in 15- than 7-day old WT samples. Thus, in these categories, the 15-day old data often showed an apparent gene expression change from mutant to WT that cannot be attributed to the mutation directly, rather relates to the developmental change in WT. Therefore, we ascertained the effect of mutation from the 7-day old data. At this age, mutants and WT possess the same organs, e.g., cotyledons and hypocotyl, differing only in size.

In most functional categories, with the obvious exception of the Photosynthesis and Expansin categories, more genes are up-regulated than down-regulated in all three emf allele samples (Table II). EMF gene activity exerts a repressive role on Flower organ, Seed, Photoreceptor, Ethylene, ABA, Stress, Cold, Heat, and Transcription factor genes.

There is more overlap in genes with changed expression between the two emf1 mutants than between emf2-1 and emf1-1. Although this may indicate differential gene regulation by EMF1 and EMF2, gene redundancy between EMF2 and its homolog VRN2 (Schubert, et al., 2005) may allow VRN2 to substitute EMF2 function in emf2-1 mutants. This is evident from the fact that, while EMF2 can interact with ABI3 (Figure 6), presumably to repress its expression, ABI3 transcripts remained low in emf2 mutants. This can be explained by the redundant function of VRN2 (Schubert et al., 2006), which would repress ABI3 in the absence of EMF2.

**EMF regulation of flowering time genes**

We have previously proposed that EMF proteins, bypassing flowering time genes, repress flower organ genes directly to prevent flowering. Indeed, EMF1 interaction with AG, AP3 and PI confirms them as direct EMF1 target genes (Calonje et al., 2008).

Nevertheless, some flowering time genes in the photoperiod, autonomous and vernalization pathways, e.g., GI, FT and FLC were up-regulated in some samples of emf mutants. In the photoperiod flowering pathway, GI activates CO, which then activates both FT and SOC1 (Michaels et al., 2005). It is curious that the increased GI expression
did not cause up-regulation of CO. Perhaps, positive regulation of CO by GI is offset by enhanced negative regulation. To date, four genes, SUPPRESSOR OF PHYA 1 (SPA1), CYCLING DOF FACTOR 1 (CDF1), RED FAR-RED INSENSITIVE 1 (RFI1) and LOV1 have been identified as CO negative regulators (Laubinger et al., 2006; Imaizumi et al., 2005; Chen and Ni 2006; Yoo et al., 2007), but only LOV1 is up-regulated in emf mutants (Table III and Figure 2). Thus, in emf mutants, reduced LOV1 repression may antagonize increased GI activation of CO.

The elevation of FT transcripts in emf2-1 without an increase of CO transcripts (Figure 2) might be explained by the reduced level of FT repressors. The increased level of GI function in emf mutants would reduce the level of TOE1, which is negatively regulated by GI (Jung et al., 2007), allowing FT level to rise. Expression of the FT repressors, TEM1 and TEM2, was also reduced in emf mutants, resulting in further elevation of FT transcripts (Table III).

The CLF-containing PRC2 is thought to mediate flowering by interacting with FLC and FT, as CLF binds to intron regions of FLC and FT and mediate the deposition of repressive histone H3K27me3 on FLC and FT chromatin (Jiang et al., 2008). In the present study, we found EMF1 and EMF2 recruited to FLC but not FT (Figure 6). As CLF interacts with EMF2 as well as VRN2 in vitro through the VEF domain shared between EMF2 and VRN2 (Chanvivattana et al., 2004; Wood et al., 2006; Chen et al., 2009), FLC may interact with the PRC2 composed of CLF/SWN-EMF2-FIE-MSI as well as that of CLF/SWN-VRN2-FIE-MSI1. Since FT is not bound by EMF2, it may interact only with the PRC2 composed of CLF/SWN-VRN2-FIE-MSI1. VRN2 and EMF2, the two Su(z)12 homologs, are partially redundant in that double mutants display a more severe phenotype than emf2 single mutants (Schubert et al., 2005). VRN2-containing PRC2 represses FLC to mediate the vernalization process (Gendall et al., 2001). It is conceivable that EMF2 also serves a redundant function to VRN2 by repressing FLC-mediated vernalization. In addition, EMF may participate in the endogenous mechanism of flowering time determination via epigenetic regulation of FLC.

The up-regulation of FT or FLC may not affect flowering time in emf mutants, as the downstream flower MADS-box genes are already de-repressed. Constitutive
expression of multiple flower *MADS-box* genes is sufficient to convert leaves into flower organs upon germination (Honma and Goto, 2001), precluding the influence of upstream flowering genes on flowering time in these plants. However, reducing without eliminating *EMF* or *CLF* activities results in weak phenotype, i.e., early-flowering, rather than *emf* mutant phenotype (Goodrich *et al.*, 1997, Aubert *et al.*, 2001; Yoshida *et al.*, 2001; Sanchez *et al.*, 2009). In these early-flowering plants, it remains possible that their flowering time may be influenced by the altered expression of flowering time genes.

**EMF1 and EMF2 target transcription factor genes to mediate vegetative development and stress response**

In each pathway regulated by EMF, some genes encode transcription factors and others are downstream genes regulated by these transcription factor. ChIP analysis showed that EMF1 and EMF2 interact with genes encoding transcription factors, e.g., *ABI3* and *LOV1*, which regulate the seed maturation program and stress-induced genes. Loss-of-repression of these genes in *emf* mutants would explain the up-regulation of their downstream genes, *AtEm1, KIN1,* and *COR15A*.

Functional analysis showed that the two EMFs promote the photosynthetic program, regulate hormone signaling, as well as cold-, heat-, drought- and other stress-induced gene programs. Among the genes related to the four major plant hormones, those in the ABA signaling pathways are most clearly repressed by EMF. ABA is essential in many aspects of vegetative development, such as stomata regulation and plant adaptation to stress conditions, like drought and low temperature (Seki *et al.*, 2007). A number of cold-related genes were up-regulated in all three *emf* alleles (Table IV and Figure 4). EMF1 and EMF2 were directly bound to *ABI3* and *LOV1*, which are genetically located upstream in cold signaling (Figure 7). EMF regulation of all categories of stress genes indicates its involvement in protection mechanisms against abiotic environmental stresses. Stress response requires rapid activation of gene expression, recently shown to involve ATP-dependent chromatin remodeling (Mlynarova *et al.*, 2007; Walley *et al.*, 2008). Our finding of EMF interaction with the key stress regulatory gene *LOV1* implies PcG involvement in epigenetic regulation of the stress-response program, probably to maintain stress genes in a silent state until stress is encountered. Future investigation of
stress regulation of EMF expression may clarify the biological function of EMF recruitment to stress genes.

Vegetative development requires not only the repression of seed and flower programs, but also of the extensive growth and differentiation needed to elaborate the shoot architecture. These processes are coordinated by the hormone signaling pathways. Expansins are key regulators of cell wall extension during growth (Li et al., 2003). Down-regulation of expansin genes in emf mutants corresponds with reduced cell size in emf mutants (Sung et al., 1992). Thus, EMF is apparently required for cell wall extension in growing seedling cells, e.g., hypocotyl and root cells. Robust vegetative development also depends on vigorous photosynthesis; and EMF is required for transcription of genes in the Photosynthesis category. The 2 EMF genes apparently are involved in the regulation of all these processes needed for successful vegetative development.

In summary, our results indicate that EMF1 and EMF2 repress both developmental and stress response pathways, primarily through direct repression of the transcription factor genes (Figure 7). Coordinated EMF regulation of these diverse programs is crucial to plant survival. Future studies on genome-wide targets of EMF in a cell- and tissue- and organ-specific manner would further elucidate the EMF-mediated epigenetic mechanisms.

**Mechanism of EMF-mediated gene silencing**

The transcriptome of *emf1* and *emf2* mutants shows global regulation of gene expression patterns by EMF, consistent with PcG’s role as master regulator of gene programs (Schwartz et al., 2006; Tolhuis et al., 2006). PcG functions to maintain gene silencing via histone modifications, primarily through H3K27 trimethylation (Schubert et al., 2006). A genome-wide study showed 4400 Arabidopsis genes were marked with H3K27me3 (Zhang et al., 2007a). PcG proteins, i.e., EMF1, EMF2 and CLF, are recruited to the regions of the flower organ identity genes, *AG, AP3,* and *PI,* in seedling nuclei that are also marked with H3K27me3. Furthermore, study of mutants showed that H3K27 trimethylation depends on EMF2, CLF and partially on EMF1 (Schubert et al., 2006; Calonje et al., 2008).
The present study identified additional EMF targets that encode transcription factors, *FLC*, *ABI3* and *LOV1*, which are marked by H3K27me3 (Zhang et al., 2007b). These findings suggest a close association of H3K27me3 with EMF-targeted silencing. However, not all genes marked by H3K27me3 are bound by EMF: *PHE1* and *FT* are marked with H3K27me3 in seedling nuclei (Zhang et al., 2007b; Jiang et al., 2008) but are not bound by EMF1 or EMF2 (Figure 6). It is possible that another PRC2 component, such as VRN2, are involved in their methylation. Whole-genome analysis of the EMF binding pattern in WT and H3K27me3 pattern in *emf* mutants will shed light on the relationship of EMF and H3K27me3 in determining target gene regulation in *Arabidopsis*. 
MATERIALS AND METHODS

Plant Materials and Growth Conditions

WT and emf mutants of Arabidopsis used in this study are from the Columbia ecotype background, and have been described in Moon et al., (2003). Seeds were surface-sterilized and plated on agar plates containing 2/5X strength Murashige and Skoog medium (Murashige and Skoog, 1962). The plates were placed for 2 days in a refrigerator and then transferred to a short day growth room (16hrs light/8hrs dark) at 21ºC. Seedlings were harvested after growth for 7- or 15 days at 21ºC, called 7- or 15-day old seedlings or seedlings 7 or 15 DAG, respectively. Ten-day old seedlings grown in short day were transplanted to soil and grown in a long day (16hrs light/8hrs dark) greenhouse maintained at 21ºC. Rosette leaves and inflorescence apex tissues were harvested about three weeks after transplantation.

Microarray Analysis

RNA isolation, cDNA synthesis, biotinylated cDNA probe synthesis and the hybridization were performed according to standard protocols with minor modification (Zhu et al., 2001). The images were acquired and quantified by MAS 5.0 (Affymetrix). Hybridization signals of perfect matched probes were condensed into a gene level expression index by a custom algorithm (Zhou et al., 2002). The average of gene expression indices of all chips was scaled to an arbitrary target of 100.

Gene Expression Analysis

Reverse transcription Polymerase Chain Reaction (RT-PCR) was performed as described previously (Moon et al., 2003). Primers used for detection of FLC, FT, CO, SOC1 and GI have been described previously (Kim et al., 2008). The other primers for RT-PCR were as follows: ACTIN8 (ATGGCCGATG CTGATGACATTC and CATCATCTCCAGCGAATCCAGC), ABI3 (ATTACCGCCAGTGATGGAGACT and GCAAAACGATCCTTCCGAGGTT), At2S3 (CACGTGAACTCCATGCAAGTCT and ATGGGGTTAGTGGCGTCATC), AtEm1 (GAAGAGCTTGATGAGAAGGCGA and TGTGACCCATCTCCTGATAACC), CBF1 (TCAAGGGCGGAGATTATTTGTCCG and
TGCCATCTCAGCGGTTTGGAAA), cor15a (TTCTTCCACAGCGGAGGCAAG and TCCTTAGCCTCTCTCTGCTTTACC), KIN1 (GAGACCAACAAGAATGCGCTTCC and TCCTTAGCCTCTCTCTGCTTTACC), LOV1 (CGCTATGATCCTTGGGAACTTC and TGATCATCCTATCAGCTCCGTT), TOE1 (GGACCAAGGCTAGAAGTTCAC and TGCTCTGTCTACGCAGTATAGC). All experiments were replicated at least three times with similar results.

Transgenic Plant Construction and Protein Expression Analysis

EMF2 promoter of 1020bp and 3’ three FLAG-tagged EMF2 cDNA were cloned into binary vector pCAMBIA1380. This construct was introduced into Agrobacterium tumefaciens strain GV3101 by the freeze-thaw method and subsequently transformed into emf2-1 heterozygous plants by floral dipping (Clough and Bent, 1998). The homozygous emf2-1 mutants rescued by EMF2 promoter::EMF2-3FLAG transgenic plants were confirmed by genotyping. Genomic DNA extracted from wild type-like hygromycin resistant transgenic plants was used to amplify the region that covers the emf2-1 mutation in the EMF2 locus using the following primers; AACAACAAATTGCAGAAGACTGAAG and CTTGGATATCATTGTCTCAGTCTTG. The resulting fragments (460bp) were digested with Hyp188III, which would cut the emf2-1 allele into two fragments, 180bp and 280bp, however, would not cut the WT EMF2 allele.

For EMF2 protein expression analysis, nuclei from different ages and tissues of WT and transgenic plants were obtained as described (Bowler et al., 2004). Nuclei were then re-suspended in SDS-PAGE loading buffer, heated and loaded on an SDS-PAGE gel. Proteins from the gel were transferred onto a membrane and EMF2-3X FLAG protein detected using anti-FLAG antibody (Sigma-Aldrich).

Chromatin Immunoprecipitation

ChIP experiments were performed as described in Calonje et al., (2008). ChIP-PCR was conducted to confirm precipitated DNA as described in Schubert et al., (2006). Primer sequences for FT-I are described in Jiang et al. (2008). For ChIP-PCR, 27, 30 and 33 cycles of PCR were used to detect enrichment of the genes. Primers used for detection of PI, ABI3, LOV1, FLC, FT-P and PHE1 were as follows:
PI (ACATAGTACGGAAGAGACCAG and TTGGTTTCGCTTTATTACACATAGCC),
ABI3 (GTGACCATTTCACATAGGAAG and CCGCTAGTATCTAAAGGTTGGT),
LOV1 (CGCTATGATCTTTGGGAACTTC and AAAGTGATAAGAGAAAGAAAGATC),
FLC (TGCGTCACAGAGAAGAAGCG and TTGCATCACTCTCCTTTACCC),
FT-P (AATTAGTGCTACCAAGTGGGA and ATCATAGGCATGAAACCCTCTAC),
and PHE1 (TACGGATGACCGCACATGCGT and CCATCCTCCTCATGCTAACA).

ACKNOWLEDGEMENTS

We thank R. L. Pan and L.J. Chen for generating some mutant samples and data analysis, and M. Yund, D. Zilberman, Robert Calderon, UC Berkeley, for helpful comments. The research was partially supported by USDA grant 03-35301-13244 to Z. R. S.

REFERENCES


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a. Number of genes showing hybridization signal >50 in at least one of the eight samples, WT and three mutants at two time points
b. Number of genes (percentage) up-regulated or down-regulated in mutants more than two fold from WT
Table II. Number of genes involved in different groups up- or down-regulated at least two fold in *emf* mutant

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### Table III. Expression levels of flowering time genes in *emf* and WT

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<sup>(a)</sup>Flower buds without protruding petals  
<sup>(b)</sup>Open flowers without siliques

### Table IV. Expression levels of seed and stress genes in *emf* and WT

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<sup>(a)</sup>Flower buds without protruding petals  
<sup>(b)</sup>Open flowers without siliques
FIGURE LEGENDS

Figure 1. Genes up-regulated or down-regulated in 3 emf mutants.

11,447 and 10,204 genes with a hybridization signal >50 in 7- and 15-day old plants, respectively, were analyzed for overlapping expression pattern among the 3 mutants. Number (percentage) of genes up- (A and C) and down-regulated (B and D) greater than two fold difference in mutants are shown.

Figure 2. RT-PCR analysis of flowering time genes in emf mutants.

RNA samples extracted from 7- and 14-day old WT and emf mutants were subjected to RT-PCR analysis. ACTIN8 was used as a loading control. Numbers presented below represent relative expression levels and were calculated using the ACTIN8 signal as a reference.

Figure 3. Overlapping seed gene expression pattern in the three emf mutants.

Sixty one and 56 of the 120 seed maturation genes up-regulated in one of the three emf mutants at 7 and 15 DAG, respectively, were analyzed for overlapping expression in two or all three emf mutants. Number (percentage) of genes up-regulated in mutants 7 (A) and 15 (B) DAG is shown.

Figure 4. RT-PCR analysis of seed and stress genes in emf mutants.

RNA samples extracted from 7- and 14-day old WT and emf mutants were subjected to RT-PCR analysis. ACTIN8 was used as a loading control. Numbers presented below represent relative expression levels and were calculated using the ACTIN8 signal as a reference.

Figure 5. EMF2promoter::EMF2-3FLAG construct and the protein expression pattern.
(A) The construct that harbors tagged \textit{EMF2} used in plant transformation is depicted. \textit{EMF2} cDNA was tagged with three \textit{FLAG} sequence and expressed under \textit{EMF2} promoter. This construct was introduced into \textit{emf2-1}, and it rescued mutant phenotype.

(B) \textit{emf2-1} mutant and rescued \textit{emf2-1} (\textit{E2-3F}) harboring \textit{EMF2promoter::EMF2-3FLAG} grown on agar plate (left), \textit{E2-3F} and WT grown in soil (right).

(C) Western analysis of nuclear protein from various tissues of WT and \textit{E2-3F} seedlings, 7 and 14 DAG, rosette leaves, inflorescence apex, and siliques of plants grown in soil. Anti-FLAG antibody was used to detect \textit{EMF2-3FLAG} fusion protein. A portion of the Coomassie-stained gels in the range of 50-100kD is shown as loading control for western blot analysis.

**Figure 6. EMF1 and EMF2 proteins interaction with target genes.**

(A) Schematic representation of gene structure of \textit{PI, ABI3, LOV1, FLC, FT} and \textit{PHE1}. Black box depicts first exon including 5’UTR and arrow indicates the region of gene amplified in ChIP analysis, -320bp to -74bp for \textit{PI}, -746bp to -586bp for \textit{ABI3}, +267bp to +393bp for \textit{LOV1}, -94bp to +232bp for \textit{FLC}, -94bp to +232bp for \textit{FT-P}, +1050bp to +1325bp for \textit{FT-I} and -59bp to +141bp for \textit{PHE1}. \textit{P} and \textit{I} denote promoter and intron regions in \textit{FT}, respectively.

(B) ChIP results showing EMF1 and EMF2 interaction with target genes. Anti-FLAG antibody was used to immunoprecipitate nuclear proteins from 14-day old transgenic plants and primers from (A) were used to amplify the DNA in the immunoprecipitated chromatin (IP). Input is pre-immunoprecipitated DNA after sonication. 30 cycles of PCR was performed using the primers covering the DNA regions shown in (A). \textit{PHE1} was used as an internal negative control. \textit{E1-3F} represents \textit{emf1-2} rescued by \textit{EMF1::EMF1-3FLAG} (Calonje \textit{et al}., 2008).

**Figure 7. A model of EMF repression of developmental and stress pathways in \textit{Arabidopsis}.**
Arrows and blocking bars indicate transcriptional activation and repression, respectively. Thick blocking bar shows EMF repression of target genes, LOV1, ABI3, PI, AG and FLC, through direct EMF-target gene interaction. Dotted arrow indicates potential EMF interaction with TEM.

SUPPLEMENTAL DATA

The following material is available in the on-line version of this article.

Supplemental data S1. Transcript level of genes in the 15 functional categories
Supplemental data S2. Selection criteria for the 15 functional categories
Supplemental data S3. ChIP-PCR analysis of EMF interaction with target genes at three PCR cycles, 27, 30 and 33.
Figure 1. Genes up-regulated or down-regulated in 3 *emf* mutants.

11,447 and 10,204 genes with a hybridization signal >50 in 7- and 15-day-old plants, respectively, were analyzed for overlapping expression pattern among the 3 mutants. Number (percentage) of genes up- (A and C) and down-regulated (B and D) greater than two fold difference in mutants are shown.
Figure 2. RT-PCR analysis of flowering time genes in emf mutants.

RNA samples extracted from 7- and 14-day old WT and emf mutants were subjected to RT-PCR analysis. ACTIN8 was used as a loading control. Numbers presented below represent relative expression levels and were calculated using the ACTIN8 signal as a reference.
Figure 3. Overlapping seed gene expression pattern in the three *emf* mutants. Sixty one and 56 of the 120 seed maturation genes up-regulated in one of the three *emf* mutants at 7- and 15 DAG, respectively, were analyzed for overlapping expression in two or all three *emf* mutants. Number (percentage) of genes up-regulated in mutants 7 (A) and 15 (B) DAG is shown.
Figure 4. RT-PCR analysis of seed and stress genes in emf mutants.

RNA samples extracted from 7- and 14-day old WT and emf mutants were subjected to RT-PCR analysis. ACTIN8 was used as a loading control. Numbers presented below represent relative expression levels and were calculated using the ACTIN8 signal as a reference.
Figure 5. *EMF2promoter::EMF2-3FLAG* construct and the protein expression pattern.

(A) The construct that harbors tagged *EMF2* used in plant transformation is depicted. *EMF2* cDNA was tagged with three *FLAG* sequence and expressed under *EMF2* promoter. This construct was introduced into *emf2-1*, and it rescued mutant phenotype.

(B) *emf2-1* mutant and rescued *emf2-1* (*E2-3F*) harboring *EMF2promoter::EMF2-3FLAG* grown on agar plate (left). *E2-3F* and WT grown in soil (right).

(C) Western analysis of nuclear protein from various tissues of WT and *E2-3F* seedlings, 7- and 14 DAG, rosette leaves, inflorescence apex, and siliques of plants grown in soil. Anti-FLAG antibody was used to detect *EMF2-3FLAG* fusion protein. A portion of the Coomassie-stained gels in the range of 50-100kD is shown as loading control for western blot analysis.
Figure 6. EMF1 and EMF2 proteins interaction with target genes. 

(A) Schematic representation of gene structure of PI, ABI3, LOV1, FLC, FT and PHE1. Black box depicts first exon including 5’UTR and arrow indicates the region of gene amplified in ChIP analysis, -320bp to -74bp for PI, -746bp to -586bp for ABI3, +267bp to +393bp for LOV1, -94bp to +232bp for FLC, -330bp ~ -70bp for FT-P, +1050bp to +1325bp for FT-I and -59bp to +141bp for PHE1. P and I denote promoter and intron regions in FT, respectively.

(B) ChIP results showing EMF1 and EMF2 interaction with target genes. Anti-FLAG antibody was used to immunoprecipitate nuclear proteins from 14-day old transgenic plants and primers from (A) were used to amplify the DNA in the immunoprecipitated chromatin (IP). Input is pre-immunoprecipitated DNA used as a positive control. ChIP was performed using the primers covering the DNA regions shown in (A). PHE1 was used as an internal negative control. E1-3F represents emf1-2 rescued by EMF1::EMF1-3FLAG (Calonje et al., 2008).
Figure 7. A model of EMF repression of developmental and stress pathways in Arabidopsis.

Arrows and blocking bars indicate transcriptional activation and repression, respectively. Thick blocking bars represent EMF repression of target genes, LOV1, ABI3, PI, AG and FLC, through direct EMF-target gene interaction. Dotted arrow indicates potential EMF interaction with TEM.