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GuaB Activity Is Required in *Rhizobium tropici* During the Early Stages of Nodulation of Determinate Nodules but Is Dispensable for the *Sinorhizobium meliloti*–Alfalfa Symbiotic Interaction

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The *gua*B mutant strain *Rhizobium tropici* CIAT8999-10T is defective in symbiosis with common bean, forming nodules that lack rhizobial content. In order to investigate the timing of the *gua*B requirement during the nodule formation on the host common bean by the strain CIAT899-10.T, we constructed gene fusions in which the *gua*B gene is expressed under the control of the symbiotic promoters *nod*A, *bac*A, and *nif*H. Our data indicated that the *gua*B is required from the early stages of nodulation because full recovery of the wild-type phenotype was accomplished by the *nod*A-*gua*B fusion. In addition, we have constructed a *gua*B mutant derived from *Sinorhizobium meliloti* 1021, and shown that, unlike *R. tropici,* the *gua*B *S. meliloti* mutant is auxotrophic for guanine and induces wild-type nodules on alfalfa and *Medicago truncatula*. The *gua*B *R. tropici* mutant also is defective in its symbiosis with *Macroptilium atropurpureum* and *Vigna unguiculata* but normal with *Leucaena leucocephala*. These results show that the requirement of the rhizobial *gua*B for symbiosis is found to be associated with host plants that form determinate type of nodules.

Additional keywords: nodule development.

Rhizobia establish symbiosis with legumes by eliciting the formation of nitrogen-fixing root nodules. This new organ develops from cell division foci triggered within young root cortical layers by signals produced by the bacteria. The signal molecules, named Nod factors, have been identified as lipochitin oligosaccharides of β1,4-linked N-acetyl-D-glucosamine having diverse chemical substitutions. The bacterial nodulation genes (*nod*) are responsible for Nod factor production and secretion. Once the plant recognizes the specific Nod signal, the plant follows a predetermined developmental pathway to form the nodule, which falls into two different types: indeterminate and determinate. Temperate legumes such as pea and alfalfa form indeterminate nodules, which are cylindrical in shape, with a persistent apical meristem responsible for nodule growth. Tropical legumes such as soybean and common bean usually form determinate nodules, which generally are spherical structures with a nonpersistent meristem. In both cases, the bacteria infect the nodule through an infection thread and enter the cytoplasm of the host cortical cells in an endocytic-like process, in which cells differentiated into bacteroids became surrounded by the peribacteroid membrane. The culmination of differentiation is the formation of cells that produce the complex nitrogenase enzyme and other important proteins that allow molecular nitrogen to be fixed as ammonia (Long 2001; Oke and Long 1999).

Although our knowledge at the molecular level of the early (i.e., nod genes and Nod factors) and late (i.e., nitrogen fixation) stages of infection is quite satisfactory, little is known about intermediate steps that correspond to nodule cell infection, release, and bacteroid differentiation. *Sinorhizobium meliloti bac*A mutants are symbiotically defective, in that the bacteria fail to be released from the infection thread (Glazebrook et al. 1993; Ichige and Walker 1997). Bacteroids of *dct*A mutants undergo degradation prior to maturation (Engelke et al. 1989). We have reported that *gua*B mutants derivative of *Rhizobium tropici* CIAT899 induce defective nodulation in common bean, in which nodule cells appear to be devoid of bacteria. Also, growth of these mutants was found to be sensitive to high temperature (Riccillo et al. 2000). The *gua*B gene encodes enzyme inosin monophosphate dehydrogenase (IMPd) which participates in the metabolic pathway of guanosine biosynthesis by converting inosin 5′-monophosphate into xanthosine 5′-monophosphate. The *gua*B *R. tropici* mutants do not require guanine for growth due to an alternative pathway via the enzyme xanthine dehydrogenase (Xdh) (Riccillo et al. 2000).

Here, symbiotic promoters which activate at different stages of the plant–bacteria interaction were used in order to control expression of the *gua*B gene during nodule development and, therefore, be able to assess the timing of *gua*B requirement for successful nodulation. Our results have shown that, in order to restore the wild-type symbiotic phenotype, *gua*B expression is required from early steps of nodule formation. Furthermore, *gua*B expression under the control of the *bac*A promoter, which controls intermediate events of nodulation, supports partial complementation of the defective phenotype shown by the GuaB$^-$ mutant. In addition, we examined the significance of the rhizobial *gua*B in different plant–bacteria associations, and found that mutations in the *gua*B of *S. meliloti* do not affect nodulation or nitrogen fixation in host plants alfalfa and

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Medicago truncatula. Unlike *R. tropici gua*B mutants, the *S. meliloti gua*B were found to be auxotrophic for guanine.

RESULTS

Expression of the *R. tropici gua*B gene under the control of symbiotic promoters.

In order to have the expression of the *R. tropici gua*B gene under the control of symbiotic promoters that activate at different stages of the host plant–rhizobia interaction, we cloned the coding sequence of *gua*B downstream from the promoters of genes *nod*A, *bac*A, and *nif*H. *nod*A promoter is activated at the very early stage of interaction when flavonoids released by the root hairs together with the rhizobial regulatory protein NodD promote expression of nodulation genes such as those of the *nod*ABC operon (Sharma and Signer 1990; Schlaman et al. 1992). The expression of the *bac*A gene and the *nif*H gene take place after the bacteria enter into the plant cell. The *bac*A gene of *S. meliloti* is expressed in the region of the nodule where bacteroids are formed and is required for differentiation (Glazebrook et al. 1993; Oke and Long 1999), whereas the *nif* genes, encoding for the nitrogenase proteins required for nitrogen fixation, are late-symbiosis genes which are induced during the ultimate steps of nodule development (Cermola et al. 2000; Taté et al. 1999).

The promoter sequences were polymerase chain reaction (PCR) amplified by using specific designed primers as described below, followed by cloning them upstream from the *gua*B coding sequence. In addition, a promoterless *gus*A gene sequence was cloned at the 3′ end of the *gua*B gene sequence in order to

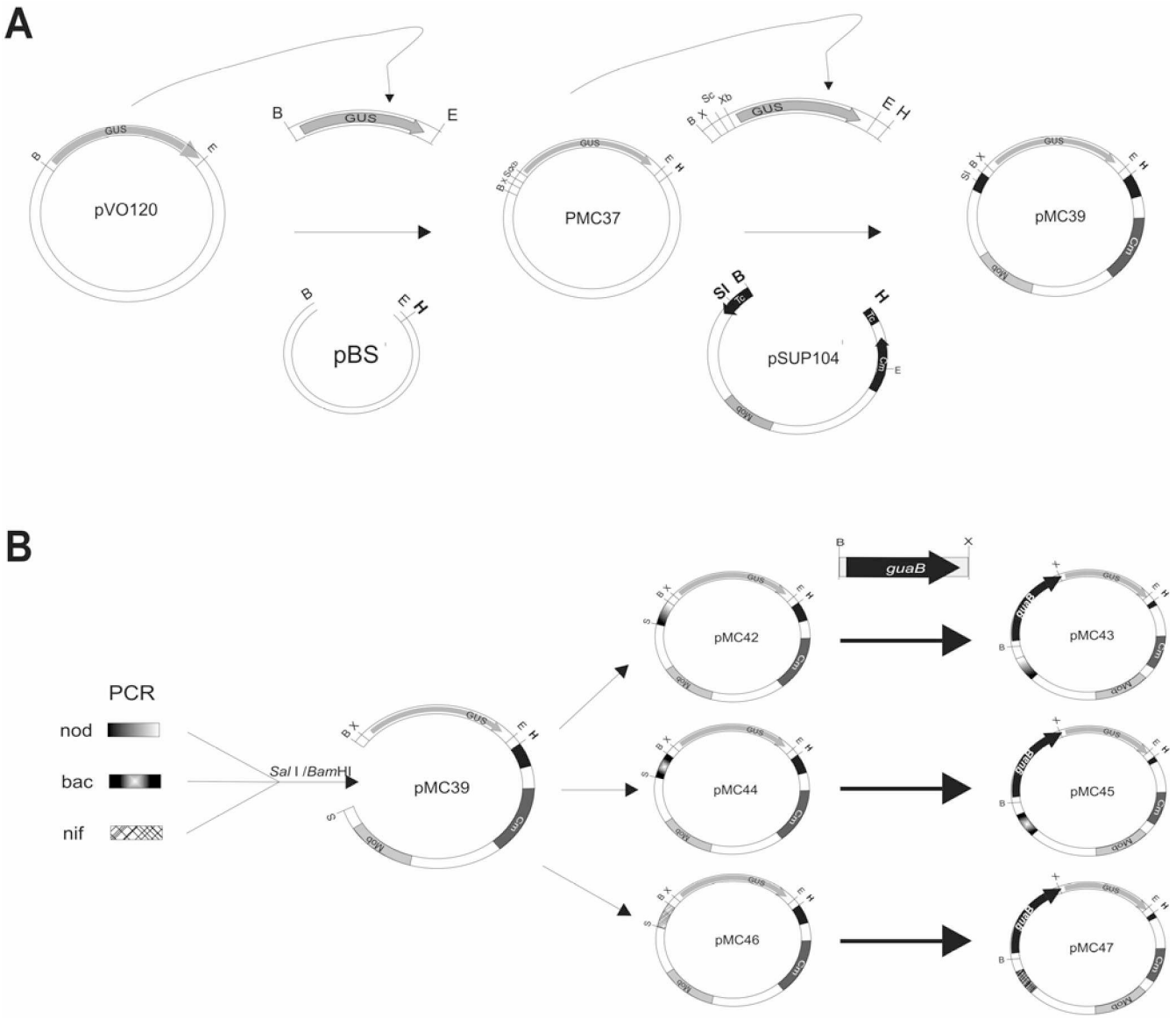

Fig. 1. Construction of gene fusions that express the *Rhizobium tropici gua*B gene under the control of symbiotic promoters. Plasmid pVO120 was the source of DNA carrying the coding sequence of the promoterless *gus*A gene and its Shine Dalgarno sequence, which were excised together as a *Bam*HI/*Eco*RI fragment and inserted into the *Bam*HI/*Eco*RI restricted plasmid pBluescript II SK (+) (Stratagene, Argentina), resulting in plasmid pMC37. This cloning step created a *Hin*dIII recognition site flanking the 3′ end of the *gus*A sequence. **A,** The *gus*A sequence was removed from pMC37 as a *Bam*HI/*Hin*dIII fragment and subcloned into plasmid pSUP104, resulting in plasmid pMC39. Each of the DNA promoter sequences were inserted individually as a *Sal*I/*Bam*HI fragment upstream from the *gus*A gene, creating the plasmids pMC42 (pr.*nod*A-*gus*A), pMC44 (pr.*bac*A-*gus*A), and pMC46 (pr.*nif*H-*gus*A). The *R. tropici gua*B coding sequence without its promoter was removed as an *Eco*RI fragment from plasmid pMC1 and cloned in plasmid PCR 2.1 TOPO (Life Technology, Argentina) in order to create recognition sites *Bam*HI and *Xho*I at the 5′ and 3′ ends, respectively. **B,** Next, the *Bam*HI/*Xho*I fragment was inserted downstream from the symbiotic promoter and upstream from the *gus*A gene, resulting in plasmids pMC43, pMC45, and pMC47, which carry the cassettes pr.*nod*A-*gua*B-*gus*A, pr.*bac*A-*gua*B-*gus*A, and pr.*nif*H-*gua*B-*gus*A, respectively.

facilitate detection of transcriptional activity driven by the symbiotic promoters. A description of these constructs is outlined in Figure 1. These constructs—cloned into the replicative vector plasmid pSUP104—were transferred into the wild-type strain *R. tropici* CIAT899 and the *gua*B mutant *R. tropici* CIAT899-10.T. Next, these transconjugants were assessed for their expression patterns in free-living conditions and during interaction with the host common bean, respectively. We previously have reported that the *gua*B mutant strain CIAT899-10.T is defective in growth at 38ºC and that both minimal medium supplemented with guanine and plasmid carrying the wild-type *gua*B sequence restored growth at 38ºC (Riccillo et al. 2000). Therefore, the three mutant strains each carrying the fusion plasmids pMC43, pMC45, and pMC47, were assayed for growth at 38ºC. None of the constructs restored the wild-type growth phenotype of strain CIAT899-10.T (Table 1), indicating that, under this condition, *gua*B is not expressed. However, it was demonstrated that strain CIAT899-10.T carrying plasmid pMC43 recovered the ability to grow at high temperature when the medium was supplemented with the *nod* inducer flavonoid naringenin (Table 1). These results were found in accordance with our expectations from previous findings by other authors, because activation of *nod* promoter takes place upon the presence of flavonoids, whereas expression of *bac*A

Table 1. Complementation of the *gua*B mutant strain *Rhizobium tropici* CIAT899-10.T by the *gua*B gene under the control of symbiosis promoters

Strain	Introduced plasmid	Supplementation[b]	Growth on minimal medium[a]	
			28ºC	38ºC
Wild-type CIAT899	None	None	+	+
Mutant CIAT899-10T	None	None	+	-
	pMC43	None	+	-
	pMC43	Naringenine	+	+
	pMC45	None	+	-
	pMC47	None	+	-

[a] Growth was determined by optical density at 600 nm.
[b] Concentration of naringenine assayed was 0.5 pM.

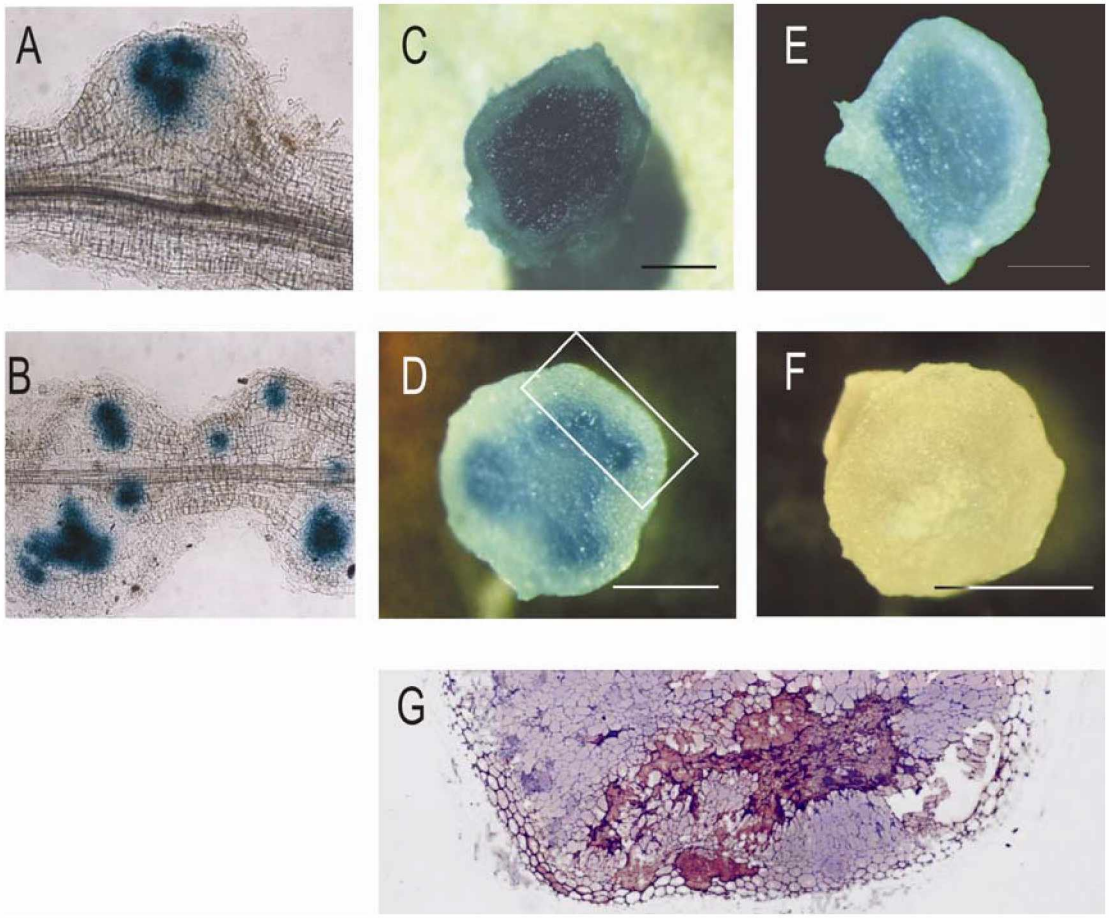

Fig. 2. *gus*A expression driven by the *nod*A, *bac*A, and *nif*H promoters in common bean roots infected with **A, C,** and **E,** the wild-type strain *Rhizobium tropici* CIAT899 and **B, D,** and **F,** the *gua*B mutant *R. tropici* CIAT899-10.T, all carrying plasmids pMC43, pMC45, and pMC47, respectively. Nodules were incubated with the substrate 5-bromo-4-chloro-3-indolyl β-D-glucuronide and potassium ferrycianide and cleared with sodium hypochlorite. **A** and **B,** β-Glucuronidase (GUS) activity in 4-day-old nodule primordium elicited by the strains carrying the pr.*nod*A-*guaB*-*gus*A fusion. **C** and **D,** Hand sections (15 days postinoculation [dpi]) of nodules formed by strains carrying the pr.*bac*A-*gua*B-*gus*A fusion. **D,** The square corresponds to the region of the nodule (shown in G) that was subjected to a closer observation after being stained with toluidine blue and viewed by bright light microscope. **E** and **F**, Hand sections of nodules (15 dpi) elicited by rhizobia containing the pr.*nif*H-*gua*B-*gus*A fusion. Note that the GUS activity was intense in wild-type nodules whereas, in the mutant nodules, it was null. Scale bars = 1 mm. **G**, Patches of invading cells surrounded by uninfected cells are visible.

and *nif*H takes place in the nodule during the symbiotic interaction with the host plant and is repressed under free-living conditions (Gage 2004; Oke and Long 1999).

In order to determine the pattern of expression of our gene fusion constructs, each driven by the symbiotic promoter of genes *nod*A, *bac*A, and *nif*H, we performed inoculation experiments on common bean by using the wild-type strain CIAT899 carrying each of the three plasmids we have constructed. Transcriptional activity in planta was monitored by X-β-glucuronidase (X-GUS) staining, and quantitative measurement of GUS activity in rhizobial cells was obtained from inoculated plants. First, results from the GUS staining assay are shown in Figures 2 and 3. Roots of plants inoculated with the strain CIAT899 (pMC43, pr.*nod*A-*guaB*) showed GUS activity at very early stages after infection, approximately 3 days postinoculation (dpi), during the curling of root hairs, and activity continued during the formation of the nodule primordium (Fig. 2A), which was detectable until approximately 6 dpi (Fig. 3B). GUS activity decreased in mature nodules and was undetectable 21 dpi (Fig. 3C and D). In roots inoculated with strain CIAT899 (pMC45, pr.*bac*A-*gua*B), GUS activity was detectable between 6 and 8 dpi and continued until 21 dpi, when nodules were fully formed (Fig. 2C). Finally, the pr.*nif*H-*gua*B-*gus*A expression was seen in roots inoculated with CIAT899 (pMC47) approximately 9 dpi and was detectable till 28 dpi (Fig. 2E).

In addition, GUS activity of rhizobial cells extracted from infected roots was determined, and the level of activity for the *R. tropici* strains harboring fusion plasmids pMC43 (pr.*nod*A-*gua*B), pMC45 (pr.*bac*A-*gua*B), and pMC47 (pr.*nifH*-*gua*B) was found to be 0.21, 0.12, and 0.14 nmoles, respectively, of *p*-nitrophenol min^{-1} per 10^4 viable cells. These levels were consistent with the data from the GUS staining described above.

Taking these results altogether, we concluded that the temporal pattern of expression of these constructs during the symbiotic interaction between *R. tropici* and common bean matches that previously reported for the bacteria from which they were identified. This also is true for the case of the *bac*A promoter which, thus far, was cloned and characterized only for *S. meliloti* (Glazebrook et al. 1993). Thus, during the interaction between *R. tropici* and common bean, we found the *nod* promoter to be the earliest promoter to be activated, followed by *bac*A and, ultimately, the *nif*H promoter. Therefore, it is reasonable to conclude that, by using these constructs, we can program the timing of the rhizobial *gua*B expression during the interaction that leads to nodule formation.

Symbiotic properties of the *gua*B *R. tropici* mutant CIAT899-10.T expressing *gua*B at different stages of symbiosis.

The three derivative strains of *R. tropici* CIAT899-10.T each carrying plasmids pMC43, pMC45, and pMC47, respectively, were tested for their symbiotic phenotype. Bean roots inoculated with these strains formed nodules, and differences in size, number, and inner color were detected among them. Plants inoculated with strain CIAT899-10.T (pMC43) formed nodules very similar in number and shape to those formed by the wild-type strain, and their roots showed GUS activity from the early stages of nodule formation after inoculation (Fig. 2A and B). In contrast, root nodules formed by strains CIAT899-10.T (pMC45) and CIAT899-10.T (pMC47) were found to be numerous and smaller in size than nodules formed by the wild-type strain. Whereas the inner part of nodules formed by CIAT899-10.T (pMC45) were light pink in color, those formed by CIAT899-10T (pMC47) were white, which suggested lower and null content of leghaemoglobin, respectively (data not shown).

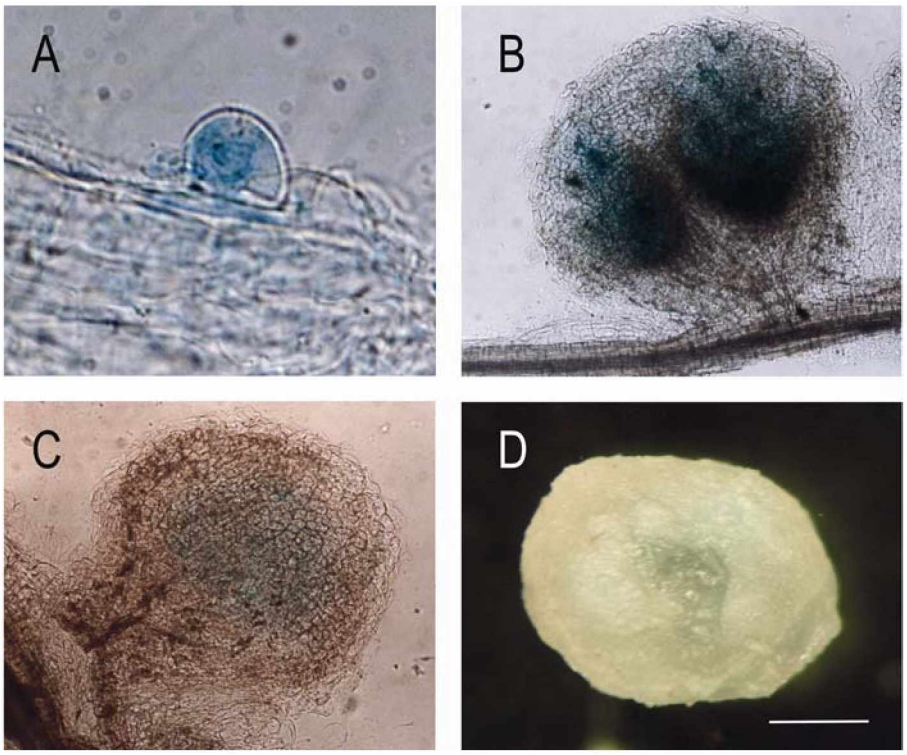

Fig. 3. Activity of the *nod*A promoter observed by β-glucuronidase (GUS) staining during initiation and formation of nodules induced by the wild type strain *Rhizobium tropici* (pMC43, pr.*nod*A-*gua*B-*gus*A) on the roots of common bean. Whole roots and nodules at **A,** 3, **B,** 4, **C,** 6, and **D,** 10 days after inoculation were incubated with the substrate 5-bromo-4-chloro-3-indolyl β-D-glucuronide and potassium ferricyanide, and cleared with sodium hypochlorite. Samples were examined by bright light microscopy. At very early stages of infection, intense GUS activity was observed on infected root hairs (A) and emerging young nodules (B), whereas the activity was low (C) and undetectable (D) in mature nodules.

The nitrogen fixation activity of the nodules was determined by using the acetylene reduction method (ARA) at 21 dpi. Percentage of wild-type activity in nodules formed by the mutant carrying plasmids pMC43 and pMC45 was found to be 85 and 8%, respectively, whereas activity of nodules formed by CIAT899-10.T (pMC47) was undetectable. The low level of activity detected in nodules induced by CIAT899-10.T (pMC45) was found to be significant and consistent in several assays (data not shown).

Additional analysis was performed 21 dpi by examination of cross-sections of resin-embedded nodules induced by strains CIAT899-10.T (pMC43), CIAT899-10.T (pMC45), and CIAT899-10.T (pMC47). Histological examination of nodules induced by strain CIAT899-10.T (pMC43, pr.*nod*A) showed them to be identical to those induced by the wild-type strain (data not shown). Nodules were full of bacteria-invaded cells, which indicated that expression of *gua*B under the control of the *nod* promoter restores the symbiotic wild-type phenotype of the mutant strain CIAT899-10.T. In contrast, nodules of CIAT899-10.T (pMC47, pr.*nif*H) were empty, devoid of bacteria inside the nodule cells (Fig. 2F), which were found to be identical to the pattern previously observed in nodules formed by the mutant strain. The nodules formed by the mutant strain carrying the plasmid pMC45 (pr.*bac*A) clearly were different from either the normal nodules or the empty nodules induced by the mutant strain CIAT899-10.T. Indeed, unlike the wild-type nodules, it was noteworthy to find a few zones of the nodule showing cells invaded by bacteria and others with spherical masses of cytoplasm-rich cells having no visible infection (Fig. 2C and D). Noninfected cells have a large accumulation of starch granules. These few cells with invading rhizobia were present as small dispersed patches surrounded by uninfected cells with high accumulation of amyloplast (Fig. 2D and G).

These results indicate that expression of *gua*B from the *bac*A promoter provides limited ability for the mutant to invade only certain and a few zones of the nodule. Furthermore, if it is assumed that the phenotypes of nodules induced by mutant strains carrying the *gua*B under the control of the promoters *nod* and *nif* represent, for us, the endpoints of the process of nodule development, we may conclude that expression of *gua*B from the *bac*A does not suffice for full nodule development, and that timing of expression of the rhizobia *gua*B is important during nodule formation.

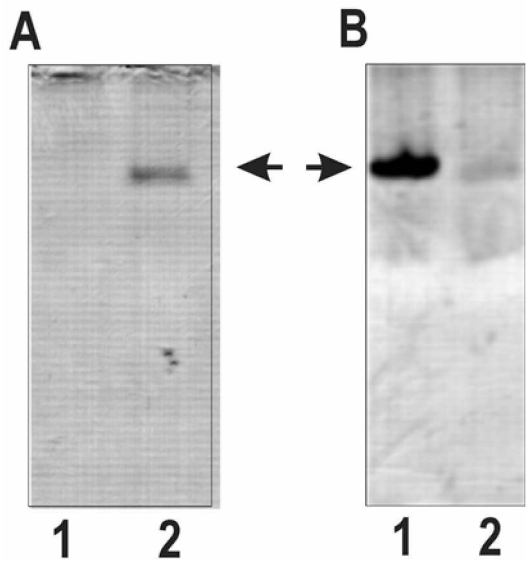

Fig. 4. Native polyacrylamide-gel electrophoresis of crude extract of *Sinorhizobium meliloti* stained by **A,** inosine monophosphate dehydrogenase (IMPd) and **B,** xanthine dehydrogenase (Xdh) activity staining. Samples containing 20 µg of protein were loaded onto each lane. Electrophoresis and staining was performed according to the procedure described by Riccillo and associates (2000) using inosine monophosphate for enzyme IMPd and xanthine for enzyme Xdh as substrates for staining activity determination of enzymes IMPd (A) and Xdh (B), respectively. Bands corresponding to the IMPd and Xdh are indicated with arrows. Lane 1, mutant strain guaB *S. meliloti* MC.1021; lane 2, wild-type strain *S. meliloti* 1021.

Investigation of the significance of *gua*B for other *R. tropici*–host associations and *S. meliloti*.

R. tropici is able to establish an efficient nodulation with legumes other than *Phaseolus vulgaris* which form determinate and indeterminate type of nodules (Martínez-Romero et al. 1999). Therefore, we carried on inoculation experiments on *Leucaena leucocephala*, *Macroptilium atropurpureum* (siratro), and *Vigna unguiculata* (cowpea) in order to assess the symbiotic phenotype of the mutant strain *R. tropici* CIAT899-10.T. All of them displayed root nodules 8 dpi. At 21 dpi, *L. leucocephala* plants were found to have nodules which did not differ in number and appearance from those formed by the wild-type strain. Unlike *L. leucocephala*, nodules formed on *M. atropurpureum* and *V. unguiculata* were found to be numerous and smaller in size. Accordingly, nodules elicited on siratro and cowpea plants displayed undetectable and low levels of nitrogenase activity, respectively (data not shown), which was in agreement with the poor growth aspect of the whole plant. This late result and those found in our earlier observation on common bean were comparable with each other. These results indicated that the negative effect of *gua*B mutations on nodulation is not a general phenomenon because nodules formed on *L. leucocephala* were found to be identical to those formed by the wild-type strain and, in addition, indicated that defective nodulation by the *gua*B strain CIAT899-10.T was found in case of the host plants that form the determinate type of nodules; namely common bean, siratro, and cowpea.

Within this framework, we wondered whether the defective nodule phenotype shown by the interaction between the *gua*B *R. tropici* mutants and common bean may also be the case for other rhizobial species in interaction with their respective host plants. Thus, a *gua*B-defective mutant (strain MC.1021) derived from the wild-type strain *S. meliloti* 1021 was obtained by fragment-specific mutagenesis of its wild-type *gua*B sequence and used in this work for further characterization. We found that, unlike the *R. tropici gua*B mutant, the *S. meliloti gua*B mutant strain MC.1021 was found to be auxotrophic for guanine. We have demonstrated that *gua*B *R. tropici* mutants behave as prototrophic due to the presence of an alternative pathway via the enzyme Xdh, bypassing the blocked step of IMPd (Riccillo et al. 2000). Therefore, IMPd and Xdh were determined in cellular extracts prepared from the wild-type *S. meliloti* strain and the *gua*B mutant strain MC.1021. The results of this analysis (Fig. 4) indicated that both the wild-type and mutant strains displayed Xdh activity, whereas only the wild-type strain was active for IMPd. This analysis added biochemical evidence that our *gua*B *S. meliloti* mutant is defective in IMPd activity. Also, it was found that the level of activity was consistently higher in extracts from the mutant than in the wild type, indicating that, in the absence of IMPd, the expression of Xdh resulted. Sukdeo and Charles (2003) reported *S. meliloti* to have two distinct loci encoding Xdh. We were unable to identify differences in the pattern of expression at the level of each locus, probably because our samples were examined on 10.5% polyacrylamide gels rather than on the 5% gels used in the work described by Sukdeo and Charles (2003), which is experimentally required in order to accomplish separation of Xdh proteins. On the other hand, we found that supplementation of the minimal medium

with the exogenous precursor adenine and hypoxanthine (which is substrate for Xdh) restored wild-type growth (Fig. 5). Allopurinol, which is a specific inhibittor of Xdh, prevented growth of the *S. meliloti gua*B mutant when added to the medium (Fig. 5). Thus, the alternative pathway for guanine biosynthesis via Xdh is functional in *S. meliloti*; however, it could be possible that the level of the cellular pool of precursors for Xdh is low and does not support prototrophic growth in case of the mutant strain.

We used *Medicago sativa* (alfalfa) and *M. truncatula* to test whether the *gua*B mutation of the *S. meliloti gua*B gene affects symbiosis. Within 21 dpi, nodules were formed in roots of alfalfa and *M. truncatula* plants inoculated with the wild-type and mutant strains, respectively. The aspect and the nitrogen fixation activity, as determined by ARA, were similar between them. Cross-sections of nodules examined by light and electron microscopy showed normal morphology and cells fully infected by rhizobia (data not shown). Altogether, these results indicated that mutations in the *gua*B gene of *S. meliloti* do not affect its symbiotic interaction with the host plants alfalfa and *M. truncatula*.

DISCUSSION

We have demonstrated that *R. tropici* requires an active *gua*B gene for the successful nodulation of common bean (Riccillo et al. 2000). In this article, we extended our studies to the timing of such requirements during infection and the effect of *gua*B on other rhizobia–legume interactions.

Our previous findings indicated that GuaB mutants were unable to be released from infection threads within nodule cells. However, considering that nodule formation is a complex process of sequential steps, the timing of such *gua*B requirement during the bacteria–host plant interaction was an unknown issue for us. Also, the ubiquitousness of this requirement among other *Rhizobium* spp.–host plant interactions represented for us another issue of interest. Hereafter, we will address our discussion to these two issues.

In order to examine the timing of the *gua*B requirement during the interaction between *R. tropici* and common bean, we used gene fusions between symbiotic promoters, which are activated at different stages of nodule formation, and the *gua*B sequence. The expression pattern of these promoters was found to follow that found in the rhizobial species from which these promoter sequences were derived (Oke and Long 1999; Schlaman et al. 1992). This also was the case for the *bac*A promoter. Until now, only the *bac*A gene of *S. meliloti* has been characterized and demonstrated to be essential during formation in alfalfa of the indeterminate type of nodule (Gage 2004; Oke and Long 1999). Our results indicated that the temporal pattern of expression of the *S. meliloti bac*A promoter in host plants that form determinate nodules is similar to that found in the indeterminate type of nodules.

These constructs represented useful tools for us to assess the effect of having the *gua*B gene active at different stages of nodulation; however, as we demonstrated earlier, mutations in the *R. tropic gua*B gene impact on the bacterial release into nodule cells mutations. It has been demonstrated that the nodulation factor which results from expression of the rhizobial common nodulation genes induces early responses such as root hair curling and infection threads, but also exerts a distal effect on the root by inducing cortical cell division and formation of a nodule primordium. This indicates that, while the bacteria are progressing in the infection threads, the root at distance initiates developmental changes

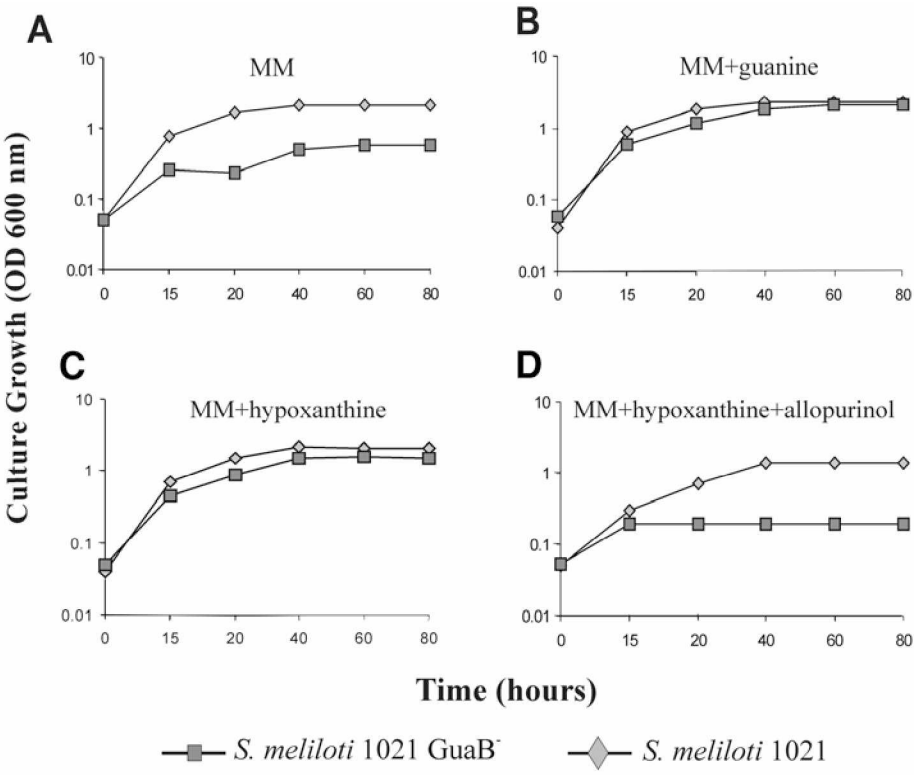

Fig. 5. Growth recovery by the *gua*B *Sinorhizobium meliloti* mutant in minimal medium (MM) supplemented with guanine and precursors of xanthine dehydrogenase. At time zero, cells of wild-type strain 1021 and mutant MC.1021 were diluted to optical density at 600 nm (OD600) = 0.08, with **A,** MM-GTS, **B,** MM-GTS supplemented with 0.14 mM guanine, **C,** MM-GTS supplemented with 0.14 mM hypoxanthine, and **D,** MM-GTS supplemented with 0.14 mM hypoxanthine and 1 mM allopurinol. Growth at 28ºC was monitored by measuring OD600. The data are the means values from three replicate experiments with standard deviation of less than 10%.

to be prepared in time to receive the rhizobia. Our data present evidence that the *gua*B gene must be active from the early stages of nodulation, as the wild-type phenotype was recovered by complementing the GuaB mutant with the *gua*B under the control of the nodulation promoter, whereas there is limited complementation by the *bac*A promoter, which is switched on after the *nod* promoter. Therefore, not only is it important to have functional the *gua*B gene, but the timing of its expression also is relevant in order to accomplish full nodulation of common bean and other plant hosts that form determinate nodules, such as *Macroptilium atropurpureum* and *V. unguiculata*.

Taté and associates (1999) have shown that the ectopic expression of the *R. etli amt*B gene involved in ammonium transport affects nodule development, altering the ability of the bacteria to invade the nodule cells. The *amt*B gene is switched off at very early stages of symbiosis. More recently, Lodwig and associates (2003) examined the symbiotic phenotype of *R. leguminosarum* bv. *viciae* double mutants in genes *aap* and *bra*, which both encode ABC-type amino-acid transporters, and found them to be negatively affected in nitrogen fixation, although nodule cells were fully occupied by rhizobia. These few examples and our results demonstrated the important role of rhizobial genes other than the specific symbiotic genes in order to complete the program of nodule development. Furthermore, our data have shown that timing also is important, which further supports the concept that the development of the symbiosis is a multistep process mediated by signal exchanges between partners in a sequential and ordered manner.

The available data on the *S. meliloti* genome enabled us to specifically target the *gua*B gene and, therefore, examine the phenotype of GuaB *S. meliloti* mutants compared with the *R. tropici gua*B mutants. Phenotypically, these mutants behave differently. The *S. meliloti gua*B mutants were found to be auxotrophic for guanine and wild type for symbiosis, whereas the *R. tropici gua*B mutants were found to be the other way round. Clearly, these results show that identical genes have different physiological impacts on different rhizobial species. Furthermore, in *S. meliloti* (unlike *R. tropici*), expression of enzyme Xdh seems to be activated in the *gua*B background. To account for these phenotypes, we propose that, in *R. tropici*, guanosine monophosphate can be replenished more efficiently from xanthine via xanthine monophosphate dehydrogenase than in *S. meliloti*, which utilizes hypoxanthine via IMPd. The function of Xdh has been studied in *Escherichia coli* by Xi and associates (2000). These authors proposed that Xdh has a prevalent role in guanine biosynthesis, which also should be the case for the guanine biosynthesis in *R. tropici*.

Our data also present evidence that the requirement of *gua*B is not a general phenomenon. Indeed, we have tested four host plants of *R. tropici* and found it to be indispensable to establish full symbiosis only on common bean, siratro, and cowpea. In contrast, the *gua*B *R. tropici* mutant as well as the *gua*B *S. meliloti* mutant was fully effective on leucaena and alfalfa, respectively. Therefore, our data demonstrated that *gua*B plays an essential role during symbiosis on certain hosts sharing the feature of forming determinate nodules, but its function is not required by other hosts that form indeterminate nodules. Although our results indicated that the effect of *gua*B is associated with the ontogeny of the two types of nodules, the genetic background underlying this difference on the plant side remains to be investigated. The host-dependent effect of rhizobial genes on symbiosis also has been shown for the *exo*B gene of *Bradyrhizobium japonicum* (Parniske et

Table 2. Bacterial strains and plasmids used in this work

Strain or plasmid	Relevant characteristics[a]	Reference
Escherichia coli		
DH5	F⁻ φ80d *lacZ* ΔM15 Δ(*lacZYA-argF*)U169 *deoR recA1 endA1 hsdR17*(r_K^- m_K^+) *phoA supE44* λ⁻ *thi-1 gyrA96 relA1*	Hanahan 1983
S17-1	*thi pro hsd*R⁻ *hsd*M⁻ *rec*A carrying a RP4 2-Tc::Mu integrated in the chromosome	Simon et al. 1983
Rhizobium tropici		
CIAT899	Sm^r; wild type, Nod$^+$Fix$^+$ on bean and leucaena	Martinez Romero et al. 1991
CIAT899-10.T	Nm^r; CIAT899 GuaB⁻:Ndv⁻ Fix⁻ on bean	Riccillo et al. 2000
Sinorhizobium meliloti		
1021	Sm^r; wild-type derivative of RCR2011	Meade et al. 1982
MC.1021	Nm^r; *gua*B mutant auxotrophic for guanine	This work
Plasmids		
pVO120	Ap^r/Km^r; nonmobilizable vector for constructing *gus*A fusions	Oke and Long 1999
pSUP104	Cm^r/Tc^r Mob$^+$; broad-host-range pACYC184 derivative	Simon et al. 1983
pK18mob	Km^r; pK18-derivative; mobilizable vector	Schäfer et al. 1994
pMC1	Cm^r; 1.5-kb *Eco*RI fragment containing promotorless *gua*B gene with Shine Dalgarno cloned in pSUP204	Riccillo et al. 2000
pMC28	Ap^r; 0.85-kb fragment of *S. meliloti gua*B gene in pGEM T-easy (Promega, Biodynamics, Argentina)	This work
pMC37	Ap^r; 1.9-kb *Bam*HI-*Eco*RI fragment containing promotorless *gus*A gene from pVO120 in pBluescript II SK (+) (Stratagene)	This work
pMC38	Ap^r/Km^r; 1.5-kb BamHI-XhoI fragment containing *gua*B gene from pMC1 in pCR 2.1 TOPO (Invitrogen, Argentina)	This work
pMC39	Cm^r; *Bam*HI-*Hin*dIII fragment containing *gus*A gene from pMC37 in pSUP104	This work
pMC40	Km^r; *Eco*RI fragment *gua*B::Km from pMC32 in pK18-mob	This work
pMC42	Cm^r; 341-bp *Sal*I-*Bam*HI fragment containing upstream sequence *nod*A gene from *R. tropici* in pMC39 (pr.*nod*A-*gus*A)	This work
pMC44	Cm^r; 288-bp *Sal*I-*Bam*HI fragment containing upstream sequence *bac*A gene from *S. meliloti* in pMC39 (pr.*bac*A-*gus*A)	This work
pMC46	Cm^r; 228-bp *Sal*I-*Bam*HI fragment containing upstream sequence *nif*H gene from *Bradyrhizobium* in pMC39 (pr.*nif*H-*gus*A)	This work
pMC43	Cm^r; 1.5-kb *Bam*HI-*Xho*I fragment containing promotorless *gua*B gene from *R. tropici* CIAT899 in pMC42 (pr.*nod*A-*gua*B-*gus*A)	This work
pMC45	Cm^r; promotorless *gua*B gene in pMC 44 (pr.*bac*A-*gua*B-*gus*A)	This work
pMC47	Cm^r; promotorless *gua*B gene in pMC 46 (pr.*nif*H-*gua*B-*gus*A)	This work

[a] Ap, Cm, Km, Nm, Tc, and Sm denote ampicillin, chloramphenicol, kanamycin, neomycin, tetracycline, and streptomycin resistance, respectively; pr., promoter.

al. 1994) and the *typ*A gene of *S. meliloti* (Kiss et al. 2004). On the other hand, it was shown that the inhibitory effect of salicylic acid on nodulation also is dependent of the type of nodulation (van Spronsen et al. 2003).

The full role of the *gua*B gene in *R. tropici* is not yet completely understood. Nevertheless, we speculate that *R. tropici*, during its interaction with host plants that form determinate nodules, may generate a GuaB-dependent signal for the cells of the nodule primordium that causes these cells to undergo changes to prepare (set) themselves for the bacterial release from the infection threads. During nodule development, many plant genes, the so-called nodulin genes, need to be coordinately induced in the different steps of the process (Crespi and Galvez 2000). In the future, it would be interesting to examine, at the molecular level, the degree of development of nodules induced by *gua*B mutants by assessing the expression profile of nodulins that were demonstrated to be activated at different stages of nodulation and, also, to assess the symbiotic phenotype of *gua*B rhizobia which form determinate nodules but export amides instead of ureides, as is the case in the association between *Mesorhizobium loti* and *Lotus japonicum*. We believe these analyses may provide clues to the role of *gua*B during organogenesis.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture media.

Bacterial strains and plasmids used in this work are listed in Table 2. *E. coli* strains were grown at 37°C on Luria Bertani medium (Miller 1972). *R. tropici* and *S. meliloti* were grown at 30°C on either TY (Beringer 1974) or minimal GTS medium (Kiss et al. 1979). Antibiotics used were as follows: streptomycin at 400 μg ml^{-1}; neomycin at 100 μg ml^{-1}; chloramphenicol at 35 and 200 μg ml^{-1} for *E. coli* and rhizobia, respectively; tetracycline at 10 μg ml^{-1}; and kanamycin at 25 μg ml^{-1}. 5-bromo-4-chloro-3-indolyl β-D-glucuronide (X-GlucA) was added to solid media to give a final concentration of 150 μg ml^{-1}.

Synthesis of the symbiotic promoters by PCR.

For construction of fusions between each symbiotic promoter (pr.) and the *gua*B sequence, the promoter sequences of genes *nod*A, *bac*A, and *nif*H were PCR amplified by using the following oligonucleotides, respectively, which were modified in their 5′ ends to generate *Sal*I-*Bam*HI recognition sites (underlined) that facilitate directional cloning of the promoter sequences upstream from the *gua*B sequence: NodAF 5′-AGCGCGTCGAC AAGATTTAAGTCCAG-3′ and NodAR 5′-CTGAGGGATCCA TCGTGTGATTGC-3′ deduced from the *R. tropici nod*A sequence (accession number X98514); BacAF 5′-CGCCGTCGA CTCGCCTTCATGACC-3′ and BacAR 5′-GCGCGGATCCT AGGGCAATTCAC-3′ deduced from *S. meliloti bac*A sequence (accession number X73522); and NifHF 5′-TAAGCGTCGAC AGTGTTGGCATGG-3′ and NifHR 5′-ACTGAGACAAATC GGATCCTACGG-3′ deduced from the *Bradyrhizobium* sp. ANU289 *nif*H sequence (accession number K00487).

The genome of *R. tropici* CIAT899 and *S. meliloti* 1021 were used as PCR templates for the amplification of pr.*nod*A and pr.*bac*A sequences, respectively, which were prepared as previously described (Sambrook et al. 1989). The DNA template used for the amplification of the pr.*nif*H sequence was pCAM131 (Wilson et al. 1995). Plasmid DNA was prepared by alkaline method (Kragelund et al. 1995).

The size of the PCR products *nod*A, *bac*A, and *nif*H were 341, 288, and 228 bp, respectively. The PCR products were digested with endonucleases *Sal*I and *Bam*HI, and individually ligated upstream from the *gus*A gene into plasmid pMC37.

Fragment-specific mutagenesis of the *S. meliloti* 1021 *gua*B gene.

An internal fragment of 845 bp of the wild-type *gua*B gene (accession SMc00815) was amplified from the *S. meliloti* 1021 genome (Galibert et al. 2001) by PCR, using primers ORFForw (5′-AGAGGAACTGGCCATGGCGC-3′) and GuabSMR (5′-GT TGGAAAGCTTCTTCACGCG-3′), which were deduced . This DNA fragment was cloned into the vector plasmid pGEM-T (Promega, Biodynamics, Buenos Aires). Fragment *gua*B::Km was excised from pMC32 as an *Eco*RI fragment and inserted into the *Eco*RI site of the suicide vector plasmid pK18-mob, resulting in the plasmid pMC40. The plasmid pMC40 was transferred into *S. meliloti* 1021 by conjugation and transconjugants were selected by their resistance to neomycin (Nm^r) in order to isolate single-crossover cointegrates. Total DNA prepared from the Nm^r clones was examined by Southern analysis using a PCR-*gua*B probe to confirm the genotype.

Plant nodulation assay.

The host plants used in this study were P. vulgaris cv. Negro Jamapa (common bean), Macroptilium atropurpureum (siratro), V. unguiculata (cowpea), Medicago sativa cv. CUF101 (alfalfa), and M. truncatula cv.108-R. Siratro seed first were pretreated in distilled water at 80°C for 5 min. Then, the seed were surface sterilized in ethanol 95% (vol/vol) for 1 min, followed by soaking in sodium hypochlorite (8 g liter–1) for 30 min, washed extensively with sterile water, and germinated in darkness at 28°C on a petri dish containing water agar. Seedlings were transferred into pots with sterile vermiculite and cultured in a growth chamber maintained at 80% relative humidity and 28°C with a 16-h daylight period. Plantlets (1 week old) were inoculated by using a rhizobial suspension in Fahraeus's medium of approximately 108 rhizobia per milliliter (Vincent 1970). Common bean and siratro plants were harvested 3 and 5 weeks after inoculation, respectively. Nitrogenase activity was determined by the ARA. The ethylene produced was quantified 30 and 60 min after incubation in 10 % acetylene (vol/vol) at room temperature.

Staining for GUS activity.

Histochemical analysis of GUS activity in nodules was performed according to the protocol of Wilson and associates (1995). When possible, nodules were removed from the roots; if not, the whole root segment was stained. Root and nodule tissues were immersed in phosphate buffer (Jefferson 1987) containing X-GlucA at 150 μg/ml and 1 mM potassium ferricyanide and vacuum infiltrated. Samples were incubated at 37°C overnight and cleared with 3% sodium hypochlorite aqueous solution prior to their examination. For the quantitative determination of GUS activity on samples of nodule bacteria, rhizobial cells from infected roots were collected as follows: nodule primordium and nodules were harvested at 7 and 14 dpi, respectively, and used immediately for isolation of bacteria by crushing them in saline buffer (50 mM sodium phosphate, pH 7.0). This homogenate was transferred into a 1.5-ml Eppendorf tube and the plant debris were sedimented by pulse centrifugation in a bench-top microfuge. Independent samples were withdrawn from the supernatant containing rhizobia to assay β-glucuronidase activity and to obtain viable cell counts by plating on TY plus chloramphenicol medium. GUS activity was determined according to the procedure of Reeve and associates (1998) for rhizobia cells growing in liquid medium.

Electron and optical microscopy.

The nodules harvested 21 dpi were fixed in 50 M potassium phosphate buffer (pH 7.2) containing 2% paraformaldehyde

and 2.5% glutaraldehyde. After 3 h, the nodules were postfixed for 1 h in the same buffer containing 2% osmium tetroxide, rinsed three times in the same buffer, dehydrated by passing through a series of graded ethanol at 0°C, and embedded in Epon-Araldita resin. Ultrathin sections were stained with uranyl acetate 2% (wt/vol) and lead citrate and observed in a Jeol JEM 1200 EX transmission electron microscope. Semithin sections (1 to 2 mm thick) of the same samples were used for optical observation after staining with toluidine blue in 0.5% borate buffer.

Determination of IMPd and Xdh activity in polyacrylamide gel.

The IMPd and Xdh activities were detected in polyacrylamide gel electrophoresis under nondenaturing conditions according to the procedure described by Miyamoto and associates et al. (1998), using inosin monophosphate or xanthine, respectively, as substrates for staining and 10.5% polyacrylamide gel. The protein concentration was determined by the method of Lowry and associates (1951) with bovine serum albumin as standard.

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