Genome-wide identification, characterization, and expression analysis of superoxide dismutase (SOD) genes in foxtail millet (*Setaria italica* L.)

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Abstract

Superoxide dismutases (SODs) play important roles in plant growth, development, and response to abiotic stresses. Despite SOD gene families have been identified in various plant species, little is known in foxtail millet (*Setaria italica* L.). In this study, a systematic analysis of SOD gene family was performed in foxtail millet and the expression pattern of SOD genes in response to abiotic stressors was analyzed at the whole-genomic level. Eight SOD genes were identified in foxtail millet, including 4 *Cu/ZnSODs*, 3 *FeSODs*, and 1 *MnSOD*. These *SiSODs* are unevenly distributed across 5 of the 9 chromosomes. Phylogenetic analysis showed that SOD proteins could be divided into two major categories (*Cu/ZnSODs* and *Fe-MnSODs*), containing seven subgroups, from foxtail millet and other plant species. SOD genes have conserved motif and exon/intron composition in the same subgroup among *Setaria italica*, *Setaria viridis*, and *Oryza sativa*. Additionally, many *cis*-elements that respond to different stressors were distributed at different densities in the promoters of 8 *SiSODs*. The expression patterns of *SiSODs* in different tissues and different abiotic stressors indicated that the *SiSODs* may play important roles in reactive oxygen species scavenging, caused by various stressors in foxtail millet. This study provides a foundation for the further cloning and functional verification of the SOD gene family response to environmental stimuli in foxtail millet.

Keywords Foxtail millet · Superoxide dismutase (SOD) · Genome-wide analysis · Abiotic stress · Expression pattern

Introduction

Reactive oxygen species (ROS), such as hydroxyl radical ($\cdot\text{OH}$), superoxide (O_2^-) and hydrogen peroxide (H_2O_2), are a series of byproducts that are produced by the metabolism of cells. ROS is necessary for organisms when it keeps at a

reasonable level. It can be used as signaling molecules in different organisms to affect different physiological processes in plants (Gechev et al. 2006). The generation and cleanup of ROS are in balance under normal conditions during plant growth and development (Alscher et al. 2002). However, plants are often exposed to various environmental stressors, including drought, salinity, extreme temperature, herbicides, heavy metals, diseases, and pests that would cause excessive accumulation of ROS. Excessive accumulation of ROS can damage biological macromolecules of living organisms, such as lipids, proteins, and nucleic acids, it can also, further result in the membranes lesions, metabolic disturbance and even cell death (Apel and Hirt 2004). To overcome ROS toxicity and protect from damage caused by the oxidative stress, plants have developed efficient mechanisms to alleviate the damage. Some protective enzymes, such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), glutathione peroxidase (GPX) and peroxiredoxin (PrxR) can maintain the dynamic balance of ROS concentration in plants through scavenging the excessive ROS caused by adverse environmental conditions (Sugimoto

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et al. 2014). Superoxide dismutase (SOD, EC 1.15.1.1) is the first enzyme involved in antioxidant enzyme system in plants. SODs could decrease the damage caused by ROS through catalyzing the dismutation of superoxide radicals to H_2O_2 and O_2 (Tepperman and Dunsmuir 1990). SODs are a group of metalloenzymes, based on the types of binding metal ions; plant SODs are mainly classified into three categories: iron SOD (FeSOD), manganese SOD (MnSOD), and copper/zinc SOD (Cu/ZnSOD) (Fink and Scandalios 2002). In plants, nuclear genes are responsible for encoding SOD proteins, but these SODs could be detected in different cellular compartments. Cu/ZnSODs are mainly located in the cytosol, chloroplasts, peroxisomes or in the extracellular space. FeSODs are present majorly in chloroplasts and possibly in the cytosol. MnSODs are located in the mitochondria and in different types of peroxisomes (Pilon et al. 2011). Recently, the SOD gene families have been characterized at the genome-wide level in many plant species, including *Arabidopsis thaliana*, *Musa acuminata*, *Sorghum bicolor*, *Oryza sativa*, *Gossypium spp*, *Solanum lycopersicum*, *Pyrus bretschneideri* and *Medicago truncatula* (Nath et al. 2014; Feng et al. 2015, 2016; Filiz and Tombuloglu 2015; Zhang et al. 2016; Wang et al. 2017, 2018; Song et al. 2018). The research results showed that different types of SOD genes have different expression patterns under various environmental stress conditions. Moreover, the same type of SOD genes did not always play the same role in different species. However, the SOD gene family has not been well studied in foxtail millet (*Setaria italica* L.).

Foxtail millet exhibited high tolerance to abiotic stresses, which is predominantly cultivated for food and fodder in arid and semi-arid regions, especially in China and India (Lata et al. 2013). As a diploid C_4 crop species with a small genome, foxtail millet has been considered as a model species for studying C_4 photosynthesis, abiotic stress tolerance, and biofuel traits (Muthamilarasan and Prasad 2015). The present study was performed to identify the SOD gene family members in the foxtail millet genome and to characterize them using in silico tools, and to analyze their expression patterns during stress treatments; our results should help to comprehensively understand the putative roles of *SiSODs* in response to abiotic stresses in this important crop.

Materials and methods

Plant materials, growth conditions, and stress treatments

Healthy seeds of the foxtail millet cultivar ‘Yu Gu 18’ were obtained from Anyang Academy of Agriculture Sciences (Anyang, China). Foxtail millet seeds were germinated on moist filter paper for 24 h at 28 °C and then planted in a

soil mixture (nutrient soil: vermiculite, 1:1, v/v) in a growth chamber with 16-h light/8-h dark, 27 ± 1 °C, and 60% relative humidity. For salt, drought and cold treatments, the roots of 3-week-old seedlings were immersed into a quarter strength (1/4) of Murashige and Skoog (MS) liquid medium supplemented with 250 mM NaCl, 20% (w/v) PEG6000 (polyethylene glycol) and 4 °C. The plants were sampled at 0, 1, 4, 12 and 24 h after treatments. For gene expression analysis, the leaves, stems, roots, and spica of plants were harvested. All samples in each treatment were harvested and stored at -80 °C for further analysis.

Identification of SOD genes in foxtail millet

To identify SOD genes in foxtail millet, all published SOD gene sequences of *Arabidopsis* and rice were used as query sequences to blast in the foxtail millet genome database (https://phytozome.jgi.doe.gov/pz/portal.html#!info?alias=Org_Sitalica) with the default settings, except that the *E* value of $\leq e^{-10}$. Then the potential SOD gene sequences were examined for copper/zinc (PF00080) and iron/manganese (PF00081, PF02777) SOD domains by the Pfam server (<http://pfam.xfam.org/>). Then, the physicochemical characteristics of *SiSOD* proteins were predicted through the ProtParam tool (<https://web.expasy.org/protparam/>), including amino acid number, instability index, molecular weight (MW), and theoretical isoelectric point (pI) (Wilkins et al. 1999). Subcellular localization of SOD proteins in foxtail millet was predicted through the WoLF PSORT (<https://wolfpsort.hgc.jp/>) and CELLO v.2.5 (<http://cello.life.nctu.edu.tw/>).

Conserved motifs and gene structure analysis of *SiSODs*

Conserved motifs of SOD proteins were analyzed through MEME (<http://meme.sdsc.edu/meme/intro.html>) with the default settings, except that the optimum width from 10 to 150 and the number of motifs was set to 10. The coding and genomic sequences of SOD genes of *Setaria italica*, *Setaria viridis*, and *Oryza sativa* were downloaded from JGI database (<https://phytozome.jgi.doe.gov/pz/portal.html>), and gene structure was analyzed through Gene Structure Display Server (GSDS) (<http://gsds.cbi.pku.edu.cn/>).

Chromosomal location and gene duplication

Chromosomal location information of the *SiSODs* was obtained from foxtail millet genome database and showed with the Graphical Geno Types software. Gene duplication was identified based on the following criteria: (1) the length of alignable sequence covers > 80% of the longer gene; and

Table 1 Physico-chemical and biochemical characteristics of *SiSODs* in *Setaria italica*

Gene name	Transcript ID	ORF length (bp)	Length (aa)	Genomic location	MW (Da)	pI	Instability index	Predicted Pfam domain	Subcellular prediction
<i>SiCSD1</i>	Seita.2G422500.1	456	151	Chr2: 47,643,063 – 47,645,616 –	15078.72	5.60	16.83	CZ	Cytoplasm
<i>SiCSD2</i>	Seita.6G251600.1	606	201	Chr6: 35,750,683 – 35,753,193 +	20302.78	5.34	22.81	CZ	Chloroplast
<i>SiCSD3</i>	Seita.9G403600.1	459	152	Chr9: 46,291,517 – 46,295,065 –	15151.76	5.76	22.53	CZ	Cytoplasm
<i>SiCSD4</i>	Seita.9G488200.1	492	163	Chr9: 52,740,444 – 52,742,965 +	16599.51	6.82	22.07	CZ	Cytoplasm
<i>SiFSD1</i>	Seita.4G011900.1	933	310	Chr4: 751,406 – 753,452 –	35104.14	9.32	51.41	IMA,IMC	Chloroplast
<i>SiFSD2</i>	Seita.4G031200.1	777	258	Chr4: 2,020,703 – 2,023,953 +	29821.09	7.17	56.01	IMA,IMC	Mitochondrion
<i>SiFSD3</i>	Seita.9G123900.1	1128	375	Chr9: 7,769,709 – 7,772,247 –	42238.27	5.93	46.72	IMA,IMC	Chloroplast
<i>SiMSD</i>	Seita.3G286500.1	705	234	Chr3: 27,117,933 – 27,122,896 –	25310.87	7.10	24.03	IMA,IMC	Mitochondrion

Si, *Setaria italica*; aa, amino acid; chr, chromosome; MW, theoretical molecular weight of protein; pI, isoelectric points of protein; CZ, Cu/Zn-superoxide dismutase (SOD); IMA, Fe/Mn-SODs alpha-hairpin domain; IMC, Fe/Mn-SODs C-terminal domain

(2) the similarity of the aligned regions > 80% (Yang et al. 2008).

Phylogenetic and promoter *cis*-elements analysis of *SiSODs*

To obtain the phylogenetic relationship of SOD proteins among different plant species, a total of 51 SOD protein sequences were identified from *Setaria italica*, *Setaria viridis*, *Arabidopsis thaliana*, *Oryza sativa*, *Sorghum bicolor*, and *Glycine max*. Multiple sequence alignments of these SOD protein sequences were performed using the ClustalW software with default parameters. MEGA 5.0 software was performed to construct an enrooted phylogenetic tree via neighbor-joining method with 1000 replicates as previously described (Tamura et al. 2011). The tree was viewed with ITOL (<http://itol.embl.de/>). To determine the possible function of *SiSODs*, 2000 bp sequences immediately upstream from the start codon of each gene was analyzed through PlantCARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html>) (Lescot et al. 2002).

Real-time qRT-PCR analyses of *SiSODs*

Total RNAs were isolated from different tissues of foxtail millet using TRIzol reagent (Tiangen, Beijing, China). Before reverse transcription, the quality of RNA samples was checked by agarose gel electrophoresis. First-strand cDNA synthesis was performed using a FastKing RT kit (Tiangen, Beijing, China). Real-time quantitative reverse transcription-PCR (qRT-PCR) was performed on an ABI 7500 Fast (Applied Biosystems, Foster City, CA, USA) using SuperReal PreMix Plus Reagent (Tiangen, Beijing, China). Relative abundance of mRNA was determined by qRT-PCR according to the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001). *Actin 7* gene (AF288226.1) was used as a reference gene. Three biological replicates were

performed for each gene. The primers for qRT-PCR are listed in supplementary Table S1.

Results

Identification of SOD genes in foxtail millet genome

A total of eight SOD genes were identified in foxtail millet, including 4 *Cu/ZnSODs* (*SiCSD1*, 2, 3 and 4) with a copper-zinc domain, 3 *FeSODs* (*SiFSD1*, 2 and 3) and 1 *MnSOD* (*SiMSD*) with an iron/manganese SOD alpha-hairpin domain and an iron/manganese SOD C-terminal domain, respectively (Table 1). Some important characteristics of SOD genes and proteins in foxtail millet are shown in Table 1 through physicochemical analysis, including the length of amino acid sequences, isoelectric point (pI), molecular weight (MW), genomic location, instability index and subcellular localizations. The instability index indicates whether a protein will be stable in a test tube (≤ 40 , probably stable; > 40 , probably not stable). The majority of *SiSOD* proteins were predicted to be stable except *SiFSDs*. Subcellular localizations of *SiSOD* proteins were determined through CELLO v.2.5 and WoLF PSORT servers. In foxtail millet, Cu/ZnSODs were predicted to localize in the cytoplasm (*SiCSD1*, 3 and 4) and chloroplasts (*SiCSD2*), MnSODs in mitochondria (*SiMSD*), and FeSODs in chloroplasts (*SiFSD1* and 3) and mitochondria (*SiFSD2*) (Table 1).

Phylogenetic analysis of *SiSOD* proteins

To investigate the phylogenetic relationships of SOD proteins between *Setaria italica* and other plants, an unrooted phylogenetic tree was constructed based on the 51 SOD proteins. The SOD proteins from different plant species

were divided into two major categories, Cu/ZnSODs and Fe-MnSODs (Fig. 1). The former category contained three subgroups (a, b, and c), and the latter category was separated into four subgroups (d, e, f and g). As shown in Fig. 1, phylogenetic analysis shows that SiSODs were distributed in each subgroup, except for subgroup d, which included only dicotyledonous FeSODs. SiCSD1, SiCSD3 and other plants' cytosolic Cu/ZnSODs formed subgroup a. SiCSD4 and other plants' cytosolic Cu/ZnSODs formed subgroup c, while SiCSD2 was clustered with other plants' chloroplastic Cu/ZnSODs in subgroup b, respectively. SiMSD and other plants' mitochondrial MnSODs formed subgroup e. In subgroup f, SiFSD2, SvFSD2 and SbFSD2 were predicted to be localized in the mitochondrion, but OsFSD2, GmFSD4 and AtFSD3 were predicted to be localized in the Chloroplast. SiFSD1, SiFSD3 and other plants' chloroplastic FeSODs formed subgroup g. In addition, SOD proteins in each subgroup were separated into mono- and dicot-specific branches; this suggests the independent evolution of SODs after the divergence of monocot and dicot plants. Additionally, we also found that all SiSODs showed closer relationships to SOD proteins from monocotyledonous plants than to those from dicotyledonous plants.

Conserved motifs and clustering analysis of SiSOD proteins

To explore the motif compositions and phylogenetic relationship of the SOD proteins of *Setaria italica*, *Setaria viridis* and *Oryza sativa*, an unrooted phylogenetic tree was generated, and ten conserved motifs were identified by MEME (Table S2). Motif 1 and 8 were related to the SOD_Cu domain (IPR001424), and the other motifs were related to the Mn/Fe_SOD_N domain (IPR019831), Mn/Fe_SOD_C domain (IPR019832) and Mn/Fe_SOD domain (IPR001189), respectively (Table S2). Strikingly, the same subgroup tended to have similar motif distribution patterns, which was in line with the phylogenetic analysis. Motif 1 and 2 were related to copper/zinc SOD domains and located in Cu/ZnSODs (subgroup a, b and c). Four motifs (motif 2, 3, 4 and 5) were related to iron/manganese SOD domains, were shared in Fe-MnSODs (subgroup e, f and g), and motif 6 and 9 were only shared in subgroup f and g, while motif 7 and 10 were only present in subgroup g (Fig. 2).

Chromosomal distribution and gene structure analysis

Five of the nine chromosomes, harbored *SiSODs* (Fig. 3a). There was only one *SiSOD* in chromosome 2, 3 and 6, while

Fig. 1 Phylogenetic tree of SOD proteins from the genomes of different plant species. Two groups (Cu/ZnSODs, Fe-MnSODs) were identified and the tree was divided into seven subgroups (a, b, c, d, e, f and g) based on the high bootstrap values. Subgroups a, b, c, d, e, f and g were marked in purple, yellow, red, green, blue, pink, and orange, respectively

Fig. 2 Conserved motifs and phylogenetic relationship analysis of SOD proteins. Phylogenetic analysis of the SOD proteins from *Setaria italica*, *Setaria viridis* and *Oryza sativa* is shown on the left.

chromosome 4 and 9 possessed two and three *SiSODs*, respectively. Gene duplication analysis shows that two duplication events, *SiCSD1/SiCSD3* and *SiFSD1/SiFSD3* were segmental duplication events based on the chromosomal distribution of the *SiSODs*.

Structural analysis of SOD genes of *Setaria italica*, *Setaria viridis*, and *Oryza sativa* were performed using the GSDS server (Fig. 3b). As shown in Fig. 3b, SOD genes in the same subgroup exhibited similar numbers of exons/introns and exon length, and the intron numbers of SOD genes were within the ranges of 5–9, respectively. In the *Cu/ZnSODs*, subgroup a and c possessed 6 introns, except that the *OsCSD1* possessed 9 introns. Subgroup b had a strictly conserved intron number of 7. In the subgroup e, all the *MnSODs* possessed 5 introns. In the *FeSODs*, subgroup f and g also possessed 7 introns, except that *SiFSD1* possessed 8 introns.

Analysis of putative promoters of *SiSODs*

Potential *cis*-acting elements are shown in Fig. 4. The light-responsive *cis*-elements accounted for the majority of elements in *SiSOD* promoter. Thirty different types of light-responsive elements were widely distributed in the eight *SiSOD* promoters, which indicated that *SiSODs* could be regulated by different light stimuli. Additionally, some stress-responsive *cis*-elements were identified in the *SiSOD* promoter regions, including WUN-motif, ARE, LTR, HSE, TC-rich, MBS and Box-W1, which were associated with wound stress, anaerobic induction, low-temperature, heat stress, defense stress, drought stress and fungal elicitors, respectively. We also found hormone-responsive *cis*-elements existing in the *SiSOD* promoters, which were involved

The conserved motifs analysis of the SOD proteins is shown on the right. Different motifs are indicated with a specific color

with ABA, auxin (IAA), ethylene, gibberellin (GA), methyl jasmonate (MeJA) and salicylic acid (SA) responses respectively, such as ABRE, AuxRR-core, ERE, GARE-motif, CGTCA-motif and TCA-element (Table S3). In addition, some *cis*-elements involved in circadian control were identified in *SiCSD1*, *SiFSD1* and *SiCSD4* gene sequences. In conclusion, we found different types and numbers of *cis*-elements that were distributed in distinct *SiSOD* promoters, indicating that *SiSOD* genes may play different roles during life cycle and response to various stressors and hormone treatments in foxtail millet.

Tissue-specific expression profiles of *SiSODs*

To explore the function of SOD genes during foxtail millet growth and development, qRT-PCR analysis was employed to analyze the tissue-specific expression profiles of *SiSODs*.

All *SiSODs* were expressed in the tested tissues, although the *SiFSD1* and *SiFSD3* showed extremely low expression levels in root tissues (Fig. 5). The expression of *SiCSD1* and *SiCSD3* displayed similar patterns; in all tested tissues, both of them were expressed more than other *SiSODs*. *SiCSD1* and *SiCSD3* were more highly expressed in root, spica, and stem, especially in spica. The expression of *SiCSD2* was relatively higher in leaf, spica and stem, and exhibited the highest expression level in stem. In addition, *SiCSD4* was consistently expressed in all four tissues, and displayed similar expression patterns in root, leaf, spica, and stem. *SiFSD1*, *SiFSD2* and *SiFSD3*, all of them were *FeSODs*, exhibited a relatively higher expression in leaf, spica and stem; however, the expression levels were low in roots. *SiMSD* was expressed highly in spica and stem, and moderately expressed in root, and had the lowest expression level

Fig. 3 Chromosomal distribution and gene structure of SOD genes. **a** Chromosomal distribution of SOD genes in foxtail millet. *SiSODs* were plotted onto five chromosomes of foxtail millet. The scale is in megabases (Mb). The chromosome numbers are indicated at the top of each chromosome. The numbers at right indicates the position

of *SiSODs*, and the names of the respective genes are given in left. Segmentally duplicated gene-pairs are connected with a red line. **b** Gene structure of SOD genes from *Setaria italica*, *Setaria viridis*, and *Oryza sativa*. Exons and introns are shown as yellow boxes and thin lines, respectively. UTRs are shown with blue boxes

Fig. 4 *Cis*-elements analysis of putative *SiSOD* promoters. Different *cis*-elements with the same or similar functions are shown in the same color

Fig. 5 Tissue-specific expression profiles of *SiSODs*. The tested tissues were leaf, root, spica, and stem. Data shown were the mean of three independent repeated experiments $\pm$ standard deviation. Error bars represent standard deviations from three independent biological replicates

in the leaf. In addition, some *SiSODs* had relatively lower expression levels in almost all tissues under normal conditions. This suggests that they may express at specific developmental stages or under special stress conditions.

Abiotic stress induced SOD gene expression in foxtail millet

Because of the remarkable salt tolerance and water usage efficiency of foxtail millet, the identification of mechanisms associating with stress tolerance in this species is important. The expression levels of most *SiSODs* were significantly altered under drought, salt and cold stress, presenting a complex regulation.

Under drought stress, the expression levels of all *SiSODs* were decreased after 1 h and then increased (Fig. 6a). The expression levels of *SiFSD1*, *SiFSD2* and *SiMSD* were significantly increased (fold change > 2) under drought treatment, and reached the highest at 12 h, 4 h and 4 h of treatments, respectively. In addition, the expression levels of *SiCSD2* and *SiFSD3* were gradually decreased after 4 h of treatment and reached a lower level at 24 h, suggesting that these two genes may play a negative regulatory role under drought stress. The expression levels of *SiCSD1*, *SiCSD3* and *SiCSD4* did not change significantly after 4 h of drought treatment.

Under salt stress, the expression levels of all *SiSODs* were also decreased after 1 h of treatment and then increased (Fig. 6b). The expression of all *SiCSDs* and *SiMSD* was strongly induced by salt treatment and exhibited 3.2–21.43-folds increase in expression. The expression levels of *SiCSD1*, *SiCSD2*, *SiCSD3* and *SiMSD* reached the highest at 24 h of treatments, whereas *SiCSD4* reached the highest

at 12 h. The expression of *SiFSD1* and *SiFSD3* were slightly altered under salt stress treatment. However, *SiFSD2* displayed a different expression under salt stress, and the expression of *SiFSD2* was increased after 4 h of treatment until reached a peak at 24 h.

The expression of almost all *SiSODs*, except for *SiCSD4*, was up-regulated by cold treatment (Fig. 6c). *SiCSD1*, *SiFSD2* and *SiCSD2* reached the highest expression levels at 4 h of treatment and decreased after experiencing a longer stress treatment. *SiMSD*, *SiFSD1*, *SiFSD3* and *SiCSD3* continued to increase after 4 h and reached a peak at 12 h, then decreased afterwards.

Discussion

Environmental stresses are major challenges for plant growth and development. These stresses often lead to the production of toxic ROS. Excess ROS could result in inactivation of enzymes, breakage of DNA strands and membrane lipids (Apel and Hirt 2004). SODs act as the first line in antioxidant systems and play important roles in responding to various environmental stimuli and provide basic protection against ROS damage in plants. Therefore, SOD gene families have been identified in different plant species (Nath et al. 2014; Feng et al. 2015, 2016; Filiz and Tombuloglu 2015; Zhang et al. 2016; Wang et al. 2017, 2018; Song et al. 2018). Considering the prominent abiotic stress tolerance of the foxtail millet, characterizing the SOD gene family in foxtail millet is important.

In our study, eight SOD genes were identified in the foxtail millet genome, including 4 *Cu/ZnSODs*, 3 *FeSODs*, and 1 *MnSOD* (Table 1). *Cu/ZnSOD* is a dimeric copper- and zinc-containing protein and act as a major isozyme of

Fig. 6 Expression patterns analysis of *SiSODs* in response to various abiotic stresses by qRT-PCR. **a** Expression patterns analysis of *SiSODs* under drought stress. **b** Expression patterns analysis of *SiSODs* under salt stress. **c** Expression patterns analysis of *SiSODs*

under cold stress. Data shown were the mean of three independent repeated experiments $\pm$ standard deviation. Error bars represent standard deviations from three independent biological replicates

the superoxide dismutase family. Zinc is necessary for the structural stability and enzymatic activity of Cu/ZnSOD, and it can be obtained by passive diffusion easily. Copper is a catalytic cofactor of Cu/ZnSOD and play a catalytic role in the disproportion of superoxide, but it can only be obtained through a copper chaperone under normal conditions (Rae et al. 1999). The copper chaperone for Cu/ZnSOD (CCS) was first defined in yeast and human, and it is responsible for the delivery of Cu to the Cu/ZnSOD (Culotta et al. 1997). CCS genes have been defined and cloned in some species (Chu et al. 2005; Sagasti et al. 2014). In some reports, CCS genes were assigned to the SOD gene family for research (Feng et al. 2016; Song et al. 2018). In our study, we found that there was one CCS gene (*SiCCS*, Seita.7G206200.1) in the foxtail millet genome, but we thought that CCS and Cu/ZnSOD should belong to different gene families, so we did not assign the *SiCCS* to the superoxide dismutase gene family in foxtail millet.

Gene duplication is an important way for the expansion of gene families (Ganko et al. 2007). Therefore, gene duplication analysis of the *SiSODs* was performed according to the criteria of a previous study (Yang et al. 2008). Two segmental duplication events (*SiCSD1/SiCSD3* and *SiFSD1/SiFSD3*) were identified, no tandem duplication event was observed between these genes in foxtail millet.

Phylogenetic analysis was performed on foxtail millet and 5 other plant species, which reveals that the members of plant SOD proteins could be divided into Cu/ZnSODs and Fe-MnSODs. FeSODs and MnSODs from different plants were clustered together in Fe-MnSODs, suggesting that they might originate from common ancestral genes (Zhou et al. 2017). Based on the high bootstrap values, these two groups could be further divided into seven subgroups. Previous reports found that the clustering of SOD proteins may be associated with the subcellular locations of SODs (Song et al. 2018; Wang et al. 2018); in our study, the members clustered in the same subgroup tended to share the same subcellular localization. Conserved motifs analysis of SOD proteins also supports the phylogenetic data (Fig. 2). The same subgroup had same common motifs. It is noteworthy that SOD proteins gathered in each subgroup not only tended to have similar motif distribution patterns, but also have similar motif position and length.

In our study, gene structure analysis also showed that the SOD proteins have strong evolutionary conservation. SOD genes in the same subgroup exhibited similar numbers of exons/introns and exon length, with the exception of *OsCSD1* and *SiFSD1* (Fig. 3). A previous study has found that the intron numbers and intron–exon organizations of plant SOD genes were highly conserved and most cytosolic

and chloroplastic genes contain 7 introns (Fink and Scandalios 2002). However, our study found that the intron number of *SiSODs* varied from 5 to 9. *SiMSD* and other plants' mitochondrial *MnSODs* (subgroup e) only had 5 introns, and *Cu/ZnSODs* that were predicted to be localized in the cytoplasm (subgroup a and c) all had 6 introns. All cytosolic SOD genes (Subgroup b, f and g) and three mitochondrial *FeSODs* in subgroup f had 7 introns. The reason for this discrepancy may be due to three main mechanisms according to a previous report, including exon/intron gain/loss, exonization/pseudoexonization and insertion/deletion (Xu et al. 2012). The divergences of structure may affect their enzymatic activity and expression pattern that responds to various stress conditions.

The gene expression under various abiotic and biotic stresses needs to undergo a complicated regulation process. Characterization of transcription factors and *cis*-elements in the promoter sequences offers insights into the upstream regulation of *SiSODs*. Our results shows that many identified *cis*-elements in the promoters of *SiSODs* were related to stress-responsive incidents. For example, MBS is a MYB binding site involving in drought-inducibility, which was obtained in all 8 *SiSODs* (Table S3). MYB is a type of transcription factor which is found to be involved in regulation of plant growth and development, physiological metabolism, cell morphogenesis, and organogenesis (Cheng et al. 2009; Slabaugh et al. 2011). Additionally, many MYB genes in plants were involved in biotic and abiotic stress responses (He et al. 2016; Salih et al. 2016). Therefore, we hypothesized that *MYB* could regulate the expression of *SiSODs* under environmental stresses, especially under drought stress. DREB transcription factors were also reported to be involved in stress responses (Qin et al. 2008; Wang et al. 2014). It could bind to the dehydration responsive element (DRE) in the promoter to regulate the expression of related genes. DRE site was also located in the putative promoter regions of certain *SiSODs*, suggesting that SOD genes may also be regulated by *DREB* in foxtail millet. Therefore, analysis of putative *SiSOD* promoters will contribute to understand and identify the detailed function of *SiSODs* in the future.

To further clarify the function of *SiSODs*, gene expression patterns of *SiSODs* in different tissues were analyzed through qRT-PCR. Large amounts of ROS are often generated during organogenesis processes and reproductive metabolic processes; therefore, the expression levels of many SOD genes were relatively high in flower (Wang et al. 2017; Zhou et al. 2017). In present study, *SiCSD1* and 3 exhibited high expression in all the four tissues and showed the highest expression in spica. Many previous studies have found that most SOD genes showed the highest expression in leaf (Pilon et al. 2011; Zhou et al. 2017). However, in our study, not like in other plants, no single *SiSOD* was

expressed highest in leaf, indicating that *SiSODs* may not play a similar role in photosynthesis. In addition, some *SiSODs* (*SiMSD*, *SiFSD1*, *SiFSD2* and *SiCSD2*) showed the highest expression in stem, indicating their specific roles required in stem development. *SiCSD2* exhibited a constitutive expression and had no significant difference among the four tissues, and similar results had been reported in previous studies (Feng et al. 2016; Song et al. 2018; Zhou et al. 2017).

Many *cis*-elements in the promoters of *SiSODs* were related to various stresses. To better understand the function of *SiSODs* under various environmental stresses, we analyzed the expression levels of the eight *SiSODs* under various environmental stresses. Each *SiSOD* responded to at least one abiotic stress, including drought, salinity and cold. *Fe-MnSODs* play a vital function under drought stress, and *Fe-MnSODs* were induced in many other plants (Feng et al. 2016; Wang et al. 2017; Zhou et al. 2017). Overexpression of *TaMnSOD* in cotton also could provide additional protection under drought (Zhang et al. 2014). In our study, *SiFSD1*, *SiFSD2* and *SiMSD* were strongly induced after drought treatment, and reached the highest at 12 h, 4 h and 4 h of treatments, respectively. It indicates that *Fe-MnSODs* also play a major role in drought stress response in foxtail millet. Overexpression of *Cu/ZnSODs* enhanced salt stress resistance in *Puccinellia tenuiflora*, *Oryza sativa*, *Triticum aestivum* and *Arabidopsis* (Wang et al. 2016; Wu et al. 2016; Guan et al. 2017). The expression of all *SiCSDs* was strongly induced by salt treatment and reached the highest at 12 or 24 h in our study, suggesting that *SiCSDs* may be involved in salt response in foxtail millet. Furthermore, in our study, *SiMSD* was also strongly induced under salt stress, and exhibited 21.43-folds increase in expression, indicating that *SiMSD* maybe also participated in salt stress response as *SiCSDs* in foxtail millet. Under cold stress, the expression of almost all the SOD genes could be affected in many plants, such as *GaSODs*, *GrSODs*, *GhSODs*, *CsSODs* and *MtSODs* (Song et al. 2018; Wang et al. 2017; Zhang et al. 2016; Zhou et al. 2017). Overexpression of SOD genes in plants could also improve their tolerance to cold stress (Zhang et al. 2016). In our study, the expression of almost all *SiSODs* was up-regulated under cold stress, indicating that SOD genes are widely involved in cold stress response in plants. These results indicate that *SiSODs* may play a predominant antioxidant role in foxtail millet under abiotic stress conditions, and these genes could be selected for the further cloning and functional verification during stress responses.

Conclusions

In this study, eight SOD genes were identified in foxtail millet, including 4 *Cu/ZnSODs*, 3 *FeSODs*, and 1 *MnSOD*. Our study also provides detailed information about the SOD gene family in foxtail millet, including sequence characteristics, phylogenetic relationships, *cis*-elements distribution and expression patterns, and may further provide theoretical basis for revealing molecular mechanism of drought stress response in foxtail millet.

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Author contributions RP and JL conceived and designed the experiments. TW, HS and ZZ performed the experiments. ZL, SZ, RG and CW contributed reagents/materials/analysis tools. TW, BZ and RP wrote and revised the paper. All authors read and approved the work reported.

Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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