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Nodules Are Induced on Alfalfa Roots by *Agrobacterium tumefaciens* and *Rhizobium trifolii* Containing Small Segments of the *Rhizobium meliloti* Nodulation Region

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Regions of the *Rhizobium meliloti* nodulation genes from the symbiotic plasmid were transferred to *Agrobacterium tumefaciens* and *Rhizobium trifolii* by conjugation. The *A. tumefaciens* and *R. trifolii* transconjugants were unable to elicit curling of alfalfa root hairs, but were able to induce nodule development at a low frequency. These were judged to be genuine nodules on the basis of cytological and developmental criteria. Like genuine alfalfa nodules, the nodules were initiated from divisions of the inner root cortical cells. They developed a distally positioned meristem and several peripheral vascular bundles. An endodermis separated the inner tissues of the nodule from the surrounding cortex. No infection threads were found to penetrate either root hairs or the nodule cells. Bacteria were found only in intercellular spaces. Thus, alfalfa nodules induced by *A. tumefaciens* and *R. trifolii* transconjugants carrying small nodulation clones of *R. meliloti* were completely devoid of intracellular bacteria. When these strains were inoculated onto white clover roots, small nodule-like protrusions developed that, when examined cytologically, were found to more closely resemble roots than nodules. Although the meristem was broadened and lacked a root cap, the protrusions had a central vascular bundle and other rootlike features. Our results suggest that morphogenesis of alfalfa root nodules can be uncoupled from infection thread formation. The genes encoded in the 8.7-kilobase nodulation fragment are sufficient in *A. tumefaciens* or *R. trifolii* backgrounds for nodule morphogenesis.

The development of a nitrogen-fixing nodule is a stepwise process that can be arrested in a number of different stages (see phenotypic classification by Vincent [45]). These stages generally have been elucidated by studying plants inoculated with mutant bacteria (45), although recessive plant mutations also affect nodule formation and nitrogen fixation (2, 6, 30, 43). Using transposon mutagenesis and other molecular biological techniques, several symbiotic (*sym*) genes recently have been identified. These include genes involved in nodulation (*nod* genes) (19, 23, 26, 38) and some of the later stages of the symbiosis (*fix* and *nif* genes) (4, 10, 14, 33, 37, 39, 41, 49).

The formation of a nodule includes a series of events that involve both symbiont and host. In the successful establishment of a nodule upon an alfalfa root, the earliest events are of primary significance. The early stages include root hair colonization and adhesion, shepherd's crook formation, infection thread development and proliferation, and nodule initiation (45). Infection thread penetration and branching are reported to occur either simultaneously with or precede nodule initiation (25). The later stages of nodule formation encompass such events as bacteroid differentiation, host cell enlargement, and the commencement of nitrogen fixation (for reviews, see references 5, 17, and 29).

Several recent reports have suggested that the early stages of nodule development can become uncoupled. For example, we reported that 20 to 40 kilobases (kb) of *Rhizobium meliloti* megaplasmid DNA, some of which has been identified as encoding initial nodulation functions (26) when introduced into *Agrobacterium tumefaciens* or *Escherichia coli*, can be expressed in these foreign backgrounds and can result in the formation of ineffective nodules on alfalfa roots (21). The nodules exhibited normal meristem and vascular development, but often were devoid of bacteria and lacked infection threads. In soybean roots, Calvert et al. (in press) have observed mitotic centers that are far removed from infection threads. These data suggest that infection thread penetration may not be an obligatory step for nodule initiation.

To test this hypothesis further, we have introduced smaller subclones containing conserved nodulation sequences from *R. meliloti* into *A. tumefaciens* and *Rhizobium trifolii*. The effect of these transconjugants on nodule development on alfalfa roots indicates that nodule initiation and development can be induced by relatively few *R. meliloti* genes in non-*R. meliloti* backgrounds and that infection thread formation is not necessary for nodule meristem initiation. Moreover, infection thread penetration into root hairs and invasion of nodule tissue by bacteria require genes outside the nodulation gene cluster examined in this investigation.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. Clone pRmSL26 has been described previously (26) and encodes at least some nodulation functions of *R. meliloti*. Clones pRmJ51 and pRmJ30 were constructed by ligating internal fragments of pRmSL26 into vectors pWB5a (W. Buikema, personal communication) and pLAFR1 (15), respectively (Jacobs and Long, in press). A map of this region is shown in Fig. 1.

Plasmids pRmSL26, pRmJ30, and pRmJ51 (Jacobs and Long, in press) were conjugated from *Escherichia coli* into *A. tumefaciens* strains A136 (*onc*$^-$, cured of Ti plasmid) and A348 (*onc*$^+$) or *R. trifolii* strain Sym$^+$ or Sym$^-$ by using the helper plasmid pRK2013 (7) following a previously published

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Characteristics	Source or reference
R. meliloti		
Rm1021	Nod$^+$ Fix$^+$, wild type Su47 Sm^r	(28)
R. trifolii		
ANU794	Nod$^+$ Fix$^+$ Sym$^+$, wild type	(8)
WC4	Heat-cured Sym$^-$ derivative of ANU794, Nod$^-$ Hac$^-$	This paper
ANU843	Nod$^+$ Fix$^+$ Sym$^+$, wild type	(36)
ANU845	Sym$^-$ derivative of ANU843	(38)
A. tumefaciens		
A348	*onc*$^+$ Occ$^+$ pTiA6	(16)
A136	*onc*$^-$ Nal^r derivative of C58 cured of Ti	(46)
E. coli		
LE392	*supE supF hsdR met trp-1 lac*	(12)
Plasmids		
pRmSL26	Tc^r, pLAFR1	(26)
pRmJ30	Tc^r, pLAFR1	Jacobs and Long, in press
pRmJ1	Tc^r, pBR322	Jacobs and Long, in press
pRmJ51	Tc^r, pLAFR1	Jacobs and Long, in press
pRK2013	*rep* ColE1 *Nm*^{r}	(7)
pWB5a	IncP *repRK2* Tc^r	W. Buikema

protocol (28). *A. tumefaciens* transconjugants were selected on M9-sucrose medium (28) plus 20 μg of tetracycline per ml, and *R. trifolii* transconjugants were selected on RDM-sucrose (44) containing 10 μg of tetracycline per ml.

The Sym$^-$ *R. trifolii* strain WC4 is a derivative of ANU794 (8) that has been cured of its symbiotic plasmid by growth at elevated temperatures by the procedure of Zurkowski and Lorkiewicz (50) (Hirsch et al., unpublished results). The loss of the symbiotic plasmid was verified by Eckhardt gel analysis (11), with modification by Buikema et al. (3) (see Fig. 5A). We were unable to obtain transconjugants from the mating of ANU794 and either pRmSL26 or pRmJ30 (Hirsch et al., unpublished results).

The presence of *R. meliloti* plasmids in the *A. tumefaciens* or *R. trifolii* strains were verified by agarose gels and Southern hybridization (40) or by the Grunstein-Hogness colony hybridization procedure (18) (data not shown). Nick translational labeling of pRmJ1, which contains an internal 8.7-kb *Eco*RI fragment of pRmSL26 (Fig. 1), was by procedures previously described (35).

Plant material. Seeds of alfalfa (*Medicago sativa* L. cultivar Iroquois), white clover (*Trifolium repens* L.), and red clover (*Trifolium pratense* L.) were sterilized, planted, and inoculated as described previously (28). Seeds were also planted on medium containing 20 mM KNO_3 (42). Root hair curling analyses were performed by growing seedlings in Fahraeus slide assemblies containing liquid medium (13, 20).

Recovery and identification of bacteria from nodules. Nodules induced by the *A. tumefaciens* or *R. trifolii* transconjugants were scored, photographed, and either squashed after surface sterilization to recover the bacteria (21) or prepared for microscopy. Colonies recovered from nodules were tested for tetracycline (10 μg/ml) resistance and checked for the presence of plasmids by the same techniques used to verify the strain constructs.

Light and electron microscopy. Nodules were prepared and embedded in plastic resins for light and electron microscopy or critical point dried, mounted on stubs, and coated for scanning electron microscopy as described previously (20).

RESULTS

Bacterial plasmids and strains. Plasmid pRmSL26 previously has been shown to contain at least some nodulation gene sequences of *R. meliloti* strain 1021 (26). Insertion of transposon Tn*5* into this region inactivates these genes and results in a Hac$^-$ Nod$^-$ (root hair curling, nodule development; 45) phenotype (20). Plasmid pRmJ30 is an 8.7-kb *Eco*RI internal fragment of pRmSL26 and includes the sites into which insertional mutations have occurred (mutants 1126 and 1027). Therefore, this region is essential for nodulation (26). Plasmid pRmJ51, an internal 5.5-kb *Pst*I fragment of pRmJ30, represents part, but not all, of the DNA required for nodulation (Jacobs and Long, in press).

The parent *A. tumefaciens* strains (A348 and A136) did not include root nodules on over 100 alfalfa plants tested. Likewise, no response was observed on red and white clover roots with the parent *A. tumefaciens* strains. *A. tumefaciens* carrying the vector pLAFR1, which confers tetracycline resistance, also were unable to elicit nodule development (>100 plants tested).

The *sym* plasmid cured (Sym$^-$) strains of *R. trifolii* (WC4 and ANU845) were unable to induce root hair curling (Hac$^-$) or nodules (Nod$^-$) on roots of white and red clover (see Table 3).

Root hair curling. Very little root hair curling or deformation was observed on alfalfa roots inoculated with A136 and A348 transconjugants carrying pRmJ30 and pRmJ51 even after 14 days in the Fahraeus slide assemblies. The degree of response did not vary with the size of *R. meliloti* insert or whether the Ti plasmid was present or absent. We occasionally observed root hair branching or waviness, but we did not see marked root hair hypertrophy, "shepherd's crooks," or corkscrews as reported previously (Fig. 2A) (21). Thus, these transconjugants were Hac$^-$ on alfalfa roots. No infection threads were found in any hairs along the length of the root even after 4 weeks of observation. A similar response was observed on alfalfa root hairs after inoculation with *R. trifolii* transconjugants (Fig. 2B).

Nodule development on alfalfa roots. *A. tumefaciens* cells carrying either pRmSL26, pRmJ30, or pRmJ51 elicited the development of white, ineffective nodules on roots of alfalfa, except for A136(pRmJ51) (Table 2). In contrast to wild-type *R. meliloti* infections, where nodules appeared 7 to 14 days after inoculation, nodules induced by *A. tumefaciens* cells carrying *R. meliloti* sequences were not apparent until 3 to 4 weeks post-inoculation.

Fewer than five nodules were found on the root system of any one positively responding plant (Fig. 2C). Moreover, the

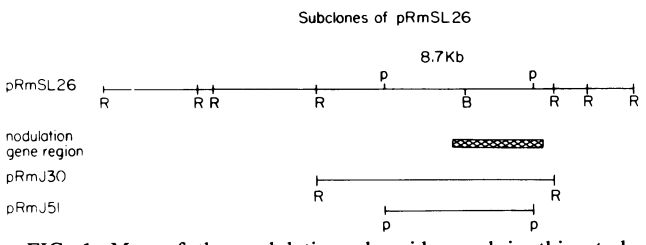

FIG. 1. Map of the nodulation plasmids used in this study. *Bam*HI and *Pst*I sites are given for the 8.7-kb fragment. Plasmid pRmSL26 is described in reference 26.

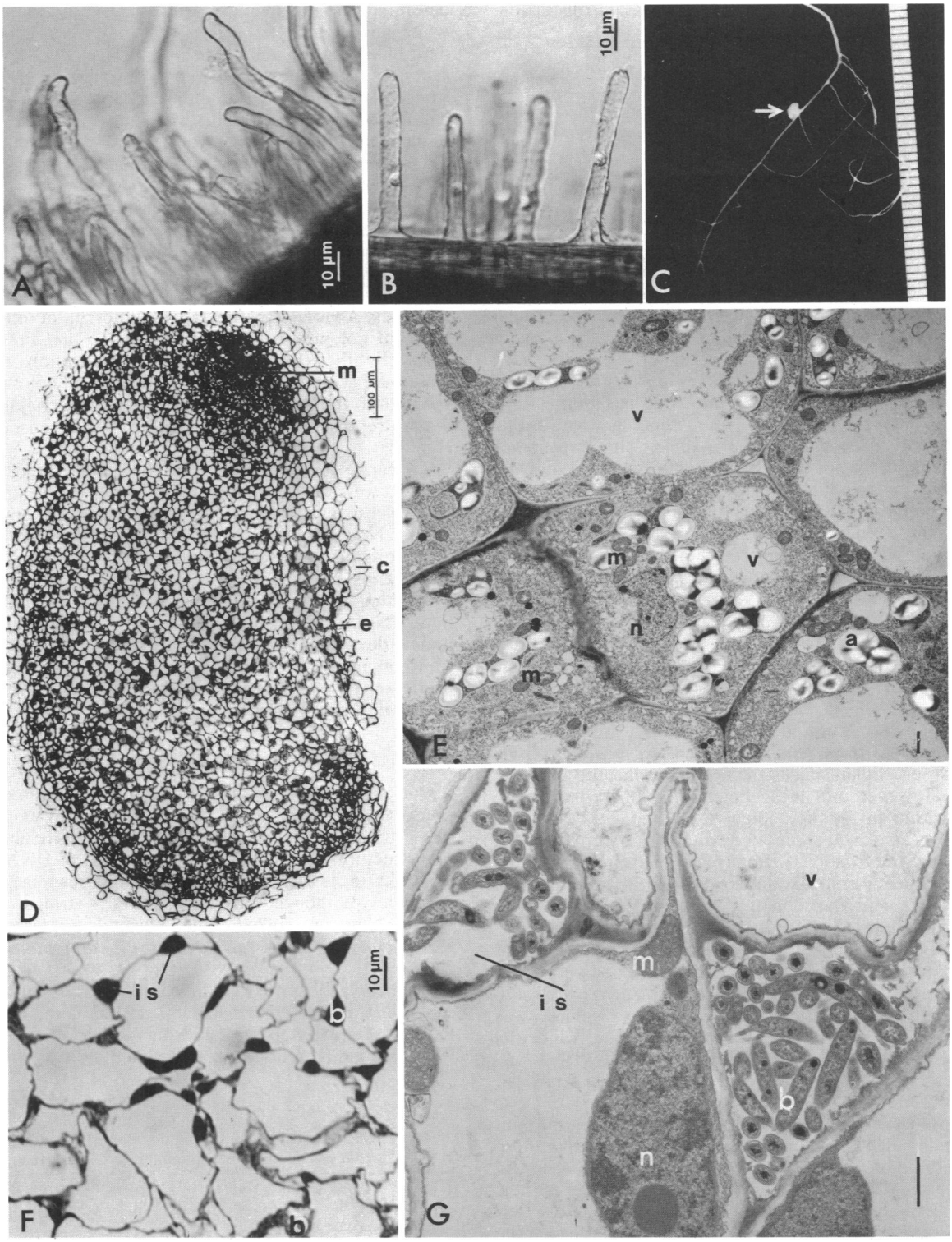

FIG. 2. Alfalfa roots. (A) Hac$^-$ response of root hairs inoculated with *A. tumefaciens* A136(pRmJ30). ×600. Bar, 10 µm. (B) Hac$^-$ response of root hairs inoculated with *R. trifolii* WC4(pRmSL26). ×600. Bar, 10 µm. (C) Tumorous appearing nodule on alfalfa root (arrow). Inoculation was with *A. tumefaciens* A348(pRmJ51). (D) Light micrograph of a section through a nodule induced by *A. tumefaciens* A348(pRmJ30). A meristem (m) is found at the distal end of the nodule. An endodermis (e) separates the main body of the nodule from the nodule cortex (c). ×110. Bar, 100 µm. (E) Transmission electron micrograph of internal cells of a nodule induced by *A. tumefaciens* A348(pRmJ30). The cells contain vacuoles (v), amyloplasts (a), mitochondria (m), and a nucleus (n), but no bacteria. ×3,800. Bar, 1 µm. (F) Light micrograph of nodule cells induced by *A. tumefaciens* A136(pRmJ30) Bacteria (b) are confined to intercellular spaces (is). ×600. Bar, 10 µm. (G) Transmission electron micrograph of cells in (F). ×9,800. Bar, 1 µm.

TABLE 2. Characteristics of *R. meliloti* clones introduced into *A. tumefaciens*

A. tumefaciens strain	Insert size (kb)	% Nodulation[a]
A348(pRmSL26)	20	15.4
A348(pRmJ30)	8.7	23.8
A348(pRmJ51)	5.5	13.6
A136(pRmSL26)	20	12.1
A136(pRmJ30)	8.7	14.6
A136(pRmJ51)	5.5	<3

[a] (Number of tubes nodulated/total number of tubes inoculated) × 100.

frequency of appearance of nodules on alfalfa roots in response to these clones was low, except for transconjugants carrying pRmJ30 (Table 2). However, the nodules exhibited a consistent morphology. They were unusually large and gall-like, averaging 2 to 3 mm in diameter, although some smaller ones developed. The tumorous morphology resulted from several discrete meristematic regions that were present on each individual nodule. This appearance contrasted with that of cylindrical nodules induced by wild-type *R. meliloti*, in which one or occasionally two meristems are located at the distal end. Unlike wild type-induced nodules, these nodules were positioned frequently at the points where lateral roots emerge (Fig. 2C).

The internal structure of nodules induced by *A. tumefaciens*(pRmJ30 or pRmJ51) transconjugants were identical. Histological examination of these nodules showed them to be devoid of bacteria and visible infection threads (Fig. 2D). The host cell cytoplasm was often displaced to the periphery of the cell, and the nodule cells contained many large amyloplasts (Fig. 2E). Several peripherally positioned vascular bundles traversed the nodule (data not shown). A few nodules induced by *A. tumefaciens-R. meliloti* hybrids showed intercellular spaces occupied by bacteria (Fig. 2F and G). However, no intracellular infection threads were present within any of the nodules examined.

Nodules also were induced on alfalfa roots by *R. trifolii*(pRmSL26) and *R. trifolii*(pRmJ30) transconjugants, but at a frequency considerably lower (4 to 10%) than that of the *A. tumefaciens* transconjugants (Table 3). The morphology of the nodules was identical to that described above.

Effect of transconjugants on clover roots. The *A. tumefaciens* and Sym$^-$ *R. trifolii* transconjugants induced very small (approximately 1-mm) protrusions on white clover roots 4 to 5 weeks after inoculation (Fig. 3A). No response was observed on red clover roots. On the other hand, white and red clover plants inoculated with strain ANU 843 (Sym$^+$ *R. trifolii*) carrying pRmSL26 and pRmJ30 developed normal, red, Fix$^+$ nodules (Fig. 3B, Table 3).

When we examined the clover root protrusions at the light and scanning electron microscope level, we found that these resembled modified lateral roots (Fig. 3D and E). A single, central vascular bundle was present rather than the multiple peripheral bundles that characterize a nodule. However, there was no defined root cap, and the cells positioned at the distal end were not densely cytoplasmic. Some of these protrusions were green; plastids with a few stacked thylakoids were evident within cortical cells (Fig. 3C).

In some protrusions, dark deposits accumulated within the host cell vacuole (Fig. 3C and D). These deposits are similar to those reported by Pankhurst (31) in tumor-like growths induced on *Trifolium pratense* roots in response to *R. trifolii* mutant strains. Based on histochemical staining, the deposits appeared to consist of protein (31). We did not determine the biochemical nature of the deposits found in white clover root cell vacuoles, but they were found to stain dark blue with toluidine blue (Fig. 3D).

A comparison between a typical lateral root and a clover root protrusion shows the differences in structure (Fig. 3F and G). No infection threads were observed in any of these protrusions, but bacteria were found within intercellular spaces near the periphery of the root.

Recovery of bacteria from nodules. Bacteria recovered from nodules were tested for tetracycline resistance and plasmid content as described above. Three or four nodules were squashed for each transconjugant, and two separate isolations for each transconjugant were tested further. Tetracycline-resistant colonies were recovered from the squashes. A variable percentage, but not all, of tetracycline-resistant colonies still contained the *R. meliloti* insert as shown by filter (Fig. 4) or colony hybridizations (Table 4). However, genomic digests indicated that all colonies corresponded to the original *A. tumefaciens* transconjugants and did not represent *Rhizobium* spp. or *E. coli* transconjugants (Fig. 5B).

To verify further that the bacteria recovered from the nodules were not contaminating rhizobial species, the tetracycline-resistant colonies were reinoculated onto alfalfa plants. Nodules identical in overall structure to those induced originally were developed, albeit at a lower frequency in some instances (Table 4).

Addition of nitrate and culture medium. The addition of 20 mM KNO_3 to the culture medium completely repressed nodule development elicited by *A. tumefaciens* or *R. trifolii* transconjugants in over 20 trials. Also, no swellings or protrusions were observed on inoculated cover roots supplied with 20 mM KNO_3.

DISCUSSION

Our experiments show that cloned DNA from a nodulation gene region of *R. meliloti* conferred upon either *onc*$^+$ or *onc*$^-$ strains of *A. tumefaciens* the ability to induce nodule-like structures on alfalfa roots. A segment of DNA as small as 5.5 kb in *A. tumefaciens* induced this response at a low frequency, although only in the *onc*$^+$ strains. Previous studies have introduced intact plasmids or larger cloned *Rhizobium* spp. DNA segments into *A. tumefaciens* (1, 22, 24, 42, 47).

The neoplastic growths induced by the *A. tumefaciens* and *R. trifolii* transconjugants developed from inner cortical cells that subsequently formed a distal nodule meristem. Like wild type-induced nodules, vascular bundles were located

TABLE 3. Frequency of nodule development induced by *R. trifolii* transconjugants on alfalfa and clover roots

Strain	% Nodulation[a] on:		
	Alfalfa	White clover	Red clover
ANU843	0	100	100
ANU794	0	100	100
ANU843(pRmSL26)	4.0	100	100
ANU843(pRmJ30)	7.1	100	95
ANU845	0	0	0
WC4	0	0	0
WC4(pRmSL26)	9.5	0	0
ANU845(pRmJ30)	4.0	0	0
WC4(pRmJ30)	10	0	0

[a] (Number of tubes nodulated/total number of tubes inoculated) × 100.

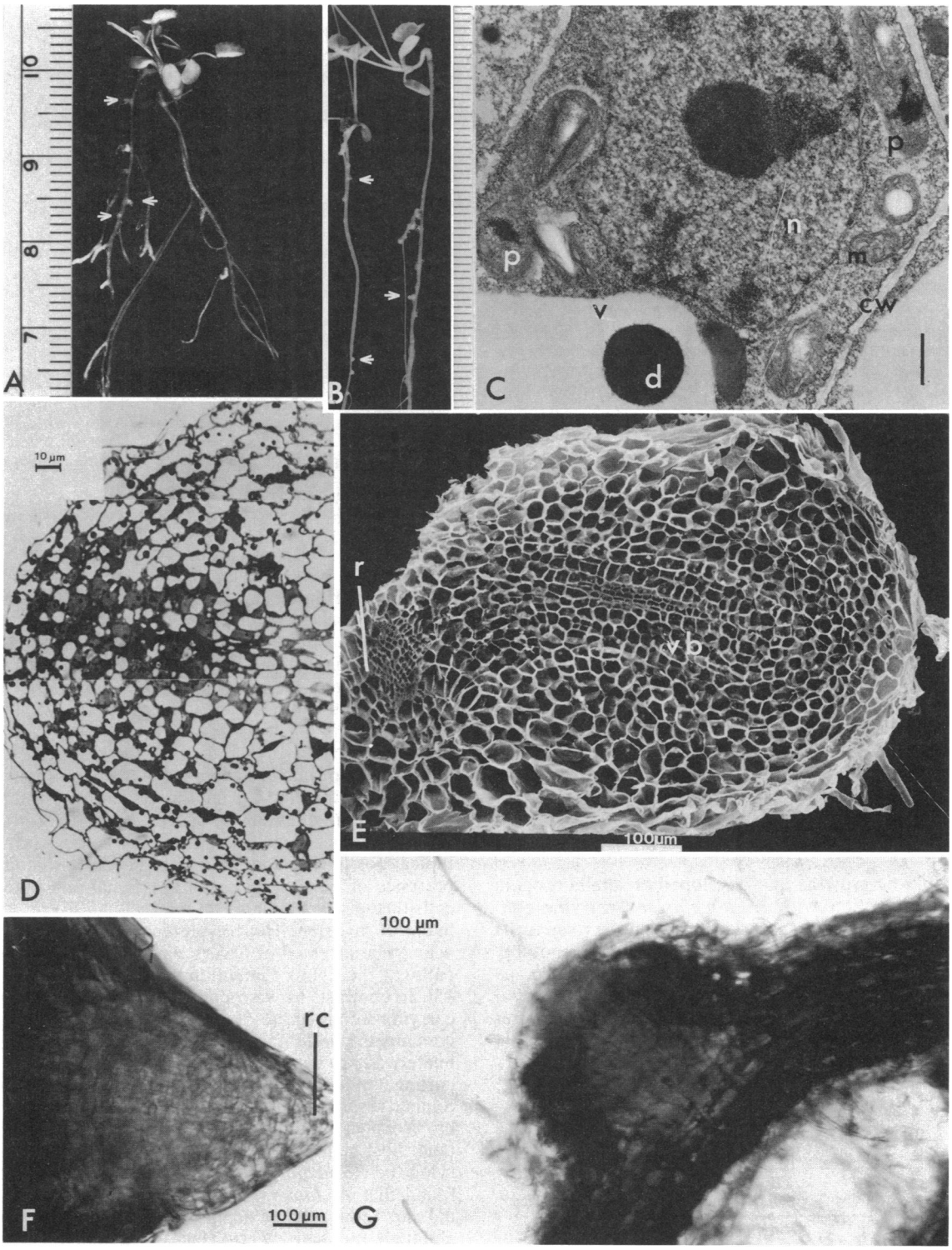

FIG. 3. Clover roots. (A) Protrusions (arrows) induced on white clover roots by *R. trifolii* WC4(pRmSL26). (B) Nodules (arrows) induced on white clover roots by *R. trifolii* ANU843(pRmJ30). (C) Transmission electron micrograph enlargement of cells from (D). The plastids (p) contain stacked lamellae, little starch, and phytoferritin. The cell possesses a nucleus (n), mitochondria (m), and cell walls (cw), but no bacteria. An electron-dense deposit (d) is present in the vacuole (v). ×9,800. Bar, 1 µm. (D) Light micrograph of a longitudinal section of a clover root protrusion induced by *A. tumefaciens* A136(pRmSL26). ×400. Bar, 10 µm. (E) Scanning electron micrograph of a longitudinal cut through a clover root protrusion induced by *A. tumefaciens* A348(pRmJ51). The vascular bundle (vb) connection to the main root (r) is maintained. ×140. Bar, 100 µm. (F) Lateral root of white clover. The root meristem is covered by a root cap (rc). ×95. Bar, 100 µm. (G) Clover root protrusion induced by *A. tumefaciens* A348(pRmSL26). ×95. Bar, 100 µm.

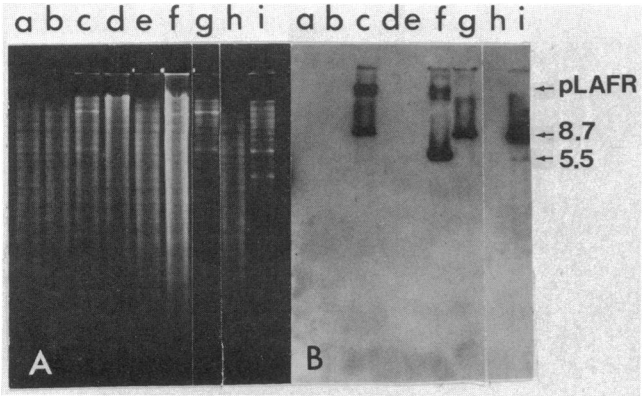

FIG. 4. (A) Agarose gel of *Eco*RI-digested total DNA isolated from *A. tumefaciens* parent strains (lanes a and b), recovered bacteria (lanes c through f, h) and controls (lanes g and i). Lanes: a, A136; b, A348; c, A136(pRmJ30); d, A136(pRmSL26); e, A136(pRmJ51); f, A348(pRmJ51); g, pRmSL26; h, A136(pRmSL26); i, pRmJ1. (B) Autoradiogram prepared from nitrocellulose filter from gel shown in (A) and probed with pRmJ1.

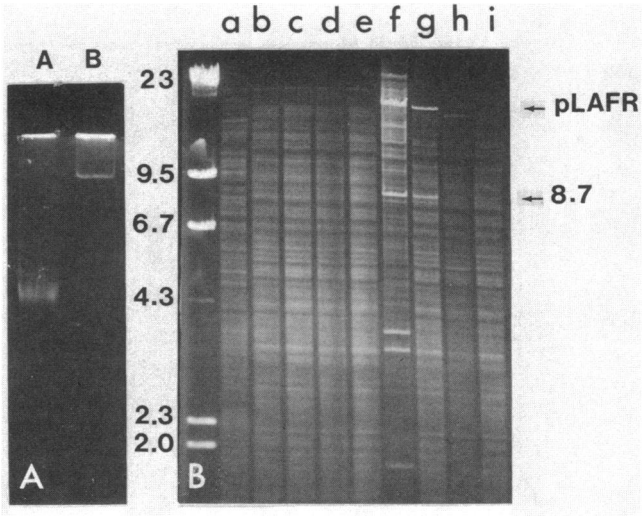

FIG. 5. (A) Eckhardt gel analysis of (A) *R. trifolii* WC4 and (B) *R. trifolii* ANU794. (B) Agarose gel of *Eco*RI-digested total DNA isolated from parent (lanes b and c), recovered (lanes d, e, and g through i), and control (a and f) colonies. Lanes: a, *R. meliloti* Rm1021; b, *A. tumefaciens* A348; c, *A. tumefaciens* A136; d, A348(pRmSL26); e, A136(pRmSL26); f, pRmJ1; g, A136(pRmJ30); h, A348(pRmJ51); i, A136(pRmJ51).

peripherally, and the body of the nodule was separated from the cortex by an endodermis. Furthermore, nodules were induced only on alfalfa, except for the Sym^+ strains of *R. trifolii* carrying *R. meliloti* nodulation genes. These elicited nodule formation on both hosts, albeit at a much lower frequency on alfalfa.

The transconjugant-induced nodules differed from wild-type and mutant *R. meliloti*-induced nodules in several important features, however. First, several discrete meristems often were evident which gave the nodule a tumorous appearance. Nodules induced by wild-type *R. meliloti* generally have one or occasionally two meristems. Second, the internal cells were devoid of bacteria and lacked infection threads. Third, fewer than five nodules developed per root system, an observation which contrasts with previous reports that noted that numerous nodules are formed on roots after inoculation with ineffective strains of *Rhizobium* spp. (27, 49).

The neoplastic growths that developed on alfalfa roots in response to infection with *A. tumefaciens* carrying *Rhizobium* spp. nodulation sequences were not crown galls elicited by the presence of the *A. tumefaciens* genotype. Several observations support this statement. First, onc^- as well as onc^+ *A. tumefaciens* strains containing *R. meliloti* clones induced nodules on alfalfa roots, although the frequency was greater by the onc^+ strains. Second, nodule development was repressed when nitrate was added to the medium. Furthermore, octopine was not detected in the nodules induced by A348 transconjugants (A. Hirsch, D. Drake, and G. Riedel, unpublished results). Nodule morphology is very distinctive; the growth developed on alfalfa roots in response to the *A. tumefaciens* and *R. meliloti* transconjugants fulfill the developmental criteria of what defines a nodule.

Roots inoculated with *A. tumefaciens*(pRmJ30) and *A. tumefaciens*(pRmJ51) developed nodules without infection threads or intracellular bacteria. Bacteria were found occasionally within intercellular spaces, but did not invade roots through the usual mechanism of root hair penetration. The typical position of nodules at lateral root junctions and the presence of bacteria between epidermal and outer cortical cells further substantiates an intercellular (5) or crack entry mode of invasion. Hence, these heterologous bacteria appear to be arrested at a very early stage in the nodulation pathway, i.e., before infection thread formation (hence Inf^-; 45). In contrast, as we reported earlier (21), *A. tumefaciens* carrying a cointegrate of pRmSL26 and pRmBE2 (pRmBE2 contains the *nod*, *nif*, and intervening region and is approximately 50 kb) induced nodules with a Hac^+ Inf^+ Bar^- (hair curling, infection thread, bacteria release; 45) phenotype. Similar results regarding the Inf^+ phenotype were reported by Wong et al. (47), who found infection threads in the outermost cells of nodules induced by *A. tumefaciens* carrying the *R. meliloti* plasmid. However, Truchet et al. (42) found that *A. tumefaciens* transconjugants carrying pSym did not induce bona fide infection threads in root hairs, although the hairs were Hac^+. Threadlike structures or infection traces related to intercellular spaces infiltrated by agrobacteria were found. The alfalfa nodules, however, were devoid of bacteria and resembled those described here for *A. tumefaciens* transconjugants carrying pRmJ30 and pRmJ51.

The more frequent induction of nodules by A348(pRmJ30) and A348(pRmJ51) compared with the onc^- transconjugants may result from uncharacterized genes on the Ti plasmid.

TABLE 4. Verification of tetracycline-resistant bacteria recovered from nodules induced by *A. tumefaciens-R. meliloti* hybrids

A. tumefaciens strain	Colony hybridization[a]	% Nodule development[b]
A348(SL26)	43/93	6.4
A136(SL26)	3/60	5
A348(pRmJ30)	48/100	8.4
A136(pRmJ30)	NT[c]	10
A348(pRmJ51)	NT	10
A136(pRmJ51)	NT	10

[a] Number positive/total colonies probed.
[b] (Number of tubes nodulated/total number of tubes inoculated) × 100.
[c] NT, Not tested. Positive results were obtained by Southern filter hybridization.

DNA homology between parts of the Ti plasmid and the *Rhizobium* spp. plasmids has been reported (19, 32), and this may indicate some shared functions. We found previously that pRmSL26 and cointegrate plasmids in *E. coli* induced nodules on alfalfa roots at a low frequency (21). However, *E. coli* or *R. trifolii* strains carrying pRmJ30 induced nodules either not at all or at a very low frequency (≤10%) respectively, whereas virulent *A. tumefaciens*(pRmJ30) elicited nodule development on 19 out of 80 tubes inoculated. Whether the lowered frequency in *E. coli* or *R. trifolii* backgrounds compared with *A. tumefaciens* is because of lack of function or poor expression of the *R. meliloti* insert in these backgrounds as opposed to better expression in or shared function with the *A. tumefaciens* genome is unknown.

Our study strongly suggests that the morphogenesis of nodules can be uncoupled from infection thread formation. The genes encoded in the 8.7- and 5.5-kb fragments appeared to suffice in a virulent *A. tumefaciens* background for nodule morphogenesis, and the 8.7-kb plasmid sufficed even in an *onc*$^-$ strain. That no infection threads formed indicates that the genes of the 8.7-kb region are not sufficient for root hair entry of alfalfa via infection threads. However, the presence of bacteria in the intercellular spaces of *A. tumefaciens*-induced nodules implies that some genes in pRmJ30 influence *Rhizobium* spp. interactions with plant cell walls.

Genetic studies have indicated that *R. meliloti nod* genes are located in two clusters. Kondorosi et al. (23) found that one of these, which is equivalent to pRmJ30, represents conserved nodulation functions and appears not to encode all of the information necessary for the nodulation of alfalfa. The other cluster lying between the 8.7-kb region and *nif* affects host specificity (23). The 8.7-kb region appears to encode "common" nodulation genes in that it hybridizes with corresponding nodulation regions from other *Rhizobium* spp. (A. Kondorosi and E. Kondorosi, personal communication; 34) and functions on other plant hosts (S. Long and R. Fisher, unpublished results). Therefore, this cluster may code for "universal" nodulation functions. The host specificity genes present elsewhere may encode information necessary for the expression of hair curling and infection thread formation phenotypes, steps which have been postulated to play a primary role in host specificity (48). Our previous results (21), whereby Hac$^+$ Inf$^+$ Bar$^-$ nodule phenotype is elicited by *A. tumefaciens* transconjugants carrying this segment of *R. meliloti* DNA, further substantiate this hypothesis.

If bacterial invasion via root hair penetration is not a prerequisite for nodule formation, one possibility is that the "common" nodulation genes of the *R. meliloti* megaplasmid alone can initiate nodule development by stimulating mitoses in the inner root cortex. Thus, the stages of root infection (i.e., bacterial attachment, root hair curling, penetration and proliferation of infection threads, and bacterial release into host cells) and nodule morphogenesis (mitoses in the root inner cortex, establishment of a meristem, and nodule formation), which in normal *Rhizobium* spp.-legume symbiosis are linked intimately, can be uncoupled. We are investigating these correlations further.

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