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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 N_2 to NH_3. 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* (Kosslak *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

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that controls cultivar-specific nodulation 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 mu*dII*1734 as described previously (Meinhardt *et al.*, 1993). We used sequencing to confirm that minimu 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 pRfDH421 (Table 1). An 8·0 kb *Eco*RI 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 *Bam*HI/*Hin*dIII 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 RfCB26. 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.*, 1995). Antigen–antibody complexes were detected with ^{125}I-labelled Protein A as described by Harlow & Lane (1988).

Random mutagenesis of strain HH103. Random Tn*5-lac* mutagenesis of a streptomycin-resistant derivative of strain HH103 (Buendía-Clavería *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

Strain or plasmid	Relevant characteristics	Source
E. coli DH5α	Plasmid host	Promega
R. fredii		
USDA191	Wild-type; Fix$^+$ nodules on Peking and McCall soybean	Keyser *et al.* (1982)
USDA191nodD1$^-$	USDA191 containing a Km resistance cassette in *nodD1*	Appelbaum *et al.* (1988)
USDA191nodD2$^-$	USDA191 containing a Km resistance cassette in *nodD2*	Appelbaum *et al.* (1988)
RfCB26	USDA191 *nolX*::mu26	This study
USDA257	Wild-type; Fix$^+$ nodules on Peking soybean, Inf$^-$ on McCall soybean	Keyser *et al.* (1982)
HH103	Wild-type; Fix$^+$ nodules on Peking and McCall soybean	
Plasmids		
pGEM-7Zf(+)	Multicopy sequencing vector; Ap^R	Promega
pRK415	Wide host range IncP1 plasmid; Tc^R	Keen *et al.* (1988)
pBluescript II KS(−)	Cloning vector; Cm^R	Stratagene
pLAFR1	Wide host range IncP1 cosmid; Tc^R	Friedman *et al.* (1982)
pSB10	pGEM-7Zf(+) carrying *nolXWBTUV* of USDA191 on a 8·0 kb *Eco*RI insert; Ap^R	This study
pRfDH421	pGEM-7Zf(+) carrying *nolXWBTUV* of USDA257 on a 4·2 kb *Bam*HI insert; Ap^R	Meinhardt *et al.* (1993)
pSB15	pRK415 carrying *nolXWBTUV* of USDA191 on an 8·0 kb *Eco*RI insert; Tc^R	This study
pSB15-26	pSB15 carrying a minimu insertion in *nolX*; Tc^R, Km^R	This study
pHBK320	pRK415 carrying *nodD1* of USDA257 on a 3·0 kb *Eco*RI insert; Tc^R	Krishnan *et al.* (1995)
pHBK330	pRK415 carrying *nodD2* of USDA257 on a 6·0 kb *Eco*RI insert; Tc^R	Krishnan *et al.* (1995)
pSB1	pLAFR1 carrying *nolXWBTUV* of USDA191 on an 8·0 kb *Eco*RI insert; Tc^R	This study

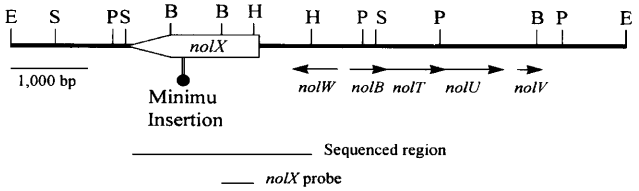

Fig. 1. Restriction map of the *nolXWBTUV* region of *R. fredii* strain USDA191. The positions of *nolX*, the sequenced *nolX* promoter region, the restriction fragment used in hybridizations to detect *nolX* message and the site of minimu insertion in pSB15-26 are indicated. The locations of *nolW* 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, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S, *Sma*I.

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 *Bam*HI fragment from strain USDA257. One cosmid, designated pSB1, contained a hybridizing 8·0 kb *Eco*RI 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 8·0 kb, *nolXWBTUV*-containing *Eco*RI fragment of strain USDA257 (Meinhardt *et al.*, 1993). The *nolX* promoter of strain USDA257 lies within a 648 bp *Hin*dIII fragment (Kovács *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 *Bam*HI/*Hin*dIII 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 (Kovács *et al.*, 1995). Genistein-induced cells of strain USDA191 produced a single

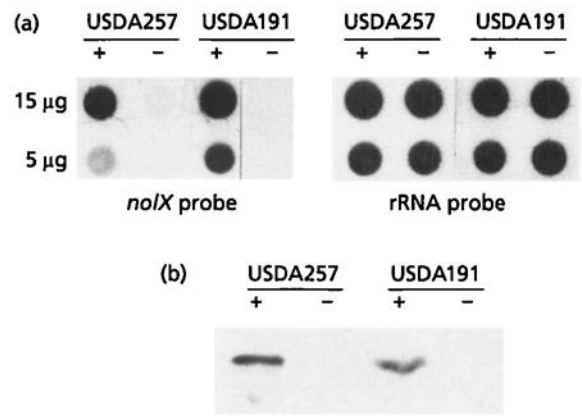

Fig. 2. Expression of *nolX* in *R. fredii*. (a) RNA dot-blot analysis. A *nolX*-specific probe was hybridized to mRNA samples from genistein-induced (+) and uninduced (−) cells of strains USDA257 and USDA191. The amounts of RNA blotted in each dot are given on the left. RNA concentrations were verified by hybridization with a ribosomal DNA probe as indicated. (b) Immunochemical 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.

reactive polypeptide that was indistinguishable from the 64 kDa NolX protein of strain USDA257 (Fig. 2b).

Expression of *nolX* under symbiotic conditions was assessed with strain RfCB26, a derivative of strain USDA191 with a sym-plasmid-borne minimu insertion within the coding region of the gene (Fig. 1). Control McCall nodules containing wild-type strain USDA191 remained colourless, but the bacteroid-containing zones of nodules harbouring the USDA191 mutant stained intensely for β-galactosidase activity (data not shown), as is the case with similarly stained McCall nodules containing a *nolX–lacZ* fusion in strain USDA257 (Meinhardt *et al.*, 1993). *nolX* thus is expressed by strain USDA191, both *in planta* and *ex planta*.

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 a marker-exchanged copy of the *nolX–lacZ* gene fusion in the sym plasmid. Wild-type strain USDA191 had essentially no β-galactosidase activity (Table 2). The presence of the plasmid-borne *nolX–lacZ* gene fusion led to a substantial basal level of enzyme activity, which was elevated nearly sixfold by treatment with the flavonoid inducer. Genistein-dependent induction of *nolX* 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.

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 immunochemical 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* 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, Tn*5-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*. This conclusion is based on evidence from experiments in which genomic DNA of strain SVQ118 was digested with *Eco*RI, which cleaves the transposon once within *lacZ*. Fragments of 8 to 9 kb were ligated into pBluescript II KS(−), and a 17-mer oligonucleotide with homology to the transposon was synthesized. It corresponded to positions 11–27 bp with respect to the end of the element and was used to initiate sequencing, which confirmed that the transposon resides within *nolX*. Expression of β-galactosidase activity by mutant SVQ118 was elevated 5·6-fold in the presence of 1 µM genistein.

Table 2. The flavonoid-inducibility of *nolX* is dependent on *nodD1* and *nodD2*

Bacterial construct	β-Galactosidase activity (Miller units ± SD)*		Fold-induction
	1 μM genistein	Uninduced	
USDA191(pSB15-26)	5850·2 ± 144·1	984·4 ± 23·4	5·9
USDA191NodD1⁻(pSB15-26)	500·0 ± 5·0	553·6 ± 6·7	0·9
USDA191NodD2⁻(pSB15-26)	1228·8 ± 104·0	644·2 ± 18·2	1·9
USDA191	2·6 ± 0·2	1·4 ± 0·4	
RfCB26	2481·7 ± 134·5	97·4 ± 28·7	25·5
RfCB26(pHBK320)	1136·0 ± 183·0	115·6 ± 86·1	9·8
RfCB26(pHBK330)	1700·9 ± 184·8	1379·6 ± 130·3	1·2

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

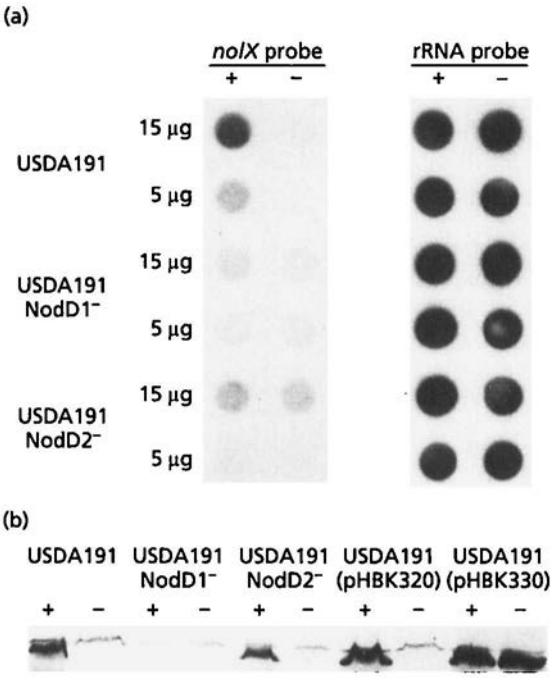

Fig. 3. Expression of *nolX* by *R. fredii* USDA191 is influenced by *nodD1* and *nodD2*. (a) RNA dot-blot analysis. A *nolX*-specific probe was hybridized to mRNA samples from genistein-induced (+) and uninduced (−) cells of strain USDA191 and from *nodD1* and *nodD2* mutants of this strain. RNA concentrations 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 (Zylicz & Georgopoulos, 1984).

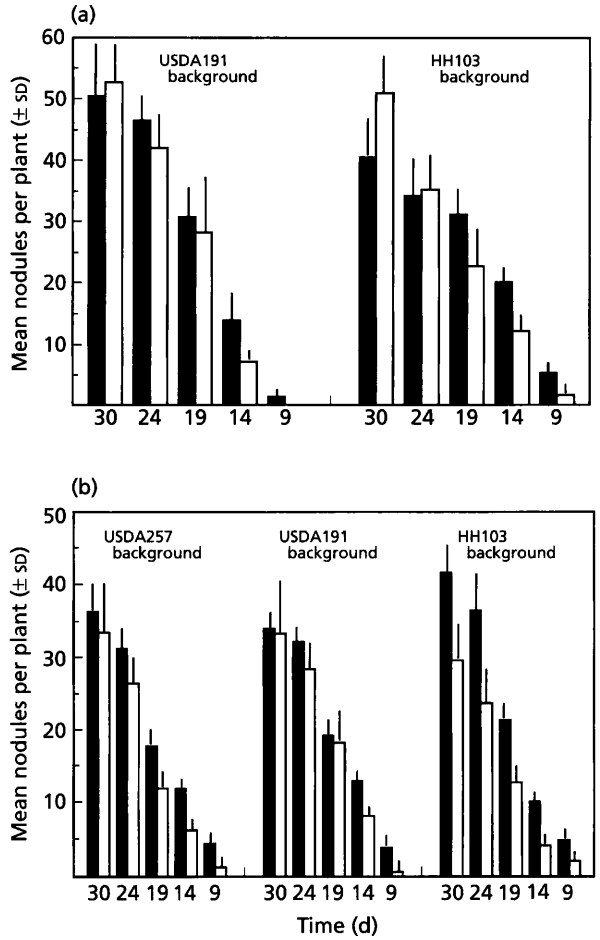

Fig. 4. Rates of nodulation of Williams (a) and Peking (b) soybean by wild-type *R. fredii* strains USDA191, USDA257 and HH103 (■), and their corresponding *nolX* mutants (□). Six plants from two separate Leonard jars were harvested at each time point.

Inactivation of *nolX* influences nodulation

The availability of *nolX* 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 *nolX* 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 × Peking combination remained significantly less than the final number in the HH103 × Peking combination (Fig. 4).

We also examined nodulaton 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 uninfectible cultivars, including McCall (Heron *et al.*, 1989; Meinhardt *et al.*, 1993). An 8·0 kb *Eco*RI fragment with homology to *nolXWBTUV* is nevertheless present in all strains of *R. fredii*, including those unimpaired in their abilities to nodulate McCall 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 McCall 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 McCall.

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.*, 1995; Van Gijsegem *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-nonspecific *R. fredii* strains could reflect the presence or absence of a biologically active extracellular factor that blocks nodulation of McCall. *R. fredii* secretes 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 Tn*5* 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* (Boundy-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* (Boundy-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 (Martínez *et al.*, 1990).

The availability of *nolX* mutants of strains HH103 and USDA191 allowed us to systematically examine the impact of this gene on symbiosis. Inactivation of *nolX* 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 *nolX*. 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 *nolX* is intact but allowed to proceed when the gene is inactivated. This differential phenotype is not just triggered by strain USDA257, and thus *nolX* 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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