***Bradyrhizobium japonicum* Mutants with Enhanced Sensitivity to Genistein Resulting in Altered *nod* Gene Regulation**

H. Ip,¹ F. D'Aoust,² A. A. Begum,² H. Zhang,³ D. L. Smith,³ B. T. Driscoll,² and T. C. Charles¹

¹Department of Biology, University of Waterloo, 200 University Avenue West, Waterloo, Ontario N2L 3G1, Canada; ²Department of Natural Resource Sciences, McGill University, 21,111 Lakeshore Road, Ste-Anne-de-Bellevue, Quebec H9X 3V9, Canada; ³Department of Plant Science, McGill University, 21,111 Lakeshore Road, Ste-Anne-de-Bellevue, QC H9X 3V9, Canada

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Bradyrhizobium japonicum mutants with altered *nod* gene induction characteristics were isolated by screening mutants for genistein-independent *nod* gene expression. Plasmid pZB32, carrying a *nodY::lacZ* transcriptional gene fusion, was introduced into *B. japonicum* cells that had been subjected to UV mutagenesis. Ten independent transformants producing a blue color on plates containing 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside but lacking genistein, indicative of constitutive expression of the *nodY::lacZ* reporter gene, were isolated. β -Galactosidase activity assays revealed that while all of the 10 strains were sensitive to low concentrations of genistein, none exhibited truly constitutive *nodY::lacZ* expression in liquid culture. Soybean plants inoculated with three of the mutants were chlorotic and stunted, with shoot dry weights close to those of the uninoculated plants, indicating the absence of nitrogen fixation. Differences in the kinetics of *nodY::lacZ* expression and lipochitin oligosaccharide Nod signal production suggested that the strains carried different mutations. Some of these strains may be useful in mitigating the low root zone temperature-associated delay in soybean nodulation at the northern extent of soybean cultivation.

Soybean, a plant of subtropical origin, is now one of the most important crops cultivated in temperate North America. In the symbiotic association with *Bradyrhizobium japonicum*, soybean plants can fix nitrogen at rates of up to 200 kg ha⁻² year⁻¹ (Smith and Hume 1987), eliminating the need for environmentally and economically costly nitrogen fertilizers. The generation of effective soybean root nodules is a complex and highly regulated process that requires production and exchange of specific molecular signals between the host plant and the bacterial symbiont (Stacey 1995). One of the first interactions to take place is the secretion of isoflavones, such as genistein (Kosslak et al. 1987), by the soybean plant. Genistein induces the expression of the *B. japonicum* nodulation (*nod*) genes, by stimulating either the NodD₁ transcriptional activator or the NodV/W two-component regulatory system, or both, resulting

in induction of *nod* gene transcription (Göttfert et al. 1990; Loh et al. 1997; Stacey 1995). The products of the *nod* genes mediate the synthesis of lipochitin oligosaccharide (LCO) Nod factors (Long 1996). Nod factors are responsible for direction of the early stages of nodule development (Sanjuan et al. 1992).

Consistent with its subtropical origins, the soybean plant requires root zone temperatures (RZT) between 25 and 30°C for optimal establishment of symbiotic activity. At the northern-most limit for production of this crop in North America, low spring soil temperatures ranging between 10 and 15°C at 10-cm depth inhibit nodule development, and this inhibition is the major limiting factor for soybean production. At low RZT, both the accumulation of *nod* gene-inducing isoflavone compounds in the soybean root (Zhang and Smith 1996) and the sensitivity of the *nod* regulon to induction by these compounds (Zhang et al. 1996) are substantially reduced. Exogenous application of genistein results in short circuiting of the plant–bacterium signaling and has been proven an effective means of overcoming the adverse effects of low RZT on nodulation and N₂ fixation (Zhang and Smith 1995, 1997). Mutants in which the *nod* genes are expressed in the absence of the plant *nod* gene induction signals might similarly be able to overcome the low RZT inhibition of nodulation, and would then be a good alternative to the direct application of genistein in the field. The goal of the current study was thus to construct *B. japonicum* strains in which the *nod* genes are expressed in the absence of inducing molecules from the plant.

RESULTS

Isolation of mutants and initial characterization.

Colonies of *B. japonicum* strain ZB977, which contains the *nodY::lacZ* fusion plasmid pZB32 in the USDA110 strain background, are white on tryptone yeast (TY) tetracycline 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal) agar-solidified medium but are blue on the same medium amended with 5 μ M genistein. In contrast, USDA110 (parental strain, does not carry pZB32) forms white colonies either in the presence or absence of genistein. USDA110 cultures were subjected to UV treatments that resulted in up to 99% mortality. To identify mutants in which the *nod* regulon is expressed in the absence of genistein, pZB32 was introduced into the mu-

Corresponding author: Trevor C. Charles; Telephone: +519 888 4567 ext. 5606; Fax: +519 746 0614; E-mail: tcharles@uwaterloo.ca

tant pools by electroporation, and transformants selected on TY tetracycline X-Gal agar. Blue-pigmented transformants were single-colony streak-purified twice on the same medium. To confirm the phenotypes of the putative *nod*-constitutive mutants, the strains were cured of the *nodY::lacZ* reporter plasmid by screening colonies for spontaneous loss of tetracycline resistance, and then pZB32 was reintroduced by conjugation. Ten transconjugant colonies that appeared blue on TY tetracycline X-Gal plates were isolated. These strains, designated Bj30050 to Bj30059 (Bj30050yz to Bj30059yz if containing the reporter plasmid), thus appeared to be mutants in which the introduced *nodY::lacZ* fusion was expressed in the absence of genistein. All 10 mutants exhibited growth similar to the parental strain on minimal medium containing either succinate, glucose, or mannitol as sole carbon source (data not shown).

Measurements of β -galactosidase activity were carried out to quantify the expression levels of the *nodY::lacZ* gene fusion within the 10 different mutants containing the reporter plasmid. Initial assays carried out under optimal temperature (25°C) in the absence of genistein revealed that the *nodY::lacZ* gene fusion was not expressed constitutively in any of the mutants (data not shown). However, all of the mutants were found to have much higher expression of the *nodY::lacZ* gene fusion, compared with the wild-type ZB977, in the presence of 5 μ M genistein after a 6 h period of induction. These results suggested that the mutants were highly sensitive to plant signals such as genistein.

Symbiotic phenotypes of the mutants.

Soybean nodulation assays were performed (Table 1) to determine the symbiotic phenotype of the mutants cured of pZB32. All 10 mutants were able to induce nodules on soybeans at 25°C. There was considerable variation, however, in the apparent levels of nitrogen fixation. Plants in association with three of the mutants (Bj30051, Bj30056, and Bj30057) appeared chlorotic and stunted. Plants inoculated with the other seven mutants appeared similar to plants inoculated with the wild-type strain.

Induction of *nod* genes at different temperatures and genistein levels.

Based on the initial characterization, five representative strains (Bj30050yz, Bj30053yz, Bj30056yz, Bj30057yz, and Bj30058yz) were chosen for more detailed characterization. The *nodY::lacZ* expression profiles over time in these strain backgrounds were compared with that of ZB977 under different combinations of temperature (15 and 25°C) and genistein concentrations (0.1 and 5 μ M). Optimal induction conditions (5 μ M genistein, 25°C) were based upon previous work (Zhang et al. 1996). The suboptimal temperature (15°C) is 2°C below the critical nodulation-suppressing RZT that was previously reported (Zhang and Smith 1994). The suboptimal genistein concentration (0.1 μ M) was based on an earlier report (Smit et al. 1992).

Prior to the addition of genistein, none of the five mutants showed *nodY::lacZ* expression levels above the basal level of the wild-type control (Fig. 1). After 12 h under optimal conditions (25°C, 5 μ M genistein), each of the mutants exhibited *nodY::lacZ* expression levels above that of the ZB977 wild-type strain, ranging from 129 to 155% of the ZB977 value (Table 2). At the lower 0.1 μ M genistein concentration at

25°C, the mutants exhibited *nodY::lacZ* expression levels ranging from four to seven times the ZB977 value under the same conditions. Induction of the *nod* genes therefore appears to be highly sensitive to genistein at 25°C.

At the suboptimal temperature of 15°C and the lower genistein concentration of 0.1 μ M, all of the five mutants exhibited at least twofold greater *nodY::lacZ* expression than ZB977 under the same conditions, with one mutant, Bj30050yz, having 10 times the wild-type activity under these conditions and a full 40% of the activity exhibited by the wild-type strain under optimal conditions (Table 2). At 15°C with the higher 5 μ M genistein concentration, four of the five mutant strains exhibited *nodY::lacZ* expression levels reduced to $1/3$ to $1/2$ of the levels for the same strains at 25°C with the same 5 μ M genistein concentration (Table 2). In fact, at the higher genistein concentration, low temperature had a greater inhibitory effect on the expression in these four mutants than it does on the wild-type strain. Only strain Bj30058yz had a higher level of *nodY::lacZ* expression than that of ZB977 under these conditions; indeed, expression in strain Bj30058yz with 5 μ M genistein was higher at 15°C than it was at 25°C.

Kinetics of *nod* gene induction in the mutant strains.

While none of the five mutants had *nod* gene induction kinetics identical to each other, or to ZB977, all of these strains demonstrated a similar general pattern. After an initial lag, they took approximately 2 to 4 h to reach maximum rate (Fig. 1, Table 3). After 9 to 12 h of induction, *nodY::lacZ* expression levels either dropped or leveled off (data not shown). At 0.1 μ M genistein, each of the mutants had much greater rates of *nod* gene induction than the wild type, regardless of temperature (Table 3, Fig. 1). For strains Bj30056yz and Bj30058yz, rate of induction under optimal conditions (25°C, 5 μ M genistein)

Table 1. Shoot dry weight values of soybean plants inoculated with *Bradyrhizobium japonicum* mutants

Inoculated strain	SDW ^a	%Wild type	Symbiotic phenotype ^b
USDA110	1.86 $\pm$ 0.08	100	Fix ⁺
Uninoculated	1.19 $\pm$ 0.04	64	Fix ⁻
Bj30050	1.73 $\pm$ 0.08	93	Fix ⁺
Bj30051	0.99 $\pm$ 0.04	53	Fix ⁻
Bj30052	1.69 $\pm$ 0.12	91	Fix ⁺
Bj30053	1.92 $\pm$ 0.13	103	Fix ⁺
Bj30054	1.92 $\pm$ 0.07	103	Fix ⁺
Bj30055	1.96 $\pm$ 0.10	105	Fix ⁺
Bj30056	1.14 $\pm$ 0.08	61	Fix ⁻
Bj30057	1.22 $\pm$ 0.01	66	Fix ⁻
Bj30058	1.39 $\pm$ 0.07	75	Fix ^{+/-}
Bj30059	1.92 $\pm$ 0.13	103	Fix ⁺

^a Shoot dry weight (SDW) values are presented as mean mg per plant $\pm$ standard error of the mean. Eight replicates were done for USDA110 and the uninoculated plants, and four replicates were done for the rest. Each replicate consisted of one pot containing two plants, and the minimum number of plants per strain was eight. Data are of one experiment and were consistent upon repetition. Plants were harvested 28 days after inoculation.

^b The ability to fix nitrogen (Fix) was determined by evaluation of the SDW and appearance of the plants. Fix⁺ plants appeared green and healthy (similar to those inoculated with USDA110), Fix⁻ plants appeared chlorotic and stunted (similar to plants not inoculated with *B. japonicum* (uninoculated control)), and plants that were intermediate were scored as Fix^{+/-}. Root nodules were present on all plants inoculated with *B. japonicum* strains but not on the uninoculated controls.

was at least twice as rapid compared with the wild type. Bj30058yz was also the only strain to show a significantly higher rate of induction than the wild type under each of the tested conditions, particularly at 15°C, 0.1 μ M genistein, although the rate at 15°C with 0.1 μ M genistein was relatively low compared with the other mutants. At 15°C, each of the mutants had higher rates of induction than the wild type, regardless of genistein concentration. These observations indicate that the β -galactosidase activities following 12 h of induction, reported in Table 2, reflect the relative rates of induction of *nodY::lacZ* in each of the strains.

LCO production by the mutant strains.

In the absence of genistein, there was no detectable LCO production by any of the five mutant strains or the wild type (data not shown). When 0.1 μ M genistein was present, all five mutant strains were found to produce at least twice as much LCO as produced by USDA110 at the corresponding temperature (Table 4). At 25°C, Bj30058 produced nearly five times as much LCO as the wild type. At 15°C, Bj30056, Bj30057, and Bj30058 each produced more than five times the level of the wild type. This is an intriguing result, considering Bj30056 and Bj30057 were found to give a Fix⁻ phenotype on

Fig. 1. Effects of genistein and temperature on initial rate of *nodY* expression. β -Galactosidase activities resulting from expression of the *nodY::lacZ* fusion on pZB32 was measured in the wild-type and mutant strain backgrounds. **A**, Wild type (ZB977); **B**, Bj30050yz; **C**, Bj30053yz; **D**, Bj30056yz; **E**, Bj30057yz; and **F**, Bj30058yz. Conditions were 5 μ M genistein at 25°C (closed circle); 5 μ M genistein at 15°C (open circle); 0.1 μ M genistein at 25°C (closed triangle), and 0.1 μ M genistein at 15°C (open triangle). Each value represents the mean $\pm$ standard error of triplicate measurements.

soybean. Together with the results from the β -galactosidase assays, these results indicate that the mutants are, in general, more sensitive to genistein with respect to *nod* gene induction and subsequent production of LCO.

DISCUSSION

Earlier studies had shown that induction of the *nodY* operon in strain USDA110 by moderate levels of genistein was reduced at lower temperatures (Zhang et al. 1996). Higher genistein levels (20 μ M) resulted in substantially increased levels of *nod* gene expression at the low temperatures. In a subsequent paper (Zhang and Smith 1996), the authors proposed that nodulation delays at low RZT were due, in part, to the reduced sensitivity of *B. japonicum* to plant-derived isoflavone signals, and, in part, to the decreased production of isoflavone signals by the plant. This led to the idea that genetic uncoupling of the *nod* gene-encoded LCO production from the requirement for activation by isoflavone might circumvent the low RZT inhibition of nodulation.

The initial objective of this work was to develop constitutive *nod* gene expression mutants by UV mutagenesis, in order to overcome the delay of nodulation of soybean caused by suboptimal RZTs early in spring. Intriguingly, each of the characterized mutants turned out to be genistein ultrasensitive but not genistein independent when *nodY::lacZ* expression was assayed in aqueous culture. Consistent with this, no detectable levels of LCO Nod factor were produced by the mutants without genistein addition and they produced higher levels of Nod factor whenever genistein was added to the culture. Perhaps the mutants were ultrasensitive to trace amounts of inducing compounds present in the agar in the solid media. Other aromatic compounds not found in soybean roots, such as xanthone molecules, were previously found to be inducers of the *nod* genes in *B. japonicum* (Cunningham et al. 1991; Yuen et al. 1995). Because the mutants required much lower

levels of genistein for *nodY* operon induction at both optimal and low temperatures and exhibited greater rates of *nod* gene activation, we concluded that the mutants have enhanced sensitivity to genistein for *nod* gene induction.

The five mutants that were characterized in more detail can be classified according to kinetics of *nodY::lacZ* induction and symbiotic phenotype. Each mutant has distinguishing *nodY::lacZ* expression kinetics, reflecting that they are all distinct isolates. However, they can be roughly divided into three groups. From initial induction rates of *nodY::lacZ* expression (Fig. 1), it is apparent that Bj30050 and Bj30053 respond similarly to the four different induction conditions. The effect of low temperature on *nodY::lacZ* induction was greater than that of suboptimal genistein level (Fig. 1). The kinetics in terms of maximum rate of induction and the time required to reach the maximum rate are very similar for the two strains (Table 3), except that at the lower genistein concentration, low temperature affects expression in Bj30053 more than it does in Bj30050. Similarly, Bj30056 and Bj30057 can be grouped

Table 4. Production of lipochitooligosaccharide (LCO) by *Bradyrhizobium japonicum* strains

Strain	25°C		15°C	
	LCO ^a	%Wild type ^b	LCO	%Wild type
USDA110	1,650	100	979	59
Bj30050	3,640	221	2,250	136
Bj30053	5,370	325	4,440	269
Bj30056	6,840	415	6,230	376
Bj30057	6,380	387	5,670	344
Bj30058	8,140	493	5,640	342

^aLipochitooligosaccharide (LCO) production ($\times 10^{-12}$ μ g cell⁻¹) from cultures induced with 0.1 μ M of genistein. LCO production expressed as mean values of triplicate measurements. The standard deviation values were within 10% of the mean for all determinations.

^bRelative values expressed as a percentage of the USDA110 value at 25°C.

Table 2. Effects of temperature and genistein concentration on *nodY::lacZ* expression

Strain	5 μ M genistein at 25°C		5 μ M genistein at 15°C		0.1 μ M genistein at 25°C		0.1 μ M genistein at 15°C	
	Activity ^a	%Wild type ^b	Activity	%Wild type	Activity	%Wild type	Activity	%Wild type
ZB977	271 $\pm$ 5	100	187 $\pm$ 8	69	18.6 $\pm$ 0.5	7	10.4 $\pm$ 0.4	4
Bj30050yz	380 $\pm$ 20	140	123 $\pm$ 5	46	122 $\pm$ 1	45	109 $\pm$ 3	40
Bj30053yz	351 $\pm$ 14	129	100 $\pm$ 5	37	136 $\pm$ 5	50	41.3 $\pm$ 0.9	15
Bj30056yz	419 $\pm$ 18	155	141 $\pm$ 3	52	72.4 $\pm$ 2	27	61.4 $\pm$ 0.7	23
Bj30057yz	381 $\pm$ 10	141	156 $\pm$ 7	58	86.7 $\pm$ 2	32	52.5 $\pm$ 0.5	19
Bj30058yz	320 $\pm$ 2	118	372 $\pm$ 19	137	99.1 $\pm$ 2.2	37	21.9 $\pm$ 0.2	8

^a β -Galactosidase activity values (Miller units) are the means of triplicate assays $\pm$ standard error. Activity was measured following 12 h of induction.

^b Relative values are expressed as a percentage of the ZB977 value at 25°C with 5 μ M genistein.

Table 3. Maximum rates of induction of *nodY::lacZ*

Strain	5 μ M genistein at 25°C		5 μ M genistein at 15°C		0.1 μ M genistein at 25°C		0.1 μ M genistein at 15°C	
	Induction ^a	Time ^b	Induction	Time	Induction	Time	Induction	Time
ZB977	86	1.9	37	2.8	6.88	2.1	0.48	8.8
Bj30050yz	80.4	2.5	20.3	3.8	43	2.5	12.1	3.8
Bj30053yz	88.4	2.1	21.3	3.2	44.4	2.3	6.82	3.8
Bj30056yz	190	1.7	29.1	3.5	44.5	1.5	9.24	3.8
Bj30057yz	115	2.5	34.5	3.2	43.8	2.7	9.2	3.3
Bj30058yz	268	2.1	72.4	2.8	45.7	2.9	1.68	3.2

^a Maximum rate of induction of the *nodY::lacZ* gene fusion (Miller units per h), measured from slope of initial rates in Figure 1, determined by fitting the best dose-response curve up to 6 h.

^b Time (h) required to reach the maximum rate of induction.

together as another class (Fig. 1, Table 3). For these two strains, the kinetic effect of low temperature was similar to that of suboptimal genistein level, and the two effects were clearly additive, as they are in the wild-type strain. The LCO production levels are also similar under different conditions (Table 4). Moreover, soybean nodules formed by these strains were both Fix⁻. The fifth mutant strain, Bj30058, does not belong to either of the first two classes. Its *nodY::lacZ* expression phenotype is distinct from the other four mutants. It is far less sensitive to induction by suboptimal levels of genistein, and the adverse effect of low temperature is only observed at the low genistein concentration.

It was initially predicted that some or all of these mutants might have Fix⁻ phenotypes due to constitutive *nod* gene expression within the bacteroid. Elevated levels of expression of the *Rhizobium leguminosarum* bv. *viciae* *nod* genes in nodulo, whether from a multicopy plasmid (Knight et al. 1986) or as the result of *nodD* mutant alleles that cause enhanced sensitivity to inducer (Burn and Johnston 1987), resulted in Fix⁻ phenotype. Suppression of *nod* gene expression in wild-type bacteroids (Schlaman et al. 1991) has also been demonstrated. In *B. japonicum*, severe inhibition of *nodD*₁ and *nodY* gene expression (up to 90%) occurred when specific organic acids such as L-malate and fumarate were present in the culture (Yuen and Stacey 1996). Since these organic acids are recognized as the primary carbon sources used by the bacteroids, such results suggested a mechanism by which *nod* gene expression is inhibited in bacteroids. Although none of our mutants exhibited constitutive *nod* gene expression, three (Bj30051, Bj30056, and Bj30057) out of ten were Fix⁻. Considering the nature of UV mutagenesis, the possibility of more than one mutation contributing to the observed phenotype must not be discounted. Determination of the in nodulo *nod* gene expression of these three mutants should aid in the investigation of the *nod* gene regulation in bacteroids.

The underlying mechanism of *nod* gene regulation has been recognized as complex. Our collection of characterized novel mutants covering a spectrum of *nod* induction will be a useful tool for further studies on the fundamental mechanisms of *nod* gene regulation and the basis for disruption of nodulation at suboptimal RZT. Investigation of the genetic bases of the mutant phenotypes will contribute toward the understanding of the gene regulatory system. Of particular interest will be determination of whether there are any alterations in expression or activation of the regulatory *nod* genes (e.g., *nodD*₁, *nodVW*) in these mutants, perhaps akin to the *R. leguminosarum* bv. *viciae* *nodD* class IV mutants described by Burn and associates (1987, 1989). Such studies should lead to resolution of the mechanistic nature of the enhanced sensitivity and provide further insight into the sophisticated *nod* gene regulation during symbiosis. Finally, one or more of the mutant strains might prove to be of practical use and commercial value as inoculant that is not subject to the low RZT nodulation inhibition effect.

MATERIALS AND METHODS

Bacterial strains and culture.

B. japonicum strains USDA110 and ZB977 were obtained from G. Stacey, University of Tennessee. ZB977 is USDA110 containing the pZB32, a tetracycline-resistant IncP plasmid that carries a *nodY::lacZ* translational gene fusion. Broth cul-

tures of *B. japonicum* were grown in Erlenmeyer flasks with shaking (200 rpm) or in 16 × 150-mm test tubes on a Rollerdrum (New Brunswick Scientific Co., Edison, NJ, U.S.A.), at 28 to 30°C (unless otherwise indicated) using the following media: yeast mannitol (YM) (Somasegaran and Hoben 1994), yeast salts (YS) (Bishop et al. 1976), and TY (Beringer 1974). For plates, the media were solidified with 1.5% (wt/vol) Difco Bacto agar (Difco Laboratories, Detroit). Minimal agar was *Rhizobium* minimal medium (Broughton et al. 1986) supplemented with 15 mM mannitol, succinate, or glucose. Media were supplemented, as needed, with tetracycline (20 µg/ml), X-Gal (80 µg/ml), and genistein (4', 5, 7-trihydroxyisoflavone; Upstate Biotechnology, Lake Placid, NY, U.S.A.) at the indicated concentrations. Frozen permanent stocks were made using 7% dimethylsulfoxide and kept at -70°C.

UV mutagenesis and transformation of *B. japonicum* mutant pools.

Aliquots (5 ml) of USDA110 culture grown to an optical density at 600 nm (OD₆₀₀) of 0.5 to 0.7 in YM broth were dispensed into 100-mm-diameter glass petri dishes. Following exposure to 30,000 µJ of UV energy in a Stratalinker (Stratagene, La Jolla, CA, U.S.A.), resulting in up to 99% mortality, 1 ml of fresh YM broth was added, and the cells were incubated for 3 to 5 days in the dark. The irradiated cultures were subcultured into TY broth and incubated until an optical density of 0.4 to 0.6 was achieved, and then the cells were rendered electroporation competent (Cangelosi et al. 1991). Plasmid pZB32 DNA was isolated from ZB977 (USDA110 carrying pZB32) by the alkaline lysis method (Sambrook et al. 1989). The cells were transformed with this pZB32 DNA via electroporation (Gene Pulser; Bio-Rad Laboratories, Mississauga, Canada), using the method described by Hattermann and Stacey (1990). Following electroporation, the cells were immediately suspended in 1 ml of YM broth and incubated overnight. Transformants were selected by spreading on TY plates containing X-Gal and tetracycline. Blue colonies appearing after 7 days of incubation were isolated for further analysis. To verify the phenotypes, the strains were cured of pZB32, and then the reporter plasmid was reintroduced by conjugation using *Escherichia coli* S17.1 (Simon et al. 1983) carrying pZB32 as donor. Strains that did not appear blue on X-Gal medium were discarded. Ten independent strains with verified phenotypes were retained for study.

β-Galactosidase assays.

Cultures of strains carrying pZB32 were grown in 100 ml of YS broth plus tetracycline, to an OD₆₀₀ of 0.1 to 0.2. The cultures were equilibrated to the testing temperatures (15 and 25°C) for 2 h, after which time genistein was added to the appropriate concentration. Aliquots (1 ml) were removed at each time interval and stored in 1.5-ml microcentrifuge tubes at -70°C until β-galactosidase activity was determined. Triplicate β-galactosidase assays were performed for each sample using the method of Miller (1992) at a temperature of 37°C with *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as substrate. The A₄₂₀ and OD₆₀₀ readings were obtained using a 96-well plate reader spectrophotometer (Spectra Max 190; Molecular Devices, Sunnyvale, CA, U.S.A.).

Soybean nodulation assays.

Seeds of *Glycine max* (L.) Merrill cv. Bayfield were surface sterilized as described previously (Nieuwkoop et al. 1987).

After germination for 72 h, the seedlings were transferred to Leonard assemblies (Leonard 1943) containing vermiculite saturated with Jensen's nitrogen-free medium (Jensen 1942). Each assembly received four seedlings, with four replicas per strain tested. Three days after transfer, the seedlings were thinned to two per pot and inoculated with approximately 10^8 cells of *B. japonicum* culture diluted in sterile distilled water. Plants were grown in a Conviron growth chamber (Conviron Co., Winnipeg, Canada) at 25°C with a 16-h photoperiod and were watered with sterile distilled water when required. Observations were taken after 28 days.

Determination of LCO production.

LCO was measured by high-pressure liquid chromatography (HPLC) (Sanjuan et al. 1992). *B. japonicum* cultures were grown in 600 ml of YM to OD₆₂₀ of 0.4 to 0.6, equal volumes were divided into four flasks (150 ml), genistein was added, and the cultures were grown for 3 days at 15 or 25°C. After the final OD₆₂₀ was measured, the cultures were extracted with 40 ml of HPLC-grade butanol by shaking for 5 min, and then left to stand overnight to separate the two phases. A 25-ml aliquot of the upper phase was collected with a glass pipette, placed in a 250-ml evaporation flask, and stored at 4°C. The volume of the extract was reduced to 2 to 3 ml by evaporation at 80°C using a Yamato RE 500 Rotary Evaporator (Yamato Scientific American Inc., Orangeburg, NY, U.S.A.), and then 4 ml of 18% acetonitrile was added. The extracts were stored, in the dark, in glass tubes sealed with Parafilm at 4°C until analysis. The samples were analyzed by HPLC, using a Vydac C18 reversed-phase column (0.46 × 25 cm, 5 µm; Vydac, Hesperia, CA, U.S.A.) and a flow rate 1.0 ml/min. An isocratic run with 18% acetonitrile (AcN/H₂O; wt/wt) was conducted for at least 10 min to establish a baseline prior to each measurement. For 45 min following the loading of each sample, an isocratic elution with 18% of AcN was conducted to ensure the removal of most nonpolar light fractions, and then a linear gradient of up to 82% AcN was applied over 70 min. The retention time of the LCOs was 84 to 86 min, and values were extrapolated from peak areas.

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