DOI: 10.1007/s10535-016-0614-6 BIOLOGIA PLANTARUM 60 (3): 401-409, 2016

Overexpression of the repressor gene *PvFRI-L* from *Phyllostachys violascens* delays flowering time in transgenic *Arabidopsis thaliana*

S.N. LIU[1,2], L.F. ZHU[2], X.C. LIN[2]*, and L.Y. MA[1]*

Forestry College, Beijing Forestry University, Beijing 100083, P.R. China[1]
The Nurturing Station for the State Key Laboratory of Subtropical Silviculture, Zhejiang Agriculture and Forestry University, Lin'an, Hangzhou 311300, Zhejiang, P.R. China[2]

Abstract

The gene *FRIGIDA* (*FRI*) is floral repressor and plays a key role in the timing of *Arabidopsis* flowering. To study the function of *FRI*-like genes in bamboo, we isolated a *FRI* family gene from bamboo *Phyllostachys violascens* and named it *PvFRI-L*. Sequence alignment and phylogenetic analysis show that the PvFRI-L protein belongs to the FRL3 (III) subfamily from monocots and contains a conserved FRIGIDA domain. PvFRI-L was located in the nucleus of onion epidermal cells. *PvFRI-L* was expressed in all tested organs of flowering and non-flowering bamboo plants with a higher expression in non-flowering than in flowering plants. Overexpression of *PvFRI-L* in *Arabidopsis* caused late flowering by downregulating *flowering locus T* and upregulating *flowering locus C*. A P-box, the binding site involved in gibberellin response, was found only in the promoter region of *PvFRI-L* but not in that of *FRI*. Furthermore, *PvFRI-L* expression in the leaves of *Ph. violascens* seedlings was downregulated with gibberellic acid treatment. Taking together, our observation suggests that *PvFRI-L* may be flowering repressor and its delaying floral timing may be regulated by gibberellic acid in bamboo.

Additional key words: Arabidopsis thaliana, bamboo, gibberellic acid.

Introduction

Flowering bamboo differs from many other plants as it is hard to predict flowering time. It may take up to 120 years to bloom but it could occur next month. Moreover, bamboo dies after flowering (Janzen 1976). Very often, bamboo has a long vegetative phase, however, it can be induced to flower under stress conditions such as a high irradiance or drought (Gielis *et al*. 1997, Franklin 2004). Although it is poorly understood what genes dominate bamboo flowering, it is well known that in the model plant *Arabidopsis thaliana* floral repressors play important roles in flowering time by preventing reproductive development (Roux *et al*. 2006, Yant *et al*. 2009). It is suspected that bamboo may have homologous repressors to inhibit precocious flowering, unless it is influenced by an environmental stress (Gielis *et al*. 1997, Franklin 2004).

Arabidopsis contains at least 20 repressors, among them *FRIGIDA* (*FRI*) has received a wide attention (Yaron *et al*. 1998, Yant *et al*. 2009, Jarillo and Pineiro 2011). The *FRI* encodes a 609 amino acid protein with a coiled-coil domain (Johanson *et al*. 2000, Michaels *et al*. 2004). It delays flowering time by increasing the expression of *flowering locus* C (*FLC*), which acts as floral repressor as well (Michaels and Amasino 1999, Shindo *et al*. 2005). The FRIGIDA superfamily contains plant proteins that are similar to the FRIGIDA protein expressed in *Arabidopsis*. It has five distinct FRI subfamilies including FRI (I), FRL1/2 (II), FRL3 (III), FRL4/4a/4b (IV), and FRL5 (V) (Risk *et al*. 2010). *FRL1* and *FRL2* have redundant roles in promoting *FLC*

Submitted 26 March 2015, *last revision* 15 December 2015, *accepted* 22 December 2015.
Abbreviations: FRI - FRIGIDA; *FLC* - *flowering locus C*; *FT* - *flowering locus T*; GA - gibberellic acid; GFP - green fluorescent protein; IPTG - isopropyl-thiogalactoside; MBP - maltose binding protein; ORF - open reading frame; qPCR - quantitative PCR; WT - wild-type.
Acknowledgments: This study was supported by the National Natural Science Foundation of China [Nos. 31270715 and 31000295], the Ministry of Science and Technology of China [No. 2012CB723008] and the Top Key Discipline of Forestry of Zhejiang Province [KF201304].
* Corresponding authors; fax: (+86) 057163743863, e-mails: linxcx@163.com, maluyi@bjfu.edu.cn

expression in the presence of *FRI* (Michaels *et al*. 2004). *FRL1* is functional in *Arabidopsis* cv. Columbia, and *FRL2* in cv. Landsberg erecta (Schlapi 2006). However, the roles of other *FRI* subfamilies remain unclear.

Studies of *FRI* family members have been focused on dicotyledon plants, dominantly on *Arabidopsis* and with a less attention on *Thellungiella halophila*, *Brassica oleracea,* and *Medicago sativa* (Fang *et al*. 2008, Irwin *et al*. 2012, Chao *et al*. 2013). These studies show that the heterologous expression of *FRI*-like genes causes late flowering in *Arabidopsis*, and these genes demonstrate a conserved function as repressor of flowering time in eudicots. However, the *FRI* family genes in monocots are only annotated (in the *NCBI* database), and their actual physiological functions largely remain to be evaluated. Furthermore, it is unclear whether the *FRI* family members are homologous between monocots and dicots in the aspect of regulating flowering.

To explore a possible role of the bamboo *FRI* family genes in flowering, we isolated a *FRI* family gene from *Phyllostachys violascens* named *PvFRI-L*. It belongs to the *FRL3* (III) subfamily. We characterized the gene and determined its expression pattern in flowering and non-flowering bamboo. Furthermore, the *PvFRI-L* function was determined by overexpressing it into wild-type *Arabidopsis*. All of our results would provide valuable information for understanding the molecular mechanism in bamboo flowering.

Materials and methods

Plants: Bamboo (*Phyllostachys violascens* Rivière; *Poaceae*: *Bambusoideae*) plants were grown in the Bamboo Garden of the Zhejiang A&F University. *Arabidopsis thaliana* L. cv. Columbia (Col) was used for plant transformation to investigate the function of *PvFRI-L*. Wild type (WT) and transgenic plants were cultivated under a 16-h photoperiod, an irradiance of 200 μmol m^{-2} s^{-1}, day/night temperatures of 22/18 °C, and an air humidity of 60 %.

Isolation of *PvFRI-L* and its promoter: The total RNA was isolated from leaves using the RNAiso plus kit (*TaKaRa*, Dalian, China) according to the manufacturer's instructions. The first-strand cDNA was synthesized with the use of reverse transcriptase *M-MLV* (*TaKaRa*). The DNA was acquired by the cetyltrimethyl ammonium bromide (CTAB) method (Doyle and Doyle 1987). The full-length open reading frame (ORF) of the *PvFRI-L* gene and its promoter were amplified by PCR with primers (Table 1) designed by using the *Ph. edulis* transcriptome database (Peng *et al*. 2013). The amplified fragments were separated by electrophoresis on a 1 % (m/v) agarose gel and stained with ethidium bromide.

Primers were designed by using *Vector NTI* and *Premier Primer 5*. Related proteins were obtained by a *BLAST* search of the *NCBI* database. Phylogenetic analysis involved the neighbor-joinning method in *MEGA 5.0* with bootstrap values from 1 000 replications (Tamura *et al*. 2011). The software *ProtParam* from *ExPASy* (http: //exPasy. org) was used to analyze physical and chemical properties of the protein. The subcellular location of the protein was predicted by using *WoLF PSORT* (http://www.genscript.com/psort/wolf_psort.html). The *cis*-acting regulatory elements in the promoter were predicted by using *PlantCARE* data (Lescot *et al*. 2002).

***Expression analysis of PvFRI-L*:** The total RNA was extracted from different tissues of the flowering and non-flowering plants. Real time qPCR primers were designed on the basis of the *PvFRI-L* full-length ORF, with *PheUBC18* as internal control gene (Qi *et al*. 2013, Table 1). Amplification by PCR was conducted using the *CFX96TM* real-time PCR detection system (*Bio-Rad*, Hercules, USA). A 2× *ComSYBR* qPCR mix was used as reference. The amplification procedure was 95 °C for 3 min followed by 40 cycles of amplication (95 °C for 10 s, 55 °C for 20 s, and 72 °C for 20 s). The reactions were performed in 0.02 cm^3 of mixtures consisting of 0.01 cm^3 of a 2× *ComSYBR* qPCR mix, 0.0005 cm^3 of each forward or reverse primer (Table 1), 0.001 cm^3 of a cDNA template, and 0.008 cm^3 of double distilled H_2O. The data were analyzed by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Subcellular location of PvFRI-L: The full-length coding sequence of *PvFRI-L* was cloned into the CaMV *35S-GFP* vector. The *PvFRI-L* terminator codon (TAG) was not included, allowing the system to generate a PvFRI-L-GFP fusion protein for investigating subcellular location in onion epidermal cells. The particle bombardment method was adopted for transient expression assays (Wang *et al*. 1988). The onion epidermal cells were visualized and recorded by confocal laser scanning microscopy (*LSM510*, *Zeiss*, Germany).

Binary plasmid construction and analysis of transgenic plants: For transgenic expression experiments, the full-length ORF of the *PvFRI-L* gene was linked with the *pC1301* vector to form a binary plasmid *35S::PvFRI-L*. To generate transgenic *Arabidopsis* plants, the *Agrobacterium tumefaciens* L. strain GV3101 transformed with the plasmid *35S::PvFRI-L* was used to infect *Arabidopsis* by the floral dip method (Clough and Bent 1998). The transformed *Arabidopsis* seeds were selected on a half strength Murashige and Skoog solid medium containing 50 μg cm^{-3} kanamycin. Positive transgenic plants were confirmed by genomic PCR. The total number of rosette leaves from 70 plants was

measured in the T_1 generation to determine flowering time. The expressions of *flowering locus T* (*FT*) and *flowering locus C* (*FLC*) genes in the transgenic plants in three T_1 lines and WT plants were analyzed by real time qPCR with gene-specific primers (Table 1). A *SAND* family gene (including *Sp100*, *AIRE-1*, *NucP41/75*, and *DEAF-1* genes) (TAIR ID: AT2G28390) was used as control (Udvardi *et al*. 2008, Hong *et al*. 2010). A 2× *ComSYBR* qPCR mix was used for amplification under the following steps of 95 °C for 3 min followed by 40 cycles of amplification (95 °C for 10 s, 60 °C for 20 s, and 72 °C for 20 s). The reaction products were analyzed and used as the indicator of *PvFRI-L* expression.

Prokaryotic expression and purification of *PvFRI-L*: A pair of primers was designed according to the sequences of the *PvFRI-L* gene and the *pMal-c2X* vector that is tagged with the maltose binding protein (MBP) sequence (Table 1). The plasmid was used as template for PCR reaction and the amplified fragment was purified using a DNA gel extraction kit. The purified PCR product was digested with restriction enzymes BamHI and SalI for 6 h, then ligated into the *pMal-c2X* vector with T4 DNA ligase to construct a recombinant plasmid *PvFRI-L-MBP*. The positive *PvFRI-L-MBP* plasmid was transformed into Transetta (DE3) *Escherichia coli* cells.

The positive transformation colonies were cultured overnight at 37 °C in Luria-Bertani (LB) media containing 50 μg cm^{-3} ampicillin. The bacteria were further inoculated into 20 cm^3 of the fresh LB with a 100 fold dilution. When absorbance at 600 nm reached 0.4 - 0.6, the culture was divided into four parts. Isopropyl-thiogalactoside (IPTG) was then added to each of the three parts with a final concentration of 400 μM, leaving one part as control. To optimize expression conditions, the bacteria were induced under 37 and 20 °C for 5 and 12 h, respectively. The cells were collected by centrifugation, and then lysed by sonication (on/off 8/6 s with a 20 % of power). All samples were detected by 12 % (m/v) SDS-PAGE.

Next, the PvFRI-L-MBP fusion protein was purified from the supernatant by using a dextrin sepharose high performance medium according to the manufacturer's instructions, and the eluent was collected and analyzed by SDS-PAGE.

Treatment with gibberellic acid: *Ph. violascens* seedlings were produced by asexual reproduction and grown in a greenhouse. A gibberellic acid stock was prepared in 95 % ethanol with a final concentration of 0.4 g dm^{-3}. Treatments with GA included spraying leaves of the seedlings with a GA solution [0.04 g dm^{-3} GA, 0.02 % (v/v) Silwett-77] once a day for 10 consecutive days. Water was used as mock solution. The treatment was performed with two biological replicates. After completion of the assays, the total RNA was extracted from leaves and used for detection of *PvFRI-L* expression.

Table 1. Oligonucleotide sequences used for expression analysis and cloning in this study.

Genes encoding	Sequence (5'→3')	Purpose
PvFRI-L	F: ATGGCGACGGAAGCTACTGTT R: CTATGGTTGCTGGGAAGGGTGGTGA	cloning
PvFRI-L	F: GGATCCATGGCGACGGAAGCTACTGTT R: GTCGACCTATGGTTGCTGGGAAGGGTGGTGA	prokaryotic expression
PvFRI-L	F: CTCCTCGTCTCCTTCCCGAA R: GTCCGTTGCTGTAAATGTGTGG	real time qPCR
FLC	F: GAGAATAATCATCATGTGGGAGC R: CAACCGCCGATTTAAGGTGG	real time qPCR
FT	F: AGGCCTTCTCAGGTTCAAAACAAGC R: TGCCAAAGGTTGTTCCAGTTGTAGC	real time qPCR
PheUBC18	F: CTCCTCGTCTCCTTCCCGAA R: GTCCGTTGCTGTAAATGTGTGG	real time qPCR
SAND family protein	F: CAGACAAGGCGATGGCGATA R: GCTTTCTCTCAAGGGTTTCTGGGT	real time qPCR

Results

The full length of *PvFRI-L* contained 1 842 bp that encoded a protein of 613 amino acids with a theoretical pI value of 6.51, and a molecular mass of 67.3 kDa. Sequence comparison of the putative PvFRI-L protein with these obtained from the *NCBI* database revealed a conserved FRIGIDA domain between 165 and 467 amino acids (Fig. 1 Suppl.). Phylogenetic analysis of the PvFRI-L protein and its orthologs shows that it was highly homologous to FRI-like proteins from the grass family such as *Brachypodium distachyon*, *Setaria italica*,

and *Oryza brachyantha* (Fig. 1). This classifies PvFRI-L as member of the FRL3 (III) subfamily as other FRI-like proteins belong to the group.

The transcripts of *PvFRI-L* were detected in all examined tissues including leaves (young and mature), culms, flowers, shoots, and rhizomes of flowering and non-flowering *Ph. violascens* (Fig. 2). *PvFRI-L* expression was significantly lower in leaves (young and mature), shoots, and rhizomes of flowering than of non-flowering plants, with no difference in culms. In the flowering plants, *PvFRI-L* expression was highest in flowers and lowest in young leaves. In the non-flowering plants, *PvFRI-L* expression was highest in shoot and lowest in young leaves and rhizome.

To characterize the expression pattern of *PvFRI-L*, its promoter sequence was analyzed and compared with that

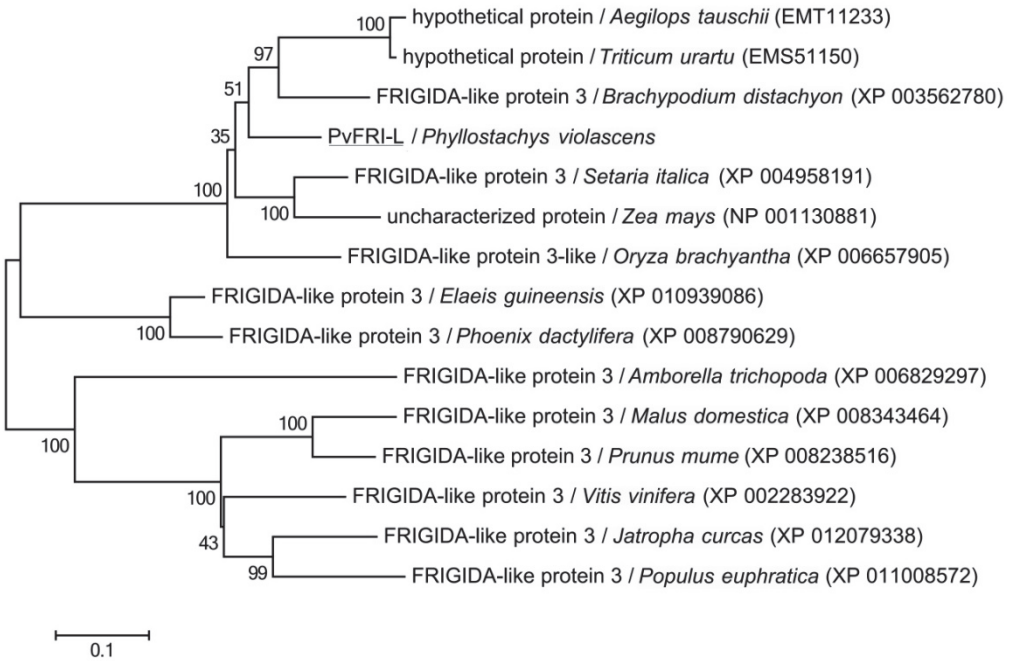

Fig. 1. The phylogenetic analysis of the PvFRI-L protein. A phylogenetic tree was generated by using *MEGA5.0*. Bootstrap values are provided to indicate reliability at each node.

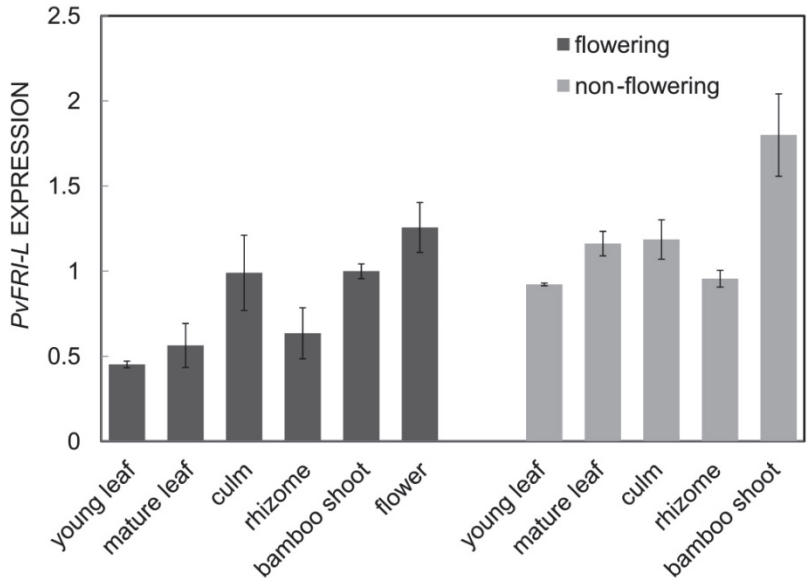

Fig. 2. The relative expression levels of *PvFRI-L* in different tissues of flowering and non-flowering *Phyllostachys violascens.* Data are means ± SDs from three biological replicates.

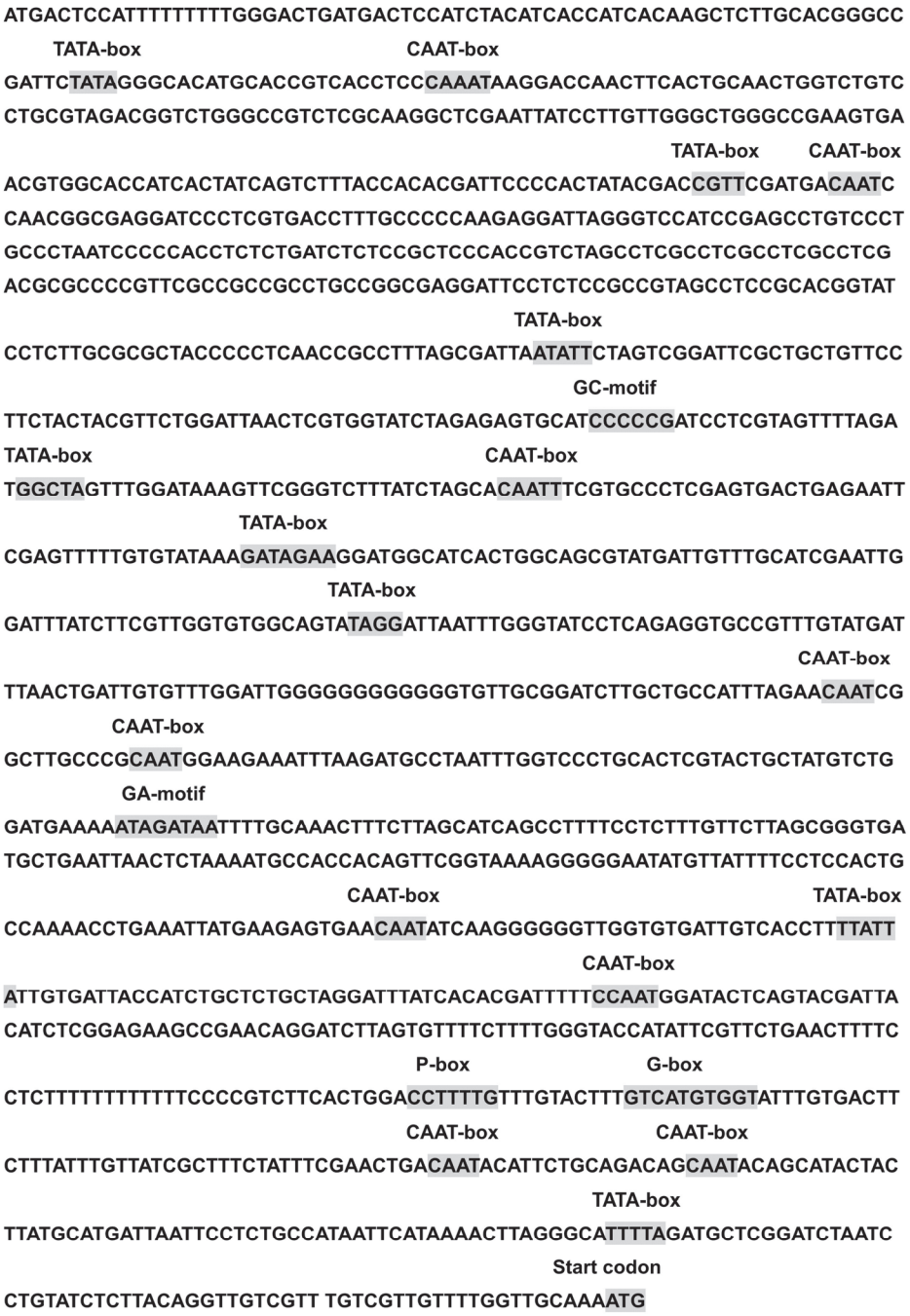

Fig. 3. A partial sequence of the promoter from *PvFRI-L*. The *gray colour* indicates specific elements of the promoter.

of *FRI* from *Arabidopsis*. A 1 500-bp fragment upstream of the start codon of the *PvFRI-L* gene was amplified, and a 1 500-bp promoter sequence of *FRI* was obtained from the *NCBI* database. Sequence alignment reveals that the two promoters shared a 36.06 % similarity. To investigate potential regulatory *cis*-acting elements, we characterized the promoter sequences using *PlantCARE* (Fig. 3). Both the promoters exhibited typical TATA-box and CAAT-box core elements. Other elements were also found such as those associated with a light response and hormones: Box4, G-Box, GAG- motif, TCA-motif, or TGA-motif (Table S1). However, P-box, 4cl-CMA2b, AC-I, AC-II, ATCT-motif, AUXRR-core, L-box, MNF1, MSA-like, and Sp1 were found to exist only in the *PvFRI-L* promoter (Table 1 Suppl.).

To determine the subcellular location of the PvFRI-L protein, we adopted the particle bombardment method. The green fluorescent protein (GFP) was used as marker in the *in vivo* targeting experiment. As shown in Fig. 4, the fusion protein PvFRI-L-GFP was localized in the

nucleus of the onion epidermal cells, whereas the control GFP was uniformly distributed in the whole onion cell.

We overexpressed *PvFRI-L* under the control of the CaMV *35S* promoter in *Arabidopsis* (WT) to determine its function. A total of 70 independent transgenic lines were generated and their phenotypes were analyzed in the T_1 generation. The *35S::PvFRI-L Arabidopsis* plants showed a late flowering phenotype (Fig. 5*A*,*B*,*E*). We further investigated expression of the floral integrators *FT* and *FLC* in four independent T_1 plants and found an increased transcription of *FLC* in the transgenic lines ($P < 0.05$), with a significantly decreased *FT* expression as compared to WT *Arabidopsis* (Fig. 5*C*,*D*).

PvFRI-L protein solubility was analyzed using a

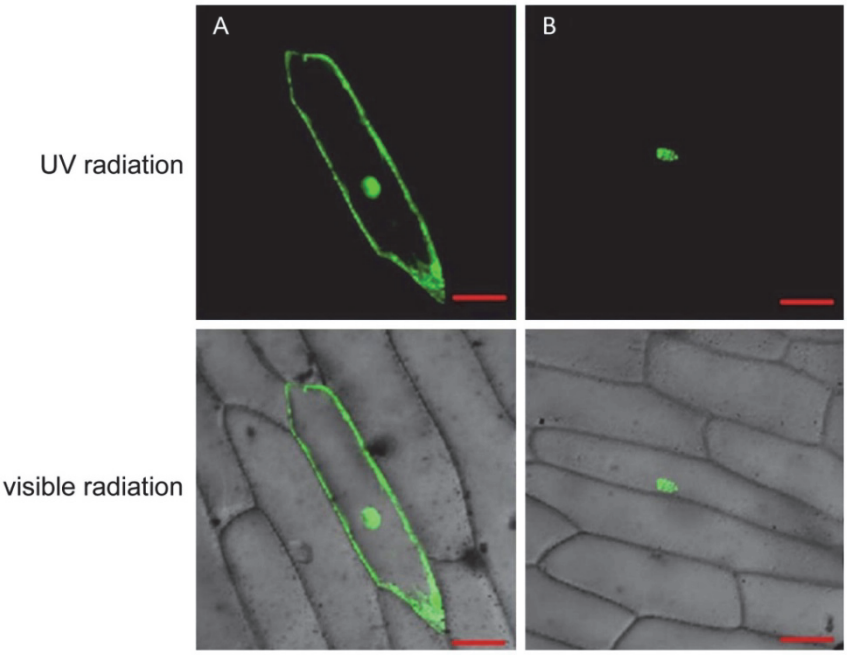

Fig. 4. The subcellular location of PvFRI-L in onion epidermal cells. *A* - Fluorescence of the control green fluorescent protein (GFP) plasmid is distributed throughout the cell; *B* - Fluorescence of the PvFRI-L-GFP fusion plasmid is strongly detected in the nucleus. *Bar* = 100 μm.

prokaryotic expression system. Although the expressions of *PvFRI-L* at 37 and 20 °C were equally high, the active fusion protein could be obtained only in the culture grown at 20 °C (Fig. 6*A*). The fusion protein had a molecular mass of 109.3 kDa and consitituted a 67.3 kDa PvFRI-L protein and 42 kDa tag protein. The soluble PvFRI-L-MBP fusion protein was purified with a dextrin sepharose high performance (Fig. 6*B*) medium. The protein sequence was further verified by *MALDI-TOF/TOF* (data not shown).

Discussion

In *Arabidopsis*, *FRI* acts as repressor and plays a key role in regulating flowering time. We cloned a gene from *Ph. violascens* named *PvFRI-L*. The protein was sorted into the FRIGIDA family for containing a FRIGIDA core domain. Phylogenetic analysis reveals that PvFRI-L is closely related to the FRI-like protein from the grass family and belongs to the FRL3 (III) subfamily. PvFRI-L was located in the nucleus similarly as *Arabidopsis* FRI (Hu *et al*. 2014). In *Arabidopsis*, *FRI* controls flowering time by regulating *FLC* and *FT*, either directly or indirectly. In the current study, the *35S::PvFRI-L* transgenic *Arabidopsis* showed a phenotype of a delayed flowering time. Relative to the WT plants, expression of *FLC* was upregulated, whereas *FT* expression was downregulated in the transgenic plants. These observations agree with previous findings for many *FRI*-like genes (Fang *et al*. 2008, Irwin *et al*. 2012, Chao *et al*. 2013). Therefore, we propose that *PvFRI-L* delays flowering time by regulating *FT* and *FLC* in transgenic *Arabidopsis*, either directly or indirectly. Our data further suggest that *PvFRI-L* is conserved in gene sequence, tissue expression pattern, and physiological function with many reported *FRI*-like genes in dicots. Thus, *PvFRI-L* might also bean important repressor of bamboo flowering.

The members of *FRI* subfamilies I and II are found in dicots but not in monocots (Risk *et al*. 2010). Our results suggest that monocot *PvFRI-L* has a functional homolog

with *FRI*-like genes of the *FRI* (I) subfamily in dicots. It is possible that the functions of *FRL3* (III) subfamily genes in monocots are similar to *FRI* (I) subfamily genes in dicots. Although this remains to be evaluated, the similarities and differences in *FRI* family members between monocots and dicots might result from evolutional divergence.

PvFRI-L was widely expressed in different tissues of *Ph. violascens*, similarly to reported *FRI*-like genes in dicots (Risk *et al*. 2010, Irwin 2012; Fig. 2). In the flowering plants, *PvFRI-L* expression was highest in flowers, whereas other *FRI*-like genes were expressed at

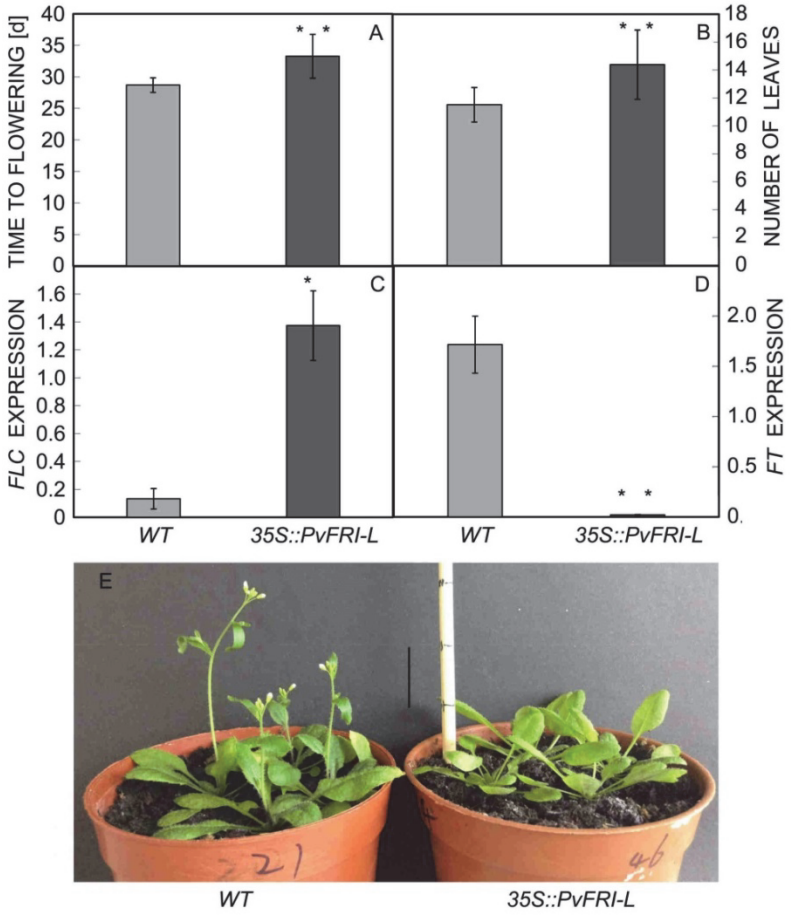

Fig. 5. Differences between transgenic plants and wild type (WT) *Arabidopsis thaliana*. *A* - Days to flowering for T_1 *35S::PvFRI-L* transgenic *Arabidopsis* plants and WT plants grown under long-day conditions (n = 70); *B* - the number of rosette leaves in T_1 *35S::PvFRI-L* transgenic and WT plants (n = 70); *C,D* - the relative expressions of *FLC* and *FT* genes in *35S::PvFRI-L* transgenic and WT plants (n = 3); *E* - a delayed flowering phenotype of *PvFRI-L* transgenic plants in comparison with WT plants. Means ± SDs, * and ** indicate significant differences at $P < 0.05$ and $P < 0.01$, respectively.

a low level in flowers. To elucidate whether the differential expression patterns of *PvFRI-L* and *FRI* were associated with regulation of their promoters, we analyzed the promoter sequences of *PvFRI-L* and *FRI*. It is known that *cis*-acting regulatory elements are important molecular switches and are involved in transcriptional regulation of gene activities controlling various biological processes in response to environmental stresses (Yamaguchi and Shinozaki 2006). There are many *cis*-acting elements in the promoter sequences of *PvFRI-L* and *FRI* (Table 1 Suppl.). Notably, the P-box, a gibberellin-responsive element, was found only in the promoter of *PvFRI-L* (Table 1 Suppl.). To determine whether GA affected *PvFRI-L* expression, the *Ph. violascens* plants were treated with GA. The mRNA level of *PvFRI-L* in leaves was significantly lower under the GA than mock treatment (Fig. 2 Suppl.). This result suggests that GA downregulated *PvFRI-L* expression *via* its promoter. Since a low concentration of GA is beneficial for flower bud differentiation of *Ph. violascens* (Lu *et al*. 2012), it may affect bamboo flowering by regulating *PvFRI-L* expression.

As important repressor in *Arabidopsis*, *FRI* responses to vernalization *via* regulating *FLC* (Clarke and Dean 1994, Michaels and Amasino 1999). Ectopic expression of *PvFRI-L* led to late flowering by mediating *FLC* expression in *Arabidopsis*. However, no *FLC* orthologs were found in monocots (Martin 2005), and bamboo

flowering may not have a vernalization requirement (Lin *et al*. 2010). *PvFRI-L* may play a role in regulating bamboo flowering through a specific pathway such as the GA pathway. This could explain in part why bamboo flowering is different from many other plants.

The full-length FRI and FRL1 proteins are insoluble when expressed in the *E.coli* system. However, a soluble protein can be expressed when the N- or C- terminal residues are deleted (Risk *et al. 2010*). But, this may impact subsequent functional and structural studies. Here, we report a strategy for expression of the soluble full-length protein. The protein was first fused with an MBP-tag in its N-terminus by using the prokaryotic expression vector *pMAL-c2X* and then expressed in *E. coli* Transetta (DE3) cells. Further successful purification of PvFRI-L would allow an *in vitro* functional and structural analyses possible.

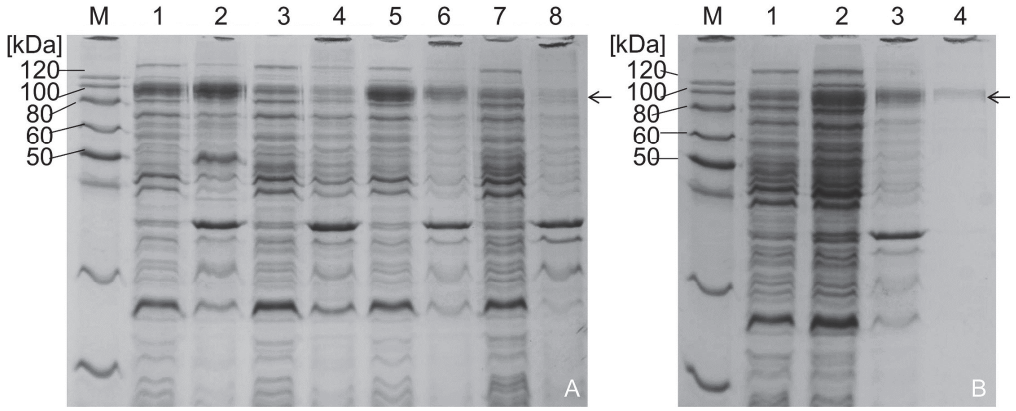

Fig. 6. The prokaryotic expression and purification of PvFRI-L. *A* - SDS-PAGE of the PvFRI-L-pHTT fusion protein in Transetta (DE3) *E. coli* cells. M: marker; *lanes* 1, 3, 5, 7: supernatant; *lanes* 2, 4, 6, 8: precipitation; *lane*s 1, 2: induced at 37 °C with isopropyl-thiogalactoside (IPTG); *lanes* 3, 4: cells grown at 37 °C without IPTG; *lanes* 5, 6: induced with IPTG at 20 °C; *lanes* 7, 8: cells grown at 20 °C without IPTG; *B* - *lane* 1: uninduced; *lane* 2: supernantant after induction; *lane* 3: pellet after induction; *lane 4*: purified PvFRI-L-MBP fusion protein by a dextrin sepharose high performance medium. The *arrows* indicate the location of the PvFRI-L-MBP fusion protein.

In summary, our results indicate that the structure, expression, and function of *PvFRI-L* are evolutionarily conserved between bamboo and eudicots. *PvFRI-L* may be important floral repressor in regulating bamboo flowering and this may be achieved through a response to GA. Our data provide valuble information for understanding the molecular mechanism of bamboo flowering.

References

Andersen, C.H., Jensen, C.S., Petersen, K.: Similar genetic switch systems might integrate the floral inductive pathways in dicots and monocots. - Trends Plant Sci. **9**: 105-107, 2004.

Clarke, J. H., Dean, C.: Mapping *FRI*, a locus controlling flowering time and vernalization response in *Arabidopsis thaliana*. - Mol. gen. Genet. **242**: 81-89, 1994.

Chao, Y., Yang, Q., Kang, J., Zhang, T., Sun, Y.: Expression of the alfalfa *FRIGIDA*-LIKE gene, *MsFRI-L*, delays flowering time in transgenic *Arabidopsis thaliana*. - Mol. Biol. Rep. **40**: 2083-2090, 2013.

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

Doyle, J.J., Doyle, J.L.: A rapid DNA isolation procedure for small quantities of fresh leaf tissue. - Phytochem. Bull. **19**: 11-15, 1987.

Fang, Q., Liu, J., Xu, Z., Song, R.: Cloning and characterization of a flowering time gene from *Thellungiella halophila*. - Acta biochim. biophys. sin. (Shanghai) **40**: 747-753, 2008.

Franklin, D.C.: Synchrony and asynchrony: observations and hypotheses for the flowering wave in a long-lived semelparous bamboo. - J. Biogeogr. **31**: 773-786, 2004.

Gielis, J., Goetghebeur, P., Debergh, P.: Morphological and biochemical aspects of flowering in bamboo-the development of model systems. - In: Chapman, G. (ed.): The Bamboos. Pp. 179-186. Linn. Soc., London 1997.

Hong, S.M., Bahn, S.C., Lyu, A. Jung, H.S., Ahn, J.H.: Identification and testing of superior reference genes for a starting pool of transcript normalization in *Arabidopsis*. - Plant Cell Physiol. **51**: 1694-1706, 2010.

Hu, X., Kong, X., Wang, C., Ma, L., Zhao, J., Wei, J., Zhang, X.M., Loake, G.J., Zhang, T.C., Huang, J.J., Yang, Y: Proteasome-mediated degradation of FRIGIDA modulates flowering time in *Arabidopsis* during vernalization. - Plant Cell **26**: 4763-4781, 2014.

Irwin, J.A., Lister, C., Soumpourou, E., Zhang, Y., Howell, E.C., Teakle, G., Dean, C.: Functional alleles of the flowering time regulator *FRIGIDA* in the *Brassica oleracea* genome. - BMC. Plant Biol. **12**: 21, 2012.

Janzen, D.H.: Why bamboos wait so long to flower. - Annu. Rev. Ecol. Syst. **7**: 347-391, 1976.

Jarillo, J.A., Piñeiro, M.: Timing is everything in plant development. The central role of floral repressors. - Plant Sci. **181**: 364-378, 2011.

Johanson, U., West, J., Lister, C., Michales, S., Amasino, R., Dean, C.: Molecular analysis of *FRIGIDA*, a major determinant of natural variation in *Arabidopsis* flowering time. - Science **290**: 344-347, 2000.

Lescot, M., Déhais, P., Thijs, G., Marchal, K., Moreau, Y., Van de Peer, Y., Rouze, P., Rombauts, S.: *PlantCARE*, a database of plant *cis*-acting regulatory elements and a portal to tools for in silico analysis of promoter sequences. - Nucl. Acids Res. **30**: 325-327, 2002.

Lin, X.C., Yuan, X.L,. Lin, R., Shi, Y., Fang, W.: [Studies on floral biology of *Phyllostachys violascens*.] - J. Fujian College Forest. **30**: 333-337, 2010. [In Chin.].

Livak, K.J., Schmittgen, T.D.: Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. - Methods **25**: 402-408, 2001.

Lu, Y.T., Yuan, X.L., Lin, X.C., Fang, W.: [Endogenous hormone changes during floral bud morphological differentiation of *Phyllostachys violascens.*] - J. Zhejiang A & F Univ. **29**: 161-165, 2012. [In Chin.].

Martin, J.: Isolation and characteriztion of flowering genes in ryegrass (*Lolium perenne* L.). - Ph.D. Thesis, Royal Veterinary and Agricultural Univ., Copenhagen 2005.

Michaels, S.D., Amasino, R.M.: FLOWERING LOCUS C encodes a novel MADS domain protein that acts as a repressor of flowering. - Plant Cell **1**: 949-956, 1999.

Michaels, S.D., Bezerra, I.C., Amasino, R.M.: FRIGIDA-related genes are required for the winter-annual habit in *Arabidopsis*. - Proc. nat. Acad. Sci. USA **101**: 3281-3285, 2004.

Peng, Z.H., Lu, Y., Li, L., Zhao, Q., Feng, Q., Gao, Z.M., Lu, H.Y., Hu, T., Yao, N., Liu, K.Y., Fan, D.L., Guo, Y.L., Li, W.J., Lu, Y.Q., Weng, Q.J., Zhou, C.C., Zhang, L., Huang, T., Zhao, Y., Zhu, C.R., Liu, M.X., Yang, X.W., Wang, T., Miao, K., Zhuang, C.Y., Cao, X.L., Tang, W.L., Liu, G.S., Liu, Y.L., Chen, J., Liu, Z.J.,Yuan, L.C., Huang, X.H., Lu, T.T., Fei, B.H., Ning, Z.M., Han, B., Jiang, Z.H.: The draft genome of the fast-growing non-timber forest species moso bamboo (*Phyllostachys heterocycla*). - Nat. Genet. **45**: 456-461, 2013.

Qi, F.Y., Hu, T., Peng, Z.H., Gao, J.: [Screening of reference genes used in qRT-PCR and expression analysis of *PheTFL1* gene in moso bamboo.] - Acta Bot. Boreal. **33**: 0048-0052, 2013. [In Chin.].

Risk, J.M., Laurie, R.E., Macknight, R.C., Day, C.L.: FRIGIDA and related proteins have a conserved central domain and family specific N- and C- terminal regions that are functionally important. - Plant mol. Biol. **73**: 493-505, 2010.

Roux, F., Touzet, P., Cuguen, J., Le Corre, V.: How to be early flowering: an evolutionary perspective. - Trends Plant Sci. **11**: 375-381, 2006.

Schlapi, M.R.: *FRIGIDA LIKE2* is a functional allele in Landsberg erecta and compensate for a nonsense allele of *FRIGIDA LIKE1*. - Plant Physiol. **142**: 1728-1738, 2006.

Shindo, C., Aranzana, M.J., Lister, C., Baxter, C., Nicholls, C., Nordborg, M., Dean, C.: Role of *FRIGIDA* and *FLOWERING LOCUS C* in determining variation in flowering time of *Arabidopsis*. - Plant Physiol. **138**: 1163-1173, 2005.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S.: *MEGA5*: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. - Mol. Biol. Evol. 2**8**: 2731-2739, 2011.

Udvardi, M.K., Czechowski, T., Scheible, W.R.: Eleven golden rules of quantitative RT-PCR. - Plant Cell **20**: 1736-1737, 2008.

Wang, Y.C., Klein, T.M., Fromm, M., Cao, J., Sanford, J.C., Wu, R.: Transient expression of foreign genes in rice, wheat and soybean cells following particle bombardment. - Plant Mol. Biol. **11**: 433-439, 1988.

Yamaguchi, S.K., Shinozaki, K.: Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. - Annu. Rev. Plant Biol. **57**: 781-803, 2006.

Yant, L., Mathieu, J., Schmid, M.: Just say no: floral repressors help *Arabidopsis* bide the time. - Curr. Opin. Plant Biol. **12**: 580-586, 2009.

Yaron, Y., Levy, Caroline, D.: The transition to flowering. - Plant Cell **10**:1973-1989, 1998.