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RESEARCH ARTICLE

SUPEROXIDE DISMUTASE FAMILY GENES IN WATERMELON AND THEIR RESPONSES TO DIFFERENT ABIOTIC STRESSES

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KEYWORDS

abiotic stress, expression analysis, phylogeny, SOD, superoxide dismutase, watermelon

HIGHLIGHTS

- A total of 8 *SOD* genes from watermelon were identified and bioinformatically analyzed.
- The SOD proteins from watermelon and other different plant species can be classified into five groups consistent with their metal cofactors.
- *ClSOD* genes exhibited distinctive tissue-specific and abiotic stress responsive expression patterns.

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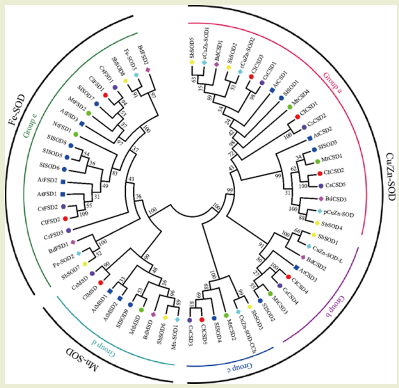
GRAPHICAL ABSTRACT



ABSTRACT

Superoxide dismutase (SOD) is an important enzyme in the antioxidant system of plants and plays a vital role in stress responses by maintaining the dynamic balance of reactive oxygen species (ROS) concentrations. Genome-wide analysis of the *SOD* gene family in various plant species has been conducted but little is known about this gene family in watermelon (*Citrullus lanatus*). Here, eight *SOD* genes were identified in the watermelon genome and are designated *ClCSD1-5*, *ClFSD1-2* and *ClMSD* according to their metal cofactors. Phylogenetic analysis shows that SOD proteins from various plant species can be classified into five groups and members in the same group possess the same metal cofactor and similar subcellular localizations. Expression analysis of the *ClSOD* genes indicates that they had tissue-specific expression patterns with high expression in different tissues including the leaves, flowers and fruits. In addition, the expression of *ClSOD* genes differed appreciably under salinity, drought and abscisic acid (ABA) treatments, indicating that they may be involved in ROS scavenging under different abiotic stresses via an ABA-dependent signaling pathway. These results lay the foundation for elucidating the function of *ClSOD* genes in stress tolerance and fruit development in watermelon.

1 INTRODUCTION

Plants are constantly exposed to numerous abiotic and biotic stresses including high and low temperatures, drought, salinity, high solar radiation, metal toxicity, UV radiation and pathogen infection[1,2]. These environmental stimuli can lead to the generation of a number of reactive oxygen species (ROS) such as singlet oxygen (O_2), superoxide anion radicals (O_2^-) and hydrogen peroxide (H_2O_2) which can cause oxidative damage to cells impairing cellular components and ultimately affecting the growth and development of plants[3,4]. A range of enzymatic and nonenzymatic antioxidant defense systems have evolved in plants to mitigate ROS toxicity and maintain the dynamic balance of ROS concentrations[5–8]. Superoxide dismutase (SOD, EC 1.15.1.1) is the first line of defense against ROS by catalyzing the decomposition of toxic O_2^- to O_2 and H_2O_2, which can be further degraded by peroxidase and catalase[9].

Plant SODs can be classified into three types based on prosthetic metals, namely copper-zinc SOD (Cu/ZnSOD), iron SOD (FeSOD) and manganese SOD (MnSOD)[6,10]. The *SOD* genes often constitute a multigene family and all three types of SOD are present in plants with different subcellular localizations, including the cytoplasm, peroxisome, chloroplasts, mitochondria, nuclei or extracellular spaces[10–12]. Genome-wide identification of the *SOD* gene family has been conducted in recent years in a range of plant species such as tomato (*Solanum lycopersicum*)[13], *Gossypium* species[6,14], cucumber (*Cucumis sativus*)[15], *Medicago truncatula*[9], foxtail millet (*Setaria italica*)[16], pear (*Pyrus bretschneideri*)[17], Japanese larch (*Larix kaempferi*)[18], tea (*Camellia sinensis*)[19] and wheat (*Triticum aestivum*)[20]. These studies report the basic characteristics and spatiotemporal expression profiles of the *SOD* family genes, demonstrating that the family members are pivotal for multiple developmental plant processes. For example, *PbrCSD5* and *PbrFSD1* might be closely associated with the postharvest ripening process of Fengshui pear[17] and *Xanthoceras sorbifolium SOD* genes have been found to be involved in ovule development after fertilization[21].

Numerous studies show that plant *SOD* genes are important regulators of responses to various environmental stimuli by protecting cells from oxidative damage by ROS[8,11,22]. For example, overexpression of *TmMnSOD* from *Triticum monococcum* in yeast and *Escherichia coli* cells increased tolerance to abiotic stresses and metal toxicity[23]. Overexpression of the *Oxya chinensis OcMnSOD* was recently found to enhance the tolerance of *E. coli* to chlorpyrifos-induced oxidative stress[24]. Also, overexpression of different *SOD* genes may lead to enhanced tolerance to single or multiple abiotic stresses in transgenic plants. For example, overexpression of a *Puccinellia tenuiflora Cu/ZnSOD* gene in transgenic yeast and *Arabidopsis* enhanced their tolerance to saline-sodic stress[25]. Higher NaCl and $NaHCO_3$ stress tolerance was also observed in transgenic rice plants overexpressing *OsCu/Zn-SOD*[26]. Overexpression of *CsCSD1* from cucumber was demonstrated to enhance salinity tolerance via an ABA-dependent signaling pathway in transgenic *Arabidopsis*[1]. Overexpression of three *Larix SOD* genes (*LkSOD2*, *LkSOD4* and *LkSOD6*) conferred tolerance to salt stress in transformed *Arabidopsis*[18].

Although the *SOD* gene family has been widely studied in many plant species this family has not been systematically investigated in watermelon (*Citrullus lanatus* cv. Xinong 8), an economically important crop worldwide. The growth and development of watermelon can be adversely affected by various abiotic and biotic stresses, particularly drought and salinity[27–29]. Here, we have systematically analyzed the *SOD* gene family in watermelon using phylogenetic analysis, multiple sequence alignment, and analysis of the conserved motifs, gene structures and promoter sequences. Expression analysis was conducted to examine the expression of watermelon *SOD* genes in different tissues, during fruit development, and under drought, salinity and abscisic acid (ABA) treatments. The results will provide valuable information in further dissecting the potential role of watermelon *SOD* genes in stress tolerance.

2 MATERIALS AND METHODS

2.1 Genome-wide identification of the *SOD* gene family in watermelon

The watermelon *SOD* gene family members were first identified by a BLASTp search against the watermelon (97103) v1 proteome, with all known *Arabidopsis* and rice SOD proteins as queries[30,31]. In addition, Hidden Markov Model profiles of Cu/ZnSOD (PF00080) and Fe-MnSOD (PF00081 and PF02777) were downloaded from the Pfam tool and used as queries to conduct an HMMER search with HMMER 3.0 software against the watermelon proteome with an E-value setting of 1e-5. All the predicted watermelon SOD proteins were subjected to Pfam and SMART tools to check that they contained the SOD domains (PF00080, PF00081 and PF02777). Sequences without the SOD domains were removed.

2.2 Analysis of physicochemical properties and conserved motifs

Based on the ProtParam tool, selected physicochemical properties of watermelon SOD proteins, specifically protein length, molecular weight (MW), and isoelectric point (pI), were determined. Subcellular localization was predicted by the ProtComp 9.0 server. The conserved motifs of the watermelon SOD proteins were analyzed with the MEME program using the parameters of 10 motifs and the motif analysis results have been displayed using the TBtools software[32].

2.3 Sequence alignment and evolutionary analysis

Sequence alignment was conducted with Clustal Omega with default parameters[33], using full-length SOD protein sequences from different plants. The alignment was then imported into MEGA 7.0 to create a neighbor-joining phylogenetic tree with bootstrap tests repeated 1000 times[34].

2.4 Chromosomal location, gene structure and promoter sequence analysis

The positions of watermelon *SOD* genes were obtained in the watermelon (97103) v1 genome and the illustration was drawn using the MapInspect software. Duplication analysis (segmental and tandem duplications) of watermelon *SOD* genes was conducted with the MCScanX software[35]. Gene structure analysis was conducted by retrieving the CDS and gDNA sequences of each watermelon *SOD* gene from the watermelon genome and their exon-intron arrangements were analyzed using the GSDS program. Promoter sequence analysis was done by obtaining the 1-kb upstream region from the translation start code (ATG) of each watermelon *SOD* gene as the promoter region from the watermelon genome and the phytohormone- and stress-responsive *cis*-elements were analyzed using the PlantCARE program.

2.5 Transcriptome analysis of the watermelon *SOD* genes

The raw RNA-seq data of both the flesh and rind at the four critical fruit development stages 10, 18, 26 and 34 days after pollination (DAP) were retrieved from the NCBI SRA database under BioProject SRP012849 and then the expression of watermelon *SOD* genes was estimated as RPKM values following a previous study[36]. The raw RPKM values were logarithmically transformed and heat maps were generated using TBtools[32].

2.6 Expression analysis of watermelon *SOD* genes by quantitative RT-PCR (qRT-PCR)

Watermelon cv. Xinong 8 seeds were germinated and grown in plastic pots containing fertilized soil. The pots were then placed in a greenhouse at 25/19°C day/night, 12:12 h light:dark photoperiod, 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$ light intensity and 70% RH. Tissue expression pattern analysis of the watermelon *SOD* genes was conducted by collecting different plant parts (roots, leaves, stems, flowers and fruits) from two-month-old plants. Three abiotic stress treatments (drought, salinity and ABA) were applied as described previously[27]. In brief, four-leaf-stage watermelon plants were transferred into Hoagland solution containing 200 $mmol \cdot L^{-1}$ NaCl or 20% PEG-6000 (w/v) to induce salt stress and drought stress, respectively. The ABA treatment was set up by spraying 100 $\mu mol \cdot L^{-1}$ ABA on the leaves of four-leaf-stage plants. The leaves were collected after different durations of treatment (0, 1, 3, 9 and 24 h). Total RNA was isolated using a total RNA Miniprep Kit (Axygen Biosciences, Corning, NY) and about 1 μg RNA was used for first-strand cDNA synthesis with a ReverTra Ace qPCR-RT kit (Toyobo, Osaka, Japan) according to the manufacturers' protocols. qRT-PCR analysis was conducted by assaying each gene in three biological replicates and three technical replicates under the experimental conditions described in our previous study[27]. The relative expressions of detected genes were analyzed by the $2^{-\Delta\Delta Ct}$ method using *β-actin* gene (Cla007792) for normalization[27] and the expression at 0 h was set at a value of 1.0. The relative expressions of the genes were compared with those of the control (0 h) using Tukey's test and $P < 0.05$ was assumed as significantly different. The sequences of primer pairs for qRT-PCR analysis are listed in Table S1.

3 RESULTS

3.1 Identification of *SOD* family genes in the watermelon genome

BLASTp and HMMER searches were used to identify SOD family members in the watermelon genome. After the removal of redundant sequences all protein sequences were confirmed by Pfam and SMART programs for the presence of a SOD domain and a total of eight *SOD* genes were identified, comprising five *Cu/ZnSOD* genes, two *FeSOD* genes and one *MnSOD* gene (Table 1). Details of the gene designations, chromosomal position, CDS length, protein length, MW, pI, and subcellular localization of each SOD family member are given in Table 1. The watermelon *SOD* genes ranged from 411 to 888 bp in CDS length and encoded proteins ranged from 136 (ClCSD5) to 295 (ClFSD2) aa in length. The MW of the watermelon SOD proteins ranged from 14.27 (ClCSD5) to 33.29 (ClFSD2) kDa, and the pI from 4.49 (ClCSD5) to 8.72 (ClMSD). According to the subcellular localization, ClCSD proteins were predicted to be in the chloroplast or cytoplasm. All ClFSD proteins were in the

Table 1 Features of *SOD* family genes identified in watermelon

Gene	Gene ID	Map position/bp	CDS length/bp	Protein length (aa)	MW/kDa	pI	Subcellular location
ClCSD1	Cla019840	Chr2:26640708-26642932	459	152	15.44	5.44	Cytoplasm
ClCSD2	Cla008698	Chr2:31797559-31799755	654	217	22.11	6.27	Cytoplasm
ClCSD3	Cla011299	Chr3:27370429-27372442	459	152	15.12	5.99	Cytoplasm
ClCSD4	Cla012125	Chr4:15855163-15857016	450	149	14.97	7.30	Chloroplast
ClCSD5	Cla001158	Chr10:3928563-3929302	411	136	14.27	4.49	Cytoplasm
ClFSD1	Cla011317	Chr3:27587644-27592715	804	267	30.92	8.09	Chloroplast
ClFSD2	Cla010691	Chr7:28869168-28873752	888	295	33.49	7.04	Chloroplast
ClMSD	Cla008101	Chr3:141384-143031	708	235	26.13	8.72	Mitochondrion

chloroplasts and ClMSD was located in the mitochondria (Table 1).

3.2 Phylogenetic characterization of *SOD* family genes in watermelon and other plant species

Insights into the evolutionary relationships of *SOD* family genes in watermelon with those in other plant species were gained by constructing a phylogenetic tree based on the alignment of SOD protein sequences from *C. lanatus*, *Arabidopsis thaliana*[30], *Oryza sativa*[31], *Brachypodium distachyon*[37], *S. lycopersicum*[13], *Sorghum bicolor*[38], *C. sativus*[15] and *M. truncatula*[9]. The phylogenetic results show that the SOD proteins could all be divided into five groups (Groups a–e) (Fig. 1). Among these groups, members from Groups a–c were Cu/ZnSODs while those from Groups d and e were MnSODs and FeSODs, respectively. As in other plant species the ClSODs were distributed in each group. ClCSD1, ClCSD2 and ClCSD3 were clustered in Group a, while ClCSD4 and ClCSD5 fell into Groups b and c, respectively. All SOD members in the three groups, a, b and c, were Cu/Zn-SOD proteins (Fig. 1). In addition, ClMSD was clustered together with other MnSOD proteins in Group d, whereas ClFSD1 and ClFSD2 together with other FeSOD proteins were placed in Group e (Fig. 1).

3.3 Characterization and conserved motif analysis of ClSOD proteins

Analysis of putative ClSOD protein sequences using the Pfam database predicted that all ClCSD proteins contained the Cu-ZnSOD domain (Sod_Cu, PF00080), whereas the Fe-MnSOD alpha-hairpin domain (Sod_Fe_N, PF00081) and Fe-MnSOD C-terminal domain (Sod_Fe_C, PF02777) were present in the ClMSD and ClFSD proteins (Fig. 2(a)).

Using MEME a total of 10 conserved motifs were identified and are shown in Fig. 2(b) and Table S3. Motifs 1, 2 and 4, which were widely present in ClCSD proteins, were annotated as the Cu-ZnSOD domain (Table S3). However, ClCSD5 was lacking in motifs 2 and 4. In addition, motifs 3, 5, 6, 8 and 9 were conserved domains of Fe-SODs and Mn-SODs. Motif 10 was found only in ClCSD5 and ClFSD1 (Fig. 2(b)).

The conserved SOD signatures and residues of ClSOD proteins were further analyzed. The multiple sequence alignment results show that two conserved Cu/ZnSOD signatures (GFH[VLI]H [AES][LY]GDTT and GNAG[EGA]R[ILV][CAG]CG) were present in nearly all ClCSDs with the exception of ClCSD5 (Fig. 3). ClFSD1 and ClFSD2 contained the conserved FeSOD signature (AQ[VI]WNHDF[FL]WES) and metal binding domain (D[MV]WEHAYY) whereas ClMSD had only the metal binding domain. In addition, nearly all ClSODs possessed conserved residues, especially His, which is necessary for metal binding (Fig. 3).

3.4 Gene structure analysis of *ClSOD* genes

The structural features of the *ClSOD* genes were determined by comparing their CDS and gDNA sequences. This comparison shows that *ClSOD* genes had different intron numbers ranging from one (*ClCSD5*) to seven (*ClFSD1*) (Fig. 4), with most genes containing five or six introns. For example, *ClCSD4* and *ClMSD* possessed five introns, and *ClCSD1*, *ClCSD2*, *ClCSD3* and *ClFSD2* had six introns each (Fig. 4).

3.5 Chromosomal distribution and duplication analysis of watermelon *SOD* genes

The eight *ClSOD* genes were differently and unevenly distributed

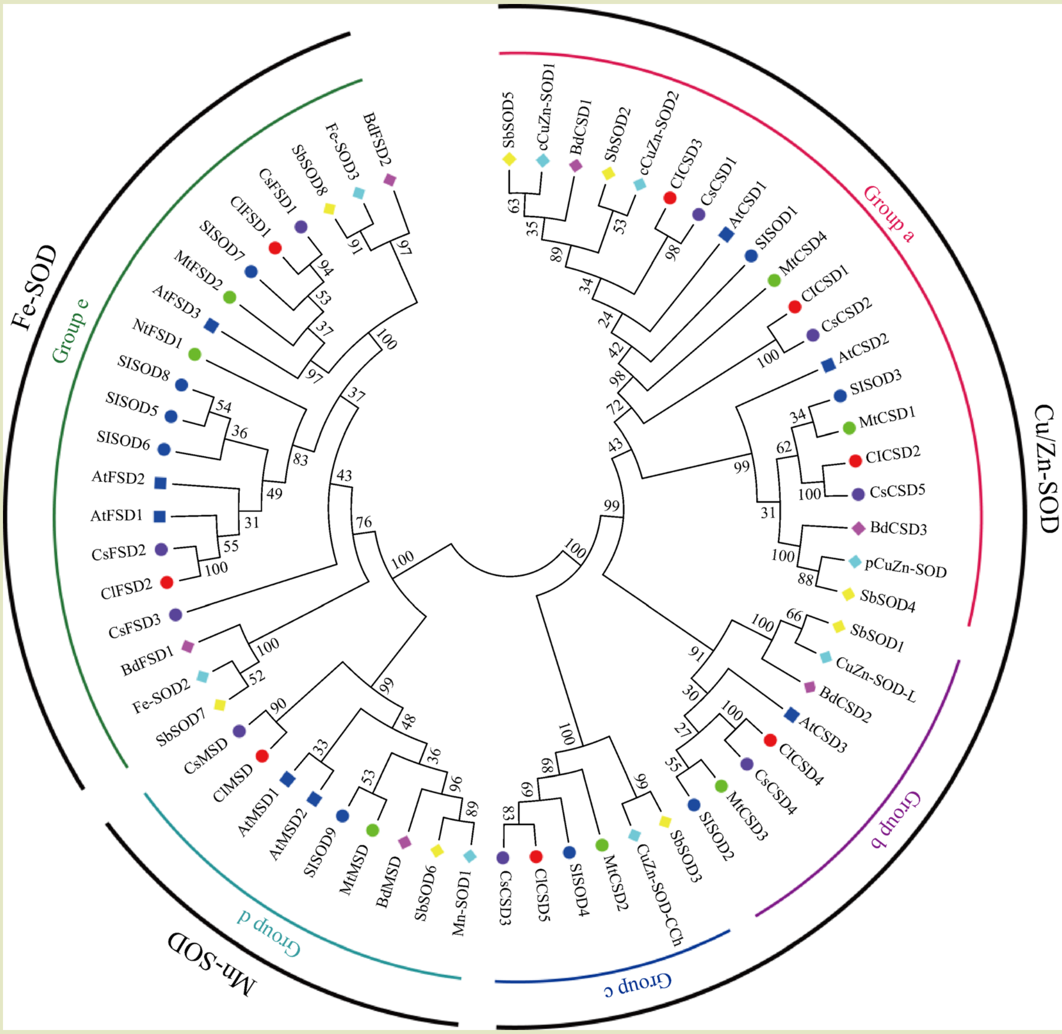


Fig. 1 Phylogenetic analysis of SOD proteins in watermelon and other plant species. The phylogenetic tree was constructed using Clustal Omega and MEGA 7.0 by the neighbor-joining method with 1000 bootstrap repeats. The protein sequences used for creation of the phylogenetic tree are listed in Table S2. Cl, *Citrullus lanatus*; At, *Arabidopsis thaliana*; Sl, *Solanum lycopersicum*; Cs, *Cucumis sativus*; Mt, *Medicago truncatula*; Sb, *Sorghum bicolor*; Bd, *Brachypodium distachyon*.

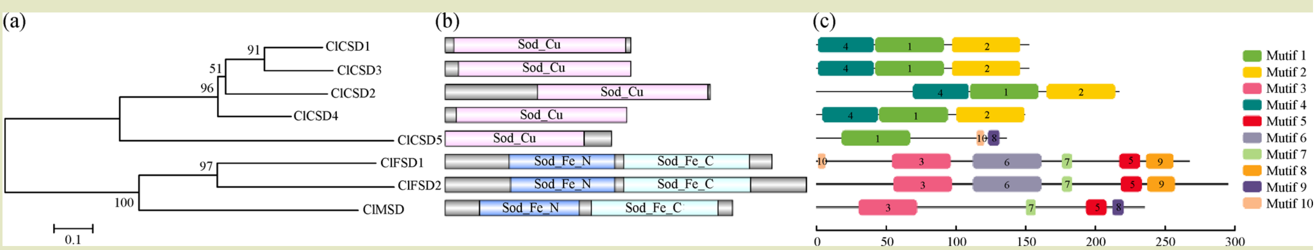


Fig. 2 (a) Phylogenetic relationships, (b) structures and (c) conserved motifs of watermelon SOD proteins. Multiple alignments were conducted with Clustal Omega using full-length watermelon SOD protein sequences, and the phylogenetic tree was created with MEGA 7.0 using the NJ method with bootstrap tests repeated 1000 times. The colored boxes indicate different conserved motifs, and their positions in each watermelon SOD protein sequence are displayed proportionally.

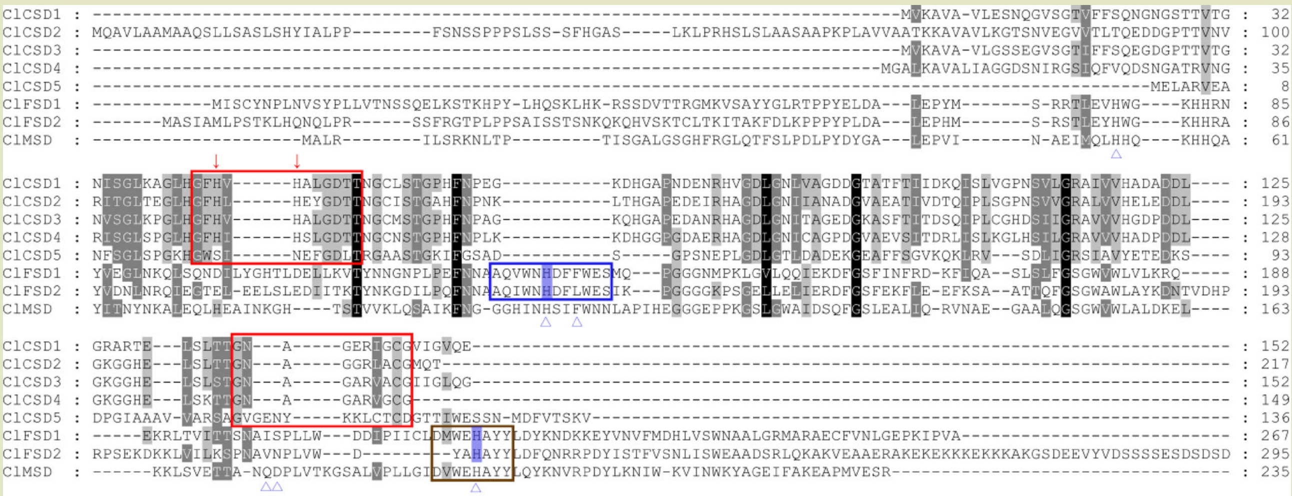


Fig. 3 Multiple sequence alignment of ClSOD protein sequences. Two conserved Cu/ZnSOD signatures (GFH[VLI]H[AES][LY]GDTT and GNAG[EGA]R[ILV][CAG]CG) are indicated with the red box. The metal binding sites of Cu/ZnSODs for Cu^{2+} and Zn^{2+} are indicated with red arrows. The conserved FeSOD signature (AQ[VI]WNHDF[FL]WES) and metal binding domain (D[MV]WEHAYY) are boxed with blue and brown, respectively. Two conserved His residues in FeSODs are indicated with blue, while six conserved residues (His, Phe, Gln and Asp) are indicated with blue boxes.

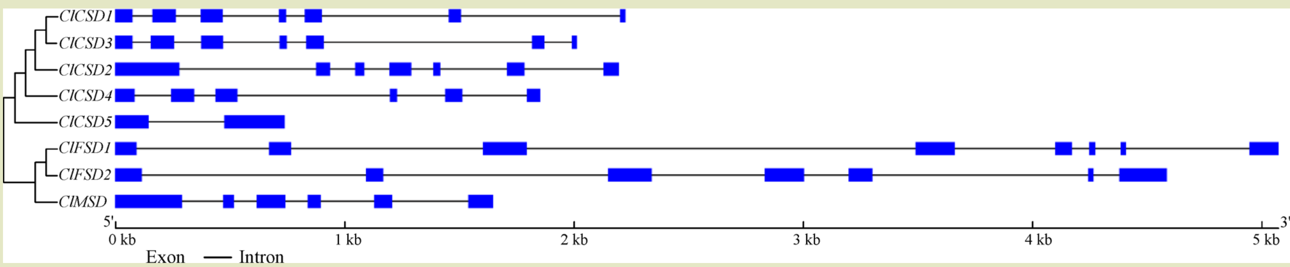


Fig. 4 Exon-intron structure of watermelon *SOD* genes based on the phylogenetic relationship. Exons and introns are indicated by blue boxes and black lines, respectively, and their lengths in each gene are displayed proportionally.

on five chromosomes in the watermelon genome. Chromosome 3 had the most genes (*ClMSD*, *ClFSD1*, and *ClCSD3*); chromosome 2 had two genes; and chromosomes 4, 7 and 10 each had one gene (Fig. 5). No tandem duplications were found in these genes. However, a pair of *ClSOD* genes, *ClCSD1* and *ClCSD3*, were found as segmental duplication genes (Fig. 5).

3.6 Analysis of *cis*-elements in *ClSOD* genes

The potential functions of *ClSOD* genes were clarified by extracting a 1-kb genomic sequence upstream of the translation start code (ATG) in each *ClSOD* gene and analyzing using the PlantCARE database. A total of six types of stress-responsive *cis*-elements were found, and the promoters of seven *ClSOD* genes were found to possess one or more AREs (anaerobic induction elements), which comprised the most *cis*-elements (Fig. 6). In addition, five other stress-responsive *cis*-elements were found in the promoters of several *ClSOD* genes such as LTR, MBS, W-box, WUN-motif and TC-rich repeats, suggesting that *ClSOD* genes are associated with the stress response (Fig. 6). Seven types of phytohormone-responsive *cis*-elements were also found in the *ClSOD* gene promoters including ABRE, ERE, CGTCA-motif, TCA-element, TGA-element, P-box and TATC-box, which are related to ABA, ethylene, methyl jasmonate, salicylic acid, auxin and gibberellin responses, respectively. It was noted that *ClFSD2* had the largest number of ERE elements in its promoter region, indicating that this gene may have a primary function in the ethylene response.

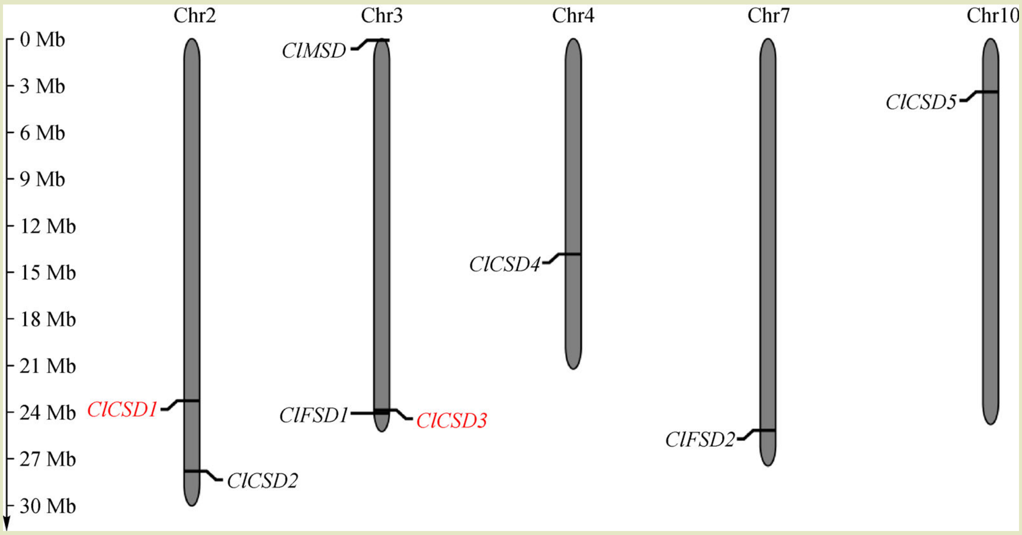


Fig. 5 Chromosomal locations of the watermelon *SOD* genes. Segmentally duplicated *SOD* genes are indicated in red.

	Stress-responsive element						Hormone-responsive element						
	LTR	MBS	W-box	ARE	WUN-motif	TC-rich repeats	ABRE	ERE	CGTCA-motif	TCA-element	TGA-element	P-box	TATC-box
ClCSD1				3	2		2					1	
ClCSD2		2	1	2			1	1					1
ClCSD3	1			1		1	3		1		1		
ClCSD4		1		1		1			2				
ClCSD5		2		2	1		1		1	1			
ClFSD1				1	1	1				1		1	
ClFSD2								7				1	
ClMSD		1		1				1	2				1

Fig. 6 Distribution of stress- and hormone-responsive elements in 1-kb promoter regions of *ClSOD* genes. LTR, low temperature-responsive element; MBS, MYB binding site involved in drought-inducibility; W-box, WRKY binding site involved in abiotic stress and defense response; ARE, *cis*-element essential for the anaerobic induction; WUN-motif, wound-responsive element; TC-rich repeats, *cis*-element involved in defense and stress responsiveness; ABRE, ABA-responsive element; ERE, ethylene-responsive element; CGTCA-motif, MeJA-responsive element; TCA-element, salicylic acid-responsive element; TGA-element, auxin-responsive element; P-box and TATC-box, gibberellin-responsive element.

3.7 Expression patterns of *ClSOD* genes in watermelon plant parts

The possible functions of *ClSOD* genes were analyzed by examining the expression patterns of selected *ClSOD* genes in five major plant parts (roots, stems, leaves, flowers and fruits) using qRT-PCR. *ClCSD2* and *ClCSD4* had higher expression in leaves than in other parts, and a relatively higher transcript level of *ClCSD5* was also found in leaves (Fig. 7(a)). In addition, *ClCSD1*, *ClCSD3* and *ClCSD5* were mostly (and at the highest level of expression) in fruits (Fig. 7(a)), suggesting that these

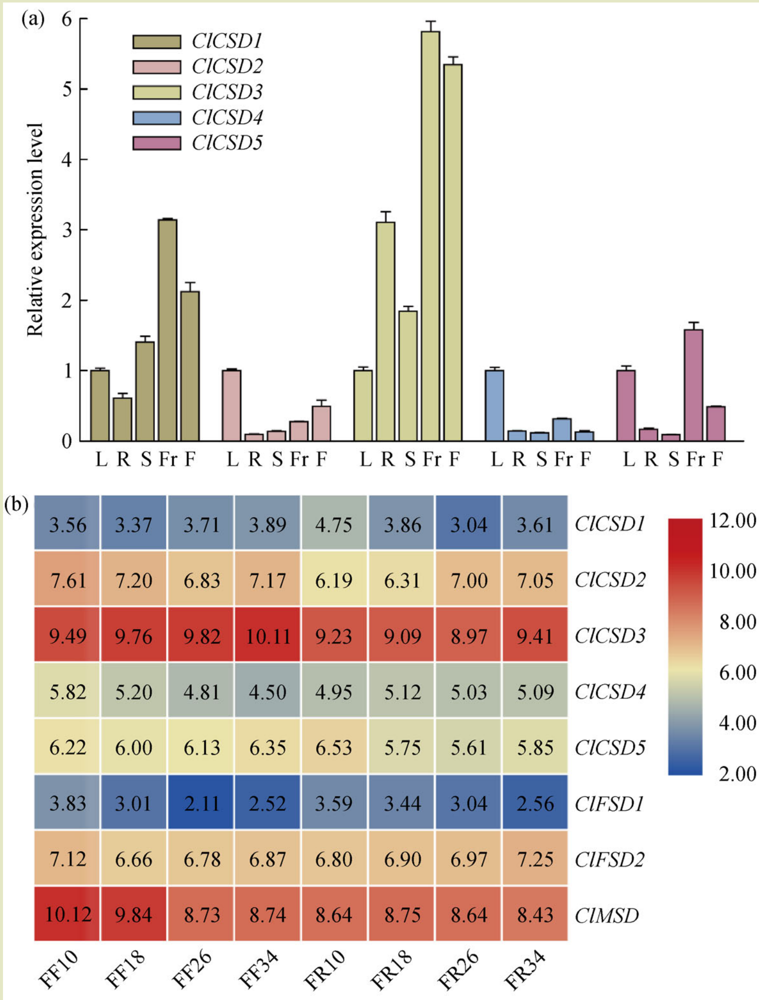


Fig. 7 Expression patterns of selected *ClSOD* genes (a) in watermelon tissues and (b) during fruit development. L, leaves; R, roots; S, stems; Fr, fruits; F, flowers; FF and FR are fruit flesh and fruit rind at 10, 18, 26, and 34 days after pollination, respectively. For transcriptome analysis the gene expression levels were calculated with the logarithmic method involving $\log_2$(RPKM + 1).

genes may be involved in fruit development. The expression patterns of the *ClSOD* genes during fruit development were also examined based on the RNA-seq data, but no *ClSOD* genes had observably upregulated expression during fruit development (Fig. 7(b); Table S4). *ClCSD4* and *ClMSD* expression was significantly lower at 26 and 34 DAP in the flesh; *ClCSD1* expression clearly decreased i at 26 and 34 DAP in the rind and substantially lower at the later stages of both flesh and rind development (Fig. 7(b)). These results indicat possible functions of these genes in watermelon fruit development.

3.8 Expression pattern analysis of *ClSOD* genes in response to different abiotic stresses

The potential roles of *ClSOD* genes in response to various abiotic stresses were examined by observing the expression of *ClSOD* genes under salinity, drought and ABA treatments using qRT-PCR. Nearly all *ClSOD* genes were significantly upregulated or downregulated under the salinity treatment with the exception of *ClCSD5* (Fig. 8). Of these, five genes were significantly upregulated and the expression of *ClFSD1*, *ClFSD2* and *ClMSD* reached a maximum after 1 h, whereas expression of *ClCSD1* and *ClCSD3* was highest after at 3 and 24 h, respectively. However, *ClCSD2* and *ClCSD4* were clearly downregulated in response to salinity stress (Fig. 8). Under the drought treatment the expression of *ClCSD1*, *ClCSD3*, *ClCSD5* and *ClMSD* was significantly upregulated whereas that of *ClCSD2*, *ClCSD4*, *ClFSD1* and *ClFSD2* declined strongly at all elapsed times (Fig. 9). The expression of all *ClSOD* genes was induced by the ABA treatment and reached a peak after 24 h (Fig. 10). These results indicate that the *ClSOD* genes may participate in the responses to a number of different stresses.

4 DISCUSSION

Here, a comprehensive genome-wide identification of the *SOD* gene family in watermelon was conducted. Eight *SOD* genes comprising five *Cu/ZnSOD* genes, two *FeSOD* genes and one *MnSOD* gene were identified in the plant genome (Table 1), corresponding to three dominant plant *SOD* gene types. The number of *SOD* genes in each type was comparable to that in other plant species such as rice (5 *Cu/ZnSODs*, 2 *FeSODs* and 1 *MnSOD*)[31], cucumber (5 *Cu/ZnSODs*, 3 *FeSODs* and 1 *MnSOD*)[15], and *M. truncatula* (4 *Cu/ZnSODs*, 3 *FeSODs* and 1 *MnSOD*)[9], and tea (7 *Cu/ZnSODs*, 2 *FeSODs* and 1 *MnSOD*)[19]. In addition, phylogenetic analysis allows the SOD proteins to be classified into five groups consistent with their

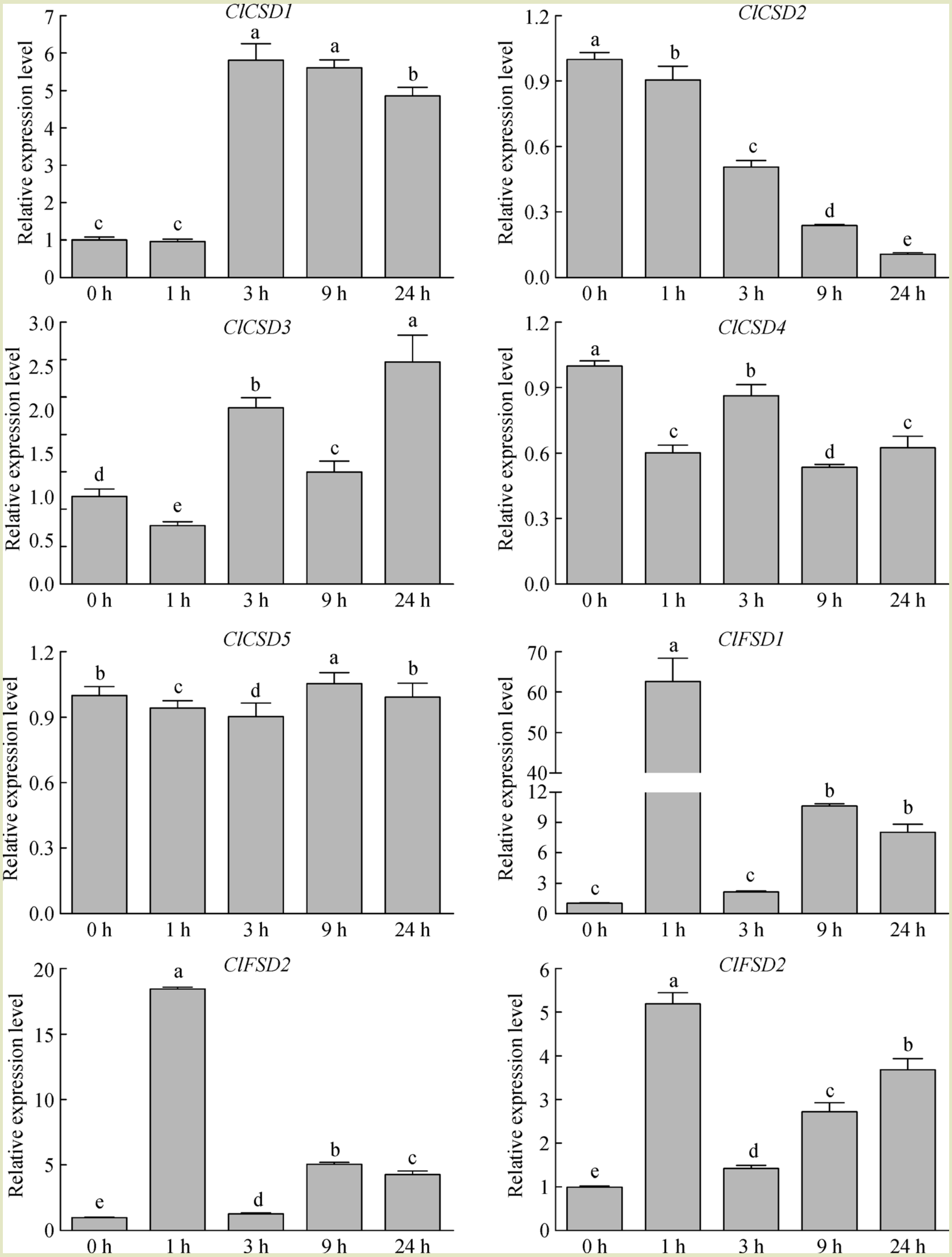


Fig. 8 Expression pattern analysis of *ClSOD* genes in response to salinity stress. Mean values with the same letters above the bars are not significantly different (Tukey's multiple range tests, $P < 0.05$) for different treatment elapsed times.

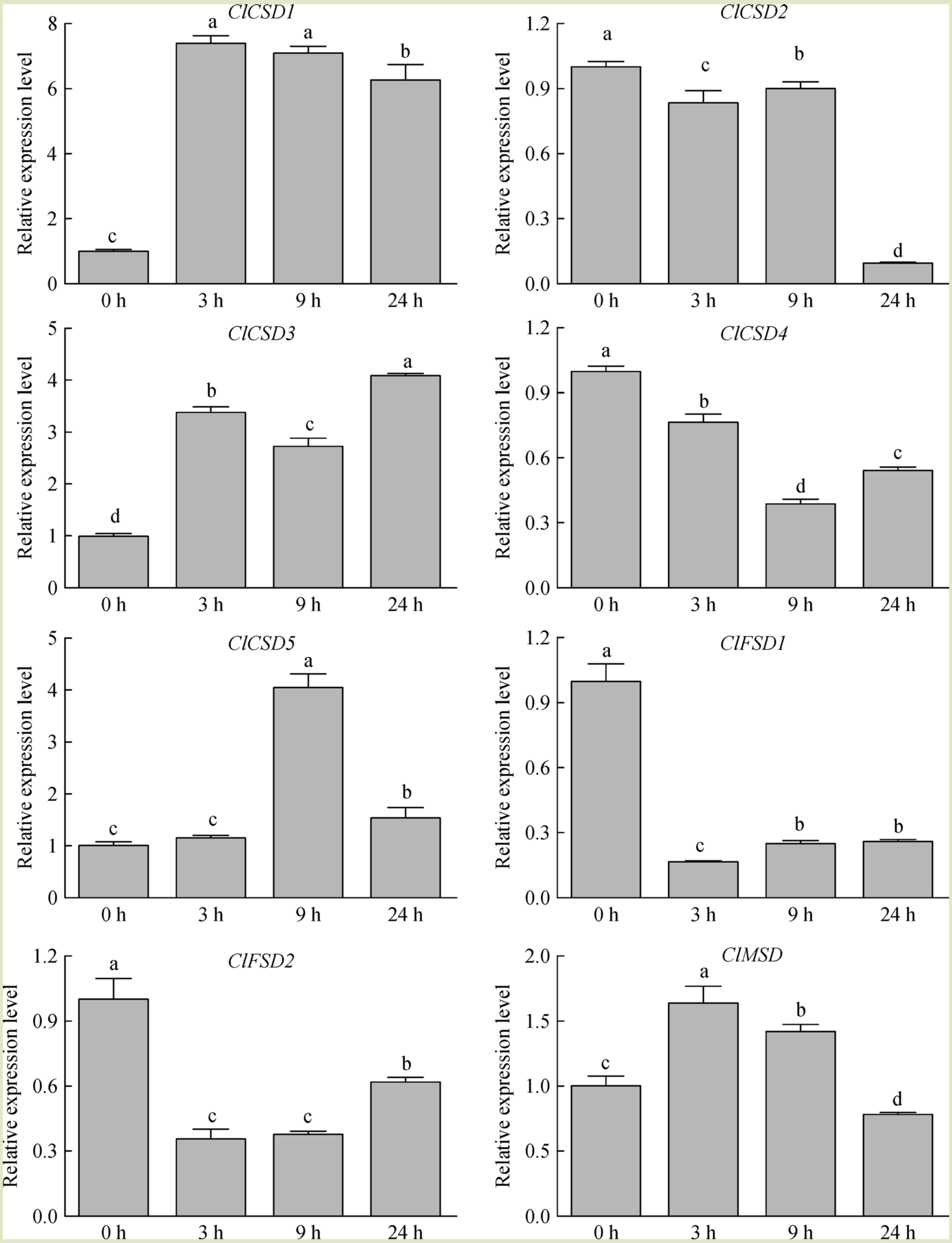


Fig. 9 Expression patterns of *ClSOD* genes under drought treatment determined by qRT-PCR. Mean values with the same letter above the bars are not significantly different (Tukey's multiple range tests, $P < 0.05$) between different treatment elapsed times.

metal cofactors (Fig. 1). Groups a-c and d-e constitute the Cu/ZnSODs and Fe-MnSODs, respectively, and were separated by a high bootstrap value (Fig. 1). Watermelon Cu/ZnSOD and Fe-MnSOD proteins had similar arrangement of conserved motifs such as Cu/ZnSOD and FeSOD signatures, and metal binding domains (Fig. 2; Fig. 3). In addition, the SODs that are evolutionarily clustered together always have the same subcellular localizations. For example, ClCSD1, ClCSD2 and ClCSD3, together with SlSOD1, MtSOD4, cCuZn-SOD2, CsCSD1 and CsCSD2, were all in Group a (Fig. 1; Table 1), and all were predicted to be cytoplasmic CSDs[9,13,15,31]. ClMSD was in Group d with other mitochondrial MnSODs such as MnSOD1[31], SbSOD6[38], SlSOD9[13], CsMSD[15] and MtMSD[9]. Moreover, most *ClSOD* genes had five or six introns

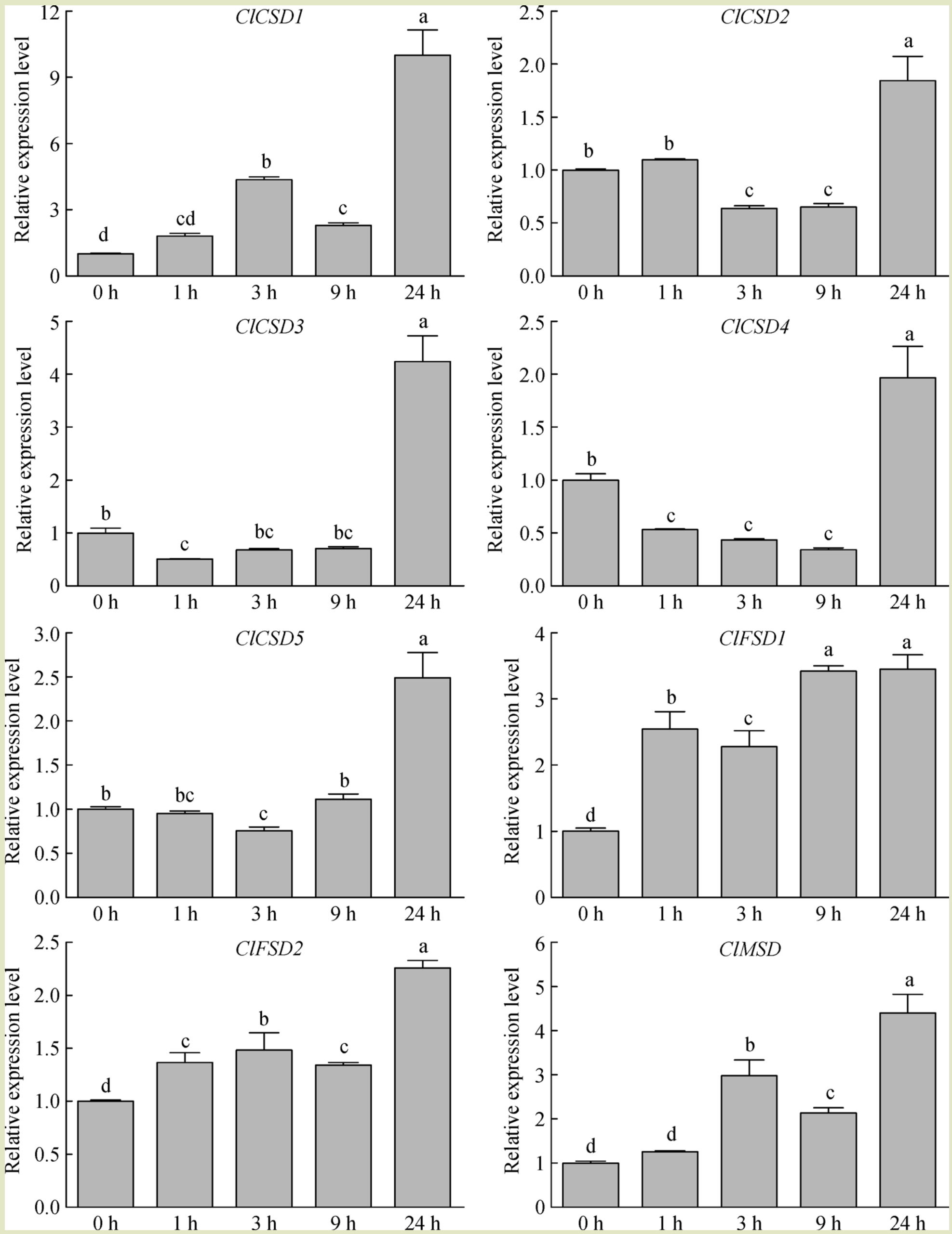


Fig. 10 Expression pattern analysis of *ClSOD* genes in response to ABA treatment. Different mean values with the same letter above the bars are not significantly different (Tukey's multiple range tests, $P < 0.05$) between different treatment elapsed times.

(Fig. 4), and this is consistent with the results of previous studies, for example on *B. distachyon*[37], sorghum[38], tomato[13] and wheat[20]. These SOD members in different plant species share common gene structure and domain architecture, demonstrating that plant *SOD* family genes have been fairly conserved during evolution.

Numerous studies show that the expression of plant *SOD* genes always has a certain degree of temporal and spatial specificity. For example, four *M. truncatula* genes (*MtCSD1*, *MtCSD2*, *MtCSD4* and *MtMSD*) had the highest expression in seeds[9]. Likewise, the expression of *PbrCSD2-3*, *PbrCSD5-6* and *PbrFSD1-3* was found to be maximum in seeds[17]. In addition, *MtCSD3* and *MtFSD1* had relatively high expression in leaves compare with other plant parts[9]. Here, all five *ClCSD* genes were highly expressed in leaves, especially *ClCSD2*, *ClCSD4* and

ClCSD5 (Fig. 7(a)), and similar results have been found in other plant species including tomato[13], cotton[6], and cucumber[15]. Leaves have a fundamental role in maintaining the life of plants through photosynthesis[14] and therefore these *SOD* genes may participate in scavenging the ROS generated from photosynthesis. In addition, *ClCSD1*, *ClCSD3* and *ClCSD5* have considerably higher mRNA contents in the fruits and flowers (Fig. 7(a)), common reproductive organs, and large amounts of ROS are also produced in these tissues[14]. It was noted that four genes (*ClCSD1*, *ClCSD4*, *ClFSD1* and *ClMSD*) exhibited significant declines in transcripts during the development of flesh and/or rind (Fig. 7(b)), consistent with previous reports showing that SODs contribute to fruit ripening processes[17,39]. In summary, the *ClSOD* genes also have tissue-specific expression patterns, indicating their important functions in specific tissues and developmental stages of watermelon.

SOD is an efficient ROS scavenger that has a vital regulatory role in responses to different abiotic stresses in plants, and many *SOD* genes have been found to have differential expression patterns in response to abiotic stresses[6,15,16,20]. In the present study a number of hormone- and stress-responsive *cis*-elements were identified in the promoters of *ClSOD* genes (Fig. 6), indicating the importance of these genes in hormone and stress responses. Hence, we further examined their levels of expression under three stress treatments comprising drought, salinity and ABA. The qRT-PCR shows that under salinity and drought stress the expression of three *ClSOD* genes (*ClCSD1*, *ClCSD3* and *ClMSD*) was significantly induced (Fig. 8; Fig. 9), suggesting that they contribute positively in response to these stresses. Generally, the gene expression and enzyme activities of SOD may be upregulated under stress conditions to enhance the tolerance of plants to different stresses. However, the expression of *ClCSD2* and *ClCSD4* was conspicuously downregulated under salinity and drought stress (Fig. 8; Fig. 9), indicating their negative functions in response to abiotic stresses. Downregulation of the *SOD* gene has also been reported in other plant speices such as *Gossypium hirsutum*[14] and wheat[20]. In addition, the expression of *ClFSD1* and *ClFSD2* was significantly upregulated by salinity stress but downregulated by drought stress (Fig. 8; Fig. 9), suggesting their different roles in response to the two stresses, and watermelon may have developed diverse regulatory mechanisms to tolerate salinity and drought stress. ABA is important in plant response to abiotic stresses, and overexpression of some *SOD* genes can confer tolerance to diverse stresses via an ABA-dependent pathway such as *CsCSD1*[1], *AtSOD* or *CmSOD*[40]. Here, the expression of all *ClSOD* genes was affected by ABA treatment (Fig. 10), indicating that these genes are involved in the abiotic stress response, possibly via an ABA-dependent signaling pathway.

5 CONCLUSIONS

Here, the *SOD* gene family in watermelon was comprehensively analyzed. A total of eight *SOD* family genes were identified comprising five *Cu/ZnSOD* genes, two *FeSOD* genes and one *MnSOD* gene. Their phylogenetic relationships, conserved motifs and residues, gene structures, and stress- and hormone-responsive *cis*-elements in the promoter regions were examined. In addition, RNA-Seq data and qRT-PCR were used to examine the expression patterns of the *ClSOD* genes in different tissues and during fruit development, as well as under different abiotic stresses. Our findings provide a theoretical basis for further functional identification of the *ClSOD* genes in stress tolerance and fruit development in watermelon.

Supplementary materials

The online version of this article at https://doi.org/10.15302/J-FASE-2020350 contains supplementary materials (Tables S1–S4).

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Compliance with ethics guidelines

Yong Zhou, Linjuan Ouyang, Dahu Zhou, Yicong Cai, and Haohua He declare that they have no conflicts of interest or financial conflicts to disclose. This article does not contain any studies with human or animal subjects performed by any of the authors.

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