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# Glutamine synthetase I-deficiency in *Mesorhizobium loti* differentially affects nodule development and activity in *Lotus japonicus*

Sirinapa Chungopast[a,b], Pilunthana Thapanapongworakul[a,c], Hiroyuki Matsuura[a], Tan Van Dao[a,d], Toshimasa Asahi[a], Kuninao Tada[a], Shigeyuki Tajima[a], Mika Nomura[a,*]

[a] *Faculty of Agriculture, Kagawa University, Miki-cho, Kita-gun, Kagawa 761-0795, Japan*
[b] *Faculty of Agriculture at Kamphaeng Saen, Kasetsart University, Nakorn Pathom 73140, Thailand*
[c] *Faculty of Agriculture, Chiang Mai University, 50200 Chiang Mai, Thailand*
[d] *Faculty of Biology, Hanoi National University of Education, 136 Xuan Thuy Road, Hanoi, Vietnam*

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ABSTRACT

In this study, we focused on the effect of glutamine synthetase (GSI) activity in *Mesorhizobium loti* on the symbiosis between the host plant, *Lotus japonicus*, and the bacteroids. We used a signature-tagged mutant of *M. loti* (STM30) with a transposon inserted into the GSI (mll0343) gene. The *L. japonicus* plants inoculated with STM30 had significantly more nodules, and the occurrence of senesced nodules was much higher than in plants inoculated with the wild-type. The acetylene reduction activity (ARA) per nodule inoculated with STM30 was lowered compared to the control. Also, the concentration of chlorophyll, glutamine, and asparagine in leaves of STM30-infected plants was found to be reduced. Taken together, these data demonstrate that a GSI deficiency in *M. loti* differentially affects legume–rhizobia symbiosis by modifying nodule development and metabolic processes.



## Introduction

Nitrogen fixation by rhizobium–legume symbiosis provides a significant proportion of the available nitrogen in the biosphere, making this relationship agronomically and ecologically important. Ammonium is the primary stable product of nitrogen fixation, in which bacteroids secrete ammonium into the plant, and it is assimilated into glutamate by glutamine synthetase (GS) and glutamate synthase (GOGAT) (Cullimore and Bennett, 1988; Temple et al., 1998). Bacteroids simply provide the plant with ammonium because the major assimilating pathways through GS and GOGAT are repressed in bacteroids (Brown and Dilworth, 1975; Kurz et al., 1975; Cullimore and Bennett, 1991; Temple et al., 1998). However, the nitrogen nutrient exchange between the plant cytosol and bacteroids is more complex. Recently, an amino-acid cycle has been reported to be essential for symbiotic nitrogen fixation by rhizobium in pea nodules (Lodwig et al., 2003; Prell et al., 2009). Given the importance of amino acid transport into bacteroids to support $N_2$ fixation, understanding the role of the endogenous synthesis of amino acids inside bacteroids by the GS/GOGAT is important. GOGAT and GSI/GSII double mutants of *Bradyrhizobium japonicum* are $Fix^-$, whereas GOGAT and GSI/GSII double mutants of *Sinorhizobium meliloti* are $Fix^+$ (Osburne and Signer, 1980; O'Gara et al., 1984; Carlson et al., 1987; deBruijn et al., 1989; Lewis et al., 1990). These results clearly indicate that *B. japonicum* needs to make glutamine and/or glutamate to form effective $Fix^+$ nodules, and *S. meliloti* does not. However, there is no report of the function of ammonium assimilation in *Mesorhizobium loti* and *Lotus japonicus* symbiosis. To understand the role of ammonium assimilation in $N_2$ fixation by legume bacteroids, mutating the GS/GOGAT pathway is essential. On the RhizoBase website (http://genome.microbedb.jp/rhizobase/Mesorhizobium), we found nine proposed GS genes in *M. loti* MAFF30309: mll0343 (glutamine synthetase I, GSI), mlr0339 (glutamine synthetase II, GSII), mll7307 (glutamine synthetase III, GSIII), mlr6210 (GSIII), mll5148, mll3074, mll7254, mll6521, and mll4187. To understand the function of ammonium assimilation in more detail, we used a rhizobial mutant produced using a signature-tagged mutagenesis (STM) technique. Shimoda et al. (2008) constructed a large-scale random mutagenesis of *M. loti* using STM. STM is based on transposon insertional mutagenesis that allows for large numbers of mutants to be analyzed simultaneously. This analysis is accomplished by tagging each mutant with a unique short DNA sequence to subsequently distinguish different mutants with unique signature tags. Sequencing the transposon insertion sites enabled the collection of defined sets of transposon mutants.

*Abbreviations:* ARA, acetylene reduction activity; GS, glutamine synthetase; STM, signature-tagged mutagenesis; wpi, weeks post-inoculation; WT, wild-type.

* Tel.: +81 87 891 3135; fax: +81 87 891 3021.
*E-mail address:* nomura@ag.kagawa-u.ac.jp (M. Nomura).

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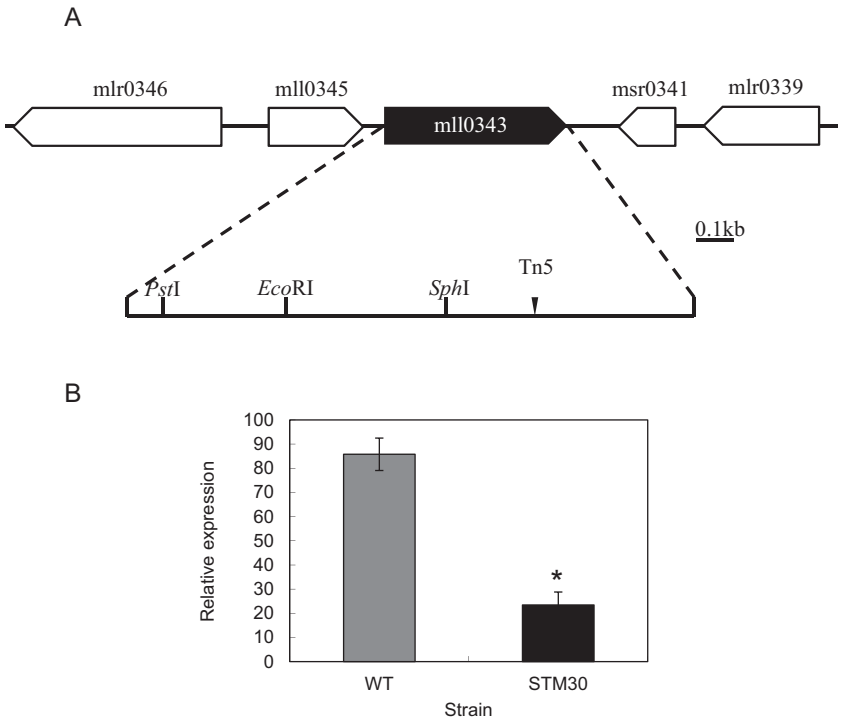


**Fig. 1.** The STM30 transposon insertion mutant for *M. loti* and GSI expression in the bacteroids. A transposon (Tn5) was inserted in the glutamine synthetase I (GSI, mll0343) gene (A). Each RNA was isolated from the bacteroids to synthesize the cDNA and then qRT-PCR was performed (B). The expression was normalized to *sigA*. All of the data are shown as ±SD. Statistically significant differences compared with the wild-type bacteroid are indicated with * ($P < 0.01$).

These mutants were identified using the RhizoBase database. We searched various GS mutants from the database and found that the STM30 mutant had a transposon inserted in the GSI (mll0343) gene (Fig. 1). In this study, we focused on GSI in *M. loti* and found that GSI (mll0343) deficiency in *M. loti* induced early nodule senescence.

## Materials and methods

### *Bacterial strains and medium*

The *Mesorhizobium loti* MAFF303099, a gram-negative, nitrogen-fixing, symbiotic bacterium for *Lotus japonicus*, was used in this study. The transposon insertion mutant strain of *M. loti*, STM30, was generated using the signature-tagged mutagenesis (STM) technique described by Shimoda et al. (2008). STM30 had a Tn inserted at base pair 399 of the 1410-bp GSI gene (mll0343; Fig. 1A). The *M. loti* strain *M. loti* strains were cultured in tryptone-yeast extract (TY) liquid medium (Beringer, 1974) at 28 °C.

### *Plant growth conditions*

Seeds from *L. japonicus* GIFU (Borjigin et al., 2011) were scarified using sandpaper to remove the seed coats. Then, 1% sodium hypochlorite solution containing 0.01% Tween-20 was used to surface sterilize the seeds for 10 min. Vermiculite was placed in the upper compartments of Magenta Jars containing a half-diluted B&D nitrogen-free nutrient solution (Broughton and Dilworth, 1971). Twenty-five seeds were cultured in each jar and germinated under sterile conditions. The jars were placed in a growth cabinet (EYELA FLI-2000) at 24 °C and exposed to 16-h/light and 8-h/dark cycles. After seven days, the plants were inoculated with 10 mL of *M. loti* ($1 \times 10^9$ cells $mL^{-1}$). These plants were examined at one, two, four, and seven weeks post-inoculation (wpi).

### *Assay for acetylene reduction*

To assay the nitrogenase activity in the nodules, the acetylene reduction activity (ARA) *in vivo* was measured. Intact whole plants bearing nodules or collected nodules were placed in a 70-mL glass vial and incubated at 25 °C with 7 mL of acetylene. After a 30-min incubation, the amount of ethylene produced was measured by gas chromatography (GC-8A Shimadzu) as previously described (Suganuma et al., 1998).

### *Free amino acid measurement*

For the amino acid analysis, the bacteroids were isolated from *L. japonicus* nodules as previously reported (Kouchi et al., 1991; Hoa et al., 2004). The nodules, leaves and isolated bacteroids were ground with a mortar and pestle in 80% ethanol, respectively. Then, the samples were heated to 80 °C, dried, and suspended in 0.02 M HCl. The amino acids were analyzed using a Hitachi amino acid analyzer (L-8900BH, Hitachi).

### *RNA extraction and quantitative real-time PCR*

The bacteroids were isolated from the nodules at 4 wpi as described previously (Hoa et al., 2004). The RNA from the bacteroids was isolated using the RNAwiz (Life Technologies) and then purified using RNeasy spin columns (Qiagen). For the RNA extraction from the nodules, 0.1 g of the nodules were ground with a mortar and pestle followed by total RNA isolation using the RNeasy Plant Mini Kit (Qiagen). Each RNA was converted to cDNA using the PrimeScript 1st strand cDNA synthesis kit (Takara). The resulting cDNA was amplified with specific primer pairs (0343Fw, 5′-CGACCAGATCGACAGCTACA-3′; 0343Rv, 5′-ATTTCAAAGCGCATCACCTC-3′; sigAFw, 5′-CATCTCCATCGCCAAGAAAT-3′; sigARv, 5′-GAACTTATCGACCGCCTTCA-3′; LjCysFw, 5′-GAG CAG ATT GGG GTG AAG AA-3′; CysRv, 5′-ATC CAC ATC CCT TTG CAT TC-3′; Ubi Fw, 5′-ATG CAG