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Functional Comparative Study of *Arabidopsis thaliana* Glutamine Synthetase Gene *GLN1;1* and *GLN1;5* in Response to Salt Stress during Germination

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Abstract In this study, molecular biology methods were use to analyze germinating overexpression transgenic plants and mutants of *Arabidopsis thaliana* glutamine synthetase genes *GLN1;1* and *GLN1;5* in response to salt stress, and activity change of Arabidopsis glutamine synthetase. The results showed *AtGLN1;1* and *AtGLN1;5* performed different expression profiles in different tissues, growth stages and under salt stress. The overexpression of *AtGLN1;5* gene could contribute to strengthen germination and growth progress of Arabidopsis under saline environment.

Keywords *Arabidopsis thaliana*; Glutamine synthetase; Salt stress; Seed germination

Introduction

It is a key period for plants from germination to seedling stage. In this stage, plants are vulnerable and most sensitive to environment (Miller, 1987), and it is a crucial stage of adapting the environment and keeping reproduction (Omami et al., 1999).

Salt stress is one of the important environment factors that affects germination and seedling growth of plants (Baskin and Baskin, 1998), the response to salt stress of plants during germination and seedling stage reflects their mechanism adapting the stress environments. The impacts to seedlings from saline are generally through osmotic effect and ionic effect (Zhang et al., 2014). The osmotic effect triggers the reduction of osmotic potential that prevent seeds and seedlings absorbing the water then interfere germination and growth of the plant (Levitt, 1980), and the ionic effect directly poisons the plant through Na^+, K^+ and other saline ions (Ungar, 1978).

NH_4^+ is a key ion in living cells, and also an important intermediate product of other compounds in the cell.

NH_4^+ and NO_3^- are also main nitrogen resources of plants and microorganisms, however, when the NH_4^+ is over accumulated, it will be toxic to the cell (Britto and Kronzucker, 2002). The accumulation of NH_4^+ in the plant cell could originated form the high NH_4^+ concentration of environment and degradation of proteins in senescent tissues, furthermore, protein degradation caused by reactive oxygen species (ROS) production in salt stress would also accumulate excess NH_4^+ in the plant (Britto and Kronzucker, 2002).

Glutamine synthetases (GSs), which participate in nitrogen metabolic process, are widely exiting in all the organisms. The GSs could assimilate the redundant NH_4^+ to glutamic acids and produce glutamines, thus we speculate GS would contribute to remit the NH_4^+ toxicity induced by salt stress.

In plants, glutamine synthetases are classified in two types, the cytoplasmic type (GS1) and the plastid type (GS2). In *Arabidopsis thaliana*, there are 6 members in the GS family, 5 GS1s (*GLN1;1*, *GLN1;2*, *GLN1;3*, *GLN1;4* and *GLN1;5*) and 1 GS2 (*GLN2*) (Ishiyama

et al., 2004).

In previous studies, expression profiles and localization of GSs in many plant species were examined (Brugière et al., 1999, 2000; Kichey et al., 2005, Lothier and Gaufichon, 2009; Nord-Larsen, 2009, Peterman and Goodman, 1991, Teixeira and Fidalgo, 2009, Vance et al., 1994), and there are some studies reported the stress-resistant and nitrogen-utilization function of GS in some species (Cai et al., 2009, Fuentes et al., 2001, Habash et al., 2001, Martin et al., 2006, Jing et al., 2004, Migge et al., 2000, Oléa et al., 2004, Oliveira et al., 2002, Pageau et al., 2006, Pérez-García et al., 1998, Tavernier et al., 2007). However, specific analysis of *AtGLNs* is lacking of exploring expression patterns and their function in resisting salt stress during germination and seedling stage.

In this study, the germinating *Arabidopsis thaliana* were used to investigate expression profiles and functions under NaCl stress of *AtGLN1;1* and *AtGLN1;5* genes. We discovered that *AtGLN1;1* and *AtGLN1;5* performed different expression profiles in different tissues, growth stages and under salt stress and overexpression of *AtGLN1;5* gene could contribute to strengthen germination and growth progress of Arabidopsis under saline environment.

1 Results and Discussion

1.1 Expression profiles of AtGLN1;1 gene

The wild-type Arabidopsis plants (Columbia-0, Col-0) after 60 d growing were selected for expression analysis. Roots, stems, rosette leaves, flowers, fruits and dry seeds were collected separately, 0.1 g of each tissue or organ were used to extracting total RNA respectively. Expression of *AtGLN1;1* in these tissues and organs were determined by quantitative real-time (qRT-PCR) and the result is as shown in the Figure 1.

As shown in Figure 1, expression of *AtGLN1;1* in all the 6 tissues and organs could be examined, its expression pattern in dry seeds was obviously the highest, and the expression in rosette leaves and fruits were also higher than in the other three. It could be observed that *AtGLN1;1* may play a role from the beginning stages of germination and function mostly

in the dormancy seeds. On the basic of this possibility, we further tested its expression in the germinating seeds and seedling stage plants.

Dry seeds, seeds of 36 h after germination and 3-week-old seedlings were used in analyzing expression of *AtGLN1;1* by RT-PCR (Figure 2).
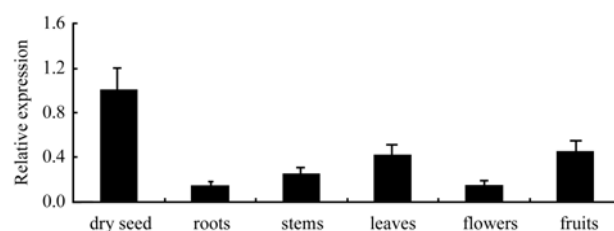

Figure 1 Real-time PCR analysis of *AtGLN1;1* gene expression in different tissues and organs of *Arabidopsis thaliana*
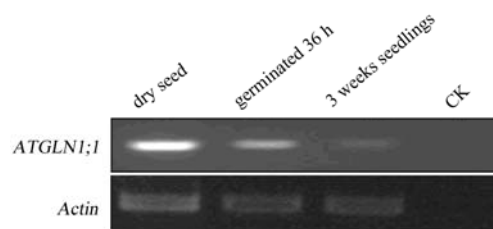

Figure 2 Expression analysis of *AtGLN1;1* in germination and seedling stage by RT-PCR detection

The expression in the dry seeds was still the highest in these 3 stages, and the expression down-regulated gradually following the growth progress, but still staying at a relatively high level during germination (Figure 2). For further ensure this results and examine more detailed, qRT-PCR analysis were conducted to analysis the expression of *AtGLN1;1* in these 3 periods (Figure 3), and the result was similar to that tested by RT-PCR.
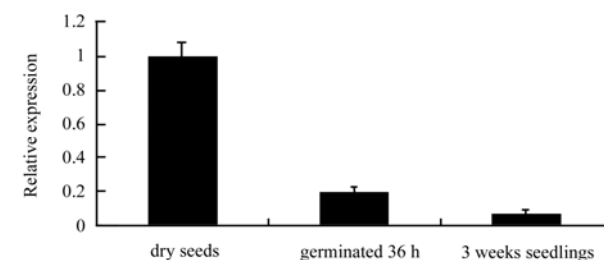

Figure 3 Expression analysis of *AtGLN1;1* in germination and seedling stage by qRT-PCR detection

Considering its highest expression existed in dry seed, we speculate that the high expression may occurred in the endosperm before germination, and reduced after germination with the consumption of endosperm. However, this hypothesis could not be confirmed in this study and more researches are required for it in the future.

1.2 Expression profiles of *AtGLN1;5* gene

For *AtGLN1;5*, we also firstly analyzed its tissue-specific expression (Figure 4). The expression in dry seeds of *AtGLN1;5* and *AtGLN1;1* were similar, which is much higher than in other tissues and organs, but the others were different. The expression of *AtGLN1;5* in the three nutritional organs (roots, stems, rosette leaves) were extremely lower than in the dry seed and the other two reproduction organs (flowers and fruits) (Figure 4).
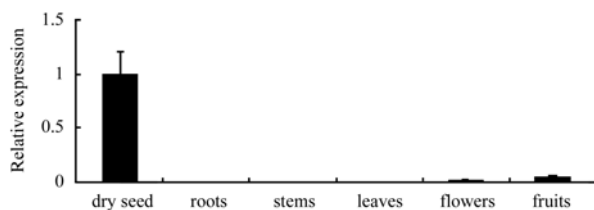

Figure 4 Real-time PCR analysis of *AtGLN1;5* gene expression in different tissues and organs of *Arabidopsis thaliana*
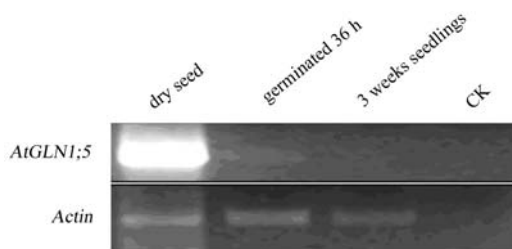

Figure 5 Expression analysis of *AtGLN1;5* in germination and seedling stage by RT-PCR detection
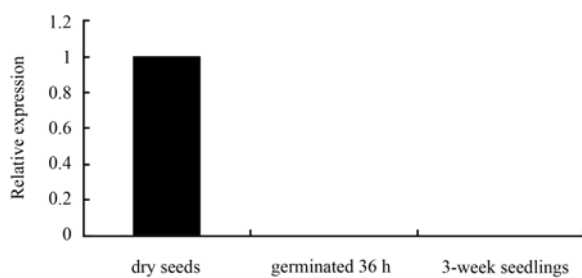

Figure 6 Expression analysis of *AtGLN1;5* in germination and seedling stage by qRT-PCR detection

We speculate that expression of *AtGLN1;5* accumulates in the process of seed formation and finally reach the peak when the mature seeds formed. And for researching expression profiles of *AtGLN1;5* during germination and seedling stage, we performed the same analysis with RT-PCR and qRT-PCR (Figure 5 and 6).

Expression of *AtGLN1;5* reduced sharply just after germination, that it was hard to detect after germinating 36 h and almost disappeared in seedling stage (Figure 5 and Figure 6). Therefore it is suggested that *AtGLN1;5* only functions in the formation of seeds and the initial of germination, and also play its most important role in dry seeds.

The reason of the rapid reduction of *AtGLN1;5* after germination maybe that it had already finished its most function before germinating, and according to the hybridization signal during TR stage, we think *AtGLN1;5* might work earlier than *AtGLN1;1*.

1.3 Analysis of *AtGLN1;1* and *AtGLN1;5* in response to salt stress

The transcripts of *AtGLN1;1* and *AtGLN1;5* were much abundant in seeds and they expressed at the very start of germination, we firstly analyzed the expression of *AtGLN1;1* and *AtGLN1;5* in the wild type seeds after 48-hour germination treated with different concentration of NaCl by RT-PCR and qRT-PCR (Figure 7 and Figure 8).

As shown in the figure, in the primary stage of germination, expression of *AtGLN1;1* was up-regulated first and then down-regulated with the NaCl concentration increased, and when treated with 200 mM NaCl, the expression of *AtGLN1;1* was almost the same as non-treated plants or even higher (Figure 7). But when the environmental NaCl concentration is over 150 mM, the wild type Arabidopsis could not survive for the normal growth, thus we think when germinating seeds were suffering the salt stress that was insufficient to kill the plant, the expression of *AtGLN1;1* would be down-regulated; and when NaCl concentration is high enough to prevent Arabidopsis growing, the expression will be up-regulated, however, the mechanism now is hard to explain.
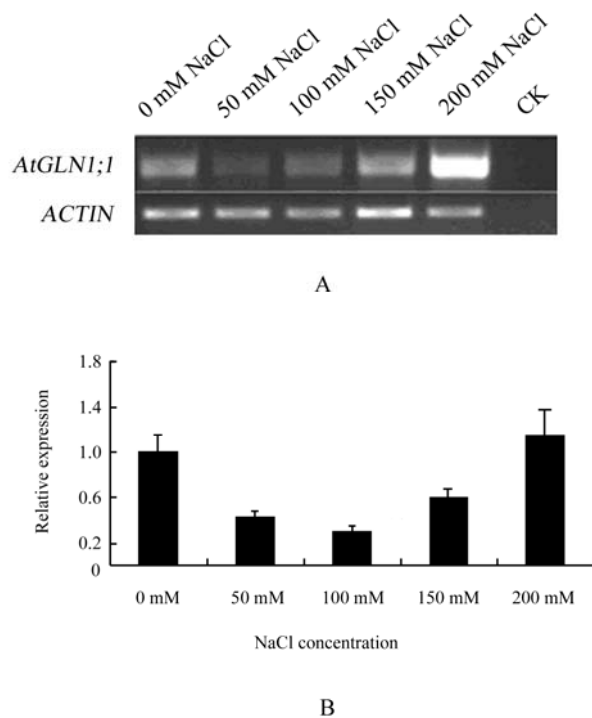
Figure 7 Expression level of *AtGLN1;1* in response to different NaCl concentration of seeds after 48 h germination
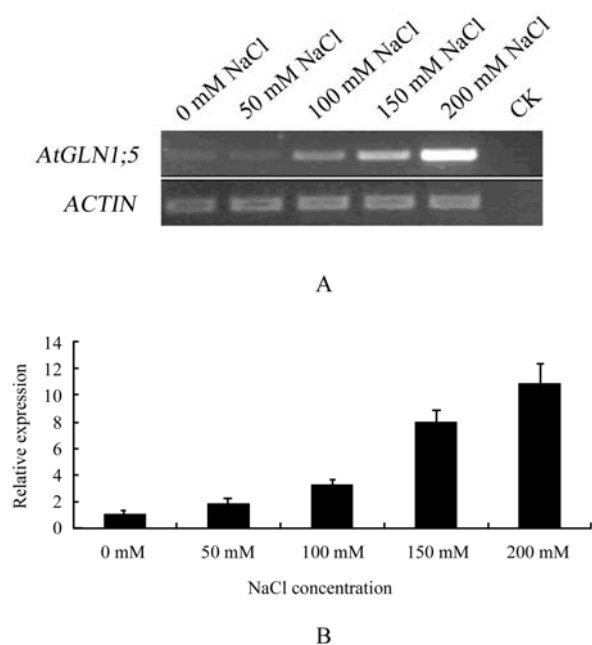
Note: A: RT-PCR analysis; B: qRT-PCR analysis

Figure 8 Expression level of *AtGLN1;5* in response to different NaCl concentration of seeds after 48 h germination

Note: A: RT-PCR analysis; B: qRT-PCR analysis

The response of *AtGLN1;5* under salt treatment is much different from that of *AtGLN1;1*. As shown in the Figure 8, in the germinating seeds, the expression level of *AtGLN1;5* increased sharply with the NaCl concentration raising up and there was a positive correlationship (Figure 8). The response of *AtGLN1;5* to NaCl could be separated to 2 parts: the first part is under the concentration that Arabidopsis could survive (0~100 mM), for that in this period *AtGLN1;5* is originally with a very high expression level, we think *AtGLN1;5* may contribute to the growth and development of Arabidopsis in this concentration range; the second part is with NaCl concentration that Arabidopsis would not finish its life cycle (150~200 mM), though the variation tend could be joining to the previous concentration, while separately considered, the expression changes is similar to that of *AtGLN1;1* (Figure 7), although we had not analyzed other members of *AtGLNs*, this similar variation tendency of *AtGLN1;1* and *AtGLN1;5* maybe indicating their functional similarity under this NaCl concentration range.

Considering the temporal specificity of gene expression, we analyzed the expression profile at different germination time points treated with 100 mM NaCl comparing to non-treated plants and dry seeds by using qRT-PCR detection (Figure 9 and Figure 10).

Figure 9 Comparative analysis of *AtGLN1;1* expression between NaCl-treated and non-treated Arabidopsis seeds after different germination time by qRT-PCR

The response of *AtGLN1;1* to NaCl treatment was not remarkable, except the very beginning of germination, there was no significant differences between in treated and in non-treated seed (Figure 9). *AtGLN1;5*

performed a quite distinguished status when comparing the treated and non-treated seeds. As time went on, the expression of *AtGLN1;5* in non-trated seeds fell down immediately, but in the seeds treated with NaCl it could stay at a stable level and was much higher than that in non-treated seeds (Figure 10). So it could be observed that the differences of *AtGLN1;1* and *AtGLN1;5* reflect not only in degrees but also in temporal specificity.
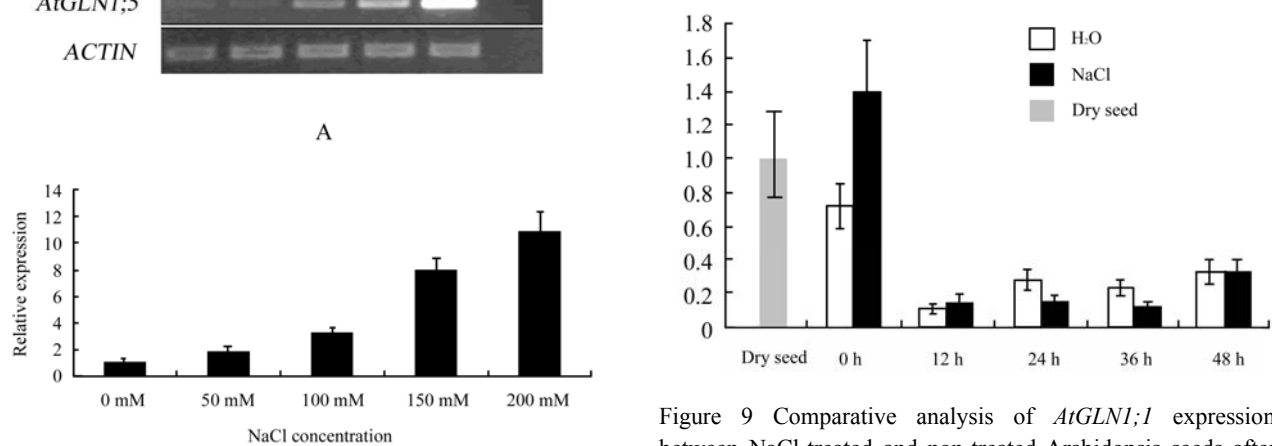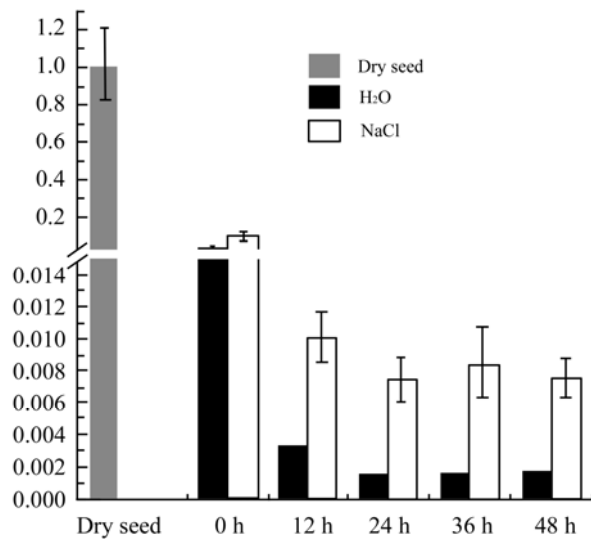

Figure 10 Comparative analysis of *AtGLN1;5* expression between NaCl-treated and non-treated Arabidopsis seeds after different germination time by qRT-PCR

For further analyzing the response of Arabidopsis glutamine synthetase to salt treatment and whether *AtGLN1;1* or *AtGLN1;5* plays a dominant role among all the GSs during germination, the activity of total glutamine synthetase of germinating Arabidopsis under NaCl treatment were determined (Figure 11).

As shown in Figure 11, no matter for concentration gradients or for time gradients, the variation trends of GS activity almost approaches that of AtGLN1;1 expression profiles under NaCl treatment (Figure 7 and 9). Therefore, we speculated although *AtGLN1;5* shows a more significant change after salt treatment in germinating seeds, but it maybe not the most dominant for its low expression level (Figure 5 and 6). For the similarity of GS activity and *AtGLN1;1* expression profile under NaCl treatment, *AtGLN1;1*

may possess the dominant place among AtGLNs when in response to salt stress, but it is different between the variation of GS activity under normal condition and *AtGLN1;1* expression (Figure 11B), so we speculate that there would be other GS members functioned together during germination but the total activity was inhibited under salt stress.
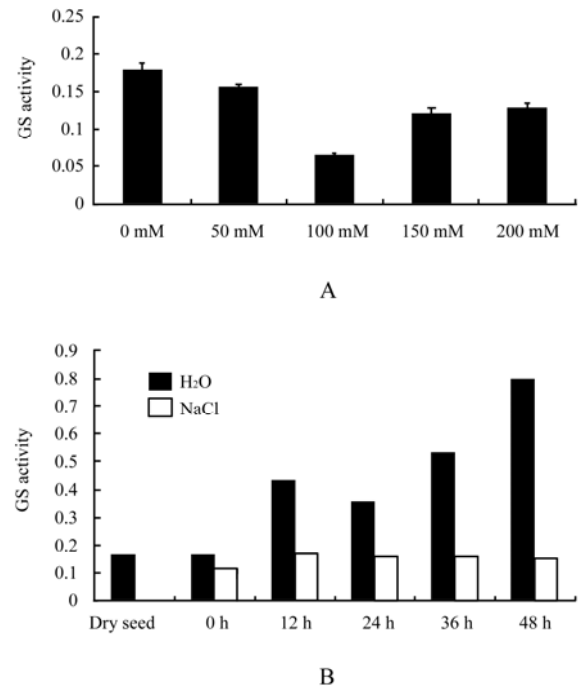

Figure 11 Activity of Arabidopsis glutamine synthetase under salt treatment during germination
Note: A: Activity of Arabidopsis glutamine synthetase in response to different concentration NaCl; B: Activity of Arabidopsis glutamine synthetase in response to 100 mM NaCl treatment after different germination time

In consequence, when germinating Arabidopsis is under salt stress, different GS genes might show different expression profile, buy may function together on protein level, meanwhile in the very beginning of germination, there maybe inreplaceable functions of *AtGLNs* in resisting salt stress. However, the detail mechanism is still need to investigate, studies of other members of *AtGLNs* in response to salt stress and their analysis in other growth stages are also need to be further researched.

2 Material and Methods
2.1 Materials
Columbia-0 ecotype *Arabidopsis thaliana* was

supported by Alkali Soil Natural Environmental Science Center (ASNESC); Northeast Forestry University (Harbin, China); DNase I, Reverse-transcript Kit, Taq DNA polymerases were purchased from TaKaRa Biotechnology; quantitative real-time PCR (qRT-PCR) reaction system was purchase from TansGen Biotech; all the primers synthesized in this paper were designed by Primer Premier 5.0 software, primer synthesis and DNA sequencing were done by BGI-tech.

2.2 NaCl treatment to Arabidopsis seeds

Seeds of Arabidopsis were sterilized then spread on the surface of agrose plate medium containing 0 mM, 50 mM, 100 mM, 150 mM or 200 mM NaCl at 4℃, after 2-day vernalization then transferred into a 22℃ constant temperature incubator for the further culture. Seed germinated for 0 h, 12 h, 24 h, 36 h and 48 h were rapidly frozen by liquid nitrogen respectively, immediately grind into powders under liquid nitrogen frozen or stored at -80℃ refrigerators.

2.3 RNA extraction and synthesis of fist-strand cDNA

Extraction of total RNA of Arabidopsis seeds was used the improved method referenced from Suzuki et al. (Suzuki et al., 2004). The detail content is: add some RNA extracting solution to the grinded samples, centrifuged at 4℃, 13 000 rpm for 5 min, get the supernatant; add equivoluminal solution of phenol, chloroform, isoamyl alcohol (v:v:v=25:24:1), mixed sufficiently, centrifuged at 4℃, 13 000 rpm for 10 min, get the supernatant; add equivoluminal chloroform, mixed sufficiently, incubating at RT for 3 min, get the supernatant; and 8 M LiCl solution of 1/3 volume of the supernatant, mixed sufficiently, precipitated at -20℃ overnight, centrifuged at 4℃, 13000 rpm for 15 min, remove the supernatant; use 75% ethyl alcohol to wash the precipitation, centrifuged at 4℃, 13 000 rpm for 5 min, remove the supernatant, drying in the air, add some RNase-free water to dissolve the RNA.

The fist-strand cDNA was synthesized according to protocol of Reverse-transcript Kit (TaKaRa).

2.4 Reverse transcription PCR analysis

Arabidopsis *ACTIN* gene was selected as internal reference, the sequences of primers used in the experiments are: 5'-GAAAATGGCTGATGGTGAAG-3' and 5'-CATAGATAGGAACAGTGTGG-3' for *ACTIN*; 5'-CTATAAGTACTACTCTTCATATCTC-3' and 5'-TAATGTTGATCCCAGCGTATAAG-3' for *AtGLN1;1*; 5'-AATCATCTTCTTTCTCTTTCGGAAC-3' and 5'-AAAGTCTAAAGCTTAGAGGATGGTG-3' for *AtGLN1;1*. 20 μL reaction system was conducted with GO Taq polymerase (TaKaRa).

Amplification of *ACTIN* and *AtGLN1;1* were performed with the procedure: 94℃ for 30 s, 55 ℃ for 30s, 72℃ for 1 min, 30 cycles in total. Amplification of *AtGLN1;5* was performed with the procedure: 94℃ for 30 s, 58 ℃ for 30s, 72℃ for 1 min, 30 cycles in total.

2.5 Quantitative real-time PCR analysis

Arabidopsis *TUBULINβ* gene was selected as internal reference, the sequences of primers used in the experiments are: 5'-ACACCAGACATAGTAGCAGAAATCAAG-3' and 5'-GAGCCTTACAACGCTACTCTGTCTGTC-3' for *TUBULINβ*; 5'-CATCAACCTTAACCTCTCAGACTCCA-3' and 5'-ACTTCAGCTGCAACATCAGGGTTGCT-3' for *AtGLN1;1*; 5'-TCTCCTAAACCTTGATCTATCAGACA-3' and 5'-CTCTTCAGCCTTCACATTGGGATGAT-3' for *AtGLN1;5*.

qRT-PCR reactions were performed on a MxPro-Mx3000P system with each tube of 20 μL reaction system containing TransStart Green qPCR SuperMix (TransGen Biotech) with the procedure: 95℃ for 30 s,60 ℃ for 30s, 72℃ for 30s, 40 cycles in total.

2.6 Activity analysis of GS

The method of analyzing total glutamine synthetase activity is the same as Yu and Zhang (Yu and Zhang, 2012).

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