BIOLOGIA PLANTARUM 56 (3): 516-520, 2012

Over-expressing *GsGST14* from *Glycine soja* enhances alkaline tolerance of transgenic *Medicago sativa*

Z.-Y. WANG[1,2], F.-B. SONG[1,2], H. CAI[3], Y.-M. ZHU[3]*, X. BAI[3], W. JI[3], Y. LI[3], Y. HUA[3]

Northeast Institute of Geography and Agroecology, Chinese Academy of Sciences, Changchun, 130012, P.R. China[1]
Graduate University of Chinese Academy of Sciences, Beijing, 100049, P.R. China[2]
Plant Bioengineering Laboratory, Northeast Agricultural University, Harbin, 150030, P.R. China[3]

Abstract

Glutathione-S-transferases (GSTs) are ubiquitous enzymes that play a key role in stress tolerance and cellular detoxification. The GST gene *GsGST14* selected from the gene expression profiles of *Glycine soja* under alkaline stress was transformed into alfalfa (*Medicago sativa* L.). Transgenic alfalfa plants showed 1.73 - 1.99 times higher GST activity than wild-type plants. Transgenic alfalfa grew well in the presence of 100 mM $NaHCO_3$, while wild-type plants exhibited chlorosis and stunted growth, even death. There were marked changes in malondialdehyde content and relative membrane permeability caused by alkaline stress in non-transgenic lines compared to transgenic lines. The results indicate that the gene *GsGST14* could enhance alkaline resistance in transgenic alfalfa.

Additional key words: glutathione S-transferase, $NaHCO_3$, transgenic alfalfa, wild soybean.

Introduction

Alfalfa (*Medicago sativa* L.) is one of the most important leguminous forage crops worldwide. Saline-alkaline stress significantly limits the productivity of alfalfa due to its adverse effects on growth, formation of nodules, and symbiotic nitrogen-fixation capacity. Saline-alkaline stress may lead to the formation of reactive oxygen species (ROS). To protect against the toxicity of reactive oxygen, aerobic organisms are equipped with an array of defense mechanisms (Sun *et al*. 2010, Wang *et al*. 2009), including one based on glutathione S-transferases (GSTs) (Katsuhara *et al*. 2005). GSTs catalyze the conjugation of glutathione (GSH, γ-Glu-Cys-Gly) with a broad variety of electrophilic compounds, which facilitate their metabolism, sequestration, or removal (Dalton *et al*. 2009). GSTs are important responders to many abiotic and biotic stresses. Studies have shown that GST transgenic plants enhance resistance to low temperature (Huang *et al*. 2009), drought (George *et al*. 2010), salt (Ji *et al*. 2010) and oxidative stresses (Zhao *et al*. 2006).

In this study, the alkaline stress-responsive gene *GsGST14,* of wild soybean, was selected from gene expression profiles of plants under alkaline stress, which had been established in our laboratory previously (Ge *et al*. 2010a,b). Transgenic alfalfa plants over-expressing *GsGST14* were obtained and their alkaline tolerance was analyzed according to stress-related physiological parameters. This study provides a way to improve alkaline tolerance in alfalfa, an important forage legume.

Materials and methods

RNA was isolated from a wild soybean (*Glycine soja* Sieb. & Zucc. cv. G07256) under alkaline (50 mM $NaHCO_3$) stress (Ge *et al.* 2010a,b). The full-length *GsGST14* cDNA sequence was obtained by RT-PCR using primers designed from *GmGST14*. Briefly, total RNA was isolated from whole *G. soja* seedlings with *Trizol* (*Invitrogen*, Carlsbad, CA, USA), according to the manufacturer's instructions, and the first-strand cDNA was synthesized using a *SuperScript*^{III} reverse transcriptase kit. The sequences of the gene-specific primers were: 5'-GAAGGAAGA TGGGAAGCGAAG-3' and 5'-ACAGAGACTTAGCTA GTATGATTCCCTG-3'. The PCR products were cloned

Received 17 April 2011, *accepted* 26 July 2011.
Abbreviations: GPDH - glyceraldehyde-3-phosphate dehydrogenase; GSH - glutathione; GST - glutathione-S-trasferase; MDA - malondialdehyde; MS - Murashige and Skoog; RT-PCR - reverse transcription polymerase chain reaction.
* Corresponding author; fax: (+86) 451 55191776, e-mail: ymzhu2001@neau.edu.cn

into the *pGEM-T* cloning vector (*Promega*, Madison, WI, USA) and subjected to sequencing.

The coding region of the *GsGST14* sequence was inserted into *pCAMBIA3300* under the control of the cauliflower mosaic virus (CaMV) 35S promoter, with the binding enhancer E12 (pCEB-GsGST14). The *Bar* gene was used as the selectable marker, and the recombinant vector was transformed into *Agrobacterium* strain EHA105. Cotyledonary nodes of *Medicago sativa* L. were transformed with recombinant *Agrobacterium* harboring pCBE-GsGST14 by co-cultivation. The transformed plants were selected using 0.5 mg dm^{-3} glufosinate ammonium (Hua 2011). Regenerated shoots were rooted on 1/2 Murashige and Skoog (MS) medium, transferred into soil, and grown under a 16-h photoperiod, irradiance about 600 μmol m^{-2} s^{-1}, temperature of 24 ± 2 °C and relative humidity of 80 ± 5 %. Genomic DNA was isolated from the fresh leaves of transformed and non-transformed plants using a modified CTAB method (Doyle and Doyle 1990). A fragment bearing the 35S promoter and *Bar* gene was amplified using specific primers: 5'-CCT GTGCCTCCAGGGAC-3' and 5'-GCGGTCTGCACC ATCGTC-3'.

Total RNA was extracted from 200 mg of young leaves, from transformed lines and non-transformed wild-type plants, with an *RNAprep* pure plant kit (*Tiangen Biotech*, Beijing, China). RNA samples were reverse-transcribed using a *SuperScriptTM*III reverse transcriptase kit (*Invitrogen*). A 286-bp fragment of the alfalfa glyceraldehyde-3-phosphate dehydrogenase (*GPDH*) gene was used as an internal control (Bao *et al.* 2009). The reverse-transcription reaction volume was 0.025 cm^3. A 498-bp fragment was amplified using a pair of *GsGST14* gene-specific primers: 5'-GGAAGCGAAGAAGTGA AGC-3' and 5'-CCAAATAGGAAGCCAGTAAG-3'. The PCR products were electrophoresed on a 1.2 % agarose gel containing ethidium bromide.

T_0 transgenic alfalfa lines and the non-transformed (wild-type) plants were propagated from stem cuttings (Bao *et al.* 2009). When adventitious roots had formed, plants of similar size were chosen for further culture. For alkaline stress treatments, plants were transplanted to plastic culture pots containing soil and grown under a 16-h photoperiod, irradiance about 600 μmol m^{-2} s^{-1}, temperature of 24 ± 2 °C and relative humidity of 60 ± 5 %. Plants were watered every 2 d with 1/8 Hoagland's nutrient solution for 4 weeks, and then the nutrient solution was supplemented with $NaHCO_3$ at final concentrations 0, 50, or 100 mM. The pH of nutrient solution was 7.0 without $NaHCO_3$, 8.0 and 8.5 with 50 and 100 mM $NaHCO_3$, respectively. After $NaHCO_3$ treatments for 14 d, the plants were used for further analyses.

The chlorophyll content was determined in 80 % (v/v) acetone extract according to the method of Lichtenthaler and Wellburn (1983). The absorbance at 663 and 645 nm was measured using a spectrophotometer (*UV-2550*, *Shimadzu*, Japan).

Total protein was extracted from the leaves of transgenic alfalfa plants and non-transformed plants using a protocol adapted from Rao (1997). Protein content was determined by Coomassie blue binding with bovine serum albumin (BSA) as the standard. GST activity was measured spectrophotometrically with the artificial substrate 1-chloro-2,4-dinitrobenzene (CDNB) as described by Mauch and Dudlar (1993). The amount of enzyme producing 1 nmol min^{-1} of conjugated product (GSH-CDNB) was defined as one unit. SOD was measured as described by Zhao (1993). In this assay, one unit of SOD was defined as the amount required to inhibit the photoreduction of nitroblue tetrazolium by 50 %.

The malondialdehyde (MDA) content was determined using a thiobarbituric acid protocol (Peever *et al*. 1989). The absorbance at 450, 532, and 600 nm was determined using *UV-2550* spectrophotometer.

Leaf cell membrane damage was determined as the relative membrane permeability of leaf cells, using a conductivity meter (*DDSJ-308A*, Precision & Scientific Instruments, Shanghai, China) according to the method described by Gibon *et al*. (1997) with slight modifications. The relative membrane permeability (RMP) was calculated using the equation: RMP [%] = $S_1/S_2 \times 100$, where S_1 and S_2 refer to conductivity of alfalfa fresh leaves and boiled leaves, respectively.

All data were analyzed by one-way *ANOVA* using *SPSS 13.0* (*SPSS*, Chicago, USA) statistical software.

Results and discussion

Using an *Affymetrix® Soybean GeneChip®*, transcriptional profiling of *Glycine soja* under 50 mM $NaHCO_3$ treatment was conducted in our laboratory (Ge *et al*. 2010a,b). The alkali stress-responsive genes of wild soybean were selected according to the gene expression profiles. Among the differentially expressed genes, a gene encoding a GST was identified. The 672-bp cDNA was amplified by RT-PCR and sequenced. Sequence analysis showed that the full-length sequence encoded 224 amino acids, with a predicted molecular mass of 25.8 kDa. National Center for Biotechnology Information (NCBI) *BLASTX* analysis showed the deduced amino acid sequences were highly similar to several previously isolated plant GSTs (Fig. 1). Analysis of the phylogenetic tree revealed that the GST was closely related to *Glycine max* GmGST14 (Fig. 1*B*), and was therefore named GsGST14 (GenBank accession number JF790974). Conserved domain analysis in *NCBI* revealed that GsGST14 belonged to a tau class GST and had two functionally important domains, the conserved N-terminal domain and a more divergent C-terminal domain. GSH binds to the N-terminal domain (G-site) while the hydrophobic substrate occupies a pocket in the C-terminal domain (H-site).

The *GsGST14* gene was introduced into an alfalfa

cv. Zhao Dong by *Agrobacterium tumefaciens*-mediated transformation under the control of the CaMV 35S promoter (Fig. 2). Glufosinate ammonium-resistant independent transgenic lines were generated. A total of 7 *GsGST14* transgenic lines were identified by PCR (Fig. 3). To detect the expression of *GsGST14* in transgenic alfalfa lines, RT-PCR was performed on young leaves of the transgenic and wild-type alfalfa. *GsGST14* expression was observed in transgenic alfalfa lines 14-4 and 14-6 (Fig. 4), and these were then chosen for further physiological assays.

The responses of alfalfa transgenic lines (14-4 and 14-6) and wild-type plants to alkaline stress were examined. Under standard culture conditions, there was no

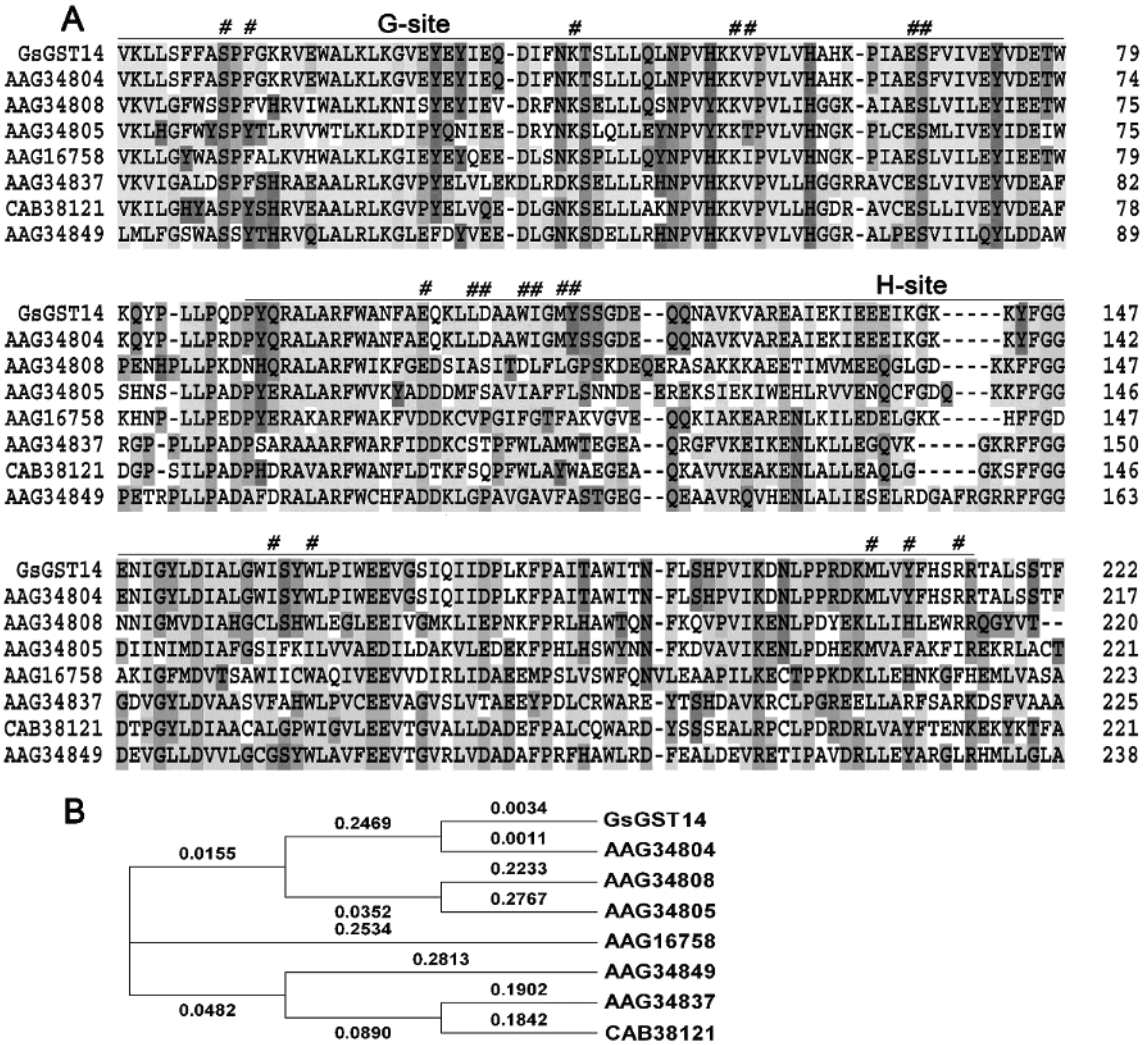

Fig. 1. *A* - Alignment of the deduced amino acid sequence of *GsGST14* with plant homologs: *Glycine soja* GsGST14; *Glycine max* GST14, AAG34804; *Glycine max* GmGST18, AAG34808; *Glycine max* GmGST15, AAG34805; *Solanum lycopersicum* putative GST T3, AAG16758; *Zea mays* GST 41, AAG34849; *Zea mays* GST 29, AAG34837; and *Zea mays* GST7 protein, CAB38121. Identical amino acid residues are *shadowed in black* and similar amino acid residues are *shadowed in gray*. Amino acids marked with ''#'' represent the characteristic residues in the conserved G-site and variable H-site. *B* - Phylogenetic analysis of the GsGST14 amino acid sequence and homologous sequences in other species.

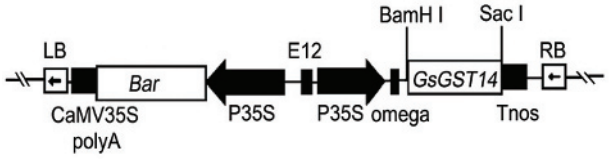

Fig. 2. Schematic representation of a construct used for *Agrobacterium tumefaciens*-mediated transformation of the *GsGST14* gene.

noticeable difference between the transgenic lines over-expressing *GsGST14* and the non-transformed plants. After 14 d under treatment with 50 or 100 mM $NaHCO_3$, both the wild-type and transgenic plants showed growth retardation in a dose-dependent manner, but the retardation was more apparent in wild-type plants. At 100 mM $NaHCO_3$, wild-type plants exhibited chlorosis and even death, whereas both the 14-4 and 14-6 transgenic

lines continued to grow well. To further study the difference between transgenic and wild-type alfalfa under alkaline treatment, shoot length and chlorophyll content were measured under different $NaHCO_3$ concentrations. Shoot length and chlorophyll content of all plants decreased gradually with increased alkaline concentrations, but the wild-type plants were significantly smaller than transgenic plants under the same $NaHCO_3$ concentration. For example, in the presence of 100 mM $NaHCO_3$, the shoot length and chlorophyll content of transgenic lines were 1.5 - 1.65 and 2.02 - 2.14 folds higher, respectively, than those of the wild-type plants (Fig. 5*A*,*B*).

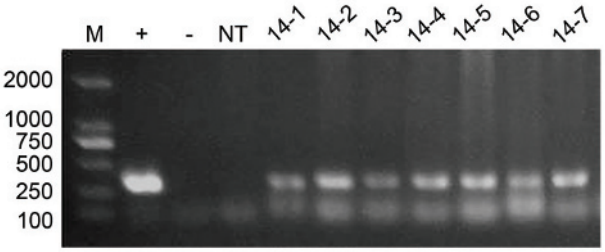

Fig. 3. Detection of T_0 transgenic plants from glufosinate ammonium-resistant lines by PCR. Specific PCR products of 287 bp were detected using a 1.2 % agarose gel containing ethidium bromide. M: DNA marker DGL 2000, +: plasmid as positive control, -: negative control, NT: non-transgenic control; 14-1 - 14-7: T_0 transgenic lines.

GST activity of crude extracts from the seedlings was measured in both the wild-type and transgenic plants (Fig. 5*C*). The transgenic alfalfa lines 14-4 and 14-6 showed 1.99 and 1.73 times higher GST activity, respectively, than the non-transformed plants under normal conditions. After alkali treatment, although GST activity progressively increased in all experimental lines, the assay revealed a marked increase in transgenic lines ($P < 0.01$). These result suggested that the increased alkaline tolerance might be related to higher GST activity in transgenic plants, due to the over-expression of *GsGST14*.

To study the influence of GST activity of other oxygen scavenging systems, SOD activity was determined (Fig. 5*D*). It was found that the SOD specific activity increased under alkaline stress. Although there was no significant difference between the transgenics and wild type plants under normal conditions, 1.66 and 1.51 folds higher SOD activity was observed in 14-4 and 14-6 lines, respectively, compared to wild-type plants under 100 mM $NaHCO_3$. The results may imply that over-expression of

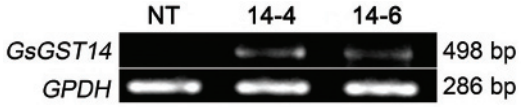

Fig. 4. RT-PCR analysis of *GsGST14* expression in leaves of control and transgenic alfalfa. NT: non-transformed plants; 14-4, 14-6: transgenic plants over-expressing *GsGST14*. Expression of the gene *GPDH* used as an internal control.

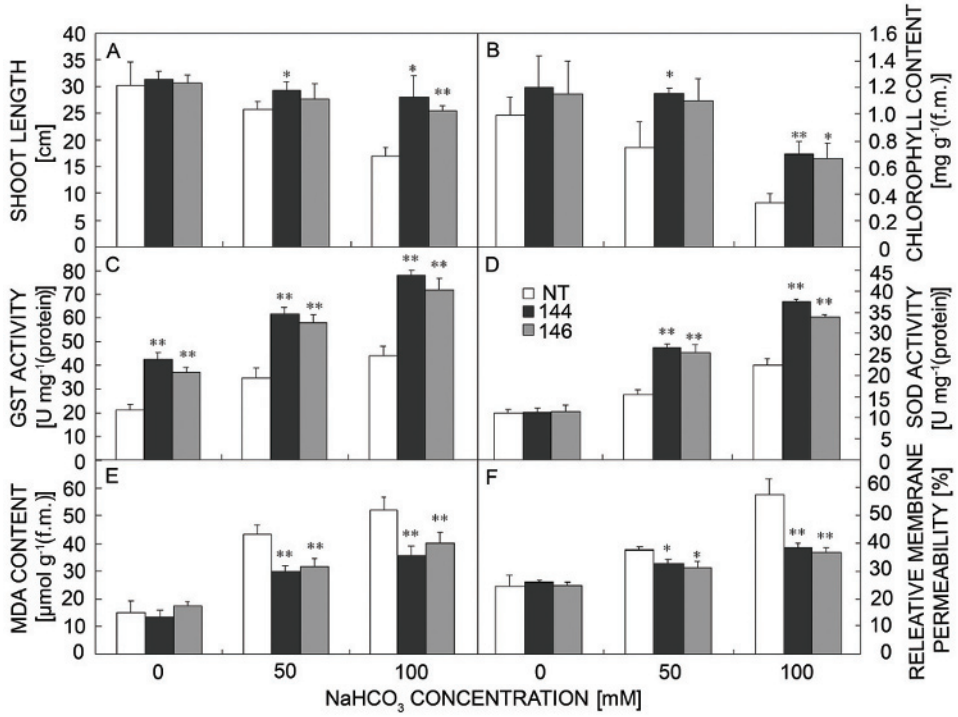

Fig. 5. Shoot length (*A*), chlorophyll content (*B*), GST activity (*C*), SOD activity (*D*), MDA content (*E*) and relative membrane permeability in leaves (*F*) of transgenic and wild-type alfalfa plants treated with 50 and100 mM $NaHCO_3$ for 14 days. Values are means ± SD ($n = 6$) and *bars* indicate SD. * and ** indicate significant differences at $P < 0.05$ and $P < 0.01$, respectively. NT: non-transgenic controls; 14-4, 14-6: transgenic plants over-expressing *GsGST14*.

GsGST14 could improve the reactive oxygen scavenging system of transgenic alfalfa lines, and decrease the effects of alkali stress injury on plant. Additionally, it seems that the co-regulation of GST and other antioxidant enzymes could be part of a system for maintaining the balance between the detrimental and beneficial roles of H_2O_2 in plant cells (Shigeoka *et al.* 2002). This is similar to previous studies that transferred GST genes into tobacco plants, enhancing tolerance to salt stress or heavy metals in the transgenic plants through increased ROS scavenging enzymes, including SOD (Zhao *et al*. 2006, Diao *et al.* 2011, Dixit *et al.* 2011).

Alkaline stress can cause oxidative damage to cell membranes. MDA content and relative membrane permeability of plant leaves are indicators of oxidative stress and membrane damage resulting from alkaline stress. It was shown that MDA content increased proportionally with alkali concentration, but a significant decrease in MDA content occurred in transgenic compared to non-transgenic plants treated with both 50 mM and 100 mM $NaHCO_3$ for 14 d (Fig. 5*E*). The changes in relative membrane permeability (Fig. 5*F*) were very similar to those of MDA during alkaline stress. These results indicate that the reduced oxidative damage caused by alkali stress in transgenic alfalfa might be related to the elevated levels of GST and SOD activity.

In conclusion, the *GsGST14* gene was isolated by *G. soja* alkaline stress transcriptome profiling and belongs to the tau class of GST family proteins. Overexpressing the *GsGST14* gene in *M. sativa* increased the GST and SOD activities and resulted in enhanced alkaline tolerance in transgenic plants. This was shown by greater shoot length and chlorophyll content, and lower stress-induced content of MDA and relative membrane permeability in transgenic alfalfa.

References

Bao, A.K., Wang, S.M., Wu, G.Q., Xi, J.J., Zhang, J.L., Wang, C.M.: Overexpression of the *Arabidopsis* H^+-PPase enhanced resistance to salt and drought stress in transgenic alfalfa (*Medicago sativa L.*). - Plant Sci. **176**: 232-240, 2009.

Dalton, D.A., Boniface, C., Turner, Z., Lindahl, A., Kim, H.J., Jelinek, L., Govindarajulu, M., Finger, R.E., and Talor, C.G.: Physiological roles of glutathione S-transferases in soybean root nodules. - Plant Physiol. **150**: 521-530, 2009.

Diao, G., Wang, Y., Wang, C., Yang, C.: Cloning and functional characterization of a novel glutathione S-transferase gene from *Limonium bicolor*. - Plant mol. Biol. Rep. **29**: 77-87, 2011.

Dixit, P., Mukherjee, P.K., Ramachandran, V., Eapen, S.: Glutathione transferase from *Trichoderma virens* enhances cadmium tolerance without enhancing its accumulation in transgenic *Nicotiana tabacum*. - PLoS ONE **6**: e16360, 2011.

Doyle, J.J., Doyle, J.L.: Isolation of plant DNA from fresh tissue. - Focus **12**: 13-15, 1990.

George, S., Venkataraman, G., Parida, A.: A chloroplast-localized and auxin-induced glutathione S-transferase from phreatophyte *Prosopis juliflora* confer drought tolerance on tobacco. - J. Plant Physiol. **167**: 311-318, 2010.

Ge, Y., Li, Y., Zhu, Y.M., Bai, X., Lv, D.K., Guo, D.J., Ji, W., Cai, H.: Global transcriptome profiling of wild soybean (*Glycine soja*) roots under $NaHCO_3$ treatment. - BMC Plant Biol. **10**:153, 2010a.

Ge, Y., Li, Y., Lv, D.K., Bai, X., Ji, W., Cai, H., Wang, A.X., Zhu, Y.M.: Alkaline-stress response in *Glycine soja* leaf identifies specific transcription factors and ABA-mediated signaling factors. - Funct. Integr. Genomics **11**: 369-379, 2010b.

Gibon, Y., Larher, F.: Cycling assay for nicotinamide adenine dinucleotides: NaCl precipitation and ethanol solubilization of the reduced tetrazolium. - Anal. Biochem. **251**: 153-157, 1997.

Hua, Y.: [Cultivation and alkaline tolerance analysis of transgenic alfalfa with *GsPPCK1* and *GsPPCK3* gene.] - PhD Thesis, Northeast Agriculture University, Beijing 2011. [In Chinese]

Huang, C., Guo, T., Zheng, S.C., Feng, Q.L., Liang, J.H., Li, L.: Increased cold tolerance in *Arabidopsis thaliana* transformed with *Choristoneura fumiferana* glutathione S-transferase gene. - Biol. Plant. **53**: 183-187, 2009.

Ji, W., Zhu, Y.M., Li, Y., Yang, L., Zhao, X.W., Cai, H., Bai, X.: Over-expression of a glutathione S-transferase gene, GsGST, from wild soybean (*Glycine soja*) enhances drought and salt tolerance in transgenic tobacco. - Biotechnol. Lett. **32**: 1173-1179, 2010.

Katsuhara, M., Otsuka, T., Ezaki, B.: Salt stress-induced lipid peroxidation is reduced by glutathione S-transferase, but this reduction of lipid peroxides is not enough for a recovery of root growth in *Arabidopsis*. - Plant Sci. **169**: 369-373, 2005.

Lichtenthaler, H. K., Wellburn, A.R.: Determination of total carotenoids and chlorophylls *a* and b of leaf extracts in different solvents. - Biochem. Soc. Trans. **11**: 591-592, 1983.

Mauch, F., Dudlar, R.: Differential induction of distinct glutathione-S-transferases of wheat by xenobiotics and by pathogen attack. - Plant Physiol. **102**: 1193-1201, 1993.

Peever, T.L., Higgins, V.J.: Suppression of the activity of non-specific elicitor from *Cladosporium fulvum* by intercellular fluids from tomato leaves. - Physiol. mol. Plant Pathol. **34**: 471-482, 1989.

Rao, M.V., Paliyath, G., Ormrod, D.P.: Influence of salicylic acid on H_2O_2 production, oxidative stress, and H_2O_2-metabolizing enzymes. - Plant Physiol. **115**: 137-149, 1997.

Shigeoka, S., Ishikawa, T., Tamoi, M., Miyagawa, Y., Takeda, T., Yabuta, Y., Yoshimura, K.: Regulation and function of scorbate peroxidase isoenzymes. - J. exp. Bot. **53**: 1305-1319, 2002.

Sun, W.H., Duan, M., Li, F., Shu, D.F., Yang, S., Meng, Q.W.: Overexpression of tomato tAPX gene in tobacco improves tolerance to high or low temperature stress. - Biol. Plant. **54**: 614-620, 2010.

Wang, Y., Yang, Z.M., Zhang, Q.F., Li, J.L.: Enhanced chilling tolerance in *Zoysia matrella* by pre-treatment with salicylic acid, calcium chloride, hydrogen peroxide or 6-benzylaminopurine. - Biol. Plant. **53**: 179-182, 2009.

Zhao, F.Y., Zhang, H.: Salt and paraquat stress tolerance results from co-expression of the *Suaeda salsa* glutathione S-transferase and catalase in transgenic rice. - Plant Cell Tissue Organ Cult. **86**: 349-358, 2006.

Zhao, K.F.: [Salt Tolerance Physiology of Plant.] - Sci. Technol. Press, Beijing 1993. [In Chinese]