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Research article

Genome-wide profiling of expression and biochemical functions of the *Medicago* glutathione S-transferase gene family

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ABSTRACT

Glutathione *S*-transferases are ubiquitous enzyme in plants, playing vital roles in several physiological and developmental processes. In this study we identified 73 GST genes from the genome of *Medicago truncatula*. The *Medicago* GSTs were divided to eight classes with tau and phi being the most numerous. Six clusters were found on four *Medicago* chromosomes. The local gene duplication mainly contributed to the expansion of this large gene family. Functional divergence was found in their gene structures, gene expression patterns, and enzyme properties. A genomic comparative analysis revealed lineage-specific loss/gain events between *Medicago* and *Glycine*. This study offered new insights into the evolution of gene family between closely related species.

1. Introduction

Glutathione *S*-transferases (GSTs; EC 2.5.1.18) are ubiquitous in all organisms, being multifunctional proteins encoded by a large gene family. Based on amino acid sequence similarity and gene organization, eight classes have been identified among the plant GSTs, including phi, tau, theta, zeta, lambda, dehydroascorbate reductase (DHAR), tetrachlorohydroquinone dehalogenase (TCHQD) and γ-subunit of the eukaryotic translation elongation factor 1B (EF1Bg) (Dixon and Edwards, 2010a; Lan et al., 2009; Oakley, 2005). Recently, we identified two new GST classes (hemerythrin and iota) in nonvascular plants (Liu et al., 2013). Tau and phi GSTs are the most abundant in vascular plants (Dixon and Edwards, 2010a; Liu et al., 2013). GSTs mainly detoxify the xenobiotic by catalyzing the conjugation of reduced glutathione (GSH) to an extensive range of hydrophobic and electrophilic substrates (Frova, 2003). Except for detoxification functions, GSTs also play crucial roles in several physiological and developmental processes, including stress resistance, isomerization, secondary metabolism, signal transduction, and protection against UV radiation, oxidative damages and heavy metals (Dixon and Edwards, 2010b; Edwards et al., 2011; Sharma et al., 2004).

Medicago truncatula is a small annual legume, and its whole genome has been sequenced (Young et al., 2011). This species has a small diploid genome, a rapid generation time and prolific seed production. *M. truncatula* is a close relative of alfalfa (*M. sativa*), a widely cultivated crop with limited genomics tools and complex autotetraploid genetics (Young et al., 2011). Now, *M. truncatula* is studied as a model organism for legume biology. *M. truncatula* and *Glycine max* genomes have undergone a common whole-genome duplication (WGD) event, occurring approximately 59 Ma (Cannon et al., 2015; Schmutz et al., 2010). After the *Medicago-Glycine* split, *Glycine* genome has undergone a WGD event occurring approximately 13 Ma, while *Medicago* genome has not (Schmutz et al., 2010; Young et al., 2011). Thus, comparison of gene evolution between *M. truncatula* and *G. max* makes it ideal for studying the evolutionary and functional dynamics of duplicate genes following polyploidy.

In this study, we identified 73 GSTs from the genome of *Medicago truncatula*, and mainly focused on GST enzyme functions towards several herbicides, toxic compounds and peroxides. Functional divergences among all the GST genes were also characterized by integrating the gene structure, gene expression pattern, and enzymatic properties. Additionally, our previous study examined GST genes in *Glycine* (Liu et al., 2015). By comparison of GST gene evolution between *M. truncatula* and *G. max*, this study revealed lineage-specific loss/gain event and divergences in substrate specificity among the orthologous/paralogous GST gene pairs between *M. truncatula* and *G. max*. Taking together, this study provides new insights into the evolution of gene family between the legume genomes.

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2. Materials and methods

2.1. Identification of GST genes from Medicago truncatula genome

To identify GST genes in *Medicago*, TBLASTN searches of the *Medicago* genome database *Medicago truncatula Mt4.0v1* (http://www.phytozome.net/search.php) were performed using 55 full-length GST protein sequences of *Arabidopsis thaliana* (Dixon and Edwards, 2010a) and 81 of *Populus trichocarpa* (Lan et al., 2009). Manual reannotation was performed to rectify incorrect start codon predictions, splicing errors, missed or extra exons, fused genes, split genes, and incorrectly predicted pseudogenes. The reannotated sequences were analyzed using an NCBI conserved domain search (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) to confirm the presence of typical GST N- and C-terminal domains in their protein structure. The predicted GST genes were then amplified from the mRNA from *Medicago*, cloned into the pEASY-T3 vector (TransGen Biotech, Beijing, China), and sequenced to verify the gene sequences. Primers used in gene amplifications were listed in Supplementary Table S1. The nomenclature for *Medicago* GSTs followed the system suggested by Dixon et al. (2002) for plant GSTs; a univocal name was assigned to each *Medicago* GST gene, consisting of three italic letters denoting the source organism, the family name (e.g., *MtrGSTU*, *MtrGSTF*, *MtrGSTT*, *MtrGSTZ*, *MtrGSTL*, *MtrDHAR*, *MtEF1Bγ*, and *MtTCHQD*, corresponding to tau, phi, theta, zeta, lambda, DHAR, EF1Bγ, and TCHQD classes, respectively) and a progressive number for each gene (e.g., *MtrGSTU1*).

2.2. Phylogenetic analyses and promoter analysis

Full-length GST protein sequences were aligned by MUSCLE software (http://www.drive5.com/muscle/) and manually adjusted by BioEdit (Hall, 1999). The phylogenetic trees were reconstructed using a maximum-likelihood procedure in PHYML software with the Jones, Taylor, and Thornton (JTT) amino acid substitution model (Guindon and Gascuel, 2003). One hundred bootstrap replicates were performed in each analysis to obtain the confidence support. GRX2 protein from *Escherichia coli* was used as an outgroup for phylogenetic analysis.

The 1000 bp promoter sequences of all identified GST genes were obtained from the phytozome database. Elements in the promoter were identified from the PlantCARE database (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/).

2.3. Expression of Medicago GST genes under normal conditions and abiotic stress

Medicago truncatula cv. Jemalong line A17 seeds were scarified with concentrated sulfuric acid, rinsed and sterilized with 2% sodium hypochlorite, and vernalized at 4 °C for 3 days on moist and sterile filter paper. Germinated seedlings were transplanted to potting soil and placed in a growth chamber set to the following conditions: 16-h/8-h light/dark regime, 200 lE m^2 sec^1 light irradiance, 24 °C and 40% relative humidity. The plants were analyzed at four developmental stages: unifoliate leaf (vegetative emergence, VE); four leaves (vegetative stage, VS1); shoot ramification of order 1 (VS2); shoot ramification of order 2 (VS3) for investigating of the expression patterns of *Medicago* GST genes under normal conditions. Materials were gathered from seven tissues including lateral roots, main root, cotyledons, unifoliate leaf, bud, trifoliate leaves, and stem under normal conditions on three plant replicates. In order to investigate the response of *Medicago* GSTs to stress treatments, three replicate VS3 seedlings were then irrigated and sprayed using 0.5% (m/v) atrazine solution, 1 mM CDNB solution, 0.5% (v/v) H_2O_2 solution for 12 h and 1 mM salicylic acid (SA) solution for 24 h, respectively. After treatments, total RNA was isolated from lateral roots, main root, bud, trifoliate leaves, and stem. Total RNA was treated with RNase-free DNase I (Promega) and reverse transcribed into cDNA using an RNA PCR Kit (AMV) version 3.0 (TaKaRa). 73 specific primer pairs were designed for PCR analysis based on the multiple sequences alignment of all *Medicago* GST gene sequences (Supplementary Table S2). PCR conditions were optimized to consist of an initial denaturation of 3 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 40 s at 60 °C and 1 min at 72 °C, and a final extension of 3 min at 72 °C. In all PCR analyses, *Medicago* actin gene (GenBank entry: XM_003625217.1) was used as an internal control. After PCR, PCR products from each sample were analyzed using 1% agarose gel and were validated by DNA sequencing.

2.4. Expression and purification of recombinant Medicago GST proteins

For investigation of the enzymatic functions of *Medicago* GST proteins, 49 *Medicago* GST genes including 40 tau, 8 phi and 1 DHAR, were subcloned into pET-30a expression vectors (Novagen) to obtain an N-terminal 6 × His tag. The primers used to construct the GST expression vectors are listed in Supplementary Table S1. Colonies containing appropriate inserts were identified by sequencing.

Escherichia coli BL21 (DE3) cells harboring pET-30a/GST plasmids were cultured overnight, diluted 1:100, and grown until the optical density (A_{600}) reached 0.5. A final concentration of 0.1 mM isopropyl-β-D-thiogalactopyranoside was added to induce synthesis of the recombinant GST proteins. Then, 10 h after induction, cells were harvested by centrifugation (8,000*g*, 3 min, 4 °C), resuspended in binding buffer (20 mM sodium phosphate, 0.5 M NaCl, and 20 mM imidazole, pH 7.4), and disrupted by cold sonication. In each case, the homogenate was then subjected to centrifugation (10,000*g*, 10 min, 4 °C). The resulting particulate material and a small portion of the supernatant were analyzed by SDS-PAGE. The rest of the supernatant was loaded onto a Nickel-Sepharose High Performance column (GE Healthcare Bio-Sciences) preequilibrated with binding buffer. The GST proteins that bound to the Nickel-Sepharose High Performance column were eluted with elution buffer (20 mM sodium phosphate, 0.5 M NaCl, and 500 mM imidazole, pH 7.4).

2.5. Enzyme assays and kinetics of GST enzymes

GST activities (at 25 °C) toward CDNB, DCNB, and NBC was measured as described by Habig et al. (1974); GST activity toward NBD-Cl was measured as described by Ricci et al. (1994); and that toward DHA, fluorodifen, and Cum-OOH was measured as described by Edwards and Dixon (2005). The dependence of GST activity on temperature was measured using CDNB as the substrate at various temperatures from 15 °C to 70 °C at 5 °C intervals. Protein concentrations were determined by measuring A_{280} (Layne, 1957). SDS-PAGE was performed on 10% separating gels and 5% stacking gels. Protein concentrations in the enzyme preparations were determined by measuring the absorbance at 280 nm. The apparent K_m values for GSH were determined using GSH concentrations ranging from 0.02 to 1.0 mM and a fixed CDNB concentration of 1.0 mM. The apparent K_m values for CDNB were determined using concentrations of CDNB, ranging from 0.04 to 1.0 mM and a fixed GSH concentration of 1.0 mM. The kinetic parameters were derived from nonlinear regression analysis by the Hyper32 program available at http://hyper32.software.informer.com/.

3. Results and discussion

3.1. Sequence and structural characteristics of Medicago GSTs genes

Seventy-three full-length genes encoding putative GSTs proteins were identified from *Medicago truncatula* genome (Supplementary Table S3). Among the 73 putative GST genes, *MtrGSTU48* was considered to be putative pseudogene, based on the presence of a stop codon occurring prematurely. After removing the stop codon, the full-length sequence was included in the phylogenetic and gene expression analyses. All the predicted GST proteins encoded by these 73 genes shared the

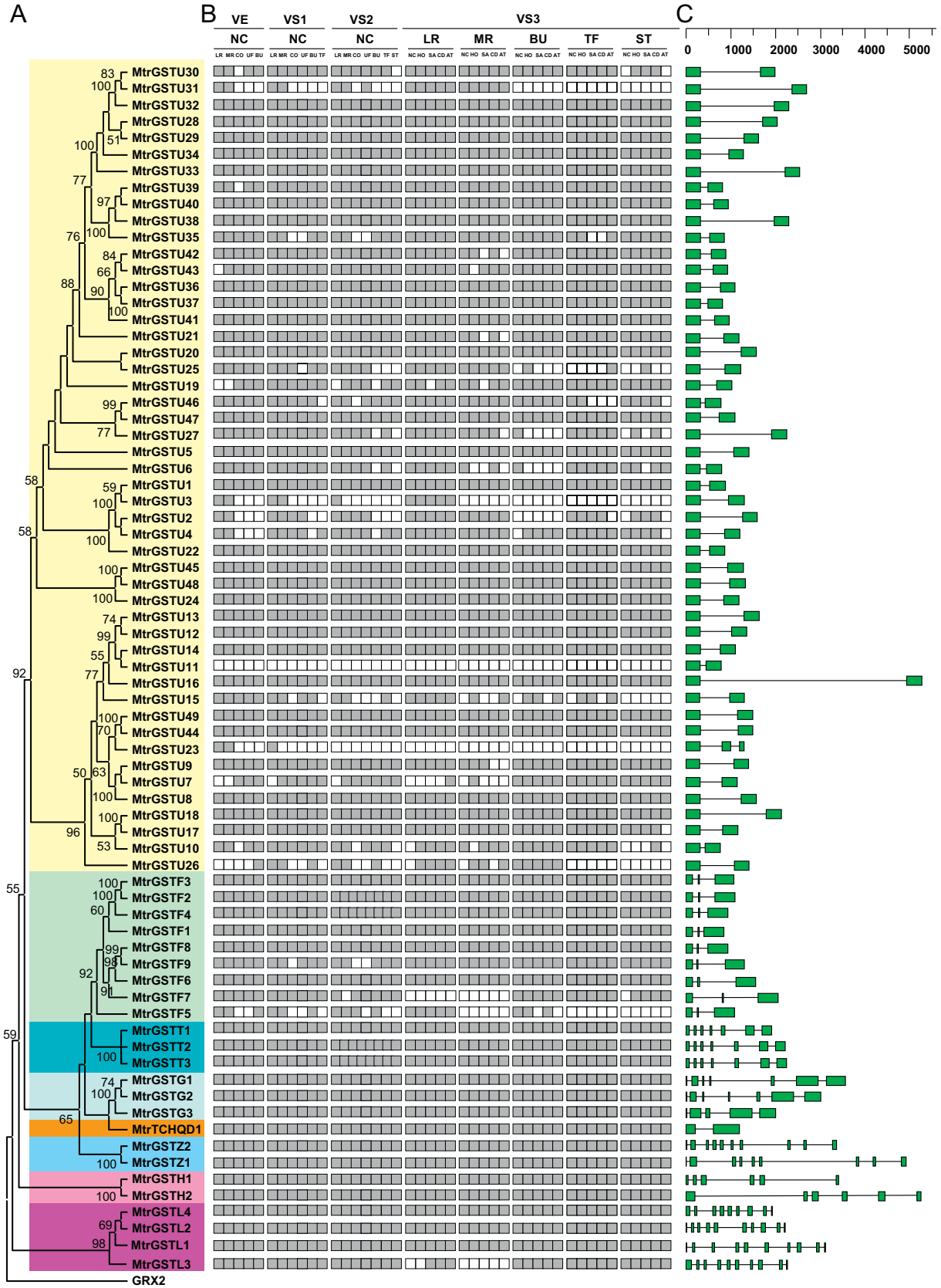


Fig. 1. Phylogenetic tree of *Medicago* GST genes (A), their expression patterns (B) and gene structures (C). In (A), Numbers at each node in the phylogenetic tree are bootstrap values, and only values higher than 50% are shown. Different GST classes are shaded with different colors. In (B), the grey box indicates positive detection of gene expression in the corresponding tissues under normal growth conditions (NC) and in response to H_2O_2 (HO), salicylic acid (SA), CDNB (CD), and atrazine (AT) stress treatments during four development stages. In (C), Exons are shown as boxes and introns are shown as lines. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

typical GST N- and C-terminal domains. Based on the NCBI conserved domain search, *Medicago* GSTs were initially classified into eight classes: the tau, phi, DHAR, theta, zeta, lambda, EF1Bγ, and TCHQD classes (Fig. 1A). The tau and phi classes were the most numerous, being represented by 49 and 9 genes, respectively. Phylogenetic analyses showed that the 73 *Medicago* GSTs were also grouped into eight distinct groups, corresponding to the classes identified by domain structures.

Identification of the exon-intron organization toward 73 *Medicago* GST genes revealed conserved gene structure within each GST class (Fig. 1C). All 49 tau GST genes contained a one-intron and two-exon structure except for *MtrGSTU23*, which had a two-intron and three-exon structure. All 9 phi GST genes contained a two-intron and three-exon structure. *MtrGSTL1, 2, 3 and MtrGSTZ2* contained ten exons, while *MtrGSTZ1 and MtrGSTL4* contained nine exons. *MtrGSTT1, 2, 3, MtrGSTG1 and 2* contained seven exons, while *MtrGSTG3* contained five exons. MtrGSTH1 and 2 had six exons.

3.2. Genomic organization of the Medicago GST gene family

Genomic organization analysis showed that 72 full-length GSTs were physically located in eight *Medicago* chromosomes (Fig. 2). Distribution of the GST genes among the chromosomes appeared to be uneven. Chromosomes 6 harbored only one GST gene (*MtrGSTU27*) whereas chromosome 7 harbored 17 GSTs. A total of six clusters of tandem arranged GST genes were observed on chromosomes1, 2, 7 and 8. Among these six clusters, four were tau GST clusters, one was phi cluster, and the other one was theta cluster. Clusters III and IV were the largest clusters, harboring 10 and 11 tau GST genes respectively.

Lineage-specific expansion of gene family arose from large- or small-scale gene duplication. In this study, among 49 *Medicago* tau GSTs, 30 (61%) were presented in four tandem clusters, indicating the tandem duplication played a major role in expansion of tau GSTs. Previous studies had also shown that tandem duplication played a dominant role in the expansion of GST genes in *Populus*, soybean, *Arabidopsis,* and rice (Dixon and Edwards, 2010a; Jain et al., 2010; Lan et al., 2009; Liu et al., 2015).

3.3. Molecular evolution of the GST gene family in Medicago and Glycine

Medicago and soybean shared the ancient WGD event (59 Ma). After this ancient WGD event, *Medicago* did not undergo any WGD events, while soybean genome underwent a recent Glycine-specific WGD event (13 Ma) (Schlueter et al., 2004; Schmutz et al., 2010; Vanneste et al., 2014). To investigate the lineage-specific expansion of GST genes in *Medicago* and *Glycine* genome, we performed a phylogenetic analysis of all GSTs from *Medicago* and *Glycine*. Nodes that were presumed to be indication of the divergence point between *Medicago* and *Glycine* were labeled with circles on the phylogenetic tree (Supplementary Figs. S1 and S2). These nodes represented the most recent common ancestral genes before the split. Some GST genes might have been present in the most recent common ancestor (MRCA) of *Medicago* and *Glycine* but were later lost in either *Medicago* or *Glycine*.

Among the eight classes, the tau and phi GSTs were the most numerous. There were at least 28 tau ancestral GST genes in the most recent common ancestor (MRCA) of *Medicago* and *Glycine* (Fig. 3). After the split, *Medicago* gained 26 genes and lost 5 genes, resulting in 49 tau GST genes, while *Glycine* gained 40 genes and lost 5 genes, resulting in 63 tau GST genes. For phi GST, *Medicago* and *Glycine* had 9 and 14 genes, respectively. There were at least 9 ancestral phi GST genes in the MRCA of *Medicago* and *Glycine* (Fig. 3). After the split, *Medicago* gained 3 genes and lost 3 genes, resulting in 9 phi GST genes, while *Glycine* gained 7 genes and lost 2 genes, resulting in 14 phi GST genes.

Except for theta GSTs, gene numbers of other type GSTs in *Glycine* were bigger than that in *Medicago*. The Glycine-specific WGD resulted in expansion of GST gene numbers in soybean genome. Our previous study had outlined the expansion of GST gene numbers in soybean genome by the Glycine-specific WGD event. In the soybean genome, 72

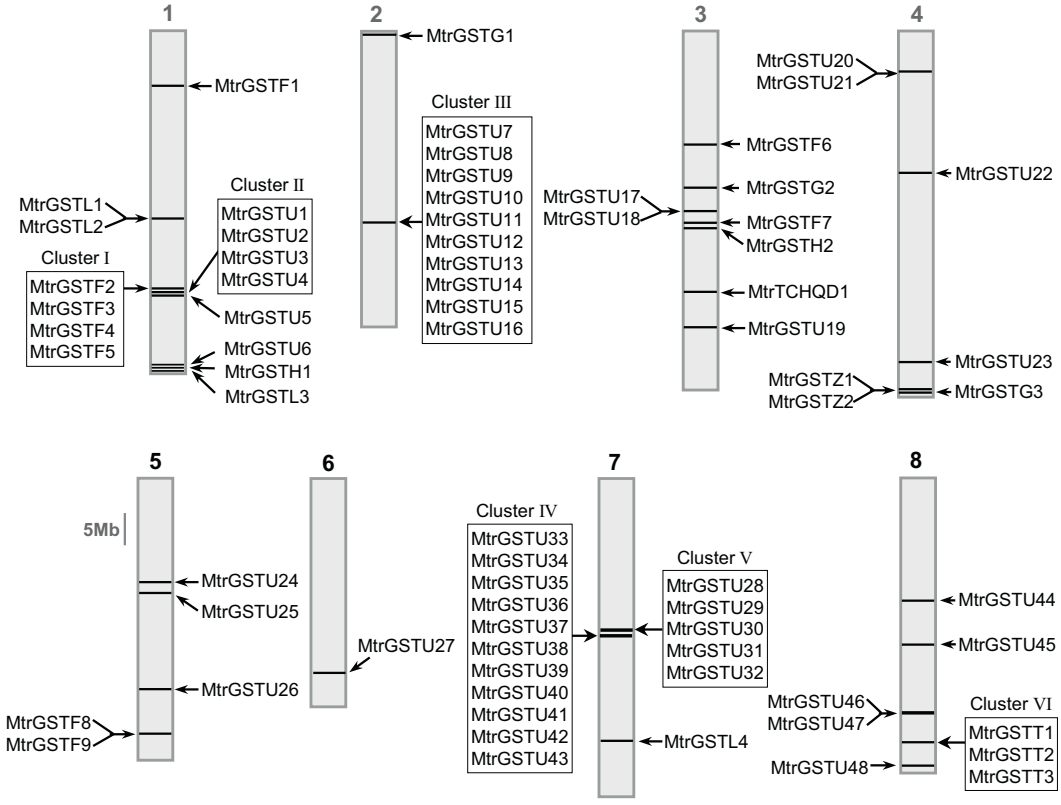


Fig. 2. Genomic localization of *Medicago* GSTs on eight chromosomes.

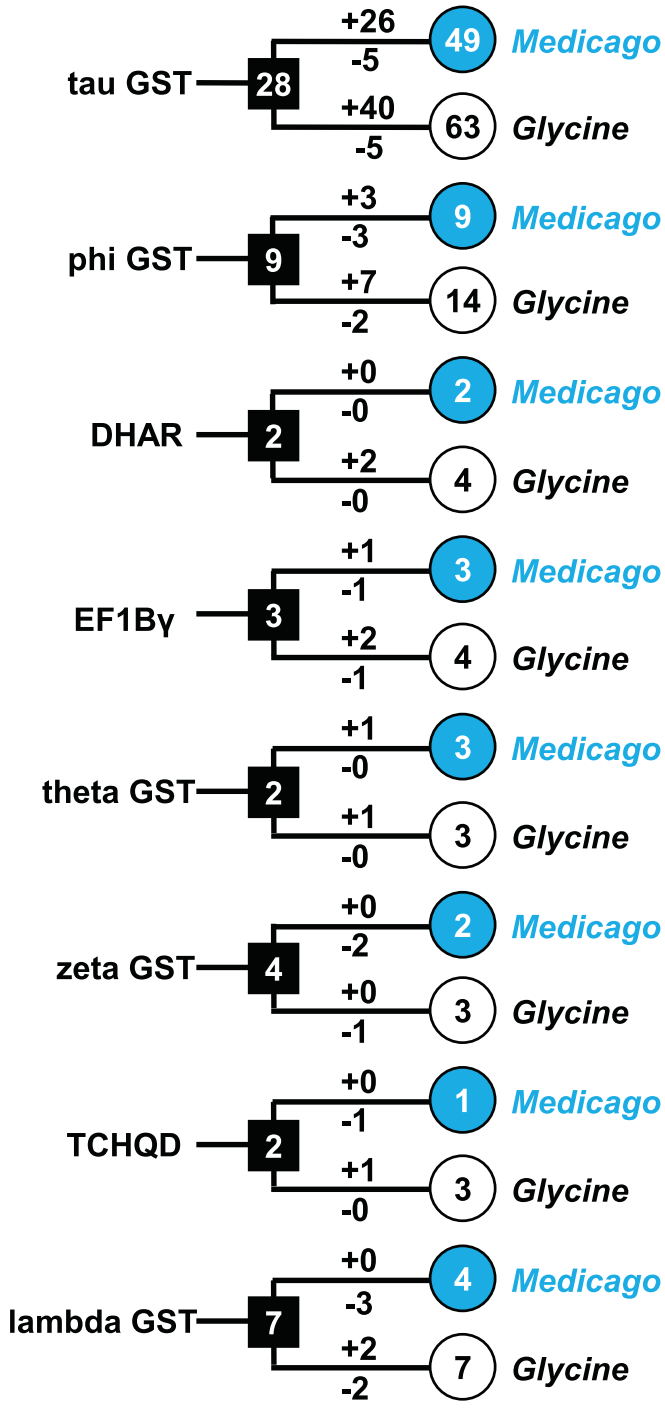


Fig. 3. The copy number changes of *Medicago* and *Glycine* GSTs genes. Numbers in circles and rectangles represent the numbers of GST genes in extant and ancestral species, respectively. Numbers on branches with plus and minus symbols represent the numbers of gene gains and losses, respectively.

GST duplicated gene pairs were formed in the soybean genome by the recent Glycine-specific WGD event (Liu et al., 2015). Among these 72 GST duplicated gene pairs, 20 pairs had been retained in the soybean genome. For example, there were two ancestral DHAR genes in the MRCA genome of *Medicago* and *Glycine*. After the split, Glycine-specific WGD resulted in four DHAR genes in *Glycine*, while *Medicago* genome still contained two DHARs.

3.4. Expression of Medicago GSTs genes under normal growth condition and abiotic stress

The expression patterns of 73 *Medicago* GSTs genes in seven different tissues under normal conditions throughout the growth stages (VE, VS1, VS2, and VS3) were analyzed by reverse transcription-PCR (Fig. 1B). To investigate the response of *Medicago* GSTs to stress treatments, we examined the expression patterns of all 73 GSTs under four stress treatments (H_2O_2, atrazine, 1-chloro-2, 4-dinitrobenzene, and salicylic acid applications) for the five different tissues of the VS3 growth stage. Among the 73 *Medicago* GSTs, 52 were expressed in all tissues under all growth conditions. Only one gene (*MtrGSTU11*) was neither expressed in any tissue nor in response to any treatment applied in this study. The rest 20 genes were selectively expressed in response to a specific treatment and/or in a specific tissue.

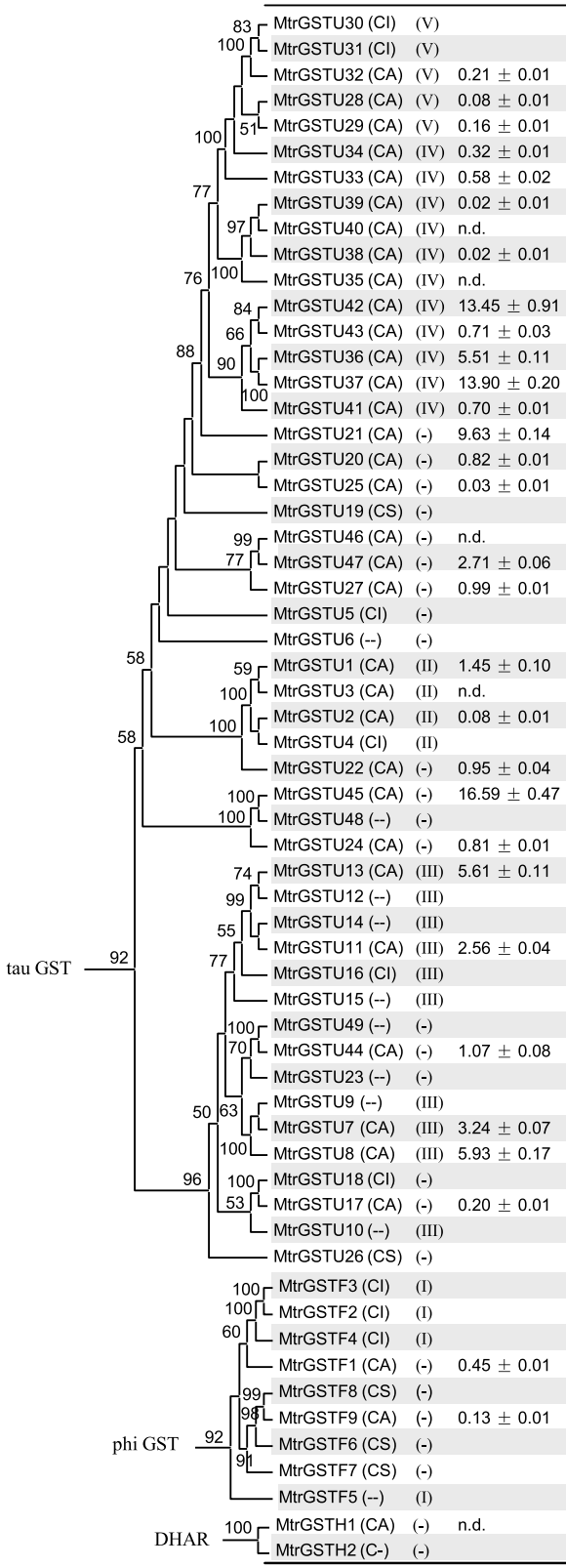
The expression patterns of the DHAR, EF1Bγ, theta, TCHQD, and zeta GSTs were homogenous, expressed in all tissues examined under all growth conditions, whereas variation in expression patterns was found among tau, phi and lambda GSTs. Among the nine phi GSTs, six (*GSTF1, 2, 3, 4, 6*, and *8*) were expressed in all tissues examined under all growth conditions, while *MtrGSTF5, 7*, and *9* were selectively expressed (Fig. 1B). However, substantially more variation in expression patterns was found among the tau class members (Fig. 1B). Of the 49 tau GSTs, 30 were expressed in all tissues under all growth conditions at all development stages, while only one (*MtrGSTU11*) was not expressed in any tissues or in response to any treatments applied in this study. The other 18 tau GSTs were selectively expressed either in response to a specific treatment and/or in a specific tissue and/or at a specific development stage. Some GST genes showed preferential accumulation of transcripts in a given tissue/organ or developmental stage or specific treatment. For instance, *MtrGSTU3* showed root-specific expression patterns, expressed only in the lateral roots and main root during VE and VS1 stages, and in the lateral roots during VS2 and VS3 stages. *MtrGSTU23* was expressed only in the lateral roots and main root during VE stage, and in the lateral roots during VS1 stage. The root-specific expression might suggest specific roles for these genes in root development. Expression of *MtrGSTU7* was not detected under normal growth conditions at any stage in lather root, but only expressed in response to treatment with atrazine, indicating that it was stress-induced.

The expression pattern was investigated among members of six tandem-arrayed GST clusters, which was classified into two different categories. In the first category, all genes in a cluster were expressed in all tissues, being represented by cluster VI. In the second category, genes in the same cluster were differentially expressed, being expressed in all tissues or preferentially expressed in specific tissues or not expressed in any tissues. This category was found in the rest five clusters.

3.5. Biochemical characteristics of the Medicago GSTs

Forty-nine *Medicago* GST proteins were expressed and purified for their catalytic activities, including 40 tau, 8 phi and 1 DHAR. Nine GSTs (MtrGSTU4, 5, 16, 18, 30, 31, MtrGSTF2, 3 and 4) were expressed as inclusion bodies, and the other 40 GSTs were expressed as soluble proteins in *Escherichia coil*. However, 5 purified proteins were instable in buffer, including MtrGSTU19 and 26, MtrGSTF6, 7 and 8, which were not included in the analysis for biochemical activities.

Thirty-five soluble *Medicago* GST proteins were assayed for activities toward seven substrates: 1-chloro-2,4-dinitrobenzene (CDNB), 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl), fluorodifen, 1,2-dichloro-4-nitrobenzene (DCNB), 4-nitrobenzyl chloride (NBC), cumene hydroperoxide (Cum-OOH), and dehydroascorbic acid (DHA). Among the 32 purified tau GSTs listed in Fig. 4, 28 showed specific activity toward CDNB, 27 toward NBD-Cl, 14 toward DCNB, 13 toward NBC, 25 toward Cum-OOH, 21 toward fluorodifen, and 1 toward DHA. Six tau GSTs had enzymatic activity toward six substrates, 6 toward five



GST	Cluster	Specific activities (μmol min^{-1} mg^{-1}) to each substrate						
		CDNB	NBD-Cl	DCNB	NBC	Fluorodifen	Cum-OOH	DHA
MtrGSTU30 (Cl)	(V)							
MtrGSTU31 (Cl)	(V)							
MtrGSTU32 (CA)	(V)	0.21 ± 0.01	0.52 ± 0.01	n.d.	0.16 ± 0.01	0.11 ± 0.01	0.76 ± 0.03	n.d.
MtrGSTU28 (CA)	(V)	0.08 ± 0.01	0.11 ± 0.01	n.d.	n.d.	n.d.	0.01 ± 0.01	n.d.
MtrGSTU29 (CA)	(V)	0.16 ± 0.01	0.18 ± 0.01	0.04 ± 0.01	0.10 ± 0.01	0.27 ± 0.07	0.02 ± 0.01	n.d.
MtrGSTU34 (CA)	(IV)	0.32 ± 0.01	0.88 ± 0.01	n.d.	n.d.	1.32 ± 0.02	0.01 ± 0.01	n.d.
MtrGSTU33 (CA)	(IV)	0.58 ± 0.02	0.40 ± 0.01	n.d.	0.18 ± 0.01	1.44 ± 0.02	0.05 ± 0.01	n.d.
MtrGSTU39 (CA)	(IV)	0.02 ± 0.01	0.10 ± 0.01	n.d.	n.d.	n.d.	n.d.	n.d.
MtrGSTU40 (CA)	(IV)	n.d.	n.d.	n.d.	n.d.	n.d.	3.81 ± 0.12	n.d.
MtrGSTU38 (CA)	(IV)	0.02 ± 0.01	0.08 ± 0.01	0.02 ± 0.01	n.d.	n.d.	n.d.	n.d.
MtrGSTU35 (CA)	(IV)	n.d.	0.02 ± 0.01	n.d.	n.d.	n.d.	n.d.	n.d.
MtrGSTU42 (CA)	(IV)	13.45 ± 0.91	4.10 ± 0.08	0.09 ± 0.01	1.18 ± 0.03	14.05 ± 0.17	0.09 ± 0.01	n.d.
MtrGSTU43 (CA)	(IV)	0.71 ± 0.03	0.02 ± 0.01	0.04 ± 0.01	n.d.	0.03 ± 0.01	0.03 ± 0.01	n.d.
MtrGSTU36 (CA)	(IV)	5.51 ± 0.11	2.57 ± 0.03	0.11 ± 0.01	0.45 ± 0.02	2.65 ± 0.08	0.14 ± 0.01	n.d.
MtrGSTU37 (CA)	(IV)	13.90 ± 0.20	1.83 ± 0.09	0.08 ± 0.01	0.42 ± 0.01	6.18 ± 0.03	0.18 ± 0.01	n.d.
MtrGSTU41 (CA)	(IV)	0.70 ± 0.01	0.64 ± 0.03	n.d.	0.13 ± 0.04	1.60 ± 0.07	0.12 ± 0.01	n.d.
MtrGSTU21 (CA)	(-)	9.63 ± 0.14	7.00 ± 0.13	n.d.	0.54 ± 0.01	0.29 ± 0.03	0.02 ± 0.01	n.d.
MtrGSTU20 (CA)	(-)	0.82 ± 0.01	0.67 ± 0.02	n.d.	n.d.	0.32 ± 0.01	0.01 ± 0.01	n.d.
MtrGSTU25 (CA)	(-)	0.03 ± 0.01	0.06 ± 0.01	n.d.	n.d.	0.09 ± 0.01	n.d.	n.d.
MtrGSTU19 (CS)	(-)							
MtrGSTU46 (CA)	(-)	n.d.	n.d.	0.05 ± 0.01	n.d.	n.d.	0.12 ± 0.01	n.d.
MtrGSTU47 (CA)	(-)	2.71 ± 0.06	3.57 ± 0.10	0.03 ± 0.01	2.49 ± 0.21	n.d.	0.09 ± 0.01	n.d.
MtrGSTU27 (CA)	(-)	0.99 ± 0.01	0.09 ± 0.01	n.d.	n.d.	0.13 ± 0.01	0.12 ± 0.01	n.d.
MtrGSTU5 (Cl)	(-)							
MtrGSTU6 (--)	(-)							
MtrGSTU1 (CA)	(II)	1.45 ± 0.10	0.21 ± 0.03	n.d.	0.14 ± 0.01	3.87 ± 0.06	n.d.	n.d.
MtrGSTU3 (CA)	(II)	n.d.	n.d.	n.d.	0.12 ± 0.02	0.32 ± 0.01	n.d.	n.d.
MtrGSTU2 (CA)	(II)	0.08 ± 0.01	n.d.	n.d.	n.d.	0.18 ± 0.01	0.06 ± 0.01	n.d.
MtrGSTU4 (Cl)	(II)							
MtrGSTU22 (CA)	(-)	0.95 ± 0.04	n.d.	0.02± 0.01	n.d.	1.44 ± 0.01	0.27 ± 0.01	n.d.
MtrGSTU45 (CA)	(-)	16.59 ± 0.47	0.36 ± 0.01	0.13 ± 0.01	n.d.	1.70 ± 0.05	n.d.	n.d.
MtrGSTU48 (--)	(-)							
MtrGSTU24 (CA)	(-)	0.81 ± 0.01	0.48 ± 0.02	0.05 ± 0.01	0.27 ± 0.09	114.30 ± 6.80	1.73 ± 0.02	n.d.
MtrGSTU13 (CA)	(III)	5.61 ± 0.11	1.49 ± 0.04	0.08 ± 0.01	0.21 ± 0.03	1.32 ± 0.01	0.03 ± 0.01	n.d.
MtrGSTU12 (--)	(III)							
MtrGSTU14 (--)	(III)							
MtrGSTU11 (CA)	(III)	2.56 ± 0.04	1.44 ± 0.03	n.d.	n.d.	0.59 ± 0.01	0.05 ± 0.01	n.d.
MtrGSTU16 (Cl)	(III)							
MtrGSTU15 (--)	(III)							
MtrGSTU49 (--)	(-)							
MtrGSTU44 (CA)	(-)	1.07 ± 0.08	1.03 ± 0.04	n.d.	n.d.	n.d.	0.01 ± 0.01	n.d.
MtrGSTU23 (--)	(-)							
MtrGSTU9 (--)	(III)							
MtrGSTU7 (CA)	(III)	3.24 ± 0.07	0.11 ± 0.01	0.12 ± 0.01	n.d.	n.d.	0.10 ± 0.01	n.d.
MtrGSTU8 (CA)	(III)	5.93 ± 0.17	2.31 ± 0.12	0.10 ± 0.01	n.d.	n.d.	0.02 ± 0.01	n.d.
MtrGSTU18 (Cl)	(-)							
MtrGSTU17 (CA)	(-)	0.20 ± 0.01	0.07 ± 0.01	n.d.	n.d.	n.d.	0.07 ± 0.01	n.d.
MtrGSTU10 (--)	(III)							
MtrGSTU26 (CS)	(-)							
MtrGSTF3 (Cl)	(I)							
MtrGSTF2 (Cl)	(I)							
MtrGSTF4 (Cl)	(I)							
MtrGSTF1 (CA)	(-)	0.45 ± 0.01	0.05 ± 0.01	n.d.	0.52 ± 0.02	0.44 ± 0.02	0.08 ± 0.01	0.01 ± 0.01
MtrGSTF8 (CS)	(-)							
MtrGSTF9 (CA)	(-)	0.13 ± 0.01	0.03 ± 0.01	n.d.	n.d.	n.d.	n.d.	n.d.
MtrGSTF6 (CS)	(-)							
MtrGSTF7 (CS)	(-)							
MtrGSTF5 (--)	(I)							
MtrGSTH1 (CA)	(-)	n.d.	n.d.	n.d.	0.25 ± 0.07	n.d.	n.d.	29.75 ± 0.74
MtrGSTH2 (C-)	(-)							

Fig. 4. Phylogenetic tree and enzyme activities of *Medicago* GST proteins. Values shown are mean ± SD, as calculated from three replicates. Each GST name is suffixed with a key describing the associated analysis: C, successfully cloned; A, purified GST protein assayed; I, recombinant protein totally insoluble; S, purified recombinant protein instable in buffer; dash, analysis not performed. n.d., no activity detected.

Table 1
Kinetic constants of the Medicago truncatula GSTs for CDNB and GSH conjugation reactions. Values shown are means ± SD, calculated from three replicates; Dispersed GSTs not grouped into any cluster are indicated with "–".

Cluster	GSTs	K_m^{GSH} (mM)	k_{cat}^{GSH} (S^{-1})	$(k_{cat}/K_m)^{GSH}$ (mM^{-1} S^{-1})	K_m^{CDNB} (mM)	k_{cat}^{CDNB} (S^{-1})	$(k_{cat}/K_m)^{CDNB}$ (mM^{-1} S^{-1})
II	MtrGSTU1	0.16 ± 0.01	2.12	13.25	0.54 ± 0.01	3.18	5.89
II	MtrGSTU2	1.01 ± 0.14	0.18	0.18	5.49 ± 0.36	0.82	0.15
III	MtrGSTU8	0.40 ± 0.01	14.99	37.48	1.62 ± 0.05	28.49	17.59
III	MtrGSTU13	0.35 ± 0.01	12.46	35.60	1.45 ± 0.03	21.71	14.97
III	MtrGSTU20	0.08 ± 0.01	1.02	127.50	0.15 ± 0.01	0.83	5.53
III	MtrGSTU21	0.05 ± 0.01	13.64	272.80	3.02 ± 0.09	49.64	16.44
–	MtrGSTU22	0.48 ± 0.04	1.75	3.65	3.89 ± 0.12	5.24	1.35
–	MtrGSTU24	0.48 ± 0.01	1.10	2.29	0.55 ± 0.02	1.11	2.02
–	MtrGSTU27	0.20 ± 0.01	1.57	7.85	3.04 ± 0.09	6.26	2.06
V	MtrGSTU29	2.01 ± 0.23	0.63	0.31	6.36 ± 0.55	1.58	0.25
V	MtrGSTU32	0.54 ± 0.03	0.20	0.37	3.24 ± 0.67	1.32	0.41
IV	MtrGSTU33	0.13 ± 0.01	0.79	6.08	0.05 ± 0.01	0.85	17.00
IV	MtrGSTU34	1.58 ± 0.04	0.97	0.61	0.98 ± 0.01	1.69	1.72
IV	MtrGSTU37	0.49 ± 0.02	34.58	70.57	6.16 ± 0.06	159.46	25.89
IV	MtrGSTU41	2.24 ± 0.03	2.42	1.08	2.59 ± 0.15	4.86	1.87
IV	MtrGSTU42	0.35 ± 0.01	25.35	72.43	3.79 ± 0.20	72.78	19.20
IV	MtrGSTU43	1.41 ± 0.10	2.53	1.79	2.08 ± 0.12	5.19	2.50
–	MtrGSTU45	0.25 ± 0.01	29.53	118.12	10.17 ± 0.05	266.77	26.23
–	MtrGSTU47	0.29 ± 0.01	3.29	11.34	0.11 ± 0.01	2.90	26.36
–	MtrGSTF1	0.19 ± 0.01	0.56	2.95	4.32 ± 0.16	3.04	0.70
–	MtrGSTF9	2.53 ± 0.04	0.79	0.31	1.03 ± 0.03	0.61	0.59

substrates, 9 toward four substrates, 6 toward three substrates, 3 toward two substrates and 2 toward only one substrate. For the 2 purified phi GSTs listed in Fig. 4, they both had activity toward CDNB and NBD-Cl, but no activity toward DCNB. Neither the 2 phi GSTs nor the 32 tau GSTs showed activity toward DHA. In contrast, the DHAR GST (MtrGSTH1) did. It showed high activity toward DHA but no activity toward any of the other six substrates used in this study except NBC.

Cluster IV was the largest cluster, harboring 11 tau GST genes. The enzyme activities of these GSTs toward CDNB varied from 0 to 13.45 mmol/min per mg, while the enzyme activity toward NBD-Cl varied from 0 to 4.10 mmol/min per mg. In this cluster, MtrGSTU36, 37 and 42 showed activity toward six substrates, MtrGSTU33, GSTU41, and 43 showed activity toward five substrates, MtrGSTU34, 38, and 39 showed activities toward four, three, and two substrates, respectively. However, MtrGSTU35 and 40 showed activities toward only one substrate. Thus, diversification in enzyme specificity and activity toward different substrates had apparently evolved among GSTs in the cluster IV.

This study examined the kinetics characteristic of the *Medicago* GSTs using CDNB and GSH as substrates (Table 1). Among 21 *Medicago* GSTs examined in this study, the apparent K_m^{GSH} values of 15 GSTs were ranged from 0.1 to 0.5 mM, indicating that the majority had similar and high affinities for GSH. This is consistent with the highly conserved nature of the N-terminal domain of the GSTs (Lan et al., 2009). Owing to the less conserved C-terminal domain of plant GSTs (Lan et al., 2009), where the enzyme binds the 2nd hydrophobic substrate, both substrate affinities (K_m^{CDNB}) and catalytic efficiency (k_{cat}/K_m^{CDNB}) varied significantly among the tau GSTs. The apparent K_m^{CDNB} values fell within the range of 0.11–10.17 mM, and the apparent k_{cat}/K_m^{CDNB} values ranged from 0.15 to 26.36 mM^{-1} S^{-1}.

Distinct variations in both affinity and catalytic efficiency were also observed among the tandem-arrayed GSTs toward different substrates. For instance, among the six tau GSTs (MtrGSTU33, 34, 37, 41, 42, and 43) examined in cluster IV, affinity (K_m) for GSH and CDNB varied 17-fold and 123-fold, respectively, and their catalytic efficiency (k_{cat}/K_m) varied 119-fold and 15-fold, separately.

Previous studies showed that genes involved in stress responses had an elevated probability of retention in a single-lineage fashion following tandem duplication, and the tandem duplicates were likely important for adaptive evolution to rapidly changing environments (Hanada et al., 2008). In plants, GSTs can protect cells from a wide range of biotic and abiotic stresses, including pathogen attack, xenobiotic and heavy metal toxins, oxidative stress and UV radiation (Agrawal et al., 2002; Kampranis et al., 2000; Loyall et al., 2000). They involved in the transport of toxic secondary products and cell signalling during stress responses. All 11 tau GSTs in cluster IV showed gene expressions and enzyme activities, indicating these GSTs were not pseudogenes. However, these 11 tau GSTs showed obvious divergences in gene expressions and enzyme activities toward different substrates, indicating functional divergences. This divergence might contribute to the retention of these tandem duplicates. On the other hand, possessing tandem GST duplicates with diverse activities to a wide range of substrates is likely to have high adaptive value, enabling perennial plants to respond to the diverse environmental challenges they are likely to encounter.

Author contributions

Conceived and designed the experiments: Qing-Yin Zeng. Performed the experiments: Xue-Min Han and Zhi-Ling Yang. Analyzed the data: Hai-Ling Yang and Yan-Jing Liu. Wrote the paper: Xue-Min Han and Qing-Yin Zeng.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.plaphy.2018.03.004.

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