Research Article
Plant Genetics

Genome-wide analysis of the maize superoxide dismutase (SOD) gene family reveals important roles in drought and salt responses

Jing Liu[1,2*], Lijuan Xu[1,2*], Jian Shang[1,2], Xiaolin Hu[1,2], Haitao Yu[1,2], Hongying Wu[1,2],
Wenben Lv[1] and Yang Zhao[1,2] (iD)

[1]*Anhui Agricultural University, School of Life Sciences, National Engineering Laboratory
of Crop Stress Resistance Breeding, Hefei, China.*
[2]*Anhui Agricultural University, Maize Engineering Technology Research Center of Anhui Province,
School of Life Sciences, Hefei, China.*

Abstract

Superoxide dismutase proteins (SODs) are antioxidant enzymes with important roles in abiotic stress responses. The SOD gene family has been systematically analyzed in many plants; however, it is still poorly understood in maize. Here, a bioinformatics analysis of maize *SOD* gene family was conducted by describing gene structure, conserved motifs, phylogenetic relationships, gene duplications, promoter *cis*-elements and GO annotations. In total, 13 *SOD* genes were identified in maize and five members were involved in segmental duplication. Phylogenetic analysis indicated that SODs from maize and other plants comprised two groups, which could be further classified into different subgroups, with most members in the same subgroup having the same subcellular localization. The *ZmSOD* promoters contained 2-10 stress-responsive *cis*-elements with different distributions. Heatmap analysis indicated that *ZmSODs* were expressed in most of the detected tissues and organs. The expression patterns of *ZmSODs* were investigated under drought and salt treatments by qRT-PCR, and most members were responsive to drought or salt stress, especially some *ZmSODs* with significant expression changes were identified, such as *ZmCSD2* and *ZmMSD2*, suggesting the important roles of *ZmSODs* in abiotic stress responses. Our results provide an important basis for further functional study of *ZmSODs* in future study.

Keywords: Maize, *SOD*, phylogenetic analysis, expression patterns, abiotic stress.

Received: January 28, 2021; Accepted: July 2, 2021.

Introduction

Reactive oxygen species (ROS) are inevitable products in the process of cellular metabolism that act as signal molecules to regulate many physiological processes in plants (Gechev *et al.,* 2006). However, abiotic stresses, such as drought, salt and extreme temperature, often induce the production and accumulation of ROS in plant cells (Karuppanapandian *et al.,* 2011), and the presence of excess ROS negatively affects cell growth and even leads to cell death (Mittler, 2002; Lee *et al.,* 2007). Efficient mechanisms have been established to cope with ROS toxicity during the long-term evolution of plants. For example, many studies have shown that some enzymes that remove ROS, such as superoxide dismutase (SOD), peroxidase (POD), catalase (CAT) and glutathione peroxidase (GPX), can protect plants from various abiotic stresses (Mittler *et al.,* 2004; Sugimoto *et al.,* 2014).

SODs are the first defense of the plant antioxidant system, and play important roles in protecting plants against oxidative stress (Nath *et al.,* 2014; Wang *et al.,* 2017). They mitigate

ROS hazards by catalyzing the conversion of superoxide (O^{2-}) into hydrogen peroxide (H_2O_2) and molecular oxygen (O_2) under oxidative stress (Gopavajhula *et al.,* 2013), and play significantly roles in protecting the stability of cell membrane and slowing oxidative damage (Karuppanapandian *et al.,* 2011). SODs are very widespread in living organisms. In plants, genome-wide analysis of SOD family genes has been performed in many species, including Arabidopsis (Kliebenstein *et al.,* 1998), rice (Nath *et al.,* 2014), wheat (Jiang *et al.,* 2019), sorghum (Filiz and Tombuloglu, 2015), upland cotton (Wang *et al.,* 2017), and Medicago (Song *et al.,* 2018). These studies have indicated that SODs are encoded by a small gene family; for example, seven *SOD* genes were found in Medicago, and eight members were reported in both of the Arabidopsis and rice genomes. SODs are metalloenzymes, whose proteins require metal cofactors to have catalytic activity (Forman and Fridovich, 1973). Based on the type of metal cofactor, plant SODs can be divided into three groups, iron SODs (FeSODs), manganese SODs (MnSODs), and copper/zinc SODs (Cu/ZnSODs) (Alscher *et al.,* 2002; Fink and Scandalis, 2002).

Increasing numbers of studies have indicated that *SOD* genes have important roles in response to abiotic stresses (Wang *et al.,* 2004; Pilon *et al.,* 2011; Asensio *et al.,* 2012; Verma *et al.,* 2019). With the development of high-throughput sequencing technology, the expression patterns of SOD family genes in stress responses have been extensively studied. For example,

nine *SOD* genes were identified in tomato, and most members showed altered expression under salt and drought stresses according to microarray data analysis (Feng *et al.,* 2016). In Medicago, differential expression was detected for most of the seven *MtSOD* genes under various stress treatments based on microarray analysis and high-throughput sequencing (Song *et al.,* 2018). In foxtail millet, the expression patterns of *SOD* genes were detected under drought, salt, and cold treatments by quantitative real-time PCR (qRT-PCR), and each *SOD* was found to respond to at least one abiotic stress (Wang *et al.,* 2018b). Importantly, the biological functions of some *SOD* genes involved in stress responses have been demonstrated in transgenic plants. For example, Zhang *et al.* (2014) showed that overexpression of a *Tamarix albiflonum SOD* gene, *TaMnSOD*, can improve cotton's tolerance to drought stress by enhancing root development and the regulation of superoxide scavenging (Zhang *et al.,* 2014). In wheat, overexpression of the *TaSOD2* gene increased salt resistance in transgenic wheat and Arabidopsis plants (Wang *et al.,* 2016).

As an important cereal crop around the world, maize (*Zea mays* L.) has been widely used in genetics and evolution research. However, the growth and yield of maize were seriously affected by various abiotic stresses, and identifying stress-responsive genes and applying them in molecular breeding is one of the effective ways to cope with abiotic stress. Although several *SOD* genes have been identified in maize, systematic analysis of this family has not been reported at whole genome level with the latest genome data, especially for their functional roles in abiotic stress responses (Cannon *et al.,* 1987; Zhu and Scandalios, 1994; Sytykiewicz, 2014). In this study, 13 maize *SOD* genes (*ZmSODs*) were identified in the current genome, and systematic analysis was performed using bioinformatics method. The expression patterns of the 13 genes were also investigated in maize seedlings under drought and salt treatments. The results lay an important foundation for further evolutionary research of plant *SOD* gene family and provide useful information for identification of key *ZmSODs* in response to abiotic stress.

Material and Methods

Identification of *ZmSOD* genes in maize

To identify SOD-encoding proteins in the maize genome, Hidden Markov Model (HMM) profiles of the Cu/ZnSOD domain (PF00080) and Fe/MnSOD domains (N-terminal domain, PF00081; C-terminal domain, PF02777) were initially obtained from the Pfam database (http://pfam.xfam.org/) (Finn *et al.,* 2006). Subsequently, we used the HMM profile of each type of SOD proteins as a query to execute a local BLASTP search against the maize genome (v4) (*p*-value = 0.001). All candidate sequences that met the standards were analyzed in the Pfam database to confirm that each sequence contained the related domains. Redundant sequences were removed based on alignments using the ClustalW software (Thompson *et al.,* 1994), and the non-redundant members were used for further analysis. The ExPASy (https://web.expasy.org/protparam/) and WoLF PSORT online tools (https://www.genscript.com/wolf-psort.html) (Horton *et al.,* 2007) were used to predict physico-chemical characteristics and subcellular localizations, respectively. Based on their positions in the genome annotations, the chromosomal distributions of the maize *SOD* genes were displayed from top to bottom on the chromosomes using the MapInspect software. Gene duplication analysis of *ZmSODs* was performed according to previous study (Si *et al.,* 2019).

Phylogenetic analysis of SOD proteins

To analyze the phylogenetic relationships of SOD proteins among different plants, the full-length amino acid sequences of 37 SOD proteins from maize, Arabidopsis, foxtail millet and rice were used to construct a phylogenetic tree with the MEGA 5.05 (Molecular Evolutionary Genetics Analysis) software. Arabidopsis, foxtail millet and rice SOD sequences were obtained from Joint Genome Institute (http://www.phytozome.net) according to previous studies (Kliebenstein *et al.,* 1998; Nath *et al.,* 2014; Wang *et al.,* 2018b). The phylogenetic tree was built using the neighbor-joining method with 1,000 bootstrap replicates, and the same method was used to construct an unrooted phylogenetic tree of the ZmSOD proteins.

Conserved motif, gene structure and promoter analysis of *ZmSOD*s

Multiple Em for Motif Elicitation (MEME) (http://meme-suite.org/tools/meme) was used to discover conserved motifs among the 13 maize SOD proteins (Bailey *et al.,* 2009). The motif number was set to 10 and the width of motifs was 6 to 50. The detected motifs were annotated using the Pfam database. Gene Structure Display Server (http://gsds.cbi.pku.edu.cn/index.php) was used to analyze the gene structure by comparing the coding sequence (CDS) with the genomic sequence of each predicted ZmSOD (Hu *et al.,* 2015). To predict putative stress-responsive *cis*-elements in the promoter regions of *ZmSODs*, the 2,000 bp flanking sequences upstream from the transcription start site (ATG) of each *ZmSOD* was obtained from the maize genomic sequence, and these promoter sequences were analyzed using PlantCARE (Lescot *et al.,* 2002).

Expression patterns of *ZmSODs* in different tissues and organs

To determine the expression patterns of the *SOD* genes in maize tissues and organs, the publicly available transcriptome data published by Walley *et al.* (2016) for 23 different developmental stages, were downloaded from MaizeGDB (http://www.maizegdb.org/). The fragments per kilobase of transcript per million mapped (FPKM) values were transformed and used to draw a heat map of *ZmSODs* as described in our previous study (Zhao *et al.,* 2019).

Plant material and stress treatments

The expression levels of the *ZmSODs* were investigated in maize seedlings under abiotic stress conditions. Seeds of the maize inbred line B73 were washed with sterile water three times, and placed in vermiculite for germination until the coleoptile grew to about 2 cm in length. Then, seeds with consistent germination were selected, washed with water, and placed on plastic tanks containing Hoagland's nutrient solution in a plant growth chamber at 28 °C/23 °C (day/night) with a

16-h light/8-h dark photoperiod. At the three-leaf stage, the seedlings were used for drought and salt treatments to explore the possible functional roles of the *ZmSODs* in response to abiotic stress. Hoagland's nutrient solution containing 20% (m/v) PEG-6000 or 200 mM NaCl was used for drought and salt treatment, respectively. At 0, 3, 6, 12, and 24 h after treatment, the third leaf of each seedling was harvested, and immediately frozen in liquid nitrogen, and stored at -80°C for RNA extraction.

RNA isolation and quantitative real-time PCR (qRT-PCR) analysis

Total RNA was extracted from the seedling samples using AG RNAex Pro Reagent (Accurate Biology, China). RNA quality and concentration were assessed by 1% agarose gel electrophoresis and P200+ Series Micro Volume Spectrophotometers (Pultton, USA), respectively. First-strand cDNA was generated from 1 µg of total RNA using HiScript® III RT SuperMix for qPCR (+ gDNA wiper) (Vazyme, China) according to the manufacturer's instructions. Gene-specific primers were designed using the Primer3Plus online tool, and the NCBI database was used to verify the specificity of the primers (Table S1). qRT-PCR reactions were carried out as described in our previous study (Zhao *et al.*, 2019). The maize *GAPDH* gene (accession number: NM_001111943.1) was utilized as an internal control for normalizing expression levels. Three biological and three technical repeats were performed for each gene.

Results

Identification of SOD proteins in maize

Using the local BLASTP program, a total of 13 non-redundant SOD proteins were obtained with predicted Cu/ZnSOD or Fe/MnSOD domains after confirmation with the Pfam database. The number of SOD proteins in the maize genome was significantly higher than in Arabidopsis and rice. Based on their phylogenetic relationships, chromosomal distributions and metal cofactors, the 13 *SOD* genes were named *ZmCSD1-ZmCSD6*, *ZmFSD1-ZmFSD5*, *ZmMSD1* and

ZmMSD2. According to the physico-chemical characteristics predicted by the Expasy tool, we found that the protein lengths, molecular weights (MWs), and isoelectric points (pI) of the ZmSOD members had large ranges. The protein lengths of the ZmSODs ranged from 152 to 386 aa, and the molecular weights of the ZmSODs varied from 15.07 to 42.87 kDa. The isoelectric points of the ZmSODs ranged from 5.33 to 8.84. According to the subcellular localization predictions, the highest number of members was localized in mitochondria, including ZmFSD1, ZmFSD2, ZmFSD3, ZmFSD5, ZmMSD1 and ZmMSD2, while only three proteins (ZmCSD3, ZmCSD4 and ZmFSD4) were localized in chloroplasts. In addition, four proteins, including ZmCSD1, ZmCSD2, ZmCSD5, and ZmCSD6, were localized in cytoplasm (Table 1).

Phylogenetic relationships and gene structure

The full-length ZmSOD sequences were aligned and used to construct an unrooted phylogenetic tree to analyze their phylogenetic relationships. The result indicated that the 13 ZmSODs could be divided into two groups (I-II) with high bootstrap value support, indicating their conserved phylogenetic relationships (Figure 1A). To further support the phylogenetic relationships of the ZmSODs, gene structure analysis was performed for the 13 *ZmSODs* using GSDS online tool (Figure 1B). We found that intron numbers in the genomic sequences of the *ZmSODs* ranged from 4 to 7. Three *ZmSODs* (*ZmCSD3*, *ZmCSD5* and *ZmFSD4*), contained seven introns, while *ZmFSD1* and *ZmMSD1* contained four introns. According to the phylogenetic tree and gene structure analysis, we found that the gene pair *ZmCSD2-ZmCSD6* exhibited a highly similar exon-intron organization pattern, suggesting their close relationship.

To further investigate the phylogenetic relationships of SOD proteins in dicot and monocot plants, a phylogenetic tree was constructed based on an alignments of 37 full-length protein sequences, including 13 sequences from maize, 8 from foxtail millet (SiCSD1, SiCSD2, SiCSD3, SiCSD4, SiFSD1, SiFSD2, SiFSD3 and SiMSD), 8 from rice (cCuZn-SOD1, cCuZn-SOD2, CuZn-SOD-L, pCuZn-SOD, CuZn-SOD-CCh, Fe-SOD3, Fe-SOD2 and Mn-SOD1), and 8 from Arabidopsis

Table 1 – Sequence characteristics of the 13 *SOD* genes identified in maize.

| Gene name	Sequence ID	Protein length (aa)	MW (kDa)	pI	Subcellular predicted
ZmCSD1	Zm00001d028232_T004	163	16.82	6.23	Cytoplasm
ZmCSD2	Zm00001d029170_T003	152	15.17	5.83	Cytoplasm
ZmCSD3	Zm00001d031908_T001	206	20.90	5.45	Chloroplast
ZmCSD4	Zm00001d002611_T002	308	32.11	5.33	Chloroplast
ZmCSD5	Zm00001d022505_T001	233	24.92	5.91	Cytoplasm
ZmCSD6	Zm00001d047479_T003	152	15.07	5.64	Cytoplasm
ZmFSD1	Zm00001d014632_T001	154	17.80	6.58	Mitochondrion
ZmFSD2	Zm00001d036135_T003	284	32.43	8.84	Mitochondrion
ZmFSD3	Zm00001d045384_T003	201	23.02	7.14	Mitochondrion
ZmFSD4	Zm00001d045538_T001	386	42.87	5.63	Chloroplast
ZmFSD5	Zm00001d025106_T003	186	21.50	6.65	Mitochondrion
ZmMSD1	Zm00001d037859_T001	213	22.84	6.71	Mitochondrion
ZmMSD2	Zm00001d009990_T001	243	26.44	6.71	Mitochondrion
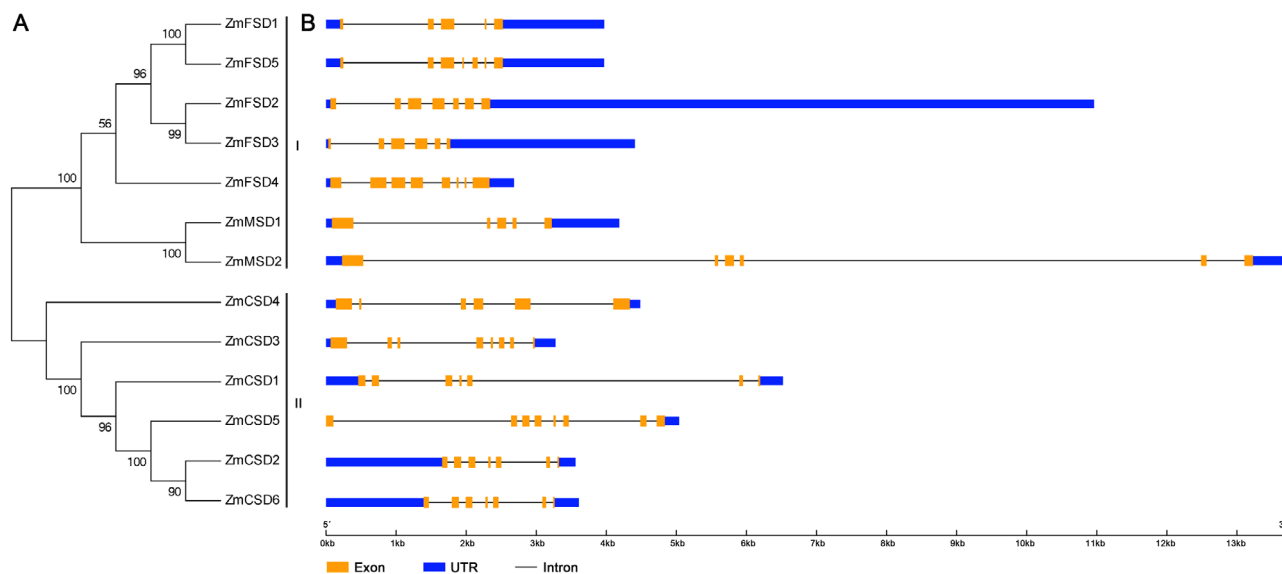
Figure 1 – Phylogenetic relationships and gene structures of maize SOD proteins. A. Unrooted tree of the 13 ZmSODs. The tree was constructed with 1,000 bootstrap replicates by the neighbor-joining method using the MEGA5.05 software. B. Exon-intron structures of the *ZmSOD*s.

(AtCSD1, AtCSD2, AtCSD3, AtFSD1, AtFSD2, AtFSD3, AtMSD1 and AtMSD2). According to the phylogenetic tree, the 37 SOD proteins could be divided into two groups: group I (Fe/MnSODs) and group II (Cu/ZnSODs), which was consistent with the types of domains they contained (Figure 2). Group I contained 19 SOD proteins, which could be further divided into three subgroups (a-c). In group II, 18 SOD proteins were divided into four subgroups (d-g). We found that most SOD proteins clustered in the same subgroups shared the same predicted subcellular localization (Table 1). For example, ZmFSD1, ZmFSD2, ZmFSD3, ZmFSD5 and other plants' mitochondrial FeSODs formed subgroup a. ZmCSD2, ZmCSD5 and ZmCSD6 were contained in subgroup g, and these ZmSODs and other plant SOD proteins were predicted to be localized in the cytoplasm. In each subgroup, we found that the ZmSODs exhibited closer relationships with foxtail millet or rice members than those of Arabidopsis. We also constructed a maximum likelihood (ML) tree with the same SOD protein sequences using MEGA 5.05 software, and the results were largely consistent with the phylogenetic relationships in the NJ tree (Figure S1), which further supported the reconstruction of the NJ tree.

Conserved motifs, chromosomal distributions and gene duplications

MEME was used to investigate the conserved motifs among ZmSODs, and 10 motifs were identified (Table S2). For the Cu/ZnSOD proteins, motifs 2 and 3 encoding the Cu/ZnSOD domain (PF00080) were detected in each of the six ZmCSDs except ZmCSD4 (Figure 3). Motif 1 encoding a Fe/MnSOD domain (N-terminal domain, PF00081) was found in each of the Fe/MnSOD proteins, while Motif 5 encodes a Fe/MnSOD domain (C-terminal domain, PF02777), was detected in ZmFSD1, ZmFSD2 and ZmFSD5. Notably, ZmSODs in the same phylogenetic group tended to have similar motif distribution patterns, which further supported the phylogenetic classification. The chromosomal locations of the

*ZmSOD*s were obtained from the maize genome database. As shown in Figure 4A, eight of the 10 chromosomes harbored *ZmSOD*s; no *ZmSOD* genes were found on chromosomes 3 or 4. Most of the genes were distributed on chromosomes 1, 6 and 9, while chromosomes 2, 5, 7, 8 and 10 each contained only one *SOD* gene. Gene duplications, including tandem and segmental duplications, were investigated to explore the potential expansion mechanism of *ZmSOD* family. According to the syntenic analysis, five genes (*ZmCSD2*, *ZmCSD5*, *ZmCSD6*, *ZmFSD2* and *ZmFSD3*) were involved in the segmental duplication, and no tandem duplications were detected in *ZmSOD* gene family (Figure 4B).

Promoter analysis of *ZmSOD*s

Increasing evidence indicates that *SOD* genes play important roles in responses to abiotic stresses. To explore the possible regulatory mechanisms of *ZmSOD*s involved in stress responses, the putative stress-responsive *cis*-elements were investigated in the promoter sequences of the *ZmSOD*s. Four *cis*-elements, including the abscisic acid responsiveness element (ABRE), dehydration-responsive element (DRE), MYB binding site involved in drought-inducibility (MBS) and low temperature-responsive element (LTR), were detected in this study. The number of detected *cis*-elements in the 13 promoter regions ranged from 2 to 10 (Figure 5). Each of the *ZmFSD5* and *ZmCSD5* promoters had 10 *cis*-elements, respectively, while *ZmCSD4* had the least number (2). The distributions of *cis*-elements in the *ZmSOD* promoters showed significant differences, which might suggest the different roles or regulatory mechanisms of *ZmSOD*s in responses to abiotic stresses.

Gene Ontology (GO) enrichment analysis of *ZmSOD*s

To explore the possible functional roles of the *ZmSOD*s, GO terms for these genes were annotated using the clusterProfiler R package (Yu *et al.,* 2012), and divided into three categories, including cellular component, molecular function, and biological
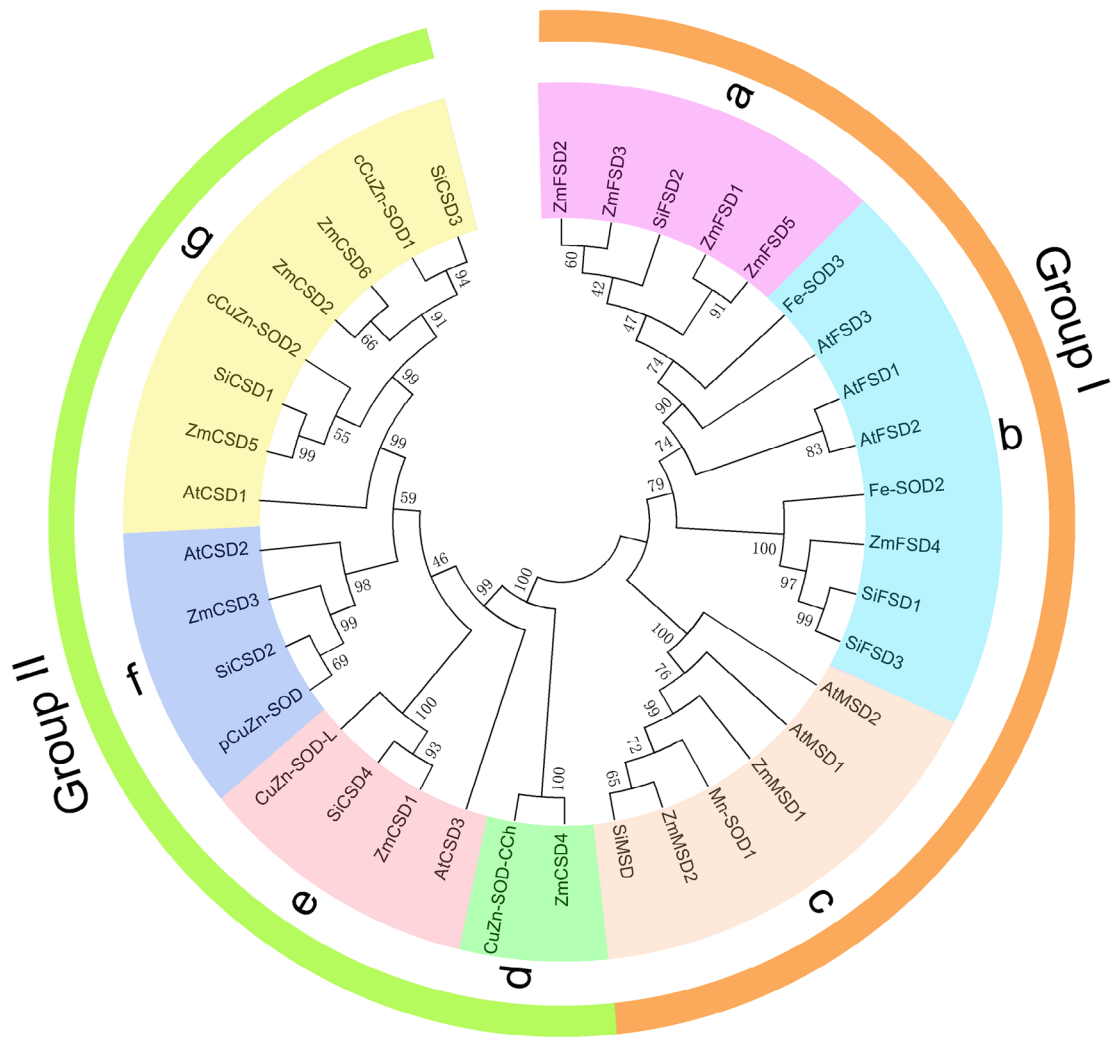
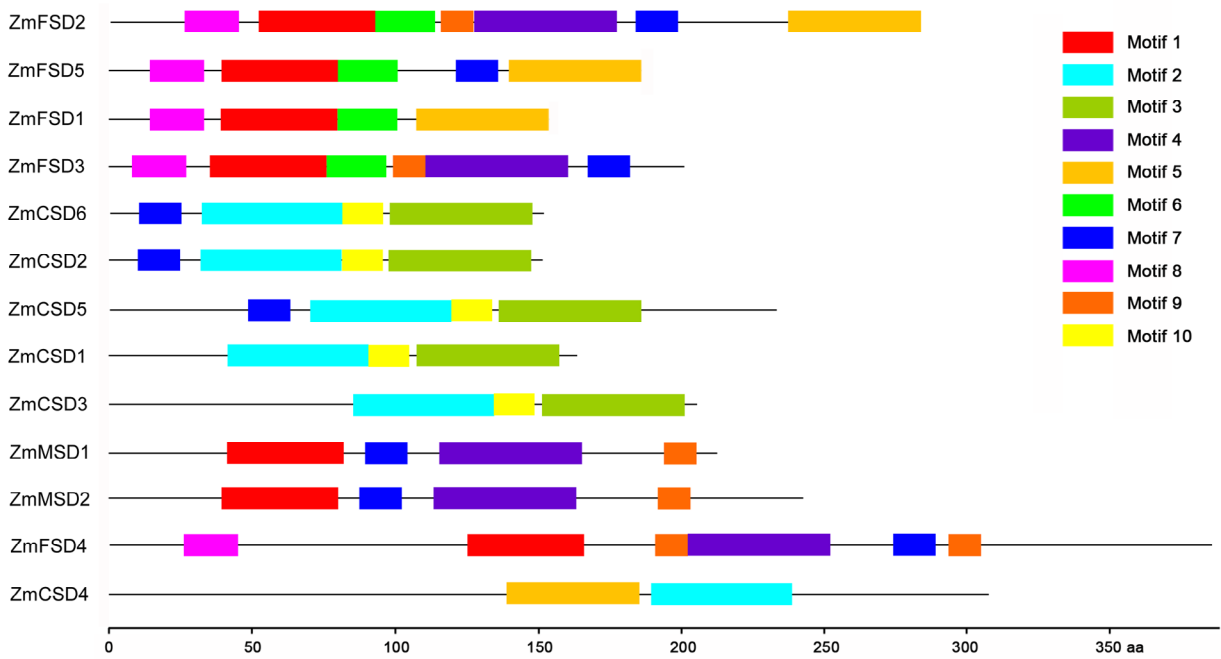**Figure 2** – Phylogenetic relationships of 37 SOD proteins from different plant species.

Figure 3 – Conserved motif analysis of ZmSOD proteins.
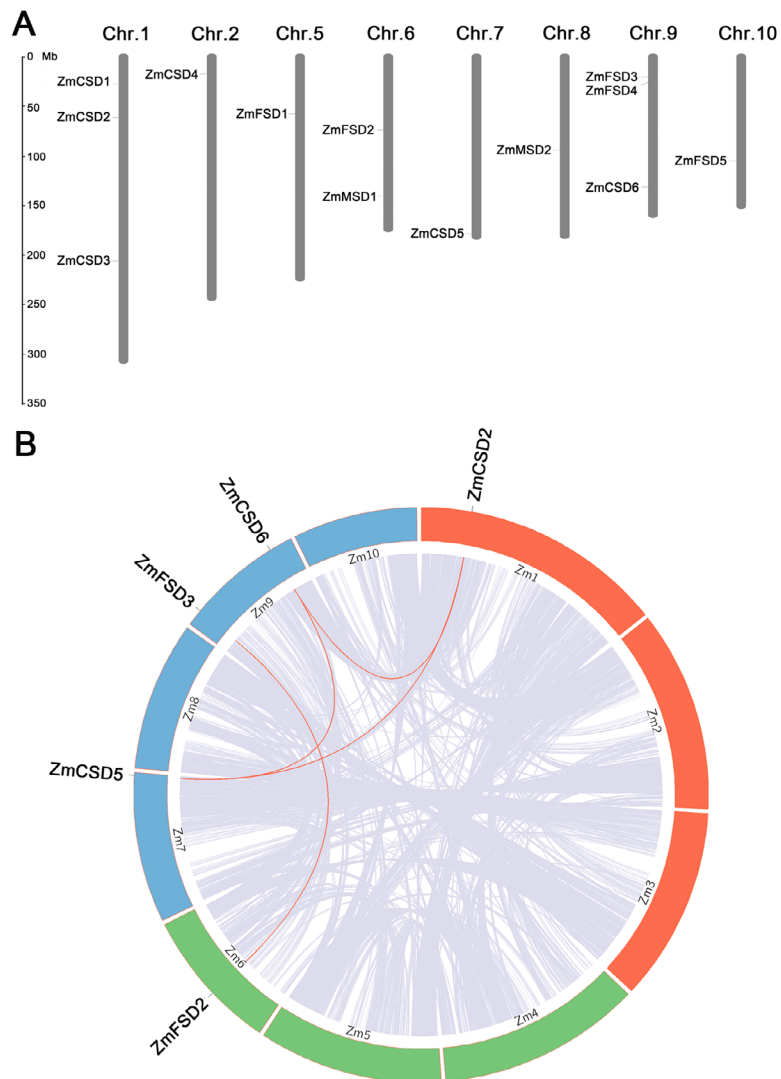
Figure 4 – Chromosomal locations and syntenic analysis of the *ZmSOD* genes.A. Chromosomal locations of the 13 *ZmSODs*. B. Synteny and gene duplication analysis among *ZmSODs* in the maize genome.
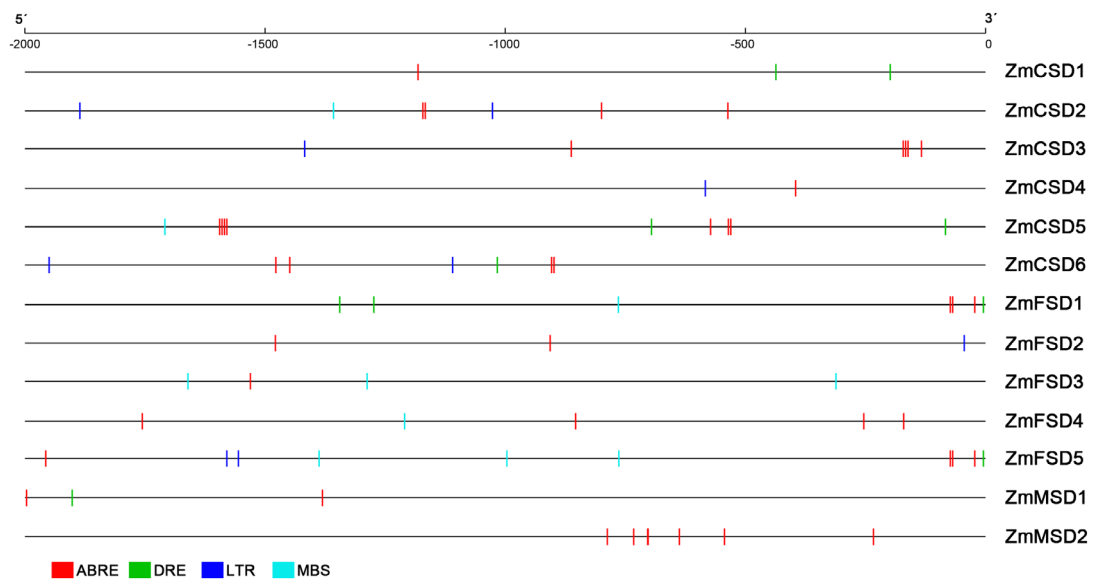

Figure 5 – *Cis*-elements in the promoter regions of the *ZmSOD* genes. Four types of putative stress-responsive *cis*-elements, including ABRE, DRE, LTR and MBS, were shown with different colors.

process (Figure 6). The results indicated that the *ZmSODs* were significantly (adjusted *p*-value < 0.05) enriched in 17 GO terms. Six GO terms, including extracellular space (GO:0005615), chloroplast nucleoid (GO:0042644), extracellular region (GO:0005576), mitochondrial matrix (GO:0005759), thylakoid (GO:0009579) and peroxisome (GO:0005777), were enriched in the cellular component category. Three GO terms, including superoxide dismutase activity (GO:0004784), copper ion binding (GO:0005507), and manganese ion binding (GO:0030145), were enriched in the molecular function category. Eight GO terms, including response to reactive oxygen species (GO:0000302), response to hydrogen peroxide (GO:0042542), response to iron ion (GO:0010039), response to osmotic stress (GO:0006970), protein homotetramerization (GO:0051289), response to herbicide (GO:0009635), metal ion transport (GO:0030001) and response to abscisic acid (GO:0009737), were enriched in the biological process category. We noted that the highest number of *ZmSODs* (12) was enriched in the "superoxide dismutase activity" molecular function term, three genes were enriched in the "response to reactive oxygen species" biological process term, and one gene was enriched in the "response to osmotic stress" biological process terms. These results suggested that *ZmSODs* have significant roles in the responses to abiotic stress.

Expression patterns of *ZmSOD*s at different developmental stages

To explore the possible functions of the *ZmSOD*s, the expression patterns of the 13 *ZmSOD*s were analyzed in 23 different tissues and organs using publicly available transcript data (Walley *et al.,* 2016). All the 13 *ZmSODs* showed detectable expression levels in most of the 23 tissues and developmental stages (Figure 7) with different expression patterns. According to their expression levels, the 13 *ZmSODs* could be divided into two groups. The first group included 7 members (*ZmFSD3*, *ZmCSD1*, *ZmCSD4*, *ZmFSD2*, *ZmFSD4*, *ZmFSD1* and *ZmFSD5*) with low expression, while the second group exhibited relatively higher expression, including *ZmCSD3*, *ZmMSD2*, *ZmCSD5*, *ZmMSD1*, *ZmCSD2* and *ZmCSD6*. We noted that *ZmMSD* genes had high expression levels in all stages except B73 mature pollen while *ZmFSD* genes had low expression levels in most stages. In addition, some *SOD* genes showed similar expression patterns that reflected their close relationships, especially for two pairs of genes (*ZmCSD2* and *ZmCSD6*, and *ZmFSD1* and *ZmFSD5*), which might suggest their similar functions in plant growth and development.

Expression analysis of *ZmSOD*s under drought and salt treatments

The expression levels of the *ZmSOD*s were investigated under drought and NaCl treatments using qRT-PCR to understand their possible roles in responses to abiotic stresses. Under drought stress, expression levels of *ZmCSD2*, *ZmCSD5* and *ZmMSD2* were significantly up-regulated with large fold changes at the 6, 12 and 24 h, respectively, while *ZmFSD2* was significantly down-regulated at three of the four time points (Figure 8A). Notably, some *ZmSODs* exhibited significant differences across the four time points. For example, *ZmCSD3* expression was significantly up-regulated at 3 h, but

significantly down-regulated expression was observed at 6, 12 and 24 h. *ZmFSD4* expression was significantly up-regulated at 12 h, but significantly down-regulated was shown at 24 h after drought treatment. In addition, some *ZmSODs* exhibited significant up- or down-regulated expression only at particular time points, such as *ZmCSD1* and *ZmFSD5*. Under salt stress, we found that expression levels of *ZmCSD1*, *ZmCSD2*, *ZmCSD4*, *ZmCSD5*, *ZmMSD1* and *ZmMSD2* were significantly up-regulated at all of the four time points (Figure 8B). As observed under drought stress, significant expression differences were observed for some *ZmSODs* across the four time points, for example, *ZmFSD3* exhibited significantly up-regulated expression at 12 and 24 h, and significantly down-regulated expression was observed at 3 h. In addition, significantly up-regulated expression was only observed at particular time points for *ZmCSD3* and *ZmFSD2*. Under salt treatment, we found that the expression levels of most *ZmSODs* exhibited larger fold changes than that observed under drought stress. These findings suggested the important roles of *ZmSODs* in responses to drought or salt stress, but may have different regulatory mechanisms.

Discussion

Environmental stresses, such as drought, heat and salinity, have serious effects on plant growth and development. Studies have indicated that ROS accumulation can causes oxidative stress because the equilibrium of oxidative reactions is disrupted by abiotic stresses (Apel and Hirt, 2004; Quan *et al.,* 2008). Toxic ROS can greatly harm the survival of plants, such as through inactivation of enzymes and membrane lipids (Apel and Hirt, 2004). SOD is one of the antioxidant enzymes that have been demonstrated to have important roles in protecting plant cells from oxidative damage (Fink and Scandalis, 2002). *SOD* family genes have been studied in several plant species, and identification of the maize *SOD* genes and determination of their functional roles in responses to drought and salt stresses will provide excellent gene resources for resistance breeding to various abiotic stresses.

Studies indicated that the number of *SOD* family genes has a large difference among different plant species. For example, 8 *SOD* genes were identified in both of rice and foxtail millet, while 26 members were identified from the whole genome of wheat. A total of 13 *SOD* genes were identified in the current maize genome, and further divided into two major types. Although there are large differences in the genome sizes of different plant species, the number of *SOD* family genes is not proportional to the genome size. It is believed that gene duplications, including tandem and segmental duplications, have important roles in the expansion of plant gene families (Cannon *et al.,* 2004). The different numbers of *SOD* family genes should be mainly attributable to the ratio of gene duplications. Our study indicated that five *ZmSODs* were involved in the segmental duplication, which suggests that segmental duplication plays an important role in the expansion of maize *SOD* gene family.

Phylogenetic analysis indicated that the 37 SOD proteins from four species were divided into two groups, termed group I (Fe/MnSODs) and group II (Cu/ZnSODs). As observed in other studies, FeSODs and MnSODs from different plants were
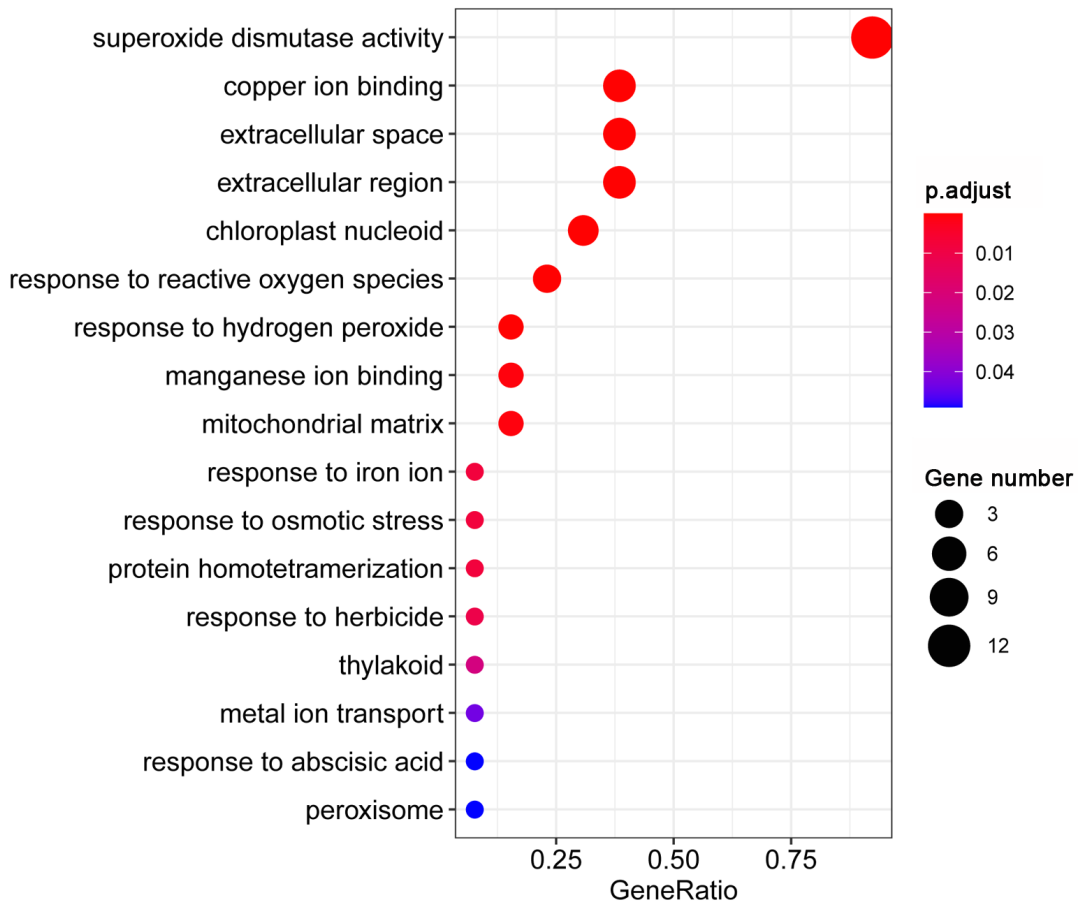
Figure 6 – Gene Ontology (GO) enrichment analysis of the *ZmSOD* genes.
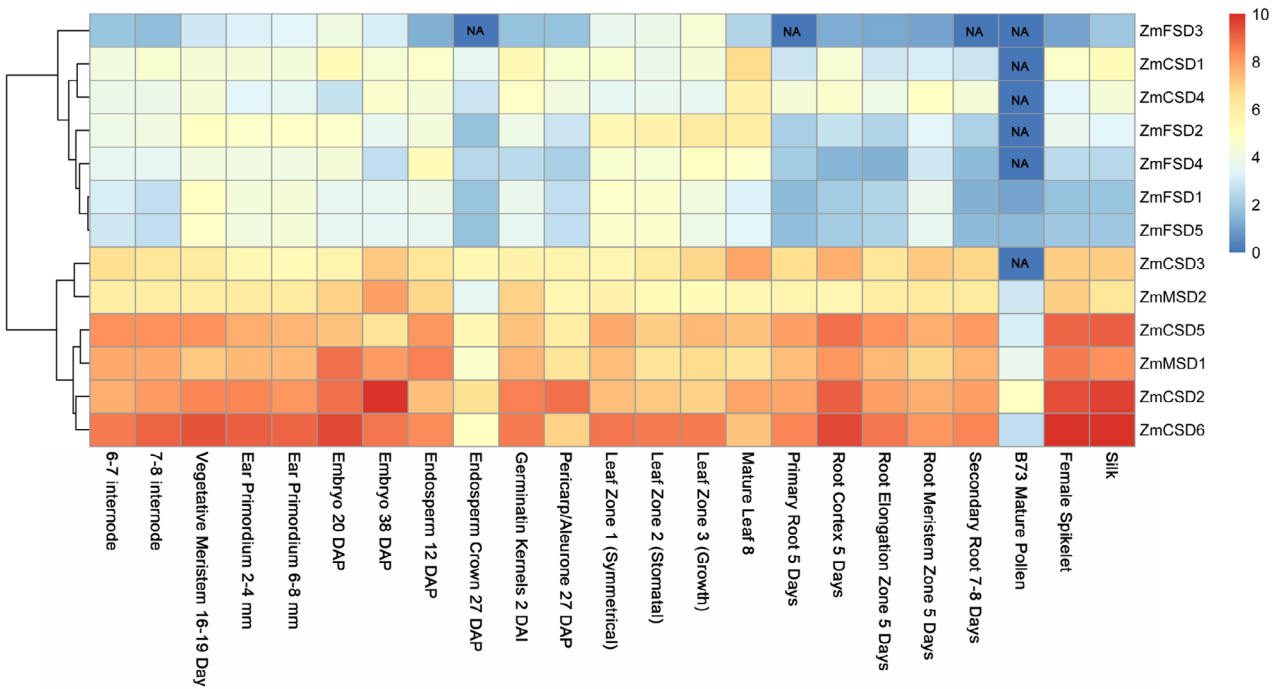

Figure 7 – Expression pattern analysis of the *ZmSOD* genes in different tissues and organs using transcript data. Expression patterns of the 13 *ZmSODs* were investigated in 23 different tissues and organs using publicly transcriptome data. FPKM showed "NA" (not available) was replaced by FPKM = 0, and all FPKM values were transformed into $\log_2$ (FPKM+1) to create the heatmap.
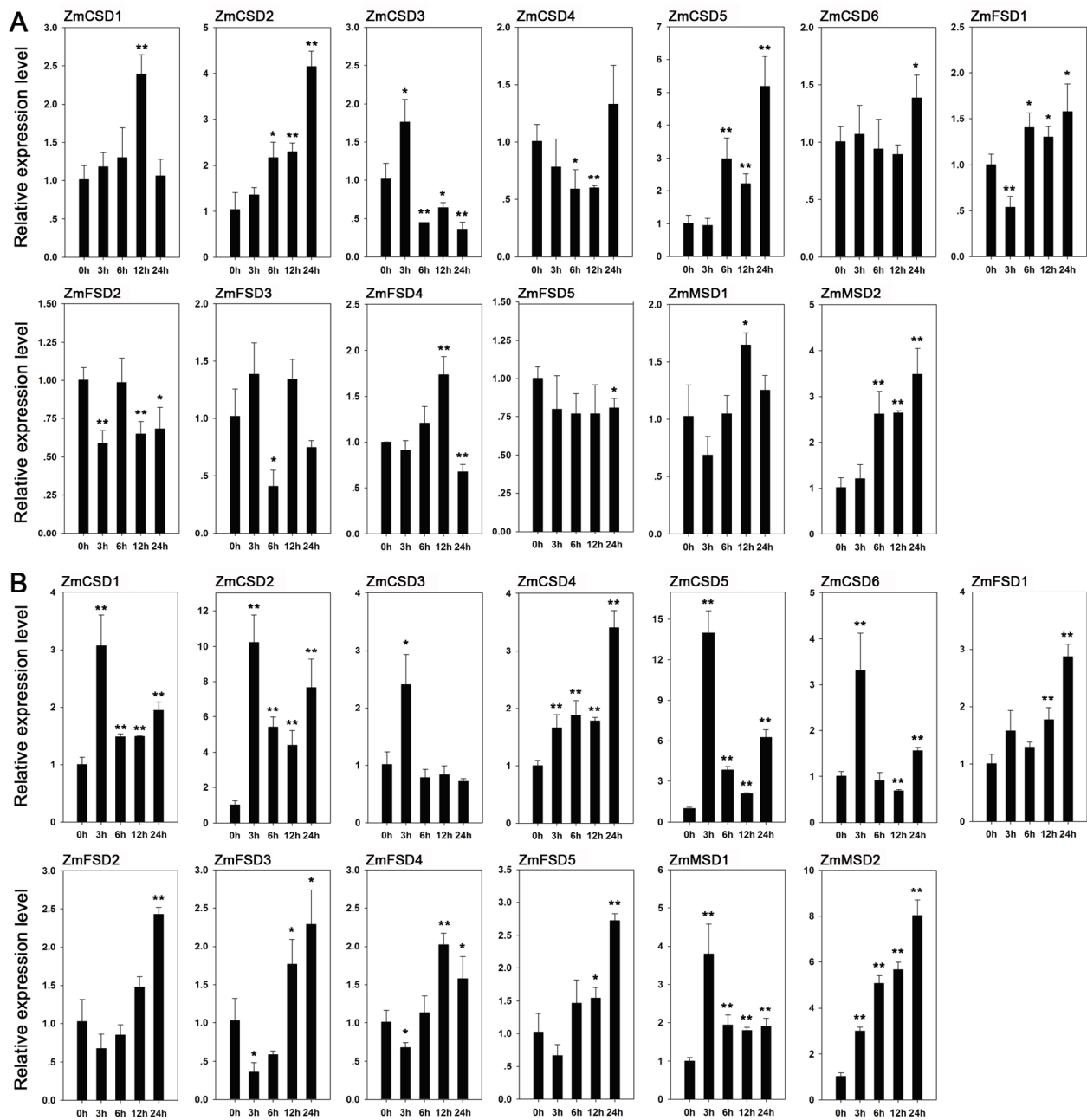
Figure 8 – Expression pattern analysis of *ZmSOD* genes under drought (A) and salt (B) treatments by qRT-PCR. The X-axis is the time course of treatment, and seedlings were sampled at 0 (CK), 3, 6, 12 and 24 h after drought or salt treatment, respectively. The Y-axis shows the relative expression levels.

clustered together in group I with high bootstrap value support, suggesting that these genes may share common ancestral genes (Wang *et al.,* 2017; Song *et al.,* 2018). SODs from monocots (maize, foxtail millet, and rice) and dicots (Arabidopsis) tended to cluster separately, which was consistent with the evolutionary relationships of these species. The findings also suggested the independent evolution of plant *SOD* genes after the divergence of monocots and dicots. Interestingly, some maize SODs exhibited close phylogenetic relationships with their orthologs from other species than their paralog proteins, suggesting that the ortholog pairs may have originated from a common ancestor and diverged after the divergence of the grass genome. Our study was consistent with previous studies that *SOD* genes clustered in one phylogenetic branch tended

to have the same subcellular localization (Song *et al.,* 2018; Wang *et al.,* 2018b), further supporting the phylogenetic reconstruction and the conserved evolution of plant *SOD* genes.

Cis-elements in gene promoters are important for transcriptional regulation (Hernandez-Garcia and Finer, 2014). Therefore, stress-responsive *cis*-elements, including ABRE, DRE, MBS and LTR, were investigated in the promoters of the 13 *ZmSODs*. Previous studies indicated these stress-responsive *cis*-elements have important roles in regulating abiotic stress responses. For example, expression of *RD29A* was induced by drought, salt, ABA and low-temperature, both of DRE and ABRE elements were found in its promoter (Shinozaki and Yamaguchi-Shinozaki, 2000; Narusaka *et al.,* 2003). In maize, mutant analysis indicated that MBS element in *ZmSO*

promoter was important for ABA- and drought-induced expression (Xu *et al.,* 2019). In addition, LTR was shown to be involved in regulation of cold responsive, and it was also found in some drought or salt responsive gene promoters (Wang *et al.,* 2018a; Xie *et al.,* 2019). We found that at least one type of the four stress-responsive *cis*-elements was detected in each of the *ZmSODs*, suggesting the important roles of the *ZmSODs* in abiotic stress responses. However, different distribution patterns were found among the 13 *ZmSODs*, even for the gene pair *ZmCSD2-ZmCSD6*. These findings may indicate different regulatory mechanisms of the *ZmSODs* in response to abiotic stress.

Plant growth and development is frequently threatened by various environmental stresses (Zhu, 2002; Gong *et al.,* 2020; Lozano-Juste *et al.,* 2020). At present, the expression patterns of *SOD* genes in response to abiotic stress have been investigated in many species, and the functional studies of some genes involved in stress response have also been demonstrated by biological experiments (Zhang *et al.,* 2014; Wang *et al.,* 2016). According to the expression levels of *ZmSODs* under stress treatments, we found that most of the *ZmSODs* showed significant response to drought or salt stress treatment. Meanwhile, the significant expression differences among the 13 *ZmSODs* were also found. Some members exhibited similar expression patterns under different stresses. For example, significantly up-regulated expression of *ZmCSD2* and *ZmMSD2* was detected under both of drought and salt treatments, suggesting their conserved functional roles of these genes in response to abiotic stresses. However, different expression patterns between drought and salt treatments were also found. For example, *ZmCSD4* exhibited significantly down-regulated expression at 6 and 12 h under drought stress, but significantly up-regulated expression was detected across the four time points under salt stress. In foxtail millet, the expression of *SiMSD* was significantly induced by drought and salt stresses, respectively (Wang *et al.,*2018b). *ZmMSD2* had a close phylogenetic relationship with *SiMSD*, and also exhibited significantly up-regulated expression under drought and salt stresses, which may indicate their conserved functions in stress responses. Importantly, we should note that some *ZmSODs* were up-regulated with a large fold changes under drought or salt treatment, such as *ZmCSD2*, *ZmCSD5* and *ZmMSD2*. Our study provided an important foundation for the selection of important functional genes and application in stress resistance breeding in maize.

Conclusions

In this study, 13 maize *SOD* genes were identified using the BLASTP program and systematic bioinformatics analysis was performed for these members. The 13 *ZmSODs* were distributed on 8 of the 10 maize chromosomes, and five members were involved in segmental duplication, suggesting that segmental duplication plays an important role in the expansion of maize *SOD* gene family. SOD proteins from maize and three other plants members were divided into two groups (Fe/MnSODs and Cu/ZnSODs) according to phylogenetic analysis, which can be further classified into different subgroups. At least one of the four detect stress-responsive *cis*-elements was identified in each of the *ZmSODs*. Transcriptome data analysis showed that *ZmSODs* were expressed in most of the detected tissues and organs. Furthermore, qRT-PCR analysis indicated that most of the *ZmSODs* were responsive to drought or salt stress treatments, especially some genes with significant expression changes were identified. Our results lay an important foundation for further identifying important members and investigating the molecular functions of *ZmSODs* involved in abiotic stress responses.

Acknowledgements

Conflict of Interest

The authors declare that they have no competing interests.

Author Contributions

JL, LJX and YZ conceived and designed the research; JL, JS, HTY, HYW and WBL performed the bioinformatics analysis; JL, LJX and XLH conducted the gene expression experiments; JL, LJX and YZ wrote the manuscript. All authors read and approved the final manuscript.

References

Alscher RG, Erturk N and Heath LS (2002) Role of superoxide dismutases (SODs) in controlling oxidative stress in plants. J Exp Bot 53:1331-1341.

Apel K and Hirt H (2004) Reactive oxygen species: Metabolism, oxidative stress, and signal transduction. Annu Rev Plant Biol 55:373-399.

Asensio AC, Gil-Monreal M, Pires L, Gogorcena Y, Aparicio-Tejo PM and Moran JF (2012) Two Fe-superoxide dismutase families respond differently to stress and senescence in legumes. J Plant Physiol 169:1253-1260.

Bailey TL, Boden M, Buske FA, Frith M, Grant CE, Clementi L, Ren JY, Li WW and Noble WS (2009) MEME SUITE: Tools for motif discovery and searching. Nucleic Acids Res 37:W202-W208.

Cannon RE, White JA and Scandalios JG (1987) Cloning of cDNA for maize superoxide dismutase 2 (SOD2). Proc Natl Acad Sci U S A 84:179-183.

Cannon SB, Mitra A, Baumgarten A, Young ND and May G (2004) The roles of segmental and tandem gene duplication in the evolution of large gene families in *Arabidopsis thaliana*. BMC Plant Biol 4:10.

Feng K, Yu JH, Cheng Y, Ruan MY, Wang R, Ye Q, Zhou G, Li Z, Yao Z, Yang Y *et al.* (2016) The SOD gene family in tomato: Identification, phylogenetic relationships, and expression patterns. Front Plant Sci 7:1279.

Filiz E and Tombuloglu H (2015) Genome-wide distribution of superoxide dismutase (SOD) gene families in *Sorghum bicolor*. Turk J Biol 39:49-59.

Fink RC and Scandalis JG (2002) Molecular evolution and structure-function relationships of the superoxide dismutase gene families in angiosperms and their relationship to other eukaryotic and prokaryotic superoxide dismutases. Arch Biochem Biophys 399:19-36.

Finn RD, Mistry J, Schuster-Böckler B, Griffiths-Jones S, Hollich V, Lassmann T, Moxon S, Marshall M, Khanna A, Durbin R *et al.* (2006) Pfam: Clans, web tools and services. Nucleic Acids Res 34:D247-D251.

Forman HJ and Fridovich I (1973) On the stability of bovine superoxide dismutase. The effects of metals. J Biol Chem 248:2645-2649.

Gechev TS, Breusegem FV, Stone JM, Denev I and Laloi C (2006) Reactive oxygen species as signals that modulate plant stress responses and programmed cell death. Bioessays 28:1091-1101.

Gong Z, Xiong L, Shi H, Yang S, Herrera-Estrella LR, Xu G, Chao D-Y, Li J, Wang P-Y, Qin F et al. (2020) Plant abiotic stress response and nutrient use efficiency. Sci China Life Sci 63:635-674.

Gopavajhula VR, Chaitanya KV, Khan PAA, Shaik JP, Reddy PN and Alanazi M (2013) Modeling and analysis of soybean (Glycine max. L) Cu/Zn, Mn and Fe superoxide dismutases. Genet Mol Biol 36:225-236.

Hernandez-Garcia CM and Finer JJ (2014) Identification and validation of promoters and cis-acting regulatory elements. Plant Sci 217-218:109-119.

Horton P, Park K-J, Obayashi T, Fujita N, Harada H, Adams-Collier CJ and Nakai K (2007) WoLF PSORT: Protein localization predictor. Nucleic Acids Res 35:W585-W587.

Hu B, Jin J, Guo A-Y, Zhang H, Luo J and Gao G (2015) GSDS2.0: An upgraded gene feature visualization server. Bioinformatics 31:1296-1297.

Jiang W, Yang L, He Y, Zhang H, Li W, Chen H, Ma D and Yin J (2019) Genome-wide identification and transcriptional expression analysis of superoxide dismutase (SOD) family in wheat (Triticum aestivum). PeerJ 7:e8062.

Karuppanapandian T, Moon J-C, Kim C, Manoharan K and Kim W (2011) Reactive oxygen species in plants: Their generation, signal transduction, and scavenging mechanisms. Aust J Crop Sci 5:709-725.

Kliebenstein DJ, Monde RA and Last RL (1998) Superoxide dismutase in Arabidopsis: An eclectic enzyme family with disparate regulation and protein localization. Plant Physiol 118:637-650.

Lee S-H, Ahsan N, Lee K-W, Kim D-H, Lee D-G, Kwak S-S, Kwon S-Y, Kim T-H and Lee B-H (2007) Simultaneous overexpression of both CuZn superoxide dismutase and ascorbate peroxidase in transgenic tall fescue plants confers increased tolerance to a wide range of abiotic stresses. J Plant Physiol 164:1626-1638.

Lescot M, Déhais P, Thijs G, Marchal K, Moreau Y, Van de Peer Y, Rouze P and Rombauts S (2002) PlantCARE, a database of plant cis-acting regulatory elements and a portal to tools for in silico analysis of promoter sequences. Nucleic Acids Res 30:325-327.

Lozano-Juste J, Alrefaei AF and Rodriguez PL (2020) Plant osmotic stress signaling: MAPKKKs meet SnRK2s. Trends Plant Sci 25:1179-1182.

Mittler R (2002) Oxidative stress, antioxidants and stress tolerance. Trends Plant Sci 7:405-410.

Mittler R, Vanderauwera S, Gollery M and Van Breusegem F (2004) Reactive oxygen gene network of plants. Trends Plant Sci 9:490-498.

Narusaka Y, Nakashima K, Shinwari ZK, Sakuma Y, Furihata T, Abe H, Narusaka M, Shinozaki K and Yamaguchi-Shinozaki K (2003) Interaction between two cis-acting elements, ABRE and DRE, in ABA-dependent expression of Arabidopsis rd29A gene in response to dehydration and high-salinity stresses. Plant J 34:137-148.

Nath K, Kumar S, Poudyal RS, Yang YN, Timilsina R, Park YS, Nath J, Chauhan PS, Pant B and Lee C-H (2014) Developmental stage-dependent differential gene expression of superoxide dismutase isoenzymes and their localization and physical interaction network in rice (Oryza sativa L.). Genes Genom 36:45-55.

Pilon M, Ravet K and Tapken W (2011) The biogenesis and physiological function of chloroplast superoxide dismutases. Biochim Biophys Acta 1807:989-998.

Quan L-J, Zhang B, Shi W-W and Li H-Y (2008) Hydrogen peroxide in plants: A versatile molecule of the reactive oxygen species network. J Integr Plant Biol 50:2-18.

Shinozaki K and Yamaguchi-Shinozaki K (2000) Molecular responses to dehydration and low temperature: Differences and cross-talk between two stress signaling pathways. Curr Opin Plant Biol 3:217-223.

Si WN, Hang T, Guo M, Chen Z, Liang Q, Gu L and Ding T (2019) Whole-genome and transposed duplication contributes to the expansion and diversification of TLC genes in maize. Int J Mol Sci 20:5484.

Song J, Zeng L, Chen R, Wang Y and Zhou Y (2018) In silico identification and expression analysis of superoxide dismutase (SOD) gene family in Medicago truncatula. 3 Biotech 8:348.

Sugimoto M, Oono Y, Gusev O, Matsumoto T, Yazawa T, Levinskikh MA, Sychev VN, Bingham GE, Wheeler R and Hummerick M (2014) Genome-wide expression analysis of reactive oxygen species gene network in Mizuna plants grown in long-term spaceflight. BMC Plant Biol 14:4.

Sytykiewicz H (2014) Differential expression of superoxide dismutase genes in aphid-stressed maize (Zea mays L.) seedlings. PLoS One 9:e94847.

Thompson JD, Higgins DG and Gibson TJ (1994) CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673-4680.

Verma D, Lakhanpal N and Singh K (2019) Genome-wide identification and characterization of abiotic-stress responsive SOD (superoxide dismutase) gene family in Brassica juncea and B. rapa. BMC Genomics 20:227.

Walley JW, Sartor RC, Shen ZX, Schmitz RJ, Wu KJ, Urich MA, Nery JR, Smith LG, Schnable JC, Ecker JR et al. (2016) Integration of omic networks in a developmental atlas of maize. Science 353:814-818.

Wang B, Lüttge U and Ratajczak R (2004) Specific regulation of SOD isoforms by NaCl and osmotic stress in leaves of the C3 halophyte Suaeda salsa L. J Plant Physiol 161:285-293.

Wang C-T, Ru J-N, Liu Y-W, Li M, Zhao D, Yang J-F, Fu J-D and Xu Z-S (2018a) Maize WRKY transcription factor ZmWRKY106 confers drought and heat tolerance in transgenic plants. Int J Mol Sci 19:3046.

Wang M, Zhao X, Xiao Z, Yin X, Xing T and Xia G (2016) A wheat superoxide dismutase gene TaSOD2 enhances salt resistance through modulating redox homeostasis by promoting NADPH oxidase activity. Plant Mol Biol 91:115-130.

Wang T, Song H, Zhang B, Lu Q, Liu Z, Zhang S, Guo R, Wang C, Zhao Z, Liu J et al. (2018b) Genome-wide identification, characterization, and expression analysis of superoxide dismutase (SOD) genes in foxtail millet (Setaria italica L.). 3 Biotech 8:486.

Wang W, Zhang X, Deng F, Yuan R and Shen F (2017) Genome-wide characterization and expression analyses of superoxide dismutase (SOD) genes in Gossypium hirsutum. BMC Genomics 18:376.

Xie Z, Nolan TM, Jiang H and Yin Y (2019) AP2/ERF transcription factor regulatory networks in hormone and abiotic stress responses in Arabidopsis. Front Plant Sci 10:228.

Xu Z, Wang M, Guo Z, Zhu X and Xia Z (2019) Identification of a 119-bp promoter of the maize sulfite oxidase gene (ZmSO) that confers high-level gene expression and ABA or drought inducibility in transgenic plants. Int J Mol Sci 20:3326.

Yu G, Wang L-G, Han Y and He Q-Y (2012) ClusterProfiler: An R package for comparing biological themes among gene clusters. OMICS 16:284-287.

Zhang D-Y, Yang H, Li X-S, Li H-Y and Wang Y-C (2014) Overexpression of *Tamarix albiflonum* TaMnSOD increases drought tolerance in transgenic cotton. Mol Breeding 34:1-11.

Zhao Y, Hu F, Zhang X, Wei Q, Dong J, Bo C, Cheng B and Ma Q (2019) Comparative transcriptome analysis reveals important roles of nonadditive genes in maize hybrid An'nong 591 under heat stress. BMC Plant Biol 19:273.

Zhu D and Scandalios JG (1994) Differential accumulation of manganese-superoxide dismutase transcripts in maize in response to abscisic acid and high osmoticum. Plant Physiol 106:173-178.

Zhu J-K (2002) Salt and drought stress signal transduction in plants. Annu Rev Plant Biol 53:247-273.

Supplementary Material

The following online material is available for this article:

Figure S1 – Maximum likelihood phylogenetic tree of 37 SOD proteins from different plant species. The Maximum Likelihood (ML) tree was constructed.

Table S1 – Gene-specific primers used for qRT-PCR in this study.

Table S2 – Conserved motif sequences of maize SOD proteins.