Comparative Expression of the CygoSTK Gene in Common Cymbidium goeringii and the Peculiar-Flower Cultivar 'Tianpeng Mudan' Postprint

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Abstract

To investigate the molecular mechanisms regulating floral organ development in Cymbidium goeringii and its mutant-flower variety, a D-class MADS-box gene CygoSTK (GenBank accession number MH917912.1) with a cDNA length of 849 bp was cloned from flower buds of both common Cymbidium goeringii and the mutant-flower variety 'Tianpeng Mudan' using a homologous cloning approach. The gene sequence was highly conserved between the two Cymbidium goeringii types, containing a complete ORF of 705 bp that encodes an STK lineage MADS-box transcription factor composed of 234 amino acid residues. Structural analysis revealed that the CygoSTK transcription factor comprises a highly conserved MADS domain (1~57) and a secondarily conserved K domain (91~172), with its C-terminal transcription activation region containing two highly conserved motifs: the AGI motif and the AGII motif. Furthermore, qPCR analysis of CygoSTK gene expression in different floral organs of Cymbidium goeringii and 'Tianpeng Mudan' demonstrated that CygoSTK expression was highest in the ovaries of both common Cymbidium goeringii and 'Tianpeng Mudan', significantly exceeding its expression in other floral organs of the respective varieties (LSD, $P < 0.05$). These findings indicate that the CygoSTK gene exhibits strong functional conservation and primarily participates in ovary development in Cymbidium goeringii.

Full Text

Preamble

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Title: Expression Comparison of CygoSTK Gene in *Cymbidium goeringii* and Abnormal Flower Variety 'Tian Peng Mu Dan'

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Abstract

To investigate the molecular mechanisms regulating floral organ development in *Cymbidium goeringii* and its abnormal-flower varieties, we cloned a D-class MADS-box gene, *CygoSTK* (GenBank accession MH917912.1), from flower buds of both common *C. goeringii* and the abnormal-flower variety 'Tian Peng Mu Dan' using a homologous cloning approach. The gene sequence was highly conserved between the two varieties, containing a complete 705 bp open reading frame (ORF) that encodes a 234-amino acid STK-lineage MADS-box transcription factor. Structural analysis revealed that the CygoSTK transcription factor contains a highly conserved MADS domain (amino acids 1–57) and a moderately conserved K domain (amino acids 91–172), with its C-terminal transcriptional activation region harboring two highly conserved motifs: the AGI motif and AGII motif. Quantitative PCR (qPCR) analysis of *CygoSTK* expression in different floral organs showed that transcript levels were highest in the ovary of both common *C. goeringii* and 'Tian Peng Mu Dan', significantly exceeding expression in all other floral organs (LSD, $P < 0.05$). These findings demonstrate strong functional conservation of *CygoSTK*, which primarily participates in ovary development in *C. goeringii*.

Keywords: *Cymbidium goeringii*, *CygoSTK* gene, MADS-box, flower development, real-time quantitative PCR

Introduction

Cymbidium goeringii, a terrestrial orchid in the family Orchidaceae, has been cultivated in China for over 2,000 years and remains one of the most beloved national orchid species (Xiang et al., 2018). Wild populations are primarily distributed in the Yangtze River basin and southwestern China, with additional distribution in Japan and the Korean Peninsula (Chen et al., 2016). Valued for its elegant foliage, refined floral morphology, subtle coloration, and delicate fragrance, *C. goeringii* holds significant ornamental and economic importance in Southeast Asia (Han et al., 2018; Zuo et al., 2017). China's long history of cultivation has yielded rich natural variants and numerous elite germplasm resources distinguished by diverse flower colors, petal types, inflorescence forms, and foliar characteristics, fostering a profound culture of orchid appreciation. Developing new *C. goeringii* varieties with high ornamental value is scientifically and economically important for enriching germplasm resources and promoting national orchid culture. Abnormal-flower varieties, prized for their rarity and

unique morphology, have long been sought after by enthusiasts and represent an important breeding direction.

Among traditional *C. goeringii* cultivars, 'Tian Peng Mu Dan' exemplifies the peony-type abnormal flower, characterized by increased numbers of labella, absence of anther caps on the gynostemium, and a peony-like appearance at anthesis that has captivated Chinese growers. Previous research on the orchid *Orchis italica* demonstrated that its *SEEDSTICK* (*STK*) homolog, *OitaSTK*, plays a crucial role in maintaining normal development of the gynostemium, ovules, and labellum (Salemme et al., 2013). In *Arabidopsis*, *STK* is a D-class MADS-box gene involved in regulating ovule and seed development, with predominant expression in ovules and seeds (Hundertmark et al., 2008). Investigating the expression patterns and functions of *STK* genes in *C. goeringii* will not only elucidate the molecular basis of floral organ morphogenesis, providing genetic resources for flower-type improvement and molecular breeding, but also contribute to our understanding of floral evolution in orchids. Using common *C. goeringii* and the abnormal-flower variety 'Tian Peng Mu Dan' as experimental materials, we cloned the D-class MADS-box gene *CygoSTK* and compared its expression patterns between the two varieties to analyze the relationship between expression differences and floral morphology variation, thereby illuminating the molecular mechanisms through which *CygoSTK* regulates *C. goeringii* flower development.

Materials and Methods

1.1 Experimental Materials

Tian Peng Mu Dan were cultivated in an artificial climate chamber at the College of Horticulture and Gardening, Yangtze University, Jingzhou, Hubei. In January 2018, freshly opened flowers were collected. For common *C. goeringii*, sepals, petals, labella, pollinia, gynostemia, and ovaries were dissected. For 'Tian Peng Mu Dan' , sepals, petals, labella, gynostemia, and ovaries were separated. All samples were rapidly frozen in liquid nitrogen and stored at -80 °C.

1.2.1 RNA Isolation and First-Strand cDNA Synthesis

Total RNA was extracted from flower buds of both *C. goeringii* and 'Tian Peng Mu Dan' using the EASYspin Plus Complex Plant RNA Kit (Aidlab Biotechnologies, Beijing). First-strand cDNA was synthesized using M-MLV reverse transcriptase (TaKaRa) according to the manufacturer' s protocols.

1.2.2 Cloning of *CygoSTK* Gene

Based on conserved sequences in the 5 and 3 untranslated regions (UTRs) of *STK* homologs from orchid species available in GenBank, primers were designed

to amplify the full-length *STK* homolog from *C. goeringii* (Table 1). Amplification protocols and identification of positive clones followed the methods of Liu and Yu (2012). Primer synthesis and sequencing were performed by Sangon Biotech (Shanghai).

1.2.3 Sequence Structure Analysis of *CygoSTK*

The complete open reading frame (ORF) of *CygoSTK* was translated and subjected to BlastP analysis on NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Twenty STK homologous proteins from diverse angiosperms were selected for phylogenetic tree construction using the neighbor-joining (NJ) method in MEGA5.0 (Table 2). Additionally, nine STK homologs from eight species—including *Arabidopsis thaliana*, *Petunia* × *hybrida*, and *Dendrobium thyrsiflorum* (Table 3)—were aligned using ClustalW in BioEdit 7.2 to analyze the domain architecture of CygoSTK.

1.2.4 Expression Analysis of *CygoSTK*

Gene-specific primers targeting non-conserved regions of *CygoSTK* were designed, with *CygoActin* (GU181354.1) serving as the reference gene (Table 1). After verifying primer specificity, quantitative real-time PCR (qPCR) was performed on a CFX96 system (BIO-RAD, USA) to examine tissue-specific expression. The 20 L reaction mixture contained 10 L ChamQ SYBR qPCR Master Mix, 0.4 L each of forward and reverse primers, 0.4 L cDNA template, and 8.6 L ddH O. Cycling conditions were: 95 °C for 1 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 15 s. The ChamQ SYBR qPCR Master Mix kit was purchased from Novozymes (Nanjing). Relative expression levels were calculated using the 2^(-ΔΔCt) method, and statistical analysis was performed with SPSS 17.0.

Results and Analysis

2.1 Floral Structure Analysis of Two *Cymbidium* Varieties

The flower of common *C. goeringii* consists of three sepals, two petals, one specialized labellum, two pollinia borne on a single gynostemium, and an ovary formed from three fused carpels, with pollinia attached to the gynostemium. In contrast, 'Tian Peng Mu Dan' produces four sepals, two or more petals, two or more labella, a gynostemium lacking pollinia at its apex, and an ovary with normal external morphology (Figure 1 [Figure 1: see original paper]).

2.2 Cloning of Full-Length *CygoSTK* cDNA

The full-length *CygoSTK* cDNA is 849 bp, comprising a 111 bp 5 UTR, a 705 bp ORF encoding a 234-amino acid STK-like transcription factor, a stop codon, and a 33 bp 3 UTR. The gene was designated *CygoSTK* (GenBank accession

MH917912.1). Sequence analysis confirmed that the nucleotide sequences cloned from both varieties were identical.

2.3 Protein Sequence Alignment and Phylogenetic Analysis

Phylogenetic reconstruction (Figure 2 [Figure 2: see original paper]) revealed that CygoSTK clusters with nine STK homologs from ten angiosperm species within the STK lineage, representing an ortholog of *Arabidopsis* STK with 57.87% sequence similarity. CygoSTK groups with monocot STK homologs in a subclade, showing the closest relationship (88.46% similarity) to the orchid *Dendrobium thyrsiflorum* STK homolog DthyrAG2, reflecting established species relationships. Sequence alignment (Figure 3 [Figure 3: see original paper]) demonstrated that CygoSTK possesses a highly conserved MADS domain (amino acids 1–57), a moderately conserved K domain (amino acids 91–172), and a less conserved I region (amino acids 58–90). The M, I, and K domains comprise 57, 33, and 82 amino acids, respectively, while the C-terminal region contains 62 amino acids including two highly conserved motifs (AGI and AGII) and a monocot-specific MD motif at the extreme C-terminus (Figure 3). These features confirm that CygoSTK belongs to the D-class MADS-box protein family.

2.4 Tissue-Specific Expression of *CygoSTK* in Floral Organs

qPCR analysis (Figure 4 [Figure 4: see original paper]) showed that *CygoSTK* is expressed in petals, labella, pollinia, gynostemia, and ovaries of common *C. goeringii*, with only weak transcription detected in sepals. Expression was highest in the ovary, significantly exceeding levels in all other floral organs (LSD, $P < 0.05$), followed by the gynostemium, which showed significantly higher expression than petals, labella, and pollinia (LSD, $P < 0.05$). Expression in labella was also significantly higher than in petals and pollinia, though no significant difference was observed between pollinia and petals. In 'Tian Peng Mu Dan' , *CygoSTK* was primarily expressed in sepals, labella, gynostemia, and ovaries, with only faint signals in petals. Similar to common *C. goeringii*, expression was highest in the ovary (significantly above all other organs; LSD, $P < 0.05$), followed by the gynostemium (significantly higher than sepals and labella; LSD, $P < 0.05$). Sepal expression was significantly higher than labellum expression. Comparing expression between varieties, *CygoSTK* showed pronounced expression in 'Tian Peng Mu Dan' sepals but only weak signals in common *C. goeringii* sepals, whereas the opposite pattern was observed in petals. Additionally, expression in labella was significantly higher in common *C. goeringii* than in 'Tian Peng Mu Dan' (LSD, $P < 0.05$). While expression in gynostemia did not differ significantly between varieties, *CygoSTK* expression in the ovary of common *C. goeringii* was significantly higher than in 'Tian Peng Mu Dan' (LSD, $P < 0.05$).

Discussion

This study identified *CygoSTK*, a MADS-box gene associated with floral organ development in both common *C. goeringii* and the abnormal-flower variety 'Tian Peng Mu Dan' . Amino acid alignment, domain architecture analysis, and phylogenetic reconstruction confirmed that CygoSTK contains the canonical MADS and K domains characteristic of highly conserved D-class MADS-box proteins.

In angiosperms, altered expression patterns of MADS-box genes often significantly impact plant growth and development (Kramer et al., 2004). In the model plant *Arabidopsis*, *STK* is predominantly expressed in ovaries and regulates ovule and seed development (Hundertmark et al., 2008). In the Amaryllidaceae species Chinese narcissus (*Narcissus tazetta* var. *chinensis*), the *STK*-like gene *NtSTK* is mainly expressed in the pistil (Wu et al., 2015). In the Rosaceae species double-flowered cherry (*Prunus lannesiana* 'Albo-rosea'), the *STK* homolog *PrseSTK* is expressed in sepals, stamens, and pistils, with ectopic expression in sepals leading to ectopic ovules on the calyx tube and contributing to morphological differences between single and double flowers (Liu and Li, 2015). In the palm oil palm (*Elaeis guineensis*), the *STK* homolog *SHELL* regulates both fruit shape development and seed oil biosynthesis (Singh et al., 2013).

Among orchids, the *STK* homolog *EpMADS23* in *Erycina pusilla* shows significantly higher expression in gynostemia than in other floral tissues (Dirks-Mulder et al., 2017). In *Phalaenopsis equestris*, the *STK*-like gene *PeMADS7* is exclusively expressed in the gynostemium at relatively late developmental stages, and *PeMADS7*-transgenic *Arabidopsis* exhibits early flowering, upward-curled leaves, and increased seed abortion (You et al., 2012). The *STK* homolog *DcOAG2* in *Dendrobium crumenatum* is expressed in gynostemia, ovaries, and pollinia, primarily regulating ovary development (Xu et al., 2010). In *Dendrobium thyrsiflorum*, the *STK* homolog *PhalAG2* is expressed in labella, gynostemia, and ovaries, co-regulating ovary development with C-class genes (Song et al., 2006). Similarly, the *STK* homolog *DthyrAG2* in *Dendrobium* is expressed in labella, gynostemia, and ovaries, playing an important role in late ovule development (Skipper et al., 2006).

In this study, *CygoSTK* was expressed in labella, pollinia, gynostemia, and ovaries of common *C. goeringii*, with highest expression in ovaries, indicating its crucial regulatory role in ovary formation. In 'Tian Peng Mu Dan' , high *CygoSTK* expression in sepals may be associated with the increased number and morphological variation of sepals, though the underlying regulatory mechanism requires further investigation. Collectively, the expression patterns in both varieties demonstrate that *CygoSTK* functions primarily in gynostemium and ovary development, with ovary expression significantly exceeding that in other organs. Thus, *CygoSTK* exhibits strong functional conservation and plays a major role in regulating ovary development in *C. goeringii*. Further investigation of this

gene will provide valuable insights for morphological modification of orchid floral organs and targeted breeding.

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Note: Figure translations are in progress. See original paper for figures.