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Full Length Article

Cloning and Activity Analysis of Grape *VvCIPK10* Promoter

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Abstract

CBL/CIPK signaling system plays an important role in plant stress response proceeding. *VvCIPK10* gene responds rapidly to low temperature and other stresses at transcriptional level. To find out how *VvCIPK10* responds to the low temperature reaction, we cloned *VvCIPK10* promoter gene in grapevine. Predictive analysis of cis-acting elements revealed that *VvCIPK10* promoter sequence was rich in TATA-box and CAAT-box elements, as well as cis-acting elements related to light response, hormone response elements and low temperature response elements. After *VvCIPK10* promoter fusion with GUS, the recombinant vector was transformed into tobacco leaves. GUS activity analysis showed that the promoter was active and its activity was induced by low temperature. The results of promoter truncation deletion test showed that 169 bp at 3' end of *VvCIPK10* promoter was the key fragment in response to low temperature, and the low temperature response element played a decisive role in this fragment.

Keywords: Grape; Low temperature; Promoter; *VvCIPK10*

Introduction

Grape is one of the important fruit trees widely cultivated in the world. Its berries are not only rich in minerals, but also contain higher level of resveratrol, which promotes human health and the improvement of living standards (Guo *et al.* 2019a; Yu *et al.* 2019; Ni *et al.* 2020). China is one of the origins of grapes, which are now cultivated throughout the country. In China, mainly European grapes are produced commercially which show good quality characteristics but with poor cold resistance (Guo *et al.* 2019c). In most areas north of the Yellow River in China, grapes need to be buried in the soil to prevent them from freezing in the winter. This increase production costs (Guo *et al.* 2019b). Therefore, it has important theoretical significance and practical value to study grape cold resistance. In recent years, great progress has been made in the study of cold resistance of grapes. Chai *et al.* (2019) established a low-temperature exothermic technology system for evaluating cold resistance of grape, identified the cold resistance of grape germplasms from different sources. Yao *et al.* (2017) cloned *VpPUB24* and *VpHOS1* from Chinese wild *Vitis pseudoreticulata*. The results showed that *VpHOS1* promoted the degradation of VpICE1 protein, while *VpPUB24* promoted the accumulation of VpICE1 protein by inhibiting the accumulation of VpHOS1 protein, resulted in the enhancement of cold resistance of transgenic plants. Yu *et al.* (2017) cloned *VaERD15* gene from *Vitis amurensis*. Transgenic *Arabidopsis* and *V. vinifera* cv. Red Globe showed that this gene could significantly improve the cold resistance of plants. Xu *et al.* (2014) cloned two *VaICE* genes from *Vitis amurensis*, and the expression of *VaICE* was induced by low temperature. The transgenic *VaICE1/2* Arabidopsis was more tolerant to low temperature.

In the early stage, we cloned *VvCIPK10* gene from grapes cv. Thompson Seedless, which coded for calcineurin B subunit interacting protein kinase. Its autophosphorylation activity depended on Mn^{2+}, not Mg^{2+} and Ca^{2+}. The autophosphorylation activity of *VvCIPK10* was inhibited by EDTA ($C_{10}H_{16}N_2O_8$) (Yu *et al.* 2016; Yan *et al.* 2017). *VvCIPK10* was expressed in all tissues of grape, mainly in grape roots and leaves. After low temperature treatment, *VvCIPK10* showed an induced expression pattern (Yu *et al.* 2016). In this study, the promoter of *VvCIPK10* was cloned from grape, the sequence and activity of the promoter were analyzed to provide theoretical basis for further exploring the molecular effects of *VvCIPK10* in grape cold resistance.

Materials and Methods

Experimental material

Grapes cv. Thompson Seedless was used as plant material for this experiment. The clone vector pGEM-T easy was

from Promega Company, and the plasmid extraction kit and gel recovery kit were sourced from Beijing Tiangen Biochemical Technology Co., Ltd. Gene sequencing and primer synthesis were completed in Nanjing Jinweizhi Biotechnology Co., Ltd. Promoter transient expression vectors pC039-0GUS and pC0390 35S::GUS were preserved in our laboratory.

Primer design and DNA extraction

The primers were designed according to the genomic sequence of Pinot Noir (*Vitis vinefera* L.). The primers were VvCIPK10-Pro-F (5'-CAAAGTGGACTTCTTCACCAC-3') and VvCIPK10-Pro-R (5'-ATGGTATCCAGAGATCGAACAC-3'). To truncate the *VvCIPK10* promoter, the primers were designed as follows: VvCIPK10Δ1-F: CTTATCTCACCACTATCAAATAAG, VvCIPK10Δ2-F: CCAAACATTCTAAATGTGGTATAAC, VvCIPK10Δ3-F: TAGTTTCATTCCGCAATGTGGA, VvCIPK10Δ4-F: CTATTAGTAAACAGACACGTGG, VvCIPK10Δ5-F: CAGCTATTTAATAACGATTGGAC. The genomic DNA of grape was extracted using the CTAB (Cetyl trimethyl ammonium bromide) method (Xu *et al.* 2010).

Cloning and sequence analysis of *VvCIPK10* promoter

PCR was performed with the use of PrimeSTAR HS DNA Polymerase using 100 ng grape genomic DNA as template. The reaction system was as follows: 5 × PrimeSTAR Buffer 5 μL, dNTP mixture 2 μL, HS DNA Polymerase 0.25 μL, DNA template 100 ng, primer 1 μL each, ddH_2O supplemented to 50 μL. The reaction procedure was: 98°C 15 s, 58°C 30 s, 72°C 2 min, 32 cycles. PCR products were recovered by agarose gel electrophoresis, and PCR products were recovered, connected to pGEM-T-easy vector and transformed into DH5 alpha competent cells. After screened with Amp antibiotics, positive clones were picked up and sequenced. Bioinformatics analysis of *VvCIPK10* promoter sequence was performed in PLACE (https://www.dna.affrc.go.jp/PLACE/?Action=newplace) and PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

Construction of promoter transient expression vector

BamHI and protective bases (5'-GGGGGATCCCAAAGTGGACTTCTTCACCAC-3') were added at the 5'end of primer VvCIPK10-Pro-F.PstI and protective bases (5'-GGGCTGCAGATGGTATCCAGATCGAACAC-3') were added to the 5'end of the primer VvCIPK10-Pro-R.PstI and protective bases (5'-GGGCTGCAGCTTATCTCACCACTATCAAATAAG) were added to the 5'end of the primer VvCIPK10Δ1-F.PstI and protective bases (5'-GGGCTGCAGCCAAACATTCTAAATGTGGTATAAC) were added to the 5'end of the primer VvCIPK10Δ2-F.PstI and protective bases (5'-GGGCTGCAGTAGTTTCATTCCGCAATGTGGA) were added to the 5'end of the primer VvCIPK10Δ3-F.PstI and protective bases (5'-GGGCTGCAGCTATTAGTAAACAGACACGTGG) were added to the 5'end of the primer VvCIPK10Δ4-F.PstI and protective bases (5'-GGGCTGCAGCAGCTATTTAATAACGATTGGAC) were added to the 5'end of the primer VvCIPK10Δ5-F. Using pGEM-T/ProVvCIPK10 plasmid as template, the PCR reaction was carried out according to the instructions of Prime STAR HS DNA Polymerase kit. The PCR products and pC0390GUS empty particles were digested by BamHI and PstI and the products of digestion were recovered by 1.2% agarose gel electrophoresis and the target fragments and large segment vectors were recovered. DH5alpha competent cells were transformed after ligation reaction. After screening by Amp antibiotics, positive clones were selected and sequenced. The recombinant vectors were named pC0390 VvCIPK10::GUS, pC0390 VvCIPK10Δ1::GUS, pC0390 VvCIPK10Δ2::GUS, pC0390 VvCIPK10Δ3::GUS, pC0390 VvCIPK10Δ4::GUS, and pC0390 VvCIPK10Δ5::GUS. PC0390GUS was used as negative control and pC039035S::GUS as positive control. The recombinant vector and control vector were transformed into Agrobacterium tumefaciens GV3101 by freeze-thaw method.

Transient transformation and GUS activity analysis of tobacco leaves

Agrobacterium-mediated instantaneous transformation of tobacco leaves was studied by Xu *et al.* (2010). The transformed tobacco plants were cultured at room temperature of 26°C ± 1°C and in 12 h light per day. After cultured for 24 h, transgenic plants were treated at low temperature. These transformed plants were cultured at the temperature of 4°C ± 1°C and in 12 h light per day for 24 h. GUS enzyme activity was determined by fluorescence quantitative analysis (Jefferson 1987). S.P.S.S.17.0 software was used to analyze the data.

Results

Cloning and sequence analysis of grape *VvCIPK10* promoter

Primer of *VvCIPK10* promoter was designed (GenBank: AM482921) according to Pinot Noir genome sequence. After PCR amplification, the band with the expected fragment size was obtained and the sequence of this promoter was verified at 1683 bp. Multiple sequence alignments revealed that there were only 6 SNPs differences between the genome sequences of Thompson Seedless

grape *VvCIPK10* and Pinot Noir. Predictive analysis of promoter cis-acting elements revealed that the promoter sequence contained basic elements and some specific elements, including 78 TATA-boxes and 26 CAAT-boxes. Specific elements included 5 light-responsive elements, 6 ERF transcription factor binding elements (ERE), 2 anaerobic-related elements, 2 ABA response elements, 1 salicylic acid response element, 1 auxin response element, 1 gibberellin response element, 1 cryogenic response element and 1 defense and adversity related element (Fig. 1).

Construction of transient expression vector

The recombinant instantaneous expression vector pC0390 VvCIPK10::GUS was constructed by double digestion of pGEM-T/VvCIPK10 plasmid and connected it to the corresponding digestion site of pC0390 GUS. The recombinant vector was further identified by double enzyme digestion after PCR detection, and the enzyme digestion results obtained the fragment with the same size as *VvCIPK10* promoter (Fig. 2), indicating that *VvCIPK10* promoter was connected to the instantaneous expression vector. The instantaneous expression vector pC0390 VvCIPK10::GUS and the control vector were transformed into agrobacterium, and positive clones were obtained through resistance screening. The positive clones were identified by PCR, and the positive clones were used for promoter activity analysis test.

Activity analysis of *VvCIPK10* promoter

Agrobacterium tumefaciens containing recombinant vector pC0390 VvCIPK10::GUS, positive control vector pC0390 35S:: GUS and negative control pC0390GUS were transformed into tobacco leaves, and GUS enzyme activity was detected after 48 h *in vitro*. The results showed that the positive control showed strong enzyme activity, the activity of recombinant vectors was lower than that of positive control; while, negative control had no activity (Fig. 3). Previous studies showed that the expression of *VvCIPK10* was induced by cold stress. Therefore, we tested whether the promoter activity of *VvCIPK10* was induced or not by cold stress. After transforming tobacco leaves with recombinant vector pC0390 VvCIPK10::GUS for 24 h, the transformed leaves were induced and cultured at 4°C for 24 h. The results showed that cold stress increased the activity of *VvCIPK10* promoter.

***VvCIPK10* promoter truncation analysis**

In order to further determine the key elements of *VvCIPK10* promoter induced by cold stress, we successively truncated the promoter sequence from the 5 'end, and transformed agrobacterium by connecting different missing fragments to pC0390GUS carrier. After infecting tobacco leaves, the promoter was induced and cultured at 4°C for 24 h.

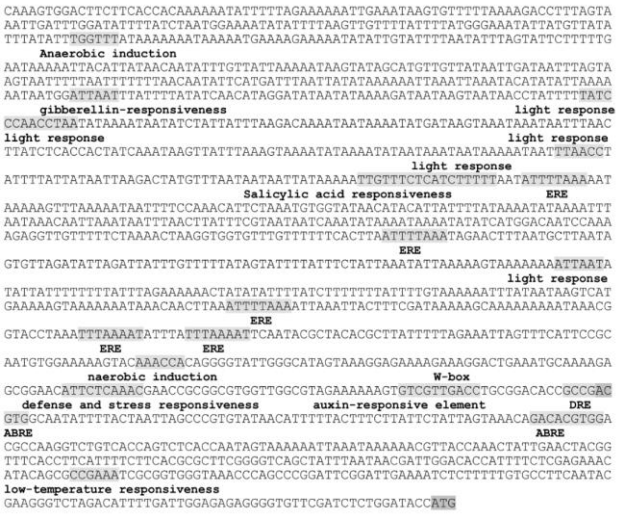

Fig. 1: Sequence analysis of the *VvCIPK10* promoter in grapevine

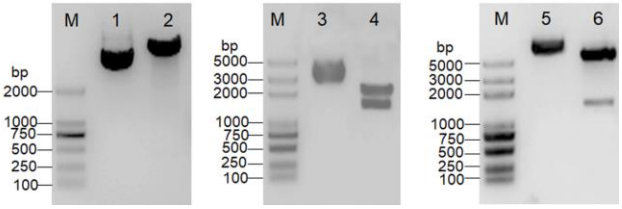

Fig. 2: The electrophoresis results of construction of plant transient expression vector

Lane M: DNA molecular weight standard; Lane 1: Empty vector pC0390GUS; Lane 2: Double enzyme digestion of vector pC0390GUS; Lane 3: pGEM-T/VvCIPK10 plasmid; Lane 4: Double enzyme digestion of pGEM-T/VvCIPK10; Lane 5: Recombinant vector of pC0390 VvCIPK10::GUS; Lane 6: Double enzyme digestion of recombinant vector of pC0390 VvCIPK10::GUS

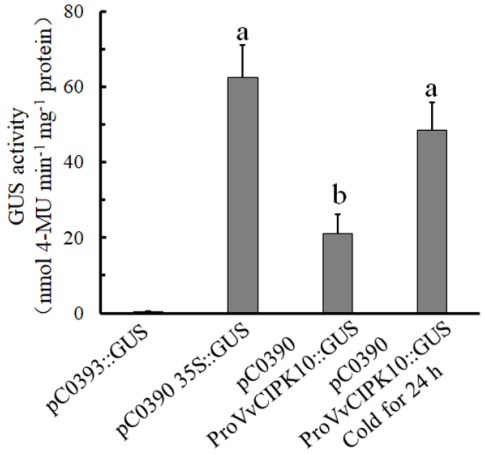

Fig. 3: Analysis of GUS enzyme activity in tobacco leaves after *VvCIPK10* promoter transformation ($P < 0.05$)

GUS activity analysis showed that all the missing fragments had certain activity, and there was no significant difference between these missing fragments and the activity of *VvCIPK10* promoter (Fig. 4). The deletion fragment of

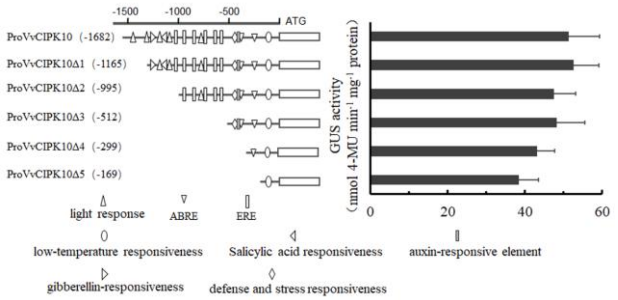

Fig. 4: Analysis of GUS enzyme activity in tobacco leaves after *VvCIPK10* promoter transformation ($P < 0.05$)

VvCIPK10Δ5 was 169 bp in length and it showed strong activity. Combined with the analysis results of cis-acting elements, it was found that the fragment contained a cis-acting element related to low temperature response, indicating that the cis-acting element played a key role in the response of *VvCIPK10* promoter to low temperature.

Discussion

Plant response to cold signaling pathway is very complicated. When plants sensed cold signals, COLD/RGA1 triggered Ca^{2+} and activated the phosphorylation of downstream key factors OST and MAPK3. The key factors of phosphorylation further activated downstream key transcription factors ICEs and CBFs, which leaded to the up-regulation of cold resistance related gene expression and enhanced plant cold resistance (Guo *et al.* 2018; Tan *et al.* 2019). CBL/CIPK signaling system was a unique Ca^{2+} dependent stress regulation signal transduction system in plants. This system included perception Ca^{2+} concentration changes of CBL protein (calcineurin B-like proteins) and interacted with the protein CIPK (CBL-interacting protein kinase), the signal system played an important role in the process of plant stress response (Luan 2009; Mo *et al.* 2018). Many studies have shown that CBL/CIPK signaling system is mainly involved in drought and salt stress response, while a few studies have shown that it was involved in low temperature response (Kudla *et al.* 2018). Yu *et al.* (2016) cloned *VvCIPK10* gene from Thompson Seedless grape. The results of expression analysis showed that *VvCIPK10* gene could respond to drought, low temperature and other stresses, in which the expression reached its maximum at 6 h under low temperature stress. These results indicated that *CIPK* played an important role in the cold resistance of grapes.

To investigate how *VvCIPK10* responds to low temperature reaction, we cloned the promoter of this gene in grapes. The *VvCIPK10* promoter was found to have strong activity after transient transformation in tobacco leaves by constructing transient expression vectors. The activity of *VvCIPK10* promoter was enhanced under low temperature treatment. After a series of short deletions of *VvCIPK10* promoter, it was found that the VvCIPK10Δ5 deletion fragment had strong activity, and had no significant difference with the full length of *VvCIPK10* promoter. The analysis of cis-acting elements of *VvCIPK10* promoter showed that a cryogenic response element and an abscisic acid response element were included in the VvCIPK10Δ5 deletion fragment. In *Arabidopsis*, *VIN3* was a key gene for vernalization, and its promoter contained a low temperature response element (Bond *et al.* 2011). Activity analysis was conducted after promoter deletion, and it was found that the low-temperature response element played a key role in *Arabidopsis thaliana* response to low-temperature sensing vernalization, and the low-temperature response element could not be detected without the cis-acting element (Finnegan *et al.* 2011). *HvCBF1* encoded a transcription factor AP2, which was induced by low temperature but not induced by drought and ABA. Transcriptional activation test showed that *HvCBF1* could bind to low temperature response element (CCGAAA) and activated the expression of downstream cold-related genes (Xue 2002). An abscisic acid response element was also included in the VvCIPK10Δ5 deletion fragment. The core sequence of the element was ACGTGG/T. Previous studies have shown that the cis-acting element was mainly involved in drought and high salt stress, and no literature has reported that the cis-acting element was involved in low temperature stress response (Shinozaki and Shinozaki 2000; Freitas *et al.* 2019). These results indicated that 169 bP at the 3'end of *VvCIPK10* promoter was the key segment of *VvCIPK10* in response to low temperature, and the cis-acting element of low temperature response played a decisive role in this segment.

Conclusion

Grape *VvCIPK10* promoter was active and its activity was induced by low temperature. 169 bP at the 3'end of *VvCIPK10* promoter was the key segment of *VvCIPK10* in response to low temperature, and the cis-acting element of low temperature response played a decisive role in this segment.

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