The soybean cultivar specificity gene nolX is present, expressed in a nodD-dependent manner, and of symbiotic significance in cultivar-nonspecific strains of Rhizobium (Sinorhizobium) fredii

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Rhizobium (now Sinorhizobium) fredii is a symbiotic nitrogen-fixing bacterium that can nodulate soybean in a cultivar-specific manner. This process is governed by a set of negatively acting nodulation genes termed nolXWBTUV. These genes prevent R. fredii strain USDA257 from infecting soybean cultivars such as McCall, but they do not block nodulation of cultivar Peking. R. fredii strain USDA191 contains DNA sequences that hybridize to nolXWBTUV, yet it forms normal nitrogen-fixing nodules on both McCall and Peking soybean. These sequences were isolated and their structure and function examined in comparison to nolXWBTUV of strain USDA257. Restriction maps of the two loci are identical, as is a 2-4 kb DNA sequence that corresponds to nolX and its promoter region. Expression of nolX by strain USDA191 is flavonoid-dependent in culture and readily detectable in nodules. The gene is not inducible in a mutant of strain USDA191 that lacks the regulatory nodD1 gene, and its expression is greatly attenuated in a nodD2 mutant. nolX is also present and flavonoid-inducible in HH103, a second R. fredii strain that nodulates McCall soybean normally. Inactivation of nolX in strain HH103, USDA191 or USDA257 leads to retardation of initial nodulation rates on soybean cultivars such as Peking and to acquisition of the capacity to form nitrogen-fixing nodules on two species of Erythrina. nolX is thus of symbiotic significance in all three strains, even though it regulates soybean cultivar specificity only in strain USDA257.

Keywords: Rhizobium (Sinorhizobium) fredii, Glycine max, nodD, nolX, symbiosis

INTRODUCTION

Rhizobium (now Sinorhizobium) fredii is a nitrogen-fixing bacterial symbiont of several dozen legume species, including the soybean (Keyser et al., 1982; Krishnan & Pueppke, 1994b). Like other rhizobia, it stimulates cortical cells of host roots to divide and it enters root hairs by means of tubular infection threads (Heron & Pueppke, 1984; Chatterjee et al., 1990). Bacterial cells are eventually released into the developing nodule, where they acquire the capacity to reduce N₂ to NH₃. As in other legume-Rhizobium associations, initiation of nodules by R. fredii is dependent on exchange of flavonoid and Nod factor signals between the symbiotic partners and is controlled by the bacterial regulatory gene nodD (Kossak et al., 1987; Appelbaum et al., 1988; Krishnan & Pueppke, 1991; Bec-Ferté et al., 1994). Strains of R. fredii differ greatly in their capacities to nodulate soybean cultivars (Keyser et al., 1982). The model strain in our laboratory, USDA257, can nodulate primitive lines such as Peking, but it fails to infect many advanced cultivars including McCall (Heron & Pueppke, 1984; Balatti & Pueppke, 1992). In contrast, nonspecific strains such as USDA191 and HH103 form nitrogen-fixing nodules on all tested soybean cultivars, including both Peking and McCall (Heron & Pueppke, 1984; Dowdle & Bohlool, 1985).

We have identified a cluster of genes in strain USDA257
that controls cultivar-specific nodule formation in a negative fashion. Inactivation of any one of these six genes, which are collectively designated nolXWBTUV, allows mutant bacteria to form fully normal, nitrogen-fixing nodules on McCall and other soybean cultivars that are not ordinarily infected (Heron et al., 1989; Meinhardt et al., 1993). The nolXWBTUV locus has been isolated and characterized, and we know that the six genes are organized into three transcriptional units: nolBTUV, nolW and nolX (Meinhardt et al., 1993; Kovács et al., 1995). Expression of nolW is constitutive, but that of nolX and nolBTUV is induced by flavonoid signals of the type known to trigger expression of other nodulation genes in strain USDA257 (Krishnan & Pueppke, 1991; Meinhardt et al., 1993). Curiously though, the cultivar specificity genes of R. fredii strain USDA257 are not associated with nod box promoters of the type thought essential for mediating flavonoid-inducibility in other Rhizobium spp. (Pueppke, 1996). Although the precise function of nolXWBTUV in strain USDA257 is not yet fully understood, amino acid homologies suggest that some of the genes may encode components of a specialized secretory system (Meinhardt et al., 1993; Van Gijsegem et al., 1995).

Using nolXWBTUV of strain USDA257 as a hybridization probe, we have detected homologous sequences in all tested strains of R. fredii, including those that nodulate McCall soybean (Meinhardt et al., 1993; Rodriguez-Navarro et al., 1996; H. B. Krishnan & S. G. Pueppke, unpublished data). The sequences are also present in broad-host-range strain NGR234 (Balatti et al., 1995), but they are apparently absent in other more distantly related rhizobia (Meinhardt et al., 1993). These observations raise a set of intriguing questions about the symbiotic significance of nolXWBTUV. Do R. fredii strains such as USDA191 lack cultivar specificity because of natural disruptions in the nolXWBTUV region, or are there essential accessory genes in strain USDA257 that differ from their counterparts in cultivar-nonspecific strains? And does nolXWBTUV have a symbiotic role in cultivar-nonspecific strains, one that is unrelated to cultivar specificity in soybean? We have begun to address these questions by focusing on nolX of strain USDA191. We have examined nolX and the sequences preceding it, assessed expression of the gene in culture and in nodules, and established that flavonoid inducibility of the gene is nodD-dependent. nolX is also present and expressed in a second cultivar-nonspecific strain, HH103. This new observation allowed us to systematically examine the consequences of inactivating nolX on the symbiotic properties of three R. fredii strains: USDA191, USDA257 and HH103.

METHODS

Bacterial strains and plasmids. Table 1 lists bacterial strains and plasmids used in this study. The nolX-lacZ gene fusion in pSB15-26 was created by mutagenizing pSB15 with mutH734 as described previously (Meinhardt et al., 1993). We used sequencing to confirm that minimal was in the same orientation as nolX and to pinpoint the insertion at position +626 with respect to the translational start site of the gene. Rhizobia were grown on a reciprocal shaker at 30 °C in yeast extract/mannitol (YEM) medium (Vincent, 1970), and Escherichia coli was cultured in Luria–Bertani (LB) broth at 37 °C (Sambrook et al., 1989). When appropriate, antibiotics were added at the following concentrations (µg ml⁻¹): ampicillin, 50; kanamycin, 50; tetracycline, 10. Bacteria were maintained as stocks in 7.5% (v/v) glycerol at −70 °C.

Manipulation of nucleic acids. Plasmid isolation, restriction digestions, DNA ligation, dot-blot and filter hybridizations, and other procedures with nucleic acids followed the general protocols of Sambrook et al. (1989). mRNA was extracted as described by Wang & Stacey (1991). DNA was sequenced with Sequenase Version 2.0 and DNA probes were radioactively labelled with the Random Primed DNA Labelling Kit (both from US Biochemicals).

Cloning and analysis of the nolXWBTUV locus of strain USDA191. A genomic library of DNA from strain USDA191 was constructed in cosmid pLAFR1 as described previously (Heron et al., 1989). The library was screened with labelled insert from plasmid pRIDH421 (Table 1). An 8.0 kb EcoRI fragment from cosmid pSB1 hybridized intensely with the probe and was of the expected size (Meinhardt et al., 1993). This fragment was subcloned into narrow- and broad-host-range vectors as pSB10 and pSB15, respectively (Table 1).

Expression of the nolX allele of strain USDA191. R. fredii cells were induced routinely by culture in the presence of 1 µM genistein (Krishnan & Pueppke, 1991). Expression of nolX was assessed by three methods. In the first, β-galactosidase activity of a nolX–lacZ fusion, either on plasmid pSB15-26 or after marker-exchange, was measured spectrophotometrically (Miller, 1972). In the second, RNA was dot-blotted onto filters and hybridized with a radioactively labelled BamHI/HindIII fragment (Fig. 1) that is internal to the nolX reading frame. Equivalence of RNA loading was verified by probing with a labelled fragment corresponding to ribosomal RNA (Scott-Craig et al., 1991). The third method involved histochemical staining of sections from McCall nodules containing strain RCB26. Seedlings were cultured as described by Krishnan & Pueppke (1991) and sections stained in situ as described by Boivin et al. (1990).

Immunological detection of NolX. Bacteria were cultured in 5 ml aliquots of YEM medium, with or without 1 µM genistein. After incubation for 24 h, cells were harvested by centrifugation at 11000 g for 10 min. The pellets were suspended in 200 µl SDS sample buffer, boiled for 3 min and centrifuged briefly; 10 µl aliquots were loaded onto a 12% SDS-polyacrylamide gel and electrophoresed (Laemmli, 1970). The gel was blotted to nitrocellulose, which was subsequently probed with anti-NolX antibodies that had been raised against a NolX–glutathione-S-transferase fusion protein (Kovács et al., 1994). Antigen–antibody complexes were detected with 125I-labelled Protein A as described by Harlow & Lane (1988).

Random mutagenesis of strain HH103. Random Tn5–lac mutagenesis of a streptomycin-resistant derivative of strain HH103 (Buendia-Claveria et al., 1989) was carried out as described by Simon et al. (1983). About 2800 kanamycin-resistant transconjugants were screened for β-galactosidase activity that could be induced by the flavonoid naringenin, a known activator of the expression of nolX in strain USDA257 (Meinhardt et al., 1993).

Symbiotic phenotypes. Nodulation tests with soybean (Glycine max cultivars Williams and Peking) were carried out in Vermiculite/Perlite mixtures by the method of Rodriguez-Navarro et al. (1996). Symbiotic responses of Erythrina
Table 1. Strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Relevant characteristics</th>
<th>Source</th>
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<tbody>
<tr>
<td><strong>E. coli DH5α</strong></td>
<td>Plasmid host</td>
<td>Promega</td>
</tr>
<tr>
<td><strong>R. fredii</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>USDA191</td>
<td>Wild-type; Fix+ nodules on Peking and McCall soybean</td>
<td>Keyser et al. (1982)</td>
</tr>
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<td>USDA191:nodD1</td>
<td>USDA191 containing a Km resistance cassette in nodD1</td>
<td>Appelbaum et al. (1988)</td>
</tr>
<tr>
<td>USDA191:nodD2</td>
<td>USDA191 containing a Km resistance cassette in nodD2</td>
<td>Appelbaum et al. (1988)</td>
</tr>
<tr>
<td>RfCB26</td>
<td>USDA191 nolX::mu26</td>
<td>This study</td>
</tr>
<tr>
<td>USDA257</td>
<td>Wild-type; Fix+ nodules on Peking soybean, Inf- on McCall</td>
<td>Keyser et al. (1982)</td>
</tr>
<tr>
<td>HH103</td>
<td>Wild-type; Fix+ nodules on Peking and McCall soybean</td>
<td></td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pGEM-7Zf(+)</td>
<td>Multicopy sequencing vector; Ap霉素</td>
<td>Promega</td>
</tr>
<tr>
<td>pRK415</td>
<td>Wide host range IncP1 plasmid; Tc霉素</td>
<td>Keen et al. (1988)</td>
</tr>
<tr>
<td>pBluescript II KS(—)</td>
<td>Cloning vector; Cm霉素</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pLAFR1</td>
<td>Wide host range IncP1 cosmid; Tc霉素</td>
<td>Friedman et al. (1982)</td>
</tr>
<tr>
<td>pSB10</td>
<td>pGEM-7Zf(+) carrying nolXWBTUV of USDA191 on a 8.00 kb</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>EcoRI insert; Ap霉素</td>
<td></td>
</tr>
<tr>
<td>pRfDH421</td>
<td>pGEM-7Zf(+) carrying nolXWBTUV of USDA257 on a 4.2 kb</td>
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<td>BamHI insert; Ap霉素</td>
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<tr>
<td>pSB15</td>
<td>pRK415 carrying nolXWBTUV of USDA191 on an 8.00 kb EcoRI</td>
<td>This study</td>
</tr>
<tr>
<td></td>
<td>insert; Tc霉素</td>
<td></td>
</tr>
<tr>
<td>pSB15-26</td>
<td>pSB15 carrying a minimu insertion in nolX; Tc霉素, Km霉素</td>
<td>This study</td>
</tr>
<tr>
<td>pHBK320</td>
<td>pRK415 carrying nodD1 of USDA257 on a 3.0 kb EcoRI insert;</td>
<td>Krishnan et al. (1995)</td>
</tr>
<tr>
<td></td>
<td>Tc霉素</td>
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<tr>
<td>pHBK330</td>
<td>pRK415 carrying nodD2 of USDA257 on a 6.0 kb EcoRI insert;</td>
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<td>Tc霉素</td>
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</tr>
<tr>
<td>pSB1</td>
<td>pLAFR1 carrying nolXWBTUV of USDA191 on an 8.00 kb EcoRI</td>
<td>This study</td>
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<tr>
<td></td>
<td>insert; Tc霉素</td>
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Fig. 1. Restriction map of the nolXWBTUV region of R. fredii strain USDA191. The positions of nolX, the sequenced nolX promoter region, the restriction fragment used in hybridizations to detect nolX message and the site of minimu insertion in pSB15-26 are indicated. The locations of nolW and nolBTUV were inferred by analogy to strain USDA257. ORF4, a small open reading frame that lies between nolU and nolV (Meinhardt et al., 1993) is not shown. B, BamHI; E, EcoRI; H, HindIII; P, PstI; S, Smal.

variegata and E. vespertilio (Krishnan & Pueppke, 1994a) were assessed as described previously (Krishnan & Pueppke, 1991).

RESULTS

Structure and expression of nolX

The nolXWBTUV locus of strain USDA191 was retrieved from a genomic library in cosmid pLAFR1 by screening with a 4.2 kb nolXWBTUV-containing BamHI fragment from strain USDA257. One cosmid, designated pSB1, contained a hybridizing 8.00 kb EcoRI fragment. This fragment, which was indistinguishable from that detected earlier in genomic digests (Meinhardt et al., 1993), was subcloned as pSB10. Its restriction map (Fig. 1) was the same as that of the 8.00 kb nolXWBTUV-containing EcoRI fragment of strain USDA257 (Meinhardt et al., 1993). The nolX promoter of strain USDA257 lies within a 648 bp HindIII fragment (Kovacs et al., 1995). The corresponding fragment from strain USDA191, as well as the entire nolX coding region (Fig. 1) were sequenced and found to be identical to the homologous sequences from strain USDA257 (accession number U77701 in the EMBL/GenBank/DDBJ nucleotide sequence data libraries).

Several experiments were performed to determine if nolX is expressed in strain USDA191. In the first, RNA was isolated from cells that had been cultured for 4-5 h in the presence or absence of 1 µM genistein. Dot-blot analysis with an internal BamHI/HindIII fragment as probe (Fig. 1) confirmed that the gene is flavonoid-inducible in the USDA191 background (Fig. 2a). Accumulation of nolX was monitored by probing protein extracts of uninduced and flavonoid-induced cells with anti-NolX antibodies (Kovacs et al., 1995). Genistein-induced cells of strain USDA191 produced a single...
a nolX-specific probe was hybridized to mRNA samples from
USDA257 and USDA191. The amounts of RNA blotted in each
dot are given on the left. RNA concentrations were verified by
hybridization with a ribosomal DNA probe as indicated. (b)
64 kDa NolX protein of strain USDA257 (Fig. 2b).

Expression of nolX in strain USDA191 is dependent
upon nodD

nodD mutants of strain USDA257 are not available so it
has not been possible to determine if expression of nolX
is under the control of these regulatory genes. We
employed nodD1 and nodD2 mutants of strain
USDA191 and a plasmid-borne nolX-lacZ gene fusion
to test this hypothesis. Plasmids bearing nodD1 or
nodD2 were also mobilized into strain RfCB26, which
contains nolX, and only slightly more
abundant in induced and uninduced cells of the nodD2
mutant (Fig. 3a). These results are in accord with data
obtained with the nolX-lacZ gene fusion (Table 2).
Western blots failed to detect NolX in cells of the nodD1
mutant, but they did reveal low levels of this protein in
cells of the nodD2 mutant (Fig. 3b). We also monitored
NolX levels in cells containing extra plasmid-borne
copies of nodD1 or nodD2. Although reduced levels of
NolX were not readily apparent in genistein-induced
cells of USDA191(pHBK320), synthesis of NolX in cells
of USDA191(pHBK330) was constitutive.

nolX is present and expressed in R. fredii strain
HH103

While we were in the process of characterizing nolX of
strain USDA191, we began screening random, Tn5-lac
insertions in strain HH103 for flavonoid-inducibility.
Like strain USDA191, HH103 forms nitrogen-fixing
nodules on all tested soybean cultivars (Dowdle &
Bohlool, 1985). Four such mutants were identified, and
we discovered that the transposon insertion in one of
them, strain SVQ118, lies within nolX. nolX transcripts were abundant in genistein-
induced cells of wild-type strain USDA191 (Fig. 3a). They
were barely detectable in induced or uninduced
cells of the nodD1 mutant, and only slightly more
abundant in induced and uninduced cells of the nodD2
mutant (Fig. 3a). These results are in accord with data
obtained with the nolX-lacZ gene fusion (Table 2).
Western blots failed to detect NolX in cells of the nodD1
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NolX levels in cells containing extra plasmid-borne
copies of nodD1 or nodD2. Although reduced levels of
NolX were not readily apparent in genistein-induced
cells of USDA191(pHBK320), synthesis of NolX in cells
of USDA191(pHBK330) was constitutive.

Expression of nolX in R. fredii. (a) RNA dot-blot analysis.
A nolX-specific probe was hybridized to mRNA samples from
genistein-induced (+) and uninduced (−) cells of strains
USDA257 and USDA191. The amounts of RNA blotted in each
dot are given on the left. RNA concentrations were verified by
hybridization with a ribosomal DNA probe as indicated. (b)
Immunoochemical detection of NolX. Total protein extracts from
genistein-induced (+) and uninduced (−) cells were
electrophoresed, transferred to nitrocellulose and probed with
antibodies that had been raised against NolX from strain
USDA257.

Extra plasmid-borne copies of nodD1 or nodD2 also
greatly influenced flavonoid-inducibility of nolX.
Expression of the plasmid-borne nolX-lacZ fusion in
strain RfCB26 was enhanced more than 25-fold by
genistein (Table 2). Plasmid pHBK320, which contains
nodD1, reduced this response by more than half, but
plasmid pHBK330, which contains nodD2, had just the
opposite effect. Expression of nolX in the absence of
flavonoid inducer was as high as that achieved by strain
RfCB26(pHBK320) in the presence of inducer, and more
than 10-fold higher than that in control strain RfCB26
(Table 2). This high basal level of expression was not
enhanced by flavonoid treatment, indicating that extra
copies of nodD2 trigger constitutive expression of nolX.

RNA dot-blots and immunoochemical analysis of cellular
proteins were used to independently confirm the
significance of nodD1 and nodD2 for expression of
nolX. nolX transcripts were abundant in genistein-
induced cells of wild-type strain USDA191 (Fig. 3a).
They were barely detectable in induced or uninduced
cells of the nodD1 mutant, and only slightly more
abundant in induced and uninduced cells of the nodD2
mutant (Fig. 3a). These results are in accord with data
obtained with the nolX-lacZ gene fusion (Table 2).
Western blots failed to detect NolX in cells of the nodD1
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cells of the nodD2 mutant (Fig. 3b). We also monitored
NolX levels in cells containing extra plasmid-borne
copies of nodD1 or nodD2. Although reduced levels of
NolX were not readily apparent in genistein-induced
cells of USDA191(pHBK320), synthesis of NolX in cells
of USDA191(pHBK330) was constitutive.

Expression of nolX in strain USDA191 was differentially sensitive to nodD1
and nodD2. Although insertional inactivation of nodD1
abolished the inducibility of nolX, disruption of nodD2
attenuated the response, to a level about one-third of
that achieved with cells containing both regulatory
genes.
Table 2. The flavonoid-inducibility of no1X is dependent on nodD1 and nodD2

<table>
<thead>
<tr>
<th>Bacterial construct</th>
<th>β-Galactosidase activity (Miller units ± SD)*</th>
<th>Fold-induction</th>
</tr>
</thead>
<tbody>
<tr>
<td>USDA191(pSB15-26)</td>
<td>5850 ± 2144-1</td>
<td>5.9</td>
</tr>
<tr>
<td>USDA191NodD1*(pSB15-26)</td>
<td>3000 ± 50</td>
<td>0.9</td>
</tr>
<tr>
<td>USDA191NodD2*(pSB15-26)</td>
<td>1228 ± 1044</td>
<td>1.9</td>
</tr>
<tr>
<td>USDA191</td>
<td>26 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>RfCB26</td>
<td>2481 ± 1345</td>
<td>25.5</td>
</tr>
<tr>
<td>RfCB26(pHBK320)</td>
<td>1136 ± 1830</td>
<td>9.8</td>
</tr>
<tr>
<td>RfCB26(pHBK330)</td>
<td>1700 ± 1848</td>
<td>1.2</td>
</tr>
</tbody>
</table>

* Values are the means of two independent experiments, each with three replicates.

Fig. 3. Expression of no1X by R. fredii USDA191 is influenced by nodD1 and nodD2. (a) RNA dot-blot analysis. A no1X-specific probe was hybridized to mRNA samples from genistein-induced (+) and uninduced (−) cells of strain USDA191 and from nodD1 and nodD2 mutants of this strain. RNA concentrations are given on the left and were verified by hybridization with a ribosomal DNA probe as indicated. (b) Immunochemical detection of NolX. Total protein extracts from genistein-induced (+) and uninduced (−) cells of strain USDA191 and nodD mutants derived from it were electrophoresed, transferred to nitrocellulose, and probed with antibodies that had been raised against NolX from strain USDA257. The minor band that is evident in all lanes is probably contaminating DnaK (Zylcic & Georgopoulos, 1984).

Inactivation of no1X influences nodulation

The availability of no1X mutants of strain USDA191, USDA257 and HH103 allowed us to systematically assess the influence of genetic background on the symbiotic significance of this gene. We inoculated two soybean cultivars, one that can be nodulated by strain USDA257 and one that is not. Inactivation of no1X retarded initial nodulation rates in all strain × cultivar combinations (Fig. 4). These differences were most...
apparent with plants examined 9 and 14 d after inoculation. With all strain pairs but one, final nodule numbers on plants that had been inoculated with a wild-type strain were equivalent to those on plants that had been inoculated with the corresponding nolX mutant. The relationship between strain HH103 and mutant SVQ118 was the single exception. Even at 30 d after inoculation, the number of nodules in the SVQ118 x Peking combination remained significantly less than the final number in the HH103 x Peking combination (Fig. 4).

We also examined nodulation of Erythrina, a genus of woody legumes that has interesting and complex symbiotic relationships with R. fredii (Krishnan & Pueppke, 1994a). Strain USDA257 fails to nodulate several Erythrina spp. but, as is the case with McCall soybean, mutation of nolXWBTUV can convert these incompatible interactions to fully compatible associations with nitrogen-fixing nodules (Krishnan & Pueppke, 1994a). Two of these species, E. variegata and E. vespertilio, were inoculated with the three wild-type strains and the corresponding nolX mutants. The parental strains failed to respond, but in each case the mutants produced large, leghaemoglobin-containing nodules.

**DISCUSSION**

Nodulation of soybean cultivars by R. fredii strains is specific, but in a differential sense. A few strains, including USDA191 and HH103, are compatible with all tested cultivars, but most fail to nodulate the majority of cultivars that have been examined (Keyser et al., 1982; Heron & Pueppke, 1984; Balatti & Pueppke, 1992). nolXWBTUV was first identified in USDA257, a strain of the latter type. Insertional inactivation of any of the six genes in this locus allows infection to proceed and nitrogen-fixing nodules to develop on all of the normally nitrogen-fixing nodules to develop on all of the normally noninfectible cultivars, including McCull (Heron et al., 1993). An 8.0 kb EcoRI fragment with homology to nolXWBTUV is nevertheless present in all strains of R. fredii, including those unimpaired in their abilities to nodulate McCull soybean (Meinhardt et al., 1993).

The presence of nolX and probably the entire cultivar specificity region in strains such as USDA191 and HH103 can be rationalized by either of two alternative hypotheses. Firstly, it is possible that undetected mutations lead to structural defects in one or more of the proteins encoded by nolXWBTUV of cultivar-nonspecific strains. By analogy with the known symbiotic behaviour of nolXWBTUV mutants of USDA257, such defects would allow nodulation of McCull to proceed. Alternatively, the cultivar specificity of strain USDA257 may depend both on nolXWBTUV and on one or more additional loci that are active in strain USDA257 but not USDA191, i.e. nolXWBTUV may be necessary but not sufficient to preclude nodulation of McCull.

Although we at first favoured the more simple model (Chatterjee et al., 1990), several lines of indirect and direct evidence are in better accord with the alternative. First, neither the data presented here nor additional unpublished sequencing has revealed any structural abnormalities in nolXWBTUV of strain USDA191. Second, amino acid sequence alignments have established significant homology between several of the protein products of nol genes and the products of genes that encode type III secretion pathways in Gram-negative pathogens (Meinhardt et al., 1993; Preston et al., 1993; Van Gijssegem et al., 1995). These pathways release proteins that interact directly with the plant or animal host to condition compatibility or incompatibility (Salmond, 1994). By analogy to these systems, the distinction between cultivar-specific and cultivar-non-specific R. fredii strains could reflect the presence or absence of a biologically active extracellular factor that blocks nodulation of McCull. R. fredii sequesters proteins upon receipt of flavonoid signals, and these molecules are obvious candidates to function as blockers (Krishnan & Pueppke, 1993; Krishnan et al., 1995). Third, we have recently subjected strain USDA257 to additional Tn5 mutagenesis and screened for acquisition of the ability to form nitrogen-fixing nodules on McCall soybean (Heron et al., 1989). Four of the kanamycin-resistant isolates that were purified from such nodules harboured single transposon-insertions in regions of the genome other than nolXWBTUV (unpublished data). This is the first direct evidence that nolXWBTUV is one of several loci that block the appearance of nitrogen-fixing nodules on McCall soybean.

**R. fredii** is apparently unique among rhizobia because it contains two classes of flavonoid-dependent genes. Genes such as nodABC are preceded by conventional nod box promoters (Krishnan & Pueppke, 1991), but other nod genes, including nolJ (Boundary-Mills et al., 1994), nolX and nolBTUV (Meinhardt et al., 1993), are not. NodD is the positive transcriptional activator of nod box-associated genes (Schlaman et al., 1992; Pueppke, 1996). It imposes control by binding to the nod box and, after perception of the appropriate flavonoid signal, by bending the DNA helix (Fisher & Long, 1993).

We used defined nodD1 and nodD2 mutants of strain USDA191 to investigate the relative contributions of these two regulatory genes to the expression of nolX. Both nodD1 and nodD2 control genistein-dependent expression of nolX, but in a rather complex manner. Flavonoid induction depends absolutely on nodD1, but it is reduced significantly when copy number of this gene is elevated. In contrast, about 30% of normal flavonoid inducibility is retained in the absence of nodD2, and extra copies of this gene trigger constitutive expression of nolX. This pattern of nodD-dependency differs fundamentally from that of nolJ, which requires only nodD1 and is insensitive to nodD2 (Boundary-Mills et al., 1994). Taken together, these data underscore the great intricacy of the R. fredii system in comparison to the relative simplicity of other rhizobia, which sometimes rely on a single copy of nodD and a few sets of nod-box-associated genes (Martinez et al., 1990).
The availability of noX mutants of strains HH103 and USDA191 allowed us to systematically examine the impact of this gene on symbiosis. Inactivation of noX led to a slight retardation of initial nodulation rates in all five tested combinations with soybean cultivars, but the ultimate number of nodules was usually not influenced by noX. These sorts of mild negative impacts on nodulation rates are a rather widespread symbiotic response to inactivation of a variety of nod genes in various Rhizobium spp. (Martínez et al., 1990; Pueppke, 1996). Nodulation of two species of Erythrina however is blocked when noX is intact but allowed to proceed when the gene is inactivated. This differential phenotype is not just triggered by strain USDA257, and thus noX functions dually, as a cultivar specificity determinant with soybean and in a more generalized sense with at least one other legume host.

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