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Cloning and Expression Analysis of the DcSKP1 Gene in Carnation

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Abstract

The SKP1 gene is a core component of the SCF E3 ubiquitin ligase protein complex and is involved in diverse biological processes. However, the carnation SKP1 gene has not been cloned. In this study, RT-PCR combined with RACE technology was employed to isolate and clone the full-length cDNA sequence of a meiosis-related SKP1 gene from carnation (Dianthus caryophyllus) anthers, designated as DcSKP1 (GenBank accession number MK931293). The results demonstrated that the full-length cDNA sequence of the DcSKP1 gene is 962 bp, containing an ORF of 567 bp that encodes 188 amino acids. Protein sequence alignment revealed that DcSKP1 contains a highly conserved TPEE motif, as well as Skp1_{POZ} and Skp1 domains, and clusters with Arabidopsis SKP1 in a single branch. Quantitative real-time PCR analysis of the expression pattern of the carnation DcSKP1 gene showed that DcSKP1 is expressed in all tissues, with higher expression levels in anthers compared to stem and leaf tissues, and the highest expression in young anthers, decreasing as anther development progresses. These findings suggest that the DcSKP1 gene may play an important role in carnation meiosis.

Full Text

Preamble

Clone and Expression Analysis of DcSKP1 in *Dianthus caryophyllus*

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Abstract

The SKP1 gene is a core component of the SCF (Cul1-Rbx1-Skp1-F-box) E3 ubiquitin ligase protein complex and participates in numerous biological processes. However, the SKP1 gene of carnation has not yet been cloned. In this study, we isolated and cloned a full-length cDNA sequence of a meiosis-related SKP1 gene from the anthers of carnation (*Dianthus caryophyllus*) using RT-PCR combined with RACE technology, designated as *DcSKP1* (GenBank accession number MK931293). The results showed that the full-length *DcSKP1* cDNA sequence was 962 bp, containing an open reading frame (ORF) of 567 bp that encodes 188 amino acids. Protein sequence alignment revealed that DcSKP1 possesses a highly conserved TPEE motif, as well as Skp1_{POZ} and Skp1 domains, and clusters on the same branch as *Arabidopsis* SKP1. Expression pattern analysis of *DcSKP1* in carnation using quantitative real-time PCR demonstrated that the gene is expressed in all tissues, with higher expression levels in anthers than in stem and leaf tissues. Expression was highest in young anthers and decreased as anther development progressed. These findings suggest that *DcSKP1* may play an important role in meiosis in carnation.

Keywords: *Dianthus caryophyllus*, DcSKP1, meiosis, bioinformatics analysis, gene expression analysis

Introduction

Numerous cellular events, including signal transduction, cell cycle regulation, and transcriptional mechanisms, are primarily controlled by ubiquitin-mediated protein degradation (Hershko & Ciechanover, 1998; Ning et al., 2016). Ubiquitination is a three-enzyme cascade process in which ubiquitin is first activated by ubiquitin-activating enzyme (E1), then transferred to ubiquitin-conjugating enzyme (E2), and finally conjugated to lysine residues of substrate proteins with the assistance of ubiquitin ligase (E3), which confers substrate specificity (Liu et al., 2002). Multiple ubiquitin molecules can attach to existing ubiquitin molecules on protein substrates to form polyubiquitin chains, and polyubiquitinated proteins are typically degraded by the 26S proteasome. The SCF complex constitutes the largest known family of E3 ubiquitin protein ligases (Schulman et al., 2000), and SCFs participate in the ubiquitination of key proteins, controlling fundamental biological processes such as cell cycle progression and transcriptional regulation (Sun et al., 2017). *Arabidopsis* SKP1-like 1 (ASK1) encodes an SCF subunit that links Cullin and F-box proteins (Yang et al., 1999). Previous studies have shown that ASK1 can interact with F-box proteins (Takahashi et al., 2004). Since these F-box proteins play important roles in different pathways (Zheng & Li, 2011), ASK1, as a key component of SCFs, is widely expressed in *Arabidopsis* and likely performs essential functions in many developmental and physiological processes (Lu et al., 2016; Ye, 2014). *Arabidopsis ask1* mutants exhibit defects in male meiosis, floral organ development, and vegetative growth (Zhao et al., 2001; Zhao et al., 2006; Zhao et al., 2015). The earliest detectable defect in *ask1-1* occurs during the leptotene-to-zygotene transition, where nu-

cleoli fail to migrate to the nuclear periphery and homologous chromosomes do not synapse (Wang et al., 2004). The ASK1-1 mutation also leads to increased recombination frequency in *Arabidopsis* male meiosis (Wang & Yang, 2006) and male sterility (Yang et al., 1999).

Carnation is one of the four major cut flowers with high yield and economic value. Current carnation breeding primarily relies on traditional hybrid breeding, including interspecific and intraspecific hybridization. Natural bud sports or radiation-induced mutations are also used as breeding methods. Additionally, transgenic technology has been employed to cultivate blue carnations (Fukui et al., 2003) and lines with enhanced anti-senescence (Yu & Bao, 2004), resistance to fusarium wilt (Hu et al., 2018), and heat tolerance (Wu, 2014). However, research on regulating genes involved in carnation meiosis to create superior carnation varieties is limited, which greatly hinders the progress of carnation breeding. The SKP1 gene regulates floral organ development and meiosis, but the carnation SKP1 gene has not yet been cloned. This study aims to clone the carnation SKP1 gene and investigate its expression patterns in anthers, stems, and leaves, thereby laying a foundation for understanding the molecular mechanisms of carnation floral development and applying this knowledge to carnation breeding.

Materials and Methods

Plant Materials

Carnation (*Dianthus caryophyllus* 'Rogate') was collected from the Baofeng Base of Yunnan Jichuang Horticulture Technology Co., Ltd. In September 2018, carnation stems, leaves, and flower buds at different developmental stages were collected. Young anthers were dissected with forceps, snap-frozen in liquid nitrogen, and stored at -80°C for later use.

Gene Cloning and Bioinformatics Analysis

DcSKP1 Gene Cloning Anthers stored at -80°C were ground with magnetic beads in an automatic sample grinder for 60 seconds. The beads were quickly removed, and 1 mL of Trizol reagent (Invitrogen, USA) was added to the centrifuge tube. Total RNA was extracted following the Trizol kit protocol. First-strand cDNA synthesis was performed using the PrimeScript™ RT reagent Kit with gDNA Eraser (Takara) according to the manufacturer' s instructions. Specific primers were designed based on the published carnation genome sequence (http://carnation.kazusa.or.jp/blast.html). The middle fragment was amplified first, followed by 3 -RACE and 5 -RACE using the SMARTer® RACE 5 /3 Kit (Takara, Japan) according to the manufacturer' s protocol. Finally, full-length primers were designed to amplify the complete cDNA sequence including the entire ORF. Primer sequences are listed in Table 1 .

Bioinformatics Analysis Homologous sequences of the target gene were analyzed using the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST). Amino acid similarity alignments of this gene and SKP1 genes from other species were performed using DNAMAN software, and a phylogenetic tree was constructed using MEGA 6.0 software. The molecular weight and theoretical isoelectric point of the protein were predicted using ExPASy ProtParam. Subcellular localization was predicted online using PSORT II, signal peptide prediction was performed using SignalP 4.1, phosphorylation sites were predicted using NetPhos 2.0, transmembrane structures were predicted using TMHMM 2.0, and hydrophilicity was analyzed using ProtScale software.

Gene Expression Analysis

Flower buds measuring 1.1–1.2 cm, 1.3–1.4 cm, and 1.5–1.6 cm in length were selected. Anthers were dissected, and stem and leaf tissues were collected in triplicate and snap-frozen in liquid nitrogen. RNA was extracted and reverse-transcribed into cDNA, which was diluted 10-fold and stored at -20°C as a template for quantitative real-time PCR. *GAPDH* was used as the reference gene with primers GAPDH 579-F and GAPDH 788-R. *DcSKP1* primers were Dca36455-1153F and Dca36455-1265R (Table 1). Three biological and technical replicates were performed, and data were analyzed and plotted using SPSS 12.0 software.

Results

Cloning of the Full-Length DcSKP1 cDNA Sequence

Using cDNA from carnation anthers as a template for PCR amplification, we first amplified the middle fragment, then used 3 -RACE and 5 -RACE to amplify the 3 and 5 ends of *DcSKP1*. Based on the assembled full-length cDNA sequence, primers were designed to amplify the complete *DcSKP1* cDNA sequence including the entire ORF (Figure 1 [Figure 1: see original paper]). Sequencing of the middle fragment yielded a 500 bp sequence (Figure 1A). The 5 and 3 ends yielded sequences of 376 bp (Figure 1B) and 567 bp (Figure 1C), respectively. Assembly of these three fragments produced a full-length *DcSKP1* cDNA sequence of 962 bp. Subsequent amplification of the complete cDNA including the ORF yielded a 921 bp sequence (Figure 1D) that was identical to the assembled sequence. These results indicate that the full-length *DcSKP1* cDNA is 962 bp, containing a 567 bp ORF that encodes 188 amino acids. This gene was designated *DcSKP1* (GenBank accession number MK931293).

DcSKP1 Amino Acid Sequence Alignment and Phylogenetic Analysis

Multiple sequence alignment of Skp1 proteins from various species using DNAMAN software revealed that DcSKP1 contains a highly conserved TPEE motif (amino acids 157–160), which is likely involved in glycosylation-related post-translational modifications. The carnation protein also possesses a Skp1_{POZ}

domain (amino acids 18–77) and a Skp1 domain (amino acids 139–186) (Figure 2 [Figure 2: see original paper]). SKP1 proteins can be classified into two types of proteins and three types of genes (Kong et al., 2010). Type I proteins are encoded by Ia and Ib genes and contain two conserved regions (Skp1_{POZ} and Skp1) and two variable regions. Type II proteins are encoded by type II genes and differ significantly from type I proteins, containing an additional C-terminal region. Ia genes contain a single intron, whereas Ib genes lack introns, and type II genes have multiple introns at various positions (Min et al., 2012). The *DcSKP1* gene isolated in this study contains one intron and is classified as a type I protein and Ia gene.

A phylogenetic tree was constructed using the maximum likelihood method to compare the DcSKP1 amino acid sequence with SKP1 genes from other species. The results showed that DcSKP1 clusters on the same branch as *Arabidopsis* SKP1 (Figure 3 [Figure 3: see original paper]), indicating the highest homology between these two genes.

Bioinformatics Analysis of DcSKP1

Analysis of amino acid composition and physicochemical properties revealed that DcSKP1 has a molecular formula of $C_{913}H_{1443}N_{237}O_{317}S_7$, a theoretical isoelectric point of 4.52, and a molecular weight of 21,036.32 Da. The protein contains 19 positively charged residues and 38 negatively charged residues, with a predicted instability index of 47.83, classifying it as an unstable protein. The amino acid composition of DcSKP1 is dominated by Glu (approximately 11.7%) and Leu, with Thr comprising about 10.1%. Subcellular localization prediction indicated that DcSKP1 is likely localized to the nucleus (52.2%), cytoplasm (26.1%), Golgi apparatus (4.3%), cytoskeleton (4.3%), peroxisome (4.3%), plasma membrane (4.3%), and mitochondria (4.3%). Signal peptide prediction showed no peak values (Figure 4 [Figure 4: see original paper]), indicating that DcSKP1 lacks a signal peptide and is not a secreted protein. Transmembrane structure prediction revealed no transmembrane helices, confirming that DcSKP1 is a non-transmembrane protein. Phosphorylation site prediction identified 48 potential phosphorylation sites (score > 0.5), including 16 serine, 12 threonine, and 1 tyrosine site (Figure 5 [Figure 5: see original paper]). Hydrophobicity and hydrophilicity analysis indicated that DcSKP1 is most likely a hydrophilic protein (minimum: -2.489, maximum: 2.011) (Figure 6 [Figure 6: see original paper]).

Quantitative Expression Analysis of DcSKP1

Quantitative real-time PCR results showed that *DcSKP1* is expressed in all three developmental stages of carnation anthers, but expression gradually decreases over time. Expression was highest in anthers from 1.1–1.2 cm flower buds (Stage 1), while expression in anthers from 1.3–1.6 cm buds (Stage 2 and Stage 3) was significantly lower. Stage 1 expression differed significantly from Stage 2 and Stage 3. Expression in stems and leaves was even lower and significantly

different from that in anthers (Figure 7 [Figure 7: see original paper]). These results suggest that *DcSKP1* is associated with anther development, particularly with meiosis.

Discussion

SCF complexes participate in a wide range of biological processes, including signal transduction, transcription, and cell cycle regulation (Song, 2011; Sun et al., 2017). The SCF complex was first discovered in yeast and plays a crucial role in the ubiquitination and degradation of cell cycle substrates (Feldman et al., 1997). The SCF complex consists of three subunits: Cullin (CDC53), SKP1, and F-box protein. SKP1 is the core component of the SCF E3 ubiquitin ligase protein complex and participates in various biological processes. In this complex, SKP1 functions as an adaptor protein that binds to both Cullin and F-box proteins. SKP1 can interact with numerous F-box proteins to ubiquitinate different substrate-specific factors (Vierstra & Smalle, 2004). The tobacco SKP1 gene contains a Skp1 domain (Zhang et al., 2007; Xia et al., 2015), and subcellular localization studies have shown that NtSKP1 protein is expressed in both cytoplasmic and nuclear compartments (Zhang et al., 2009). Carnation DcSKP1 possesses Skp1_{POZ} and Skp1 domains, as well as a highly conserved TPEE motif that is likely involved in glycosylation-related post-translational modifications. Bioinformatics prediction indicates that DcSKP1 is primarily localized to the nucleus.

The *Arabidopsis* SKP1 (*atk1-1*) mutant exhibits reduced male fertility, fewer viable pollen grains, and abnormal male gametophyte meiosis. Male meiosis in the *atk1-1* mutant produces an abnormal number of microspores of different sizes, and meiotic spindles lack the normal bipolar structure of typical spindles. These abnormal spindles may cause abnormal chromosome segregation and subsequent spore and pollen abnormalities, and the incorrect alignment of *atk1-1* chromosomes at metaphase I may also be related to these abnormal spindles (Chen et al., 2002). Additionally, ASK1 suppresses recombination during pollen meiosis (Wang & Yang, 2006). Studies in wheat have shown that a sharp decrease in SKP1 expression may disrupt normal cell division, thereby affecting pollen grain growth and development and leading to premature death. Concurrently, a significant reduction in SKP1 protein levels would impair the formation of SCF complexes, block the ubiquitin-proteasome pathway, and result in delayed degradation of cell cycle inhibitor proteins, ultimately affecting pollen viability and causing male sterility (Song, 2011). In this study, *DcSKP1* expression was high in young anthers and decreased as anthers developed, suggesting that *DcSKP1* may participate in the meiotic process in carnation.

Rice OmSKP1 is primarily localized to the nucleus and is expressed in both vegetative (leaf) and reproductive (flower) tissues, with significantly higher expression in flowers than in leaves, indicating that OmSKP1 mainly functions in floral tissues (Fan, 2013). Wheat TSK1 gene is strongly expressed in meristematic tissues such as anthers, young roots, and floral apical meristems, suggest-

ing its involvement in plant cell division-related processes (Li, 2006). In wheat, the relative expression of SKP1 gradually decreases in both fertile and sterile plants as anther development progresses (mononuclear, binuclear, and trinuclear stages). However, at the same developmental stage, SKP1 expression is higher in fertile anthers than in sterile anthers (Song, 2011). In *Arabidopsis*, ASK1 is continuously and widely expressed throughout reproductive and vegetative growth, with higher expression in regions of active mitosis and meiosis (Porat et al., 1998). ASK1 expression fluctuates during the meiotic cell cycle, peaking from leptotene to pachytene, suggesting that this period is critical for ASK1 function (Wang & Yang, 2006). Strawberry FaSKP1-1 is expressed in various plant tissues including roots, leaves, stems, petals, styles, receptacles, pollen, and fruits (Yin et al., 2016). In this study, we investigated the expression pattern of *DcSKP1* and found that it is expressed in all tissues, with significantly higher expression in anthers than in stem and leaf tissues. These results suggest that *DcSKP1* is associated with anther development and may participate in the meiotic process in carnation. This study provides a foundation for investigating the causes of pollen abortion, understanding the signaling pathways regulating meiosis, and breeding superior plant varieties in carnation.

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