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Flavonoid Metabolism by Rhizobia – Mechanisms and Products

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Abstract

A number of *Rhizobium* species and biovars have been shown to degrade *nod* gene-inducing flavonoids by mechanisms which originate in a cleavage of the C-ring of the molecule. The initial products of these reactions are new open and (re) closed ring flavonoid structures. Subsequent C-ring fissions yield a variety of conserved A- and B-ring monocylic hydroaromatics. Studies with *Bradyrhizobium japonicum* and its isoflavonoid *nod* gene inducers, daidzein and genistein, have identified a wide range of C-ring modification products and conserved A- and B-ring metabolites in extracts of cells and culture supernatants. Some of these, such as liquiritigenin, naringenin, coumestrol and umbelliferone could play a role in *nod* gene regulation or other recognition mechanisms operating in the early phases of communication between the symbiotic partners.

Keywords: Flavonoids, rhizobia, degradation, legumes

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1. Introduction

In common with many other soil microorganisms rhizobia are capable of catabolizing a variety of aromatic compounds. Monocylic aromatics and hydroaromatics, such as benzoate and shikimate, can be degraded to 3-oxoadipate, via catechol or protocatechuate, prior to entry into the tricarboxylic acid cycle (Chen et al., 1984; Parke and Ornston, 1984). Protocatechuate itself is a universal growth substrate for rhizobia. The possibility that rhizobia could catabolize polycyclic flavonoids responsible for *nod* gene induction has been raised as a consequence of experiments which showed that such compounds also induced expression of *Rhizobium* genes with unknown functions and showing no homologies with nodulation gene promoters (Sadowsky et al., 1988; Perret et al., 1994). The first demonstration of flavonoid catabolism by *Rhizobium* involved utilization of the flavan-3-ol catechin by an isolate from *Leucaena leucocephala* with formation of phloroglucinol carboxylic acid and protocatechuate (Gajendiran and Mahadevan, 1988). Rao et al. (1991) subsequently reported that *R. loti* could cleave the C-ring of the pentahydroxy flavone quercetin by means of a novel mechanism which yielded phloroglucinol and protocatechuate among the degradation products. Studies with other *Rhizobium* species/biovars and their respective *nod* gene inducers confirmed that flavonoid degradation is a common metabolic feature in members of this genus (Rao and Cooper, 1994). In this paper we present further evidence for C-ring modification and fission during interactions between rhizobia and their flavonoid or isoflavonoid *nod* gene inducers.

2. Materials and Methods

Bacteria

Rhizobia employed in this study and their sources were as follows: *Rhizobium leguminosarum* bv trifolii, strain P3 (this laboratory); *Rhizobium leguminosarum* bv viciae, strain RBL5601 (Department of Plant Molecular Biology, Leiden University, The Netherlands); *Rhizobium meliloti*, strain RM41 and *Bradyrhizobium japonicum*, strain USDA 110 *spc* 4 (Botany Department, University of Marburg, Germany). Strains were grown individually at 25°C for 72 h in shake flask cultures in a flavonoid-free medium with mannitol substituting for arabinose as principal carbon source (Rao et al., 1991). Inocula for test media were prepared by centrifugation and washing (twice) followed by resuspension of cells in sterile water.

Test media

Rhizobium strains were grown in the medium described by Rao et al. (1991) and *Bradyrhizobium japonicum* was grown in the minimal medium of Tully (1985), modified by the omission of p-aminobenzoic acid, pyridoxine and nicotinic acid and the inclusion of D-xylose (10 mM) as principal carbon source. Flavonoids (Table 1) were obtained from Apin Chemicals (Abingdon, UK) and checked for purity by HPLC and GC. Single compounds were added to the test media from filter-sterilised stock solutions in methanol to give a final concentration of 10 μM. Media were dispensed in 1-litre amounts in 3-litre Erlenmeyer flasks, inoculated with ca 1.5×10^3 viable cells ml^{-1} and incubated in the dark at 25°C for up to 48 h on a rotary shaker (200 rpm). Control treatments, necessary for confirmation of bacterial flavonoid transformation, were also included.

Analysis of culture supernatants

After incubation, flask contents were centrifuged (8,000 x g for 10 min) and the supernatants were extracted with ethyl acetate (x6) after saturation with sodium chloride. After drying with Na_2SO_4 and concentration on a rotary evaporator, residues were taken up in 0.5 ml of methanol and treated with excess ethereal diazomethane at 0°C. After 12 h the solvents, together with excess diazomethane, were evaporated and the remaining residues were re-dissolved in 0.5 ml of diethyl ether prior to analysis by GC-MS (Rao et al., 1991). Additionally, bulked supernatant extracts from genistein-supplemented *B. japonicum* cultures were analysed by HPLC using the protocols described by Graham (1991) for separation of aromatic metabolites.

3. Results and Discussion

When incubated with rhizobia, flavonoids with OH substitutions at the 5 and 7 positions of the A ring (naringenin, luteolin) yielded phloroglucinol and/or phloroglucinol carboxylic acid as conserved A-ring products, whereas 7, 4'-dihydroxyflavone, with a single OH substitution at the 7 position, yielded resorcinol (Table 1). A greater variety of conserved B-ring metabolites was detected including p-coumaric (4-hydroxycinnamic), p-hydroxybenzoic, protocatechuic, phenylacetic and caffeic acid (Table 1). In all cases, degradation of the three ring structure was initiated by fissions in the central (C) ring. In the case of the flavanone naringenin, incubation for 2 h with *R. leguminosarum* bv viciae permitted the transient detection by GC-MS of C-ring

Table 1. Principal degradation products detected in extracted culture supernatants after incubation of *nod* gene-inducing flavonoids and isoflavonoids with rhizobia

Organism	Flavonoid[1]/isoflavonoid[2] substrate	Principal degradation products derived from: Conserved A-ring	Conserved B-ring
R. leguminosarum bv. viciae	Naringenin[1]	Phloroglucinol	p-Coumaric acid p-Hydroxybenzoic acid
R. leguminosarum bv. trifolii	7, 4'-Dihydroxyflavone[1]	Resorcinol	p-Hydroxybenzoic acid
R. meliloti	Luteolin[1]	Phloroglucinol Phloroglucinol carboxylic acid	Caffeic acid Protocatechuic acid Phenylacetic acid
B. japonicum	Daidzein[2]	Resorcinol	p-Coumaric acid p-Hydroxybenzoic acid Phenylacetic acid
B. japonicum	Genistein[2]	Phloroglucinol Phloroglucinol carboxylic acid	p-Coumaric acid p-Hydroxybenzoic acid Phenylacetic acid

modification products, in the form of new chalcone ($M^{+}314$) and flavanone ($M^{+}300$) structures, in derivatized supernatant extracts. When incubated with *R. meliloti*, luteolin yielded several new closed C-ring metabolites, despite the concurrent existence of C-ring fission and the formation of five monocyclic aromatic degradation products (Fig. 1).

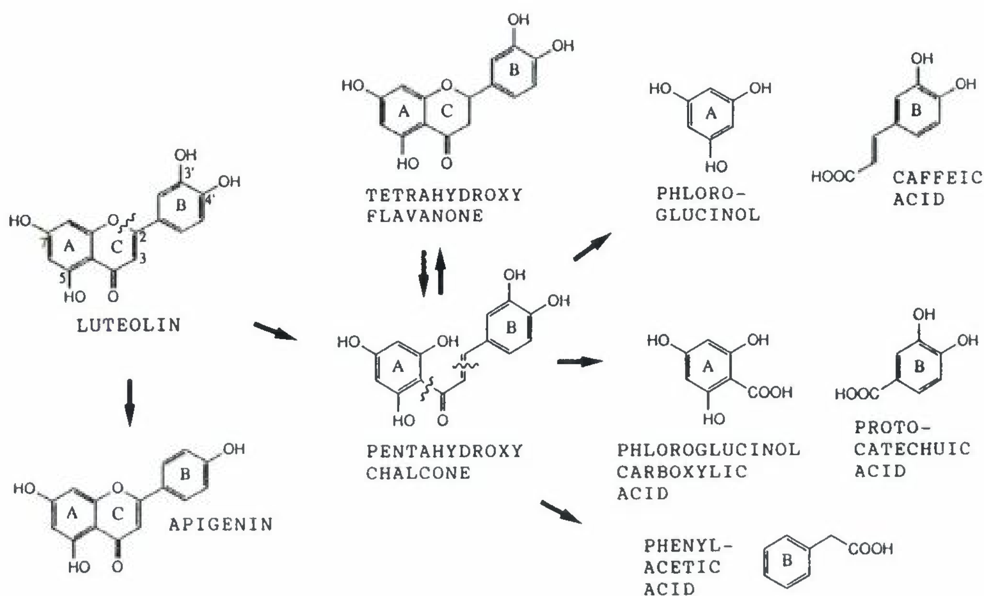

Figure 1. Proposed degradation pathway of luteolin by *Rhizobium meliloti*.

The same basic pattern of degradation was also seen when the isoflavone *nod* gene inducers daidzein and genistein were incubated with *Bradyrhizobium japonicum*. For example, several C-ring modification products were detected when daidzein was incubated with *B. japonicum*; these included isoliquiritigenin (open C-ring) and liquiritigenin (closed C-ring). The principal C-ring fission products in this system (Table 1) were resorcinol (conserved A-ring product), 4-hydroxybenzoic acid and p-coumaric acid (conserved B-ring products). Another potential A-ring derivative, umbelliferone (or its satellite compound 4-methyl umbelliferone), was also detected in culture supernatant extracts. A proposed degradation pathway for daidzen by *B. japonicum* is presented in Fig. 2. None of the principal A- or B-ring derived degradation products was detected in cell extracts, but daidzein-treated *B. japonicum* cells

did contain liquiritigenin, coumestrol and umbelliferone as well as unmetabolized daidzein. An HPLC analysis of supernatant extract from genistein-supplemented medium after incubation with *B. japonicum* was also performed. Peak identities were obtained from comparisons of their UV absorption spectra and mass spectra from GC-MS analyses with those of authentic compounds. Quantitation of these data showed that significant amounts of isoflavonoid were metabolised in a 24 h period (Fig. 3). Our results indicate that flavonoid and isoflavonoid *nod* gene inducers can be catabolized by rhizobia via similar C-ring fisson mechanisms. Products encompass both open and closed C-ring modification structures in addition to monocyclic hydroaromatics. The process can be viewed as a reversal of plant flavonoid biosynthesis. Flavonoid degradation via C-ring fission has been previously reported for only two other bacterial genera: *Eubacterium* and *Clostridium* (Krumholz and Bryant, 1986; Winter et al., 1989). However, the ensuing degradation products were different from the rhizobial metabolites.

The results raise several questions concerning the origin of previously reported new flavonoid structures in legume root exudates and the consequences of degradation for *nod* gene regulation. There is a remarkable similarity

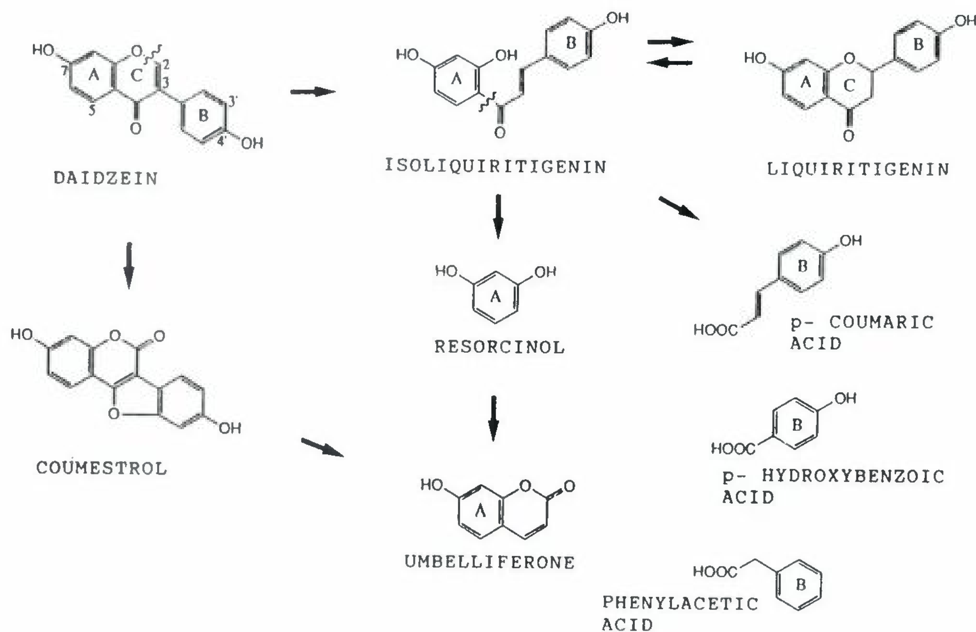

Figure 2. Proposed degradation pathway of daidzein by *Bradyrhizobium japonicum*.

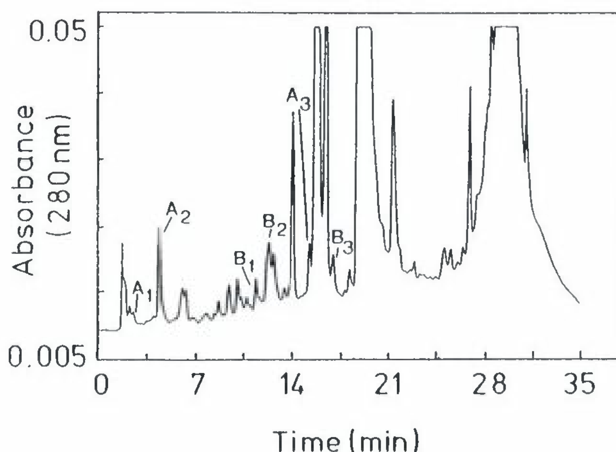

Figure 3. HPLC chromatogram of extracted supernatant of genistein-supplemented medium after incubation with *B. japonicum* for 24 h. Initial genistein concentration was 10 μM and individual product concentrations (nM) were: A1, phloroglucinol (280); A2, phloroglucinol carboxylic acid (1560); A3, umbelliferone (690); B1, p-hydroxybenzoic acid (980); B2, p-coumaric acid (1354); B3, phenylacetic acid (550).

between the C-ring modification products from naringenin catabolism and the new chalcone and flavanone structures reported by Recourt et al. (1991) in *Vicia sativa* root exudates after inoculation of the plant with *R. leguminosarum* bv viciae. This suggests that bacterial degradation, rather than *de novo* plant synthesis and release, could account for the occurrence of at least some of these compounds. The formation of inducer-derived metabolites could have implications for the control of *nod* gene induction in rhizobia. For example, isoliquiritigenin is a powerful inducer for *B. japonicum* (Kape et al., 1992) whereas umbelliferone is a potent *nod* gene inhibitor (Kosslak et al., 1990).

The formation of a variety of monocyclic hydroaromatics could be of significance for other rhizosphere phenomena. Control of interstrain competition for soybean nodulation by *B. japonicum* can be achieved in the presence of specific *nod* gene inhibitors (Cunningham et al., 1991) and it is also known that products such as p-coumaric acid possess chemotactic properties towards *B. japonicum* (Kape et al., 1991).

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