Effects of Flavonoids Released Naturally from Bean (Phaseolus vulgaris) on nodD-Regulated Gene Transcription in Rhizobium leguminosarum bv. phaseoli

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Nine flavonoid aglycones released from black bean (Phaseolus vulgaris 'P1165426CS') seeds and roots induced nodC::lacZ transcription in Rhizobium leguminosarum bv. phaseoli strains containing extra cloned copies of the regulatory genes nodD1, nodD2, or nodD3 from that biovar. Individual flavonoids generally induced highest levels of nodC::lacZ transcription (Iₘ₉₅) with extra copies of nodD2, and the concentration required for half-maximum induction (I₅₀) was lowest with extra copies of nodD1 genes. One apparently unique feature of R. l. bv. phaseoli is that naturally released flavonoids with very diverse structures induce nod genes. For all three nodD genes, two compounds exuded from roots, genistein and naringenin, produced much higher levels of nodC::lacZ transcription than other flavonoids, but this fact was not explained by increased transcription of the nodD genes themselves. The remaining seven flavonoid aglycones showed reproducibly different capacities to induce nodC::lacZ transcription, but all were considerably less powerful inducers than genistein and naringenin in strains with extra copies of each of the nodD genes. Tests with glycosides of the nod-gene inducers showed that glycosides, which are normally released by bean, had lower I₅₀ values than the corresponding aglycones with all nodD genes. Additive interactions observed between the strong nod-gene inducer genistein and the weak inducer eriodictyol remain to be explained at the molecular level.

Additional keywords: anthocyanins, anthocyanidins, flavanones, flavonols, nodulation.

Certain plant flavonoids are highly effective inducers of nodABC transcription in Rhizobium and Bradyrhizobium, and interactions between flavonoids and rhizobia contribute to the specificity of symbioses involving these bacteria (reviewed by Long 1989; Young and Johnston 1989). These processes require the protein product of the regulatory gene nodD. Active flavonoids have been isolated from many legumes, and detailed studies of several legumes show that individual species can release numerous aglycone nod-gene inducers. Thus, alfalfa (Medicago sativa L.) (Hartwig et al. 1990a; Maxwell et al. 1989), vetch (Vicia sativa subsp. nigra L.) (Recourt et al. 1991; Zaat et al. 1989), and common bean (Phaseolus vulgaris L.) (Hungria et al. 1991a,b) release at least five to nine different flavonoid nod-gene inducers.

Although exudation of an appropriate flavonoid signal from plants apparently is the first step toward root nodule formation and symbiotic N₂ fixation, there is no clear understanding of how legumes profit by releasing more than one nod-gene-inducing flavonoid. The presence of three nodD genes in Rhizobium meliloti Dangeard and Rhizobium leguminosarum bv. phaseoli Jordan (Davis and Johnston 1990a; Györgypal et al. 1988; Honma and Ausubel 1987) suggests that various flavonoids released from their host plants, alfalfa and bean, may activate different NodD proteins. This concept is supported in the alfalfa symbiosis by the observation that 4,4'-dihydroxy-2'-methoxychalcone in root exudate activates gene products from both nodD1 and nodD2 (Hartwig et al. 1990b), whereas other flavonoids activate only the NodD1 protein (Hartwig et al. 1990b; Honma et al. 1990). Common bean roots and seeds release natural nod-gene inducers belonging to four different classes of flavonoids (Hungria et al. 1991a,b). Structurally diverse flavonoids from commercial sources also induce nod genes in R. l. bv. phaseoli (Davis and Johnston 1990b). The nod-gene-inducing activity of so many flavonoid structures in R. l. bv. phaseoli may be explained by two possible mechanisms. Either these molecules activate different NodD proteins, or all nodD gene products in R. l. bv. phaseoli are activated by a wider range of flavonoid structures than NodD proteins in R. meliloti. The purpose of this study was to test for specific interactions between nod-gene inducers released by common bean and the three nodD gene products in R. l. bv. phaseoli.

MATERIALS AND METHODS

Bacterial strains and plasmids. Derivatives of R. l. bv. phaseoli strain 4292 containing various combinations of a nodC::lacZ or nodD::lacZ fusion plus extra cloned copies of different nodD genes (Davis and Johnston 1990a,b) were used (Table 1). Strains were grown on rifampicin (50 µg ml⁻¹), tetracycline (2 µg ml⁻¹), kanamycin (150 µg ml⁻¹), and streptomycin (150 µg ml⁻¹) to maintain plasmids.

Assays. We assayed flavonoid-dependent transcription of nod genes as β-galactosidase activity (Miller 1972) by using techniques described previously (Hungria et al...
1991a). In *nodC::lacZ* assays, control bacteria that had not been exposed to flavonoids were subtracted spectrophotometrically from flavonoid-treated cultures of the same genotype. In *nodD::lacZ* assays, the effects of cloned copies of *nodD* genes on β-galactosidase activity were tested at the relatively high flavonoid concentration of 5 μM. Because of possible colorimetric interference by some flavonoids, a 5-μM control solution of the test flavonoid was subtracted spectrophotometrically from each assay culture. Bacteria not exposed to flavonoids were measured against the standard buffer solution.

The aglycones of natural *nod*-gene inducers released from bean seeds and roots were purified as described previously (Hungria *et al*. 1990a,b). When commercial standards were used in the assays, they were purified by high-performance liquid chromatography immediately before use. Flavonoid concentrations were calculated spectrophotometrically relative to an assay volume of 475 μl with known extinction coefficients (log ε) (Hungria *et al*. 1990a,b). Concentrations of flavone, 5-hydroxyflavone, 7-hydroxyflavone, chrysin (5,7-dihydroxyflavone), and galangin (3,5,7-trihydroxyflavone) (Spectrum Chemical Mfg. Corp., Gardenia, CA) were calculated with log ε values of 4.20 at 297 nm, 4.35 at 272 nm, 4.50 at 308 nm, 4.08 at 318 nm, and 4.07 at 360 nm, respectively (Mabry *et al*. 1970; Weast 1969).

The maximum β-galactosidase induction (*I* max) produced by each flavonoid was determined directly from saturating flavonoid concentrations in the assays. The half-maximum induction value (*I* 50) was then calculated in nanomolar units (nM) by graphical methods. All experiments were repeated at least twice with three replicates for each treatment. Reported values are representative of bacterial strain and flavonoid effects, and absolute means varied less than 12% between replicates and experiments.

### RESULTS

**Induction of *nodC::lacZ* transcription by flavonoid aglycones in the presence of different *nodD* genes.** Assays of natural *nod*-gene-inducing flavonoids (10 nM–10 μM) from bean seeds and roots showed that aglycones of all flavonoids induced *nodC::lacZ* transcription with each of the three *nodD* genes found in *R. l. bv. phaseoli* (Table 2). Uninduced control cultures produced the following mean values (β-galactosidase, U) across numerous experiments: strain 1, 200; strain 2, 870; strain 3, 480; strain 4, 675. When data for *I* max and *I* 50 values in Table 2 were averaged separately for flavonoids released from seeds and roots, there was no statistically significant difference between those groups of compounds for *I* max values with any *nodD* gene. Similar comparisons for mean *I* 50 values, however, showed that root flavonoids produced half-

### Table 1. Derivatives of *Rhizobium leguminosarum* bv. *phaseoli* 4292 used in this study

<table>
<thead>
<tr>
<th>Strain*</th>
<th>Plasmids*</th>
<th>Extra genes</th>
<th>Plasmid 1</th>
<th>Plasmid 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>pIJ1737 pKT230</td>
<td>nodC::lacZ</td>
<td>Vector alone</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>pIJ1737 pIJ1730</td>
<td>nodC::lacZ</td>
<td>nodD1</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>pIJ537 pIJ1731</td>
<td>nodC::lacZ</td>
<td>nodD2</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>pIJ537 pIJ1732</td>
<td>nodC::lacZ</td>
<td>nodD3</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>pIJ1734 pKT230</td>
<td>nodD1::lacZ</td>
<td>Vector alone</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>pIJ1734 pIJ1730</td>
<td>nodD1::lacZ</td>
<td>Vector alone</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>pIJ1735 pKT230</td>
<td>nodD1::lacZ</td>
<td>Vector alone</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>pIJ1735 pIJ1731</td>
<td>nodD1::lacZ</td>
<td>Vector alone</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>pIJ1736 pKT230</td>
<td>nodD1::lacZ</td>
<td>Vector alone</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>pIJ1736 pIJ1732</td>
<td>nodD1::lacZ</td>
<td>Vector alone</td>
<td></td>
</tr>
</tbody>
</table>

* (Parent strain 4292 contained Sym plasmid pRP2JL.
* Construction of plasmids pIJ734, pIJ1735, pIJ1736, and pIJ1737 is published (Davis and Johnston 1990b). The cloning vector pKT230 was used to introduce *nodD* genes in plasmids pIJ1730, pIJ1731, and pIJ1732.

### Table 2. Maximum *nod*-gene-inducing activity (*I* max) for the aglycones of flavonoids released naturally from bean seeds and roots, and concentrations required for half-maximum induction (*I* 50)

<table>
<thead>
<tr>
<th>Flavonoid</th>
<th><em>I</em> max (U)*</th>
<th><em>I</em> 50 (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>nodD1</td>
<td>nodD2</td>
<td>nodD3</td>
</tr>
<tr>
<td>Seed rinse</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Delphinidin</td>
<td>(3,3',5,5'-pentahydroxyflavilium)</td>
<td>432</td>
</tr>
<tr>
<td>Petunidin</td>
<td>(3,3',5,5'-pentahydroxy-5'-methoxyflavilium)</td>
<td>573</td>
</tr>
<tr>
<td>Malvidin</td>
<td>(3,4',5,7,8,8'-trans-hexahydroxy-3',5'-dimethoxyflavilium)</td>
<td>619</td>
</tr>
<tr>
<td>Myricetin</td>
<td>(3,3',5,5',7'-pentahydroxyflavone)</td>
<td>499</td>
</tr>
<tr>
<td>Quercetin</td>
<td>(3,3',5,5'-pentahydroxyflavone)</td>
<td>666</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>(3,4',5,7,8,8'-trans-hexahydroxyflavone)</td>
<td>827</td>
</tr>
<tr>
<td>Root exudate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genistein</td>
<td>(4',5,7-trihydroxyisoflavone)</td>
<td>1,031</td>
</tr>
<tr>
<td>Eriodictyo</td>
<td>(3',4,5'-tetrahydroxyflavanone)</td>
<td>650</td>
</tr>
<tr>
<td>Naringenin</td>
<td>(4',5,7-trihydroxyflavone)</td>
<td>808</td>
</tr>
</tbody>
</table>

* Assays measured β-galactosidase activity produced from *nodC::lacZ* fusions in *Rhizobium leguminosarum* bv. *phaseoli* strain 4292 with extra copies of *nodD1*, *nodD2*, or *nodD3*. Each value was calculated from means of three replicate assays at 11 flavonoid concentrations from 10 nM to 10 μM. Uninduced control values from bacteria of the same genotype were subtracted in all assays.
maximum induction of nodC::lacZ at significantly lower concentrations with each of the three nodD genes.

Equimolar mixtures of naringenin and genistein (10–100 nM), the most active inducers in the presence of nodD2, increased β-galactosidase activity significantly more (P ≤ 0.05) than predicted by an additive model of the two compounds (Fig. 1). Similar results were obtained for R. l. bv. phaseoli strains that contained extra copies of nodD1 or nodD3 (data not shown). Mixtures of genistein or naringenin with eriodictyol or malvidin (10–100 nM) also gave significantly more β-galactosidase activity in the presence of extra nodD1, nodD2, and nodD3 genes than that predicted from an additive model (data not shown).

When increasing amounts of the weak root nod-gene inducer, eriodictyol, were combined with a 200-nM concentration of the strong inducer, genistein, the effectiveness of the genistein was not decreased even at a 7:1 ratio of eriodictyol/genistein (Fig. 2). Rather, eriodictyol increased β-galactosidase activity significantly over that of 200 nM genistein alone.

**Effects of flavonoid structure on nodC::lacZ induction.** Within each class of natural bean flavonoids, increasing the number of free hydroxyl groups on the B ring (prime-numbered C atoms) decreased the I_max value and increased the I_90 value (Table 2). Genistein, the only isoflavone nod-gene inducer identified from bean, had the largest I_max and lowest I_90 of all natural bean flavonoids with all nodD genes, except for the comparison of I_90 values with naringenin in the presence of extra nodD2 genes (Table 2). At least one hydroxyl group must be present on the simple flavone molecule to activate a NodD protein in R. l. bv. phaseoli (Table 3). Although 5-hydroxyflavone activated NodD2 protein, only 7-hydroxyflavone induced nodC::lacZ transcription with extra copies of nodD1 or nodD3.

**Effects of glycosylation on flavonoid activity.** Seven of the nine nod-gene-inducing flavonoids studied here are released naturally from bean in glycosylated forms (Hungria et al. 1991a,b). Comparisons between hydrolyzed (aglycone) and unhydrolyzed (intact glycoside) flavonoids purified from bean seed and root exudates showed that the I_max of flavonoid glycosides with each nodD gene was statistically similar to data reported in Table 2 for aglycones (data not shown). The I_90 values averaged across all flavonoids, however, were increased significantly by hydrolysis (P ≤ 0.05), more than 80% in the presence of nodD1 or nodD3 and 44% for bacteria containing extra copies of nodD2 (data not shown).

**Induction of nodD genes by flavonoid aglycones.** Assays designed to test which flavonoids present in the previously studied bean exudate were responsible for inducing nodD1 transcription (Davis and Johnston 1990b) showed that at least two aglycones from both seed rinse (malvidin and kaempferol) and root exudate (genistein and naringenin) produce that response (Table 4). None of the four flavonoids induced transcription of either nodD2::lacZ or nodD3::lacZ fusion.

**DISCUSSION**

Data from this study show that flavonoids released naturally from seeds or roots of common bean (P. vulgaris)

![Fig. 2. Effects of eriodictyol in the presence or absence of 200 nM genistein on induction of nodC::lacZ in Rhizobium leguminosarum bv. phaseoli containing extra copies of nodD2. Means ± SE are from three replicates. SE bars are obscured by symbols for most data points.](image)

**Table 3. Maximum nod-gene-inducing activity (I_max) of selected flavones and the concentrations required for half-maximum induction (I_90)**

<table>
<thead>
<tr>
<th>Flavonoid</th>
<th>nodD1</th>
<th>nodD2</th>
<th>nodD3</th>
<th>nodD1</th>
<th>nodD2</th>
<th>nodD3</th>
<th>nodD1</th>
<th>nodD2</th>
<th>nodD3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flavone</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>7-Hydroxyflavone</td>
<td>380</td>
<td>900</td>
<td>480</td>
<td>540</td>
<td>660</td>
<td>1,110</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>5-Hydroxyflavone</td>
<td>0</td>
<td>620</td>
<td>0</td>
<td>510</td>
<td>660</td>
<td>970</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>Chrysin (5,7-dihydroxyflavone)</td>
<td>495</td>
<td>1,180</td>
<td>535</td>
<td>510</td>
<td>660</td>
<td>970</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Assays measured β-galactosidase activity produced from nodC::lacZ fusions in Rhizobium leguminosarum bv. phaseoli strain 4292 with extra copies of nodD1, nodD2, or nodD3. Each value was calculated from means of three replicate assays at six flavonoid concentrations from 50 nM to 5 μM. Uninduced control values from bacteria with the same genotype were subtracted in all assays.

*Not applicable in the absence of inducing activity.*
(Hungria et al. 1991a,b) activate each of the three nodD gene products present in R. l. bv. phaseoli to induce transcription of a nodC::lacZ fusion (Table 2). One major point demonstrated here is that activation of the three NodD proteins in R. l. bv. phaseoli depends less on flavonoid structure than do comparable processes in R. meliloti (Hartwig et al. 1990b). This minimal flavonoid specificity of NodD proteins in R. l. bv. phaseoli may represent one step toward the extreme case of flavonoid-independent nod gene transcription reported for some nodD mutants (Burn et al. 1987; Spanik et al. 1989). A second unusual result from this study is the observation that combining genistein with flavanones released naturally from bean roots produces synergistic or additive effects on nodC::lacZ transcription (Figs. 1, 2).

Structurally different flavonoids produced two- to sevenfold changes in $I_{\text{max}}$ and $I_{S0}$ values with individual NodD proteins (Table 2). The most discernible effect of flavonoid structure was the very high $I_{\text{max}}$ value for genistein, the only isoflavone nod-gene inducer identified from these beans (Table 2). Comparisons among the numerous anthocyanidins, flavonols, and flavanones released by bean showed that decreasing the number of free hydroxyls on the B ring (prime-numbered C atoms) markedly increased nod-gene-inducing activity as measured by both $I_{\text{max}}$ and $I_{S0}$ values. However, because galangin, which lacks B-ring hydroxyls, induced nod gene transcription to only 60% of the $I_{\text{max}}$ for kaempferol (data not shown), the 4'-hydroxylation on the B ring contributes substantially to nod-gene-inducing activity. All aglycones of bean nod-gene inducers have free hydroxyl groups at the C-5 and C-7 positions, but either hydroxyl activates NodD2 protein (Table 3). Thus, although a hydroxyl group in the C-7 position could be a common binding site for stimulatory and inhibitory compounds in other rhizobial species (Djordjevic et al. 1987; Kossak et al. 1987; Le Strange et al. 1990; Peters and Long 1988; Zaat et al. 1989), that hydroxyl is not required for nod gene induction in R. l. bv. phaseoli cells containing extra copies of nodD2 (Table 3). Glycosylation on the C-3 hydroxyl decreased the $I_{S0}$ value for R. l. bv. phaseoli, but whether this structural change reflects an interaction with NodD protein or an increased uptake during the assay is not known.

Results from this work emphasize the widely recognized need for a greater understanding of how flavonoids interact with NodD proteins. Not all NodD proteins in R. l. bv. phaseoli were activated to the same extent by individual, naturally released flavonoids. Thus, the nodD2 gene product generally had a higher $I_{\text{max}}$ than either the NodD1 or the NodD3 proteins (Tables 2, 3). Tests with combinations of some flavonoids produced greater than additive increases in nodC::lacZ transcription (Fig. 1), but the extremely positive synergisms produced by combinations of some other natural inducers in R. meliloti (Hartwig et al. 1990b) were not observed for any of the three nodD gene products with the flavonoid combinations tested. One possible interpretation of these results is that flavonoids activate NodD proteins by interacting at two sites. The eriodictyol-genistein results in Figure 2 are consistent with this possibility, but more specific biochemical measurements are needed to test that hypothesis.

Previous observations on the regulation of nodD transcription by bean exudate (Davis and Johnston 1990b) were extended by tests of individual, natural nod-gene inducers from bean (Table 4). Constitutive expression of nodD2 and nodD3 was not increased significantly by 5-μM treatments with four flavonoid aglycones (malvidin, kaempferol, genistein, and naringenin) released by seeds and roots. A higher level of basal nodD1 transcription, however, was nearly doubled ($P \leq 0.05$) by each of the flavonoids tested. These results are consistent with those reported for similar genetic constructions in R. l. bv. phaseoli strain 8401 (Davis and Johnston 1990b), which lacks the Sym plasmid pR2P2II present in strains used in this study. The stimulatory effect of the root exudate flavonoids genistein and naringenin on nodD1-dependent transcription of nodD1 indicates that this poorly understood phenomenon probably occurs in the root zone, as well as in the previously studied presence of crude seed rinse (Davis and Johnston 1990b). The absence of a significant stimulation of nodD2 transcription by either genistein or naringenin (Table 4) eliminates that mechanism as a possible explanation for the extremely high $I_{\text{max}}$ values measured for those compounds in the presence of extra copies of nodD2 (Table 2).

The capacity of R. l. bv. phaseoli nodD gene products to be activated by a broad range of flavonoids probably has evolved for at least two reasons. First, beans have a wide variety of seed colors that are associated with the presence of many different flavonoids (Feenstra 1960). Therefore, rhizobia that are induced to form root nodules by the widest array of those compounds would be favored in selection. Second, genetic instability in R. l. bv. phaseoli (Martinez et al. 1990) may result in losses of nodD genes, and strains in which the remaining NodD proteins can

### Table 4. Effects of major flavonoids released from bean seeds and roots on transcription of nodD::lacZ fusions in Rhizobium leguminosarum bv. phaseoli strain 4292

<table>
<thead>
<tr>
<th>Strain</th>
<th>lacZ fusion</th>
<th>Extra nodD</th>
<th>β-galactosidase activity (U) induced by indicated flavonoids&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Naringenin</th>
<th>LSD&lt;sub&gt;α&lt;/sub&gt;&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>nodC</td>
<td>None</td>
<td>196</td>
<td>264</td>
<td>221</td>
</tr>
<tr>
<td>5</td>
<td>nodD1</td>
<td>None</td>
<td>160</td>
<td>169</td>
<td>157</td>
</tr>
<tr>
<td>6</td>
<td>nodD1</td>
<td>nodD1</td>
<td>917</td>
<td>1,629</td>
<td>1,599</td>
</tr>
<tr>
<td>7</td>
<td>nodD2</td>
<td>None</td>
<td>521</td>
<td>554</td>
<td>566</td>
</tr>
<tr>
<td>8</td>
<td>nodD2</td>
<td>nodD2</td>
<td>582</td>
<td>666</td>
<td>607</td>
</tr>
<tr>
<td>9</td>
<td>nodD3</td>
<td>None</td>
<td>282</td>
<td>293</td>
<td>274</td>
</tr>
<tr>
<td>10</td>
<td>nodD3</td>
<td>nodD3</td>
<td>301</td>
<td>315</td>
<td>296</td>
</tr>
</tbody>
</table>

<sup>a</sup>Flavonoids were tested at a concentration of 5 μM in six replicates.

<sup>b</sup>Value for comparing significant flavonoid effects ($P \leq 0.05$) in each strain, unless indicated as being nonsignificant (NS).
be activated by numerous flavonoids would be favored in
selection. Now that the actual flavonoids released into the
seed and root zones have been identified (Hungria et al.
1991a,b), detailed tests of regulatory mechanisms that
control R. l. bv. phaseoli and other microbes in the rhizo-
sphere can begin.

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