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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* 'PI165426CS') 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 (I_{max}) with extra copies of *nodD2*, and the concentration required for half-maximum induction (I_{50}) 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 I_{50} 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 N_2 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.*

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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., Gardena, 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.

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

Strain[a]	Plasmids[b]	Extra genes	
		Plasmid 1	Plasmid 2
1	pIJ1737 pKT230	*nodC::lacZ*	Vector alone
2	pIJ1737 pIJ1730	*nodC::lacZ*	*nodD1*
3	pIJ1737 pIJ1731	*nodC::lacZ*	*nodD2*
4	pIJ1737 pIJ1732	*nodC::lacZ*	*nodD3*
5	pIJ1734 pKT230	*nodD1::lacZ*	Vector alone
6	pIJ1734 pIJ1730	*nodD1::lacZ*	*nodD1*
7	pIJ1735 pK230	*nodD2::lacZ*	Vector alone
8	pIJ1735 pIJ1731	nodD2::*lacZ*	*nodD2*
9	pIJ1736 pKT230	*nodD3::lacZ*	Vector alone
10	pIJ1736 pIJ1732	*nodD3::lacZ*	*nodD3*

[a]Parent strain 4292 contained Sym plasmid pRP2JI.
[b]Construction of plasmids pIJ1734, 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.

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 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})

Flavonoid	I_{max} (U)[a]			I_{50} (nM)		
	nodD1	*nodD2*	*nodD3*	*nodD1*	*nodD2*	*nodD3*
Seed rinse						
Delphinidin (3,3′4′5,5′,7-hexahydroxyflavylium)	432	690	347	808	1,124	1,622
Petunidin (3,3′,4′,5,7-pentahydroxy-5′-methoxyflavylium)	573	824	598	603	989	1,535
Malvidin (3,4′,5,7-tetrahydroxy-3′,5′-dimethoxyflavylium)	619	1,066	616	474	919	839
Myricetin (3,3′,4′,5,5′,7-hexahydroxyflavone)	499	607	880	1,044	1,350	2,341
Quercetin (3,3′,4′,5,7-pentahydroxyflavone)	666	788	986	614	1,167	2,018
Kaempferol (3,4′,5,7-tetrahydroxyflavone)	827	1,273	1,018	484	1,008	1,866
Root exudate						
Genistein (4′,5,7-trihydroxyisoflavone)	1,031	4,458	1,573	216	374	484
Eriodictyol (3′,4′,5,7-tetrahydroxyflavanone)	650	911	919	483	902	1,352
Naringenin (4′,5,7-trihydroxyflavanone)	808	2,441	1,253	232	279	673

[a]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 ($P \leq 0.05$) 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_{50} value (Table 2). Genistein, the only isoflavone *nod*-gene inducer identified from bean, had the largest I_{max} and lowest I_{50} of all natural bean flavonoids with all *nodD* genes, except for the comparison of I_{50} 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_{50} values averaged across all flavonoids, however, were increased significantly by hydrolysis ($P \leq 0.05$), 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.

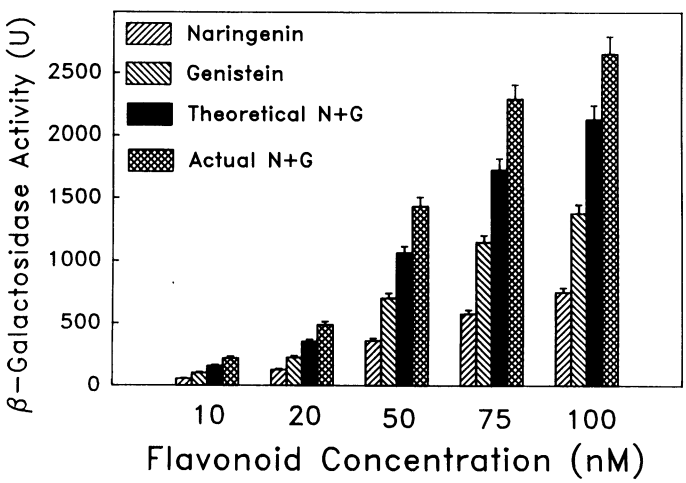

Fig. 1. Effects of genistein (G) and naringenin (N), separately and together, on induction of *nodC::lacZ* in *Rhizobium leguminosarum* bv. *phaseoli* containing extra copies of *nodD2*. Concentrations reflect those of separate flavonoids; flavonoid combinations contained twice the indicated concentration. Means ± SE are from three replicates.

DISCUSSION

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

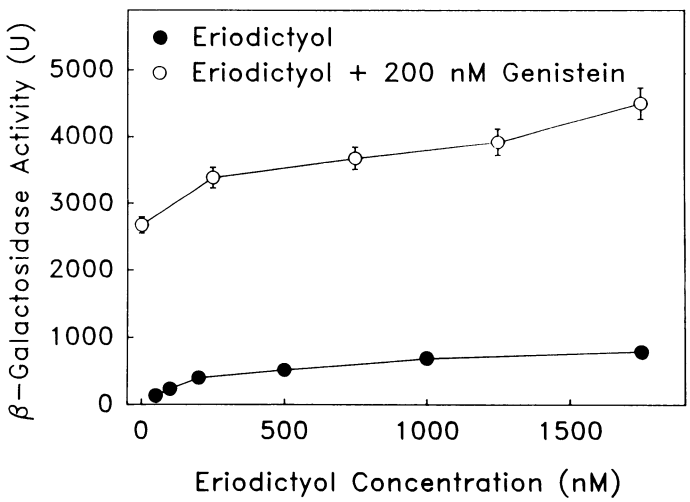

Fig. 2. Effects of eriodictyol in the presence or absence of 200 nM genistein on induction of *nodC::lacZ* in *Rhizobium leguminosarum* bv. *phaseoli* containing extra copies of *nodD2*. Means ± SE are from three replicates. SE bars are obscured by symbols for most data points.

Table 3. Maximum *nod*-gene-inducing activity (I_{max}) of selected flavones and the concentrations required for half-maximum induction (I_{50})

	I_{max} (U)[a]			I_{50} (nM)		
Flavonoid	***nodD1***	***nodD2***	***nodD3***	***nodD1***	***nodD2***	***nodD3***
Flavone	0	0	0	NA[b]	NA	NA
7-Hydroxyflavone	380	900	480	540	660	1,110
5-Hydroxyflavone	0	620	0	NA	1,180	NA
Chrysin (5,7-dihydroxyflavone)	495	1,180	535	510	660	970

[a] 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.

[b] 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; Spaink *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_{max} and I_{50} values with individual NodD proteins (Table 2). The most discernible effect of flavonoid structure was the very high I_{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_{max} and I_{50} values. However, because galangin, which lacks B-ring hydroxyls, induced *nod* gene transcription to only 60% of the I_{max} for kaempferol (data not shown), the 4′-hydroxyl 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; Kosslak *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_{50} 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_{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 pRP2JI 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_{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* (Martínez *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

Strain	*lacZ* fusion	Extra *nodD*	β-galactosidase activity (U) induced by indicated flavonoids[a]					$LSD_{0.05}$[b]
			None	Malvidin	Kaempferol	Genistein	Naringenin	
1	*nodC*	None	196	264	244	298	273	32
5	*nodD1*	None	160	169	157	188	177	NS
6	*nodD1*	*nodD1*	917	1,629	1,599	1,611	1,548	218
7	*nodD2*	None	521	554	566	581	570	NS
8	*nodD2*	*nodD2*	582	666	607	674	621	NS
9	*nodD3*	None	282	293	274	301	288	NS
10	*nodD3*	*nodD3*	301	315	296	308	299	NS

[a] Flavonoids were tested at a concentration of 5 μm in six replicates.
[b] 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 rhizosphere can begin.

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