agronomy

Article

Role of ZmGST Gene Family Involved in Nicosulfuron Stress Tolerance Revealed by Genomic and Transcriptomic Analyses

Xiaomin Liu [1], Dan Zhao [2], Xian Xu [1], Libing Yuan [3], Bochui Zhao [1], Binghua Li [1], Xinli Guo [1] and Guiqi Wang [1,*]

[1] Key Laboratory of Crop Cultivation Physiology and Green Production of Hebei Province, Institute of Cereal and Oil Crops, Hebei Academy of Agriculture and Forestry Sciences, Shijiazhuang 050035, China
[2] Biological Control Centre of Plant Pathogens and Plant Pests of Hebei Province, College of Plant Protection, Agricultural University of Hebei, Baoding 071001, China
[3] Plant Protection Institute, Hebei Academy of Agricultural and Forestry Sciences, Baoding 071001, China
* Correspondence: wt3326@sina.com

Abstract: Glutathione S-transferases (GST) are a large family of polymorphous proteins that play important roles in herbicide detoxification and stress response. Nicosulfuron is the most broadly used herbicide in maize fields, and it can cause different injuries to maize varieties, but little is studied about the systemic and comprehensive GST gene family responding to nicosulfuron stress in maize. In this research, pre-treatment with glutathione S-transferase inhibitor 4-chloro-7-nitrobenzoxadiazole (NBD-Cl) increased nicosulfuron phytotoxicity to both sensitive and tolerant maize genotypes. A total of 55 *ZmGST* genes belonging to six major sub-classes were identified in the maize genome and named according to the nomenclature system. Based on phylogenetic analyses, highly conserved gene structure and motif distribution were detected in the same class. Chromosome mapping results showed that *ZmGST* genes were distributed over the 10 chromosomes unevenly. There were thirteen and eight gene pairs identified as tandem and segmental duplication events, respectively, which played important roles in the expansion of the GST gene family in maize. RNA-seq and qRT-PCR analyses showed that there were great dissimilarities in *ZmGST* gene expression patterns between the tolerant and sensitive maize plants. More highly expressed *ZmGST* genes were found in the tolerant than in the sensitive without nicosulfuron stress. However, under 60 g a.i. ha^{-1} nicosulfuron stress, more *ZmGST* genes were significantly upregulated in HB41 than in HB09. This study provided experimental evidence showing that glutathione S-transferases were involved in nicosulfuron stress in maize. It will contribute to the further functional analysis of the GST gene family in maize.

Keywords: glutathione S-transferases (GST); *Zea mays* L.; nicosulfuron; genome-wide analysis; herbicide stress

check for updates

Citation: Liu, X.; Zhao, D.; Xu, X.; Yuan, L.; Zhao, B.; Li, B.; Guo, X.; Wang, G. Role of ZmGST Gene Family Involved in Nicosulfuron Stress Tolerance Revealed by Genomic and Transcriptomic Analyses. *Agronomy* **2022**, *12*, 2598. https://doi.org/10.3390/agronomy12112598

Academic Editor: Manish K. Pandey

Received: 25 September 2022
Accepted: 19 October 2022
Published: 22 October 2022

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Copyright: © 2022 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).

1. Introduction

Nicosulfuron is a selective herbicide belonging to the sulfonylurea family (Group 2). It has been one of the most widely utilized post-emergence herbicides in maize fields, for its high selectivity, broad weeding spectrum, short soil residual activity, and low mammalian toxicity since it was introduced in China in 1998 (Cholette et al., 2019; Huang et al., 2019). Nicosulfuron can inhibit branched-chain amino acid biosynthesis by blocking the acetolactate synthase enzyme in sensitive plants and alter the ascorbate-glutathione metabolism cycle and lipid peroxidation, which induces symptoms ranging from chlorosis, stunting, severe leaf malformations to death of the entire plant. It has been demonstrated that sensitivities to nicosulfuron are significantly different among various maize lines, which based, on their differential metabolism and detoxification ability, the tolerant plants can rapidly convert nicosulfuron into nonphytotoxic forms [1].

Plant glutathione S-transferases (GST, EC 2.5.1.18) represent a complex and multifunctional enzyme super family. They can biotransform a broad range of endogenous and exogenous electrophilic compounds, which include different types of herbicides. Maize GSTs have been well documented to catalyze the conjugation of tripeptide glutathione (GSH) with a wide variety of herbicides, such as chlorotriazine, chloroacetanilide, and thiocarbamate classes, to protect themselves from phytotoxicity [2]. To date, various plant GST genes have been reported, including 55 in *Arabidopsis thaliana* [3], 79 in *Oryza sativa* [4], 101 in *Glycine max* [5], 330 in *Triticum aestivum* (Wang, et al. [6]), 84 in *Hordeum vulgare* [7], 49 in *Gossypium arboreum*, 81 in *Solanum lycopersicum* [8], 114 in *Helianthus annuus* [9], etc. Based on sequence similarity, immunological reactivity, kinetic properties, and structural conformation, plant GSTs can be classified into four to fourteen classes, eight of which are more widespread, including Theta (GSTT), Phi (GSTF), Tau (GSTU), Zeta (GSTZ), Lambda (GSTL), γ-subunit of translation elongation factor 1B (EF1Bγ), tetrachlorohydroquinone dehalogenase (TCHQD), and dehydroascorbate reductase (DHAR) [10,11].

Plant GSTs are believed to play an important role in the phase II herbicide detoxification pathway [12–14]. In the 1970s, GSTs in maize were first reported to be responsible for the conjugation of photosystem II inhibitor atrazine with GSH, which could protect maize from injury [15]. Since then, they have been revealed to participate in the detoxification of different classes of herbicides like alachlor, fluorodifen, fomesafen, atrazine, etc. [16]. Plant Tau and Phi classes were demonstrated to be closely associated with the detoxification of different herbicides, which was attributed to their class specificities in substrate preference [14,17]. Phi class GSTs in *Oryza sativa* and *Phaseolus vulgaris* were reported to display high specific activity toward chloroacetanilide herbicides, which included alachlor, acetochlor, and metolachlor [18,19], while the Tau class GSTs showed significant activity toward fluorodifen, a diphenylether herbicide [20]. Many weed populations have been identified to exhibit herbicide resistance because of the transcription enhancement of the Phi and Tau class GST genes, such as *Lolium rigidum* [21], *Amaranthus palmeri* [22], *Beckmannia syzigachne* [23], and *Alopecurus aequalis* [24].

In this study, a combined computational strategy comprising HMM (Hidden Markov Model) profile scan coupled with BLAST analysis was employed to identify *ZmGST* genes from the *Zea mays* genome database. Their characteristics of conserved motifs, gene structure, and gene duplication for different classes were also analyzed. Moreover, comprehensive expression pattern analysis of *ZmGST* genes was performed in two maize genotypes, HB09 (tolerant) and HB41 (sensitive), under nicosulfuron treatment to reveal possible candidate *ZmGST* genes that responded to herbicide stress.

2. Materials and Methods

2.1. Effect of NBD-Cl Addition to Nicosulfuron

Based on previous research, NBD-Cl (glutathione S-transferase inhibitor) at a rate of 0.27 kg a.i. ha^{-1} was selected to further understand the key role of glutathione S-transferases in plant response to herbicide stress [25,26]. HB09 and HB41 maize seeds were collected from the Institute of Cereal and Oil Crops, Hebei Academy of Agriculture and Forestry Sciences, China. Maize plants were grown under greenhouse conditions (27 °C/24 °C day/night and 16 h/8 h light/dark) with two plants per pot. Nicosulfuron was applied at 0, 30, 60, and 90 g a.i. ha^{-1} when the maize plants were at three- to four-leaf stage. NBD-Cl was applied 48 h before nicosulfuron application. Non-herbicide-treated controls were included in each experiment for comparison. At 21 days after treatment (DAT), surviving plants were counted, cut at the soil surface, and dried for 5 days in a forced air oven at 65 °C, and then dry weights were recorded. The dry weight data per plant was expressed as a percentage of the non-treated controls for each treatment. There were 3 replicate pots per treatment, and the experiment was carried out twice in the controlled glasshouse.

2.2. Identification, Sequence Analysis, and Nomenclature of ZmGSTs

To identify putative ZmGSTs, the HMM model for GST (GST_N, PF02798; GST_C, PF00043) was obtained from the PFAM database. An HMM search and a keyword 'GST' search were conducted to retrieve all GST family members from the B73 maize genome database (B73 RefGen_v4). All the candidate ZmGSTs were then looked up in the National Center for Biotechnology Information (NCBI) Conserved Domains Database to confirm the presence of typical GST N- and C-terminal domains. All identified GST proteins were given the prefix "Zm" for *Zea mays* after the subclass identifier and were numbered progressively according to the previously described nomenclature system [3]. Chromosomal location, strand position, CDS coordinate (from 5 to 3′), length of gene, cDNA, and CDS were retrieved from the Maize Genetics and Genomics Database (MaizeGDB). Physiochemical properties like polypeptide length, pI, and molecular weight were calculated using the ExPASy Prot-Param (http://web.expasy.org/protparam/) (accessed on 17 October 2022) tool.

2.3. Gene Structure, Chromosomal Location, and Phylogenetic Analysis of ZmGSTs

The exon-intron structures of the ZmGST gene family were identified using online GSDS 2.0 (Gene Structure Display Server, http://gsds.cbi.pku.edu.cn/) (accessed on 18 May 2022). The conserved motifs of GST gene family members in *Zea maize* were analyzed by the MEME online website (http://meme-suite.org/tools/meme) (accessed on 6 June 2022) with the default settings except for the maximum number of motifs and width, which were set to 10 and 100. The physical location of *ZmGST* genes was collected from the Maize Genetics and Genomics Database (MaizeGDB) and the positions of these *ZmGST* genes were plotted on ten *Zea mays* chromosomes. The distribution maps of *ZmGST* genes on the chromosome were drawn using the biological software Mapchart. Duplication events were predicted by blastp search (e-value10^{-10}) with $\geq$80% sequence identity in the MaizeGDB Database.

2.4. Phylogenetic and Synteny Analysis of ZmGSTs

To understand the evolutionary relationship among ZmGST proteins, GST protein sequences from three plant species, including *Arabidopsis thaliana*, *Oryza sativa*, and *Zea mays*, were aligned using Clustal X. A molecular phylogenetic tree was constructed in MEGA7.0 using the NJ (neighbor joining) procedure with 1000 bootstrap replications. Complete deletion mode was used to access tree topology and reliability.

2.5. Transcriptome Sequencing, Gene Expression Analysis of ZmGST Genes

The two contrasting maize genotypes were cultivated to a three- to four-leaf stage according to the method described above, and then treated uniformly by spraying with nicosulfuron at a rate of 60 g a.i. ha^{-1}. The young leaf tissue samples of treated and untreated maize plants were harvested aseptically after 48 h, frozen immediately with liquid nitrogen, and stocked in a $-80\,^{\circ}C$ freezer. Total RNA extraction, cDNA synthesis, and quantitative real-time PCR were performed as requested. Illumina HiSeq X Ten sequencing technology was applied to detect key candidate genes associated with nicosulfuron stress. Three biological replicates were used for RNA-seq. All sequencing data were saved in the NCBI Sequence Read Archive (SRA) database under the accession number PRJNA 858937. The expression profile of *ZmGST* genes under nicosulfuron stress was obtained from the database. The transcript abundances were calculated and quantified for each sample using the reads per kilobase per million mapped reads (RPKM). Finally, the average RPKM values were log-transformed and used to generate the heatmap of *ZmGST* gene expression condition by TBtools with the hierarchical clustering method.

2.6. Quantitative RT-PCR Validation Experiments

Total RNA was extracted using TRIzol reagents according to the provided protocol (Invitrogen, Waltham, MA, USA). The first strand of cDNA was synthesized using an oligo (dT) primer and a Maxima First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham,

MA, USA). The maize *UPF1* gene was used as a housekeeping gene for normalization [27]. PCR was performed using SYBR Green (7500 Applied Bio Systems, Foster, CA, USA) The reaction conditions were as follows: 95 °C for 30 s and 40 cycles (95 °C for 10 s, 56 °C for 30 s, and 72 °C for 60 s). The mRNA levels for each gene were calculated using the $2^{-\Delta\Delta CT}$ method. The primer pairs were designed by Primer 3 software and synthesized by Sangon Biotech (Shanghai, China) [28] (Table S1), and each reaction was carried out in triplicate.

3. Results

3.1. Effect of GST Inhibitors on Nicosulfuron Photoxicity to Maize Plants

Our previous study demonstrated that HB09 and HB41 had greatly different phenotypic responses to nicosuluron [29]. In this study, the pot experiments showed that there was no obvious growth reduction for both of the two genotypes when treated with only the GST inhibitor 4-chloro-7-nitrobenzoxadiazole (NBD-Cl) at 270 g a.i. ha^{-1}. NBD-Cl followed by nicosulfuron both enhanced its phytotoxicity to HB09 and HB41. HB09 did not display a significant effect from either nicosulfuron treatment alone or the combination of NBD-Cl and nicosulfuron when compared to the non-treated control. However, it significantly enhanced nicosulfuron phytotoxicity to HB41. Nicosulfuron application rates at 30, 60, and 90 g a.i. ha^{-1} resulted in a reduction of biomass by 30.2%, 47.2%, and 64.0%, respectively. NBD-Cl followed by 30, 60, and 90 g a.i. ha^{-1} nicosulfuron decreased biomass by 55.6%, 68.9%, and 79.4%, respectively, (Figure 1). It seemed that pre-treatment with NBD-Cl inhibited more GST activity in sensitive maize than in tolerant maize after nicosulfuron application. These results indicated that there were dissimilar GST responses under nicosulfuron stress in the tolerant and sensitive maize plants.

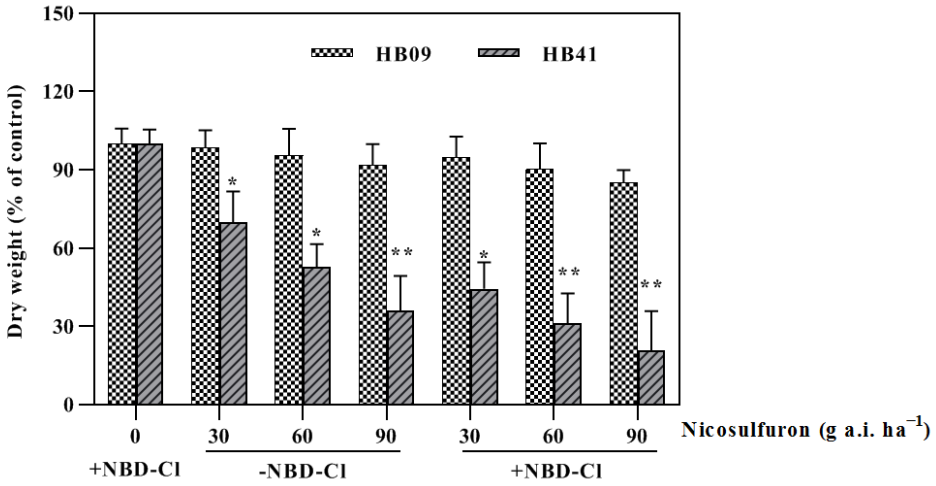


Figure 1. Effects of GST inhibitions (NBD-Cl) on maize growth response to nicosulfuron 21 DAT. The error bars represent the standard deviation (S.D.) of the mean of three independent replicates. Compared to the control group, statistically significant differences were referenced as * $p < 0.05$ and ** $p < 0.01$ by the *t*-test.

3.2. Identification and Characterization of the GST Gene Family in Zea mays

Further genome-wide analysis of the GST gene family results showed that there were fifty-five *ZmGST* genes encoding putative GST proteins in the *Zea mays* genome (B73 RefGen_v4). Based on their conserved sequences and homology to the known AtGSTs which had already been assigned to GST classes, these GST proteins were categorized into six major sub-classes: Phi (13 members), Tau (32 members), Zeta (3 members), DHAR (2 members), TCHQD (1 member), and EF1Bγ (4 members) (Table S2). The Phi and Tau classes were found to have the most abundant, with 13 and 32 members, while the TCHQD class had only 1 member. Basically, the open reading frames of the 55 *ZmGST* genes ranged from 417 (*ZmGSTU17*) to 1314 bp (*ZmEF1γ4*), with deduced protein lengths ranging from 138 to 437 amino acids. The predicted molecular weights were between 15.1 and 49.5 kDa,

and the isoelectric point (pI) values ranged from 4.85 (*ZmGSTU24*) to 9.39 (*ZmGSTF10*), in which 80% of the members were acidic proteins and 20% were basic proteins.

3.3. Gene Structure, Chromosomal Localization, and Gene Duplication of ZmGST Genes

To compare the structural components of 55 *ZmGST* genes, the exons and introns were mapped using the Gene Structure Display Server (GSDS) program (Figure 2a,b). The structure analysis revealed that there were great variations among the diverse *ZmGST* genes. There were 1–9 exons in ZmGST proteins, with the maximum number of exons in all three Zeta subfamily members. All members of the EF1Bγ subfamily have seven exons. There were 2–6 exons in the other subfamily members. The number of introns in the open reading frame of different *ZmGST* transcripts ranged from 0 to 8. Ten distinct conserved motifs were predicted by analyzing the conserved motifs of ZmGST proteins using the MEME program (Figure 2c, Table 1). The results indicated that the length of these motifs ranged from 21 to 80 amino acids. All the Tau class ZmGST proteins were found to contain motifs 3 and 10, and all the Phi class ZmGST proteins shared motifs 2 and 8. Motif 7 was a unique conserved motif for the EF1Bγ subfamily, and motifs 1 and 5 were only found to exist in the Tau class ZmGST proteins except for ZmGSTU17.

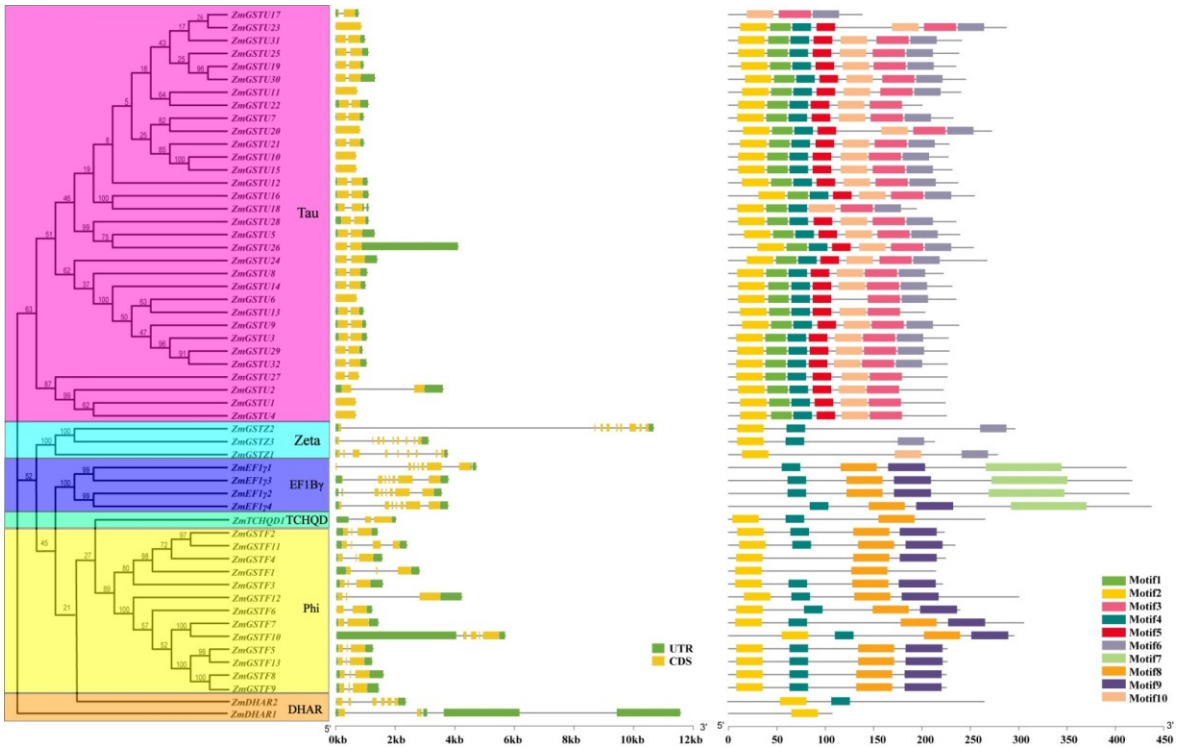


Figure 2. Phylogenetic relationships, gene structures, and protein domains of the ZmGST gene family. (**a**) Six ZmGSTs clusted into six groups in an unrooted ML tree constructed by MEGA7.0; (**b**) The intron/exon structures of ZmGSTs were analyzed by comparing genomic and cDNA sequences. CDS, UTR, and introns were represented by orange boxes, green boxes, and gray lines, respectively. The sizes of CDS and introns can be estimated using the ratio of the bottom; (**c**) Ten conserved motifs of ZmGSTs were generated by the online MEME tool. Different motifs were displayed using the different colored boxes. The lengths of proteins and motifs were estimated using the scale at the bottom.

Table 1. List of the putative motifs of ZmGST proteins.

Motif	Width	Best Possible Match
1	23	NKSELLLRSNPVHKKVPVLIHGG
2	29	GVWTSPFVIRVRIVLNLKGLAYEYVEEDL
3	35	GKPFFGGDSVGYVDVVLGGLLGWVRASEELHGVRP
4	21	KPVCESQIIVQYIDEAFAGTG
5	21	LPADPYERAVARFWAAYIDDK
6	29	IDAARTPLLAAWMERFCELDAAKAVLQDV
7	80	DWKRLYSNTKTNFREVAIKGFWDMYDPEGYSLWFC-DYKYNDENTVSFVTMNKVGGFLQRMDLCRKYAFGKMLVVGSEPPF
8	39	DPAVIAENEDKLKQVLDVYDEILSKNEYLAGDEFTLADL
9	40	SSDRGRKLFTARKHVARWYDKISTRDSWRQVIKMQREHPG
10	29	VFRGRTAEEMAEAARQAVAALETLEQAFR

Analysis of gene distribution in the *Zea mays* chromosome showed that *ZmGST* genes are spread throughout the genome. The *ZmGST* genes were distributed over the 10 chromosomes (Figure 3). The distribution of these genes was highly uneven across different chromosomes, ranging from 1 to 15 genes per chromosome. A maximum number of 15 genes were found to be located on chromosome 1, which was followed by 12 genes on chromosome 3. In addition, over half of the Tau class genes were clustered on chromosomes 1 and 3. Only 1, 2, 3, and 4 genes were found to be localized on chromosomes 2, 4, 10, and 5, respectively. According to the criterion of tandem duplication, we confirmed 13 tandem duplicated genes formed seven gene clusters distributed on three different chromosomes, and eight of them were Tau class members; the other five were from the Phi class. Furthermore, eight segmental duplication events were detected. Both of the two kinds of duplication events were considered to contribute to the ZmGST gene family expansion in Zea mays.

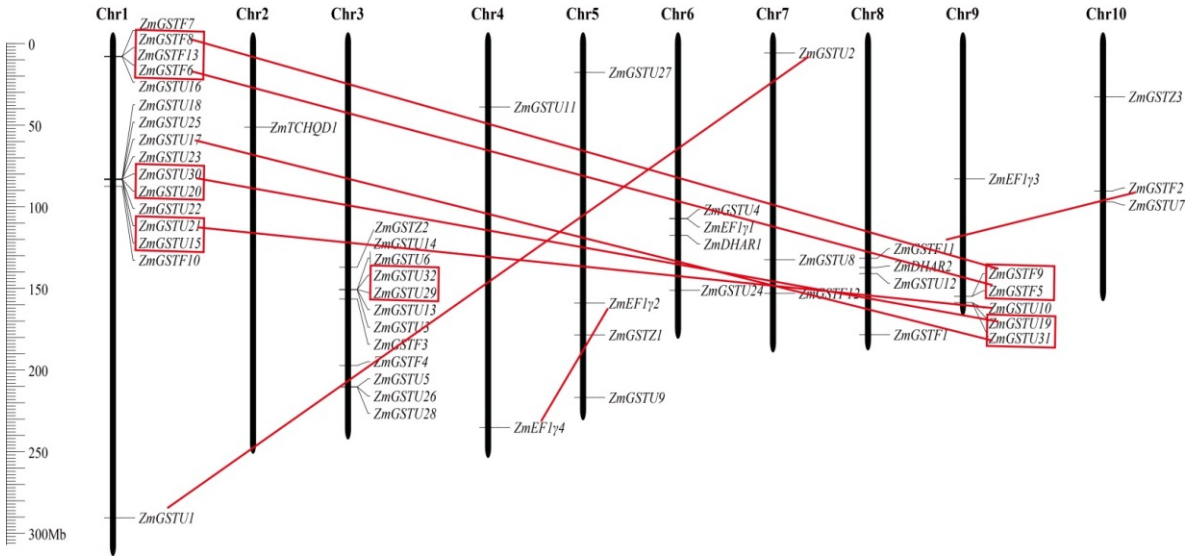
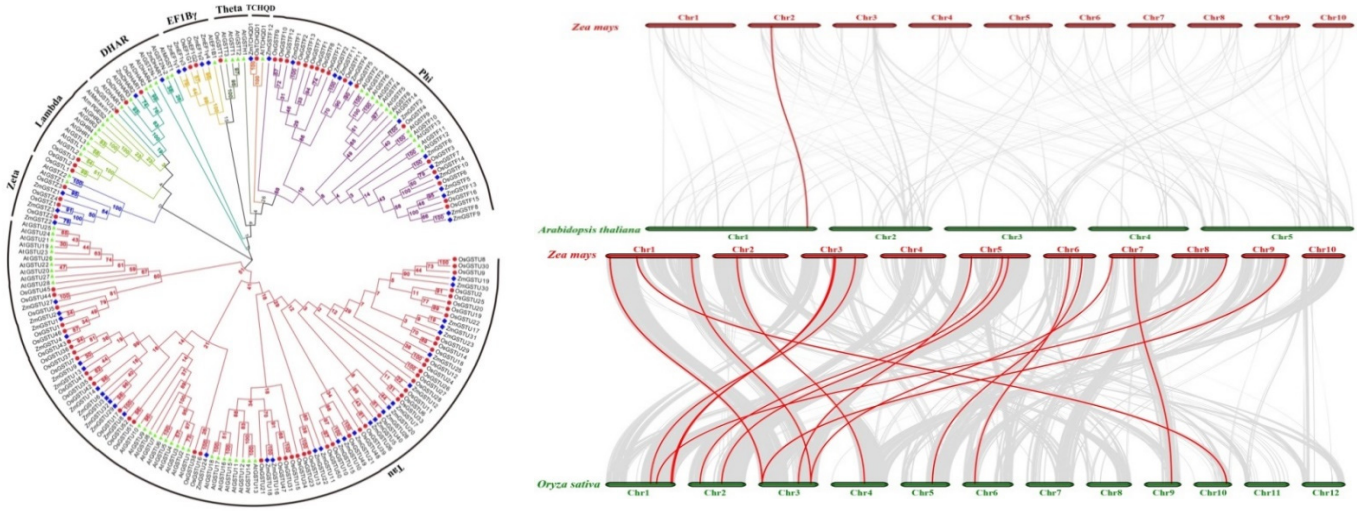


Figure 3. Chromosomal distribution of *ZmGST* genes in the *Zea mays* genome plotted using Mapchart software. Chromosome numbers were indicated on top of chromosomes, and the size of the chromosome was indicated by a vertical scale (Mb). Tandem duplicated genes were marked by red rectangles. Segmental duplicated gene pairs were connected with red lines.

3.4. Phylogenetic and Synteny Analysis of GSTs in Selected Species

To explore the evolutionary relationships of the ZmGST proteins, known GST proteins from GST super families characterized in *Oryza sativa* and *Arabidopsis* were collected. All the putative GST proteins from the three species were aligned to generate the unrooted phylogenetic trees using the Clustal X and MEGA tools with the neighbor joining method (Figure 4a). The phylogenetic tree showed that most of the GST proteins from three species within the same sub-classes were clustered together and formed a monophyletic group. The phylogenetic analysis results showed that the Tau class was the largest and most abundant with 32, 49, and 28 members in *Zea mays*, *Oryza sativa*, and *Arabidopsis*, respectively. Under a large superclade, all Tau members were distributed into different small clades, which indicated that there were internal variations among Tau members. The Phi class formed the second largest clade. EF1Bγ was the third class in *Zea mays* with four members. No Theta or Lambda members were identified. To elucidate the evolution of the GST gene families, comparative syntenic maps were constructed within the three representative species. Based on the syntenic relationship between *Zea mays* and *Oryza sativa*, a total of 20 orthologous GST gene pairs were identified, while only 1 gene pair was found between *Zea mays* and *Arabidopsis thaliana* (Figure 4b). These significant differences between monocots and dicots are possibly owing to the extensive gene duplication events that they underwent after their divergence.



(**a**) (**b**)

Figure 4. Phylogenetic and synteny analysis among *Zea mays*, *Oryza sativa*, and *Arabidopsis thaliana*. (**a**) Phylogenetic trees and classification of glutathione transferase proteins of the published GST proteins. The phylogenetic trees were constructed based on multiple sequence alignment using Clustal X followed by the maximum-likelihood method using JTT model MEGA7.0 software. Highlighted genes are represented with a diamond symbol. The blue color represents *ZmGST* genes. (**b**) Synteny analysis of *GST* genes. The gray lines in the background indicate the collinear blocks within *Zea mays* and the other two plant genomes. The red-colored lines highlight the syntenic *GST* gene pairs.

3.5. Expression Profiles of Potential Nicosulfuron Stress-Responsive ZmGST Genes

We performed RNA-seq experiments to further investigate the diversification of *ZmGST* gene expression patterns in HB41 and HB09 in response to nicosulfuron stress. RPKM ≤ 1 in all the samples was considered as not expressed. Finally, forty-seven *ZmGST* genes were screened from the transcriptome database, and heatmap analysis was used to visualize the expression profiles of these genes in the tolerant and sensitive maize plants under nicosulfuron treatment. Based on the expression profiles, all genes were divided into four different modules. Module 1 from *ZmGSTF6* to *ZmEF1γ3* exhibited a high expression level after nicosulfuron treatment in HB41, among them, *ZmGSTU25*,

ZmGSTU8, and *ZmGSTU4* had 2.4-, 2.0-, and 1.8-fold higher expression in HB41 forty-eight hours after 60 g a.i. ha^{-1} nicosulfuron treatment, respectively. Module 2 from *ZmGSTU6* to *ZmGSTU32* showed higher levels of expression in untreated HB41 than in HB09. *ZmGSTU11*, *ZmGSTU20*, *ZmGSTZ3*, and *ZmGSTU6* displayed 7.3-, 4.4-, 3.2-, and 2.2-fold higher expression in HB41 than HB09. *ZmDHAR1* and *ZmGSTU14* were clustered along a small branch. In Module 3, expression levels of these two genes were greatly induced after nicosulfuron treatment, with 3.1- and 1.5-fold in HB41, and 4.0- and 1.7-fold in HB09, respectively. The large gene cluster of Module 4 from *ZmGSTU5* to *ZmGSTU22* (28 of 47) had the highest expression levels in the untreated tolerant maize HB09 than in the sensitive HB41. Compared with HB41, Tau class *ZmGST* gene members such as *ZmGSTU24*, *ZmGSTU29*, *ZmGSTU28*, and *ZmGSTU19* exhibited 204.6-, 105.7-, 20.2-, and 11.3-fold higher expression, while Phi class *ZmGST* gene members such as *ZmGSTF4*, *ZmGSTF7*, *ZmGSTF10*, and *ZmGSTF2* exhibited 10.7-, 4.6-, 3.9-, and 2.4-fold higher expression in HB09 (Figure 5). We speculated that there were more highly expressed *ZmGST* genes that belonged to Tau classes in the tolerant maize plants than the sensitive ones, and they were greatly involved in the responses to nicosulfuron stress.

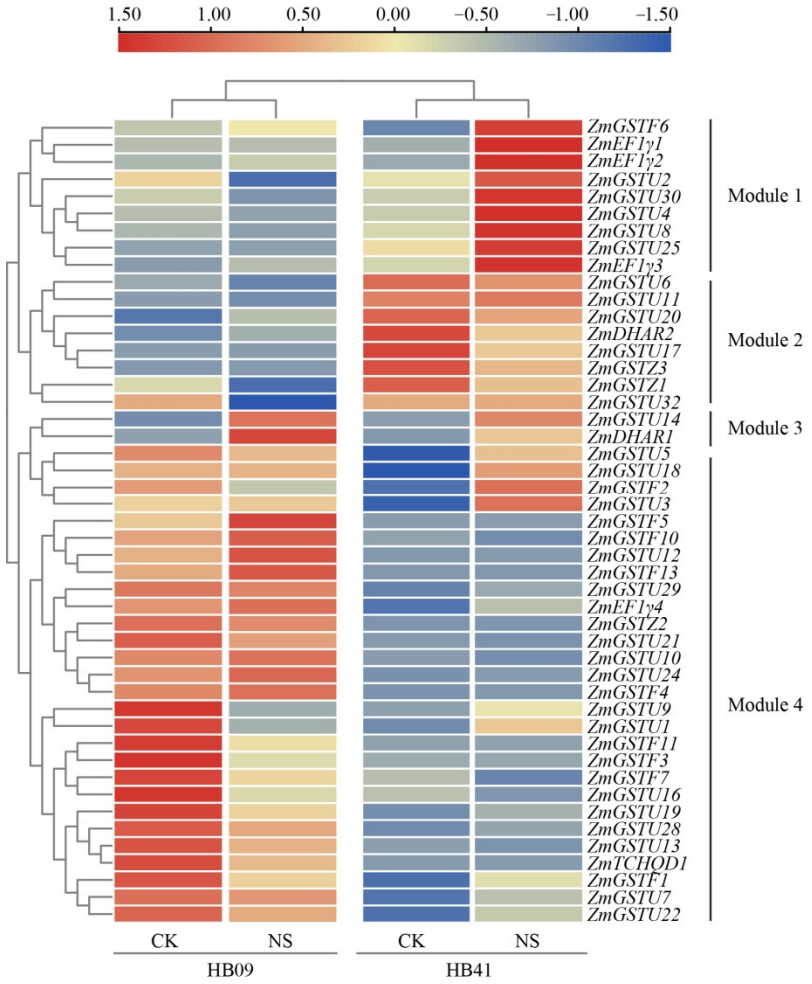


Figure 5. Hierarchical clustering of gene-expression data of *ZmGST* genes in *Zea mays* under nicosulfuron stress. The color bar represents the relative signal intensity values, with the color ranging from blue to red indicating low–high expression abundance. The *ZmGST* gene names are mentioned on the right, and the two maize genotypes and the treatment are indicated at the bottom of each column. CK, untreated control; NS, nicosulfuron treated at 60 g a.i. ha^{-1}.

3.6. Expression of ZmGSTs Using qRT-PCR

In addition, to confirm the accuracy and reproducibility of gene expression patterns observed in the above-described RNA-seq experiments, we selected nine *ZmGST* genes, including *ZmGSTU1*, *ZmGSTU3*, *ZmGSTU9*, *ZmGSTU14*, *ZmGSTU22*, *ZmGSTU25*, and *ZmGSTU28* in the Tau subclass, *ZmGSTF2* in the Phi subclass, and *ZmDHAR1* in the DHAR subclass for further validation. We analyzed their expression files using quantitative real-time PCR, with *UPF1* as the reference gene. Overall, we got basically similar gene expression pattern results using the two methods. *ZmGSTU1*, *ZmGSTU3*, *ZmGSTU9*, *ZmGSTU22*, *ZmGSTU28,* and *ZmGSTF2* exhibited higher expression in HB09 than HB41, while after the application of 60 g a.i. ha^{-1} nicosulfuron, most of them were significantly upregulated in the sensitive genotype compared with the tolerant. Expression of *ZmDHAR1* and *ZmGSTU14* was highly stimulated by nicosulfuron stress in both HB41 and HB09 (Figure 6).

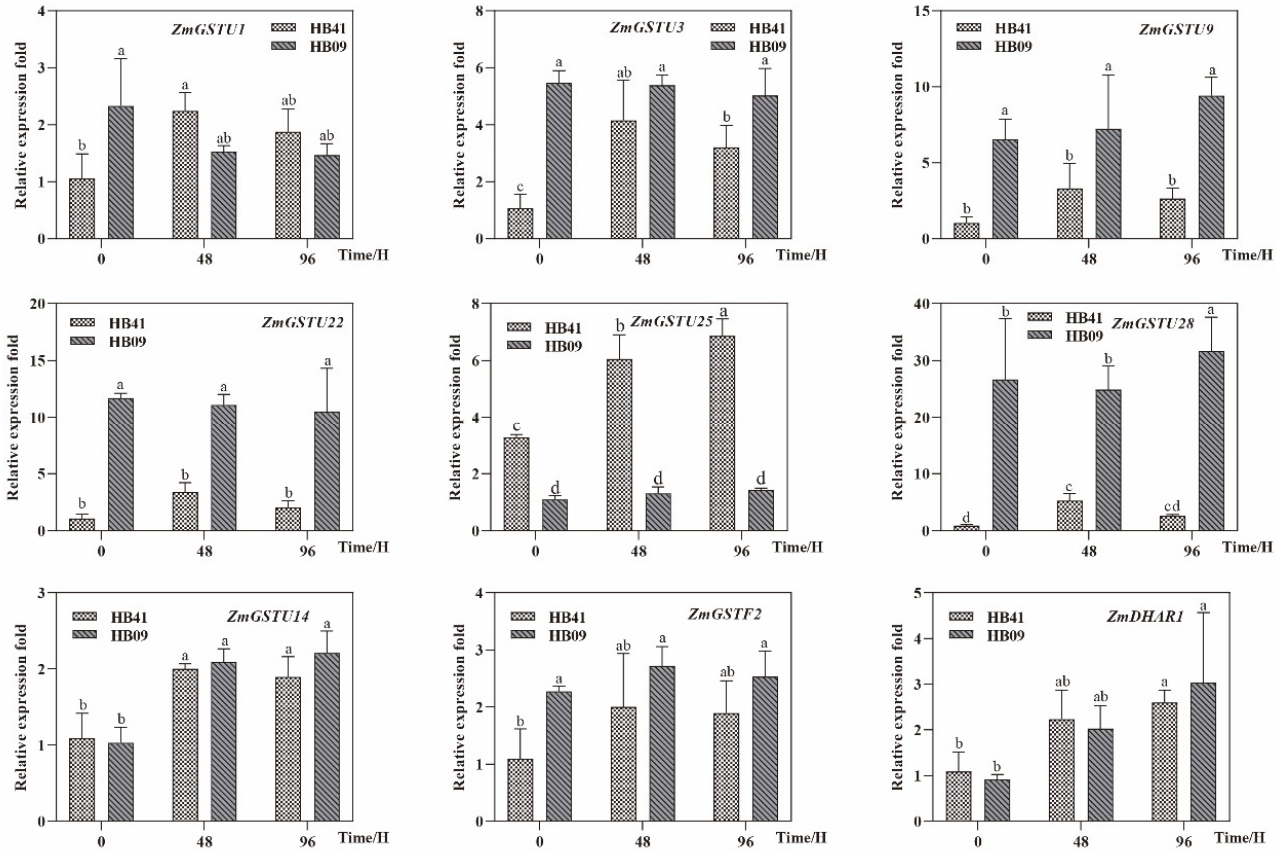


Figure 6. Relative expression of the nine selected glutathione S-transferase genes in two maize genotypes after 60 g a.i. ha^{-1} nicosulfruon treated 0, 48, and 96 h using quantitative real-time PCR. Expression levels were normalized to UPF1, and error bars represent the standard deviation of the means of three independent replicates. Different letters above the error bars show significant differences at $p < 0.05$ based on Duncan's multiple range tests.

4. Discussion

Herbicides and other abiotic stress factors such as cold, heat, and drought could significantly enhance the accumulation of reactive oxygen species (ROS) in plant leaves, which would result in cell death [30]. Wang et al. found that nicosulfuron could greatly reduce the growth of waxy maize because it could induce the reduction of electron transfer activity and result in the overproduction of ROS [1]. Glutathione transferases, NADPH oxidases, superoxide dismutase (SOD), and peroxidase (POD) are well known for their ROS scavenging roles [31]. Glutathione S-transferases have also been thought to be associated with herbicide metabolism, and they are considered to be one of the most important determinant factors

of herbicide selectivity in crops and weeds [32]. Normally, the expression levels of GSTs are much higher in cereal crops than in weeds [13]. The GST-mediated metabolism has been previously reported to confer atrazine resistance in *Amaranthus tuberculatus*, and no activity of NBD-Cl was observed in the resistant biotype [33]. NBD-Cl has been demonstrated to inhibit the expression of phi (F) class of GSTs in blackgrass (*Alopecurus myosuroides* Huds.) [34]. In our study, we found that 60 g a.i. ha^{-1} nicosulfuron (recommended use rate) could cause great damage to the sensitive maize plants, with severe chlorosis, leaf veins turning purple, followed by the death of the entire plant. While it caused no obvious injury to the tolerant maize plants. Preprocessing with NBD-Cl greatly increased nicosulfuron photoxicity to the sensitive maize plants, while no impact on the response was perceived in the tolerant maize. Therefore, we speculated that nicosulfuron sensitivity in maize was greatly associated with glutathione S-transferases.

In 2000, McGonigle et al. identified 42 GST clones named *ZmGST* I to *ZmGST42* categorized into I, II, and III types in maize [35]. With the publication of the genome sequence of *Arabidopsis thaliana*, phylogenetic relationships of the plant GST super families were constructed, and a unifying nomenclature based on the well-established mammalian GST system was also adopted [13,36]. In our study, we demonstrated that there were at least 55 *ZmGST* genes belonging to six major sub-classes in the *Zea mays* L. var. B73 genome. All of the *ZmGST* genes were renamed following the suggested nomenclature system by Edwards [37]. The Tau and Phi classes were found to contain the most numerous members, with 32 and 13 *ZmGST* genes, respectively. They were considered to be specific to the plant and had the most abundant genes [36]. Tau GSTs were the largest group in angiosperms, gymnosperms, and ferns, and there were 58, 28, 52, 35, and 47 Tau GSTs once reported in the poplar, *Arabidopsis*, rice, loblolly pine, and *Selaginella moellendorffii* genomes, respectively [38]. No tau GSTs were found in the *Physcomitrella patens* genome, and Phi class GSTs were the most numerous with 10 members [39]. Recently, Munyampundu et al. identified similar Phi class GST sequences in ascomycete fungi, myxobacteria, and protists [40].

High level sequence similarities within the same GST classes were revealed through phylogenetic analysis in the three representative species, including *Zea mays*, *Oryza sativa*, and *Arabidopsis thaliana*. Structural component analysis indicated that most ZmGST proteins in the same phylogenetic class had similar gene structure and motif distribution. The rapid expansion of the GST gene family in plants was thought to be primarily due to the expansion of the Tau and Phi classes. These large-scale expansions within the Tau and Phi classes were considered to be related to their important defense role in response to changes in various environmental factors [41]. Tandem duplication has been reported to play a crucial role in the expansion of Tau and Phi GSTs in *Glycine max*, *Arabidopsis thaliana*, and *Oryza sativa* genomes [5,36,42]. In this research, we identified eight pairs of segmental duplications and seven pairs of tandem duplicated genes. Eight of thirty-two (25%) *ZmGSTU* genes consisted of four clusters, and five of thirteen (38%) *ZmGSTF* genes consisted of three clusters.

Historically, GSTs from the Tau (GSTU) and Phi (GSTF) classes were always considered to play important roles in biotic and abiotic stress tolerance in plants, including their rapid conjugation and detoxification of numerous herbicides in crops and weeds [43]. Further comprehensive and global gene expression of *ZmGST*s results indicated that there were great dissimilarities in *ZmGST* expression patterns that existed in both of the two genotypes for the fast defence responses against nicosulfuron. Much more highly expressed *ZmGST* DEGs (15 of 55) were detected in tolerant maize than in the sensitive genotype without nicosulfuron stress. After the application of nicosulfuron, more *ZmGST* genes were significantly induced in HB41, and most of them were the Tau class members, which were considered to participate in the responses to nicosulfuron stress. While for the tolerant genotype, there was only one upregulated *ZmGST* DEG which was in accordance with their phenotypic responses. Li et al. found that the expression of *ZmGSTU1*, *ZmGSTU2*, *ZmGSTU3*, *ZmGSTF1*, and *ZmGSTF3* were expressed higher in the metolachlor tolerant maize compared to the sensitive and involved in metolachlor detoxification [44]. *ZmGSTF1*

was once reported to display remarkable catalytic activity for alachlor and participate in its detoxifying process [45].

5. Conclusions

In short, glutathione S-transferase was demonstrated to be associated with nicosulfuron stress response in maize. A total of 55 *ZmGST* genes were comprehensively identified in the *Zea mays* L. var. B73 genome through the genome-wide analysis. The sequences of the GST orthologous groups were highly conserved. Based on phylogenetic analysis, six classes were categorized with the most numerous Tau and Phi *ZmGST* genes. Tandem gene duplication in these two classes resulted in the expansion of GST gene families in maize. Transcriptome results showed that there were different expression patterns of *ZmGST* genes in the tolerant and sensitive maize genotypes with or without nicosulfuron treatment, which were considered to possess different stress responses against herbicide stress.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy12112598/s1, Table S1: Primers for qRT-PCR of the differentially expressed ZmGST genes; Table S2: List of identified ZmGST genes in *Zea mays* along with their detailed genomic and proteomic information.

Author Contributions: Conceptualization, X.L. and G.W.; experimental, X.X. and B.Z.; manuscript—draft, X.L., B.L. and X.G.; data analysis—support, D.Z. and L.Y. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the Natural Science Foundation of Hebei Province (C2022301006), HAAFS Agriculture Science and Technology Innovation Project (2022KJCXZX-LYS-13), and Fundamental Research Funds of Hebei Academy of Agriculture and Forestry Sciences (2021060204).

Data Availability Statement: Primers for qRT-PCR and information of identified *ZmGST* genes were uploaded as Supplementary Files.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Wang, J.; Zhong, X.; Li, F.; Shi, Z. Effects of nicosulfuron on growth, oxidative damage, and the ascorbate-glutathione pathway in paired nearly isogenic lines of waxy maize (*Zea mays* L.). *Pestic. Biochem. Phys.* **2018**, *145*, 108–117. [CrossRef] [PubMed]
2. Cummins, I.; Dixon, D.P.; Freitag-Pohl, S.; Skipsey, M.; Edwards, R. Multiple roles for plant glutathione transferases in xenobiotic detoxification. *Drug Metab. Rev.* **2011**, *43*, 266–280. [CrossRef] [PubMed]
3. Dixon, D.P.; Edwards, R. Glutathione Transferases. In *Arabidopsis Book*; The American Society of Plant Biologists: Rockville, MD, USA, 2010; Volume 8, p. e0131.
4. Jain, M.; Ghanashyam, C.; Bhattacharjee, A. Comprehensive expression analysis suggests overlapping and specific roles of rice glutathione S-transferase genes during development and stress responses. *BMC Genom.* **2010**, *11*, 73. [CrossRef] [PubMed]
5. Liu, H.J.; Tang, Z.X.; Han, X.M.; Yang, Z.L.; Zhang, F.M.; Yang, H.L.; Liu, Y.J.; Zeng, Q.Y. Divergence in Enzymatic Activities in the Soybean GST Supergene Family Provides New Insight into the Evolutionary Dynamics of Whole-Genome Duplicates. *Mol. Biol. Evol.* **2015**, *32*, 2844–2859. [CrossRef]
6. Wang, R.; Ma, J.; Zhang, Q.; Wu, C.; Zhao, H.; Wu, Y.; Yang, G.; He, G. Genome-wide identification and expression profiling of glutathione transferase gene family under multiple stresses and hormone treatments in wheat (*Triticum aestivum* L.). *BMC Genom.* **2019**, *20*, 986. [CrossRef]
7. Rezaei, M.K.; Shobbar, Z.-S.; Shahbazi, M.; Abedini, R.; Zare, S. Glutathione S-transferase (GST) family in barley: Identification of members, enzyme activity, and gene expression pattern. *J. Plant Physiol.* **2013**, *170*, 1277–1284. [CrossRef]
8. Islam, M.S.; Choudhury, M.; Majlish, A.-N.K.; Islam, T.; Ghosh, A. Comprehensive genome-wide analysis of Glutathione S-transferase gene family in potato (*Solanum tuberosum* L.) and their expression profiling in various anatomical tissues and perturbation conditions. *Gene* **2018**, *639*, 149–162. [CrossRef]
9. Ma, L.; Zhang, Y.; Meng, Q.; Shi, F.; Liu, J.; Li, Y. Molecular cloning, identification of GSTs family in sunflower and their regulatory roles in biotic and abiotic stress. *World J. Microb. Biot.* **2018**, *34*, 109. [CrossRef]
10. Lallement, P.A.; Brouwer, B.; Keech, O.; Hecker, A.; Rouhier, N. The still mysterious roles of cysteine-containing glutathione transferases in plants. *Front. Pharmacol.* **2014**, *5*, 192. [CrossRef]
11. Nianiou-Obeidat, I.; Madesis, P.; Kissoudis, C.; Voulgari, G.; Chronopoulou, E.; Tsaftaris, A.; Labrou, N.E. Plant glutathione transferase-mediated stress tolerance: Functions and biotechnological applications. *Plant Cell Rep.* **2017**, *36*, 791–805. [CrossRef]

12. Strom, S.A.; Hager, A.G.; Concepcion, J.C.T.; Seiter, N.J.; Davis, A.S.; Morris, J.A.; Kaundun, S.S.; Riechers, D.E. Metabolic Pathways for S-Metolachlor Detoxification Differ Between Tolerant Corn and Multiple-Resistant Waterhemp. *Plant Cell Physiol.* **2021**, *62*, 1770–1785. [CrossRef] [PubMed]
13. Dixon, D.P.; Skipsey, M.; Edwards, R. Roles for glutathione transferases in plant secondary metabolism. *Phytochemistry* **2010**, *71*, 338–350. [CrossRef] [PubMed]
14. Labrou, N.E.; Papageorgiou, A.C.; Pavli, O.; Flemetakis, E. Plant GSTome: Structure and functional role in xenome network and plant stress response. *Curr. Opin. Biotech.* **2015**, *32*, 186–194. [CrossRef] [PubMed]
15. Frear, D.; Swanson, H.R. Biosynthesis of S-(4-ethylamino-6-isopropylamino-2-s-triazino) glutathione: Partial purification and properties of a glutathione S-transferase from corn. *Phytochemistry* **1970**, *9*, 2123–2132. [CrossRef]
16. Zhang, J.J.; Yang, H. Metabolism and detoxification of pesticides in plants. *Sci. Total Environ.* **2021**, *790*, 148034. [CrossRef]
17. Georgakis, N.; Poudel, N.; Papageorgiou, A.C.; Labrou, N.E. Comparative structural and functional analysis of phi class glutathione transferases involved in multiple-herbicide resistance of grass weeds and crops. *Plant Physiol. Bioch.* **2020**, *149*, 266–276. [CrossRef]
18. Chronopoulou, E.; Madesis, P.; Asimakopoulou, B.; Platis, D.; Tsaftaris, A.; Labrou, N.E. Catalytic and structural diversity of the fluazifop-inducible glutathione transferases from *Phaseolus vulgaris*. *Planta* **2012**, *235*, 1253–1269. [CrossRef]
19. Cho, H.Y.; Kong, K.H. Study on the biochemical characterization of herbicide detoxification enzyme, glutathione S-transferase. *Biofactors* **2007**, *30*, 281–287. [CrossRef]
20. Chronopoulou, E.; Madesis, P.; Tsaftaris, A.; Labrou, N.E. Cloning and Characterization of a Biotic-Stress-Inducible Glutathione Transferase from *Phaseolus vulgaris*. *Appl. Biochem. Biotech.* **2014**, *172*, 595–609. [CrossRef]
21. Goggin, D.E.; Cawthray, G.R.; Flematti, G.R.; Bringans, S.D.; Lim, H.; Beckie, H.J.; Busi, R. Pyroxasulfone-Resistant Annual Ryegrass (*Lolium rigidum*) Has Enhanced Capacity for Glutathione Transferase-Mediated Pyroxasulfone Conjugation. *J. Agric. Food Chem.* **2021**, *69*, 6414–6422. [CrossRef]
22. Rangani, G.; Noguera, M.; Salas-Perez, R.; Benedetti, L.; Roma-Burgos, N. Mechanism of Resistance to S-metolachlor in *Palmer amaranth*. *Front. Plant Sci.* **2021**, *12*, 652581. [CrossRef] [PubMed]
23. Pan, L.; Gao, H.; Xia, W.; Zhang, T.; Dong, L. Establishing a herbicide-metabolizing enzyme library in Beckmannia syzigachne to identify genes associated with metabolic resistance. *J. Exp. Bot.* **2016**, erv565.
24. Zhao, N.; Li, W.; Bai, S.; Guo, W.; Yuan, G.; Wang, F.; Liu, W.; Wang, J. Transcriptome Profiling to Identify Genes Involved in Mesosulfuron-Methyl Resistance in *Alopecurus aequalis*. *Front. Plant Sci.* **2017**, *8*, 1391. [CrossRef] [PubMed]
25. Pan, L.; Guo, Q.; Wang, J.; Shi, L.; Yang, X.; Zhou, Y.; Yu, Q.; Bai, L. *CYP81A68* confers metabolic resistance to ALS and ACCase-inhibiting herbicides and its epigenetic regulation in *Echinochloa crus-galli*. *J. Hazard Mater.* **2022**, *34*, 128225. [CrossRef] [PubMed]
26. Chen, W.; Wu, L.; Wang, J.; Yu, Q.; Bai, L.; Pan, L. Quizalofop-p-ethyl resistance in *Polypogon fugax* involves glutathione S-transferases. *Pest Manag. Sci.* **2020**, *76*, 3800–3805. [CrossRef]
27. Lin, F.; Jiang, L.; Liu, Y.; Lv, Y.; Dai, H.; Zhao, H. Genome-wide identification of housekeeping genes in maize. *Plant Mol. Biol.* **2014**, *86*, 543–554. [CrossRef]
28. Kõressaar, T.; Lepamets, M.; Kaplinski, L.; Raime, K.; Andreson, R.; Remm, M. Primer3_masker: Integrating masking of template sequence with primer design software. *Bioinformatics* **2018**, *34*, 1937–1938. [CrossRef]
29. Liu, X.; Bi, B.; Xu, X.; Li, B.; Tian, S.; Wang, J.; Zhang, H.; Wang, G.; Han, Y.; McElroy, J.S. Rapid identification of a candidate nicosulfuron sensitivity gene (*Nss*) in maize (*Zea mays* L.) via combining bulked segregant analysis and RNA-seq. *Theor. Appl. Genet.* **2019**, *132*, 1351–1361. [CrossRef]
30. Boulahia, K.; Carol, P.; Planchais, S.; Abrous-Belbachir, O. *Phaseolus vulgaris* L. seedlings exposed to prometryn herbicide contaminated soil trigger an oxidative stress response. *J. Agric. Food Chem.* **2016**, *64*, 3150–3160. [CrossRef]
31. Yang, G.; Wang, Y.; Xia, D.; Gao, C.; Wang, C.; Yang, C. Overexpression of a GST gene (*ThGSTZ1*) from Tamarix hispida improves drought and salinity tolerance by enhancing the ability to scavenge reactive oxygen species. *Plant Cell Tissue Organ Cult.* **2014**, *117*, 99–112. [CrossRef]
32. Nandula, V.K.; Riechers, D.E.; Ferhatoglu, Y.; Barrett, M.; Duke, S.O.; Dayan, F.E.; Goldberg-Cavalleri, A.; Tétard-Jones, C.; Wortley, D.J.; Onkokesung, N. Herbicide Metabolism: Crop Selectivity, Bioactivation, Weed Resistance, and Regulation. *Weed Sci.* **2019**, *67*, 149–175. [CrossRef]
33. Shergill, L.S.; Bish, M.D.; Jugulam, M.; Bradley, K.W. Molecular and physiological characterization of six-way resistance in an *Amaranthus tuberculatus* var. rudis biotype from Missouri. *Pest Manag. Sci.* **2018**, *74*, 2688–2698. [CrossRef] [PubMed]
34. Cummins, I.; Wortley, D.J.; Sabbadin, F.; He, Z.; Coxon, C.R.; Straker, H.E.; Sellars, J.D.; Knight, K.; Edwards, L.; Hughes, D. Key role for a glutathione transferase in multiple-herbicide resistance in grass weeds. *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 5812–5817. [CrossRef] [PubMed]
35. McGonigle, B.; Keeler, S.J.; Lau, S.M.; Koeppe, M.K.; O'Keefe, D.P. A genomics approach to the comprehensive analysis of the glutathione S-transferase gene family in soybean and maize. *Plant Physiol.* **2000**, *124*, 1105–1120. [CrossRef] [PubMed]
36. Dixon, D.P.; Lapthorn, A.; Edwards, R. Plant glutathione transferases. *Genome Biol.* **2002**, *3*, 1–10. [CrossRef]
37. Edwards, R.; Dixon, D.P.; Walbot, V. Plant glutathione S-transferases: Enzymes with multiple functions in sickness and in health. *Trends Plant Sci.* **2000**, *5*, 193–198. [CrossRef]

38. Monticolo, F.; Colantuono, C.; Chiusano, M.L. Shaping the evolutionary tree of green plants: Evidence from the GST family. *Sci. Rep.* **2017**, *7*, 14363. [CrossRef]
39. Liu, Y.J.; Han, X.M.; Ren, L.L.; Yang, H.L.; Zeng, Q.Y. Functional divergence of the glutathione S-transferase supergene family in *Physcomitrella patens* reveals complex patterns of large gene family evolution in land plants. *Plant Physiol.* **2013**, *161*, 773–786. [CrossRef]
40. Munyampundu, J.P.; Xu, Y.P.; Cai, X.Z. Phi class of glutathione S-transferase gene superfamily widely exists in nonplant taxonomic groups. *Evol. Bioinform.* **2016**, *12*, EBO-S35909. [CrossRef]
41. Frova, C. Glutathione transferases in the genomics era: New insights and perspectives. *Biomol. Eng.* **2006**, *23*, 149–169. [CrossRef]
42. Soranzo, N.; Gorla, M.S.; Mizzi, L.; De Toma, G.; Frova, C. Organisation and structural evolution of the rice glutathione S-transferase gene family. *Mol. Genet. Genom.* **2004**, *271*, 511–521. [CrossRef] [PubMed]
43. Sylvestre-Gonon, E.; Law, S.R.; Schwartz, M.; Robe, K.; Keech, O.; Didierjean, C.; Dubos, C.; Rouhier, N.; Hecker, A. Functional, Structural and Biochemical Features of Plant Serinyl-Glutathione Transferases. *Front. Plant Sci.* **2019**, *10*, 608. [CrossRef] [PubMed]
44. Li, D.; Gao, Q.; Xu, L.; Pang, S.; Liu, Z.; Wang, C.; Tan, W. Characterization of glutathione S-transferases in the detoxification of metolachlor in two maize cultivars of differing herbicide tolerance. *Pestic. Biochem. Phy.* **2017**, *143*, 265–271. [CrossRef]
45. Karavangeli, M.; Labrou, N.E.; Clonis, Y.D.; Tsaftaris, A. Development of transgenic tobacco plants overexpressing maize glutathione S-transferase I for chloroacetanilide herbicides phytoremediation. *Biomol. Eng.* **2005**, *22*, 121–128. [CrossRef] [PubMed]