Flavonoid Inducers of Nodulation Genes Stimulate *Rhizobium fredii* USDA257 to Export Proteins into the Environment

Hari B. Krishnan and Steven G. Pueppke

Department of Plant Pathology, University of Missouri, Columbia, MO 65211 U.S.A.
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Genistein, an isoflavone from the roots of soybean (*Glycine max*), is among the most powerful inducers of the expression of nodulation genes in *Rhizobium fredii* USDA257, a nitrogen-fixing symbiont of this plant. We show here that concentrations of this compound as low as 100–500 nM also stimulate cells of USDA257 to excrete five major proteins, of molecular masses of 54, 39, 36, 20, and 8.9 kDa. Other known inducers of nodulation genes in *R. fredii*, including luteolin and naringenin, also elicit accumulation of this set of proteins in USDA257, but the noninducers biochanin A and quercetin are inactive in induction of protein secretion. [^{35}S]Methionine is incorporated into each of the proteins, but none contains detectable polysaccharide. Export of an array of proteins in response to nodulation gene inducers is characteristic of *R. fredii* and depends on the presence of an intact symbiosis plasmid. *R. leguminosarum* bv. *phaseoli, Bradyrhizobium japonicum,* and broad host range *Rhizobium* sp. strain NGR234 each elaborate a single genistein-induced extracellular protein, but the response is lacking when several other nitrogen-fixing species are exposed to their nodulation gene inducers. The N-terminal sequence of the 36-kDa protein, MetTyrSerSerLysThrGlySerAlaSerGlnSerThr, differs from that of all known nodulation genes, and the conservation of the N-terminal methionine indicates that this protein probably is exported without N-terminal processing.

Additional keywords: common *nod* genes; symbiosis plasmid.

Legume-*Rhizobium* symbioses channel massive quantities of atmospheric nitrogen into both agricultural and nonagricultural plant species, and thus are of fundamental economic and ecological significance. Early in the symbiosis, the host releases flavonoid signals that are perceived by the bacteria and trigger the coordinated expression of a series of nodulation (*nod*) genes (Long 1989; Martinez *et al.* 1990). In most *Rhizobium* species, these genes lie on a large symbiosis or sym plasmid. The common *nod* genes, *nodABC*, are found in all rhizobia and are required for biosynthesis of the core molecules of lipo-oligosaccharide Nod factors that deform root hairs and initiate host cell dedifferentiation (Lerouge *et al.* 1990; Spaink *et al.* 1991; Schultze *et al.* 1992). Additional loci, including *nolFE, nodH, nodM, nodPQ,* and *nodL*, appear to structurally modify the basic Nod factors, so that they deform root hairs and elicit cortical cell divisions in a host-specific manner (Roche *et al.* 1991; Spaink *et al.* 1991).

Both the common and the host-specific *nod* genes are under the positive control of the regulatory gene, *nodD* (Györgypal *et al.* 1991). Rhizobia such as *R. tropici* and *R. leguminosarum* bv. *viciae* and *R. l.* bv. *trifolii* contain single copies of *nodD*; others, including *R. l.* bv. *phaseoli, R. meliloti,* and *R. fredii*, have two or three related *nodD* loci. In each of these organisms, at least one of the NodD proteins is believed to be activated by flavonoid signals from the host and then to complex with *cis*-regulatory elements termed *nod* boxes. As a consequence, the adjacent *nod* genes become transcriptionally active and fulfill their nodule initiation and host specificity functions (Long 1989; Györgypal *et al.* 1991; Schlaman *et al.* 1992).

Although the nucleic acid sequences of many *nodD*-inducible genes are available, and the deduced amino acid sequences of the proteins have been examined extensively (Györgypal *et al.* 1991), the cellular levels of Nod proteins in rhizobia usually are too low to permit direct biochemical characterization. The naringenin- and hesperetin-inducible 30-kDa NodO protein of *R. l.* bv. *viciae* is an exception to this generalization (deMaagd *et al.* 1988, 1989a, 1989b; Economou *et al.* 1990; Scheu *et al.* 1992). This protein, which binds Ca^{2+} and is excreted without N-terminal cleavage, plays an accessory role in definition of host range of this biovar. Thus, although nodO mutants generally remain Nod^+ (Economou *et al.* 1989), they become Nod^- on vetch if *nodFE* is inactivated (Downie and Surin 1990). Identification of most other Nod proteins, however, has relied on coupled transcription/translation systems and expression of cloned genes and gene fusions in *E. coli* (Dusha *et al.* 1986; Fisher *et al.* 1987). These experiments have facilitated antibody production and have demonstrated that NodA is cytoplasmic (Schmidt *et al.* 1986; Johnson *et al.* 1989), whereas NodE (Spaink *et al.* 1989), NodI (Schlaman *et al.* 1990), and NodC (John *et al.* 1988; Johnson *et al.* 1989) are membrane-associated. NodD, in contrast, is partitioned both in the membrane and the cytosol (Schlaman *et al.* 1989).

R. fredii is a species known primarily as a symbiont

Address correspondence to: Steven G. Pueppke, Department of Plant Pathology, University of Missouri, Columbia, MO 65211 U.S.A.

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of soybean, but, in fact, it nodulates dozens of other legume species (Keyser *et al.* 1982; S. G. Pueppke and W. J. Broughton, unpublished observations). Both copies of *nodD* of this organism have been sequenced and functionally analyzed (Appelbaum *et al.* 1988), as have *nodABC* (Krishnan and Pueppke 1991b) and the host specificity genes *nolC* (Krishnan and Pueppke 1991a) and *nodSU* (Krishnan *et al.* 1992). The selectivity of *R. fredii* strain USDA257 for specific soybean cultivars is an area of particular emphasis in our laboratory (Balatti and Pueppke 1992a, 1992b). As part of these studies, we have recently discovered that the flavonoid inducers of *nodABC* from *R. fredii* USDA257 have a second major biological activity: They stimulate the rapid accumulation of a set of proteins that is exported to the environment. Here we describe these proteins, define the flavonoid- and species-specificity of their induction, and show that their accumulation is sym plasmid dependent.

RESULTS

Flavonoid inducers of *nodABC* elicit accumulation of extracellular proteins by *R. fredii* USDA257.

A *lacZ* fusion to *nodC* of *R. fredii* USDA257 was induced by low concentrations of several symbiosis-signaling molecules, including the trihydroxyisoflavone, genistein (Krishnan and Pueppke 1991b). At a concentration of 1 μM, this compound failed to detectably alter the levels of cytoplasmic, periplasmic, or residual cellular proteins in USDA257 (Fig. 1). Although genistein appeared to induce a new membrane protein of about 62 kDa (bracketed in Fig. 1), its modulating effect on extracellular proteins was considerably more apparent. Culture fluids from uninduced cells contained relatively low background levels of proteins, which could be resolved into about 25 bands on one-dimensional denaturing gels. Genistein induced the export of five distinct extracellular proteins into these cultures (Fig. 1). All were prominant, and their relative molecular masses were estimated to be 54, 39, 36, 20, and 8.9 kDa. [^{35}S]Methionine was incorporated into each of the five proteins, but none stained with the periodic acid-Schiff reagent (data not shown). This ruled out the possibility that some of the genistein-induced bands represent lipopolysaccharide contaminants and made it unlikely that any of the proteins contained carbohydrate. Thus a symbiosis signal that is known to be elaborated by soybean roots (Cho and Harper 1991; Graham 1991) could trigger a soybean symbiont to synthesize an array of proteins and target them for release into the environment.

Flavonoid concentrations that were adequate to induce *nodC* were sufficient to elicit the appearance of the five extracellular proteins, and the structural specificity for induction corresponded to that for *nod* gene activation.

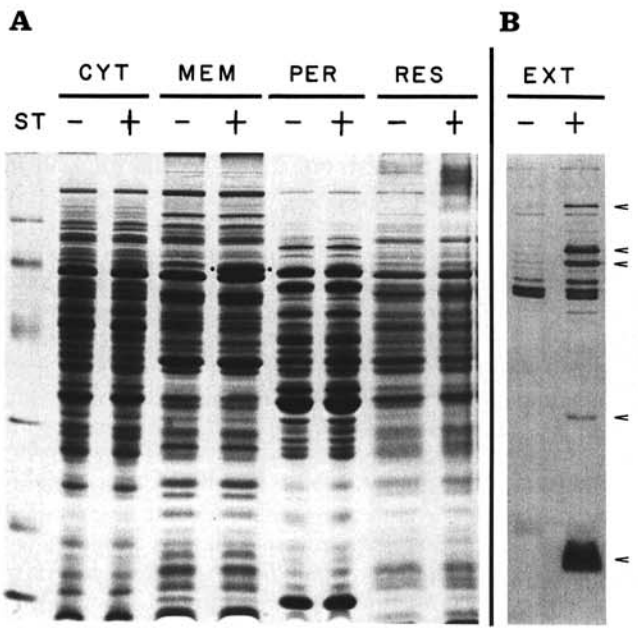

Fig. 1. Subcellular fractionation of proteins from flavonoid-induced cultures of *Rhizobium fredii* USDA257. Cultures were induced for 36 hr with 1 μM genistein, and proteins were isolated and fractionated as described in Materials and Methods. Lanes marked − and + contain extracts from uninduced and induced cells, respectively. The protein fractions are coded as follows: CYT = cytoplasmic; MEM = membrane-associated; PER = periplasmic; RES = residual; EXT = extracellular. **A,** Coomassie blue-stained proteins after electrophoresis on a 12.5% polyacrylamide gel. The molecular weight standards in the lane marked ST are, in descending order, of 97.4, 66.2, 45.0, 31.0, 21.5, and 14.4 kDa. The induced membrane protein is bracketed between dots. **B,** Silver-stained proteins after electrophoresis on a 15% polyacrylamide gel. Genistein-induced extracellular proteins of 54, 39, 36, 20, and 8.9 kDa are marked with arrowheads on the right, in descending order.

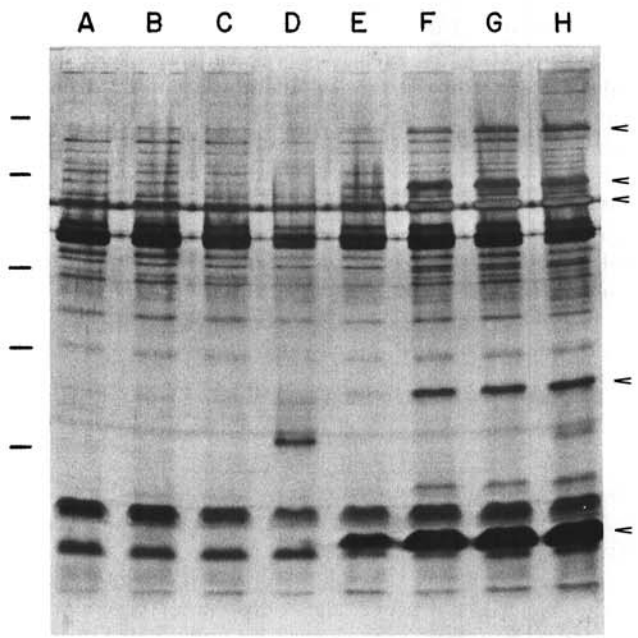

Fig. 2. Concentration-dependence of extracellular protein induction in *Rhizobium fredii* USDA257 by genistein. Extracellular proteins were prepared after induction of cultures for 36 hr as described in Materials and Methods and fractionated on a 15% polyacrylamide gel. Final genistein concentrations were: A, 0 (control); B, 100 pM; C, 500 pM; D, 10 nM; E, 100 nM; F, 500 nM; G, 1 μM; H, 10 μM. In descending order, the bars on the left mark the positions of molecular weight standards of 66.2, 45.0, 31.0, 21.5, and 14.4 kDa. The genistein-induced proteins are indicated with arrowheads on the right.

The activity of genistein was concentration dependent, with a threshold between 100 and 500 nM (Fig. 2). The five proteins appeared coordinately as the concentration of genistein was increased, and they first became visible at concentrations known to be half-maximal for induction of *nodC* (Krishnan and Pueppke 1991b). Genistein and the flavone luteolin, both of which were powerful activators of *nodC* expression, induced virtually indistinguishable arrays of proteins (Fig. 3). The flavone naringenin, a considerably less active inducer of *nodC*, and two inactive compounds, the isoflavone biochanin A and the flavonol quercetin (Krishnan and Pueppke 1991b), did not stimulate extracellular protein accumulation (Fig. 3). At the concentrations tested, none of these treatments significantly influenced bacterial multiplication rates (data not shown), and thus their effects could not be attributed to generalized growth responses.

The only known extracellular Nod protein, NodO, bound Ca^{2+} (Economou *et al.* 1990), a cation known to influence *nod* gene expression (Richardson *et al.* 1988; Howieson *et al.* 1992) and Ca^{2+} is known to influence the ability of strain USDA257 to nodulate soybean (Balatti *et al.* 1991). Ca^{2+} concentrations as high as 1 mM failed to directly induce the accumulation of the five exported proteins in cultures of USDA257, and concentrations as high as 500 μM also failed to significantly influence the induction of the proteins by 1 μM genistein (Fig. 4). Induction of the five proteins by genistein was inhibited, however, by higher concentrations of Ca^{2+} (data not shown).

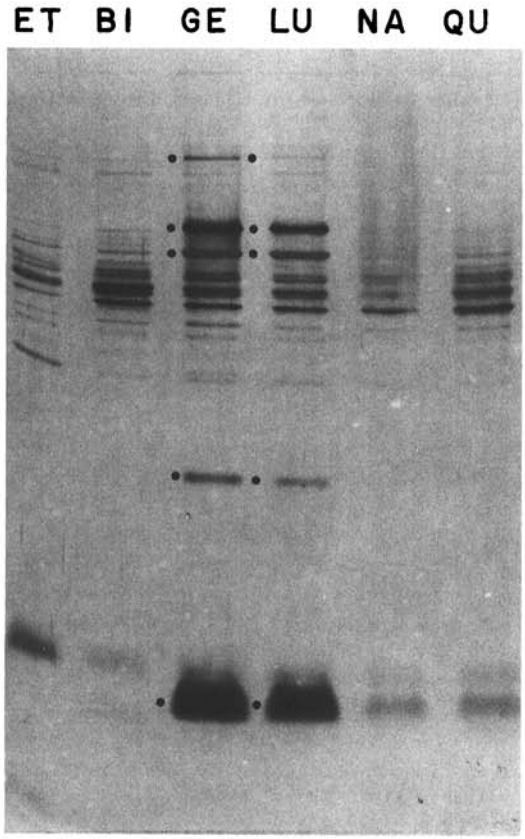

Fig. 3. Flavonoid specificity of extracellular protein induction in *Rhizobium fredii* USDA257. Cells were grown in the presence of 1 μM inducer for 36 hr, and extracellular proteins were prepared as described in Materials and Methods and fractionated on a 15% polyacrylamide gel. Cells were induced as follows: ET = ethanol control; BI = biochanin A; GE = genistein; LU = luteolin; NA = naringenin; QU = quercetin. The five extracellular proteins in the genistein lane are marked with dots.

Induction of extracellular proteins requires a sym plasmid and is strain-specific.

R. fredii strains USDA193 and USDA205 are siblings of USDA257. Each of these strains produced the 39-, 36-, 20-, and 8.9-kDa extracellular proteins that are characteristic of USDA257 (Fig. 5). Both strains also exported a higher molecular weight protein, but their sizes were different from that of the 54-kDa protein of USDA257. None of these changes was apparent in genistein-treated cultures of IA728, a sym plasmid-cured derivative of USDA193, or in USDA205-1A03, which had a sym plasmid deletion that removed both copies of *nodD*, but left *nodABC* intact. The absence of the broad protein band at 8.9 kDa was especially striking in these comparisons, as was the constitutive, flavonoid-independent appearance of new, low molecular weight bands in the mutants (Fig. 5).

Rhizobium strains varied substantially in their capacities to elaborate extracellular proteins in response to treatment with flavonoids, a fact that could explain why the phenomenon has escaped attention in the past. For example, the culture filtrates of *R. l.* bv. *trifolii* BAL are essentially protein-free, and a *nod* gene inducer for this species, naringenin (Bender *et al.* 1988), does not stimulate the detectable export of any new proteins (Fig. 6). *R. tropici* UMR1173, *R. meliloti* RCR2011, and *R. l.* bv. *viciae* PF2 each produces a single major extracellular protein in culture, but as is the case with *R. l.* bv. *trifolii*, the inducers luteolin (Peters *et al.* 1986) and naringenin (Zaat *et al.*

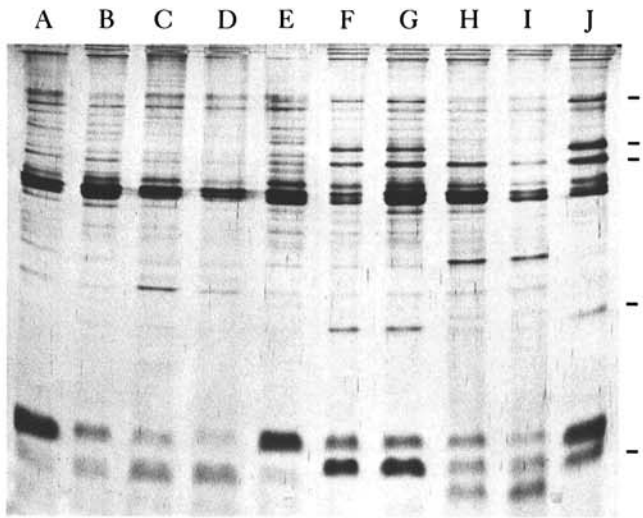

Fig. 4. Effect of calcium on the flavonoid-inducible export of proteins by *Rhizobium fredii* USDA257. Cells were grown for 24 hr in the absence (lanes A–E) or the presence (lanes F–J) of 1 μM genistein. Calcium concentrations were as follows: 250 μM (lanes A and F); 500 μM (lanes B and G); 750 μM (lanes C and H); 1 mM (lanes D and I); none (lanes E and J). Extracellular proteins were prepared as described in Materials and Methods and fractionated on a 15% polyacrylamide gel.

1987) have no effect. The NodO protein, which is characteristic of some strains of *R. l.* bv. *viciae* (deMaagd *et al.* 1989b), is conspicuously absent in extracts of strain PF2. In contrast, broad host range strain NGR234, *B. japonicum* USDA110, and *R. l.* bv. *phaseoli* 127K14 produce basal arrays of extracellular proteins not unlike that elaborated by USDA257, but each strain accumulates just one major new protein in response to genistein treatment (bracketed between dots in Fig. 6).

N-terminal sequence of the 36-kDa protein.

The N-terminal of the 36-kDa protein was sequenced, yielding the following 13 amino acid residues: MetTyrSer-SerLysThrGlySerAlaSerGlnSerThr. It is highly unusual for processed proteins to contain N-terminal methionine residues (vonHeijne 1987), and thus the protein was most likely excreted without N-terminal processing. The sequence did not match the N-terminal of any known *nod* gene product in the SwissProt Version 20 database. This includes the 30-kDa NodO protein, the only Nod protein that is known to be excreted (deMaagd *et al.* 1989a, 1989b). The FASTN algorithm did, however, detect a 77% match between ser4 and thr13 of the 36-kDa protein and ser3 and thr11 of a 22.3-kDa, tissue-specific protein from ovarian follicle cells of *Drosophila melanogaster* (Burke *et al.* 1987).

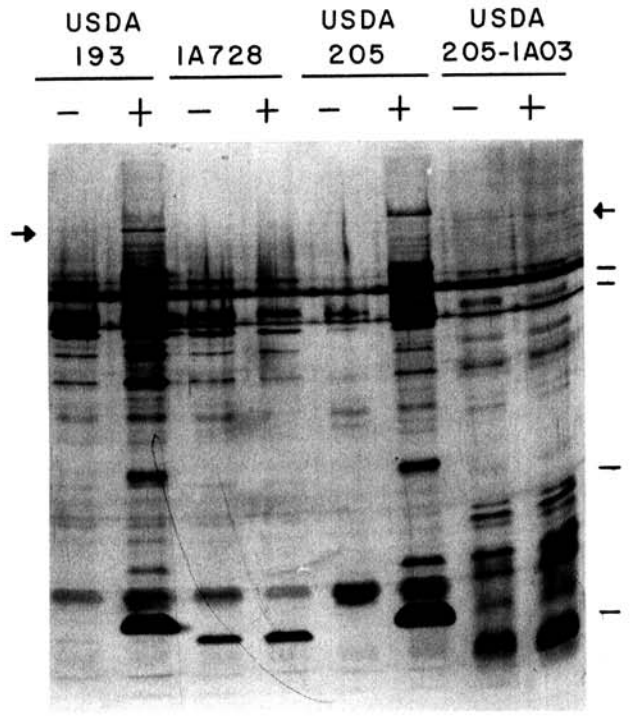

Fig. 5. Extracellular protein induction by genistein in *Rhizobium fredii* is strain- and sym plasmid-dependent. After induction by 1 μM genistein for 36 hr, extracellular proteins were prepared as described in Materials and Methods and fractionated on a 15% polyacrylamide gel. Lanes marked – and + contain extracts of uninduced and induced cells, respectively. The positions of the genistein-induced proteins are marked with bars. The arrows mark the induced high molecular weight proteins.

DISCUSSION

Metabolites from the roots of a potential host legume elicit major shifts in gene expression by rhizobia. This process appears to be initiated when the constitutive NodD protein encountered flavonoid signals from the plant and activated an array of inducible *nod* genes (Györgypal *et al.* 1991). Some of these genes, including *nodABC, nodFE, nodL, nodM,* and *nodPQ,* are known or suspected to encode enzymes that participate in the synthesis of Nod factors (Lerouge *et al.* 1990; Baev *et al.* 1991; Roche *et al.* 1991; Spaink *et al.* 1991; Marie *et al.* 1992). These lipo-oligosaccharide compounds trigger two of the earliest steps in symbiotic development: reorientation of the growth of root hairs and induction of centers of mitosis in the host root cortex (Truchet *et al.* 1991). Other Nod genes, including *nodM, nodT,* and *nodI,* are believed to help regulate the efficiency of nodulation and to define host range, but their precise biochemical functions remain obscure (Martinez *et al.* 1990).

Nothing is presently known about the Nod factors of *R. fredii*, but several flavonoids, including genistein, are potent inducers of *nodD*-dependent *nod* genes within this species, just as they are in other rhizobia (Krishnan and Pueppke 1991b). We showed here that genistein caused *R. fredii* USDA257 and several sibling strains to coordinately export an array of new extracellular proteins, which could accumulate to high levels in the extracellular milieu. Elicitation of this response was limited to authentic inducers of *nodABC*, and the low signal concentrations that were sufficient to activate the common *nod* genes also were sufficient to trigger protein excretion. In addition, strains lacking either a sym plasmid or with deleted *nodD1* and *nodD2* failed to respond. These characteristics of induction, in combination with the final destination of

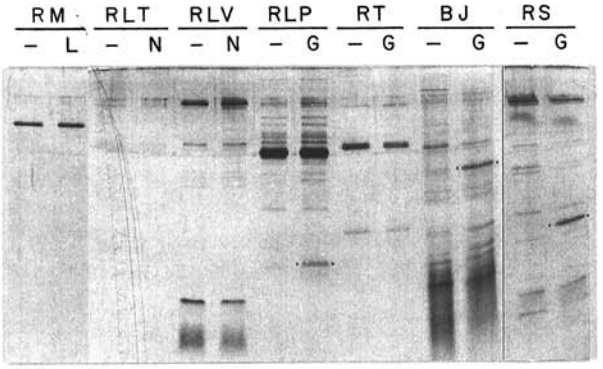

Fig. 6. Diversity of the response of *Rhizobium* species to treatment with flavonoid inducers of *nodABC*. Cells were harvested 36 hr after induction with 1 μM flavonoid, and extracellular proteins were fractionated on a 15% polyacrylamide gel as described in Materials and Methods. Lanes marked – contain extracts of uninduced cells; lanes marked L, N, and G contain extracts of cells induced with luteolin, naringenin, and genistein, respectively. RM = *Rhizobium meliloti* RCR2011; RLT = *R. leguminosarum* bv. *trifolii* BAL; RLV = *R. l.* bv. *viciae* PF2; RLP = *R. l.* bv. *phaseoli* 127K14; RT = *R. tropici* UMR1173; BJ = *Bradyrhizobium japonicum* USDA110; RS = *Rhizobium* sp. NGR234. Major genistein-induced proteins are bracketed between dots.

the proteins, make them prime candidates to intervene during some aspect of nodule initiation. We currently are examining this possibility, as well as determining whether genes other than *nodD* influence protein excretion.

The coordinated elicitation of an array of extracellular proteins by flavonoid signals from the host does not represent a generalized phenomenon in nodulating organisms. We detected it only in *R. fredii*, a unique *Rhizobium* species with a broad host range for legume species and a marked cultivar specificity for its host of isolation, soybean (Keyser *et al.* 1982). Three other rhizobia, including two broad host range organisms, *Rhizobium* sp. NGR234 and *B. japonicum* USDA110, nonetheless produced single major extracellular proteins in response to treatment with their *nod* gene inducers. *R. l.* bv. *viciae* also elaborates a single protein, NodO (deMaagd *et al.* 1989a, 1989b; Economou *et al.* 1990), which is the product of an inducible *nod* gene. NodO is exported without N-terminal processing via a hemolysin-type system (Scheu *et al.* 1992), but its production is restricted to a subset of *R. l.* bv. *viciae* strains (PF2 does not appear to be one of them). Based on size considerations, none of the proteins that we detected is likely to be NodO.

We obtained preliminary structural data on the N-terminal of the 36-kDa *R. fredii* protein, and they provided two important clues about its function. First, an N-terminal methionine was present, indicating that this extracellular protein was probably not processed. Second, the sequence differed from that of all known *nod* gene products. The locus responsible for the 36-kDa protein thus is a potentially novel *nod* gene, and it appears to constitute the second *Rhizobium* protein that is exported without N-terminal cleavage.

There are several possible mechanisms by which the extracellular proteins could mediate a bacterial response to the plant's nodulation signal. They may, for example, have roles in biosynthesis of low molecular weight bacterial molecules that function in nodule initiation. The potential involvement of the extracellular *R. fredii* proteins in Nod-factor production is debatable, however, because the known members of this class of compounds are synthesized by cytoplasmic and membrane-bound enzymes. Other Nod metabolites of uncertain function have nevertheless been identified, and they could be synthesized or otherwise modified by extracellular enzymes (Philip-Hollingsworth *et al.* 1991; Spaink *et al.* 1992). The proteins also may have important enzymatic activities in the rhizosphere. These include the direct structural modification of flavonoid signals from the plant (Hartwig and Phillips 1991; Rao *et al.* 1991) and the possible feedback stimulation or inhibition of further flavonoid release (vanBrussel *et al.* 1990; Recourt *et al.* 1991). In addition, they may participate in the adsorption of rhizobia to root hairs, perhaps by enzymatically unmasking specific root hair receptors. It also is conceivable that the proteins themselves function as return signals to the plant, triggering responses required for nodule initiation.

In the context of this discussion, it is important to consider that many of the types of flavonoids that induce *nod* genes also possess other sorts of biological activities (VanEtten and Pueppke 1976). These include modulation of responses as diverse as root-hair streaming (Popovici and Reznik 1976), the activity of protein kinases (Akiyama *et al.* 1987; Geahlen *et al.* 1989), and bacterial growth (Gnanamanickam and Smith 1980; Gnanamanickam and Mansfield 1981). We do not yet know all of the effects of compounds such as genistein on bacterial cells, and so we cannot yet rule out the possibility that the extracellular proteins have additional or alternative functions only indirectly related to nodulation. Given the relative ease with which the proteins can be purified, however, all of these hypotheses are testable—and we are beginning to sort them out.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

R. fredii strains and *B. japonicum* USDA110 originally were from the culture collection of the U. S. Department of Agriculture, Beltsville, MD (Keyser and Griffin 1987). IA728 is a Nod$^-$ derivative of strain USDA193 that has been cured of its sym plasmid (Ramakrishnan *et al.* 1986). USDA205-1A03 is a Nod$^-$ mutant of strain USDA205 (Keyser and Griffin 1987); based on Southern hybridizations, it lacks *nodD1* and *nodD2*, but retains *nodABC* (our unpublished observations). The sources of other strains of rhizobia were as follows: *R. meliloti* RCR2011 from J. Dénarié, CNRS-INRA, Toulouse, France; *R. l.* bv. *phaseoli* 127K14 from the Nitragin Co., Milwaukee, WI; *R. l.* bv. *trifolii* BAL from D. H. Hubbell, University of Florida, Gainesville; *R. l.* bv. *viciae* PF2 from T. A. Lie, Agricultural University of Wageningen, Netherlands; *R. tropici* UMR1173 from E. Martínez, UNAM, Cuernavaca, Mexico; *Rhizobium* sp. NGR234 from W. J. Broughton, University of Geneva, Switzerland.

Rhizobia were stored on slants of YEM medium (Vincent 1970). Liquid cultures for analysis of protein expression were initiated in a prescribed manner. First, 5-ml YEM starter cultures were prepared from slants and incubated overnight at 28° C on a rotary shaker at 160 cycles per minute. Aliquots of 100 μl were transferred from these cultures to a series of 125-ml Erlenmeyer flasks, each containing 25 ml of YEM medium. Cultures were induced with 2.5 μl of absolute ethanol containing 10 mM flavonoid, to yield a final inducer concentration of 1 μM. Controls received 2.5 μl of absolute ethanol only. Test flavonoids included genistein and luteolin (both from ICN Pharmaceuticals), and biochanin A, naringenin, and quercetin (all from Sigma Chemical Co.). Inducer concentrations in some experiments were reduced by dilution of stock solutions before use. Induced cultures were incubated as before for 36 hr before proteins were extracted.

The effect of calcium on genistein-induced export of proteins was monitored by cultivating USDA257 in a defined gluconate-mannitol medium (Balatti *et al.* 1991) that had been adjusted to pH 6.2 with MES. Cultures contained from 250 μM to 1 mM calcium chloride and were induced with 1 μM genistein. These cultures were incubated for 24 hr before proteins were extracted.

Isolation and analysis of flavonoid-induced proteins.

Bacterial cells were harvested by centrifugation at 7,700 × *g* for 10 min. In some experiments, cells were separated into soluble, membrane, periplasmic, and residual fractions as described (Glenn and Dilworth 1979; Krishnan and Pueppke 1991a). Proteins were precipitated from culture fluids by the addition of three volumes of acetone. Floating polysaccharides from mucoid strains were removed immediately by spooling with a glass rod, before incubating the mixtures overnight at −20° C. Precipitated proteins were collected by centrifugation at 12,000 × *g* for 15 min. The precipitates were air-dried and dissolved in 400 μl of SDS sample buffer, and 30-μl aliquots were electrophoresed in SDS-polyacrylamide gels, essentially as described by Laemmli (1970).

Proteins were stained with Coomassie Brilliant Blue R-250 or silver (Morrissey 1981), and polysaccharides were visualized after treatment with the periodic acid-Schiff reagent (Segrest and Jackson 1972). The N-terminal sequence of the 36 kDa, flavonoid-inducible protein was obtained following western transfer of the electrophoretically purified polypeptide onto a Millipore Immobilon PVDF membrane (Matsudaira 1987). Labeling of cultures with [^{35}S]methionine (1,120 Ci/mmol) and fluorographic detection of radioisotope-labeled proteins was as described (Krishnan and Pueppke 1991b).

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