Elaboration of flavonoid-induced proteins by the nitrogen-fixing soybean symbiont *Rhizobium fredii* is regulated by both *nodD1* and *nodD2*, and is dependent on the cultivar-specificity locus, *nolXWBTUV*

H. B. Krishnan, Chen-lan Kuo† and S. G. Pueppke

Author for correspondence: S. G. Pueppke. Tel: +1 314 882 2643. Fax: +1 314 882 0588.
e-mail: plantsgp@mizzou1.missouri.edu

Department of Plant
Pathology, University of
Missouri, Columbia, MO
65211, USA

Genistein and other flavonoids from host legumes are known to stimulate cells of the nitrogen-fixing soybean symbiont *Rhizobium fredii* to synthesize Nod factors, which function as signals during nodule initiation. Flavonoids also trigger *R. fredii* to secrete a set of signal-responsive (SR) proteins into the environment. By insertion mutagenesis, we showed that secretion of SR proteins by this organism has an absolute dependence on the regulatory gene *nodD1*. We isolated and sequenced *nodD1* and *nodD2* of *R. fredii* USDA257 and constructed strains containing additional, plasmid-borne copies of these genes. Extra copies of *nodD1* had no effect on secretion of SR proteins, but extra copies of *nodD2* rendered the process constitutive. Extracts from seeds of the soybean cultivars McCall and Peking can substitute for purified flavonoids as inducers of SR proteins. The *nolXWBTUV* locus is known to control cultivar-specific nodulation of McCall soybean in a negative, flavonoid-dependent manner. Inactivation of any of these genes prevented SR proteins from accumulating in culture fluids. Protein secretion in response to host signals was a characteristic of nine out of ten *R. fredii* strains tested. Immunological probes failed to detect SR3 or SR5 in mature soybean or cowpea nodules. Although the functions of these proteins remain unknown, their potential role in symbiosis is strengthened by the discovery that their accumulation depends on *nodD1*, *nodD2* and *nolXWBTUV*.

Keywords: *Rhizobium fredii*, *nodD*, *nolXWBTUV*, nodulation, *Glycine max*

INTRODUCTION

The legume–*Rhizobium* symbiosis represents a biologically specialized partnership between the roots of higher plants and bacteria from the soil. Rhizobia are allowed to invade the root cortex, where they initiate meristematic activity that leads to the appearance of root nodules. Nitrogen is fixed within these structures and then made available to the plant. Communication between the legume root and rhizobia can commence well before cells of the potential symbionts establish actual physical

contact, and it is mediated by sets of signals that function in a reciprocal fashion, activating gene expression in both partners (Fisher & Long, 1992). Most of the known signal compounds from plants are flavonoid derivatives, which can be released by both seeds and roots (Phillips, 1992). One of their major roles is to trigger expression of inducible nodulation or *nod/nol* genes in rhizobia, a process dependent on the regulatory gene *nodD* (Györgypal *et al.*, 1991; Schlaman *et al.*, 1992). NodD proteins bind to specialized regulatory regions termed *nod* boxes, which are positioned upstream of inducible *nod* genes and serve as transcriptional activators. The products of a number of inducible *nod* genes are known to control the synthesis of a set of return signals, the Nod factors (Dénarié *et al.*, 1992; Spink, 1994). These oligochitin derivatives are released by flavonoid-induced rhizobia, and they complete the communication circuit, activating

Present address: Program in Plant Biology, University of California, Davis, CA 95616, USA.

Abbreviation: SR, signal-responsive.

The GenBank accession numbers for *nodD1* and *nodD2* are L38459 and L38460, respectively.

gene expression and provoking morphological responses by the plant. *Rhizobium* cells then can invade and cause the production of root nodules.

We are examining nodulation genes and signalling processes in *Rhizobium fredii*, a nitrogen-fixing symbiont of soybean and other legumes such as cowpea (*Vigna unguiculata*) and pigeon pea (*Cajanus cajan*) (Keyser *et al.*, 1982; Heron & Pueppke, 1984). The species is of particular usefulness for such studies because the ability of strains such as USDA257 to nodulate soybean is highly specific. Cultivars such as Peking and Davis form normal nitrogen-fixing nodules, but others, including McCall and Viçoja, do not (Heron & Pueppke, 1984; Balatti & Pueppke, 1992). Such cultivar-specificity is dependent on a complex sym plasmid locus, *nodXWBTUV*, which regulates this process in a negative manner (Heron *et al.*, 1989; Meinhardt *et al.*, 1993; Kovács *et al.*, 1995) that is *nodD*-dependent (unpublished data).

The isoflavones genistein and daidzein are elaborated in high concentration by soybean seedlings, and they appear to function as signals to *R. fredii* (Kosslak *et al.*, 1987; Cho & Harper, 1991; Graham, 1991). Genistein and daidzein are potent *nodD*-dependent inducers of nodulation genes such as *nodABC*; they are among the flavonoids that prompt the synthesis of oligochitin Nod factors (Krishnan & Pueppke, 1991b; Bec-Ferté *et al.*, 1994), and they are involved in regulation of the cultivar-specificity locus *nodXWBTUV* (Kovács *et al.*, 1995). We recently discovered that genistein and other flavonoid signals also stimulate *R. fredii* strain USDA257 to secrete a set of five proteins into the cellular milieu (Krishnan & Pueppke, 1993). These proteins, which we call signal-responsive (SR) proteins, have molecular masses of 54, 39, 36, 20 and 8.9 kDa, and we refer to them as SR1, SR2, SR3, SR4 and SR5, respectively. Although their functions in *R. fredii* are yet to be determined, they are targeted to the right location and appear at the appropriate time to have some role in mediating symbiotic interactions with host legumes. Here we address a series of key questions about the expression of SR proteins in *R. fredii*.

METHODS

Bacterial strains. Wild-type strains of *R. fredii* were from the culture collection of the United States Department of Agriculture, Beltsville, MD, USA (Keyser & Griffin, 1987). Mutant strain 257DH4, which contains Tn5 in the cultivar-specificity gene, *nodU*, has been described previously (Heron *et al.*, 1989). Mutants 191NodD1⁻ and 191NodD2⁻ are derivatives of strain USDA191 and were constructed by inserting a kanamycin resistance cassette into the unique *Bam*HI site of *nodD1* and *nodD2*, respectively (Appelbaum *et al.*, 1988). Mutant 191NodD1⁻NodD2⁻ contains the omega interposon in *nodD1* and a kanamycin resistance cassette in *nodD2* (Boundy-Mills *et al.*, 1994). Mutants of *R. fredii* USDA257 with mini-mu insertions in *nodXWBTUV* have been described previously (Meinhardt *et al.*, 1993).

Molecular manipulation of *nodD1* and *nodD2*. *nodD1* and *nodD2* of *R. fredii* USDA257 were cloned from cosmids pRK1309 and pRK1489, respectively, into pTZ19U (Krishnan & Pueppke, 1991b). pD13 contained *nodD1* on a 3.0 kb *Eco*RI insert, and pD26 contained *nodD2* on a 6.0 kb *Eco*RI insert.

Restriction maps of these two fragments were identical to those of the fragments containing *nodD1* and *nodD2* of strain USDA191 (Appelbaum *et al.*, 1988). The relationships between the genes of strain USDA257 and those of strain USDA191 were confirmed by DNA sequencing. The insert of pD13 was subcloned into pGEM7Zf⁺ as 1.45 and 1.55 kb *Eco*RI/*Bam*HI fragments, and that of pD26 was subcloned into the same vector as 1.6 and 4.4 kb *Eco*RI/*Bam*HI fragments. Double-stranded DNA sequencing in both directions from the *Bam*HI sites was carried out by the dideoxy chain-termination procedure (Sanger *et al.*, 1977) with Sequenase version 2.0 from US Biochemicals. A total of 1120 bp from *nodD1* and 996 bp from *nodD2* was sequenced and found to be identical to the published sequences of the corresponding *nodD* alleles of strain USDA191 (Appelbaum *et al.*, 1988). The *Eco*RI inserts of pD13 and pD26 were cloned into pRK415 (Keen *et al.*, 1988) to create pHBK320 and pHBK330. These plasmids were mobilized triparentally into strain USDA191 to create strains with extra, plasmid-borne copies of *nodD1* and *nodD2*, respectively.

Induction and detection of SR proteins. Bacterial cells were induced in a standardized manner with starter cultures as inoculum for 5 ml test cultures (Krishnan & Pueppke, 1993). The latter were harvested 36 h after treatment with 1 µM genistein (Sigma) and 10 µCi (370 kBq) [³⁵S]methionine (1120 Ci mmol⁻¹, New England Nuclear). Proteins were precipitated by adding 3 vols ice-cold acetone. After overnight incubation at -20 °C, proteins were recovered by centrifugation at 12000 g for 15 min, air-dried, and resuspended in 400 µl SDS-sample buffer (Laemmli, 1970). Aliquots of 30 µl were subjected to electrophoresis on 15% (w/v) SDS-polyacrylamide gels (Laemmli, 1970; Krishnan & Pueppke, 1993). ³⁵S-labelled proteins were detected by fluorography (Bonner & Laskey, 1974).

Seed extracts from soybean (*Glycine max*) cultivars Peking (from the University of Missouri Bradford Research Farm) and McCall (from Eric Pueppke, Erie, ND, USA), were obtained by shaking 10 g seedlots overnight at 150 cycles min⁻¹ in 50 ml 50% (v/v) aqueous ethanol. The seeds were then removed and the liquid centrifuged at 22000 g for 15 min. Supernatant solutions were sterilized by passage through 0.45 µm filters and stored frozen. The abilities of extracts to induce expression of a *nodC-lacZ* gene fusion were then established (Krishnan & Pueppke, 1991b), thus circumventing errors possibly introduced by differing recovery rates during seed extraction. Optimized inducer concentrations, 2 µl McCall extract ml⁻¹ culture and 0.5 µl Peking extract ml⁻¹ culture were employed in subsequent experiments. Proteins were detected by SDS-PAGE as described above.

Immunological analysis. Proteins for use as antigens were prepared essentially as described above from large-scale liquid cultures of USDA257 that had been grown for 3 d in the presence of 1 µM genistein. The proteins were fractionated by preparative SDS-PAGE (Laemmli, 1970) and stained briefly with Coomassie blue. Gel slices containing SR3 and SR5 were excised and washed extensively with 100 mM Tris/HCl, pH 8.0. Proteins from the gel slices were recovered by extraction with 100 mM Tris/HCl, pH 8.0, containing 1% SDS, 0.1 mM EDTA, and 150 mM NaCl. They were collected by acetone precipitation, dried briefly *in vacuo*, and dissolved in 50 mM Tris/HCl, pH 8.0, containing 0.5% SDS. Antibodies were raised in female New Zealand albino rabbits as described previously (Krishnan & Okita, 1986).

Nodules were collected from cowpea (cv. Pink Eye Purple Hull; seed from Hastings Seed Co.) and soybean (cv. Davis; seed from Missouri Foundation Seeds) plants 20 d after inoculation with *R. fredii* USDA257. Frozen nodules (5 g from each legume)

were ground to a fine powder under liquid nitrogen with a pestle and mortar. The powder was extracted with 20 ml grinding buffer, which consisted of 50 mM Tris/HCl, pH 7.5, containing 5 mM MgCl₂, 1 mM DTT, 200 mM NaCl, 1 mM EDTA, 1% SDS, and 0.01% PMSF. The slurry was centrifuged at 22000 *g* for 15 min. The supernatant solution was brought to 75% (w/v) saturation with ammonium sulfate and placed on ice for 4 h. The precipitated proteins were recovered by centrifugation as above. The pellet was redissolved in 1 ml 50 mM Tris/HCl, pH 6.8, containing 0.1 mM DTT and 0.1 mM EDTA, and dialysed against 2 l of the same buffer. The dialysed extract was clarified by centrifugation and an equal volume of 2× SDS-sample buffer added.

Nodule proteins were resolved by SDS-PAGE as described above. Protein blot analysis followed the protocols of Burnett (1981). Nitrocellulose membranes were incubated with anti-serum that had been diluted 1:100 in 10 mM Tris/HCl, pH 7.5, containing 150 mM NaCl and 5% (w/v) nonfat dry milk powder, followed by incubation with 1 µCi ¹²⁵I-labelled Protein A. Immunoreactions were visualized after autoradiography with intensifying screens at -80 °C.

SR proteins were also localized immunologically in bacteria. Cells from genistein-induced cultures of strain USDA257 and mutant 257DH4 were harvested by centrifugation as described above, and proteins separated into soluble, membrane, and periplasmic fractions (Krishnan & Pueppke, 1991a). Aliquots of 100 µg protein from each fraction and from culture fluids were then spotted onto nitrocellulose membranes and probed with antibodies (Burnett, 1981).

RESULTS

The appearance of SR proteins depends on both *nodD1* and *nodD2*

We first sought to determine if expression of the SR proteins, like that of inducible *nod* genes (Györgypal *et al.*, 1991; Schlaman *et al.*, 1992), is dependent on *nodD*. *R. fredii* strains contain two copies of this gene, *nodD1* and *nodD2* (Appelbaum *et al.*, 1988). We induced strain USDA191 and mutants 191NodD1⁻, 191NodD2⁻ and 191NodD1⁻NodD2⁻ with genistein and electrophoretically fractionated extracellular proteins. Uninduced cultures of these organisms contained as many as 16 extracellular proteins that could be labelled with ³⁵S-methionine and that were not greatly influenced by genistein treatment (Fig. 1). Many of these bands were not apparent in earlier experiments, where proteins were detected by the less sensitive method of silver-staining (Krishnan & Pueppke, 1993). Parental strain USDA191 responded to genistein by producing the five SR proteins that had been detected previously (Krishnan & Pueppke, 1993), as well as several new protein bands. Although all five SR proteins were evident as major protein bands, SR2 and SR3 were only partially resolved in gels containing ³⁵S-labelled proteins.

The five SR proteins were differentially influenced by inactivation of *nodD1* and *nodD2* (Fig. 1). SR proteins were not inducible in cultures of mutant 191NodD1⁻ or 191NodD1⁻NodD2⁻, indicating that *nodD1* is essential for this process. All five proteins, however, were present at apparently normal levels in fluids from induced cultures of mutant 191NodD2⁻. The effect of elevated copy number of *nodD1* and *nodD2* is shown in Fig. 2. Plasmid

Fig. 1. Production of SR proteins is dependent on the alleles of *nodD*. Lanes labelled '+' and '-' contain ³⁵S-labelled proteins from genistein-induced and control cells, respectively, of each strain. Proteins were detected by fluorography.

Fig. 2. SR proteins are regulated by the copy number of *nodD2*. Lanes labelled '+' and '-' contain ³⁵S-labelled proteins from genistein-induced and control cells, respectively, of each strain. Proteins were detected by fluorography. pHBK320 and pHBK330 contain *nodD1* and *nodD2*, respectively.

pHBK320, which contains *nodD1*, had no impact on SR protein production by strain USDA191 – SR proteins were absent from uninduced cultures and inducible by genistein. In marked contrast to this observation, *nodD2*-containing plasmid pHBK330 released SR protein pro-

Fig. 3. Seed extracts of soybean cultivars McCall (M) and Peking (P) stimulate production of SR proteins. ^{35}S -labelled proteins from uninduced cells of *R. fredii* USDA257 (C) and from cells induced with 1 μM genistein (G) were included as negative and positive controls, respectively. Proteins were detected by fluorography.

duction from its dependence on the flavonoid signal, so that the proteins were constitutive.

Soybean seed extracts are efficient inducers of SR proteins

We selected *R. fredii* USDA257 and soybean cultivars Peking and McCall to determine if host signals can substitute for purified genistein as inducers of SR proteins. USDA257 produces nitrogen-fixing nodules on soybean cultivar Peking, but not on cultivar McCall (Heron & Pueppke, 1984; Balatti & Pueppke, 1992). The system thus allowed us to monitor inducer activity from a host species and simultaneously determine if such activity is related to cultivar specificity. Cultures were induced with standardized amounts of seed extract (see Methods) and ^{35}S -labelled extracellular proteins fractionated by SDS-PAGE. At concentrations optimal for induction of a *nodC-lacZ* gene fusion, signals in McCall and Peking seed extracts also triggered the accumulation of SR1, SR2, SR3 and SR5 in cultures of strain USDA257 (Fig. 3). SR4 was not detectable, and the levels of SR5 appeared to be relatively low compared to those in genistein treatments. Although this may indicate that the seed extracts are differentially effective as inducers, the relative intensities of bands may fluctuate from experiment to experiment (cf. Fig. 1, 2, and 3; Krishnan & Pueppke, 1993).

Production of SR proteins depends on *noIXWBTUV*

Although we detected no cultivar specificity in inducer activities in McCall and Peking seed extracts, we thought it important to consider a related question: does *noIXWBTUV*, a gene cluster that negatively regulates

Fig. 4. Production of ^{35}S -labelled SR proteins is dependent on *noIXWBTUV*. Strain USDA257 is included as the positive control and the remaining lanes contain proteins from individual mutants harbouring mini- μ insertions in *noIB*, *noIT*, *noIU*, *noIV*, *noIW* and *noIX* as indicated. Dots mark the positions of marker proteins of 97.4, 66.2, 45.0, 31.0, 21.5 and 14.4 kDa. Proteins were detected by fluorography.

Fig. 5. *noIU*-negative mutant 257DH4 does not produce SR3 and SR5. Proteins were separated into soluble (Sol), membrane (Mem), periplasmic (Per), and extracellular (Ext) fractions and spotted onto nitrocellulose membranes. Lanes labelled '+' and '-' contain proteins from flavonoid-induced and control cells, respectively, of strain USDA257 or mutant 257DH4. The blots were probed with antibodies against SR3 (left panel) or SR5 (right panel) and protein-antibody conjugates detected by autoradiography after treatment with ^{125}I -labelled Protein A.

cultivar specificity of strain USDA257 for McCall soybean, influence SR proteins? Curiously, inactivation of *noIU*, *noIV*, *noIW* or *noIX* greatly depressed levels of all extracellular proteins, both in the presence and absence of genistein (Fig. 4). SR proteins were not detectable in these

Fig. 6. Nine of ten *R. fredii* strains produce SR proteins upon induction with genistein. Lanes labelled '+' and '-' contain ^{35}S -labelled proteins from flavonoid-induced and control cells, respectively. Proteins were detected by fluorography.

mutants. Although inactivation of *noI*B or *noI*T did not obviously influence overall levels of extracellular proteins, these mutants specifically failed to elaborate the five SR proteins upon induction with 1 μM genistein. Secretion of SR proteins by mutant 257DH4, which contains the Tn5 insertion that originally defined the *noI*XWBTUV locus (Heron *et al.*, 1989), was also noninducible by genistein (data not shown), confirming that the lack of response cannot be attributed to nonspecific perturbations conditioned by the presence of the mini-mu element in the genome.

We employed mutant 257DH4 to determine whether the *noI*XWBTUV locus influences synthesis of the SR proteins or just their export from bacterial cells. Cultures were induced with genistein and bacterial proteins separated into soluble, membrane, periplasmic and extracellular fractions. These were spotted onto nitrocellulose membranes and probed with antibodies against SR3 and SR5. Fig. 5 shows that culture fluids of the mutant lack these two SR proteins, and it additionally confirms that bacterial cells are devoid of these proteins. Thus, disruption of *noI*U, at least, abolishes the production of SR3 and SR5.

SR proteins are characteristic of *R. fredii*

Protein export in response to flavonoids was established previously for *R. fredii* USDA193, USDA205 and USDA257 (Krishnan & Pueppke, 1993). The data shown in Fig. 6 confirm that this phenomenon is, with the notable exception of USDA192, generalized in this symbiotic species. Nine of the ten *R. fredii* strains tested appeared to export all five of the originally described SR proteins in response to the inducer. SR1, SR4 and SR5 are clearly resolved, but SR2 and SR3 again are difficult to discern from other bands in these gels. In each case, there were subtleties in the response as revealed by the sensitive fluorography technique. This is particularly true in the gel region containing proteins of > 30 kDa, where poorly resolved bands other than those corresponding to the standard set of five SR proteins are visible.

Fig. 7. Mature soybean and cowpea nodules lack SR3 and SR5. Gels were loaded with proteins from uninduced cultures of strain USDA257 (lane 1), strain USDA257 induced with 1 μM genistein (lane 2), extracts of 20-d-old cowpea nodules (lane 3), extracts of 20-d-old Davis soybean nodules (lane 4) and root tissue from nodulated Davis soybean plants (lane 5). The immunoblots were exposed to antisera against SR3 and SR5 and protein-antibody conjugates detected by autoradiography after treatment with ^{125}I -labelled Protein A.

SR proteins are not present in nodules

We used immunological methods to investigate whether two of the proteins, SR3 and SR5, are detectable *in planta*. As expected, antibodies directed against these proteins failed to react with proteins from uninduced bacterial cultures (Fig. 7). Antibodies against SR3 reacted strongly with a 36 kDa protein from induced cultures, but they also cross-reacted with two smaller and one larger protein. Antibodies against SR5 were specific, detecting only an 8.9 kDa protein from genistein-treated cultures (Fig. 7). Immunoblot experiments with these antibodies confirmed that although SR3 and SR5 are readily detectable components of induced bacterial cultures, neither is detectable in mature nodules of cowpea and Davis soybean, or for that matter, control soybean roots (Fig. 7).

DISCUSSION

Compelling evidence from several model systems now underscores the key role of flavonoids as signals in the legume-*Rhizobium* symbiosis (Phillips, 1992). The regulatory gene *nodD* is indispensable in this process. NodD protein binds to *nod* box promoter sequences in the absence of flavonoids, but transcription of downstream *nod/nol* genes can proceed only upon exposure to signal compounds. Many of these flavonoid-induced genes have important roles in the biosynthesis of return signals, the Nod factors, but others do not participate in this process (Dénarié *et al.*, 1992; Spaink, 1994). Only one such *nodD*-dependent gene, *nodO*, is known to encode an extracellular protein (deMaagd *et al.*, 1988, 1989a, 1989b; Economou *et al.*, 1990). This protein is produced by 20 of 29 strains of *Rhizobium leguminosarum* bv. *viciae* that have been examined, but not by other *Rhizobium* spp. (deMaagd *et al.*, 1989a). *nodO*-negative mutants nodulate normally, but in a *nodE*-negative genetic background, which itself conditions reduced nodulation, disruption of *nodO* leads to further decreases in nodulation (Economou *et al.*, 1990, 1994; Downie & Surin, 1990). *nodO* from *R. leguminosarum* bv. *viciae* can also broaden the host range of *R. leguminosarum* bv. *trifolii*, but again only in a *nodE*-negative background (Economou *et al.*, 1994). Neither the symbiotic function of *nodO* nor the basis for its interaction with *nodE* is understood, but NodO protein does create ion channels in membranes and thus probably has some role in mediating communication with the host (Sutton *et al.*, 1994).

Our observations confirm that *R. fredii*, too, elaborates extracellular proteins in a *nodD*-dependent manner. As is the case with *R. leguminosarum* bv. *viciae*, some strains lack this property, and the function of the proteins remains unclear. Nevertheless, there are several fundamental distinctions between the two systems. *R. leguminosarum* bv. *viciae* contains a single copy of *nodD*, and the appearance of NodO is absolutely dependent on it (deMaagd *et al.*, 1988). *R. fredii*, in contrast, possesses two copies of this gene, *nodD1* and *nodD2*. Although these alleles encode polypeptides that are nearly 70% homologous to one another (Appelbaum *et al.*, 1988), *nodD1* appears to be of greater symbiotic significance than *nodD2*. Inactivation of *nodD1* conditions severe nodulation defects on soybean, but *nodD2* mutants yield normal nodule numbers. Nevertheless, nodulation rate is delayed, and nodulin levels, plant cell size and bacteroid densities are all reduced in nodules containing the *nodD2* mutant (Appelbaum *et al.*, 1988). *nodD2* is also functional *ex planta* – extra, plasmid-borne copies of this gene cause colonies to become nonmucoid, apparently by inhibiting synthesis of extracellular polysaccharides (Appelbaum *et al.*, 1988).

The impact of *nodD1* and *nodD2* on SR proteins varies somewhat from that on nodulation and colony morphology: *nodD1* exerts an overriding plus/minus influence on appearance of the proteins, but the dosage of *nodD2* also affects the process, rendering the proteins constitutive when copy number is elevated. These observations are consistent with the view that signal perception

by *nodD1* and *nodD2* of *R. fredii* leads to an array of responses, one of which is the appearance of new proteins in the cellular milieu. More so than the observations of Appelbaum *et al.* (1988), our results underscore the probable involvement of *nodD2* as a modulator of symbiotic responses. The functional basis for these effects is not known, but there is potential for competitive and cooperative interactions between different NodD proteins at the *nod* box (Györgypal *et al.*, 1991), and these are likely to be of great significance in fine tuning of the nodulation process.

Cultivar specificity is a second fundamental distinction between the *R. leguminosarum* bv. *viciae* and the *R. fredii* systems. If any of six genes known to negatively regulate cultivar specificity in *R. fredii* USDA257 is inactivated, all five SR proteins fail to appear in cultures. The predicted molecular masses of the products of the *nolXWBTUV* locus do not correspond to those of the SR proteins, and thus it is unlikely that the cultivar specificity genes themselves encode the extracellular proteins. Mutations in *nolB* and *nolT* seem to specifically influence the SR proteins. In *nolU*-, *nolV*-, *nolW*- and *NolX*-negative mutants however, accumulation of all secreted proteins is severely attenuated, so the effect seems to be nonspecific. Nevertheless, SR3 and SR5 are absent in cells of a *nolU*-negative mutant, and thus this gene appears to both generally depress protein export and specifically prevent production of SR proteins.

Collectively, our data reveal greater complexity in the SR protein response than was previously suspected. For example, the sensitive fluorographic method used here detects additional proteins that are genistein-inducible and others whose levels are elevated following treatment with the flavonoid. Although these proteins were not visible in the silver-stained gels that were examined previously (Krishnan & Pueppke, 1993), they should probably be considered as part of the SR protein response. In addition, we have discovered that the appearance of the SR proteins is influenced to varying extents by *nodD1*, *nodD2* and *nolXWBTUV*, each of which is a known regulator of symbiotic processes (Appelbaum *et al.*, 1988; Heron *et al.*, 1989; Meinhardt *et al.*, 1993; Kovács *et al.*, 1995). Signals from soybean seeds, which are also regulators of symbiosis (Kosslak *et al.*, 1987; Cho & Harper, 1991; Graham, 1991), can trigger the appearance of SR proteins, but they appear to be absent from mature nodules, which would also be expected to contain such flavonoid signals. These observations imply that induction of SR proteins is a finely tuned process that is related to signal-response circuits that function in symbiosis. The next step will be to purify nondenatured proteins, so that the fundamental question of function can be addressed directly.

ACKNOWLEDGEMENTS

This work was supported by Competitive Research Grant no. 92-03037 from the United States Department of Agriculture, National Research Initiative. This is Journal Series no. 12313 of the Missouri Agricultural Experiment Station.

REFERENCES

- Appelbaum, E. R., Thompson, D. V., Idler, K. & Chartrain, N. (1988). *Rhizobium japonicum* USDA 191 has two *nodD* genes that differ in primary structure and function. *J Bacteriol* **170**, 12–20.
- Balatti, P. A. & Pueppke, S. G. (1992). Identification of North American soybean lines that form nitrogen-fixing nodules with *Rhizobium fredii* USDA257. *Can J Plant Sci* **72**, 49–55.
- Bec-Ferté, M.-P., Krishnan, H. B., Promé, D., Savagnac, A., Pueppke, S. G. & Promé, J.-C. (1994). Structures of nodulation factors from the nitrogen-fixing soybean symbiont *Rhizobium fredii* USDA257. *Biochemistry* **33**, 11782–11788.
- Bonner, W. M. & Laskey, R. A. (1974). A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. *Eur J Biochem* **46**, 83–88.
- Boundy-Mills, K. L., Kossak, R. M., Tully, R. E., Pueppke, S. G., Lohrke, S. & Sadowsky, M. J. (1994). Induction of the *Rhizobium fredii* *nod* box-independent nodulation gene *nolJ* requires a functional *nodD1* gene. *Mol Plant-Microbe Interact* **7**, 305–308.
- Burnett, W. N. (1981). Western blotting. Electrophoretic transfer of proteins from SDS-polyacrylamide gels to unmodified nitrocellulose and radiograph detection with antibody and radioiodinated protein A. *Anal Biochem* **112**, 195–203.
- Cho, M.-J. & Harper, J. E. (1991). Effect of inoculation and nitrogen fixation on isoflavonoid concentration in wild-type and nodulation-mutant soybean roots. *Plant Physiol* **95**, 435–442.
- Dénarié, J., Debellé, F. & Rosenberg, C. (1992). Signalling and host range variation in nodulation. *Annu Rev Microbiol* **46**, 497–531.
- Downie, J. A. & Surin, B. P. (1990). Either of two *nod* gene loci can complement the nodulation defect of a *nod* deletion mutant of *Rhizobium leguminosarum* bv. *viciae*. *Mol & Gen Genet* **222**, 81–86.
- Economou, A., Hamilton, W. D. O., Johnston, A. W. B. & Downie, J. A. (1990). The *Rhizobium* nodulation gene *nodO* encodes a Ca²⁺-binding protein that is exported without N-terminal cleavage and is homologous to haemolysin and related proteins. *EMBO J* **9**, 349–354.
- Economou, A., Davies, A. E., Johnston, A. W. B. & Downie, J. A. (1994). The *Rhizobium leguminosarum* biovar *viciae* *nodO* gene can enable a *nodE* mutant of *Rhizobium leguminosarum* biovar *trifolii* to nodulate vetch. *Microbiology* **140**, 2341–2347.
- Fisher, R. F. & Long, S. R. (1992). *Rhizobium*-plant signal exchange. *Nature* **357**, 655–660.
- Graham, T. L. (1991). Flavonoid and isoflavonoid distribution in developing soybean seedling tissues and in seed and root exudates. *Plant Physiol* **95**, 594–603.
- Györgypal, Z., Kiss, G. B. & Kondorosi, A. (1991). Transduction of plant signal molecules by the *Rhizobium* NodD proteins. *BioEssays* **13**, 575–581.
- Heron, D. S. & Pueppke, S. G. (1984). Mode of infection, nodulation specificity, and indigenous plasmids of 11 fast-growing *Rhizobium japonicum* strains. *J Bacteriol* **160**, 1061–1066.
- Heron, D. S., Érsek, T., Krishnan, H. B. & Pueppke, S. G. (1989). Nodulation mutants of *Rhizobium fredii* USDA257. *Mol Plant-Microbe Interact* **2**, 4–10.
- Keen, N. T., Tamaki, S., Kobayashi, G. & Trolling, D. (1988). Improved broad-host-range plasmids for DNA cloning in Gram-negative bacteria. *Gene* **70**, 190–197.
- Keyser, H. H. & Griffin, R. F. (1987). *Beltsville Rhizobium Culture Collection Catalogue*. Beltsville: Agricultural Research Service.
- Keyser, H. H., Bohlool, B. B., Hu, T. S. & Weber, D. F. (1982). Fast-growing rhizobia isolated from root nodules of soybean. *Science* **215**, 1631–1632.
- Kossak, R. M., Bookland, R., Barkei, J., Paaren, H. E. & 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.
- Kovács, L. G., Balatti, P. A., Krishnan, H. B. & Pueppke, S. G. (1995). Transcriptional organization and expression of *nolXWBTUV*, a locus that regulates cultivar-specific nodulation of soybean by *Rhizobium fredii* USDA257. *Mol Microbiol* in press.
- Krishnan, H. B. & Okita, T. W. (1986). Structural relationships among rice glutelin polypeptides. *Plant Physiol* **81**, 748–753.
- Krishnan, H. B. & Pueppke, S. G. (1991a). *nolC*, a *Rhizobium fredii* gene involved in cultivar-specific nodulation of soybean, shares homology with a heat-shock gene. *Mol Microbiol* **5**, 737–745.
- Krishnan, H. B. & Pueppke, S. G. (1991b). Sequence and analysis of the *nodABC* region of *Rhizobium fredii* USDA257, a nitrogen-fixing symbiont of soybean and other legumes. *Mol Plant-Microbe Interact* **4**, 512–520.
- Krishnan, H. B. & Pueppke, S. G. (1993). Flavonoid inducers of nodulation genes stimulate *Rhizobium fredii* USDA257 to export proteins into the environment. *Mol Plant-Microbe Interact* **6**, 107–113.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- deMaagd, R. A., Wijffelman, C. A., Pees, E. & Lugtenberg, B. J. J. (1988). Detection and subcellular localization of two sym plasmid-dependent proteins of *Rhizobium leguminosarum* biovar *viciae*. *J Bacteriol* **170**, 4424–4427.
- deMaagd, R. A., Spaik, H. P., Pees, E., Mulders, I. H. M., Wijffels, A., Wijffelman, C. A., Okker, R. J. H. & Lugtenberg, B. J. J. (1989a). Localization and symbiotic function of a region of the *Rhizobium leguminosarum* sym plasmid pRL1JI responsible for a secreted, flavonoid-inducible 50-kilodalton protein. *J Bacteriol* **171**, 1151–1157.
- deMaagd, R. A., Wijffels, A. H. M., Spaik, H. P., Ruiz-Sainz, J. E., Wijffelman, C. A., Okker, R. J. H. & Lugtenberg, B. J. J. (1989b). *nodO*, a new *nod* gene of the *Rhizobium leguminosarum* biovar *viciae* sym plasmid pRL1JI, encodes a secreted protein. *J Bacteriol* **171**, 6764–6770.
- Meinhardt, L. W., Krishnan, H. B., Balatti, P. A. & Pueppke, S. G. (1993). Molecular cloning and characterization of a sym plasmid locus that regulates cultivar-specific nodulation of soybean by *Rhizobium fredii* USDA257. *Mol Microbiol* **9**, 17–29.
- Phillips, D. A. (1992). Flavonoids: plant signals to soil microbes. *Recent Adv Phytochem* **26**, 201–231.
- Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **74**, 5463–5467.
- Schlaman, H. R. M., Okker, R. J. H. & Lugtenberg, B. J. J. (1992). Regulation of nodulation gene expression by NodD in rhizobia. *J Bacteriol* **174**, 5177–5182.
- Spaik, H. P. (1994). The molecular basis of the host specificity of the *Rhizobium* bacteria. *Antonie Leeuwenhoek* **65**, 81–98.
- Sutton, J. M., Lea, E. J. A. & Downie, J. A. (1994). The nodulation-signaling protein NodO from *Rhizobium leguminosarum* biovar *viciae* forms ion channels in membranes. *Proc Natl Acad Sci USA* **91**, 9990–9994.

Received 23 February 1995; revised 11 May 1995; accepted 23 May 1995.