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Bradyrhizobium japonicum Rhizobitoxine Mutants with Altered Host-Range on *Rj4* Soybeans

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Genotype-specific nodulation (GSN) of *Rj4* soybean is defined by limited nodulation with *Bradyrhizobium japonicum* strain USDA 61, but normal nodulation with other strains, e.g., USDA 110. Strain USDA 61 is a rhizobitoxine-producing *Bradyrhizobium* for which we had previously isolated rhizobitoxine null and *in planta* overproducing strains. The null mutant nodulated *Rj4* plants at a rate similar to strain USDA 61. Surprisingly, the *in planta* overproducing mutants nodulated *Rj4* plants at a rate similar to nonrestricted strains of *Bradyrhizobium*. These results suggested that rhizobitoxine was involved in the ability of USDA 61 derivatives to overcome the *Rj4* nodulation restriction. Rhizobitoxine is an inhibitor of ethylene biosynthesis, and ethylene has been shown to be detrimental to nodulation. Therefore, experiments were performed to test whether ethylene was involved in mediating the GSN response of *Rj4* soybeans either directly or indirectly via the induction of a plant defense response. The data indicate that *Rj4* soybeans do not respond to USDA 61 by induction of ethylene, chalcone synthase, or extensin, which would be indicative of a defenselike response. Moreover, the exogenous addition of inhibitors of ethylene biosynthesis did not restore nodulation to USDA 61 inoculated *Rj4* soybeans. Therefore, ethylene does not appear to be the primary mediator of GSN in *Rj4* soybean. Because nodulation host range has been shown to be affected by the structure of Nod factors produced by rhizobia, we analyzed by thin-layer chromatography the Nod factors produced by strain USDA 61 and rhizobitoxine-mutant derivatives. In those mutants that nodulate *Rj4* plants, minor Nod factors appeared different and the distribution of major Nod factors was shifted towards a compound that comigrates with the primary compound produced by the unrestricted strain, USDA 110. This suggests that mutations leading to overproduction of rhizobitoxine in USDA 61 have the pleiotropic effect of modifying the profile of Nod metabolites produced. This latter effect may explain the nodulation of *Rj4* soybean by USDA 61 derivatives.

Additional keywords: nitrogen fixation, symbiosis, toxins.

Plant-microbe interactions are often defined by their host range, and a great deal of effort is being put forth to understand the mechanisms of host range in both mutualistic and parasitic interactions. In the *(Brady)rhizobium*-legume symbiosis, a given species of rhizobia is limited in the species of legumes that it can nodulate. There are also examples of host-range limitations within a plant species that are referred to as genotype-specific nodulation (GSN). In GSN, single dominant genes in the plant limit nodulation by specific strains of rhizobia, while allowing normal nodulation with other strains. For example, in *Bradyrhizobium*-soybean symbiosis, three GSNs have been described, *Rj2* (Caldwell 1966), *Rj3* (Vest 1970), and *Rj4* (Vest and Caldwell 1972). The soybean *Rj2* gene restricts or limits nodulation by strain USDA 7, and the *Rj3* gene limits nodulation by strain USDA 33. Unfortunately, the *Rj3* GSN restricted strain has been lost (Devine and O'Neill 1989). The initial characterization of *Rj4* GSN demonstrated ineffective nodulation by strain USDA 61 (Vest and Caldwell 1972), but the number and diversity of strains restricted by *Rj4* has been expanded to strains related to USDA 61 (Devine *et al.* 1990), and to unrelated strains that are members of the USDA 123 serogroup (Sadowsky and Cregan 1992).

In addition to the *Rj2–4* genes in soybean, Cregan and Keyser (1986) identified a single gene in plant introduction lines of soybean that restricted nodulation by certain members of the USDA 123 serogroup of *B. japonicum*. Sadowsky *et al.* (1991) subsequently showed that a single bacterial gene, *nolA,* allowed productive nodulation even in the presence of the restrictive plant gene. Other rhizobial GSN genes have been identified. For example, the first report of such a gene was in *Rhizobium leguminosarum* bv. *viciae* strain TOM where a single gene, *nodX,* was identified that allowed this strain to nodulate Afghanistan pea (Davis *et al.* 1988; Gotz *et al.* 1985; Hombrecher *et al.* 1984; Lie 1978). Genetic crosses between European pea and Afghanistan pea revealed that nodulation restriction of *R. l.* bv. *viciae* strains was determined by a single recessive plant gene (Holl 1975). In another case, Pueppke and colleagues found that *R. fredii* strain USDA 257 nodulates soybean cultivar Peking normally but cannot nodulate cultivar McCall (Heron and Pueppke 1984, 1987). The plant genetic character found in cultivar McCall that controls nodulation restriction has not been defined as dominant or recessive. Heron *et al.* (1989) isolated five mutants of strain USDA 257 in which the host range was extended to include cultivar McCall. These mutants are interesting in that they appear to identify single bacterial GSN genes that act dominantly to restrict nodulation of specific genotypes of soybean.

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Genotype-specific interactions are also evident in plant pathogenesis. Many plant-pathogen interactions are governed by the gene-for-gene hypothesis. In these interactions, dominant resistance genes in the plant are only effective in stopping infection if the invading pathogen expresses complementary avirulence genes (Keen 1990). In the absence of the host resistance gene or the microbial avirulence gene, infection and disease symptoms proceed. Plant recognition of the pathogen avirulence gene product results in a hypersensitive (HR) plant defense response which contains the infection. Some of the components of the HR defense response are the differential induction of ethylene biosynthesis (Reinhardt *et al.* 1991), and differential accumulation of phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS) (Bell *et al.* 1986), and extensin RNA (Roby *et al.* 1985). Whereas there may be several pathways to induce PAL, CHS, and extensin expression, these genes are all induced by ethylene (Ecker and Davis 1987).

An unexplored area of GSN in *(Brady)rhizobium*-legume symbiosis is the plant response to inoculation with restricted strains. A defenselike response by the plant might lead to limitations in nodule formation. In some special cases, defenselike responses by the plant in *(Brady)rhizobium*-legume symbiosis have been identified. For example, Werner *et al.* (1985) showed that PAL and CHS mRNA as well as the phytoalexin glyceollin, accumulated to greater amounts in ineffective soybean nodules. More recently, Estabrook and Sengupta-Gopalan (1991) reported an increase in PAL and CHS mRNA expression upon soybean inoculation. This work is also consistent with the earlier report of the increased synthesis of flavonoids by vetch upon infection with *Rhizobium* (Van Brussel *et al.* 1990).

Recently, the key signal molecules involved in the induction of early nodulation responses in legumes were identified. These signal molecules or Nod factors are substituted lipo-oligosaccharides of *N*-acetylglucosamine (Lerouge *et al.* 1990). The addition of these compounds at very low levels (e.g., 10^{-11} M) induces root hair curling and cortical cell divisions on the appropriate legume host (Truchet *et al.* 1991). Nod factors isolated from *R. l.* bv. *viciae* can induce abnormal thick short roots on *Vicia saliva* subsp. *nigra* (L.) Ehrh. (Spaink *et al.* 1991). Interestingly, normal root growth and nodulation pattern can be restored to these treated plants by the use of an ethylene biosynthesis inhibitor (Zaat *et al.* 1989). Therefore, at least in this case, there appears to be a connection between *Rhizobium* signaling to the plant host and the induction of ethylene production. Another example of the effect of ethylene on nodulation is shown by the poorly nodulating *sym5* mutant of pea. This mutant exhibits normal root growth but requires the presence of ethylene biosynthesis inhibitors or antagonists to restore normal nodulation (Fearn and LaRue 1991). In this mutant, nodule formation arrests prior to cortical cell divisions (Guinel and LaRue 1991). This illustrates that nodulation can be particularly sensitive to ethylene and this effect could play a role in GSN. Therefore, the negative effects of ethylene on nodulation could be due either to the induction of defense-related genes or to the inhibition of DNA replication and cell division (Apelbaum and Burg 1972).

With regard to the role of ethylene in plant-microbial symbiosis, it is interesting to note that two plant symbionts synthesize a phytotoxin, rhizobitoxine, which inhibits the formation of ethylene (Mitchell and Frey 1988; Owens *et al.* 1971; Owens and Wright 1965). One of these symbionts, *Bradyrhizobium japonicum*, has both mutualistic and parasitic characteristics (Ruan and Peters 1992), whereas the second, *Pseudomonas andropogonis* (Smith) Stapp, is a parasitic symbiont (Mitchell *et al.* 1986). The role for rhizobitoxine in bradyrhizobial symbiosis is not clear, but as outlined above, the ability to inhibit ethylene biosynthesis may be beneficial in some symbiotic interactions. We have recently isolated rhizobitoxine mutants of *Bradyrhizobium* allowing the direct investigation of the role of rhizobitoxine in symbiosis. One group of mutants fails to make toxin in culture and *in planta*, whereas a second group fails to make toxin in culture but does make toxin *in planta* (Ruan and Peters 1992). Specifically, in this paper we explore the role of rhizobitoxine in mediating a GSN interaction with *Rj4* soybean.

MATERIALS AND METHODS

Bacterial strains and cultures. Bacterial strains used in this study are listed in Table 1. *B. japonicum* was maintained on and cultured in YEMG medium (5.0 g of mannitol, 5.0 g of sodium gluconate, 0.5 g of yeast extract, 0.5 g of K_2HPO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$, and 0.1 g of NaCl per liter at pH 6.8) supplemented with 1% Casamino Acids at 28–30° C. Kanamycin was added to the media at 150 μg/ml. Inocula were prepared by diluting log phase cultures (OD_{600} = 0.3–0.6) to an appropriate density with sterile water. Cell densities were confirmed by viable cell count.

Nodulation assays. Soybean (*Glycine max* L.) cultivars used in this study were Williams(*rj4*) and the near isogenic genetic lines BARC-2(*Rj4*) and IBARC-3(*rj4*) developed by Devine and O'Neill (1986). Seeds were surface sterilized by treatment with 20% sodium hypochlorite for 20 min followed by 1 min in 70% ethanol and washed thoroughly in sterile water. Seeds were then imbibed and allowed to germinate aseptically for 2 days in Magenta boxes (Magenta Co., Chicago) containing 0.6% agar in water. Germinated seeds were transferred to modified Leonard jars constructed from Magenta boxes containing vermiculite or into plastic growth pouches (Northrup King Seed Co., Minneapolis, MN). Plants were inoculated 3–4 days postgermination with

Table 1. Bacterial strains used in this study

Strain	Phenotype[a]	Source or reference
Bradyrhizobium japonicum		
USDA 110	wild type, Rtx^-	Bauer[b]
USDA 61	wild type, Rtx^-	Kuykendal[c]
RX18E	USDA 61 Tn*5* Rtx^-	(Ruan and Peters 1992)
RX21D	USDA 61 Tn*5* Rtx^+	(Ruan and Peters 1992)
RX20B	USDA 61 Tn*5* Rtx^{++}	(Ruan and Peters 1992)
TX17G	USDA 61 Tn*5* Rtx^{+++}	(Ruan and Peters 1992)
RX19E	USDA 61 Tn*5* Rtx^{+++}	(Ruan and Peters 1992)
RX19H	USDA 61 Tn*5* Rtx^{+++}	(Ruan and Peters 1992)

[a] The superscript character denotes rhizobitoxine production in nodules on soybean cultivar Williams, −, no RTX; +, 1–20 μg RTX/g nodule; ++, 20–100 μg RTX/g nodule; +++, greater than 100 μg RTX/g nodule.
[b] Ohio State University.
[c] USDA, Beltsville, MD.

10^6 cells per milliliter for pouch experiments and 10^8 cells per milliliter for Leonard jar experiments. Plants were watered with sterile Jensen's nitrogen-free solution (Vincent 1977) and grown in a plant growth chamber with 400 μE of light during a light/dark cycle of 16/8 hr at 28°/18° C and 70–80% relative humidity. Roots of plants grown in pouches were not specifically shielded from light. Nodules were counted following a time schedule for pouch experiments and at 35 days postinoculation in the Leonard jar experiments.

For nodulation assays including the ethylene biosynthesis inhibitors rhizobitoxine (RTX) and aminoethoxyvinylglycine (AVG; Sigma, St. Louis, MO), plants were grown in pouches as described above. However, RTX and AVG were included in the 1 ml of inoculum at 250 μM. The volume of a wet pouch is approximately 10 ml, so the inhibitors were diluted 10-fold to their desired final concentration of 25 μM.

For experiments using silver or cobalt, plants were grown in modified Leonard jars. Silver sulfate and cobalt nitrate were added at 10 and 100 μM, respectively, 2 days postinoculation. Because of toxicity of these metals, the Leonard jars were flushed twice with distilled water 1 day after treatment. Toxicity studies of silver and cobalt on *Bradyrhizobium* strains USDA 61 and USDA 110 were performed by growing the bacteria in various concentrations of the chemicals in YEMG and determining the cell number after 5 days of growth.

Isolation and purification of RTX. RTX was isolated from the cultures of *Bradyrhizobium* by a modification of the procedure previously described (Owens and Wright 1965). The supernatant from a 1-L stationary-phase culture of *Bradyrhizobium* strain USDA 61 grown in YEMG was filtered through a 0.25-mm filter. The filtrate was passed over a Dowex-50W column (2.5 × 30 cm) with the resin in the NH_4+ form at a flow rate of 2 ml/min. The column was washed with 10 volumes of distilled water and RTX was eluted using a gradient from 0 to 0.1 N NH_4OH at 2 ml/min. To eliminate NH_4OH, 100-μl samples were taken from each fraction, dried under vacuum, and resuspended in 100 μl of sterile water. Fractions were assayed for RTX content using an RTX enzyme assay as previously described (Ruan and Peters 1991). RTX from fresh nodules formed by various *Bradyrhizobium* strains was determined using the procedure described previously (Ruan and Peters 1991). The amount of RTX in a 50-μl sample of culture or nodule extract was determined by comparison to a standard curve of authentic RTX, which was kindly provided by Robin Mitchell, Department of Scientific and Industrial Research, Auckland, New Zealand.

Ethylene assays. Ethylene accumulation was determined for plants inoculated with different strains of *Bradyrhizobium* or treated with ethylene inhibitors. Two-day-old seedlings were inoculated with 10^8 cells per plant or treated with 25 μM AVG or RTX. For ethylene assays, three root tips of 2- to 7-day-old seedlings were cut 2–4 cm in length and placed in a 2-ml reaction vial (Pierce, Rockford, IL) containing 100 μl of water. Nodules of 20-day-old plants were isolated and 15–25 nodules were placed in a 2-ml vial containing 100 μl of water. Vials were sealed with Teflon/silicone disks (Pierce) and kept in the dark for 2 hr at 25° C. Ethylene was sampled by removing 1 ml of gas. Ethylene was measured using a 2-m × 1/8-in. activated alumina 60/80 column in a Varian gas chromatograph 3700 with a flame ionization detector. Amounts of ethylene were estimated by comparison to an ethylene standard.

RNA isolation and northern (RNA) blot analysis. Total RNA was isolated from soybean roots or nodules of plants grown in pouches as described above (Haffner *et al.* 1978). RNA samples (10 μg) were separated by electrophoresis in a 1.2% agarose gel containing formaldehyde, transferred onto nylon filters (Hybond N; Amersham, Arlington Heights, IL), and hybridized with radioactively labeled DNA probe (Lehrach *et al.* 1977; Thomas 1980). Probes used were pCHS1 for a bean chalcone synthase (Ryder *et al.* 1984), and pDC5A1 for a carrot extensin (Chen and Varner 1985). Labeled probe was synthesized using the Random Primer DNA Labeling System (BRL, Gaithersburg, MD) and α-^{32}P-dATP (Amersham). Filters were hybridized in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 26% formamide, 0.5% SDS, 5× Denhardt's, 100 μg/ml of salmon sperm DNA. Filters were washed at 42° C in 2× SSC, 0.5% SDS for 2 hr and at 0.5× SSC, 0.5% SDS for 2 hr. Filters were exposed to X-ray film (Kodak X-Omat AR) with intensifying screens.

Detection of nodulation factors. Nodulation factors were examined using thin-layer chromatography (TLC) essentially as described by Spaink *et al.* (1992). Wild-type and mutant strains were grown on RDY (Bishop *et al.* 1976) in a shaking incubator at 30° C to an OD_{600} of 0.5–0.6. Cells were collected by centrifugation at 12,000 × *g* and washed in a minimal medium (MM) (Barbour *et al.* 1991) containing sodium glutamate as nitrogen source and no additional carbon source. The culture was diluted to an optical density of 0.1 in MM and allowed to grow for 5–6 hr. Fifty microcuries of ^{14}C-acetate (56 mCi/mmol, ICN Radiochemicals) and genistein (to 2 μM) (ICN Biomedicals, Cleveland, OH) were added to 1 ml of each culture. Bacteria were further cultured at 30° C for 16–20 hr. Culture supernatants were extracted with 1/2 volume butanol. Butanol fractions were collected and air dried. Dry residues were resuspended in 15 μl of butanol, and aliquots were applied to octadecyl reverse-phase TLC plates (Sigma, St. Louis, MO). Plates were developed using 50:50 acetonitrile/water as the solvent, as described previously Spaink *et al.* (1992). TLC plates were exposed to X-ray film (Kodak X-Omat AR) for 3 days. Chromatographs were analyzed using a Molecular Dynamics PhosphorImager (Sunnyvale, CA).

Assays for induction of rhizobitoxine synthesis. Wild-type and mutant strains were grown in YEMG in a shaking incubator at 30° C to an OD_{600} of 0.5–0.6. Cultures were then diluted to an OD of 0.1 in YEMG and genistein was added to a final concentration of 2 μM. Cultures were incubated for an additional 48 hr. RTX assays were done in triplicate as described previously (Ruan and Peters 1991).

RESULTS

Plant defense response. Given the similarities between GSN and the gene-for-gene interactions of plants and pathogens, we sought to determine if soybeans responded differ-

entially to restricted and nonrestricted strains of *Bradyrhizobium*. A differential response of *Rj4* soybeans to restricted strain USDA 61 could affect nodulation. To characterize the host response to inoculation, we looked at expression of two defense-inducible genes, CHS and extensin, and ethylene accumulation in root tissue of BARC-2(*Rj4*) plants inoculated with nonrestricted (USDA 110) and restricted (USDA 61) strains of *B. japonicum*. We characterized an initial response by looking at roots soon after inoculation and at a time later in symbiosis by looking at mature nodules.

We measured CHS RNA levels in root RNA using northern blot analysis at 3 and 6 hr after inoculation. These times were chosen because previously CHS had been shown to be induced by plant pathogens at these times (Ryder *et al.* 1984). In root RNA, the CHS probe hybridized to one size message of 1.8 kb, which was slightly more abundant in the root 6 hr after inoculation as compared to 3 hr and control (Fig. 1A). However, there was no difference in the expression of CHS between the restricted and nonrestricted cases. For extensin, the amount of RNA was unchanged at all times examined (Fig. 1B). None of the multiple extensin transcripts hybridizing to the genomic carrot probe were differentially induced upon inoculation. These results for CHS and extensin were independent of inoculum dose because inocula of 10^6, 10^7, and 10^8 cells per milliliter all gave similar results (data not shown).

CHS and extensin mRNA levels were also monitored in 35-day mature nodules. An additional CHS mRNA was observed in nodule RNA that was not present in root RNA. As shown in Figure 1, the CHS probe hybridized to a 3-kb CHS RNA in addition to the 1.8-kb RNA. In nodules formed on BARC-2(*Rj4*) and BARC-3(*rj4*) by USDA 61, the amounts of CHS and extensin messages in restricted (*Rj4*) and nonrestricted (*rj4*) nodules were similar. The presence of two hybridizing CHS RNA species in nodules and only one in roots is in contrast to the findings of Estabrook and Sengupta-Gopalan (1991) who found a single CHS mRNA in nodules of soybean cultivar Prize. This difference may be due to differences in cultivars as has been seen in the organization and expression of leghemoglobin genes of soybean (Brisson *et al.* 1982).

We assayed ethylene from BARC-2(*Rj4*) soybean roots uninoculated and inoculated with USDA 110 or USDA 61. No difference in the generation of ethylene was observed between control roots and roots inoculated with restricted or nonrestricted *Bradyrhizobium* (Table 2). The levels of ethylene generated were similar to those previously found in uninoculated soybean roots (Reinhardt *et al.* 1991).

These data show that *Rj4* soybean roots do not show a defenselike response to inoculation with restricted strains of *Bradyrhizobium*. Ethylene might be involved in *Rj4* GSN without observable differences in ethylene production in whole roots, as is the case with the *sym5* mutation (Fearn and LaRue 1991). We therefore explored this possibility by using *in planta* RTX overproducing mutants of USDA 61.

Rhizobitoxine *in planta* overproducing mutants nodulate *Rj4* plants. RTX is an inhibitor of ethylene biosynthesis (Owens *et al.* 1971). A test of a role for ethylene in *Rj4* GSN might be to use RTX null and *in planta* overproducing

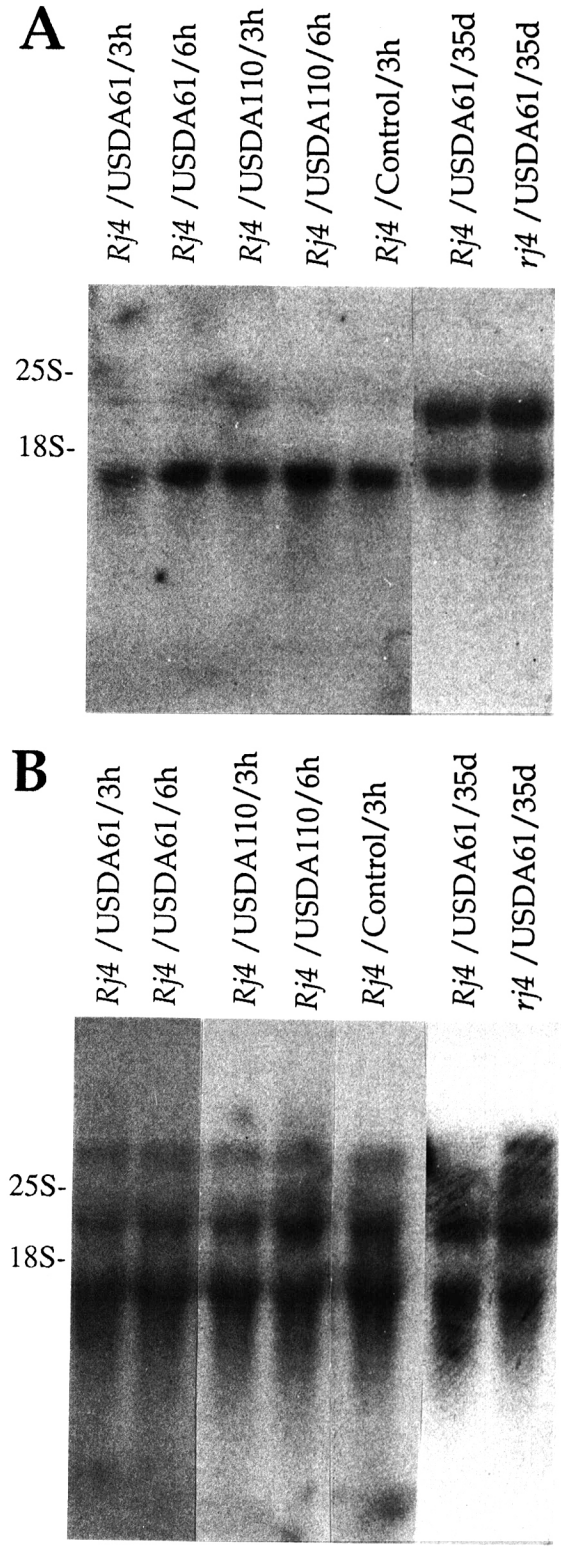

Fig. 1. Northern blot analysis of chalcone synthase and extensin RNA accumulation in soybean roots and nodules. RNA from *Rj4* roots and from *Rj4* and *rj4* nodules (35 days) was probed with, **A**, chalcone synthase and, **B**, extensin.

mutant derivatives of USDA 61 (see Table 1). The overproducing mutants are so named because, while producing no RTX in culture, they produce more RTX *in planta* than the parental strain (Ruan and Peters 1992). To determine if RTX has any effect on the *Rj4* GSN, nodulation assays using these mutants were performed on *Rj4* soybean. On BARC-2(*Rj4*), the *in planta* RTX overproducing mutants RX20B, RX19H, RX19E, and RX17G formed nodules at a rate similar to unrestricted strain USDA 110, while an RTX null mutant, RX18E, a mutant that produces low RTX *in planta*, RX21D, and wild-type USDA 61 formed only a limited number of nodules (Fig. 2). The strains USDA 110, USDA 61, and mutant derivatives of USDA 61 all formed similar numbers of nodules on BARC-3(*rj4*) (data not shown).

These results suggest a positive correlation between the amount of RTX made *in planta* and the ability of USDA 61 derivatives to nodulate *Rj4* soybeans. Because the amount of RTX produced in nodules is known to be cultivar dependent (Erdman *et al.* 1957; Johnson and Means 1960; Ruan and Peters 1992), and the original designation of the *in planta* RTX overproducing phenotype was from studies on soybean cultivar Williams (recessive *rj4*) (Ruan and Peters 1992), the RTX content of the BARC-2(*Rj4*) nodules was determined. The amounts of RTX in BARC-2(*Rj4*) nodules (Table 3) were found to be similar to those previously reported for cultivar Williams (see Table 1 and Ruan and Peters 1992). These values are consistent with the finding that the *in planta* RTX overproducing strains caused chlorosis on BARC-2(*Rj4*) plants, whereas USDA 61, RX21D, and RX18E did not cause chlorosis.

Ethylene was monitored in control roots and roots inoculated with an *in planta* RTX overproducing mutant to determine if ethylene biosynthesis was altered by these mutants at a time when nodule meristem formation would be initiated. These measurements showed no observable decrease in ethylene production at 48 and 120 hr post-inoculation (Table 2). To demonstrate that RTX synthesized by *Bradyrhizobium* does inhibit ethylene accumulation, ethylene measurements were made for 28-day-old nodules formed by wild-type strain USDA 61 and the *in planta* RTX overproducing mutant RX17G. Nodules formed by RX17G produced significantly less ethylene (8 pmol g^{-1} hr^{-1}) than nodules formed by USDA 61 (28 pmol g^{-1} hr^{-1}).

The positive correlation between RTX production *in planta* and the nodulation of *Rj4 soybeans* suggested that RTX is integral to the nodulation of *Rj4* soybeans by USDA 61. The effect of RTX could be due to inhibition of ethylene biosynthesis or due to some unidentified activity of RTX unrelated to ethylene.

Nodule formation in the presence of RTX and other ethylene inhibitors. If the *in planta* overproduction of RTX is responsible for the ability of USDA 61 RTX mutants to nodulate *Rj4* soybean, then addition of RTX should allow wild-type USDA 61 and the RTX null mutant to nodulate *Rj4* soybean. If the importance of RTX is as an inhibitor of ethylene biosynthesis, then other ethylene antagonists should also be effective. Therefore several nodulation assays were performed in the presence of RTX and other ethylene antagonists.

In pouch experiments, addition of RTX or a structural analog, aminoethoxyvinylglycine (AVG), which also inhibits ethylene biosynthesis did not result in greater numbers of nodules produced by restricted or nonrestricted strains (Fig. 3). In addition to the experiments shown here

Table 2. Ethylene generated by soybean roots inoculated with *Bradyrhizobium*[a]

Sample	Hours postinoculation		
	6	48	120
Exp 1			
Control	123	208	ND[b]
USDA 61	114	161	ND
USDA 110	125	179	ND
Exp 2			
Control	ND	119	227
USDA 61	ND	106	188
RX17G	ND	132	243
RX18E	ND	92	237

[a] Values are means of three measurements of three roots each expressed as pmol C_2H_4 g fresh $wt^{-1}h^{-1}$. No significant differences (0.05 confidence level) were observed between the means of the experiments by Welsch's approximate *t*-test.
[b] ND, not determined.

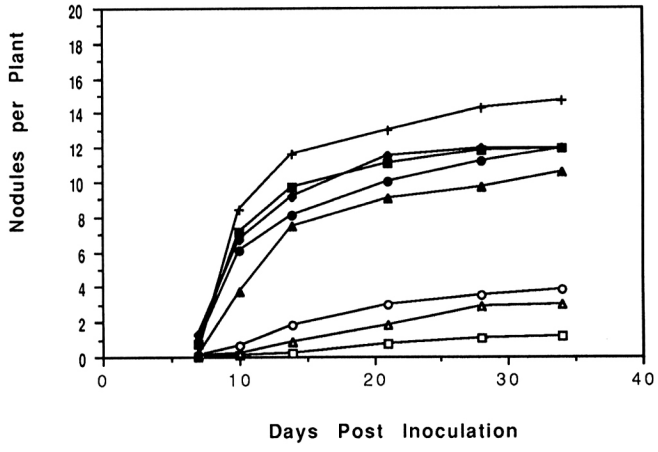

Fig. 2. Nodulation kinetics of different strains of *Bradyrhizobium* on *Rj4* plants. The symbols are for USDA 110 (+), USDA 61 (-○-), RX18E (-□-), RX21D (-△-), RX20B (-■-), RX17G (-●-), RX19H (-●-), and RX19E (-▲-). Statistical analysis of end point data is provided in Table 3.

Table 3. Nodules per plant and rhizobitoxine content of nodules from *Rj4* soybeans

Strain	Nodules/plant[a]	μg RTX/g Nodule[b]
USDA 110	14.7 a	0.2
USDA 61	3.8 b	6.6
RX18E	1.2 c	0.3
RX21D	3.0 b	13.0
RX20B	12.0 ad	75.0
RX17G	11.8 ad	176.0
RX19E	10.5 d	169.0
RX19H	12.0 ad	172.0

[a] Values are the mean of at least 20 plants. Means not followed by common letters are significantly different (0.05 confidence level) by Welsch's approximate *t*-test.
[b] Values are the mean of three measurements of nodules from at least five plants per measurement. The amount of toxin is in proportion to the severity of chlorosis displayed.

using 25 μM of the inhibitors, experiments were done with a range of inhibitor concentrations from 1 to 100 μM; all without any effect on *Rj4* GSN. Addition of RTX or AVG at 100 μM caused chlorosis on host plants. Failure of 25 μM RTX or AVG to alter *Rj4* nodulation is despite inhibition of ethylene production by 90 and 60% of control values at 6 and 48 hr posttreatment, respectively.

In addition to experiments with RTX and AVG, which both inhibit ethylene biosynthesis by inhibiting ACC synthase (Amrhein and Wenker 1979), similar nodulation assays were performed in the presence of two other ethylene antagonists that have different modes of action, cobalt (Lau and Yang 1976) and silver (Beyer 1976). Again, no difference in the number of nodules formed was observed between control samples and treated samples (data not shown).

These results clearly demonstrate that ethylene is not evolved in mediating *Rj4* GSN and, more importantly, that RTX does not directly affect *Rj4* GSN. We therefore examined Nod factors synthesized by USDA 61 and its mutant derivatives.

Nodulation factors. Because of the demonstrated importance of the structure of Nod factors in determining host range in nodulation, the Nod factors produced by USDA 61 and its RTX mutant derivatives were analyzed. Bacterial cultures were induced with genistein, and Nod factors were labeled with ^{14}C-acetate and separated by TLC (Fig. 4). The wild-type strain produced six major and several minor compounds when grown in the presence of genistein, a known nodulation gene inducer of *Bradyrhizobium* (Banfalvi *et al.* 1988; Göttfert *et al.* 1988; Kosslak *et al.* 1987; Smit *et al.* 1992). Major compounds 1 and 2 could not be distinguished during analysis using the PhosphorImager (Figs. 4 and 5). Soybean seed extract induced a similar complement of Nod factors as was induced by genistein (data not shown). The major compound of *Bradyrhizobium* USDA 110 that comigrates with the major

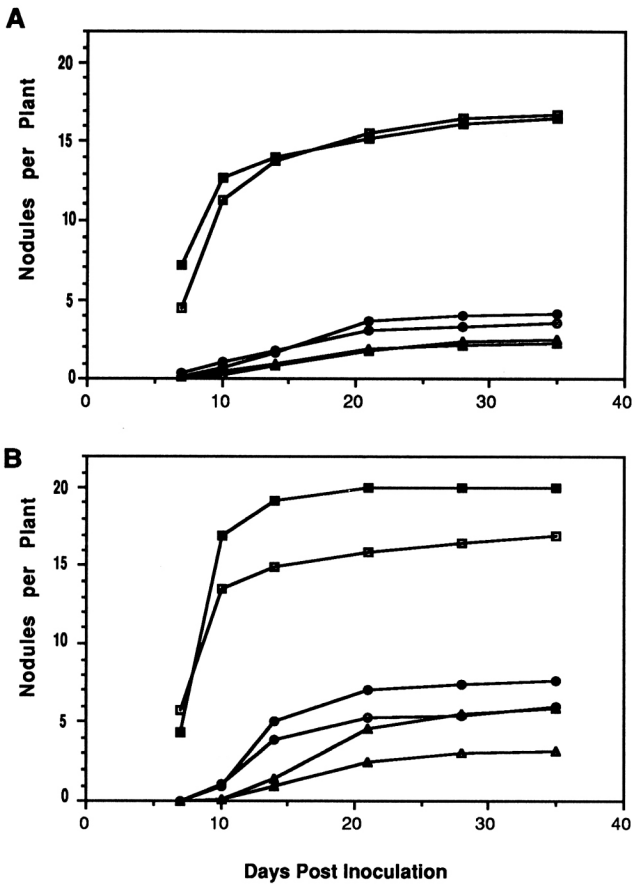

Fig. 3. Nodulation kinetics of different strains on *Rj4* plants in the presence or absence of RTX and AVG. The symbols are the same for **A,** addition of 25 μM RTX and **B,** addition of 25 μM AVG. The symbols are for USDA 110 without (-□-) and with (-■-) inhibitor; USDA 61 without (-○-) and with (-●-) inhibitor; RX18E without (-△-) and with (-▲-) inhibitor. Statistical analysis of nodule number at 35 days using Welsch's approximate *t*-test demonstrated that treatment with RTX or AVG did not significantly (0.05 confidence level) change the mean numbers of nodules formed compared to controls by any strain.

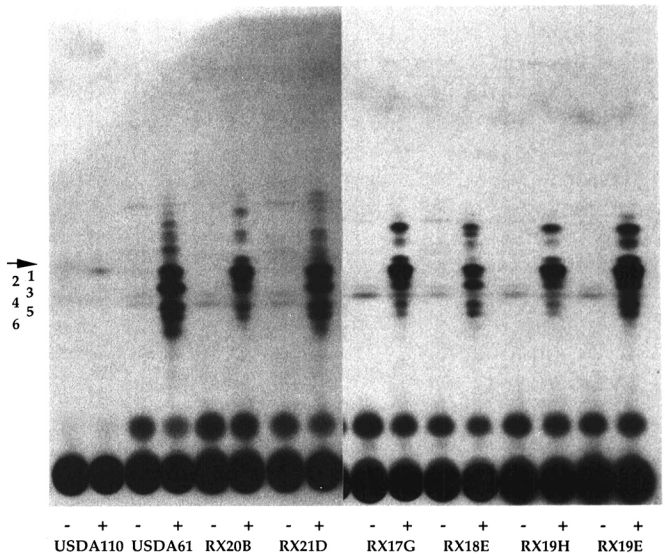

Fig. 4. Thin-layer chromatography of metabolites produced by wild-type USDA 110, USDA 61, and rhizobitoxine mutant derivatives in response to genistein. The samples are butanol extracts of USDA 110, USDA 61, and mutant derivatives cultured in the absence (−) or presence (+) of genistein (2μM).

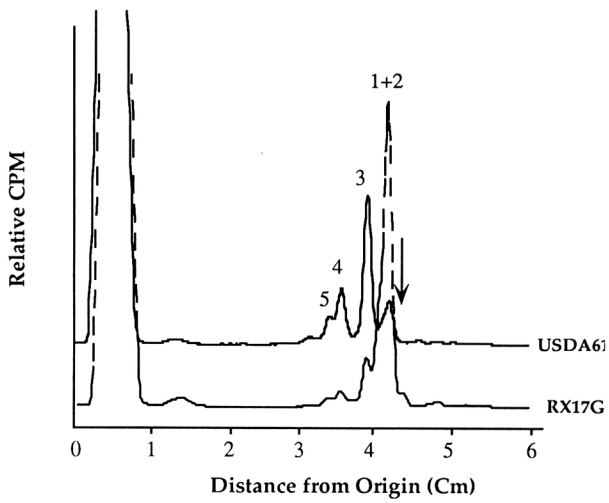

Fig. 5. A PhosphorImage analysis of Nod metabolites produced by strains USDA 61 and RX17G. Labeled peaks correspond to those in Figure 4.

compound of USDA 61 (compound 1, Fig. 4) has recently been purified and found to induce root hair curling on soybean at 10^{11} M (Sanjuan *et al.*, in press). The structure of this compound is similar to that of the Nod factors from *R. meliloti* (Roche *et al.* 1991; Schultze *et al.* 1992), and *R. l.* bv. *viciae* (Spaink *et al.* 1991). Ratios between major Nod factors **1+2, 3, 4,** and **5** were determined for all mutants. The RTX null mutant, RX18E, and the mutant that forms wild-type amounts of RTX *in planta*, RX21D, produced a similar collection of major compounds in the same proportions as wild type in the order of prevalence of $\mathbf{1+2} \leq \mathbf{3} \geq \mathbf{4} > \mathbf{5}$. For the *Rj4* nodulating mutants (RX20B, RX17G, RX19H, and RX19E), the proportions of compounds are significantly altered such that $1 + 2 > 3 > 4 > 5$. The profiles of compounds formed by USDA 61 and an overproducing mutant, as determined by PhosphorImage analysis, is shown in Figure 5. Mutants with similar *Rj4* nodulating phenotypes have profiles similar to the strains represented in Figure 5. USDA 61 and its mutant derivatives display similar growth rates and, therefore, growth stage is not a cause for the differences in Nod factors observed.

With respect to the minor compounds, there are differences between the *in planta* overproducing mutants and the strains that form no or small amounts of RTX *in planta*. An example is the minor compound next to compound 1 (arrow) which is more abundant in the *Rj4*-nodulating mutants than in the *Rj4* nonnodulating strains (Fig. 4). Another minor factor can be observed that is less abundant in the *in planta* overproducing mutants but is abundant in USDA 61 and the other mutant derivatives (Fig. 4).

Influence of nodulation gene induction on RTX production. Because we observed a difference in Nod factor abundance in *in planta* RTX overproducing mutants we tested the effects of genistein on RTX production in culture. These mutants do not produce RTX in culture. Cultures of USDA 110, USDA 61, and mutant derivatives were cultured for 2 days in the presence of 2 μM genistein. Neither USDA 110 nor any of the USDA 61 mutant derivatives formed RTX in the presence of inducer, and genistein did not alter the amount of RTX accumulated by strain USDA 61 (4.5 μM).

DISCUSSION

The occurrence of dominant plant genes that limit bacterial infection is shared in parasitic and mutualistic symbiosis. Our finding that loss of function mutations of USDA 61 extend host range to *Rj4* soybeans is the first demonstration of dominant genes in rhizobia interacting with a dominant host gene to restrict nodulation. This interaction parallels gene-for-gene plant-pathogen interactions in that interactions of dominant genes present in both symbionts result in a restriction or limitation of infection. In both of these types of interactions, absence of either the bacterial or plant dominant gene results in successful infections. In gene-for-gene interactions, a hypersensitive defense response is induced to limit infection.

Our working hypothesis was that GSN of *Rj4* soybean could be explained by the induction of a defenselike response by the plant in response to inoculation by the restricted *B. japonicum* strain USDA 61. This hypothesis was consistent with the data that *in planta* RTX overproducing mutants of strain USDA 61 possessed the ability to nodulate *Rj4* soybean, and that RTX is a known inhibitor of ethylene production (Owens *et al.* 1971). Ethylene is normally induced during a plant defense response and has the ability to induce other genes involved in such a response (Ecker and Davis 1987). However, despite the similarities of the soybean GSN to the gene-for-gene interactions described for plant-pathogen interactions, a defenselike response was not apparent in *Rj4* soybean inoculated with USDA 61. This was evidenced by the similarity in accumulation of ethylene and CHS and extensin mRNA in roots inoculated by restricted and nonrestricted strains.

Although the above data appear to rule out a defenselike response as explaining *Rj4* GSN, ethylene could mediate the *Rj4* soybean nodulation restriction by inhibiting the cell divisions necessary for the establishment of the nodule meristem (Apelbaum and Burg 1972). However, exogenous addition of inhibitors of ethylene production did not affect nodulation by strain USDA 61. Therefore, we conclude that ethylene is not the primary determinant of nodulation restriction in *Rj4* soybean. Indeed, exogenous addition of RTX also did not restore nodulation of *Rj4* soybean by strain USDA 61. These experiments suggest that RTX is not directly involved in the observed nodulation of *Rj4* soybean by *in planta* RTX overproducing strains. The exogenously supplied RTX was taken up by the plant as shown by the induction of chlorosis, but we do not know if RTX is taken up by the bacteria.

The *in planta* RTX overproducing mutants of strain USDA 61 are interesting in that they have altered host-range and that by conventional definitions in *(Brady)-Rhizobium* symbiosis would be considered host-range mutants. However, Southern blot analysis indicates that there is no homology between the USDA 110 *nod* region and the Tn*5* insertions of the RTX mutant strains (Russell *et al.* 1985 and data not shown). Recent data has indicated that host-range is largely determined by the production of specific Nod metabolites by the rhizobia that induce the early events of nodulation on a compatible legume host (Roche *et al.* 1991; Schultze *et al.* 1992; Spaink *et al.* 1991; Truchet *et al.* 1991). These Nod metabolites are substituted lipo-oligosaccharides of *N*-acetylglucosamine and require the *nod* gene products for synthesis (Lerouge *et al.* 1990; Roche *et al.* 1991; Schultze *et al.* 1992; Schwedock and Long 1990; Spaink *et al.* 1991). The specific substitutions present on the Nod factors produced by a particular rhizobia species appear to determine their specificity for a particular legume host (Roche *et al.* 1991; Schultze *et al.* 1992; Spaink *et al.* 1991; Truchet *et al.* 1991). In addition to stimulatory activity, such as induction of root hair curling and cortical cell division, it has been suggested that some Nod factors may inhibit the activities of other Nod factors (Djordjevic and Weinman 1991; Schultze *et al.* 1992). This would be similar to the observation of different flavonoids acting as inhibitors or inducers of bacterial *nod* gene expression (Djordjevic *et al.* 1987; Hartwig *et al.* 1989; Peters and Long 1988). Therefore, host specificity determined by Nod metabolites may involve a complex interplay between the recognition by the legume host of metabolites that are either

inhibitory or stimulatory to the nodulation process.

Because of the importance of Nod factors in determining host-specific nodulation, we examined the profile of Nod factor production by strain USDA 61 and derived mutants. Surprisingly, those mutants exhibiting increased RTX production also possessed an altered complement of Nod metabolites. In these *Rj4* nodulating mutants, compound 1 (Figs. 4 and 5) is made in much greater relative proportion. This compound comigrates with the major compound made by strain USDA 110 (Spaink *et al.* 1992). This strain is not restricted for nodulation of *Rj4* soybeans. Moreover this compound has been purified from strain USDA 110 and has been shown to induce root hair curling on soybean at levels as low as 10^{-11} M (Sanjuan *et al.*, in press). These data suggest two possible hypotheses. First, activation of nodule formation may require a minimum of activating compounds (i.e., compound 1 and perhaps others) which are present in too low abundance in strain USDA 61. Alternatively, certain compounds produced by strain USDA 61 (e.g. compounds 5, 4, and 3, as seen in Figs. 4 and 5) may be inhibitory to nodulation of *Rj4* soybeans, such that the relative proportion of activating versus inhibitory compounds is important in determining if nodule development proceeds. These hypotheses can be tested when the Nod metabolites produced by strain USDA 61 are purified and their biological activities on *Rj4* soybean are examined individually and in combination.

Conditions that induce Nod factor biosynthesis did not induce RTX biosynthesis by the *in planta* overproducing mutants. Therefore, the signal or metabolite that must be supplied by the plant for RTX biosynthesis by these mutants is not the known nodulation gene inducer. The fact that the RTX null mutant has a similar complement of Nod factors as wild type suggests that RTX does not directly affect Nod factor biosynthesis in culture.

The co-occurence of the *Rj4* nodulating phenotype, the *in planta* overproduction of RTX, and the altered pattern of Nod factors in strains RX20B, RX19H, RX19E, and RX17G is unlikely to be coincidence because each of these mutants represents an independent isolate and displays the same combination of phenotypes.

The occurrence of chlorosis-inducing strains of *Bradyrhizobium* was first described four decades ago. Since that time there has been no demonstration of a positive role of RTX in mutualistic symbiosis. Whereas the findings presented here do not demonstrate a positive role of RTX in mutualistic symbiosis, the results suggest that regulation of RTX biosynthesis is interconnected with Nod factor biosynthesis. It should be noted that the mutations that extend the host-range of USDA 61 also increase its parasitic character by causing chlorosis on host genotypes that would normally not show symptoms (this study and Ruan and Peters 1992). These pleiotropic mutations illustrate how closely related the mutualistic and parasitic behavior is in this strain and how delicately the symbiotic outcome is balanced. By further characterization of these *Rj4* nodulating *in planta* RTX overproducing mutants, we hope to better understand the dual character of this symbiont.

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