Genome-wide identification and expression analysis of the glutathione S-transferase (GST) family under different developmental tissues and abiotic stresses in Chinese cabbage (Brassica rapa ssp. pekinensis)

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Abstract

The glutathione-S-transferase (GST) family of proteins is ancient and versatile, and plays an important role in combating exogenous pathogens, endogenous toxicants, and various abiotic stresses. Although the GST family has been studied in many crops, few studies have been reported in Chinese cabbage (Brassica rapa ssp. Pekinensis). In the present work, genome-wide analysis of the GST family in Chinese cabbage was carried out, and the expression and functions of genes under different conditions were investigated. A total of 88 GST genes were identified and divided into seven subfamilies according to their evolutionary relationships. Tandem duplication of genes was revealed as the main mechanism of expansion in this family. Transcriptome analysis under high and low temperatures and abiotic stress conditions revealed that most GST genes respond to environmental changes to varying degrees, particularly under herbicide and cadmium stress conditions. Our findings provide a basis for analysing the functions of GST family members in Chinese cabbage, especially in response to various abiotic stresses.

Keywords: GST, Chinese cabbage, genome-wide analysis, RT-PCR, transcriptomics, abiotic stress
Introduction

Glutathione transferase, formerly known as glutathione S-transferase (Jain M1 et al. 2010), is a multifunctional soluble dimeric enzyme (Nutricati E et al. 2006) that catalyses the conjugation of the tripeptide glutathione with various hydrophobic electrophilic substrates (Liu YJ et al. 2013; Nutricati E et al. 2006). The basic function of GST, which is ubiquitous in animals, plants, and microorganisms, is the detoxification of endogenous and heterogenic compounds (Nutricati E et al. 2006; Marrs KA 1996; Dixon D P et al. 1998). GST was first discovered in animals, and shortly after the enzyme from maize was shown to catalyse the conjugation of herbicides and GSH to alleviate poisoning (Shimabukuro R H et al. 1970). Since these early studies, numerous herbicide-resistant GSTs have been identified in plants including rice, soybean, Arabidopsis, and poplar (McGonigle B et al. 2000; Lan T et al. 2009; Soranzo N 2004; Wagner U et al. 2002).

Based on sequence and structural homology, plant GSTs can usually be divided into Phi (F), Tau (U), Lambda (L), dehydroascorbate reductase (DHAR), Theta (T), Zeta (Z), and tetrachlorohydroquinone dehalogenase (TCHQD) (Liu YJ et al. 2013; Dong Y, Li C et al. 2016; Sheehan, D. et al.2001; Dixon, D. P. et al. 2002), among which Phi, Tau, DHAR, and Lambda are plant-specific (Edwards R and Dixon DP 2005). Tau and phi include the most members, and these subfamilies are mainly involved in the detoxification of exogenous toxicants (Shimabukuro R H et al. 1970; Dixon, D. P. et al. 2002). Overexpression of Tau- and Phi-like GSTs can increase the tolerance of transgenic plants to herbicides and salt stress (Liu YJ et al. 2013; Karavangeli M 2005; Benekos K et al. 2010; Jha B et al. 2011) . GSTs can also incorporate by-products of oxidative stress, thereby reducing the risk of plant oxidative stress. Overexpression of GSTs with higher glutathione peroxidase (GPOX) activity in tobacco seedlings increased the resistance of transgenic tobacco plants to low temperature and oxidative stress compared with wild-type (WT) plants, verifying for the first time the role of Tau GST in cold tolerance and antioxidant stress in plants (Roxas VP et al. 1997) . In conclusion, both Phi and Tau GSTs display significant functional diversity, both in protecting plants from damage and maintaining intracellular metabolic balance, whereas DHAR and Lambda GSTs act as thiotransferases, and are not conjugated to GSH (Dixon, D. P. et al. 2002). High temperature treatment of rice seedlings can increase DHAR mRNA and protein levels, indicating that DHAR confers high temperature protection.

In addition, evidence suggests GST plays a role in plant development. For example, studies in some model and ornamental plants show that GSTs participate in accumulation of anthocyanidins in the vacuole, which are mainly involved in the transformation of flower colour (Kitamura S et al. 2012). In Arabidopsis thaliana, GST clearly expressed in different organs, and expression is altered in response to biological and abiotic stresses (Kao CW et al. 2016; DeRidder BP and Goldsbrough PB 2006; Moons A 2005; Sappl PG et al.2004). The use of photoaffinity labelling proved that GST is a cytokine- and auxin-binding protein (Zettl R et al. 1994) which regulates their activities by binding to different hormones. In addition, accumulation of reactive oxygen species (ROS) may be induced under stress.
conditions, and GST activity is increased under stress conditions to protect plants against ROS, suggesting GSTs play a very important role as antioxidants (Moons A 2003; Itzhaki H et al. 1994; Kiyosue T et al. 1993). In addition to oxidative stress, GSTs respond differently to environmental changes representing biological and abiotic stresses. Overexpression of Tau in Arabidopsis enhances tolerance to salt and oxidative stress (Dong Y, Li C et al. 2016; Sharma, R et al. 2014), and 35 of 56 GSTU GSTs in Sorghum mediate tolerance to cold and salt stress (Chi Y et al. 2011). Previous studies on the GST family have focused on vascular plants, but Tau-like proteins have not been identified in non-vascular plants, indicating different evolutionary patterns during evolution (Liu YJ et al. 2013).

Although GSTs in other crops have been thoroughly investigated, structural and functional analysis of GSTs in Chinese cabbage has not been reported. Recent publication of the Chinese cabbage genome sequence and related information makes it possible to analyse GSTs at the whole genome level. This was performed in the present work using bioinformatics methods, and all GST family members were classified, chromosomally located, quantitatively analysed in terms of gene expression under different stress conditions, and functionally verified. Analysis of the genome of Chinese cabbage is of great significance for improving resistance and quality in this important crop species.

Materials and Methods

Database searching, sequence analysis, and nomenclature
We obtained genome sequences of B. rapa from the Brassica database (BRAD, http://brassicadb.org/brad/) (Wang C et al. 2015; Wang X et al. 2011). In order to identify all members of the GST family in B. rapa, we downloaded 57 Arabidopsis thaliana GST sequences from the TAIR database (http://www.arabidopsis.org/), and performed systematic BLAST homology searches in the Brassica database, with an expected value (e-value) cut-off of 1.0 (Huang W et al. 2015; Wang C et al. 2015). In addition, we searched for syntenic genes between A. thaliana and B. rapa (Table S1), manually removed redundant genes, and tested candidate gene sequences using the Pfam database (http://Pfam.sanger.ac.uk/). Nomenclature of Chinese cabbage GSTs followed the system suggested previously for A. thaliana (Dixon, D. P. et al. 2002); a univocal name was assigned to each GST gene, consisting of a letter for the subfamily class, such as GSTU, F, T, Z, and L, corresponding to Tau, Phi, Theta, Zeta, and Lambda, with a number for each gene.

Phylogenetic analysis and conserved motif analysis
In order to group GSTs, phylogenetic trees were produced using full-length sequences of GST proteins from B. rapa and Arabidopsis. Phylogenetic and molecular evolutionary genetic analyses were conducted using MEGA6 (http://www.megasoftware.net/) with the neighbour-joining (NJ) method (Wang C et al. 2015; Tamura K et al. 2013), with 1000 bootstrap replicates (Wang C et al. 2015). We used the online software MEME (http://meme.sdsc.edu/meme/) to detect conserved motifs in GST
proteins (Bailey TL et al. 2009).

Location of GST genes on chromosomes and structural analysis

The position of each GST gene on the 10 chromosomes was determined from the Brassica database and positions marked on each chromosome using MapChart (Voorrips RE 2002). The exon positions of BrGST genes were analysed by GSDS (http://gsds.cbi.pku.edu.cn/) (Hu B et al. 2014).

Plant material, growth conditions, and abiotic stress treatments

We selected Chinese cabbage DH FT as the experiment material, and placed seeds on a petri dish to germinate. After germination, some seedlings were transferred to a soil-vermiculite (3:1) plug tray and placed in a controlled environment growth chamber (Wang C et al. 2015; Zhang G et al. 2015). Others were transferred to a hydroponic culture with Hoagland hydroponic formula as a nutrient solution. Artificial growth conditions were 25/15°C, with a 12 h/12 h day/night photoperiod and 50–60% relative humidity for 1 week (Huang W et al. 2015; Zhang G et al. 2015). The discovery of the GST gene family was linked to their role in resistance to herbicides, and plant GSTs play an important role in cadmium stress. We therefore sprayed the foliage of plants in plug trays with glyphosate or CdCl2 (10 mg/L) at the four-leaf stage. In addition, after researching appropriate conditions, hydroponic plants were transferred from normal conditions to 4°C or 38°C for cold and heat treatment, respectively, or treated with 15% (w/v) polyethylene glycol (PEG) 6000 and 250 mM NaCl for osmotic stress (Wang C et al. 2015). Root, stem, leaf, petal, bud samples were collected at five time points for each treatment at 3 h intervals and stored at -80°C until needed for RNA extraction.

Transcriptome analysis under abiotic stress

Total RNA was isolated from tissue samples using TRIzol Reagent (Invitrogen, USA) according to the manufacturer’s instructions. To construct the cDNA library, polyA-containing mRNA was isolated from total RNA using oligo (dT) beads and broken into short fragments by the addition of fragmentation buffer. These short RNA fragments served as templates to synthesise first-strand cDNA using random hexamer primers prior to second-strand cDNA synthesis (Huang S. et.al 2017). End repair of dscDNA with phosphate at the 5’ end and polyA at the 3’ end was followed by ligation with adaptors possessing a polyT overhang at the 3’ end. Two specific primers were used to amplify the ligation product, which was denatured by heating (Huang S. et.al 2016), and the single-stranded DNA was cyclised using a splint oligo and DNA ligase. Finally, library products were sequenced using an Illumina HiSeq 2000 platform.

RNA purification, and expression analyses using real-time RT-PCR

In order to investigate the expression of representative GST genes in Chinese cabbage in various
tissues and following different abiotic stress treatments, total RNA was isolated using an RNA kit (TIANGEN, Beijing, China) according to the manufacturer’s instructions. RNA was reverse-transcribed into cDNA using a FastQuant RT Kit with gDNase (TIANGEN). cDNAs were diluted 1:50 with ddH2O and used as a template for RT-PCR. Primers were designed using Primer Premier software (version 5.0) and are listed in Table S2. The actin gene was used as an internal control to normalise the expression levels of target genes. The reaction was performed using an Applied Biosystems Quantsstudio 6 Flex Real-time PCR System (Life Technologies, USA) according to the supplier’s protocol((Huang S. et.al 2015). Reaction mixtures (50 µl) contained 21 µl ddH2O, 2 µl of diluted cDNA product from reverse-transcription PCR, 2 µl gene-specific primers, and 25 µl UltraSYBR Mixture (Low ROX, CWBIO, Beijing, China). Thermal cycling involved denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and annealing/elongation at 60°C for 60 s. Melting curve analysis was performed at 95°C for 15 s, 60°C for 60 s, 95°C for 15 s, and 60°C for 15 s. All real-time PCR samples were tested using at least three biological and three technical replicates, and relative gene expression was calculated by the 2-∆∆Ct method (Livak KJ et al. 2001).

**Results**

**The GST gene family in Chinese cabbage**

In *Arabidopsis*, there are 57 members of the GST gene family(Chen IC et al. 2007). The *Arabidopsis* GST protein sequences were obtained from the TAIR database, and BLASTp and BLASTn screening identified 92 homologous GST genes in the Chinese cabbage genome. Subsequent collinearity analysis confirmed 77 genes displaying collinearity with *Arabidopsis*. Domain analysis identified 14 genes with only an N-terminal GST domain, two genes with only a C-terminal GST domain, and 61 genes with both domains. So coupled with a series of previous analysis identified 79 members of the GST family in *B. rapa*. However, it is worth mentioning that there are other nine genes displaying a collinear relationship with Arabidopsis GSTs did not appear to possess a GST-specific domain. In this article, the nine genes are temporarily attributed to the members of the GST family. So there are 88 GST family members. But in the next study the nine genes are not discussed for the time being. Expansion of the number of GST genes relative to Arabidopsis indicates that tandem replication may have occurred during evolution. Gene location, predicted gene position, ORF length, protein length, and chromosome location for all 79 Chinese cabbage GSTs are listed in Table 1 (1).

**Table 1 The GST gene family in *B. rapa***

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<td>Chromosomal distribution of GST genes in Chinese cabbage</td>
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| MapChart software was used to map the 79 GST genes shown in Fig. 1. BrGSTU4 was not clearly located on a chromosome, but all other GST genes could be located on the 10 chromosomes of Chinese cabbage. Chromosome 7 contains the greatest number (15 genes), while only one (BrGSTU21) is located on chromosome 1. Tandem gene duplication during evolution appears to have led to several gene clusters on chromosomes.
Fig. 1. Chromosomal distribution of GST genes in Chinese cabbage. The 78 GST genes are distributed on all 10 chromosomes. The scale on the left shows the physical distance of the gene on the chromosome.

Phylogenetic analysis of the GST family in Chinese cabbage

In order to analyse the evolutionary relationships of GSTs in Chinese cabbage, a phylogenetic tree was constructed using full-length protein sequences from *A. thaliana* and *B. rapa* (Fig. 2). The results showed that Chinese cabbage GSTs can be divided into seven subfamilies, corresponding to the *A. thaliana* sequences, with Tau and Phi accounting for the vast majority (40 and 22, respectively). The Zeta class has three members, the Dehy, and Lambda classes each include four genes, the Theta class contain five genes, and the Tetrach class is represented by a single GST. In order to maintain consistent naming of the GST family in Chinese cabbage, we named genes based on the principles applied previously to other species, using U, F, Z, T, DHAR, L, and TCHQD to represent Tau, Phi, Zeta, Theta, Dehy, Lambda, and Tetrach (Peng Z et al. 2014), followed by a unique number for members of each subgroup. As shown in the figure, genes of the same subfamily are close together, indicating similar evolution of GST genes in *Arabidopsis* and *B. rapa*. 
Fig. 2. Phylogenetic trees based on full-length protein sequences of GSTs from *Arabidopsis* and Chinese cabbage. Trees were constructed using the neighbour-joining method in MEGA6. Different colours represent different subgroups.

**Gene structure of GSTs in Chinese cabbage**

As shown in Fig. 3, the number of exons differs between GST family members in Chinese cabbage. While most genes include two or three exons, *BrGSTU2* contains only one, and *BrGSTL2* includes 15 exons. In the DHAR subgroup, all members have three exons except *BrDHAR2*, and there are three exons in *BrGSTF*, but *BrGSTF19* and *BrGSTF7* have two exons, and *BrGSTF21*, *BrGSTF22*, and *BrGSTF8* contain four exons. *BrGSTU6*, *BrGSTU8*, *BrGSTU17*, and *BrGSTU31* belonging to the *BrGSTU* subgroup have three exons, while the rest of the members in this subfamily have two exons. Members of the other subfamilies (*BrGSTL*, *BrGSTT*, and *BrGSTZ*) contain multiple exons.
Fig. 3 Gene structure of GSTs in Chinese cabbage analysed by the online software GSDS. Exons and introns are indicated by coloured boxes and thin lines, respectively.

**Conserved motif identification in Chinese cabbage GSTs**

The MEME online tool was used to analyse conserved motifs in the 79 GSTs in Chinese cabbage, with a maximum number of predicted conserved motifs of 20, and all other settings were default values (Krajewski MP et al. 2013). The longest motif was 50 amino acids, and the shortest was 15 residues (Fig. 4). The distribution of the 20 conserved motifs was further analysed, and none of the genes contain all 20 conserved motifs. Indeed, all genes include between one and eight of the motifs. Specifically, 74 genes contain motif No. 1, 68 genes contain motif No. 5, motifs 2, 4, 7, 10, 12, and 16 are specific for the BrGSTU class, and motifs 3, 6, 11, 18, and 15 are specific for the BrGSTF, BrGSTU, and BrGSTZ classes. Motif 19 is present only in the BrGSTU BrGSTT classes, and motif 14 is specific to the BrGSTZ and BrGSTF classes.

Table 2 Distribution of conserved motifs in GSTs in Chinese cabbage. The MEME online tool was used to detect 20 conserved motifs (indicated by different coloured boxes).

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Transcriptome and expressional pattern analysis under abiotic stress conditions

Expression of BGPEQ-500 RSRNA-Seq under four stress treatments (high temperature, low
temperature, NaCl, and PEG) was analysed with RPGM as the unit of expression. Five BrGST genes were not detected following temperature stress (BrGSTU3, BrGSTU38, BrGSTF21, BrGSTF22, BrGSTU19). Analysis of the thermal map of the temperature stress treatment (Fig. 4a), with the extension of processing time, the expression level of some genes changed. In high temperature stress, relative to 0h, the expression level of 18 genes was changed at 3h, 5 of them were up regulated (BrGSTU12, BrGSTF5, BrGSTZ1, BrDHAR4, BrGSTU39), 13 down regulated, and 7 genes were up-regulated (BrGSTU12, BrGSTU40, BrGSTF5, BrGSTU13, BrGSTU10, BrGSTF11, BrGSTL2) and 11 expression decreased at 12h. Under low temperature stress, there were 3 gene expression changes after 3h treatment, of which only BrGSTU16 up-regulated expression, the other two (BrGSTU30, BrGSTU1) decreased, but at 12h, there were 19 genes expression response to stress changes, 7 up regulation and 12 down regulation.

In the osmotic stress treatment, only BrGSTF21 was not detected, and a heatmap at different time points under NaCl and PEG treatment (Fig. 4b) revealed that the expression of some genes also changed with the extension of treatment time, under salt stress, 29 gene expression changes at 3h, the expression of only two genes (BrGSTF12, BrGSTF11) were down-regulated, the rest were up-regulated, the expression changes of 30 genes at 12h, 6 genes were down-regulation and others were up-regulated.

In the treatment of drought stress, 23 genes responded to environmental changes at 3h, and five of them (BrGSTU17, BrTCHQD1, BrGSTF6, BrGSTU35, BrGSTF12) were down regulated and 18 up-regulated. 28 genes were changed at 12h, 12 of them were down regulated, and 16 were up-regulated.
(a)
Fig. 4 Heatmap expression analysis of temperature (a) and osmotic stress (b) in Chinese cabbage.

Expression levels at 0, 3, and 12 h were mapped. The coloured bar on the right indicates the level of expression.

Increasing evidence suggests plant GSTs are involved in stress responses, especially to herbicide and cadmium stress. Changes in gene expression in the leaves of plants subjected to different stress treatments were therefore measured, based on related genes in Arabidopsis, 20 GSTs in Chinese cabbage potentially related to abiotic stress were selected, and the results showed that most of the 20 selected GSTs were gradually up-regulated with increasing exposure time to herbicides (Fig. 5). This trend was particularly obvious for BrGSTF9, BrGSTU12, BrGSTU8, and BrGSTF1. However, for BrGSTU39, BrGSTU13, and BrGSTF16, expression was highest at 9 h, then gradually decreased with increasing duration of exposure. Interestingly, for BrGSTF6, BrGSTF2, and BrGSTU15, expression was highest at 3 h, and it was 1569-fold higher than controls in the case of BrGSTF2, suggesting this gene is particularly sensitive to herbicide stress.
Expression of GSTs was also altered in response to cadmium stress (Fig. 5). Expression of \textit{BrGSTU12} and \textit{BrGSTF15} was highest at 3 h, but only ~4–6-fold higher than controls, and there were no obvious differences at other time points. Expression of most genes was slightly up-regulated at 3 h, although expression of \textit{BrGSTF2} was maximal at 12 h, although only 5.7-fold above levels in controls.

**Fig. 5** Analysis of the expression of 20 selected GSTs in herbicide and cadmium stress. a,b,c The relative expression ratios of GST genes under herbicide stress. d, f The relative expression ratios of GST genes under cadmium stress.

**Differential expression of Chinese cabbage GSTs in different tissues**

Fluorescent quantitative analysis revealed different levels of gene expression in different tissues (Fig. 6 7). Most GST genes were expressed more highly in the roots than in other parts, especially \textit{BrGSTU33}, \textit{BrGSTF15}, \textit{BrGSTF17}, \textit{BrGSTF10}, \textit{BrGSTU25}, and \textit{BrGSTU20}, suggesting they may be related to receptors in root tissue that are more sensitive to environmental changes. Expression of \textit{BrGSTU20} was significantly higher in stems than in other parts. \textit{BrGSTU33}, \textit{BrGSTU13} was expressed most highly in the bud, and \textit{BrGSTF1} expression was much higher in petals than in other parts.
Fig. 6  Analysis of the expression of 20 selected GSTs in different tissues

Discussion

Sequencing of the complete genome of Chinese cabbage provides an opportunity to investigate the GST family at the whole genome level. Using bioinformatics methods, 79 GST genes were identified and divided into seven subfamilies with different numbers of members in each subgroup, indicating a non-uniform distribution. This evident diversity likely underlies diversity of GST function, and identification of the entire gene family provides genetic resources for functional studies. Apart from a single gene in the scaffold region, the 78 GST genes in Chinese cabbage are widely distributed across all 10 chromosomes, indicating a broad common ancestor. Although it is not yet possible to locate the ancestral gene using current second-generation sequencing technology, future developments in this area will likely allow it to be localised to a specific chromosomal position.

Studies have shown that members of certain plant gene families are clustered or dispersed on chromosomes by tandem repeat extension or fragment duplication (Liu YJ et al. 2013). The GST family in Chinese cabbage appears to have expanded via both mechanisms. The large number of highly conserved motifs in both domains of the GST enzymes indicates important functional roles that have been strictly preserved during evolution.
Plant GSTs are multifunctional proteins encoded by a large gene family that has been divided into seven classes (Tau, Phi, Theta, Lambda, DHAR, TCHQD) in previous studies (Oakley, 2005; Dixon and Edwards, 2010). Among the seven categories, Tau, Phi, Lambda, and DHAR are unique to plants. To date, GSTs have been studied in A. thaliana, rice, soybean, poplar, small moss, and other plants. For example the rice genome has undergone several rounds of genome-wide replication during evolution, which has greatly expanded the GST gene family (Chi Y et al. 2010). Determination of the chromosomal distribution of the GST gene family a series of clusters scattered across all chromosomes, but most tandem events appear to have occurred in the Tau class, even though only 28 members of the Tau class are predicted in A. thaliana, compared with 52 in rice (Jain M et al. 2010). In the no-vascular small moss, 37 GST genes were identified and divided into 10 classes, including two new genera (hemerythrin and itao) (Liu YJ et al. 2013), but members of the Tau category were not identified, further suggesting it is unique to vascular plants. Specific gene losses may have occurred during evolution; in the published Chinese cabbage database, the GST family is divided into seven classes. Based on comparison of 57 GST gene sequences in A. thaliana, 79 GSTs were identified in Chinese cabbage. Thus, an additional genes encoding GST proteins were identified in the present study, indicating extensive gene duplication during evolution (Huang W et al. 2015). Most of the newly discovered GST genes are members of the Tau class, including BrGSTU22, BrGSTU8, and BrGSTU9 on chromosome 4 resulting from tandem repeats during the evolutionary process. Plotting the predicted distribution of GST family members on the chromosomes indicates different degrees of tandem gene replication on different chromosomes. Members of the Tau class therefore played an important role in the evolution of the entire GST gene family. Construction of a phylogenetic tree based Arabidopsis and Chinese cabbage GSTs resulted in division into seven subfamilies. GST family members have two terminal domains, but nine GST genes displaying a significant collinear relationship with Arabidopsis thaliana GSTs were identified in the present work, indicating that they may have evolved from counterparts in the Arabidopsis genome. However, domain detection suggests the nine genes lack GST family-specific domains, some parts of the gene sequences may have been altered, deleted, or replaced during evolution, that potentially explaining why, although further verification is clearly needed.

Analysis of the expression patterns of the 20 selected GST genes revealed differences in tissue distribution, indicating a variety of functions in the growth and development of Chinese cabbage. For example, expression of GSTU20 was higher in stems than in other parts, while GSTU13 and GSTU34 expression was highest in buds, and GSTF1 as expressed highly in petals. In addition, differences in the expression of these genes were observed following stress treatments. Under herbicide stress, GSTF6, GSTF2, and GSTU15 were down-regulated at 3 h after treatment, and expression decrease with increasing stress duration. Expression of these genes was more sensitive than that of others, which could be relevant for improving plant growth and development.
Conclusion

Members of the GST family are important detoxification enzymes. The identification of the entire
GST family in Chinese cabbage in the present work will aid future research on the functions of
individual GST enzymes and the GST family as a whole.

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