Molecular cloning and characterization of a cytoplasmic cyclophilin gene in sugarcane

Youxiong Que1, Jinxian Liu1,2, Liping Xu1, Jinrong Guo1, Jingsheng Xu1 and Rukai Chen1

1Key Lab of Sugarcane Genetic Improvement, Ministry of Agriculture, Fujian Agriculture and Forestry University, Fuzhou 350002, Fujian Province, China.
2Department of Tea Science and Biology, Wuyi University, Wuyishan 354300, Fujian Province, China.

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Cyclophilins are ubiquitous proteins with an enzymatic activity of peptidyl-prolyl cis-trans isomerase (PPIase), which play important roles in a variety of stress responsiveness. In this study, we reported the cloning and characterization of a full-length cytoplasmic cyclophilin gene in sugarcane. Sequence analysis showed the cDNA of this gene (GenBank accession number: GQ246462), termed as Sc-CyP, was 904 bp long, including a 519 bp complete ORF, the 5' UTR of 74 bp and 3'UTR of 311 bp, plus a typical AATAA motif and poly (A) tail. It encoded the 172 amino acid polypeptide with a molecular weight of 18.4 KD and the isoelectric point of 8.68. The Sc-CyP encoding protein had the conserved site Trp128 (W128) ubiquitous of all cyclophilins in eukaryotes and the KSGKPLH48-54 region specific to cytoplasmic cyclophilins in plants. SDS-PAGE analysis and PPIase assay revealed that the expression product, with PPIase activity, was a fusion protein with a molecular weight about 25 and 18.4 kD of Sc-CyP plus 7 kD of His•Tag peptides. In real-time qPCR analysis, the Sc-CyP gene showed induced expression under PEG, NaCl, SA and H2O2 stresses, indicating it a stress-related gene for drought and salt stress, signal transduction and disease resistance response in sugarcane.

Key words: Sugarcane (Saccharum officinarum), cyclophilin, PPIase, real-time quantitative PCR.

INTRODUCTION

Immunophilins, including cyclosporin A, FK506 and rapamycin are defined as receptors for immune-suppressive drugs. FK506- and rapamycin-binding proteins were abbreviated as FKBP, while the cyclosporin A was referred to as cyclophilins (CyPs) (He et al., 2004). Cyclophilins constituted a family of abundant and highly conserved proteins which appeared to be ubiquitous in organisms, ranging from bacteria to plants and animals (Galat, 1999). The first cyclophilin was identified as the specific target of the immunosuppressant cyclosporin A (CsA) in mammalian-cells (Handscharmer T et al., 1984). Since then, a series of cyclophilins had been identified and annotated in higher plants, especially in monocotyledonous plants (Opiyo and Moriyama, 2009). These proteins belonged to the cluster of immunophilins which possessed an endogenous enzymatic activity. They exhibited peptidyl-prolyl cis-trans isomerase (PPIase or rotamase) activity, which catalyzed cis-trans isomerization of the amide bond between a proline residue and the preceding amino acid residue and they could also participate in the rate-limiting step in protein folding whose process could be accelerated by the rotamase of cyclophilin (Brandts et al., 1975).

To date, the diverse functions of cyclophilins have been reported (He et al., 2004; Opiyo and Moriyama, 2009). In animal cells, the CyP/CsA complex was found to inhibit the Ca2+-dependent phosphatase activity of the calcineurin protein (Schreiber, 1991). In yeast, genetic analysis of CyP-deficient mutants revealed that cyclophilin was critical for the recovery from heat shock (Sykes et al., 1993). Besides, cyclophilins were reported to play a role in a wide variety of processes, including cell
division (Faure et al., 1998), transcription regulation (Rycyzyn and Clevenger, 2002), signaling (Brazin et al., 2002) and so on. In plants, cyclophilin genes existed as a large gene family, with 29 members in Arabidopsis thaliana (Romano et al., 2004). Plant cyclophilins required various proteins to execute different kinds of physiological processes, with a striking feature of various expression modes in response to different abiotic or biotic stresses (Godoy et al., 2000; Sharma and Singh, 2003; Opiyo and Moriyama, 2009). Marivet et al. (1994) found that the transcription of a bean cyclophilin gene was up-regulated due to environmental or pathogenic stresses, indicating this gene may be important for signal transduction or protein folding under these stresses (Marivet et al., 1994). Research on a cyclophilin gene in maize revealed that it expressed at a basal level in all tissues and the expression could be enhanced by treatment of salicylic acid (SA), which acted as an important signal molecule responsible for induction of plant defense response (Marivet et al., 1995). It was also demonstrated that CyP/GsA complex may reactivate Ca\(^{2+}\)-inactivated K\(^{+}\) channel by inhibiting phosphatase activity in guard cells of Vicia faba, leading to changes in guard cell turgor, which suggested that a conserved Ca\(^{2+}\)-dependent signaling pathway was also existed in plants (Luan et al., 1993).

To our knowledge, there is not any report on the isolation and characterization of a sugarcane cyclophilin gene, which highlights the meanings of the ongoing exploitation of cyclophilin genes in sugarcane. In this study, we reported the cloning and characterization of a cyclophilin gene in sugarcane, aimed to lay the foundation for further research and application of this gene in sugarcane molecular breeding through genetic engineering.

**MATERIALS AND METHODS**

**Materials and treatment**

Sugarcane varieties FN 22, sugarcane stem full-length cDNA library, E. coli DH5\(\alpha\) and BL21 (DE3) and the prokaryotic expression vector pET29a (+) were provided by key lab of Sugarcane varieties FN 22, sugarcane stem full-length cDNA library (Private Foundation for further research and application of this China.

The large-scale sequencing and bioinformatics analysis were conducted for sugarcane stem full-length cDNA library (Private bulletin). Those clones which showed high homology to cyclophilin genes in NCBI database were selected and sequenced completely to obtain the full-length cDNA sequence of a cyclophilin gene in sugarcane.

For the full-length cDNA sequence of the cyclophilin gene, ORF was predicted with the online tool ORF Finder from NCBI (http://www.ncbi.nlm.nih.gov/orf/soft.html). The BLAST program in NCBI was used for the homology analysis. Besides, the ProtParam (http://cn.expasy.org/tools/protparam.html) was applied to analyze the basic property of the encoding protein. Multiple sequence alignment and phylogenetic analysis were performed with DNA MAN software (version 6.0) and MEGA3.1. InterProScan (http://mbi.au.uk/InterProScan) and SMART (http://smart.embl-heidelberg.de) were used to analyze the putative domain of the encoding protein. The gene sub-cellular localization was carried out with SubLoc V1.0 (http://www.bioinfo.tsinghua.edu.cn/SubLoc/) and the signal peptide prediction was performed with SignalP3.0 (http://www.cbs.dtu.dk/services/SignalP/). SOPMA was adopted for the secondary protein structure prediction (http://npsa-pbil.ibcp.fr/cgi-bin/npsa:automat.pl?page=npsa_sopma.html) and the analysis of the repetitive sequence of the amino acid was performed with the REP searcher (http://www.embl-heidelberg.de/~andrade/papers/rep/search.html).

**Construction of prokaryotic expression vector of Sc-CyP gene**

pET29a (+) was used as the prokaryotic expression vector and the specific primer pairs amplifying the ORF region of Sc-CyP gene were designed. The primer sequences were as follows, of which the underlined parts were the restriction endonuclease sites of the repetitive sequence of the amino acid was performed with the REP searcher (http://www.embl-heidelberg.de/~andrade/papers/rep/search.html).

**Obtaining and sequence analysis of a full-length cDNA sequence of Sc-CyP gene**

The primers were synthesized by Shanghai Sangon Biological Engineering Technology and Services CO., Ltd. With plasmid DNA extracted from the library clone containing Sc-CyP gene as the template, the PCR amplification was carried out. The PCR reaction was composed of 5.0 \(\mu\)l × PCR buffer, 4.0 \(\mu\)l 2.5 mmol·L\(^{-1}\) dNTPs; 2.0 \(\mu\)l 10 mmol·L\(^{-1}\) forward and reserves primers respectively; 2.0 \(\mu\)l plasmid DNA; 0.25 \(\mu\)l Taq enzyme (5 U·\(\mu\)l\(^{-1}\)). The PCR reaction was pre-denaturation for 5 min at 94°C; denaturing for 30 s at 94°C; annealing for 30 s at 55°C and extending for 1 min at 72°C, 30 cycles; followed by finally extending for 10 min at 72°C. When the reaction finished, 1% agarose gel electrophoresis was performed and the target PCR product was recovered. After receiving the double digestion with EcoRI and SalI, the obtained ORF sequence was ligated by T4 DNA ligase into pET29a (+) which had also executed the double digestion with EcoRI and SalI. The
Expression profile of Sc-CyP gene under various exogenous stresses

25S rRNA was selected as the control gene in the Real-time qPCR analysis (Que et al., 2009c). According to the sequence of Sc-CyP gene and 25S rRNA (BG536525), two pairs of real-time qPCR primers were designed with GenScript online PCR primers designs tool (http://www.genscript.com/cgi-bin/tools/primer_genescipt.cgi). The forward and reverse primers of Sc-CyP gene were 5'-GGAGGGTGGGGTGCCGATTTTT-3’ and 5’-ACTTCTCCGGGTAGTGGGACCCTACCTT-3’, respectively. For the 25S rRNA, they were 5'-GGAGGGTGGGGTGCCGATTTTT-3’ and 5’-CCATTTGTGGGTTTGCCAAATCC-3’, respectively.

The 20 µl reverse transcription system contained 4 µl 25 mmol·L⁻¹ MgCl₂, 2.0 µl 5× RT buffer solution, 2.0 µl 10 mmol·L⁻¹ dNTPs, 0.5 µl RNase inhibitor, 0.5 µl random primers (0.5 µg), 15 U AMV reverse transcriptase and 4.0 µl total RNA (1.0 µg) as the template and RNase free H₂O as the supplement. Reverse transcription conditions were as follows: kept at room temperature for 10 min, then incubated at 42°C for 15 min and 95°C for 5 min and then kept at 5°C for 5 min. In real-time qPCR analysis, ABI PRISM7500 real-time PCR system was used. For real-time qPCR amplification, the RT-PCR product was chosen as the template and the volume of the reaction system was 25 µl, including SYBR Primix Ex TaqTM (2×), 12.5 µl; Rox reference dye II, 0.5 µl; forward and reverse primers (10 mmol·L⁻¹), 0.5 µl, respectively; cDNA template, 2.5 µl and sterile water, 8.5 µl. Three replicates were set for each sample. The PCR reaction conditions were pre-denaturation at 95°C for 10 s and 40 cycles with 94°C 5 s, 60°C 25 s. When the reaction was completed, the melting curve was analyzed. The method of 2⁻ⁿTN was adopted to analyze the real-time qPCR results (Livak and Schmittgen, 2001; Que et al., 2009d).

RESULTS

Cloning and sequence analysis Sc-CyP gene

According to the results of large-scale sequencing and bioinformatics analysis, one EST sequence which had 95% homology to the sorghum cyclophilin gene (EU722309) was obtained from the sugarcane stem full-length cDNA library. The corresponding library clone of this EST was then completely sequenced and the full-length cDNA sequence of a cyclophilin gene in sugarcane was obtained and termed as Sc-CyP. It had been submitted to the GenBank with the accession number GQ246462. Sequence analysis showed that the Sc-CyP gene had a full length of 904 bp, ORF (open reading frame) length of 519 bp, 5’ UTR (un-translated region) of 74 bp and 3’UTR of 311 bp and the typical AATAA motif and poly(A) tail could also be found in the 3’UTR (Figure 1).

The primary structure of the predicted Sc-CyP protein is shown in Figure 1. The ORF encoded 172 aa with the molecular weight of 18.4 kDa and isoelectric point of 8.68. The Sc-CyP encoding protein in sugarcane had a highly conserved functional site Trp128 (W128) present in all cyclophilins in eukaryotes (Liu et al., 1991), and the 7 amino acid residues region of KSGKPLH48-54, which was specific to cytoplasmic cyclophilins in plants, was also found (Lippuner et al., 1994). Protein domain prediction indicated that Sc-CyP encoding protein had the conserved structure domain of cyclophilin (Figure 2). With SubLoc v1.0, it was located in the cytoplasm.

Sequence homology analysis of Sc-CyP gene

The blastn analysis indicated that the homology of the sugarcane cyclophilin gene (Sc-CyP) with the sorghum cyclophilin gene (EU722309) and with the corn peptide prolyl cis-trans isomerase gene (PPI) (EU967603) was 88% (818/920) and 88% (757/856), respectively. It also showed in blastn analysis that the homology of the Sc-CyP gene with that of the cyclophilin genes from sorghum bicolor (ACD93011), Zea mays (ACG35201), Dasyxypium villosum (ABU56608), Triticum aestivum (AAS17067), Oraya sativa (AAA57045), Gerbera (ABV26711), Ricinus communis (CAC80550) and Picea abies (CAB80166) was 95% (165/172), 95% (165/172), 87% (149/171), 86% (148/171), 86% (149/172), 83% (144/172), 83% (143/171) and 84% (145/172), respectively. Multiple alignment analysis of amino acid sequences of the cyclophilins encoded by Sc-CyP gene and those from other plant species was performed using DNAMAN software and the results are shown in Figure 3. It indicated that the amino acid sequences of all these cyclophilins had the similar length. What is more, the Sc-CyP encoding protein had rather high homology with the cyclophilins from other plant species, especially in the region KSGKPLH, which should be the key for its enzymatic activity.

The phylogenetic tree of the cyclophilin encoded by Sc-CyP gene and the cyclophilins of other 15 plant species was constructed using the MEGA3.1 software. Figure 4 shows that the Sc-CyP and the cyclophilin of P. abies, Z.
### Figure 1.
Nucleotide sequence of Sc-CyP gene and primary structure of Sc-CyP protein. Capital and small letters represent amino acid and nucleotide, respectively; * shows stop codon; the underline shows the polyadenylation signal AATAA; the big frame shows the conservative sequence of the plant cytoplasmic cyclophilin; the small frame shows conservative amino acid of cyclophilin in all kinds of organisms.

### Figure 2.
Predicted domain of Sc-CyP protein.

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**mays**, *Sorghum bicolor*, *O. sativa*, *T. aestivum* and *D. villosum* belongs to the same category, indicating that these cyclophilins may evolve from a common ancestor through different pathways. Besides, the Sc-CyP and the cyclophilin of *Z. mays* were classified into one group and that from *D. villosum* and *T. aestivum* were in another group.
group, which suggested that the evolutionary relationship between the two within one group was the closest.

**Construction of the Sc-CyP gene prokaryotic expression vector**

After the PCR product of the ORF amplification of Sc-CyP gene was recovered, it received the BamH I and Sal I digestion. The target fragment was then recovered and used to ligate into the pET29a (+) which also received double digestion of BamH I-Sal I and a positive recombinant was transformed into the E. coli DH5a competent cell. Through PCR amplification and double digestion with BamH I-Sal I, the fragment with the same size as that of the target fragment could be obtained from the positive clone pET29a-ScCyP. The results of the agarose gel electrophoresis are shown in Figure 5, indicating the construction of the prokaryotic expression vector pET29a-ScCyP of the Sc-CyP gene was successful.

**SDS-PAGE analysis of prokaryotic expression product and enzyme activity assay**

As shown in Figure 6, the target protein began to express in the second hour with the molecular weight of about 25 kD, which was basically consistent with the deduced
Figure 4. Phylogenetic tree of cyclophilins isolated from different plant species.

Figure 5. Validation of pET29a-ScCyP by PCR and double digestion with BamHI + Sal I. A: M, DL2000; Lane 1, negative control; Lane 2, PCR product; B: M, DL2000; 1, digested product with BamHI + Sal I.

molecular weight of the fusion protein of 25.4 kD, that was 18.4 kD of Sc-CyP plus 7 kD of His • Tag peptides. Enzyme activity assay demonstrated that the purified prokaryotic expression protein exhibited PPIase activity and this kind of PPIase activity could be inhibited by FK506 and rapamycin. Though the Sc-CyP gene had the successful expression within E. coli BL2, this target protein could not be expressed in the empty vector, while no wrong coding or reading-frame shift of amino acid sequences were found, all of which further confirmed the
successful construction of this prokaryotic expression vector.

**Expression profile of Sc-CyP gene under various exogenous stresses**

Real-time qPCR was used to examine the expression profile of Sc-CyP gene under different kinds of exogenous stresses. Figure 7 shows that the Sc-CyP gene expression was induced during the whole period under all the stresses of PEG, NaCl, SA and H$_2$O$_2$, with various expression profiles under different kinds of exogenous stresses. Under PEG stress, the induced expression of Sc-CyP gene had the trend of “increasing-declining-increasing-declining”. At the time point of 3 h, the Sc-CyP gene expression was strongly induced, reaching more than 24 times that of the control at 0 h. Then the expression began to decrease, which lasted until the time point of 6 h. From the time point of 6 h on, the expression of Sc-CyP gene increased again. The peak was found at the time point of 24 h, which was more than 72 times that of the control, and after that it began to drop again. But even at the time point of 72 h, the expression was still more than 12 times that of the control. Under NaCl stress, the induced expression of Sc-CyP gene was strong and just at the time point of 3 h, more than 40 times expression that of the control at 0 h was observed. It reached the peak at 18 h at which the expression was more than 630 times that of the control. Although, it began to decrease later, at the following three time points, it was still more than 97 times that of the control. Under the stress of SA, the expression of Sc-CyP gene had the “increasing-declining-increasing-declining” trend. At the time point of 12 h, the induced expression of Sc-CyP gene was not very strong. However, it was rather strong at the time point of 24 h with the expression 5.7 times that of the control at 0 h. Although, the expression began to decrease after the time point of 24 h, it was still more than twice that of the control. It reached the peak at 72 h, which was more than 6 times that of the control. Under the stress of H$_2$O$_2$, the induced expression of Sc-CyP gene had the trend of “increasing-declining-increasing-declining”. At the time point of 3 h, the Sc-CyP gene expression was more than 2.5 times that of the control at 0 h. The peak was found at the time point of 24 h which was more than 6 times that of the control. Although, the expression was induced during the period of 48 to 72 h, it began to decrease after that which was to some extent equal to that of the control at 0 h. Based on the real-time qPCR analysis earlier, it could be concluded that the Sc-CyP gene showed induced expression under all the four kinds of exogenous stresses, PEG, NaCl, SA and H$_2$O$_2$. This indicated that the Sc-CyP gene cloned in this study was stress-related and it may play the protective roles of resistance in sugarcane.

**DISCUSSION**

Cyclophilin is a kind of intracellular binding protein which exits widely in a variety of biological cells and has high affinity with the cyclosporine A (CsA). According to their subcellular localization, plant cyclophils could be divided into several types, such as cytoplasmic, chloroplast and mitochondrial (Chou and Gasser, 1997). So far, a lot of cyclophilin genes had already been cloned in A. thanalia, G. max and several other crops and also received in-depth studies (He et al., 2004; Opiyo and Moriyama, 2009). Previous studies of cytoplasmic cyclophils in mammals showed that they played a role in drug-induced immunosuppression by inhibiting calcineurin phosphatase activity in Ca$^{2+}$-dependent signaling pathway (Crabtree and Clipstone, 1994). In addition, there were at least five kinds of cytoplasmic cyclophils

![Figure 6. Expression of pET29a-ScCyP in E. coli BL21. M, protein maker; Lane 1-3, pET29a-ScCyP induction for 6, 4 and 2 h respectively; Lane 4, pET29a-ScCyP without induction; Lane 5, pET29a (+) induction for 6 h; Lane 6, pET29a (+) without induction; Lane 7, BL21 without induction.](image-url)
in *A. thaliana*, indicating that cytoplasmic cyclophilins may play even more diverse roles in plants than previously suspected (Chou and Gasser., 1997). However, there is not any report about studies on cyclophilin genes in sugarcane.

In this study, a full-length cytoplasmic cyclophilin gene in sugarcane was obtained (GenBank accession number: GQ246462). Using the PET-29a (+)/BL21 (DE3) expres-
At the cytoplasmic cyclophilin gene.

When the expression level of cyclophilin in Grecian foxglove was enhanced, the freezing tolerance of the plant under low temperature was increased. Godoy et al. (2000) found that the potato cyclophilin responded to SA and bacterial stimulation. Chen et al. (2007) demonstrated that the cyclophilin expression in Lepidium sativum could be induced by salt, ABA, H$_2$O$_2$ and heat shock stress and also by fungal infection. Previous studies revealed that PEG and NaCl could produce osmotic stress and the exogenous PEG or NaCl treatment could thus be used as the simulated condition of drought stress (Jia et al., 2008), while SA and H$_2$O$_2$ were proved to play important roles in signal transduction and resistance response of plant at early stages, such as hypersensitive response (HR) and systemic acquired resistance (SAR) (Wu et al., 1995; Scott et al., 1999). In this study, it was demonstrated in real-time qPCR analysis that when sugarcane received the simulated drought or salt stress, PEG and NaCl stresses, the expression of Sc-CyP gene was greatly induced and thus, it was inferred that the Sc-CyP gene played the protective role under the drought and salt stress. What is more, the expression of Sc-CyP gene could also be improved significantly under the stresses of H$_2$O$_2$ and SA, which suggested that the Sc-CyP gene played a certain role under exogenous stresses similar to the H$_2$O$_2$ and SA as well, mostly probably a role in signal transduction and disease resistance response in sugarcane.

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