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Overexpression of the *StP5CS* gene promotes nodulation and nitrogen fixation in vegetable soybean under drought stress

X.X. Wang, F. Gao, S.P. Yang, J.Y. Gai and Y.L. Zhu*

National Center for Soybean Improvement,
Nanjing Agricultural University, Nanjing 210 095, China.
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ABSTRACT

The *StP5CS* (GenBank accession number: JN606861) T_6 homozygous transgenic lines (HTLs) of vegetable soybean [*Glycine max* (L.) Merrill] were grown using vermiculite pot culture to determine whether *StP5CS* overexpression would enhance nodulation and symbiotic nitrogen fixation (SNF) in two T_6 HTLs (17W-1, 17W-2) under drought conditions. The growth performance, nodule development and seed weight of T_6 HTLs were significantly better than those of wild type (WT) plants. The proline levels in various tissues of T_6 HTLs were higher than WT plants. The concentrations of total ureide, total N, leghemoglobin (Lb) and the activity of glutamine synthetase (GS, EC 6.3.1.2) in the T_6 HTLs were significantly increased. Moreover, the relative expression levels of five key nodulation- and SNF-associated genes (i.e., *GmENOD40-1*, *GmENOD40-2*, *GmLba*, *GmGS1β1* and *GmGS1β2*) were significantly higher in T_6 HTLs. In conclusion, overexpression of *StP5CS* enhances nodulation and SNF in transgenic vegetable soybean under drought stress conditions.

Key words: Drought stress, Nitrogen fixation, Nodulation, *StP5CS* gene, Vegetable soybean.

INTRODUCTION

Rhizobium-legume symbiotic nitrogen fixation (SNF) plays an important role in biological nitrogen fixation; this symbiosis serves as the major source of fixed nitrogen for legume hosts and agricultural crops, allowing them to thrive in nitrogen-deficient soil (diCenzo *et al*. 2015). However, SNF is an extremely complex biological process which is very sensitive to drought stress (Amela *et al*. 2018). Marino *et al*. (2007) conducted a root-separation test on nodulated peas and showed that the root nodules subjected to drought stress had a decreased ability to fix N_2, while the activity of key enzymes and the malic acid content in root nodules were significantly decreased.

N_2-fixing leguminous plants have evolved a variety of adaptive mechanisms to increase nitrogen uptake by the plants and maintain high N_2-fixing efficiency under drought conditions (Morais *et al*. 2012). The cyclic amino acid proline has improved plant tolerance to drought in a number of crops (Kaur and Asthir, 2015). SNF and nitrogen metabolism are energy expensive processes in legume nodules, proline can serve as a source of energy for N_2 fixation in order to compensate for drought stress on the respiratory system (Kim and Nam, 2013).

Drought stress severely limits the growth and production of vegetable soybeans [*Glycine max* (L.) Merrill] (Gian *et al*. 2014; Kusvuran and Dasgan, 2017). In our previous studies, a novel *StP5CS* gene from salt-tolerant *Solanum torvum* Swartz was successfully isolated (Zhang *et al*. 2015) and bioinformatic analysis showed that *StP5CS* plays an important role in proline synthesis. Previous work has shown that overexpression of *StP5CS* can promote the accumulation of proline in transgenic vegetable soybean (Zhang *et al*. 2015). In the present study, two *StP5CS* T_6 HTLs were grown in vermiculite to investigate whether the overexpression of *StP5CS* gene can enhance nodulation and N_2-fixing ability of transgenic vegetable soybean plants under drought conditions.

MATERIALS AND METHODS

Plant materials: In our previous report, Zhang *et al*. (2015) obtained two T_4 HTLs of vegetable soybean [*Glycine max* (L.) Merrill] expressing *StP5CS* from the host cultivar 'NY-1001'. In this study, these two T_4 HTLs were further self-pollinated for two generations to obtain two T_6 HTLs coded as 17W-1 and 17W-2. These two lines were tested for N_2 fixation under drought conditions in comparison to WT plants derived from *StP5CS*-negative plants from the T_1 generation.

Rhizobium inoculation, plant growth and drought treatment: According to the methods for rhizobium inoculation and the culture of vegetable soybean seedlings, previously established in our laboratory, the germinated seeds were incubated with *Bradyrhizobium japonicum* (Zhu *et al*. 2016) for 30 minutes and then immediately transplanted into plastic boxes [32 cm (length) × 22 cm (width) × 12 cm (height)] containing sterilized vermiculite as substrate (five plants per box). The boxes were randomly placed under

*Corresponding author's e-mail: ylzhu@njau.edu.cn

controlled environmental conditions: day/night temperature of 25/20°C photoperiod of 16 h light/8 h dark, light intensity of 600 μmol · m^{-2} · s^{-1} and relative humidity of 70-80%. The seedlings were irrigated with 600 mL per box of nitrogen-free nutrient solution (Broughton and Dilworth, 1971) every three days for a period of ten days and after that the seedlings were subjected to drought treatment.

The moisture of the vermiculite was adjusted to 18% using a substrate moisture measuring meter (SMMM) (TZS, Zhejiang Top Instrument, China) based on a preparatory trial for the drought experiment (Biabani and Sajadi, 2018). During the three-week period of drought stress, the substrate moisture in the cultivating pots was monitored and adjusted twice per day, at 8:00 AM and 5:00 PM, using an SMMM while slowly adding fresh nutrient solution to each pot until moisture value reached 18%. The seedlings were exposed to 18% substrate moisture for three weeks. Upon termination of the drought stress, the plastic boxes were evenly divided into three groups. Nodules were sampled from group one plants to extract RNA for qRT-PCR analysis and were stored at -80°C. Samples of three biological replicates were taken at each time. Plants from group two were used for measuring the relevant physiological parameters and five biological replicates were taken at each time. Plants from group three were recovered from the drought stress by watering with 600 ml of nutrient solution every two days until the plants had grown for 80 days and the seeds reached maturity.

Determination of growth performance and nodulation parameters: Plant drought injury was recorded by visual observation of wilting index. Wilting index was scored from 1 to 5 using the method of Pathan *et al*. (2014). A measurement of plant vegetative growth of the T_6 HTLs and WT plants under drought conditions was conducted using the method of Zhu *et al*. (2016). Seed yield was recorded at maturity.

Determination of biochemical parameters: The proline concentration in various tissues was measured according to the method of Bates *et al*. (1973). Glutamine synthetase (GS) activity was determined using a kit from Suzhou Kiming Biotechnology Co., China. The content of leghemoglobin (Lb) in nodules was measured by the pyridinogen chromogenin method (Riley and Dilworth, 2010). Total N content was determined by the semi-micro Kjeldahl method using a nitrogen analyzer (Kjeltec 2300, FOSS, Sweden). Ureide quantification was carried out as described by Van (1970).

Quantitative Real-time PCR analysis: Total nodule RNA was isolated from both T_6 HTLs and WT plants by using TRIzol Reagent (Life Technologies, USA). The first cDNA strand was synthesized using the PrimeScript RT reagent kit (TaKaRa) with 1 μg of extracted RNA (TaKaRa). Quantitative real-time PCR was performed using SYBR® Premix Ex Taq™ II9 (TaKaRa). *GmCons6* (forward 5′-AGATAGGGAAATGGTGCAGGT3′; reverse, 5′-CTAAT GGCAATTGCAGCTCTC-3′) (Libault *et al*. 2008) was used as an internal reference gene for the following five genes involved in nodulation and N_2 fixation in vegetable soybean: two early nodulin genes *GmENOD40-1* (forward, 5′-TCTC TCTTGAGTGGCAGAAGCA-3′; reverse, 5′-TGGAGT CCATTGCCTTTTCG-3′) and *GmENOD40-2* (forward, 5′-GAGTGGCGGAAGCAGATACAC-3′; reverse, 5′-CTACA TAGCCATAGAGACCCCAATG-3′) (Kouchi and Hata, 1993); one leghemoglobin gene *GmLba* (forward, 5′-AACATTCCTCAATACAGCGTTGTG-3′; reverse, 5′-CCCGTGAGCTTAGGATTAG-3′) (She *et al*. 1993) and two cytosolic glutamine synthetase genes *GmGS1β1* (forward, 5′-GCTTTTCTTAGTAGGATTTGGTCTC-3′; reverse, 5′-TAACAATCGGAAAACGAGGGA-3′) and *GmGS1β2* (forward, 5′-GTGGAAGCCATGAGCAAAA CT-3′; reverse, 5′-CGAGGGAAAGGAATAGAAAACA-3′) (Masalkar *et al*. 2010). *ELF1b* (forward 5′ -GTTGAAAA GCCAGGGGACA-3′; reverse, 5′-TCTTACCCCTTGAG CGTGG-3′) (Le *et al*. 2012) was also used as a reference gene to compare the expression profiles of the *StP5CS* (forward 5′ -TGATATCTCGGCTGGCTCTAAAGG-3′ ; reverse, 5′ -GTTCCACAATCAGGTCATTAAGAC-3′) (Zhang *et al*. 2015) in response to drought stress. The relative expression levels of the concerned genes were enumerated using the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak, 2008).

Statistical analysis: The experimental data were examined by a one-way analysis of variance (ANOVA) using the IBM SPSS 20 statistical software (IBM Crop, USA), with Duncan's new multiple range test at $P < 0.05$. Graphs were generated using Origin 8.0 (Electronic Arts Inc, USA) and Excel 2010 software.

RESULTS AND DISCUSSION

Differences in physiological indices between the *StP5CS* overexpression and WT plants: The transcript levels of *StP5CS* were determined by using qRT-PCR, *StP5CS* was expressed in T_6 HTLs nodules but not in WT plants (Fig 4A).

As shown in Table 1, after drought treatment, both the T_6 HTLs and the WT plants exhibited drought injury, but the wilting index of the T_6 HTLs was lower than that of the WT plants. The dry weight of shoots and roots, the fresh weight and dry weight of the nodules of the T_6 HTLs were significantly higher than those of the WT plants. Moreover, transgenic plants demonstrated better pod-setting development. The superior growth performance of the T_6 HTLs was observed as compared to WT plants under drought conditions (Fig 1).

The above results showed that the overexpression of *StP5CS* improved drought resistance of vegetable soybean. Serraj *et al*. (1999) demonstrated that the drought-tolerant soybean cultivar Jackson possesses higher nodule biomass

Table 1: Plant biomass production and nodulation parameters of the T_6 homozygous transgenic lines (HTLs) and wild type (WT) plants grown in pot culture after three weeks drought treatment.

Plantline	Wilting index	Shoot DW (g · plant^{-1})	Root DW (g · plant^{-1})	Number of pods	Nodule DW (mg · plant^{-1})	Nodule FW (g · plant^{-1})
17W-1	1.41±0.32b[z]	0.32±0.06a	0.14±0.03a	25.0±3.0a	35.80±4.80a	0.20±0.05a
17W-2	1.57±0.45b	0.35±0.02a	0.16±0.05a	28.0±3.0a	39.42±3.08a	0.21±0.06a
WT	4.30±0.42a	0.08±0.05b	0.09±0.02b	18.0±3.0b	27.05±2.94b	0.09±0.03b

[z]Data are means ± standard deviation (SD) of five replicates. Different lowercase letters represent significant differences between plant lines based on Duncan's new multiple range test ($P < 0.05$). 17W-1 and 17W-2, T_6 homozygous transgenic lines; FW, fresh weight; DW, dry weight.

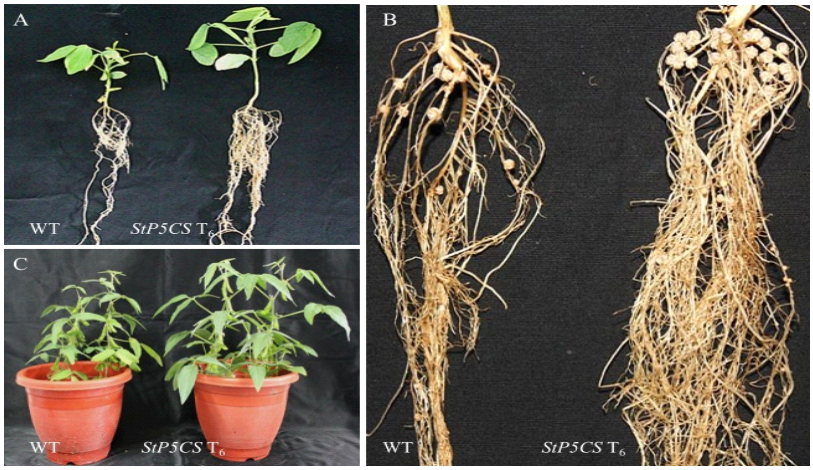

Fig 1: The phenotypes of the whole plants (A) and nodules (B) of T_6 HTLs and WT plants after three weeks of drought treatment and the plants were recovered by irrigation with salt-free nutrient solution for continued growth at the pod-setting stage (C).

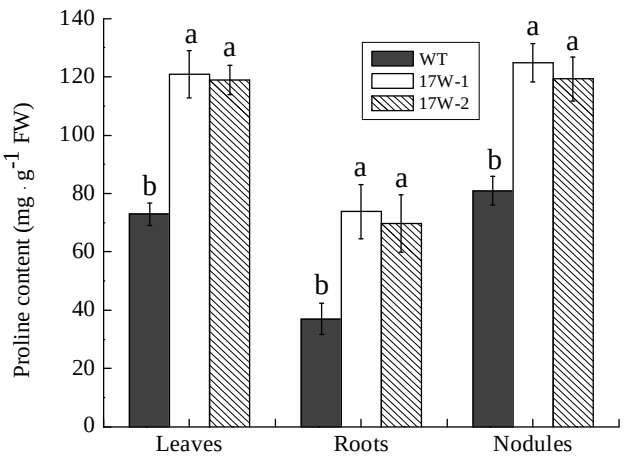

Fig 2: Proline contents in different organs of T_6 HTLs and WT plants after three weeks of drought treatment. Sampling replications and the method of significance test were described in MATERIALS AND METHODS.

and stronger N_2 fixation capacity than that of the drought-sensitive cultivar Biloxi. In the present study, the T_6 HTLs had a greater biomass production and higher seed yield than those of WT plants, indicating overexpression of *StP5CS* improved N_2-fixation in the transgenic plants.

***StP5CS* overexpression led to higher proline levels and higher total ureide accumulation in T_6 HTLs:** The T_6 HTLs accumulated higher levels of proline in each plant tissue than WT plants (Fig 2), indicating overexpression of *StP5CS* enhances proline accumulation.

In legume nodules, N_2-fixation is an energy-intensive enzymatic reaction, proline metabolism in nodules has been proposed to be a means of energy transfer which can be used as an energy source for N_2 fixation. Proline accumulates in plant cells, including those of legume roots and nodules, in response to various abiotic and biotic stresses (Kim and Nam, 2013). Proline may chaperone proteins and subcellular structures or act as a singlet oxygen quencher to alleviate the osmotic shock that affects nodule functions (Shinde *et al*. 2017). Higher levels of proline in T_6 HTLs resulting from the functional expression of *StP5CS* can enhance nodule functions by improving the drought tolerance of vegetable soybean.

T_6 HTLs showed higher N and total ureide accumulation: The total N concentrations of whole plants and total ureide concentrations in stems of the T_6 HTLs were higher than those of the WT plants (Fig 3).

In soybean, ureides are products of SNF, synthesized by the plant in the nodules and exported to other tissues as a nitrogen source (Duran and Todd, 2012). Ureide concentration is often used as an important indicator to measure the N_2 fixation ability of legumes (Herridge, 1982).

In this study, the T_6 HTLs exhibited higher ureide concentrations in the stems and higher whole plant N concentrations than WT plants, suggesting that the T_6 HTLs maintained higher ammonium assimilation efficiency under drought stress.

***StP5CS* overexpression promoted nodulation and SNF related gene expression:** The transcript levels of five key genes (i.e., *GmENOD40-1*, *GmENOD40-2*, *GmLba*, *GmGS1β1* and *GmGS1β2*) were significantly higher in T_6 HTLs than in WT plants (Fig 4B). This leads to T_6 HTLs exhibited higher GS activity and higher Lb levels under drought stress (Fig 5).

Two early nodulin genes, namely, *GmENDO40-1* and *GmENDO40-2*, are highly expressed in the cortical dividing cells in the early stage of soybean nodule development, play a predominant role in the process of nodule development (Kouchi and Hata, 1993). The increased expression of two early nodulin genes *GmENDO40-1* and *GmENDO40-2* might be one of the mechanisms underlying the promotion of nodulation in T_6 HTLs, resulting in a higher nodule biomass to promote SNF and plant growth in the T_6 HTLs.

Lb has a strong affinity for oxygen and its basic function is to absorb the oxygen in nodules, creating anoxic

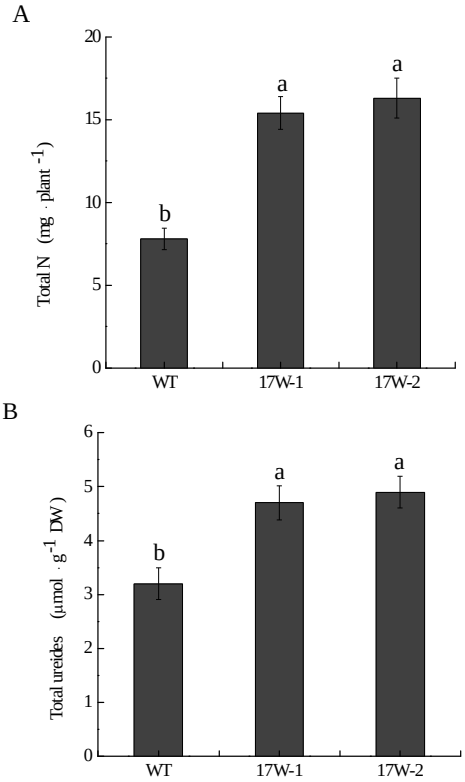

Fig 3: Total N (A) and total ureide concentrations (B) of T_6 HTLs and WT plants after three weeks of drought treatment. Sampling replications and the method of significance test were described in MATERIALS AND METHODS.

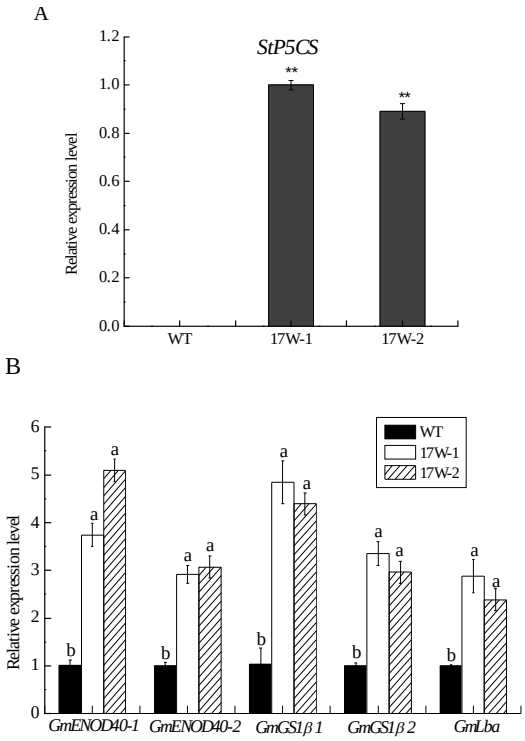

Fig 4: Relative expression levels by qRT-PCR of *StP5CS*, *GmENOD40-1*, *GmENOD40-2*, *GmLba*, *GmGS1β1* and *GmGS1β2* in T_6 HTLs and WT plants after three weeks of drought treatment. Sampling replications and the method of significance test were described in MATERIALS AND METHODS.

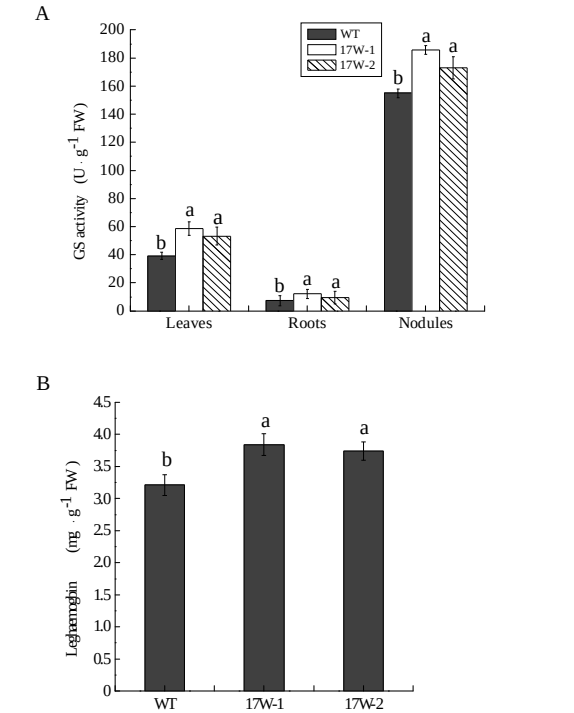

Fig 5: GS activities (A) and Lb concentrations (B) of T_6 HTLs and WT plants after three weeks of drought treatment. Sampling replications and the method of significance test were described in MATERIALS AND METHODS.

conditions for N_2 fixation by the rhizobia and preventing nitrogenase inactivation by oxygen (Shimoda *et al*. 2010). Overexpression of *StP5CS* increased the expression level of *GmLba*, contributing to Lb accumulation in the T_6 HTLs and facilitating the N_2-fixing activity of the T_6 HTLs under drought conditions.

GS is a key enzyme in the first step of nitrogen assimilation, GS1 increases the activity of the GS enzyme (Cabeza *et al*. 2014). Researchers have shown that *GmGS1β1* and *GmGS1β2* are endemic genes of leguminous plants and that their expression levels are affected by the external environment. Thus, *GmGS1β1* and *GmGS1β2* are important molecular targets for the regulation of N_2 assimilation by environmental factors (Cai *et al*. 2013). The higher expression levels of *GmGS1β1* and *GmGS1β2* in the T_6 HTLs indicated that the T_6 HTLs maintain higher N_2 assimilation efficiency under drought stress, which is beneficial to the growth and development of vegetable soybeans.

CONCLUSION

To summarize, overexpression of *StP5CS* promotes the accumulation of proline in T_6 HTLs, resulting in both maintaining the optimal nodule function under drought stress and the enhancement of the expression of five key genes involved in nodulation and SNF: *GmENOD40-1*, *GmENOD40-2*, *GmLba*, *GmGS1β1* and *GmGS1β2*. These factors work together to promote the nodulation and N_2-fixation capacity of the T_6 HTLs in vegetable soybean under drought conditions.

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REFERENCES

Amela, B., Sihem, T., Faiza, O. and Mohamed, O.S. (2018). Characterization of *rhizobia* from root nodule and rhizosphere of *Vicia faba* in Algeria. *Legume Res.* **41:** 624-628.

Bates, L.S., Waldren, R.P. and Teare, I.D. (1973). Rapid determination of free proline for water-stress studies. *Plant Soil*. **39:** 205-207.

Biabani, A. and Sajadi, S. J. (2018) Effect of moisture content on biophysical characteristics of chickpea cultivars. *Legume Res*. **41**: 432-435.

Broughton, W.J. and Dilworth, M.J. (1971). Control of leghaemoglobin synthesis in snake beans. *Biochem J*. **125:** 1075-1080.

Cabeza, R.A., Liese, R., Lingner, A., Von, S.I., Neumann, J., Salinas-Riester, G., Pommerenke, C. and Schulze, J. (2014). RNA-seq transcriptome profiling reveals that *Medicago truncatula* nodules acclimate N_2 fixation before emerging P deficiency reaches the nodules. *J Exp Bot*. **65:** 6035-6048.

Cai, H., Xie, W. and Lian, X. (2013). Comparative analysis of differentially expressed genes in rice under nitrogen and phosphorus starvation stress conditions. *Plant Mol Biol Rep*. **31:** 160-173.

diCenzo, G.C., Zamani, M., Cowie, A. and Finan, T.M. (2015). Proline auxotrophy in *Sinorhizobium Meliloti* results in a plant-specific symbiotic phenotype. *Microbiology*. **161:** 2341-2351.

Duran, V.A. and Todd, C.D. (2012). Four allantoinase genes are expressed in nitrogen-fixing soybean. *Plant Physiol Bioch*. **54:** 149-155.

Gian, L., Yumi, C., Yi, J.Y., Jongwook, C., Myungchul, L., Ma, K.H., Sokyoung, L., Jinwoong, C. and Jungro, L. (2014). Genetic diversity and population structure of Korean soybean collection using 75 microsatellite markers. *Korean J Cropence Sci*. **59:** 492-497.

Herridge, D.F. (1982). Relative abundance of ureides and nitrate in plant tissues of soybean as a quantitative assay of nitrogen fixation. *Plant Physiol*. **70:** 1-6.

Kaur, G. and Asthir, B. (2015). Proline: a key player in plant abiotic stress tolerance. *Biol Plantarum*. **59:** 609-619.

Kim, G.B. and Nam, Y.W. (2013). A novel $\Delta^{(1)}$-pyrroline-5-carboxylate synthetase gene of *Medicago* truncatula plays a predominant role in stress-induced proline accumulation during symbiotic nitrogen fixation. *J Plant Physiol*. **170:** 291-302.

Kouchi, H. and Hata, S. (1993). Isolation and characterization of novel nodulin cDNAs representing genes expressed at early stages of soybean nodule development. *Mol Gen Genet*. **238:** 106-119.

Kusvuran, S. and Dasgan, H.Y. (2017) Effects of drought stress on physiological and biochemicalchanges in *Phaseolus vulgaris* L. *Legume Res*. **40**: 55-62.

Le, D.T., Aldrich, D.L., Valliyodan, B., Watanabe, Y., Ha, C.V., Nishiyama, R., Guttikonda, S.K., *et al*. (2012). Evaluation of candidate reference genes for normalization of quantitative RT-PCR in soybean tissues under various abiotic stress conditions. *Plos One*. **7:** e46487.

Libault, M., Thibivilliers, S., Bilgin, D.D., Radwan, O., Benitez, M., Clough, S.J. and Stacey, G. (2008). Identification of four soybean reference genes for gene expression normalization. *Plant Genome*. **1:** 44-54.

Marino, D., Frendo, P., Ladrera, R., Zabaiza, A., Puppo, A., Arrese-lgor, C. and González, E.M. (2007). Nitrogen fixation control under drought stress. Localized or systemic? *Plant Physiol*. **143:** 1968-1974.

Masalkar, P., Wallace, I.S., Jin, H.H. and Roberts, D.M. (2010). Interaction of cytosolic glutamine synthetase of soybean root nodules with the C-terminal domain of the symbiosome membrane nodulin 26 aquaglyceroporin. *Plant Physiol*. **143:** 1968-1974.

Morais, M.C., Panuccio, M.R., Muscolo, A. and Freitas, H. (2012). Salt tolerance traits increase the invasive success of *Acacia longifolia* in Portuguese coastal dunes. *Plant Physiol Bioch*. **55:** 60-65.

Pathan, S.M., Lee, J., Sleper, D.A., Fritschi, F.B., Sharp, R.E., Carter Jr, T.E., Nelson, R.L., King, C.A., Schapaugh, W.T., Ellersieck, M.R., Nguyen, H.T. and Shannon, J.G. (2014). Two soybean plant introductions display slow leaf wilting and reduced yield loss under drought. *J Agron Crop Sci*. **200:** 231-236.

Riley, I.T. and Dilworth, M.J. (2010). Cobalt requirement for nodule development and function in *Lupinus-angustifolius* L. *New Phytol*. **100:** 347-359.

Schmittgen, T.D. and Livak, K.J. (2008). Analyzing real-time PCR data by the comparative C_T method. *Nat Protoc*. **3:** 1101-1108.

Serraj, R., Sinclair, T.R. and Purcell, L.C. (1999). Symbiotic N_2 fixation response to drought. *J Exp Bot*. **50:** 143-155.

She, Q., Sandal, N.N., Stougaard, J. and Marcker, K.A. (1993). Comparative sequence analysis of cis elements present in *Glycine max* L. leghemoglobin *lba* and *lbc3* genes. *Plant Mol Biol*. **22:** 931-935.

Shimoda, Y., Shimoda-Sasakura, F., Kucho, K., Kanamori, N., Nagata, M., Suzuki, A., Abe, M., Higashi, S. and Uchiumi, T. (2010). Overexpression of class 1 plant hemoglobin genes enhances symbiotic nitrogen fixation activity between *Mesorhizobium loti* and *Lotus japonicus*. *Plant J*. **57:** 254-263.

Shinde, S.S., Kachare, D.P., Satbhai, R.D. and Naik, R. M. (2017) Water stress induced proline accumulation and antioxidative enzymes in groundnut (*Arachis hypogaea* L.). *Legume Res*. **41**: 67-72.

Van, d.D.C. (1970). Differential analyses of glyoxylate derivatives. *Anal Biochem*. **33:** 143-157.

Zhang, G.C., Zhu, W.L., Gai, J.Y., Zhu, Y.L. and Yang, L.F. (2015). Enhanced salt tolerance of transgenic vegetable soybeans resulting from overexpression of a novel Δ^1-pyrroline-5-carboxylate synthetase gene from *Solanum torvum* Swartz. *Hortic Enviro Biote*. **56:** 94-104.

Zhu, W.L., Yang, L.F., Yang, S.P., Gai, J.Y. and Zhu, Y.L. (2016). Overexpression of rice phosphate transporter gene *OsPT2* enhances nitrogen fixation and ammonium assimilation in transgenic soybean under phosphorus deficiency. *J Plant Biol*. **59:** 172-181.