Molecular and Functional Characterization of Broccoli EMBRYONIC FLOWER 2 Genes

Mao-Sen Liu¹, Long-Fang O. Chen¹*, Chun-Hung Lin¹, Ying-Mi Lai¹, Jia-Yuan Huang¹ and Zinmay Renee Sung²*,

¹Institute of Plant and Microbial Biology, Academia Sinica, Taipei, 11529, Taiwan
²Department of Plant and Microbial Biology, University of California, Berkeley, CA 94720, USA
*Corresponding authors: Zinmay Renee Sung; E-mail, sungr@berkeley.edu; Fax, +1-510-642-4995; Long-Fang O. Chen;
E-mail, ochenlf@gate.sinica.edu.tw; Fax, +88-622-782-7954

Polycomb group (PcG) proteins regulate major developmental processes in Arabidopsis. EMBRYONIC FLOWER 2 (EMF2), the VEFS domain-containing PcG gene, regulates diverse genetic pathways and is required for vegetative development and plant survival. Despite widespread EMF2-like sequences in plants, little is known about their function other than in Arabidopsis and rice. To study the role of EMF2 in broccoli (Brassica oleracea var. italicca cv. Elegance) development, we identified two broccoli EMF2 (BoEMF2) genes with sequence homology to and a similar gene expression pattern to that in Arabidopsis (AtEMF2). Reducing their expression in broccoli resulted in aberrant phenotypes and gene expression patterns. BoEMF2 regulates genes involved in diverse developmental and stress programs similar to AtEMF2 in Arabidopsis. However, BoEMF2 differs from AtEMF2 in the regulation of flower organ identity, cell proliferation and elongation, and death-related genes, which may explain the distinct phenotypes. The expression of BoEMF2.1 in the Arabidopsis emf2 mutant (Rescued emf2, Rem2) partially rescued the mutant phenotype and restored the gene expression pattern to that of the wild type. Many EMF2-mediated molecular and developmental functions are conserved in broccoli and Arabidopsis. Furthermore, the restored gene expression pattern in Rescued emf2 provides insights into the molecular basis of PcG-mediated growth and development.

Keywords: Brassica oleracea var. italicca • Broccoli • EMBRYONIC FLOWER 2 • Flowering • Polycomb Group Protein.

Abbreviations: AG, AGAMOUS; AP3, APETALA3; asBoEMF2.1, broccoli harboring 35S:antisenseBoEMF2.1; CDS, coding sequence; CLF, CURLY LEAF; COR15A, COLD REGULATED 15A; EMF2, EMBRYONIC FLOWER 2; EXPA, EXPASIN A; FIE, FERTILIZATION INDEPENDENT ENDOSPERM; FIS2, FERTILIZATION INDEPENDENT SEED 2; FLC, FLOWERING LOCUS C; FT, FLOWERING LOCUS T; LTP, LIPID TRANSFER PROTEIN; MEA, MEDEA; MS, Murahige and Skoog; MSI1, MULTICOPY SUPPRESSOR OF IRA1; PcG, polycomb group; PI, PISTILLATA; PRC, polycomb repressive complex; qRT–PCR, quantitative real-time reverse transcription–PCR; RACE, rapid amplification of cDNA ends; Rescued emf2, Rem2 mutant harboring 35S:BoEMF2.1; RFLP, restriction fragment length polymorphism; SEP3, SEPALATA3; Su[z]12, Suppressor of Zeste 12; SVP, SHORT VEGETATIVE PHASE; SWN, SWINGER; TF2, TERMINAL FLOWER 2; transWT, 35S:BoEMF2.1 containing WT; VRN2, VERNALIZATION2; WT, wild type.

The nucleotide sequences reported in this paper has been submitted to GenBank under the accession numbers JQ412719 (BoEMF2.1 cDNA), JQ412720 (BoEMF2.2 cDNA), JQ412722 (BoEMF2.1 genomic DNA) and JQ412723 (BoEMF2.2 genomic DNA). The Arabidopsis and broccoli microarray data were submitted to Gene Expression Omnibus (GEO) database under the accession numbers GSE36943 and GSE36963, respectively.

Introduction

Polycomb group (PcG) proteins are epigenetic repressors in eukaryotes known to maintain the silent states of their target genes (Morey and Helin 2010, Margueron and Reinberg 2011). PcG proteins form multiprotein complexes, such as polycomb repressive complex 1 and 2 (PRC1 and PRC2), which modify histone moieties and remodel chromatin structures to repress transcriptional activities (Schuettengruber et al. 2007). PRC2 has histone methyl transferase activity that trimethylates histone 3 lysine 27 (H3K27) on target genes, whereas PRC1 catalyzes histone 2A (H2A) monoubiquitination (Muller and Verrijzer 2009). Together, these PcG confer stable gene silencing (Morey and Helin 2010, Margueron and Reinberg 2011).

PRC2, first described in Drosophila, is composed of four core proteins (Muller et al. 2002). In plants, Arabidopsis proteins homologous to components of Drosophila PRC2 are required for three developmental functions (Calonje and Sung 2006, Hennig and Derkacheva 2009, Holec and Berger 2012). Arabidopsis FERTILIZATION INDEPENDENT ENDOSPERM (FIE) and MULTICOPY SUPPRESSOR OF IRA1 (MSI1) are
homologs to Drosophila Extra sex comb (ESC) and p55, respectively. CURLY LEAF (CLF), MEDEA (MEA) and SWINGER (SWN) are homologous of Enhancer of Zeste (E[z]), which catalyzes the trimethylation of H3K27 on target genes. EMBRYONIC FLOWER 2 (EMF2), VERNALIZATION 2 (VRN2) and FERTILIZATION INDEPENDENT SEED2 (FIS2) are homologous to Suppressor of Zeste 12 (Su[z]12) (Calonje and Sung 2006, Hennig and Derkacheva 2009, Holec and Berger 2012). The three Arabidopsis homologous proteins VRN2, EMF2 and FIS2 share several amino acid sequence domains with their Drosophila Su[z]12 homologs, including the VEF5 domain (Yoshida et al. 2001). These three proteins also play a major role in determining the biological function of the three PRC2 complexes by regulating specific target genes. EMF2, together with MSI1, FIE and CLF or SWN (EMF2/CLF or SWN/FIE/MSI1), maintains a specific growing pattern by repressing the expression of the flower MADS-box genes AGAMOUS (AC), APETALA 3 (AP3) and PISTILLATA (PI) (Moon et al. 2003, Calonje and Sung 2008). FIS2/MEA/FIE/MSI1 regulates Arabidopsis seed development by repressing PHERES1 during gametophyte and endosperm development (Kohler et al. 2003). Likewise, VRN2/CLF or SWN/FIE/MSI1 is involved in vernalization-mediated flowering by regulating FLOWERING LOCUS C (FLC) (Sung and Amasino 2004, Wood et al. 2006, De Lucia et al. 2008).

Similarly, Arabidopsis proteins homologous to Drosophila PRC1 components such as the RING-finger homolog AtRING1A/B and AtBMI1A/B are required for maintaining cell identity in Arabidopsis (Sanchez-Pulido et al. 2008, Xu and Shen 2008, Bratzel et al. 2010). These proteins interact with LIKE HETEROCROMATIN PROTEIN1 (LHP1)/TERMINAL FLOWER2 (TFL2) and EMF1, and catalyze histone 2A (H2A) monoubiquitination (Bratzel et al. 2010, Chen et al. 2010). In Arabidopsis the EMF2-like sequences exist in many plants (Chen et al. 2009, Luo et al. 2009); however, their function has been studied only in the model plants Arabidopsis (Luo et al. 1999, Gendall et al. 2001, Yoshida et al. 2001) and rice (Luo et al. 2009). AtEMF2, BoEMF2 proteins are quite different from the other two Arabidopsis Su[z]12 homologs, AtVRN2 and AtFIS2 (Chen et al. 2009): an N-terminal cap, an N-terminal domain, E5–10 (exon 5–exon10), a C2H2 domain, E15–17 (exon 15–exon 17) and a C-terminal acidic-W/M (VEFS) domain (Fig. 1C). The VEF5 domains for BoEMF2.1 and BoEMF2.2 share 87.4% identity to each other, and 93.7 and 88.2%, respectively, to AtEMF2. The two BoEMF2 proteins are quite different from the other two Arabidopsis Su[z]12 homologs, AtVRN2 and AtFIS2 (Chen et al. 2009). AtVRN2 and AtFIS2 show only 30.2 and 19.1% protein identity to AtEMF2, respectively. Of the protein domains, both AtVRN2 and AtFIS2 lost the N-terminal cap region, and the E5–10 and E15–17 domains present in AtEMF2 and the BoEMF2s. In addition AtFIS2 protein acquired a specific aspartic acid-rich (D-rich) domain at the N-terminus and a serine-rich (S-rich) domain flanking the C2H2 and VEF5 domains (Fig. 1C).

To investigate the function of EMF2 in other plant species, we studied the close relative of Arabidopsis, broccoli (Brassica oleracea var. italica) EMF2 (BoEMF2) genes, BoEMF2.1 and BoEMF2.2, were cloned by combining chromosome walking and rapid amplification of cDNA ends (RACE) with primers based on Arabidopsis sequences (Supplementary Fig. S1, Supplementary Table S1). BoEMF2.1 and BoEMF2.2 are both composed of 22 exons (Fig. 1A). Their coding regions are 1,896 and 1,890 nucleotides, and show 90.6 and 86% similarity, respectively, to Arabidopsis EMF2 (AtEMF2). Southern blot analysis with a probe specific to BoEMF2.1 exon 10–14 (Fig. 1A) that showed 81% similarity to that of BoEMF2.2 confirmed the cloning of two copies of BoEMF2s in the broccoli genome (Fig. 1B). XbaI, SacI, Ncol and EcoRV were chosen to digest broccoli genomic DNA. There are two XbaI, two SacI, three Ncol and one EcoRV cutting site on the BoEMF2.1 genome whereas there are three SacI, three Ncol and one EcoRV cutting site on the BoEMF2.2 genome. As shown in Fig. 1B, digestions of broccoli genomic DNA with XbaI, SacI, Ncol and EcoRV all produced one strong and one weak band. The strong band represented the DNA fragment of BoEMF2.1 and the weak one represented that of BoEMF2.2 (Fig. 1B) due to the fact that the probe is 100% match to the BoEMF2.1 sequence.

Protein sequences of BoEMF2.1 and BoEMF2.2 show 82.2% identity to each other, and 90.8 and 83.2%, respectively, to AtEMF2. In addition to their high protein identity, BoEMF2s and AtEMF2 share a common domain organization (Chen et al. 2009): an N-terminal cap, an N-terminal domain, E5–10 (exon 5–exon10), a C2H2 domain, E15–17 (exon 15–exon 17) and a C-terminal acidic-W/M (VEFS) domain (Fig. 1C). The VEF5 domains for BoEMF2.1 and BoEMF2.2 share 87.4% identity to each other, and 93.7 and 88.2%, respectively, to AtEMF2.

Real-time quantitative reverse transcription–PCR (qRT–PCR) of the expression of the BoEMF2 genes with Brassica Actin (BoActin) used as a control revealed ubiquitous expression in all organs studied (Fig. 1D). Both genes were most highly expressed in flower buds relative to roots, leaves, stems and
Fig. 1 Characterization of BoEMF2.1 and BoEMF2.2. (A) Gene structure of the two BoEMF2 genes. The box represents the exon and the connecting gray line represents the intron of the two genes. The 5’- and 3’-non-coding regions are shown as boxes with diagonal lines. The restriction sites of XbaI, SacI, NcoI and EcoRV, and the region for probe synthesis for Southern blot analysis are indicated. The scale bar represents 1 kbp of nucleotides. (B) Southern blot analysis. Genomic DNA was digested with use of XbaI, SacI, NcoI and EcoRV, and detected with a BoEMF2.1-specific digoxigenin labeling probe on a PVDF membrane. Arrows indicate the enzyme-digested DNA fragments. The size of the marker in base pairs is indicated at the left. (C) Protein identity and domain organization. The N-terminal domain (N-ter) is composed of two parts including an N-terminal cap (cap), E5–10, C2H2 zinc finger, E15–17 and VEFS domain conserved in At- and BoEMF2s. The D-rich and S-rich domain are specific to AtFIS2. Protein identity is indicated at the right. The scale bar represents the length of the protein in amino acids. (D) Tissue-specific expression pattern. The y-axis indicates the relative mRNA level normalized to BoActin. Data are the mean with SD from three independent experiments.
siliques. The expression pattern for both is similar to that of AtEMF2.

Thus, BoEMF2 genes are similar to their Arabidopsis homolog in terms of sequence, domain organization and gene expression pattern. We knocked down the expression of the BoEMF2 genes in broccoli to study their biological function and introduced BoEMF2.1 into Arabidopsis emf2 mutants to determine whether BoEMF2.1 can rescue the AtEMF2 function.

Reducing BoEMF2 activity affected vegetative and flower development in broccoli

To investigate the role of BoEMF2 in broccoli growth and development, we used reverse genetics to knock down the expression of the BoEMF2 genes by introducing the full-length coding sequence (CDS) of antisense BoEMF2.1 under the control of a constitutive Cauliflower mosaic virus promoter 35S (35S:antisenseBoEMF2.1) into broccoli. Compared with plants transformed with the empty vector (vector-only control), T1 and T2 generations of transgenic broccoli harboring 35S:antisenseBoEMF2.1 (asBoEMF2.1) showed aberrant phenotypes during development (Fig. 2; Supplementary Fig. S2). For the T1 plants, 6 weeks after germination, leaves tended to curl upwards. The extent of curl was inversely related to the level of endogenous BoEMF2 genes (asBoEMF2.1). H, broccoli head. P, pistil. Bars represent 1 cm.

Figure 2 Phenotypes and expression of BoEMF2 genes in asBoEMF2.1 transgenic plants. (A) Six-week-old vector-only control and three types of asBoEMF2.1 transgenic broccoli are shown in the upper panel: type I asBoEMF2.1-41, type II asBoEMF2.1-29 and type III asBoEMF2.1-8. The lower panel shows the mRNA level of BoActin and BoEMF2 genes in control and transgenic plants. PCR cycles for BoEMF2.1, BoEMF2.2 and BoActin were 30, 30 and 24, respectively. The Arabic numbers indicate the expression of genes relative to that of BoActin, used as a control. The arrows indicated the curled leaves. (B) Adult plants at the inflorescence stage of control (1), type I asBoEMF2.1 (2) and type II asBoEMF2.1 (3). Elongated inflorescence (4), flower bud (6), open flower (7 and 8) of control (left) and type I asBoEMF2.1 (right). An enlarged view of the red box in 2B-4, showing a fused pistil-like structure (FP) on the apex of a type I asBoEMF2.1 inflorescence (5). Inflorescence (9) and abnormal flower (10) of type II asBoEMF2.1. H, broccoli head. P, pistil. Bars represent 1 cm.
Type I plants showed a few mildly curly leaves and expression of BoEMF2.1 reduced as compared with the wild type (WT) level (asBoEMF2.1-41 in Fig. 2A; asBoEMF2.1-1, -7, -10, -16, -23, -25, -39 and -41 in Supplementary Fig. S2), whereas the RNA level of BoEMF2.2 in these plants was not as affected as in type I plants. Type II asBoEMF2.1 plants were dwarf, had tightly curled leaves and showed almost completely knocked-out expression of BoEMF2.1 and a clear reduction in the asBoEMF2.2 level (asBoEMF2.1-29 in Fig. 2A; asBoEMF2.1-14, -29 and -32 in Supplementary Fig. S2). Type III asBoEMF2.1 plants had a few tightly curled leaves that wilted as new leaves emerged and died prematurely within a few days in the soil. Interestingly, unlike the emf2 mutation that greatly reduced internode and petiole elongation in Arabidopsis, type III plants appeared to have a normal petiole and internode length (asBoEMF2.1-8 in Fig. 2A). These plants had non-detectable BoEMF2.1 levels and almost completely knocked-out expression of BoEMF2.2 (asBoEMF2.1-8 in Fig. 2A; asBoEMF2.1-5 and -8 in Supplementary Fig. S2).

The vector-only control, type I and type II asBoEMF2.1 plants could undergo reproductive growth and produce flowers. The asBoEMF2.1 broccoli flowered earlier than control plants (Table 1): 69.0 ± 5.4 d after germination and producing 12.8 ± 1.4 leaves as compared with 87.7 ± 5.2 d with 16.2 ± 1.0 leaves for controls. Compared with control plants (Fig. 2B-1), asBoEMF2.1 type I (Fig. 2B-2) and type II (Fig. 2B-3) plants produced thinner stems, fewer leaves, abnormal flowers and, sometimes, a determinate inflorescence (Fig. 2B-4, -5). These features resulted in an abnormal inflorescence profile in asBoEMF2.1 plants (Fig. 2B-4).

Type I asBoEMF2.1 plants produced many flowers, thus forming a small broccoli head (Fig. 2B-2). The sepals are not fully enclosed to protect the inner flower organs in the flower bud (Fig. 2B-6). In the open flowers, the petals and sepals were irregular in shape and smaller (Fig. 2B-7, -8). Occasionally, fused pistil-like structures were observed on the top of the inflorescence, which suggests determinate growth (Fig. 2B-4, -5). Few seeds could be collected from type I plants. Type II asBoEMF2.1 plants produced many to a few flower buds (Fig. 2B-9) with imperfect flowers, usually lacking petals and stamens (Fig. 2B-10), with barely any seed produced.

In summary, asBoEMF2.1 showed knocked-down endogenous BoEMF2.1 and BoEMF2.2 expression, which is consistent with the sequence similarity of the two BoEMF2 genes. Knocking down both BoEMF2 genes caused more severe phenotypes than knocking down only BoEMF2.1, so the two BoEMF2 genes must have redundant functions and have a dosage effect on leaf morphologic features, vegetative growth and flower development in broccoli. **Global gene expression pattern of asBoEMF2.1 transgenic broccoli**

To study the underlying molecular mechanism of the aberrant phenotypes caused by the reduced BoEMF2 expression, we studied the global gene expression pattern of 11-day-old asBoEMF2.1 and the vector-only control by microarray assay (Redman et al. 2004). The 11-day-old asBoEMF2.1 plants with one to two true leaves were used to determine BoEMF2 RNA levels. Individual seedlings were genotyped to identify those with knocked-down expression. Only type I and type II asBoEMF2.1 broccoli were found but not type III plants because of their delayed germination and slow growth. In comparing the gene expression pattern for the asBoEMF2.1 transgenic and vector-only controls, we identified 2,973 genes up-regulated and 3,369 genes down-regulated in asBoEMF2.1. Similar to the Arabidopsis emf2 mutant, asBoEMF2.1 broccoli showed ectopic expression of genes in most of the 15 functional categories described by Kim et al. (2010) (Table 2; Supplementary Table S2). About 16% of genes ectopically expressed in emf2 were also ectopically expressed in asBoEMF2.1 broccoli, including the flower organ identity genes AG and SEPATALLA3 (SEP3), the flowering time gene FLOWERING LOCUS T (FT), the seed maturation LIPID TRANSFER PROTEIN family genes (LTP) and late embryo abundant protein genes (LEA), genes encoding transcription factors and genes involved in hormone and stress responses (Table 2). The ectopic expression of these flower organ identity genes is consistent with the abnormal flower development and early flowering of asBoEMF2.1 broccoli (Fig. 2).

To confirm the microarray results, we used RT–PCR to examine the expression of broccoli SEP3 (BoSEP3), BoAG and BoPI. The Affymetrix ATH1 chip experiment showed BoSEP3 and BoAG up-regulation, but not BoPI, in asBoEMF2.1 (Supplementary Table S2). RNA was extracted from two independent 11-day-old asBoEMF2.1 transgenic plants (G1 and G2), the 11-day-old vector-only control (C), and flower (CF) and silique (CSI) from a vector-only control plant. The BoEMF2 mRNA levels were reduced in the two asBoEMF2.1 broccoli seedlings. The G1 seedling showed a reduced BoEMF2 mRNA level relative to the control: 18% for BoEMF2.1 and 48% for BoEMF2.2, and the G2 seedling showed complete knockout of BoEMF2.1 and 35% BoEMF2.2 expression as compared with the vector-only control (Fig. 3). In the control broccoli, BoSEP3 and BoAG were preferentially expressed in flower (CF) and silique (CSI), whereas BoPI expression was specific to flowers. In the 11-day-old asBoEMF2.1, both BoSEP3 and BoAG were up-regulated but not BoPI, which was repressed, as in control seedlings (Fig. 3). The RT–PCR results for flower organ identity genes were consistent with the microarray results and suggest that BoEMF2.1 and AtEMF2 regulate similar, but not identical, target genes.
BoEMF2.1 partially rescued the Arabidopsis emf2 phenotype

The two BoEMF2 genes are similar to AtEMF2 in sequence, protein domain organization and expression pattern. Because the expression of BoEMF2.1 was higher than that of BoEMF2.2 in broccoli and the former showed higher sequence similarity to AtEMF2 than BoEMF2.2, we used BoEMF2.1 to investigate whether it can substitute for the AtEMF2 function. We generated a 35S::BoEMF2.1 full-length CDS construct and introduced it into Arabidopsis emf2 mutants to determine whether BoEMF2.1 can restore normal seedling and adult development in emf2. Hygromycin-resistant transgenic plants harboring the emf2 mutation were identified by restriction fragment length polymorphism (RFLP) genotyping (Supplementary Fig. S3A) as described (Calonje et al. 2008).

Analysis of the phenotypes of the homozygous emf2 mutants harboring 35S::BoEMF2.1 (Rescued emf2) grown under short-day conditions showed that BoEMF2.1 partially rescued emf2 phenotypes (Fig. 4, Table 3).

At 7 d after germination, emf2 mutants showed short hypocotyls, petioleless, and small and oval-shaped cotyledons (Chen et al. 1997). BoEMF2.1 partially rescued the hypocotyl and petiole elongation in emf2 mutants (Fig. 4A, Table 3). The petioleless cotyledons of emf2 were slightly curled upwards, whereas the cotyledons of Rescued emf2 were curled downwards along the proximodistal axis, and the WT cotyledons were flat and round (Fig. 4A).

At 15 d, WT seedlings produced four petiolated rosette leaves and the cotyledons continued to grow (Fig. 4B, C), whereas the cotyledons of emf2 stopped growing, and no petiolated rosette leaves were produced (Fig. 4D, E). Rescued emf2 produced petiolated rosette leaves to varying extents (Fig. 4F, G). The hypocotyl was longer for Rescued emf2 than emf2 but shorter than for the WT (Fig. 4A–G, Table 3). The 15-day-old WT and Rescued emf2 were transplanted into the soil for further observation, and emf2 seedlings remained on agar plates because they cannot survive in soil. The Rescued emf2 produced about six petiolated rosette leaves, which were smaller than those of the WT (Fig. 4H–K) before flowering at about 30 d after germination; emf2 mutants flowered at about 22 d and produced no rosette leaves, and the WT flowered at about 104 d and produced >64 rosette leaves (Table 3). Therefore, BoEMF2.1 partially rescued vegetative growth in emf2, although the rescued plants were smaller and flowered early. The Rescued emf2 produced a normal, although smaller inflorescence than the WT (Fig. 4J, K). emf2 plants produced terminal flowers with a large pistil and dwarfed short stamens (Fig. 4L). Rescued emf2 showed indeterminate growth and secondary inflorescences developed from the axillary meristems (Fig. 4J, K), like WT plants.
Flower development in emf2 was partially rescued by the constitutive expression of BoEMF2.1. Rescued emf2 produced flower organs of relatively normal size: unlike petal-less and extremely dwarfed stamens in emf2, petals were present, although smaller; the pistil was only slightly taller than stamens (Fig. 4M, N). However, partially rescued flowers did not produce seeds and the siliques did not elongate spontaneously (Fig. 4O), probably because of the slightly shorter filaments, smaller anthers and fewer pollen produced than the WT. Therefore, we performed manual pollination by placing the entire anther from the same flower or the same plant onto the pistil and generated many seeds in siliques of relatively normal size (Fig. 4O), but still fewer than the WT.

Thus, broccoli BoEMF2.1 can substitute for Arabidopsis AtEMF2 in performing developmental functions in part at every stage of Arabidopsis development.

**BoEMF2.1 partially restored the expression of misexpressed genes in the emf2 mutant**

To understand the molecular basis of the partial gene rescue, we studied the global gene expression pattern of Rescued emf2 seedlings. The genotype and expression of BoEMF2.1 were
characterized to ensure that the Rescued emf2 plants selected based on morphological features were indeed the emf2 mutants harboring BoEMF2.1 (Supplementary Fig. S3A, B). RNA was extracted from 7-day-old Rescued emf2, emf2, WT, and WT-like transgenic seedlings harboring BoEMF2.1 (transWT) for microarray analysis as described in the Materials and Methods.

We first examined the emf2 expression pattern using the 22 K Affymetrix ATH1 microarray and found 1,490 and 2,461 genes up- and down-regulated, respectively, by >2-fold in emf2. About 94% of the genes misregulated in the current study were detected on the custom GeneChip reported by Kim et al. (2010). The two independent data sets showed an overlap of 393 and 223 genes up- and down-regulated, respectively, in emf2 seedlings (Fig. 5A; Supplementary Table S3), which represent a robust set of EMF2-regulated genes. As reported by Kim et al. (2010), we found misexpression of genes in emf2 in the 15 functional categories (Supplementary Tables S3, S4), including flower organ identity genes AP1, AP3, SEP3 and AG; seed maturation genes LTP3 and LTP4; cell growth genes EXPANSIN A (EXPA); flowering time genes FT, FLC, SHORT VEGETATIVE PHASE (SVP); a COLD REGULATED 15A (COR15A) gene; and the heat-shock protein (HSP) HSP70 gene.

We then studied the global gene expression pattern in the Rescued emf2. The control used was transWT, which is a transgenic plant harboring BoEMF2.1 in the WT background and phenotypically indistinguishable from the WT, and hence a better control than the WT, which does not harbor any transgene. A comparison of the gene expression patterns of Rescued emf2 normalized to transWT and emf2 normalized to WT revealed 1,122 of the 1,490 genes up-regulated in emf2 (75%) no longer up-regulated and 1,899 of the 2,461 down-regulated genes in emf2 (77%) no longer down-regulated. Of the robust set of EMF2-regulated genes, 257 of the 393 genes up-regulated in emf2 (65%) were no longer up-regulated (Fig. 5B) and 158 of the 223 down-regulated genes in emf2 (71%) were no longer down-regulated (Fig. 5B), which suggests that 35S:BoEMF2.1 restored the misregulation of many genes in emf2. Further analysis showed that the expression of genes misregulated in all functional categories was restored, for many, to near-normal levels (Table 4; Supplementary Table S4).

The reduced expression of flower organ identity genes AP1, AP3 and PI, and changed expression of flowering time genes FT and SVP are consistent with the normal flower organ development and delayed flowering, respectively, in emf2 harboring BoEMF2.1. BoEMF2.1 also restored the expression of the gibberellin acid synthesis gene GIBBERELLIN 2-OXIDASE 2 (GA2OX2), stress-related genes C-REPEAT/DRE BINDING FACTOR 1 (CBF1) and COR15A, and transcription factors TEOSINTE BRANCHED1, CYCLOIDEA AND PCF TRANSCRIPTION FACTOR (TCP), NO APICAL MERISTEM (NAM) and CUP SHAPED COTYLEDON (CUC). Many of these genes are direct targets of EMF1 and their expression is repressed by EMF1 and PRC2 (Kim et al. 2010, Kim et al. 2012). A change in the expression pattern of EMF-interacting Arabidopsis genes with BoEMF2.1 suggests the ability of broccoli BoEMF2.1 to act as a member of Arabidopsis PRC2 in silencing Arabidopsis genes.

Despite a high percentage of genes with expression restored by BoEMF2.1 in emf2, several flower MADS-box genes, AG, PI and SEP3, were still up-regulated in Rescued emf2 seedlings (Supplementary Table S4). To verify the microarray results, we performed qRT–PCR analysis of select genes in transWT, WT, emf2 and Rescued emf2. We confirmed the increased

![Fig. 5 BoEMF2.1 restoration of genes misexpressed in emf2. (A) Venn diagrams of genes up-regulated (left) and down-regulated (right) by >2-fold in emf2 mutants in the current study (yellow) and in Kim et al. (2010) (green). (B) Pie charts of restoration of emf2 misexpressed genes by the expression of BoEMF2.1 in emf2 mutants. The number (ratio) of genes with expression restored (blue) and not restored (red).](https://academic.oup.com/pcp/article-abstract/53/7/1217/1906440)
Table 4 Restoration of expression of up- and down-regulated genes in 7-day-old emf2 by BoEMF2.1

| Category                  | No. of genes investigated | Genes misexpressed in emf2 | No. of genes misexpressed<sup>a</sup> | No. of genes with restored expression in rescued emf2<sup>b</sup> | Example of genes with expression restored to near-normal level |
|---------------------------|---------------------------|----------------------------|----------------------------------------|---------------------------------------------------------------|---------------------------------------------------------------|
| Flower organ identity     | 22                        | Up                         | 13                                     | 11                                                            | PI, SEP1, AP3, SHP1, SEP2, CRC, STK, AP1, SHP2 MBP2, SPL4, CRC |
|                           |                           | Down                       | 0                                      | 0                                                             |                                                               |
| Flowering time            | 20                        | Up                         | 4                                      | 3                                                             | FT, ELF4                                                      |
|                           |                           | Down                       | 5                                      | 4                                                             | SVP, AGL31, RAV1                                               |
| Seed                      | 108                       | Up                         | 35                                     | 30                                                            | LTP gene family, LEA, OLEO2, AIR1, SAG21, 2S seed storage gene, CRU2, CRU3 |
|                           |                           | Down                       | 19                                     | 17                                                            | NIK3, OLEO4                                                   |
| Auxin                     | 99                        | Up                         | 4                                      | 3                                                             | Auxin-responsive protein genes                                |
|                           |                           | Down                       | 21                                     | 21                                                            | DFL2, IAA2, IAA30, PIN3, PIN4, PIN7                          |
| Gibberellin               | 13                        | Up                         | 3                                      | 2                                                             | GA2OX2, RGL2                                                  |
|                           |                           | Down                       | 4                                      | 4                                                             | GA-responsive protein genes                                   |
| Ethylene                  | 53                        | Up                         | 7                                      | 7                                                             | ERF2, ethylene-responsive genes                               |
|                           |                           | Down                       | 9                                      | 9                                                             | EIL1, ERS2, EFE, ERF8                                        |
| ABA                       | 12                        | Up                         | 2                                      | 0                                                             |                                                               |
|                           |                           | Down                       | 2                                      | 2                                                             | AFB1, AFB4                                                   |
| Cold                      | 19                        | Up                         | 5                                      | 4                                                             | COR15A                                                       |
|                           |                           | Down                       | 3                                      | 3                                                             | CBF1, CBF2, COR15B                                            |
| Stress                    | 46                        | Up                         | 2                                      | 2                                                             | Zinc finger family protein genes                              |
|                           |                           | Down                       | 6                                      | 6                                                             | Dehydration responsive genes                                  |
| Heat                      | 47                        | Up                         | 10                                     | 9                                                             | HSP17, HSP18, HSP70, HSP101                                   |
|                           |                           | Down                       | 4                                      | 4                                                             | DNAJ, HSFC1                                                  |
| Photosynthesis            | 49                        | Up                         | 0                                      | 0                                                             |                                                               |
|                           |                           | Down                       | 10                                     | 10                                                            | PSII system genes                                             |
| Photoreceptor             | 28                        | Up                         | 1                                      | 1                                                             |                                                               |
|                           |                           | Down                       | 3                                      | 3                                                             | MYB3/RA2X2, ELIP1, PIF3                                       |
| Histone                   | 35                        | Up                         | 1                                      | 1                                                             | histone H1-3                                                  |
|                           |                           | Down                       | 2                                      | 2                                                             | histone H2A 12, histone 2B                                    |
| Expansin                  | 20                        | Up                         | 5                                      | 3                                                             | EXPA7, EXPA18, EXPA20                                         |
|                           |                           | Down                       | 10                                     | 9                                                             | EXPA1, 3, 5, 6, 8, 10, 11, 15, EXPB3                          |
| Transcription factor      | 542                       | Up                         | 67                                     | 56                                                            | NAC1, AF1, CUC3, COL1, COL9, NAM                              |
|                           |                           | Down                       | 68                                     | 58                                                            | TCP family genes                                              |

<sup>a</sup> Genes with ≥2-fold up- or down-regulated expression in emf2.<br>
<sup>b</sup> Genes with up- or down-regulated expression in emf2 with expression restored ≥2-fold by BoEMF2.1.

ABF, ABC/RIC ACID RESPONSIVE ELEMENT-BINDING FACTOR; AF1, NAC DOMAIN CONTAINING PROTEIN 2; AGL23, AGAMOUS-LIKE 23; CBF, C-REPEAT/DRE BINDING FACTOR; COL, CONSTANS-LIKE; COR, COLD-REGULATED; CRC, CRABS CLAW; CRU, CRUCIFERIN; CUC3, CUP SHAPED COTYLEDON3; DBF2, DWARF IN LIGHT 2; DNAJ, DNAJ HEAT SHOCK N-TERMINAL DOMAIN-CONTAINING PROTEIN; EFE, ETHYLENE-FORMING ENZYME; ELF1, ETHYLENE-INSENSITIVE3-LIKE 1; ELF4, EARLY FLOWERING 4; ELIP1, EARLY LIGHT-INDUCIBLE PROTEIN 1; ERF, ERF DOMAIN PROTEIN; ER52, ETHYLENE RESPONSE SENSOR 2; EXP, EXPANSIN A; EXPB, EXPANSIN B; GA2OX2, Gibberellin 2-OXIDASE 2; HSFC1, HEAT SHOCK TRANSCRIPTION FACTOR C1; HSP, HEAT SHOCK PROTEIN; IAA, INDOLE-3-ACETIC ACID INDUCIBLE; MBP2, MYROSINASE BINDING PROTEIN 2; NAC1, NAC DOMAIN CONTAINING PROTEIN 1; NAM, NO APICAL MERISTEM; NIK3, NPH.INTERACTING KINASE 3; MYB3/RA2X2, MYB DOMAIN PROTEIN 38/REGULATOR OF AUXILLARY MERISTEMS 2; OLEO, OLEOSIN; PIF, PHYTOCHROME INTERACTING FACTOR 3; PIN, PIN-FORMED; RAV1, RELATED TO AB13/VPI 1; RGL2, RGA-LIKE 2; SAG21, SENESENCE-ASSOCIATED GENE 21; SHP, SHATTERPROOF; SPL6, SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 4; STK, SEEDSTICK; TCP, TEOSINTE BRANCHED1, CYCLOIDEA AND PFC TRANSCRIPTION FACTOR.
expression of flower MADS-box genes AP3 and PI and the seed genes LTP3 and LTP4 in emf2 but not AG, and SEP3 restored in part with broccoli EMF2.1 (Fig. 6). Similarly, the down-regulated cell growth genes EXPA3 and EXPA8 and the flowering time gene SVP were restored in part in emf2 with BoEMF2.1.

In summary, the restoration of misregulated gene expression is associated with phenotypic rescue, that suggests that genes with restored expression in the Rescued emf2 are at least in part responsible for the emf phenotypes.

## Discussion

### Two BoEMF2 genes show functional conservation and redundancy in broccoli

EMF2-like sequences common in plants (Chen et al. 2009, Luo et al. 2009) have functions distinct from their derived sequences, VRN2 and FIS2 (Chen et al. 2009). The two BoEMF2 genes were identified by their high sequence identity and their similar gene expression patterns to AtEMF2 (Fig. 1). The similar phenotypes between emf2 mutants and asBoEMF2.1 broccoli (Fig. 2, Table 1) and the partial rescue of emf2 mutants by BoEMF2.1 further confirmed their identity. The enhanced asBoEMF2.1 phenotypes by knocking down both BoEMF2 genes implied their functional redundancy. Reducing EMF2 activities in Arabidopsis and broccoli caused misexpression of genes in all 15 functional categories including flower MADS-box, hormone signaling and EXPA genes, indicating the conserved molecular function of BoEMF2 with that of AtEMF2 in mediating epigenetic gene silencing (Kim et al. 2010, Kim et al. 2012). Luo et al. (2009) reported a T-DNA insertion mutant of rice EMF2 that flowered earlier than wild-type rice and produced abnormal flowers. Thus, EMF2 is functionally conserved in regulating flowering time and organ development in Arabidopsis (Yoshida et al. 2001), rice (Luo et al. 2009) and broccoli.

### Reducing BoEMF2 activity affected vegetative and reproductive development in broccoli

The relatively weak phenotypes displayed by type I and II asBoEMF2.1 broccoli resemble the weak phenotypes of Arabidopsis impaired in PcG components, such as clf mutants (Schubert et al. 2006), tfl2 mutants (Larsson et al. 1998, Exner et al. 2009) and Arabidopsis expressing antisense EMF1 under the control of the LEAFY promoter in leaf primordia of LFY:asEMF1 transgenic plants (Sanchez et al. 2009). Indeed, these plants can produce vegetative leaves but are smaller in size, and flower early. In addition, leaves are curly around the edges in clf and LFY:asEMF1 plants. The curly leaf observed in asBoEMF2.1 may be attributed to the ectopic expression of the flower MADS-box genes such as AG, because the curly leaf phenotype is restored in ag clf double mutants of Arabidopsis (Goodrich et al. 1997). The early flowering and abnormal flower organ phenotypes of type I and II asBoEMF2.1 broccoli are consistent with the ectopic expression of flower MADS-box genes such as broccoli AG and SEP3 (Fig. 3). SEP3, a class E flower organ identity gene, enhances the expression of AP3, PI and AG to specify flower organ identity (Immink et al. 2009, Kaufmann et al. 2009) and is involved in flowering time control by its regulation of flowering time genes such as SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1 (SOC1), AGAMOUS LIKE 24 (AGL24) and SVP in the inflorescence (Kaufmann et al. 2009).

The microarray data of the type I and type II asBoEMF2.1 broccoli might display a mild gene expression change due to a slight reduction of the activities of BoEMF2s. The strong phenotype displayed by type III transgenic broccoli is, in principle, comparable with that of the Arabidopsis emf2 mutant but differs from emf2 in that the broccoli seedlings produced 3–5 leaves, showed petiole and internode elongation (asBoEMF2.1-8 in Fig. 2A) and died quickly without producing any flower organs. Limited availability of type III asBoEMF2.1 plants precluded microarray analysis to elucidate the molecular basis of these phenotypic differences. However, microarray analysis of the type I and type II plants revealed similar as well as divergent functions or gene programs in the two species. For example, 13 flower organ identity genes were misexpressed in the Arabidopsis emf2 mutant while eight were misexpressed in asBoEMF2.1 (Table 4, Supplementary Table S2). Ectopic expression of fewer flower organ identity genes may have allowed an abbreviated vegetative development in type III asBoEMF2.1, which could not occur in Arabidopsis emf2. Unlike emf2, asBoEMF2.1 can produce an elongated internode and petiole. This may be related to the fact that fewer auxin category and EXP family

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**Fig. 6** qRT–PCR analysis of gene expression in Rescued emf2. Verification of gene expression based on microarray data by qRT–PCR in 7-day-old Arabidopsis seedlings. 35S:BoEMF2.1 containing transgenic WT (transWT), WT, emf2 and Rescued emf2. The y-axis represents the relative mRNA level of genes normalized to UBIQUITIN 10 in log value. Genes analyzed are shown on the x-axis. Data are the mean with SD from three independent experiments.
genes, which are involved in hypocotyl elongation (Gray et al. 1998), and cell wall loosening and cell expansion (Sampedro and Cosgrove 2005), respectively, are down-regulated in asBoEMF2.1 (Supplementary Tables S2, S4). In addition, the death-related genes, VASCULAR ASSOCIATED DEATH 1 and RADICAL-INDUCED CELL DEATH 1, were up-regulated >2-fold in type II asBoEMF2.1 broccoli, but remained at a normal level in the Arabidopsis emf2 mutant (Supplementary Table S5). This may be the reason for the early wilting and death of type III asBoEMF2.1 broccoli.

BoEMF2.1 restored WT seedling and adult development in Arabidopsis emf2

The restoration of nearly 70% genes misexpressed in emf2 mutants by BoEMF2.1 (Fig. 5) explained the partial rescue of hypocotyl, petiole and cotyledon phenotypes of emf2 to that of the WT (Fig. 4). These included the restoration of the genes in auxin and other hormone categories, as well as most of the EXPA genes (Table 4, Supplementary Table S4), which are important for cell elongation and expansion in broccoli (Gray et al. 1998, Sampedro and Cosgrove 2005).

BoEMF2.1 also rescued the expression pattern of flowering time genes, FT and SVP (Supplementary Tables S3, S4), and flower MADS-box genes, thus contributing to the delayed flowering in the Rescued emf2. Of the 13 ectopically expressed flower organ identity genes in emf2, five were no longer ectopically expressed and, except for AG and SEP3, the expression of the remaining genes was greatly reduced in the Rescued emf2 (Table 2, Supplementary Table S4). However, Rescued emf2 still flowers earlier than does the WT, possibly because of the ectopic expression of AG and SEP3 (Fig. 6), as SEP3 both activates flower organ identity genes and promotes flowering (Kaufmann et al. 2009). The partial phenotypic and molecular rescue of emf2 by asBoEMF2.1 may be attributed in part to the use of a 3SS promoter instead of the EMF2 to drive BoEMF2.1 expression.

Protein–protein interaction between EMF2 and CLF, which possesses methyl transferase activity, through the conserved 3' VEFS domain in EMF2 is part of the PRC2 multiprotein complex that mediates gene silencing in Arabidopsis (Chanthivattana et al. 2004). Hence, the fact that BoEMF2.1 restored EMF2’s function suggests that BoEMF2.1 can interact with the Arabidopsis CLF and participate in the Arabidopsis PRC2 action. This is consistent with its 93.7% sequence identity in the VEFS domain to that of AtEMF2.

However, unlike BoAG and BoSEP3 in broccoli, BoEMF2.1 cannot repress the expression of AG and SEP3 in Arabidopsis, suggesting that these two genes require a more stringent condition for PRC2-mediated repression than the genes with restored expression. As a result, BoEMF2.1 cannot fulfill its role in the PRC2 action. The other possibility is that additional proteins, such as EMF1, might have evolved in Arabidopsis to cooperate with PRC2 in repressing the expression of AG and SEP3, but not with the PRC2 containing BoEMF2.1. Future investigations at the protein structure–function level will address the question of why BoEMF2.1 can restore some but not all misexpressed genes in the Rescued emf2, thereby elucidating the diverse EMF2-mediated repression mechanisms.

Materials and Methods

Plant material and growth conditions

Seeds of broccoli (B. oleracea var. italica cv. Elegance) were purchased from Know-You Seed Company (Kaohsiung, Taiwan), germinated on wet filter paper, transferred to soil in 7-in. pots and grown under a 14/10-h photoperiod at 75–110 μmol m⁻² s⁻¹ illumination at 20°C.

Arabidopsis thaliana cv. Columbia (Col) was used as the WT. The emf2 mutant constituted the emf2-1 allele in the A. thaliana Col background (Chen et al. 1997). Arabidopsis seeds were sown directly in soil or surface-sterilized, placed on a 0.8% agar plate containing half-strength Murashige and Skoog (MS) salt and 1.5% sucrose with or without 20 μg ml⁻¹ hygromycin, cold stratified at 4°C for 2–3 d, then transferred to a growth chamber with an 8/16-h photoperiod at 75–150 μmol m⁻² s⁻¹ illumination at 21°C for germination and development. Fifteen-day-old seedlings grown on an agar plate were transferred into soil and grown under the short-day condition for observation of bolting, flowering and silique development.

DNA and RNA extraction

For Southern blot analysis, genomic DNA was extracted from the second leaf from the shoot apex of 1-month-old broccoli by use of the Plant Genomic DNA Extraction Kit (Viogene). For gene cloning experiments, genomic DNA was extracted from the broccoli inflorescence shoot.

RNA was extracted from most Arabidopsis and broccoli tissue by the TRIzol Reagent method (Invitrogen). To extract RNA from siliques, siliques were ground into powder in liquid nitrogen with use of a mortar and a pestle, and RNA was extracted with the use of extraction buffer (100 mM Tris–HCl, pH 8, 1.4 M NaCl, 20 mM EDTA, pH 8 and 2% cetyltrimethylammonium bromide) as described (Asif et al. 2000). DNA was eliminated by use of the TURBO DNA-free kit (Ambion). The DNA remaining was reverse transcribed by use of the Transcriptor First Strand cDNA Synthesis Kit (Roche) to obtain cDNA products.

Cloning of broccoli EMF2 (BoEMF2) genes

Genomic DNA and cDNA prepared from the inflorescence of broccoli was used for broccoli EMF2 gene cloning. We used a combination of chromosome walking and RACE with the BD GenomeWalker Universal Kit (Clontech) and FirstChoice RLM-RACE kit (Ambion), respectively.

Primers designed to clone BoEMF2 genes are given in Supplementary Table S1.

Cloning began with RT–PCR with the primer pairs for EP1 and EP2 based on the Arabidopsis EMF2 (AtEMF2) sequence to obtain a broccoli cDNA fragment of about 500 nucleotides...
(Supplementary Fig. S1). The following EP primers were designed with broccoli sequences obtained during cloning. We used EP3 and EP4 primers designed with the broccoli 500 bp cDNA fragment to obtain the 3’ sequence of the cDNA by 3’-RACE and EP5 and EP6 to obtain the 5’ sequence of genomic DNA by chromosome walking. With the broccoli genomic DNA sequence (the red part in Supplementary Fig. S1) obtained with EP5 and EP6, we designed EP7 and EP8 primers to generate another cDNA fragment to form a contig with the 3’ cDNA sequence obtained with primers EP3 and EP4. Further 3’-RACE was performed with EP9 and EP10 primers to extend the broccoli EMF2 5’-cDNA sequence. Finally, EP11 containing a start codon and EP12 containing a stop codon were designed to obtain the full-length coding region of BoEMF2.1. However, sequencing results for this full-length BoEMF2.1 coding region showed sequence contamination, so we searched for another EMF2-like sequence (BoEMF2.2) (see below).

To clone the BoEMF2.2 gene, primers EP13 and EP14 specific for a newly identified partial sequence of the EMF2-like sequence were designed to obtain a cDNA fragment of about 210 nucleotides (Supplementary Fig. S1). With this short sequence, EP15 paired with EP16 and EP17 paired with EP18 were designed for 3’ end and 5’ end chromosome walking to obtain the 3’ end and 5’ end genomic sequences, respectively. Further 3’-RACE was performed with EP13 and EP16 primers to obtain the 5’-cDNA sequence for BoEMF2.2. The following primers were used to produce the partially overlapping 5’-cDNA sequences: EP17 and EP19, based on the BoEMF2.2 genomic sequence; EP11, shared with both BoEMF2 genes; and EP20, specific to the BoEMF2.2 genomic sequence (Supplementary Fig. S1). The final 5’-RACE and chromosome walking involved the EP21 and EP22 primers to obtain the 5’-untranslated and 5’-genomic sequences, respectively. A contig of these cDNA and genomic DNA fragment was then reconstructed (Supplementary Fig. S1).

Southern blot analysis

In total, 10 μg of total DNA was digested with restriction enzymes, separated on a 1.2% agarose gel, blotted onto a nitrocellulose membrane, hybridized with a gene-specific probe and imaged by use of X-ray film. The restriction enzymes were selected with the criterion of no cutting site resides in the gene sequences. The gene-specific probes were labeled with digoxigenin by PCR (Neuhausurl and Neuhaus 1993).

Sequence analysis

All sequences were retrieved from the NCBI (http://www.ncbi.nlm.nih.gov/) or TAIR (http://www.arabidopsis.org/) database. Sequence analysis involved use of Vector NTI (Invitrogen).

Plasmid construction, transformation, and screening for transgenic broccoli and rescued emf2 Arabidopsis seedlings

Plasmid construction. The Cauliflower mosaic virus promoter 35S (35S) sequence of pBI121 was amplified by PCR with the primers 35S-BaF (TTTGGATCCAGATATTTACCTTTTCAATT) and 35S-SaR (AAAGTCGACCGTTCCTCTC) with BamHI and SalI cutting sites, respectively. The amplified 35S promoter fragment was then introduced into pCAMBIA1380 vector by use of the BamHI and SalI sites, with selection markers of ampicillin for microbes and hygromycin for plants to create a pCAMBIA1380-35S-promoter2 vector.

For BoEMF2 knockdown experiments in broccoli, RNA extracted from broccoli inflorescence was used to synthesize cDNA. Using the cDNA as template, ABoEMF2-SpF (AAAACTAGTATGGCCGCTTCTGTC) and ABoEMF2-SaR (AAAGTCGACGGACGTGTTCTC) with Spel and SalI cutting sites, respectively, were used to clone the full-length CDS of antisense BoEMF2.1. The obtained antisense BoEMF2.1 sequence was subcloned into pCAMBIA1380-35S-promoter2 vector by use of the Spel and SalI cutting sites to generate a 35S promoter-triggered antisense BoEMF2.1 expression vector.

For the Arabidopsis emf2 rescue experiment, primer pairs for BoEMF2-SaF (AAAAATCGACGGAGCATTCCG) and BoEMF2-SpR (AAACTAGTATGGCCGCTTCTGTC) with Spel and SalI cutting sites, respectively, were used to clone the sense BoEMF2.1 gene from broccoli inflorescence cDNA. The cloned BoEMF2.1 sequence was then subcloned into pCAMBIA1380-35S-promoter2 vector by use of the SalI and Spel cutting sites to create a 35S promoter-triggered BoEMF2.1 expression vector.

Transformation of antisense BoEMF2.1 into broccoli. Cotyledons and hypocotyls of 3-day-old broccoli seedlings were cut into pieces and infected with the Agrobacterium tumefaciens strain GV3101 harboring 35S:antisenseBoEMF2.1 as described (Chen et al. 2001). Transgenic broccoli was screened by ability to grow on 20 μg ml⁻¹ hygromycin and plasmid specific-PCR.

emf2 rescue by BoEMF2.1. Because homozygous emf2 is lethal, we introduced 35S:BoEMF2.1 into heterozygous emf2 plants, which were identified by allele-specific RFLP genotyping (Calonje et al. 2008) and transformed with A. tumefaciens strain GV3101 harboring 35S:BoEMF2.1 by the floral dipping method (Clough and Bent 1998). T1 seeds from transformed heterozygous emf2 plants (T0) were surface-sterilized and screened on an agar plate containing 20 μg ml⁻¹ hygromycin. Hygromycin-resistant seedlings were transplanted into soil to obtain T2 seeds and plants. T3 plants homozygous for 35S:BoEMF2.1 and harboring the Arabidopsis emf2 mutation were identified by producing 100% hygromycin-resistant progeny and allele-specific RFLP genotyping.

RT–PCR analysis

The second leaves of 4-week-old antisense BoEMF2.1 broccoli (asBoEMF2.1) and vector-only control were harvested, and the expression of the two BoEMF2 genes was determined to confirm the knockdown efficiency of the endogenous BoEMF2 genes (Fig. 3). In total, 2 μg of total RNA was used to synthesize...
the first-strand cDNA, and PCR analysis involved primers specific to BoEMF2.1 and BoEMF2.2 (Supplementary Table S1). The expression of broccoli actin (BoActin; accession No. AF111812) was a control.

To study gene expression in asBoEMF2.1 broccoli, 11-day-old seedlings of the asBoEMF2.1 T1 generation (G1), the T2 generation (G2), vector-only control seedlings and flower and siliques of control plants (Fig. 4) were harvested for RNA extraction and cDNA preparation. In total, 2 μg of total RNA was used for first-strand cDNA synthesis. The cDNA was diluted five times in contraction before PCR analysis with gene-specific primers (Supplementary Table S1). The PCR products were separated on a 1.5% agarose gel and stained with ethidium bromide. Images were obtained by use of a Bio-Rad Gel Doc EQ system, and the intensity of DNA bands was quantified with use of Quantity One (Bio-Rad).

qRT–CR analysis

For qRT–PCR analysis of BoEMF2 genes in broccoli, roots and second expanded leaf and stem were harvested from 4-week-old broccoli grown in soil; flower buds and siliques were collected after flowering and silique formation. For Arabidopsis, RNA was extracted from 7-day-old seedlings of the 35S::BoEMF2.1-containing WT (transWT), WT, emf2 and Rescued emf2.

Primers for genes were designed for qRT–PCR (Supplementary Table S1) with the ABI Prism 7500 sequence detection system (Applied Biosystems) at 58°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 s and 60°C for 1 min. The relative gene expression was determined by the comparative CT method, with the expression of UBQ10 (At4g05320) used as an internal control for Arabidopsis and BoActin for broccoli. The mean value of 2^(-ΔΔCT) (ΔCT = Cg - Cc, gene of interest - Cc, control gene) was determined from three independent experiments.

Microarray experiments and analysis

For broccoli, the seedlings of asBoEMF2.1 transgenic plants and vector-containing control were grown on half-strength MS agar containing 1.5% sucrose and harvested at 11 d after germination. RNA was extracted by the TRIzol reagent method (Invitrogen), and RT–PCR was performed to determine the endogenous BoEMF2 level. The asBoEMF2.1 line with knocked-down expression of BoEMF2s and the vector-only control (Fig. 3) were used for microarray analysis.

For Arabidopsis, 7-day-old 35S::BoEMF2.1-containing WT (transWT) and Rescued emf2 seedlings grown on hygromycin-containing agar plates were harvested and sorted by cotyledon phenotype: transWT and Rescued emf2 with round flat and curled outwards cotyledons, respectively (Fig. 4). The seedlings of WT and emf2 grown on MS-only plates were collected and sorted by round flat and small oval shaped cotyledons, respectively (Fig. 4). After confirming the genotype by allele-specific RFLP (Calonje et al. 2008) and BoEMF2.1 expression (Supplementary Fig. S2), microarray analysis was performed.

For microarray experiments, 10 μg of total RNA was used as a template for cDNA synthesis, cRNA was biotin labeled by in vitro transcription then fragmentation by use of the GeneChip Expression Analysis Technical Manual rev5 (Affymetrix). In total, 10 μg of labeled cRNA was hybridized to the 22 K ATH1 GeneChip (Redman et al. 2004) at 45°C for 16.5 h. Washing and staining involved use of Fluidic Station-450, and genechips were scanned by use of the Affymetrix GeneChip Scanner 7 G. The mean gene expression indices for all chips were scaled to an arbitrary target of 500.

For broccoli gene expression analysis, only genes with hybridization signals ≥60 in at least one of the two samples, the vector-only and the asBoEMF2.1 broccoli, were analyzed. For Arabidopsis, only genes with hybridization signals ≥50 in at least one of four plant samples, emf2, WT, Rescued emf2 and transWT, were analyzed. The hybridization signal ratio of fold change of gene expression in broccoli was analyzed by the ratio of asBoEMF2.1 to vector-only control, with a ratio ≥2 or ≤0.5 used to identify up- or down-regulated genes, respectively. The fold change of gene expression in Arabidopsis was based on the ratio of emf2 to the WT over that of Rescued emf2 to transWT, with a ratio ≥2 or ≤0.5 used to identify up- or down-regulated genes, respectively.

Supplementary data

Supplementary data are available at PCP online.

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