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Induction of *Bradyrhizobium japonicum* common *nod* genes by isoflavones isolated from *Glycine max*

(symbiosis/nodulation/*nodC–lacZ* fusion/soybeans)

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ABSTRACT **The early events in legume nodulation by *Rhizobium* spp. involve a conserved gene cluster known as the common *nod* region. A broad-host-range plasmid (pEA2-21) containing a *Bradyrhizobium japonicum nodDABC–lacZ* translational fusion was constructed and used to monitor *nod* gene expression in response to soybean root extract. Two inducing compounds were isolated and identified. Analysis using ultraviolet absorption spectra, proton nuclear magnetic resonance, and mass spectrometry showed that the two inducers were 4′,7-dihydroxyisoflavone (daidzein) and 4′,5,7-trihydroxyisoflavone (genistein). Induction was also seen with some, but not all, of the flavonoid compounds that induce *nod* genes in fast-growing *Rhizobium* strains that nodulate clover, alfalfa, or peas. When pEA2-21 was introduced into *Rhizobium trifolii*, it was inducible by flavones but not by daidzein and genistein. In *Rhizobium fredii*, pEA2-21 was induced by isoflavones and flavones. Thus, the specificity of induction appears to be influenced by the host-strain genome.**

Members of the bacterial genus *Rhizobium* form symbiotic associations with leguminous plants that result in the formation of nitrogen-fixing root nodules. In three agronomically important *Rhizobium*/legume associations, *R. trifolii*/clover, *R. meliloti*/alfalfa, and *R. leguminosarum*/pea, important bacterial nodulation genes (1–4) and plant compounds that induce them (5–7) have been identified. In these associations flavones (5–7) or flavanones (7) have been found to induce the *nodABC* genes, as well as other *nod* genes involved in host specificity (1). Isoflavones have been found to inhibit the induction of *nodABC* in *R. leguminosarum* (7).

The *Bradyrhizobium japonicum*/soybean symbiosis is of considerable agricultural importance. In contrast to *Rhizobium* spp., *Bradyrhizobium* species are slow-growing (8), nitrogen-fixation and nodulation genes are located on the chromosome (9) and not on plasmids (10–12), and less is known about the genetic requirements for nodulation (13–15). In particular, the compounds produced by the soybean host that interact with the common *nod* genes have not been characterized.

In this study, a *nodABC–lacZ* translational fusion was used to monitor *nod* gene expression in *B. japonicum* in response to soybean root extract. Two major components from soybeans (*Glycine max* cv. Williams) were isolated that induced the expression of the *nodABC–lacZ* fusion when it was present in the soybean-nodulating bacteria *B. japonicum* and *Rhizobium fredii*, but not when it was present in *R. trifolii*.

MATERIALS AND METHODS

Strains and Plasmids. Standard procedures (16) were used for DNA manipulations. A *Hin*dIII fragment containing the *nod* region of *B. japonicum* USDA 123 was cloned in both orientations into the single *Hin*dIII site of plC19R (17). *Bam*HI–*Bgl* II fragments containing the *nod* genes and flanking polylinker sequences were then cloned into the *Bam*HI site of the broad-host-range vector pGD926 (18) resulting in pEA2-21 and pEA4-10 (Fig. 1). pEA2-21 and pEA4-10 were mobilized from *Escherichia coli* to *Bradyrhizobium* and *Rhizobium* strains by standard triparental crosses (18). The recipient strains *B. japonicum* 123spc1 and 110spc4, *R. trifolii* ANU843spc, and *R. fredii* 191spc1 are spontaneous spectinomycin-resistant mutants of wild-type strains. ANU-843spc was obtained from B. Rolfe (Canberra, Australia); 110spc4 was from H. Hennecke (Zurich); 123spc1 was from T. McLoughlin (Agrigenetics Advanced Science Company); and wild-type USDA 191 was from D. Weber (Beltsville, MD).

Plant Extract. Seeds (200 g) of *G. max* cv. Williams were surface-sterilized for 10 min in 10% Clorox, rinsed once with sterile water, immersed for 5 min in 1% HCl, and rinsed six times with sterile water. Seeds were plated on 1% yeast extract/mannitol (19) agar and germinated for 3 days in the dark at 27°C. The seedlings were extracted once with 9:1 methanol/water (24 hr) followed by 1:1 methanol/water (24 hr) (20, 21). The two methanol fractions were combined and rotary-evaporated to dryness. The resulting material was dissolved in water and sterilized through a 0.22-μm Corning cellulose acetate filter. A portion of the original extract was reserved (fraction I), and the rest was extracted three times with anhydrous ethyl ether (20, 21). Both the ether-soluble (fraction II) and aqueous-soluble (fraction III) fractions were rotary-evaporated to dryness. Fraction II was dissolved in 1.0 ml of absolute ethanol plus 99.0 ml of water and filter-sterilized. For biological assays, the pH was adjusted to 6.5. Fraction III was dissolved in 150 ml of water, 75 ml was reserved, and the remaining 75 ml was subjected to acid hydrolysis (6% HCl) (22, 23) followed by five extractions with anhydrous ethyl ether to yield an aqueous-soluble (fraction IV) and an ether-soluble (fraction V) fraction. Both fractions were rotary-evaporated to dryness. Fraction IV was dissolved in water, adjusted to pH 6.5, and filter-sterilized. Aliquots of fraction V were dissolved in methanol for chemical analyses or in ethanol for bioassays. An aliquot of fraction I was acid-hydrolyzed to remove *O*-glycosides (20), yielding fraction I_{HCl}.

Analytical Methods. The five fractions (I–V) from soybean seedlings were fractionated by high-pressure liquid chromatography (HPLC) using a μBondapak C_{18} reverse-phase column (Phenomenex, Rancho Palos Verdes, CA; 30 cm × 3.9 mm). Elution was with a 50–100% methanol gradient in water, over 20 min at a flow rate of 1 ml/min. The eluate was monitored at 254 nm. Two components from fraction V that induced expression of pEA2-21 were repurified by HPLC on

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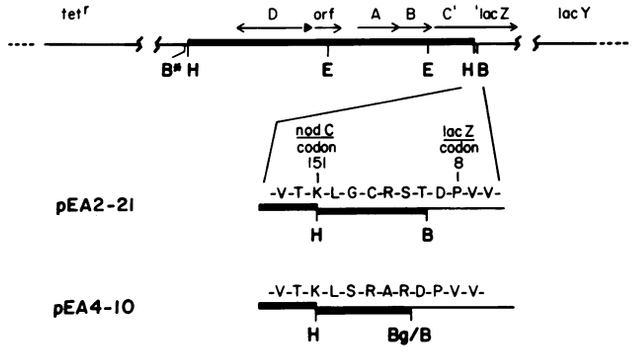

FIG. 1. Structure of pGD926 derivatives containing *nodC–lacZ* fusions. A 4.3-kilobase *Hin*dIII fragment containing USDA 123 *nod* genes (thick line) was cloned into the *Bam*HI site of pGD926 (thin line). The use of two alternative *Hin*dIII/*Bam*HI linker sequences resulted in two different *nodC–lacZ* fusion plasmids, pEA2-21 and pEA4-10. The amino acid sequences (standard one-letter symbols) of the *nod–lac* junctions are shown. Restriction enzyme abbreviations are H, *Hin*dIII; E, *Eco*RI; B, *Bam*HI; Bg, *Bgl* II; and B*, which designates a *Bam*HI site in pEA4-10 and a hybrid *Bam*HI/*Bgl* II site in pEA2-21. tet^R refers to the tetracycline resistance marker in pGD926.

an Alltech Hypersil silica 5-μm straight-phase column (4.6 mm × 250 mm) by isocratic separation using 90:10 chloroform/methanol at a flow rate of 1 ml/min. Ultraviolet–visible spectroscopy, mass spectrometry, and proton nuclear magnetic resonance (^{1}H NMR) spectroscopy were used for analysis of the compounds. Low- and high-resolution electron-impact mass spectometry was done on a Kratos MS 50, TC ultra-high-resolution instrument (Kratos Analytical Instruments, Ramsey, NJ), using a direct-insertion probe with a source temperature of either 140°C or 250°C, at the University of Wisconsin (Madison). Samples for ^{1}H NMR were prepared in di($^2H_3$)methyl sulfoxide (Aldrich) and analyzed on a Bruker (Bellerica, MA) 400-mHz NMR at the University of Wisconsin (Madison).

β-Galactosidase Assays. Assays for the induction of the *nodC–lacZ* fusion product were performed on toluene-permeabilized cells as described by Miller (22). For induction, cells were grown to early logarithmic phase and induced for 21 hr in either AIEHM medium (23), for *B. japonicum* strains, or TM medium (1), for *R. fredii* and *R. trifolii* strains. Three replicate cultures were assayed for each compound or fraction tested. Fractions were assayed for inducing activity by adding 1.5 ml of a fraction (or dilutions of a fraction) to 1.5 ml of cells. HPLC-purified components (of fractions I and V) or synthetic compounds were assayed by diluting 1.5 ml of cells with 1.5 ml of medium and adding desired concentrations of the HPLC-purified material or synthetic compound from stock solutions made up in absolute ethanol.

Sources of Flavonoid Compounds. Quercetin, umbelliferone, chrysin, 17β-estradiol, estrone, and diethylstilbestrol were obtained from Sigma; prunetin and formononetin from Spectrum Chemical (Gardena, CA); naringenin, apigenin, and biochanin A from Aldrich; daidzein, genistein, kaempferol, and luteolin from ICN; coumestrol from Kodak; and trigonelline from American Tokyo Kasei (Portland, OR). J. Norris (University of Texas, Austin) kindly provided samples of 7-hydroxyisoflavone, 5,7-dihydroxyisoflavone, 3′,4′,7-trihydroxyisoflavone, and 3′,4′,5′,7-tetrahydroxyisoflavone (baptigenin). 4′,7-Dihydroxyflavone was synthesized according to published procedures (24).

RESULTS

In several *Rhizobium* and *Bradyrhizobium* strains, the *nodABC* genes form an operon preceded by a conserved DNA sequence, the "*nod* box" (15, 25, 26), which is located near the transcription initiation site (27). In USDA 123, a 4.3-kilobase *Hin*dIII fragment contains *nodD*, the *nod* box, a potential open reading frame (*orf*), *nodA*, *nodB*, and the amino-terminal end of *nodC* (E.R.A., J.B., and D. Thompson, unpublished data). This *Hin*dIII fragment was cloned into pGD926 to create plasmids pEA2-21 and pEA4-10 (Fig. 1). The resulting *nodC–lacZ* fusion proteins retained activity, making it possible to monitor *nodC* expression by the β-galactosidase assay. The two plasmids behaved identically in all *nod*-expression experiments carried out to date (data not shown).

In preliminary experiments, growth of USDA 123(pEA2-21) in an aqueous soybean root exudate preparation resulted in a 3- to 7-fold increase in β-galactosidase activity compared to untreated cells. The active component in the exudate was heat-stable (1 hr, 121°C) and could be removed by passage over activated charcoal (data not shown). Based on these preliminary observations, an extract was obtained from 3-day-old seedlings soaked in methanol/water. This extract (fraction I) was partitioned into ether and acid-hydrolyzed to yield fractions II–V.

All of the fractions were tested for induction of the *nodC–lacZ* fusion in USDA 123 (Fig. 2). On a μg/ml basis, the ether fractions (II and V) were 100–1000 times more active than the aqueous fractions (I, III, and IV). Hydrolyzed

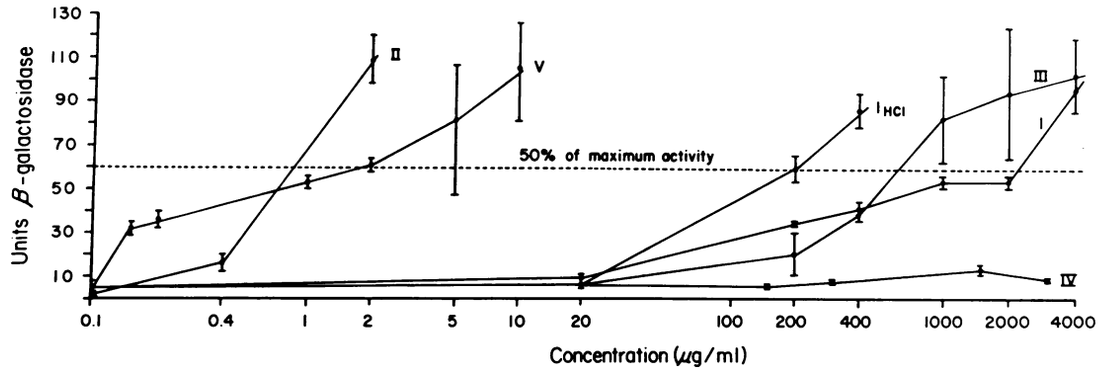

FIG. 2. Response of *nodC–lacZ* fusion to soybean root extract fractions. Values for β-galactosidase activity are given as the mean ± SD for *B. japonicum* strain USDA 123(pEA2-21). Controls consisted of cells grown in the absence of inducers (values represented at 0.1 μg/ml) and minus-cell controls. Fractions: I, original methanol extract; I_{HCl}, original methanol extract hydrolyzed for 1 hr with 6% HCl; II, ether-soluble material from fraction I; III, aqueous-soluble material from fraction I; IV, aqueous-soluble material following hydrolysis and ether extraction of fraction III; V, ether-soluble material obtained after hydrolysis of fraction III. Concentrations of the fractions tested are shown on a logarithmic scale in μg/ml. Total material recovered in fractions: I, 7.78 g; II, 0.04 g; III, 6.16 g; IV, 2.91 g; V, 0.10 g.

fraction I (I_{HCl}) was 5–10 times more active than unhydrolyzed fraction I. Concentrations greater than 10 μg/ml for fractions II and V, 400 μg/ml for fraction I_{HCl}, and 2000 μg/ml for fractions I and III had an inhibitory effect on the growth of USDA 123 (data not shown).

Fig. 3 shows HPLC profiles for fractions I and V, as well as the β-galactosidase activity associated with each peak. Significant activity was observed with two components (X and Y) that were eluted at 60–70% methanol in fraction I and with two components (H and I) that were eluted at 65–80% methanol in fraction V. Two minor components that were eluted at 80–88% methanol in fraction V also showed activity.

Ultraviolet and visible absorption spectra of the four major components suggested that they were isoflavonoids (20, 21). Furthermore, components X and H, and components Y and I, gave identical ultraviolet absorption spectra (data not shown). Spectra comparisons (Fig. 4 *Insets*) of the two unknowns with those of published isoflavones (21) tentatively identified component H as 4',7-dihydroxyisoflavone (daidzein), and component I as 4',5,7-trihydroxyisoflavone (genistein). Both component H and synthetic daidzein (Fig. 4*A Inset*) had absorption maxima at 248.3 nm and 300.8 nm and showed identical bathochromic shifts to 255.0 nm and 324.2 nm upon addition of sodium methoxide. Similarly, component I and synthetic genistein had absorption maxima at 260.8 nm, which shifted to 272.5 nm upon the addition of sodium methoxide (Fig. 4*B Inset*).

Electron-impact mass spectroscopy was used to confirm the identity of components H and I (Fig. 4). The daidzein standard and component H (Fig. 4*A*) showed essentially identical mass spectra with the molecular ion (M+) at *m/z* 254 and major fragmentation ions at 137 and 118. Genistein and component I (Fig. 4*B*) showed identical mass spectra with M+ at *m/z* 270 and major fragmentation ions at 118 and 153. These are the same fragmentation ions reported by Porter *et al.* (28) for daidzein and genistein. Daidzein and component H showed identical ^1H NMR spectra, as did genistein and component I (data not shown). The biological activity of synthetic daidzein and genistein and of components H and I was tested over a range of concentrations. Induction of the *nodABC–lacZ* fusion in USDA 110(pEA2-21) by purified and synthetic inducers was statistically indistinguishable (Fig. 5).

Flavonoid compounds similar in structure to daidzein and genistein were tested for their ability to induce *nodABC–lacZ* (Table 1). Of the compounds tested, 7-hydroxyisoflavone and 5,7-dihydroxyisoflavone were as effective as daidzein and genistein for induction of *nodABC–lacZ* in *B. japonicum*. Furthermore, a 4-hr exposure to these compounds was sufficient to detect induction of the *nodABC–lacZ* fusion in USDA 110 or USDA 123 (data not shown). No induction in the *B. japonicum* background was observed with luteolin, chrysin, naringenin, quercetin, umbelliferone, baptigenin, 3',4',7-trihydroxyisoflavone, or estrogen analogs of daidzein and genistein (29).

When the *nodABC–lacZ* fusion from USDA 123 was transferred into *R. trifolii*, no induction was observed with soybean root extract or any of the isoflavones tested. However, flavones previously identified as inducers for *R. trifolii nod* genes (6) were able to induce expression of the *B. japonicum nodABC–lacZ* genes in *R. trifolii*. In the *R. fredii* background, induction of the *B. japonicum nodABC–lacZ* fusion was observed with most of the flavonoid compounds tested. Thus, the range of compounds able to induce expression in *R. fredii* was considerably broader than that observed for either *B. japonicum* or *R. trifolii*.

DISCUSSION

We have identified the isoflavones daidzein and genistein as the major components in soybean root extract responsible for inducing the *nod* genes in *B. japonicum*. Both of these compounds have been previously identified in soybean flour (30), root extracts (28), and exudates (31). In *R. meliloti*, *R. trifolii*, and *R. leguminosarum*, flavones (5, 6) or flavanones (7) have been identified as the components of legume exudates that induce the *nod* genes. Firmin *et al.* (7) identified daidzein and genistein as potent antagonists of *nod* gene induction in *R. leguminosarum*.

Activation of *B. japonicum nodABC–lacZ* fusions by daidzein and genistein shows a concentration dependence (Fig. 5) similar to that reported for the induction of fast-growing rhizobia with flavones (5–7). However, at concentrations greater than 5 μM, both daidzein and genistein inhibited the growth of the cells. Isoflavonoids have been reported to function as either phytoalexins or their precursors (28). It is of interest that isoflavonoids as a class include substances associated with defense mechanisms against microbial pathogens and substances recognized as signals for induction of symbiotic genes in *B. japonicum* and *R. fredii*.

Some of the structural features required for induction of *B. japonicum nodABC–lacZ* genes can be inferred from the results presented in Table 1. It appears that hydroxylation at

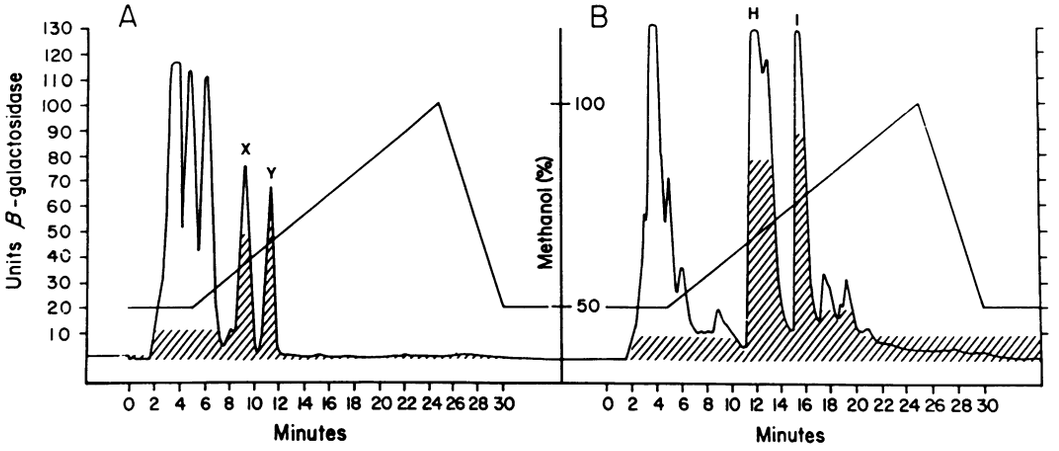

FIG. 3. Reverse-phase HPLC of extract (fraction I) and ether-extracted compounds (fraction V). (*A*) Elution profile of fraction I. (*B*) Elution profile of fraction V. For HPLC separation, 1.0 ml of fractions I and V were dried down and brought up in 1.0 ml of 50% methanol in water. A 500-μl aliquot of fraction I was injected and monitored at 254 nm. For fraction V, 100 μl was injected. β-Galactosidase activity (hatching) is shown for *B. japonicum* USDA 123(pEA2-21). Cells grown in the absence of inducers had less than 9 units of β-galactosidase activity.

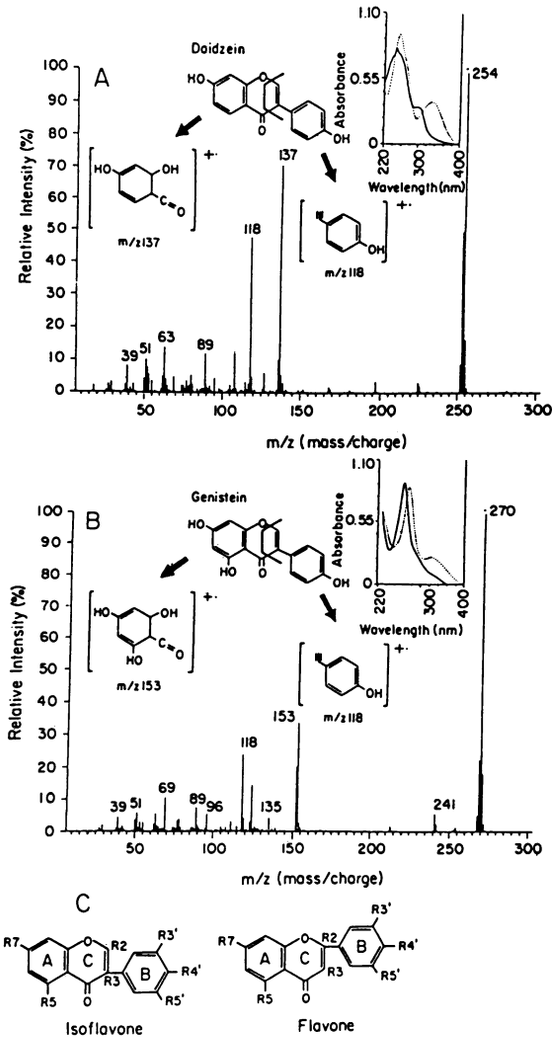

FIG. 4. Analytical chemical analysis of purified components H and I. Ultraviolet absorption spectra (*Insets*) and high-resolution electron-impact mass spectra are shown for purified H (*A*) and I (*B*). Ultraviolet and visible absorption spectra are shown in methanol (solid line) and methanol plus sodium methoxide (dotted line). The absorption spectrum of each was first measured in methanol, then two drops of sodium methoxide were added to a 1.0-ml sample and the absorption spectrum was measured again. Fragmentation of component H (*A*) occurs via a retro Diels–Alder process to produce *m/z* 118 and 153 ions. Same type of fragmentation produces *m/z* 118 and 137 ions for component I (*B*). The structures of purified H and I are shown in the respective panels. (*C*) Basic carbon skeleton of isoflavones and flavones tested for induction. A, C, and B rings are indicated. Positions where replacement of an H with either an OH or OCH_3 group occurred are shown. Isoflavones: daidzein (R7 = R4′ = OH); genistein (R7 = R5 = R4′ = OH); 7-hydroxyisoflavone (R7 = OH); 5,7-dihydroxyisoflavone (R7 = R5 = OH); baptigenin (R7 = R3′ = R4′ = R5′ = OH); biochanin A (R7 = R5 = OH; R4′ = OCH_3); formononetin (R7 = OH, R4′ = OCH_3); prunetin (R5 = R4′ = OH; R7 = OCH_3). Flavones: 4′,7-dihydroxyflavone (R7 = R4′ = OH); apigenin (R7 = R5 = R4′ = OH); luteolin (R7 = R5 = R3′ = R4′ = OH); chrysin (R7 = R5 = OH).

the 7 position on the isoflavone skeleton is sufficient for induction (Fig. 4*C*). Whether or not any hydroxylation on the A ring is required is not known because unsubstituted isoflavone has not yet been isolated in nature (21, 29). Methylation of the hydroxyl at the 7 position (e.g., prunetin) or the 4′ position (e.g., formononetin) significantly decreased expression of the *nodABC–lacZ* genes. Induction by a flavone molecule appears to require hydroxylation of the A and B rings. Chrysin, which is hydroxylated at the 5 and 7

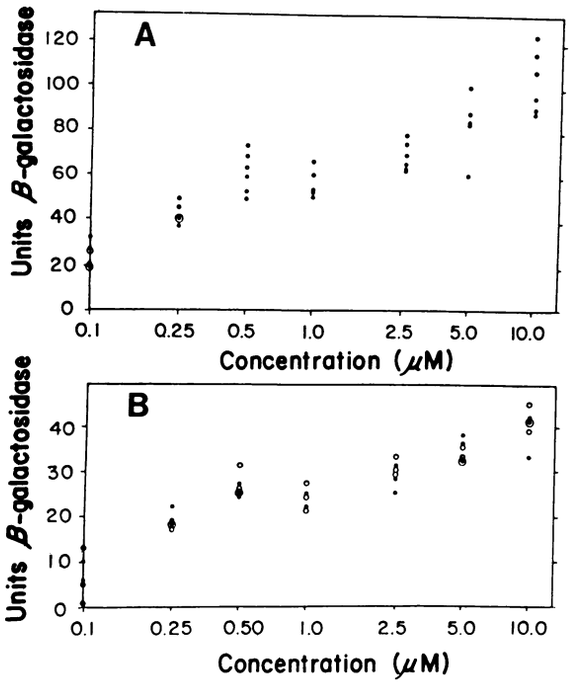

FIG. 5. Comparison of inducing activity of purified and synthetic inducers. (*A*) Component H (○) and synthetic daidzein (●). (*B*) Component I (○) and synthetic genistein (●). Units of β-galactosidase, corrected for background activity in the absence of inducers, are plotted on the vertical axis versus concentration of inducers. Values shown are for *B. japonicum* strain 110(pEA2-21) and are from a single experiment.

positions, did not induce pEA2-21, whereas both 4′,7-dihydroxyflavone and apigenin were able to induce pEA2-21. Hydroxylation at either the 3′ or 5′ carbons on the B ring results in loss of inducing ability for both isoflavones or flavones.

Flavonoids are commonly found as *O*-glycosides in nature; glycosylation makes them less reactive and more water-soluble, allowing for storage of these compounds in the plant cell vacuole (20). Approximately 75% of daidzein and genistein appear to be glycosylated, based on the recovery of the aglycone forms in fractions II and V. Eldridge (30) has also reported that the glucosides of daidzein and genistein accounted for over 50% of the total isoflavone content in soybean flours. Further, the inducing activity of fraction I was observed to increase after acid hydrolysis (Fig. 2), suggesting that the glycosylated forms are less active as inducers.

Introduction of the *B. japonicum nodDABC–lacZ* genes into *R. trifolii* resulted in induction only by flavones (Table 1). Isoflavones and soybean root extract were ineffective for induction in this background. Thus, the presence of the *nodDABC* genes from *B. japonicum* did not alter the spectrum of inducers already reported for *R. trifolii nod–lac* fusions (6). In the *R. fredii* background, induction of pEA2-21 was observed with both flavones and isoflavones, the spectrum of which was broader than that for either *B. japonicum* or *R. trifolii*. Interestingly, *R. fredii* shares similarities with both the fast-growing rhizobia and *B. japonicum*, which may explain, from an evolutionary standpoint, the less stringent requirements of *R. fredii* for plant factors from different legume hosts.

nodD is a regulatory gene that is constitutively expressed and is required for induction of other *nod* genes (32–34). Recently, it has been reported that different *nodD* genes show different inducer specificities (34). It might be expected that acquisition of a *B. japonicum nodD* would broaden the

Table 1. β-Galactosidase expressed by *nodABC–lacZ* fusions in response to flavonoid compounds

Compounds*	β-Galactosidase activity,† units			
	B. japonicum		*R. fredii*	
	USDA 110	USDA 123	USDA 191	*R. trifolii* ANU843
Background	10 ± 3	8 ± 5	25 ± 3	9 ± 4
SRE I	72 ± 20	95 ± 13	229 ± 8	16 ± 5
Isoflavones				
Daidzein	63 ± 6	76 ± 8	210 ± 23	17 ± 6
Genistein	67 ± 5	65 ± 2	196 ± 16	15 ± 1
7-Hydroxy-	56 ± 4	69 ± 2	226 ± 16	ND
5,7-Dihydroxy-	68 ± 5	110 ± 16	217 ± 7	ND
Baptigenin	12 ± 1	11 ± 1	ND	ND
3′,4′,7-Trihydroxy-	14 ± 1	9 ± 1	ND	ND
Biochanin A	37 ± 3	14 ± 2	163 ± 7	12 ± 6
Formononetin	19 ± 5	11 ± 1	183 ± 21	12 ± 3
Prunetin	20 ± 2	20 ± 3	138 ± 17	ND
Flavones				
4′,7-Dihydroxy-	43 ± 4	30 ± 4	201 ± 9	90 ± 10
Apigenin	21 ± 2	38 ± 12	194 ± 15	80 ± 10
Luteolin	7 ± 1	4 ± 1	182 ± 10	62 ± 6
Chrysin	6 ± 0	7 ± 1	179 ± 2	45 ± 10
Flavanone/flavonols				
Naringenin	8 ± 0	4 ± 1	181 ± 3	54 ± 3
Quercetin	7 ± 1	4 ± 1	46 ± 6	10 ± 2
Kaempferol	20 ± 2	18 ± 9	71 ± 10	ND
Coumestans				
Coumestrol	27 ± 4	13 ± 2	227 ± 9	12 ± 1
Umbelliferone	8 ± 1	4 ± 1	44 ± 2	10 ± 1

*All compounds were used at 5 μM, with the exception of soybean root extract fraction I (SRE I), which was used at 2 mg/ml. Other compounds tested with no detectable activity were β-estradiol (5 μM), estrone (5 μM), diethylstilbestrol (5 μM), and trigonelline (50 μM).

†Values represent means ± SD of three replicates. ND, not determined.

inducer spectrum for *R. trifolii*. The observed inability of pEA2-21 to be induced by isoflavones in *R. trifolii* could result from lack of proper synthesis or function of the *B. japonicum nodD* gene product in *R. trifolii*. Alternatively, *nodD* may not be the sole determinant of inducer specificity. A further complication is that *R. fredii* USDA 191 and *B. japonicum* USDA 123 each contain two different *nodD* genes (ref. 35; E.R.A., D. Thompson, and M. Maroney, unpublished data).

The results shown here indicate that genes invoived in the earliest steps in nodule formation are not fully expressed in the culture conditions traditionally used in laboratory studies of the *B. japonicum*/soybean symbiosis and in the preparation of commercial inocula for use in agriculture.

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