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Molecular cloning and characterization of a novel gene encoding an EF-hand calcium-binding protein related to fruit seedlessness of grapevine

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ABSTRACT

EF-hand calcium-binding protein (*CBP*) gene plays a critical role during embryonic development in animals, but it remains unclear whether this gene possesses similar function in plants. In this study, a novel *CBP* gene was isolated from *Vitis vinifera* cv. Thompson Seedless and designated as *VvCBP1*. Using a combination of sequence structural investigation, expression pattern analysis of different embryo development in grapevine, and silencing assay of *VvCBP1* homolog in tomato, the functional role of *VvCBP* gene in embryo development of seedless grapevine was studied. Structural investigations showed that *VvCBP1* is 907 bp in length, and contains two EF-hand motifs. Expression pattern of this gene is different in embryo between seedless and seeded grapevine species. Moreover, the silencing of homology gene led to the seeds number significantly decreased in tomato. These data demonstrate clearly that *VvCBP1* plays an important role in embryo development of seedless grapevine.

1. Introduction

Seedlessness is an economically important quality trait in grapevine. Seedless grapevines are of great interest for fresh fruit consumption and raisin production worldwide. Two different types of seedlessness have been observed in grape genetic resources: parthenocarpy and stenospermocarpy (Stout, 1936). In stenospermocarpic varieties, pollination and fertilization occur as normal, but the embryo and/or endosperm abort two to four weeks after fertilization (Ledbetter and Ramming, 1989). It has been previously reported that the property of seedlessness was controlled by a single or a few recessive genes (Bouquet and Danglot, 1996; Spiegel-Roy and Sahar, 1990; Stout, 1936), although the possibility of dominant control has also been suggested (Roytchev, 1998; Stout, 1937).

In previous studies, marker-assisted selections for seedlessness in grapevine have been well-documented (Doligez et al., 2002; Lahogue et al., 1998; Yang et al., 2005a). Many of them displayed quantitative characteristics (Doligez et al., 2002; Lahogue et al., 1998), while the SCAR marker selection was reported to be a dominant allele (Yang et al., 2005b). In recent years, numerous essential genes for seed and embryo development have been intensively studied, many of which remain uncharacterized and are currently annotated to encode proteins with unknown functions (Albert et al., 1999; Griffith et al., 2007; Kwong et al., 2003; Stone et al., 2001; Tzafrir et al., 2004). In addition, the molecular and cellular mechanisms of embryo abortion and seedlessness during grapevine berry development remain to be uncovered. A recent differential expression analysis indicated that a chloroplast chaperonin 21 (ch-Cpn21) gene from *Vitis vinifera* cv. Thompson Seedless, identified by silencing assays in tobacco and tomato fruits, was essential for grape seed development (Hanania et al., 2007). Subsequently, the same group has isolated a ubiquitin extension gene S27a using similar approach, whose overexpression resulted in abnormal plant regeneration and inhibited shoot development. Moreover, S27a silencing in embryogenic callus led to cell necrosis and callus death (Hanania et al., 2009). Evidence from these results suggests that the gene functions in regulation of developing organs of grapevine.

EF-hand calcium-binding proteins (CBPs) consist of large protein family genes, which are found in a number of plant species.

Abbreviations: CBPs, calcium-binding proteins; EST, expressed sequence tag; RACE, rapid amplification of cDNA end; RT-PCR, reverse transcription polymerase chain reaction; SCAR, sequence characterized amplification region; SSH, suppression subtractive hybridization; TRV, tobacco rattle virus; VIGS, virus-induced gene silencing.

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Previous studies on EF-hand CBPs indicated that the family member can sensor the cellular Ca^{2+} concentration and transduct the Ca^{2+} signals to trigger downstream response (Gifford et al., 2007). It has also been reported that EF-hand CBPs were involved in various developmental processes including root, floral organ and reproductive development (Capoen et al., 2009; Dumas and Gaude, 2006; Kaothien et al., 2005; Li et al., 2007; Poulter et al., 2008; Yoon et al., 2006), hormone regulated cellular activities (cell division and elongation, stomatal closure/opening) (Kim et al., 2006; Lanteri et al., 2006), biotic and abiotic stress-induced defense responses (Albrecht et al., 2003; Chung et al., 2004; Kobayashi et al., 2007; Lecourieux et al., 2006; Pandey et al., 2007; Yang and Poovaiah, 2000).

In our previous study, a cDNA library of *V. vinifera* cv. Thompson Seedless related to embryo abortion at different development stages was constructed, and 3908 EST sequences were obtained. A combination of sequence analysis and RT-PCR assay revealed that one EST sequence (GenBank accession no. GR905727) possesses EF-hand calcium-binding protein domain and displayed a different expression profile from seeded grapevine species. This finding promotes us to further investigate the structure and functional characterization of the gene. Our results demonstrated that the full-length cDNA, designated *VvCBP1*, possesses two EF-hand calcium-binding motifs, which shares high similarity with that of the tomato. Moreover, using *tobacco rattle virus* (TRV)-mediated virus-induced gene silencing (VIGS) technology, we silenced the tomato homolog of *VvCBP1* in tomato fruits, which led to the number of transformation tomato seed significantly decrease. The results suggest that *VvCBP1* may be tightly associated with fruit seedlessness of grapevine.

2. Materials and methods

2.1. Plant materials and growth conditions

V. vinifera cvs. Thompson Seedless and Pinot Noir were cultivated in the Grape Repository of Northwest A & F University, Yangling, Shaanxi, China under the natural environment with normal management. Embryos of *V. vinifera* cvs. Thompson Seedless and Pinot Noir were sampled after full-bloom stage of 15 d, 20 d, 25 d, 30 d, 35 d, 40 d, 45 d, and 50 d during embryo development, and stored at −80 °C before use.

Lycopersion esculentum cv. Micro-Tom used for VIGS were grown in the greenhouse under the condition of 25/20 °C and 70% humidity with a 14/10 h light/dark period.

2.2. Cloning the full-length cDNA of VvCBP1 and sequence analysis

A cDNA library of *V. vinifera* cv. Thompson Seedless berry at different stages of embryo development was previously constructed and a total of 3908 high quality ESTs were generated (Zhang, 2007). Bioinformatic analysis indicated that one of these ESTs sequence possesses EF-hand calcium-bind protein, and further expression analyses displayed a different expression profile from seeded grapevine in embryo development. Gene-specific primers (*VvCBP1*-5′: 5′-CAAC CACTTCTCTATGTACTCTTCTAC-3′; *VvCBP1*-R: 5′-CAAGCCCCACGGAG GCTCCAGCCACC-3′) were designed to the EST sequence to amplify full-length cDNA from *V. vinifera* cv. Thompson Seedless. Total RNA was isolated from each sample stage from *V. vinifera* cvs. Thompson Seedless and Pinot Noir according to the method previously described (Zhang et al., 2003), and cDNA synthesized using PrimeScript reverse transcriptase kit (TaKaRa, Dalian, China) according to the manufacture's instructions. RACE techniques were employed to obtain full-length cDNA sequence using the SMART RACE cDNA Amplification Kit (Clontech). The PCR products was cloned into pMD18-T vector (TaKaRa) and sequenced.

The nucleotide sequence analysis was performed using BLASTN and BLASTX programs of NCBI (http://www.ncbi.nlm.nih.gov/BLAST). Chromosomal location of *VvCBP1* gene was predicted by BLAT server at the Genoscope Genome Browser (http://www.genoscope.cns.fr/blat-server/cgi-bin/vitis/webBlat). The protein conserved domain was analyzed using Smart (http://smart.emblheidelberg.de/smart/change_mode.pl) and ExPASy (http://au.expasy.org/tools/). Amino acid sequence alignment and phylogenetic tree were generated by ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html). The sequences used for bioinformatics analysis are *Lycopersion esculentum* (GenBank accession no. BT014117), *Arabidopsis thaliana* (NP_196037), *Populus trichocarpa* (XP_002308014), *Medicago truncatula* (ACJ84993), *Ricinus communis* (XP_002534120), *Saccoglossus kowalevskii* (XP_002737488), *Oryza sativa* (NP_001066106), *P. trichocarpa* (XP_002308014), *Juglans nigra* (ACN39566), *Zea mays* (NP_001147282), *Pisum sativum* (CAB63845), *Sorghum bicolor* (XP_002450240), *Picea sitchensis* (ABR17289) and *Glycine max* (ACU14400).

2.3. Construction of silencing plasmids

To produce the VIGS vector for knock-down of *VvCBP1* homolog in tomato (*LeCBP1*, accession no. BT014117), a 167 bp fragment of *LeCBP1* corresponding to 269–435 nt was amplified using the primers 5′-TCCCGTTCCGTCTCCCTAC G-3′ and 5′-CCTTCGGTGGTTTGTTCGGATC-3′. The resultant PCR products were confirmed by DNA sequencing and cloned into pTRV2 to generate pTRV2-*LeCBP1*.

2.3.1. Virus infection by Agrobacterium-mediated infiltration

For VIGS assay, pTRV1, pTRV2 and pTRV2-*LeCBP1* were respectively transformed into *Agrobacterium tumefaciens* strain GV3101 by electroporation. Briefly, single colony harboring each of the construct was isolated and grown overnight in 50 ml LB medium supplemented with the appropriate antibiotics (28 °C, 200 rpm). Cells were harvested, resuspended in infiltration media (10 mM $MgCl_2$, 10 mM MES pH 5.6, 200 mM acetosyringone) and adjusted to OD_{600} of 1.0. Subsequently, pTRV1 was mixed (1:1 v/v) either with pTRV2 or with pTRV2-*LeCBP1* in which the former was control. Mixtures were incubated in a dark incubator at RT for 3 h with gentle agitation. The cultures were then infiltrated into the carpopodium of tomato fruits (10 d after pollination) that still attached to the plant using 1 ml needleless syringe (Fu et al., 2005).

2.3.2. RT-PCR analysis

Total RNA were extracted from grapevine embryos or tomato fruit samples according to the protocol previously described (Zhang et al., 2003). Reverse transcription was carried out with 2.5 μg of total RNA after DNase treatment using PrimeScript Reverse Transcriptase (TaKaRa). Semi-quantitative RT-PCR was performed using PrimeScript™ RT-PCR Kit (TaKaRa). Grapevine *GAPDH* gene (GenBank accession no. EF192466) was used as internal control. Primers for *VvCBP1* and *GAPDH* were 5′-GCTGAGAGAGGCACTGATGAG-3′ and 5′-GC AATAAGAAATGGCAAGACG-3′, and 5′-GGAGCTGAATTTGTTGTTGAG-3′; 5′-CATTAACTCCAACAACG-AACATAGG-3′), respectively.

To determine the transcriptional level of *LeCBP1* in tomato fruit, a pair of primers (5′-ATCAGGATGGGAGTGGGTTA-3′ and 5′-CAGCAATTCCAGGA TAGAAGGT-3′) was used. Tomato *Ubiqutin* gene (GenBank accession no. AK246708) was used as internal control with specific primers (5′-ACTGATTTCTCTCCTCCAGAC-3′; 5′-GAGTGCCCTAATGCTGAGTGCG-3′). RT-PCR products were separated on 1.2% agarose gels, EtBr stained, and quantified by