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Identification and Characterization of the Glutathione Peroxidase (GPX) Gene Family in Watermelon and Its Expression under Various Abiotic Stresses

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Abstract: Plant glutathione peroxidase (GPX) is an important antioxidant enzyme to maintain H_2O_2 homeostasis and regulate plant response to abiotic stress. In this paper, we present the first report of a genome-wide identification of *GPX* genes in watermelon. A total of six genes (*ClGPX1–ClGPX6*) were identified, which were unevenly located on four chromosomes of the watermelon genome. Based on phylogenetic analysis, the *GPX* genes of *Arabidopsis*, rice, cucumber, and sorghum were classified into four groups. Through analyzing the promoter regions of *ClGPX* genes, many development-, stress-, and hormone-responsive *cis*-acting regulatory elements were also identified. Expression pattern analysis by qRT-PCR indicated that all *ClGPX* genes were actively expressed in flowers and fruits, and exhibited relatively lower expression in other tissues, particularly roots and stems. In addition, the expression of *ClGPX* genes was significantly induced by salt, drought, and cold stresses, as well as abscisic acid (ABA) treatment at different time points, suggesting that they may be involved in response to abiotic stress and ABA. Taken together, our findings demonstrated that *ClGPX* genes might function in watermelon development, especially in flower and fruit tissue, as well as in response to abiotic stress and hormones.

Keywords: watermelon (*Citrullus lanatus*); *GPX*; gene family; gene expression; abiotic stress; phylogeny

1. Introduction

Plants are exposed to various abiotic stresses including drought, salt, and extreme temperature, which can induce the generation of reactive oxygen species (ROS) that seriously affect plant growth and development. To survive under these stresses, plants develop various enzymatic and non-enzymatic mechanisms to protect themselves from the adverse environmental effects caused by ROS [1,2]. Among the antioxidant enzymes, glutathione peroxidase (GPX, EC 1.11.1.9, also designated as GPX-like (GPXL) for the *Arabidopsis thaliana* (L.) isoforms) is an efficient ROS scavenger that belongs to the non-haeme thiol peroxidase family and uses glutathione (GSH) and thioredoxin (Trx) as reducing substrates [3]. Compared with mammal GPXs, plant GPXs have a preference to Trx instead of GSH as the reducing substrate [4,5]. In addition, plant GPXs contain cysteine (Cys) in their active sites, while most of mammal GPXs harbor a selenocysteine (SeCys) residue instead of Cys [3,6,7].

In recent years, increasing evidence has shown that plant GPX proteins played important roles in regulating plant responses to various abiotic stresses such as salt, drought, heat, cold, and oxidative stress. For example, eight *GPX* genes were identified in *Arabidopsis*, most of which were regulated to different degrees by salt and drought treatments not only in *Arabidopsis* wild-type (WT) plants but also

in *atgpx* mutants [8,9]. Both *atgpx3* and *atgpx8* mutants exhibited higher sensitivity to drought and osmotic stress than the WT plants [10,11]. Silencing the mitochondrial *GPX1* in rice impaired normal plant development and both photosynthesis and photorespiration under salinity conditions [12,13]. *OsGPX5*-knockdown plants also displayed enhanced sensitivity to salt stress and impaired seed germination and plant development [14]. In *Pennisetum glaucum*, the *PgGPx* transcript was observably up-regulated in response to salinity and drought stresses, and the overexpression of *PgGPx* increased the tolerance against multiple abiotic stresses in *E. coli* and rice [6]. Additionally, the overexpression of *NnGPX* from *Nelumbo nucifera* also resulted in improved salt tolerance in rice [15]. Moreover, some *GPX* genes have also been found to regulate different plant growth and development processes, such as root architecture [13,16], shoot development [13,17], stomatal control [18,19], regeneration [17,20], photosynthesis [12], immune responses [18], and hormone signaling [16,19,21]. In general, the above studies indicated that plant *GPX* genes played vital roles in regulating plant development, stress response, and hormone signaling.

The *GPX* gene family has been intensively identified and investigated in many plant species, including *Arabidopsis thaliana* (L.) [22], *Lotus japonicus* [23], *Thellungiella salsuginea* [24], *Oryza sativa* [9], *Gossypium hirsutum* [25], *Cucumis sativus* [5], *Sorghum bicolor* [26], and *Triticum aestivum* [27]. To obtain a more comprehensive understanding of the *GPX* gene family in watermelon, we identified six watermelon *GPX* genes in silico and analyzed their tissue-specific expression as well as their functions in response to various abiotic stresses including salt, drought, and cold stresses, as well as abscisic acid (ABA) treatment. The findings may provide valuable information for functional studies of watermelon *GPX* genes in the future.

2. Materials and Methods

2.1. Identification and Sequence Analysis of ClGPXs

To comprehensively annotate *GPX* genes in watermelon, all reported GPX protein sequences, including eight in *Arabidopsis* and five in rice, were retrieved from the *Arabidopsis* Information Resource (http://www.Arabidopsis.org) and the Rice Genome Annotation Database (http://rice.plantbiology.msu.edu/). The 13 sequences were blasted against the watermelon genome database (Version1, http://www.icugi.org) using the BLASTP program with default settings to obtain putative GPX sequences in watermelon. Additionally, the hidden Markov model (HMM) profile of the GPX domain (Pfam: PF00255) from the Pfam database (http://pfam.xfam.org/) was used to identify candidate GPX sequences. The e-value was set as 1×10^{-5}. After exclusion of the redundant sequences, the remaining sequences were determined by Pfam.

2.2. Multiple Sequence Alignments and Phylogenetic Analysis

Glutathione peroxidase from watermelon, *Arabidopsis*, rice, cucumber, and sorghum were aligned using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) with default settings. Based on the neighbor-jointing (NJ) method, a neighbor-joining phylogenetic tree was constructed with the help of the MEGA 5.0 software program using the maximum-like hood method with a bootstrap value of 1000 [28].

2.3. Gene Structure Analysis and Chromosomal Location

Gene structure (including exons and introns) analysis of the *GPX* genes was performed using the gene structure display server (GSDS, http://gsds.cbi.pku.edu.cn/) by aligning their coding sequence (CDS) to the genomic DNA (gDNA) sequences. Chromosomal location of *GPX*s was determined by the information obtained from the Cucurbit Genomics Database and was then visualized in a Circos map using CIRCOS software (http://circos.ca).

2.4. Prediction of Promoter Cis-Acting Regulatory Elements

Cis-elements were identified within the 1500-bp genomic DNA sequence before the start codon (ATG) for each gene from the watermelon genome database. The plant putative *cis*-elements in the promoter regions of the watermelon *GPX* genes were identified using the Plant Cis-Acting Regulatory Element (PlantCARE) (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

2.5. Plant Materials and Treatments

Watermelon (*Citrullus lanatus* (Thunb.) Matsum. & Nakai) 'Xinong-8' variety was used in this study. For tissue-specific analysis, germinated seeds were directly sown in the greenhouse in pots containing a peat–vermiculite mixture (23:1, *v*/*v*) obtained from Jiangxi Agriculture University, Nanchang, China (115°83′ E, 28°76′ N), which were placed under the following conditions: a 12-h photoperiod, a temperature of 25/19 °C (day/night) temperature, and a light intensity of 600 μmol·m^{-2}·s^{-1}. The samples of different watermelon tissues (the root, stem, expanding leaf, mature leaf, stem apex, fruit, and flower) were sampled separately from two-month-old watermelon plants.

For drought and salt stress treatments, watermelon seedlings at the four-leaf stage were cultured in Hoagland solutions containing PEG-6000 (20%, *w*/*v*) and 200 mM NaCl, respectively, under a photoperiod of 12 h at 25 °C (day) and 12 h at 19 °C (night), a photosynthetic photon flux density (PPFD) of 200 μmol·m^{-2}·s^{-1} supplied from fluorescent tubes, and a relative humidity (RH) of 70% in growth chambers. The low-temperature treatment was carried out at 4 °C under the same photoperiod and light conditions. For ABA treatment, 100 μM ABA was sprayed on the four-true-leaf watermelon seedlings. The leaves were sampled at 0, 1, 3, 9, and 24 h after treatments. All samples were frozen in liquid nitrogen and used for the following expression analyses.

2.6. RNA Extraction and Quantitative RT-PCR (qRT-PCR)

Total RNA from all samples was extracted using the total RNA Miniprep Kit (Axygen Biosciences, Union City, CA, USA) according to the manufacturer's protocol. Total RNA (1 μg) was reverse-transcribed for the synthesis of cDNA using the ReverTra Ace qPCR-RT Kit (Toyobo, Osaka, Japan) according to the manufacturer's instructions. qRT-PCR was performed using the iCycler iQTM Real-time PCR Detection System (Bio-Rad, Hercules, CA, USA) with SYBR Green PCR Master Mix (Takara, Tokyo, Japan). The primers used in this analysis are described in Supplementary Table S1. The watermelon *Actin* gene (Cla007792) was used as an internal reference. Each treatment was repeated using three independent biological replicates and three technical replicates. The relative expression levels of *GPX* genes were calculated following the $2^{-\Delta\Delta Ct}$ method according to the method used by Livak and Schmittgen [29].

2.7. Statistical Analysis

Data were statistically analyzed using analysis of variance (ANOVA), and the means were tested for significant treatment differences ($p < 0.05$) using Tukey's test.

3. Results

3.1. Identification of GPX Family Members in Watermelon

Based on BLAST searches against the watermelon genome using sequences of *Arabidopsis* and rice GPX proteins as queries, a total of six putative *GPX* genes were identified, which were renamed as *ClGPX1*–*ClGPX6* based on their order on the chromosomes (Table 1). All of them contained the GPX protein family domain (PF00255), indicating that they belong to the *GPX* gene family. The gDNA length of the *ClGPX*s ranged from 873 bp (*ClGPX4*) to 3188 bp (*ClGPX3*), encoding polypeptides ranging from 124 to 241 amino acids. The deduced ClGPX proteins had theoretical molecular weight (MW) values ranging from 13.82 to 26.38 kDa and calculated isoelectric point (pI) values from 5.17

to 9.14 (Table 1). In addition, the grand average of hydropathicity (GRAVY) values of the ClGPX proteins ranged from −0.459 (ClGPX5) to −0.082 (ClGPX3), indicating that all of them were hydrophilic proteins. The prediction of subcellular localization using WoLF PSORT suggested that the ClGPX proteins were mainly located in chloroplast, as well as in some other locations, such as nucleus, cytosol, mitochondria, or extracellular space (Table 1).

3.2. Comparison of Deduced GPX Proteins in Watermelon

The identities between two ClGPX proteins ranged from 15.58% (ClGPX1 and ClGPX4) to 73.05% (ClGPX2 and ClGPX5) (Table 2). To characterize the ClGPX proteins, a multiple sequence alignment of the amino acid sequences of ClGPXs was carried out with Clustal Omega. The results showed that all of these GPXs contained three completely conserved Cys residues as well as three highly conserved motifs, namely, GKVLLIVNVASXCG (GPX signature 1), ILAFPCNQF (GPX signature 2), and WNFXKF, except for ClGPX1 and ClGPX4, which missed the first Cys residue, and lacked the GPX signature 2 and WNFXKF, respectively (Figure 1). In addition, three other conserved domains and four highly potential catalytic sites (Cys, Gln, Trp, and Asn) were also present in the amino acid sequences of the majority of the ClGPXs (Figure 1).

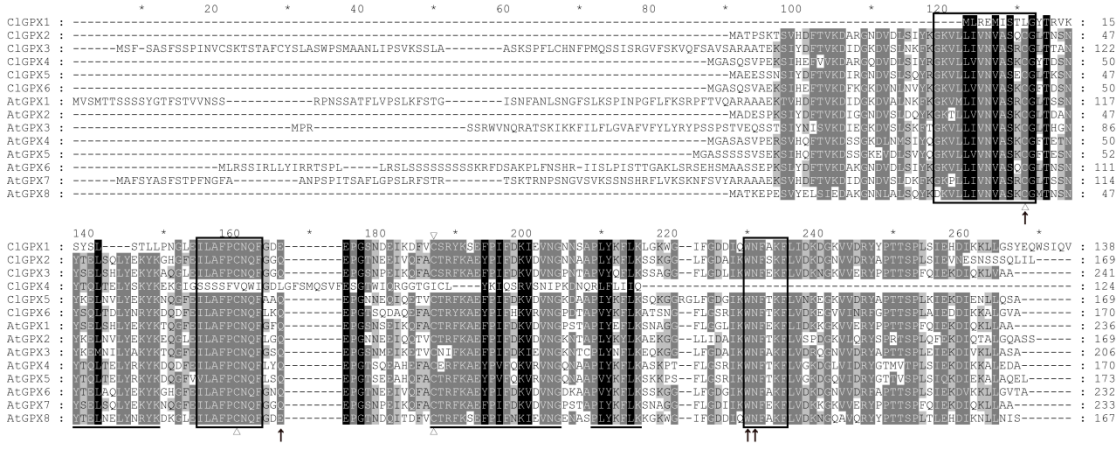

Figure 1. Multiple sequence alignment of GPX protein sequences from watermelon and *Arabidopsis*. Sequence alignments were obtained using Clustal Omega. The three boxes indicate the GPX signature motifs 1, 2, and WNFXKF. Three other conserved domains are underlined. Three conserved Cys residues replaced by Sec in mammalian GPXs are marked by triangles. *Arrows* represent the conserved amino acid residues (Cys, Gln, Trp, and Asn) involved in the potential catalytic sites.

Table 1. A list of watermelon glutathione peroxidase (*GPX*) gene family members and their related information.

Genes	Locus Name	Chromosomal Location (5′–3′)	gDNA Size (bp)	CDS Size (bp)	Protein Physicochemical Characteristics				Subcellular Prediction
					Length (aa)	MW (kDa)	pI	GRAVY	
ClGPX1	Cla011456	Chr1: 2581077 .. 2582952 (−)	1876	417	138	15.87	5.17	−0.332	Chlo/Mito
ClGPX2	Cla011457	Chr1: 2588184 .. 2589821 (−)	1638	510	169	18.63	5.69	−0.297	Chlo/Nucl
ClGPX3	Cla021039	Chr5: 24166857 .. 24170044 (−)	3188	726	241	26.38	9.14	−0.082	Chlo
ClGPX4	Cla006080	Chr7: 2894455 .. 2895327 (+)	873	375	124	13.82	8.91	−0.202	Chlo/Extr
ClGPX5	Cla010856	Chr7: 30465660 .. 30468005 (+)	2346	510	169	19.07	7.60	−0.459	Chlo/Nucl
ClGPX6	Cla014745	Chr9: 5467241 .. 5470257 (+)	3017	513	170	19.08	8.87	−0.342	Chlo/Cyto

The subcellular prediction of watermelon GPX proteins was performed with the WoLF PSORT tool (http://www.genscript.com/psort/wolf_psort.html). gDNA, genomic DNA; bp, base pair; CDS, coding sequence; aa, amino acid; MW, molecular weight; pI, isoelectric point; GRAVY, grand average of hydropathicity; Chlo, chloroplast; Extr, secreted; Nucl, nucleus; Cyto, cytosol; Mito, mitochondria.

Table 2. The percentage of ClGPX proteins' amino acid identity.

	ClGPX1	ClGPX2	ClGPX3	ClGPX4	ClGPX5	ClGPX6
ClGPX1	100					
ClGPX2	57.14	100				
ClGPX3	59.23	68.07	100			
ClGPX4	15.58	42.48	33.62	100		
ClGPX5	56.49	73.05	68.07	39.82	100	
ClGPX6	49.62	58.68	61.54	47.41	61.08	100

3.3. Phylogenetic Relationships of GPX Genes in Watermelon and Other Plant Species

To examine the evolutionary relationships among the GPXs, a neighbor-joining (NJ) phylogenetic tree was constructed using 32 GPX proteins from five different plant species. As a result, these GPX proteins could be clustered into four groups (Groups a–d) based on their sequence similarities (Figure 2). ClGPX1 and ClGPX3 fell into Group a; ClGPX3 fell into Group b; ClGPX4 fell into Group c; and ClGPX5 and ClGPX6 fell into Group d. It is noteworthy that ClGPX proteins were distributed in each group with homologs from the other four plant species, especially cucumber. In addition, the sequences of ClGPXs were more closely related to cucumber GPXs (CsGPXs) and *Arabidopsis* GPXs (AtGPXs) than to rice GPXs (OsGPXs) and sorghum GPXs (SbGPXs) (Figure 2).

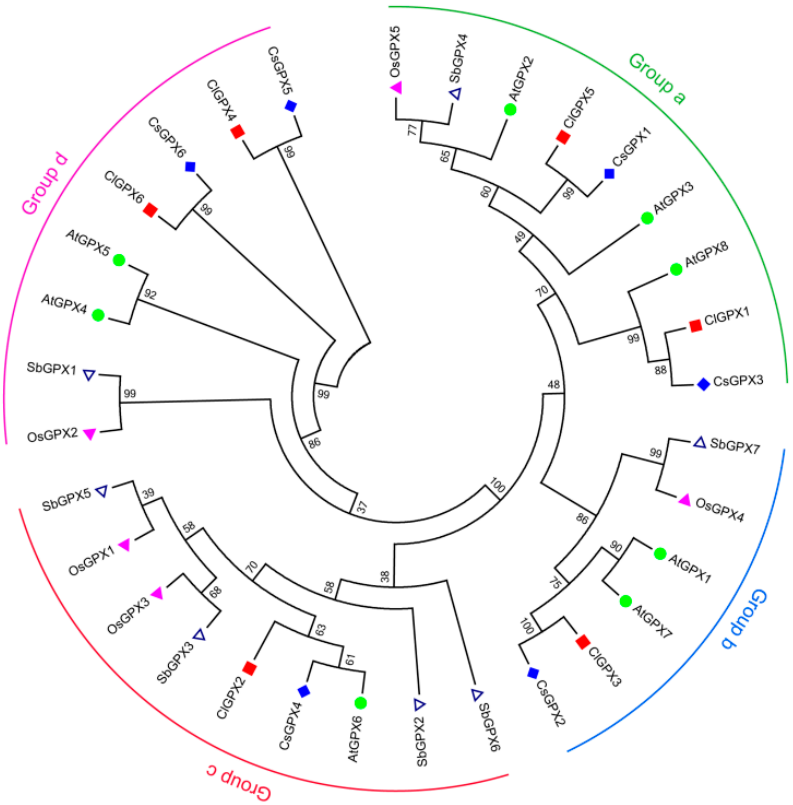

Figure 2. Phylogenetic relationships of the *GPX* gene family from watermelon and different plant species. The six cucumber, eight *Arabidopsis*, five rice, and seven sorghum GPX protein sequences were aligned by Clustal Omega and a phylogenetic tree was generated with the help of MEGA 5.0 using the neighbor-joining (NJ) method. The bootstrap value was set as 1000 replicates. The protein IDs of the GPX members are listed in Table S2.

3.4. Gene Structures and Chromosomal Localizations of ClGPXs in Watermelon

The gene structures of the *ClGPX* genes were determined using the GSDS by aligning the ORF sequences with the corresponding genomic sequences. As a result, *ClGPX3*–*ClGPX6* possessed six exons and five introns (Figure 3), which was similar to the gene structure of the *GPX* genes from *Arabidopsis* [22], *T. salsuginea* [24], cotton [25], and cucumber [5], suggesting a high degree of conservation in the gene structure among plant species. However, *ClGPX1* and *ClGPX2* contained only three and four introns, respectively.

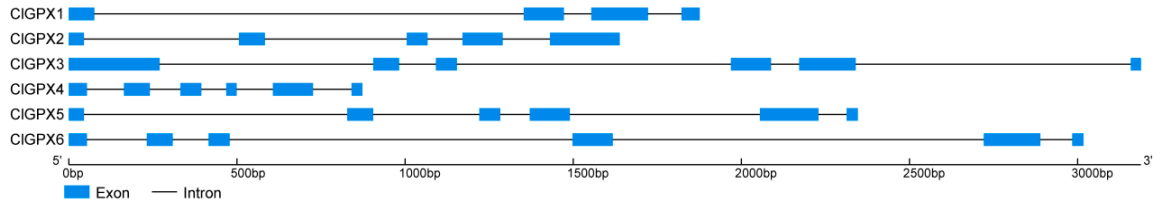

Figure 3. Exon-intron structure analysis of *ClGPX* genes in watermelon. Gene structure analysis was carried out with the gene structure display server (GSDS). The blue boxes and black lines indicate exons and introns, respectively. The lengths of exons and introns of each *ClGPX* gene were displayed proportionally and can be estimated using the scale at the bottom.

In silico mapping of *ClGPX*s on chromosomes revealed that these genes were randomly distributed across the four watermelon chromosomes. Among them, chromosomes 1 and 7 were found to contain two *ClGPX* genes, whereas chromosomes 5 and 9 possessed one gene, respectively. In addition, a tandem duplication event was detected in chromosome 1 (*ClGPX1* and *ClGPX2*) (Figure 4).

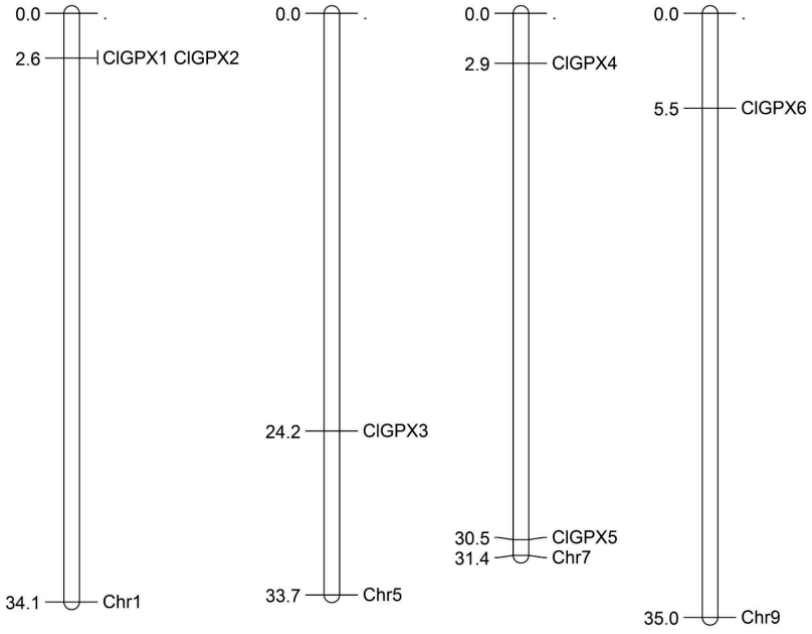

Figure 4. Chromosomal distribution of watermelon *GPX* genes. The chromosome numbers and its size are shown at the bottom of each bar. Chromosomal distances are presented in Mb.

3.5. Bioinformatics Analysis of ClGPX *Promoters*

A number of putative *cis*-acting regulatory elements were found in the promoter sequences of *ClGPX* genes by PlantCARE. For example, seven kinds of *cis*-elements related to different developmental processes, such as meristem development (CAT-box), circadian control (circadian), zein metabolism regulation (O2-site), endosperm development (GCN4_motif and Skn-1_motif), shoot development (as-2-box), and flavonoid biosynthesis (MBSII), were detected in the promoter regions of some *ClGPX* genes (Figure 5, Table S3), implying that *ClGPX* genes may have tissue-specific expression in watermelon. In addition, nine kinds of stress-responsive elements were present in the promoter regions of most *ClGPX* genes, such as heat stress-responsive element (HSE), MYB binding site involved in drought-inducibility (MBS), defense- and stress-responsive element (TC-rich repeats), element for maximal elicitor-mediated activation (AT-rich sequence), WRKY binding site involved in abiotic stress and defense response (W-box), low-temperature-responsive element (LTR), elicitor-responsive element (ELI-box3), wound-responsive element (WUN-motif), and anaerobic induction element (ARE).

It is worth noting that all of the promoter sequences of *ClGPX* genes possessed the *cis*-elements related to gibberellin (GARE-motif and/or P-box), implying that *ClGPX* genes are responsive to gibberellin (Figure 5). Moreover, several hormone-responsive *cis*-elements were also identified in the promoter regions of some *ClGPX* genes, such as CGTCA-motif, AuxRR-core, ABRE, ERE, and TCA-element, which reflect plant responses to methyl jasmonate (MeJA), auxin, ABA, ethylene, and salicylic acid, respectively.

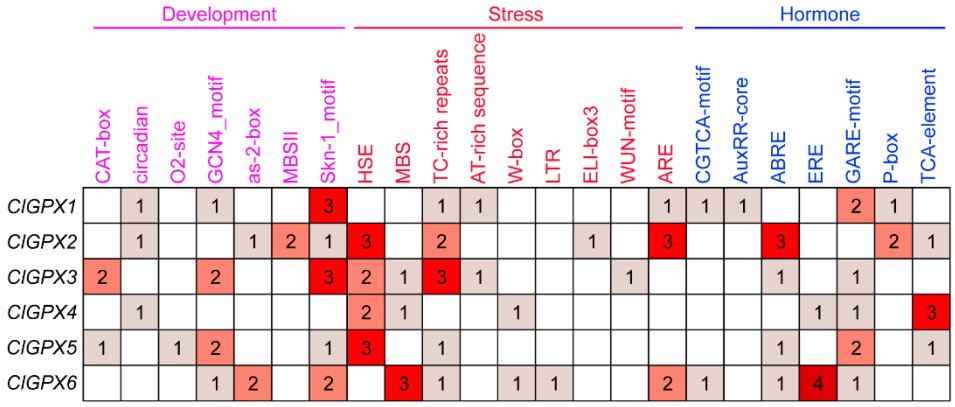

Figure 5. Prediction of *cis*-acting regulatory elements present in the promoters of *ClGPX* genes.

3.6. Tissue-Specific Expression of ClGPX Genes in Watermelon

To predict the possible functions of *ClGPX* genes in organ development, expression analysis in different tissues (mature and expanding leaf, root, stem, stem apex, flower, and fruit) was performed by qRT-PCR. The expression level of each gene in mature leaf was set as 1.0. The results showed that the six *ClGPX* genes were expressed in all tested tissues with divergent expression patterns. Among them, *ClGPX1* showed the highest expression in fruits, moderate expression in flowers and expanding leaves, and weak expression in other tissues, particularly the roots (Figure 6A). The expression patterns of *ClGPX2*, *ClGPX4*, *ClGPX5*, and *ClGPX6* were similar, with higher expression in flowers and fruits, and relatively lower expression in other tissues (Figure 6B,D–F). *ClGPX3* showed relatively higher expression in flowers and mature leaves, moderate transcript abundance in flowers and roots, and relatively lower expression in other tissues (Figure 6C). These results indicated that *ClGPX* genes may play important roles in various physiological and developmental processes of watermelon.

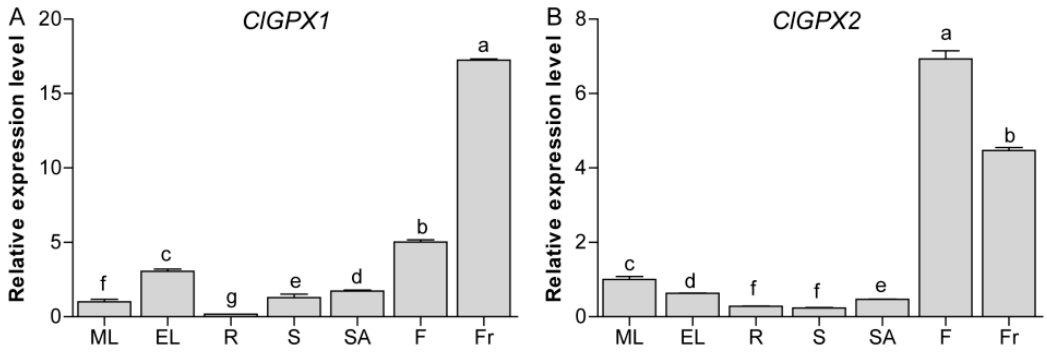

Figure 6. *Cont.*

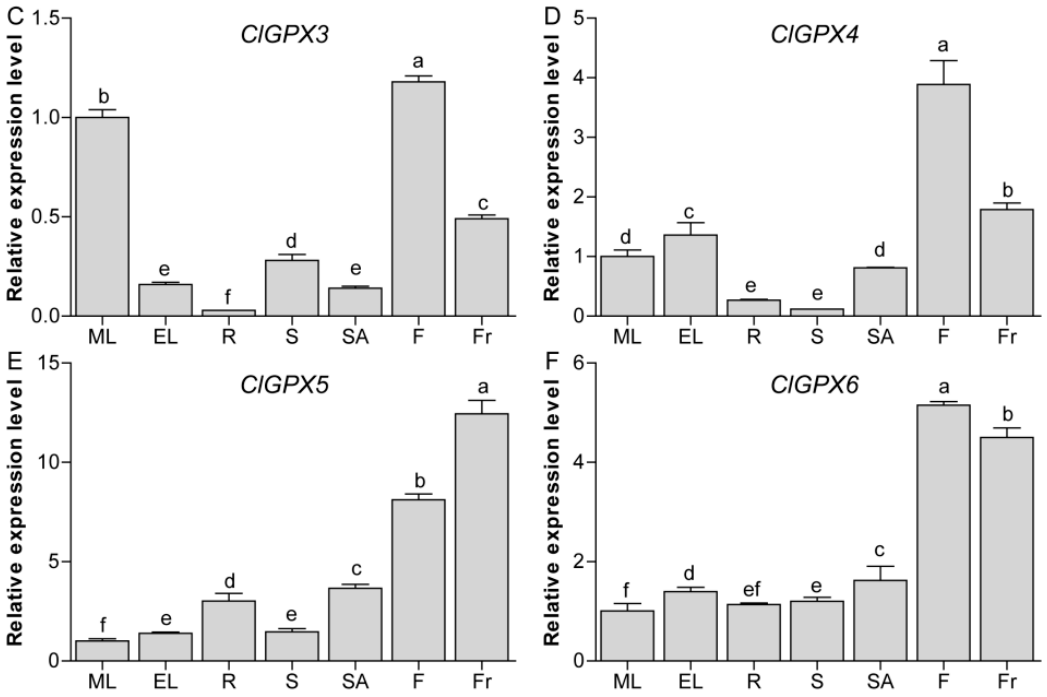

Figure 6. Expression profiles of *ClGPX* genes across different tissues in watermelon. The amount of *ClGPX* mRNA was normalized to that of *actin*. Error bars were SD of three biological replicates. Different letters indicate statistically significant differences (Tukey's test with a $p < 0.05$). ML, mature leaves; EL, expanding leaves; R, roots; S, stems; SA, stem apexes; F, flowers; Fr, fruits.

3.7. Expression Profiles of ClGPX Genes in Response to Abiotic Stress and ABA Treatments

To gain insights into the potential functions of *ClGPX* genes in response to abiotic stress, the expression of *ClGPX* genes was examined by qRT-PCR under cold, salt, drought, and ABA treatments. All *ClGPX* genes were induced but displayed differentially expression patterns under these conditions (Figure 7). Under cold stress conditions, the transcription of *ClGPX1* sharply increased at the early time point (1 h) and decreased at subsequent time points, whereas that of *ClGPX2* gradually increased and reached the maximum at 9 h, followed by a decrease at 24 h (Figure 7A). *ClGPX3–6* showed similar expression patterns under cold stress: their expression increased gradually and reached the maximum at 3 h, but decreased thereafter (Figure 7A). Under salt stress, the expression of all *ClGPX* genes was obviously up-regulated (Figure 7B). The expression of *ClGPX1–6* reached the maximum at 9 h (5.53-fold), 24 h (7.24-fold), 24 h (5.77-fold), 3 h (7.91-fold), 3 h (8.35-fold), and 3 h (2.86-fold), respectively (Figure 7B). Among them, the expression of *ClGPX2* and *ClGPX5* showed gradual increases at early time points (1 h and 3 h), followed by a decrease at 9 h, and then a significant increase again at 24 h (Figure 7B). All *ClGPX* genes were induced to different degrees by drought (PEG) treatment (Figure 7C). Among the six *ClGPX* genes, *ClGPX1* was dramatically up-regulated and its expression reached the highest level at 1 h (115.25-fold), which was remarkably higher than that of other *ClGPX* genes (Figure 7C). The transcription of *ClGPX2* was induced gradually and reached the highest level (19.74-fold) at 24 h, whereas *ClGPX3–6* reached their highest levels at 3 h (6.04-fold), 1 h (10.17-fold), 9 h (34.36-fold), and 9 h (5.71-fold), respectively (Figure 7C). Under ABA treatment, the expression of all *ClGPX* genes was induced gradually until 1 h or 3 h, and subsequently declined as the treatment continued (Figure 7D). These results indicated that *ClGPX* genes may play important roles in regulating abiotic stress response.

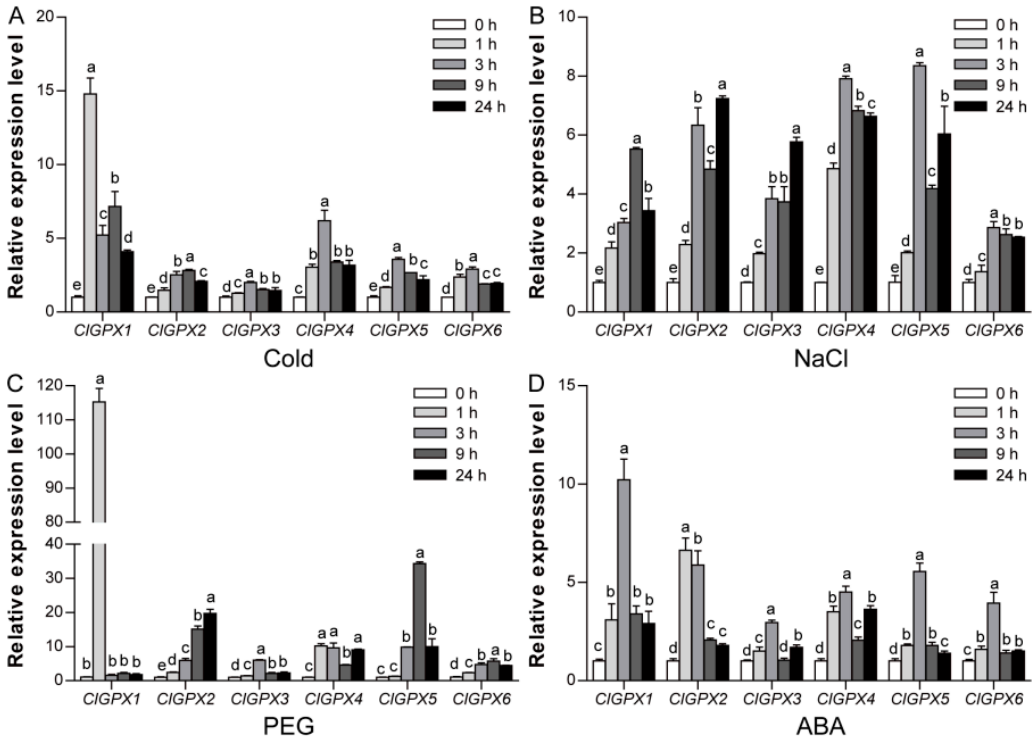

Figure 7. qRT-PCR analysis of the expression profiles of *ClGPX* genes in the leaves of watermelon under (**A**) cold, (**B**) salt, (**C**) drought, and (**D**) ABA treatments. The expression value of control sample (0 h) was normalized to 1. Error bars were SD of three biological replicates. Different letters indicate statistically significant differences (Tukey's test with a $p < 0.05$).

4. Discussion

GPX proteins have been reported to be encoded by a multigene family in many plant species. For example, 8 *AtGPX*s in *Arabidopsis* [9], 5 *OsGPX*s in rice [9], 13 *GhGPX*s in cotton [25], 6 *CsGPX*s in cucumber [5], and 14 *TaGPX*s in bread wheat [27] were identified using a bioinformatics approach. In this study, six *ClGPX* genes were identified in the watermelon genome. Although several segmental duplications have been found in different plant genomes [5,30], no segmental duplication event has been identified in the watermelon genome. In contrast, like in cucumber [5], a tandem duplication event was present between *ClGPX1* and *ClGPX2* in watermelon (Figure 4), indicating that tandem duplication makes major contributions to the expansion of the watermelon *GPX* gene family.

Similar to GhGPXs, no ClGPXs were predicted to be located in endoplasmic reticulum and peroxisome (Table 1), where ROS is produced at high levels, implying that some other antioxidant enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), or catalase (CAT) function in these cellular compartments [25]. Like other plant GPX proteins, the majority of ClGPX proteins in watermelon contain three conserved modules, namely GPX signature 1, GPX signature 2 and WNFXKF (Figure 1). In addition, the Cys residue located in the GPX signature 1 is the specific structure of selenium-independent plant GPXs [21], and is also present in most of the ClGPX proteins, except for ClGPX1, in which Cys is replaced by Leu (L). This phenomenon has never been reported before, suggesting that ClGPX1 may have particular functions in watermelon. The other two Cys residues were present in most of the ClGPX proteins except for ClGPX4, and were shown to be essential for Trx regeneration and enzyme catalysis [6]. Other evolutionarily conserved sequences such as Y(T/S/K)(Q/E)LXXLYX(K/R)YK, C(T/S)R(F/Y)K(A/S)E(Y/F)P(I/V)F(D/Q)K(V/I)(D/E/R)VNG and P(L/V/I)YKFLK, and active sites of enzymes including Gln (Q), Trp (W), and Asn (N), were also found in nearly all of the ClGPX protein sequences (Figure 1). These findings indicate that the protein

structures and active sites of GPXs are highly conserved among plant species, which is essential for the sensing of redox potential in plants [27,31].

The phylogenetic tree showed that GPXs from watermelon, cucumber, *Arabidopsis*, rice, and sorghum were classified into four groups (Figure 2), which was consistent with the classification in other plant species [24–27]. Interestingly, each group contained one or more GPXs from every plant species, and each ClGPX was highly related to its homologs in the other four plant species, especially in cucumber, implying that the common ancestor was composed of pairs of potential orthologs. In addition, the ClGPXs were closer to GPXs in dicots (cucumber and *Arabidopsis*) than to those in monocots (rice and sorghum) (Figure 2), indicating that the *GPX* genes may have originated before the divergence of monocot and dicot lineages and then evolved separately. Moreover, several members in the same group had the same subcellular localizations. For example, ClGPX3 was clustered together with AtGPX1, AtGPX7, SbGPX7, and other GPXs localized in chloroplast (Table 1, Figure 2) [18,26], implying that they may have similar functions in chloroplast. Furthermore, gene structure analysis revealed that most of the *ClGPX* genes possessed five introns (Figure 3), and their exon lengths were similar to those of their homologous genes in cucumber [5], *T. salsuginea* [24], cotton [25], *Arabidopsis* and rice [5], suggesting the conservation of their functions. However, fewer introns were also found in certain genes of watermelon. For example, *ClGPX1* and *ClGPX2* contained three and four introns, respectively (Figure 3). Similar results were also observed in other plant species, including *Populus trichocarpa* (Potri.001G105100, four introns) [30], rice (*OsGPX1*, four introns) [5], sorghum (*SbGPX3*, three introns; *SbGPX1*, four introns) [26], and bread wheat (*TaGPX1-A1*, two introns; *TaGPX5-B2*, four introns) [27]. This phenomenon indicates that the expansion of the *GPX* gene family may be due to the intron loss during gene duplication, which may influence the functional divergence of the *GPX* family genes.

The *cis*-element analysis of *ClGPX* promoters and tissue expression patterns of *ClGPX* genes provided insights into their functions in watermelon plant development. In this study, the identification of seven types of *cis*-elements involved in different developmental processes suggests that *ClGPX* genes may have tissue-specific expression patterns (Figure 5). In addition, all six *ClGPX* genes showed high expression levels in flowers and fruits (Figure 6), indicating their possible roles in the flower and fruit development of watermelon. This finding is consistent with the results of previous studies. For example, in cotton, nearly all *GhGPX*s showed the highest transcripts in flowers [25]. In particular, *ClGPX2* and *ClGPX4* showed similar expression patterns in most of the tested tissues (Figure 6B,D), implying that they may have similar or redundant functions. Notably, several *ClGPX* genes such as *ClGPX1*–*ClGPX4* displayed relatively lower expression in roots, possibly because they are located in the chloroplast, implying that they may play a particular role in scavenging ROS under photo-oxidative stress [18,25]. The various expression patterns of *ClGPX* genes indicate that they may play different functional roles at particular developmental processes in watermelon.

Increasing evidence indicates that plant *GPX*s play a positive role in plant response to abiotic stresses, such as salinity, drought, heat, and cold [5,25,27). Our results showed that a number of stress- and hormone-responsive *cis*-elements are present in *ClGPX* promoters (Figure 5, Table S3), implying that *ClGPX* genes may be involved in multiple hormone and stress responses. Moreover, we found that the expression of all *ClGPX* genes was up-regulated by salinity, drought, and cold (Figure 7), which corresponds to the results for other plant species. Many studies have revealed the significant increases in mRNA levels of *GPX* genes under various stress conditions. For example, almost all *TsGPX*s were significantly up-regulated at least at one time point in *T. salsuginea* roots under NaCl and PEG treatments [24]. The transcript of a *GPX* gene from *P. glaucum* (*PgGPx*) was highly induced in response to salinity and drought stresses [6]. Similarly, enhanced expression levels of *CsGPX*s and *TaGPX*s under various stress conditions have been reported in cucumber [5] and bread wheat [27], respectively. In addition, the differential expression patterns of *ClGPX* genes in response to stress conditions suggest that watermelon may have evolved diverse mechanisms in order to adapt to different abiotic stresses. Furthermore, the transcriptions of *ClGPX* genes were up-regulated by

ABA treatment (Figure 7), and most of the *ClGPX* promoters contained up to 3 ABA-responsive elements (ABREs) (Figure 5), indicating that *ClGPX* genes may be involved in stress response through an ABA-dependent signal pathway. Hence, our results show that all *ClGPX* genes appear to participate in the growth and development of different tissues. Moreover, *ClGPX* genes have various expression patterns under different stresses, indicating that they have specific functions in response to stress.

5. Conclusions

In conclusion, a total of six *ClGPX* genes were found in the watermelon genome. The *ClGPX* genes were differentially expressed in various tissues, suggesting that they played specific roles in watermelon development. The results of gene expression analysis under different abiotic stresses (cold, PEG, and NaCl) and ABA treatments revealed that all *ClGPX* genes may be involved in stress responses and an ABA signaling pathway. These findings will lay the foundation for investigating the functions of *GPX* genes in watermelon.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/8/10/206/s1, Table S1: Primers used for qRT-PCR, Table S2: GPX protein sequences used for phylogenetic tree analysis, Table S3: The predicted promoter *cis*-acting regulatory elements of *ClGPX* genes.

Author Contributions: Y.Z. and Y.Y. designed the research. Y.Z. and J.L. executed the experiments. Y.Y., J.W., and W.Y. analyzed and discussed the data. Y.Y. and Y.Z. wrote the manuscript.

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References

1. Ahmad, P.; Jaleel, C.A.; Salem, M.A.; Nabi, G.; Sharma, S. Roles of enzymatic and nonenzymatic antioxidants in plants during abiotic stress. *Crit. Rev. Biotechnol.* **2010**, *30*, 161–175. [CrossRef] [PubMed]
2. You, J.; Chan, Z. ROS regulation during abiotic stress responses in crop plants. *Front. Plant Sci.* **2015**, *6*, 1092. [CrossRef] [PubMed]
3. Bela, K.; Horvath, E.; Galle, A.; Szabados, L.; Tari, I.; Csiszar, J. Plant glutathione peroxidases: Emerging role of the antioxidant enzymes in plant development and stress responses. *J. Plant Physiol.* **2015**, *176*, 192–201. [CrossRef] [PubMed]
4. Navrot, N.; Collin, V.; Gualberto, J.; Gelhaye, E.; Hirasawa, M.; Rey, P.; Knaff, D.B.; Issakidis, E.; Jacquot, J.P.; Rouhier, N. Plant glutathione peroxidases are functional peroxiredoxins distributed in several subcellular compartments and regulated during biotic and abiotic stresses. *Plant Physiol.* **2006**, *142*, 1364–1379. [CrossRef] [PubMed]
5. Zhou, Y.; Hu, L.; Ye, S.; Jiang, L.; Liu, S. Genome-wide identification of glutathione peroxidase (GPX) gene family and their response to abiotic stress in cucumber. *3 Biotech* **2018**, *8*, 159. [CrossRef] [PubMed]
6. Islam, T.; Manna, M.; Reddy, M.K. Glutathione peroxidase of *Pennisetum glaucum* (PgGPx) is a functional Cd^{2+} dependent peroxiredoxin that enhances tolerance against salinity and drought stress. *PLoS ONE* **2015**, *10*, e0143344. [CrossRef] [PubMed]
7. Koh, C.S.; Didierjean, C.; Navrot, N.; Panjikar, S.; Mulliert, G.; Rouhier, N.; Jacquot, J.P.; Aubry, A.; Shawkataly, O.; Corbier, C. Crystal structures of a poplar thioredoxin peroxidase that exhibits the structure of glutathione peroxidases: Insights into redox-driven conformational changes. *J. Mol. Biol.* **2007**, *370*, 512–529. [CrossRef] [PubMed]
8. Bela, K.; Riyazuddin, R.; Horváth, E.; Hurton, Á.; Gallé, Á.; Takács, Z.; Zsigmond, L.; Szabados, L.; Tari, I.; Csiszár, J. Comprehensive analysis of antioxidant mechanisms in *Arabidopsis* glutathione peroxidase-like mutants under salt- and osmotic stress reveals organ-specific significance of the AtGPXL's activities. *Environ. Exp. Bot.* **2018**, *150*, 127–140. [CrossRef]

9. Islam, T.; Manna, M.; Kaul, T.; Pandey, S.; Reddy, C.S.; Reddy, M.K. Genome-wide dissection of *Arabidopsis* and rice for the identification and expression analysis of glutathione peroxidases reveals their stress-specific and overlapping response patterns. *Plant Mol. Biol. Rep.* **2015**, *33*, 1413–1427. [CrossRef]
10. Gaber, A. *Arabidopsis* glutathione peroxidase 8 is a key enzyme in response to environmental stresses. *Arab. J. Biotechnol.* **2011**, *14*, 213–224.
11. Miao, Y.; Guo, J.; Liu, E.; Li, K.; Dai, J.; Wang, P.; Chen, J.; Song, C. Osmotically stress-regulated the expression of *glutathione peroxidase 3* in *Arabidopsis*. *Chin. Sci. Bull.* **2007**, *52*, 127–130. [CrossRef]
12. Lima-Melo, Y.; Carvalho, F.E.; Martins, M.O.; Passaia, G.; Sousa, R.H.; Neto, M.C.; Margis-Pinheiro, M.; Silveira, J.A. Mitochondrial GPX1 silencing triggers differential photosynthesis impairment in response to salinity in rice plants. *J. Integr. Plant Biol.* **2018**, *58*, 737–748. [CrossRef] [PubMed]
13. Passaia, G.; Fonini, L.S.; Caverzan, A.; Jardim-Messeder, D.; Christoff, A.P.; Gaeta, M.L.; de Araujo Mariath, J.E.; Margis, R.; Margis-Pinheiro, M. The mitochondrial glutathione peroxidase GPX3 is essential for H_2O_2 homeostasis and root and shoot development in rice. *Plant Sci.* **2013**, *208*, 93–101. [CrossRef] [PubMed]
14. Wang, X.; Fang, G.; Yang, J.; Li, Y. A thioredoxin-dependent glutathione peroxidase (OsGPX5) is required for rice normal development and salt stress tolerance. *Plant Mol. Biol. Rep.* **2017**, *35*, 333–342. [CrossRef]
15. Diao, Y.; Xu, H.; Li, G.; Yu, A.; Yu, X.; Hu, W.; Zheng, X.; Li, S.; Wang, Y.; Hu, Z. Cloning a glutathione peroxidase gene from *Nelumbo nucifera* and enhanced salt tolerance by overexpressing in rice. *Mol. Biol. Rep.* **2014**, *41*, 4919–4927. [CrossRef] [PubMed]
16. Passaia, G.; Queval, G.; Bai, J.; Margis-Pinheiro, M.; Foyer, C.H. The effects of redox controls mediated by glutathione peroxidases on root architecture in *Arabidopsis thaliana*. *J. Exp. Bot.* **2014**, *65*, 1403–1413. [CrossRef] [PubMed]
17. Passaia, G.; Caverzan, A.; Fonini, L.S.; Carvalho, F.E.L.; Silveira, J.A.G.; Margis-Pinheiro, M. Chloroplastic and mitochondrial *GPX* genes play a critical role in rice development. *Biol. Plant* **2014**, *58*, 375–378. [CrossRef]
18. Chang, C.C.C.; Ślesak, I.; Jordá, L.; Sotnikov, A.; Melzer, M.; Miszalski, Z.; Mullineaux, P.M.; Parker, J.E.; Karpińska, B.; Karpiński, S. *Arabidopsis* chloroplastic glutathione peroxidases play a role in cross talk between photooxidative stress and immune responses. *Plant Physiol.* **2009**, *150*, 670–683. [CrossRef] [PubMed]
19. Miao, Y.; Lv, D.; Wang, P.; Wang, X.C.; Chen, J.; Miao, C.; Song, C.P. An *Arabidopsis* glutathione peroxidase functions as both a redox transducer and a scavenger in abscisic acid and drought stress responses. *Plant Cell* **2006**, *18*, 2749–2766. [CrossRef] [PubMed]
20. Faltin, Z.; Holland, D.; Velcheva, M.; Tsapovetsky, M.; Roeckel-Drevet, P.; Handa, A.K.; Abu-Abied, M.; Friedman-Einat, M.; Eshdat, Y.; Perl, A. Glutathione peroxidase regulation of reactive oxygen species level is crucial for in vitro plant differentiation. *Plant Cell Physiol.* **2010**, *51*, 1151–1162. [CrossRef] [PubMed]
21. Zhai, C.Z.; Zhao, L.; Yin, L.J.; Chen, M.; Wang, Q.Y.; Li, L.C.; Xu, Z.S.; Ma, Y.Z. Two wheat glutathione peroxidase genes whose products are located in chloroplasts improve salt and H_2O_2 tolerances in *Arabidopsis*. *PLoS ONE* **2013**, *8*, e73989. [CrossRef] [PubMed]
22. Rodriguez Milla, M.A.; Maurer, A.; Rodriguez Huete, A.; Gustafson, J.P. Glutathione peroxidase genes in *Arabidopsis* are ubiquitous and regulated by abiotic stresses through diverse signaling pathways. *Plant J.* **2003**, *36*, 602–615. [CrossRef] [PubMed]
23. Ramos, J.; Matamoros, M.A.; Naya, L.; James, E.K.; Rouhier, N.; Sato, S.; Tabata, S.; Becana, M. The glutathione peroxidase gene family of *Lotus japonicus*: Characterization of genomic clones, expression analyses and immunolocalization in legumes. *New Phytol.* **2009**, *181*, 103–114. [CrossRef] [PubMed]
24. Gao, F.; Chen, J.; Ma, T.; Li, H.; Wang, N.; Li, Z.; Zhang, Z.; Zhou, Y. The glutathione peroxidase gene family in *Thellungiella salsuginea*: Genome-wide identification, classification, and gene and protein expression analysis under stress conditions. *Int. J. Mol. Sci.* **2014**, *15*, 3319–3335. [CrossRef] [PubMed]
25. Chen, M.; Li, K.; Li, H.; Song, C.P.; Miao, Y. The glutathione peroxidase gene family in *Gossypium hirsutum*: Genome-wide identification, classification, gene expression and functional analysis. *Sci. Rep.* **2017**, *7*, 44743. [CrossRef] [PubMed]
26. Akbudak, M.A.; Filiz, E.; Vatansever, R.; Kontbay, K. Genome-wide identification and expression profiling of ascorbate peroxidase (*APX*) and glutathione peroxidase (*GPX*) genes under drought stress in sorghum (*Sorghum bicolor* L.). *J. Plant Growth Regul.* **2018**, *37*, 925–936. [CrossRef]
27. Tyagi, S.; Himani Sembi, J.K.; Upadhyay, S.K. Gene architecture and expression analyses provide insights into the role of glutathione peroxidases (GPXs) in bread wheat (*Triticum aestivum* L.). *J. Plant Physiol.* **2018**, *223*, 19–31. [CrossRef] [PubMed]

28. Tamura, K.; Peterson, D.; Peterson, N.; Stecher, G.; Nei, M.; Kumar, S. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol. Biol. Evol.* **2011**, *28*, 2731–2739. [CrossRef] [PubMed]
29. Livak, K.J.; Schmittgen, T.D. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* **2001**, *25*, 402–408. [CrossRef] [PubMed]
30. Ozyigit, I.I.; Filiz, E.; Vatansever, R.; Kurtoglu, K.Y.; Koc, I.; Ozturk, M.X.; Anjum, N.A. Identification and comparative analysis of H_2O_2-scavenging enzymes (ascorbate peroxidase and glutathione peroxidase) in selected plants employing bioinformatics approaches. *Front Plant. Sci.* **2016**, *7*, 301. [CrossRef] [PubMed]
31. Passaia, G.; Margis-Pinheiro, M. Glutathione peroxidases as redox sensor proteins in plant cells. *Plant Sci.* **2015**, *234*, 22–26. [CrossRef] [PubMed]