Biotechnol Lett (2010) 32:1961–1968
DOI 10.1007/s10529-010-0362-1

ORIGINAL RESEARCH PAPER

Functional conservation of the plant *EMBRYONIC FLOWER2* gene between bamboo and *Arabidopsis*

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Received: 22 June 2010 / Accepted: 21 July 2010 / Published online: 31 July 2010
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Abstract A novel *EMBRYONGIC FLOWER2* gene, *DlEMF2*, was isolated from bamboo, *Dendrocalamus latiflorus*. It is 2518 bp in length encoding a protein of 629 amino acids. It was expressed in all organs and at a higher level in shoot tissue than in inflorescences. Transgenic *Arabidopsis* plants expressing antisense *DlEMF2* displayed a spectrum of early-flowering phenotypes. Quantitative real-time PCR experiments indicated that the antisense transgene *DlEMF2* reduced endogenous *AtEMF2* transcript levels leading to the flowering time reduction. Besides, transforming the bamboo *DlEMF2* gene into *Arabidopsis emf2-1* mutant could rescue phenotype in vegetative and reproductive developmental stages, which reveals that EMF2 has functional conservation between monocots and eudicots.

Keywords Anti-sense · Bamboo *EMF2* · Rapid amplification of cDNA ends · Real-time PCR · Rescue

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Introduction

In higher plants, the developmental phase changing from vegetative growth to flowering is regulated by a complex gene network. Different from other plants, woody bamboos have a long vegetative stage, with reproduction cycles varying 3 to 120 years (Janzen 1976) and bamboo forests die en masse after gregarious flowering. This peculiar characteristic of bamboo has intrigued mankind since ancient times, and remains mystic. In *Arabidopsis*, the loss-of-function *emf* mutants (*emf1*, *emf2*) skip vegetative development and produce small inflorescence whose lateral buds produce only flowers and no additional inflorescences (Sung et al. 1992). Since the *AtEMF2* gene played a major role in maintaining vegetative phase through

repression of the flowering program, we hypothesized that its bamboo homolog(s) would be involved in maintaining the unusually long vegetative phases of bamboos. In *Arabidopsis*, the *EMF2*, *VERNALISATION* (*VRN2*) and *FERTILISATION INDEPENDENT ENDOSPERM2* (*FIS2*) genes encode Su (z)12-related proteins known as EMF2 protein complex, belonging to Polycomb-group (Pc-G) proteins (Birve et al. 2001; Calonje et al. 2008). Polycomb group (PcG)-mediated gene silencing is a common developmental strategy for maintaining stably inherited repression of target genes and involves different protein complexes known as Polycomb-repressive complexes (PRCs) (Birve et al. 2001). Further study proposed that *Arabidopsis* EMF2 protein complex is equivalent to *Drosophila* PRC2 in terms of subunit composition and biochemical function (Chanvivattana et al. 2004; Schubert et al. 2005, 2006).

Some 70 partial ESTs and 20 full length cDNAs of *EMF2* have been found in higher plants, most of them being from eudicots; in monocots, there are some *EMF2* homologs have been identified, including two rice homologs of *EMF2* (Li et al. 2006; Luo et al. 2009). However, whether *EMF2* homologs in monocots particularly bamboo has functions similar to eudicot *EMF2* homologs is still not clear, and this study will fill this gap in our understanding of the *EMF2* class and enrich our knowledge of mechanisms of bamboo flowering.

Materials and methods

Plant materials and growth conditions

Dendrocalamus latiflorus used in this study is a tropical bamboo widely cultivated in southern China and of great economic value. *Arabidopsis thaliana*, ecotype Columbia, was used to evaluate the biological function of the gene due to the difficulties of genetic manipulation in bamboos. *Arabidopsis* seeds were germinated on MS medium (pH = 5.7) containing 1% (w/v) sucrose and grown under long daylight at 22 ± 2°C.

Cloning and sequencing of full-length *DlEMF2* cDNA

Oligonucleotides used for rapid amplification of cDNA ends were designed based on the multi-alignment result of EMF2 genes of *Triticum aestivum*, *Oryza stativa* and *A. thaliana* from the GenBank database. Total RNA was extracted from the shoots, leaves, roots, stems and inflorescences of *D. latiflorus* using Trizol reagent according to the supplier's (Gibco) instructions. The total RNA extracted from shoots was used for RACE, and those from the others were used for tissue-specific expression.

Kits of 3′-and 5′-RACE System (BD) for rapid amplification of cDNA ends were used in the 3′-end and 5′-end cloning of *D. latiflorus*. In order to obtain two specific primers for RACE, firstly, we designed a pair of primers EMF2S (5′-GACKGAAGAYTTYDCTTGYCC-3′), and EMF2A (5′-CGCCATCWACTTCATCTTCACTATC-3′) to amplify a conserved internal cDNA fragment. Reverse transcription and PCR amplification were conducted in terms of the manufacturer's instruction (Invitrogen). According to this conserved sequence, two specific primers EMF3E (5′-GTTCTTCCATTCTCACAGGG-3′) and EMF5E (5′-ATCGGTCTATCGGAGAATGCCC-3′) were designed to amplify the missing 5′-and 3′-terminal fragments. In the 3′-RACE, 3 μg of total RNA were reversely transcribed using adapter primer (5′-AAGCAGTGGTATCAACGCAGAGTAC$(T)_{30}$VN-3′). Amplification of 3′-cDNA end was carried out using gene-specific primer EMF3E and universal amplification primer (5′-CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT-3′) by PCR in 50 μl. In the 5′-RACE, RT, dC-tailing and PCR amplification were conducted in terms of the 5′-RACE kit instruction. 3 μg total RNA mixture was reverse-transcribed using 5′-RACE CDS primer. PCR was performed with primer EMF5E and UPM using 2.5 μl dC-tailed cDNA as template in 50 μl reaction mixture. All of the amplified PCR products were purified and cloned into pGEM-T vector followed by sequencing.

Gene expression

RNA was isolated from various tissues with Trizol reagent. cDNAs were generated by RT-PCR, using Superscript II reverse transcriptase (Invitrogen). For semi-quantitative RT-PCR, equal amounts of RNA (1 μg) from bamboo shoots, leaves, roots, stems and inflorescences were used. For all PCR reactions, bamboo actin gene was used as an internal control to show the consistent amount of beginning RNA for RT-PCR.

Quantitative real-time PCR

Using Trizol solution, three total RNA was isolated from two early-flowering transgenic plants and one wild type plant as control. For reverse transcription, according to the manufacturer's instructions (Invitrogen), 5 μg total RNA for each samples were digested with RNase-free DNase I (Roche) before they were used for reverse transcription. Quantitative real-time PCR was conducted as described by Guo et al. (2006). Two Primers for the quantitative real-time PCR are RT-F (5′-CAAAACTCTGCTCTGCTGCG-3′) and RT-R (5′-AGAGACCAGAACCAATACAC-3′), and use UBQ10 as control.

Plasmid construction and plant transformation

We constructed sense and anti-sense DlEMF2 plasmid as follows. For sense expression vector, the 1.9 kb *DlEMF2* cDNA fragment (covering the entire ORF) amplified by primers P3 (5′-GCGTCTAGAGCGAG GATG-3′) and P4 (5′-GCGCTCGAGGAACCAATA CACG-3′) was inserted into a modified pJIM19 vector in the sense orientation. To create the antisense expression vector, the *C*-terminal acidic-W/M domain of *DlEMF2* (which has 83% similarity to *AtEMF2*) was amplified by primers Anti-F(5′-GCGCTCGAGA AATGGGATTTCTGTAG-3′) and Anti-R (5′-GCGT CTAGAGACCAGAACCAATACAC-3′) and subcloned into pJIM19. Next, the constructs were transformed into *Agrobacterium tumefaciens* strain GV3101 and the positive clones were used for transformation. Resistant plants were confirmed by the following two strategies: (1) kanamycin resistance screening, and (2) semi-quantitative RT-PCR. T1 seeds from confirmed primary transformants were planted on 1/2 MS medium containing 50 mg kanamycin l^{-1} for selection of transgenic progeny. T2 seeds were plated on the same medium for screening of homozygous T2 plants.

Complementation experiment

As *emf2-1* homozygous mutants are sterile, we transferred above sense *DlEMF2* construct into *Agrobacterium tumefaciens* GV3101 and transformed into *emf2-1* heterozygous plants (Columbia ecotype). The transgenic plants were then screened on 2/5 MS medium with 50 mg kanamycin l^{-1} and their insertions were confirmed by PCR.

Assessment of *DlEMF2* gene rescued *Arabidopsis emf2-1* mutant

emf2-1 heterozygotes and *DlEMF2/emf2-1* segregating progeny were subjected to phenotype analysis and PCR-based genotyping. Putative *DlEMF2/emf2-1* plants were geminated in 2/5 MS plates to follow Mendelian segregation. Genomic DNA extracted from the progeny was used to amplify the region containing the site for *emf2-1* point mutation in the *EMF2* locus. The primers used were: P1 (AAC AAC AAA TTG CAG AAG ACT GAA G) and P2 (CTT GGA TAT CAT TGT CTC AGT CTT G). The *emf2-1* mutation introduced an *Hpy*188III site. Specifically, the G-to-A transition at position 2994 bp in the cDNA generated a cleaved amplified polymorphic sequence marker (CAPS) not present in the wild type allele. Thus, *Hpy*188III would not cut the wild type's 460 bp fragment, but would cut the *emf2-1* product into 180 bp and 280 bp fragments.

Results and discussion

Cloning and sequencing of full-length *DlEMF2* cDNA

The complete cDNA of *DlEMF2* was 2518 bp in length (GenBank Accession No. DQ251440). Except for a 249-bp 5′-UTR and a 382-bp 3′-UTR, the cDNA of *D. latiflorus* possessed an 1887 bp ORF from 250 to 2136 nt. The predicted amino acid sequence of *DlEMF2* was aligned with the homologous protein sequences from *Oryza sativa*, *Triticum aestivum*, *Zea mays*, *Arabidopsis thaliana* and *Hordeum vulgare* using ClustalX program (Fig. 1). Three conserved regions, C_2H_2-type zinc finger motif, *N*-terminal basic domain and *C*-terminal acidic-W/M domain have been found among these plants. Using these methods, we found only a single *EMF2* homolog in *D. latiflorus*, although future work will include Southern blot analyses to verify its uniqueness.

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                 |← N-terminal basic domain →| |← →| |← C2H2-typezinc finger region →|
OsEMF2    (67) YCKPVELYNIIQRRSIKNPAFLQRCLLYKIHARRKKR (104) ***(246)*** (350) TEVTEDFSCPFCLVPCGSFKGLGCHLNASHDLFHYEFW (388)
TaEMF2         YCKPVELYNIIQRRAIRNPAFLQRCLHYKIHASRKKR      ***(246)***      TEVTEDFACPFCLVKCGSYKGLGCHLNSSHDLFHFEFW
ZmEMF2         YCKPVELYNIIQRRAMKNPLFIQRCLLYNIHARRKKR      ***(246)***      TEVTEDFSCPFCYVRCGSFKGLGCHLNSSHDLFHYEFW
DlEMF2         YCKPVELYNIIQRRAIKNPAFLQRCLLYKIHARRKKR      ***(246)***      TEVTEDFSCPFCLVSCGSFKGLGCHLNSSHDLFHFEFW
AtEMF2         YCKPVELYNIIQRRAIRNPLFLQRCLHYKIEAKHKRR      ***(246)***      TEVTEDFSCPFCLVKCASFKGLRYHLPSTHDLLNFEFW
HvEMF2         YCKPVELYNIIQRRAIRNPAFLQRCLHYKIHASRKKR      ***(246)***      TEVTEDFACPFCLVKCGSYKGLGCHLNSSHDLFHFEFW
Consensus      YCKPVELYNIIQRR   NP F QRCL Y I A  K R                       TEVTEDF CPFC V C S KGL  HL   HD L  EFW

                 |← →| |← C-terminal acidic-W/M domain
OsEMF2    ***(149)*** (537) RQFFHSHRAQPMALEQVFSDRDSEDEVDDDIADFEDRRMLDDFVDVTKDEKLIMHMWNSFVRKQRVLADGHIPWACEAFSQFHGQ
TaEMF2    ***(149)***       RQFFHSHRAQPMALEQVFSDRDSEDEVDDDIADFEDKRMLEDFVDVTDDEKLIMHMWNSFVRKQRVLADGHIPWACEAFSRLHGK
ZmEMF2    ***(149)***       RQFFHSHRAQPMQLEQVFSDRDSEDEVDDDIADFEDRRMLDDFVDVTKDEKLIMHMWNSFVRKQRVLADGHIPWACEAFSQLHGR
DlEMF2    ***(149)***       RQFFHSHRAQPMALEQVFSDRDSEDEVDDDIADFEDRRMLDDFVDVTKDEKLIMHMWNSFVRKQRVLADGHIPWACEAFSRLHGQ
AtEMF2    ***(149)***       RQFFHSHRAQPMALEQVLSDRDSEDEVDDDVADFEDRRMLDDFVDVTKDEKQMMHMWNSFVRKQRVLADGHIPWACEAFSRLHGP
HvEMF2    ***(149)***       RQFFHSHRAQPMALEQVFSDRDSEDEVDDDIADFEDRRMLDDFVDVTNDEKLIMHMWNSFVRKQRVLADGHIPWACEAFSRLHGK
Consensus                   RQFFHSHRAQPM LEQV SDRDSEDEVDDD ADFED RML DFVDVT DEK  MHMWNSFVRKQRVLADGHIPWACEAFS  HG

                                                                  →|
OsEMF2    (622) ELVQNPALLWCWRFLWSNSGTTVYWMREPMPATQFLKATTEA (663)
TaEMF2          HLVQNPPLLWSWRFLMIK-----LWNHSLLDARAMNVCGTIL
ZmEMF2          QLIQNPALLWGWRFFMIK-----LWNHNILDARTMNTCNTVL
DlEMF2          QLVQNSALLRCWRFFMIK-----LWNHSLLDARTMNTCNTIL
AtEMF2          IMVRTPHLIWCWRVFMVK-----LWNHGLLDARTMNNCNTFL
HvEMF2          HLVQNPPLLWGWRFLMIK-----LWNHSLLDARAMNVCGTIL
Consensus                L   WR            W      A      T
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Fig. 1 Amino acid alignment of *DlEMF2* and other *EMF2* proteins. The predicted amino acid sequence of *DlEMF2* was aligned with the homologous protein sequences from *Oryza sativa*, *Triticum aestivum*, *Zea mays*, *Arabidopsis thaliana* and *Hordeum vulgare*. It has three conserved regions, a C_2H_2-type zinc finger motif, an *N*-terminal basic domain, and a *C*-terminal acidic-W/M domain. The three conserved domains are emphasized with gray background. The consensus sequence is given in *underlined bold* type below

Organ-specific expression pattern of *DlEMF2*

Its organ-related expression pattern showed that the transcripts of *DlEMF2* were relatively abundant in all organs, somewhat higher in shoots and lower in inflorescences (Fig. 2). This expression pattern is similar to *AtEMF2* (Yoshida et al. 2001), but much different from the expression pattern of *OsEMF2*, which was abundant in the inflorescence and shoot meristems, but much lower in leaf, root, immature seed and callus organs (Li et al. 2006).

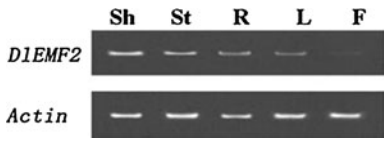

Fig. 2 Organ-specific expression pattern of the *DlEMF2* gene. Semi-quantitative PCR analysis for the organ-specific expression pattern of *DlEMF2* in five selected organs: *Sh* shoot, *St* stem, *R* root, *L* leaf, *F* inflorescence. A bamboo actin gene was used as an internal control

Suppression of *EMF2* in antisense transgenic plants

We generated transgenic *Arabidopsis* plants expressing antisense or sense *DlEMF2* under the control of the CMV 35S promoter to study whether *DlEMF2* activity regulates flowering. We produced 51 independent transgenic plants containing the antisense construct pJIM19-anti-DlEMF2, 27 (53%) of which displayed early-flowering phenotypes, whereas 15 (29%) transformants showed wild-type-like phenotype under long-day conditions. Others showed unrelated

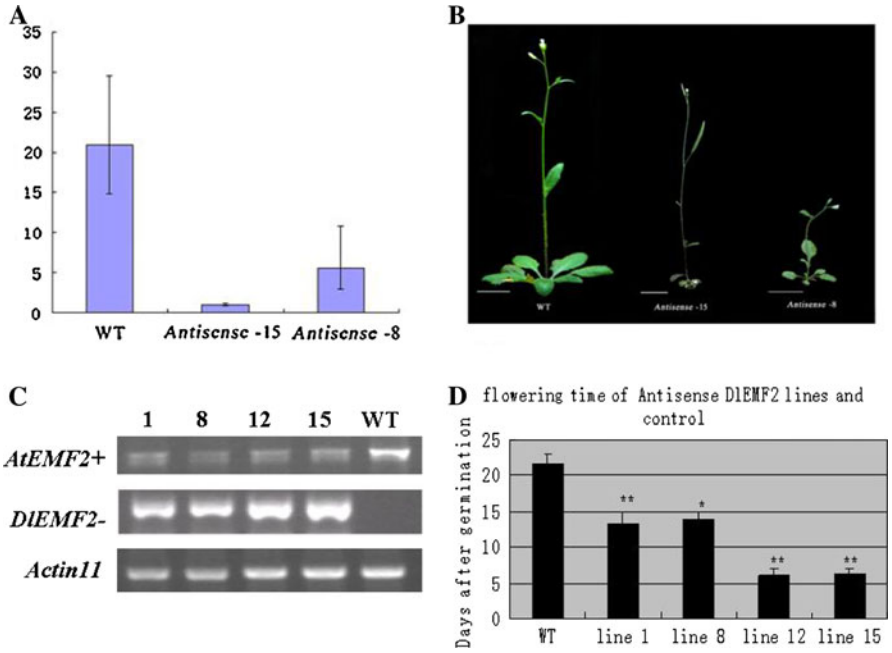

Fig. 3 Analysis of anti-sense *DlEMF2* transgenic plants. **a** Quantitative real-time PCR showed the relative expression level of endogenous *AtEMF2* in WT (control) and antisense *DlEMF2-C* lines. Antisense-15 and Antisense-8 represent strong and weak phenotypes of early flowering plants, respectively. The numbers on the *y*-axis indicate the relative expression level of *AtEMF2*. **b** A 4-week-old wild-type plant and two independent antisense *DlEMF2-C* transgenic plants used in result A. *Bar* = 1 cm. **c** Antisense (*DlEMF2-*) and endogenous (*AtEMF2+*) RNA levels in transgenic plants. Semiquantitative RT-PCR analysis was performed using total RNA as template. *ACTIN11* RNA was amplified as a control. Four independent early-flowering plants were tested. WT was the Columbia wild type accession. **d** Flowering times of control and pJIM19-antiDlEMF2 lines. The heading time was measured and statistically analyzed using one-tailed student's *t* test (*$P < 0.05$; ** $P < 0.01$). Data were presented as mean ± SE ($n > 15$)

morphological abnormalities. A gradient of the early-flowering phenotype was observed, which we divided into two categories: strong-type (bolted after developing 4 or 5 rosette leaves) and weak-type (bolted after developing 7 or 8 rosette leaves). The semi-quantitative RT-PCR showed that the endogenous *AtEMF2* mRNA level was significantly reduced (Fig. 3c). Quantitative real-time PCR revealed that the strong-type accumulated lower endogenous *AtEMF2* levels than the weak-type (Fig. 3a, b). The early-flowering phenotype of T2 generation was unaltered. The statistical analysis of flowering time was shown in Fig. 3d. In contrast to the severe phenotypes of the antisense *AtEMF2* transgenic plants in *Arabidopsis* (Yoshida et al. 2001), the antisense *DlEMF2* lines have normal floral organs and are fertile (Fig. 4). Conversely, in sense-transgenic plants, we did not observe late-flowering phenotypes among 60 T1 transformants. We did observe four early-flowering plants with curly leaves and abnormal flower organs (data not shown). After two generations screening, we obtained pJIM19-DlEMF2 T2 plants, which showed around 3:1 Kanamycin resistance. This demonstrated that the insertion is a single copy and the gene is recessive. The gene deficiency detection (Fig. 5a) and the statistical results (Fig. 5b) showed out the over-expression lines of DlEMF2 have not obvious phenotype about flowering time.

In *Arabidopsis*, the epigenetic mechanism required for maintaining vegetative development involves the putative EMF2 protein complex which comprises four core proteins. As EMF2 protein is one of these four members, enhancement of only EMF2 protein component in this complex might be insufficient to repress the reproductive program, leading to late flowering. On the other hand, knocking-out any one of the components such as *EMF2* expression could disable the complex and cause early flowering, as observed in our anti-sense and sense transgenic plants.

Fig. 4 Early flowering of pJIM19-antiDlEMF2 plants. **a** Similar siliques of WT (*left*) and pJIM19-antiDlEMF2 plants (*right*). *Bar* = 1 mm. **b** Dissected floral organs of WT (*left*) and antisense plants (*right*). *Bar* = 1 mm

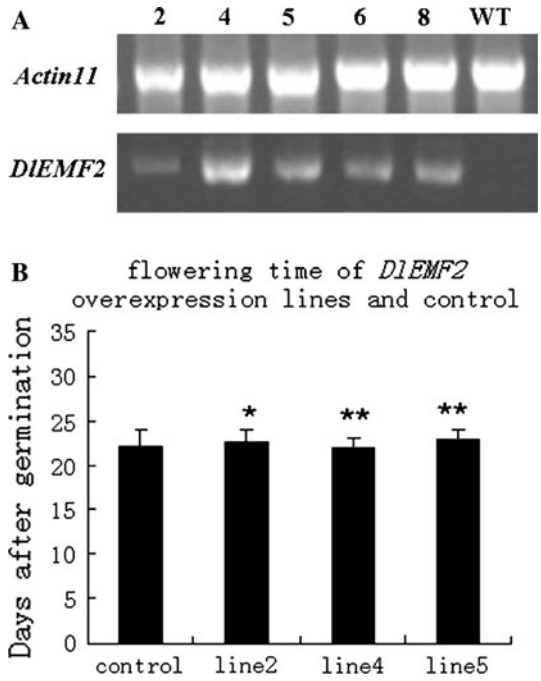

Fig. 5 Expression level and flowering time of pJIM19-DlEMF2 lines. **a** Sense (*DlEMF2*) RNA levels in transgenic plants. Semiquantitative RT-PCR analysis was performed using total RNA as template. *ACTIN11* RNA was amplified as a control. Four independent sense transgenic lines were tested. WT was the Columbia wild type accession. **b** Flowering time of WT (control) and over-expression lines (line 2, 4, 5). The heading time was measured and statistically analyzed using one-tailed student's t test (* $P < 0.05$; ** $P < 0.01$). Data were presented as mean ± SE ($n > 15$)

Bamboo *DlEMF2* gene rescued *Arabidopsis emf2-1* mutant

We transformed the pJIM19-DlEMF2 construct into nine putative *emf2-1* heterozygotes. The homozygous *emf2-1* plants were easy to differentiate from WT, as they did not produce a normal rosette upon germination but rather produced several cauline leaves with no petioles and multiple floral buds (Fig. 6A). The nine putative transgenic plants of the T1 generation were selected on MS medium containing 50 mg l^{-1} Kanamycin. We found no *emf2-1*-like phenotypes in the T1 generation, suggesting that *DlEMF2* expression had rescued the *emf2-1* mutant in seedling stage. We randomly selected three kanamycin resistant plants from each of nine T1 lines to get 27 T2 lines.

The transgenic T2 generation seeds were plated on MS medium and identified by phenotype observation and molecular method detection. We found four lines for which the ratio of WT-like plants to *emf2-1* mutant-like plants was approximately 15:1 (line 2, 91:6; line 4, 90:6; line 5, 88:5; line 9, 92:6), suggesting that *DlEMF2* could rescue *Arabidopsis emf2-1* mutant, since otherwise the ratio would have been 3:1. From the four lines, we selected one line (line 9 shown in Fig. 6B) with which to verify *emf2-1* point mutation by PCR-based genotyping (see Materials and methods; Fig. 6C). We extracted genomic DNA extracted from 70 wild-type-like plants from line 9, amplified the diagnostic PCR product with P1 and P2, and then incubated the PCR products with *Hpy*188III. Among them eight plants showed two bands of 280 and 180 bp (Fig. 6D). Amplifying *DlEMF2* bands from genomic DNA in the same lines showed 100% positive results (data not shown). This result indicated that these plants were homozygous for the *emf2-1* mutation but also contained the transgene. This assay confirmed the presence of

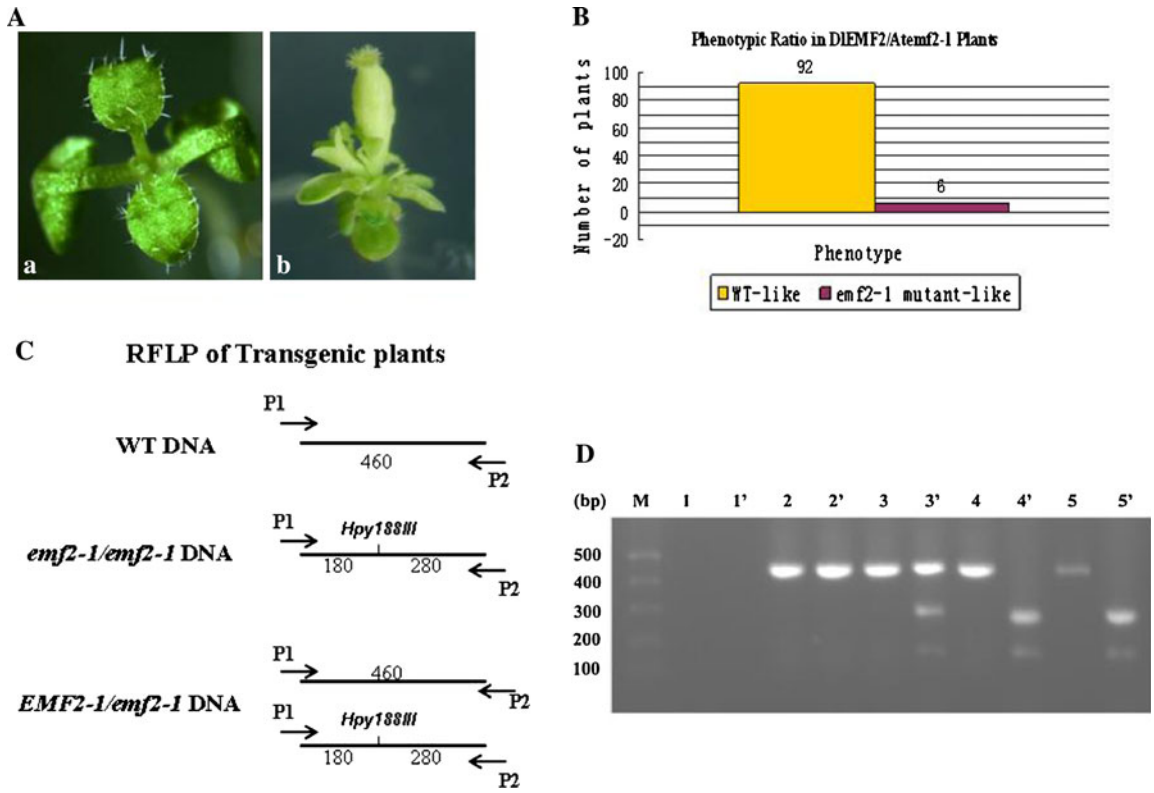

Fig. 6 *DlEMF2* expression rescued the *Arabidopsis emf2-1* mutant. **A** Segregating phenotypes in the progeny of heterozygous *Arabidopsis emf2-1* plants harboring the *DlEMF2* transgene. The two observed phenotype classes were (*a*) WT-like and (*b*) *emf* mutant-like. **B** Segregation ratio of the two phenotypes of T2 plants of Line 9 (six plants being homozygous *emf2* mutants harboring the *DlEMF2* transgene). **C** Allele-specific RFLPs created by the *emf2-1* CAPS marker. The figure illustrates the PCR fragments, the primers (P1 and P2 arrows), the restriction enzyme sites, and the predicted length (bp) of the fragments for each genotype (wild type, *emf2-1* homozygous mutant, and the *EMF2*/*emf2-1* heterozygote). **D** Genotypic analysis of *DlEMF2*/*emf2-1*/*emf2-1*. 1–5: PCR products of mock, WT, *EMF2*/*emf2-1*, and two independent lines of *DlEMF2*/*emf2-1*/*emf2-1*. 1′–5′: the bands resulting from *Hpy*188III digestion of the PCR products

emf2-1 individuals whose flowering phenotypes were rescued by the *DlEMF2* transgene.

We planted these 70 WT-like plants of line 9 in soil, and all grew completely normally for two weeks. After this time, five of the plants began to flower. These plants produced curly leaves, weak stems, and mutant flowers often producing abnormal petals, one or two stigmas, and six anthers (data not shown). Our PCR-based genotyping found that these plants contained the transgene but were also homozygous wild type at the *EMF2* locus (data not shown). This discovery led us to conclude that these phenotypes likely resulted from the co-suppression of *AtEMF2* by the *DlEMF2* transgene. The remaining 65 plants grew normally throughout the vegetative and reproductive phases.

In conclusion, EMF2 mediated flowering inhibition is the core procedures of plant epigenetic silence system. DlEMF2, as a bamboo homolog, can rescue *Arabidopsis emf2-1* mutant phenotype, revealed that EMF2 homologs from *Arabidopisis* and *D. latiflorus* showed remarkable conservation, not only in nucleotide and protein sequences, but also in its function.

Acknowledgments This research was supported by the Knowledge Innovation Program of the Chinese Academy of Sciences (KSCX2-YW-N-029), the National Basic Research Program of China (973 Program, 2007CB411600) and the National Natural Science Foundation of China (30770154). We are indebted to Prof. Renee Sung, Dr. Caihong Yu, and Dr. Renhong Wu for their valuable help. We also appreciate Dr. Zachary Larson-Rabin for critical reading of the manuscript.

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