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RESEARCH ARTICLE

Functional analysis of *PI*-like gene in relation to flower development from bamboo (*Bambusa oldhamii*)

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Abstract

Bamboo flowering owns many unique characteristics and remains a mystery. To investigate the molecular mechanisms underlying flower development in bamboo, a petal-identity gene was identified as a *PISTILLATA* homologue named *BoPI* from *Bambusa oldhamii* (bamboo family). Expression analysis showed that *BoPI* was highly expressed in flower organs and gradually increased during flower development stage, suggesting that *BoPI* played an important role in flower development. Ectopic expression of *BoPI* in *Arabidopsis* caused conversion of sepals to petals. *35S::BoPI* fully rescued the defective petal formation in the *pi*-1 mutant. BoPI could interact with BoAP3 protein *in vitro*. These results suggested that *BoPI* regulated flower development of bamboo in a similar way with *PI*. Besides flower organs, *BoPI* was also expressed in leaf and branch, which revealed that *BoPI* may involve in leaf and branch development. Similar to other MIKC-type gene, *BoPI* contained the C-terminal sequence but its function was controversial. Ectopic expression of the C-terminal deletion construct (*BoPI*-Δ*C*) in *Arabidopsis* converted sepals to petals; BoPI-Δ*C* interacted with BoAP3 on yeast two-hybrid assay, just like the full-length construct. The result implied that the C-terminal sequence may not be absolutely required for organ identity function in the context of *BoPI*.

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Introduction

Our understanding about floral organ formation mainly comes from the studies performed on the model eudicots species, *Antirrhinum* and *Arabidopsis*, on which the quartetic ABCDE model has been established to explain floral organ determination (Theissen 2001). Class B genes function specifically in the petal and stamen development (Kramer *et al.* 1998), though it contains only two members including *PISTILLATA* (*PI*). *PI* gene encodes a protein belonging to the MADS-box super family which typically contains four different functional domains. The 56 amino acid MADS domain has DNA-binding activity (Shore and Sharrocks 1995) while the K domain consisting of three putative amphipathic a-helices, mediates APETALA3 (AP3)–PI interaction (Yang *et al.* 2003). The I domain, located between the MADS and K domains is found indispensable for dimerization and functional specificity for *Arabidopsis* MADS proteins AP3, PI, AGAMOUS (AG) and APETALA1 (AP1) (Riechmann *et al.* 1996). The C-terminal domain contains a conservative PI motif in PI-homologues proteins whose function is controversial. It has been reported that the C-terminal sequence is dispensable for activity of *PI*-orthologous genes in *Arabidopsis*, pea (*Pisum sativum*) and legume (*Medicago truncatula*) (Berbel *et al.* 2005; Piwarzyk *et al.* 2007; Benlloch *et al.* 2009), whereas other study indicates that the C-terminal motif sequence is valuable for *PI* homologue function in regulating perianth organ formation in lily (Chen *et al.* 2012). These results suggest that the conserved motif sequence in the C-terminus of the *PI*-homologues genes may conduct divergent function among different species but need more data to substantiate.

Bamboo is a monocot plant that belongs to the grass family. Different from rice and maize, which are members of the grass family, bamboo owns many peculiar characteristics in flowering. It has long juvenile phase and usually dies after flowering. More remarkably, the initial of flowering is synchronous in a large scale and only a few species are annual flowering or periodic flowering. Further, there are many flowers exist in the inflorescence. Bamboo floral structures consist of lemmas, paleas, lodicules, stamens

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and pistils, which are also different from those in eudicots and nongrass monocots. Till date the mechanism of bamboo flowering remains unclear. To investigate the molecular mechanisms underlying flower development in bamboo, we isolated a *PI*-like gene from *B. oldhamii* and studied its expression pattern. CaMV *35S::BoPI* transgenic *Arabidopsis* assay was performed to analyse its putative characteristic and function. The C-terminal sequnence of *BoPI* was also investigated. This study will lay foundation to understand the bamboo flowering.

Materials and methods

Plant materials

Green shoots of *B. oldhamii* were cultured and proliferated in basal medium (Lin *et al.* 2010). *A. thaliana* wild-type (ecotype Columbia) and transgenic plants were grown in a controlled room temperature under 22°C with 16 h light / 8 h dark. The *Arabidopsis* pi-1 mutant line (CS77) in the Landsberg background was obtained from the *Arabidopsis* Biological Resource Center, Ohio State University, Columbus, USA.

Isolation of PI homologue in B. oldhamii

Total RNA was extracted from floral buds at various stages of development using RNAiso-plus (TaKaRa, Dalian, China) following instructions of the manufacturer. First-strand cDNA was synthesized from 1 μg of the DNase I-treated RNA, using oligo (dT) 18 adaptor primer and M-MLV reverse transcriptase (TaKaRa, Dalian, China). Isolation of the 3′ end of *BoPI* was carried out through a 3′RACE using the 3′full-RACE Core Set ver. 2.0 kit (TaKaRa, Dalian, China) following the manufacturer's protocol and gene-specific primer GSPBoPI (5′-AGATCA GTGTGCTGTGCGAC-3′). 5′ partial cDNA of *BoPI* was isolated by using the 5′ full-RACE kit (TaKaRa, Dalian, China) by the protocol from the manufacturer, and gene-specific primers APGSP1 (5′-TCGTCAGCCCATTCTCA AGT-3′) and APGSP2 (5′-GCTTCTCATCCCACAGTATC TT-3′). The full-length cDNA sequences of *BoPI* were amplified by PCR using forward primer BoPIF (5′-CTTGT GCGTCGCTAGCTGGA-3′) and reverse primer BoPIR (5′-TACAGAGCCAGTGGTGGATG-3′).

Sequencing and phylogenetic analyses

Sequence alignment and phylogenetic tree construction involved the use of MEGA 4. Multiple sequence alignment of protein sequences for class B MADS-box proteins retrieved from the NCBI server (http://www.ncbi.nlm.nih.gov/) involved use of ClustalW with default parameters. The phylogenetic tree was constructed using the neighbour-joining (NG) method with the parameters bootstrap (1000 replicates), p-distance model and pairwise gaps / missing data.

Quantitative real-time PCR (qRT-PCR)

RNA of lemma, palea, lodicules, stamen, pistil, leaves and branches in flowering bamboo and vegetative bamboo as well as different stages of flower buds were extracted using RNAiso Plus (TaKaRa, Dalian, China) following the manufacturer's instructions. First-strand cDNA was synthesized with PrimeScript®RT Master Mix (Perfect Real Time, TaKaRa, Dalian, China) according to the user manual. These cDNAs were used as templates for qRT-PCR.

For qRT-PCR, the procedure was carried out using SYBR Premix Ex *Taq*II (TaKaRa). Reactions were performed in 25 μL mixtures; each reaction contained 12.5 μL 1× SYBR GreenPCR Master Mix with 0.5 μL ROXII as a reference dye for real-time PCR, 1 μL forward primer (10 μM), 1 μL reverse primer (10 μM) and 50 ng of cDNA. No template controls were run to determine contamination and level of primer dimmer formation.

qRT-PCR reactions were run on a CFX96 detection system (Bio-Rad, Hercules, USA). The amplification condition was 95°C for 10 min, followed by 40 cycles of amplification (95°C for 10 s, 57°C for 30 s). Primers BPF (5′-AGCAAG ATGTCGCCCTGAGT-3′) and BPR (5′-CGGTAGGAAGA TTCAGCAGCC-3′) were used for *BoPI*. Primers BactinF (5′-GAGATGAACAAGAGGTCGG-3′) and BactinR (5′-GGTGGAGTCAATGATGAG-3′) were used as a normalization control. Ct values were determined after automatic adjustment of the baseline and manual adjustment of the fluorescence threshold. Data were analysed by CFX Manager software (ver. 1.6). Gene expression was analysed by normalized expression ($\Delta\Delta C_T$) using reference gene.

Plant transformation and transgenic plant analysis

Full-length *BoPI* and truncated *BoPI (BoP-ΔC*) were obtained by PCR amplification using forward primer 5′-GGGCCCATGGGGCGCGGGAAGATCGA-3′ and two reverse primers using TGA stop code 5′-TCTAGACTAGTT GTTCTCCTGCAGGT-3′ and 5′-TCTAGACTAGGCGAC ATCTTGCTGGT. The full-length cDNAs for *BoPI* and *BoPI-ΔC* were cloned into the binary vector *pCAMBIA*1301 under the control of the cauliflower mosaic virus (*CaMV*) *35S* promoter. Constructs were introduced into *Arabidopsis* by the floral-dip method (Clough and Bent 1998). Transformants were selected by using 50 μg mL^{-1} kanamycin and further verified by PCR.

Yeast two-hybrid analysis

Full-length cDNA for *BoPI*, *BoPI-ΔC*, *BoPI* (IKC) and *BoAP3* was obtained by PCR. The primer sequences for *BoAP3* were forward (5′-GGAATTCCATATGATGGG

GCGCGGCAAG-3′) and reverse (5′-CCGGAATTCTTAA CCGAGGCGCAGGTCGC-3′) and for *BoPI* and *BoPI-ΔC* were forward (5′-GGAATTCCATATGATGGGGCGCGG GAAGATCGA-3′) and two reverse primers (5′-CCGGA ATTCCTAGTTGTTCTCCTGCAGGT-3′) and (5′-CCGGA ATTCCTAGGCGACATCTTGCTGGT-3′). For *BoPI* (IKC) were forward (5′-CCGGAATTCATGACCTCGCTATCAAG AAT-3′) and reverse (5′-GGAATTCCATATGATGGGGCGCG GGAAGATCGA-3′). PCR fragments were ligated into the plasmid pGBKT7 (binding domain vector) or pGADT7 (activation domain vector) provided by the Matchmaker Two-Hybrid System (Clontech, Mountain View, USA). Recombinant plasmids were transformed into yeast by lithium acetate method (Gietz *et al.* 1992). A total of 2 μL yeast culture was plated on Ade–His–Leu–Trp + Xgal plates.

Results

BoPI gene isolation and sequencing

An expressed-sequence tag (EST) sequence with homology to *PI* was obtained from a *B. oldhamii* cDNA library which we built previously (Lin *et al.* 2010). The full-length sequence was obtained by rapid amplification of cDNA ends and the corresponding gene named *BoPI*. It was 1037 bp in length with 109-bp 5′-untranslated and 301-bp 3′-untranslated regions and a poly (A) tail in 5′-end. The open reading frame (ORF) encoded a protein with 209 amino acids. Alignment of the deduced amino acid sequence with that of class B MADS proteins from other species revealed a consensus PI motif (figure 1). A phylogenetic tree analysis classified BoPI protein into the subclade of monocot PI-type genes (figure 2).

Expression pattern of BoPI in floral buds and tissues

The expression of *BoPI* in different organs revealed that *BoPI* was expressed in all organs investigated and reached the highest level in floral organs (figure 3a). Further, *BoPI* was detected through three stages of floral bud development (figure 3b) and increased gradually with the development of the floral buds (figure 3c). In flower organ (figure 3d), *BoPI* was mainly expressed in lodicules and stamens (figure 3e). Besides lodicules and stamens, *BoPI* was detected in lemmas, paleas and pistils at relatively low level (figure 3e).

Ectopic expression of BoPI in wild type and pi Arabidopsis

To investigate whether *BoPI* could regulate petal development in eudicots, we transformed *Arabidopsis* with a recombined construct contained full-length cDNA of *BoPI* driven by the *CaMV 35S* promoter. We obtained 20 independent *35S::BoPI* transgenic *Arabidopsis* plants, which were phenotypically distinguishable from nontransformed wild-type plants. In wild-type flowers, the first whorl of green sepals remained tightly associated after flower opening (figure 4, a&b), whereas in transgenic plants, the first whorl sepals

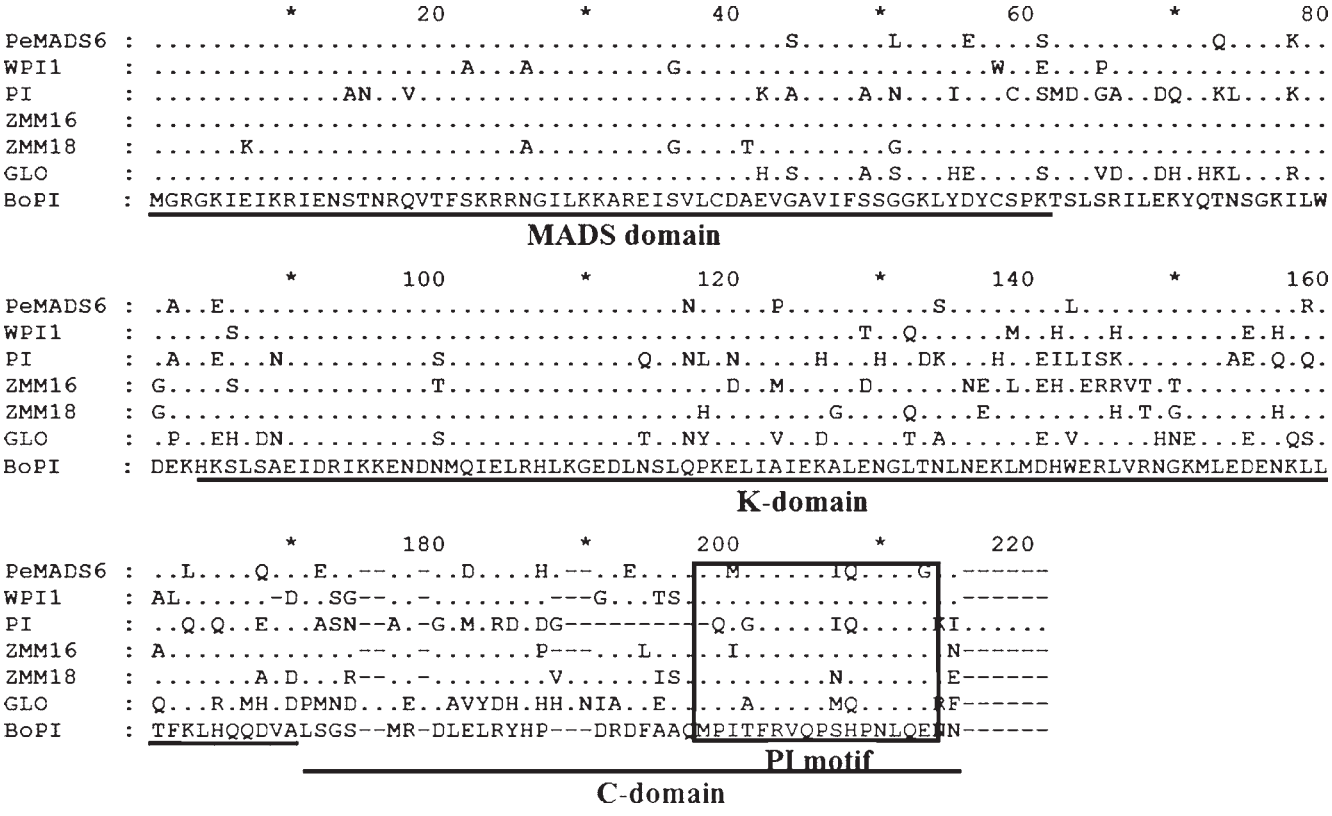

Figure 1. Sequence alignment of *BoPI* cloned from *B. oldhamii* and related class B MADS-domain proteins.

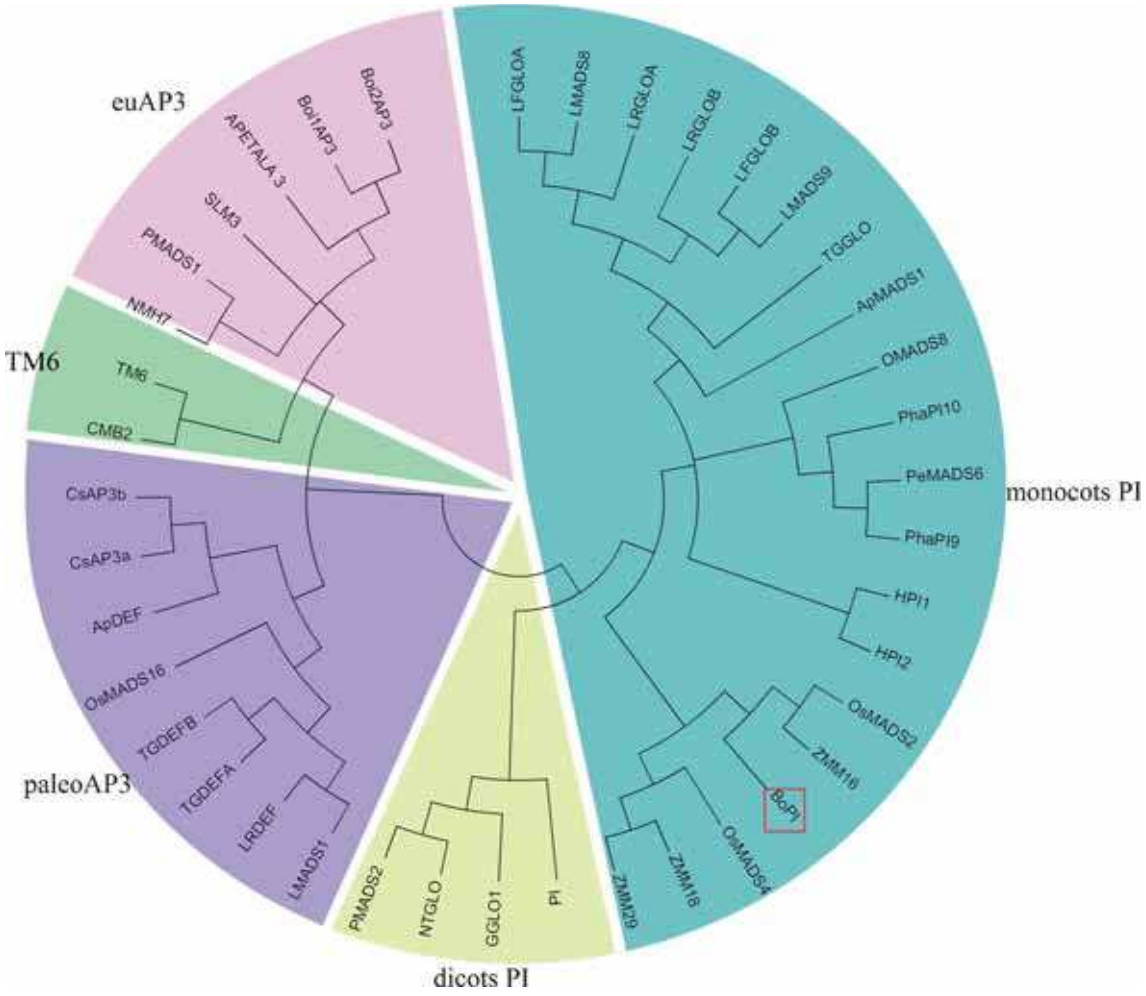

Figure 2. Phylogenetic analysis of class B MADS-domain proteins based on their amino acid sequences. AP3 (NM_115294), ApDEF (AB177941), ApGLO (AB079259), Boi1AP3 (U67453), Boi2AP3 (U67455), CMB2 (L40405), CsAP3a (AY948339), CsAP3b (AY948340), GGLO1 (AJ009726), HPI1 (AF134114), HPI2 (AF134115), LFGLOA (AB359186), LFGLOB (AB359187), LMADS1 (AF503913), LMADS8 (HQ698550), LMADS9 (HQ698551), LRDEF (AB071378), LRGLOA (AB071379), LRGLOB (AB071380), NMH7 (L41727), NTGLO (X67959), OMADS8 (HM140842), OsMADS2 (NM_001051547), OsMADS4 (NM_001062125), OsMADS16 (NM_001065095), PeMADS6 (AY678299), PhaPI9 (AY748818), PhaPI10 (AY771991), PI (NM_122031), PMADS1 (X69946), PMADS2 (X69947), SLM3(X80490), TGDEFA (AB094965), TGDEFB (AB094966), TGGLO (AB094967), TM6 (DQ539417), ZMM16 (AJ292959), ZMM18 (AJ292960) and ZMM29 (AJ292961).

were converted into petal-like structures and completely separated during flower opening (figure 4, c&d).

To investigate if *BoPI* could restore the petal-defective phenotype in *pi-1* mutant, we transformed *Arabidopsis pi-1* mutant plants with a recombinant construct containing *BoPI* driven by the *CaMV 35S* promoter. Seven plants were phenotypically distinguished from nontransformed wild-type plants (figure 4, e & f). In these *35S*::*BoPI* transgenic *pi-1* plants, the defective petal and stamen formation was fully restored (figure 4f). Further, these transgenic plants showed conversion of the first whorl sepals to petal-like structures (figure 4f).

Function analysis of the C-terminal sequence of BoPI

To gain insight into the function of C-terminal sequence of *BoPI*, the binary vector carrying truncated *35S*::*BoPI-ΔC* was introduced into *Arabidopsis* by *Agrobacterium*-mediated transformation. We obtained 15 independent *35S::BoPI-ΔC* transgenic *Arabidopsis* plants (figure 5, a&b). All plants were phenotypically distinguishable from untransformed wild-type plants. This transgenic phenotype was also observed in *35S::BoPI* plants (figure 4, c&d). PI and AP3 interact with each other on yeast two-hybrid assay (Honma and Goto 2001; Yang *et al.* 2003). To determine whether the C-domain of BoPI was necessary for heterodimer formation of the BoPI–BoAP3 complex, we tested the interaction of BoAP3 using three versions of BoPI: full-length BoPI (PI-MIKC), a MADS-deleted form (BoPI-IKC) and a C-domain deleted form (BoPI-MIK or BoPI-Δ*C*) (figure 5c). Full-length BoPI (PI-MIKC) and the C-domain deleted form (BoPI-MIK) interacted with the full-length BoAP3 but not the MADS-deleted form (BoPI-IKC) (figure 5d).

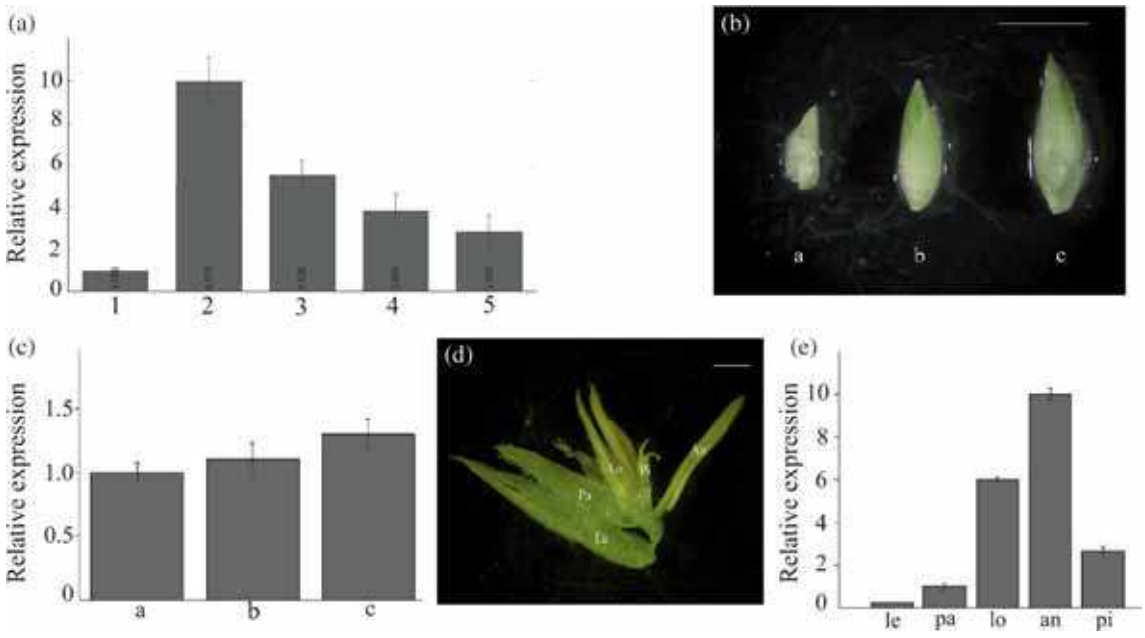

Figure 3. QRT-PCR analysis of *BoPI* expression. (a) The relative quantification in different tissues is shown at the bottom of each bar. 1, branch (flowering bamboo); 2, floral organs (flowering bamboo); 3, leaf (flowering bamboo); 4, branch (vegetative bamboo); 5, leaf (vegetative bamboo). (b) Flower buds in different stages. (c) The relative quantification in different stages of flower buds. (d) Flower organs are enclosed by two bracts called the palea (Pa) and lemma (Le). The perianth of each floret is represented by two transparent scales called lodicules (Lo). Generally three anthers (An) and a pistil (Pi), (e) The relative quantification in floral organs is shown: le, lemma; pa, palea; lo, lodicules; an, anthers; pi, pistil. Bar = 0.5 cm for b, bar = 0.1 cm for d.

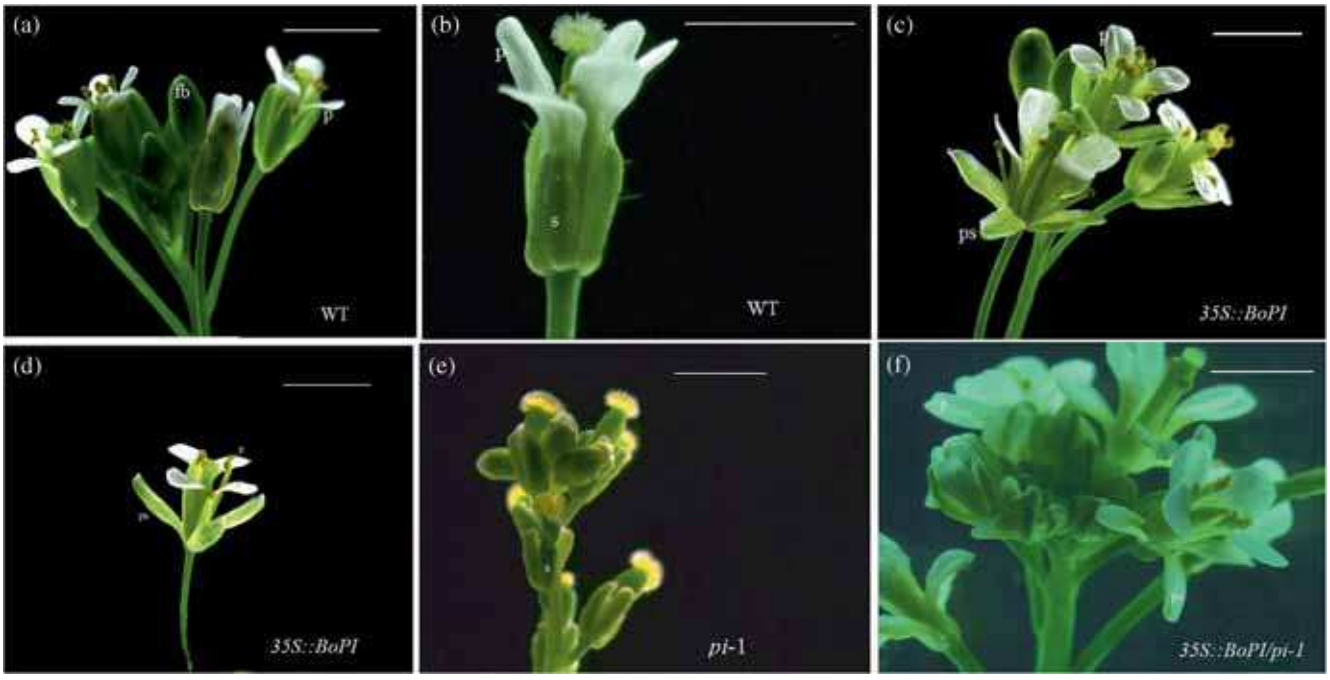

Figure 4. Phenotypic analysis of transgenic *A. thaliana* plants (*35S::BoPI*; *35S::BoPI-ΔC*). (a) A wild-type inflorescence contains flower buds (fb) and mature flowers with normal first whorl sepals (s) and second whorl petals (p). (b) Close-up observation of wild-type *Arabidopsis* flower which consists of four whorls of organs. (c) A *35S::BoPI* inflorescence contains petal-like sepals (ps) in the first whorl and normal petals (p) in the second whorl of the flower. (d) Close-up view of a *35S::BoPI* transgenic *Arabidopsis* flower, petal-like sepals (ps) and normal petals (p) were produced in the first and second whorls of the flowers, respectively. (e) A *35S::BoPI-ΔC* inflorescence contains petal-like sepals (ps) in the first whorl and normal petals (p) in the second whorl of the flower. (f) Close-up view of *35S::BoPI-ΔC* transgenic *Arabidopsis* flower, petal-like sepals (ps) and normal petals (p) were produced in the first and second whorls of the flowers, respectively. Bar = 0.2 cm for a, b, c, d, e, f.

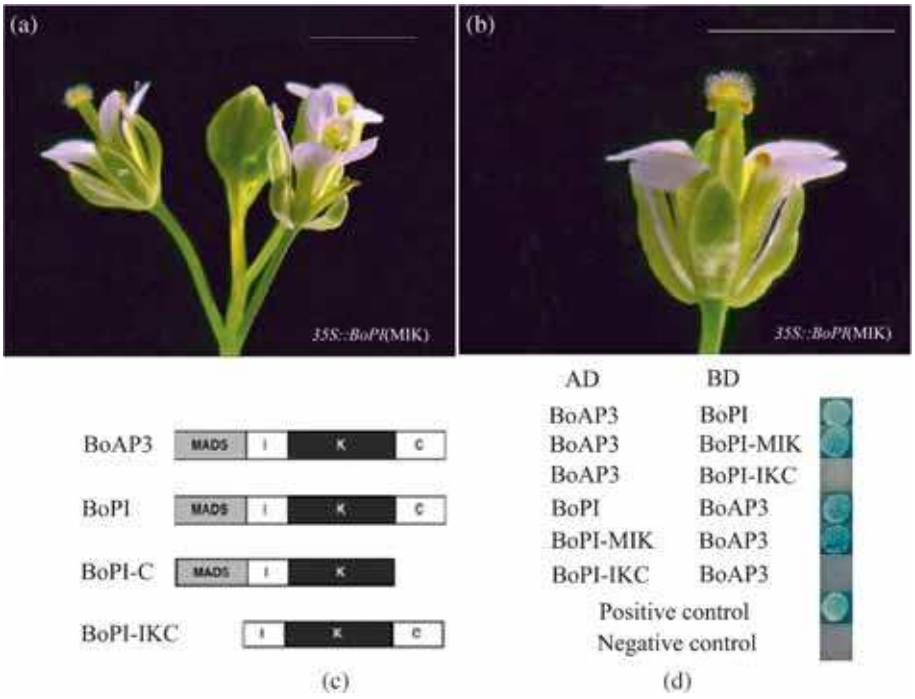

Figure 5. Phenotypic analysis of transgenic *Arabidopsis* (*35S::BoPI-ΔC*) and analysis of heterodimer formation between BoPI and BoAP3. (a) *35S::BoPI-ΔC* inflorescence contained petal-like sepals (ps) in the first whorl and normal petals (p) in the second whorl of the flower. (b) Close-up of *35S::BoPI-ΔC* transgenic *Arabidopsis* flower: petal-like sepals (ps) and normal petals (p) produced in the first and second whorls of the flowers, respectively. Bar = 0.2 cm for a, b. (c) Domain structure of BoPI and BoAP3 on yeast two-hybrid assay. (d) Interaction patterns of class B proteins in *B. oldhamii*.

Discussion

To investigate the flower development of bamboo, we isolated *BoPI* a *PI* homologous gene in this study. Based on the sequence alignment and phylogenetic analysis, *BoPI* was found to be closely related to grass *PI* homologous. Research on grass species, such as rice and maize, had shown that there were at least three *PI*-like genes in maize, *Zmm16*, *Zmm18* and *Zmm29* (Munster *et al.* 2001) and two in rice, *OsMADS4* and *OsMADS2* (Chung 1995). Yet, we only identified one *PI*-like gene in bamboo (*B. oldhamii*) by screening the cDNA library (Lin *et al.* 2010), this was probably due to low quantity sequencing. We then screened the transcriptome database in ma bamboo (*Dendrocalamus latiflorus*) and moso bamboo (*Phyllostachys heterocycla*) (Liu *et al.* 2012; Peng *et al.* 2013) and found that there were two *PI*-like genes in these species (data not shown). These results suggested that bamboo may have two *PI*-like genes.

To date, a lot of *PI* homologous genes have been isolated in monocot species but their expression pattern are different. *LMADS8* and *LMADS9* in lily (*Lilium longiflorum*) were strongly expressed in first and second whorls (Chen *et al.* 2012), while some *PI* homologous genes were expressed in all four flower organs, such as *TGGLO* in tulip and *PeMADS6* in *Phalaenopsis equestris* (Kanno *et al.* 2003; Tsai *et al.* 2005). *OsMADS4* and *OsMADS2* were detected in stamens and carpels in rice but maize *PI*-like genes were expressed in lodicules, stamens and carpels (Chung 1995; Munster *et al.* 2001). These results suggested that *PI* homologous genes may not be strictly restricted in regulating petal and stamen identity development during the flower development. For instance, a modified ABC model was proposed to explain *TGGLO* which was expressed in whorl 1 in tulip (Kanno *et al.* 2007). *BoPI* was detected in all whorls of flower organs (figure 3e), which demonstrated that *BoPI* may involve in floral organs development other than petal development. In addition, *BoPI* was not only expressed in flower organs but also in leaf and branch (figure 3a). Class B genes were often thought to be flower-specific in *Arabidopsis* and *Antirrhinum*, but in tomato they were also involved in other processes, such as fruit development (Busi *et al.* 2003). These data indicated that *BoPI* may play a role in the development of branches and leaves.

Studies on eudicot species, such as *Arabidopsis* and *Antirrhinum* demonstrated that functions of *PI* genes were highly correlated with their expression patterns; therefore, expression patterns may be good indicators of gene function (Ma and dePamphilis 2000). In flower organ, *BoPI* was mainly expressed in lodicules and stamens, suggesting that this gene played an important role in specifying lodicules and stamens development. Functional analyses suggested that ectopic expression of *BoPI* in transgenic wild type caused the first whorl sepals converted into petal-like structures (figure 4, c & d) and agreed with the phenotype in

Arabidopsis caused by constitutive expression of *PI* (Krizek and Meyerowitz 1996). The clearest demonstration that *BoPI* was a functional homologue to the B-function gene *PI/GLO* came from the functional complementation analysis. The *35S*::*BoPI* transgenic plants in *pi-1* mutant background restored petals and stamens defects, and produced petal-like sepals (figure 4f), suggesting that *BoPI* could regulate petal and stamen development in *Arabidopsis*. In addition, petal-like sepals were observed in *35S::BoPI*/*pi-1* probably due to the constitutive expression of *BoPI* driven by CaMV35S promoter. Lodicules are grass-specific organs and have been proposed to be homologous to eudicot petals. But this proposition remains controversial (Irish 2009). Similar position between petal and bamboo lodicules in floral organ, and high level expression of *BoPI* in lodicules, as well as the role of *BoPI* in regulating petal development in *Arabidopsis* supported the idea that lodicules represented eudicot petals.

Our results of constitutive expression in transgenic plants showed that *BoPI* worked as a functional homologue to *PI* both in bamboo and *Arabidopsis*. Similar result was observed in maize, *Zmm16*, a *PI*-like gene in maize, could also substitute for the endogenous *PI* gene of *Arabidopsis* to rescue the *pi-1* mutant *Arabidopsis* (Whipple *et al.* 2004). These data suggested that *PI* homologues shared remarkable functional conservation between monocots and eudicots.

Functional analysis and yeast two-hybrid analysis (figure 5) revealed that the truncated version (*BoPI-*ΔC) could function as *BoPI* and also displayed a positive interaction with BoAP3. This result suggested that C-terminal domain may be dispensable for floral organ identity function in the context of BoPI. The outcome of our experiments was different from lily (Chen *et al.* 2012). In their experiments, they showed that *PI* homologue function in regulating perianth organ formation. The function of C-terminal sequence require more detailed analyses.

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