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Molecular Cloning and Expression Analysis of C Function Gene *CjPLE* in Double Flower Varieties of *Camellia japonica*

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Abstract The C function gene in the classic ABC model is the important factor that determines the stamen and pistil organs in the floral axis, and is also the regulatory gene of floral meristem termination differentiation. A 901 bp cDNA sequence of C function genewas cloned from flower bud of *Camellia japonica*, named *CjPLE* (MF278983), which contained an opening reading frame of 780 bp. The "Genome Walking" experiment showed that it contained two introns. Through phylogenetic analysis, we found that it formed a PLE subclass with *TAG1* of *Solanum lycopersicum* and PLE of *Antirrhinum majus*. The results of real-time PCR showed that *CjPLE* gene had high expression in the carpel of wild *Camellia japonica*. In peony type double-flower varieties 'Hongluzhen' and 'Zhuangyuanhong', the highest expression of *CjPLE* gene was in stamens, while in 'Rongqiu', the expression of *CjPLE* gene in inner petals and stamens were both high. The above results indicate that *CjPLE* is a homologous gene of the PLE branch in which belongs to C function gene in *Camellia japonica*, and might play a role in regulating the development of floral organs.
Keywords *Camellia japonica*; *CjPLE* gene; Real-time PCR; Gene structure; Phylogenetic analysis

Camellia japonica is one of the ten famous traditional flowers in China, which belongs to *Camellia*, Theaceae. The flower is colorful with ever green leaves, and usually regarded as the ornamental plants. Several studies about flower color have been carried out in *Camellia*. Through the research of flower gene *CnF3H* in *Camellia nitidissima* by Zhou (2015), it was found that the gene was most expressed in stamen, and the expression levels of the other parts from high to low were petals, sepals, pistils and bracts. Through the research on three *Camellia F3H* genes by Fan et al. (2016), *CcF3H* was found to be more stable in structure and more conservative in sequence that could be used as the most effective gene of overexpressed exogenous genes for the inheritance and breeding of Camellia floral colors. After a long period of artificial domestication, the ornamental variety of *Camellia japonica* contains abundant flower pattern changes, but the double flower phenotype of stamen petalody was in the majority. The formation of double flowers is affected by various factors and multiple pathways are found to be involved in double flower formation. Among these, the MADS-box gene family plays a key role in regulating the development of flower organs, which usually concentrates on floral organ development, flowering regulation and diversity of angiosperms (Alvare et al., 2000; Becker and Theissen, 2003; Kaufmann et al., 2005; Hemming and Trevaskis, 2011).

ABC model was initially put forward by Coen and coauthors in last century (Coen and Meyerowitz, 1991). According to the model, the properties of typical flower organs (sepal, petal, stamen, carpel) is mainly regulated by coordinated regulation of function genes of A, B and C (Carpenter and Coen, 1990; Bowman et al., 1991; Coen and Meyerowitz, 1991). Until 1995, the model was developed to ABCD model by Colombo et al. (1995). While at the beginning of this century, a more complex ABCDE model was developed. In general, A, B and C genes separately regulate the development of sepal, petal and stamen, respectively. Moreover, these three classes of genes can co-regulate the development of flower organs. A and B genes can regulate the development of petal together as well as B and C genes regulate the development of stamen. While D gene independently regulates the growth of ovule as well as E gene plays a role in the process of the development of petal, stamen, endoderm and

ovule (Ferrario et al., 2004).

All regulatory genes in "ABCDE" model belong to MADS-box gene family except A gene *APALATA2*. MADS-box gene is a very important transcriptional regulatory factor. In angiosperm, C function gene *AGAMOUS* (*AG*) is the largest branch of MADS-box. However, in eudicotyledon *Arabidopsis thaliana* and *Antirrhinum majus*, two core branches were proved to exist at the same time: *euAG* and *PLENA* (*PLE*), and functional studies had found that these branches were also involved in the development of internafloral whorls of organs (Fourquin and FerrNdiz, 2012). After a long period of artificial domestication, the ornamental variety of camellia contains abundant changes of flower patterns. Double-flower camellia includes 5 types: semidouble type, peony type, rose type, amenone form and complete double type. It is still not clear about how ABCDE genes regulate the formation of double flower. The study on *Camellia japonica* gene *CjAPL1* and *CjAPL2* found that the expression level of A function genes were correlated with the degree of double flower, but overexpressed *Arabidopsis* plants didn't have the double flower phenotypes (Sun et al., 2003a). The study on C gene *CjAG* of *Camellia japonica* found that two irreversible evolution paths existed in the expression changes of C function genes during the formation of double flower in *Camellia japonica*: (1) The expression level of C functional gene in complete double *Camellia japonica* 'Shibaxueshi' was entirely lost which was the same as the other double flowers species; (2) In amenone type ('Jinpanlizhi'), instead of being inhibited, the expression level of C function genes increased, and ectopic expressed in the inner petals. In this study, focusing on the PLE branch of C function genes, gene cloning and evolution analysis were carried out in *Camellia japonica*. Combined with the gene expression pattern in wild single and double flower varieties, the regulation of *CjPLE* in flower development and double flower formation was discussed.

1 Results and Analysis

1.1 Molecular cloning of the full-length *CjPLE* gene

Through the local BLAST comparison, a full length of 1 019 bp gene numbered c56140.graph_c0, homologous with PLE\, was discovered in transcriptome data of *Camellia japonica* bud (Li et al., 2017), of which the open reading frame (ORF) was 738 bp and encoded a total of 245 amino acids. The 5' non-coding region was 107 bp and 3' non-coding region was 174 bp. Based on this sequence information, PCR primer was designed outside the ORF of the gene, and a 901 bp sequence was obtained through specific PCR amplification. Using ORF Finder to analyze *CjPLE* gene sequence, the result showed that the gene ORF was 780 bp and could encoded a complete protein. The 5' non-coding region was 60 bp and 3' non-coding region was 61 bp. Using BioEdit to compare the protein sequences of these two genes, the result showed that c56140.graph_c0 had more 14 amino acids than cloned *CjPLE* while other amino acid was identical. The above results indicated that the cloned *CjPLE* gene was a full-length gene and had been submitted to NCBI, the accession numbered was MF278983.

Through the analysis of protein structure encoded by *CjPLE* gene by CD-Search on NCBI, we found that the encoded protein contained a K-box (E-value: 9.74e-31) domain and a highly conservative region in the specific MADS-box (E-value: 9.42e-46) domain (Figure 1).

1.2 Sequence alignment and phylogenetic analysis of *CjPLE* and related genes in different plants

To investigate the evolutionary relationship between *CjPLE* and other species, 18 protein sequences, which had high homology with *CjPLE* (referring to the phylogenetic tree of Alice Tadiello et al.), were selected to construct NJ (Neighbor-joining) phylogenetic tree using MEGA 7.0, (Figure 2). The result showed that *CjPLE* had the closest evolutionary relationship with *TAG1* from tomato, and formed a PLE sub branch with *Antirrhinum majus AmPLE* (Alice et al., 2009).

1.3 Gene structure

Through the specific PCR amplification, the cDNA sequence of *CjPLE* was obtained. To study the complete structure of the gene, we constructed 4 libraries using Universal GenomeWalkerTM 2.0 kit by taking wild

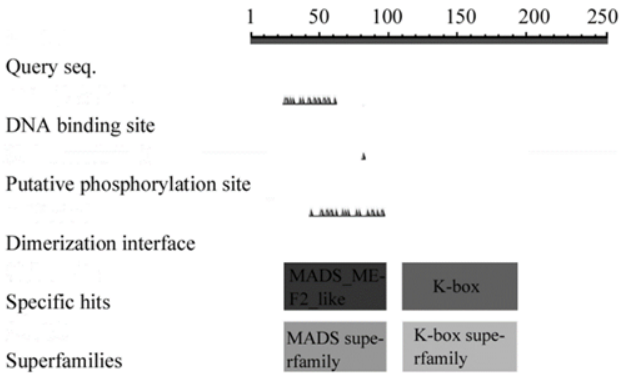

Figure 1 Conserved domains of coded protein from CjPLE gene

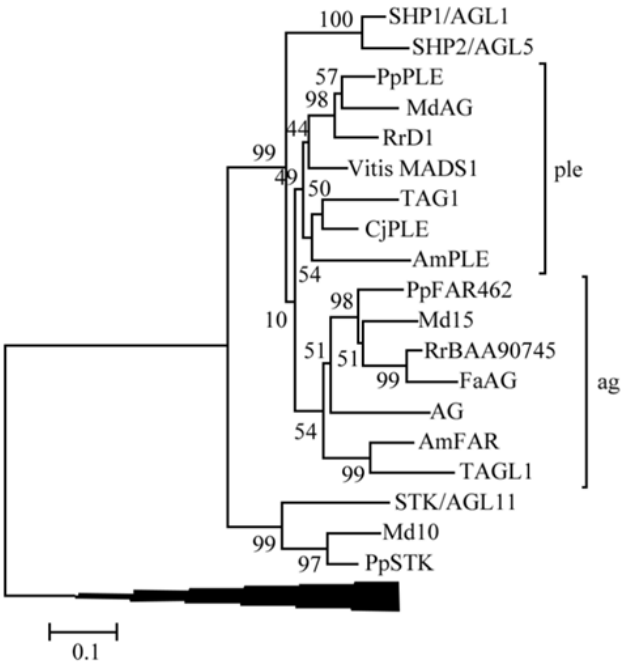

Figure 2 Phylogenetic analysis of *CjPLE* gene and related sequences in other plant species

Note: The proteins in this phylogenetic tree and their accession numbers: Apple (*Malus domestica*), '*MdAG*'AF401637, '*Md15*'CAC80858, '*Md10*'CAA04324; *Arabidopsis*, '*SHP/AGL1*'NP_191437, '*SHP2/AGL5*'NP_850377, '*AG*'NP_567569, '*STK/AGL11*'NP_001078364; Tomato (*Solanum lycopersicum*), '*TAGL1*'AAM33099, '*TAG1*'AAM33101; Peach (*Amygdalus persica*), '*PpPLE*'FJ88413, '*PpFAR462*'FJ184275, '*PpSTK*'ABQ85556; Rose (*Rosa rugosa*), '*RrD1*'AB025643; Snapdragon (*Antirrhinum majus*), '*AmPLE*'AAB25101; Strawberry (*Fragaria ananassa*), '*FaAG*'AAD45814; The outside stray are showed in black part (Alice et al., 2009)

Camellia japonica as a template. Besides, two pairs of specific primers were designed to amplify target gene. However, only one pair of primers successfully amplified (Table 1). The primers were directed towards the 5' terminal and 3' terminal of the gene, respectively, and contained a repeating area, which ensured the acquisition of whole sequence.

Genome walking result showed that the full length of the gene was 1 195 bp with two small introns of which were 33 bp and 118 bp, respectively (Figure 3).

1.4 Expression analysis of *CjPLE* in floral organs of wild and double flower cultivars of *Camellia japonica*

MADS-box domains were detected in *CjPLE* through analysis. To further determine the gene function, real-time PCR experiment was carried out using cDNA extracting fromwild *Camellia japonica*and several peony double flower cultivars 'Hongluzhen', 'Rongqiu', 'Zhuangyuanhong'. The expression level of the gene in wild *Camellia*

Table 1 Primer sequence for cloning and expression of *CjPLE* gene in *C. japonica*

Primer name	Primer sequence (5'-3')	Function
P-1	TTGTGACCATCCCTTTTCCT	
P-2	TCAGGGGCAGATACAGAACA	Specific PCR amplification
GSP3	CCAAGTCACTTTTTGTAAGCGCCGCAA	
GSP4	GCTGAGAGCTTCTCCCAAAATGTGCCT	Genome walking
PLE-Fa	ACTGTTGTTGGCGTACTCGTAAAG	
PLE-Ra	CGAAGTCGCCCTTATCGTCTT	Real-time PCR
GAPDH-F	GGGAATCCTTGGTTACACTGAG	
GAPDH-R	ACCCCATTCGTTGTCATACC	Primers of the reference gene

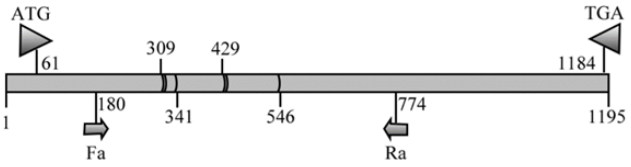

Figure 3 The structure chart of *CjPLE* gene

japonica flower bud was used as reference to calculate the relative expression level (Figure 4; Figure 5). *CjPLE* gene expression was different in various floral organs of wild *Camellia japonica*, of which the highest expression was carpel. While in peony double flower cultivars 'Hongluzhen' and 'Zhuangyuanhong', the gene had the highest expression level in stamen and had a higher expression level in inner petals and stamen in 'Rongqiu'.

2 Discussion

2.1 *CjPLE* is a PLE-like C function gene in *Camellia japonica*

Related studies have been carried out on different genes of ABCDE in *Camellia japonica*, and the current cloned A function genes included *CjAPL1* and *CjAPL2* (Sun et al., 2013b); B function genes included *CjDEF*, *CjGLO1*, *CjGLO2* and *CjTM6* (Viaene et al., 2009); C function gene included *CjAG*. Based on the transcriptional information of *Camellia japonica* flower organs (Li et al., 2017), a full length 901 bp gene of PLE lineage *CjPLE*

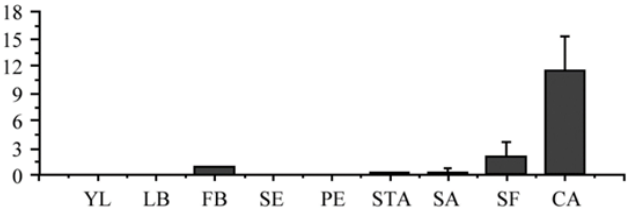

Figure 4 Relative expression level of *CjPLE* gene in different floral organs of wild *Camellia japonica*

Note: YL: Young leaf; LB: Leaf bud; FB: Flower bud; SE: Sepal: PE: Petal; STA: Stamen; SA: Semet; SF: Filament; CA: Carpel

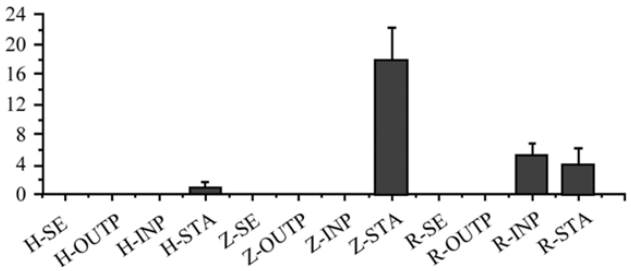

Figure 5 Relative expression level of *CjPLE* gene in different floral organs of 'Hongluzhen', 'Zhuangyuanhong' and 'Rongqiu'

Note: H-SE: Sepals of 'Hongluzhen'; H-OUTP: Out-petals of 'Hongluzhen'; H-INP: In-petals of 'Hongluzhen'; H-STA: Stamens of 'Hongluzhen'; Z-SE: Sepals of 'Zhuangyuanhong'; Z-OUTP: Out-petals of 'Zhuangyuanhong'; Z-INP: In-petals of 'Zhuangyuanhong'; Z-STA: Stamens of 'Zhuangyuanhong'; R-SE: Sepals of 'Rongqiu'; R-OUTP: Out-petals of 'Rongqiu'; R-INP: In-petals of 'Rongqiu'; R-STA: Stamens of 'Rongqiu'

was obtained by homologous cloning. Sequence analysis indicated that the ORF length of the gene was 780 bp. The 5' non-coding region was 60 bp and 3' non-coding region was 61 bp. The gene complete coded a protein containing 260 amino acids, which contained highly conservative region of specific MADS-box domain and K-box domain. *CjPLE* had the closest evolutionary relationship with *TAG1*, and formed PLE subbranch with *AmPLE*. Through genomic amplification and sequence analysis, we obtained and analyzed the gene structure of *CjPLE*, and the results showed that the gene contained two small introns, 33 bp and 118 bp respectively, which was inconsistent with the structure of C function gene in *Arabidopsis thaliana* and *Antirrhinum majus*. The analysis of MAD-box family of the whole genome of jujube showed that the genetic structure of different family members was variated from 1 to 12 introns, indicating that the *CjPLE* gene of *Camellia japonica* might have the function ambiguity (Zhang et al., 2017).

2.2 *CjPLE* is a potential regulator in the control of double flower development in Camellia

Real-time fluorescence quantitative PCR was used to analyze the relative expression level of *CjPLE* gene in different flower organs of wild *Camellia japonica*, 'Hongluzhen', 'Zhuangyuanhong' and 'Rongqiu'. The gene was highly expressed in the carpels of wild *Camellia japonica*. The gene expression was the highest in the stamens of the peony type double flower varieties 'Hongluzhen and 'Zhuangyuanhong', and was higher in inner petals and stamens of 'Rongqiu'. The study on C function gene *CjAGL6* found that the expression level of *CjAGL6* gene was highest in stigmas, followed was petal stamens, filaments and ovary, and lowest in sepals and petal sepals (Sun et al., 2013a). *CjPLE* had the same expression pattern with *CjAGL6*, the general trend was that the expression level was low in outer organs while was high in inner organs. These analyses indicated that the expression pattern of *CjPLE* conformed to the functional action of C function genes and might play a regulatory role in the development of different double-flower.

3 Materials and Methods

3.1 Materials

The plant materials used in this research were taken from camellia conservation plot at Research Institute of Subtropical Forestry in Chinese Academy of Forestry (Hangzhou, China). The flower buds were stored in -80°C refrigerator after liquid nitrogen refrigeration grinding.

3.2 RNA extraction and cDNA synthesis of *Camellia japonica*

The total RNA of *Camellia japonica* bud was extracted by EASYspin Plus Plant RNA Kit (Elade, Beijing), and the first strand of cDNA was then synthesized by reverse transcriptase (Fevmentas, Canada). Through the local BLAST comparison, cDNA sequence that was homologous with PLE was found in transcriptional database of *Camellia japonica* bud, and primers P-1 and P-2 were designed using Primer 5.0 for specific PCR (Table 1). The target fragment was recovered by gel and connected to T-vector pMD20 (TaKaRa, Dalian), transformed into *E. coli* DH 5α (TaKaRa, Dalian), and positive monoclonal wsa selected for sequencing.

3.3 Sequence homologous alignment and phylogenetic tree analysis

Online BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was applied for homologous sequence alignment of *CjPLE* gene, and the cDNA opening reading frameof the gene was searched online using ORF Finder (Web). BioEdit was used for sequence assembly of cDNA full-length and alignment of amino acid sequence. Phylogenetic tree was constructed and analyzed by MEGA7.0.

3.4 *CjPLE* gene structure determined by Genome walking

The full-length *CjPLE* was amplified using the Universal GenomeWalker^{TM} 2.0 kit. Theyoung leaf DNA of wild *Camellia japonica* was extracted with the CTAB DNA rapid extraction kit (Aidlab) of, and the DNA quality was detected by 0.6% agarose gel electrophoresis.

25 μL DNA (0.1 μg/μL) was taken for enzyme digestion by *Dra* I, *EcoR* V, *Pvu* II and *Stu* I restriction enzymes (as blank control), respectively. The treatment conditions were: 37°C for 2 h, lightly mixed for 5~10 s and kept in

37°C overnight (16~18 h). The enzyme digestion was detected the completeness by 0.6% agarose gel electrophoresis. The DNA was purified and recycled by NucleoSpin Gel and PCR Clean-Up kit. The designed specific primers GSP3 and GSP4 were designed to amplified the sequence by TD-PCR.

3.5 Fluorescent quantitative PCR

Total RNA of various floral organs of*Camellia japonica*, 'Hongluzhen', 'Rongqiu' and 'Zhuangyuanhong' were extracted by EASYspin Plus Plant RNA Kit. Then, each of them was taken 800 mg to synthesize the first strand of cDNA with reverse transcriptase. According to the known full-length sequence of *CjPLE* gene, fluorescence quantitative primers PLE-Fa and PLE-Ra were designed (Figure 1). Using GAPDH as internal reference genes, GAPDH-F and GAPDH-R were designed as internal specific primers. The reagent of fluorescence quantitative reaction was SYBR® Premix Ex *Taq*™ II and the instrument was ABI 7300 Real-time PCR (America). The reaction was in 95° Cfor 30 s, 95°C for 5 s and 60°C for 31 s, repeating 40 cycles. Repeat every test and sample for 3 times, and the data was analyzed by related software of ABI 7300 Real-time PCR, Excel 2003 and $2^{-\Delta\Delta CT}$ method (Sun et al., 2013b).

Authors' contributions

LT was the experiment designer and executor of this research who analyzed the data and wrote the first draft of this thesis. LJY, LXL, FZQ and YW took part in the test result analysis and thesis modification. NS and YHF were the constructors and chargers of this project, who direct experiment design, data analysis, paper writing and revision. All authors read and proved the final paper.

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