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Comparative Analyses of Superoxide Dismutase (SOD) Gene Family and Expression Profiling under Multiple Abiotic Stresses in Water Lilies

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Abstract: Plants in their natural habitat frequently face different biotic and abiotic stresses, which lead to the production of reactive oxygen species (ROS) that can damage cell membranes, cause peroxidation and deterioration of macromolecules, and ultimately result in cell death. Superoxide dismutase (SOD), a class of metalloenzymes, is primarily found in living organisms and serves as the principal line of defense against ROS. The *SOD* gene family has not yet been characterized in any species of water lily from the genus *Nymphaea*. The present study aims to conduct a genome-wide study to discover *SOD* genes in four representative water lily species. In our present comparative study, we discovered 43 *SOD* genes in the genomes of four water lily species. The phylogenetic investigation results revealed that *SOD* genes from water lily and closely related plant species formed two distinct groups, as determined by their binding domains with high bootstrap values. Enzymatic ion-binding classified the *SOD* gene family into three groups, *FeSOD*, *Cu/ZnSOD*, and *MnSOD*. The analysis of gene structure indicated that the *SOD* gene family exhibited a relatively conserved organization of exons and introns, as well as motif configuration. Moreover, we discovered that the promoters of water lily *SODs* contained five phytohormones, four stress-responsive elements, and numerous light-responsive *cis*-elements. The predicted 3D protein structures revealed water lily *SODs* form conserved protein dimer signatures that were comparable to each other. Finally, the RT-qPCR gene expression analysis of nine *NcSOD* genes revealed their responsiveness to heat, saline, cold, cadmium chloride, and copper sulphate stress. These findings establish a basis for further investigation into the role of the *SOD* gene family in *Nymphaea colorata* and offer potential avenues for genetic enhancement of water lily aquaculture.

Keywords: *NcSOD* genes; sequence analysis; abiotic stresses; expression pattern

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1. Introduction

Plants residing in their native environments often encounter a multitude of stress factors, including elevated salinity levels, prolonged drought periods, high temperatures, and the presence of heavy metals. These stressors exert notable influences on the plants' overall growth, development, and productivity [1,2]. Under stress, plants adapt their homeostatic mechanisms by generating an excess of reactive oxygen species (ROS) within their cells. ROS are primarily generated in various parts of the plant cell, including the plasma membrane, peroxisomes, apoplast, cell walls, endoplasmic reticulum, mitochondria, and chloroplasts [3]. These ROS are toxic free radicals that can oxidize proteins, damage cell membranes, and cause harm to DNA when formed in excessive amounts [4,5]. The occurrence of stresses in plants inevitably leads to the production of ROS, such as peroxide

radicals (HOO−), hydrogen peroxide (H_2O_2), and singlet oxygen ($^1O_2$). For instance, several potent scavengers of active oxygen have the ability to mitigate environmental stresses by regulating the expression of genes belonging to enzyme reaction families, such as superoxide dismutase (*SOD*), catalase (*CAT*), peroxidase (*POD*), glutathione peroxidase (*GPX*), and peroxidase (*PrxR*) [6–9]. Plants have developed effective and intricate antioxidant defense mechanisms comprising a variety of enzymatic and non-enzymatic antioxidants to manage the harmful effects of ROS. Among various antioxidant enzymes, *SOD*, a group of metalloenzymes, is predominantly present in living creatures. In managing environmental cues, *SOD*s show a vital part in the physio-biochemical processes of plants via acting as the primary defense against ROS [3]. In plants, *SOD* enzymes are encoded by a family of genes that are classified based on their metal cofactors: (*FeSOD*), (*Cu/ZnSOD*), (*MnSOD*), and (*NiSOD*) [10–12]. Among them *NiSOD* is predominantly found in cyanobacteria, streptomyces, and marine organisms, but has not yet been documented in plants [13,14]. Iron and manganese superoxide dismutase are mainly present in lower plants, whereas copper and zinc are found in higher plants [15]. Such *SODs* are usually dispersed in different cell parts [16]. In the main, *Cu/ZnSODs* are localized in the cytosol, peroxisomes, and chloroplasts. *MnSODs* are found inside mitochondria and *FeSODs* are usually found in the peroxisomes and chloroplasts [17,18].

SODs have been demonstrated in recent studies to secure plants against abiotic stress factors including cold, drought, heat, salinity, ethylene and abscisic acid [19–22]. Various findings have demonstrated that *SOD* genes might be transcribed and induced in many plants in different stress circumstances [23,24]. In recently published articles, the *SOD* gene family under various abiotic and hormones stress situations in *Brassica napus* [25], *Zostera marina* [26], *Salvia miltiorrhiza* [27], and *Hordeum vulgare* [28] were reported. Furthermore, different stress conditions can result in varied expression patterns of diverse forms of *SOD* genes. For example, tomatoes (*Solanum lycopersicum*) exhibit specific patterns of regulation in their *SOD* genes; for instance, under salt stress, *SlSOD1* is a single gene among the nine *SlSOD* genes that shows significant upregulation, while *SlSOD2, SlSOD5, SlSOD6*, and *SlSOD8* are also regulated. However, in drought conditions, the expression levels of four genes among the nine, namely "*SlSOD2, SlSOD5, SlSOD6*, and *SlSOD8*," are observed to be high [23]. Moreover, the expression profiles of the identical *SOD* gene type varied in the presence of stress. For instance, the studies revealed that the expression of *MnSODs* in *Arabidopsis* remained unchanged during oxidative stress, while scientists observed a considerable alteration in the expression of *MnSODs* in *Zostera marina*, peas (*Pisum sativum*), and wheat (*Triticum aestivum*) during salinity stress [26,29–31]. The findings imply that diverse *SOD* genes unveil distinct expression patterns in reaction to varying environmental stresses. In addition, scientists have revealed that the regulation of *SOD* expression may involve various miRNAs and alternative splicing mechanisms [32,33].

Water lilies are the most significant ornamental waterscape plants in the world. It is a perennial aquatic plant of the order Nymphaeales, genus *Nymphaea* in the family *Nymphaeaceae*. *Nymphaea* (*Nymphaeaceae*), also called flowering plants, are angiosperms with large and showy flowers. There are more than 60 species in the world, mostly distributed in tropical, subtropical, and temperate regions. They have curved or rounded and variously notched waxy-coated leaves on long stalks, usually grow on the water, and surround flowers. Each plant can grow approximately 70 to 80 flowers. The aquaculture of water lily, flowers can also be used as fresh cut flowers, in tea, in dried flower crafts, and in textile production. Water lilies have garnered significant attention from scientists, researchers, and entrepreneurs worldwide due to their immense economic, medicinal, and cultural value. While these plants hold great importance in phylogenetic research, the accessibility to comprehensive genetic and genomic information remains somewhat limited [34]. Since we released the first water lily (*Nymphaea colorata*) genome sequence in 2020 [35], the *SOD* gene family has not yet been discovered in any species of water lily.

To fill in this gap, the present study aims to conduct a comparative genome-wide study to discover *SOD* genes in representative water lily species genomes. Our analysis included

the characterization of their phylogenetic connections, conserved motifs, cis-elements, gene structure, expression analysis, protein-protein interaction and 3D structures, in order to decode its structural characteristics and functions under stresses.

2. Materials and Methods

2.1. Retrieval of SOD Gene Family in Water Lily Species

To investigate the *SOD* gene family in water lilies, the Blastp search method was employed, utilizing the *Arabidopsis SOD* sequence as a query to examine the entire genome of each water lily species individually [36]. In our study, we used two methodologies, protein blast and the hidden Markov model (HMM), to detect *SOD* genes in four water lily species in which the genome sequences of two species *Nymphaea colorata*, and *Nymphaea thermarum*, are available online; while the other two, i.e., *Nymphaea minuta*, and *Nymphaea mexicana*, have unpublished genome sequences. For BLASTP, we utilized eight *A. thaliana SOD* amino acid sequences (AT1G08830.1/*AtCSD1*, AT2G28190.1/*AtCSD2*, AT5G18100.1/*AtCSD3*, AT4G25100.1/*AtFSD1*, AT5G51100.1/*AtFSD2*, AT5G23310.1/*AtFSD3*, AT3G10920.1/*AtMSD1*, and AT3G56350.1/*At00MSD2*) as the query, with an e-value set to 1×10^{-5}. We obtained these eight *AtSODs* amino acid sequences from the *Arabidopsis* genome database TAIR (http://www.arabidopsis.org/ accessed on 5 January 2023). To identify conserved domains *SOD_Cu* (PF00080), *SOD_Fe_C* (PF00081) and *SOD _Mn* (PF02777), we performed scans of specific amino acid sequences using the web resources Pfam (Pfamv34.0-19178pSSMs) protein domain database (http://pfam.xfam.org/ accessed on 8 January 2023), and SMART (http://smart.embl-heidelberg.de/ accessed on 9 January 2023) [37].

2.2. Analysis of Physicochemical Features and Subcellular Localization

In order to anticipate the physicochemical characteristics of *SOD* proteins in water lily species, such as amino acid count (A.A), theoretical isoelectric point (pI), molecular weight (kDa), Pfam Domains, Functional annotations, and grand average of hydropathicity (GRAVY), we employed the ProtParam website accessible at http://web.expasy.org/protparam/ accessed on 13 January 2023 [38]. In order to predict the subcellular localization of *SOD* proteins, we employed the WoLF PSORT (https://wolfpsort.hgc.jp/ accessed on 17 January 2023) [39] and ProtComp 9.0 server (http://linux1.softberry.com/ accessed on 18 January 2023) [27].

2.3. Phylogenetic Analysis and Conserved Motifs

We generated a phylogenetic tree to investigate the evolutionary connections of the water lily *SOD* gene family. This was done using protein sequences from *N. colorata*, *N. mexicana*, *N. minuta*, *N. thermarum*, and *A. trichopoda* (*Amborella trichopoda*). First, we aligned the protein sequences using MUSCLE with default parameters [39]. Then, we used the MEGA11 software (https://megasoftware.net/home accessed on 27 January 2023) to make a phylogenetic tree using the Neighbor-Joining algorithms. We assigned confidence levels to each branch of the tree using bootstrap tests (1000). We used the MEME server (https://meme-suite.org/meme/db/motifs accessed on 2 February 2023) with default settings to detect conserved motifs in the protein sequences of water lily *SODs* [40].

2.4. Prediction of the Cis-Regulatory Elements in the Promoter

To examine the potential cis-elements in the promoters of water lily *SODs*, we obtained the 2 Kb sequence upstream of start codons from each species' genome. Then we used the PlantCARE website (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/ accessed on 6 February 2023) [41], to examine the promoter sequence of each gene, and then we created figure using TBtools (V 1.068).

2.5. Examination of the 3D Structures of Water Lily SOD Proteins

The comprehension of a protein's functions requires a detailed understanding of its 3D structure. To this end, we analyzed the predicted 3D structures of four water lily species,

using the online servers SOPMA and SWISS MODEL, both available through ExPASy at https://www.expasy.org/ accessed on 15 February 2023. Finally, we applied the UCSF Chimera visualization tool to visualize the 3D structures [42].

2.6. Analysis of SOD Gene Structure of Water Lily

The Gene Structure Display Server (GSDS; http://gsds.cbi.pku.edu.cn/index.php accessed on 19 February 2023) program was used to visualize the organization of exons and introns in the *SOD* genes of water lilies [27].

2.7. Analysis of Potential Protein Interaction

To make the *SOD* protein interaction network, we utilized STRING 11.0 (https://string-db.org/cgi/input.pl accessed on 4 March 2023) tool for this purpose [43].

2.8. Expression Profiling of NcSOD Genes in Pollen and Ovule

The expression pattern of the *NcSOD* gene family was obtained from our own RNA-seq raw data (unpublished). All 9 *NcSOD* genes' expression levels were explored in 1 day mature pollen, and 0, 1, 2, and 3 days mature ovule. The expression heatmap was constructed using TBtools (V 1.068, https://github.com/CJ-Chen/TBtools/ accessed on 9 March 2023), in which the color bar from light yellow to a dark red exhibited less to high levels of expression, and light blue to dark blue shows less or no expression of *NcSOD* genes.

2.9. Plant Materials and Abiotic Stresses

To examine how *NcSOD* members respond to different abiotic stresses, we grew *N. colorata* mature plants in water tub filled with tap water under an open environment. The plants were then subjected to various stress treatments, including 250 mM NaCl, 200 µM $CuSO_4$, and 2.5 mM $CdCl_2$, as well as being treated with cold stress at 8 °C and heat stress at 42 °C. Each treatment was performed with three independent biological replicates, and each sample was collected from at least five individual plants. Leaves from the plantlets were collected at 0, 2, 4, and 6 h for the salt, heat, cold, and heavy metal stress experiments. After collection, all samples were instantly frozen in liquid nitrogen and preserved at −80 °C until total RNA isolation.

2.10. RNA Isolation and Real-Time Quantitative PCR Expression Analysis

RNA extraction was performed using the RNAprep Pure Plant Kit (TIANGEN, Beijing, China). The concentration of the samples was determined using a NanoDrop 2000 C spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The genomic DNA, was removed by DNase I treatment, followed by cDNA synthesis using the QuantiTect Reverse Transcription Kit (Qiagen, Shanghai, China). The RT-qPCR expression was performed on the Roche LightCycler 96 PCR system, following the recommended guidelines for the ChamQTM SYBR RT-qPCR Master Mix (Vazyme Biotech Co., Ltd., Sanya, China). For each RT-qPCR, the expression level of the actin gene in *N. colorata* was employed to standardize the RNA samples. Three biological replicates for each sample were employed for RT-qPCR, analysis with actin as internal control. Gene-specific primers for *NcSODs* and Nc-actin in the RT-qPCR system were designed using the online NCBI Primer-BLAST Program and their specificity was confirmed using the Oligo Calculator online tool (http://mcb.berkeley.edu/labs/krantz/tools/oligocalc.html accessed on 5 April 2023). The primers were synthesized by NANSHAN BIOTECH, (Sanya), and listed in (Table S1). The $2^{-\Delta\Delta CT}$ method was used to analyze the RT-qPCR gene expression data [44].

3. Results

3.1. Genome-Wide Analysis of SOD Gene Family in Four Water Lily Species

In this comparative study, we discovered 43 *SOD* genes in the genomes of four water lily species. Protein sequences of eight *A. thaliana* (*AtSODs*) were used as queries and removed the repetitive redundant sequences (Table S2), and 9–15 genes were obtained

for each species, for example three diploid water lilies *N. colorata* (9 *SODs*), *N. thermarum* (10 *SODs*), *N. minuta* (9 *SODs*), and a tetraploid water lily *N. mexicana* (15 *SODs*) (Table 1; Table S3). After conducting domain scrutiny, we identified 15 proteins with a *Cu/Zn-SODs* domain (Pfam; 00080), 19 with a *Fe-SODs* domain (Pfam; 00081), and 9 with a *Mn-SODs* domain (Pfam; 02777) in water lily species. These results are consistent among all species and contain all *SOD* genes and domains with sub-families.

Table 1. Characteristics of the *SOD* genes from four water lily species.

Plant Species	Transcript ID	Gene Name	Pfam Domains	Protein Length (A.A)	Functional Annotations	MW (kDa)	pI	Subcellular-Localization	GRAVY
Nymphaea colorata	Nycol.F01435	NcCSD1	PF00080	161	Cu/Zn-SOD	16.337	7.19	Cytoplasmic	−0.121
	Nycol.E01211	NcCSD2	PF00080	170	Cu/Zn-SOD	17.134	5.72	Cytoplasmic	−0.108
	Nycol.L00920	NcCSD3	PF00080	223	Cu/Zn-SOD	22.902	5.96	Chloroplast	0.025
	Nycol.A03620	NcFSD1	PF00081	198	Fe-SOD	23.178	6.23	Mitochondrial	−0.409
	Nycol.B01638	NcFSD2	PF00081	275	Fe-SOD	31.321	9.27	Chloroplast	−0.328
	Nycol.D01220	NcFSD3	PF00081	239	Fe-SOD	26.823	5.66	Chloroplast	−0.437
	Nycol.D01221	NcFSD4	PF00081	308	Fe-SOD	35.007	5.31	Chloroplast	−0.618
	Nycol.J01291	NcFSD5	PF00081	249	Fe-SOD	27.667	7.86	Chloroplast	−0.335
	Nycol.L00218	NcMnSD1	PF02777	260	Mn-SOD	29.073	7.71	Mitochondrial	−0.332
Nymphaea mexicana	NM3G27.30	NMCSD1	PF00080	135	Cu/Zn-SOD	13.478	5.45	Cytoplasmic	−0.246
	NM27G140.23	NMCSD2	PF00080	177	Cu/Zn-SOD	18.221	6.82	Cytoplasmic	−0.071
	NM26G14.21	NMCSD3	PF00080	117	Cu/Zn-SOD	12.166	6.41	Cytoplasmic	0.156
	NM16G174.41	NMCSD4	PF00080	135	Cu/Zn-SOD	13.478	5.45	Cytoplasmic	−0.246
	NM15G96.16	NMCSD5	PF00080	253	Cu/Zn-SOD	26.721	4.68	Cytoplasmic	−0.091
	NM15G40.65	NMFSD1	PF00081	231	Fe-SOD	25.785	6.8	Mitochondrial	−0.298
	NM9G74.16	NMFSD2	PF00081	259	Fe-SOD	29.11	6.39	Chloroplast	−0.324
	NM5G64.29	NMFSD3	PF00081	259	Fe-SOD	29.12	7.02	Chloroplast	−0.306
	NM6G47.44	NMFSD4	PF00081	306	Fe-SOD	34.951	5.39	Chloroplast	−0.588
	NM5G249.61	NMFSD5	PF00081	297	Fe-SOD	33.521	5.34	Chloroplast	−0.607
	NM14G120.45	NMMnSD1	PF02777	259	Mn-SOD	28.748	8.7	Mitochondrial	−0.284
	NM8G53.44	NMMnSD2	PF02777	271	Mn-SOD	31.044	9.05	Chloroplast	−0.302
	NM7G151.45	NMMnSD3	PF02777	247	Mn-SOD	28.178	7.09	Chloroplast	−0.323
	NM5G249.14	NMMnSD4	PF02777	236	Mn-SOD	26.48	5.38	Cytoplasmic	−0.408
	NM6G47.40	NMMnSD5	PF02777	240	Mn-SOD	26.914	5.39	Cytoplasmic	−0.414
Nymphaea minuta	Nmin13g00218	NminCSD1	PF00080	160	Cu/Zn-SOD	16.397	6.86	Cytoplasmic	−0.15
	Nmin06g00456	NminCSD2	PF00080	122	Cu/Zn-SOD	12.78	8.88	Cytoplasmic	−0.115
	Nmin08g00862	NminCSD3	PF00080	320	Cu/Zn-SOD	33.427	5.13	Cytoplasmic	0.104
	Nmin08g01659	NminFSD1	PF00081	231	Fe-SOD	25.827	6.8	Mitochondrial	−0.348
	Nmin00g05403	NminFSD2	PF00081	237	Fe-SOD	26.373	7.17	Cytoplasmic	−0.278
	Nmin02g01006	NminFSD3	PF00081	259	Fe-SOD	29.041	7.71	Mitochondrial	−0.303
	Nmin05g01512	NminFSD4	PF00081	362	Fe-SOD	40.905	5.15	Cytoplasmic	−0.552
	Nmin01g00323	NminMnSD1	PF00081	230	Mn-SOD	24.608	7.96	Cytoplasmic	−0.155
	Nmin05g01511	NminMnSD2	PF02777	238	Mn-SOD	26.783	5.51	Cytoplasmic	−0.421
Nymphaea thermarum	KAF3774564.1	NtCSD1	PF00080	161	Cu/Zn-SOD	16.75	5.36	Cytoplasmic	0.05
	KAF3777549.1	NtCSD2	PF00080	267	Cu/Zn-SOD	28.061	9.47	Mitochondrial	−0.023
	KAF3779999.1	NtCSD3	PF00080	267	Cu/Zn-SOD	28.053	4.86	Cytoplasmic	−0.122
	KAF3781552.1	NtCSD4	PF00080	158	Cu/Zn-SOD	17.099	5.51	Cytoplasmic	−0.124
	KAF3786936.1	NtMnSD1	PF02777	248	Mn-SOD	27.721	7.86	Mitochondrial	−0.352
	KAF3791217.1	NtFSD1	PF00081	274	Fe-SOD	31.403	9.3	Mitochondrial	−0.343
	KAF3782520.1	NtFSD2	PF00081	259	Fe-SOD	28.995	7.71	Mitochondrial	−0.285
	KAF3793027.1	NtFSD3	PF00081	306	Fe-SOD	34.987	5.39	Mitochondrial	−0.635
	KAF3793028.1	NtFSD4	PF00081	238	Fe-SOD	26.841	5.66	Cytoplasmic	−0.415
	KAF3779312.1	NtFSD5	PF00081	351	Fe-SOD	39.341	10.15	Cytoplasmic	−0.468

The biochemical and physiological characteristics of all *SODs* were investigated by calculating various parameters (Table 1). Protein length in the four representative water lily species ranged from 117 to 362 amino acids. Consequently, the molecular weight was found to be from 12.166 to 40.905 kDa. Most of the species investigated in this study displayed acidic properties, exhibiting pI values among 4.68 to 10.15. Furthermore, the predicted GRAVY values of *SOD* proteins were negative, showing that they are hydrophilic.

According to the subcellular localization prediction, the majority of water lily *CSDs* were found in the cytoplasm, while only a few localized in the chloroplast. Water lily *FSDs*

and *MnSDs* were specifically located in the chloroplast and mitochondria, respectively (Table 1).

Furthermore, an NCBI domain search was conducted to perform domain-based analysis on all *SOD* proteins to acquire data and TBtools was then used to make the domain structures. As a result of this analysis, the presence of the *SOD* family was verified on all chosen protein sequences (Figure 1).

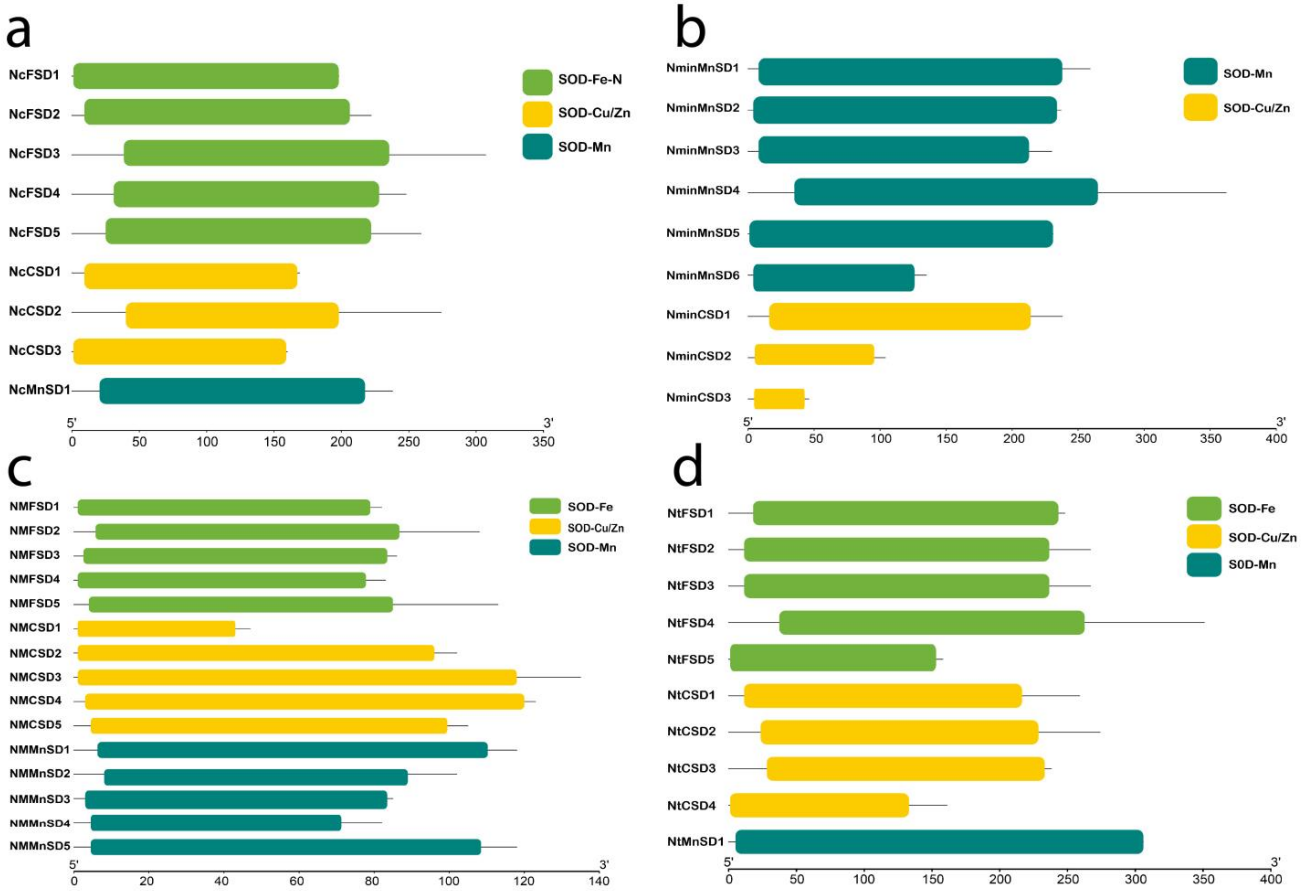

Figure 1. Symbolic *SOD* domain structures of four water lily species: (**a**) *Nymphaea colorata*; (**b**) *Nymphaea minuta*; (**c**) *Nymphaea mexicana*; and (**d**) *Nymphaea thermarum*. Among four species only *Nymphaea minuta* contains Mn and Cu/Zn, while others constitute all three subfamilies of *SOD*.

3.2. Phylogenetic Relationships and Conserved Motif Analysis in Representative Water Lily Species

To uncover the evolutionary relations among *SODs* in various plant species, a phylogenetic tree was created using the complete protein sequences. The present research investigated the evolutionary relationships among genes of *NcSODs*, *NtSODs*, *NminSODs*, *NMSODs*, and *AmtSODs*. By considering the domains (*Fe-SODs*, *Cu/Zn-SODs*, and *Mn-SODs*) and analyzing a phylogenetic tree, a total of 50 *SODs* were classified into two main groups (Figure 2a). To assess the structural diversity of water lily *SOD* proteins and predict their functions, we utilized the MEME software to analyze their full-length protein sequences and identify conserved motifs. By examining conserved motifs in the *SOD* family, this analysis confirmed the categorization and evolutionary connections among *SOD* genes within the water lily species. The investigation revealed that 10 conserved motifs were present in all species (Figure 2b). The detailed information about the identified motifs, including their names, sequences, and widths is displayed in Table S4. The number of conserved motifs in *SOD* proteins ranged from two to six, and their distribution aligned with the groups. The *Cu-SOD* and *Fe-SOD* groups had only one motif in two genes (Figure 2b). Furthermore, *MnSODs* and *FeSODs* were classified in the similar group and subcluster,

whereas *Cu/ZnSODs* were placed in a distinct group. Interestingly, motifs 1, 2, 4, and 9 were estimated to be particular to *Cu/Zn-SOD* whereas motifs 3, 5, 6, and 10 were exclusive to the *MnSODs* and *FeSODs* groups. In conclusion, the reliability of group arrangements was strongly supported by analyzing conserved motif patterns and phylogenetic relationships between water lily species. This suggests that water lily *SODs* proteins possess highly conserved amino acid residues within groups. Consequently, it is reasonable to infer that proteins with analogous structures and motifs may have similar efficient roles.

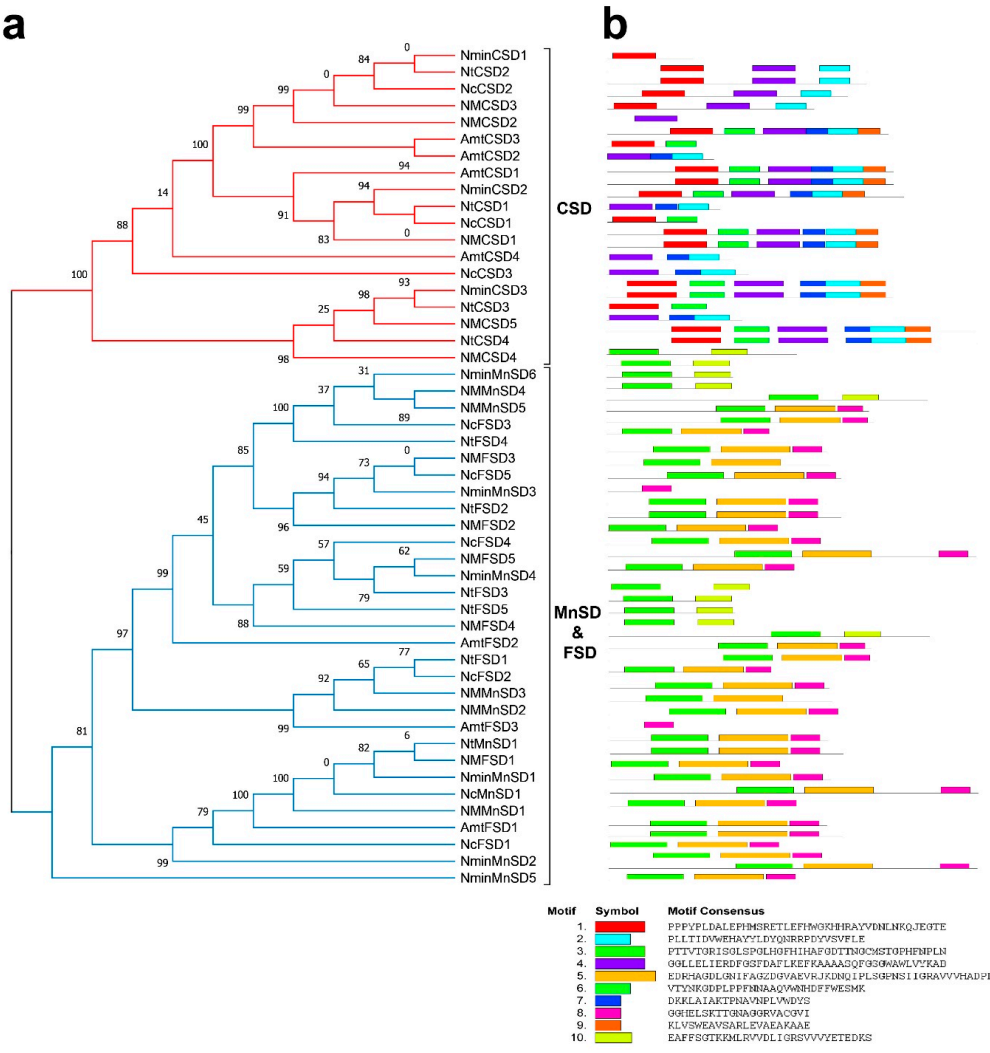

Figure 2. Classification of *SOD* genes according to their subfamilies: (**a**) A neighbor-joining phylogenetic tree; (**b**) Conserved motif analysis. The motifs supported the two subfamilies which are mentioned in tree. Reliability of group arrangements was strongly supported by analyzing conserved motif patterns and phylogenetic relationships between water lily species. Different types of motifs represented by differently colored boxes.

3.3. Analyses of Cis-Elements in Water Lily SOD Gene Promoters

Retrieving cis-regulatory elements from the promoter regions of water lily *SODs* enabled the differentiation of gene functions and regulatory roles. By using the PlantCARE database, an analysis was conducted on the 2 kb upstream region from the start codon of individual *SOD* gene. Based on current findings, the cis-elements were classified into three categories: light related, stress related, and hormone response elements (Figure 3).

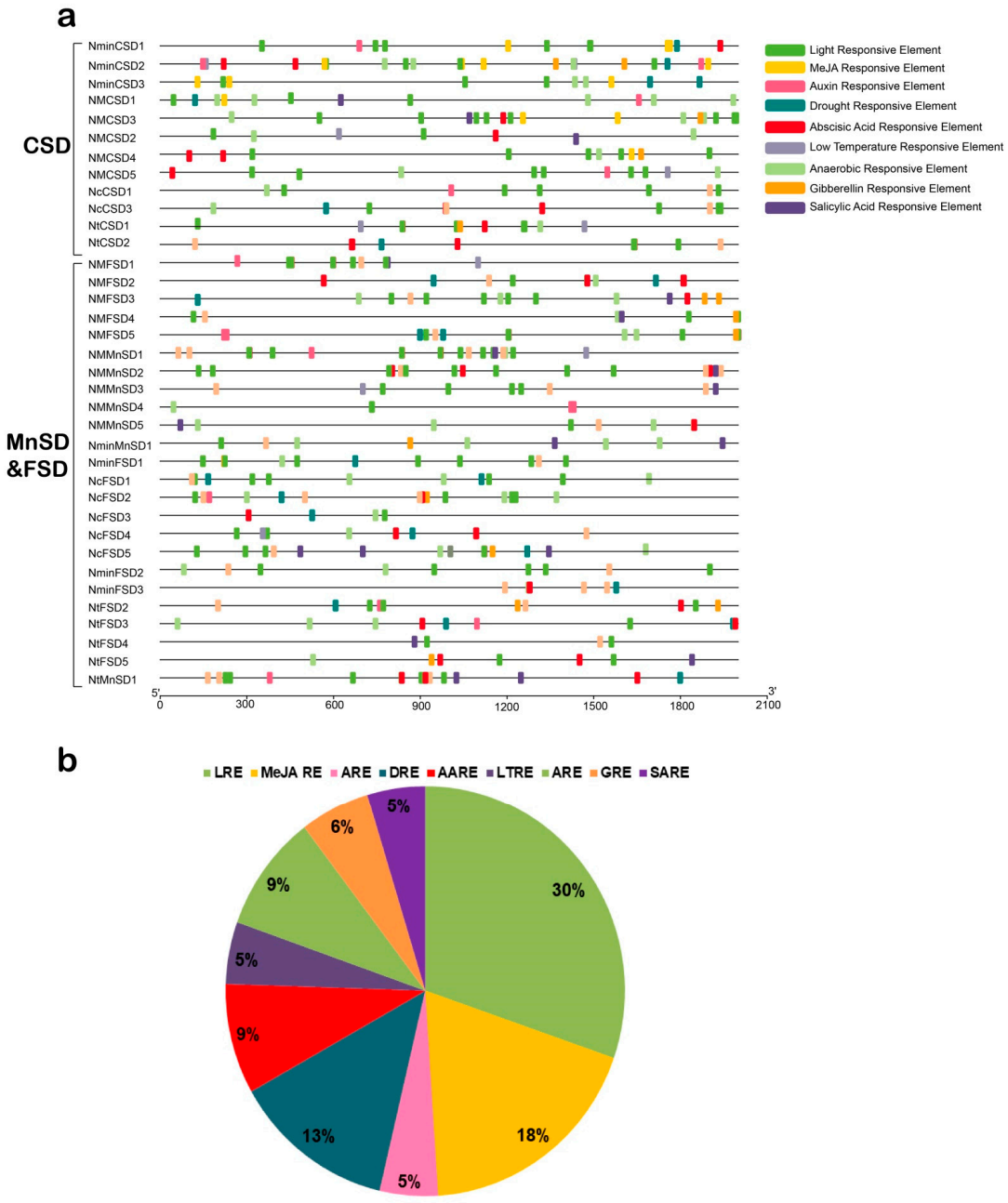

Figure 3. Exploration of abiotic-stress related cis-regulatory components in the water lily *SOD* promoter regions. (**a**) Various hormone-associated and stress-responsive elements are explored. (**b**) The size of pie chart corresponds to the ratio of the respective promoter element. Cis-elements that share functional similarity are represented by the same colors.

In this study, five phytohormone-associated elements (Salicylic acid (SA), Gibberellin, Auxin, Abscisic acid (ABA), and Methyl jasmonate (MeJA), were detected, including ABRE, TCA-components, TGACG-and CGTCA-motif, TATC-box, P-box, etc. Moreover, four stress-responsive components (drought, low-temperature, light, and anaerobic) were recognized, such as LTR, ARE, TCT-motif, LAMP-element, MBS, etc (Figure 3). Generally many light reactive components were detected to be extensively dispersed in same group species, and demonstrating the important part of water lily *SODs* in response to light stress. Comparative analysis of the findings revealed that the *SOD* promoter cis-elements in water lily species can exhibit a significant response to abiotic stresses and can play a role in regulating plant growth and development and stress response.

3.4. 3D Structure Analysis of Water Lily SOD Proteins

The examination of a protein's structure holds a great importance in understanding its function. We used SWISS MODEL and SOPMA online tools with the default search options, to predict the 3D structures of proteins. This study involved the prediction of three-dimensional models for four water lily proteins. The generated models were downloaded for the purpose of visualizing the 3D structures. The helices are represented by yellow, while the sheets or strands are represented by green (Figure 4). Proteins that fall under specific groups exhibit related structural evenness. The *MnSD* and *FSD* subfamilies share a nearly identical structure, with an equal amount of helices and sheets. Similarly, proteins in the *CSD* subfamily also possess an analogous structure.

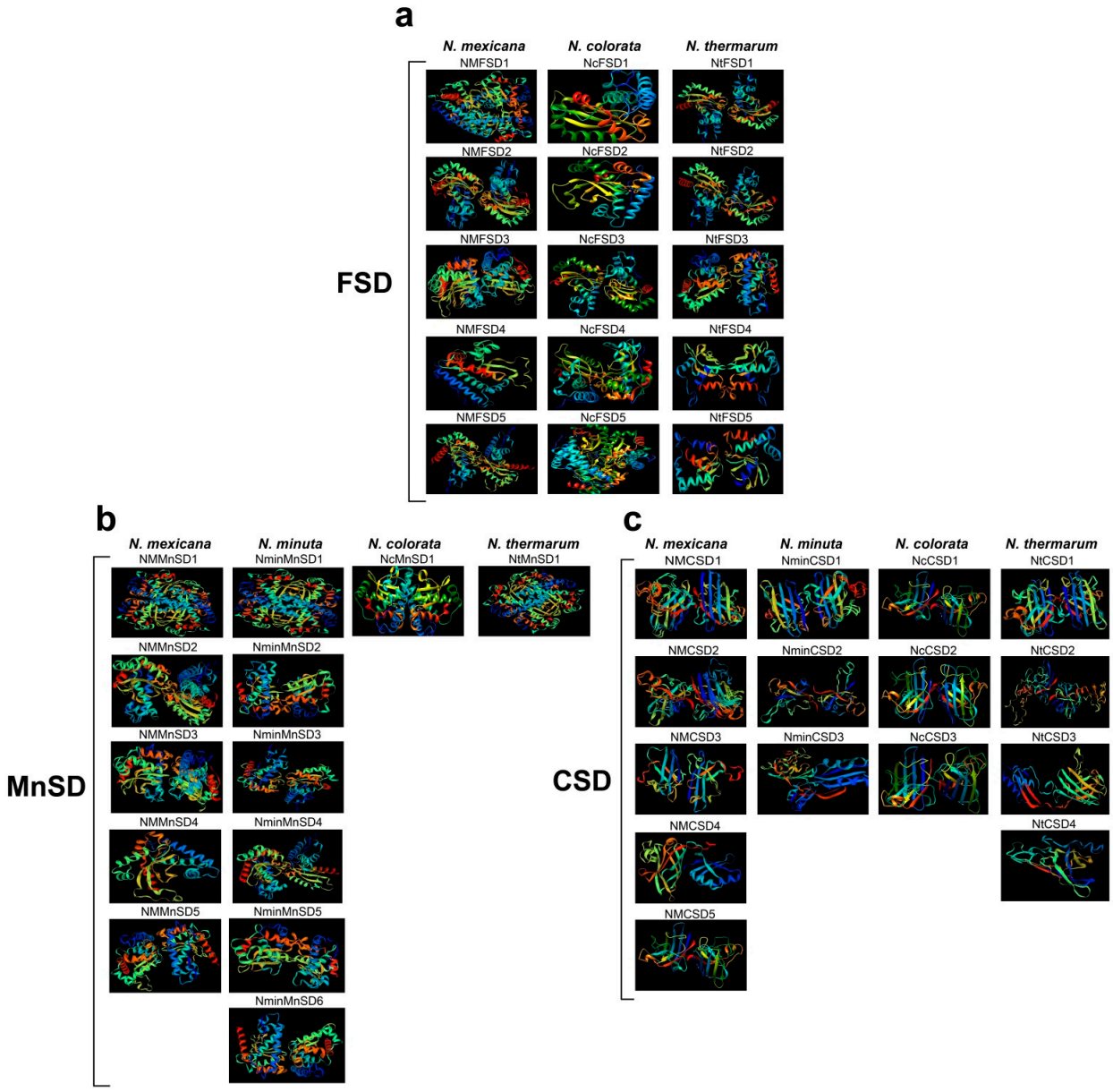

Figure 4. The 3D structures of four water lilies' (*Nymphaea colorata*, *Nymphaea thermarum*, *Nymphaea minuta*, and *Nymphaea mexicana*) *SOD* proteins categorized based on their sub families. (**a**) Represents the 3D structures of *FSD* subfamily; (**b**) Represents the 3D structures of *MnSD* subfamily; (**c**) Represents the 3D structures of *CSD* subfamily in all species. The final models are displayed, with diverse colors representing various sheets, domains, and helices. Note: In group (**a**) the *Nymphaea minuta* has no *FSD* subfamily.

3.5. Analysis of Exon-Intron Structure of NcSOD

The analysis of exon-intron structure of *NcSOD* genes was performed to elucidate the structural characteristics of species (Figure 5). *NcSOD* genes displayed varied exon-intron organizational patterns, with introns ranging from 5 (*NcFSD5*) to 9 (*NcFSD4*). The number of exons in *NcSOD* differs from 1 (*NcFSD1*) to 9 (*NcFSD2*). In one *NcFSD1* gene, introns are absent, and there is only one exon. The gene structure investigation revealed that the *SOD* gene family displayed a relatively conserved exon/intron organization.

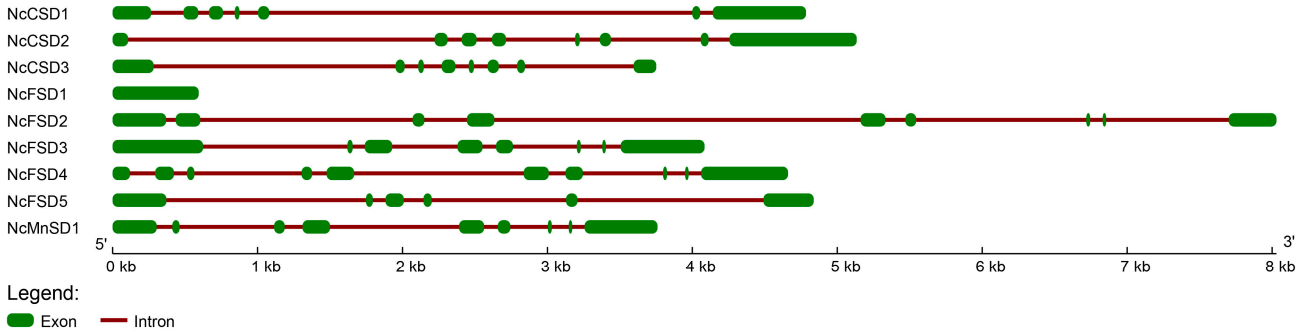

Figure 5. Gene structure of *NcSOD* shows conserved exon/intron organization.

3.6. Expression Examination of NcSOD Genes in Reproductive Organs

The *SOD* gene family has a crucial role in plant growth, development, and response to stress. In order to investigate their specific biological functions in *N. colorata*, we observed the expression patterns of the 9 *NcSOD* genes in pollen and ovules using our own unpublished RNA-seq raw data. Under normal growth conditions, not all predicted genes in the *N. colorata SOD* family were expressed. Our analysis revealed that *NcFSD3*, *NcFSD5*, and *NcMnSD1* were highly expressed in ovules at 0, 1, 2, and 3 days, while showing relatively lower expression in pollen on day 1 (Figure 6; Table S5). *NcCSD1* and *NcCSD2* were moderately expressed in ovules and pollen throughout all days. *NcFSD2* and *NcFSD4* showed a moderate expression in ovule but exhibited no expression in pollen. Both *NcFSD1* and *NcCSD3* showed no expression levels in both ovules and pollen. Generally, results exhibited that genes from all three subfamilies, i.e., Fe, Mn and Cu, play essential roles in *N. colorata* reproduction, growth, and development.

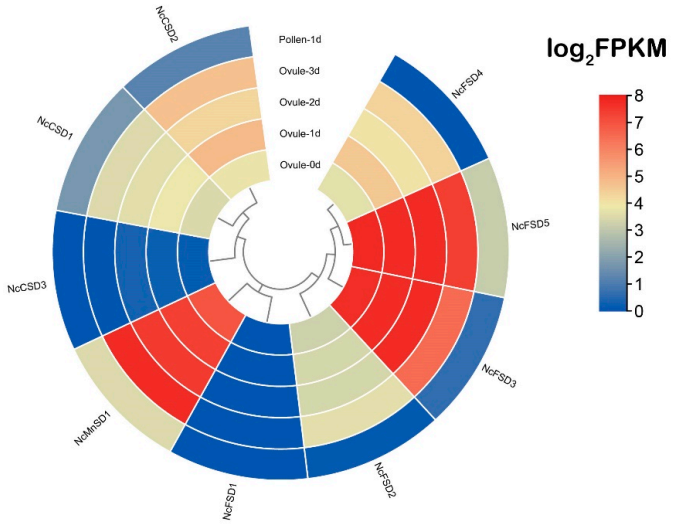

Figure 6. Expression of the *NcSOD* genes was analyzed in pollen and ovule samples at four different time-points: 0 d, 1 d, 2 d, and 3 d. The expression bar from light blue to dark blue shows less or no expression of *NcSOD* genes. The light yellow to a dark red color exhibited less to high level of expression of these genes.

3.7. Potential NcSOD Protein–Protein Interaction

The potential *NcSOD* protein–protein interaction was analyzed via "STRING"11.0 (https://string-db.org/cgi/input.pl accessed on 25 April 2023). As shown in Figure 7, among nine *NcSOD* genes, seven *SOD* proteins participate in strong interaction networks. Interestingly, we observed that different proteins co-regulate each other to respond to stress conditions. For example, *NcCSD3*, *NcFSD1* and *NcFSD4* are upregulated after 2 h under cold stress (Figure 8c). In water lilies, they potentially exert a regulatory function by forming protein complexes to improve cold tolerance and cope with various stresses.

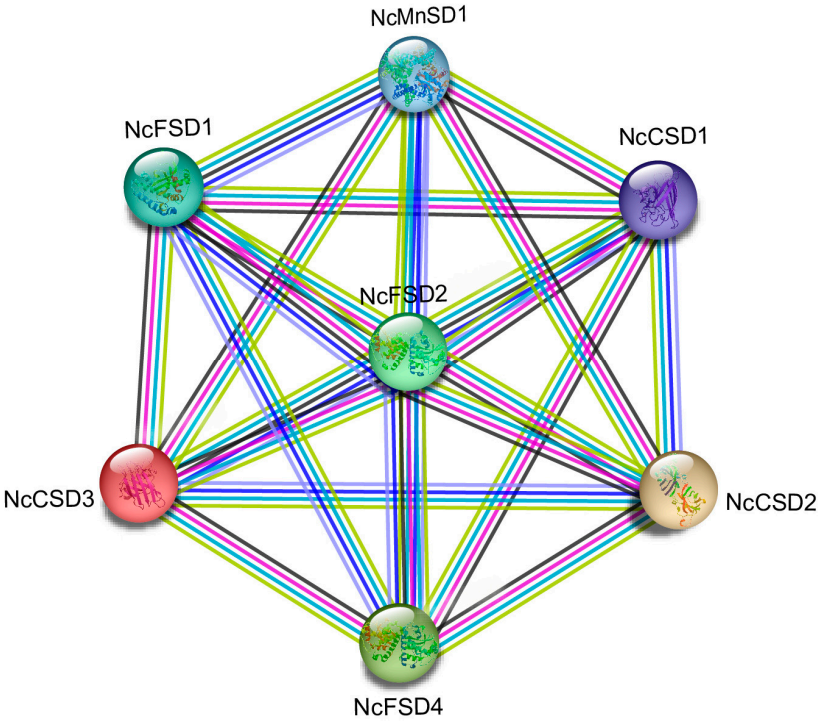

Figure 7. Protein interaction linkage among the seven *SOD* genes from *Nymphaea colorata*. Different colored lines show the interaction of the genes.

3.8. Real-Time Quantitative PCR (RT-qPCR) Analysis of NcSOD Genes under Abiotic Stresses

In order to know the function of *SODs*, we employed RT-qPCR to examine the expression patterns of the *SOD* gene under various stress conditions like salinity, heat, cold, and heavy metals (copper sulphate and cadmium chloride). Substantial variations were perceived in the expression levels of the *NcSOD* genes across various treatments, indicating a complex and dynamic nature of their expression patterns.

Salt treatment strongly induced the expression of all *NcCSDs*, peaking at 2 and 4 h. Our study found high expression of *NcCSDs* at 6 h, suggesting its involvement in salt response in *N. colorata*. Additionally, *NcMnSD1*, *NcFSD1*, *NcFSD2*, and *NcFSD5* were strongly induced and highly expressed under salt stress, implying their potential participation in the salt stress response (Figure 8a).

During the heat stress condition, the levels of expression of all *NcSOD* genes were upregulated at both 2 h and 4 h, with the exception of *NcFSD4*, which initially showed a decrease at 2 h and then an increase at 4 h. Following the 6 h treatment, the expression levels of the various genes showed variation (Figure 8b).

Under the cold treatment, distinct expression profiles were observed among all *NcSODs*. *NcCSD3*, *NcFSD1*, and *NcFSD4* exhibited upregulated expression at almost all time points, and reached their maximum expression at 2 and at 4 h, while *NcCSD1* and *NcCSD2* expression was slightly low. On the other hand, the remaining members showed down-regulated expression (Figure 8c).

NcSOD genes showed a positive response against heavy metals. In response to the copper sulphate treatment, the expressions of *NcSOD* genes exhibited variations at different time points. *NcCSD3* and *NcFSD1* displayed high expression at 2, 4 and 6 h, while other genes were low-expressed and different levels of expression were recorded. Furthermore, the highest expression levels for all genes were observed at the 6 h treatment (Figure 8d). During the cadmium chloride treatment, all *NcSOD* genes were upregulated at 2 h. Notably; *NcCSD3* exhibited consistently high expression levels across all time points, as shown in the figure. At the 4 h and 6 h treatment, all genes experienced a gradual increase and demonstrated robust expression levels (Figure 8e). These findings can enhance our comprehension of *NcSOD* genes across various environmental conditions.

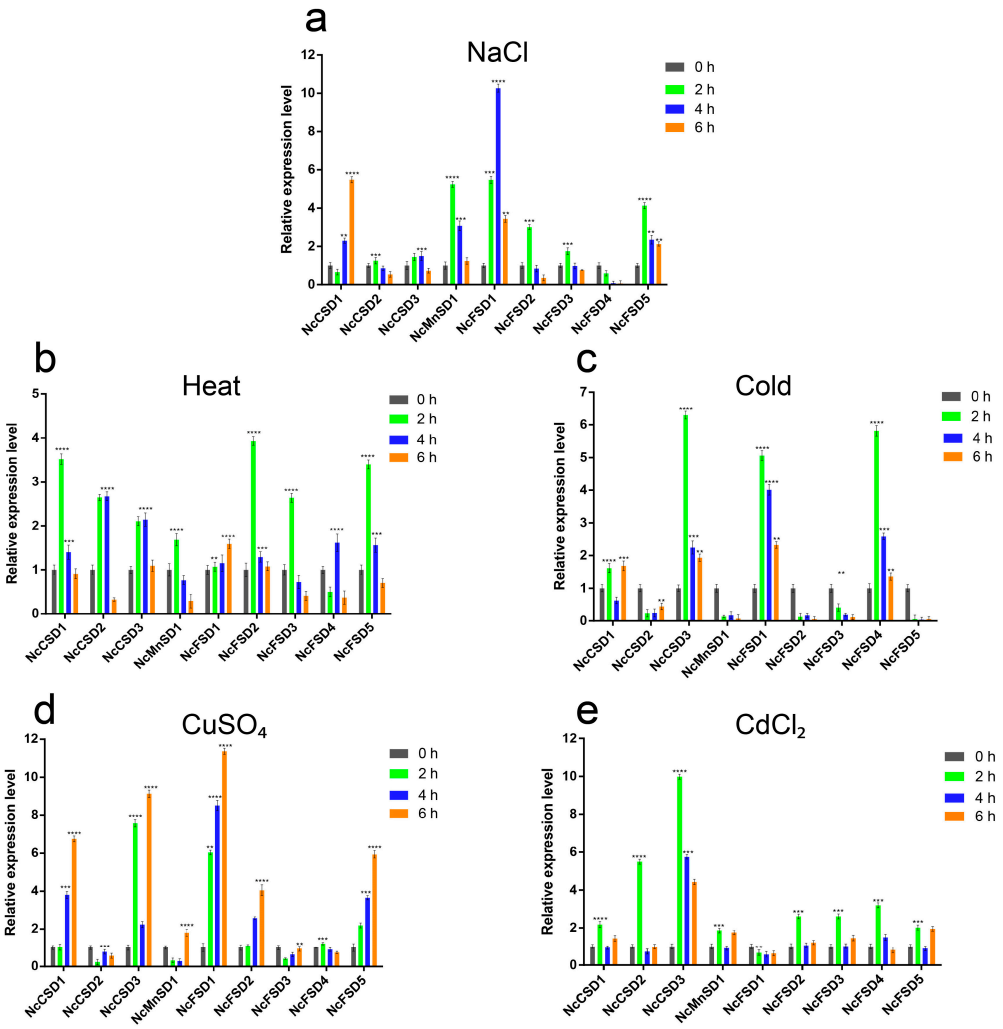

Figure 8. RT-qPCR analysis of the expression patterns of *NcSOD* genes in the leaves under various abiotic stresses: (**a**) salt; (**b**) heat; (**c**) cold; (**d**) $CuSO_4$; and (**e**) $CdCl_2$ stress (0 (CK), 2, 4, and 6 h). Data presented as means, ± standard error, $n = 3$; statistically significant differences are exhibited by asterisks ($p \leq 0.05$), according to the LSD test.

4. Discussion

Water lilies, with their significant ornamental, economic, medicinal, and cultural value, face challenges stemming from various abiotic stressors. However, through a combination of scientific research, technological innovations, and sustainable practices, we can optimize the growth and production of water lilies while preserving their aesthetic and functional benefits [45]. *SODs* have been demonstrated in recent studies to secure plants against abiotic stress factors including cold, drought, heat, salinity, ethylene, and abscisic acid [20–25].

In last several years, various plant species have been found to contain *SOD* family genes. For example, the aquatic sea grass (*Zostera marina)* has five *SOD* genes [26], *Medicago truncatula* [17] and barley (*Hordeum vulgare*) contain seven genes [28], sorghum (*Sorghum bicolor*) has eight genes [9], tomato (*Solanum lycopersicum*) has nine genes, and grapevine (*Vitis vinifera*) has ten genes [46]. Thus, we explored this family in four representative water lily species and checked the expression analysis in *Nymphaea colorata*.

In the present study, 43 *SOD* genes were identified in four water lily species, including 19 *Fe-SODs*, 15 *Cu/Zn-SODs*, and 9 *Mn-SODs* in all species (Table 1). The genes were classified into three major groups according to their binding domain (Figure 1). The number of genes in various water lily species was similar to that in cucumbers (*Cucumis sativus*) (9) and grapes (*Vitis vinifera*) (10), but fewer genes than the polyploidy crops cotton (*Gossypium hirustum*) (18) and wheat (*Triticum aestivum*) (26). However, the number of genes that encode Fe, Cu/Zn, and *Mn-SOD* differ among various species. For instance, *N. colorata* has three *Cu/Zn-SODs*, five *Fe-SODs*, and one *Mn-SOD*. The variation in *SOD* family member number could be due to the changes in genome sizes among species.

Previous research has shown that *Cu/ZnSODs* are consistently acidic, while *Fe-MnSODs* can be acidic or basic [42]. Most of the species investigated in this study displayed acidic properties. The results of subcellular localization of *SOD* proteins revealed that *Cu/Zn-SODs* are likely to be expressed in the cytoplasm, but *Mn-SODs* and *Fe-SODs* are expressed in mitochondria and chloroplasts, respectively, consistent with previous studies on *SODs* [18]. These distinct cellular locations enable *Fe-SODs*, *Cu/Zn-SODs*, and *Mn-SODs* to collaborate with one another to maintain the balance of free radicals in cells by functioning in different cellular parts.

Previous studies have indicated that the majority of cytoplasmic and chloroplast *SODs* comprise seven introns [13]. However, in our study it was revealed that *NcSOD* had a variable amount of exons ranging from 1 to 9. Furthermore, the number of introns in *NcSOD* varied from 5 to 9 (Figure 5). Notably, Figure 5 indicates that *NcFSD1* comprises only one exon and lacks introns. The variability in the gene structure of *SODs* may arise from the mechanisms involving the insertion or deletion of exons and introns [47].

Various research studies have demonstrated that *SOD* genes from distinct species are divided into three subfamilies [12]. In our study, we examined the evolutionary connections of *SOD* proteins in *N. colorata*, *N. thermarum*, *N. minuta*, *N. mexicana*, and *A. trichopoda* which categorized within three subfamilies (Figure 2a): *Fe*, *Cu/Zn*, and *Mn-SOD*. Within the phylogenetic tree, the three subfamilies were classified into two distinct groups: *Cu/ZnSODs* and *Fe-MnSODs*. *FeSODs* and *MnSODs* were clustered together, and a high bootstrap value separated them. The water lily *SODs* exhibited a strong clustering relationship with closely related species, while showing less affinity with outgroup. This suggests that this gene family has undergone relatively conserved evolution. The presence of specific domains suggests a basis for classifying these genes and the possibility of shared ancestral genes. In cotton (*Gossypium hirustum)*, the *MSD* and *FSD* families were found to have originated from a common ancestor, while the *CSD* subfamily developed independently. As a result, the two major groups expanded separately, as reported by Wang [48].

The analysis of promoters unveiled the existence of three main kinds of cis-components related to light, abiotic stress, and hormones response. Additionally, there were cis-elements associated with tissue-specific expression and developmental processes. Significant quantities of light-responsive cis-components were identified within *SOD* gene promoters, indicating the potential involvement of *SODs* in the abiotic stress response. Numerous investigations indicated the participation of *SOD* genes in the abiotic stress response across diverse plant species, including maize (*Zea mays*), *Pennisetum glaucum*, *Dendrobium catenatum*, and *Arabidopsis* [36,49–51]. Moreover, *SOD* gene promoters were found to contain a range of cis-elements linked to abiotic stress responses, including ARE, ABRE, MBS, ERE, Box-4, and TC-rich repeats. These cis-elements potentially contribute to the regulation of gene expression under diverse stress conditions. Among plant species like *Arabidopsis*,

banana (*Musa paradisiaca*), rice (*Oryza sativa*), tomato (*Solanum lycopersicum*), poplar (*Populus angusti-folia*), and cotton (*Gossypium herbaceum*), the majority of *SOD* genes exhibit inducibility in response to various abiotic stresses [4,36,52–55]. Under various abiotic and hormones stress situations the *SOD* gene family were recently identified by many researchers in various different types of plants like in *Brassica napus* [25], *Zostera marina* [26], *Salvia miltiorrhiza* [27], and *Hordeum vulgare* [28].

The 3D structures of water lily *SOD* proteins remain relatively conserved, similar to conserved domains, gene structure, and phylogeny. The findings indicate that water lily *SODs* genes potentially perform diverse functions across various tissues and genotypes. These results supported earlier anticipated three dimensional structures of *SODs* in *G. arboretum* [54], sorghum (*Sorghum bicolor*) [9], rice (*Oryza sative*) [52], and in *Gossypium raimondii*. The preceding investigation demonstrated that metal ion binding active sites and the formation of conserved disulfide bonds within individual subunits contribute to protein stability, specificity, and dimerization [56].

To determine the specific expression profiles of *NcSOD* genes during various stages of development, we utilized RNA-seq data from ovules and pollen at various developmental stages. By analyzing the RNA-sequencing data from *N. colorata*, and examined the expressions of the 9 *NcSOD* genes in pollen and ovules at different days post-anthesis. Our analysis revealed that *NcFSD3*, *NcFSD5*, and *NcMnSD1* were highly expressed in ovules at 0, 1, 2, and 3 days, while showing relatively lower expression in pollen on day 1 (Figure 6). While *NcFSD1* and *NcCSD3* showed no expression levels in both ovules and pollen that are in agreement with earlier findings [46].

The RT-qPCR analyses offer valuable insights into the potential role of *NcSODs* in reaction to diverse stresses. Our research revealed significant changes in the expression levels of nine *NcSODs* in varied stress environments, suggesting their crucial regulatory role in response to stress and possible functional interconnections. Overexpressing *Cu/ZnSODs* improved salinity stress resistance in *Triticum aestivum, Oryza sativa, Puccinellia tenuiflora*, and *Arabidopsis* [57,58]. Salt treatment strongly induced the expression of all *NcCSDs*, peaking at 2 h and 4 h. Our study found high expression of *NcCSD* at 6 h, suggesting its involvement in salt response in *Nymphaea colorata*. Additionally, *NcMnSD1*, *NcFSD1*, *NcFSD2*, and *NcFSD5* were strongly induced and highly expressed under salt stress, implying their potential participation in the salt stress response, similar to *NcCSDs*, in *N. colorata* (Figure 8a). Particularly, most *NcSOD* genes exhibited upregulation throughout heat treatment, with some displaying analogous expression patterns (Figure 8b). During cold treatment, distinct expression profiles were observed among all *NcSODs. NcCSD3, NcFSD1*, and *NcFSD4* exhibited upregulated expression at almost all time points, and reached their maximum expression at 2 h and at 4 h, while *NcCSD1* and *NcCSD2* were slightly expressed. On the other hand, the remaining members showed down-regulated expression (Figure 8c). These conclusions are consistent with previous findings, which reported a notable increase in *SOD* activity in rapeseed (*Brassica napus*) under cold stress conditions [59]. *NcSOD* genes showed a positive response against heavy metals. In response to the copper sulphate treatment, the expressions of *NcSOD* genes exhibited variations at different time points (Figure 8d). During the cadmium chloride treatment, all *NcSOD* genes were upregulated at 2 h. Notably, *NcCSD3* exhibited consistently high expression levels across all time points, as showed in the (Figure 8e). Nevertheless, certain genes within the nine *NcSODs* exhibited a pattern of initially increasing and subsequently decreasing expressions in response to both heavy metal treatments. Similar results were also reported in reaction to heavy metals treatment in several plants [27]. However, experimental verification is still needed to fully elucidate the roles of *NcSODs* regulatory networks, and their interaction mechanism, under different abiotic stresses.

5. Conclusions

In conclusion, this study conducted a comprehensive genome-wide analysis of the *SOD* gene family in four representative water lily species, resulting in the identification of 43 water lily *SODs*. The gathered information, encompassing exon-intron structure, cis-components, protein features, phylogenetic relations, and expression profiles of *N. colorata*, has shed light on the significant roles played by *NcSOD* genes in responding to salt, heat, cold, and heavy metal stresses. Findings of this systematic investigation provide a valuable resource for future functional research on *NcSOD* proteins in biological processes and lay a solid foundation for stress-resistant breeding of *N. colorata*. To further deepen our understanding of *NcSODs*' functions, our future studies will focus on gene engineering and comprehensive analysis, integrating genomics, transcriptomics, proteomics, and metabolomics.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/horticulturae9070781/s1, Table S1: the list of primer was used for gene expression analysis by RT-qPCR, Table S2: the protein sequences of SOD family genes in *Arabidopsis thaliana*, Table S3: the protein sequences of SOD family genes in four representative water lilies, Table S4: the information of identified 10 motifs in water lily SOD proteins, Table S5: The transcriptome data of *Nymphaea colorata* from ovules and pollen at various developmental stages.

Author Contributions: F.C. and W.U.K. conceived and designed this project. W.U.K. performed the analyses. L.U.K. and D.C. participated in the data analyses. W.U.K. carried out the experimental study and wrote the draft manuscript. F.C. and W.U.K. checked and revised the manuscript. All authors have read and agreed to the published version of the manuscript.

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