RESEARCH Open Access

CsPI from the perianthless early-diverging Chloranthus spicatus show function on petal development in Arabidopsis thaliana

Kunmei Su1*, Zhenhuan Li1 and Zhiduan Chen2*

Abstract

Background: In the floral ABC model, B-class genes comprised of DEFICIENS (DEF)/APETALA3 (AP3) and GLOBOSA (GLO)/PISTILLATA (PI) had been proposed to involve in second and third whorl floral organ development. However, less is known about the function of B-class genes from early-diverging angiosperms. Chloranthaceae is one of the early-diverging angiosperm families. In this study, we characterized the role of the PI-like gene CsPI cloned from Chloranthus spicatus which have the simplest perianthless bisexual flowers.

Results: The expression profile analysis reveals high levels of CsPI mRNA in stamens in Chloranthus spicatus, with weak distribution in leaves and other floral organs. Nevertheless, CsPI rescued both stamen and petal development in Arabidopsis thaliana pi-1 mutants and caused partially conversion of sepals into petaloid organs in wild-type Arabidopsis thaliana plants. Yeast two-hybrid analysis showed that CsPI can form not only homodimers but also heterodimers with proteins encoded by Arabidopsis thaliana and Chloranthus spicatus AP3-like genes.

Conclusions: These results suggested that CsPI has an ancestral function on stamen development and that CsPI has capability to specify petal development in Arabidopsis thaliana. The finding indicates that the activity of the encoded PI-like proteins is highly conserved between the early-diverging Chloranthus and Arabidopsis. Moreover, our results appear to suggest that B-function genes may not play a role in perianth development in Chloranthus spicatus.

Keywords: Early-diverging angiosperm; B-class gene; CsPI; Perianthless; Petal development

Background

In plants, MADS-box genes are of particular interest because of the large size of the family and the critical developmental roles the members are known to play (Theissen et al. 2000). In the model plant Arabidopsis thaliana, five classes of MADS-box genes were involved in determining the development of floral organ identity. Functions of these genes have been summarized in the ABCDE model, which holds that different A, B, C, D and E class MADS-domain proteins interact to form functional “ternary” or “quartet” protein complexes that are responsible for establishing the various floral organ identities (Egea-Cortines et al. 1999; Honma and Goto 2001; Smaczniak et al. 2012; Theissen and Saedler 2001). In this model, the A class genes APETALA1 (AP1) and APETALA2 (AP2) control sepal formation; A, B [APETALA3 (AP3), PISTILLATA (PI)] and E (SEPALATA1/2/3) class genes together regulate petal formation; B, C [AGAMOUS (AG)] and E class genes control stamen formation; C and E class genes regulate carpel formation; and the D class genes SEEDSTICK (STK) are involved in ovule development (Theissen 2001; Theissen and Saedler 2001).

Numbers of MADS-box genes have already been identified in almost every group of flowering plants, including early-diverging angiosperms. These MADS-box genes involved in flower development provided convenience for further studies on the evolution of flowers. Up to now, a huge variety of inflorescence and floral morphologies are found among flowering plants. Phylogenetic studies based on morphology and genes have demonstrated that the origin and early diversification of flowers during evolution may have significantly contributed to

* Correspondence: primer673@126.com; zhiduan@ibcas.ac.cn
1 School of Environment and Chemistry Engineering, Tianjin Polytechnic University, Tianjin 300387, China
2 State Key Laboratory of Systematic and Evolutionary Botany, Institute of Botany, Chinese Academy of Sciences, Xiangshan, China

© 2014 Su et al; licensee Springer. This is an open access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.
the sudden occurrence of diverse angiosperms in a relatively short time span during the Early Cretaceous. Therefore, the MADS-box gene family controlling flower development in early-diverging plants gains more and more attention.

Among the early-diverging angiosperms, the ANITA groups (ANITA is the acronym of Amborella, Nymphaeaceae, Illiciales, Trimeniaceae and Austrobaileyaceae), which have undifferentiated perianth, are suggested the earliest extant angiosperms by phylogeny analysis (Hansen et al. 2007; Soltis et al. 2007a; Qiu et al. 1999; Zanis et al. 2002). Following this earliest diverging grade, Chloranthaceae is sister to the magnoliids and together this group is sister to a large clade that includes eudicots and monocots (Hansen et al. 2007; Moore et al. 2007). In fact, the family Chloranthaceae has been placed in many different positions in phylogenetic trees based on morphology and gene sequences, for example Piperales, Laurales, Magnoliidae, Austrobaileyales (reviewed by Hansen et al. 2007). The family Chloranthaceae contains four extant genera (Chloranthus, Sarcandra, Ascarina, and Hedyosmum) and approximately 70 species. Each of the four extant genera (Chloranthus, Sarcandra, Ascarina, and Hedyosmum) has distinctive morphological

In the floral ABC model, B-class genes comprised of DEFICIENS (DEF)/APETALA3 (AP3) and GLOBOSA (GLO)/PISTILLATA (PI) had been proposed to involve in second and third whorl organ development. In eudicots, functions of AP3-like and PI-like genes are basically conserved in petal and stamen development (for review Soltis et al. 2007b; Becker and Theissen 2003). In the core eudicot A. thaliana, single mutant of AP3 and PI caused the homeotic transformation of petals to sepals in the second whorl and of stamens to carpels in the third whorl (Jack et al. 1992; Goto and Meyerowitz 1994). In basal eudicots Aquilegia vulgaris and Papaver somniferum (Ranunculales), B-class genes are also found to be necessary for the development of both petals and stamens (Drea et al. 2007; Kramer et al. 2007). In the basal eudicot California poppy (Eschscholzia californica), mutant of the PI-lineage gene SEI shows homeotic changes characteristic of floral homeotic B class mutants (Lange et al. 2013). In monocots, heterologous expression studies suggested that B-class genes play the same role as in eudicots, although data from heterologous expression studies are difficult to interpret (Bartlett and Specht 2010). silky1 (si1), a mutant of Zea mays AP3-like gene, shows homeotic conversions of stamens into carpels and lodicules into palea/lemma-like structures (Ambrose et al. 2000). Consistent with this, Silky1 and Zmm16 (PI-like gene of Zea mays), are also able to rescue petal development in A. thaliana ap3 and pi mutant, respectively (Whipple et al. 2004). The PI homologs from Agapanthus praecox and Elaeis guineensis, monocot flowers with petaloid inner perianth organs, also have been shown to rescue the pi-1 mutant of A. thaliana (Nakamura et al. 2005; Adam et al. 2007). These data appear to suggest that the function of B-class genes is conserved in monocots and eudicots. However, less is known about the function of B-class genes in early-diverging angiosperms. Therefore, we preferentially selected the B class genes from the early-diverging angiosperm Chloranthus spicatus for functional analysis.

In Chloranthus spicatus, the AP3-like gene CsAP3 has been investigated through in situ hybridization expression analyses and transformation experiments. CsAP3 is exclusively expressed in male floral organs, but is not detected in the dome-shaped spike primordia, bract primordial and leaves (Li et al. 2005). Only weak complementation was seen in the third floral whorl (stamen), nevertheless, no complementation was seen in the second floral whorl (petal) when CsAP3 was expressed in A. thaliana ap3-3 mutant plants (Su et al. 2008). No ectopic gain-of-function in the fourth floral whorl was observed when CsAP3 was ectopically expressed in wild-type A. thaliana plants. However, less research work on the function of the PI-like gene from C. spicatus was reported although complete coding sequence of CsPI has already been isolated previously (Su et al. 2008). Therefore, functional analysis of CsPI is necessary.

To investigate the role of the PI-like gene CsPI in floral development, the expression pattern was analyzed using quantitative real-time PCR analysis. To complement the results of the expression pattern analyses, we transformed 35S::CsPI into wild-type A. thaliana plants and 5D3::CsPI into the pi-1 mutant plants. To explore how they worked, we tested interactions of proteins by employing the yeast two-hybrid system.

Methods

Plant material and RNA extraction
C. spicatus used in our experiments were cultivated in the Botanical Garden, Institute of Botany, Chinese Academy of Sciences, Beijing. Total RNA was prepared using Trizol (Invitrogen). Then poly(A) mRNA were purified using Oligotex mRNA Mini Kit (Qiagen) and the first-strand cDNA was synthesized with Superscript III (Invitrogen) (Su et al. 2008).

Vectors construction

Full-length CsPI cDNA sequence fragment was cloned into the binary vector pCAMBIA 1301 (Cpgbiotech). Primers YCsPI and PTA were used in PCR amplification. The cauliflower mosaic virus (CaMV) 35S promoter (Benfey and
Chua 1990) was fused to the cDNA to drive nearly ubiquituous expression of all the transgenes in a wild-type background. Furthermore, to avoid ectopic expression of these transgenes, in another series of experiments the A. thaliana AP3 promoter 5D3 was used to drive expression of the transgenes in whorls 2 and 3 of developing A. thaliana flowers in the pi-1 mutant background (Lamb and Irish 2003). The promoter sequence was amplified by PCR from DNA extracted from leaves of wild-type A. thaliana using primers in our previous studies (Su et al. 2008).

**A. thaliana** transformation and genotyping

The plasmid constructs were transformed into wild-type Landsberg erecta A. thaliana plants and pi-1 mutant plants respectively, by the floral dip method (Clough and Bent 1998).

Seeds of the transgenic A. thaliana plants were selected on solid 0.5 × MS medium (Murashige and Skoog 1962) containing 50 mg/L rifampicin at 4°C for 2 days, and then were transferred to the greenhouse under long-day condition (16 h light/8 h dark) at 22°C for 10 days. As the control, seeds of wild-type A. thaliana were cultured on solid 0.5 × MS medium as described above. Subsequently, the wild-type and transgenic seedlings were transplanted to soil and were cultured at 22°C with 16 h light and 8 h dark.

Homozygous pi-1 plants were identified using a dCAPS marker, in which BspHI cuts the wild-type sequence (Lamb and Irish 2003), but the site is abolished by the pi-1 mutation. All observed phenotypes were heritable and segregated as dominant traits. Morphological analysis was performed on the T1 generation.

**Primers used in experiments**

Primers used in our experiments were all showed in Table 1.

**Quantitative real-time PCR analysis**

Total RNA was extracted from roots, stems, leaves, bracts, stamens and carpels of C. spicatus for expression pattern analysis of CsPI. For their constitutive and complementary expression analysis, total RNA was extracted from the inflorescences of A. thaliana carrying transgenic constructs. After the purification of RNA samples, first-strand cDNA was synthesized with Superscript™ III Reverse Transcriptase (Invitrogen) in a 20μl reaction volume. Each kind of sample was prepared three times as described above. Quantitative real-time PCR was performed with the iQ SYBR Green supermix (Bio-Rad) in a Rotor-gene 3000 classic real-time PCR machine (Corbett Research). PCR conditions were 15 min at 95°C, followed by 40 cycles of 30 s at 94°C, 30 s at 56°C and 30 s at 72°C. To detect the expression pattern of CsPI in C. spicatus, the C. spicatus housekeeping gene 18S rRNA was used to normalize the amount of the cDNAs added to the reaction. To analysis the expression of CsPI in wild-type and pi-1 mutant A. thaliana, the A. thaliana housekeeping gene ACTIN was used as normalization control. Specific primer pairs were designed with the help of Beacon Designer 4 software (Premier Biosoft International). These primers include CsPIReTi-F2, CsPIReTi-R2. In each experiment, two standard curves were applied for the relative quantification of the cDNA copies. Each sample was analyzed three times to determine reproducibility.

**SEM observation**

All flowers collected from the transgenic wild-type A. thaliana plants were immediately fixed with FAA (formalmin: acetic acid: 50% ethanol = 5: 6: 89). Then these flowers were dried and coated as described previously (Xu et al. 2005), and observed with a Hitachi S-800 scanning electron microscope (SEM).

**Yeast two-hybrid assays**

Yeast two-hybrid assays were performed using the GAL4-based MATCHMAKER Two-Hybrid System (Clontech). Saccharomyces cerevisiae strain AH109, GAL4 activation domain (AD) expression vector pGADT7 and GAL4 DNA-binding domain (DNA-BD) expression vector pGBK7 were used. Full-length cDNA of CsAP3, CsPI, were amplified with Ncol restriction enzymes digest site overlapping the start codon and BamHI at the 3’ end. EcoRI and BamHI sites were introduced to generate

---

**Table 1 Primers used in this paper**

| Name of primers | Sequence of primers |
|-----------------|---------------------|
| CsPIReTi-F2     | 5′-GGGTTAATGCTATCATCTTGACATC-3′ |
| CsPIReTi-R2     | 5′-ATGGTCTTGTGGATGGAACAGGAA-3′ |
| qActup          | 5′-CGTATTGACGAAAGGATACTAC-3′ |
| qActdown        | 5′-CACATCTGGAGAGGTGTCTAC-3′ |
| 18S primerF     | 5′-CCGGTACCCATCCACGAAAGAA-3′ |
| 18S primerR     | 5′-TCGACACTCCGGCGTC-3′ |
| AtPINde1        | 5′-GATCTCTATGAGGTGAGATAGAAAG-3′ |
| AtPINNoM        | 5′-TGATTGAATTCGTGTTCTTCCAT-3′ |
| YCsAP3          | 5′-CCGGCCTAGGGAAGAAGGATT-3′ |
| CsAP3NoM        | 5′-TCTATCATATGTGAGCGCTAC-3′ |
| YCsPI           | 5′-CGGGCCATGAGGTGGAGATAC-3′ |
| CsPINoM         | 5′-TGTCGAGATCTCTTGGGCTCC-3′ |
| AtAP3Nd1        | 5′-GATCTCATATGAGCGAGGAAAG-3′ |
| AtAP3NoM        | 5′-TCTATGAGATCTCCGCTAACAC-3′ |
| PIINT-2         | 5′CCCAATTTATGATATCTGTCAG-3′ |
| PI-1            | 5′-TACCAAGAATGTTATGCGACAGAAATACATCATG-3′ |
| PTA             | 5′-CCGGATCTCAGAGCGCCCG(T)17-3′ |
MADS-deleted CsAP3 and CsPI, for cloning into pGADT7 and pGBKT7, respectively. All constructs were verified by restriction enzymes analyses and sequencing. The yeast strain AH109 was transformed with above constructs according to the manufacturer’s protocol of small-scale LiAc yeast transformation procedure. Confirmation of the transformants and interaction analyses were performed as previously described (Shan et al. 2006; Su et al. 2008). The transformants co-transformed plasmids of AP3 and PI in absence of MADS domain from A. thaliana were used as a positive control (Yang et al. 2003). The transformants containing plasmids pGADT7 and pGBKT7 were used as a negative control.

Results
Expression patterns of CsPI in C. spicatus
In order to get a clue about the function of CsPI, mRNA accumulation was analyzed by quantitative real-time PCR. As shown in Figure 1, CsPI mRNA was absent in roots and stems. Only weak expression of CsPI was found in leaves and bracts (Figure 1). Some expression was expressed in carpels and the strongest expression was detected in stamens (Figure 1). The expressing quantity of CsPI in stamens was 3 times what in carpels. These data suggested that CsPI was expressed broadly in C. spicatus. The expression pattern is similar to those of the PI-like genes from other early-diverging angiosperms (Kim et al. 2005; Lv et al. 2012).

Ectopic expression of CsPI in wild-type A. thaliana
To further explore the function of CsPI in floral development, we transformed wild-type A. thaliana plants with the cDNA under the control of the cauliflower mosaic virus (CaMV) 35S promoter. We obtained 42 A. thaliana transgenic plants, 26 of which displayed homeotic changes. The vegetative organs of these plants were normal, and no effect in flowering time was detected (data not shown). Phenotypic alterations were observed only in flowers. Flowers of these 35S::CsPI transgenic plants seemed to have two whorls of petals (Figure 2E and F). Sepals in the first whorl were partially converted into petaloid organs (Figure 2E, F, G). These petaloid structures expanded like petals although its size was smaller than that of petals (Figure 3E and F). Moreover, flowers of some 35S::CsPI transgenic plants, such as line 13 and 19 showed 5 petals and 5 petaloid sepals (Figure 2F). Noticeably different from those of the wild-type flowers, margins of these petaloid sepals consist of white tissue and surface were smooth (Figure 2F and G, compare F with A and G with B separately). Examination by SEM revealed that the surface of these regions in the 35S::CsPI transgenic plants was a mosaic composed of both sepal and petal cells, while these cells were similar in shape and size (Figure 2H, compare H with C, D). However, flowers of 35S::CsPI-3 and 35S::CsPI-25 were similar to wild-type A. thaliana. To find whether the severe phenotypes were correlated with CsPI expression in the transgenic plants, quantitative real-time PCR analysis was performed. Transgenic lines with only 4 petals and 4 petaloid sepals, represented by 35S::CsPI-5 and 35S::CsPI-15, showed lesser RNA expression of CsPI than 35S::CsPI-13 and 35S::CsPI-19 (Figure 3). However, the expression of CsPI in these 4 lines was obviously higher than what in lines 35S::CsPI-3 and 35S::CsPI-25. These data demonstrated that the accumulation levels of CsPI transcripts in different lines are consistent with phenotypic alterations.

Functionality of CsPI in pi-1 mutants of A. thaliana
In addition to the wild-type A. thaliana, CsPI was also transformed into A. thaliana pi-1 mutant plants. In this transformation experiments, the A. thaliana AP3 promoter 5D3 was used to drive expression of CsPI in whorls 2 and 3 of developing A. thaliana flowers in the pi-1 mutant background (Lamb and Irish 2003).

We obtained 21 independent 5D3::CsPI transgenic pi-1 plants. Among of them, 10 (47.6%) showed full rescue and 4 (19%) showed strong rescue. In flowers of fully rescued plants, petals had the shape of wild-type petals but were somewhat smaller (Figure 4D). Moreover, the epidermal cells of rescued petals (Figure 4I) resembled those of the wild-type which were characteristically rounded (Figure 4I). Petals of strongly rescued flowers were small and green (Figure 4C), with the epidermal petal cells which were more similar to those of wild-type petals than sepals (Figure 4H). The third-whorl floral organs of fully rescued flowers were not fully extended stamens with

![Figure 1 The detection of the expression of CsPI](http://www.as-botanicalstudies.com/content/55/1/21)
fertile pollen grains (Figure 4D), while the third floral whorl of strongly rescued flowers was mosaic organs between carpel and stamen (Figure 4E). Weak rescue was also seen for 7 (33.3%) lines, in which neither stamens nor petals were rescued (Figure 4B).

Here, transgene expression was also determined by quantitative real-time PCR, which demonstrated that level of phenotypic rescue is correlated with the expression level of transgene (Figure 5). For example, the expression of CsPI was clearly higher in fully rescued 5D3::CsPI-13 and 5D3::CsPI-20 than in strongly rescued 5D3::CsPI-7, while the expression of CsPI was clearly lesser in weakly rescued 5D3::CsPI-2 and 5D3::CsPI-5 than in strongly rescued 5D3::CsPI-7.

Interaction pattern analysis of CsPI
To investigate the interaction patterns of CsPI proteins to learn how they worked, yeast two-hybrid assays were performed. As positive control, we investigated the interaction between A. thaliana AP3 and PI proteins, which was marked as AtAP3 and AtPI respectively. As negative controls, we detected the growth of transformants co-transformed with the fusion plasmid containing the protein and the pGADT7 or the pGBKT7 free vector.

In our experiments, interaction patterns of the full-length and the MADS-deleted CsPI, CsAP3, AtPI and AtAP3 were tested. As negative controls, we demonstrated that transformants co-transformed with the fusion plasmid containing the protein and the pGADT7 or the pGBKT7 free vector did not grow on the selective medium (Figure 6H, I). As positive control, the MADS-deleted AtPI and AtAP3 sequence formed heterodimers (Figure 6) (Yang et al. 2003). Dimerization could not be observed for full-length CsPI, CsAP3, AtPI and AtAP3 (data not shown). However, the MADS-deleted CsPI can form heterodimers with AtAP3 and CsAP3 (Figure 6 A and B). Since specificity of heterodimerization is largely based on the sequence of the I-domain and K-domain (Kaufmann et al. 2005; Riechmann et al. 1996; Yang et al. 2003), this applies very likely also to the full length...
(MIKC) sequence. Moreover, the MADS-deleted CsPI can also form homodimerization (Figure 6C), a feature which has been found also for some other AP3-like and PI-like proteins of non-core eudicots, including monocots such as lily (Lilium) and tulip (Tulipa), but not in core eudicots (Hsu and Yang 2002; Su et al. 2008; Tzeng et al. 2004; Winter et al. 2002). However, the MADS-deleted protein AtPI was not able to interact with itself (Figure 6G).

Discussion

According to the ABCDE model, B class genes, including both PISTILLATA (PI) and APETALA3 (AP3) homologs, contribute to petal and stamen development. Functional analysis concentrated on monocots and eudicots suggested that the function of the B-class genes is conserved. In this study, we demonstrated the functional conservation of the PI-like genes between the early-diverging angiosperm C. spicatus and A. thaliana.
To identify the function of CsPI in *C. spicatus*, we detected the expression pattern through quantitative real-time PCR. CsPI was expressed in a broad range, including the leaves, bracts, stamens and carpels. The expression pattern of CsPI was different from that of the other *C. spicatus* B-class gene CsAP3, which was found to be exclusively expressed in stamens (Li et al. 2005). The expression pattern was also different with that of the core eudicots *AP3*/PI genes, which are expressed restricted to the second and third whorls (reviewed by Kim et al. 2005). However, this pattern was consistent with those of their counterparts in early-diverging angiosperms. Kim et al. also found that PI transcripts were detected in petals, stamens and carpels in early-diverging such as in *Amborella trichopoda* and *Nuphar advena* (Kim et al. 2005). Similarly, *AcPI* in monocot *Ananas comosus* was expressed in stems, leaves, bracts and sepals, petals, stamens and carpels (Lv et al. 2012). The broader range of expression of *PI* homologs is inferred to be the ancestral pattern for all angiosperms (Kim et al. 2005). However, it is worth mentioning that strong expression of CsPI was only detected in stamens. Although MADS-box gene function is often correlated with gene expression pattern, transient and/or weak expression does not correspond to a known genetic function (reviewed by Kim et al. 2005). Therefore, CsPI may mainly function on stamen development in *C. spicatus*. Compatible with this hypothesis, the complementation of the third whorl floral organs of the *pi*-1 mutant plants were observed when CsPI was expressed under the control of the *AP3* promoter *SD3*. The phenotype is also observed in *pi* mutant plants transformed with *PI* and the *PI*-like gene *Zmm16* from maize under the control of the *A. thaliana* *AP3* promoter (Lamb and Irish 2003; Piwarzyk et al. 2007; Whipple et al. 2004). These results suggested that the *PI*-like gene CsPI from the early-diverging *C. spicatus* conserved the function on stamen development.

Most interestingly, CsPI also showed function on the petal development when it was expressed in wild-type or *pi* mutant *A. thaliana* plants. Like to those of the *A. thaliana* *pi* mutant plants expressing *PI* or the *PI*-like gene *Zmm16* (Lamb and Irish 2003; Piwarzyk et al. 2007; Whipple et al. 2004; Yang et al. 2003), the second whorl floral organs were rescued when *SD3::CsPI* was transformed into *pi*-1 mutant plants. In line with this, the *3SS::CsPI* transgenic plants exhibited a partial conversion of sepals to petaloid organs. This phenotype is similar to that of the *3SS::PI* *A. thaliana* plants. It has been reported that the *3SS::PI* *A. thaliana* also modifies sepals into petaloid organs but no ectopic stamen is formed (Krizek and Meyerowitz 1996; Lamb and Irish 2003; Yang et al. 2003). The only slight difference is that flowers of some *3SS::CsPI* plants showed an increase in the number of the first and the second floral organs. This can be attributed to the different expression levels as shown in quantitative real-time PCR analysis and northern blot analysis. Alternatively, the expression level of CsPI may be correlated with the number of petals.

As to why CsPI showed functions in *A. thaliana* similar to those of *PI*, it is possibly provided by the yeast two-hybrid analysis, which revealed that CsPI proteins can form heterodimers with AtAP3 proteins. It has been reported that the *A. thaliana* *AtAP3* gene was faintly expressed in the first floral organ as well as in the second and the third floral organs (Jack et al. 1992; Smaczniak et al. 2012). Therefore, the fact of transforming sepal into petaloid structures or rescue the second and the third whorl of the *pi*-1 mutant plants might be due to the same fact as that of the *A. thaliana* genes, both *AP3* and *PI* should be present together with *SEP* genes (Krizek and Meyerowitz 1996).

Alternatively, homodimers of CsPI may also be able to act to specify petals with AtAP3. As shown, CsPI can form homodimers besides heterodimers. This feature also has been found for some other class B proteins of gymnosperms and non-core eudicots (Chen et al. 2012; Hsu and Yang 2002; Liu et al. 2013; Liu et al. 2010; Su et al. 2008; Tseng et al. 2004; Winter et al. 2002; Yang et al. 2003), but not in core eudicots. For example, proteins transformed by *Lilium longiflorum* *PI*-like genes *LMADS8* and *LMADS9* can also form homodimers besides heterodimers (Chen et al. 2012). It’s worth noting that flowers of the *A. thaliana* overexpressed the *Lilium longiflorum LMADS8*/9 (PI-like) also showed partially transformation of sepals to petaloid organs and homodimers.
of LMADS8/9 were able to bind to the CARG1 of AtAP3 (Chen et al. 2012). Moreover, C-terminal deleted HoPI (PI-like) proteins from Hedyosmum orientale (Chloranthaceae) lost the petal identity function in A. thaliana as they failed to form homodimers (Liu et al. 2013). For these facts, we can’t exclude such a scenario for homodimers of CsPI to act in petal development in A. thaliana. This interaction pattern may represent an ancient flexible interaction of AP3 and PI homologous proteins (Liu et al. 2013).

This finding that CsPI has capability to specify petal identity in A. thaliana was compatible with the view that the perianthless state of C. spicatus is derived rather than ancestral (Li et al. 2005). As to the loss of petals, we prefer the hypothesis that the B function, which requires the concerted expression of AP3 and PI homologues, may not contribute to petal development in Chloranthaceae (Liu et al. 2013). In H. orientale (Chloranthaceae), HoPI was broadly expressed in all floral organs, whereas HoAP3 was restricted to stamens (Liu et al. 2013). In perianthless C. spicatus, CsPI reported here, was also broadly expressed in all floral organs, but CsAP3 was restricted to stamens (Li et al. 2005). Therefore, the overlap of AP3 and PI homologue expression is limited to the stamens in Chloranthaceae. Yet, coordinated expression of the AP3- and PI-like genes is correlated with the identity of petaloid organs (reviewed by Liu et al. 2013). These data appear to suggest that the main reason for the loss of petals in Chloranthaceae may not be the floral homeotic B-function. Nonetheless, we still can’t rule out the possibility that changes in cis-regulatory elements or trans-regulatory factors that regulate B-class genes are causally linked to the greatly reduced perianth in Chloranthus (Li et al. 2005). As shown in this paper, some 35S::CsPI plants showed an increase in the number of the first and the second floral organs. These plants showed expression of CsPI which was much higher than that of other plants. The data implied that weak expression of B-class genes in C. spicatus may be correlated with the reduction of perianth. Consistent with this hypothesis, it has been reported that independent petal losses within buttercup family (Ranunculaceae) were strongly associated with decreased or eliminated expression of a B-class gene, APETALA3-3 (AP3-3) (Zhang et al. 2013). It would be interesting to investigate, therefore, whether there are specific cis-regulatory elements controlling the expression of CsAP3 and CsPI in petals.

Conclusions

CsPI retained the ancestral function in stamen identity and showed capability to specify petal development in A. thaliana. These data suggested that the role of PI-like gene was conserved in the early-diverging angiosperm Chloranthus spicatus and the core-eudicot Arabidopsis thaliana. CsPI can form homodimers besides heterodimers and they may both be involved in petal development in A. thaliana. Moreover, it seems likely that the loss of petals maybe not directly caused by the floral homeotic B-function in Chloranthus spicatus.

Competing interests

The authors declare that they have no competing interests.

Authors’ contributions

KS and ZL carried out the molecular genetic studies, participated in the sequence alignment; KS and ZC drafted the manuscript. All authors read and approved the final manuscript.

Acknowledgments

The authors are grateful for the financial support of the National Natural Science Foundation of China (Nos. 30900092) and the State Key Laboratory of Systematic and Evolutionary Botany (LSEB 2012-03).

Received: 7 July 2013 Accepted: 23 December 2013
Published: 4 February 2014

References

Adam H, Jouannic S, Orieux M, Morcillo F, Richaud F, Duval Y, Tregear JW (2007) Functional characterization of MADS box genes involved in the determination of oil palm flower structure. J Exp Bot 58:1245–1259
Ambrose BA, Lerner DR, Ciceri P, Padillo CM, Yanofsky MF, Schmidt RJ (2000) Molecular and genetic analyses of the silky1 gene reveal conservation in floral organ specification between eudicots and monocots. Mol Cell 5:5569–579
Bartlett ME, Specht CD (2010) Evidence for the involvement of GLOBOSA-like gene duplications and expression divergence in the evolution of floral morphology in the Zingiberales. New Phyol 187:521–541
Becker A, Theissen G (2003) The major clades of MADS-box genes and their role in the development and evolution of flowering plants. Mol Phylogenet Evol 29:464–489
Benfey PN, Chua NH (1990) The cauliflower mosaic virus 35S promoter: combinatorial regulation of transcription in plants. Science 250:959–966
Chen MK, Hsieh WP, Yang CH (2012) Functional analysis reveals the possible role of the C-terminal sequences and PI motif in the function of lily (Lilium longiflorum) PISTILLATA (PI) orthologues. J Biol Chem 6:941–961
Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J 16:735–743
Drea S, Hileman LC, de Martino G, Irish VF (2007) Functional analyses of genetic pathways controlling petal specification in poppy. Development 134:4157–4166
Egea-Cortines M, Saedler H, Sommer H (1999) Ternary complex formation between the MADS-box proteins: SQDMOSA DEFICIENS and GLOBOSA is involved in the control of floral architecture in Antirrhinum majus. EMBO J 18:5370–5379
Goto K, Meyerowitz EM (1994) Function and regulation of the Arabidopsis floral homeotic gene PISTILLATA. Gene 134:4157–4166
Hansen DR, Dastidar SG, Cai ZQ, Penafior C, Kuehl JV, Boore JL, Jansen RK (2007) Phylogenetic and evolutionary implications of complete chloroplast genome sequences of four early-diverging angiosperms: Buxus (Buxaceae), Chloranthus (Chloranthaceae), Dioscorea (Dioscoreaceae), and Illicium (Schisandraceae). Mol Phylogenet Evol 45:547–563
Honna T, Goto K (2001) Complexes of MADS-box proteins are sufficient to convert leaves into floral organs. Nature 409:525–529
Hsu HF, Yang CH (2002) An Orchid (Oncidium Gower Ramsey) AP3-like MADS box gene regulates floral formation and initiation. Plant Cell Physiol 43:1198–1209
Jack T, Brockman LL, Meyerowitz EM (1992) The homeotic gene APETALA3 of Arabidopsis thaliana encodes a MADS box and is expressed in petals and stamens. Cell 68:683–697
Kaufmann K, Melzer R, Theissen G (2005) MIRK-type MADS-domain proteins: structural modularity, protein interactions and network evolution in land plants. Gene 347:183–198
Kim S, Koh J, Yoo MJ, Kong HZ, Hu Y, Ma H, Soltis PS, Soltis DE (2005) Expression of floral MADS-box genes in basal angiosperms: implications for the evolution of floral regulators. Plant J 43:724–744
Kramer EM, Holappa L, Gould B, Jaramillo MA, Setnikov D, Santiago PM (2007) Elaboration of B gene function to include the identity of novel floral organs in the lower eudicot Aquilegia. Plant Cell 19:750–766
Krzek BA, Meyerowitz EM (1996) The Arabidopsis homeotic genes APETALA3 and PISTILLATA are sufficient to provide the B class organ identity function. Development 122:11–22

Lamb RS, Irish VF (2003) Functional divergence within the APETALA3/PISTILLATA floral homeotic gene lineages. Proc Natl Acad Sci U S A 100:6558–6563

Lange M, Orathokhao S, Lange S, Melzer R, Theissen G, Smyth DR, Becker A (2013) The serena B class floral homeotic mutant of California Poppy (Eschscholzia californica) reveals a function of the enigmatic R Motif in the formation of specific multicellular MADS domain protein complexes. Plant Cell 25(2):438–453

Li GS, Meng Z, Kong HZ, Chen ZD, Theissen G, Lu AM (2005) Characterization of candidate class A, B and E floral homeotic genes from the perianthless basal angiosperm Choranthus spicatus (Chloranthaceae). Dev Genes Evol 215:437–449

Liu CJ, Zhang J, Zhang N, Shan HY, Su KM, Zhang JS, Meng Z, Kong HZ, Chen ZD (2010) Interactions among proteins of floral MADS-Box genes in basal eudicots: implications for evolution of the regulatory network for flower development. Mol Biol Evol 27(5):1598–1611

Liu SJ, Sun YH, Du XQ, Xu QJ, Wu F, Meng Z (2013) Analysis of the APETALA3- and PISTILLATA-like genes in Hedysorum orientale (Chloranthaceae) provides insight into the evolution of the floral homeotic B-function in angiosperms. Ann Bot 112:1239–1251

Lv LL, Duan J, Xie JH, Liu YG, Wei CB, Liu SH, Zhang JX, Sun GM (2012) Cloning and expression analysis of a PISTILLATA homologous gene from pineapple (Ananas comosus). J Integr Plant Biol 54:1039–1053

Moore MJ, Bell CD, Solis PS, Solis DE (2007) Using plastid genome-scale data to resolve enigmatic relationships among basal angiosperms. Proc Natl Acad Sci U S A 104(49):19363–19368

Murashige T, Skoog FA (1962) Revised medium for rapid growth and bio-assays with tobacco tissue culture. Plant Physiol 15:473–497

Nakamura T, Fukuda T, Nakano M, Hasebe M, Kameya T, Kanno A (2005) The modified ABC model explains the development of the petaloid perianth of Agapantus praecox ssp. orientalis (Agapanthaceae). Plants 58:435–448

Piwazkry E, Yang YZ, Jack T (2007) Conserved C-terminal motifs of the Arabidopsis proteins APETALA3 and PISTILLATA are dispensable for floral organ identity function. Plant Physiol 145(4):1495–1505

Qiu YL, Lee J, Bernasconi-Quadroni F, Solts DE, Solts PS, Zanis M, Zimmer EA, Chen Z, Savolainen V, Chase MW (1999) The earliest angiosperms: evidence from mitochondrial, plastid and nuclear genomes. Nature 402:404–407

Riechmann JL, Krizek BA, Meyerowitz EM (1996) Dimerization specificity of the Arabidopsis APETALA1 gene. Plant Mol Biol 29:181–195

Su KM, Zhao SZ, Shan HY, Kong HZ, Chen ZD, Meng Z (2006) Conservation and divergence of candidate class B genes in Arabidopsis thaliana (Lazaridaceae). Dev Genes Evol 216:785–795

Stenzel S, Lommatzsch A, Krieger-Kozuch M, Chmiel R, Solms T (2013) A homeotic P class gene is involved in floral homeotic gene expression from the early developmental stage in sugar beet (Beta vulgaris). Ann Bot 112:1091–1099

Su KM, Zhang J, Zhang N, Shan HY, Kong HZ, Su KM, Wang J (2014) Floral homeotic gene expression is highly correlated with loss of petals in Arabidopsis thaliana. Proc Natl Acad Sci U S A 111:5074–5079

Winter KL, Weiser C, Kaufmann K, Bohne A, Kirchner C, Kanno A, Saedler H, Theissen G (2002) Evolution of class B floral homeotic proteins: obligatory heterodimerization originated from heterodimerization. Mol Biol Evol 19:587–596

Xu YY, Wang XM, Li J, Li JH, Wu JS, Walker JC, Xu ZH, Chong K (2005) Activation of the WUS gene induces ectopic initiation of floral meristems on mature stem surface in Arabidopsis thaliana. Plant Mol Biol 57:773–784

Yang Y, Fanning L, Jack T (2003) The K domain mediates heterodimerization of the Arabidopsis floral organ identity proteins, APETALA3 and PISTILLATA. Plant Cell 15:33–47

Zanis MJ, Solis DE, Solts PS, Mathews S, Donoghue MJ (2002) The root of the angiosperms revisited. Proc Natl Acad Sci U S A 99:6848–6853

Zhang R, Guo CC, Zhang WS, Wang PP, Li L, Duan XS, Du QG, Zhao L, Shan HY, Hodges SA, Kramer EM, Ren Y, Kong HZ (2013) Disruption of the petal identity gene APETALA3-3 is highly correlated with loss of petals within the buttercup family (Ranunculaceae). Proc Natl Acad Sci U S A 110:5074–5079