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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 (qRT-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: ɣ-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 plant-rhizobium 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^{o}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 ± 5^{o}C and 20 ± 5^{o}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 μmol 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^{o}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 TTTT	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 μg 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 ($p \leq 0.05$) 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 pI of 5.91 (Fig. 1). The amino acid

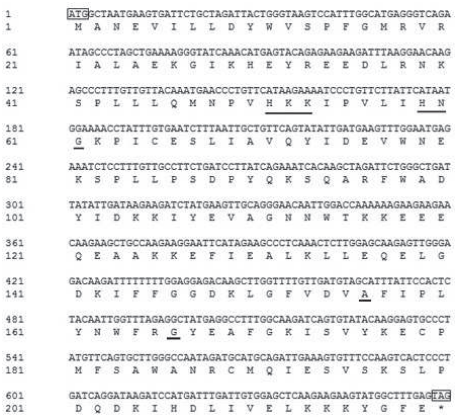

Fig. 1. Coding sequence (CDS) and deduced amino acid sequence. Boxes represent start and end codon. Conserved amino acid of tau class GST is underlined

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

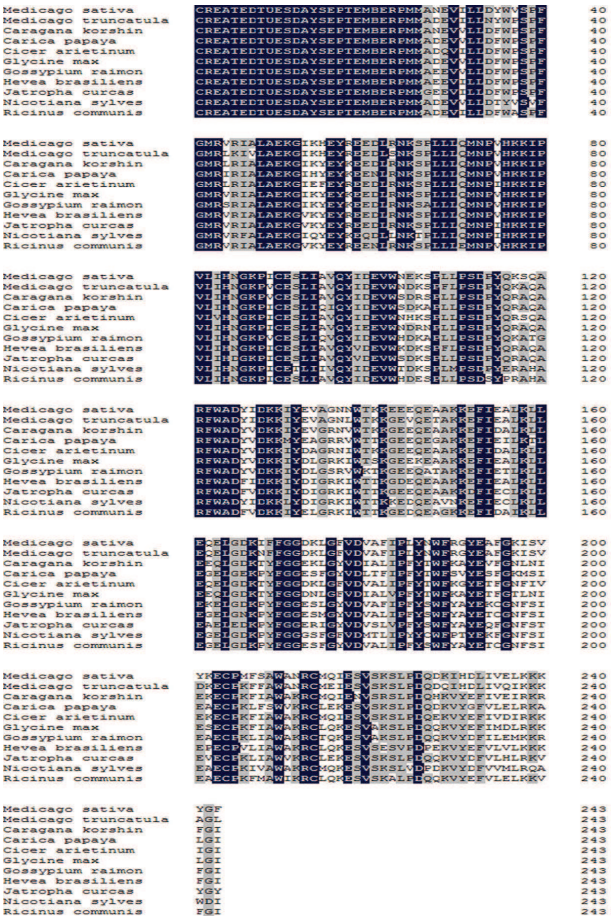

Fig. 2. Amino acid sequence alignment of *MsGST* from alfalfa with GST from other plant species. The black shading represents conserved residues and gray shading represents similar residues in more than 80% sequences

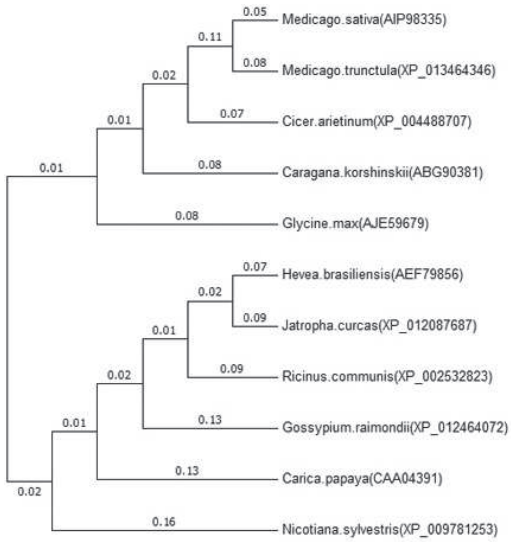

Fig. 3. Phylogenetic tree of *MsGST* protein with other selected plant species

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,

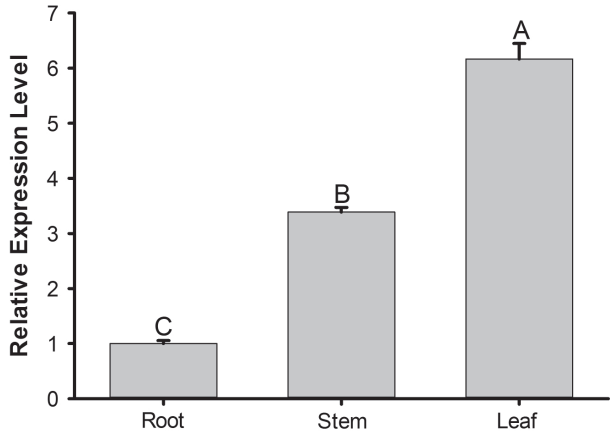

Fig. 4. Relative expression levels of *MsGST* in alfalfa different tissues. The relative level of mRNA was normalized to *Medicago* Actin gene. Bars represent the means ± SE (n=3). Bars with different letters demonstrate significant difference ($p \leq 0.05$)

MsGST mRNA was expressed in the root, stem and leaf, while the expression levels were significantly different ($p \leq 0.01$). 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 ($p \leq 0.05$) 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

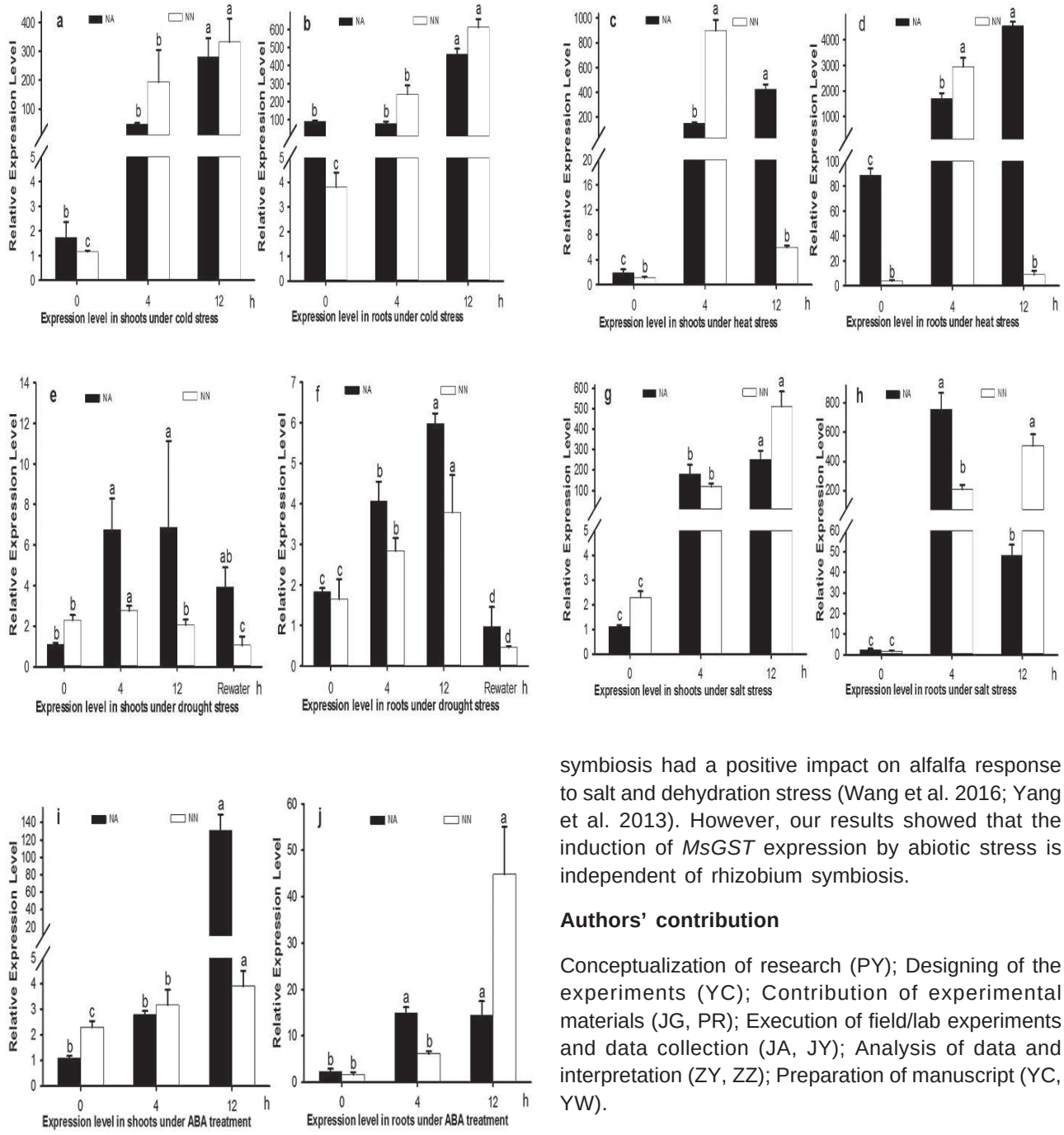

Fig. 5. Relative expression levels of *MsGST* induced by ABA treatment and abiotic stresses in alfalfa inoculated with rhizobium (NA) and not-inoculated rhizobium (NN). Rewater means that plants were put into nutrition solution for 12h after 12-hour-drought stress. The relative level of mRNA was normalized to that of the *Medicago* Actin gene. Bars represent the means ± SE (n=3). Bars with different letters demonstrate significant difference ($p \leq 0.05$) between the same group

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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