

**Research Report**

**AtGS1.2 Gene May Enhance Salt Stress Tolerance in Seed Germination and Overexpression of Arabidopsis thaliana**

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**Abstract**

Glutamine synthetase (GS) is the key enzyme of ammonium assimilation in plants. In this research, we focus on GS1.2 (AtGS1.2) in Arabidopsis thaliana, and study the function of ammonium assimilation in relieving salt stress. Semi-quantitative PCR was employed to investigate the expression of AtGS1.2 in different time. The expression level of AtGS1.2 under the salt-treatment during seed germination stage was analyzed by northern blot method. The result indicated that the expression of AtGS1.2 was induced by 100 mM NaCl treatment during seed germination, comparing with control. We also obtained the over-expression lines of AtGS1.2 to analyze the functions at salt stress. According to the results, we can infer that AtGS1.2 in Arabidopsis plays an important role in the defense of salt stress during seed germination and seedling stages.

**Keywords**

Arabidopsis; Glutamine synthetase; NaCl stress; Seed germination

**Introduction**

Glutamine synthetase (Glutamine synthetase, GS) is a key enzyme of ammonium assimilation in plants (Hirel, McNally et al. 1984), which is widely distributed in microorganisms, higher plants and animals. GS can catalyze the assimilation of ammonium ions to glutamine. There are two types of glutamine synthetase in higher plants: GS1 which is located in the cytosol (Kamachi, Yamaya et al. 1992) and GS2 which is located in the chloroplast. In higher plants, GS1 codes cytoplasmic glutamine synthetase, and there are five subtypes (Melo, Lima et al. 2003), mainly involved in assimilation of ammonium in the root, reactivation during leaf senescence and the transportation of synthetic glutamine in vascular system; and in most plants (Lam, Coschigano et al. 1996); GS2 is a chloroplast glutamine synthetase, which is mainly expressed in photosynthetic tissues, assimilate the nitrogen of light breathing. In ontogeny, the difference of GS1 gene expression and the response to external stress showed that GS1 isoforms played specific roles in the plant life cycle (Peat and Tobin. 1996).

Nitrogen is an essential mineral nutrients to sustain crop production increasing (Derkx, Orford et al. 2012), which directly affects the growth and yield of plants (Liu, Wang et al. 2009). Nitrogen is very important and regulator of carbon and nitrogen metabolism in root growth, leaf grown (Hockin, Mock et al. 2012). Plants mostly absorb soluble nitrogen, such as ammonium (NH₄⁺), nitrate (NO₃⁻), Nitrite (NO₂⁻), amino acids and urea (Becker, Carryol et al. 2000). In aerobic and water well soil, NO₃⁻ is the main form of inorganic N (Ishiyama, Inoue et al. 2004, Masalkar and Roberts 2015), so the roots of plants preferentially absorb NO₃⁻. In contrast, the nitrification process is blocked under certain circumstances, which is due to lack of oxygen and/or acidic environment. In this situation, NH₄⁺ takes up a dominant position, and therefore plants are more likely to absorb NH₄⁺ (Yu and Zhang 2012).

The toxic effects caused by salt stress is one of the important environmental factors that influence the seed germination (Parida and Das 2005) and seedling growth, even more it can affect the entire life process plant. Salinity stress can reduce the photosynthetic...
rate of plant leaves, effectively inhibit the absorption of moisture in roots (Jiang, Yang et al. 2007), which causes an acceleration in energy consumption making that premature of plants. Eventually plant will died because of nutrient starvation. Whether Seeds can germinate successfully in Saline Soil is the prerequisite for successful planting, and different seeds to salinity stress tolerance (Zhou, Shi et al. 2012).

The main influences of salt stress on seed germination are synergistic effect, negative effect, and complete inhibition effect (Zhu 2001). Hu researched Bromus inermis cv. Xilinguole and Bromus stamineus under the treatment with single salt as Na₂SO₄, NaCl, MgCl₂. It showed that the initial germination time and the time required for germination of two kinds of seeds were prolonged with the improvement of salt concentration. At the same time, the rate of germination came down, while the single salt damage rate increased. Yuan et al. discovered that a low concentration of mixed salt (Na₂SO₄) solution could promote the germination of Panicum virgatum seed, and the promter action reduced by the increasing of salt concentration (Yuan, Gu et al. 2013). The seed germination will be inhibited by a high concentration of salt. Chen et al. discovered that the level of ammonium caused by NaCl will be reduced by the increasing of GS activity in tomato leaf (Chen, Shen et al. 2004). Mohamed Debouba proved that under salt stress (100 mM NaCl), ammonium ion content significantly decreased in the Arabidopsis leaves, and the ammonium ion content in roots is almost as twice as the control group (Debouba, Dguimi et al. 2013).

Many studies have been reported about the response of plant under salt stress during seeding period in many species of wild-type plants including Arabidopsis, but there are less reporting directly about the germination period and overexpression of plants response to salt stress. Therefore, the study focus on seed germination stage and chose overexpression plants to directly research the function of GS under salt stress. In this study, we researched the response of AtGS1.2 under salt stress during seed germination, and also analyzed the expression level of AtGS1.2 under salt stress in mRNA level. At the same time, we deeply analyzed the tolerance of AtGS1.2 transgenic lines under salt stress. We explored the role of AtGS1.2 in salt stress response, in order to have a depth understanding of AtGS1.2.

1 Materials and Methods
1.1 Plant materials and stress treatments
Arabidopsis thaliana wild type (WT) and Arabidopsis thaliana GS1.2 (AtGS1.2) transgenic T₃ generation seeds were supported by Alkali Soil Natural Environmental Science Center (ASNEC) in Northeast Forestry University. 3-week-old WT seedlings were immediately frozen in liquid nitrogen for RNA isolation for gene cloning. WT seeds in 1/2MS solid medium and transgenic T₃ generation seeds in 1/2MS solid medium with 40 mg/L kanamycin for screening two weeks, collected the whole stalk immediately frozen in liquid nitrogen for RNA isolation and Northern blot analyses and for DNA isolation and Southern blot analyses.

WT seeds were treated at 0, 100 mM NaCl solid sodium, then immediately collected frozen in liquid nitrogen at 0, 6, 12, 24, and 48 h. The sample was used for RNA isolation and reversing transcription to cDNA for semi-quantitative PCR analyses.

WT and transgenic T₃ generation seedlings were grown in 1/2MS solid medium containing 0 mM NaCl, 100 mM NaCl, 125 mM NaCl, 150 mM NaCl solid medium for 14 d in a culture room at 25 °C with a light cycle of 8 h light and 16 h dark. Each plate was separated into four places, and each place planted 21 seeds. Three independent biological replicates were performed. The growth and development of transgenic and WT seedlings were observed, then the length of roots was measured. These data were presented as the mean ± standard error from three independent experiments. Statistical analysis performed using software SPSS 13.0. LSD t-test was used to compare the mean values of different groups (P<0.05).

1.2 Gene Cloning and Sequence analysis
The MAT sequences of different species were searched from the GenBank in the National Center for Biotechnology Information (NCBI). The sequences for AtGS1.2 (GenBank No. NM_105291) were amplified by PCR using gene specific primers from Arabidopsis cDNA. For AtGS1.2 gene, the specific primers were sense primer 5′-GCAACCATGAG-TCTTCTTGC-3′ and antisense primer 5′-CTTCTCAA-GGGTCCAGAGGA-3′. Total WT RNA was
extracted using TRizol reagent and reverse transcription to cDNA. PCR was performed in a 20 μL reaction volume containing 11.6 μL ddH₂O, 2 μL 10× PCR buffer (plus Mg²⁺), 3.2 μL dNTP (2.5 mM), 1 μL of each primer (10 μM), 1 μL cDNA as template, and 0.2 μL Taq (TaKaRa, Japan). The PCR amplification program was as follows: 3 min at 94 °C, 35 cycles for 30 s at 94 °C, 30 s at 58 °C, and 1 min at 72 °C, as well as a final extension for 10 min at 72 °C. Sequences of resulting amplification products were determined.

Nucleic acid and amino acid sequence of AtGS1.2 (GenBank No. NM_105291) were analyzed according to NCBI (http://www.ncbi.nlm.nih.gov) and ExPASy databases. Complete amino acid sequences of different plant sources were obtained from NCBI database, the Homology alignment of multiple sequences were done with BioEdit software and phylogenetic tree was constructed with MEGA3.1 software. Dnastar Lasergene V7.1 (http://dnastar.com/tproducts-lasergene.aspx) analyzed the open reading frame (ORF) and the deduced amino acid. The alignments of multiple sequences were used by Clustal X 1.83.

1.3 Semi-quantitative PCR analyses
The trace DNA in Arabidopsis total RNA was digested with DNAase, and the first-strand cDNA was synthesized according to the Prime Script RT reagent Kit (TaKaRa Inc., China) instruction. Real-time fluorescent quantitative PCR was done in the ABI 7500 (Applied Biosystems Inc., USA) PCR instrument using SYBR Green Master mix (TaKaRa Inc., China). The special gene primers for semi-quantitative PCR were sense primer 5'-CACAAGATTCAATCTTCTTGTTC-3' and antisense primer 5'-GTTCACATCC-TTCTGCAACAAAG-3', and the actin primers were sense primer 5'-AACCTGTTGGATCCTGCAACTG-3' and antisense primer 5'-TGATCCCTTCTGCAG GTTCACC-3'. The PCR reaction system was 20 μL, which containing 10 μL of 20×Sybr Green mix, 0.5 μL of each primer (10 μmol·L⁻¹), 1 μL of cDNA, 8 μL of ddH₂O. Amplification conditions were as follow: 95 °C pre-denaturaled for 5 min and then 95 °C for 30 s and 60 °C for 30 s and 72 °C for 90 s with 30 cycles.

1.4 Transformation of Arabidopsis
The coding regions of AtGS1.2 were sub cloned into the plant transformation binary vector pBI121 (Clontech) digested with Xba I and Sac I to construct the plasmid. The constructs were introduced into Agrobacterium tumefaciens strain EHA105. Arabidopsis was transformed by the floral dip method (Clough and Bent 1998). The transgenic plants were confirmed by northern blot and southern blot.

1.5 Identification of AtGS1.2-overexpressing plants
Northern blot and Southern blot methods were used to identify the transgenic plants.

5 μg RNA was separated on a 1 % agarose gel containing 6 % formaldehyde, and transferred to Hybond-N membranes, using a digoxigenin-labeled AtGS1.2 cDNA probe (Roche), and then detected with the CDP-Star by Luminescent Image Analyzer LAS-4000 (Fujifilm, Japan). The specific primers used for Northern blot were as follow: GS1.2-northern-F, 5'-TGTTAACCTTGAC ATCTCA- GACAACAGT-3' and GS1.2-northern-R, 5'-ACTTC AGCAATAACATCAGGGTACG A-3' and the full length of the probe is 375 bp.

The total DNA was extracted using CTAB reagent. 10 μg DNA was digested from restriction enzyme (Sac I) at 37 °C overnight, and separated on 1 % agarose gel, and transferred to Hybond-N membranes, using a digoxigenin-labeled AtGS1.2 cDNA probe (Roche), and then detected with the CDP-Star by LAS-4000 (Fujifilm, Japan). The specific primers used for Southern blot were as same as the Northern blot ones.

2 Results and Discussion
2.1 Sequence analysis of GS gene
The full-length cDNA sequence of AtGS1.2 was 1,107 bp, encoding a deduced protein of 356 amino acids. The protein had a predicted molecular weight of 39.2 kDa and its isoelectric point was 5.078. The amino acid sequence of AtGSs shared 50–82 % high identities with the previously published GS sequences of other species. A Phylogenetic tree was constructed to analyze the genetic relationship between AtGSs and other GSs (Figure 1). The result showed that in the system evolution of genetic relationship, GS gene was divided into six categories, and close genetic relationship between species of the GS gene types of similarity was higher. Such as GS1.2 genes in this study, cruciferous Brassica napus and Camelina sativa GS1.2 genes were very highly similar to arabiadopsis thaliana GS1.2.
Polygenetic relationship of Glutamine synthetase with various GSs from other species. Bootstrap values are calculated 1,000 times; values below 50% are not included. The polygenetic tree was constructed by neighbor-joining method with the amino acid sequence using MEGA3.1. The following sequences are included: Arabidopsis thaliana: AtGS1.1 (NM_123119.3), AtGS1.2 (NM_105291), AtGS1.3 (NM_112663.2), AtGS1.4 (NM_121663), AtGS1.5 (NM_103743.2); Camelina sativa: CsGS1.1 (NC_025696.1), CsGS1.2 (NC_025700.1), CsGS1.3 (NC_025685.1), CsGS1.4 (NC_025692.1); Brassica napus: BnGS1.1 (AGC24238.1), BnGS1.2 (CDY38104.1); Oryza sativa: OsGS1.1 (NP_001054133.1), OsGS1.2 (NP_001049424.1), OsGS1.3 (NP_001048045.1), OsGS2 (NP_001051067.1)

Gene expression characteristics showed that the expression was not detected in dry seeds, but in 36 h seedlings and 3-week-old plants was detected by northern blot. There was the highest expression in 3-week-old plants (Figure 2a), so we chose 3 weeks as the best period for cloning. To investigate that AtGS1.2 gene was related to salt stress at seeds germination, semi-quantitative PCR was used to analyze the expression of AtGS1.2 at seed germination. The result showed that AtGS1.2 rose in direct proportion at the germination time both at salt stress and no stress (Figure 2b). Compared with the control (0 h), the expression rose at 24 h treatments, and the highest expression occurred at 48 h at 100 mM NaCl stress. The expression was higher at 100 mM salt stress than no stress at the same germination time. We considered that AtGS1.2 may be salt stress tolerance relative gene.

2.2 Over-expression of GS1.2 in Arabidopsis Enhanced Tolerance to NaCl Stress

We generated Arabidopsis transgenic plants which over-expressed AtGS1.2 gene. Four independent T3 generation lines transgenic lines of each gene were identified by Northern blot analysis (Figure 3a). Northern analysis showed that each of these lines expressed AtGS1.2 in plants. The transgenic plants showed higher expression than WT plants. Three independent T3 generation lines transgenic lines of each gene were identified by Southern blot analysis (Figure 3b). Southern analysis showed that the accumulation various copies of the transgenic plants. To further verify the tolerance of AtGS1.2 transgenic at salt stress treatment, the WT and AtGS1.2 transgenic plants of Arabidopsis seeds germination, root length and phenotypic were analyzed on 1/2 MS culture medium supplement different concentration of NaCl. On control medium, germination was not
noticeably different between WT and three T₃ generation transgenic lines (4, 10, and 11) over-expressing AtGS1.2 (Figure 3c). However, in the presence of 125 mM NaCl, germination was significantly inhibited in the WT plants, but only moderately inhibited in the transgenic lines (Figure 3c,d). The growth of WT was significantly weaker than three transgenic plants at 125 mM NaCl treatment. Fourteen days after seed germination, measurements confirmed that root lengths of the AtGS1.2 transgenic lines were higher than those of WT plants under stress conditions (Figure 3e). The germination rate on the same day of the transgenic plants was higher than WT plants at 125 mM NaCl treatment, it showed that AtGS1.2 over-expressed can decrease germination time (Figure 3e). Apparently as a result of the over-expression of AtGS1.2, transgenic plants had enhanced the tolerance of salt stress.

Figure 3 AtGS1.2 enhances the tolerance of Arabidopsis plants to salt stress. (A) Expression of AtGS1.2 genes in wild-type (WT) and T₃ generation transgenic lines. 5 μg total RNA extracted from 2-week-old seedlings were analyzed by Northern blot. (B) Expression of AtGS1.2 genes in wild-type (WT) and T₃ generation transgenic lines. 10 μg total DNA extracted from 2-week-old seedlings were analyzed by Southern blot. M: λ DNA. (C) Relative salt tolerance of wild-type (WT) and the T₃ generation transgenic plants over-expressing AtGS1.2 gene under salt stresses. WT and transgenic seeds germinated on 1/2MS solid medium agarose plates supplemented with different concentrations of 0, 100, 125, 150 mM NaCl, for 14 d. (D) Effects of salts on root lengths of wild-type (WT) and the T₃ generation transgenic plants over-expressing the AtGS1.2. 14-day-old WT and transgenic seedlings grown on 1/2MS were with 0, 100, 125, 150 mM NaCl. (E) The root lengths of WT, AtGS1.2-transgenic plants after the treatment as indicated. The germination value was observed the sample which was treated with 125 mM NaCl for 6 days. Each experiment was repeated three times. Each value represents the mean ± SE of 21 plants.

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