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# IDENTIFICATION OF *GS1a, GS1b* AND *GS1c* GENES FROM *EICHHORNIA CRASSIPES* AND THEIR TRANSCRIPT ANALYSIS IN RESPONSE TO DIFFERENT NITROGEN SOURCES

**MINGHUI FU*, LIHUA JIANG AND GUOHUA YAN**

*Department of Bioengineering, Guangdong University of Technology, Guangzhou 510006, PR China*
** Corresponding author's email: mhfugd@126.com*

**Abstract**

*Eichhornia crassipes* was one of the fastest growing plants on earth. It can assimilate nitrogen very efficiently. Glutamine synthetase (GS) is a key enzyme in nitrogen metabolism. Thus research of GS in *Eichhornia crassipes* could help to explain the mechanism of the ability of *E. crassipes* to assimilate nitrogen. In this study, we cloned three cytosolic GS cDNAs, GS1a, GS1b and GS1c from the roots of *E. crassipes* using the RACE method, studied the characterizations with the yeast complementation experiment and analyzed the expressions of GS1a, GS1b and GS1c with qRT-PCT in response to different nitrogen sources in roots and leaves. They were approximately 1400-1500 nucleotide encoding proteins with 354-356 amino acids that functionally complemented the mutant yeast ΔGln for growth on ammonium as the sole source of nitrogen. Their relative expression level was markedly up-regulated under the status of nitrate as the only nitrogen source except that of GS1b in roots, which was only up-regulated less than one fold, and GS1a in leaves, which was up-regulated under condition of nitrogen deprivation. All these results suggested that *E. crassipes* has at least three different cytosolic GS1 genes which have typical conserved domains of GS gene and can functionally complement the mutant yeast that is deprived of the GS gene. All these *E. crassipes* GS1 genes have different expression patterns in response to different nitrogen sources.

**Key words:** *Eichhornia crassipes*, Glutamine synthetase, Clone, Expression.

## Introduction

Glutamine synthetase (GS) is the first enzyme of a plant to turn inorganic nitrogen into organic nitrogen. It associates with glutamate synthase (GOGAT) to assimilate ammonia into glutamine (Gln) and glutamate (Glu), which are the major nitrogen donors of higher plants in the biochemical synthesis of many nitrogenous compounds. Thus, GS is the key enzyme of nitrogen metabolism in plants (Miflin & Habash, 2002). It is closely related with the plant's utilization of nitrogen (Man *et al*., 2005; Sun *et al*., 2005), the production and the quality of the plant (Fuentes *et al*., 2001) and the plant's stress resistance (Rana *et al*., 2008; Lee *et al*., 2013).

There are many GS isozymes in higher plants. According to their subcellular localization, they can be divided into two types. One is cytosolic GS (GS1), and the other is plastidic GS (GS2). GS1 is encoded by multigenes, where it has been found that three GS1 genes exist in rice (Tabuchi *et al*., 2005), five in maize (Sakakibara *et al*., 1992b; Martin *et al*., 2006), five in *Arabidopsis thaliana* (Ishiyama *et al*., 2004), two in potato (Teixeira *et al*., 2005) and three in *Populus tremula* (Castro-Rodríguez *et al*., 2011, 2015). GS2 is usually encoded by a single gene. Only in *Medicago sativa*, a second GS2 gene was identified (Seabra *et al*., 2010). The subunit molecular weight of GS2 was about 44-45 kDa, and that of GS1 was less than 38-40 kDa. In the N-terminal domain of the GS2 subunit there is a signal peptide encoded by 50 amino acids that can direct GS2 into the plastid, while there is a special conserved region that is encoded by 16 amino acids in its C-terminal domain. Both GS1 and GS2 play different roles in the plant. In different species there are different numbers of GS1 isoenzymes, which are found in different types of cells and tissues according to their differential physiological functions. GS1 is usually found in heterotrophic organs such as the roots, stems, seeds, nodules, fruits and flowers, where it assimilates ammonium taken up from the soil, released from stress response, senescence and seed germination (Castro-Rodríguez *et al*., 2011). Conversely, the chloroplastic GS2 was detected in photosynthetic tissues where it assimilated the ammonium released from nitrate/ nitrite reduction and photorespiration (Betti *et al*., 2014).

*Eichhornia crassipes* is an invasive aquatic plant, native to Amazon basin. It is one of the most productive weeds in the globe. Though its uncontrolled reproduction can lead to an ecological unbalance, an appropriate amount of *E. crassipes* can also be used to remediate eutrophic lakes and rivers (Wang *et al*., 2013), breeding waste-water (Chen *et al*., 2010) and industrial waste-water (Casabianca *et al*., 1995) due to its strong assimilation of nitrogen and phosphorus (Reddy *et al*., 1989, 1990), rapid reproduction and accumulation of heavy metals (Deng *et al*., 2004; Odjegba & Fasidi, 2007; Caldelas *et al*., 2009). However, the mechanism of its efficient uptake and utilization of nitrogen and phosphorus is unclear. The uptake of nitrogen is dependent on the ammonium transporters in the plasma membrane. In our previous research, a novel ammonium transporter gene had been cloned and its expression had been analyzed (Li *et al*., 2014). After uptake, there is a utilization process in which GS is the most important enzyme. In this current paper, we have cloned three complete GS genes from *E. crassipes*, characterized their function with a mutant yeast complement experiment and analyzed their expression in different organs using RT-PCR.

## Methods and Materials

**RNA extraction:** *E. crassipes* was collected from the Anjian Reservoir in the campus of Guangdong University of Technology in Guangzhou. *E. crassipes* was not endangered or protected species, so no specific permission was required for this research. Total RNA of from *E. crassipes* roots was extracted by RNAiso plus kit made by TAKARA, and agarose gel electrophoresis was used to detect the integrity of RNA.

**GS1a, GS1b and GS1c gene fragments amplified with degenerate primers:** A prime-script$^{TM}$ RT-PCR kit (TAKARA) was used to reverse transcribe the total RNA (Jiang *et al*., 2014). PCR assays were completed with root cDNA as the template and two pairs of primers (P1 and P2, P3 and P4) (Table 1) as degenerate primers respectively. These two pairs of primers were designed based on conserved sequences of GS1 from other plants. In this PCR procedure, the program was 5min at 94°C, followed by 30 cycles of 30s at 94°C, 30s at 53°C, 1min at 72°C, and the finally step was 10min at 72°C. The PCR product was cloned into a pMD19-T vector after it was purified by an agarose DNA purification kit (TAKARA), and then transformed and sequenced (Invitrogen).

**Rapid amplification of cDNA ends (RACE) and cloning**: The unknown 3' end and 5' end were cloned by RACE method, and then the full length of gene sequence was obtained. RNA was extracted, and then it was used to prepare 3' or 5' adaptor ligated cDNA with 3' or 5' full RACE kit (TAKARA). This cDNA was used as a template in the following nested PCR reactions. All specific primers (Table 1) in RACE were designed according to the above known sequences.

**5'RACE:** Using the 5'RACE out primer and the 5'RACE specific outer primer (P5, P10, P14 in Table 1), the first program was 94°C for 3min, and then 20 cycles of 94°C for 30s, 40°C for 30s with a 0.5°C increase at each cycle and 72°C for 1min, and the last was 72°C for 10min. Using the 5'RACE inner primer, and the 5'RACE specific inner primer (P6, P11, P15 in Table 1), the second program was 94°C for 3min, and then 30 cycles of 94°C for 30s, 40°C /44°C /48°C /52°C /56°C for 30s and 72°C for 1min, and the last step was 72°C for 10min.

**3'RACE:** The first amplification was completed at the same conditions as those described for the 5'RACE first reaction using the 3'RACE out primer and the 3'RACE specific outer primer (P7, P12, P16 in Table 1).The second and the third amplifications of GS1a were conducted at the same conditions as that described for the 5'RACE second reaction using the 3'RACE inner primer and the 3'RACE specific inner primer (P8, P9, P13, P17 in Table 1). The second reaction of GS1b and GS1c was a somewhat different from that of GS1a. The first step was 94°C for 3min, and then 16 cycles of 94°C for 30s, 59°C for 30s with a 0.5°C dampening temperature at each cycle and 72°C for 1min. After that, there were 19 cycles of 94°C for 30s, 51°C for 30s and 72°C for 1min. The final step was 72°C for 10min.

**Full-length cDNAs cloned:** The intermediate fragment, 3 'end and 5' end fragment were spliced into the full length of a gene. According to the spicing full-length sequence, the special primers (P18-P23, Table 1) were designed to amplify the full-length cDNA. The PCR program was at 94°C for 3min, and then 30 cycles of 94°C for 30s, 55°C for 30s, 72°C for 1min. The last step was 72°C for 10 min. The full-length cDNA was cloned and then sequenced. The program of GS1b and GS1c were the same as that of the second reaction described for 3'RACE of GS1b and GS1c.

**Sequence analysis:** The full-length nucleotide sequences were blasted against a non-redundant nucleotide database (Altschul, 1997) and translated by the translation tool from EXPASY (http://web.expasy.org/translate/). The program Protparam (Wilkins *et al*., 1999) was used to analyze the physico-chemical properties of GS1a, GS1b and GS1c. The conserved domains were analyzed by Prosite (Sigrist *et al*., 2002, 2013) and Smart (Schultz *et al*., 1998). TargetP (Emanuelsson *et al*., 2007) was used to identify their cellular localization. All these translated sequences were aligned using the program ClustalX-1.83 (Thompson *et al*., 1997).

**Yeast growth and function complementation:** The wild-typeBY4741 (Mata his3Δ1, leu2Δ0, met15Δ0, ura3Δ0), and the mutant strain Y25454 (Mata/a, his3Δ1/his3Δ1, leu2Δ0/leu2Δ0, lys2Δ0/LYS2, MET15/met15Δ0, ura3Δ0/ura3Δ0, and YPR035w:kanMX4/YPR035w) were purchased from EUROSCARF (Germany). A haploid strain of Δgln1 was achieved by sporulation of the strain Y25454 (Kassir & Simchen, 1991; Bernard *et al*., 2008). The Δgln1, Mata his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, and YPR035w: kan MX4 could grow on media that was appended with L-glutamine. The yeast sporulation process was carried out by the standard procedures (Kassir & Simchen, 1991). Competent Δgln1 cells were prepared according to specific kit assays and procedures (Fun Genome, Beijing). The full-length cDNAs of GS1a, GS1b and GS1c were respectively linked into the yeast vector pYES2. The recombinant vector was then transformed into competent yeast cells. Yeast transformants were selected by *ura* minus medium at first, and then transferred to yeast nitrogen base (YNB) medium (without amino acids, ammonium) appended with 0.1% glutamine or 1mM $(NH_4)_2SO_4$ as its only nitrogen source. These transformants were cultured at 30°C for 3 days.

**qRT-PCR analysis of GS1a, GS1b and GS1c expression:** The collected *E. crassipes* was precultured in a nutrient solution (i.e., 0.25 mM $Ca(NO_3)_2{\cdot}4H_2O$, 0.25 mM $KNO_3$, 0.1 mM $MgSO_4{\cdot}7H_2O$, and 0.05 mM $KH_2PO_4$) for 20 days at 25°C under conditions of 16 h of light (2000 Lx) and 8 h of darkness indoors. The solution was replaced every two days. After that, the pre-cultured *E. crassipes* was divided into three groups, cultured in distilled water, 1.8mM $NH_4Cl$ and 1.8mM $KNO_3$ respectively for 2h, 4h or 6h later. Fresh roots or fresh leaves were collected. RNA was extracted, and then reverse transcribed into cDNAs. Specific primers of GS1a, GS1b and GS1c and the primers of reference gene P24-31 (Table 1) were designed and synthesized for qPCR. There were 1μl cDNA, 1μl primer-F (10μm), 1ul primer-R (10um), 0.5μl dNTP (10mM), 2μl 10×buffer, 1μl $Mg^{2+}$, 0.2μl Taq polymerase, 0.2μl Sybgreen and 13.1μl $ddH_2O$ in each 20μl PCR reaction.

The PCR program was 95°C for 2min, and then 40 cycles of 94°C for 20s, 60°C for 20s, 72°C for 30s. GS gene expression level was evaluated by qRT-PCR using a 7500 qPCR device. All real-time PCR reactions were performed in triplicate. The results from different treatments were referenced to the *E. crassipes* actin gene (Accession number: KC505366) expression levels to avoid bias. We used the $2^{-\Delta\Delta Ct}$ method to calculate the relative expression levels of GS gene (Livak & Schmittgen, 2001).

**Table 1. Primers used in this study**

| Primer Name | Sequence(5'-3') |
|---|---|
| P1(forward degenerate primerfor GS1a, GS1b) | AAGCTGCCAAAGTGGaaytaygaygg |
| P2(reverse degenerate primer for GS1a, GS1b) | GGACCCACCTGGAACtcccaytgncc |
| P3(forward degenerate primer for GS1c) | CGATAAGATCATCGCTGAGtayathtggrt |
| P4(reverse degenerate primer for GS1c) | TAGCCTTCTTGATCACCtcraanccncc |
| P5 (5' outer specific primer for GS1a) | TCCTGCTCAATGCCATACCA |
| P6 (5' inner specific primer for GS1a) | TAGCAGTCGCACATCACAAG |
| P7 (3' outer specific primer for GS1a) | CTTGTGATGTGCGACTGCTAC |
| P8 (3' inner specific primer for GS1a) | TGATGTTGTTGCTGAAGTCCC |
| P9(3' inner specific primer for GS1a) | TATTACTGTTCTGCTGGTGC |
| P10 (5' outer specific primer for GS1b) | GGGCATCAACAATGTCACGG |
| P11 (5' inner specific primer for GS1b) | GCCACTTGACATCCTTCTGA |
| P12 (3' outer specific primer for GS1b) | TCCTGTGCTCCTCTTTGCCT |
| P13 (3' inner specific primer for GS1b) | CAGGAGGGGCAACAACATTC |
| P14 (5' outer specific primer for GS1c) | TCCTGCTCAATGCCATACCA |
| P15 (5' inner specific primer for GS1c) | TAGCAGTCGCACATCACAAG |
| P16 (3' outer specific primer for GS1c) | CGGATTCTTTGGTTGGGCTC |
| P17 (3' inner specific primer for GS1c) | ACTACGATGGTTCCAGCACT |
| P18 (forward full-length primer for GS1a) | GAGAGGGTGGGTTCTTAGTC |
| P19 (reverse full-length primer for GS1a) | GGCAAGAGAAAGAAATCACG |
| P20 (forward full-length primer for GS1b) | TCCTGTGCTCCTCTTTGCCT |
| P21 (reverse full-length primer for GS1b) | GGGCAGGAACCACCCTCTTA |
| P22 (forward full-length primer for GS1c) | CGGATTCTTTGGTTGGGCTC |
| P23 (reverse full-length primer for GS1c) | AAAGGATGGTGGTCTCAGCA |
| P24(forward primer for GS1a in qRT-PCR) | GCGTACGGCGAAGGAAACGAGCG |
| P25(reverse primer for GS1a in qRT-PCR) | GCGACCATGGACGTCACAACAT |
| P26(forward primer for GS1b in qRT-PCR) | AGTCCATGAGGAAAGAAGGAGG |
| P27(reverse primer for GS1b in qRT-PCR) | ATTGATGCTCCACGATTTGCA |
| P28(forward primer for GS1c in qRT-PCR) | ATACAGACAAGATCAATTCTTCTGG |
| P29(reverse primer for GS1c in qRT-PCR) | AACATCCTGAATTGTCCTCCTAA |
| P30(forward primer for actin in qRT-PCR) | CATTCAATGTGCCTGCCATGT |
| P31(reverse primer for actin in qRT-PCR) | GGATAGCATGTGGAAGGGCATAG |

## Result and Discussion

**Sequence cloning and analysis:** The full sequences of GS1a, GS1b and GS1c were submitted to GenBank of the NCBI and the accession numbers were KF683089, KJ881169, and KJ881170 respectively. The physico-chemical properties of three GSs are listed in Table 2. All three GS genes contained an approximate 1500 nucleotide-long open reading frame (ORF). The ORFs encoded proteins with 354-356 amino acids. The molecular weight of three proteins was about 39kDa, and the isoelectric point was about 5.5-5.9. Blast results indicated that GS1a, GS1b and GS1c shared high homology with other plant GSs (Table 3) such as *Hevea brasiliensis*, *Zea mays*, and others, by more than 80%. There were some typical conserved domains of GS such as a putative ATP-binding region, N-glycosylations, GS beta-Grasp domain and a GS catalytic domain in GS1a, GS1b and GS1c (Table 4). Target P predicted that they had no signal peptide and were present in the cytoplasm. The result of multiple alignment (Fig. 1) showed that they were all conserved sequences of amino acids, especially at the GS catalytic domain. All these indicated that these cloned genes might be GS sequences with similar and typical GS structure, and might have similar function which would be verified by the following complementary experiment with mutant yeast.

*E. crassipes* is one of the fastest growing plants known. Its uncontrolled growth can destroy the ecological balance of water body and produce serious economic and social problems. It can assimilate nitrogen very efficiently (Reddy *et al*., 1989). Nowadays, the focus of most studies is how to make use of it and how to repress its production (Aboul-Enein *et al*., 2011). However, there were little studies with regard the molecular mechanism of its nitrogen assimilation capabilities. GS is a key enzyme which incorporates ammonium to glutamate with the addition of ATP. This is a primary and crucial step from inorganic nitrogen to organic nitrogen. So GS plays an important role in the assimilation of nitrogen. The research of GS in *E. crassipes* could help to explain the mechanism of the ability of *E. crassipes* to assimilate nitrogen. Better understanding of the molecular mechanism of *E. crassipes* to assimilate nitrogen could help investigators further develop *E. crassipes* in phytoremediation of eutrophic water and to control its growth from the point of nutrition metabolism.

**Table 2. Physico-chemical properties of GS1a, GS1b and GS1c.**

| Gene name | Full length (bp) | Number of amino acid | Molecular weight (kD) | Isoelectric point |
|---|---|---|---|---|
| EcGS1a | 1434 | 356 | 39.3 | 5.52 |
| EcGS1b | 1455 | 354 | 39.0 | 5.95 |
| EcGS1c | 1508 | 356 | 39.3 | 5.94 |

**Table 3. Similarity analysis of GS1a,GS1b and GS1c with other GSs of other plants**

| Species | Accession number | Amino acid Identity (%) | | |
|---|---|---|---|---|
| | | EcGS1a | EcGS1b | EcGS1c |
| *Hevea brasiliensis* | AAB61597.1 | 93.0 | 89.6 | 91.9 |
| *Zea mays* | AFP20991.1 | 89.0 | 85.6 | 88.2 |
| *Spinacia oleracea* | ABU45501.1 | 89.9 | 86.7 | 89.6 |
| *Nicotiana attenuate* | AAR86718.1 | 89.6 | 89.0 | 89.9 |
| *Cucumis sativus* | NP_001267644.1 | 91.3 | 88.4 | 90.5 |
| *Solanum tuberosum* | AAG40237.2 | 88.4 | 87.2 | 88.7 |
| *Eutrema halophilum* | BAJ34289.1 | 86.2 | 85.9 | 85.4 |
| *Triticum aestivum* | ADW94625.1 | 88.2 | 84.5 | 87.6 |
| *Populus trichocarpa* | XP_002310666.1 | 89.6 | 87.6 | 89.6 |
| *Glycine max* | NP_001242332.1 | 90.5 | 88.1 | 89.9 |
| *Lactuca sativa* | CAA42689.1 | 87.3 | 86.7 | 87.9 |
| *Pinus sylvestris* | CAA49476.1 | 82.6 | 82.2 | 81.5 |
| *Brassica napus* | CAA73063.1 | 90.2 | 88.1 | 89.0 |

**Table 4. The conserved domains analyzed by Prosite and Smart**

| Gene | Putative ATP-binding region | N-glycosylations | GS beta-Grasp domain | GS catalytic domain |
|---|---|---|---|---|
| EcGS1a | 237-253AA | 11-14、185-188、251-254AA | 17-97 AA | 103-351 AA |
| EcGS1b | 237-253AA | 11-14、251-254AA | 17-97 AA | 103-351 AA |
| EcGS1c | 237-253AA | 11-14、185-188、251-254AA | 17-97 AA | 103-351 AA |

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GS1a        MALLTDLINLNLSDTTEKVIAEYIWIGGSGMDMRSKARTLPGPVTDPSQLPKWNYDGSST
GS1c        MALLTDLINLNLSDTTEKIIAEYIWIGGSGMDMRSKARTLPGPVTDPSQLPKWNYDGSST
GS1b        MSLLTDLINLNLSESSNKIIAEYIWVGGSGMDMRSKARTLSGPVTDPSQLPKWNYDGSST
            *:***********::::*:*******:***************.**********

GS1a        GQAPGEDSEVILYPQAIFKDPFRRGNNILVMCDCYTPAGEPIPTNNRYNAAKIFSHPDVV
GS1c        GQAPGEGSEVIIYPQAIFKDPFRRGNNILVMCDCYTPAGEPIPTNKRFNAAKIFSHPDVV
GS1b        GQAPGEDSEVILYPQAIFKDPFRRGNNILVMCDCYTPQGEPIPTNKRHNAAKIFSNPAVA
            ******.*****:*************************** ********:*.********:* *.

GS1a        AEVPWYGIEQEYTLLQKDVKWPLGWPVGGYPGPQGPYYCSAGADKAFGRDIVDAHYKACL
GS1c        VKVPWYGIEQEYTLLQKDVNWPLGWPVGGYPGPQGPYYCAAGADKAFGRDIVDAHYKACL
GS1b        AEETWFGLEQEYTLLQKDVKWPLGWPVGGFPGPQGPYYCAAGADKAFGRDIVDVHYEACI
            .:  .*:*:***********:**********:**********:**************.**:**:

GS1a        YAGINISGINGEVMPGQWEFQVGPAVGISAGDELWVARYILERITEIAGVVLSFDPKPIQ
GS1c        YAGINISGINGEVMPGQWEFQVGPAVGISAGDELWVARYILERITEIAGVVLSFDPKPIQ
GS1b        YAGINIRGINGEVMPGQWEFQVGPSVGISAGDELWVARYILERITEIAGVVLSFDPKPIQ
            ****** ******************:************************************

GS1a        GDWNGAGAHTNYSTRSMREDGGYEVIKKAIEKLGLRHKEHIAAYGEGNERRLTGRHETAD
GS1c        GDWNGAGAHTNYSTKSMREDGGYEVIKKAIEKLGLRHKEHIAAYGEGNERRLTGRHETAD
GS1b        GDWNGAGAHTNYSTKSMRKEGGYEVIKKAIEKLGKRHKEHIAAYGEGNERRLTGRHETAD
            ***************:***:::*************** **************************

GS1a        INTFKWGVANRGASIRVGRETEKNGKGYFEDRRPASNMDPYVVTSMVAETTILWKP
GS1c        INTFKWGVANRGASIRVGRETEKNGKGYFEDRRPASNMDPYVVTSMVAETTILWKP
GS1b        INTFKWGVANRGASIRVGRDTEKAGKGYFEDRRPASNMDPYVVTSMIAETTILL--
            ********************:*** ************************:*******
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Fig. 1. Alignments of amino acid sequences of GS1a, GS1b and GS1c in *E.crassipes* using ClustalX-1.83.

**Yeast complementation experiments:** To further study the function of GS in nitrogen metabolism, we transferred the GS1a, GS1b and GS1c gene respectively into Δgln1 which can only grow in medium containing 0.1% Gln as the only nitrogen source since it is missing glutamine synthetase. GS-carrying cells and Wide-type yeast could grow on 1mM $NH_4^+$ as the only nitrogen source while the mutant could not grow on this energy source (Fig. 2). These studies revealed that GS expressed a functional protein that played an important role in the assimilation of ammonium, and did so most likely when transforming ammonium to glutamine. This was very consistent with the results of Nord-Larsen *et al*., (2009) who cloned GS genes from ryegrass and confirmed the function of GS with the yeast complementation experiments, as well as Bernard *et al*., (2008) who studied the expression, cellular localization and function of GS genes from wheat. All of the GS genes from different plants could functionally complement the mutant yeast that were deprived of the GS gene.

**Expression analyses of GS1a, GS1b and GS1c in different nitrogen sources:** The relative expression level of GS1a and GS1c from the root was up-regulated markedly under nitrate status (Fig. 3). The positive effect of ammonium on expression of GS1b from the root was also shown, although the effect was not more remarkable than that on GS1a and GS1c from the root. GS1c was a partially sensitive to nitrate, and only after two hours did it show higher expression levels. The relative expression levels of the GS1b and GS1c genes extracted from leaves were the same as that of GS1c from the root (Fig. 4). Nitrate greatly promoted the expression of both GS1b and GS1c in leaves, and only after two hours did they exhibit a higher expression level. However, the expression level of the GS1a gene from leaves was different. The relative expression level of the GS1a gene from leaves was markedly up-regulated under a nitrogen deprivation status. After six hours of nitrogen deficiency, GS1a transcript levels were promoted to levels higher than that found under ammonium or nitrate.

The relative expression level of GS gene was influenced by species, organ, tissues, developmental stage and environmental factors, which was reported by many previous papers (Nogueira *et al*., 2005; Zozaya-Hinchliffe *et al*., 2005; Zhao & Shi, 2006; Bernard *et al*., 2008; Castro-Rodríguez *et al*., 2011; Seabra *et al*., 2013; Castro-Rodríguez *et al*., 2015). Ammonium and nitrate could influence GS gene expression. This influence depended on the species, organ and the specific member of GS family examined (Sukanya & Snustad, 1994). In some cases, both ammonium and nitrate were reported to enhance the activities or the transcript level of GS genes (Kozaki *et al*., 1991; Hoelzle *et al*., 1992; Sakakibara *et al*., 1992a; Stanford *et al*., 1993), while in other cases, only ammonium (Vezina & Langlois, 1989; Sugiharto & Sugiyama, 1992) or nitrate (Hayakawa *et al*., 1990; Sakakibara *et al*., 1992a; Redinbaugh *et al*., 1993) were found to have positive effect. Our current observations showed that the nitrate nitrogen could obviously increase the relative expression of GS1a and GS1c in the root and GS1b and GS1c in the leaves of *E. crassipes*. The positive effect of ammonium on GS1b in root was not more remarkable than GS1a and GS1c in root. However, nitrogen deprivation could up-regulate the expression of GS1a in leaves from *E. crassipes*. Additionally, GS1c in the root and GS1b and GS1c in the leaves were more sensitive to nitrate than GS1a in the root, and only after two hours, the enhancement was shown. All these differential expression was regulated by different mechanism. These different response to environment factors might contribute to nitrogen metabolism homeostasis in functions that are related with the changes in environmental conditions and organ types (Castro-Rodríguez *et al*., 2015).

Our result only reported there existed differential expression patterns in roots and leaves of *E. crassipes* in response to different nitrogen sources. Attempt to discover the detailed differential expression patterns in *E. crassipes* will be in progress, and it will compare the expression level of GS genes in other organs in addition to root and leave, even in tissue and cellular level. Our experiment result seemed contrasted with the result of Li *et al*., 2008 which reported ammonium could increase the activities of GS in *E. crassipes*. In fact, our study reported the expression levels of specific GS1 genes, while Li *et al*., (2008) studied the total activities of GS in specific organs. The total GS included not only our examined GS1, but also other GSs still unidentified now. Furthermore, there existed post-transcriptal modification and post-translational modification in the procedure of GS expression. So expression level of specific GS gene was not well consistent with the total activities of GS.

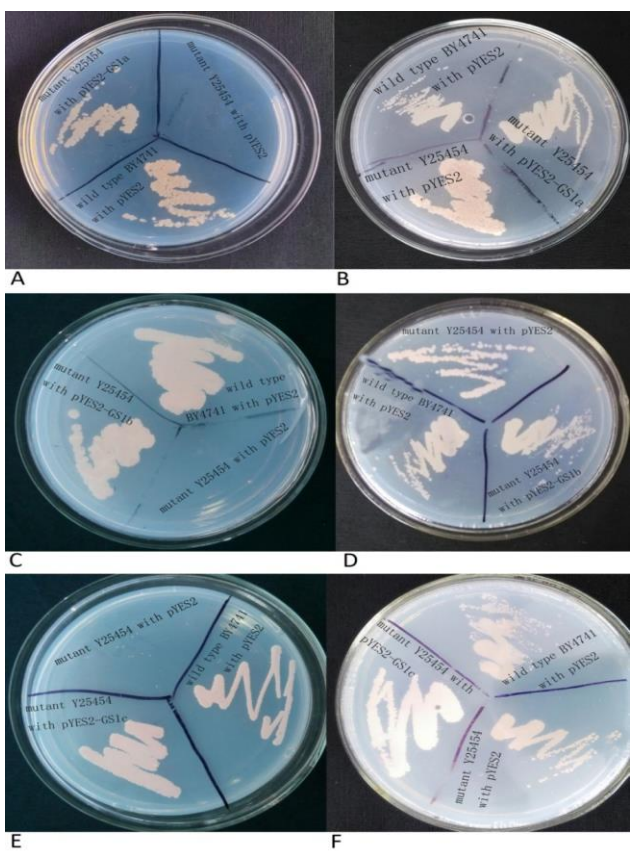


Fig. 2. Yeast complementation of mutant ΔGln1 by GS1a, GS1b, and GS1c. Images A, C and E showed the plate with YNB medium containing 1mM ammonium. Images B, D, F showed the plate with YNB medium containing 0.1% Gln. Every plate was divided into three parts. One part incubated the wild-type yeast BY4741 transformed with empty vector pYES2, the other incubated the mutant yeast ΔGln1 transformed with empty vector pYES2 and the third incubated the mutant yeast ΔGln1 transformed with the vector with GS1 gene.

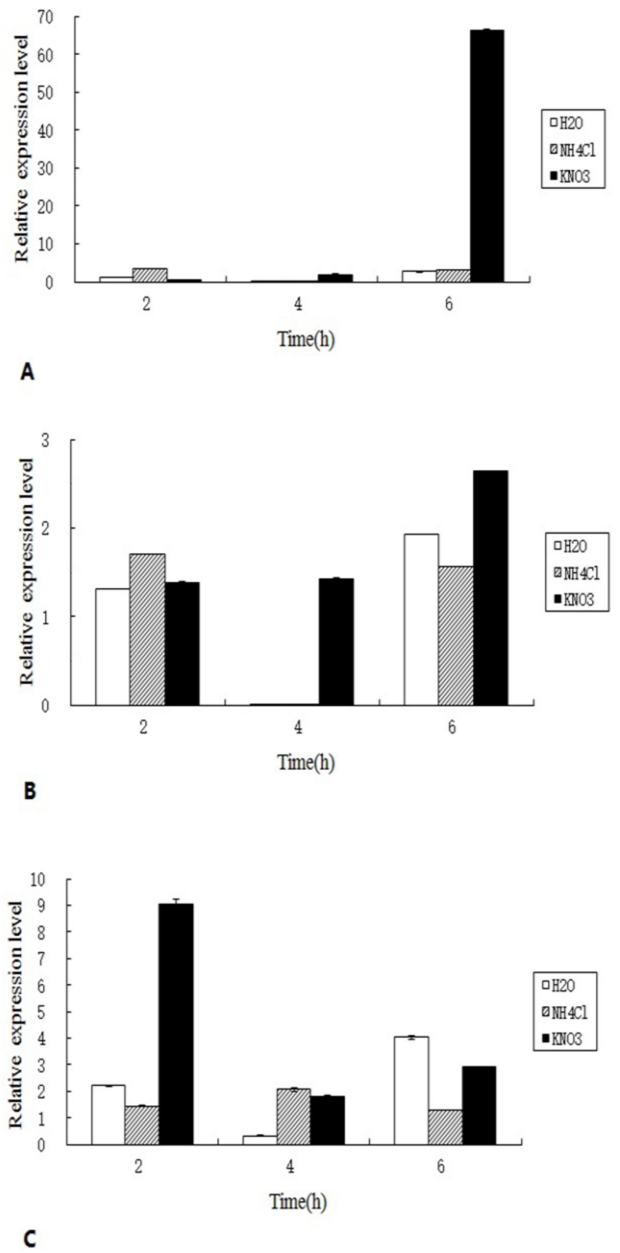


Fig. 3. The relative expression level of three GS genes from the roots under different nitrogen status. The values are given as mean ± SD. A. GS1a; B. GS1b; C. GS1c. Expression levels were normalized against the value for plants grown in pre-culture medium and collected at 0 hour. Error bars represent means of three repeats +SE (n=3).

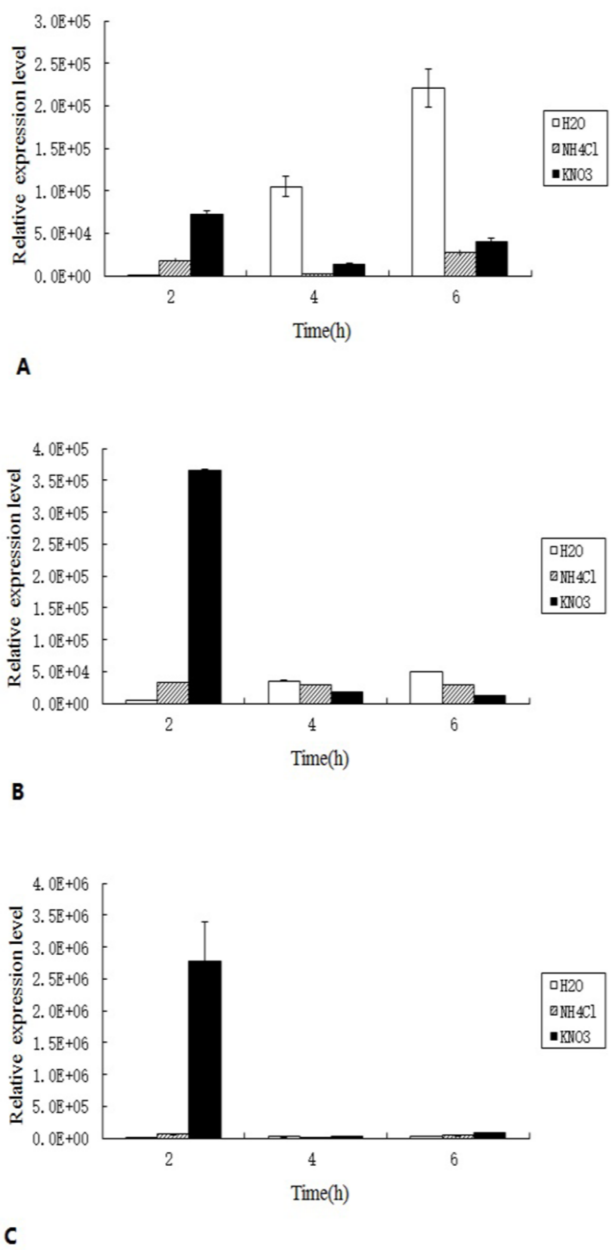


Fig. 4. The relative expression levels of three GS genes from leaves under different nitrogen status. The values are given as mean ± SD. A. GS1a; B. GS1b; C. GS1c. Expression levels were normalized against the value for plants grown in pre-culture medium and collected at 0 hour. Error bars represent means of three repeats + SE (n=3).

## Conclusions

In this study, we cloned two cytosolic GS cDNAs, GS1b and GS1c from the roots of *E. crassipes*, studied the characterizations with the yeast complementation experiment and analyzed the expressions of GS1a, GS1b and GS1c in response to different nitrogen sources in roots and leaves. These GS cDNAs were approximately 1400-1500 nucleotide encoding proteins with 354-356 amino acids that functionally complemented the mutant yeast ΔGln for growth on ammonium as the sole source of nitrogen. Their relative expression level was markedly up-regulated under the status of nitrate as the only nitrogen source except that of GS1b in roots, which was only up-regulated less than one fold, and GS1a in leaves, which was up-regulated under condition of nitrogen deprivation. All these results indicated that *E. crassipes* has at least three different cytosolic GS1 genes which have typical conserved domains of GS gene and can functionally complement the mutant yeast that is deprived of the GS gene. All these *E. crassipes* GS1 genes have different expression patterns in response to different nitrogen sources.

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