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Phytochemical and Immunocytochemical Evidence for the Accumulation of 2′-Hydroxylupalbigenin in Lupin Nodules and Bacteroids

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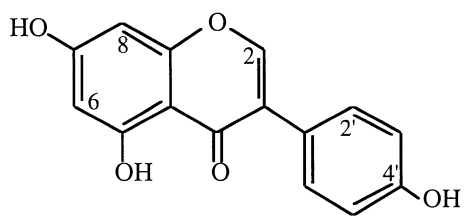
Symbiotic interaction between *Rhizobium lupini* and *Lupinus albus* promotes both qualitative and quantitative changes in the isoflavonoids of root and nodule tissues. These changes were determined by high-pressure liquid chromatography and radiolabeling with L-[U-^{14}C]phenylalanine. Root nodules from mature lupin plants accumulate prenylated derivatives of both genistein and 2′-hydroxygenistein, with the predominance of 2′-hydroxylupalbigenin, a diprenylated isoflavone. Immunogold localization, using specific polyclonal immunoglobulin Gs, revealed an association of 2′-hydroxylupalbigenin with the establishment of early symbiotic structures, as well as with the bacteroids themselves. These results, together with the fact that prenylated isoflavonoids significantly reduce the *in vitro* growth rate of a number of *Rhizobium* species, suggest that 2′-hydroxylupalbigenin may be involved in the symbiotic association of *L. albus* and *R. lupini*. This hypothesis is discussed in relation to the putative biological significance of prenylated isoflavonoids.

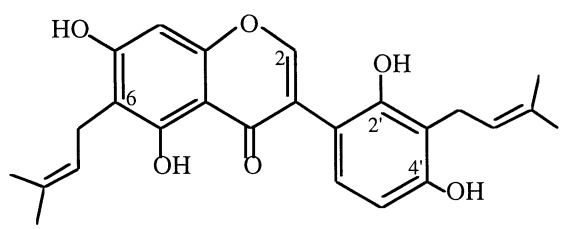
Additional keywords: anti-2′-hydroxylupalbigenin immunoglobulin G, immunocytolocalization, symbiosis.

Compatible symbiosis between legumes and *Rhizobium* or *Bradyrhizobium* spp. results in the formation of root nodules, which are structurally and biochemically specialized in nitrogen fixation (Long 1989). Various flavonoid compounds have been identified as key factors in these symbiotic interactions, acting as inducers or inhibitors of nodulation (*nod*) genes of the bacterial symbiont (Phillips 1992). In addition, flavonoid compounds are known to play a determining role in the species-specificity of host symbiont recognition, by their ability to regulate the initial infection of the host (Spaink *et al.* 1987; Van Brussel *et al.* 1990). However, little is known of the putative role of flavonoids in the postinfectional development of symbiosis, which includes the formation and functioning of nodules. Among the postinfectional events, a species-specific increase in flavonoid or isoflavonoid biosynthesis in legume roots, as triggered by *Rhizobium* association, is a well-recognized phenomenon (Parniske *et al.* 1988; Recourt *et al.* 1992; Schmidt *et al.* 1992; Savouré *et al.* 1994). Nevertheless, the flavonoid levels induced by *Rhizobium*-legume symbiosis are generally significantly lower than those measured in pathogenic interactions (Schmidt *et al.* 1992). This could be partially explained by the initial activation of a different set of host genes coding for phenylalanine ammonia-lyase and chalcone synthase, which seems to be a necessary condition for successful nodule initiation and development (Estabrook and Sengupta-Gopolan 1991). In fact, the flavonoid compounds synthesized after symbiotic infection are considered important mediators of early symbiotic events, such as the induction of meristem (Schmidt *et al.* 1992).

Prenylated isoflavonoids are among the postinfectional flavonoids for which few studies have recently been reported. Glyceollin phytoalexins have been shown to accumulate in developing nodules of soybean, whereby they may enable the host to control the bacteroid population (Parniske *et al.* 1991b; Karr *et al.* 1992). Interestingly, glyceollins are formed by the prenylation of pterocarpans which, in turn, originate



Genistein



2′-Hydroxylupalbigenin

Fig. 1. Structures of genistein and 2′-hydroxylupalbigenin.

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from daidzein, a 5-deoxyisoflavone (Heller and Forkmann 1994), the known inducer of *nod* genes in *Bradyrhizobium*– and *Rhizobium–Glycine max* symbiosis (Kosslack *et al.* 1987; Sadowsky *et al.* 1988). In addition, isoflavonoid phytoalexins, such as glyceollins, have been shown to reduce the *in vitro* growth rate of *Bradyrhizobium japonicum* and *Rhizobium lupini* symbionts (Pankhurst and Biggs 1980; Parniske *et al.* 1991a).

In contrast with most legumes which accumulate prenylated isoflavones and prenylated pterocarpans postinfectionally, lupin roots constitutively express the synthesis of a variety of mono- and diprenylated derivatives of the isoflavones genistein and 2′-hydroxygenistein (Fig. 1 and Table 1) (Tahara *et al.* 1984, 1989, 1990). Whereas the flavonoid signals of most symbiotic associations have been identified (Phillips 1992), nothing is known of the role of flavonoids and isoflavonoids in the nodulation of lupin. Preliminary experiments in this laboratory indicated the presence of a variety of prenylated isoflavones in lupin nodules, as well as the roots from which they arose. This raised the question as to whether these metabolites are synthesized *de novo* in the nodules or whether they are imported from the adjacent root tissue. We demonstrate in this study the biosynthesis and accumulation of the entire complement of lupin isoflavonoids in the nodules. This phenomenon is also supported by an immunocytochemical study using a monospecific antibody raised against 2′-hydroxylupalbigenin, the most abundant diprenylated isoflavonoid found in the nodules.

Table 1. *Lupinus albus* isoflavonoids

Isoflavone group	Generic name
Glucosides	
Genistein glucoside	5,7,4′-Trihydroxyisoflavone 7-*O*-glucoside
2′-Hydroxygenistein glucoside	5,7,2′,4′-Tetrahydroxyisoflavone 7-*O*-glucoside
Aglycones	
Genistein	5,7,4′-Trihydroxyisoflavone
2′-Hydroxygenistein	5,7,2′,4′-Tetrahydroxyisoflavone
Genistein monoprenyls[a]	
Wighteone	6-Prenyl-5,7,4′-trihydroxyisoflavone
Isowighteone	3′-Prenyl-5,7,4′-trihydroxyisoflavone
Lupiwighteone	8-Prenyl-5,7,4′-trihydroxyisoflavone
2′-Hydroxygenistein monoprenyls	
Luteone	6-Prenyl-5,7,2′,4′-tetrahydroxyisoflavone
Licoisoflavone A	3′-Prenyl-5,7,2′,4′-tetrahydroxyisoflavone
Diprenyls	
Lupalbigenin	6,3′-Diprenyl-5,7,4′-trihydroxyisoflavone
2′-Hydroxylupalbigenin	6,3′-Diprenyl-5,7,2′,4′-tetrahydroxyisoflavone

[a] Prenyl = 3,3-dimethylallyl.

RESULTS

Lupinus albus isoflavonoids.

Chromatographic separation of isoflavone aglycones and glucosides, as well as the mono- and diprenylated derivatives of both genistein and 2′-hydroxygenistein, was achieved by means of a high-pressure liquid chromatography (HPLC) gradient elution protocol (Gagnon *et al.* 1992b). This protocol provided a reliable, qualitative and quantitative estimate of lupin isoflavonoids within a single chromatographic run. The 11 isoflavonoids analyzed (Table 1) were grouped to represent the glucosides, aglycones, genistein monoprenyls, 2′-hydroxygenistein monoprenyls, and the diprenyls of both aglycones.

The early stages of germination of lupin seedling roots are characterized by an isoflavonoid pattern which consists mostly of isoflavone glucosides and, to a lesser extent, of their aglycones, with traces of their prenylated derivatives. Since there is a constant release of isoflavones in the root exudates, lupin isoflavones appear to be in a state of flux during early germination. In addition, pulse feeding of sterile lupin roots with labeled precursors, at different stages of seedling growth, demonstrated the biosynthesis of these compounds, as well as their rapid turnover in the root system (Gagnon 1993).

Isoflavonoid composition of lateral roots and nodules.

The isoflavonoid composition of nodules secured from 6-week-old plants and of the lateral roots harboring them was compared with that of 2- and 6-week-old uninfected lateral roots (Table 2). Isoflavone glucosides appeared to be the predominant metabolites in the uninfected 2- and 6-week-old roots, constituting approximately 90 and 67%, respectively, of total isoflavonoids; the remainder consisted mostly of aglycones and traces of prenylated compounds. Amounts of total isoflavonoids identified were 2.3 and three times greater in 6-week-old uninfected and infected roots, respectively,

Table 2. Isoflavonoid composition (nmol/mg fresh weight) of lateral roots and nodules[a]

	Lateral roots			Nodules (6 wk old)		
	Uninfected		Infected			
Isoflavonoid group[b]	2 wk old	6 wk old	6 wk old	0–2 mm in diameter	2–4 mm in diameter	>4 mm in diameter
Glucosides	1.51 b	2.52 a	0.69 a	0.89 a	0.21 e	0.54 b
Aglycones	0.12 a	0.79 b	3.42 a	1.87 c	0.66 b	0.92 f
Genistein monoprenyls	<0.00 d	0.16 a	0.40 a	1.30 d	0.76 e	0.54 b
2′-Hydroxygenistein monoprenyls	0.03 c	0.02 f	0.16 b	1.43 d	0.45 f	0.39 b
Lupalbigenin	0.03 a	0.23 b	0.33 b	1.40 c	0.98 c	0.63 c
2′-Hydroxylupalbigenin	<0.00 a	0.03 f	0.13 a	2.19 e	1.41 c	1.39 c
Total identified	1.69	3.75	5.13	9.08	4.47	4.41

[a] Values are averages of triplicate samples, with standard deviations indicating the following confidence levels: a, <5%; b, <10%; c, <15%; d, <20%; e, <25%; and f, >25%.

[b] See Figure 1 and Table 1 for individual isoflavones included in each group.

than in young lateral roots. However, it is interesting to note that this increase in total isoflavonoids resulted in a higher level of aglycones (66% of total isoflavonoids) in lateral roots harboring nodules, whereas the uninfected roots did not exhibit such an increase (Table 2).

HPLC of nodule metabolites indicated that small nodules (0–2 mm in diameter) contained twice the amount of total isoflavonoids identified in nodules of intermediate size (2–4 mm in diameter) or large size (>4 mm in diameter) (Table 2). The relative abundance of isoflavonoid compounds was quite similar for nodules of all sizes, except for the mono- and diprenylated compounds, which accounted for 67–80% of total isoflavonoids in nodules of all sizes. The high levels of 2′-hydroxylupalbigenin in nodule tissues are notable; they constituted 24–32% of total isoflavonoids at all maturation stages.

Incorporation of L-[U-^{14}C]phenylalanine into nodule isoflavonoids.

The incorporation of a phenylalanine label into isoflavonoids from nodules of different sizes indicated *de novo* synthesis of these metabolites. It should be noted that the specific activities shown in Table 3 take into account the endogenous pools of isoflavonoids originally present in the nodules (Table 2). The high specific activities of isoflavonoids, especially the glucosides, in the small and intermediate-sized nodules reveal their high rate of biosynthetic activity, compared with the large nodules. However, the low label incorporation into prenylated compounds is a reflection of the large pool size of these metabolites in the nodules, most notably that of 2′-hydroxylupalbigenin in the small nodules (Table 2). Moreover, the fact that prenylation is a terminal enzymatic reaction in the biosynthesis of prenylated isoflavonoids (Heller and Forkmann 1994) accounts for their low specific activity, compared with that of the glucosides (Table 3).

Immunocytolocalization of 2′-hydroxylupalbigenin.

The results of immunodetection assays indicate that the anti-2′-hydroxylupalbigenin immunoglobulin (Ig) Gs were highly specific for the hapten, and no cross-reactivity was detected with the other isoflavonoid analogs tested (Fig. 2). Assays conducted with preimmune serum as primary antibody gave negative results (data not shown).

Treatment of nodule sections with anti-2′-hydroxylupalbigenin IgGs resulted in a specific labeling associated with the occluding material filling the intercellular spaces of the variable oxygen diffusion barrier (Fig. 3A). A few clusters of gold label were occasionally present within the cytoplasmic area adjacent to the intercellular spaces (Fig. 3A). These cellular features of the inner cortex correspond perfectly with those previously described for lupin nodules (Iannetta *et al.* 1993). Examination of the invasion zone reveals a consistent association of gold particles with the core of more than 50% of the bacteroids, whereas the peribacteroid spaces and membranes are almost devoid of the label (Fig. 3B and Table 4). Senescent and necrotic bacteroids were not labeled (results not shown). High magnification of bacteroids shows that the gold particles are precisely located in a central fibrillar system, which appears to surround one or several successive electron-dense nucleoid regions (Fig. 3C). Furthermore, intrabacteroidal polar bodies, when present, were not labeled. The ultrastructural bacteroid features described above correspond with those previously reported for the fibrillar material, nucleoid regions, and polar bodies of *Lotus* nodules (Wood *et al.* 1985). Control treatments performed on nodule tissue sections did not reveal any significant labeling (Fig. 3D).

Table 3. Specific activities (dpm/nmol) of isoflavonoids in nodules[a]

Isoflavonoid group	Nodule diameter		
	0–2 mm	2–4 mm	>4 mm
Glucosides	1,121	1,676	1,002
Aglycones	104	253	113
Genistein monoprenyls	232	198	172
2′-Hydroxygenistein monoprenyls	162	404	164
Lupalbigenin	130	54	22
2′-Hydroxylupalbigenin	78	45	24

[a] Specific activities of isoflavonoids in nodules that had been exposed to L-[U-^{14}C]phenylalanine for 24 hr, as determined by liquid scintillation counting of high-pressure liquid chromatography fractions corresponding to isoflavone peaks. Amounts of isoflavonoids were similar to those reported in Table 2.

DISCUSSION

Root nodules are symbiotic organs resulting from bacterial infection of legume root tissue, and their stages of maturation are reflected by their increase in size (Hirsh 1992). They are found predominantly on lateral roots or at the junction of primary and lateral roots (Libbenga and Bogers 1974; Caétano-Annollès *et al.* 1991). Analysis of isoflavonoids indicates that active synthesis of these compounds takes place in young, developing nodules, since the increase in nodule tissue during maturation may not be accompanied by isoflavonoid synthesis, thus resulting in lower amounts of nodule metabolites relative to tissue weight. Furthermore, the decrease in isoflavonoid content during the maturation of nodules may be due to an increased release of these metabolites into the rhizosphere, especially the aglycones and prenylated derivatives. This phenomenon has also been shown to occur in intact lupin roots (Gagnon 1993) and in root cells grown in culture (Gagnon *et al.* 1992a).

Both HPLC and thin-layer chromatography (TLC) showed that lupin nodules have a different isoflavonoid composition from that of the lateral roots. In fact, the diprenyl and, to a lesser extent, the monoprenyl derivatives are the predominant isoflavonoids in the nodules. Whereas 2′-hydroxylupalbigenin constituted more than 24% of total isoflavonoids in

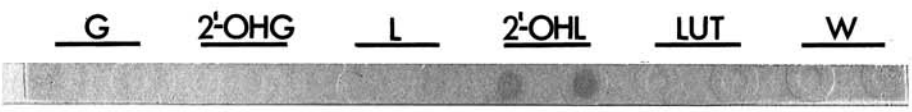

Fig. 2. Immunodetection assay (dot blot) of the cross-reactivity of anti-2′-hydroxylupalbigenin immunoglobulin G with other prenylated and nonprenylated isoflavonoids found in *Lupinus albus* root tissues. G, genistein; 2′-OHG, 2′-hydroxygenistein; L, lupalbigenin; 2′-OHL, 2′-hydroxylupalbigenin; LUT, luteone; W, wighteone. See Table 1 and Figure 1 for structures.

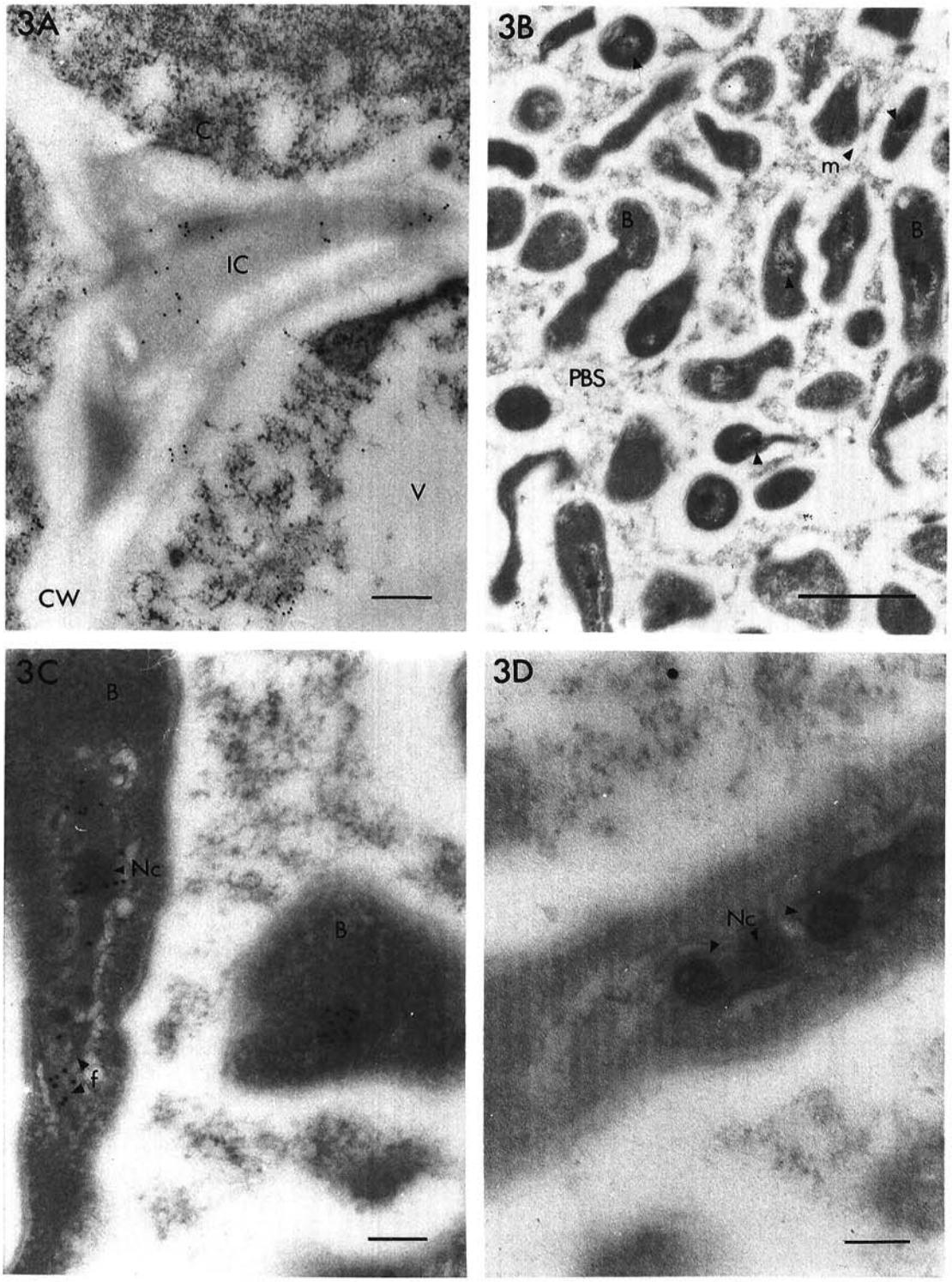

Fig. 3. A, Transmission electron micrograph of the inner cortex of a lupin nodule after immunogold labeling with anti-2′-hydroxylupalbigenin immunoglobulin (Ig) G. The gold label is specifically associated with the occluding material filling the intercellular space (IC), with clusters of the label occasionally present in the cytoplasmic areas (C) adjacent to the intercellular spaces; bar = 0.2 μm. CW, cell wall; V, vacuole. **B,** Transmission electron micrograph of bacteroids (B) in the invasion zone after immunogold localization with anti-2′-hydroxylupalbigenin IgGs; bar = 1 μm. View of a group of bacteroids typical of those generally found in cells of the invasion zone. Bacteroids show a specific labeling of their inner core (arrows), whereas the peribacteroid spaces (PBS) and membranes (m) are almost devoid of gold particles. **C,** Labeling of bacteroids (B) reveals a strong association of the gold particles with a central fibrillar system, sometimes surrounding an electron-dense nucleoid region (Nc); bar = 0.1 μm. When present, the polar bodies were always devoid of gold particles. **D,** Transmission electron micrograph of control bacteroids after immunogold labeling with anti-2′-hydroxylupalbigenin IgGs that were previously incubated with their hapten antigen; bar = 0.05 μm. The label is absent from the nucleoid regions (Nc) of the bacteroids.

small nodules, the monoprenyls and the diprenyl derivative lupalbigenin decreased as nodule size increased. In contrast, lateral roots contained lower amounts of prenylated isoflavonoids than the nodules they harbored. However, the relative abundance of aglycones in lateral roots suggests that these compounds may act as a reservoir of metabolites for further prenylation and possible transport to the nodules.

The incorporation of a phenylalanine label into isoflavonoids from nodules of different sizes indicates that the nodules contain the entire enzyme complement involved in the biosynthesis of prenylated isoflavonoids (Dewick 1994). Taken together, these results suggest that the increase in the level of prenylated compounds in the nodules, especially 2′-hydroxylupalbigenin, is associated with bacterial infection that may be related to a state of "symbiotic stress." A similar situation has been reported in soybean, in which glyceollin I accumulates in nodules as nodule function decreases. This phytoalexin was also present in neighboring lateral roots, but at a much lower level than in the nodules (Karr *et al.* 1992).

The localization of 2′-hydroxylupalbigenin in the cortical cells of nodules corroborates the phytochemical data and the incorporation of the phenylalanine label into isoflavonoids from excised nodules, and it suggests that the biosynthesis of this diprenylated isoflavone in the cortex is triggered by the symbiotic process. The gold label is located on the occluding material of intercellular spaces of the variable oxygen diffusion barrier (Fig. 3A). This material, which has been shown to be synthesized *de novo* during nodule development and in response to stress factors, results in part from the accumulation of glycoproteins (Iannetta *et al.* 1993; de Lorenzo *et al.* 1993). A molecular cross-linking between 2′-hydroxylupalbigenin and these glycoproteins may be envisaged, similar to the cross-linking of phenolic compounds and *de novo* synthesis of glycoproteins in other plant-microbe interactions, both pathogenic (Clarke 1982; Friend 1981; Fry 1983; Hahlbrock and Scheel 1989) and symbiotic (Grandmaison *et al.* 1993). In general, this type of cross-linked matrix is believed to increase the plant's resistance to further microbial attack (Clarke 1982; Friend 1981; Fry 1983; Hahlbrock and Scheel 1989). We suggest, therefore, that the association of 2′-hydroxylupalbigenin with glycoproteins in such symbiotic tissue may be in the form of a cross-linked matrix that secures the nodule from further microbial invasion and lowers the rate of oxygen diffusion. Moreover, the presence of the gold label in the cytoplasm, adjacent to the intercellular spaces, suggests that 2′-hydroxylupalbigenin may be synthesized in the cortical cells and then released into the intercellular spaces, where it accumulates. The dense association of 2′-hydroxylupalbigenin with the perinucleoid fibrillar material indicates that prenylated isoflavones may play a role in the intracellular metabolism of the bacteroids. The fact that, within the same cell, some bacteroids are not labeled suggests that binding of the diprenylated isoflavone to the nucleoid region of the bacteroid corresponds to a biochemical event that is not chronologically synchronized in the bacteroidal population. Such a role may be related to some regulatory function, since nucleoid areas to which the fibrillar material is structurally connected are presumably made of DNA (Wood *et al.* 1985).

Table 4. Morphometric analysis of labeling observed in infected cortical cells of lupin nodules

	Mean ± SD
Total number of bacteroids per field[a] (n = 20)	57 ± 6.5
Bacteroids with gold label (%) (n = 1,100)	66.5 ± 15.7
Number of gold particles per bacteroid (n =1,100)	9.2 ± 6.5
Number of gold particles on interbacteroidal space per field (n = 20)	35 ± 12.7

[a] The field area at 3,500× was 100 μm^2.

It has recently been shown that 2′-hydroxylupalbigenin (10 μM) significantly reduces the *in vitro* growth rate of *R. loti,* a symbiont compatible with lupin, as compared with the nonprenylated aglycone 2′-hydroxygenistein (Gagnon 1993). In addition, the pterocarpans glyceollin and phaseollin (Parniske *et al.* 1991a; Pankhurst and Biggs 1980) have been reported to inhibit, respectively, *in vitro* growth of *B. japonicum* and *in vitro* growth of *R. japonicum* and *R. lupini.* Taken together, these results suggest a role for these metabolites in the regulation of nodulation. Work in progress is aimed at the identification of inducers and inhibitors of *nod* genes in *Rhizobium*-lupin symbiotic interaction.

MATERIALS AND METHODS

Plant material.

Seeds of white lupin, *Lupinus albus* L. cv. Kievskij, were generously supplied by Satoshi Tahara, Hokkaido University, Sapporo, Japan. Surface-sterilized seeds were soaked for 24 hr and germinated on vermiculite under aseptic conditions in an incubator at a light intensity of 350–400 E m^{-2} s^{-1} for 16 hr followed by 8 hr of darkness, at maximum and minimum temperatures of 26 and 20° C and 50% relative humidity. One-week-old plants raised under greenhouse conditions were inoculated with commercial inoculum of *R. lupini* (Liphatech, Milwaukee, WI).

Plants grown in the greenhouse were thoroughly washed with tap water, and their nodules were harvested and segregated by size (0–2, 2–4, and more than 4 mm in diameter). Each size-group was randomly subdivided into three samples for HPLC.

Chemicals: Isoflavonoids and radiolabeled substrate.

Lupin isoflavonoids (Table 2) were a generous gift from Satoshi Tahara, Hokkaido University, Sapporo, Japan. Uniformly labeled L-[U-^{14}C]phenylalanine (specific activity 554.8 mCi/mmol) was purchased from ICN Biomedicals (Costa Mesa, CA). All HPLC solvents were filtered with Millipore filters and degassed before use.

Incorporation studies with L-[U-^{14}C]phenylalanine.

Label incorporation was performed on nodules sliced into halves and placed in contact with L-[U-^{14}C]phenylalanine (0.02 μCi/mg of tissue, fresh weight) for 24 hr. At the end of the metabolic period, tissue sections were thoroughly washed with water and then extracted with aqueous MeOH (as described in the following section) for HPLC.

Tissue extraction and sample preparation.

A glass rod was used to homogenize lateral roots or nodules in Eppendorf tubes with washed sand and an appropriate volume of 80% MeOH. The resulting homogenate was centrifuged in an Eppendorf microfuge for 2 min at 10,000 rpm,

and the pellet was re-extracted two times, first with 80% MeOH and then with absolute MeOH. The combined liquid of the methanolic extracts was evaporated in a Speed Vac concentrator, and the residue was resuspended in an aliquot of 80% MeOH proportional to the weight of the extracted tissue. The final extract was centrifuged for 2 min prior to HPLC injection. To monitor radiolabel incorporation during HPLC, fractions corresponding to isoflavonoid peaks were collected, the solvent was evaporated, and scintillation fluid (EcoLite, ICN) was added for counting. Radioactivities (in dpm/nmol) of different fractions were correlated with their corresponding isoflavonoid peaks.

HPLC and TLC of isoflavonoids.

Isoflavonoids were chromatographed on a Merck reverse-phase C18 LiChrospher 100 column, 250 × 4 mm i.d. (particle size 5 μm), as previously described (Gagnon *et al.* 1992b), with the modification described below. Chromatography was carried out at a flow rate of 1 ml/min, with 45% solvent A (0.5% methanolic acetic acid) in 55% solvent B (0.5% aqueous acetic acid) for 2 min. This was followed by a gradient increase to 100% solvent A in 23 min, and then isocratic conditions were maintained for a further 10 min. Data acquisition was carried out using a System Interface Module and Baseline 810 software, operated on a 25-mHz 386SX PC. Qualitative analysis (peak identification) was based on the retention time and the proportional absorbance of peaks at 254, 280, and 340 nm. Quantification was based on the surface area of individual peaks at 254 nm and their molar extinction coefficients. TLC of tissue extracts was carried out according to the method described by Tahara *et al.* (1984; results not shown).

Production and characterization of antibodies.

A hapten-carrier protein was prepared by coupling 2′-hydroxylupalbigenin to bovine serum albumin via a *p*-aminohippuric acid bridge (Erlanger 1980). The purified, dialyzed conjugate was used to raise polyclonal antibodies, by the use of a previously described protocol (Lamoureux *et al.* 1986). IgGs were precipitated with 80% $(NH_4)_2SO_4$, desalted, and then affinity-purified on a protein A column (High-Trap, Bio-Rad). Characterization of IgG specificity and cross-reactivity was performed by absorbing 2′-hydroxylupalbigenin or other flavonoid analogs on a nitrocellulose membrane in a dot blot apparatus (BioRad). The membrane was first equilibrated in phosphate-buffered saline (PBS), pH 7.2, and dried prior to adsorption of each of the following antigens: genistein, 2′-hydroxygenistein, lupalbigenin, 2′-hydroxylupalbigenin, luteone, and wighteone (Table 1), dissolved in PBS, pH 7.2, containing 4% dimethyl sulfoxide. The membrane was dried before treatment with a Western blotting kit (BioRad) according to the manufacturer's instructions, in which Tris–saline buffer and gelatin were replaced by PBS and defatted milk as blocking agent. Anti-2′-hydroxylupalbigenin IgGs were used at a concentration of 180 μg ml^{-1}, and goat-anti-rabbit IgGs conjugated to alkaline phosphatase were the secondary antibodies (diluted 1:1,500 in PBS-Tween). A control assay was conducted simultaneously, using preimmune serum as the primary antibody.

Microscopy.

Sections of small nodules (approximately 1 mm in diameter) were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2, for 6 hr at 4°C, and then were thoroughly rinsed with the same buffer. After dehydration in ethanol series, samples were embedded in LR White resin (hard), and ultrathin sections (60 nm) were mounted on 150-mesh Formvar-coated nickel grids. The latter were first floated on a drop of PBS-blocking solution containing 5% defatted milk and then were incubated on a drop of affinity-purified anti-2′-hydroxylupalbigenin IgGs, diluted in 50 mM PBS, pH 7.2, at a concentration of 180 μg ml^{-1}. Sections were thoroughly washed with PBS prior to incubation in a moist chamber on a drop of protein A–gold complex (10 nm in diameter; OD_{525} = 0.005). After being rinsed with distilled water, they were contrasted with a saturated solution of uranyl acetate containing 0.5% (v/v) acetic acid for 15 min and then with 0.02% lead citrate for 5 min. The sections were examined in a Phillips 410 electron microscope at 80 kV. Specificity of labeling was assessed with the following control tests: 1) incubation with anti-2′-hydroxylupalbigenin IgGs which were previously absorbed with 100 μM of the hapten in PBS, followed by protein A–gold, 2) incubation with preimmune serum, followed by protein A–gold, and 3) incubation with protein A–gold suspension alone. Labeling and control experiments were repeated on 20 root and nodule ultrathin sections obtained from five tissue samples.

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LITERATURE CITED

Caétano-Annollès, G., Paparozzi, E. T., and Gresshoff, P. M. 1991. Mature nodules and root tips control nodulation in soybean. J. Plant Physiol. 137:389-396.

Clarke, D. D. 1982. The accumulation of cinnamic amides in the cell walls of potato tissue as an early response to fungal attack. Pages 321-322 in: Active Defense Mechanism in Plants. R. K. S. Wood, ed. Plenum Press, New York.

de Lorenzo, C., Iannetta, P. P. M., Fernandez-Pascual, M., James, E. K., Lucas, M. M., Sprent, J. I., Witty, J. F., Minchin, F. R., and de Felipe, M. R. 1993. Oxygen diffusion in lupin nodules. II. Mechanisms of diffusion barrier operation. J. Exp. Bot. 44:1469-1474.

Dewick, P. M. 1994. Isoflavonoids. Pages 117-239 in: The Flavonoids—Advances in Research Since 1986. J. B. Harborne, ed. Chapman and Hall, London.

Erlanger, B. F. 1980. The preparation of antigenic hapten-carrier conjugates: A survey. Pages 85-104 in: Methods in Enzymology: Immunological Techniques. Part A, Vol. 70. H. Van Vunakis and J. J. Langone, eds. Academic Press, New York.

Estabrook, E. M., and Sengupta-Gopolan, C. 1991. Differential expression of phenylalanine-ammonia-lyase and chalcone synthase during soybean nodule development. Plant Cell 3:299-308.

Friend, J. 1981. Plant phenolics, lignification and plant disease. Pages 197-261 in: Progress in Phytochemistry. Vol. 7. J. B. Harborne and T. Swain, eds. Pergamon Press, New York.

Fry, S. C. 1983. Oxidative phenolic coupling reactions cross-link hydroxyproline-rich glycoprotein molecules in the plant cell wall. Pages 59-72 in: Current Topics in Plant Biochemistry and Physiology. D. D. Randall, D. G. Blevins, R. L. Larson, and B. J. Rapp, eds. University of Missouri Press, Columbia.

Gagnon, H. 1993. The isoflavones of *Lupinus albus:* Their constitutive and environmentally induced accumulation and secretion, and their effect on bacterial growth. M.Sc. thesis, Concordia University, Montreal.

Gagnon, H., Séguin, J., Bleichert, E., Tahara, S., and Ibrahim, R. K. 1992a. Biosynthesis of white lupin isoflavonoids from [U-^{14}C]L-phenylalanine and their release into the culture medium. Plant Physiol. 100:76-79.

Gagnon, H., Tahara, S., Bleichert, E., and Ibrahim, R. K. 1992b. Separation of aglucones, glucosides and prenylated isoflavones by high-performance liquid chromatography. J. Chromatogr. 606:255-259.

Grandmaison, J., Furlan, V., van Calsteren, M.-R., and Olàh, G. M. 1993. Characterization and localization of plant phenolic derivatives likely involved in the pathogen resistance of endomycorrhized roots. Mycorrhiza 3:155-164.

Hahlbrock, K., and Scheel, D. 1989. Physiology and molecular biology of phenyl-propanoid metabolism. Annu. Rev. Plant Physiol. Plant Mol. Biol. 40:347-369.

Heller, W., and Forkmann, G. 1994. Biosynthesis. Pages 399-425 in: The Flavonoids—Advances in Research Since 1986. J. B. Harborne, ed. Chapman and Hall, London.

Hirsch, A. M. 1992. Developmental biology of legume nodulation. New Phytol. 122:211-237.

Iannetta, P. P. M., de Lorenzo, C., James, E. K., Fernandez-Pascual, M., Sprent, J. I., Lucas, M. M., Witty, J. F., de Felipe, M. R., Minchin, F. R. 1993. Oxygen diffusion in lupin nodules. 1. Visualization of diffusion barrier operation. J. Exp. Bot. 44:1461-1467.

Karr, D. B., Emerich, D. W., and Karr, A. L. 1992. Accumulation of the phytoalexin, glyceollin in root nodules of soybean by effective and ineffective strains of *Bradyrhizobium japonicum.* J. Chem. Ecol. 18: 997-1008.

Kosslak, R. M., Bookland, R., Barkei, J., Paren, H. E., and Appelbaum, E. R. 1987 Induction of *Bradyrhizobium japonicum* common *nod* genes by isoflavones isolated from *Glycine max.* Proc. Natl. Acad. Sci. USA 84:7428-7432.

Lamoureux, S. W., Vacha, W. E. K., and Ibrahim, R. K. 1986. Localization of partially methylated flavonol glucoside in *Chrysosplenium americanum.* I. Preparation and properties of a trimethylflavonol glucoside antibody. Plant Sci. 44:169-173.

Libbenga, K. R., and Bogers, R. J. 1974. Root nodule morphogenesis. Pages 430-472 in: The Biology of Nitrogen Fixation. A. Quipsel, ed. North-Holland, Amsterdam.

Long, S. R. 1989. Rhizobium legume nodulation: Life together in the underground. Cell 56:203-214.

Pankhurst, C. E., and Biggs, D. R. 1980. Sensitivity of *Rhizobium* to selected isoflavonoids. Can. J. Microbiol. 26:542-545.

Parniske, M., Ahlborn, B., and Werner, D. 1991a. Isoflavonoid inducible resistance to the phytoalexin glyceollin I in soybean *Rhizobia.* J. Bacteriol. 173:3432-3439.

Parniske, M., Fischer, H. M., Hennecke, H., and Werner, D. 1991b. Accumulation of the phytoalexin glyceollin I in soybean nodules infected by a *Bradyrhizobium japonicum nifA* mutant. Z. Naturforsch. 46C:318-320.

Parniske, M., Pausch, G., and Werner, D. 1988. Changes in flavonoid pattern of root hairs of *Glycine max* in response to symbiotic infection with *B. japonicum.* Page 466 in: Nitrogen Fixation: Hundred Years After. H. Bothe, F. J. de Bruijn, and W. E. Newton, eds. Fisher Verlag, Stuttgart.

Phillips, D. A. 1992. Flavonoids: Plant signals to soil microbes. Recent Adv. Phytochem. 26:201-232.

Recourt, K., van Tunen, A., Mur, L., van Brussel, A., Lugtenberg, B., and Kijne, J. 1992. Activation of flavonoid biosynthesis in roots of *Vicia sativa* subsp. *nigra* plants by inoculation with *Rhizobium leguminosarum* biovar *viciae*. Plant Mol. Biol. 19:411-420.

Sadowsky, M. J., Olson, E. R., Foster, V. E., Kosslak, R. M., and Verma, D. P. S. 1988 Two host inducible genes of *Rhizobium fredii* and characterization of the inducing compound. J. Bacteriol. 170:171-178.

Savouré, A., Magyar, Z., Pierre, M., Brown, S., Schultze, M., Dudits, D., Kondorosi, A., and Kondorosi, E. 1994. Activation of the cell cycle machinery and the isoflavonoid biosynthesis pathway by active *Rhizobium meliloti nod* signal molecules in *Medicago microcallus* suspensions. EMBO J. 13:1093-1102.

Schmidt, P. E., Parniske, M., and Werner, D. 1992. Production of the phytoalexin glyceollin I by soybean roots in response to symbiotic and pathogenic infection. Bot. Acta 105:15-18.

Spaink, H. P., Wijffelman, C. A., Pees, E., Okker, R. J. H., and Lugtenberg, B. J. J. 1987. *Rhizobium* nodulation gene *nodD* as a determinant of host specificity. Nature 328:337-340.

Tahara, S., Ingham, J. L., Nakahara, S., Mizutani, J., and Harborne, J. B. 1984. Fungitoxic dihydrofuranoisoflavones and related compounds in white lupin, *Lupinus albus.* Phytochemistry 23:1889-1900.

Tahara, S., Orihara, S., Ingham, S., and Mizutani, J. 1989. Seventeen isoflavonoids from *Lupinus albus* roots. Phytochemistry 28:901-911.

Tahara, S., Shibaki, S., Ingham, J. L., and Mizutani, J. 1990. Further isoflavonoids from white lupin roots. Z. Naturforsch. 45C:147-153.

van Brussel, A. A. N., Recourt, K., Pees, E., Spaink, H. P., Tak, T., Wijffelman, C. A., Kijne, J. W., and Lugtenberg, B. J. J. 1990. A biovar specific signal of *Rhizobium leguminosarum* bv. *viciae* induces nodulation genes inducing activity in root exudates of *Vicia sativa* subsp. *nigra.* J. Bacteriol. 172:5394-5401.

Wood, S. M., Layzell, D. B., Newcomb, W., and Pankhurst, C. E. 1985. A morphometric study of effective nodules induced by *Rhizobium loti* and *Bradyrhizobium* sp. (*Lotus*) on *Lotus pedunculatus.* Can. J. Bot. 63:43-53.