The *Rhizobium leguminosarum* bv. *viciae* VF39 γ -aminobutyrate (GABA) aminotransferase gene (*gabT*) is induced by GABA and highly expressed in bacteroids

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A *Rhizobium leguminosarum* bv. *viciae* VF39 gene (*gabT*) encoding a γ -aminobutyrate (GABA) aminotransferase was identified, cloned and characterized. This gene is thought to be involved in GABA metabolism via the GABA shunt pathway, a theoretical bypass of the 2-oxoglutarate dehydrogenase complex. Mutants in *gabT* are still able to grow on GABA as a sole carbon and nitrogen source. 2-Oxoglutarate-dependent GABA aminotransferase activity is absent in these mutants, while pyruvate-dependent activity remains unaffected. This indicates that at least two enzymes with different substrate specificities are involved in the GABA metabolism of *R. leguminosarum* bv. *viciae* VF39. The *gabT* promoter was cloned into a newly constructed, stable promoter-probe vector pJP2, suitable for the study of transcriptional GUS fusions in free-living bacteria and during symbiosis. Under free-living conditions the *gabT* promoter is induced by GABA and repressed by succinate. Transcriptional regulation is mediated by GabR in a repressor-like manner. During symbiosis with the pea host plant *gabT* is induced and highly expressed in the symbiotic zone. Nodules induced by *gabT* mutants, however, are still effective in nitrogen fixation.

Keywords: carbon metabolism, GABA shunt, symbiotic expression, promoter-probe vector, pJP2

INTRODUCTION

During symbiosis between *Rhizobium* spp. and the legume host plant, the bacteria are released into a new environment, the plant cell, in which they differentiate into nitrogen-fixing bacteroids. The bacteroids remain enclosed in a plant-derived peribacteroid membrane, which constitutes a selective barrier between the micro-symbiont and the plant cytosol. Nutrient exchange between the symbiotic partners takes place across this barrier.

C₄-dicarboxylic acids are thought to be the only source of carbon skeletons available to the bacteroids in

sufficient amounts to support the energetically expensive process of nitrogen fixation. These dicarboxylic acids are derived from the plant's glycolysis. Phosphoenolpyruvate (PEP) is carboxylated via PEP carboxylase (EC 4.1.1.31) to oxaloacetate and then further reduced by malate dehydrogenase (EC 1.1.1.37) to malate (Streeter, 1991; Vance & Heichel, 1991). The C₄-dicarboxylic acids are taken up by the bacteroids and presumably directly channelled into the TCA cycle. Atmospheric nitrogen is fixed to ammonia by the nitrogenase enzyme complex, which is expressed in the bacteroids in response to the low oxygen concentration present in the nodule. The majority of the ammonia produced is transported to the plant host cell, where it is assimilated and incorporated into amino acids. Part of the ammonia however appears to be assimilated by the bacteroids themselves in order to maintain their metabolism. Two potential ammonia-assimilating enzymes, alanine dehydrogenase (EC 1.4.1.1) and glutamate dehydrogenase (EC 1.4.1.2) both show high activities in mature bacteroids of *Sinorhizobium meliloti* (Miller *et al.*,

Abbreviations: GABA, γ -aminobutyrate; GUS, β -D-glucuronidase; PEP, phosphoenolpyruvate; PNP, *p*-nitrophenol; SSDH, succinate semialdehyde dehydrogenase; TCA, tricarboxylic acid; X-Gal, 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside; X-Glc, 5-bromo-4-chloro-3-indolyl β -D-glucuronic acid.

The GenBank accession number for the sequence determined in this work is AF335502.

1991). Alanine is, as recently discovered, also an excretion product of anaerobically isolated mature bacteroids in soybean and pea (Waters *et al.*, 1998; Allaway *et al.*, 2000).

The role of glutamate, which generally appears in large quantities in the nodules (Miller *et al.*, 1991; Vance & Heichel, 1991; Salminen & Streeter, 1992), remains unclear. A reason for glutamate accumulation could be the inhibition of the bacterial 2-oxoglutarate dehydrogenase complex during symbiosis. This complex is part of the TCA cycle and converts 2-oxoglutarate to succinyl-CoA. It is sensitive to the redox charge of the cell because of inhibition by the concentration of NADH (Streeter, 1991). The high NADH level in bacteroids is probably due to oxygen limitation, which is also a prerequisite for effective functioning of the bacteroids' nitrogenase (McDermott *et al.*, 1989; Dunn, 1998; Poole & Allaway, 2000). In symbiosis, 2-oxoglutarate could be directly aminated by glutamate dehydrogenase, or transaminated by any aminotransferase using 2-oxoglutarate as the receiving oxoacid. Consistent with this, mutations in the 2-oxoglutarate dehydrogenase complex in *Rhizobium leguminosarum* bv. *viciae* 3841 lead to the synthesis and excretion of large quantities of glutamate (Walshaw *et al.*, 1997).

A possible sink for glutamate in bacteroids includes the GABA shunt pathway. This is a theoretical bypass of the 2-oxoglutarate dehydrogenase complex, in which glutamate is decarboxylated to γ -aminobutyric acid (4-aminobutyrate; GABA) by glutamate decarboxylase (EC 4.1.1.15). The amino residue of GABA is removed by GABA aminotransferase (EC 2.6.1.19) leading to succinate semialdehyde, which is further oxidized to succinate by succinate-semialdehyde dehydrogenase (EC 1.2.1.16). The GABA aminotransferase is a pyridoxal-phosphate-dependent enzyme that transfers the amino residue of GABA to an oxoacid. In the case of 2-oxoglutarate as the receiving oxoacid, this reaction would also cover the first step of the pathway, the amination of 2-oxoglutarate to glutamate. The existence of a GABA shunt pathway in *Rhizobium* spp. has been often discussed (McDermott *et al.*, 1989; Miller *et al.*, 1991; Dunn, 1998; Poole & Allaway, 2000). However, it has never been confirmed to operate during symbiosis, as corresponding mutants were not available.

In this paper, we report the identification, cloning and analysis of the *R. leguminosarum* bv. *viciae* VF39 *gabT* gene, encoding 2-oxoglutarate-dependent GABA aminotransferase. We constructed mutants and analysed *gabT* expression in free-living state and in bacteroids. We show that while *GabT* is not essential for growth on GABA or symbiotic nitrogen fixation, it is specifically induced in the nodule, suggesting a role in bacteroid metabolism.

METHODS

Bacterial strains, plasmids and media. All bacterial strains and plasmids used in this work are listed in Table 1.

Escherichia coli strains were grown at 37 °C in Luria–Bertani medium (Maniatis *et al.*, 1982). *Rhizobium leguminosarum* strains were grown at 29 °C in TY complex medium (Beringer, 1974), Vincent minimal medium (VMM) (Vincent, 1970) or acid minimal salt medium (AMS) (Poole *et al.*, 1994), supplemented with 0.2% (w/v) GABA or glutamate as carbon and nitrogen source. NH_4Cl (0.1%, w/v) was added as nitrogen supply when succinate was given as carbon source.

To adjust the pH, TY media were buffered with 50 mM MES (for pH 5.3 and 6.0), with 50 mM MOPS (pH 7.0) or with 50 mM TABS (pH 8.0). 5-Bromo-4-chloro-3-indolyl β -D-galactopyranoside (X-Gal) was added at a final concentration of 40 $\mu\text{g ml}^{-1}$.

For β -D-glucuronidase (GUS) activity, TY solid media were supplemented with 5-bromo-4-chloro-3-indolyl β -D-glucuronic acid (X-Glc) at a final concentration of 40 $\mu\text{g ml}^{-1}$.

Antibiotics were used as needed for *E. coli* strains at the following final concentrations: ampicillin 100 $\mu\text{g ml}^{-1}$; kanamycin 25 $\mu\text{g ml}^{-1}$; tetracycline 10 $\mu\text{g ml}^{-1}$. For *R. leguminosarum*, antibiotics were added at final concentrations of: 500 $\mu\text{g ml}^{-1}$ (streptomycin); 80 $\mu\text{g ml}^{-1}$ (neomycin) and 10 $\mu\text{g ml}^{-1}$ (tetracycline).

Preparation of cell extracts. Cultures of *R. leguminosarum* VF39 WT or mutant strains were grown overnight on AMS (400 ml) or VMM (250 ml), supplemented with GABA and glutamate and were harvested at an OD_{600} of approximately 0.5 by centrifugation (8000 r.p.m.; 10 min; 4 °C; Sorvall SLA-3000). The pellet was suspended in 50 ml 20 mM phosphate buffer (pH 7.2) and centrifuged again. Then the cells were resuspended in 4–8 ml breaking buffer (40 mM HEPES, 1 mM DTT at pH 7.0) and either crushed twice in a French pressure cell at 70 MPa or sonicated for 3 min at 90 W on ice. Cell debris was removed by centrifugation (17000 r.p.m.; 25 min; 4 °C; Sorvall SS34). The cell extracts were kept on ice and enzyme activities were measured within 2 h after preparation. Protein concentrations were determined by the method of Bradford (1976) and compared to a BSA standard (0–20 $\mu\text{g ml}^{-1}$). Protein concentrations in the prepared cell extracts were between 1 and 3 mg ml^{-1} .

Enzyme assays. 2-Oxoglutarate-dependent GABA aminotransferase was measured with 150 mM GABA and 50 mM 2-oxoglutarate (disodium salt) as substrates, as earlier described by Bartsch (1990). The assay was performed in a final volume of 500 μl , using 250 μl cell extract. Glutamate formation was determined by reduction of NAD^+ in a coupled glutamate dehydrogenase (GDH) assay. In a final volume of 1.5 ml [40 mM hydrazine; 50 mM glycine (pH 7.6); 2.7 mM NAD^+ ; 3 units GDH (Sigma, G-2626)], the complete GABA aminotransferase sample (500 μl) was incubated for 1.5 h at 37 °C. NADH production was measured at 340 nm in a spectrophotometer. Assays were done in duplicate with a 'minus enzyme' control for each extract. Results were compared to a standard curve (with 0–120 nmol glutamate per sample in duplicate), prepared freshly every day.

Pyruvate-dependent GABA aminotransferase activity was measured with 150 mM GABA and 50 mM pyruvate (sodium salt) as substrates. The assay was performed as described for 2-oxoglutarate-dependent GABA aminotransferase with the following changes. Ten microlitres of cell extract was used in a final assay volume of 500 μl . Alanine production was measured in a final volume of 1.5 ml [40 mM hydrazine; 50 mM glycine (pH 9.0); 2.7 mM NAD^+ ; 0.5 units alanine dehydrogenase (Sigma, A-7653)].

Table 1. Bacterial strains and plasmids

Strain or plasmid	Description	Reference
Bacteria		
S17-1	<i>E. coli</i> 294::[RP4-2(Tc::Mu) (Km::Tn7)] <i>pro res ΔrecA</i> , Tc ^r	Simon <i>et al.</i> (1983)
VF39	<i>R. leguminosarum</i> bv. <i>viciae</i> wild-type, Sm ^r	Priefer (1989)
VF39-TP2	VF39 <i>ΔlacZ</i> , Sm ^r Tc ^r	T. Patchkowski, RWTH-Aachen
PH10	VF39-TP2, <i>gabT</i> ::Tn5-B20	V. Lipka, RWTH-Aachen
VF39-19T	pK19mob <i>gabT</i> plasmid integration mutant	This work
VF39-18R	pK18mob <i>gabR</i> plasmid integration mutant	This work
Plasmids		
pWKR102A	<i>mob</i> , Gm ^r Cm ^r	Colonna-Romano <i>et al.</i> (1990)
pWKR102A::Tn5-B20/8	pWKR102::Tn5-B20	V. Lipka, RWTH-Aachen
pSVB28	pUC8 derivative with new polylinker region, Ap ^r	Arnold & Pühler (1988)
pSVB28-PH10 <i>Sall</i>	pSVB28 with 10·7 kb <i>Sall</i> fragment <i>gabT</i> ::Tn5-B20	This work
pTR102	Mini-RK2, Ap ^r Tc ^r	Weinstein <i>et al.</i> (1992)
pTR102-d	Deletion of pTR102, Ap ^r Tc ^r	This work
pCCOGUS	pUC19 derivative with a promoterless GUS cartridge, Ap ^r	Axelos <i>et al.</i> (1989)
pTR102-GUS	pTR102-d with promoterless GUS cartridge, Ap ^r Tc ^r	This work
pJP2	pTR102 GUS with artificial MCS, Ap ^r Tc ^r	This work
pJP2- <i>gabT</i>	pJP2 with a 572 bp fragment containing the <i>gabT</i> promoter	This work
pK18/19mob	pK18/19 derivatives, <i>mob</i> , Km ^r	Schäfer <i>et al.</i> (1994)
pK19mob- <i>gabT</i>	pK19mob with a 394 bp <i>Ecl136II/EcoRI gabT</i> fragment	This work
pK18mob- <i>gabR</i>	pK18mob with a 392 bp <i>Sall/HindIII gabR</i> fragment	This work

Succinate-semialdehyde dehydrogenase (SSDH) was measured with 2·5 mM succinate semialdehyde and 1 mM NAD⁺ as substrates (Laura Green, University of Missouri, personal communication). The assay was performed in 50 mM Tris buffer at pH 8·0, 1 mM DTT and 10 mM KCN, to avoid reoxidation of NADH by cytochrome *c* oxidase. Reduction of NAD⁺ was measured spectrophotometrically at 340 nm.

GUS and *lacZ* activity assays. Precultures grown on TY medium were harvested and transferred to VMM containing the different substrates. The cultures were started at an OD₆₀₀ of 0·2 and showed a basic level of induction of about 100 nmol *p*-nitrophenol (PNP) min⁻¹ per OD₆₀₀ unit (Fig. 3, *t* = 0). GUS was assayed as previously described by Boesten *et al.* (1998). After 20 min the assay was stopped and cleared of cell debris by centrifugation (13 000 r.p.m.; 10 min). The release of PNP was measured spectrophotometrically at 415 nm. Enzyme activities were calculated and expressed in nmol PNP min⁻¹ per OD₆₀₀ unit.

β-D-Galactosidase (*lacZ*) was assayed similarly, with 50 μl *o*-nitrophenyl *β*-D-galactopyranoside. *o*-Nitrophenol was detected at 420 nm.

DNA techniques. Standard DNA techniques were carried out according to Maniatis *et al.* (1982). Total DNA was prepared by the CTAB method described by K. Wilson in Ausubel *et al.* (1987). Plasmid DNA from cultures was purified with the NucleoSpin Plasmid kit (Macherey-Nagel). DNA fragments from agarose gels were isolated using the NucleoSpin Extract kit (Macherey-Nagel) according to the manufacturer's instructions.

Construction of the pJP2 promoter-probe vector. The basis of the pJP2 promoter-probe vector is the broad-host-range, mobilizable plasmid pTR102 (Weinstein *et al.*, 1992), a mini-RK2 derivative, containing *par* functions ensuring plasmid stability.

First, the *Bam*HI, *Pst*II and *Hind*III restriction sites of pTR102 were deleted by *Bam*HI and *Hind*III restriction, Klenow fill-in and religation, leading to vector pTR102-d. Then a promoterless *uidA* gene (GUS) from pCCOGUS (Axelos *et al.*, 1989) was cloned into the *Pst*II and *Sac*I sites of pTR102-d, leading to pTR102-GUS. An artificial multiple cloning site was inserted into the *Nco*I and *Kpn*I sites of pTR102-GUS. Two complementary oligonucleotides with the following sequences were synthesized (MWG-Biotech): 5'-CAT GGA TCC AAG CTT CTC GAG CTC TAG ACT GAG GTA AT-3' and 5'-CAT GAT TAC CTC AGT CTA GAG CTC GAG AAG CTT GGA TCC ATG GTA C-3' (Fig. 1b). The fragment contains a ribosome-binding sequence (GAGG, 5 bp upstream of the ATG), which was a consensus compiled from a number of known *R. leguminosarum fix* and *nif* genes. Stop codons were introduced in all reading frames to prevent translational gene fusions to the GUS protein. Finally several single-cutting restriction sites were added to facilitate cloning of DNA fragments.

The resulting plasmid pJP2 (Fig. 1a) was tested for background levels of GUS activity in *R. leguminosarum* VF39 under free-living conditions in TY medium and during symbiosis with the pea host plant. In both cases only negligible activities were observed, which demonstrated the absence of vector promoters reading into the *uidA* gene.

Sequence analyses. Appropriate restriction sites within the *gabT* region were used to clone 300–800 bp fragments into vector pUC19. Sequencing of the resulting recombinant plasmids was done for both strands by the chain-termination method using a Thermo Sequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech) and IRD700/800 labelled standard primers (universal and reverse) according to the manufacturer's instructions. Extension products were separated and detected on a LI-COR 4200L sequencer. An IRD700-labelled IS50

Fig. 1. Physical and genetic map of the pJP2 promoter-probe vector. (a) The vector encodes tetracycline (*tetR/tetA*) and ampicillin (*bla*) resistance. *trfA*, RK2 replication initiation gene; *oriV*, RK2 origin of vegetative replication; *oriT*, RK2 origin of transfer. The *parCBA* and *parDE* operons encode genes that ensure plasmid stability in the absence of antibiotic selection. *uidA*, *E. coli* GUS gene. (b) Oligonucleotides used to introduce stop codons (bold) and single-cutting restriction sites (underlined) which were added to facilitate the cloning of DNA fragments. The ATG start codon of the GUS gene (shaded, in 3'-direction) is part of the GTAC 5'-overhang (right side). The *NcoI* restriction site (right side) was not reconstituted upon cloning into pTR102-GUS.

Fig. 2. Organization of the 2.5 kb *SalI* fragment containing *gabT* and construction of the plasmid integration mutants VF39-18R and VF39-19T. (a) Physical and genetic map of the *gabRTD* region on the 2535 bp *SalI* fragment. The site of the Tn5-B20 insertion in mutant PH10 is marked by a triangle. Additional *Sau3AI* and *EcoRI* sites between bp 606 and 2535 are not indicated. (b) The 572 bp *Sau3AI* fragment was used for promoter studies in promoter-probe vector pJP2-*gabT*. (c) The 392 bp *SalI/HindIII* fragment was cloned into pK18mob (pK18mob-*gabR*) to generate the plasmid integration mutant VF39-18R. (d) The 394 bp *EcoRI/EcoRI* fragment was cloned into pK19mob (pK19mob-*gabT*) to generate the plasmid integration mutant VF39-19T.

primer (5'-CGG GAA AGG TTC CGT TCA GGA CG-3') was used to sequence fragments flanking the Tn5-B20.

Nucleotide sequence comparisons were done by the NCBI BLAST2 package (Altschul *et al.*, 1997) against the NCBI-nr database.

Construction of plasmid integration mutants. A 394 bp *EcoRI/EcoRI* fragment (Fig. 2d) was used for the con-

struction of the *gabT* integration mutant VF39-19T. The fragment was cloned into the *SmaI/EcoRI* sites of pK19mob, resulting in pK19mob-*gabT*. The plasmid was transferred from *E. coli* S17-1 to *R. leguminosarum* VF39 and recombined into the genomic *gabT* region via single crossover. Total DNA of the resulting clones was digested with *SalI* and with *EcoRI*, separated by gel electrophoresis, transferred onto Qia-brane nylon membranes (Qiagen) by vacuum blotting (Pharmacia)

and correct integration was verified by Southern hybridization at 68 °C with a PCR-derived (using universal and reverse primers and pK19mob-*gabT* as template) and digoxigenin-11-dUTP-labelled *gabT* probe (DIG DNA Labelling Kit; Boehringer). The integration mutant was designated VF39-19T and used for further studies.

To generate a *gabR* mutant, a 392 bp *Sall/HindIII* fragment (Fig. 2c) was cloned into the *Sall/HindIII* sites of pK18mob, yielding pK18mob-*gabR*. After plasmid integration into the VF39 genome, the corresponding mutant VF39-18R was isolated and correct mutagenesis was tested by Southern hybridization.

Plant cultivation, thin sections and staining. The pea *Pisum sativum* cv. Rondo was used as a host plant in this work. Seeds were sterilized in concentrated sulphuric acid for 15 min, washed three times with sterile water and germinated on sterile vermiculite. Five seedlings were then transferred to sterile vermiculite in 10 × 10 cm plastic boxes and grown for 3 d.

Bacteria were pre-grown on solid medium in the presence of the appropriate antibiotics. A loop of cells was resuspended in 3 ml sterile water and used for inoculation. Plants were grown at 22 °C under artificial light for 16 h a day.

Nodules were harvested after 28 d and prepared for GUS activity assays and histochemical staining.

Bacteroid suspensions for GUS assays were prepared as follows. Five to ten fresh nodules were collected in 1 ml 50 mM sodium phosphate buffer (pH 7.0). The nodules were crushed with an appropriately shaped metal rod. Plant tissue was removed by centrifugation (5 min; 1250 r.p.m.). The supernatant was cleared (5 min; 4500 r.p.m.) and the pellet resuspended in 500 µl sodium phosphate buffer. The resulting bacteroid suspension was directly used for GUS activity assays as described above.

For thin sectioning, fresh nodules were sliced under 50 mM sodium phosphate buffer (pH 7.0) into 80 µm sections using a Leica VT1000S vibratome. The sections were incubated in staining buffer (50 mM sodium phosphate buffer pH 7.0; 0.1% Triton X-100; 5 mM K₃[Fe(CN)₆]; 5 mM K₄[Fe(CN)₆]) containing 0.02% X-Glc until clear blue staining appeared. For strongly expressed fusions the staining time was about 30–60 min. The reaction was stopped by fixing the sections in 1.25% glutaraldehyde in 50 mM sodium phosphate buffer (pH 7.0). Pictures were taken on an Olympus B201 bright-field microscope.

RESULTS

Cloning and identification of the *R. leguminosarum* bv. *viciae* VF39 GABA aminotransferase gene

A *R. leguminosarum* bv. *viciae* VF39 derivative with reduced *lacZ* background activity (VF39-TP2; T. Patschkowski, RWTH-Aachen) was mutagenized with transposon Tn5-B20 (Simon *et al.*, 1989). This transposon carries a promoterless *lacZ* gene at the end of the left IS50 border. Integration of the Tn5-B20 into a gene may produce a transcriptional fusion to *lacZ* and thus allow the expression of the interrupted gene to be studied.

In an initial approach to identify promoters and genes induced under acidic and/or alkaline conditions, about

11 000 mutants were tested for growth and expression on solid TY medium at different pH values (pH 5.3–8.0). None of them was impaired in growth.

One mutant, PH10, showed a more intense blue staining on media containing X-Gal at pH 5.3 and was selected for further investigations. In liquid TY medium *lacZ* expression was induced twofold at pH 5.3 when compared with the expression level at pH 6.0 (V. Lipka, RWTH-Aachen, unpublished).

The Tn5-B20 transposon (8.2 kb) was cloned from the genome of mutant PH10 and was found to be located on a 10.7 kb *Sall* fragment (pSVB28-PH10*Sall*). After restriction analyses and further subcloning, the 2.5 kb of chromosomal DNA flanking the transposon insertion were sequenced entirely. The sequence has been deposited at the NCBI GenBank (accession no. AF335502).

An ORF encoding 426 aa interrupted by the Tn5-B20 insertion showed good homologies to several 4-aminobutyrate (GABA) aminotransferase genes and was therefore designated as *gabT*. The best homology was found to the *Mesorhizobium loti* mlr5521 gene annotated as '4-aminobutyrate aminotransferase' (Kaneko *et al.*, 2000; NCBI accession no. NP106172), which encodes a protein 81% identical over 429 aa to the *R. leguminosarum* *gabT* gene product. The *E. coli* *goaG* gene, located on the Kohara clone #257 at min 29.1–29.6 of the *E. coli* K-12 chromosome and annotated as 'GABA or ornithine aminotransferase' (Jovanovic & Model, 1997; NCBI accession no. P50457), showed the second-best result at the protein level, with 61% identity over 421 aa, and the third-best similarity was found with the *Pseudomonas aeruginosa* PAO1 *gabT* locus (Stover *et al.*, 2000; NCBI accession no. AAG03655), exhibiting 57% identity over 426 aa. A second *E. coli* 'GABA aminotransferase' gene, located on the Kohara clone #443 at min 59.8–60.2 of the K-12 chromosome (NCBI accession no. P22256), showed the fourth-best homology. The identity was 56% over 426 aa. This gene is part of the *gabCDTP* region (Bartsch *et al.*, 1990), which encodes gene products responsible for uptake (*gabP*) and degradation (*gabDT*) of GABA.

Downstream of the *R. leguminosarum* *gabT* gene, the 5'-end of another ORF was discovered, encoding a putative protein with homologies to the N-terminus of several SSDHs (data not shown); SSDH is the next enzyme in the GABA shunt pathway. This ORF was therefore designated as *gabD'* (Fig. 2). A third ORF identified upstream of *gabT* and orientated in the opposite direction showed homologies to *merR*-type transcriptional regulators and thus may specify a regulator for *gabTD* expression. It was tentatively named as *gabR'*.

The phenotype of *gabT* mutants

The *R. leguminosarum* PH10 Tn5-B20 mutant was tested for growth in liquid VMM containing GABA as sole carbon and nitrogen source. Growth rates of the

Table 2. Phenotypes of *R. leguminosarum* VF39 and several mutants

Phenotype	VF39	PH10	VF39-19T	VF39-18R
Growth in liquid VMM+GABA (doubling time, h)*	16.0 (± 1.8)	16.0 (± 1.8)	17.8 (± 0.6)	14.3 (± 0.9)
2-Oxoglutarate-dependent GABA aminotransferase activity [nmol glutamate min ⁻¹ (mg protein) ⁻¹] [†]	1.32 (± 0.38)	−0.09 (± 0.20)	0.09 (± 0.15)	6.82 (± 1.17)
Pyruvate-dependent GABA aminotransferase activity [nmol alanine min ⁻¹ (mg protein) ⁻¹] [†]	213 (± 33)	227 (± 27)	203 (± 22)	148 (± 17)
SSDH activity [nmol NADH min ⁻¹ (mg protein) ⁻¹] [†]	182 (± 17)	199 (± 32)	155 (± 26)	228 (± 22)

* Growth rates were calculated from cultures (VMM, 0.2% GABA) in the exponential growth phase. The data are means from three independent cultures, with SD in parentheses.

[†] Enzyme activities are means from four independent cultures (VMM, 0.2% GABA + glutamate), with SD in parentheses.

mutant showed no difference from the wild-type (Table 2).

The ability of PH10 to utilize GABA as a sole carbon and nitrogen source posed the question of a second GABA aminotransferase gene in this strain. Therefore the enzyme activity of the GABA aminotransferase, using GABA as substrate and either 2-oxoglutarate or pyruvate as amino-accepting oxoacid, was tested in mutant and wild-type. Both activities could be demonstrated in the wild-type strain. The *gabT* mutant showed a total absence of 2-oxoglutarate-dependent GABA aminotransferase activity (Table 2), suggesting that the identified gene indeed encodes the only 2-oxoglutarate-dependent GABA aminotransferase in *R. leguminosarum* VF39. Pyruvate-dependent GABA aminotransferase activity, however, was not affected by the *gabT* mutation. This demonstrates the existence of a second gene encoding a pyruvate-dependent GABA aminotransferase with no 2-oxoglutarate-dependent activity.

To confirm that the absence of 2-oxoglutarate-dependent GABA aminotransferase from the PH10 extracts was indeed due to the mutation in the *gabT* gene, a plasmid integration mutant (VF39-19T) was generated. This mutant showed the same phenotypes as the PH10 strain.

To see if the downstream *gabD* gene is similarly affected by the *gabT* mutation, SSDH activity was also measured. The wild-type strain and all mutants showed similar SSDH levels (Table 2).

In symbiosis with the host *P. sativum* both *gabT* mutants produced normal, pink nodules and showed nitrogen fixation activities comparable to the wild-type.

Free-living and symbiotic expression of *gabT*

To study *gabT* regulation, a 572 bp *Sau3AI* fragment (Fig. 2b) containing the putative *gabT* promoter region was cloned into the *Bam*HI site of the promoter-probe vector pJP2. The *Sau3AI* fragment contains the complete intergenic region between *gabT* and the predicted *gabR'* gene. It also includes 77 nucleotides of the 5'-end of the *gabT* coding region.

Fig. 3. Induction of the *gabT* promoter in wild-type VF39. (a) GUS activity was monitored over a period of 27 h in liquid cultures (VMM, 0.2% of different carbon and nitrogen sources: ▲, GABA; ■, GABA + glutamate; ◆, GABA + succinate; □, glutamate; ◇, succinate + ammonium). (b) Corresponding growth curves. Data are means from three independent cultures. Standard deviation never exceeded 16% of the mean.

The fusion plasmid pJP2-*gabT* was transferred into *R. leguminosarum* VF39 and the induction of the *gabT* promoter was monitored during growth on different carbon and nitrogen sources. As shown in Fig. 3, GABA, as the substrate for the GABA aminotransferase, induces transcription of *gabT*, although only transiently. An initial threefold induction was observed, which declined to the basal level within 24 h. Glutamate, which is thought to be an intermediate in the GABA shunt pathway and a possible precursor of GABA, did not

induce the *gabT* promoter and neither did succinate, the end-product of the pathway. Succinate, however, caused a reduction of the basal level of transcriptional activity.

Putrescine, which has a structural similarity to GABA, was also found to induce the *gabT* promoter, but it did not support growth as a sole carbon source (data not shown). Ornithine, as a possible precursor of putrescine, or as an alternative substrate for the aminotransferase, supported good growth, but did not mediate induction of the *gabT::uidA* gene fusion (data not shown).

The effect of dual carbon sources was also examined. In combination with GABA, glutamate led to prolonged expression of *gabT* (Fig. 3), compared to the transient expression with GABA alone. Succinate in combination with GABA decreased the induction seen with GABA alone.

The expression of the *gabT* promoter was also studied during symbiosis with pea as a host plant. It was found to be induced in the proximal part of the infection zone (II), next to interzone II–III, and strongly expressed in the late symbiotic zone (results not shown). The symbiotic activity of the *gabT::uidA* fusion was 813 ± 63 nmol PNP min⁻¹ per OD₆₀₀ unit (mean $\pm$ SD; $n = 3$), which is comparable to the magnitude of maximum free-living induction by GABA and glutamate (Fig. 3).

Role of *gabR* in *gabT* expression

To investigate a possible function of GabR in *gabT* regulation, a plasmid integration mutant of *gabR* (VF39-18R) was constructed (see Methods). When pJP2-*gabT* was introduced into this mutant derivative, deep blue colonies were formed on TY plates containing X-Glc, while the wild-type carrying this plasmid produced only slightly bluish colonies after several days of growth. Quantitative measurements of GUS activity confirmed a constitutively high activity of the *gabT* gene fusion in the *gabR* mutant background. Overnight cultures in liquid TY medium resulted in GUS activities of about 700 nmol PNP min⁻¹ per OD₆₀₀ unit, whereas the wild-type showed only about 100 nmol PNP min⁻¹ per OD₆₀₀ unit (Fig. 3; $t = 0$). Consistent with this, 2-oxoglutarate-dependent GABA aminotransferase activity in protein extracts from the mutant was about five times higher than in the wild-type (Table 2). These results indicate that *gabT* expression is significantly enhanced in a *gabR* mutant background and that GabR acts as a repressor modulating *gabT* transcription.

When tested for growth in VMM liquid medium containing GABA as a sole nitrogen and carbon source, the *gabR* mutant VF39-18R showed no difference from the wild-type or the *gabT* mutants (Table 2).

DISCUSSION

In this work we have identified and characterized the *gabT* gene from *R. leguminosarum* bv. *viciae* VF39. The *gabT* gene encodes an 2-oxoglutarate-dependent GABA

aminotransferase, an enzyme of the GABA shunt pathway, which is a theoretical bypass for the 2-oxoglutarate dehydrogenase complex. Although homology comparisons with the *R. leguminosarum* *gabT* sequence revealed several putative GABA aminotransferases in the databases, published work on the genes and mutants in bacteria is rare. Except for the *gabT* gene of *E. coli* K-12 (Bartsch *et al.*, 1990), most of the data encountered are annotations from various genome projects. This is the first report of a well-characterized mutant in one of the GABA shunt enzymes in the genus *Rhizobium*.

E. coli K-12 wild-type strains (CS101A and CS8A) have been found to be unable to grow on GABA as a sole source of carbon and nitrogen (Dover & Halpern, 1971). However, UV-induced mutants are able to use GABA as a sole nitrogen source, and in these mutants GABA aminotransferase and SSDH activity were both increased six- to ninefold. These mutants were proposed to be regulatory mutants. A second generation of UV-induced mutations permitted growth on GABA as sole source of carbon and nitrogen (Dover & Halpern, 1971), and led to increased uptake of GABA. These results indicate that in *E. coli* K-12 the GABA shunt can serve as a metabolic pathway for GABA degradation.

In contrast to *E. coli*, *R. leguminosarum* bv. *viciae* VF39 grows on GABA as a sole source of carbon and nitrogen. The *gabT* mutants are not impaired in growth on GABA even though the activity of 2-oxoglutarate-dependent GABA aminotransferase is absent. However, pyruvate-dependent GABA aminotransferase activity is unaffected by the *gabT* mutation and may be responsible for continued growth on GABA. This implicates the presence of at least two genes encoding GABA aminotransferases with distinct substrate specificities in *R. leguminosarum* bv. *viciae*.

Enzyme activities of 2-oxoglutarate and pyruvate-dependent GABA aminotransferase were earlier presented from *Sinorhizobium meliloti* bacteroids by Miller *et al.* (1991) and from *Bradyrhizobium japonicum* bacteroids by Kouchi *et al.* (1991). The question of whether one or two enzymes were responsible for the different activities was not solved in these reports.

The pyruvate-dependent GABA aminotransferase activity in the *gabT* mutants of *R. leguminosarum* VF39 maintains a functioning GABA shunt pathway. The GABA shunt has often been discussed to play a role in rhizobial carbon metabolism during symbiosis, as the 2-oxoglutarate dehydrogenase complex is thought to be inhibited by excess of reducing equivalents under the oxygen-limiting conditions present in the nodule (McDermott *et al.*, 1989; Vance & Heichel, 1991; Dunn, 1998; Poole & Allaway, 2000).

Miller *et al.* (1991) reported relatively high GABA concentrations [31 nmol (mg protein)⁻¹] in bacteroids of *S. meliloti*. Similarly, Vance & Heichel (1991) found GABA to be present in whole root nodules at concentrations of about 400–8000 nmol (g fresh wt)⁻¹. They further discussed a role for GABA in regulation of

cytoplasmic pH during microaerobic and anaerobic conditions. Assuming that HCO_3^- serves as a substrate for nodule PEP carboxylase, acidosis resulting in excess H^+ produced during hydration of dark CO_2 fixation could be toxic to host cells. Decarboxylation of glutamate to GABA could therefore be a significant sink for protons. This could explain why the *gabT* transposon mutant described here was initially identified by a screen for genes induced at low pH values.

To investigate *gabT* expression in *R. leguminosarum* bv. *viciae* during symbiosis, we constructed a new promoter-probe vector pJP2, which is maintained stably even in the absence of selective pressure. Vector stability is traditionally tested via re-isolation of rhizobia from crushed nodules and plating on selective media (Weinstein *et al.*, 1992; Dombrecht *et al.*, 2001). However, for *S. meliloti* the differentiation from free-living bacteria to bacteroids is irreversible and bacteroids do not regenerate viable cells on growth medium (McRae *et al.*, 1989). Re-isolation from crushed nodules only results in growth of free-living or transforming bacteria but not bacteroids. We therefore believe that plasmid loss can be best visualized in stained thin sections of whole nodules, and it was never observed in the case of pJP2.

In pea nodules, the *R. leguminosarum* VF39 *gabT* promoter is induced directly before interzone II–III, and the gene is highly expressed throughout the late symbiotic zone III. This suggests a role of the GABA aminotransferase in the metabolism of the bacteroids. During symbiosis with the pea host, however, *gabT* mutants (PH10 and VF39-19T) did not show any phenotypes distinctly different from VF39. Also, expression of the *gabT::uidA* fusion in the mutant VF39-19T was not affected on the plant (data not shown).

To evaluate the possible role of the GABA shunt in bacteroid metabolism, a double mutant in both GABA aminotransferases may be required. Therefore a *gabT* deletion mutant is now being used as a recipient in a second transposon mutagenesis. Screening for mutants unable to grow on GABA may lead to a strain affected in pyruvate-dependent GABA aminotransferase activity.

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