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Soybean Nodulating Rhizobia Modify *nod* Gene Inducers Daidzein and Genistein to Yield Aromatic Products that Can Influence Gene-Inducing Activity

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Degradation of *nod* gene-inducing isoflavones daidzein or genistein by soybean-nodulating *Bradyrhizobium japonicum* USDA 110*spc*4, *Rhizobium fredii* HH103, and *Rhizobium* sp. NGR234 was compared using gas chromatographic and mass spectrometric analyses of derivatized culture medium supplied with the gene inducers as substrates. The principal features of these biotransformations are an initiation of a C-ring fission mechanism and the formation of a number of structurally different subspecies of de novo flavonoids. The supernatants from the early phase of bacterial cultures contained C-ring modification products coumestrol (coumestan), liquiritigenin (flavanone), and umbelliferone (coumarin), together with a key intermediate chalcone, isoliquiritigenin. *B. japonicum* cell extracts contained coumestrol, liquiritigenin, and umbelliferone together with residual amounts of unmetabolized daidzein or genistein. Degradation products phloroglucinol, phloroglucinol carboxylic acid, or resorcinol, derived due to conservation of the A-ring, and the principal B-ring derived metabolites *p*-coumaric acid (4-hyroxy cinnamic acid) or 4-hydroxy benzoic acid and phenylacetic acid were also detected in the culture filtrates. Changes in the amounts of root exudate isoflavonoids during incubation with individual rhizobial strains and the detection of coumestrol and isoliquiritigenin when *B. japonicum* was separately incubated with the isoflavone glycosides daidzin and genistin in a nitrogen-free plant growth medium confirmed the degradative activity and provided additional evidence that microbial biotranformations of preexisting flavonoid pools can contribute towards the flavonoid composition of the rhizosphere. Rhizobial cells accumulated some of the inducer-derived metabolites whose planarity or hydroxylation patterns can influence gene induction. Two products in particular, umbelliferone and phenylacetic acid isolated via HPLC, affected *nod* gene expression in *B. japonicum* USDA 110*spc*4. The inducer-derived microbial metabolites reported here may also be involved in the development of phytoalexin resistance by rhizobia, chemotactic responses and in distinguishing a symbiotic from a pathogenic invasion.

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The establishment of a nitrogen-fixing symbiosis between a legume and a bacterium from the family Rhizobiaceae is the culmination of a complex series of bidirectional molecular signals initiated by the release of flavonoids from the roots of the host plant. These products of the phenyl propanoid pathway induce the transcriptional activation of rhizobial *nod* genes via an interaction with the *nodD* gene product(s) (Peters et al. 1986; Redmond et al. 1986), resulting in the synthesis by the bacterium of a host-specific lipooligosaccharide reverse-signal molecule (Nod factor) which causes hair curling and cortical cell division in the plant root. The molecular basis of the regulation and functions of rhizobial *nod* genes has been recently reviewed by Gottfert (1993) and Spaink (1994).

In the particular case of soybean (*Glycine max*) the principal *nod* gene-inducing compounds released from roots belong to a subclass of flavonoids, the isoflavonoids, and include the isoflavones genistein and daidzein and the coumestan, coumestrol (D'Arcy-Lameta 1986; Banfalvi et al. 1988; Kosslak et al. 1987). A chalcone, isoliquiritigenin, is also a minor component of soybean root exudate which possesses strong *nod* gene-inducing potential (Kape et al. 1992). In addition to inducing expression of *nod* genes, isoflavonoids have also been shown to induce resistance in *Bradyrhizobium japonicum* and *Rhizobium fredii* to the soybean phytoalexin, glyceollin (Parniske et al. 1991). Another indication that isoflavonoids could influence the expression of genes other than those directly involved in Nod factor synthesis was found in a study by Krishnan and Pueppke (1993). They identified an array of new proteins in the growth medium of *R. fredii* following treatment with genistein at *nod* gene-inducing concentrations.

In a recent study (Rao and Cooper 1994) we showed that *R. fredii* could metabolize genistein to yield a variety of monophenolic products derived from the conserved A- and B-rings of the isoflavone. Here we present further evidence for isoflavone degradation by *R. fredii*, *B. japonicum,* and another soybean-nodulating bacterium, *Rhizobium* sp. NGR234. We also show that some products generated during genistein degradation have an inhibitory influence on *nod* gene expression in *B. japonicum.*

RESULTS

Degradation products identified via direct GC-MS analyses.

Metabolites of daidzein and genistein were broadly categorized into (i) C-ring modification products in which the basic flavonoid ring system was conserved and (ii) C-ring fission products in which either the A- or B-ring was conserved. The rhizobia exhibited differences in the accumulation of individual degradation products in the media supernatants (Table 1).

C-ring modification: Bradyrhizobium japonicum USDA 110*spc*4 yielded isoliquiritigenin (open C-ring) and liquiritigenin (closed C-ring) as the major C-ring modification products of daidzein. Liquiritigenin was not detected in the culture supernatant extracts from *R. fredii* HH103 or *Rhizobium* sp. NGR234. In contrast, genistein yielded naringenin chalcone and naringenin as open and closed C-ring de novo flavonoid structures, respectively. However, the chalcone was not detected in *R. fredii* HH103 and *Rhizobium* sp. NGR234 culture filrates. Coumestrol, a member of the isoflavonoid subclass coumestans and also categorized as a C-ring modification product, was detected in all culture extracts obtained during the early phase of rhizobial growth.

C-ring fission products: Based on the numbered position of hydroxyl group substitutions present on A- and B-ring carbon (*C*) atoms of the original isoflavone substrate (see Fig. 2 below), characteristic products resulted from the flavonoid C-ring fission. Thus, daidzein, with a single hydroxylation in its A-ring (*C*7), yielded resorcinol as a conserved A-ring product, while genistein, with two hydroxyls in its A-ring (*C*5 and *C*7), yielded phloroglucinol and phloroglucinol carboxylic acid as the A-ring derived metabolites. A presumed A-ring derivative, umbelliferone (or its satellite compound 4-methyl umbelliferone), was also detected in all culture supernatant extracts. The B-rings of both gene inducers had the same single hydroxyl group substitution (*C*4′) and the conserved B-ring products, 4-hydroxy benzoic acid and 4-hydroxy cinnamic (*p*-coumaric) acid were found in all supernatant extracts. Quantitation of HPLC data from *B. japonicum* supernatant extracts showed that significant amounts of each *nod* gene inducer were metabolized in a 24-h period (Table 1).

Isoflavone degradation products identified in *B. japonicum* cell extracts.

The combined methods of separation on a polyamide-coated TLC, solid-phase extraction, GC-MS and HPLC on the cell extracts from *B. japonicum* USDA 110 *spc*4 revealed the presence of several metabolites in addition to the *nod* gene inducers themselves (Table 2). For example, daidzein-treated *B. japonicum* cells accumulated liquiritigenin, while genistein-treated cells contained naringenin. Coumestrol occurred in both inducer treatments. None of the principal A- or B-ring derived degradation products identified from the supernatants was detected in either of the two cell extracts, but umbelliferone was found in both treatments.

HPLC analysis of genistein-derived metabolites in culture supernatants of *B. japonicum* USDA 110*spc*4.

HPLC of bulked supernatant extracts was used to confirm the presence and to quantify some of the metabolites detected by GC-MS, as well as to provide a series of fractions for use in subsequent *nod* gene induction assays (Fig. 1). In this example fraction 1 contained two A-ring products: phloroglucinol (A_1) and phloroglucinol carboxylic acid (A_2). Fraction 2 contained two B-ring products: 4-hydroxy cinnamic acid (B_1) and 4-hydroxy benzoic acid (B_2). Fraction 3 had an A-ring

Table 1. Microbial metabolites identified[a,b] from the supernatants of media supplemented with either daidzein or genistein and incubated with rhizobia

Metabolites	Mode of biotransformation	Molecular ion peaks of methyl ether of the metabolite	*B. japonicum* USDA 110*spc*4	*R. fredii* HH103	*Rhizobium* sp. NGR234
Daidzein derived:					
Isoliquiritigenin	C-ring modification	284, 238, 131	+	+	+
Liquiritigenin		284, 194, 120	+	–	–
Coumestrol		296, 240	+	+	+
Resorcinol	Conservation of A-ring	210, 137, 109	+ (170)	–	+
Umbelliferone		176, 148, 133	+ (520)	+	–
4-Hydroxycinnamic acid	Conservation of B-ring	192, 161, 133	+ (930)	+	+
4-Hydroxybenzoic acid		166, 135	+ (1,250)	+	+
Phenylacetic acid		150, 135, 107	+ (620)	–	–
Genistein derived:					
Naringenin chalcone	C-ring modification	314, 137, 105	+	–	–
Naringenin		314, 180, 134	+	+	+
Coumestrol		296, 240	+	+	+
Phloroglucinol	Conservation of A-ring	168, 153, 110	+ (280)	–	–
Ploroglucinol carboxylic acid		226, 211, 183	+ (1,560)	+	+
4-Methyl umbelliferone		174, 148, 133	+	+	+
Umbelliferone		176, 148, 133	+ (690)		
4-Hydroxycinnamic acid	Conservation of B-ring	192, 161, 133	+ (1,354)	+	+
Phenylacetic acid		150, 135, 107	+ (550)	–	–
4-Hydroxybenzoic acid		166, 135	+ (980)	+	+

[a] The presence (+) or absence (–) of metabolites is based on GC-MS assays carried out at 2 and 24 h.
[b] Figures in parentheses are concentrations (nM) of products detected in HPLC analyses of *B. japonicum* culture supernatant extracts after 24 h.

derived umbelliferone (A_3, UV absorbance maxima (λmax) at 326 nm and m/z = 176) and a B-ring metabolite, phenylacetic acid (B_3). The presence of umbelliferone was further confirmed by its characteristic λmax shifts after derivatization with UV shift reagents (0.2% ethanolic solution of anhydrous sodium acetate or 10% sodium ethoxide: fresh sodium in ethanol) each of which gave Δλ = 49 (λmax = 375 nm). The residual substrate genistein (G, λmax at 265 and 340 nm; m/z: 270 and molecular ion peaks at 153 and 118) occurred in fraction 4. The final fraction (5) did not contain aromatic metabolites and was disregarded for further analyses. All aromatic components detected in fractions 1 through 4 corresponded to authentic standards with regard to their UV and mass spectra. Product concentrations after 24 h of incubation ranged from 280 to 1,560 nM (Table 1) and unmetabolized genistein was present at a concentration of 2,876 nM.

Proposed degradation pathways.

These are presented for genistein and daidzein (Fig. 2). In each case a chalcone (naringenin chalcone from genistein and isoliquiritigenin from daidzein) is the first product shown, indicating that the *C*-O bond of the C-ring undergoes an initial cleavage with concomitant reduction of the *C*2–*C*3 bond in the same ring. However, detection of closed ring compounds such as naringenin from genistein or liquiritigenin from daidzein also indicated the occurrence of C-ring closure without the *C*2–*C*3 bond reduction. These open and closed ring structures represent de novo species of flavonoids which upon further fissions in the C-ring release an array of conserved A- or B-ring products. The formation of open ring compounds (e.g., chalcones) and the detection of phloroglucinol (A-ring product) and 4-hydroxycinnamic acid (B-ring product) as cometabolites from genistein suggest the operation of aryl (B-ring) migration from the *C*3 to the *C*2 carbon atom of the C-ring prior to chalcone formation and ensuing C-ring fission activities. Low isoflavonoid substrate concentrations, bacterial medium and growth conditions coupled with rapid end product formation limited our ability to detect the transient intermediates involved in this mechanism. Chalcone structures would be expected to have very weak persistency in such physiologically active environments. The proposed pathways allow for the possibility that certain products may be formed from several sources. For example, umbelliferone could arise from resorcinol, coumestrol, or *p*-coumaric acid (or all three) in the daidzein degradation scheme.

Effect of microbial metabolites on *nod* gene expression in *B. japonicum* USDA110*spc*4.

The influence of the HPLC fractions containing various genistein-derived metabolites on *nod* gene expression in *B. japonicum* USDA 110*spc*4 is summarized in Table 3. The level of *nod* gene expression was measured by assaying the β-galactosidase activity of the *B. japonicum* variant. The measurements were made in a medium containing either whole or partial HPLC fractions in the presence of 1μM of the normal inducer genistein. Fractions 1 and 2 containing the principal A- and B-ring metabolites (A_1, A_2, B_1, B_2), respectively, did not significantly alter the *nod* gene expression. Fraction 3 containing the metabolites umbelliferone (A_3) and phenylacetic acid (B_3) significantly reduced *nod* gene expression by 36%. In the presence of normal inducer genistein, a reconstituted fraction containing umbelliferone but without phenylacetic acid (fraction 3 minus B_3) reduced β-galactosidase activity by 30%, and one that excluded umbelliferone but retained phenylacetic acid (fraction 3 minus A_3) reduced the gene-inducing activity by 20%. When both metabolites were omitted from the fraction, *nod* gene expression in the presence of genistein was almost restored to the levels of other noninhibiting fractions (e.g., fraction 1), indicating the absence of any other inhibitory components in this fraction. The particular concentrations of these metabolites in the fractions may have influenced results in these assays. This hypothesis was in part tested by preparing 1/1 mixtures of the metabolites/genistein. Under these conditions umbelliferone reduced the gene-inducing activity by 50%, while phenylacetic acid produced a 29% reduction in the level of β-galactosidase activity induced by genistein alone.

Changes in isoflavonoids from soybean root exudates before and after incubation with rhizobia in N-free rooting solution.

The levels of six isoflavonoids from sterile soybean root exudate were estimated before and after incubation with either

Table 2. Isoflavone degradation products identified in *Bradyrhizobium japonicum* USDA 110*spc*4 cell extracts by HPLC after 24 h of incubation[a]

Metabolite	Derived from: Daidzein (nM)	Derived from: Genistein (nM)
C-ring modification products		
Liquiritigenin	256	–
Naringenin	–	478
Coumestrol	390	350
Ring fission products		
Umbelliferone	279	235
Conserved A- or B-ring products	–	–
Residual (unmetabolized) isoflavone	370	405

[a] All compounds quantified by HPLC after 24 h were also detectable by GC-MS after 2 h.

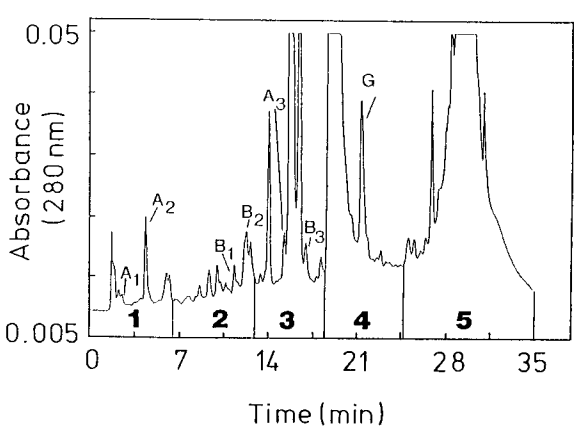

Fig. 1. High-performance liquid chromatography (HPLC) of bulk extracts of genistein (10 μM) supplemented medium following incubation with *Bradyrhizobium japonicum* for 24 h. Products and retention time (minutes): A1 = phloroglucinol (3.1 min); A2 = phloroglucinol carboxylic acid (4.3 min); A3 = umbelliferone (15.2 min); B1 = 4-hydroxybenzoic acid (11.4 min); B2 = 4-hydroxycinnamic (*p*-coumaric) acid (12.9 min); B3 = phenylacetic acid (16.7 min); G = residual genistein (21.1 min).

B. japonicum, *R. fredii*, or *Rhizobium* sp. NGR234 (Table 4). The levels of the glycosides daidzin and genistin were reduced by 14 and 12%, respectively, during incubation with *B. japonicum*, while a maximum 35% reduction in the glycosides was observed in the samples incubated with either *Rhizobium* sp. NGR234 or *R. fredii*. Variation in the levels of the aglycones daidzein and genistein was also observed. Daidzein concentration was enhanced after incubation with *B. japonicum* (15%), *R. fredii* (16%) and *Rhizobium* sp NGR234 (13%). In contrast, the amount of genistein declined after rhizobial incubation when compared to its original level in *Rhizobium*-free controls. *R. fredii*-treated root exudates produced a reduction of 34% in genistein concentration, while only a 14 and 6% decrease was recorded in samples obtained from *Rhizobium* sp. NGR234 and *B. japonicum* treatments, respectively. The level of coumestrol was enhanced by 9% after incubation with either *B. japonicum* or *Rhizobium* sp. NGR234 and by 13% after incubation with *R. fredii*. Isoliquirtigenin concentration increased by 22% after incubation with *B. japonicum* and by 10% after incubation with *Rhizobium* sp. NGR234 or *R. fredii*. Negligible changes in isoflavonoid concentrations were observed in incubated sterile controls. When *B. japonicum* USDA 110*spc*4 was incubated in a N-free plant growth medium supplemented only with daidzin or genistin the conversion of the isoflavone glycosides to aglycones and the presence of coumestrol and isoliquiritigenin were detectable, indicating the existence of microbial degradative activity.

DISCUSSION

Results from this study demonstrated that diverse soybean-nodulating rhizobia can metabolize significant quantities of the *nod* gene-inducing isoflavones daidzein and genistein via similar modes of biotransformation. In general, the strains differed only in the range of degradation products accumulating in cells or culture supernatants. The metabolites detected indicate that the degradation is initiated by means of fissions in the C-rings of the isoflavones. This type of flavonoid degradation has been reported for a number of other rhizobia (Rao et al. 1991; Rao and Cooper 1994).

An interesting feature of the catabolism of these isoflavones is the formation of a number of structurally different sub-

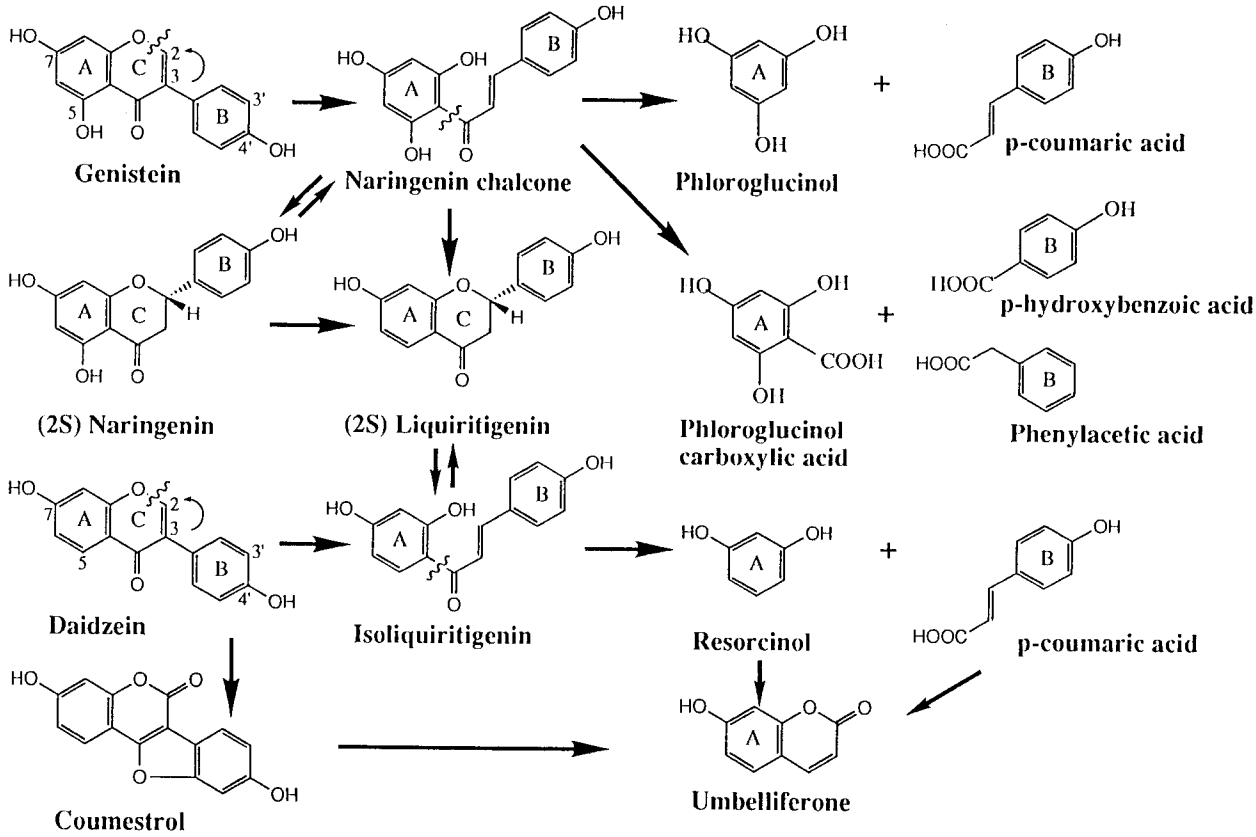

Fig. 2. Proposed degradation pathways for the catabolism of *nod* gene inducers daidzein and genistein. The rhizobial mode of biotransformation of the *nod* gene inducing isoflavones is initiated via a modification of the C-ring accompanied by aryl (B-ring) migration from the C_3 to the C_2 position. The modifications at C_2-O or C_2-C_3 bonds appear to be crucial steps in this form of C-ring cleavage mechanism. After an initial C-ring fission at the C_2-O bond, isoliquiritigenin and naringenin chalcone were the chalcones (open C-ring) formed from daidzein and genistein, respectively, with concomitant reduction of the C_2-C_3 bond. In the absence of C_2-C_3 reduction, ring closure yielded the flavanones liquiritigenin and naringenin; these intermediates possibly exist in stereo-isomeric forms and thus are depicted in an hypothetical 2S-configuration. Together with coumestrol (coumestan) and umbelliferone (coumarin) they represent new flavonoids arising from the rhizobial biotransformation of the original *nod* gene inducing isoflavones. Further fissions in the C-ring yielded conserved A- and B-ring products. The isomerization of chalcones and flavanones has been depicted with reversible arrows. In most respects, the degradative steps (fission mechanisms) displayed by rhizobia can be regarded as a reversal of plant flavonoid biosynthesis in which the A-ring, phloroglucinol (formed from 3 molecules of malonyl CoA) condenses with the B- and C-ring moiety, 4-hydroxy cinnamic acid (from 1 molecule of p-coumaroyl CoA) to yield the basic C-15 flavonoid skeletal structure. The initial C-ring modification steps involved in rhizobial flavonoid metabolism could be analogous to interconversions known in plants.

species of de novo flavonoids. Two closed C-ring modification products, coumestrol (coumestan) and liquiritigenin (flavanone), and an A-ring derived metabolite, umbelliferone (coumarin) together with a key intermediate chalcone, isoliquiritigenin (open C-ring) were detected in the supernatants during the early phase of bacterial growth. However, the closed C-ring metabolites and the coumarin persisted only in the cell extracts. Biosynthetically related new chalcones and flavanones have previously been observed in the root exudates of *Vicia sativa* subsp. *nigra* after inoculation with *Rhizobium leguminosarum* bv. *viciae* (Recourt et al. 1991) and their origin was attributed to de novo biosynthesis in roots and rapid release into the rhizosphere in reponse to inoculation. Common bean root exudates are also known to contain increased levels of coumestrol and daidzein in response to *Rhizobium* inoculation (Dakora et al. 1993). Our current findings, when taken together with those from our previous studies on flavonoid metabolism (Rao et al. 1991; Rao and Cooper 1994) provide strong support for an alternative explanation for the presence of new flavonoids in legume root exudates based on rhizobial biotransformation. Data from the present study indicate that substantial changes in the levels of root exudate isoflavonoids occur during incubation with rhizobia. Furthermore, in the same carbon- and nitrogen-free plant growth medium supplemented only with the isoflavone glycosides daidzin and genistin, and incubated with *B. japonicum*, detection of coumestrol and isoliquiritigenin together with the aglycones daidzein and genistein provided additional evidence for the operation of microbial degradative activity. These processes, together with other biological activities such as Nod factor-mediated release of flavonoids (Schmidt et al. 1994), could therefore be responsible for changes in the flavonoid content of the legume rhizosphere. Lack of added carbohydrate and nitrogen in the experiments with root exudate flavonoids makes a comparison with the culture medium experiments difficult, but it is apparent that biotransformations of naturally occurring flavonoids can occur under nutrient-limited conditions.

Table 3. Influence of complete and partial HPLC fractions on the *nod* gene-inducing activity in *Bradyrhizobium japonicum* USDA 110*spc*4 containing pRJ458 *(nod C-lacZ)*[a]

nod gene expression in presence of 1 μM of genistein		
	β-Galactosidase activity[b]	
Treatment	**Units**	**(%)[c]**
Whole Fraction I	1,240	91.8
Fraction I minus A_1, A_2	1,138	84.2
Whole Fraction II	1,222	90.5
Fraction II minus B_1, B_2	1,156	85.6
Whole Fraction III	860	63.7
Fraction III minus A_3	1,090	80.8
Fraction III minus B_3	956	70.8
Fraction III minus A_3, B_3	1,200	88.8
Whole Fractions IV	1,356	100.0
Fraction IV minus G, C	1,300	96.2
A_3 : Genistein (1:1)	678	50.2
B_3 : Genistein (1:1)	961	71.1
1 μM Genistein (control)	1,350	100.0

[a] A-ring products: A_1 = phloroglucinol, A_2 = phloroglucinol carboxylic acid, A_3 = umbelliferone. B-ring products: B_1 = 4-hydroxybenzoic acid, B_2 = 4-hydroxycinnamic acid, B_3 = phenylacetic acid. G = residual genistein, C = coumestrol.
[b] Mean variation ± 14.5 U; mean background activity of 94 U was deducted.
[c] The effect on *nod* gene-inducing activity as a percentage of activity of normal induction by 1 μM genistein (control).

The accumulation of daidzein- or genistein-derived metabolites in cells may have implications for the control of *nod* gene induction in rhizobia. Isoliquiritigenin is a strong *nod* gene inducer (Kape et al. 1992) and coumestrol can also act as a moderately effective gene inducer for *B. japonicum*, but umbelliferone and naringenin are potent *nod* gene inhibitors (Kosslak et al. 1990). The concurrent presence of some of these metabolites together with residual gene inducers in cells raises the possibility of competitive binding to NodD proteins as a mechanism for regulating *nod* gene expression. Our data also indicate that certain degradation products from *nod* gene inducers (e.g., umbelliferone and phenylacetic acid) can reduce levels of *nod* gene expression in *B. japonicum* in the presence of 1.0 μM of the normal gene inducer genistein. These results are more likely to indicate a specific effect of degradation products on *nod* gene induction than a general toxicity towards growth of the organism. In this study products were presented at nanomolar concentrations and previous work with the same strain of *B. japonicum* (Kape et al. 1991) failed to detect toxic effects from a variety of phenolic compounds at concentrations up to 100 mM.

Our findings point to a more complicated mechanism for the control of *nod* gene expression than the proposal by Hubac et al. (1994) involving enhanced retention of a single in-

Table 4. Levels of *nod* gene-inducing isoflavonoids from soybean root exudates before and after incubation with rhizobia in nitrogen- and carbon-free rooting solution

	Concentration (nM)[a]					
	Rhizobium-free[b] incubation		After incubation with			Rooting solution
Isoflavonoids detected	**Before**	**After**	***B. japonicum* USDA 110*spc* 4**	***R. fredii* HH103**	***Rhizobium* sp. NGR 234**	**+ gene-inducing glycoside[c] + *B. japonicum***
Daidzin	1,470	1,455	1,270	1,100	960	+[d]
Genistin	1,200	1,180	1,050	760	770	+
Daidzein	775	800	895	900	880	+
Genistein	1,060	1,075	995	695	905	+
Coumestrol	850	840	925	960	925	+
Isoliquiritigenin	290	285	355	320	315	+

[a] Data are means of three replicates; standard deviation in all cases < ± 5%.
[b] Uninoculated control.
[c] Daidzin (5 μM) + genistin (5 μM).
[d] + present after 36 h of incubation.

ducer molecule (luteolin) in the outer membrane of *R. meliloti*. Intensive studies using radiolabeled inducers are required for clarifying the interaction with the *nodD* gene product or other receptor molecules and the fate of the inducer in the bacterium (Peters et al. 1986).

It has been suggested that catabolism of flavonoids could be one of the earliest responses of rhizobia (Peters and Verma 1990) in a complex *nod* gene regulatory process. From our results further influences of flavonoid catabolism on the nodulation process could be proposed as follows: The open C-ring of isoliquiritigenin, derived from the degradation of daidzein or genistein, could offer increased molecular flexibility to match the optimal conformation of the NodD receptor protein required for *nod* gene regulation (Spaink 1994). Further studies at the subcellular level would be required to verify this suggestion. Isoliquiritigenin and coumestrol can have dual functions in symbiotic communication. Both compounds are potent inducers of *nod* genes or rhizobial resistance towards phytoalexins (Parniske et al. 1991) and have been implicated in the ability of *B. japonicum* to nodulate soybean (Werner et al. 1994). Plant-derived isoflavonoids such as coumestrol and its precursor daidzein, are thought to act as signaling molecules from legumes for distinguishing pathogens from symbionts (Dakora et al. 1993). A possible role for rhizobially derived coumestrol is that its phytoalexin-like structure could confer the microsymbiont with specific tolerance towards phytoalexins during the initial phase of symbiosis.

The formation of a variety of monocyclic hydroxy aromatic metabolites could have implications for other rhizosphere phenomena such as growth and competition for nodule formation. The presence of specific *nod* gene inhibitor compounds (Cunningham et al. 1991) can offer chemical control of interstrain competition for soybean nodulation by *B. japonicum*, while strain-specific *nod* gene inhibition was associated with differences in flavonoid metabolism among competing *Bradyrhizobium* strains (Kosslak et al. 1990). It is also interesting to note that rhizobia can generate products such as hydroxycinnamic acids (e.g., *p*-coumaric acid) which have been shown to possess chemotactic properties towards *B. japonicum* (Kape et al. 1991).

MATERIALS AND METHODS

Bacterial strains.

B. japonicum USDA 110*spc*4, *R. fredii* HH103, and a derivative of *B. japonicum* USDA 110*spc*4 harboring plasmid pRJ458 containing a *nodC–lacZ* fusion were obtained from the Botany Department, Marburg University, Marburg, Germany. *Rhizobium* sp. NGR234, was supplied by the Laboratory of Plant Molecular Biology, University of Geneva, Geneva, Switzerland.

Media and culture conditions for assaying degradation.

The minimal medium (Tully 1985) used for the growth of soybean rhizobia was modified by the omission of *p*-aminobenzoic acid, pyridoxine, and nicotinic acid. D-Xylose was added at 10 mM as the carbon source. Daidzein or genistein (Sigma) was added as required from a stock solution to give a final concentration of 10 μM. Strains were grown as single cultures in flavonoid-free medium, harvested by centrifugation, washed twice, and resuspended in sterile water. The strains were used to inoculate 2,000-ml volumes of media in 5.0-liter Erlenmeyer flasks. The initial cell concentration was 1.5×10^3 ml^{-1} and incubation was at 25°C on a rotary shaker. Treatments were I: medium only (control), II: medium plus diadzein or genistein (control), III: medium plus (i) *B. japonicum* USDA110*spc*4, or (ii) *R. fredii* HH103, or (iii) *Rhizobium* sp. NGR234 (controls), and IV: medium plus diadzein, or genistein plus bacterium (i, ii, iii).

Extraction of products.

Cells: *B. japonicum* USDA 110*spc*4 cells obtained from 6 liters of medium were washed (×5) in saline (0.1 M NaCl) water, freeze dried and extracted by refluxing at 40°C for 4 h using chloroform/methanol (1/1). The resulting mixture was centrifuged and the supernatants were passed through a silica gel G60 (Merck) column (25 × 150 mm). The first two column volumes were eluted with methanol/acetonitrile (80/20), followed by acidified methanol/water (1/1), and the fractions were concentrated and made up to a known volume in methanol.

Supernatants: After 2, 24, or 48 h of incubation the media and contents were centrifuged (8,000 × *g* for 10 min) and 4 liters of the cell-free supernatant from each treatment were hand extracted with ethyl acetate (×6) after saturation with sodium chloride, followed by drying with Na_2SO_4 and concentration on a rotary evaporator. Two liters of supernatant from another sample of each treatment was freeze dried. Residues were extracted with methanol and ethyl acetate and the concentrates combined with the previously hand extracted ethyl acetate residues. These methods of sample preparation were adopted to facilitate the extraction of products of varying hydrophobicity and volatility. The combined, concentrated residues were redissolved in methanol, passed through a glass column (15 × 200 mm) containing silica gel G60 and eluted with methanol:water (80/20). The eluate was freeze dried and methanol soluble portions were made up to a known volume.

A thin-layer chromatography (TLC) step provided a simple and effective means of monitoring the biotransformations of daidzein and genistein during incubation and also for distinguishing the substrate from other isoflavonoids (e.g., coumestrol) among the products. Aliquots of the concentrated extracts obtained from cells or supernants were applied to a polyamide coated TLC plate (Merck) and eluted twice with absolute ethanol. The TLC plate was thoroughly dried at room temperature between the two runs. Isoflavones daidzein and genistein moved faster than coumestrol. For qualitative purposes, isoflavones were visualized by their characteristic dark purple color under UV light, while compounds such as coumestrol exhibited a fluorescent blue, closer to the origin. Depending on quantitative availability, samples were scraped from the TLC plate surface, eluted with methanol, and subjected to a direct GC-MS analysis for structural confirmation.

To obtain full information on all metabolites, the remaining portions of the extracts from cells or supernatants were loaded into a Waters SEP-PAK C18 cartridge and a solid-phase extraction (SPE) was carried out using 80/20, 60/40, and 1/1 of methanol:water. Fractions were collected and freeze dried. Methanol soluble portions were concentrated, made up to a known volume and systematically screened for aromatic metabolites by GC-MS and HPLC coupled to a spectral array detector as described below.

Direct GC-MS identification of degradation products.

Metabolites from isoflavone degradation were identified as follows: The residues of cell or supernatant extracts (0.5 ml) were treated with an excess of ethereal diazomethane generated using a Diazald kit (Aldrich Chemicals) at 0°C. After 12 h, the solvents, along with the excess diazomethane, were evaporated and the residues were dissolved in 0.5 ml of diethyl ether prior to GC-MS analysis (Rao et al. 1991).

HPLC analyses of metabolites from isoflavone degradation by *B. japonicum*.

Cell and supernatant extracts were also analysed by high-performance liquid chromatography (HPLC) using protocols based on those described by Graham (1991a) for separation of aromatic metabolites, except that in our analyses we used a spectral array detector. The HPLC conditions were as follows: a Thermo Separation Products P2000 binary pump generated a linear gradient program of 5 to 65% acetonitrile in water (pH 3.5, adjusted with acetic acid) for 30 min, and this was followed by a stepwise reversal of the solvent program for an additional 10 min, with a flow rate of 1.5 ml min^{-1} throughout. Injections (25 µl) from the extracts were made on a Merck Lichrosorb Hibar (RP-18 10 µm, 250 mm), C18 reverse-phase column. The HPLC was linked to a Forward Optical Scanning Detector (Thermo Separation Products model UV 2000). The chromatograms and the spectral data for the injected samples and standards were collected and analyzed on a Thermo Separation Products PC1000 Datastation. Using standard UV-shift reagents (Markham 1982) metabolites were also derivatized and their characteristic UV λ max shifts verified. Multiple injections (250 µl) of bulk extracts from treatments III and IV were chromatographed under the conditions described above on a semipreparative Lichrosorb RP-18 column. The eluate was collected in the form of fractions at 6-min intervals and five such fractions were individually concentrated on a rotary vacuum pump at 40°C and the residues freeze-dried and resuspended in methanol. The component peaks were identified from their retention times, UV-spectra, and mass spectra obtained from GC-MS analyses. Using authentic compounds for identified peaks in the HPLC analyses, product concentrations were calculated from injection volumes, integrated peak areas, UV absorbance units, and extinction coefficients. Fractions were maintained either as complete composites or as corresponding fractions from which specific metabolites had been removed. These were then reconcentrated and methanol soluble portions were used for *nod* gene induction assays.

Measurement of *nod* gene expression (β-galactosidase activity).

A variant of *B. japonicum* strain 110*spc4* harboring plasmid pRJ458 and containing a *nodC–lacZ* fusion was used to assay the *nod* gene-inducing activity of the above HPLC fractions in the presence or absence of the normal *nod* gene inducers daidzein or genistein at 1 µM. The level of *nod* gene expression was determined indirectly by assaying β-galactosidase activity using the procedures described by Kape et al. (1991).

Analyses for isoflavonoid contents before and after incubation of soybean root exudate residues with or without rhizobia in N-free rooting solution.

Soybean seeds (*Glycine max* (L.) Merr. cv. Maple Arrow) were aseptically established following the methods of Kape et al. (1992) and the resulting root exudates were collected, freeze dried, and used as a source of isoflavonoids. The root exudate residue was made up to a known volume in methanol. Using the HPLC methods of Graham (1991b) for separation and identification of soybean isoflavonoids in root exudates and also the GC-MS methods described above, the initial levels of six isoflavonoids (daidzin, genistin, daidzein, genistein, coumestrol, and isoliquiritigenin) were determined. The methanolic root exudate preparation was filtered through Nalgene (0.2 µm) filter units. Washed cell suspensions (about 1.8×10^6 cells ml^{-1}) of either *B. japonicum* USDA 110*spc4*, *R. fredii* HH103, or *Rhizobium* sp NGR234 together with an aliquot of the root exudate were added to 10 ml of sterile N-free plant growth medium (Wood et al. 1983) containing no added carbon and modified as follows: NaH_2PO_4 was omitted and the rest of the medium was diluted to 1/4 strength before use. A mixture of phosphates consisting of KH_2PO_4 (67 mM) and Na_2HPO_4 (67 mM) was autoclaved separately and added at prescribed volumes (Cooper 1978) to the medium to set at pH 6.7. Incubation was carried out at 28°C for 36 h on a low speed (50 rpm) rotary shaker. An uninoculated treatment was also included for monitoring any nonmicrobial transformations. After incubation, the contents were freeze dried, and a methanol soluble portion analyzed for isoflavonoid contents. To test the extent of microbial degradative activity, the glycosides daidzin (Apin Chemicals, U.K.) and genistin (Sigma) were added at 5.0 µM each and inoculated with about 1.5×10^6 cells of *B. japonicum* USDA 110*spc4* strain in an Erlenmeyer flask containing the carbon and nitrogen free-medium. An uninoculated sample served as a control. After incubation for 36 h, the contents were extracted and analyzed via GC-MS.

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