

Cloning and expression analysis of a novel Glutathione S-transferase gene, *MsGST*, from alfalfa (*Medicago sativa*)

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Abstract

Glutathione S-transferases are important enzymes in protecting cells by scavenging reactive oxygen species induced by various stresses. In this study, a novel GST gene, MsGST (KM044312), was cloned and characterized from alfalfa. The open reading frame of MsGST contains 660bp nucleotides, encoding 219 amino acid residues. Amino acid sequence alignment indicated that the deduced MsGST protein was highly homologous to other plant tau class GST sequences. According to amino acid phylogenetic analysis, the MsGST gene was clustered into the same branch with other legume plants. Real-time quantitative PCR (gRT-PCR) revealed that the expression levels of MsGST were up-regulated in both shoots and roots under ABA treatment and various stresses, including salt, drought, cold and heat stress. The effect of nodules on MsGST gene expression indicated that the induction of MsGST expression by abiotic stress is independent of rhizobium symbiosis. In conclusion, the MsGST gene may be involved in response to different abiotic stresses in alfalfa.

Key words: *Medicago sativa*, cloning, expression, glutathione S-transferase, abiotic stress

Introduction

Glutathione S-transferases (GSTs) are important enzymes for protecting cells from stress by scavenging reactive oxygen species (ROS) (Potters 2010). GSTs can catalyze tripeptide glutathione (GSH: γ -Glu-Cys-Gly) to conjugate many electrophilic and lipophilic compounds, and the products are soluble (Yang et al. 2009). GST expression can be induced by various stress conditions (Dixon et al. 2002; Moons 2005). The phytohormones, for example, abscisic acid (ABA),

ethylene, auxin and cytokinin also can induce some GSTs (Gong et al. 2005; Moons 2005). Presently, eight classes of GSTs have been found in mammals (Frova 2003), and six classes in plants (Frova 2006), but Tau and Phi classes are only and extensively found in plants (Frova 2006; Jha et al. 2011). There are 61 GST genes in Medicago trunctula by Blast in NCBI and 49 of them belong to tau class. The Tau GSTs have been investigated in some plants. For example, overexpression of a tau class SbGST gene in transgenic tobacco improves tolerance to salt stress (Xu et al. 2015). In addition, overexpression of AtGSTU19 provides tolerance to high-salt, drought and methylviologen stress (Xu et al. 2016). The GmGSTU4 in soybean can catalyze the diphenyl ether herbicide fluordifen and has activity as glutathione-dependent peroxidase (GPOX) (Benekos et al. 2010).

Alfalfa (*Medicago sativa*), one of the most important forages in the world, is cultivated widely around the world. Alfalfa can fix nitrogen by rhizobium symbiosis in root nodules. However, the plantrhizobium system is naturally exposed to various environmental stresses such as drought (Clement et al. 2008), heat (Hungria and Kaschuk 2014) and salt (Delgado et al. 1994), which limited plant growth and nitrogen fixation. To understand whether rhizobium symbiosis has an effect on host plant tolerance to abiotic stress, we screened the differently expressing genes between alfalfa inoculated rhizobium (NA) and alfalfa not-inoculated rhizobium (NN) by DNA

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microarray technology (data not published). The functions of these candidate genes are little known. Here, one of the candidate genes, *MsGST*, was isolated and analyzed. To investigate the expression pattern of *MsGST* to abiotic stresses and nodules, the transcriptional levels of alfalfa treated with ABA treatment, salt stress, drought stress, cold stress and heat stresses were detected by qRT-PCR.

Materials and methods

Plant materials

Seeds of alfalfa (M. sativa L. cv. Baoding) were sterilized with 70% ethanol for 30s and 0.5% sodium hypochlorite solution for 10 minutes successively. Then, these seeds were rinsed 4-5 times with sterile water and germinated on wet filter paper in Petri dish at 24°C under 16 h photoperiod. After 4 days, each seedling was transplanted to one plastic pot which contains sterilized sandy soil in greenhouse in March 2016. Seedlings were watered with 1/4 strength Hoagland solution (Hoagland and Arnon 1950) for 14 days and then separated into two sets randomly. One set was inoculated with Rhizobium meliloti strain Dormal and watered with N-free nutrient solution every day (NA). The other set was not inoculated and watered with 1/4 strength Hoagland solution (NN). All plants grew at the average temperature of $30 \pm 5^{\circ}$ C and $20 \pm$ 5° C, and the relative humidity of 55 ± 5% and 70 ± 5% during day and night.

Abiotic stress treatments

After inoculation for 60 days, plants were exposed to stressful conditions. Plants were transferred into incubator at 4°C and 42°C for cold and heat treatment, separately, for 4 and 12h. For drought, salt stress and ABA treatment, sand was first gently washed away from roots. The roots were immediately wrapped with a wet tissue paper and then placed on a rack for dehydration stress for 0, 4, 12h and re-watered for 12h. The plants were put in the nutrient solution supplemented with 150mmol NaCl or 10 μ mol ABA for 4 and 12h for salt stress and ABA treatment. Shoots and roots were harvested separately and put into liquid nitrogen immediately and then stored in refrigerator at -80°C until use. The experiment was repeated three times.

Isolation and cloning of MsGST gene

RNA isolation from alfalfa leaves and complement DNA synthesis were performed using RNeasy extraction Kit and Superscript III reverse transcriptase (Invitrogen,

USA), respectively. The primers (GST-F/GST-R, Table 1) were designed with primer primier 5 according to the sequence of *Medicago truncatula* GST gene. The product was extracted and subcloned into pMD-18T vector (Takara, Japan) for sequencing. Based on the partial sequence, gene-specific primers (3'-GST, 5'-GST, Table 1) were designed to perform rapid amplification of cDNA ends using SMARTer RACE 5'/3'kit, and the full length was obtained.

Table 1. A list of primers used in this study

Primer	Sequence(5'-3')	Use
GST-F	TCACTTCCATATTCCACA	cDNA fragment cloning
GST-R	TTAGGGCACTCCTTATCTA CAC	cDNA fragment cloning
3'-GST	CTGCCAAGAAGGAATTCAT AGAAGCCC	3'RACE
5'-GST	AGATTCACAAATAGGTTTT CCAT	5'RACE
qGST-F	CTATGAGGCCTTTGGCAA GAT	Quantitative RT-PCR
qGST-R	GATCAGGGAGTGACTTGG AAAC	Quantitative RT-PCR
Actin-F	TTTGAGACTTTCAATGTG CCCGCC	Reference gene
Actin-R	TAGCATGTGGGAGTGCAT AACCCT	Reference gene

Sequence analysis and phylogeny analysis

The cDNA sequence of *MsGST* gene was analyzed on the website (http://www.ncbi.nlm.nih.gov/), getting its deduced amino acid sequence and open reading frame (ORF). A multiple amino acid sequence alignment was performed using DNAMAN and the phylogenetic tree was constructed according to the amino acid sequence alignment by neighbor-joining method (MEGA) with 1000 bootstrap replication.

Gene expression analysis

Total RNA was extracted from tissues harvested. Then, total RNA (1 μ g per reaction) was reverse transcribed into cDNA to perform qRT-PCR. The relative expression levels were calculated with method described by Livak and Schmittgen (2001) and calibrated with amplification efficiency.

Statistical analysis

Data were analyzed with IBM SPSS Statistics 18.0 software (SPSS commercial software, USA) and the significant differences ($p \le 0.05$) were determined by analysis of Duncan's test.

Results and discussion

Molecular Cloning and sequence characterization of the MsGST gene

In present study, a novel *GST* gene from alfalfa was cloned and named *MsGST* (KM044312). The length of open reading frame (ORF) of *MsGST* is 660bp, which encodes a protein of 219 amino acid residues with a predicted relative molecular mass of 25.57kD and a theoretical pl of 5.91 (Fig. 1). The amino acid

ATG	GCI	AAT	GAR	GIG	BATT	CIG	CTA	GAT	TAC	TGG	GTA	AGI	CCI	TTT	GGC	ATO	AGG	GIC	AGA
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ATA	GCC	CTA	GCT	GAL	AAG	GGT	ATC	AAA	CAT	GAG	TAC	AGA	GAA	GAA	GAT	TTP	AGO	AAC	AAG
I	A	L	A	Ε	K	G	I	K	H	E	Y	R	Ε	Ε	D	L	R	N	K
AGC	CCI	TTG	TIC	TT	ACAA	ATG	AAC	CCI	GTI	CAT	AAG	AAA	ATC	CCT	GTT	CTI	ATT	CAT	TAAT
S	P	L	L	L	8	М	N	P	v	H	ĸ	K	I	P	v	L	I	H	N
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G	K	P	I	C	E	S	L	I	A	v	Q	Y	I	D	E	v	W	N	Ε
AAA	TCI	tcci	TTG	TTO	CCT	TCT	GAT	CCI	TAT	CAG	AAA	TCA	CAR	GCT	AGA	TTC	TGG	GCT	GAT
к	s	P	L	L	P	S	D	P	Y	Q	К	s	Q	A	R	F	W	A	D
TAT	ATI	GAT	AAG	AA	ATC	TAT	GAA	GTI	GCA	GGG	AAC	AAT	TGG	ACC	AAA	AAA	GAP	GAA	GAA
Y	I	D	K	K	I	Y	Ε	v	A	G	N	N	W	Т	K	K	Έ	Ε	Ε
CAA	GAA	GCT	GCC	AA	AAG	GAA	TTC	ATA	GAA	GCC	CTC	AAA	CTC	TIG	GAG	CAJ	GAG	TIG	GGA
Q	Ε	A	A	K	K	E	F	I	Ε	A	L	K	L	L	E	Q	Ε	L	G
GAC	AAG	ATI	TTT	TTI	GGA	GGA	GAC	AAG	CTI	GGI	TTT	GTI	GAT	GT	GCJ	TTT	ATI	CCA	CTC
D	К	I	F	F	G	G	D	K	L	G	F	v	D	v	A	F	I	P	L
TAC	AAI	TGG	TTT	AG	AGGC	TAT	GAG	GCC	TTI	GGC	AAG	ATC	AGT	GTA	TAC	AAG	GAG	TGC	CCT
Y	N	W	F	R	G	Y	Ε	A	F	G	ĸ	I	s	v	x	K	Ε	С	P
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М	F	S	A	W	A	N	R	C	Μ	8	I	Ε	S	V	S	K	S	L	P
GAT	CAG	GAT	AAG	ATC	CAT	GAT	TTG	ATT	GIG	GAG	CTO	AAG	AAG	AAG	TAT	GGC	TTT	GAG	TAG
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A & A & P & L & L & P & L & L & L & L & L & L & L \\ \\ & A & A & K & K & L & F & I & L & K & L & L & L & L & L & L & L \\ \\ & C & A & A & K & K & L & L & L & L & L & L & L & L \\ \\ & A & A & K & K & L & L & L & L & L & L & L & L & L & L & L \\ \\ & A & A & L & K & L & L & L & L & L & L & L & L & L \\ \\ & A & A & L & L & L & L & L & L & L & L & L & L & L & L \\ \\ & A & \mathsf$

Fig. 1. Coding sequence (CDS) and deduced amino acid sequence. Boxes represent start and end codon. Conserved amino acid of tau class GST is underlined

sequence alignment analysis showed that *MsGST* was highly homologous with the GST of other plants and shared the identity of 89, 80, 77, 73, 70 per cent with *Medicago trunctula* (XP_013464346), *Cicer arietinum* (XP_004488707), *Caragana korshinskii* (ABG90381), *Glycine max* (NP_001237713) and *Carica papaya* CAA04391), respectively (Fig. 2), indicating that the *MsGST* protein is a typical GST protein.

Phylogenetic analysis showed the *MsGST* gene clustered into the same branch with other legume plants and was the nearest to *Medicago truntula* (Fig. 3). These results implicated the evolution of the *MsGST* and the orthologous and paralogous relationships of GST protein within the legumes. According to the previous characterization of comparative homology study, *MsGST* has several conserved amino acids of

Medicago sativa	CREATEDTUESDAYSEPTEMBERPMMANBVILIDYWVSEP	40
Medicago truncatula	CREATEDTUESDAYSEPTEMBERPMMADEVILINYWFSEF	40
Caragana korshin	CREATEDTUESDAYSEPTEMBERPMMANEVGLIDFWESEF	-9 0
Carica papaya	CREATEDTUESDAYSEPTEMBERPMMADEVOLUDFWFSEF	40
Cicer arietinum	CREATEDTUESDAYSEPTEMBERPMMADCVILIDFWESEF	40
Slycine max	CREATEDTUESDAYSEPTEMBERPMMADEVVLLDFWESEF	40
Sossypium raimon	CREATEDTUESDAYSEPTEMBERPMMAEEV ^o ll <mark>dfwese</mark> f	-12 C
Hevea brasiliens	CREATEDTUESDAYSEPTEMBERPMMAEEVILUDFWFSFF	40
Jatropha curcas	CREATEDTUESDAYSEPTEMBERPMMGPPVILLDFWESEF	40
Nicotiana sylves	CREATEDTUESDAYSEPTEMBERPMMADPVMLLDTMVSMF	-9.0
Ricinus communis	CREATEDTUESDAYSEPTEMBERPMMADEVILLDFWASEF	40
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Jacropha Curcas		
Nicotiana sylves	CMRVRFFLAERGIQYEYREGILINKIFLLLIMNPIHKKIP	80
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licer arietinum	VIMPRGRPICESLIAWOYIEEVWNHKSHILFSDFYQRSQA	120
stycine max	VIL TENGRETOPSICIAVQMIEEVWNDRNELLPSDFYQRAQA	120
Sossypium raimon	VIIHNGKPACESIIQVQYIEEVWHDKAFLIPSEPYQKATG	120
devea brasiliens	VIIHNGKPICESLIAVQYVEEVWKDKSFFIPSDEYQRAQA	120
Jatropha curcas	VITHIGKPICESLIAVQYVEVWSDKSELIPSDEYQRAQA	120
Nicotiana sylves	VITHNGKPICETLITYQYTTEVWTDKSFIMPSDFYERAHA	120
Ricinus communis	VIIHNGKPICESLIAVQYIDEVWHDESFLIPSDSYPRAHA	120
fedicago sativa	RFWADYIDKKIYEVAGNNWTKKEEEQEAAKKEFIEAIKIL	160
Medicago truncatula	REWADYIDKKIYEVAGNIWIKKGEVÇETAKKEFIFAIKIL	160
Caragana korshin	RFWADYVDKKIYEVGRNVWTKKGEEGEAARKEFIDAIRIL	160
Carica papaya	RFWADYVDKKMYEAGRRVWTIKGEEGEGARKEFIEIIKIL	160
Cicer arietinum	RFWADYVDKKIYDAGRNIWTKKGEEÇEAAKKEFIDAIKIL	160
Slycine max	REWADYODKKTYDLGRKINTSKGEEREAARREETEATKIL	160
Sossypium raimon	REWADYVDKKIYDLGSRVWKTKGEEQATAKKEFIETIKIL	160
Hevea brasiliens	REWADFIDKKIYDIGRKIWTTKGDEGEAAKKEFIEAIKIL	160
Jatropha curcas	RFWADFVDKKIYDIGRKIWTTKGEEQEAAKKEFIECIKIL	160
Nicotiana sylves	REWADYIDKKIYDIGRKIWTIKKEDQEAVNKEFIECIKIL	160
Ricinus communis	REWADFVDKKIYELGRKINTIKGEDÇEAGKKEFIDAIKLL	160
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Medicago truncatula	E 2EI GEKNEFGGDKI GEVDVAFIPI YNWFRGYEAFGKI SV	200
Caragana korshin	EEGIGEKIYFGGERIGYVDIALIPFYIWFKAYEVFGNLNI	200
Carica papaya	EGELGEKPYFGGESFGYVDLTFIPFYIWFSVYESFGKMSI	200
Cicer arietinum	EZELGEKTYFGGDKIGFVDVALIPFYIWFKGYEIFGNFIV	200
Slycine max	EEGIGEKTYFGGDNIGEVDIALVPFYIWFKAYETFGTLNI	200
Sossypium raimon	EKELGEKPYFGGESIGYVDVAFIPFYSWFYAYEKCGNFSI	200
Hevea brasiliens	ESELGNKPYEGGESMGYVDVALIPEYSWEVAYEICGNFSI	200
Jatropha curcas	EAELEEKPYFGGERIGYVDVSLVPFYSWFYAYE2FGNFST	200
Nicotiana sylves	EGBLIGEKPYFGGGSEGEVDMTLIPMYCWFPTYEKEGNFSI	200
Ricinus communis	EGENGERPYFGGESFGYVDVALIPFYSWFYAYEICGNFSI	200
No. of the second se		
Medicago sativa	YKECPMFSAWANRCMQIESVSKSLEDQDKIHDLIVELKKK	240
Medicago truncatula	DHECHKFFAWANRCMEIESVSKSLFEQDQIHDLIVQIKKK	240
Caragana korshin	ENECHKFIAWAKRCMQIENVSRSLFDQHKVYEFIVEIRKR	240
Carica papaya	EABCHKLFSWVKRCLEKESVSKSLFDQDKVYGFVLELRKA	240
Cicer arietinum	ERECERFIAWARRENQIDSVSRSLPDQERVYEFIVDIRKK	240
Slycine max	ESECERFIAWARRCLORESVARSLEEQQRVYEFIMDLRKK	240
sossypium raimon	EABCHRLIAWAKRCIQKPSVAKSLFDQQKVYDFILEMKKR	240
nevea brasiliens	EFECTVLIAWAKRCLQKPSVSESVFDPEKVYEFVLVLKKK	240
Jatropha curcas	EVECHKLIAWVKRCLEKESVSKSLFEQQKVYDFVLHLRKV	240
Nicotiana sylves	EAECEKIVAWAHRCMQHESVSKSLVDPDKVYDFVVMLRQA	240
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sossypium raimon		243
Nevea brasiliens	191	243
Jatropha curcas	IGI	243
Nicotiana syives	FOI	243
Contras Communits	1 MA	243

Fig. 2. Amino acid sequence alignment of *MsGST* from alfalfa with GST from other plant species. The black shading represents conserved residues and gray shading represents similar residues in more than 80% sequences





tau class GST (Droog 1997; Aliya et al. 2003).The first conserved section, histidine-lysine-lysine (H-K-K), was observed at position 51-53; the second conserved triplet of amino acid residue histidine-asparagine-glycine (H-N-G) was identified at position 59-61; the third conserved amino acid is phenylalanine (A) at position 156 and the fourth conserved amino acid is glycine (G) at position 165 (Fig. 1). Therefore, *MsGST* is classified as a tau class *GST* based on the phylogenetic analysis and conserved regions.

Tissue-specific expression analysis

To investigate the expression pattern of *MsGST* in alfalfa, the levels of *MsGST* mRNA in different tissues were monitored by qRT-PCR. As shown in Fig. 4,



Fig. 4. Relative expression levels of *MsGST* in alfalfa different tissues. The relative level of mRNA was normalized to *Medicago* Actin gene. Bars represent the means \pm SE (n=3). Bars with different letters demonstrate significant difference ($p \le 0.05$)

MsGST mRNA was expressed in the root, stem and leaf, while the expression levels were significantly different ($p \le 0.01$). The transcriptional level was highest in leaves followed by stems and then roots. These results suggested that the expression of *MsGST* in alfalfa was tissue-specific. By contrast, it was reported that *ZmGSTI* and *ZmGSTV* were more abundant in root than in shoot (Dixon et al. 1998).

MsGST expression was induced by abiotic stress

To investigate whether *MsGST* is a stress-responsive gene, we examined the transcript level of *MsGST* by qRT-PCR under ABA treatment and salt, drought, cold and heat stresses. As shown in Fig. 5, the expression of *MsGST* was induced by abiotic stress. The expression level increased with the time of cold

treatment in the root and shoot of NN plant, while the transcription level showed no significant difference 4h after cold stress but increased at 12h in the root and shoot of NA plant (Figs. 5a and b). A significant increase of MsGST expression level was found in NA plant under heat treatment, while the expression level increased 4h after heat stress and then decreased at 12h in NN plant (Figs. 5c and d). The expression of MsGST in NA shoot increased ($p \le 0.05$) after 4h of drought stress and maintained at a steady level after 12h. Drought stress induced an increase in MsGST expression level in NN shoot after 4h but a reduction after 12h, which was comparable with normal condition (Fig. 5e). MsGST expression level increased with the time of drought stress in the roots of both NA and NN plant (Fig. 5f). Resuming water after drought stress induced a decrease in MsGST expression level compared with normal condition, except for the NA shoot, which was comparable with normal condition. MsGST expression level increased with the time of salt stress in the shoots of NA and NN plants (Fig. 5g) The expression of MsGST was induced by ABA treatment and reached the highest level at 12h in NA and NN shoots (Fig. 5i). MsGST expression level in NA root increased 4h after ABA treatment and maintained stable at 12h, while NN root showed a dramatic increase in MsGST expression at 12h, which was 27.21 times higher than under normal condition (Fig. 5j).

The results of present study showed that MsGST expression was induced by abiotic stress implying that MsGST is a stress response gene and might participate in alfalfa response to various stresses. GST proteins are expected to be an important enzyme to protect cells from stress because of their ability to scavenge ROS (Potters 2010). Some reports also confirmed positive up-regulation of GSTs for abiotic stress resistance. For instance, in Salicornia brachiate and Arabidopsis thaliana, the expression levels of GST were up-regulated under cold stress (Jha et al. 2011; Yang et al. 1998). GST transcription in mustard leaves also increased after heat treatment (Gong et al. 2005). The abundance of MaGSTU1, MaGSTU2, and MaGSTU3 from banana was increased by cold, salt and drought treatment (Wang et al. 2013). It was also reported that GST expression levels in Arabidopsis thaliana were up-regulated during ABA treatment and salt stress (van der Kop et al. 1996; Yang et al. 1998). The expression pattern of the tau class MsGST identified here is similar to plants undergoing abiotic stress. In our previous study, we found that rhizobium







symbiosis had a positive impact on alfalfa response to salt and dehydration stress (Wang et al. 2016; Yang et al. 2013). However, our results showed that the induction of MsGST expression by abiotic stress is independent of rhizobium symbiosis.

Authors' contribution

Conceptualization of research (PY); Designing of the experiments (YC); Contribution of experimental materials (JG, PR); Execution of field/lab experiments and data collection (JA, JY); Analysis of data and interpretation (ZY, ZZ); Preparation of manuscript (YC, YW).

Declaration

The authors declare no conflict of interest.

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