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Molecular cloning and characterization of a cytoplasmic cyclophilin gene in sugarcane

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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) ubiquitious 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 H_2O_2 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 FKBPs, 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 (Handschumacher

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 immuno-philins 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 Ca^{2+}-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

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Abbreviations: PPIase, Peptidyl-prolyl cis-trans isomerase; **IPTG,** isopropyl-beta- D-thiogalactopyranoside; **ORF,** open reading frame; **UTR,** untranslated region; **HR,** hypersensitive response; **SAR,** systemic acquired resistance.

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/CsA 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* DH5α and BL21 (DE3) and the prokaryotic expression vector pET29a (+) were provided by key lab of sugarcane genetic improvement, Ministry of Agriculture, P. R. China. *EcoR* I, *Sal* I, T$_4$ DNA ligase, Taq enzyme, DNA and protein molecular marker, SYBR®Premix Ex Taq™ were from TaKaRa (Japan); Hepes, Triton X100, chymotrypsin, IPTG (isopropyl-beta-D-thiogalactopyranoside) and reverse transcription kit from Promega Corporation (USA); FK506 and rapamycin from Sigma (USA) and ABI PRISM7500 real-time PCR system was used.

Strong and evenly growing sugarcane stalks were soaked and sterilized in water with 0.1% potassium permanganate for 24 h and then planted into autoclaved fine sand in 10 trays, 40 shoots in each tray. These trays were then placed into the illumination incubator where each day they were kept under light for 12 h at 29°C and dark for 12 h at 26°C with a light intensity of 440 mol m^{-2} s^{-1}. When growing to the height of 15 cm, they were transferred into the greenhouse for cultivation and sprayed with Hongland nutrient solution (Zhang and Qu, 2003). In the six leaves period, evenly growing stalks were selected and the sand was removed. After

rehydration for 2 days, the stalks were cultivated in Hongland nutrient solution for one week and then treated as follows. For the first group, 5.0 m mol·L^{-1} SA and 10 mmol·L^{-1} H$_2$O$_2$ were sprayed onto the leaves, respectively (Que et al., 2009a). For the second group, the sugarcane roots were soaked in PEG8000 solution (25%) and NaCl solution (250 mmol·L^{-1}), respectively (Zhang, 2007). The sampling times were 0, 12, 24, 36, 48, 60 and 72 h for SA and H$_2$O$_2$ treatment, 0, 3, 6, 12, 24 and 72 h for PEG treatment and 0, 3, 18, 24, 48 and 72 h for NaCl treatment. All the samples collected were immediately fixed in liquid nitrogen and stored in a refrigerator at -85°C until RNA extraction.

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

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/gorf/gorf.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 DNAMAN software (version 6.0) and MEGA3.1. InterProScan (http://ebi.ac.uk/ InterProScan) and SMAR (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/npsaautomat.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 *EcoR* I and *Sal* I, respectively. Sc-CyP-F: 5'-TATGGATCCATGGCGA ACCCGCG-3'; Sc-CyP-R: 5'-TTAGTCGACGCTGAGCTGGCCGC-3'.

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 µl 10×PCR buffer, 4.0 µl 2.5 mmol·L^{-1} dNTPs; 2.0 µl 10 µmol·L^{-1} forward and reserves primers respectively; 2.0 µl plasmid DNA; 0.25 µl Taq enzyme (5 U·µl^{-1}); ddH$_2$O was added as the supplement and the final volume was 50 µl. PCR amplification program 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 *EcoR* I and *Sal* I, the obtained ORF sequence was ligated by T4 DNA ligase into pET29a (+) which had also executed the double digestion with *EcoR* I and *Sal* I. The

recombinant was transferred into *E. coli* DH5α. Plasmid DNA of clones selected by blue/white colony screening was extracted. The positive clone validated by PCR and double digestion was just the prokaryotic expression vector which termed as pET29a- ScCyP (Que et al., 2009b).

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

pET29a-*ScCyP* and empty pET29a(+) were both transformed into *E. coli* BL21 (DE3). The single colony was inoculated into the LB medium containing the ampicillin (100 μg·ml^{-1}) for overnight shake culture at 150 r/min at 37°C. In the following day, 1% of the medium was inoculated into the LB medium containing of 100 μg·ml^{-1} ampicillin and shake cultured. When OD600 reached 0.4 to 0.6, 1.0 ml of LB liquid medium was collected as the control and the remaining medium was added with IPTG with the final concentration 1.0 mmol·L^{-1}. The LB medium with pET29a-S*cCyP* was induced for 2, 4 and 6 h, respectively at 37°C and 1.0 ml medium was collected at each time point; the LB medium with empty pET29a (+) was induced with IPTG for 6 h and 1.0 ml corresponding medium was collected. The medium collected was used for the SDS-PAGE with 25 μl sample loading and also for peptidyl-prolyl-cis-trans-isomerase (PPIase) assay according to Blecher et al. (1996). When the electrophoresis finished, the gel was colored with Coomassie brilliant blue and imaged (Que et al., 2009b).

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* (BQ536525), two pairs of real-time qPCR primers were designed with GenScript online PCR primers designs tool (http://www.genscript.com/cgi-bin/tools/primer_ genscript.cgi). The forward and reverse primers of Sc-CyP gene were 5′-ACTTCTCGCCGTAGATGGAC-3′ and 5′-CACTACAAGGGC TCCACCTT-3′, respectively. For the 25S rRNA, they were 5′-GCAGCCAAGCGTTCATAGC-3′ and 5′-CC TATTGGTGGGGTG AACAATCC-3′, respectively.

The 20 μl reverse transcription system contained 4.0 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 H$_2$O 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 μmol·L^{-1}), 0.5 μl, respectively; cDNA template, 2.5 μl and sterile water, 8.5 μl. Three replicas 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$^{-\triangle\triangle CT}$ was adopted to analyze the real-time qPCR results (Livak and Schmittgen, 2001; Que et al., 2009d).

RUSULTS

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 of 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 kD 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 (Lippumer 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), *Dasypyrum villosum* (ABU56008), *Triticum aestivum* (AAS17067), *Oryza sativa* (AAA57045), *Gerbera* (ABV26711), *Ricinus communis* (CAC80550) and *Picea abies* (CAC81066) 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.*

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1       ACCAGATCAG ATCACACAAC AGCCGAAGGA AAATTTCCCC CAAAACCCTC CCCAAACCCT
61      AGCTTCGGAT CCCGATGGCG AACCCGCGCG TCTTCTTCGA CATGACCGTC GGCGGCGCCC
                   M  A  N  P  R  V  F  F  D  M  T  V  G  G  A  P
121     CGGCGGGGCG GATCGTGATG GAGCTGTACG CCAACGAGGT GCCCAAGACG GCCGAGAACT
         A  G  R  I  V  M  E  L  Y  A  N  E  V  P  K  T  A  E  N  F
181     TCCGCGCGCT GTGCACGGGC GAGAAGGGCG TGGGG̲G̲A̲A̲A̲T̲C̲ ̲G̲G̲G̲G̲A̲A̲G̲C̲C̲G̲ ̲C̲T̲C̲C̲ACTACA
         R  A  L  C  T  G  E  K  G  V  G │K  S  G  K  P  L  H│ Y  K
241     AGGGCTCCAC CTTTCACCGC GTCATCCCGC AGTTCATGTG CCAGGGCGGC GACTTCACCC
         G  S  T  F  H  R  V  I  P  Q  F  M  C  Q  G  G  D  F  T  R
301     GCGGCAACGG CACGGGAGGG GAGTCCATCT ACGGCGAGAA GTTCCCCGAC GAGAAGTTCG
         G  N  G  T  G  G  E  S  I  Y  G  E  K  F  P  D  E  K  F  V
361     TGCGCAAGCA CACGGGGCCC GGGGTGCTCT CCATGGCCAA CGCGGGGCCC AACACCAACG
         R  K  H  T  G  P  G  V  L  S  M  A  N  A  G  P  N  T  N  G
421     GCTCCCAGTT CTTCGTCTGC ACCGTCGAGA CCCCC̲TGGCT CGACGGCAAG CACGTCGTCT
         S  Q  F  F  V  C  T  V  E  T  P │W│ L  D  G  K  H  V  V  F
481     TCGGCCAGGT CGTCGAGGGC ATGGACGTCG TCAAGGCCAT CGAGAAGGTG GGCTCCCGCA
         G  Q  V  V  E  G  M  D  V  V  K  A  I  E  K  V  G  S  R  S
541     GCGGCTCCAC CTCCAAGGAG GTCAAGGTCG CTGACTGCGG CCAGCTCAGC TAGATCTCTG
         G  S  T  S  K  E  V  K  V  A  D  C  G  Q  L  S      *
601     GTCGTCTCGT CTCGTCTGTC CGGCCGCCCT CCCCTCCGTC ATCGTCGACT CCGCCTGCGT
661     CCGTCCCTTT TCCATTGCT  TCGATCTGAA̲ ̲T̲A̲A̲GATGATG GTGATCTGAG TGGTGGTCTG
721     TGTTGAGTCG TTCAATCATG TGTTTGTGTC GCTCGTGGTT TAATTTAGAG GTTTATTTAG
781     GCATGGATCT GCGAATCCCA TGGCGCCTCT GCTTACTTCC TGTTTCACCT GTTATGTTAT
841     GAGATCCATC TCAAGATAAT GCTATGAGAA CTGAAATTCT GTTTAAAAAA AAAAAAAAAA
|901    AAAA
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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.
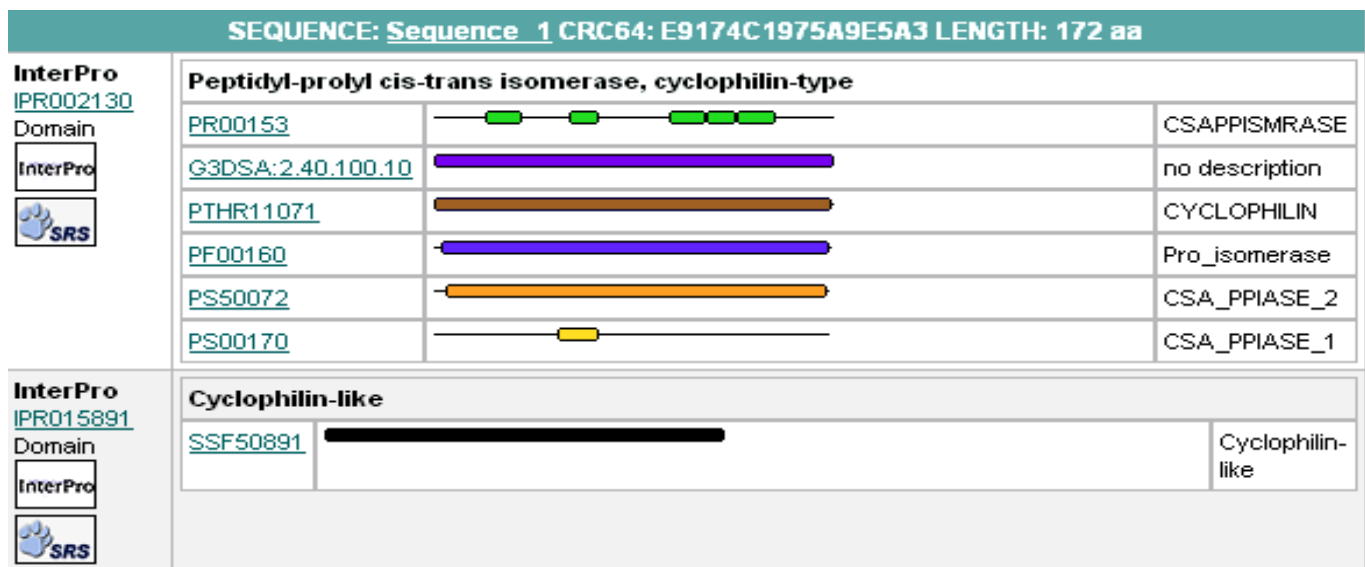

SEQUENCE: Sequence_1 CRC64: E9174C1975A9E5A3 LENGTH: 172 aa		
InterPro IPR002130 Domain	**Peptidyl-prolyl cis-trans isomerase, cyclophilin-type**	
	PR00153	CSAPPISMRASE
	G3DSA:2.40.100.10	no description
	PTHR11071	CYCLOPHILIN
	PF00160	Pro_isomerase
	PS50072	CSA_PPIASE_2
	PS00170	CSA_PPIASE_1
InterPro IPR015891 Domain	**Cyclophilin-like**	
	SSF50891	Cyclophilin-like

Figure 2. Predicted domain of Sc-CyP protein.

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
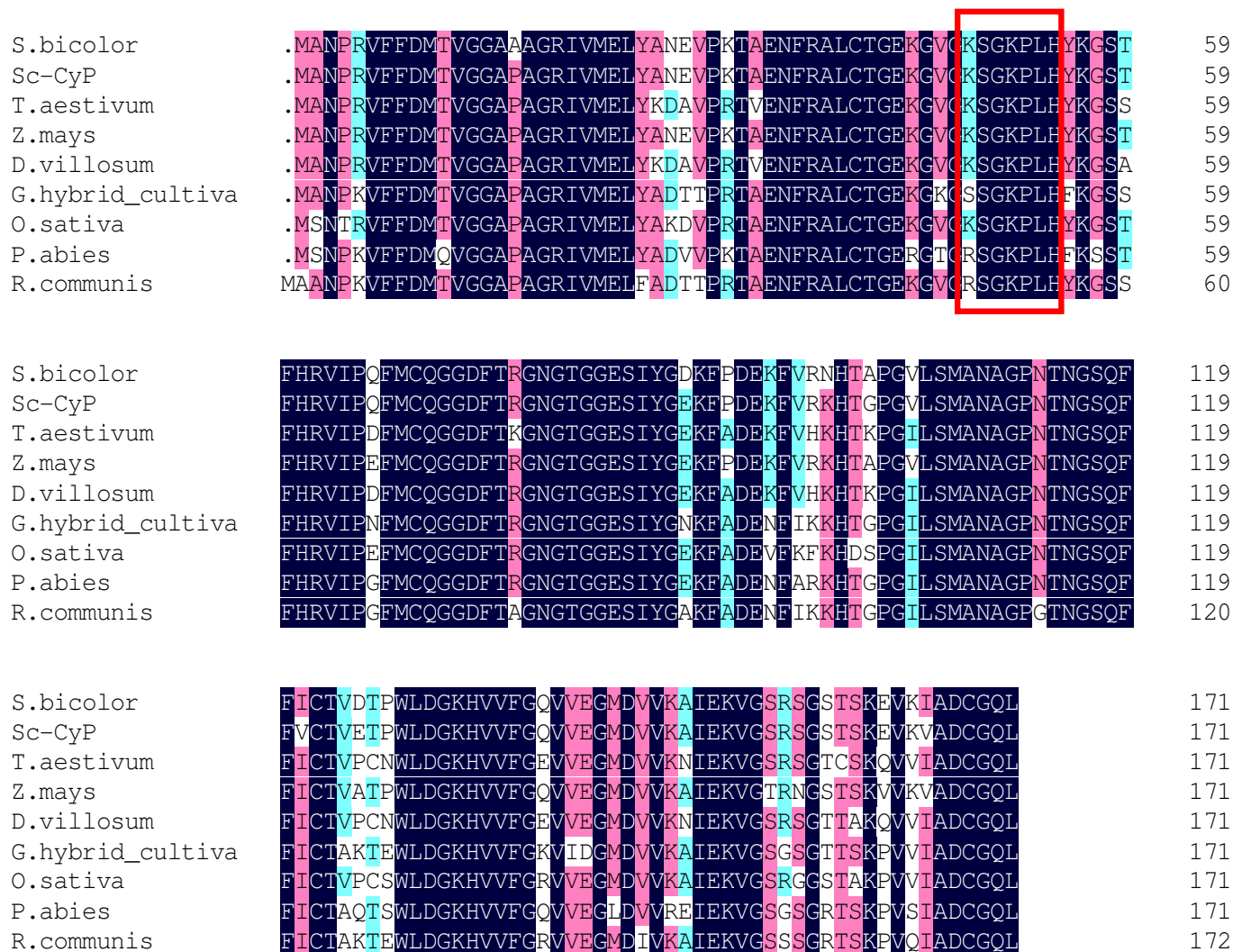
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S.bicolor        .MANPRVFFDMTVGGAAAGRIVMELYANEVPKTAENFRALCTGEKGVQKSGKPLHYKGST   59
Sc-CyP           .MANPRVFFDMTVGGAPAGRIVMELYANEVPKTAENFRALCTGEKGVQKSGKPLHYKGST   59
T.aestivum       .MANPRVFFDMTVGGAPAGRIVMELYKDAVPRTVENFRALCTGEKGVQKSGKPLHYKGSS   59
Z.mays           .MANPRVFFDMTVGGAPAGRIVMELYANEVPKTAENFRALCTGEKGVQKSGKPLHYKGST   59
D.villosum       .MANPRVFFDMTVGGAPAGRIVMELYKDAVPRTVENFRALCTGEKGVQKSGKPLHYKGSA   59
G.hybrid_cultiva .MANPKVFFDMTVGGAPAGRIVMELYADTTPRTAENFRALCTGEKGKSSGKPLHFKGSS   59
O.sativa         .MSNTRVFFDMTVGGAPAGRIVMELYAKDVPRTAENFRALCTGEKGVQKSGKPLHYKGST   59
P.abies          .MSNPKVFFDMQVGGAPAGRIVMELYADVPKTAENFRALCTGERGTQRSGKPLHFKSST   59
R.communis       MAANPKVFFDMTVGGAPAGRIVMELFADTTPRTAENFRALCTGEKGVQRSGKPLHYKGSS   60

S.bicolor        FHRVIPQFMCQGGDFTRGNGTGGESIYGDKFPDEKFVRNHTAPGVLSMANAGPNTNGSQF  119
Sc-CyP           FHRVIPQFMCQGGDFTRGNGTGGESIYGEKFPDEKFVRKHTGPGVLSMANAGPNTNGSQF  119
T.aestivum       FHRVIPDFMCQGGDFTKGNGTGGESIYGEKFADEKFVHKHTKPGILSMANAGPNTNGSQF  119
Z.mays           FHRVIPEFMCQGGDFTRGNGTGGESIYGEKFPDEKFVRKHTAPGVLSMANAGPNTNGSQF  119
D.villosum       FHRVIPDFMCQGGDFTRGNGTGGESIYGEKFADEKFVHKHTKPGILSMANAGPNTNGSQF  119
G.hybrid_cultiva FHRVIPNFMCQGGDFTRGNGTGGESIYGNKFADENFIKKHTGPGILSMANAGPNTNGSQF  119
O.sativa         FHRVIPEFMCQGGDFTRGNGTGGESIYGEKFADEVFKFKHDSPGILSMANAGPNTNGSQF  119
P.abies          FHRVIPGFMCQGGDFTRGNGTGGESIYGEKFADENFARKHTGPGILSMANAGPNTNGSQF  119
R.communis       FHRVIPGFMCQGGDFTAGNGTGGESIYGAKFADENFIKKHTGPGILSMANAGPGTNGSQF  120

S.bicolor        FICTVDTPWLDGKHVVFGQVVEGMDVVKAIEKVGSRSGSTSKEVKIADCGQL         171
Sc-CyP           FVCTVETPWLDGKHVVFGQVVEGMDVVKAIEKVGSRSGSTSKEVKVADCGQL         171
T.aestivum       FICTVPCNWLDGKHVVFGEVVEGMDVVKNIEKVGSRSGTCSKQVVIADCGQL         171
Z.mays           FICTVATPWLDGKHVVFGQVVEGMDVVKAIEKVGTRNGSTSKVVKVADCGQL         171
D.villosum       FICTVPCNWLDGKHVVFGEVVEGMDVVKNIEKVGSRSGTTAKQVVIADCGQL         171
G.hybrid_cultiva FICTAKTEWLDGKHVVFGKVIDGMDVVKAIEKVGSGSGTTSKPVVIADCGQL         171
O.sativa         FICTVPCSWLDGKHVVFGRVVEGMDVVKAIEKVGSRGGSTAKPVVIADCGQL         171
P.abies          FICTAQTSWLDGKHVVFGQVVEGLDVVREIEKVGSGSGRTSKPVSIADCGQL         171
R.communis       FICTAKTEWLDGKHVVFGRVVEGMDIVKAIEKVGSSGRTSKPVQIADCGQL         172
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Figure 3. Multiple sequence alignment of cyclophilins isolated from different plant species. Frame shows conservative sequence of the plant cytoplasmic CyP protein; ▲shows conservative amino acid of CyP protein *S. bicolor*: (ACD93011); *T. aestivum*, (AAS17067); *Z. mays*, (ACG35201); *D. ivillosum*, (ABU56008); *G. hybrid* cultiva, (ABV26711); *O. sativa,* (AAA57045); *P. abies,* (CAC81066); *R. communis* ,(CAC80550).

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. coil* 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
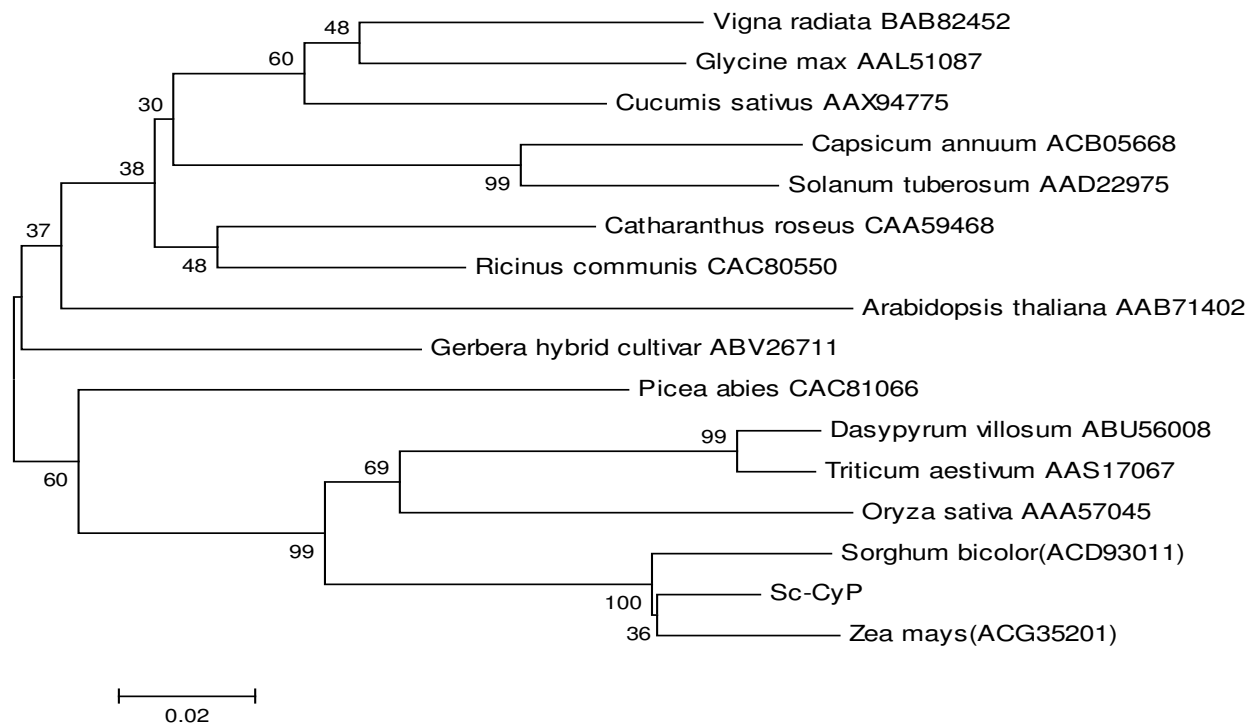
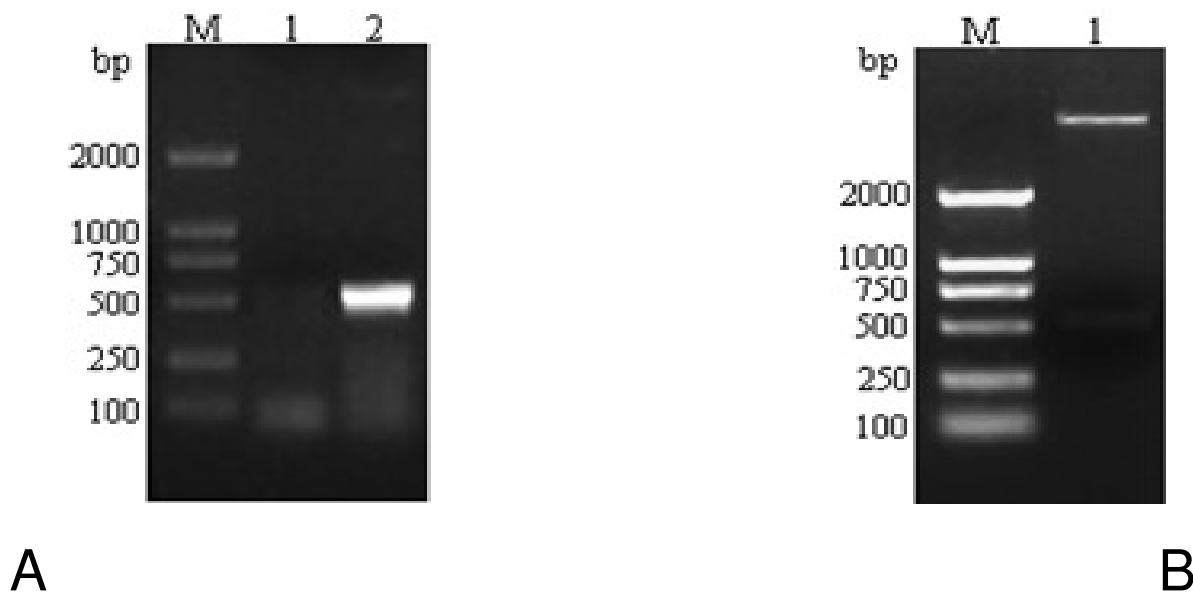**Figure 4.** Phylogenetic tree of cyclophilins isolated from different plant species.

A

B

Figure 5. Validation of pET29a-*ScCyP* by PCR and double digestion with *BamH* I+*Sal* I. A: M, DL2000; Lane 1, negative control; Lane 2, PCR product; B: M, DL2000; 1, digested product with *BamH* I+*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
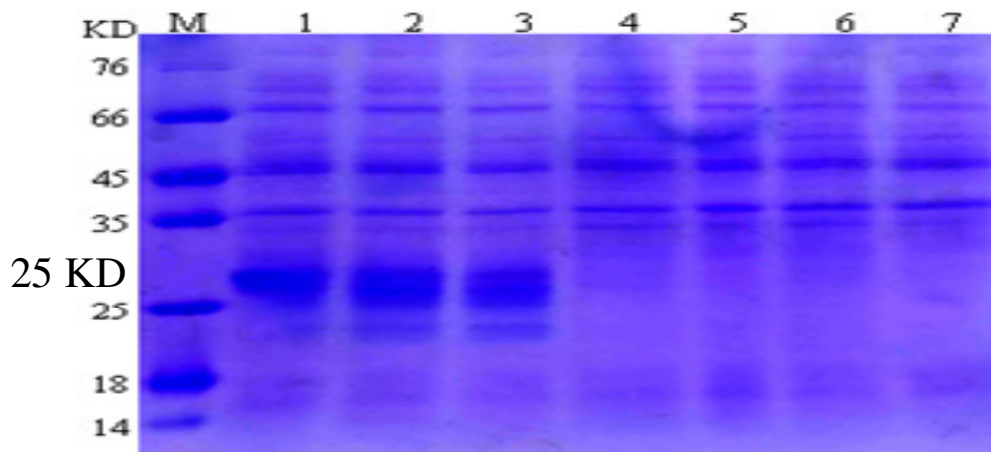
Figure 6. Expression of pET29a-*ScCyP* in *E. coli* BL21. M, protein maker; Lane 1-3, pET29a-*ScCyP* induction for 6, 4 and 2 h respectively; Lane 4, pET29a-*ScCyP* without induction; Lane 5, pET29a (+) induction for 6 h; Lane 6, pET29a (+) without induction; Lane 7, BL21 without induction.

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 exogeneous 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 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" 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 cyclophilins 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 cyclophilins in mammals showed that they played a role in drug-induced immunosuppression by inhibiting calci-neurin phosphatase activity in Ca^{2+}-dependent signaling pathway (Crabtree and Clipstone, 1994). In addition, there were at least five kinds of cytoplasmic cyclophilins
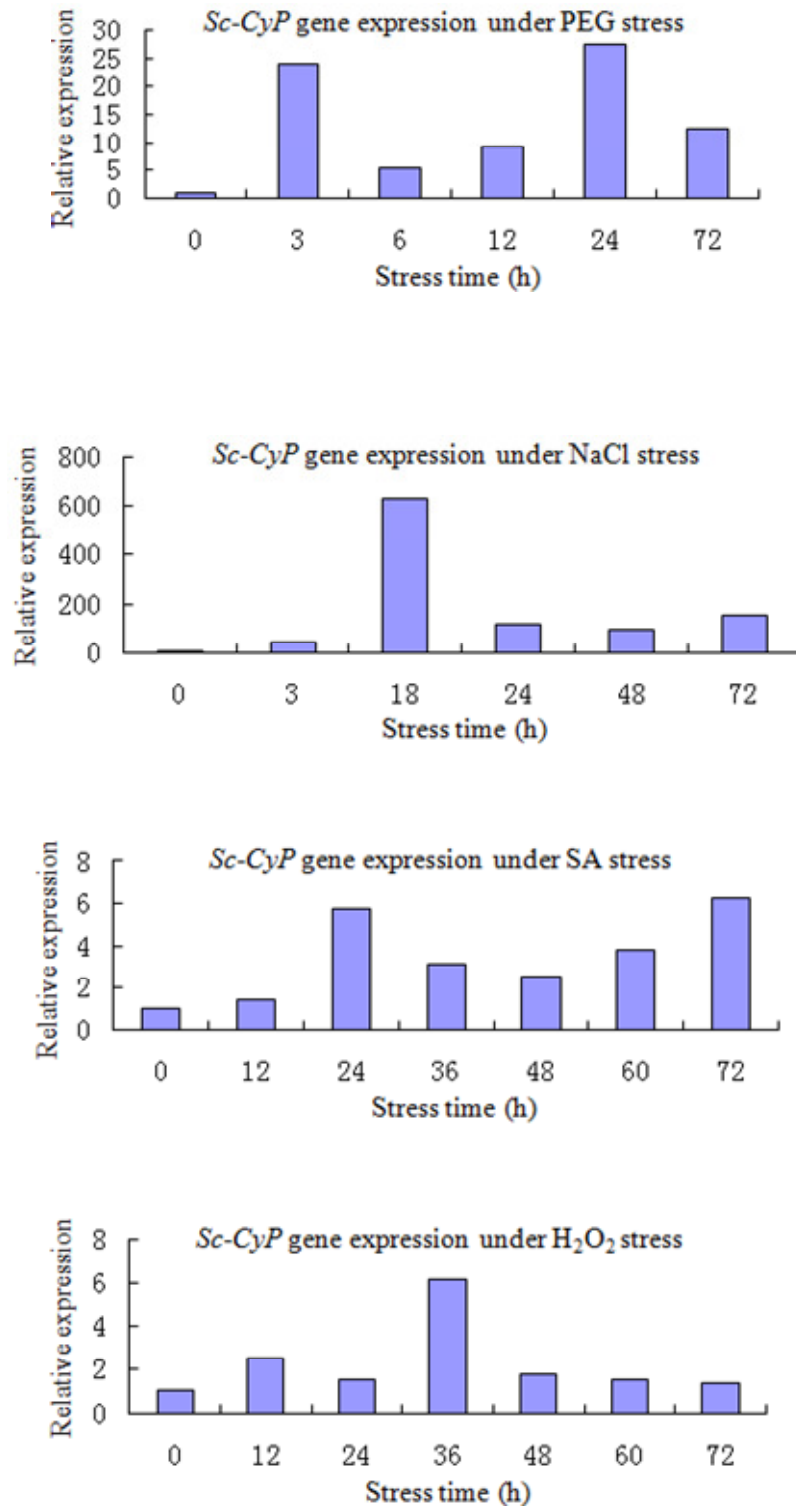
Figure 7. Expression profiles of *Sc-CyP* gene under various exogenous stresses.

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-

sion system, the prokaryotic expression vector pET29a-*ScCyP* with the *Sc-CyP* gene was successfully constructed. The prokaryotic expression product was a fusion protein of 25.4 kD, that was 18.4 kD of Sc-CyP plus 7 kD of His • Tag peptides. The PPIase activity of the prokaryotic expression product and its inhibition by the immunosuppresive drugs FK506 and rapamycin further demonstrated that the *Sc-CyP* gene obtained was indeed a kind of cyclophilin in sugarcane. Since cyclophilin comprised the PPIase activity that catalyzed the rate limiting step in protein folding, it was hypothesized that one possible function of *Sc-CyP* gene may be to act as "chaperone-like" protein to facilitate the folding of the stress-related proteins or to protect these proteins from proteolytic degradation or aggregation under exogenous stresses, as it had been speculated for some other cyclophilins (Ferreira et al., 1996). Due to the existence of one lysine residues, this KSGKPLH48-54 region would carry a positive charge, while the conservation of this region between the distantly related monocotyledonous and dicotolydenous plants indicated the strong selective pressure for maintenance of the sequence in this region (Figures 3 and 4). It was predicted by SubLoc v1.0 that the protein encoded by *Sc-CyP* gene was located in the cytoplasm and thus, it was further confirmed that the *Sc-CyP* gene identified in this study should be classified into the cytoplasmic cyclophilin gene.

Many studies showed that cyclophilins were the proteins in response to adverse stress (Godoy et al., 2000; He et al., 2004; Opiyo and Moriyama, 2009) and their expression and accumulation could be regulated by various environmental stress factors, mainly consisting of the abiotic or biotic stress factors such as mercuric chloride, abscisic acid (ABA), ethylene, salicylic acid (SA), methyl jasmonate (MJ), salt stress, cold and heat, light, drought, injury and fungal infection (Godoy et al., 2000; Nuc et al., 2001; He et al., 2004; Romano et al., 2004; Opiyo and Moriyama, 2009). Kullertz et al. (1999) revealed that 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_2O_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_2O_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_2O_2 and SA, which suggested that the *Sc-CyP* gene played a certain role under exogenous stresses similar to the H_2O_2 and SA as well, mostly probably a role in signal transduction and disease resis-tance response in sugarcane.

This study reported the molecular cloning and characterization of a full-length cyclophilin gene in sugarcane and the results should be helpful in the ongoing exploitation of cyclophilin genes in sugarcane.

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