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The GntR-Type Regulators GtrA and GtrB Affect Cell Growth and Nodulation of *Sinorhizobium meliloti*

Yi Wang[1†], Ai-Min Chen[1†], Ai-Yuan Yu[1], Li Luo[2], Guan-Qiao Yu[1], Jia-Bi Zhu[1], and Yan-Zhang Wang[1*]

[1]National Key Laboratory of Plant Molecular Genetics, Institute of Plant Physiology and Ecology, Shanghai Institutes for Biological Sciences (SIBS), Chinese Academy of Sciences, Shanghai 200032, P. R. China
[2]Biological Sciences Department, Lehman College, The City University of New York, New York 104681, USA

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GntR-type transcriptional regulators are involved in the regulation of various biological processes in bacteria, but little is known about their functions in *Sinorhizobium meliloti*. Here, we identified two GntR-type transcriptional regulator genes, *gtrA* and *gtrB*, from *S. meliloti* strain 1021. Both the *gtrA1* mutant and the *gtrB1* mutant had lower growth rates and maximal cell yields on rich and minimal media, as well as lower cell motility on swimming plates, than did the wild-type strain. Both mutants were also symbiotically deficient. Alfalfa plants inoculated with wild-type strain 1021 formed pink elongated nodules on primary roots. In contrast, the plants inoculated with the *gtrA1* and *gtrB1* mutants formed relatively smaller, round, light pink nodules mainly on lateral roots. During the first 3~4 weeks post-inoculation, the plants inoculated with the *gtrA1* and *gtrB1* mutants were apparently stunted, with lower levels of nitrogenase activity, but there was a remarkable increase in the number of nodules compared to those inoculated with the wild-type strain. Moreover, the *gtrA1* and *gtrB1* mutants not only showed delayed nodulation, but also showed markedly reduced nodulation competition. These results demonstrated that both GtrA and GtrB affect cell growth and effective symbiosis of *S. meliloti*. Our work provides new insight into the functions of GntR-like transcriptional regulators.

***Keywords*:** GntR-type transcriptional regulators, GtrA, GtrB, cell growth, nodulation

Sinorhizobium meliloti is a Gram-negative soil bacterium that is capable of establishing a symbiotic relationship with the alfalfa plant, *Medicago sativa*. A complex interplay between a bacterium and its symbiotic partner leads to the formation of a morphologically specialized plant organ, the nodule, in which *Rhizobia* carry out nitrogen fixation. During the establishment of successful symbiosis, a precise molecular dialogue between symbiotic partners is triggered when root-secreted plant flavonoids act as transcriptional activators of *rhizobium* nodulation (Long, 1996). In the ensuing process, proteins encoded by the activated *nod* genes aid in the synthesis of rhizobial signal molecules known as Nod factors, which play a pivotal role in determining the fate of symbiotic interaction (Denarie *et al*., 1996; Long, 1996). Accumulating evidence has proven that LysR-type transcriptional regulators play a crucial role in an effective symbiotic relationship (Honma and Ausubel, 1987; Mulligan and Long, 1989; Kondorosi *et al*., 1991; Swanson *et al*., 1993; Luo *et al*., 2005). Based on information from the *S. meliloti* sequencing project, it is known that GntR-type transcriptional regulators comprise the second largest family of transcriptional regulators in *S. meliloti* 1021 (Galibert *et al.*, 2001). This information suggests that the GntR family of transcriptional regulators can play an important role in symbiosis between *S. meliloti* and alfalfa. However, little is known about the functions of the GntR family of transcriptional regulators in *S. melilot*.

The GntR family of transcriptional regulators is one of the most prevalent superfamilies of bacterial transcription factors. They are generally composed of an N-terminal winged HTH (helix-turn-helix) domain, followed by a C-terminal domain that can bind a variety of ligands (Fujita and Fujita, 1987; Haydon and Guest, 1991; Rigali *et al*., 2001). Members of the GntR family of proteins are currently clustered into six subfamilies on the basis of C-terminal effector-binding and oligomerization domains, e.g., FadR, HutC, MocR, YtrA, AraR, and PlmA (Rigali *et al*., 2001; Lee *et al*., 2003). They can function as activators or repressors, although most of them are repressors. The consensus binding sequence for GntR-like transcriptional regulators is a [5'-(N)yGT(N)xAC (N)y-3'] motif, but the promoters of targeted genes are not highly conserved (Rigali *et al*., 2001).

Most of the GntR-like transcriptional regulators have been reported to be involved diverse metabolism pathways in bacteria; among these GntR-like transcriptional regulators are the FadR, HutC, MocR, and AraR subfamilies (Mota *et al*., 1991; Rigali *et al*., 2001). The YtrA subfamily has been implicated in the ATP binding cassette (ABC) transport system, which is common in prokaryotes (Yoshida *et al*., 2000). The PlmA subfamily might regulate plasmid maintenance in *Anabaena* (Lee *et al*., 2003). The AraR subfamily is involved in absorbing and utilizing the carbohy-

† These authors contributed equally to this work.
* To whom correspondence should be addressed.
(Tel) 86-21-5492-4167; (Fax) 86-21-5492-4015
(E-mail) yzwang@sippe.ac.cn

drates (Sa-Nogueira *et al*., 1997; Mota *et al*., 1999). Recent evidence suggests that some GntR-type transcriptional regulators also control the development of bacteria. DasR in *Streptomyces coelicolor* regulates the sugar phosphotransferase system and links N-acetylglucosamine metabolism to the control of development (Rigali *et al*., 2006). Agl3R in *S. coelicolor* is required for morphogenesis and antibiotic production, and controls transcription of an ABC transporter in response to a carbon source (Hillerich and Westpheling, 2006). DevA is required for development in *S. coelicolor* (Hoskisson *et al*., 2006). Mce1R is required for organized granuloma formation, which is both protective to the host and necessary for the persistence of *Mycobacterium tuberculosis* (Casali *et al*., 2006). Systematic targeted mutagenesis of *Brucella melitensis* 16M reveals a major role for GntR regulators in the control of virulence (Haine *et al.*, 2005).

The complete sequencing of the *S. meliloti* genome revealed 54 putative genes encoding GntR-type transcriptional regulators, which allowed us to conduct a systematic study of the GntR family of transcriptional regulators in *S. meliloti* (Galibert *et al*., 2001). We successfully mutated the 54 *gntR* (GntR family of transcriptional regulators) genes by plasmid insertion mutagenesis and screened for their phenotypes. During this process, we found two *gntR* mutants to be symbiotically deficient. Here, we reported the characterization of the two *gntR* genes, *gtrA*, and *gtrB*, in *S. meliloti* 1021. Mutation of *gtrA* or *gtrB* not only affects cell growth and maximal cell yield, but also impairs symbiosis with alfalfa plants. Although both mutants were able to form nitrogen-fixing nodules, they varied in terms of the timing of nodule emergence, the progression of nitrogen fixation, the number of nodules, and the nodulation competitiveness.

Materials and Methods

Secondary structure prediction

Secondary structure prediction was performed by using the compilation of the PSI-pred, Predict Protein, Sspro, and Jpred automated prediction programs on the PredictProtein server (http://cubic.bioc.columbia.edu).

Strains and culture conditions

The *Escherichia coli* DH5α strain was used for cloning, MT616 (pRK600) (Cm^r) was used as the helper strain for conjugation, and *S. meliloti* 1021 (Str^r) was used as the wild-type strain (Leigh *et al*., 1985). The *S. meliloti* phage φM12 was used for general transduction (Long *et al*., 1988).

E. coli strains were cultured on Luria-Bertani (LB) medium at 37°C, and all *S. meliloti* strains were cultured in LB/MC (LB medium supplemented with 2.5 mmol/L $MgSO_4$ and 2.5 mmol/L $CaCl_2$) (Leigh *et al*., 1985). Minimal medium Z-MGS was used to examine the growth requirements of the mutants (Zevenhuizen and Van Neerven, 1993). Agar (1.5%) was used as the solid medium. Antibiotics were supplemented as required at the following concentrations: chloramphenicol, 10 μg/ml; kanamycin, 50 μg/ml; neomycin, 200 μg/ml; and streptomycin, 500 μg/ml.

Plasmid insertion mutagenesis

Plasmid insertion mutagenesis was conducted as described by Luo *et al*. (2005). Suicide plasmid pK19mob2ΩHMB was prepared using the Wizard Plus Midipreps DNA purification system (Promega, USA), digested with restriction enzymes *Hin*dIII and *BsrG*I (New England BioLabs, USA), and purified with the Watson PCR purification kit (Watson Inc., China). The DNA fragments (~0.3 kb) containing the middle part of each putative *gntR* gene were amplified by PCR from *S. meliloti* 1021 genomic DNA with primers specific for each gene, and the enzyme cleavage sites for *Hin*dIII and *BsrG*I were introduced into the primer sequences. The primers used to amplify the partial sequence of the *gtrA* gene (the SMa0160 open reading frame) were: Primer 1; 5'-GCGTGTACAGGTGATGGCTGAACTGGAAG-3' (*BsrG*I site underlined) and Primer 2; 5'-GGGAAGCTTTCCATGTTCCCTGAACGAGA-3' (*Hin*dIII site underlined), and the primers used to amplify the partial *gtrB* gene (the SMa0222 open reading frame) were: Primer 3; 5'-GCGTGTACATAGC TGGACACGGCGCAAGA-3' (*BsrG*I site underlined) and Primer 4; 5'-GGGAAGCTTTCGGCCAGAATG CCGTTGAG-3' (*Hin*dIII site underlined). A 0.3 kb fragment that had been isolated from the PCR products was digested with *Hin*dIII and *BsrG*I, and was then ligated into pK19mob2ΩHMB precleaved with the same enzymes. The recombinant plasmids were first transformed into DH5α, and were then conjugated into wild-type strain 1021 using MT616 as a helper in a triparental mating. Successful plasmid insertion into the *gtrA* or *gtrB* gene was selected on solid LB/MC containing streptomycin and neomycin. Single colonies were streaked twice on the same selective medium. A bacterial culture inoculated from a single colony on the selective medium was lysed with *S. meliloti* phage φM12. The lysate was then introduced back into wild-type 1021 in order to select for a single mutation of the *gtrA* or *gtrB* gene.

Successful insertion was confirmed by PCR using genomic DNA isolated from the mutant *S. meliloti* cells. PCR was performed with one primer that annealed to the suicide plasmid (primer 5; 5'-CCTGGCCTTTTGCTGGCCT-3') and another primer complementary to the putative *gtrA* and *gtrB* genes (Primer 1 for the *gtrA* gene and Primer 3 for the *gtrB* gene). When the plasmid is inserted into the target gene, a DNA fragment with a predicted size will be amplified by PCR. Otherwise, the integration process will not be successful.

Swimming behavior assay

To ensure the synchronicity of the bacterial liquid cultures, all of the strains were routinely initiated from glycerol stocks. Wild-type strain 1021 and mutants were grown to exponential phase at 28°C in LB/MC, subcultured in 5 ml fresh LB/MC with appropriate antibiotics, and grown to early exponential phase (OD_{600}=0.15~0.30). Aliquots (2 μl) of the bacterial cultures (≈2×10^7 cells) were dispensed uniformly onto the surfaces of freshly prepared LB/MC agar (0.3% agar) swimming plates, and were then incubated at 28°C for 4 days.

Growth curve assay

A single colony of each bacterial strain was inoculated into a 5 ml liquid LB/MC medium with the appropriate antibiotics, and was grown to an OD_{600} of ~2.0 at 28°C. The cultures were then subcultured (1:100) with the same medium, and the cell density was measured by monitoring the optical density

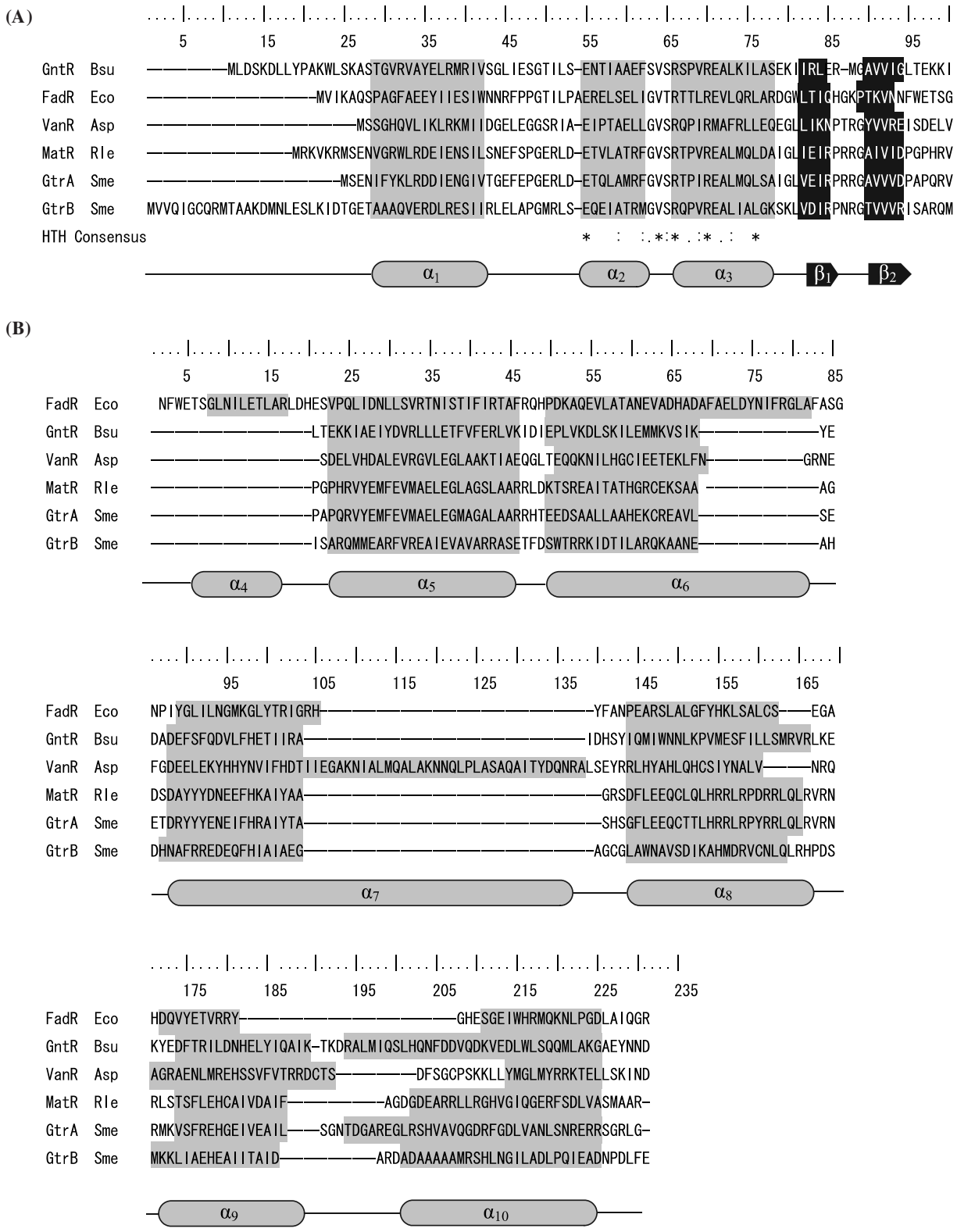


Fig. 1. (A) Structure-based sequence alignment of the N-terminal DNA-binding domain of the GtrA and GtrB protein. (B) Structure-based sequence alignment of the C-terminal domain of the GtrA and GtrB protein. Protein sequences for alignment are as follows: GntR (P10585), *Bacillus subtilis* (Bsu); FadR (P09371), *Escherichia coli* (Eco); Van (Q24839), *Acinetobacter* sp. ADP1 (Asp); MatR (Q9JP74), *Rhizobium leguminosarium* (Rle); GtrA (SMa0160) and GtrB (SMa0222), *Sinorhizobium meliloti* (Sme). The following symbols denote the degree of conservation observed in each column: (*) indicates that the residues or nucleotides in that column are identical in all sequences in the alignment, (:) means that conserved substitutions have been observed, (.) means that semi-conserved substitutions have been observed.

at 600 nm. Triplicate data were used for the measurement of each growth curve. Standard methods were used for doubling time calculations from mean optical density values during the exponential growth phase (Stanier *et al*., 1986).

Nodulation test

To test the nodulation efficiency of *S. meliloti* mutants in a greenhouse environment, surface-sterilized alfalfa (*Medicago sativa* L. cv. Iroquois) seeds were infected by soaking for 30 min in 5 ml of LB/MC containing 2×10^8 cells of wild-type strain 1021 or mutants. The infected alfalfa seeds were placed on the surfaces of autoclaved vermiculite and perlite (2:1) in one gallon pots, and were irrigated with nitrogen-free modified Fahraeus medium (FM) (Vincent, 1970). After covering the seeds with another thin layer of autoclaved mixture (with a depth of 0.5 cm), the pots were wrapped with a piece of plastic film and covered with a piece of paper. Seeds germinated after 2 days in an incubator at 28°C. The pots with seedlings were transferred to a greenhouse and watered with nitrogen-free FM liquid medium every other day. The leaf color of the plants and the number of root nodules were recorded regularly. The results presented for nodulation were replicated at least five times.

Nitrogenase assay

The nitrogenase activity was analyzed using the C_2H_2 reduction method (Wang *et al*., 1991). A gas chromatograph (type 102G; 220 V, 50 Hz, 1.5 kW; Shanghai Analytic Instrument Factory, Shanghai, China) was filled with PORAPAKN gas chromatography column packing material (Waters Associates Inc.), and the reduction reaction was performed in 1 ml of C_2H_2 at 28°C for 30 min. The fresh weights were recorded for a pool of 10 alfalfa plants, and the root tissues of these plants were sealed in a glass test tube in order to detect the nitrogenase activities.

Nodulation kinetics assay

Alfalfa seeds were surface-sterilized and germinated according to Leigh *et al*. (1985). Seven alfalfa seedlings (about 2∼3 cm in height) were arranged in each solid Jensen's (Jensen's medium, 1.5% agar) plate, which was then flooded with bacterial cells (2×10^7) the next day. Five or six plates were stacked with root tips pointing down. These plates were wrapped with aluminum foil in order to keep the root in darkness. Nodulated plants were counted twice daily after the first nodule became visible.

Nodule occupancy test

Surface-sterilized alfalfa seeds were soaked for 30 min in a mixture of a late exponential culture of wild-type 1021 and the *gtrA1* or *gtrB1* mutant (1:1). After 4 weeks, nodules from these plants were harvested, surface-sterilized, crushed, and plated on non-selective rich medium (Leigh *et al*., 1985). The colonies that had been grown in the non-selective medium were streaked on selective medium containing streptomycin and neomycin. The *gtrA1* and *gtrB1* mutants with the plasmid insertion are resistant to streptomycin and neomycin, thus allowing for an easy determination of the ratio of wild-type to mutant cells.

Results

Identification of two GntR-type transcriptional regulator genes

The *S. meliloti* Genome Sequence Project has predicted that the SMa0160 and SMa0222 open reading frames encode putative GntR-type transcriptional regulators. In this work, the SMa0160 open reading frame was designated as the *gtrA* gene (for GntR-type transcriptional regulators) and the SMa0222 open reading frame was designated as the *gtrB* gene. The predicted gene product, GtrA, encodes a 224 amino acid peptide, and the predicted gene product GtrB encodes a 242 amino acid peptide. Secondary structure analyses showed that both of the proteins consist of three α-helices and two β sheets ($\alpha_1\alpha_2\alpha_3\beta_1\beta_2$) in the N-terminal DNA-binding domain, the typical N-terminal domain of the GntR family of transcriptional regulators. The helix-turn-helix (HTH) motif is formed by helices α_2 and α_3 (Fig. 1A). Analysis of the C-terminal secondary structure showed that both GtrA and GtrB consist of an all-helical C-terminal domain, six helices (Fig. 1B), thus suggesting that they fell into the VanR subgroup in the FadR subfamily of the GntR superfamily.

Results from a PSI-BLASTP alignment search of the GenBank database show that GtrA is 64% identical to MatR of *Rhizobium leguminosarum*. MatR is a trans-acting repressor that regulates the *matABC* operon, which is involved in malonate metabolism of *R. leguminosarum* (Lee *et al*., 2000). It is interesting to find that the gene cluster upstream of *gtrA* (containing four consecutive genes SMa 0157, SMa0155, SMa051, and SMa0150) is similar to the *matABC* operon in *R. leguminosarum* (An and Kim, 1998; An *et al*., 2002). The protein encoded by SMa0150 displays the highest homology with the MatB protein (≈75% identity). Surprisingly, the region from the 388^{th} to 838^{th} residues of the protein encoded by SMa0151 has the highest similarity to the MatA protein (≈63% identity). The promoter region of SMa0157 located immediately upstream of *gtrA* is opposite to and overlaps the promoter of *gtrA* (Fig. 2A). There is a putative GntR-binding site containing 5'-TGTAT ACA-3' sequence located in the promoter region of SMa 0157, which is similar to the T.GT-$N_{(0-3)}$-AC.T motif for the FadR subfamily of transcriptional regulators. Furthermore, an inverted repeat sequence, TCTTGTA/TACAAAA, was also found in the SMa0157 promoter region, which is similar to the MatR binding site (TCTTGTA/TACACGA) from *R. leguminosarum* (Lee *et al*., 2000). According to similar situations in bioinformatics, it is speculated that consecutive gene clusters SMa0150, SMa0151, SMa0155, and SMa0157 might form an operon that is involved in malonate metabolism. These gene clusters are regulated by GtrA in a similar manner as that of MatR in *R. leguminosarum*.

GtrB protein shares no significant homology with any other protein in the currently available databases. The promoter region of upstream gene SMa0220 of the *gtrB* gene is opposite to and overlaps the promoter for the *gtrB* gene (Fig. 2B). The SMa0220 was predicted to be a putative aldehyde dehydrogenase by the *S. meliloti* sequencing project, and has been shown to have relatively high homology to *Gluconobacter oxydans* NAD(P)-dependent L-sorbosone dehy-

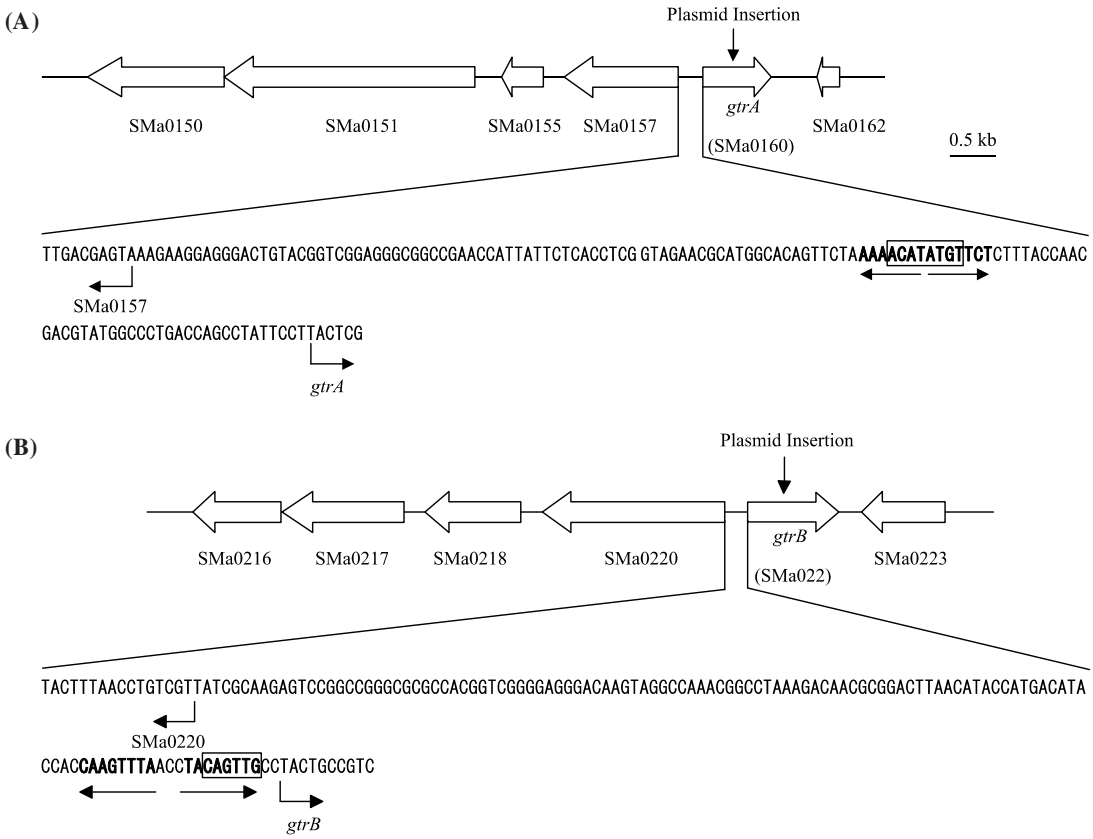


Fig. 2. Locations and surrounding genes of the *gtrA* and *gtrB* genes, showing predicted binding sites for GntR-type transcriptional regulators. GntR-binding sites are boxed; the inverted repeat sequences are indicated by arrows.

drogenase protein (≈46% identity). A typically panlindromic binding sequence, GTTGACAT/ATTTGAAC, is located in the promoter region of SMa0220; this sequence contains a *cis*-acting element, 5'-GTTGAC-3', which is recognized by the GntR superfamily. The consecutive genes upstream of SMa0220 include SMa0216, SMa0217, and SMa0218, which partially encode the ABC transporter system. Whether SMa 0220, SMa0218, SMa0217, and SMa0216 constitute an operon regulated by GtrB requires further investigation.

Construction of *gtrA1* and *gtrB1* mutants

The *gtrA* and *gtrB* genes were inactivated by plasmid insertion mutagenesis in order to investigate their functions at free-living and symbiotic stages. The two genes were mutagenized as described by Luo *et al.* (2005). To ensure that each of the mutants had a single insertion mutation in its genome, we regenerated both mutants by successfully retransducing them to the wild-type strain. The generated mutants for *gtrA* and *gtrB* were designated as *gtrA1* and *gtrB1*, respectively. A single plasmid insertion in the *gtrA1* and *gtrB1* mutants was confirmed by PCR, using one primer internal to the insertion plasmid and the other primer from the *gtrA*- or *gtrB*-specific region (data not shown). Subsequent southern hybridization of *gtrA1* and *gtrB1* genomic DNAs with a vector-derived probe further validated a unique single insertion event in the *gtrA* or *gtrB* gene (data not shown).

The SMa0162 gene downstream of *gtrA* or the SMa0223 gene downstream of *gtrB* is transcribed from its own promoter in the opposite direction of the *gtrA* or *gtrB* gene promoter (Fig. 2), so it is unlikely that the plasmid insertion in *gtrA* or *gtrB* had a polar effect on the expression of its downstream gene. Indeed, RT-PCR verified that there was no change in the expression of the SMa0162 gene in the *gtrA1* mutant compared to the wild-type strain (data not shown). Similar experimental results confirmed that the expression of SMa0223 was not affected by *gtrB* disruption (data not shown).

Phenotypes of the free-living *gtrA1* and *gtrB1* mutants

The GntR family of transcriptional regulators has been found to mainly regulate the expressions of genes involved in a variety of metabolic pathways. When plated on rich medium LB/MC and minimal medium Z-MGS with mannitol and glutamic acid as the carbon and nitrogen source, *gtrA1* and *gtrB1* mutants formed smaller colonies than did wild-type strain 1021. A low growth rate was also observed in liquid LB/MC culture, and the mutants took a longer period to reach saturation stage than did wild-type strain 1021. The doubling times of *gtrA1* and *gtrB1* in LB/MC medium were 4.67±0.86 h and 4.71±0.77 h, respectively, while that of wild-type strain 1021 was 3.24±0.39 h. These results further confirmed that *gtrA1* and *gtrB1* grew slower than the wild-type. In addition, the maximal optical density was lower in the mutants than in the wild-type, being about 4.11 and

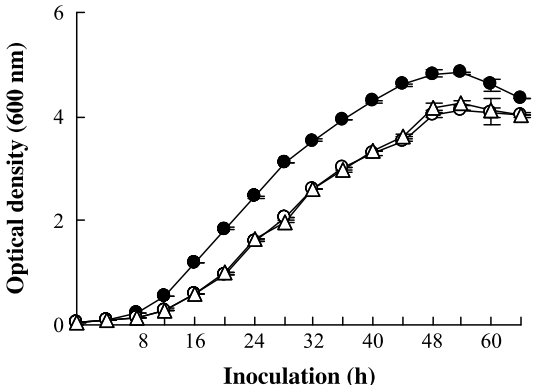
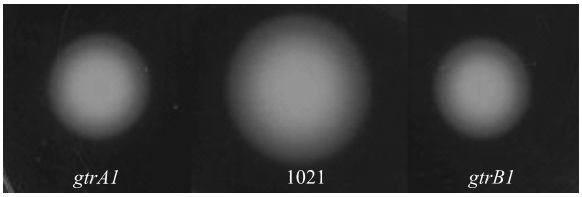


Fig. 3. Growth curve of the wild-type (●), *gtrA1* mutant (○), and *gtrB1* mutant (△). Values are means of optical density determinations of triplicate cultures, and the bars represent standard errors.



Strain	1021	*gtrA1*	*gtrB1*
Colony diameter (mm)	22±0.81	16.6±0.50	16.7±0.60

Fig. 4. Swimming phenotypes of the *gtrA1* and *gtrB1* mutants. Data presented for colony diameter grown for 4 days are means and standard errors of 100 measurements in each experiment.

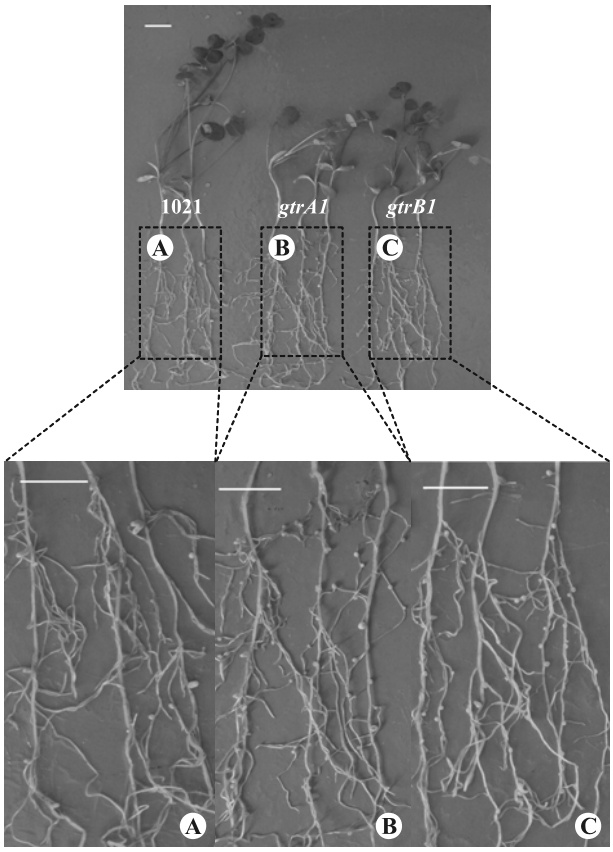


Fig. 5. Symbiotic phenotypes of the *gtrA1* and *gtrB1* mutants (20 days post-inoculation). Close-up of root nodules of the alfalfa plants inoculated with wild-type strain 1021 (A), the mutant *gtrA1* (B), and the mutant *gtrB1* (C) (Bars=1 cm).

4.27 for *gtrA1* and *gtrB1*, respectively, compared to 4.84 for the wild-type strain (Fig. 3). Similar growth patterns of *gtrA1* and *gtrB1* mutants were also observed in the minimal medium ZMGS (data not shown). These results indicate that the disruption of *gtrA* and *gtrB* reduced cell growth.

The motility of mutants was tested because this parameter of rhizobial cells has often been found to affect nodulation efficiency (Ames *et al.*, 1980). Therefore, it was necessary to examine the motility of the mutants. The experiment was conducted on swimming plates with rich medium (LB/MC with 0.3% agar) using wild-type strain 1021 as the control. The colony diameter of 1021 was 22±0.81 mm, while those of *gtrA1* and *gtrB1* were 6.6±0.50 mm and 16.7±0.60 mm, respectively (Fig. 4). The results demonstrated that *gtrA* and *gtrB* may influence cell motility either directly or indirectly.

Symbiotic phenotypes of the *gtrA1* and *gtrB1* mutants

A nodulation assay was carried out in order to explore the functions of *gtrA* and *gtrB* in symbiosis. The two defective mutants were tested for their symbiotic properties on the host plant under greenhouse conditions. The nodules induced by wild-type strain 1021 were normally pink and cylindrical (Fig. 5A), but nodules elicited by the mutants were relatively smaller, more round, and light pink in color, and a great number of small nodules were located in the lateral roots (Fig. 5B and C). During the first 3∼4 weeks post-inoculation, the plants that had been inoculated with the *gtrA1* or *gtrB1* mutants were visibly stunted and had significantly lower fresh weight than those inoculated with strain 1021 at 20 or 30 days post-inoculation. However, the difference in fresh weight between the plants inoculated with mutants and those with strain 1021 shrank at 40 days post-inoculation (Fig. 5 and 6A). These symbiotic defects paralleled with the changes in nitrogenase activity. Acetylene reduction assays showed that the nitrogenase activity of alfalfa inoculated with *gtrA1* or *gtrB1* mutants was much lower than that of those inoculated with strain 1021 at 20 or 30 days post-inoculation, but the gap dwindled gradually at 40 days post-inoculation (Fig. 6B). The changes in the nitrogenase activity of plants inoculated with mutants were consistent with those for the fresh weight of plants inoculated with mutants. These results indicated that mutations of *gtrA* or *gtrB* impaired the nodulation ability. It was simultaneously found that the number of nodules elicited by both mutants increased substantially at 20, 30, and 40 days post-inoculation (Fig. 6C). The abnormally high number of nodules elicited by *gtrA1* and *gtrB1* suggests that both mutants are able to avoid triggering host defense and demolishing the plant host's autoregulation of the nodule number. However, such apparently

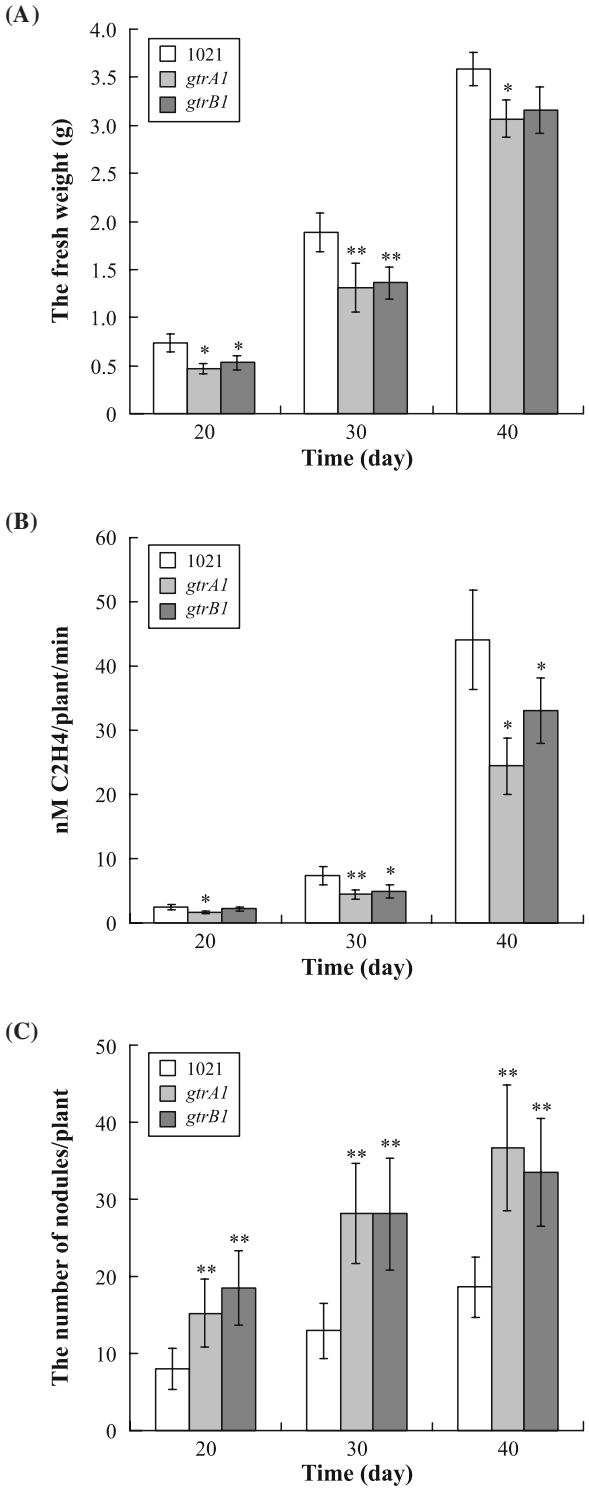


Fig. 6. (A) The fresh weight of plants inoculated with wild-type strain 1021, the *gtrA* and *gtrB1* mutants (B) nitrogenase activities of wild-type strain 1021, the *gtrA*1 and *gtrB1* mutants (C) the number of root nodules of plants inoculated with wild-type strain 1021, the *gtrA1* and *gtrB1* mutants. Each value is the mean of five repeats, with standard error expressed as bars; (*) and (**) indicate significant and highly significant levels for $P<0.05$ and $P<0.01$, respectively, of the difference between the wild-type strain and the mutant.

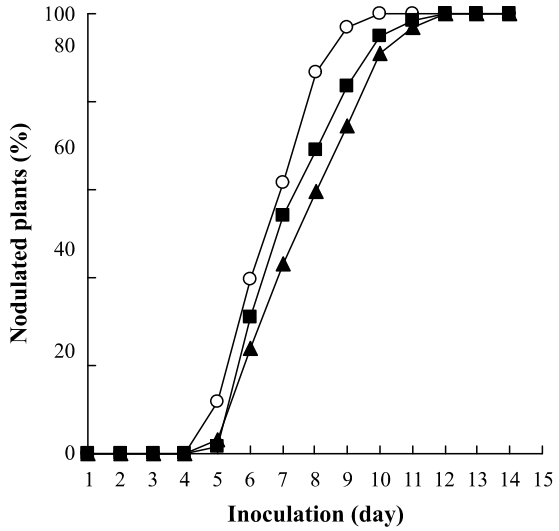


Fig. 7. Nodulation kinetics of wild-type strain 1021 (○), the *gtrA1* mutant (■) and the *gtrB1* mutant (▲). Shown is the percentage of nodulated plants following inoculation with the wild-type strain and mutants. Values are the mean of three independent experiments.

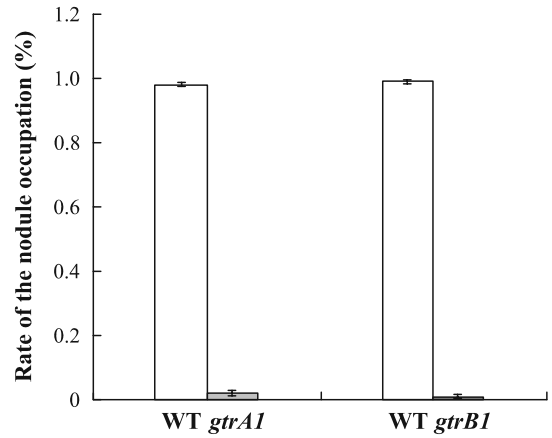


Fig. 8. Nodule occupancy assay. Data represents the percentage of nodules occupied by wild-type strain 1021 (WT, white), the *gtrA1* and *gtrB1* mutants (grey) after inoculation with 1:1 mixtures of 1021 & *gtrA1* and 1021 & *gtrB1*, respectively. Values are the mean of three independent experiments. Error bars represent standard error.

stunted growth at the early stage of nodulation elicited by *gtrA1* and *gtrB1* mutants became indiscernible with the progression of nodulation.

To explore the symbiotic deficiency in the early stage of nodulation induced by the *gtrA1* and *gtrB1* mutants, we compared their nodulation kinetics with those of wild-type strain 1021 on agar plates. The mutants and strain 1021 appear to initiate infections at the same time, but nodule formation induced by *gtrA1* and *gtrB1* mutants emerged 2 days later than those induced by wild-type 1021 (Fig. 7). The delayed nodulation kinetics suggested that both mutants became deficient in their ability to develop infection at the normal rate, rather than in their timing in initiating infections.

In addition, in investigating the association between *gtrA1* or *gtrB1* and alfalfa, it is necessary to estimate the extent to which *gtrA* and *gtrB* altered nodulation. Therefore, we performed the nodule occupancy assay in order to determine the difference in nodulation competitiveness between the mutants and the wild-type. When *gtrA1* or *gtrB1* was co-inoculated with wild-type strain 1021 on alfalfa roots in equal amounts, the nodule occupancy values were 2.02±0.008% and 97.98 ±0.008% for *gtrA1* and the wild-type, and 1.01±0.006% and 98.99±0.006% for *gtrB1* and the wild-type, respectively (Fig. 8). These results showed that mutations of *gtrA* and *gtrB* significantly reduced nodulation competitiveness.

Discussion

Two *gntR* genes, *gtrA* and *gtrB*, were identified in this study. The directional insertion mutation introduced disruption in the two *gntR* genes and generated the *gtrA1* and *gtrB1* null mutants, respectively. Both of the mutants had a slower growth rate and a lower maximal yield than those of the wild-type strain. It is highly likely that *gtrA1* and *gtrB1* were impaired in some unknown metabolic pathways, and that their growth was subsequently affected. Slower growth may result in a defective phenotype in the motility of *gtrA1* and *gtrB1*.

The symbiosis-deficient phenotypes of *gtrA1* and *gtrB1* were highly similar and complicated. In the first 3∼4 weeks post-inoculation, the plants inoculated with the *gtrA1* and *gtrB1* mutants showed slower growth accompanied by lower nitrogenase activity. This indicates that the two mutants did not form efficient symbioses with the host plants. However, at a later stage of nodulation (40 days after inoculation), the difference in growth between the plants inoculated with both mutants and those inoculated with the wild-type strain decreased. This recovery can be explained by the emergence of a great number of small, round, light pink nodules with low nitrogenase activity, which can meet the plant demand for a nitrogen source to a certain extent. We propose that both mutants may be able to elicit a defective effect on nodule number autoregulation so as to induce the formation of an abnormally great number of nodules, which can compensate for the nitrogen fixation function.

Symbiotic defects of *gtrA1* and *gtrB1* were clearly reflected in nodulation competitiveness and nodulation kinetics. Both of the mutants showed marked reductions in nodulation competition, but also showed delayed nodulation. However, both of the mutant cell types were able to reach the infection sites of the host almost simultaneously with the wild-type strain. Late nodule emergence is caused by a delayed response of the infection site to become active, or may be caused by a slower infection process. The lower number of infections can result in reduced nodulation for the mutant *gtrA1* or *gtrB1* compared to the wild-type strain.

The phenotypes that resulted from the disruption of the *gtrA* and *gtrB* genes are highly similar, but their amino acid sequences are only 31% identical, which implies that the similar phenotypes might be caused by changes in different, possibly independent, regulatory mechanisms. GtrA has high homology with the *R. leguminosarum* MatR protein, which regulates the expression of the *matABC* operon involved in malonate metabolism (Lee *et al*., 2000). Moreover, the proteins encoded by the SMa0151 and SMa0150 genes neighboring *gtrA* exhibited the highest similarity to MatA and MatB, respectively. Although no phenotype of *matR* mutation has been reported, growth reduction has been observed in clover plants infected with the Δ*matB R. leguminosarum* bv. *trifolii* mutant (An *et al*., 2002). These analyses imply that the GtrA protein might be an ortholog of the MatR protein. However, GtrB protein has very low homology with the entries in the current database.

The fact that *gtrA1* and *gtrB1* are defective in motility may explain their symbiotic phenotypes. The slow motility can further affect the locations of mutant colonies on alfalfa roots, and may result in delayed development of the infection thread compared to the wild-type. Consequently, most nodules were occupied by the wild-type cells in a co-inoculation assay. Even when the plants were only inoculated with the mutant cells, the nodules formed later did those on plants treated with the wild-type strain.

Pleiotropic effects produced by the disruption of *gtrA* and *gtrB* raise the possibility that GtrA and GtrB can repress gene transcription in response to the absence of some certain metabolites during certain physiological processes. Given the overall impact of GtrA or GtrB on nodule development, it is possible that GtrA or GtrB may act as a repressor of another repressor that is involved in cell growth. Further studies will be directed toward identifying GtrA- and GtrB-regulated genes that can influence cell growth and nodulation. Identification of the molecular mechanisms by which GtrA and GtrB regulate the targeted genes will help to better understand the role of GntR-like transcriptional regulators in symbiosis.

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