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蕙兰 *MADS* 基因 *APETALA1/FRUITFULL*-like 的克隆和时空表达特性

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摘 要：为研究兰花成花转变及花发育的调控机理，利用反转录 RT-PCR 和 RACE 的方法，从蕙兰萼片中克隆出一个 *APETALA1/FRUITFULL*-like (*AP1/FUL*-like) 基因，命名为 *CfAP11*，GenBank 登录号为 JQ031272.1。该基因编码的氨基酸序列与 MADS-box 蛋白家族中类 AP1/FUL 亚家族中球花石斛 *FRUITFULL*-like 具有较高的同源性 (84%)，系统进化分析表明该蛋白的氨基酸序列与 *AP1/FUL* 转录因子亚家族中的蛋白聚为一类。生物信息学分析推测表明，该基因编码的蛋白具有 MADS 保守域和相对保守的 K 区，二级结构中α-螺旋所占比例较高 (58.97%)，三级结构与月季、水稻和水仙非常相似。相对荧光定量 PCR 分析结果表明：*CfAP11* 在根中表达痕量，生殖期比营养期叶片中表达量低、盛花期比花蕾期花葶中表达量高，由此推测，*CfAP11* 可能与蕙兰的成花诱导、花发育有关；并发现 *CfAP11* 在盛花期花葶和子房中表达量远高于其他组织，表明其可能以某种机制参与果实的形成过程。

关键词：蕙兰，*AP1/FUL*-like，RACE，时空表达

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Molecular cloning and spatiotemporal expression of an *APETALA1/FRUITFULL*-like *MADS*-box gene from the orchid (*Cymbidium faberi*)

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Abstract: In order to identify genes involved in floral transition and development of the orchid species, a full-length *APETALA1/FRUITFULL*-like (*AP1/FUL*-like) MADS box cDNA was cloned from *Cymbidium faberi* (*C. faberi*) sepals and designated as *C. faberi APETALA1*-like (*CfAP11*, JQ031272.1). The deduced amino acid sequence of *CfAP11* shared 84% homology with a member of the *AP1/FUL*-like group of MADS box genes (AY927238.1, *Dendrobium thyrsiflorum FUL*-like MADS box protein 3 mRNA). Phylogenetic analysis shows that CfAP11 belonged to the *AP1/FUL* transcription factor subfamily. Bioinformatics analysis shows that the deduced protein had a MADS domain and a relatively conservative K region. The secondary structure of CfAP11 mainly consisted of alpha helices (58.97%), and the three-dimensional structure of the protein was similar to that of homologues in *Roza hybrida*, *Oryza sativa* and *Narcissus tazetta*. Real-time quantitative PCR (qRT-PCR) results reveal low levels of its mRNA in roots, lower levels in leaves during reproductive period than vegetative period, and higher levels in pedicels at full-blossom stage than at bud stage. These results suggest that *CfAP11* is involved in floral induction and floral development. Additionally, we observed higher levels of *CfAP11* expression in pedicels and ovaries than in other tissues during full-blossom stage, which suggests that *CfAP11* may also be involved in fruit formation in certain mechanism.

Keywords: *Cymbidium faberi*, *AP1/FUL*-like, RACE, spatiotemporal expression

Introduction

The ABCDE flower development model was established based on studies on several floral mutants in *Arabidopsis*, snapdragon and petunia. The model demonstrates the genetic interactions of the five major classes of floral homeotic selector genes referred to as class A, B, C, D, and E genes, almost all of which are MIKC-type MADS box genes. Each of these gene classes determines the identity of different floral organs: A alone specifies sepals in whorl 1; the combined activities of A and B control petals in whorl 2; B and C together regulate stamens in whorl 3; C alone controls carpels in whorl 4; D is involved in ovule development; and E is required for petal specification, stamen and carpel identity, and possibly also for ovule identity[1-2].

All MADS box proteins contain a highly-conserved amino acid (aa) motif [60 aa] located at the N-terminus that interacts with DNA and proteins such as other MADS box (M) transcription factors[3]. This domain is followed by a weakly-conserved I region (I) and a moderately-conserved region of roughly 70 aa, which is designated as a K-box. The I region and K-box are responsible for protein-protein interactions in type II MADS box proteins. The C-terminus (C) is the most variable part and is involved in ternary complex formation and transcriptional activation[4].

Recent studies have demonstrated that the functions of MADS box gene family members are not restricted to flower organ development. MADS box genes have been shown to be involved in root formation[5], initiation of flowering, determination of

meristem identity[6], embryonic development[7], and seed and fruit formation[8-9]. Furthermore, a single MADS box gene may have different functions at different developmental stages[10].

Class A-function genes, such as the *APETALA1* (*AP1*) MADS box gene, are involved in *Arabidopsis* floral determination. *AP1* expression was first observed 16 h after the start of photoinduction[11]. *AP1* from *Arabidopsis*[12] has been found to lead to the formation of sepals in whorl 1. Overexpression of *CDM111* (*AP1*) in *Arabidopsis* plants resulted in an aberrant phenotype of flower perianth segment[13]. Ectopic expression of *AP1* in wild-type *Arabidopsis* directs the conversion of vegetative and inflorescence meristems into floral meristems and the development of compound terminal flowers[14].

OMADS10 (*paleoAP1* ortholog) from *Oncidium* Gower Ramsey was expressed only in vegetative leaves and in the lip and carpel of mature flowers[15]. *MdMADS12* is an *AP1*-like gene that was expressed at similar levels in leaves, vegetative shoots, and floral tissues. In addition, it may be involved in transition from the juvenile to the adult stage[16].

Relatively few A function MADS box genes have been characterized in monocots compared to those in dicots[17-18]. Studies on the SQUA subclade genes among monocots have been mostly limited to maize and rice[19]. A few studies on Chinese orchids have been reported, but no *AP1*-like gene has yet been reported in *C. faberi*.

The Orchidaceae (with nearly 30 000 species) family is the largest family of flowering plants. Members of this family represent extreme specializations[20-21] that are possible better than any other family of flowering plants. Orchids have the greatest extent of flower morphological diversity[21] compared with other petaloid monocots such as lilies and tulips. The typical flower of a petaloid monocot is composed of five fundamentally three-fold whorls or derivatives thereof. It consists of six organs that are arranged in two whorls and represent three classes of organ identity. The first floral whorl contains three outer tepals (often called sepals). In the second floral whorl, there are two lateral inner tepals (petals) and a median inner tepal called the lip or labellum. The reproductive organs are special because they comprise a gynostemium or column, which is a compound structure formed by the fusion of male and female organs[22]. Thus, the mechanism of floral organ formation in orchids is more complex than that in other plants.

The shortening of the juvenile phase from several years to only a few months during floral transition is very helpful in studying the molecular mechanisms in orchids. Moreover, molecular analysis of orchid flower architecture has not been widely performed. The study of the molecular mechanism of flowering has been an important research issue in recent years, however, very limited molecular studies have been undertaken on orchid floral development, and no information about *AP1/FUL*-like A-function genes in *C. faberi* is currently available. In this study, we present the identification and expression analysis of a novel *AP1/FUL*-like gene in *C. faberi*.

1 Materials and methods

1.1 Plant materials

Roots, leaves, sepals, pedals, lips, columns, ovaries, and pedicels were sampled from *C. faberi* during the vegetative period, bud stage and full-blossom stage. Samples were immediately frozen in liquid nitrogen and stored at −80 °C until analysis.

1.2 RNA isolation and analysis

Total RNA was extracted from all materials using the Trizol method (Invitrogen). The integrity of RNA samples was assessed using agarose gel (1.2%) electrophoresis. RNA concentration and purity were determined based on the A_{260}/A_{280} ratio using a UC800 nucleic acid-protein analyzer (Beckman Co., USA).

1.3 Reverse transcriptase (RT) PCR

For the isolation of MADS box gene cDNA fragments, 1 μg of total RNA was used to synthesize the first strand cDNA from the poly A tail with a

T(18) primer using the 1st Strand cDNA Synthesis Kit of TaKaRa (TaKaRa Biotechnology, Dalian, China).

1.4 Isolation of *AP1/FUL*-like MADS box gene and 18S rRNA

RT-PCR was performed using *Taq* DNA polymerase (TaKaRa). Specific primers were designed based on the sequences of *AP1/FUL*-like genes in GenBank (Table 1). In addition, primers specific to *C. faberi* 18S rRNA were also designed (Table 1).

The PCR fragment of the predicted size was excised from the agarose gel and purified using a Montages DNA Gel Extraction Kit (Tiangen, Beijing, China). The purified PCR products were ligated into a pMD19-T vector (TaKaRa) for identification and sequencing.

1.5 Cloning of full-length cDNA encoding an AP1/FUL-like protein

Based on the conserved regions of AP1/FUL-like protein sequences, nested primers were designed and synthesized for rapid-amplification of cDNA ends (RACE) (Table 1).

The RACE PCR was performed using the CLONTECH SMART RACE kit. Both 500 and 700 bp products were aligned and assembled into one sequence. The assembled sequence was re-amplified and cloned into cloning vector pMD19-T. The sequence was submitted to Genbank nucleotide databases after sequence determination and verification. The ORF was determined using the NCBI ORF Finder tool.

1.6 *CfAP11* sequence analysis

The sequence *CfAP11* was analyzed using EXPASY. The nucleotide sequence, deduced amino acid sequence, and open reading frame (ORF) were analyzed. Sequence comparison was conducted through a database search using the BLAST tool. Phylogenetic analysis of CfAP11 proteins with other plant species was performed with DNAMAN using default parameters. A phylogenetic tree was constructed using the neighbor-joining method with NCBI.

1.7 Analysis of amino acid sequences

The physicochemical properties of the protein sequence were synthetically analyzed using ProtParam. The SignalP 3.0 tool was applied to predict the signal peptide sequence of CfAP11. The trans-membrane domain was predicted using TMHMM. The hydrophilicity/hydrophobicity of the CfAP11 protein was analyzed with the help of "ProtScale". Subcellular localization of CfAP11 was identified using PSORT. The secondary structure of CfAP11 was predicted using sopma and predictprotein. The CfAP11 protein structure was modeled on a SWISS-MODEL server.

1.8 Expression levels detected by real-time quantitative PCR (qRT-PCR)

The expression levels of the isolated *AP1/FUL*-like MADS box gene at different periods and in different organs were determined by qRT-PCR using the Mastercycler (Eppendorf, USA) and SYBR Premix Ex *Taq*^{TM} II qPCR Kit (TaKaRa) according to the manufacturer's instructions. The 18S rRNA gene from *C. faberi* was used as an internal control. The *CfAP11* and 18S rRNA (as internal control) primers for qRT-PCR were shown in Table 1.

Table 1 Primers used in this study

Primer names	Primer sequences (5′−3′)
AP1 forward	CTGAAG(A/C)GGAT(A/C)GAGA ACAAGAT
Reverse	CAAGCTGCTGCTC(C/A/T)A(A/G)GT GTTG
18S forward	TGGTTGATCCTGCCAGTAGT
Reverse	GTTCACCTACGGAAACCTTG
5'RACE: outer	CATTACGCAAGTCTTCCTGA
inner	GCAGGGACTTCTCCTTTCTTTG
3'RACE: outer	GAGATACCGCTATGCTGAAAGA
inner	GCTCTTCTAGGCTTAATGTGTC
qRT-PCR: *CfAP11* forward	GCATCAAAGAAAGGAGAAGTC
Reverse	GCAGGGACTTCTCCTTTCTTTG
18S forward	TTCGGTCCTATTGTGTTGGC
Reverse	TCGCAGTGGTTCGTCTTTCA

The ratio of gene-specific expression to 18S rRNA signal was defined as a relative expression. Negative control (no template) samples were processed as a routine quality control of the assay. The comparative CT method (2^{-DDCT}) was used for quantification analysis. The mean values of triplicate-independent PCR served as the CT values for both target and internal control genes.

2 Results

2.1 Cloning of *AP1/FUL*-like MADS box cDNA from *C. faberi*

The analysis of the extracted total RNA from sepals revealed that it had an A_{260} to A_{280} ratio of 1.99. In addition, the 18S and 28S rRNA bands, which characterize eukaryotes, on formaldehyde denatured agarose gels were specific. Total RNA had high purity and had not decomposed.

A homolog of the *AP1*-like MADS box cDNA designated as *CfAP11* (JQ031272.1) was isolated from *C. faberi* using a combination of PCR techniques. The *CfAP11* full-length cDNA sequence was obtained by assembling two cloned 3' and 5'-end cDNA fragments. The sequence is 705 bp in length, contains an ATG start codon and a TGA stop codon, and encodes a protein of 234 residues (AEX86945.1). Alignment of related proteins revealed that the sequences of the *CfAP11* cDNAs were more homologous in the coding region spanning the MADS (Fig. 1). Meanwhile, the I and K domains were more divergent at the C terminal domain. The C-terminus of CfAP11 proteins lacks the CFAA motif (conserved in many AP1-like genes of dicots) and the LPPWML motif (conserved in most SQUA-like genes of monocots).

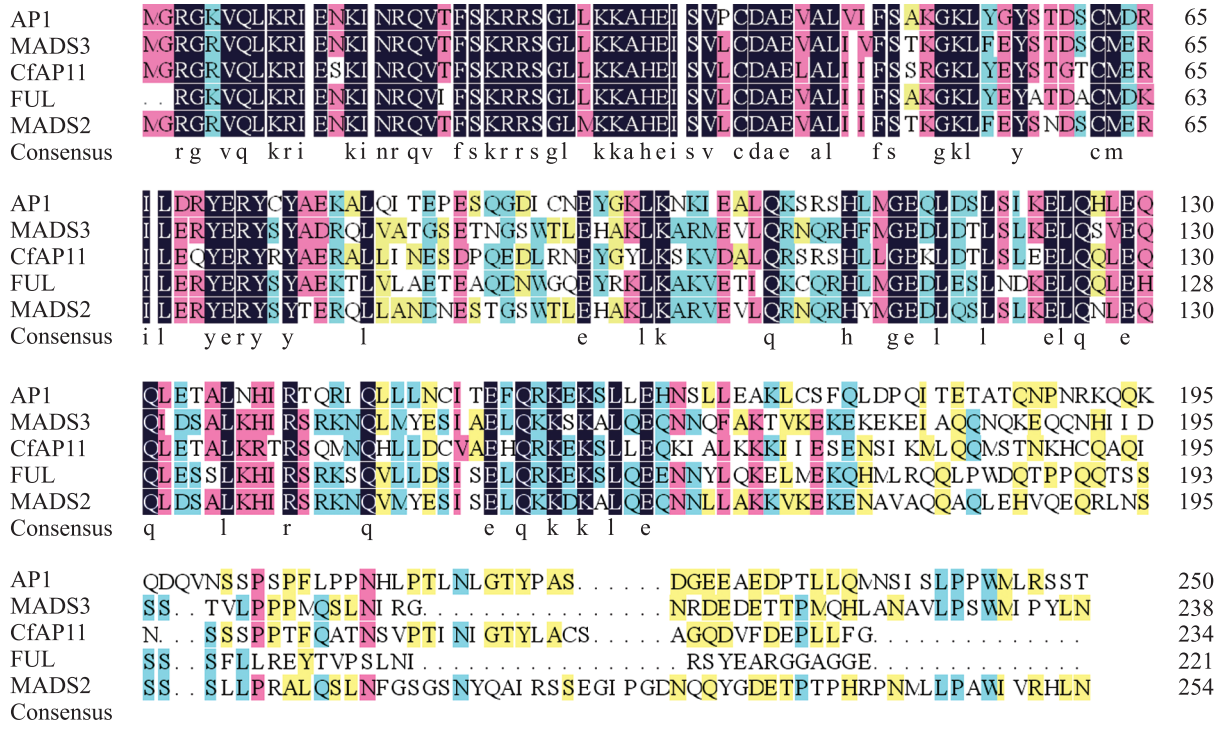

Fig. 1 Multiple-sequence alignment of the five AP1/FUL-like proteins showed that the CfAP11 protein contains a MADS (M) and a keratin-like (K) domain. CfAP11: AEX86945.1; AP1: AAZ76264.1; MADS2: AAC83170.1; MADS3: ACZ36916.1; FUL: ABG80456.

CfAP11 showed the highest sequence similarity (84%) with a member of the *AP1/FUL*-like group of MADS box genes (AY927238.1, *Dendrobium thyrsiflorum FUL*-like MADS box protein 3 mRNA). Phylogenetic analysis showed that CfAP11 belongs to the *AP1/FUL* transcription factor subfamily (Fig. 2). The genes within this clade are mostly involved in floral transition and early flower meristem development[23-25].

2.2 Physicochemical properties of the CfAP11 protein

The cloned *CfAP11* cDNA contains an ORF of 705 bp. The deduced polypeptide has 234 aa with a predicted molecular weight of 26.90 kDa and pI of 8.88. The peptide has 5 cysteine residues and relatively high levels of Leu (13.2%), Glu (9.4%), Ser (9.4%), and Gln (8.1%) residues. It is composed of a total of 3 803 atoms and has the chemical formula $C_{1169}H_{1919}N_{341}O_{364}S_{10}$. It contains 30 residues with a negative charge (Asp+Glu), which corresponds to 12.8% of the total aa amount, and 35 residues with a positive charge (Arg+Lys), which is 15.0% of the total aa amount. The N-terminal of the sequence considered has a M (Met) residue. The estimated half-life is 30 h. The instability index (II) was computed to be 56.08, indicating that the protein is unstable. The grand average of hydropathicity (GRAVY) is −0.615.

2.3 Characteristics of the CfAP11 protein in terms of hydrophilicity/hydrophobicity, transmembrane domain, and subcellular localization

SignalP analysis revealed that the CfAP11 protein does not possess a signal peptide, indicating that it is not a secretory protein. Analysis of the CfAP11 protein using the online tool TMHMM showed that it does not have an obvious transmembrane domain.

The prediction results (Fig. 3A) of hydrophilicity and hydrophobicity showed that the CfAP11 protein was at most 2.078 at position 46 and at least −2.778 at position 157. The number of hydrophilic amino acid residues was greater than that of hydrophobic amino acid residues in each peptide chain. Our findings suggest that CfAP11 is a putative unstable hydrophilic protein.

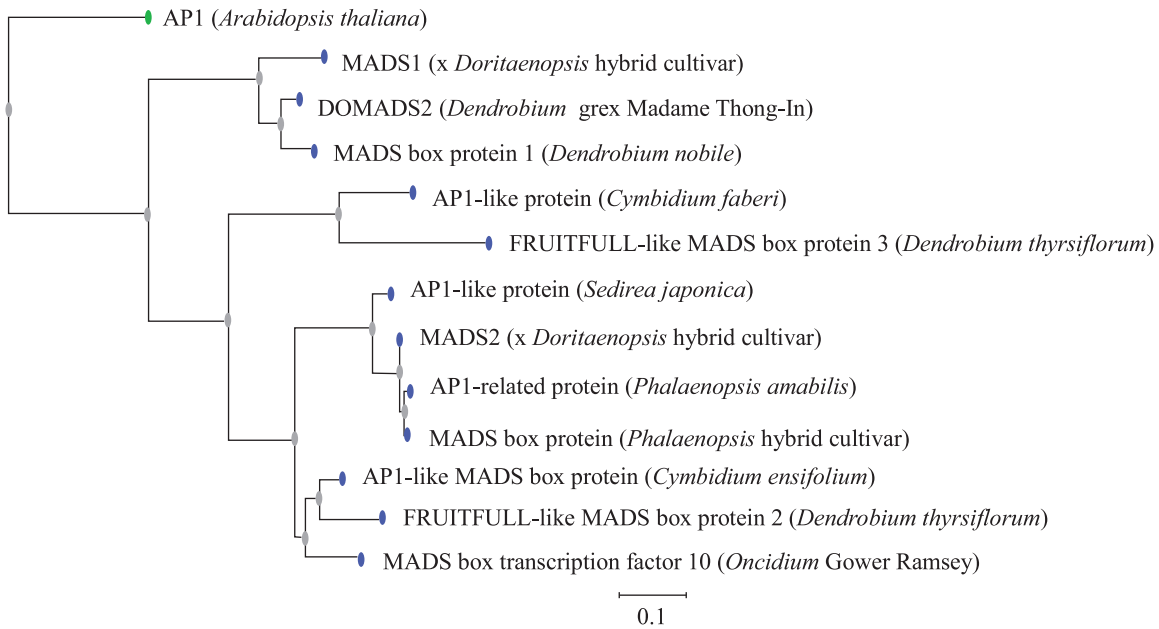

Fig. 2 Phylogenetic tree of CfAP11 MADS box protein sequences with known AP1/FUL-like MADS box protein sequences.

Prediction of the subcellular localization of CfAP11 using PSORT showed that CfAP11 is most likely localized in the mitochondria with a probability of 65.5%.

2.4 Prediction of the secondary structure and functional domains of CfAP11

Prediction of its secondary structure using the online tool sopma suggested that CfAP11 consists of random coils (29.06%), alpha helices (58.97%), extended strands (8.55%), and beta turns (3.42%). The proportions of random coils and alpha helices in the secondary structure were very high. Prediction by the predictprotein tool showed that the secondary structure of CfAP11 consists of loops (39.74%), helices (49.57%), and strands (10.68%), which concur with the predictions obtained using sopma.

Prediction and analysis of the functional domain of CfAP11 using prosite revealed a conserved MADS domain at the N-terminus that spanned from aa 1-61. Stability and conservation of the MADS domain is a vital requirement for CfAP11 protein function. The CDD online prediction tool provided by NCBI confirmed that CfAP11 belongs to the MADS superfamily.

2.5 Three-dimensional protein modeling

The final step in the bioinformatics analysis of protein subunit structures is 3D modeling using the SWISS MODEL program, which predicts protein structure based on aa sequences and comparisons with existing structures in databases. 3D views of the resulting models (Fig. 3B) were generated through online modeling on NCBI. These 3D models are visual representations of the information contained in *AP1*. They reveal the spatial arrangement of secondary structure elements like α-helices, β-strands, loops, and turns. The structure of CfAP11 contains a conserved MADS domain, as indicated by comparisons with 3D structures of *Roza hybrida*, *Oryza sativa*, and *Narcissus tazetta*.

2.6 Spatiotemporal expression analysis of *CfAP11*

Total RNA isolated from several tissues of *C. faberi* was used in further analysis of *CfAP11* gene expression. With 18S rRNA levels as the internal control, qRT-PCR was used to detect changes in *CfAP11* expression at different periods and in different organs to identify the pattern of *CfAP11* MADS box gene expression. The *CfAP11* qRT-PCR fragment was 230 bp long and that of 18S rRNA was 120 bp long. *CfAP11* expression was detected in the vegetative and reproductive periods. As shown in Figure 4, *CfAP11* mRNA expression was not limited

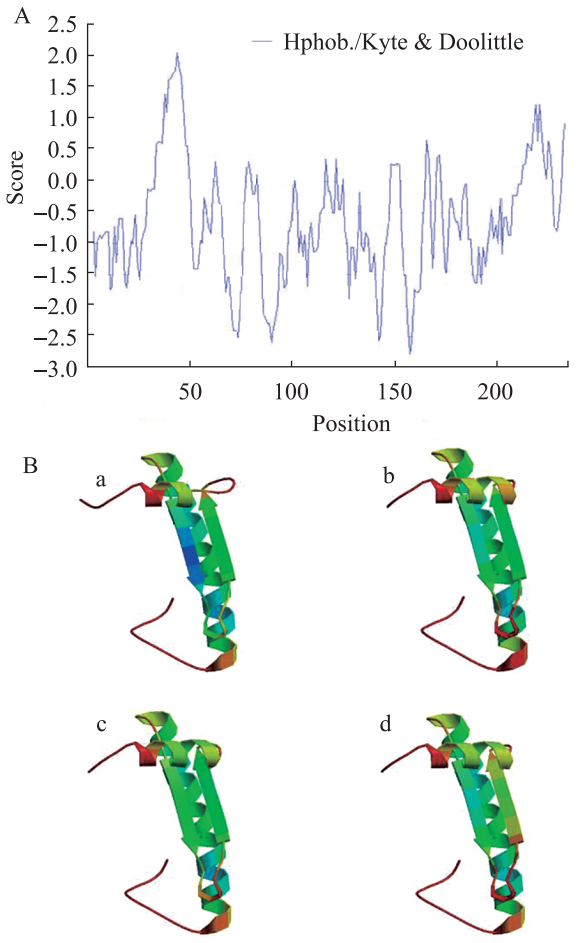

Fig. 3 Bioinformatic analysis of *CfAP11*. (A) Hydropathy analysis was performed using the Kyte and Doolittle algorithm. Positive scores indicate hydrophobic regions and negative scores indicate hydrophilic regions. (B) Comparison of CfAP11 protein spatial structure predicted by the Swiss-Model. a: *Cymbidium faberi* (AEX86945.1); b: *Roza hybrida* (FJ970028.1); c: *Oryza sativa* (AF058698.1); d: *Narcissus tazetta* (JN704304.1).

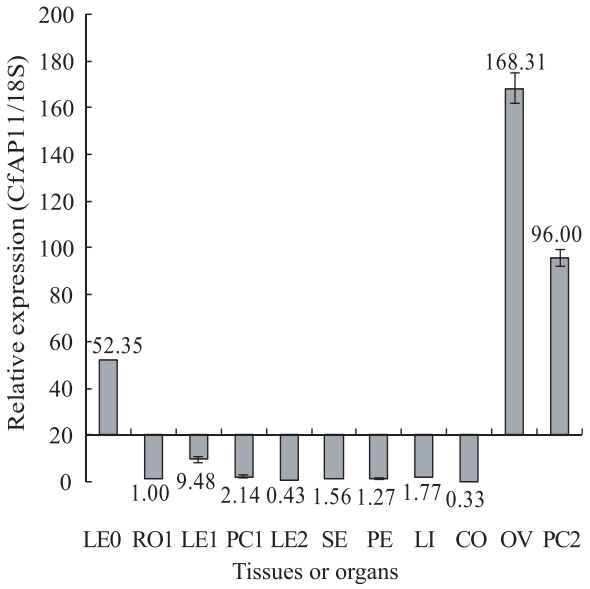

Fig. 4 Expression patterns of *CfAP11* at different stages. Relative expression was quantified by qRT-PCR and normalized by 18S rRNA. Each bar represents three repetitions from each RNA sample (LE0: leaves of vegetative period; RO1: roots of bud stage; LE1: leaves of bud stage; PC1: pedicels of bud stage; LE2: leaves of full-blossom stage; SE, sepals; PE: petals; LI: lips; CO: columns; PC2: pedicels of full-blossom stage; OV: ovaries). Error bars represent standard errors for each case.

to flower whorls. Our findings are different from those of a previous study, in which mRNAs for AP1 and SQUA were detected only in sepals and petals of mature flowers[26].

The detection of higher levels of *CfAP11* in leaves during the vegetative growth period compared with the reproductive period suggests that the gene may have a role in flower induction and initiation. These findings concur with those of function analysis of three lily *AP1*-like MADS Box genes[27].

During the reproductive growth period, *CfAP11* expression level at full-blossom stage was much higher than that at bud stage. There was high expression level of *CfAP11* in leaves at bud stage, while much higher expression was found in pedicels and ovaries at full-blossom stage. These findings indicate that *CfAP11* plays an important role during floral organ formation, which is consistent with the findings of Chen et al[27].

Expression levels of *CfAP11* in leaves gradually decreased until the plants reached full-blossom stage, which is related to earlier flowering and formation of floral organs.

Additionally, *CfAP11* mRNA was found to accumulate in the pedicels at full-blossom stage, which is consistent with the findings of Yanofsky that *AP1* mRNAs accumulated throughout the floral pedicel[28].

AP1 is expressed in the first and second whorl floral organs[12]. However, *CfAP11* was found to be expressed in all floral organs tested, including pedicels, sepals, petals, lips, columns (gynostemium, a fused structure of stigmas, styles, and stamens) and ovaries.

Many studies have shown that *AP1/FUL*-like is closely related to fruit formation[29]. At full-blossom stage, *CfAP11* mRNA expression was detected in floral organs as well as leaves and pedicels of adult flowering plants. High levels of expression were detected in ovaries and pedicels, moderate expression levels were observed in sepals, petals, and lips, while weak expression was observed in leaves and columns. Total expression level increased when the plant reached full-blossom stage. Therefore, the ability to regulate both floral initiation and flower formation was probably retained in *CfAP11*. In addition, *CfAP11* is most likely related to fruit formation.

3 Discussion

MADS box genes have been shown to be involved in floral transition both as inhibitors (*FLF/FLC* in *Arabidopsis*)[30] and inducers (*PFG* in *Petunia*)[31]. In this study, a RT-PCR approach with degenerate MADS box primers was used on *C. faberi* in order to clone *CfAP11* MADS box genes possibly involved in the juvenile-to-adult phase transition. For monocots like *C. faberi*, new insights on possible ways of manipulating the mechanisms controlling the transition and initiation of flowering

is of great interest. Knowledge of genes involved in the onset of flowering may lead to experimental protocols that shorten the juvenile phase with a relatively short vegetative period, which would be beneficial for breeding programs.

The MADS domain of the aligned proteins was found to be highly conserved. Conserved regions were also observed in the K domain. However, the C terminal domain of the aligned proteins was more divergent. Chen et al. reported that the conserved motifs (paleoAP1 or euAP1) in the C-terminus of the SQUA/AP1 subfamily of MADS box genes are not strictly necessary for their function[27]. The C-terminus of CfAP11 proteins lack the CFAA motif found in many AP1-like dicot proteins. Additionally, it is also absent in the C-terminus of the LPPWML motif conserved in most SQUA-like genes of monocots, which are closely related to *C. faberi*.

3.1 Analysis of *CfAP11* and characterization of the putative protein

Results obtained by BLAST search against the GenBank database, multiple sequence comparisons, and phylogenetic analyses suggest that the gene fragments isolated from *C. faberi* are most likely homologues of *AP1*. *CfAP11* had the highest sequence similarity (84%) with *Dendrobium thyrsiflorum* FRUITFULL-like MADS box protein 3 mRNA (AY927238.1), a member of the *AP1/FUL*-like group of MADS box genes. Therefore, *CfAP11* was categorized within the *AP1/FUL*-like subfamily in the phylogenetic tree. The MADS box genes of this group function in plant development, including control of flowering transition, determination of floral organ identity, and regulation of fruit maturation. In our efforts to isolate and study *AP1* MADS box genes involved in flower development and floral transition, we started with transgenic tobacco plants to study the functional characterization of *CfAP11*.

Genetic characteristics of *CfAP11* were analyzed online using bioinformatics tools. However, some of the results of our analyses need further confirmation. For example, the result of 3D modeling of the protein's secondary structure needs to be experimentally verified using appropriate techniques such as X-ray crystallography or NMR spectroscopy.

3.2 Quantitative evaluation of expression of *AP1/FUL*-like genes

For MADS box genes involved in flower formation, three out of four cloned sequences were found to be homologous to flower whorl-determining genes. This finding indicates that the expression of the corresponding genes is not strictly confined to reproductive tissues and may be functional in other tissues and other developmental stages as well.

AP1 regulates the transition from inflorescence shoot to floral meristems and the development of sepals and petals[32-33].

We analyzed the spatiotemporal expression pattern of *CfAP11* during vegetative and reproductive periods by qRT-PCR. Transcripts were found to be expressed at different levels. Furthermore, *CfAP11* was found to be strongly expressed in leaves during the vegetative period before flowering, indicating that *CfAP11* is likely related to floral induction.

As expected, *CfAP11* was observed to be moderately expressed in leaves and weakly in roots at bud stage, when inflorescence develops after floral transition. At full-blossom stage, *CfAP11* expression was detected in all floral organs. These findings indicate that *CfAP11* functions in floral organ formation.

Strikingly, *CfAP11* transcripts accumulated mainly in pedicels and ovaries at full-blossom stage. The increase in *CfAP11* expression levels correlates with the progressive acquisition of flowering competence. Based on these observations, we propose that *CfAP11* is involved in fruit formation and functions in a broad range of tissues.

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