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Inactivation of *nolC* Conditions Developmental Abnormalities in Nodulation of Peking Soybean by *Rhizobium fredii* USDA257

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Inactivation of *nolC* in *Rhizobium fredii* USDA257 is known to expand host range for nodulation to include the soybean cultivar McCall. We show here that *nolC* also influences symbiotic interactions with Peking, a primitive soybean cultivar that forms nitrogen-fixing nodules with USDA257. Peking nodules containing *nolC* mutants reduce less acetylene than controls; nodule ultrastructure is altered, and shoot dry weights are diminished. Rhizobia with homogenotized *nolC*::*lacZ* fusions produce enhanced levels of extracellular polysaccharides and express high constitutive levels of β-galactosidase activity. Using X-gal as a histochemical stain, we detected sustained activity of *nolC* during all stages of nodule development on Peking, including preinfection. Transcription of key plant genes is modulated in nodules induced by *nolC* mutants. Expression of leghemoglobin is reduced, and that of VuA, a member of the ENOD2 early nodulin family, is abolished. The number and levels of transcripts homologous to VuB, another early nodulin, also are influenced by the gene. We conclude that *nolC* may have multiple effects on *R. fredii* USDA257, only some of which regulate cultivar specificity in McCall.

Additional keywords: gene fusions, host specificity genes, *nod* genes.

USDA257 is a strain of the nitrogen-fixing soybean symbiont, *Rhizobium fredii* Scholla and Elkan. This fast-growing bacterial species first was discovered in China (Keyser *et al.* 1982), and it offers some unique advantages for those wishing to analyze the legume-*Rhizobium* symbiosis. Principal among these is cultivar-specificity: USDA257 produces Fix^+ root nodules on primitive soybean varieties such as Peking, but fails to do so on several agronomically advanced varieties (Keyser *et al.* 1982). Included in the latter group is McCall, which initially responds to USDA257 with normal root hair curling and associated cortical cell divisions (Chatterjee *et al.* 1990; Heron and Pueppke 1984). These developmental changes are not sustained, however, and the nodulation process is interrupted prematurely at the stage of meristem formation. The mechanisms that condition such early cessation of nodulation in certain cultivars are of great interest, but they are not well understood.

We have used transposon Tn*5* to induce mutations in USDA257 that permit nodulation of McCall and several other cultivars to proceed beyond the meristem stage (Heron *et al.* 1989). One mutant, 257DH5, produces sparse numbers of nodules on seedlings grown in plastic growth pouches; they are initiated via infection threads, but are morphologically abnormal and incapable of fixing nitrogen (Chatterjee *et al.* 1990). The negatively acting gene responsible for this phenotype, *nolC*, has been cloned and characterized. *nolC* is chromosomal and lacks homology to characterized *nod* genes, but it has strong homology to *dnaJ*, a heat-shock gene from *Escherichia coli* (Migula) Castellani and Chalmers (Krishnan and Pueppke 1991a).

In our initial comparison of mutant 257DH5 with parental strain USDA257, we were unable to detect on Peking any symbiotic or other abnormalities accompanying extension of host range to cultivars such as McCall (Heron *et al.* 1989). More recently, we have noticed differences in the nodulation behavior of parental strain USDA257 and mutant 257DH5 on vermiculite-grown Peking soybean. We now report that 257DH5 and other *nolC* mutants are distinct from parental strain USDA257, both in their interactions with soybean cultivar Peking and in their ability to produce extracellular polysaccharides in culture. We have prepared *lacZ* fusions in *nolC* and use these to document expression of the gene during nodule development. We also show that plant gene expression in Peking nodules is modulated by the activity of *nolC*.

MATERIALS AND METHODS

Preparation and analysis of *nolC* mutants. *R. fredii* USDA257 originally was from the culture collection of the U. S. Department of Agriculture, Beltsville, MD. The preparation and characterization of Tn*5*-induced mutant 257DH5 has been described (Heron *et al.* 1989; Krishnan and Pueppke 1991a). *nolC*::*lacZ* fusions were created by mutagenizing pHBK100, which contains an intact copy of *nolC* within a 4.3-kb *Eco*RI-*Hin*dIII insert (Krishnan and Pueppke 1991a) in broad host range plasmid pRK415, with minimu bacteriophage transposon mu*d*II1734 (Castilho *et al.* 1984). A screen of 32 minimu-containing plasmids for constitutive activity of *lacZ* (Miller 1972) identified two high activity fusions: nolC12 and nolC29. The site of minimu insertion in each was determined by restriction mapping and by DNA sequence analysis (Krishnan and Pueppke 1991b) with oligonucleotide primers corresponding to the termini of the insertion element. Fusions *nolC*12 and *nolC*29 were transferred to USDA257 by triparental mating

and homogenotized by plasmid incompatibility (Heron *et al.* 1989) to yield mutants 257DH512 and 257DH529, respectively.

Acetone-precipitable extracellular polysaccharides from 5-day-old cultures were isolated as described by Zdor and Pueppke (1991). Polysaccharides from three separate sets of cultures were quantified by the anthrone method with glucose as standard.

Plant tests. Seeds of *Glycine max* (L.) Merr. 'Peking' were harvested from plants grown at the University of Missouri Bradford Research Farm. Seeds with intact seedcoats were surface-disinfested and planted directly into aseptically prepared Leonard jars that had been filled with vermiculite (Krishnan and Pueppke 1991b). After 3 days, a 200-μl aliquot of bacteria from a 3-day-old, log-phase culture was pipetted aseptically onto the base of each emerging seedling. Plants were incubated in a growth chamber as described (Chatterjee *et al.* 1990), and groups of seedlings were harvested 7, 13, 18, 23, and 28 days after planting. Acetylene-reduction rates were determined by the method of Schwinghamer *et al.* (1970), and shoot dry weights were recorded. Samples of 13- and 28-day-old nodules were fixed, embedded, and examined by transmission electron microscopy as described (Chatterjee *et al.* 1990).

Expression of *nolC* during nodule development. Root segments and nodules from control plants that had been inoculated with USDA257 and from plants that had been treated with mutant 257DH512 were harvested 5–15 days after inoculation and stained with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), essentially as described by Boivin *et al.* (1990). Hand sections then were prepared and viewed under bright-field optics with an Olympus BH-2 microscope. Photos were taken with an Olympus Exposure Control Unit and Kodak Gold 400 film.

Northern hybridizations. Total RNA from nodules collected 13, 18, 23, and 28 days after inoculation was resolved on 1.5% agarose gels containing formaldehyde (Rochester *et al.* 1986). After blotting to nitrocellulose, the RNA was hybridized by standard procedures (Maniatis *et al.* 1982) with a series of DNA probes. These included: soybean leghemoglobin clone pLB23 (Fuller and Verma 1984); pVuA, a cowpea clone that is homologous to soybean early nodulin gene ENOD2 (van de Wiel *et al.* 1990; Trese and Pueppke 1991); and pVuB, a second early nodulin clone with a temporal pattern of expression different from that of pVuA (Trese and Pueppke 1991).

RESULTS

Nodules produced by mutant 257DH5 on Peking soybean are functionally abnormal. The symbiotic phenotypes of parental strain USDA257 and mutant 257DH5 are distinct on McCall soybean, but both are qualitatively Fix$^+$ on cultivar Peking (Heron *et al.* 1989). This observation led us to conclude that mutations in *nolC* condition symbiotic alterations only in advanced soybean cultivars, and we subsequently focused on these interactions. During more recent experiments, however, we noticed that Peking nodules induced by 257DH5 seemed to darken and senesce prematurely, suggesting that they might be functionally altered in comparison to controls. This possibility was confirmed in a series of comparative physiological experiments (Fig. 1). During a 28-day growth period, acetylene-reduction rates by the mutant on Peking never achieved those of the parental strain (Fig. 1A). The differences were particularly pronounced at 11 days after inoculation, when the rate due to USDA257 was four times that due to 257DH5. The high acetylene-reduction rate in plants inoculated with USDA257 declined thereafter, and by 3 wk after inoculation, they were roughly comparable to rates in plants inoculated with the mutant. Shoot dry weights reflected acetylene-reduction activities in that plants inoculated with the mutant achieved only about 60% of the weight of plants inoculated with the parental strain (Fig. 1B).

***nolC* is expressed constitutively in culture and during nodule development.** To monitor the expression of *nolC*

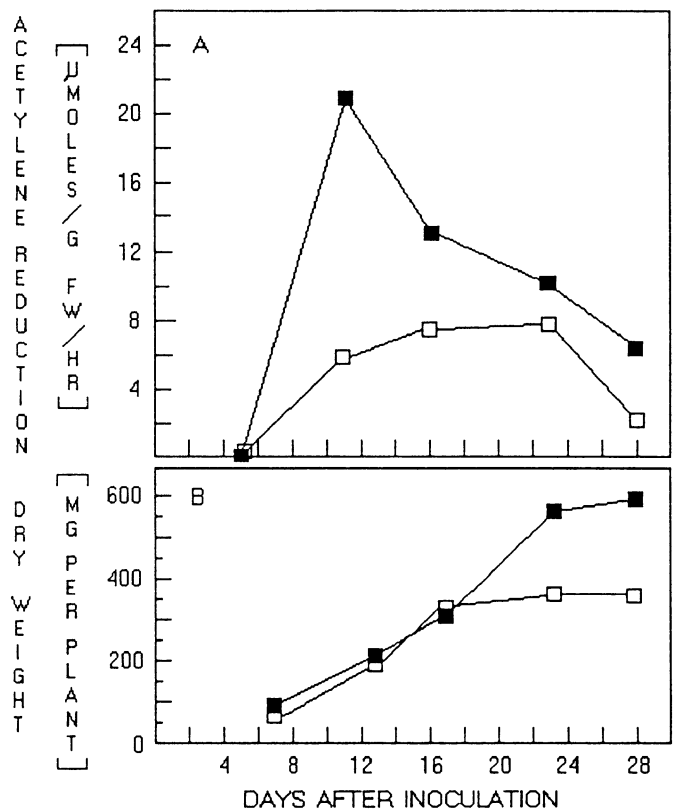


Fig. 1. Effect of *nolC* on the interaction between Peking soybean and *Rhizobium fredii* USDA257. **A,** acetylene-reduction rates; **B,** shoot dry weights. Seedlings were inoculated with either USDA257 (■) or *nolC* mutant 257DH512 (□) as described in Materials and Methods. The experiment was repeated three times, and a total of three plants (acetylene-reduction) and 25 plants (dry weight) was examined on each harvest date. Standard errors were less than 9% of means.

Table 1. Expression of *nolC*::*lacZ* fusions in culture

Bacteria	β-Galactosidase activity[a] (Miller units)	
	28°C	37°C
USDA257 (control)	3.2	2.0
nolC12	3,574 ± 572	3,533 ± 85
nolC29	41 ± 5	41 ± 7

[a] Values are the means ± standard deviations of two to three replicate experiments.

in the presence of the host plant, we randomly mutated a 4.3-kb, *nolC*-containing restriction fragment with mud*II*1734. Two of 32 mutant plasmids expressed elevated β-galactosidase activity in *E. coli*. The location of the minimu element in each was determined by sequencing, and both mutations were homogenotized into USDA257 for further testing. The minimu element is located at nucleotide position 78 (amino acid 26) and nucleotide position 1,165 (amino acid 388), respectively, in mutants 257DH512 and 257DH529. Because the entire coding region of *nolC* contains 1,176 bp (Krishnan and Pueppke 1991a), the *lacZ* fusions are in the N-terminal (*nolC*12) and the extreme C-terminal (*nolC*29) of the protein.

Table 1 gives the expression of β-galactosidase activity in the two *lacZ* fusions. Because of the sequence homology of *nolC* to a heat-shock gene, these assays were done at normal growth temperature and at 37° C. Irrespective of temperature, however, *nolC*29 was constitutively expressed at 15–20 times the control level. Expression of *nolC*12, the N-terminal fusion, also was temperature-insensitive. Levels of expression of this fusion were considerably greater, between 1,000 and 1,500 times that of the control, undoubtedly because of the proximity of the minimu element to the promoter. A series of flavonoids known to induce *nodABC* of strain USDA257 (Krishnan and Pueppke 1991b) were inactive in modulating expression of either of the *nolC*::*lacZ* fusions (data not shown).

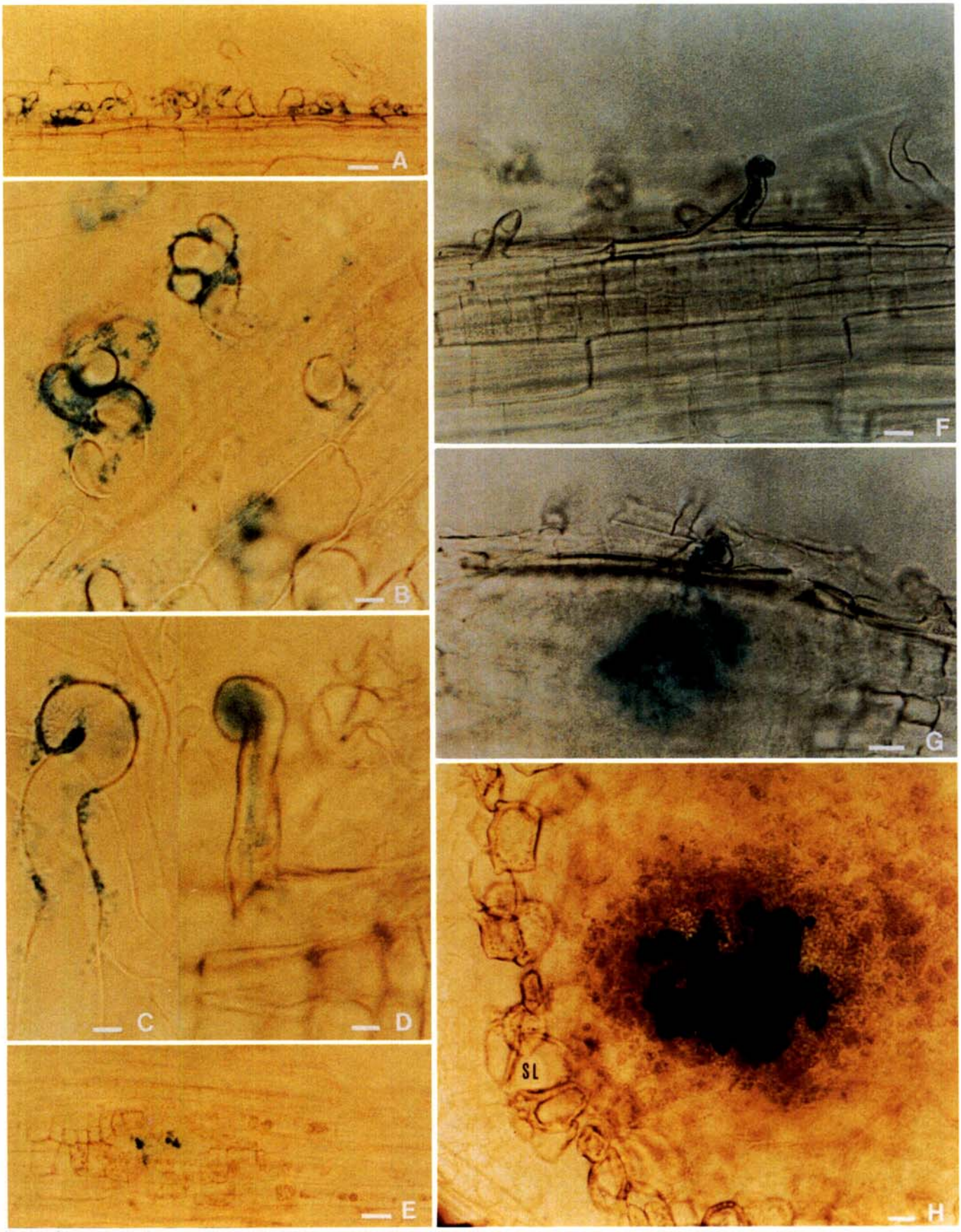
The exceptionally high levels of *nolC* expression by 257DH512 prompted us to stain inoculated roots with X-gal in an attempt both to microscopically monitor activity of the gene during nodulation of Peking and to visualize nodulation of this host by a *nolC* mutant. Although controls inoculated with USDA257 did not stain, the expression of *nolC* by the mutant was sufficient to allow visualization of blue-stained bacteria and infection structures at all stages of nodule development and to confirm that nodules are initiated in the normal fashion (Fig. 2). Thus individual rhizobia and bacterial aggregates could be resolved on root surfaces (Fig. 2A) as well as curled root hairs (Fig. 2B,C). Blue-colored infection threads were clearly discernible, both in protruding curled root hairs (Fig. 2D,F,G) and in underlying cells of developing nodules (Fig. 2E,G). Resolution of branched and ramifying infection threads within cortical cells associated with infections was especially striking (Fig. 2G), as was the level of *nolC* expression in bacteroid-containing cells of young nodules (Fig. 2H).

Mutation of *nolC* conditions altered nodule ultrastructure and plant gene expression. Figure 3 compares the internal ultrastructure of Peking nodules containing USDA257 with those containing mutant 257DH512. Nodules produced by the parental strain possessed the internal structures expected for soybean: the host cytoplasm was dense, bacteroids were closely packed, and electron-lucent poly-β-hydroxybutyrate granules had accumulated (Fig. 3A,C). In contrast, even young, 13-day-old nodules containing 257DH512 had a visibly lower bacteroid density and were characterized by the presence of starch grains (Fig. 3B), an indication of ineffective nitrogen fixation (Dart 1975). By 28 days after inoculation, these nodules showed signs of senescence (Fig. 3D). Peribacteroid membranes were dilated, the electron density of the host cytoplasm was greatly reduced, irregularly shaped vacuoles were interspersed with the peribacteroid units, and poly-β-hydroxybutyrate granules were conspicuous in their absence. Twenty-eight-day-old nodules containing the parental strain, however, remained healthy and ultrastructurally similar to 13-day-old nodules (cf. Fig. 3A,C).

The effect of *nolC* on host gene expression within nodules was monitored by northern analysis of nodule transcripts with three different nodulin clones (Fig. 4). Nodulins VuA and VuB originally were discovered because of their preferential expression in cowpea roots inoculated with USDA257, but they also appear in soybean roots inoculated with *Bradyrhizobium japonicum* (Kirchner) Jordan (Trese and Pueppke 1991). VuA-homologous transcripts were detected between 13 and 28 days after inoculation in Peking nodules produced by *R. fredii* USDA257, but not in corresponding nodules produced by mutant 257DH512 (Fig. 4). The pattern of appearance of VuB-homologous transcripts was distinctly different (Fig. 4). Although a pair of transcripts was inevitably present in nodules containing the parental strain, nodules harboring the mutant were devoid of the larger transcript. Younger nodules containing the *nolC* mutant expressed the smaller transcript, but 23- and 28-day-old nodules lacked the smaller of the VuB-homologous transcripts, too. In comparison, leghemoglobin transcripts were present in all nodules at all harvest dates. The level of expression, however, was always greater in USDA257 nodules than in corresponding 257DH512 nodules (Fig. 4).

***nolC* enhances extracellular polysaccharide production.** During the preparation and analysis of *nolC* mutants, we observed that the mucoidy of colonies appeared to differ from that of the parental strain. We consequently isolated and quantified extracellular polysaccharides from 5-day-old liquid cultures of USDA257 and the three *nolC* mutants. Concentrations of extracellular polysaccharides, expressed as micrograms ± standard deviation per 10^9 cells, were: 8.6 ± 1.9 (USDA257), 14.7 ± 1.5 (257DH5), 15.5 ± 3.2 (257DH512), and 19.9 ± 1.0 (257DH529). Thus, the levels

Fig. 2. Initiation of nodules on Peking soybean by *Rhizobium fredii* mutant 257DH512 and expression of *nolC* during nodule development. Seedlings were inoculated as described in Materials and Methods, and primary root segments were excised 5 days later and treated with X-gal as described by Boivin *et al.* (1990). Under these conditions, blue staining indicates activity of the *nolC*::*lacZ* gene fusion. Free-hand sections were examined by bright-field microscopy. **A** and **B** are longitudinal sections of curled root hairs illustrating expression of *nolC* in free-living bacteria before infection. **C** and **D** show bacteria on the surface of a curled root hair and expression of *nolC* within an infection thread growing within a curled root hair. **E** is a low magnification surface view of an infection focus, which is characterized by meristematic activity giving rise to cubical cells and a slight protuberance of the epidermis from the surface of the root. The infection threads have branched and grown into the underlying cell layer. **F** and **G** are longitudinal cross-sections showing heavy expression of *nolC* within infection threads. Whereas the infection in F is confined to the upper four cell layers, the developing nodule in G has begun to assume its spherical shape and is filled with ramifying infection threads. H is a nodule cross-section. Discrete infected cells within the interior are heavily stained. SL = sclerotized layer. Bars represent 40 μm (A, E, and H), 20 μm (B, C, and D), and 20 μm (F and G).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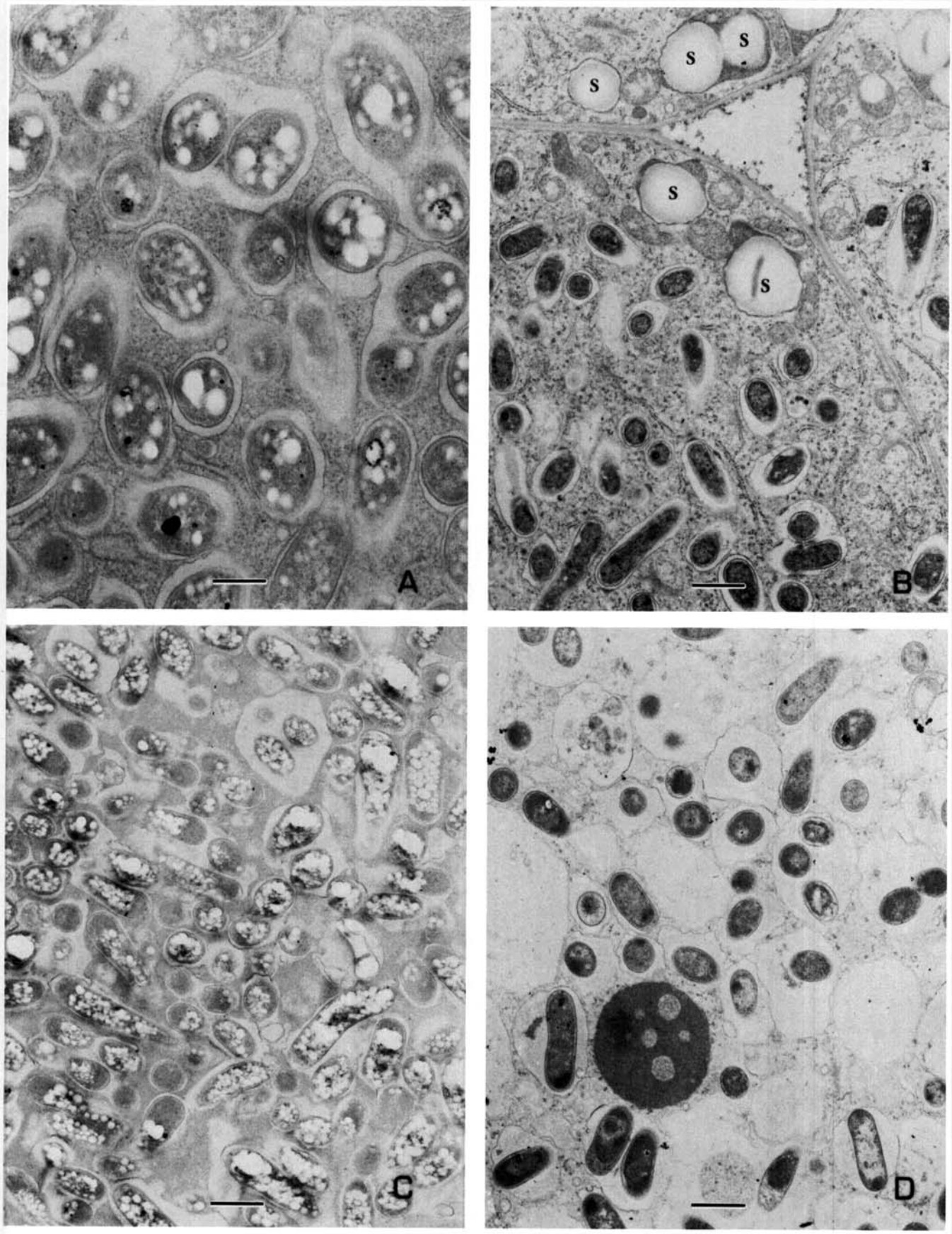

Fig. 3. Ultrastructure of nodules produced on Peking soybean by *Rhizobium fredii* USDA257 and mutant 257DH512. Seedlings were inoculated as described in Materials and Methods. **A,** USDA257, 13 days after inoculation. Bar = 0.6 μm. **B,** 257DH512, 13 days after inoculation. Bar = 1.2 μm. **C,** USDA257, 28 days after inoculation. Bar = 1.2 μm. **D,** 257DH512, 28 days after inoculation. Bar = 1.2 μm. S = starch grain.

of these materials in *nolC* mutants were between 1.7 and 2.3 times that in USDA257.

DISCUSSION

The host range of rhizobia is defined by a diverse assemblage of genes (Martinez *et al.* 1990), which includes a large subset with positive, host-specific functions. *nodL*, *nodT*, *nodX*, and the alleles of *nodD* in *R. meliloti* exemplify this group (Honma and Ausubel 1987; Davis *et al.* 1988; Györgypal *et al.* 1988; Surin and Downie 1988; Lewis-Henderson and Djordjevic 1991b). Negatively functioning host range genes make up a second subset. When inactivated, these genes allow a *Rhizobium* strain to increase the number of plants that it nodulates. Such loci have been detected in *R. meliloti* (Debellé *et al.* 1986; Horvath *et al.* 1986; Faucher *et al.* 1988), *R. leguminosarum* bv. *trifolii* (Djordjevic *et al.* 1985; Lewis-Henderson and Djordjevic 1991a), and *R. fredii* (Heron *et al.* 1989; Chatterjee *et al.* 1990; Krishnan and Pueppke 1991a, 1991b), where they function at the level of either legume species or cultivars within a species.

There are two model systems for analysis of negative control of cultivar specificity, and in both cases, manipulation of genes that regulate this process appears to have no generalized consequences for symbiosis. Thus, inactivation of *nodM* of *R. l.* bv. *trifolii* TA1 extended nodulation to Woogenellup subterranean clover (Lewis-Henderson and Djordjevic 1991b) but had no adverse effect on nodulation of other subterranean clover cultivars or of other *Trifolium* species (Surin *et al.* 1990). Similarly, we have reported that inactivation of *nolC* of *R. fredii* USDA257 extended nodulation to McCall soybean with no apparent influence on symbiosis with soybean cultivars normally nodulated by USDA257 or other legume hosts of *R. fredii* (Heron *et al.* 1989). This latter conclusion now requires revision, because we have discovered that inactivation of *nolC* in fact is detrimental to nodulation of Peking, a primitive soybean cultivar that forms normal Fix^+ nodules with USDA257 (Keyser *et al.* 1982; Heron and Pueppke 1984). These adverse effects, which include a diminished capacity for nitrogen fixation as well as ultrastructural abnormalities associated with ineffective nodules (Dart 1975), were not readily apparent after visual examination of pouch-grown seedlings (Heron *et al.* 1989). Lower plant vigor, which was substantiated as reduced shoot dry weights, was nonetheless clearly evident when plants were grown for longer periods in vermiculite.

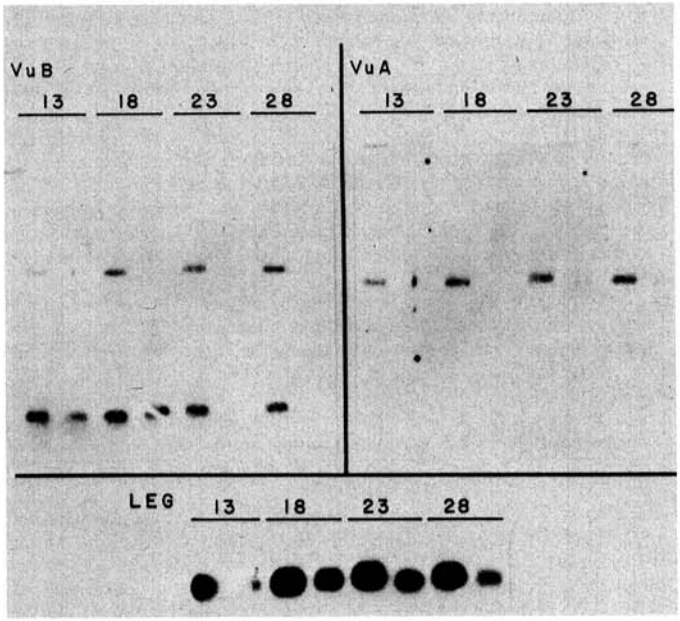

Fig. 4. Hybridization of total RNA from Peking nodules with DNA probes for nodulins pVuA, pVuB, and leghemoglobin (LEG). Seedlings were inoculated with *Rhizobium fredii* USDA257 or mutant 257DH512 as described in Materials and Methods and harvested 13, 18, 23, or 28 days later, as indicated. The left and right lanes corresponding to each harvest date were loaded with 10 μg of total RNA from plants inoculated with USDA257 and 257DH512, respectively.

Plant gene expression in Peking nodules also was influenced by *nolC*. Thus, message levels for leghemoglobin were reduced considerably in the relatively ineffective Peking nodules containing the mutant, as is the case in several other ineffective combinations (Fuller and Verma 1984; Gloudemans *et al.* 1987). Likewise, modulation of the expression of nodulins VuA and VuB in Peking was apparent. Transcripts corresponding to both of these nodulins originally were isolated from young nodulating roots of cowpea, and each is expressed in nodulating Williams soybean (Trese and Pueppke 1991). VuA has homology to ENOD2, an early nodulin that has been detected in several legumes (Franssen *et al.* 1987; Moerman *et al.* 1987; Dickstein *et al.* 1988; Strittmatter *et al.* 1989). VuB hybridized to a single, 1,800 nucleotide transcript in soybean and cowpea, and it was subject to strong, temporal regulation in cowpea roots inoculated with USDA257 (Trese and Pueppke 1991). Given the expression of ENOD2 in both poorly developed empty nodules (Dickstein *et al.* 1988; Allen *et al.* 1991) and in simple hormone-induced root outgrowths (Hirsch *et al.* 1989), it is surprising that VuA-homologous transcripts could not be detected in Peking nodules containing the *nolC* mutant, even though they were relatively well developed and fixed some nitrogen. Equally unexpected, two distinct VuB transcripts were present in Peking nodules containing USDA257. Disruption of *nolC* differentially influenced their expression: the larger transcript was absent in all nodules harboring 257DH512, regardless of their developmental stage. Levels of the smaller transcript initially were roughly equivalent in nodules produced by the parent strain and the mutant, but this message, too, ultimately disappeared. Although our nodulin data with Peking are still preliminary, they indicate that *nolC* has a major impact on plant gene expression in developing Peking nodules and that regulation of VuA (ENOD2) and VuB in Peking nodules may differ sharply from that in other systems, including cowpea and soybean varieties such as Williams.

nolC has strong sequence homology to *dnaJ*, which encodes a heat-shock gene in *E. coli*. Based on this similarity and on the known global regulatory roles for *dnaJ*, we have suggested that *nolC* may be a regulatory gene (Krishnan and Pueppke 1991a). Modulation of nodulin levels by *nolC* is consistent with this interpretation, as are two additional observations. First, *in planta* histological studies confirm that expression of *nolC* is not temporally

regulated, but rather sustained throughout nodule development, including the preinfection stage. This pattern of expression resembles that of *nodD*, a well characterized regulatory *nod* gene (Schlaman *et al.* 1991). Second, *nolC* has striking quantitative effects on extracellular polysaccharide production. Although surface polysaccharides long have been known to function in nodulation, they are structurally diverse, and their precise roles are poorly understood and dependent on particular bacteria-host combinations (Gray and Rolfe 1990; Hotter and Scott 1991). Several genes influencing cell surface polysaccharides have been discovered in *R. fredii* (Kim *et al.* 1989; Ko and Gayda 1990), and when present in elevated copy number, some, including *nodD2* (Appelbaum *et al.* 1988) and other uncharacterized plasmid-borne genes (Barbour and Elkan 1989), suppress mucoidy in a manner analogous to that of *nolC*. Moreover, and as is the case with *nolC*, inactivation of these genes has only modest effects of nodule function. We are continuing to examine the extracellular polysaccharides of *R. fredii* and their significance for symbiosis.

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