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Full Length Research Paper

Molecular cloning and expression analysis of an Mn-superoxide dismutase gene in sugarcane

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Superoxide dismutases (SODs) play an important role in stress-tolerance in plants. In this study, for the first time, a full-length cDNA sequence of *MnSOD* gene, termed as *Sc-MnSOD* (GenBank accession number: GQ246460), was obtained in sugarcane. Sequence analysis revealed that *Sc-MnSOD* gene was 919 bp long, including a 702 bp ORF, the 5' UTR of 99 bp and 3'UTR of 118 bp. It encoded the 233 amino acid residues with a molecular weight of 25.3 KD and isoelectric point of 7.11. Protein domain prediction indicated that besides the conserved domain in MnSOD, Sc-MnSOD also had the signal peptides at the sites of 1 to 26 aa at the N-terminus. With SubLoc v1.0, Sc-MnSOD was localized in the mitochondria. In homology analysis, the *MnSOD* genes from different plant species were rather conservative. SDS-PAGE analysis and enzyme activity assay showed that the prokaryotic expression product was a fusion protein with SOD enzyme activity of 726 $U \cdot mg^{-1}$. Real-time qPCR analysis demonstrated that the *e*xpression of *Sc-MnSOD* gene was greatly induced by the *Ustilgao scitaminea*, firstly induced and then inhibited by H_2O_2, and slightly influenced by SA, which suggested that it may play a role in the clearing of active oxygen and thus in disease resistance mechanism in sugarcane.

Key words: Reactive oxygen species, *Saccharum officinarum*, Mn-superoxide dismutase, prokaryotic expression, real-time quantitative PCR.

INTRODUCTION

Reactive oxygen species (ROS) are chemically-reactive molecules that contain oxygen. They could induce the synthesis of a variety of peptides and the release of intracellular Ca^{2+}, thus regulate gene expression (Cui et al., 1999). The generation and elimination of ROS in plants are in dynamic balance, which is systematically coordinated by the reaction among superoxide dismutase (SOD), peroxide (POD) and catalase (CAT) or the non-enzyme glutathione (GSH), ascorbic acid (AsA) and carotene (CAR) (Gupta and Datta, 2003; McCord et al., 1969). Therefore, the balance of ROS was reflected by the activity changes of anti-oxidative enzymes. Further more, the role of free oxygen radicals in plant resistance had attracted wide attention and it has been proved to play an important role in regulating the physiological metabolism.

Plants have evolved a complex and coordinate anti-oxidative enzyme system to scavenge ROS and protect them from injury due to the oxidative stress. Superoxide dismutase (SOD; EC1.15.1.1), one of the most effective antioxidants in plants, can catalyze the dismutation of

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Abbreviations: ROS, Reactive oxygen species; **SOD,** superoxide dismutase; **IPTG,** isopropyl-beta-D-thiogalactopyranoside; **ORF,** open reading frame; **UTR,** untranslated region; **HR,** hypersensitive response; **SAR,** systemic acquired resistance

super-oxide anion radical into hydrogen peroxide and oxygen, specifically eliminating the ROS and balancing free oxygen radicals. As the first defensive line in the anti-oxidative system against oxygen toxicity, SOD plays a great role in the development and stress resistance in plants. Since the first SOD was found in red blood cells of cattle by MoCord et al. (1969), the first plant *SOD* gene was cloned from maize in 1987 (Cannon et al., 1987). From that day on, a number of *SOD* genes have been cloned in several plant species, such as tomato (Perl-Treves et al., 1988), tobacco (Bowler et al., 1989), rice (Kaminaka et al., 1999), Arabidopsis (Kliebenstein et al., 1999), peach (Bagnoli et al., 2002), cassava (Shin et al., 2005) and lotus (Dong et al., 2009). Kurama et al. (2004) identified one MnSOD mitochondrial precursor and six Cu/ZnSOD EST clusters in the sugarcane EST genome through the sequencing of expressed sequence tags (ESTs) of a sugarcane database derived from libraries induced and not induced by pathogens, all of which were confirmed to be expressed by RT-PCR in sugarcane leaves inoculated with *Puccinia melanocephala.* To our knowledge, this is the only report about *SOD* gene identification and characterization in sugarcane. Till now, there is still no report about the molecular cloning and characterization of the full-length *SOD* gene in sugarcane. From the aforementioned, it is urgent to clone and characterize the full-length *SOD* gene in sugarcane, which should be helpful to reveal the stress resistance mechanism in sugarcane, especially the oxidative stress resistance.

In this study, on the basis of the large-scale sequencing and bioinformatics analysis of sugarcane leaf cDNA library, the full length cDNA of *Sc-MnSOD* gene was obtained. Its sequence characteristics were analyzed and its prokaryotic expression and enzyme activity assay were also conducted. Real-time qPCR technique was then applied to study the expression profile of the *Sc-MnSOD* gene under various exogenous stresses, which aimed to lay the foundation for further study and its application in sugarcane breeding through genetic engineering.

MATERIALS AND METHODS

Materials and treatment

Sugarcane variety FN 22, sugarcane leaf full-length cDNA library, *E. coli* DH*5α* and BL21 (DE3), and the prokaryotic expression vector were provided by Key Lab of Sugarcane Genetic Improvement, Ministry of Agriculture, P. R. China. Restriction enzyme *EcoR* I, *Sal* I, T_4 DNA ligase, Taq enzyme, DNA and protein molecular marker, SYBR®Premix Ex Taq™ were purchased from TaKaRa (Japan); IPTG (isopropyl-beta-D-thiogalactopyranoside) and Reverse Transcription kit were from Promega Corporation (USA); and the instrument used in the real-time qPCR analysis was the ABI PRISM7500 real-time PCR System.

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_2O_2 were sprayed on the leaves, respectively (Que et al., 2009a). For the second group, spore suspension with concentration of 5 x 10^6 ml^{-1} was inoculated through stabling bud tissues (Que et al., 2009a). The sampling times were 0, 12, 24, 36, 48, 60 and 72 h for SA and H_2O_2 treatment and 0, 12, 24, 36, 48 and 60 h for *Ustilgao scitaminea* 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 full-length cDNA of *Sc-MnSOD* gene

The large-scale sequencing and bioinformatics analysis were conducted for the sugarcane leaf cDNA library (Xu et al., 2009). The library clones containing one EST which showed high homology to cloned *SOD* gene in NCBI database were selected and sequenced completely to obtain the full-length cDNA sequence of *SOD* gene.

For the full-length cDNA sequence of *SOD* 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 properties 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 of *SOD* gene. 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-MnSOD* gene

pET29a (+) was used as the prokaryotic expression vector and the specific primer pairs amplifying the ORF region of *Sc-MnSOD* were designed. The primer sequences were as follows: *Sc-MnSOD*-F: 5'-TTGAATTCATGGCTCTCCGCAC-3′; *Sc-MnSOD*-R: 5′-GGGTCGACAGCAAGAACATTTTCG-3′, of which the underlined parts were the restriction endonuclease sites *of EcoR* I and *Sal* I, respectively.

The primers were synthesized by *Shanghai* Sangon Biological Engineering Technology & Services CO., Ltd. With plasmid DNA extracted from the library clone containing *Sc-MnSOD* 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 reverse primers, respectively; 2.0 µl plasmid DNA; 0.25 µl Taq enzyme (5 U·µL^{-1}); ddH_2O was added as the supplement and the final volume was 50 µl. PCR amplification

program was pre-denaturation for 5 min at 94°C; denaturation for 30 s at 94°C, annealing for 30 s at 55°C and extension for 1 min at 72°C, 30 cycles; followed by finally extension 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 *Eco*R I and *Sal* I, the obtained ORF sequence was ligated by T_4 DNA ligase into pET29a (+) which had also executed the double digestion with *Eco*R I and *Sal* I. The recombinant plasmid was then transferred into *Escherichia coli* DH5α. The plasmid DNA of several clones selected by blue/white colony screening was extracted. A positive clone validated by PCR and the double digestion of *Bam*H I+*Sal* I was just the prokaryotic expression vector which is termed as pET29a-*MnSOD* (Que et al., 2009b).

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

pET29a-*MnSOD* 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 100 μg ml^{-1} ampicillin and shake culture. When OD_{600} 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 of 1.0 mmol L^{-1}. The LB medium with pET29a-*MnSOD* 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. When the electrophoresis ended, the gel was colored with Coomassie brilliant blue and imaged (Que et al., 2009b). Mn-SOD enzyme activity of the medium with 6 h IPTG induction was determined by a photochemical assay system consisting of methionine, riboflavin and NBT according to Giannopolitis and Ries (1977).

Expression profile of *Sc-MnSOD* 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-MnSOD* 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-MnSOD* gene were 5′-ATGGTTAGGCCGGATTACCTGAA-3′ and 5′-CAAACAGACGAAACCCAGCA-3′, respectively. For the 25S *rRNA*, they were 5′-GCAGCCAAGCGTTCAT AGC-3′ and 5′-CCTATTGGTGGGTGAACAATCC-3′, respectively.

The 20 μl reverse transcription system contained 4.0 μl 25 mmol L^{-1} $MgCl_2$, 2.0 μl 5×RT reaction buffer, 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 μg) as the template and RNase free H_2O as the supplement. The reverse transcription program was as follows: firstly 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 amplification, the RT-PCR product was used as the template and the total volume of the reaction system was 25 μl, including 12.5 μl SYBR Primix Ex Taq™ (2×); 0.5 μl Rox reference dye II; 0.5 μl forward and reverse primers (10 μmol L^{-1}) respectively; 2.5 μl cDNA template; and 8.5 μl sterile water. Three replicas were set for each sample. The PCR reaction conditions were pre-denaturation at 95°C for 10 s, and then 40 cycles with 94°C for 5 s and 60°C for 25 s. When the reaction ended, the melting curve was analyzed. The method of $2^{-\Delta\Delta CT}$ was adopted to analyze the real-time qPCR results (Livak and Schmittgen, 2001; Que et al., 2009d).

RESULTS

Cloning and sequence analysis of *Sc-MnSOD* gene

Through the large-scale sequencing of sugarcane leaf full-length cDNA library and the bioinformatics analysis, one EST was found to be of 92% homology to the *Zea mays MnSOD* gene (accession number: AAA72022). The library clone containing this EST was then selected for complete sequencing and the full-length cDNA sequence of *MnSOD* gene in sugarcane termed as *Sc-MnSOD* was obtained, with the GenBank accession number of GQ246460. Sequence analysis showed that the length of *Sc-MnSOD* was 919 bp, including a 702 bp complete ORF, the 5' UTR (untranslated region) of 99 bp, and 3'UTR of 118 bp. Besides, the bases at the sites of -9, -6, -3 and +4 around ATG close to 5' terminus of Sc-MnSOD protein were all G, which was consistent with the Kozak principle. Further, the typical AATAA signal peptide and poly (A) signal tail region were found in the 3'UTR (Figure 1).

The predicted primary gene structure is shown in Figure 1. It showed that this gene including 702 bp ORF encoded a *MnSOD* protein of 233 amino acids with a molecular weight of 25.3 kD and isoelectric point of 7.11. Protein domain prediction revealed that besides the conserved domain possessed by the typical MnSOD, Sc-MnSOD also had the signal peptide at the N-terminus (Figure 2). It could also be predicted that the sites of 1 to 26 aa at the N-terminus had a sequence of signal peptide using SignalP3.0 (Figure 1). With SubLoc v1.0, encoding protein was assumed to be localized in the mitochondria.

Homology analysis of *Sc-MnSOD* gene

The Blastn analysis indicated a rather high homology of 96% (757/788), between *Sc-MnSOD* gene (accession number: GQ246460*)* obtained in this study and MnSOD gene in *Z. mays* (accession number: L19463). The Blastx analysis showed the homology of the *Sc-MnSOD* encoding protein with that of MnSOD from *Z. mays* (Accession number: P41980), *Triticum aestivum* (Accession number: AAB68036), *Oryza sativa japonica* (Accession number: AAA62657), *Helianthus annuus* (Accession number: ABH11434), *Nelumbo nucifera* (Accession number: AAX22235), *Pistacia vera* (Accession number: ABR29644) and *Tamarix* spp (Accession number: AAS77885) was 96 % (224/233), 89 % (208/233), 87 % (206/235),76 % (176/229),78 % (179/229),76 % (176/229) and 76 % (175/229), respectively.

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1       TGAAGCACAT AGCGAGCCGC CGCTCATCAC TCATCCCCTT CCCCGCCTCC ATCTCGGTGT
61      CACACGCATA CGCACCTAGG AGAGACAGCG AGCGAAGCCA TGGCTCTCCG CACCCTGGCA
                                                  M   A   L   R   T   L   A
121     TCGAAGAAGG CCCTATACTT CGCGCTCGGC GGCGCGGCCC GGCCGTTGGC GGCGTCCTCC
        S   K   K   A   L   Y   F   A   L   G   G   A   A   R   P   L   A   A   S   S
181     AGGGGGGTGA CGACGGTCAC GCTCCCCGAC CTCTCCTACG ACTTCGGCGC GCTGGAGCCG
        R   G   V   T   T   V   T   L   P   D   L   S   Y   D   F   G   A   L   E   P
241     GCCATCTCGG GGGAGATCAT GCGCCTGCAC CACCAGAAGC ACCACGCCAC CTACGTCGCC
        A   I   S   G   E   I   M   R   L   H   H   Q   K   H   H   A   T   Y   V   A
301     AACTACAACA AGGCGCTGGA GCAGCTCGAC GCCGCCGTCG CCAAGGGCGA CGCCTCCGCC
        N   Y   N   K   A   L   E   Q   L   D   A   A   V   A   K   G   D   A   S   A
361     GTCGTCCAGC TCCAGGGCGC CATCAAGTTC AACGGCGGCG GTCATGTGAA CCATTCAATC
        V   V   Q   L   Q   G   A   I   K   F   N   G   G   G   H   V   N   H   S   I
421     TTCTGGAAGA ACCTCAAGCC TATTAGTGAG GGTGGTGGGG AGCCACCTCA TGGGAAACTT
        F   W   K   N   L   K   P   I   S   E   G   G   G   E   P   P   H   G   K   L
481     GGCTGGGCCA TTGATGAGGA TTTTGGTTCG TTTGAAGCAC TTGTAAAGAA GATGAATGCA
        G   W   A   I   D   E   D   F   G   S   F   E   A   L   V   K   K   M   N   A
541     GAAGGCGCTG CTTTACAAGG ATCTGGATGG GTGTGGTTAG CTTTGGATAA AGAGGCAAAA
        E   G   A   A   L   Q   G   S   G   W   V   W   L   A   L   D   K   E   A   K
601     AAGCTTTCAG TTGAAACCAC AGCTAATCAG GATCCTCTGG TGACTAAAGG TGCAAGCTTG
        K   L   S   V   E   T   T   A   N   Q   D   P   L   V   T   K   G   A   S   L
661     GTTCCGCTGT TGGGGATTGA TGTCTGGGAG CATGCATACT ACCTGCAGTA CAAGAATGTT
        V   P   L   L   G   I   D   V   W   E   H   A   Y   Y   L   Q   Y   K   N   V
721     AGGCCGGATT ACCTGAACAA CATCTGGAAG GTGATTAACT GGAAATATGC TGGAGAGGTG
        R   P   D   Y   L   N   N   I   W   K   V   I   N   W   K   Y   A   G   E   V
781     TACGAAAATG TTCTTGCTTG AATTGTCTTA ATGGACAATA CCCATCTGTG CTGGGTTTCG
        Y   E   N   V   L   A   *
841     TCTGTTTGAC CATGTGAAAT AAAGATGGAC CGGACCTGTC GAGCCGCTGG ACCTTGTGCA
901     AAAAAAAAAA AAAAAAAAA
```

Figure 1. Nucleotide sequence of *Sc-MnSOD* gene and primary structure of protein. Capital and small letters represent amino acid and nucleotide, respectively; *Shows stop codon; the underline shows the signal peptide; double underlines show the polyadenylation signal AATAAA.

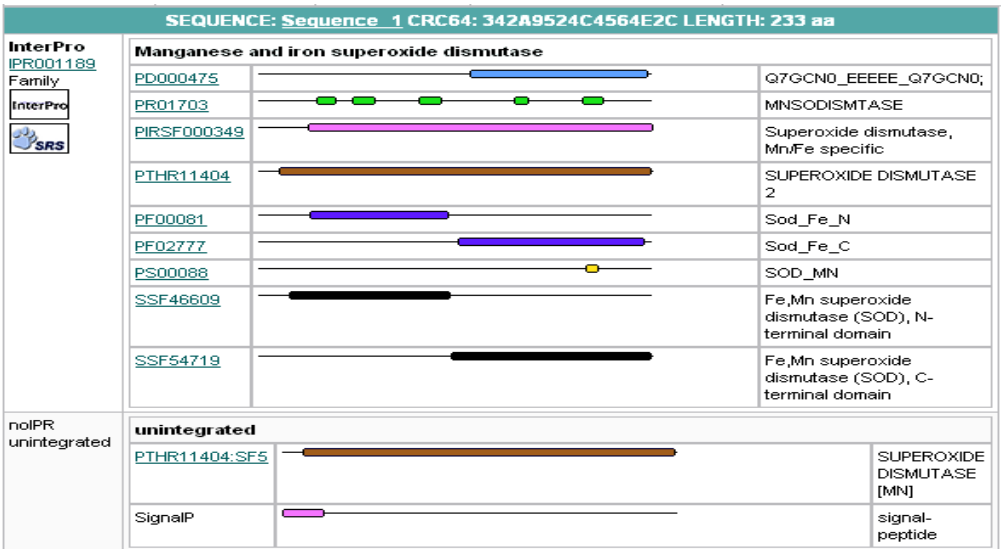

Figure 2. Predicted domain of Sc-MnSOD protein.

Multiple sequence alignment with DNAMAN among the proteins encoded by these genes showed high homology with the *Sc-MnSOD* encoding protein in sugarcane. Figure 3 shows that the amino acid sequence of the MnSOD protein after the site of 36 aa in these plants was rather conserved during the evolution, indicating that *MnSOD* gene may have potential function in the stress-tolerance mechanism in plants. In addition, the conservation between the sites of 12 and 35 aa among these sequences was relatively poor and the structure domain prediction showed that these were the cleavage sites of the signal peptide of the SOD protein.

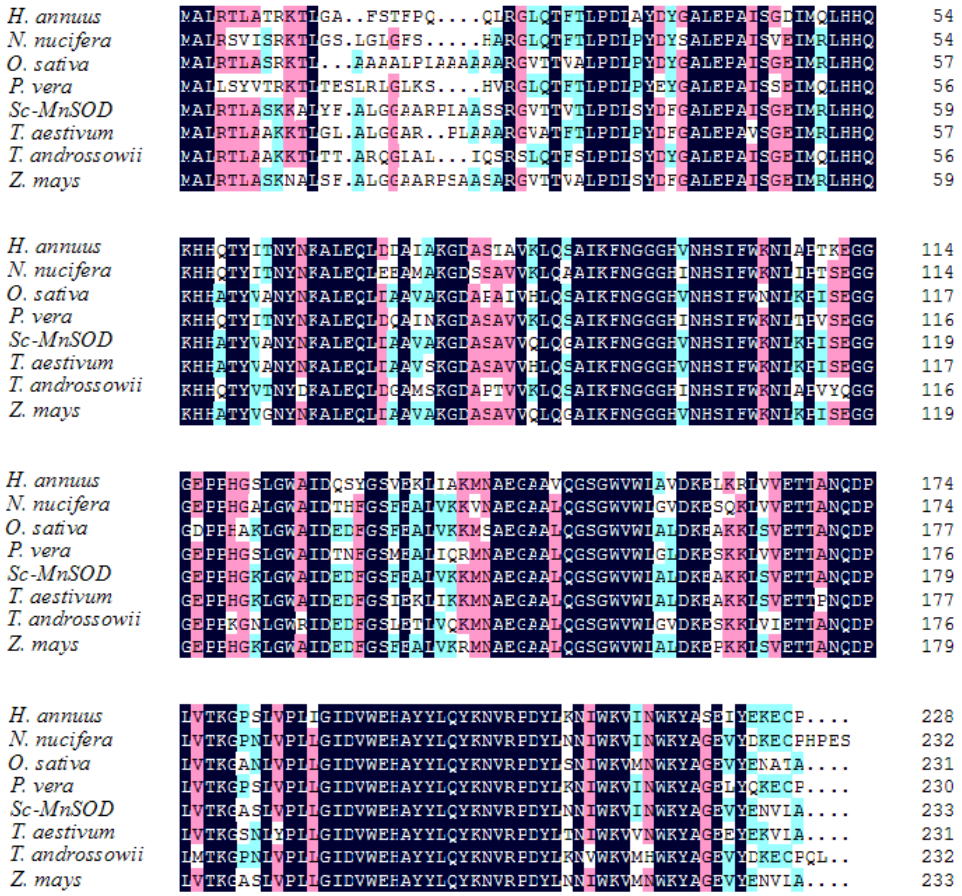

Figure 3. Multiple sequence alignment of MnSOD proteins isolated from different plant species.*H.annuus* (ABH11434); *N.nucifera* (AAX22235); *O.sativa*(AAA62657); *P.vera* (ABR29644); *Sc-MnSOD* (GQ246460); *T.aestiva* (AAB68036); *T.androssowii* (AAS77885); Z.*mays* (P41980).

Using the neighbor-joining method in MEGA3.1, together with bootstrap 1000 to make sure of the reliability, the phylogeny analysis among in sugarcane and the MnSOD of all the other 16 plant species were conducted, and the phylogenetic tree is shown in Figure 4. Overall, it showed that *Sc-MnSOD* and the *MnSOD* of *Z. mays*, *Oryza sativa*, *Triticum aestivum* and *Zantedeschia aethiopica* fell into one category, indicating that they may have evolved from the same ancestor but through different pathways. Among them, the Sc-*MnSOD* and the *MnSOD* of *Z. mays* were in the same group, suggesting the closest evolutionary relationship. The *MnSOD* of the remaining plants formed the other group, in which *MnSOD* from *Arabidopsis thaliana* and *Brassica napus* shared the closest evolutionary relationship.

Prokaryotic expression vector construction of the *Sc-MnSOD* gene

After the PCR product from the ORF amplification of *Sc-MnSOD* gene was recovered, it received double digestion by *Eco*R I and *Sal* I. The target fragment was then recovered to and ligated by T_4 DNA ligase into the prokaryotic expression vector pET29a (+) which had also received the double digestion by *Eco*R I and *Sal* I. The fragment of the same size as that of the target fragment could be obtained from the positive recombinant with PCR amplification and double digestion of *Eco*RI + *Sal* I. The results of the agarose gel electrophoresis suggested the successful construction of the prokaryotic expression vector pET29a-*MnSOD*.

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

As shown in Figure 5, the target protein encoded by *Sc-MnSOD* gene began to express in the second hour and reached the peak at the fourth hour. The molecular weight of the protein expressed in pET29a-*MnSOD* was about 32 kD, and it was consistent with the deduced molecular weight of 32.3 kD; that was the molecular weight of 25.3 kD of the *Sc-MnSOD* encoding protein

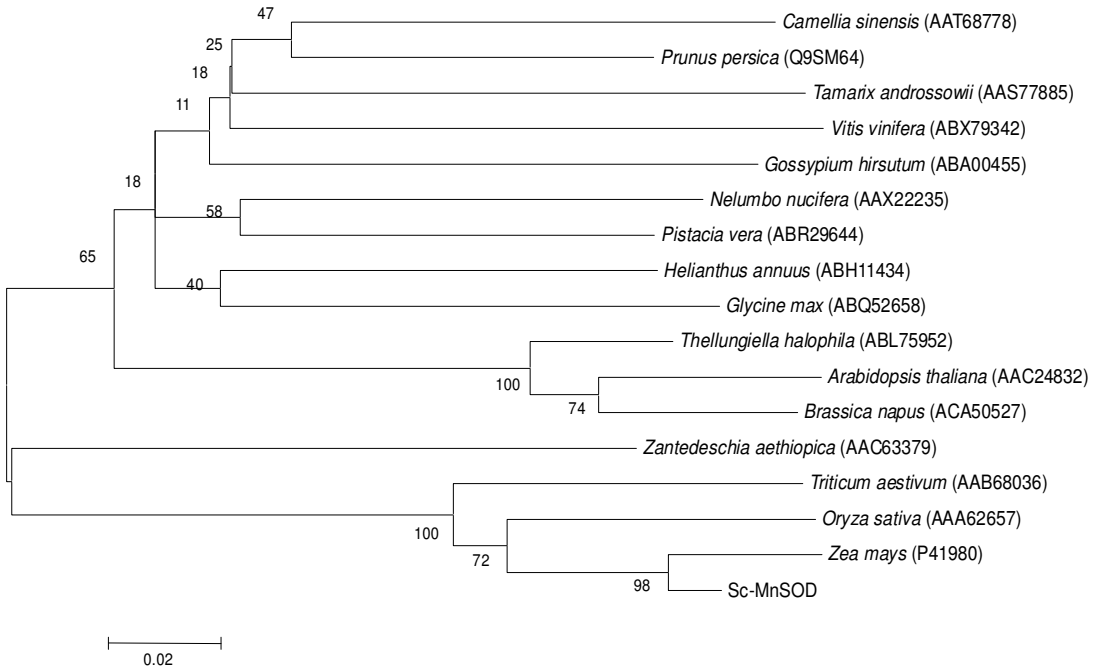

Figure 4. Phylogenetic tree of MnSOD proteins isolated from different plant species.

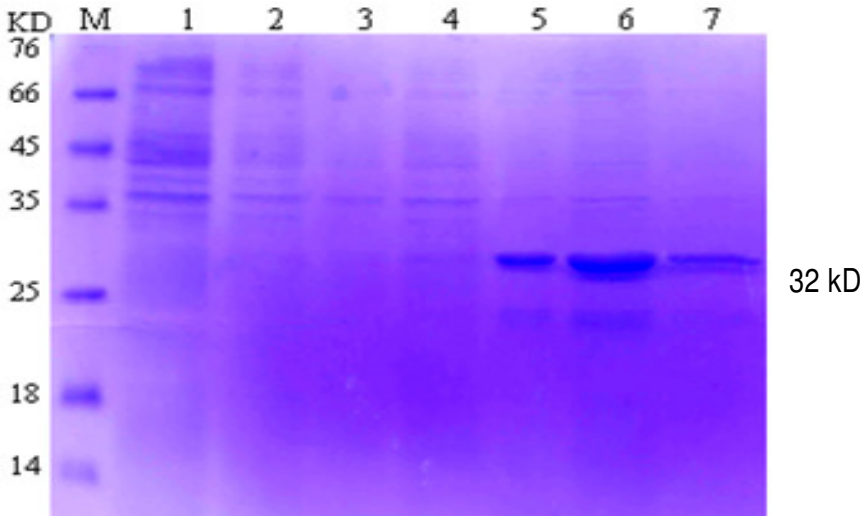

Figure 5. The expression of pET29a-*MnSOD* in *E. coli* BL21.M protein maker. 1, BL21 without induction; 2, pET29a (+) without induction; 3, pET29a (+) induction for 6 h; 4, pET29a-*MnSOD* without induction; 5 to 7, pET29a-*MnSOD* induction for 2, 4 and 6 h, respectively.

plus the molecular weight of 7 KD of the His•Tag peptides, while no target protein was expressed in the empty vector. SOD enzyme activity assay revealed that the prokaryotic expression product in pET29a-*MnSOD* showed 726 U mg^{-1} activity. From aforementioned, it indicated again that the prokaryotic expression vector was successfully constructed, without wrong coding or reading-frame shift of the amino acid sequence, and *Sc-MnSOD* was successfully expressed within the *E. coli* BL21 (DE3).

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

Real-time qPCR analysis of *Sc-MnSOD* gene expression

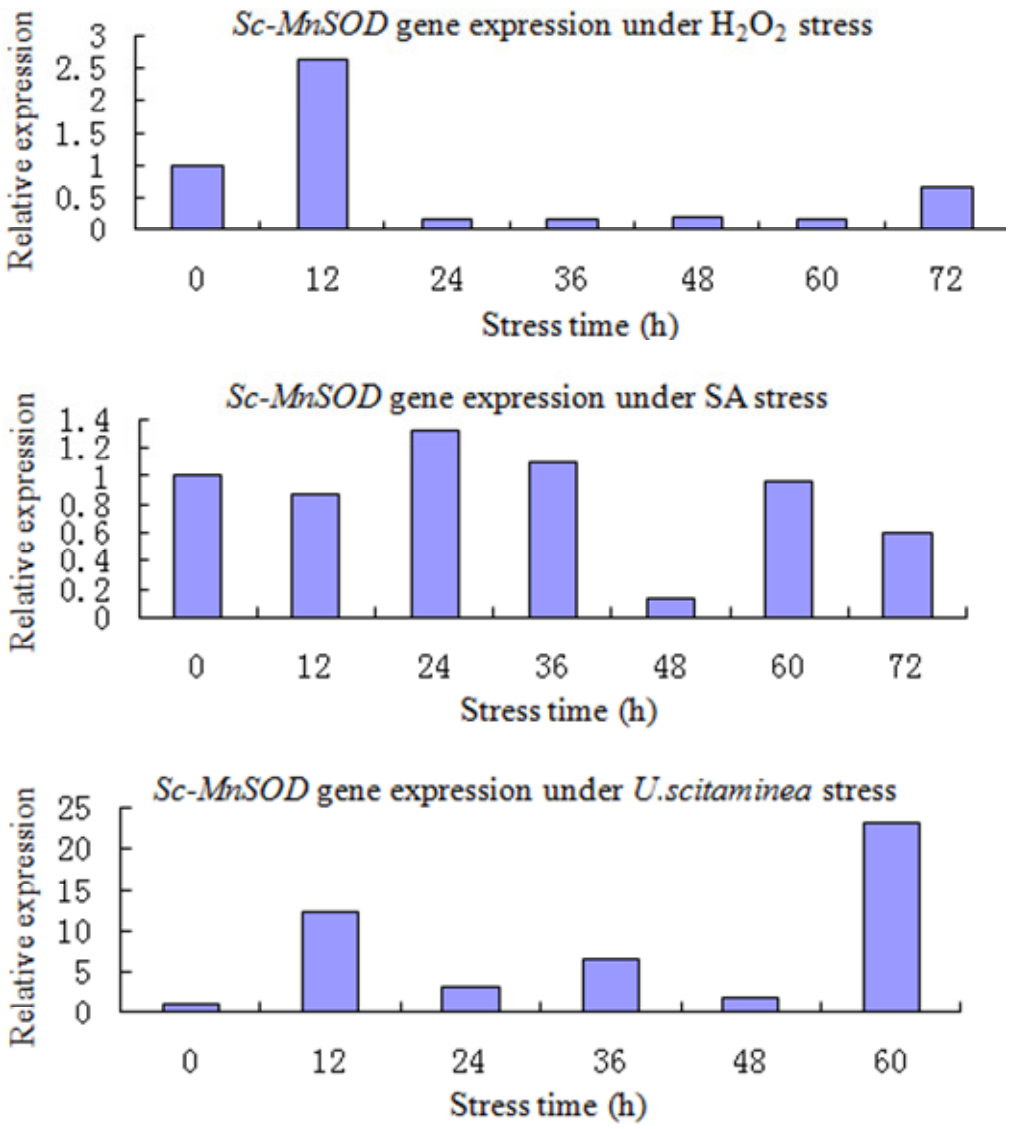

Figure 6. Expression profiles of *Sc-MnSOD* gene under different kinds of stresses.

under various exogenous stresses was conducted. Figure 6 shows that the expression profile of *Sc-MnSOD* gene was different under various exogenous stresses. Under the stress of H_2O_2, the expression of *Sc-MnSOD* gene was firstly induced. At the time point of 12 h, the expression was 2.6 times that of the control at 0 h, but it was strongly inhibited in all the following time points, especially at the time points of 24, 36, 48 and 60 h. Under SA stress, the expression was strongly inhibited at the time point of 48 h with the expression of 0.13 times that of the control at 0 h; however, at other time points, neither inhibition nor induction was obvious, with the expression value been almost equal to that of the control at 0 h. Under the stress of *U. scitaminea*, the expression of *Sc-MnSOD* gene was always strongly induced. At the time point of 12 h, the expression increased to over 12 times that of the control. Although the expression dropped later, it was still higher than that of the control at 0 h. The peak was found at the time point of 60 h, with expression value of over 23 times that of the control. Based on the results of real-time qPCR analysis earlier mentioned, it could be concluded that the *Sc-MnSOD* gene expression was always induced under the stress of *U. scitaminea*. Under H_2O_2 stress, *Sc-MnSOD* gene was firstly induced and then inhibited, while SA had only slight influence on the expression of this gene. *U. scitaminea* and H_2O_2 were assumed to induce a great number of superoxide anions and destroy the balance between the production and clearing of the active oxygen, thus harming the cells (Song et al., 2006). Since the expression of *Sc-MnSOD* gene could be induced under both kind of stresses, it was inferred that *Sc-MnSOD*

gene might play a certain role in the clearing of active oxygen and thus in the mechanism of disease resistance.

DISCUSSION

Sugarcane (*Saccharum officinarum*) is not only the most important sugar crop in the world, but also one important energy crop. However, drought, flood, high temperature, pests and diseases often undermine its production, causing great economic losses. Therefore, it is essential to carry out studies on the mechanism of sugarcane stress resistance, of which the exploitation of the stress-resistance genes in sugarcane is fundamental.

SOD, the important free radical scavenger in the organism, can clear the excessive free oxygen radicals, such as O_2^- , H_2O_2 and OH-, which are harmful to cells. Because different kinds of metal ions bound to the SOD, it could be divided into three types which are Cu/ZnSOD, MnSOD and FeSOD (Grace et al., 1990). FeSOD and MnSOD are mainly found in lower plants and Cu/ZnSOD in higher plants. Cu/ZnSOD is mainly localized in the cytoplasm and chloroplast, MnSOD in the mitochondria and FeSOD in the chloroplast. Although MnSOD and FeSOD shared high homology in terms of sequence and structure, no homology could be found between Cu/ZnSOD, MnSOD and FeSOD (Baum and Scandalios, 1979; Ma and Zhu, 2003). Among these SODs, MnSOD was the only form of SOD that had been shown to be essential for the survival of aerobic life (Carlioz and Touati, 1986). Like the MnSOD in the yeast and animals, MnSOD in plants was also closely related with the mitochondria. It was encoded by the nuclear gene, and compounded into the protein precursor in the cytoplasm, and then the protein precursor was transferred into the mitochondria to serve its function under the guide of the leader peptide (White et al., 2000).

In this study, on the basis of large-scale sequencing, a full-length *MnSOD* gene was obtained from the sugarcane leaf cDNA library. It was determined through the bioinformatics analysis to be *MnSOD* and termed as *Sc-MnSOD* (GenBank accession number: GQ246460). It encoded 233 amino acids, with an encoding protein molecular weight of 25.3 kD and isoelectric point of 7.11. In comparison with the *Mn-SOD* genes cloned in several other species, the homologies of the putative amino acid sequences were considerably high, indicating that the *Mn-SOD* genes were rather conservative among different kinds of plant species. Using SubLoc v1.0, it was predicted that the protein encoded by this gene was localized in the mitochondria, which was in accordance with the previous studies (Grace et al., 1990). In the prokaryotic expression analysis, the molecular weight of the target protein was 33 kD with SOD enzyme activity, which was consistent with the weight of the fusion protein 32.3 kD inferred from the Sc-MnSOD of 25.3 kD plus the His•Tag peptides of 7 kD. This is the first report of cloning and characterization of a full-length mitochondrial *Mn-SOD* gene in sugarcane.

Previous studies showed that most Cu/Zn-SOD and Fe-SOD were constitutively expressed, and Mn-SOD was of mainly induced expression (Kim et al., 1996; Herouart et al., 1993). Furthermore, these three kinds of SOD were functionally complementary to each other and mutually influenced in the expression. It was interesting that when the expression of one type of SOD gene in one organism was suppressed, the expression of the other two types of SOD would be increased so as to maintain the role they exerted during the development and stress resistance of that organism (Kim et al., 1996; Herouart et al., 1993). Several studies indicated that an expression increase of *MnSOD* gene in plants enhanced tolerance to many environmental stresses (Bowler et al., 1991; Wu et al., 1999; Dai et al., 2009; Bai et al., 2009; Zhang et al., 2010; Yang et al., 2009). McKersie et al. (1999) transferred the *MnSOD* gene of tobacco into *Medicago truncatula* and over-expressed it in the mitochondria. The results indicated that the total enzyme activity of transgenic plants was 2 times that of the control, thereby improved the cold-resistance ability and thus raised the yield output. Gachon et al. (2004) found that transgenic *Brassica* plants over-expressed wheat *MnSOD* gene were more tolerant to oxidative stress and aluminum toxicity. Lu et al. (2010) found that over expression of Cu/ZnSOD could enhance tolerance and recovery of potato from drought stress.

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) (Scott et al., 1999). In this study, with the real-time qPCR method, the *Sc-MnSOD* gene expression in sugarcane was analyzed under the stresses of H_2O_2, SA and *U. scitaminea*. The results suggested that the expression of *Sc-MnSOD* gene was greatly induced by the *U. scitaminea*, firstly induced and then inhibited by H_2O_2, and slightly influenced by SA. With the pathogenic *U. scitaminea* and H_2O_2 as the stress, sugarcane may produce free oxygen radicals, such as O_2^-, H_2O_2 and OH-, which are harmful to the sugarcane itself, thus bringing about the active oxygen and destroying the balance between its production and clearing, and consequently undermining the cell. The expression of *Sc-MnSOD* gene was then induced to resist the stresses posted by *U. scitaminea* and H_2O_2, which was in accordance with the results reported by Song et al. (2006).

This study would be helpful for a deeper understanding of the resistance function mechanism of the *Sc-MnSOD* gene and the application of this gene in sugarcane breeding through genetic engineering.

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