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Isolation and Characterization of the *PISTILLATA* Ortholog Gene from *Cymbidium faberi* Rolfe

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Received: 29 May 2019; Accepted: 1 August 2019; Published: 2 August 2019

Abstract: *Cymbidium faberi* Rolfe is a very popular potted plant in China, Japan and Korea where it has been cultivated for centuries. The economic value of this popular native Asian orchid could be enhanced by changes in its floral traits. In *Arabidopsis*, *PISTILLATA* (*PI*) is involved in regulating petal and stamen development. In order to investigate the possible role of the *PI* ortholog involved in floral development, we isolated *CyfaPI* from *C. faberi*. Protein alignment and a phylogenetic tree grouped *CyfaPI* in the *PI* lineage. *CyfaPI* transcripts were detected in all floral organs, but were absent in leaves. Moreover, in flowers, the highest expression level of *CyfaPI* was present in the gynostemium and the lowest level was found in anther caps. In addition, ectopic expression of *CyfaPI* in *Arabidopsis pi-1* mutant rescued petal development, and complement the development of filament-like structure (part of stamen), but failed to complement anther development in the stamen whorl. All these finding suggest that *CyfaPI* is mainly responsible for perianth and gynostemium development in *C. faberi*. Our data may help to trace the development of the gynostemium program and evolution in orchids.

Keywords: *PISTILLATA*; *Cymbidium faberi*; MADS-box gene; Floral development; Orchidaceae

1. Introduction

Cymbidium faberi Rolfe is a very popular potted plant in several countries of East Asia, and has been cultivated for centuries in China, Japan and Korea. Many artificially propagated varieties have been developed based on various flower traits including their color and number as well as the form of the perianth (sepal and petal). All of these elements contribute to the visual appreciation of orchids. The perianth is the most visually obvious structure, which is related to its primarily function as an optical attraction for pollinators. Moreover, the molecular mechanisms underlying the perianth identity program have been intensively studied in model species such as *Arabidopsis thaliana*, *Antirrhinum majus* and *Petunia hybrida* [1], and the evolution of the perianth identity has been extensively discussed recently in [2]. However, the perianth in monocots does not completely correspond to the obvious differentiation of sepal and petal whorls in model eudicots. In eudicots, the conserved function of the *PISTILLATA* (*PI*)/*GLOBOSA* (*GLO*) orthologs, such as *MtPI* from *Medicago truncatula* [3], *TrPI* from *Taihangia rupestris* [4], *CabuPI* from *Catalpa bungei* [5], is the regulation of petal and stamen development. Furthermore, each ortholog is able to fully complement the floral phenotype of the *Arabidopsis pi* mutant, and the expression pattern of each corresponds to their conserved function. In basal angiosperm, *PI*-like genes usually show broad expression zones in floral organs. For example, *HoPI_1* and *HoPI_3* from *Hedyosmum orientale* are expressed in all floral organs including the bracts and carpels. In addition, *HoPI_1* mimics the endogenous *Arabidopsis PI* and fully rescues both petal and stamen development in *Arabidopsis pi-1* mutants, while *HoPI_3* only rescues petal development, and partially rescues stamen development [6]. In another basal angiosperm *Magnolia wufengensis*, the *PI* ortholog *MawuPI* was only able to rescue filament-like structures in the third whorl, but failed to rescue petal development in

the second whorl [7]. Together, these data provide interesting evidence of a B-function evolutionary scenario among early-diverging clades of the angiosperm.

Monocots are one of the major clades of angiosperm, and the Orchidaceae family is one of the most species-rich in this clade; it includes species with highly diversified and specialized floral shapes. Previous studies have indicated that floral diversity in orchids results from changes in the regulatory regions of the *MADS*-box genes followed by gene duplication events [8], and that *PI*-like genes work together with different *AP3/AGL6* homologs involved in specifying sepal/petal/lip development in *Oncidium* and *Phalaenopsis* orchids [9]. Here, we isolated a *PI* ortholog, *CyfaPI*, from a Chinese traditional orchid *C. faberi* Rolfe. The flower of *C. faberi* consists of three whorls of floral organs, with three sepals in whorl 1, two petals and a lip in whorl 2, and a gynostemium fused by the male and female tissues in whorl 3 (Figure 1). *CyfaPI* transcripts are detected in all floral organs, but are absent in the leaves, which showed a different expression pattern to *PI*-like genes from eudicots. Further functional analysis revealed that *CyfaPI* is able to fully complement petals in whorl 2, but can only rescue filament-like structures in whorl 3 of flowers in transformed *Arabidopsis pi-1* mutants. Our data may provide the clue to help trace the gynostemium development program and evolution in orchids.

Figure 1. Flower and inflorescence of *Cymbidium faberi*. (A) Flower of *C. faberi*; (B) The gynostemium and ovary of *C. faberi*; (C) The inflorescence of *C. faberi*. Sepals (sep), petals (pet), lips (lip), anther cap (anc), gynostemium (gyn), ovary (ova). Bars: 5 mm.

2. Results

2.1. Cloning and Sequence Alignments of *CyfaPI*

The *CyfaPI* (*Cymbidium faberi* *PISTILLATA*) cDNA is 932 bp with a 633 bp ORF (Open Reading Frame) encoding a 210 amino acid (aa) protein (Genbank accession number: MK450600.1). Putative protein alignment and a phylogenetic analysis grouped *CyfaPI* into the GLO/PI lineage (Figure 2). In addition, *CyfaPI* is nested within a clade of orthologs from other monocots and is most closely related to orthologs from the Orchidaceae, such as *Oncidium* OMADS8 (97.14% identity) (Figure 2A). Computational translation shows that *CyfaPI* is a putative transcriptional regulator that contains a highly conserved 57 aa MADS-box domain (1–57) at the N-terminal region, a less conserved 64 aa K domain (87–150) in the middle region and a highly conserved 16 aa PI motif (193–208) in the variable C terminal region (151–210). Moreover, *CyfaPI* contains three putative amphipathic α -helices, K1(87–108), K2 (121–135) and K3 (143–167) subdomains that could potentially mediate protein interaction (Figure 2B) [10–12]. In addition, *CyfaPI* is 55.56%, 55.66%, 59.05%, 60.85%, 71.09%, 64.29%, 74.53%, 79.05%, and 67.14% identified to PI from *A. thaliana*, TrPI from *T. rupestris*, MtPI from *M. truncatula*, CabuPI from *C. bungei*, HoPI_1 and HoPI_3 from *H. orientale*, MAwuPI from *M. wufengensis*, and LMADS8 and LMADS9 from *Lilium longiflorum*, respectively (Figure 2B).

Figure 2. Phylogenetic analysis and sequence alignments of CyfaPI with B-class MADS-box proteins from other plant species. (A) Phylogenetic analysis of B-class MADS-box proteins; (B) Sequence alignments of CyfaPI with PI-like proteins from other plant species; the first underline indicates the MADS-box domain and the second the K domain. The PI motif in the C-terminal region is boxed. Dots represent amino acids (aa) identical to CyfaPI. Dashes indicate gaps inserted to improve the sequence. The K1, K2 and K3 helices with the (abcdefg)_n heptad repeats are also underlined.

2.2. Expression Analyses of CyfaPI in *C. faberi*

CyfaPI expression was restricted to floral organs, such as sepals, petals, lips, anther caps, gynostemium and ovaries, and was absent in juvenile leaves (Figure 3). In addition, the highest *CyfaPI*

expression was observed in gynostemium, where the expression was significantly higher than that in sepals, petals, lips, anther caps and ovaries ($LSD, p < 0.05$). Moreover, there was no significant differences in *CyfaPI* expression in the sepals and petals, where the *CyfaPI* expression in both these tissues was significantly higher than that in the lips, anther caps and ovaries ($LSD, p < 0.05$). The lowest *CyfaPI* expression was detected in anther caps, where the expression was considerably lower than that in sepals, petals, gynostemium and ovaries ($LSD, p < 0.05$), but showed no significant difference to *CyfaPI* expression in lips ($LSD, p > 0.05$) (Figure 3A). *CyfaPI* expression was also detectable in floral buds at 5 mm (early stages of flower differentiation), 8 mm (seasonal dormancy of floral buds), 10 mm (breaking dormancy and further development), 15 mm, 20 mm, 25 mm and at bud opening (Figure 3B). Moreover, *CyfaPI* expression became detectable during the early stages of flower differentiation and increased until the seasonal dormancy of floral buds. In addition, with the breaking of flower dormancy and further development, *CyfaPI* expression increased continuously and achieved its highest level ($LSD, p < 0.05$) in floral buds of 25 mm when it reached maturity, and then began to drop after the floral buds opened.

Figure 3. *CyfaPI* expression was detected by RT-qPCR in different tissues (A) and various stages of flower development (B) in *C. faberi*. (A) Expression of *CyfaPI* in the juvenile leaves (lea), sepals (sep), petals (pet), lips (lip), anther caps (anc), gynostemium (gyn) and ovaries (ova) with the *C. faberi* *ACTIN* gene used as the internal control; (B) Expression of *CyfaPI* at various stages of flower development. Different letters indicate statistically significant differences.

2.3. Ectopic Expression of *CyfaPI* in *Arabidopsis pi-1* Mutant

To further determine the role of *CyfaPI* in flower development, we attempted to rescue the loss-of-function *Arabidopsis pi* mutant (*pi-1*) via the expression of a *CyfaPI* transgene. Binary vectors carrying 35S::*CyfaPI* were introduced into *Arabidopsis* by *Agrobacterium*-mediated transformation. The transgenic *Arabidopsis* lines were confirmed by RT-qPCR and the mutant allele was identified by dCAPS genotyping (Figure S1). Seven wild-type, 11 homozygous *pi-1* and 14 heterozygote *pi-1* independent transgenic *Arabidopsis* lines were obtained. Phenotypes of the transgenic lines were assayed in a homozygous *pi-1* mutant background to evaluate whether the *CyfaPI* transgene could act as a substitute for the endogenous *PI* gene in regulating petal and stamen development. Phenotypes of transgenic lines were also analyzed in a wild-type *Arabidopsis* background to evaluate the function of *CyfaPI* in the floral development (Table 1). In addition, the *CyfaPI* expression in homozygous *pi-1* mutants and wild type transgenic *Arabidopsis* with different phenotypes was also determined (Figure 4).

Among the 11 35S::*CyfaPI* homozygous *pi-1* transformants, 3 (27.27%) lines showed strong complementation phenotypes with flowers consisting of four concentric whorls of floral organs. The flower has 4 petaloid sepals in whorl 1 (outmost ring), 4 petals in whorl 2, 3 (or 4) filament-like organs without anther attachment in whorl 3 and a normal gynoecium in whorl 4 (innermost central area) (Figure 5G,H). Two (18.18%) lines displayed medium complementation phenotypes that produced flowers with four concentric whorls of floral organs. There were 4 normal sepals in whorl 1, 4 petals in whorl 2, 3 (or 4) filament-like organs without anther attachment in whorl 3 and a normal gynoecium in whorl 4 of flowers of this complementation group (Figure 5K,L). Six lines (54.55%) failed to

display a phenotype complementation and produced flowers similar to homozygous *pi-1* mutant flowers. In addition, the flower phenotypes of transgenic *Arabidopsis* in heterozygous *pi-1* mutant and the wild-type background were also assayed, and almost all the flower-phenotype changes in transgenic *Arabidopsis* in heterozygous *Pl/pi-1* background resembled the flower phenotypes displayed by wild-type lines expressing the introduced transgene. Among a total of 7 35S:: *CyfaPI* wild-type transgenic *Arabidopsis*, 3 (42.86%) lines showed severe phenotype changes and produced flowers with sepals converted into petaloid organs in whorl 1 (Figure 5E,F), and 3 (42.86%) lines displayed medium phenotype changes and ha flowers with the first whorl sepal partly converted into petal (Figure 5I,J). In addition, the *CyfaPI* expression levels corresponded to the flower-phenotype changes in transgenic plants (Figure 4). For example, *CyfaPI* expression levels in transgenic *Arabidopsis* lines in homozygous *pi-1* background with strong complementation phenotypes ($T_1-9/pi-1$, $T_1-10/pi-1$) were significantly higher than that in homozygous *pi-1* transgenic plants with medium complementation phenotypes ($T_1-7/pi-1$, $T_1-8/pi-1$) or without complementation of petal and stamen development ($T_1-1/pi-1$, $T_1-2/pi-1$) (LSD, $p < 0.05$). Moreover, *CyfaPI* expression levels in homozygous *pi-1* transgenic plants with medium complementation phenotypes ($T_1-7/pi-1$, $T_1-8/pi-1$) were also observed to be significantly higher than that in homozygous *pi-1* transgenic plants without phenotype complementation ($T_1-1/pi-1$, $T_1-2/pi-1$) (LSD, $p < 0.05$). Similar results were also observed in wild-type transgenic *Arabidopsis*. Furthermore, three interesting flower phenotypes were also found in the 35S:: *CyfaPI* transgenic *Arabidopsis* in the heterozygous *Pl/pi-1* background. Specifically, one line produced significantly larger sized flowers, and another line produced flowers with five petals in whorl 2. The third line produced flowers with six petals in whorl 2 (Figure 6).

Figure 4. The *CyfaPI* expression was detected by RT-qPCR in the T_1 transgenic plants in wild-type and homozygous *Arabidopsis pi-1* mutant background. (A) *CyfaPI* expression was detected in wild-type transgenic *Arabidopsis*; (B) *CyfaPI* expression was detected in homozygous *pi-1* mutant transgenic *Arabidopsis*. Different letters indicate statistically significant differences.

Wild-type (WT) *Arabidopsis*; (T_1-1/WT) 35S:: *CyfaPI* transgenic line without phenotypic changes; (T_1-2/WT , T_1-3/WT , T_1-4/WT) 35S:: *CyfaPI* transgenic lines displayed weak phenotype changes in floral structure; (T_1-5/WT , T_1-6/WT) 35S:: *CyfaPI* transgenic lines displayed severe phenotype changes in floral structure; (*pi-1*) Homozygous *pi-1* mutant *Arabidopsis*; ($T_1-1/pi-1$, $T_1-2/pi-1$) 35S:: *CyfaPI* transgenic lines without phenotype complementation; ($T_1-7/pi-1$, $T_1-8/pi-1$) 35S:: *CyfaPI* transgenic lines displayed medium complementation phenotypes; and ($T_1-9/pi-1$, $T_1-10/pi-1$) 35S:: *CyfaPI* transgenic lines showed strong complementation phenotypes.

Figure 5. Phenotypes comparison of the wild-type, homozygous *pi-1* mutant, 35S:: *CyfaPI* transgenic *Arabidopsis* under a wild-type and homozygous *pi-1* mutant background. (A) Inflorescence of wild-type *Arabidopsis*; (B) Wild-type *Arabidopsis* flower with normal 4 concentric whorls of floral organs (4 sepals in whorl 1, 4 petals in whorl 2, 6 stamens in whorl 3 and a gynoecium in whorl 4); (C) Inflorescence of homozygous *pi-1* mutant *Arabidopsis*; (D) Flower of homozygous *pi-1* mutant *Arabidopsis* with two outer whorl sepals and one inner whorl gynoecium; (E) Inflorescence of 35S:: *CyfaPI* transgenic *Arabidopsis* in wild-type background displaying severe phenotype changes; (F) Flower of 35S:: *CyfaPI* transgenic *Arabidopsis* in wild-type background showing severe phenotype changes; (G) Inflorescence of 35S:: *CyfaPI* homozygous *pi-1* transgenic *Arabidopsis* showing strong complementation phenotypes; (H) Flower of 35S:: *CyfaPI* homozygous *pi-1* transgenic *Arabidopsis* showing strong complementation phenotypes; (I) Inflorescence of 35S:: *CyfaPI* transgenic *Arabidopsis* in wild-type background displaying medium phenotype changes; (J) Flower of 35S:: *CyfaPI* transgenic *Arabidopsis* in wild-type background showing medium phenotype changes; (K) Inflorescence of 35S:: *CyfaPI* homozygous *pi-1* transgenic *Arabidopsis* showing medium complementation phenotypes; (L) Flower of 35S:: *CyfaPI* homozygous *pi-1* transgenic *Arabidopsis* showing medium complementation phenotypes. sepal (sep), petaloid sepal (psep), petals (pet), stamen (sta), filament-like organ (filo), carpel (car); Bars: (C) 2 mm; (A, B, E, F, G, H, J, K) 1 mm; (D, I, L) 500 μ m.

Table 1. Summary of different phenotypes of transgenic lines.

Transgenic Lines	Phenotype of Flower	No. Plants
35S:: <i>CyfaPI</i> homozygous <i>pi-1</i> transformants	strong complementation	3(27.27%)
	medium complementation	2(18.18%)
	no complementation	6(54.55%)
	severe phenotype change	3(42.86%)
35S:: <i>CyfaPI</i> wild-type transformants	weak phenotype change	3(42.86%)
	no phenotype change	1(14.28%)

3. Discussion

Molecular studies in model species have indicated that the function of floral MADS-box genes is highly correlated with their expression patterns; therefore, the expression patterns of MADS-box genes in other species may be a good predictor [13]. In *Arabidopsis*, *PI* works together with another B-function protein *APETALA3* (*AP3*) to specify petal and stamen identity. Therefore, high levels of *PI* expression are restricted to petals and stamens [14]. Moreover, *PI* orthologs, such as *MtPI* from *M. truncatula* [3,15], *TrPI* from *T. rupestris* [4], and *CabuPI* from *C. bungei* [5], show conserved expression patterns and functions in these core eudicots. However, some *PI* orthologs from basal angiosperm species, such as *H. orientale* and *M. wufengensis*, have shown broad expression zones in floral organs and have been demonstrated to have different functions [6,7]. *H. orientale*, *HoPI_1* and *HoPI_3* showed similar expression zones, but different expression levels. In addition, *HoPI_1* fully rescued both petal and stamen development in *Arabidopsis pi-1* mutants, while *HoPI_3* could only rescue petal development as well as partially rescuing stamen development [6]. However, the *MawuPI* from *M. wufengensis* only partly rescued the stamen development in *Arabidopsis pi-1* mutants [7]. In addition, some monocots' *PI* orthologs were also found to have expressions that extended outwards from the first whorl perianth [16,17]. For examples, two *L. longiflorum* *PI* orthologs, *LMASDS8* and *LMADS9*, are expressed persistently in all the perianth whorls during flower development, but only in the stamens of young flower buds. Moreover, both genes could fully rescue petal development, but failed to complement stamen development in the *Arabidopsis pi-1* mutant [16]. Together, these data suggest that changes in expression domains or an altered sequence within the *PI* orthologs may contribute to functional divergence. *O. Gower Ramsey OMADS8*, an orchid *PI* ortholog, was expressed in all floral organs and vegetative leaves, and was able to fully complement petal and stamen development in the *Arabidopsis pi-1* mutant [17,18]. Moreover, the *Oncidium* *PI* ortholog has been demonstrated to work together with *AP3*-like and *AGL6*-like proteins to regulate sepal/petal/lip development and differentiation [9]. Similar expression and function of *PI* ortholog was also observed in *Phalaenopsis* [9,19–21]. Some *PI* orthologs from other Orchidaceae species, such as *Liparis distans*, *Phaius tankervilleae*, *Dendrobium* Spring were also found to be expressed in all floral whorls [22]. Interestingly, two copied orchid *PI* orthologs are present in *Orchis italica*, and both genes are expressed in all floral organs. In addition, the highest expression level of both genes is detected in the lateral inner tepals of the immature inflorescence, and is detected in the lips of the mature inflorescence; while the continued lowest expression level of both genes is found in the columns from immature to mature inflorescence [23,24]. In our study, *C. faberi CyfaPI* expression was found in all floral organs, but was absent in leaves. In addition, the highest expression level of *CyfaPI* was present in the gynostemium, and the lowest expression level was in the anther caps. A relatively high expression level of *CyfaPI* was also found in the sepals and petals (Figure 3A). That is, *CyfaPI* showed a different expression pattern with the *PI* orthologs from closely related species, such as *L. longiflorum* and *O. Gower Ramsey*. Moreover, protein sequence alignments suggested that *CyfaPI* is 79.05%, 67.14%, 97.14% identified with *LMASDS8*, *LMADS9* from *L. longiflorum*, and *OMADS8* from *O. Gower Ramsey*, respectively (Figure 2B). Furthermore, ectopic expression of *CyfaPI* in *Arabidopsis pi-1* mutant fully rescued petal development, and complemented filament-like structure but failed to complement anther development in the stamen whorl. Together, these data suggest that changes in expression zones or an altered sequence within the *PI* orthologs in different groups of monocots may contribute to floral morphology changes. In *C. faberi*, high *CyfaPI* expression was found in the perianth (sepal and petal) and gynostemium (fused organ of filament and column). In addition, complementary loss-of-function alleles showed that *CyfaPI* could rescue petal and filament development in *Arabidopsis pi-1* mutant. These results suggest that *CyfaPI* is mainly responsible for perianth and gynostemium development in *C. faberi*.

Specifically, one line of transgenic *Arabidopsis* in heterozygous *PI/pi-1* background produced noticeably larger sized flower, and another line produced flowers with five petals in whorl 2. The third line produced flowers with six petals in whorl 2 (Figure 6). Perhaps the future challenge is to uncover how *C. faberi* *PI* ortholog, *CyfaPI*, is involved in increasing petal number and the size of floral organs.

In addition, our experiment suggests that *CyfaPI* holds the potential for biotechnical engineering to develop new ornamental plant varieties of orchids with interesting and valuable traits.

Figure 6. Interesting flower phenotypes of 35S::CyfaPI transgenic *PI/pi-1 Arabidopsis*. (A) The large flower of 35S::CyfaPI transgenic *PI/pi-1 Arabidopsis* and the *PI/pi-1 Arabidopsis* flower (*he_pi*) with four sepals in whorl 1, four petals in whorls 2, six stamens in whorl 3 and a gynoecium in whorl 4; (B) 35S::CyfaPI transgenic *PI/pi-1 Arabidopsis* producing flower with five petals in whorl 2; (C) 35S::CyfaPI transgenic *PI/pi-1 Arabidopsis* producing flower with six petals in whorl 2. Bars: (A) 5 mm; (B) 500 µm; (C) 1 mm.

4. Materials and Methods

4.1. Plant Material

Cymbidium faberi plants were grown in ceramic flowerpots (Ø25.5 × 26 cm) full with orchid substrates in the Botanical Garden of Yangtze University under natural conditions in Jingzhou city, Hubei Province. Juvenile leaves, sepals, petals, lips, anther caps, gynostemium and ovaries were sampled when the flower buds opened, immediately frozen in liquid nitrogen and stored at −80 °C until use. In addition, floral buds measuring 5 mm (early stages of flower differentiation), 8 mm (seasonal dormancy of floral buds), 10 mm (breaking flower dormancy and further development), 15 mm, 20 mm, 25 mm long and opening floral buds were also sampled. The *pi-1* mutant *Arabidopsis* (CS77) seeds were obtained from the ABRC (*Arabidopsis* Biological Resource Center, ABRC) at the Ohio State University, USA.

4.2. Isolation and Characterization of PI Ortholog from *C. faberi*

Total RNA was extracted from different tissues as described above by using an EASYspin Plant RNA Kit (Aidlab, Beijing, China) according to the manufacturer's protocols. First-strand cDNA was synthesized from 1 µg of DNase I-treated total RNA with an oligo (dT)18 adaptor primer and M-MLV Reverse Transcriptase (TaKaRa, Shiga, Japan). The *CyfaPI* 3' end cDNA sequences were amplified with gene-specific forward primer GSPPI (5'-ATCAATAGGCAGGTGACCTTCTC-3') and 3'RACE Outer Primer (5'-TACCGTCGTTCCACTAGTGATTT-3') following the manufacturer's protocols as described in the 3'-full RACE Core Set Ver. 2.0 kit (TaKaRa, Shiga, Japan), but with 45 s annealing at 57 °C. The full length of *CyfaPI* cDNA was amplified with the forward primer CyfaPIF (5'-CTTTTGCTTCTGTTCTTGAGATC-3') and the reverse primer CyfaPIR (5'-GCACGCACATATGTCATAATCATC-3'), and the PCR amplification procedure followed Liu et al. [25]. The primer design was based on the sequence of *PI/GLO* orthologs, *CyenPI* (Genbank accession numbers: JQ326259.1) from the closely related species *C. ensifolium* (Orchidaceae). Putative protein sequences of CyfaPI and 85 B-class MADS-box proteins in NCBI Genbank (Table S1) from other angiosperms species were selected for constructing phylogenetic trees. The B-class MADS-box proteins were identified based on previously published papers and a BLAST search in the Genbank sequence database. Phylogenetic trees were constructed with MEGA5.0 software using the neighbor-joining method described by Tamura et al. [26], with the default setting, and bootstrap values were based on

1000 replicates. The full-length CyfaPI proteins consisting of complete MADS, I, K and C domains were aligned with BioEdit 7.0.9 software using the ClustalW program with default settings.

4.3. Expression Analysis of CyfaPI

For RT-qPCR analysis, total RNAs from juvenile leaves, sepals, petals, lips, anther caps, gynostemium, ovaries, floral buds of different length (5 mm, 8 mm, 10 mm, 15 mm, 20 mm, 25 mm) and opening floral buds were extracted using the procedure described above. DNA free total RNA and first-strand cDNA were prepared by using the HiScript® II Q RT SuperMix for qPCR kit (Vazyme, Nanjing China) following the manufacturer's protocols. The RT-qPCR of three biological replicates was performed with the gene-specific forward primer qCyfaPIF (5'-CACATACCTATTGCACCATC-3') and the gene-specific reverse primer qCyfaPIR (5'-GGTCATTGGCATCTGAGCTG-3') on the Line-Gene 9600 Plus Real-time PCR Detection System, with SYBR green I for transcript measurements. Amplification of *C. faberi actin* (Genbank accession numbers: JN177719.1) fragment with specific primers qCyfaactinF (5'-AAGACTTACACCAAGCCGAAGAAGATC-3') and qCyfaactinR (5'-CCAGCTCCACAGGTTGCGTTAG-3') was used as the internal control. The PCR procedure was as follows: 95 °C for 30 s, followed by 40 cycles of 95 °C for 10 s, 60 °C for 30 s, which was followed by melt curve stages of 95 °C for 15 s, 60 °C for 60 s, and 95 °C for 15 s. The experiments were repeated three times for each sample. Relative expression levels were calculated based on the $2^{-\Delta\Delta C_t}$ method [27].

4.4. Ectopic Expression of CyfaPI in *Arabidopsis pi-1* Mutant and Function Analysis

CyfaPI cDNAs containing full-ORF (open reading frame) were cloned into the pBI121 vector with *Xba*I and *Xma*I restriction enzymes in the sense orientation. The 35S::CyfaPI constructs were then transformed into heterozygous *PI/pi-1 Arabidopsis* with the floral-dip method suggested by Clough and Bent [28]. The T₁ seeds were selected and seedlings were cultivated by referring to the methods described by Li et al. [29]. Moreover, the positive transgenic lines were confirmed by RT-qPCR as described above with the same CyfaPI-gene-specific primers qCyfaPIF and qCyfaPIR suggested above. For RT-qPCR, the *A. thaliana* β -actin (Genbank accession numbers: NM_116090.3) fragment was amplified as the internal control with the forward primer qactinF (5'-AAGACTTACACCAAGCCGAAGAAGATC-3') and the reverse primer qactinR (5'-CCAGCTCCACAGGTTGCGTTAG-3'). Homozygous *pi-1* transformants were identified by dCAPS genotyping following the method suggested by Lamb and Irish [30]. Phenotypes of transgenic *Arabidopsis* in homozygous *pi-1* mutant background and wild-type background were assayed after flowering, respectively.

4.5. Statistical Analyses

All experiments were carried out with three biological replicates, and data were expressed as mean $\pm$ SE (standard error). Statistical significance was determined using one-way ANOVA with the LSD (Least significant difference) test at $p < 0.05$ level in SPSS 19.0. P values of less than 0.05 were regarded as statistically significant.

Supplementary Materials: Supplementary materials can be found at <http://www.mdpi.com/2073-4395/9/8/425/s1>. Figure S1: Genotyping and phenotype of wild-type, homozygous *pi-1* mutant and heterozygous *PI/pi-1 Arabidopsis*. Supplementary Table S1: Information on sequences selected for alignments and phylogenetic analyses from NCBI GenBank.

Author Contributions: Z.-X.L. designed the experiments, provided project supervision and revised the manuscript, Y.F. conducted the experiments and wrote the draft manuscript. All authors approved the final draft of the manuscript.

Funding: This research was supported by the Excellent Master Dissertation Cultivation Program of Yangtze University (YS2018049).

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

BLAST	Basic local alignment search tool
PCR	Polymerase chain reaction
RT-qPCR	Quantitative reverse transcription PCR
LSD	Least significant difference

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