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Nitrogen-Fixing Nodules Induced by *Agrobacterium tumefaciens* Harboring *Rhizobium phaseoli* Plasmids

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Rhizobium phaseoli **CFN299 forms nitrogen-fixing nodules in *Phaseolus vulgaris* (bean) and in *Leucaena esculenta*. It has three plasmids of 185, 225, and 410 kilobases. The 410-kilobase plasmid contains the nitrogenase structural genes. We have transferred these plasmids to the plasmid-free strain *Agrobacterium tumefaciens* GMI9023. Transconjugants containing different combinations of the *R. phaseoli* plasmids were obtained, and they were exhaustively purified before nodulation was assayed. Only transconjugants harboring the 410-kilobase plasmid nodulate *P. vulgaris* and *L. esculenta*. Nodules formed by all such transconjugants are able to reduce acetylene. Transconjugants containing the whole set of plasmids from CFN299 nodulate better and fix more nitrogen than the transconjugants carrying only the Sym plasmid. Microscopic analysis of nodules induced by *A. tumefaciens* transconjugants reveals infected cells and vascular bundles. None of the *A. tumefaciens* transconjugants, not even the one with the whole set of plasmids from CFN299, behaves in symbiosis like the original *R. phaseoli* strain; the transconjugants produce fewer nodules and have lower acetylene reduction (25% as compared to the original *R. phaseoli* strain) and more amyloplasts per nodule. More than 2,000 bacterial isolates from nodules of *P. vulgaris* and *L. esculenta* formed by the transconjugants were analyzed by different criteria. Not a single rhizobium could be detected. Our results show that *R. phaseoli* plasmids may be expressed in the *A. tumefaciens* background and direct the formation of effective, differentiated nodules.**

The bacteria belonging to the genus *Rhizobium* form nitrogen-fixing nodules in the roots of legumes. Bacterial genes controlling nodulation, host range specificity, and nitrogen fixation have been located on large plasmids called Sym plasmids. One of the approaches used to evaluate the genetic information contained in plasmids has been the transfer of these plasmids to different *Rhizobium* species or to *Agrobacterium tumefaciens*. The transfer between *Rhizobium* species has led to different results. Receptors either acquire the full capacity to nodulate and fix nitrogen in the host legumes specified by the transferred plasmid (16–18) or are only capable of nodulating but not of fixing nitrogen (16); in some cases not even nodulation has been obtained (7).

The genera *Agrobacterium* and *Rhizobium* belong to the family *Rhizobiaceae*, and members of both genera are capable of interaction with higher plants. Formation of tumors in dicotyledonous plants by *A. tumefaciens* depends on a plasmid (Ti) that carries oncogenicity determinants. When *Rhizobium* symbiotic plasmids have been transferred to *A. tumefaciens*, the recipients have gained the ability to promote the formation of non-nitrogen-fixing nodules on the appropriate host legumes (16, 19, 31, 32). A plasmid-free *A. tumefaciens* strain, GMI9023 (27), promises to be a suitable receptor for plasmids from *Rhizobium* species. We report here the formation of nitrogen-fixing nodules in *Phaseolus vulgaris* (bean) and in *Leucaena esculenta* by GMI9023 carrying different plasmids from *Rhizobium phaseoli* CFN299.

MATERIALS AND METHODS

Bacterial strains and plasmids. *R. phaseoli* CFN299 (also called UMR1026 or CENA183) was from Peter Graham, University of Minnesota, St. Paul. Also used were *Rhizobium meliloti* GMI7064 (1), *A. tumefaciens* C58 (12) and GMI9023 (27), and *Escherichia coli* S-17(pSUP5011) (28) and HB101(pRK2013) (10).

Strain construction. CFN299 derivatives carrying Tn*5-mob* (28) were obtained from matings between strains S-17 and CFN299. The transconjugants were selected for nalidixic acid (20 mg/liter) and kanamycin (50 mg/liter) resistances. The GMI9023 derivatives were obtained through the mobilization of random Tn*5-mob* mutants of CFN299 in triparental matings with a helper *E. coli* strain (HB101) carrying the mobilizing plasmid pRK2013 (10). The transconjugants were selected in LB medium (22) (where CFN299 is unable to grow) containing rifampin (200 mg/liter) and kanamycin (50 mg/liter).

Strain purification and identification. To purify *A. tumefaciens* transconjugants, bacteria were grown in liquid LB medium, and dilutions were plated for colony isolation. The 3-ketolactose production assay was carried out as described previously (21) with liquid cultures in BYLA medium (21) instead of plates. Sensitivity tests for phages S2 and S5 (13) were performed as reported previously (15). Melanin production of *Rhizobium* strains was evaluated in PY medium (23) containing tyrosine (100 mg/liter) and $CuSO_4$ (20 mg/liter).

Heat curing of plasmids. Km^s strains were obtained from various *A. tumefaciens* transconjugants after various subcultures in liquid LB medium grown to a low density at 37°C.

Nodulation and acetylene reduction assays. *P. vulgaris* cv. Negro Jamapa and *L. esculenta* seeds were surface sterilized and germinated as described previously (21). *L. esculenta* plants were grown in glass tubes or in 125-ml Erlenmeyer flasks, and beans were grown either in tubes or in 250-ml Erlenmeyer flasks with Fahraeus (9) agar medium without nitrogen. Bean plants were also grown in Leonard jars with

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TABLE 1. Nodulation and nitrogen fixation in *P. vulgaris* and in *L. esculenta* of *R. phaseoli* CFN299 and *A. tumefaciens* GMI9023 harboring different plasmids of *R. phaseoli* CFN299

Strain	*R. phaseoli* plasmids	Tn*5* localization	*P. vulgaris*				*L. esculenta*	
			Nodulation		Nitrogen fixation		Nodulation at 28°C	Nitrogen fixation[a] at 28°C
			21°C	28°C	21°C	28°C		
R. phaseoli CFN299	abc		+	+	+	+	+	+
A. tumefaciens transconjugants[b]								
A1	a	a	−	−	−	−	−	−
A2	b	b	−	−	−	−	−	−
A3	c	−	+	+	−	+	+	±
A12	ab	b	−	−	−	−	−	−
A13	ac	c	+	+	−	+	+	±
A14	ac	−	+	+	−	+	+	±
A123	abc	b	+	+	−	+	+	±

[a] +, Positive acetylene reduction obtained in all plants tested; ±, positive acetylene reduction obtained in some of the plants tested.
[b] A123 is a plant-selected transconjugant, A14 is a derivative of A123, A3 was constructed from A14, and the other transconjugants were directly obtained from crosses (see the text).

vermiculite. Plants were maintained at 26 to 28°C, unless the temperature effect was evaluated by growing bean plants at 21°C. Nodulation kinetics was followed from day 7 after inoculation up to day 18. Nitrogenase activity was measured at day 14 as described previously (21).

Isolation of bacteria from nodules. Bacteria were always isolated in medium without any antibiotic selection, PY medium, from surface-sterilized nodules. Tests for nodule surface sterility were performed. Nodules were squeezed in liquid PY, and different dilutions were plated for isolated colonies. Around 150 colonies were picked from each nodule and tested for growth in *Agrobacterium* selective medium, LB with rifampin (200 mg/liter) and streptomycin (200 mg/liter) and with kanamycin (50 mg/liter) when required.

Plasmid visualization. Plasmid profiles were analyzed by the Eckhardt procedure (8) from liquid cultures. Plasmid weight was estimated by using *A. tumefaciens* C58 as a reference.

Plasmid and DNA hybridization. Techniques for DNA isolation and electrophoresis were as described previously (21). Total DNA digests or plasmids from Eckhardt gels were transferred to nitrocellulose filters by the Southern procedure (30). Hybridization was performed as reported previously (24). pCQ152 carrying a 300-base-pair sequence from the coding region of the *nifH* gene from *R. phaseoli* CFN42 was used as a probe (25). pSup5011 (28) was used as a probe for Tn*5* localization.

Light microscopy. Slices of 2-week-old nodules of *P. vulgaris* and *L. esculenta* were fixed for 90 min with 4% glutaraldehyde and postfixed in 1% OsO_4 for 1 h by the method of Truchet et al. (31). After dehydration in a graded ethanol series, samples were embedded in Spurr low-viscosity medium (Polysciences Inc., Warrington, Pa.). Semithin sections of 1.5 μm were laid on a slide and stained by the basic fuchsin-methylene blue method (31).

RESULTS

***A. tumefaciens* transconjugants.** *R. phaseoli* CFN299 has symbiotic properties distinguishing it from other *R. phaseoli* strains. It has an extended nodulation spectrum that allows the formation of nitrogen-fixing nodules in *L. esculenta* (Table 1; see also Fig. 4). It is acid and aluminium tolerant (P. Graham, unpublished results). Its *nifH* hybridization pattern shows a single band (21) as opposed to the multiple hybridization bands (*nifH* gene reiterations) found in most *R. phaseoli* strains (21). Unlike other *R. phaseoli* strains (3, 20), we have found that it does not produce a melaninlike pigment. CFN299 harbors three plasmids of 185 kilobases (kb) (plasmid a), 225 kb (plasmid b), and 410 kb (plasmid c) (Fig. 1). The *nifH* gene from our reference *R. phaseoli* strain, CFN42, hybridizes to the 410-kb plasmid (Fig. 1). CFN299 was randomly mutagenized with Tn*5-mob*. A hundred Km^r CFN299 isolates were mated with *A. tumefaciens* GMI9023. Mobilization of the Km^r determinant to GMI9023 was obtained in 20% of these crosses. The *A. tumefaciens* transconjugants obtained were either screened for their plasmid content (and on this basis some were chosen for further colony purification; see below) or inoculated en masse in *P. vulgaris* to allow the plant to select the nodulating transconjugants. Strains isolated from the nodules of the mass inoculation experiment all proved to be *A. tumefaciens* by antibiotic resistance markers and by 3-ketolactose production (data not shown). One colony isolated from the largest red nodule was selected and included among the former *A. tumefaciens* transconjugants for exhaustive colony isolation to make sure no bacterial mixtures were to be evaluated for symbiotic properties. The *A. tumefaciens* transconjugant carrying plasmids a and b (AI2) was purified by a single passage with Tween 40 as described previously (21), since plasmid a was lost in the long purification procedure. The bacteria purified this way presented *A. tumefaciens* charac-

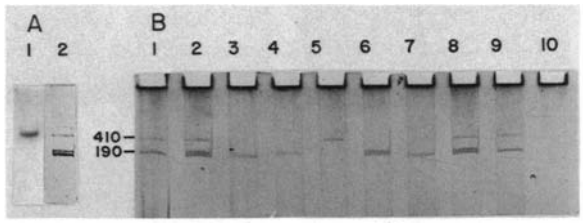

FIG. 1. (A) Lanes: 1, autoradiogram of the Southern blot of the plasmid profile of *R. phaseoli* CFN299 after hybridization with a 300-base-pair *nifH* internal sequence of a *R. phaseoli* reference strain; 2, plasmid pattern of *R. phaseoli* CFN299 obtained by the Eckhardt procedure. (B) Agarose gel electrophoresis of plasmids from strains of *A. tumefaciens*, *R. phaseoli*, and their transconjugants. Lanes: 1, C58 harboring pAtC58 (410 kb) and pTi (190 kb) used as a marker; 2, donor strain, *R. phaseoli* CFN299; 3 through 9, *A. tumefaciens* transconjugants A1, A2, A3, A12, A13, A123, and A14, respectively; 10, plasmid-free *A. tumefaciens* receptor strain GMI9023.

teristics; they were lysed by the *A. tumefaciens* phages S2 and S5, they produced 3-ketolactose, and they presented electrophoretic fingerprints of total DNA digestions not distinguishable from that of GMI9023. The plasmid patterns of these bacteria are shown in Fig. 1. Besides *A. tumefaciens* transconjugants containing single plasmids, transconjugants harboring two or three *R. phaseoli* plasmids were obtained. The transconjugant isolated in the mass inoculation experiment from a big red nodule was GMI9023 with the three plasmids (A123). The localization of Tn*5* in the strains with various plasmids was done by hybridization with Tn*5* to plasmid profiles or by the analysis of the plasmid patterns of the Km^s strains generated by subculturing or by heat curing. The Tn*5* localization is shown in Table 1. Plasmids a and c were mobilizable without Tn*5-mob* (A12, A13, A123). Plasmid c was mobilized at a much lower frequency since it had to be selected by the plant (A123). We were not able to obtain transconjugants containing only the *nif* plasmid. The construction of this strain (A3) was as follows. A spontaneous chloramphenicol-resistant mutant of A14 was obtained. A14 is a Km^s derivative from A123 that lost plasmid b during subculturing. Cm^r A14 was mated with A1, selecting for the transference of Km^r. This transconjugant, carrying plasmids a and c and containing Tn*5-mob* in plasmid a, was heat cured, and the strains containing only plasmid c were identified by their Km^s phenotype and by their plasmid electrophoretic profile.

Nodulation in *P. vulgaris* (bean). Only *A. tumefaciens* strains carrying plasmid c are capable of nodulating bean. *A. tumefaciens* strains carrying only plasmid a or b or both do not nodulate. Thus, plasmid c carries the symbiotic determinants for nodule initiation.

The nodulation of the *A. tumefaciens* transconjugants is delayed compared with that of the wild *R. phaseoli* CFN299. On the average, nodules appear on day 8 after inoculation with *R. phaseoli* CFN299, 2 days later in the case of the *A. tumefaciens* strain that has all three plasmids (A123), and around 4 days later with transconjugants harboring only plasmid c (A3) or plasmids a and c (A13 or A14).

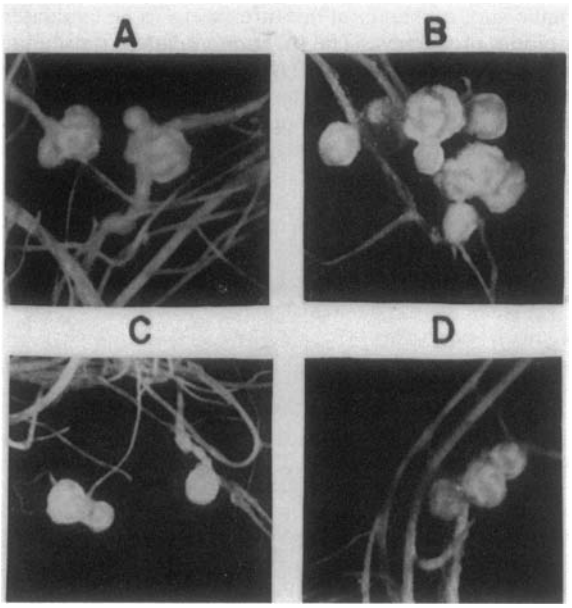

FIG. 2. Two-week-old nodules formed in *P. vulgaris* by (A) *R. phaseoli* CFN299 and *A. tumefaciens* transconjugants (B) A123, (C) A13, and (D) A3.

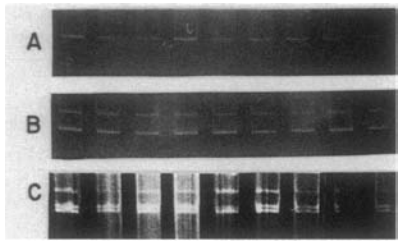

FIG. 3. Agarose gel electrophoresis of plasmids from some strains isolated from nodules of *P. vulgaris* formed by the *A. tumefaciens* transconjugants (A) A3, (B) A13, and (C) A123.

There are always fewer nodules in plants inoculated with *A. tumefaciens* transconjugants than with the original *R. phaseoli* strain. On the average, *A. tumefaciens* with the whole set of plasmids (A123) forms 50% of the nodules formed with *R. phaseoli*, and the strains containing plasmid c (A3) or plasmids a and c (A13 or A14) form just around 30%. The topology of the nodules formed by the *A. tumefaciens* transconjugants is peculiar. They appear mainly at the point of emergence of secondary roots, as opposed to the overall root distribution of *R. phaseoli* nodules.

Nodules formed by the *A. tumefaciens* transconjugants fix nitrogen. Levels of nitrogen fixation, as determined by acetylene reduction, are only 10 to 25% per plant as compared with *R. phaseoli*-inoculated plants. On the average, higher nitrogen fixation levels are obtained with the *A. tumefaciens* transconjugant that contains the three *R. phaseoli* plasmids (A123). Nodule number, nodulation kinetics, and nitrogen fixation levels may vary from one plant to another, more so with the *A. tumefaciens* inoculations than with *R. phaseoli*. Although acetylene reduction levels may be in some cases 25% per plant when compared with the original *R. phaseoli* strain, nitrogen deficiencies are observed in all bean plants inoculated with the transconjugants. However, nodules formed by *A. tumefaciens* transconjugants may be contributing to plant nitrogen, since some difference in plant development is observed with plants nodulated by *A. tumefaciens* as compared with non-nodulated plants grown in Leonard jars. Nodule development and nitrogen fixation with the *A. tumefaciens* transconjugants are dependent on plant culture temperature conditions. No acetylene reduction is obtained from nodules formed with any of the *A. tumefaciens* transconjugants when plants are grown at 21°C instead of the usual 26 to 28°C (Table 1). At 21°C nodules formed by *A. tumefaciens* are small and white, as opposed to the big red nodules formed at 28°C (Fig. 2). On the other hand, *R. phaseoli* CFN299 is capable of forming nitrogen-fixing nodules at either 21 or 28°C.

Identity of strains isolated from bean nodules. A total of 1,800 isolated colonies from 15 nodules from various bean plants inoculated with *A. tumefaciens* transconjugants A3, A13, and A123 were screened; all colonies grew in the selective medium for GMI9023. From the original selectionless plates, 150 randomly chosen colonies (10 colonies per nodule, 5 nodules per transconjugant) were all positive for 3-ketolactose production; 10 to 15 colonies per *A. tumefaciens* transconjugant were analyzed for their plasmid contents (Fig. 3), and all 10 isolates tested from nodules formed by A123 were sensitive to phages S2 and S5. In another approach, the whole nodule sets from three nitro-

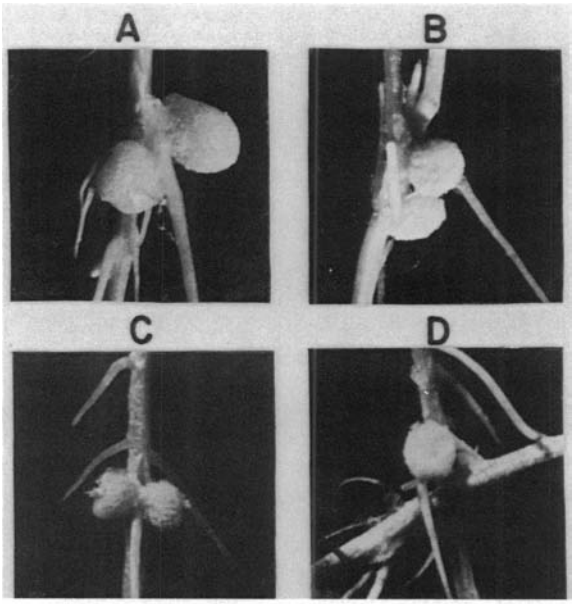

FIG. 4. Nodules formed in *L. esculenta* by (A) *R. phaseoli* CFN299 and *A. tumefaciens* transconjugants (B) A123, (C) A13, and (D) A3.

gen-fixing plants inoculated with either A3, A13, or A123 were screened, nodule by nodule, for 3-ketolactose production. All 130 nodule isolates produced 3-ketolactose. None of the isolates from nodules of a plant infected with *R. phaseoli* CFN299 produced it.

Nodulation in *L. esculenta*. The four *A. tumefaciens* transconjugants (A3, A13, A14, A123) that nodulate bean plants are also capable of nodulating *L. esculenta* (Table 1 and Fig. 4). The *A. tumefaciens* transconjugant containing the whole set of plasmids (A123) nodulates faster than do the other transconjugants. Levels of acetylene reduction of plants nodulated by *A. tumefaciens* were lower than those obtained in *P. vulgaris* with these transconjugants. In some of the *L. esculenta* plants no nitrogen fixation was obtained (Table 1).

Two hundred colonies from various *L. esculenta* nodules formed by *A. tumefaciens* were tested for antibiotic resistance markers in LB. All corresponded to *A. tumefaciens* GMI9023.

Microscopic analysis of nodules. Longitudinal sections of *P. vulgaris* nodules induced by the *A. tumefaciens* transconjugants A3, A13, and A123 present a general morphology than resembles that of wild *R. phaseoli* CFN299 nodules (Fig. 5). Infected cells are slightly less abundant than in CFN299 nodules; vascular bundles are localized in the periphery. However, there is a large number of amyloplasts in nodules induced by transconjugants not observed with the original strain (Fig. 5). Another difference is the presence of dark-stained zones in these nodules. These dark zones (arrows in Fig. 5) may correspond to intercellular bacterial proliferation, but we cannot exclude the possibility that they might represent infection threads.

L. esculenta nodules formed by *A. tumefaciens* transconjugant A3, which carries only the Sym plasmid, are also similar morphologically to CFN299 nodules (Fig. 6). They present vascular bundles in the periphery and a peculiar corky material surrounding the nodule; they contain an apical meristematic zone. Nodules of the transconjugants possess infected cells in a clearly reduced number when compared with *R. phaseoli* CFN299 nodules (Fig. 6).

Functionality of Sym plasmid in another bacterial background. Plasmid c of CFN299 has been transferred to a *R. meliloti* Fix$^-$ mutant, GMI7064 (1). At 28°C, the *R. meliloti* transconjugant containing the *R. phaseoli* Sym plasmid is able to form small, white, non-nitrogen-fixing nodules in *P. vulgaris*, whereas the original *R. meliloti* does not nodulate.

DISCUSSION

Different research groups have transferred Sym plasmids to *A. tumefaciens* and tested their symbiotic performance in the appropriate host plant. In none of these cases have nitrogen-fixing nodules been obtained; results show atypical bacterial penetration without infection threads and nodules with no intracellular bacteria (31, 33).

We have obtained *A. tumefaciens* transconjugants that induce a more complete symbiotic process leading to nodules with infected cells and vascular bundles distributed as in wild-type nodules and capable of acetylene reduction. Hooykaas et al. (14) transferred the *R. phaseoli* pSym9 plasmid to *A. tumefaciens*. They got no effective nodules. Their *R. phaseoli* plasmid would belong to the most common *R. phaseoli* Sym plasmid type, with reiterated *nif* genes and melanin production, clearly different from the Sym plasmid of CFN299. Another difference that may explain the discrepancy of results is the temperature used. Their plant culture conditions were as described by Djordjevic et al. (6); plants were grown at 21°C. We found that at this temperature no acetylene-reducing nodules were formed with *A. tumefaciens* transconjugants in beans.

The extended nodulation phenotype of CFN299 is encoded in the 410-kb plasmid (plasmid c), since this capacity may be transferred to *A. tumefaciens*. Thus, the 410-kb plasmid (plasmid c) carries host specificity genes for *L. esculenta* nodulation as well as for bean nodulation. Like plasmid c of CFN299, plasmid b contains symbiotic determinants that confer a nodulation advantage in the *A. tumefaciens* background when tested in both *L. esculenta* and *P. vulgaris*. Participation of other plasmids different from the Sym plasmid in symbiosis has been described in rhizobia. In *R. meliloti*, megaplasmid b codifies for exopolysaccharides that are involved in infection thread formation (11). In *R. phaseoli* CFN42, curing of one plasmid of lower molecular weight than the Sym plasmid reduces the nodule number and nitrogen fixation (unpublished results).

Nodules formed by the different *A. tumefaciens* transconjugants in *P. vulgaris* and *L. esculenta* develop structures characteristic of wild *R. phaseoli*-induced nodules. They have peripheric vascular bundles and infected cells. However, a reduced number of infected cells, especially in *L. esculenta*, and the presence of amyloplasts in *P. vulgaris* are in accordance with a reduced nitrogen fixation activity. In *P. vulgaris* nodulated with the *A. tumefaciens* transconjugants, the acetylene reduction activity per nodule is, on the average, half of the activity per nodule obtained with the original *R. phaseoli* CFN299. Amyloplasts would accumulate as a result of the reduced energy consumed as less nitrogen is fixed. We have also observed that *A. tumefaciens*-induced nodules have an earlier senescence than CFN299 nodules.

The *A. tumefaciens* transconjugants, even the one containing the whole set of plasmids from strain CFN299, do not behave in symbiosis like the original CFN299. This would imply that the chromosome has a symbiotic role; there are either genes lacking in *A. tumefaciens* or genes interfering with the normal symbiotic process. Chromosomal genes of *R. phaseoli* CFN42 participating in symbiosis have been

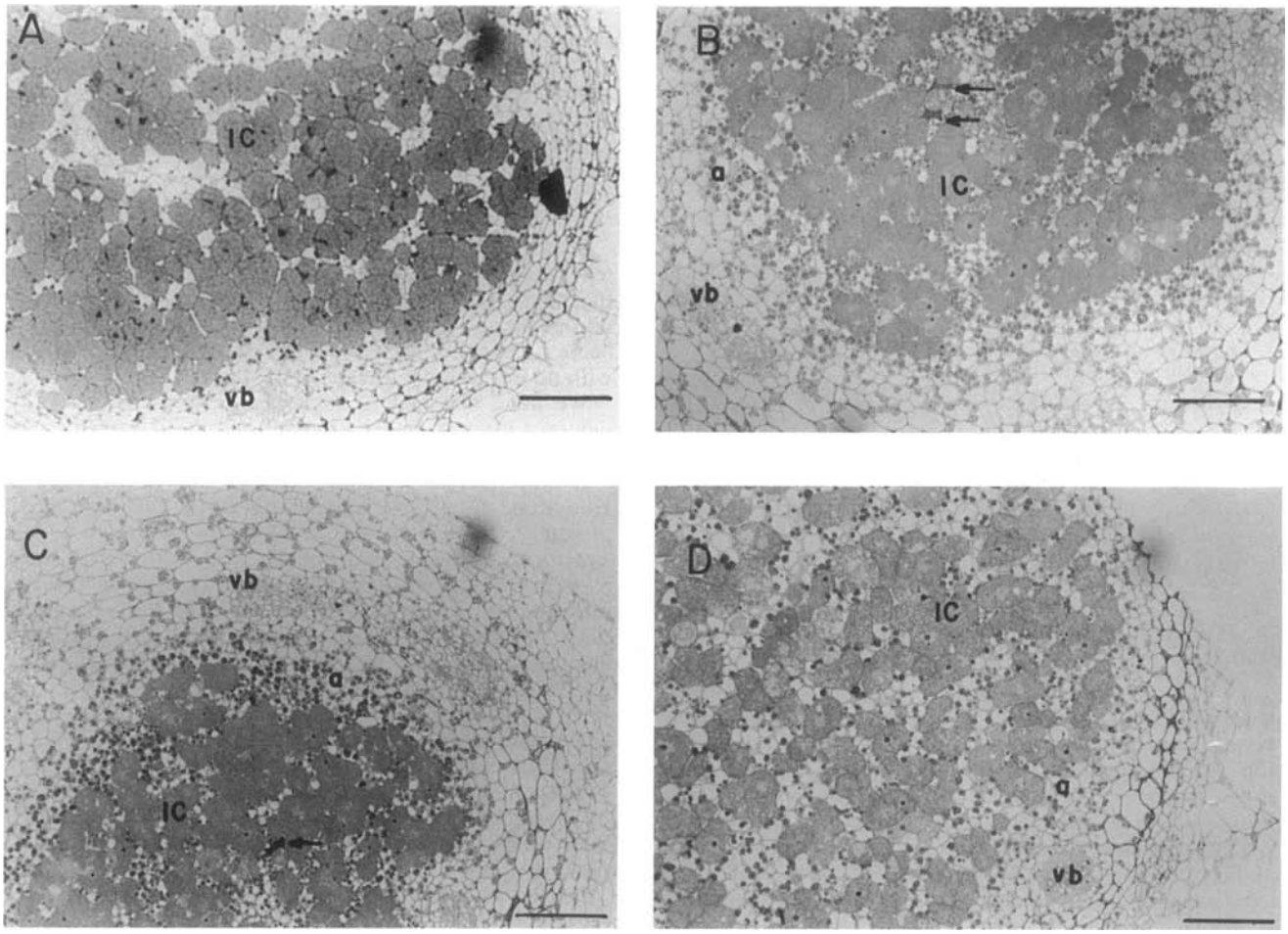

FIG. 5. Longitudinal sections of *P. vulgaris* nodules induced by (A) *R. phaseoli* CFN299 and by *A. tumefaciens* transconjugants (B) A123, (C) A13, and (D) A3. IC, Infected cells; vb, vascular bundles; a, amyloplasts. The arrows indicate darker zones (see text). Bars, 100 μm.

described (23). The importance of the bacterial background is also illustrated with the *R. meliloti* transconjugants carrying the Sym plasmid of CFN299; these do not fix nitrogen in *P. vulgaris*. *R. meliloti* has megaplasmid a (symbiotic) and megaplasmid b, whose role in the *P. vulgaris* and *L. esculenta* symbiotic process is unknown. Differential gene expression depending on the bacterial background cannot be excluded.

In a previous report, we showed that *P. vulgaris* may be nodulated effectively by a wide range of *Rhizobium* strains

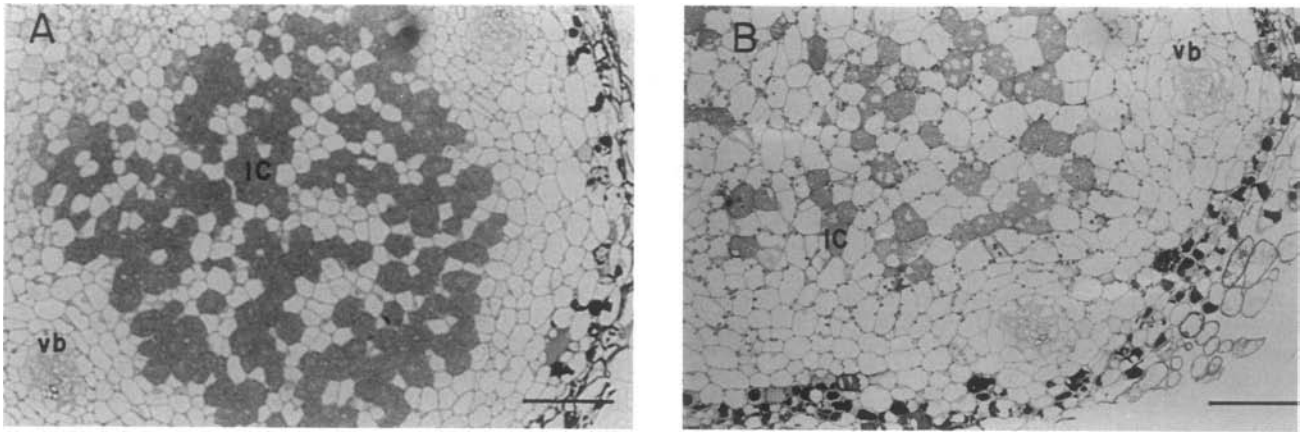

FIG. 6. Longitudinal sections of *L. esculenta* nodules induced by (A) *R. phaseoli* CFN299 and (B) *A. tumefaciens* transconjugant A3. For abbreviations, see the legend to Fig. 5. Bars, 100 μm.

isolated from different legumes (21). There are important differences among these *Rhizobium* strains (21; unpublished results). The nodule number and nitrogen-fixing levels obtained in these nonspecific strains resemble the nodulation performance of *A. tumefaciens* transconjugants in beans. Furthermore, *R. phaseoli* has been defined as a highly heterogeneous group on the basis of protein patterns (26) and antibiotic resistances (4). Taking all of these data into account, we propose that *P. vulgaris* may be quite a permissible host for nodulation, allowing many different bacteria to establish symbiosis with it. That is, symbiotic plasmids with *P. vulgaris*-nodulating genes may be contained in different bacterial backgrounds and direct the establishment of an effective symbiosis in beans.

Symbiotic genes with specificity for *P. vulgaris* seem to be present in different types of Sym plasmids. We have found that pSym of CFN299 belongs to a different compatibility group than does pSym of CFN42 (the standard reference strain with *nifH* reiterations), since we have been able to maintain both symbiotic plasmids in GMI9023 (unpublished results). Other *R. phaseoli* plasmids show differences; pSym9 is a nonconjugative plasmid of 275 megadaltons (16), pRP2J1 is a conjugative plasmid of 190 megadaltons (20), and in pSym of CFN23 symbiotic instability is associated with genetic rearrangements (29). It would be interesting to see whether nodulation genes with *P. vulgaris* specificity are equivalent. The analysis of the infection process (2, 5) induced by *R. phaseoli* CFN299 and by the transconjugants described would also be important.

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