Controlling the Expression of Rhizobial Genes During Nodule Development with Elements and An Inducer of the *lac* Operon

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A simple strategy was tested for imposing artificial regulation of rhizobial genes during nodule development. Isopropyl- β -D-1-thiogalactoside (IPTG) was added to liquid root media to sustain expression of rhizobial genes controlled by *Escherichia coli lac* promoter/operators and repressor gene *lacI*. Conversely, a rinsing protocol was devised to remove IPTG sufficiently that genes could be repressed after having been induced. *gusA* under this control exhibited clearly delineated expression and repression in both the determinate *Rhizobium etli*–*Phaseolus vulgaris* and the indeterminate *Sinorhizobium meliloti*–*Medicago sativa* symbioses. Apparently, IPTG was taken up in sufficiently undegraded concentrations that gene expression was derepressed even in interior portions of the nodule. Moreover, the rinsing protocol led to obvious repression of *gusA*. Importantly, no deleterious effects of IPTG on nodule development, infection, or nitrogen fixation were observed. An *R. etli* CE3 gene required for lipopolysaccharide O antigen and infection on bean was put under this control by means of a two-plasmid construct. When this construct was added to a strain with a null mutation in this gene, infection, nodule development, and nitrogenase activity all depended on the length of time before IPTG was rinsed from the roots after inoculation.

In the development of the root-nodule symbiosis of legumes and rhizobia, there are two major developmental programs (Oldroyd and Downie 2008; Patriarca et al. 2004). One is the formation of the organ, the nodule, by a series of plant-developmental events that generally are triggered by a lipochitoooligosaccharide Nod factor from the bacterial partner (Stacey et al. 2006; Tirichine et al. 2007). The other program is the infection process, which is manifested by a unique structure called the infection thread (Gage 2004).

By mutating bacterial genes, much has been learned regarding essential bacterial components in these processes. Generally, these genetic tests utilize null mutants. However, one problem with this approach is that it reveals only the first point in nodule development at which a component is required. Often it is suspected that a component is required at more than one step in development but that possibility cannot be tested with conventional null mutants.

One approach to resolve this conundrum is to put the production of the factor under conditional control, such that it is produced during one stage of nodulation and not at another stage. A method that has achieved success in this regard is to put a gene of interest under the control of rhizobial promoters known to be induced at different points in nodule development. Collavino and associates (2005) fused *gusA* to *nodA*, *nifH*, and *bacA* promoters and could show the effects of differential induction in this way. Another idea is to use temperature-sensitive alleles (Grzemski et al. 2005). The virtue of this approach is that the gene product itself can be inactivated. However, screening for such alleles is time consuming and often may not be possible. Fusion to temperature-sensitive promoters is a more general approach but there remains the question of the effect of temperature shift on the physiology being studied.

In the work to be described, another approach was taken, one that provides conditional control by the exogenous application of a chemical inducer, isopropyl- β -D-1-thiogalactoside (IPTG). This well-known artificial ligand of the *lac* repressor of *Escherichia coli* was used to induce expression in planta of bacterial genes fused to DNA containing *lac* regulatory elements. In principle, this approach could interrupt or activate gene expression at any particular point during the infection process of legume nodule development. First, the efficacy of this technique was tested by using IPTG to manipulate *gusA* expression during determinate *Rhizobium etli*–*Phaseolus vulgaris* and indeterminate *Sinorhizobium meliloti*–*Medicago sativa* nodule development. Next, this approach was applied to regulate *wreG*, a gene needed for bacterial O-antigen production and normal nodule development in the *R. etli*–*P. vulgaris* symbiosis.

RESULTS

Using IPTG to control *gusA* expression in rhizobia during nodule development.

R. etli strain CE424, harboring transposon mTn5SS*gusA10* in which *gusA* is under the control of the *tac* promoter and *lacI* (Wilson et al. 1995), was inoculated on *P. vulgaris*. After differing periods of IPTG supplementation in the root medium, the presence of active β -glucuronidase (GUS) in nodule sections was assayed histochemically. The results of an experiment in which nodules were sectioned and stained 9 days after inoculation (dai) are shown in Figure 1A to E. In this system, nodules emerge from the root after 6 dai. When IPTG was present in the root medium only during the first 4 dai and then washed out, GUS staining after 9 dai was confined almost exclusively to infection threads, mainly within the root hair and two cell layers below it (Fig. 1B and C). Conversely, when IPTG was

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first added at the end of 4 dai, GUS was not evident in the earliest part of the infection (Fig. 1D and E). Instead, staining was observed mainly in the center of the nodule. From these results, it was concluded that IPTG entered the developing

nodule in sufficient concentration to induce the *lacI*-controlled expression of *gusA* (e.g., giving the staining seen in Figure 1D and E). Moreover, it appeared that IPTG was effectively removed by the rinsing protocol after 4 dai, because staining

Fig. 1. *gusA* and *wreG* under the control of PO_{lac} and *lacI* were regulated during nodule development by the provision or removal of isopropyl- β -D-1-thiogalactoside (IPTG). **A to F**, Control of *gusA* in *Rhizobium etli* within *Phaseolus vulgaris* nodules. **A to E**, Sections of nodulated *P. vulgaris* roots were excised 9 days after inoculation (dai) with strain CE424, sliced by hand, and stained for β -glucuronidase (GUS) activity. **A**, IPTG was present throughout. **B**, and **C**, IPTG was present during growth of the inoculant and included in the root medium only during the first 4 dai, at the end of which time it was washed from the roots. **D** and **E**, IPTG was absent during growth of the inoculant and during the first 4 dai but was included in the root medium thereafter. **F**, A longer-term experiment, in which IPTG was added to the roots 21 dai. Four days later, nodules were removed, sliced, and stained. **G to K**, Control of *gusA* in *Sinorhizobium meliloti* within *Medicago sativa* nodules. Three weeks after inoculation with strain MM1, sections of nodulated *M. sativa* roots were excised, sliced by hand, and stained for GUS activity. IPTG was present during growth of the inoculum and included in the root medium for **G**, the first 7; **H**, the first 14; or **I**, all 21 dai. IPTG was washed from the roots and rooting medium after **G**, 7 or **H**, 14 dai. **J** and **K**, IPTG was added first on **J**, 8 or **K**, 15 dai and maintained in the root medium for the remainder of the 21 dai. **L to Q**, Quality of *P. vulgaris* nodulation depended on how long IPTG was present to sustain *R. etli wreG* expression. Seedlings were inoculated with strain CE489 (*wreG*/pJB4/pJB12) that had been grown in tryptone yeast containing 0.1 mM IPTG, except for the inoculant in **L**, which never was exposed to IPTG. In the other treatments, the root medium contained 0.1 mM IPTG until it was removed after the number of dai indicated by the numeral in the upper right of each panel. Roots were photographed at 12 dai and the cropped images shown were representative of the nodule development resulting from these respective treatments. The portion of the root shown in each case was at or near the crown where nodules first appear. A few examples of the many pseudonodules from 0 or 2 days of IPTG supplementation are indicated by arrows in **L** and **M**. **R to T**, Bacterial infection of *P. vulgaris* also reflected how long IPTG was provided to sustain *R. etli wreG* expression. *P. vulgaris* seedlings were inoculated with *R. etli* CE489 (*wreG*/pJB4/pJB12 and constitutive *gusA*). IPTG (0.1 mM) was present in the root medium for the first **R**, 2; **S**, 4; or **T**, 8 dai and then removed by the rinsing protocol. After 8 dai, representative nodulated segments from each treatment were excised from roots, sliced, and stained for the presence of active GUS. The scale bar in the lower right represents 100 μ m (**A** to **F**), 200 μ m (**G** to **K** and **R** to **T**), or 2.0 mm (**L** to **Q**). Connection of the nodule to the root is at or below the bottom in **A** to **F**, **R**, and **T**; and at or beyond the lower left corner in **G** to **K** and **S**.

indicated that GUS content declined sharply in the bacteria that were generated after that point (Fig. 1B and C). A separate experiment examined whether IPTG could reach bacteroids in older nodules. In this experiment, IPTG was absent initially and then added to the root medium at 21 dai. GUS staining was evident in central plant cells of nodules 4 days after the IPTG addition (Fig. 1F).

Comparable results were obtained with *S. meliloti* and alfalfa. For this purpose, the same transposon was used to construct a derivative of *S. meliloti* strain Rm1021 carrying *lacI*-regulated *gusA*. Representative results with this strain, MM1 (Table 1), are shown in Figure 1G to K. When IPTG was present in the root medium throughout the course of nodulation, GUS staining was observed throughout the nodule, except at the uninfected tip (Fig. 1I). When IPTG was supplied only during the first 7 dai and then rinsed out, staining at 21 dai was strongest in the oldest third of the nodule (Fig. 1G). The converse procedure, IPTG supplied only during the last 2 weeks of the experiment, led to converse staining: almost only in the younger two-thirds of the nodule at 21 dai (Fig. 1J). Likewise, if IPTG was supplied only during the last 7 days, only the youngest half of the nodule exhibited staining at 21 dai (Fig. 1K).

In neither host nor bacterial species did additions of IPTG noticeably affect the appearance and rate of nodule development or nitrogenase activity (data not shown). This was true regardless of whether these wild-type strains carried the mTn5-*gusA* insertions. Importantly also, subjecting the inoculated plants to the rinsing protocol as early as 2 dai on *P. vulgaris* or 7 dai on alfalfa (the earliest day tested) had no noticeable effect on subsequent nodule development and nitrogenase activity (data not shown).

Construction of plasmids to express *wreG*.

Gene *wreG* (Fig. 2), locus RHE_CH00748 of the *R. etli* CE3 genome (Gonzalez et al 2006), was chosen as a candidate for applying this regulation to a bacterial gene required in symbiosis. The three-letter designation for genes that specify O-antigen synthesis in *R. etli* CE3 is *wre* (Ojeda et al 2010). The for-

mer designation of *lps* was changed to *wre* to conform to the conventions of the bacterial polysaccharide genetic nomenclature (Reeves et al 1996). These genes are required for the *R. etli*-*P. vulgaris* symbiosis because the bacterium must produce near-normal amounts of lipopolysaccharide O antigen in order to infect normally and elicit normal nodule development (Noel and Duelli 2000; Noel et al 1986).

Located at the 5' end of a 28-kb *wre* gene cluster in *R. etli* CE3, *wreG* is hypothesized to encode a transferase that adds the second sugar of the O antigen (Ojeda 2009). Mutant strain CE358, derived from *R. etli* CE3, carries a Tn5 insertion in *wreG* and is unable to synthesize lipopolysaccharide (LPS) I, the "smooth" LPS that contains O antigen (Fig. 3, lane 1). Importantly, only this open reading frame (ORF) is needed to restore LPS I in this mutant, and the extent of LPS I restoration serves as a measure of the expression of a wild-type copy of *wreG* introduced into this mutant strain (Fig. 3, lanes 3 to 6). For controlling this gene, the first attempt was to insert it within the same modified mTn5 that was used for the pilot studies with *gusA*. However, this approach was unsuccessful for various technical reasons, including the unforeseen existence of a sequence rearrangement in the copy of the mTn5 used in the foregoing experiments compared with the published nucleotide sequence. Instead, the *lac* control elements were built into a plasmid system (Fig. 2).

First, the *wreG* ORF, along with its native ribosome-binding site, was inserted within plasmid pFAJ1700 (Dombrecht et al. 2001), which replicates in *R. etli*. Next, the upstream regulatory sequences of the *E. coli lac* operon (including *lacI*, *P_{lac}*, and two repressor-binding sequences, *O₃* and *O₁*) were inserted upstream of the *wreG* ribosome-binding site (Fig. 2). This stretch of DNA is essentially identical to the corresponding portion of the *lac* operon in wild-type *E. coli*, with the exception of having a mutation of the *lacI* promoter that, in *E. coli*, causes 10-fold upregulation of the *lacI* (repressor) gene (Calos 1978). The combination of this promoter mutant and otherwise wild-type *lacI* is commonly referred to as *lacI^Q*. The resulting plasmid pJB4 is shown in Figure 2. This plasmid was trans-

Table 1. Strains and plasmids

Strain or plasmid	Relevant characteristics ^a	Source or reference
<i>Rhizobium etli</i>		
CE3	Sm ^r Nal ^r ; str-1 Lps ⁺ Ndv ⁺	Noel et al. 1984
CE358	Sm ^r Km ^r Nal ^r ; str-1 <i>wreG</i> 2::Tn5 Lps ⁻ Ndv ⁻	Cava et al. 1990
CE424	CE3 with mTn5SS <i>gusA</i> 10 at unknown site, Lps ⁺ Ndv ⁺	This work
CE480	CE358 carrying pJB4 and pJB12	This work
CE489	CE480 with mTn5SS <i>gusA</i> 20 at unknown genomic site	This work
<i>Sinorhizobium meliloti</i>		
RM1021	Sm ^r Km ^r Nal ^r ; Ndv ⁺	Meade et al 1982
MM1	Sm ^r Km ^r Nal ^r Sp ^r ; Ndv ⁺ ; mTn5SS <i>gusA</i> 10 at unknown site	This work
Plasmids		
pBKS-	pBlueScriptKS ⁻ ; Ap ^r	Stratagene
pCAM110	Sm ^r Sp ^r Ap ^r ; carries mTn5SS <i>gusA</i> 10 (IPTG-inducible <i>gusA</i>)	Wilson et al. 1995
pCAM120	Sm ^r Sp ^r Ap ^r ; carries mTn5SS <i>gusA</i> 20 (constitutive <i>gusA</i>)	Wilson et al. 1995
pCR2.1	Ap ^r Km ^r ; T-A cloning vector for polymerase chain reaction (PCR) products	Invitrogen
pFAJ1700	Ap ^r Tc ^r ; IncP wide-host cloning vector	Dombrecht et al. 2001
pGEX-4T-1	Ap ^r ; <i>lacI^Q</i> O ₃ P _{lac} O ₁ P _{lac} O ₁	Kaelin et al. 1992
pJB4	Ap ^r Tc ^r ; 1.4-kb <i>Xba</i> I- <i>Pst</i> I fragment with <i>lacI^Q</i> O ₃ P _{lac} O ₁ <i>lacZ_T</i> in pJB33	This work
pJB6	Ap ^r Km ^r ; 1.3-kb PCR product with <i>lacIq</i> O ₁ in pCR2.1	This work
pJB12	Cm ^r ; 1.3-kb <i>Eco</i> RI fragment with <i>lacIq</i> O ₁ in pSa152	This work
pJB27	Ap ^r Km ^r ; 1.4-kb PCR product with <i>wreG</i> in pCR2.1	This work
pJB30	Ap ^r ; 7.8-kb <i>Eco</i> RI fragment with <i>wreG</i> in pBKS-	This work
pJB33	Ap ^r Tc ^r ; 1.4-kb <i>Xba</i> I- <i>Sac</i> I fragment with <i>wreG</i> in FAJ1700	This work
pSa152	Cm ^r ; IncW wide-host cloning vector	Tait et al. 1983
pSa322	Ap ^r ; IncW mobilizing plasmid	Tait et al. 1983
pTN2	pCOS109.11 (partial <i>wre</i> cluster in pLAFR1) harboring <i>wreG</i> 2::Tn5	Cava et al. 1990

^a Abbreviations: Sm^r, Nal^r, Km^r, Sp^r, Ap^r, and Tc^r indicate resistant to streptomycin, nalidixic acid, kanamycin, spectinomycin, ampicillin, and tetracycline, respectively; Lps⁻, deficient in O antigen; Ndv⁻, forms pseudonodules because of insufficient infection; Ndv⁺, elicits normal nodule development. The Lps and Ndv phenotypes of strains CE480 and CE489 are isopropyl-β-D-1-thiogalactoside (IPTG) dependent.

ferred into *wreG* mutant strain CE358. In the resulting strain, appreciable amounts of LPS I were produced in the absence of IPTG, such that addition of IPTG provided only modest increases in LPS I (Fig. 3, lanes 3 and 4).

In order to reduce the expression of *wreG* in the absence of inducer, additional copies of *lacI* were introduced into this genetic background on a compatible multicopy plasmid (plasmid pJB12) (Fig. 2). Addition of this plasmid resulted in substantially greater repression of *wreG*, although some expression was still detected in the absence of IPTG (Fig. 3, lane 5). Examination of relative mRNA levels in reverse-transcription polymerase chain reaction (PCR) experiments also indicated that *wreG* was highly upregulated by addition of IPTG in strains constructed in this manner but that complete repression in the absence of IPTG was not obtained (data not shown).

Strain CE480 (Table 1) is an isolate of strain CE358 carrying plasmids pJB4 and pJB12. Strain CE489 (Table 1) was derived from CE480 by introducing transposon mTn5*gusA20*, which harbors constitutively active *gusA*. With this modification, infection of *P. vulgaris* by CE489 could be monitored by GUS staining.

The effect of manipulating *wreG* expression during nodule development.

Strain CE489 was grown in tryptone yeast (TY) medium containing IPTG and then inoculated onto plants grown in

plastic pouches in which the root medium was supplemented with IPTG. On a subsequent day, IPTG was removed by pouring off the medium, rinsing the roots and pouches with water, and adding medium not containing IPTG. After treating inoculated plants for differing periods with IPTG in this way, the resulting infection and nodule development were documented by i) inspection of roots for the quality of nodule development at 12 dai (Fig. 1L to Q); ii) measurements of bulk nitrogenase activity, nodule number, and nodule weight after 12 dai (Fig. 4); and iii) GUS staining of infection in nodule slices after 8 dai (Fig. 1R to T).

Unless IPTG was present, strain CE489 grown *ex planta* made substantially less LPS I than LPS II, a form of LPS that lacks O antigen (Fig. 3, lane 5). Other strains with this LPS phenotype (low LPS I to LPS II ratio) elicit small, white pseudonodules on *P. vulgaris* in which infections cease within the root hair (Noel et al. 1986, 2000). Such pseudonodules lack nitrogenase activity and many anatomical features that define true nodules (Newman et al. 1992). When CE489 was grown in the presence of IPTG, on the other hand, the ratio of LPS I to LPS II was much closer to that of the wild type (Fig. 3, lane 6). Hence, it was expected that the extent of symbiotic development elicited by this strain would be dependent on IPTG, and it was (Figs. 1L to T and 4).

When IPTG was never added or was removed after 2 dai, the resulting nodulation at 12 dai consisted of small, white

Fig. 2. Plasmids pJB4 and pJB12. Except for the indicated DNA inserts, the sequence of pJB4 is derived from vector pFAJ1700 (Dombrecht et al. 2001) and that of pJB12 is from pSa152 (Tait et al. 1983). The expanded 2,905-nucleotide section below the plasmid maps indicates pertinent features of the 2,822-nucleotide *Xba*I-*Sac*I insert in pJB4, as well as two opposing transcriptional terminators from pFAJ1700 designed to prevent transcription into the inserted DNA from either side. Numbers indicate the nucleotide positions of the beginning and end of each genetic element, with gaps indicated by ---. Unique restriction sites *Xba*I, *Pst*I, and *Sac*I used in the cloning are centered at nucleotide positions 43, 1,480, and 2,865 in this sequence. Nucleotides 1 to 43 and 2,865 to 2,905 derive from pFAJ1700, 47 to 137 from pGEX-4T1, 138 to 1,474 ultimately from *Escherichia coli*, 1,501 to 2,813 from *Rhizobium etli*, and the remainder from polymerase chain reaction primers and vector pCR2.1. The 1,290-nucleotide insert in pJB6 used in constructing pJB12 includes an exact match of nucleotides 59 to 1,311 of this 2,905-nucleotide portion of pJB4. Abbreviations: *OPO_{lac}*, the *E. coli lac* operators O₃ and O₁ sandwiching the *E. coli lac* promoter; *lacZ_T*, truncated *lacZ* created in this work. Abbreviations on the plasmid maps are explained by Dombrecht and associates (2001) and Tait and associates (1983).

pseudonodules (Fig. 1L and M) typical of *R. etli* mutants deficient in LPS I. Essentially no larger (true) nodules (Fig. 4), no infection (Fig. 1R), and no nitrogenase activity were observed with these treatments (Fig. 4). Maintaining IPTG in the medium until the end of 4 dai, on the other hand, allowed limited infection (Fig. 1S) and uniformly much larger nodules (Fig. 1N). Nodule number measured at 12 dai was not appreciably increased and nodule weight was only modestly increased by further supplementation with IPTG beyond four days (Fig. 4). On the other hand, increases in infection and nitrogen fixation continued to accrue from maintaining IPTG in the medium until 8 dai (Figs. 1T and 4). These results are interpreted below (see Discussion) with respect to infection and its dependence on *wreG* expression.

Similar results were obtained with independent isolates constructed to have the same genotypes as CE480 and CE489. Control strains not carrying pJB4 showed neither positive nor negative effects of IPTG supplementation. Those such as strain CE424 having wild-type alleles of symbiotically important genes behaved normally (Fig. 4), whereas *wreG* mutant CE358 without pJB4 gave only pseudonodules lacking nitrogen-fixing activity when supplemented throughout an experiment with IPTG (data not shown).

DISCUSSION

IPTG was an effective inducer for bacteria during either determinate nodulation (the *R. etli*-*P. vulgaris* symbiosis) or indeterminate nodulation (the *S. meliloti*-*M. sativa* symbiosis). Importantly, IPTG itself seemed to cause no harm to the plant or to the symbiosis. Growth of plants in plastic pouches allowed for not only gene induction by presenting IPTG in the root nutrient solution but also downregulation by removing IPTG with a washing protocol.

The results with *gusA* expression (Fig. 1A to K) implied that IPTG from the root medium reached infecting bacteria in suffi-

ciently undegraded concentrations to sustain induction deep within the nodule. This was true in the apoplast (infection thread) but apparently IPTG also entered the infected plant cells, crossing a plant membrane into the symplast and two other membranes to reach bacterial cytoplasm. The clearest evidence in support of that idea was in Figure 1F, where staining implied that *gusA* in bacteria (presumably as bacteroids) already within infected cells was induced by IPTG added a week after nodule development was essentially complete. A further implication was that these bacteria or bacteroids were still active in general gene expression.

Cessation of GUS synthesis by withdrawing IPTG resulted in a sharp boundary of staining within the overall infection, particularly evident in the experiments with determinate nodulation (Fig. 1B and C). This observation is consistent with the concept that the bacteria divide only near the tips of the growing bacterial colony within the infection thread (Gage 2002, 2004; Gage et al. 1996). If bacteria throughout the infection threads divided equally after repression was triggered, all bacteria would end up with equally dilute concentrations of GUS protein. Clearly, this type of result was not observed (Fig. 1B and C). This phenomenon sharpens repression that is artificially imposed by this technique, and helps to counteract deficiencies in this technique that delay the onset of repression (as discussed below).

The point at which the staining stops in Figure 1B and C (withdrawal of IPTG after 4 dai) is roughly where an infection has reached after 6 dai on bean roots (K. D. Noel, *unpublished data*). A very rough estimate deduced from this result is that an enzyme regulated in this way will become markedly decreased at the forefront of the bacterial colony within 2 days after IPTG is removed at this early point in infection. The concen-

Fig. 3. Control of *Rhizobium etli* *wreG* expression achieved ex planta. Strains were grown in tryptone yeast broth in the presence (+) or absence (-) of 0.1mM isopropyl- β -D-1-thiogalactoside, and lipopolysaccharide (LPS) production was measured by sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis. Greater staining of LPS I relative to LPS II was interpreted as greater expression of *wreG*. Labeling below the gel image indicates the genotypes of the loaded strains, which were 1, CE358; 2, CE3; 3 and 4, CE358 carrying plasmid pJB4; and 5 and 6, CE358 carrying both plasmids pJB4 and pJB12.

Fig. 4. Nodule development and nitrogenase activity depended on the extent of *Rhizobium etli* *wreG* expression, when controlled by timed applications of isopropyl- β -D-1-thiogalactoside (IPTG). *Phaseolus vulgaris* seedlings were inoculated with *R. etli* CE489 (*wreG*/pJB4/pJB12) or *R. etli* CE424 (as a control strain that has wild-type symbiotic properties independently of IPTG). IPTG (0.1 mM) was present in the root medium through the day indicated and then removed by the rinsing protocol. At the completion of 12 days after inoculation (dai), nitrogenase activity was measured by acetylene reduction and nodules were counted and weighed. Values for strain CE489 (connected by solid lines) are the means of duplicate samples. Duplicate samples were collected on the same days for strain CE424 but did not show trends with respect to period of IPTG supplementation. Instead, the CE424 values for each parameter were averaged over all time points and are indicated by the dashed lines. In counting nodules, apparent pseudonodules were excluded. Values for nodule mass are in milligrams per plant, while those for nitrogenase activity are in nmole ethylene per minute per plant.

tration of IPTG itself must decrease even faster after the washing procedure, because it is controlling transcription of the mRNA for a stable protein. When the protein is an enzyme, the product of its action might not decrease to an undetectable or ineffective level as fast as the enzyme protein does. This possibility brings up the point that every activity being investigated could follow different time courses of effects in this system of conditional regulation.

In a symbiosis with indeterminate development, this protocol of manipulated gene expression reveals the stratified nature of the resulting organ. The tip of the nodule distal to the root is relatively free of bacteria. In the region just below the tip, plant cells are actively undergoing infection, whereas the infected cells most proximal to the root are those that had been infected first. It is to be expected that restricting IPTG application to different periods on different roots could reflect the history of infection when the staining is put together in a composite portrait, as done in this study. One illustration was that providing IPTG only in the last week before harvest resulted in almost no staining in the third of the nodule closest to the root (Fig. 1K). Bacteria were present there (Fig. 1 G to I). Hence, these bacteria were relatively inactive biosynthetically, GUS was subject to relatively high turnover (proteolysis) there, or the IPTG was not reaching their cytoplasm.

This procedure was applied to a rhizobial gene by putting *R. etli wreG* under *lacI* control. The results indicated that, as with *lacI*-regulated *gusA*, the expression of a symbiotically relevant gene can be manipulated with this approach and, thereby, test predictions of hypothetical roles in symbiosis. The extent of infection, nitrogen fixation, and nodule development all depended on the length of time that IPTG was supplied to the root system in this case. It can be inferred that *wreG* was expressed at derepressed levels within infection threads as a consequence of IPTG being presented in the root medium and that, subsequently, repression could be established by the rinsing protocol. These are two requirements that the procedure must meet to be useful.

The explanation of these particular results starts with the well-documented observation that, unless an *R. etli* strain can produce at least 50% of the wild-type amount of LPS I, only sparse and scattered pseudonodules that lack bacteroids and nitrogen fixation result on *P. vulgaris* roots (Noel et al 2000). This was basically the result if IPTG was never added or was removed at the end of 2 dai. Therefore, the elicitation of true nodules by longer supplementation implies that IPTG was sustaining the expression of *wreG*. The response to longer IPTG supplementation was markedly nonlinear, particularly as shown by the dramatic effect that occurred between 2 and 4 days of IPTG supplementation (Figs. 1M and N and 4). This result may at first seem surprising; however, it is predicted from previous studies of how *P. vulgaris* nodule anatomical development depends on the progress of infection (Ferraioli et al 2004; Newman et al 1992). These prior studies have revealed what appears to be a triggering of the developmental pathway toward nodule-type anatomy when a certain extent of infection is achieved. Even if infections stop just beyond this point, the triggered development still results in anatomy characteristic of nodules, with peripheral vasculature and a central zone that is lacking from lateral roots and pseudonodules (Ferraioli et al 2004; Newman et al 1992, 1994).

When IPTG was washed out of the root medium and rhizosphere at the end of 4 dai with the mutants carrying *wreG* under *lacI* control, it may be inferred that infection went beyond the point of triggering true nodule formation because the resulting nodulation was nearly wild type in external morphology (Fig. 1N). Moreover, nodule number and size did not increase with IPTG supplementation beyond that time (Fig. 4).

However, the resulting infection was far from complete. Longer IPTG supplementation to approximately 8 dai provided greater infection and, ultimately, greater nitrogen fixation (Figs. 1T and 4).

These results imply that rinsing to remove the IPTG caused sufficient repression that LPS I content dropped below a threshold needed for further infection. In view of the timing of the IPTG effects, a tentative conclusion is that this dependence on LPS I content continued throughout infection thread formation. Likely, but less assuredly, it continued through the process of endocytosis and into bacteroid proliferation. Providing rigorous support for this idea will require more exacting analysis, including of LPS itself. This is precisely the type of question that prompted the development of this conditional expression system. To generalize, this technique conceptually is useful for asking any bacterial component that is important early in nodule development whether and when it is needed later in development.

However, the particular constructions for providing genetic control in this study are not optimal. For instance, the plasmid system used to control *wreG* did not yield complete repression. Repression was improved by adding additional copies of *lacI* and, undoubtedly, this approach can be refined further. Even with the *lacI^Q* mutation, the promoter for *lacI* is weak in both *E. coli* and *R. etli* (Ramirez-Romero et al. 2006), and 10-fold further increases in expression can be achieved in *E. coli* using different promoters (Oehler et al. 1994). In addition, the copies of the regulated gene can be reduced to one by introducing it into the rhizobial genome on a transposon along with the two *lac* operator sequences needed for efficient repressor action. Other potential solutions are to use different promoters and promoter-operator configurations, all ultimately triggered by IPTG induction. Alternatively, it may be that systems using other inducers and having more extreme induced/repressed ratios can be applied successfully in nodule development even if the inducer is metabolized by the plant.

A more fundamental problem is that, generally, the genetic product of interest is a secondary genetic product (protein) or tertiary product (a metabolite produced by enzymatic action). In those cases, repression will not immediately eliminate the product of interest. Nevertheless, the tertiary product examined in this study (LPS) obviously is amenable to this approach.

In summary, this work already has established several useful points. IPTG added to root media can be used to induce rhizobial genes during the course of infection and nodule development. A rinsing protocol removes IPTG sufficiently that genes under *LacI* control also can be repressed after infection is well underway. This regulation works in both bean (determinate) and alfalfa (indeterminate) nodule development. Despite the desirability of improvements as noted above, the system as it exists provides enough control to show that one specific rhizobial component (LPS O antigen) continues to act in nodule development much later than the point at which a null mutant is blocked.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions.

Bacterial strains and plasmids used in this study are listed in Table 1. *R. etli* and *S. meliloti* strains were grown at 30°C in TY medium (0.5% tryptone, 0.3% yeast extract, and 10 mM CaCl₂). For these rhizobia, the following antibiotics were used at the concentration noted: streptomycin (Sm), 250 µg/ml; nalidixic acid (Nal), 30 µg/ml; kanamycin (Km), 30 µg/ml; chloramphenicol (Cm), 20 µg/ml; and tetracycline (Tc), 0.5, 1, or 5 µg/ml. *E. coli* strains were grown at 37°C in Luria-Bertani medium (1.0% tryptone, 0.5% yeast extract, 1.0% NaCl) sup-

plemented with the appropriate antibiotic: Km, 50 µg/ml; Cm, 30 µg/ml; Tc, 15 µg/ml; ampicillin (Ap), 100 µg/ml; bleomycin (Bm), 10 µg/ml; or spectinomycin (Sp), 50 µg/ml.

Cloning the *wreG* ORF.

The *wreG2::Tn5* insertion mutation in strain CE358 arose first by transposon mutation of a 30-kb expanse of cloned *R. etli* DNA in *E. coli* to give plasmid pTN2, followed by transfer to *R. etli* CE3 and homologous double recombination that replaced the wild-type with mutant allele *wreG2::Tn5* (Cava et al. 1990). To locate the mutation and to identify the mutated ORF, DNA segments in plasmid pTN2 flanking each end of the Tn5 insertion were cloned separately by digesting with *Bam*HI and selecting for Km resistance to obtain one side of Tn5 and DNA flanking it, and by digestion with *Sph*I and selection for Bm resistance to obtain the other side. Nucleotide sequence was obtained in the flanking DNA of each clone by using a primer that initiated near the end of the inverted repeat in Tn5 (TN5 anti3) (Table 2) and primers initiating in the opposite direction from the cloning vector pBlueScriptKS⁺ (BKSF0542 and BKSR0977) (Table 2). The sequence of the mutated ORF was pieced together from these sequences and from comprehensive sequencing of a complementing 7.8-kb *Eco*RI fragment cloned into plasmid pJB30. Later, this sequence was confirmed with the publication of the total chromosomal sequence of *R. etli* CFN42, the wild isolate from which strain CE3 was derived (Gonzalez et al. 2006). From this data, it was concluded that the Tn5 insertion was located 355 nucleotides downstream of the putative start codon at nucleotide 784,822 of the published genome sequence. Primers CE358BHss and CE358BHs (Table 2) and CE3 genomic template DNA were used to obtain a PCR product containing the wild-type *wreG* ORF and the putative ribosome-binding site associated with this ORF. The PCR product was cloned into plasmid pCR2.1 to obtain pJB27. The *wreG* ORF was then subcloned from pJB27 into pFAJ1700 (Dombrecht et al. 2001) using *Xba*I and *Sac*I restriction sites. The resulting plasmid, pJB33, contained a 50-nucleotide sequence from pCR2.1 in which a unique *Pst*I site, situated between the *Xba*I site and the *wreG* PCR product, was used in the subsequent insertion of *lac* control elements described below.

Construction of plasmids for conditional expression.

A PCR product carrying *E. coli lacI^Q*, *P_{lac}*, truncated *lacZα*, and two *lacO* sequences was obtained by using pGEX-4T-1 (Kaelin et al. 1992; Smith and Johnson 1988) as template and IqPCRss and IqPCRas (Table 2) as primers. This product truncates *lacZα*; therefore, reverse primer IqPCRas included an in-frame stop codon, as well as a *Pst*I site for subsequent subcloning. As an *Xba*I-*Pst*I fragment containing *lacI^Q*, *P_{lac}*, truncated *lacZα*, and two *lacO* sequences (O3 and O1, in the locations found naturally in *E. coli*) (Lewis et al 1996; Müller-Hill

1998), this product was ligated to the major *Xba*I-*Pst*I fragment of pJB33 to form plasmid pJB4 (Fig. 2).

After it became apparent that *wreG* was only partially repressed by this construct under noninducing conditions, plasmid pJB12 (Fig. 2) was constructed to provide a bacterium with extra copies of the *lac* repressor on a plasmid compatible with pJB4. A PCR product (*lacI^Q*) from pGEX-4T-1 obtained with *lacI*qss and *lacI*qas (Table 2) as primers was cloned into plasmid pCR2.1, resulting in plasmid pJB6. Exploiting *Eco*RI sites pCR2.1 on either side of the inserted PCR product, an *Eco*RI fragment containing *lacI^Q* was cloned into the *Eco*RI site of IncW vector plasmid pSa152 (Tait et al. 1983), resulting in plasmid pJB12.

Rhizobial strain construction.

IncP plasmid pJB4 was transferred from *E. coli* to *R. etli* strains by triparental conjugation, including *E. coli* MT616 (Finan et al. 1986). Transconjugants were selected by resistance to Sm, Nal, and Tc on TY agar. IncW plasmid pJB12 was transferred to strains already containing an IncP plasmid by similar triparental matings involving either helper strain MT616 or *E. coli* HB101 (Boyer and Roulland-Dussoix 1969) carrying IncW mobilizer plasmid pSa322 (Tait et al. 1983). Transconjugants were selected by resistance to Sm, Nal, and Cm. *R. etli* and *S. meliloti* strains carrying a mini-transposon insertion conferring inducible (mTn5SSgusA10) or constitutive (mTn5SSgusA20) *gusA* activity were constructed by matings with *E. coli* S17-1 λ pir (Simon et al 1983) carrying pCAM110 or pCAM120, followed by selecting Sp resistance on TY agar (Wilson et al. 1995).

Sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis.

Strains were grown to stationary phase in TY broth or TY with 0.1 mM IPTG (supplemented with antibiotics to maintain plasmids) at 30°C. Cell-pellet material extracted into sodium dodecyl sulfate gel-loading buffer was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis with a resolving gel having 18% polyacrylamide. LPS in the gels was detected by silver-periodate staining (Noel et al. 2004).

Nodulation tests and treatment with IPTG.

Bean seed (*P. vulgaris* cv. Midnight Black Turtle Soup) (Idaho Seed Bean, Twin Falls, ID, U.S.A.), were disinfected with 5% hypochlorite for 15 min, rinsed five times with sterile deionized water, germinated in the dark on water-saturated filter paper for 48 h at 30°C, and planted in plastic growth pouches (Mega International, Minneapolis, MN, U.S.A.) containing 40 ml of nitrogen-free salts solution (0.6× RBN) (Wacek and Brill 1976) supplemented with 0.1 mM IPTG as noted. Alfalfa seed (*M. sativa* cv. Vernal) (OLDS Seeds Company, Madison, WI, U.S.A.) were sterilized, germinated, and

Table 2. Primer list^a

Primer	Sequence 5' to 3'	Temperature (°C)	Comments
BKSF0542	GGATGTGCTGCAAGGCGATTAAAGT	67.60	...
BKSR0977	CTGGCAGACAGGTTTCCCGACTG	69.69	...
CE358BHs	GGGATCCGCGGTCTGTCATTTCTGT	69.50	<u>Bam</u> HI
CE358BHss	GGGATCCCTAATCGGCTAACGCGGTC	69.50	<u>Bam</u> HI
IqPCRas	CCTGCAGTCATTCCAGTCACGACGTTGTA	70.10	<u>Pst</u> I and
STOP codon			
IqPCRss	TTCTAGACAGCGAGTCAGTGAGCGAGGAAG	70.10	<u>Xba</u> I
lacIqAS	CCTGCAGAAATGCGTTGCGCTCAC	67.98	<u>Pst</u> I
lacIqSS	TTCTAGAGAGCGAGGAAGCGGAAG	66.28	<u>Xba</u> I
TN5anti3	GCCGCACGATGAAGAGCAGAAG	66.40	...

^a Underlined bases indicate a restriction enzyme sequence that was designed into the primer. The bold TCA in the IqPCRas primer confers a stop codon in the *lacZ α* gene.

planted in the same way, except that immersion in 5% hypochlorite was for 30 min. The 2-day-old seedlings were inoculated with 0.25 ml of bacteria grown at 30°C in TY broth (if no IPTG was to be included in the plant medium initially) or TY with 0.1 mM IPTG. Plants were grown at 27°C with a cycle of 14 h of light and 10 h of darkness. For experiments in which the IPTG was removed to discontinue induction, the liquid contents of the pouch were poured out, and 200 ml sterile dH₂O was added. The pouch was pressed between pieces of stiff cardboard so that the water completely filled but did not overflow the pouch, in order to immerse the entire root system, for 15 min. The water was poured off and this rinse was repeated. Nitrogen-free salts solution (40 ml of 0.6× RBN) was added to the emptied pouch and growth of the plant was continued. To add IPTG for induction at some time after planting, liquid contents were poured from the pouch and 40 ml of 0.6× RBN containing 0.1 mM IPTG was added. After specified periods of time, roots were photographed, root sections were excised for staining nodule slices, or nitrogenase activity was measured as acetylene reduction. For the latter assay, the shoots were removed, intact nodulated roots were incubated with acetylene in stoppered serum bottles, and the production of ethylene was measured by gas chromatography on a Porapak N column (Noel et al. 1982). In some experiments, after measuring acetylene reduction, nodules were stripped from the roots, weighed, and counted.

Nodule staining.

Short root segments carrying nodules were sliced away from the remainder of the root. Use of pouches allowed aseptic removal of a root segment with a surface-sterilized razor blade and continued growth of the plant if desired. The nodules were sliced longitudinally with a new section of a double-edged razor blade and then put into 0.5 ml of staining solution (X-GlcA at 0.5 mg/ml, 0.02% sodium azide, 1 mM potassium ferrocyanide, 1 mM potassium ferricyanide, Sarkosyl at 0.1 g/ml, Triton X-100 at 0.1 g/ml, 1 mM EDTA, and 50 mM sodium phosphate, pH 7.0) in a 13 × 100 mm glass test tube. After incubation at room temperature for 15 min to 2 h without shaking the tube, the staining solution was replaced with 1.0 ml of a storage solution (0.02% sodium azide, Sarkosyl at 0.1 g/ml, Triton X-100 at 0.1 g/ml, 1 mM EDTA, and 50 mM sodium phosphate, pH 7.0). The slices were then viewed by wet mount in this storage solution. The staining procedures and solutions were modifications of those described by Wilson and associates (1995).

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