Identification of *Rhizobium* Plasmid Sequences Involved in Recognition of *Psophocarpus*, *Vigna*, and Other Legumes

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Abstract. Symbiotic DNA sequences involved in nodulation by *Rhizobium* must include genes responsible for recognizing homologous hosts. We sought these genes by mobilizing the symbiotic plasmid of a broad host-range *Rhizobium* MPIK3030 (= NGR234) that can nodulate *Glycine max*, *Psophocarpus tetragonolobus*, *Vigna unguiculata*, etc., into two Nod$^-$ *Rhizobium* mutants as well as into *Agrobacterium tumefaciens.* Subsequently, cosmid clones of pMPIK3030*a* were mobilized into Nod$^+$ *Rhizobium* that cannot nodulate the chosen hosts. Nodule development was monitored by examining the ultrastructure of nodules formed by the transconjugants. pMPIK3030*a* could complement Nod$^-$ and Nif$^-$ deletions in *R. leguminosarum* and *R. meliloti* as well as enable *A. tumefaciens* to nodulate. Three non-overlapping sets of cosmids were found that conferred upon a slow-growing *Rhizobium* species, as well as on *R. loti* and *R. meliloti*, the ability to nodulate *Psophocarpus* and *Vigna*, thus pointing to the existence of three sets of host-specificity genes. Recipients harboring these *hsn* regions had truly broadened host-range since they could nodulate both their original hosts as well as MPIK3030 hosts.

RHIZOBIUM in association with leguminous plants fix nitrogen in specialized root structures called nodules. Nodules arise as a result of coordinated expression of both bacterial and plant genes. 20–30 plant proteins are specifically produced in nodules (4), and a number of *Rhizobium* genes involved in nodule development have been identified. These include genes that are required for synthesis of the enzyme nitrogenase (*nif*)[1] as well as some of the nodulation (*nod*) genes involved in root-hair curling (12, 21), an essential early nodulation step in many legumes (27). Nodulation genes are functionally conserved between *R. leguminosarum* and *R. meliloti* (2, 11, 23), and there is a 69–72% nucleotide sequence homology between the *nod* ABC genes of these two species (35, 41). In addition, DNA homology is demonstrable between the *nod* genes of *Rhizobium* spp and chromosome/plasmid DNAs of most other *Rhizobium* species (6). This DNA sequence homology between *nif* and *nod* genes of different rhizobia allowed identification of the symbiotic plasmid in the broad-host range *Rhizobium* spp MPIK3030 (= NGR 234) and localization of the *nif* and *nod* gene clusters (1, 8, 29, 33).

As speciation within the Rhizobiaceae is defined by host-range, the similarity in *nod* genes from different *Rhizobium* species suggests that they are not the sole determinant of host-specificity. Rather, another set of genes must exist that allow specific rhizobia to recognize certain hosts. Indeed, Kondorosi et al. (21) have identified a region on the *R. meliloti sym* plasmid, located between *nod* and *nif*, that appears to be involved in host-specificity. Mutations at this locus could not be complemented by the *sym*-plasmid from *R. leguminosarum* (21).

In this study we attempted to identify loci on the 460 kbp *sym*-plasmid of MPIK3030 (MPIK3030 also contains a larger, cryptic plasmid), that contain *hsn* genes for the hosts *Psophocarpus tetragonolobus*, *Vigna unguiculata*, and related legumes by mobilizing whole plasmids and cosmid-clones of the plasmids into rhizobia that cannot nodulate these hosts. Fully functional, wild-type rhizobia (i.e., can nodulate their homologous hosts) were chosen as the recipients on the assumption that their intact *nod* genes would be functional on heterologous hosts (2, 11, 23).

Materials and Methods

Bacterial Strains

All strains used are listed in Table I. In general *E. coli* strains were grown on LB (37), and *Rhizobium* isolates on TY (3), although *Rhizobium* sp CB376 was grown on rhizobial minimal medium (in gl^{-1}: mannitol, 10; K_2HPO_4,

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[1] *Abbreviations used in this paper: hsn,* rhizobial genes involved in recognition of the host legume; *fix,* genes required for symbiotic nitrogen fixation; *nif,* a class of *fix* genes required for synthesis of the enzyme nitrogenase; *nod,* genes required for nodulation; *onc,* oncogenic genes (of *Agrobacterium tumefaciens*).

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Table I. Strains and Plasmids Used

Organism	Strain/plasmid	Relevant characteristics	Source or reference
Agrobacterium tumefaciens	GV3101 = C58C1	Ti plasmid cured derivative of C58, Rif^R	22
E. coli	DH1	F^-, *recA*1	24
	FM15	F^-, *recA*	
	HB101	F^-, *recA*13, SM^R	5
	J5-3(RP4)	F^-, Ap^R, Nm^R, Tc^R	10
	SM10	C600 *recA* chromosomally int RP4 2-Tc::Mu, Km^R	39
Rhizobium sp	CB376	Nod^+ (*Lotononis bainesii*)	30
	MPIK3030 = NGR234	Nod^+ (*Vigna unguiculata*), Sm^R, Rif^R	40
	pMPIK3030*a*	460 kbp *sym* plasmid of MPIK3030	33
R. leguminosarum	JIIN6015	*nod-nif* deletion derivative of JIIN300, 5-FU^R, Sm^R, Rif^R	18
R. loti	NZP4010	Nod^+ (*Lotus pedunculatus*) = NZP2037 cured of pR1o2037*a*, Sm^R, Rif^R	32, C. E. Pankhurst[‡]
R. meliloti	AK1188 (= ZB121)	*nod-nif* deletion derivative of AK631, 5-FU^R, Rif^R	19, 21
	L5.30	Nod^+ (*Medicago sativa*), Sm^R, Rif^R	J. Denarié[§]
E. coli	pJB8	cosmid, Ap^R	17
	RG4.11	*tra*, Tc^R	28
	R68.45	*tra*, Cb^R, Nm^R, Tc^R	14
	RP4	*tra*, Cb^R, Nm^R, Tc^R	10
	RP4-4	*tra*, Ap^R, Km^S, Tc^R	15
	pWA45, 46, 54, 58, 59, 76, 88, 90* and 110*	pMPIK3030a clones in pJB8, see Table IV for further details	this paper

* pWA90 and pWA110 are identical.

[‡] Located at D.S.I.R., Palmerston North, New Zealand.

[§] Located at CNRS-INRA, Castanet-Tolosan, France.

2.05; KH_2PO_4, 1.45; NaCl, 0.15; NH_4NO_3, 0.5; $MgSO_4 \cdot 7\,H_2O$, 0.5; $CaCl_2$, 0.01; Ca-pantothenate, 1 μg; biotin, 1 μg; thiamine, 1 μg; and B & D micronutrients (9), 1 ml [pH 6.8]). *E. coli* was cultivated at 37°C and *Rhizobium* at 26°C. Antibiotics were used at the following concentrations (values for *E. coli* in parentheses) ($\mu g \cdot ml^{-1}$): ampicillin (Ap), 100 (100); carbenicillin (Cb), 100 (100); 5-fluorouracil (5-FU), 10 (not used); kanamycin (Km), 100–200 (25); neomycin (Nm), 100–200 (25); rifampicin (Rif), 100 (100); streptomycin (Sm), 250 (20); and tetracycline (Tc), 5 (10) (8).

Matings

Donor and recipient cells were grown in liquid culture to exponential phase, mixed in Eppendorf tubes (at a ratio of 1:1 in *E. coli* matings and 1:5 in *E. coli/Rhizobium* crosses) and forced together by 30-s centrifugation. After the supernatant was decanted, the cells were carefully resuspended in 50 μl medium and spread onto cellulose acetate filters (pore size 0.2 μm) on agar plates. Crosses were done under conditions that favored the slowest-growing parent. Matings were incubated from 4 to 60 h (depending on the recipient), and afterwards were resuspended and diluted in physiological saline (*E. coli* as recipient) or distilled water (for *Agrobacterium* and *Rhizobium*).

Plasmid Mobilization

As pMPIK3030*a* is not self-transmissible, it was made mobilizable by first introducing the *cis*-acting DNA recognition site for conjugative DNA transfer (Mob-site) from RP4. This was accomplished by conjugating the Tn5-Mob vector pSUP5011 (38) into MPIK3030 and selecting for Nm^R transconjugants. Introduction of RP4-4 into these Nm^R transconjugants then allowed mobilization of pMPIK3030*a*::Tn5-Mob into various *Agrobacterium* and *Rhizobium* strains using methods described by Simon (38). Cosmid clones of pMPIK3030*a* were mobilized by co-integrate formation: first, Tn5-Mob was conjugated into *E. coli* HB101 strains that contained the pJB8 cosmids and $\text{Ap}^R\text{Nm}^R\text{Sm}^R$ colonies selected. These were in turn mated with J5-3 (RP4-4), and $\text{Ap}^R\text{Sm}^R\text{Tc}^R$ colonies were selected and purified on ApNmTc-containing media. Finally, selected colonies were mated with various Rif^R rhizobia and the resulting transconjugants tested for nodulation on *Vigna unguiculata* as well as examined for plasmid content using the Eckhardt technique (13). Rhizobia were then re-isolated from the nodules, the antibiotic resistance makers checked, plasmid contents re-monitored, reactions with specific antibodies tested, and re-isolates were used to re-inoculate *V. unguiculata*. Occasionally, these re-isolates were also tested on *Glycine max*, *Lotus pedunculatus*, *Macroptilium atropurpureum*, *Medicago sativa*, and *Psophocarpus tetragonolobus*.

Plasmids

Plasmids were detected in agarose gels using the Eckhardt procedure (13) as modified by Simon (38). pMPIK3030*a* was isolated using the direct lysis procedure (6).

Cloning and Mapping Procedures

A clone-bank of pMPIK3030*a* (purified by passage through two cesium chloride gradients) was prepared in pJB8 (17, 33). Partial mapping of about one-third of pMPIK3030*a* was performed by single and double digestions with the restriction endonucleases *Bam*HI, *Eco*RI, and *Sma*I (*Xma*I), by partial digestion (*Bam*HI, *Eco*RI, and *Sma*I), by hybridization with various clones (33), and by deletion mapping. Clones that contained inserts were identified by colony hybridization (25) using nick-translated pMPIK3030*a* as the hybridization probe. Other recombinant DNA techniques are described in Broughton et al. (6) and Maniatis et al. (26).

Immunological Procedures

Antisera against the *Rhizobium* strains CB376, L5.30, MPIK3030, and NZP4010 were raised in rabbits and used to identify strains by their immunoprecipitation reactions (31).

Light and Electron Microscopy

(*a*) Root-hair curling. Fåhraeus slides were prepared by gluing (using transparent elastic silicone glue) a 24 × 60-mm glass coverslip to a normal 26 × 76-

mm microscope slide in a way that left a 1–1.5-mm gap between the two (43). The complete assemblies were autoclaved, filled with nitrogen-free B & D solution, and planted with a pre-germinated *Macroptilium atropurpureum* seedling. When root development was sufficient, the seedlings were inoculated with rhizobia and thereafter regularly examined under dark-field microscopy. Photographs were recorded on Kodak Ektachrome film. (*b*) Transmitted light- and electron-microscopy were performed as described by Wong et al. (45).

Plant Tests

Duplicate Leonard jars planted with three plants each of *Glycine max* cv Caloria, *Psophocarpus tetragonolobus* cv UPS99 or *Vigna Unguiculata* cv China (10% un-inoculated control jars as well as jars with 2.5 mM, 5 mM, and 7.5 mM KNO_3) were used to screen the transconjugants (9). In critical cases (e.g., in constructions that produced ineffective nodules), these experiments were repeated up to three times. Nodulation of *L. pedunculatus*, *M. atropurpureum*, and *M. sativa* was tested with seedlings growing on B & D agar (7, 9) in tubes. Acetylene reduction was measured on excised roots according to Williams and Broughton (44). Nodules were classed as effective if the rates of acetylene reduction were at least 10% of that obtained with plants inoculated with the wild-type MPIK3030. Partially effective nodules reduced 1–10% of that of plants inoculated with MPIK3030.

Results

Our general strategy for isolating the *hsn* genes of *Rhizobium* MPIK3030 was to mobilize first pMPIK3030*a*, then parts of pMPIK3030*a*, into rhizobia that cannot nodulate our test legumes, but were otherwise fully functional. Before we could do this, however, it was necessary to demonstrate that pMPIK3030*a* could be expressed in heterologous chromosomal backgrounds. We tested this by mobilizing pMPIK3030*a* into *A. tumefaciens* cured of the Ti-plasmid and into Nod^- derivatives of *R. leguminosarum* and *R. meliloti* carrying deletions in the *sym* plasmid (Fig. 1*a*). In each case the frequency of transfer of pMPIK3030*a*::Tn5-Mob into the different recipients was $\sim 1 \times 10^{-6}$ (Table II). Horizontal (Eckhardt) electrophoresis of a *R. meliloti* AK1188 (pMPIK3030*a*) transconjugant demonstrated the presence of pMPIK3030*a* along with the indigenous *R. meliloti* plasmids (Fig. 1*a*).

Only ~20% of *A. tumefaciens* transconjugants could nodulate *Psophocarpus* and *Vigna* and even then all nodules formed were ineffective. With *Vigna* this was because the nodules were simply callus-like outgrowths devoid of bacteroids (Fig. 2*b*). On the other hand, C58C1(pMPIK3030*a*::Tn5-Mob) transconjugants produced bacteroid-containing nodules on *Psophocarpus* (Fig. 2, *a*, *c*, and *d*). Few cortical cells were actually infected, however, and there was a massive accumulation of starch grains in the neighboring cells (Fig. 2*a*). Bacteroid density within infected cells was also low (Fig. 2*c*), although the bacteroids themselves seemed to have an intact peribacteroid membrane (Fig. 2*d*). Re-isolates from *Psophocarpus* nodules could re-nodulate both *Psophocarpus* and *Vigna*.

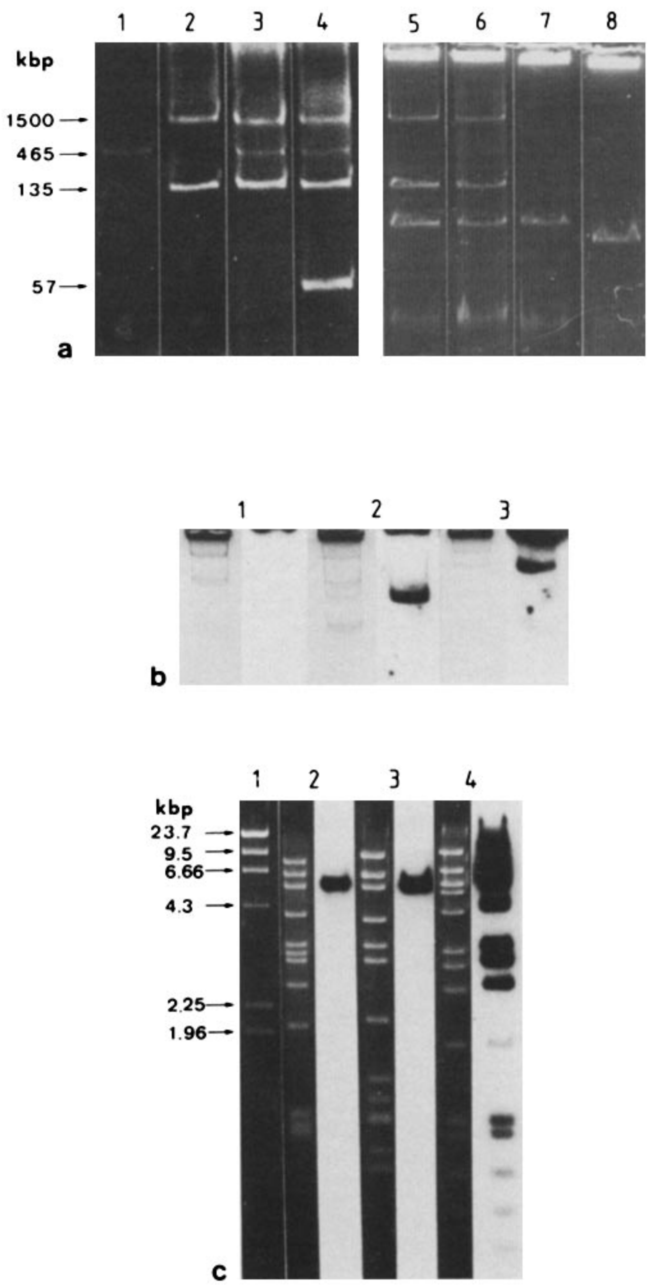

Figure 1. (*a*) Photograph of Eckhardt gel showing *R. meliloti* L5.30 and *R. loti* NZP4010 transconjugants harboring pMPIK3030*a* and various cosmid co-integrates. Lane *1*, MPIK3030; lane *2*, L5.30; lane *3*, L5.30(pMPIK3030*a*::Tn5-Mob); lane *4*, L5.30 pMPIK-3030*a*::Tn5-Mob, RP4.4); lane *5*, L5.30(pWA76::Tn5-Mob::RP4.4); lane *6*, L5.30(pWA88::Tn5-Mob::RP4.4); lane *7*, NZP4010-(pWA76::Tn5-Mob::RP4.4); lane *8*, NZP4010(pWA88::Tn5-Mob::RP4.4). kbp, kilobase pair. (*b*) Composite photograph showing hybridization of pWA88 to *R. meliloti* L5.30 transconjugants harboring a cosmid co-integrate and to pMPIK3030*a*. Lane *1*, L5.30; lane *2*, L5.30(pWA88::Tn5-Mob::RP4.4); lane *3*, MPIK3030. (*c*) Composite photograph showing hybridization of pWA88 to *Eco*RI-digested *hsn* cosmids. Lane *1*, λ DNA (*Hind*III) size markers; lane *2*, pWA54; lane *3*, pWA58; lane *4*, pWA88. The homologous bands in lanes *2*, *3*, and *4* are due to the cosmid cloning vector pJB8.

R. leguminosarum (pMPIK3030*a*) transconjugants were effective but unless they were maintained under constant antibiotic selection pressure they were unstable (Table II). For this reason they were not further studied. Presumably incompatibility exists between pMPIK3030*a* and a JIIN6015 plasmid. All *R. meliloti* (MPIK3030*a*) transconjugants tested could nodulate both *Psophocarpus* and *Vigna*, producing nodules that initially were either effective or ineffective (Table II). The major difference between infected cortical tissue of *V. unguiculata* and *P. tetragonolobus* concerned the size of the bacteroid-filled cells. Bacteroid-containing *Psophocarpus* cells had a volume about sixty times larger than those of *Vigna* (cf Fig. 3, *a* and *b*), and the bacteroid density within the *Psophocarpus* cells was greater (cf Fig. 3, *c* and *d*). In both species the peribacteroid membrane enclosed either one or

Table II. Transfer of pMPIK3030a::Tn5-Mob into Onc$^-$ Agrobacterium and into Nod$^-$ Rhizobium Strains

Recipient	Frequency of transconjugants per recipient	Nodulation of*		Remarks
		P. tetragonolobus	*V. unguiculata*	
A. tumefaciens				
C58C1	1×10^{-6}	2/2, I	2/10, I	Pseudo-nodules only on *Vigna* (Fig. 2)
R. leguminosarum				
JIIN6016	1×10^{-6}	nt	10/10, E	Unstable (incompatible)
R. meliloti				
AK1188	1×10^{-6}	8/8, I, E	10/10, I, E	Fig. 3

* Average no. of Leonard jar assemblies (three plants per jar) nodulated/no. of transconjugants tested. E, effective nodules; I, ineffective nodules; nt, not tested.

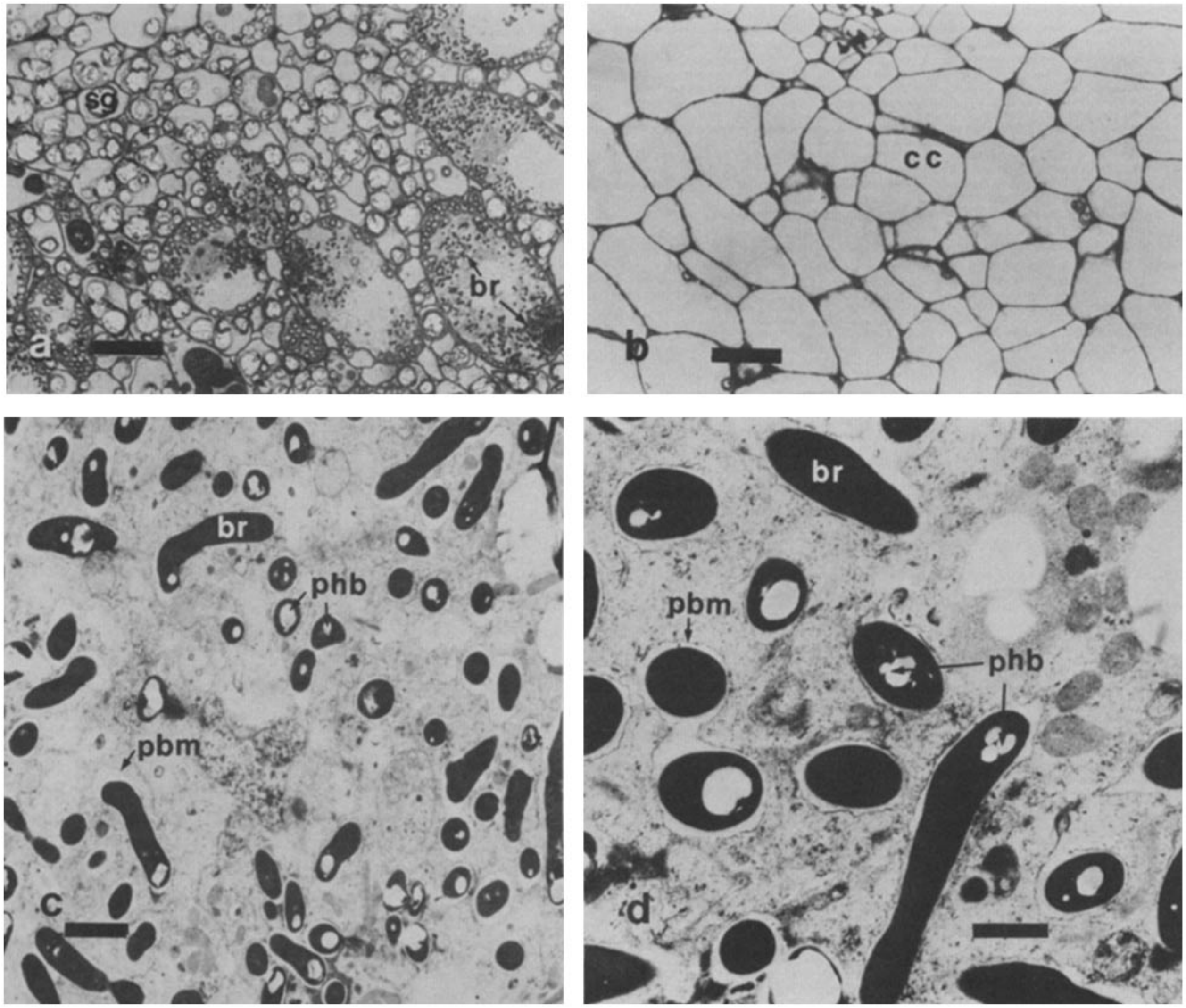

Figure 2. Light and electron micrographs of nodule tissues produced by *A. tumefaciens* C58C1(pMPIK3030*a*) transconjugants on *P. tetragonolobus* (*a*, *c*, and *d*) and *V. unguiculata* (*b*). (*a*) Light micrograph of a median longitudinal section of a 4–5-wk-old nodule. *sg*, starch grains; *br*, bacteroids. Bar, 20 μm. (*b*) Light micrograph of a median longitudinal section of a 4–5-wk-old nodule showing uninfected cortical cells (*cc*). Bar, 20 μm. (*c* and *d*) Electron micrograph of part of infected nodules showing bacteroids (*br*) and the peribacteroid membrane (*pbm*). Polyhydroxybutyrate granules (*phb*) are recognizable within the bacteroids. (*c*) Bar, 2 μm. (*d*) Bar, 1 μm.

two bacteroids (Fig. 3, *e* and *f*).

These observations demonstrate that (*a*) those genes of pMPIK3030*a* that are involved in the early stages of nodulation of *Psophocarpus* and *Vigna* can be expressed in *A. tumefaciens*, *R. leguminosarum*, and *R. meliloti*, and (*b*) in addition to the early nodulation genes, *nif* genes of pMPIK3030*a* must be expressed in *R. leguminosarum* and *R. meliloti* under control of pMPIK3030*a* promoters.

Thus, assuming the generality of the observations we only needed to find comparable recipients with fully functional

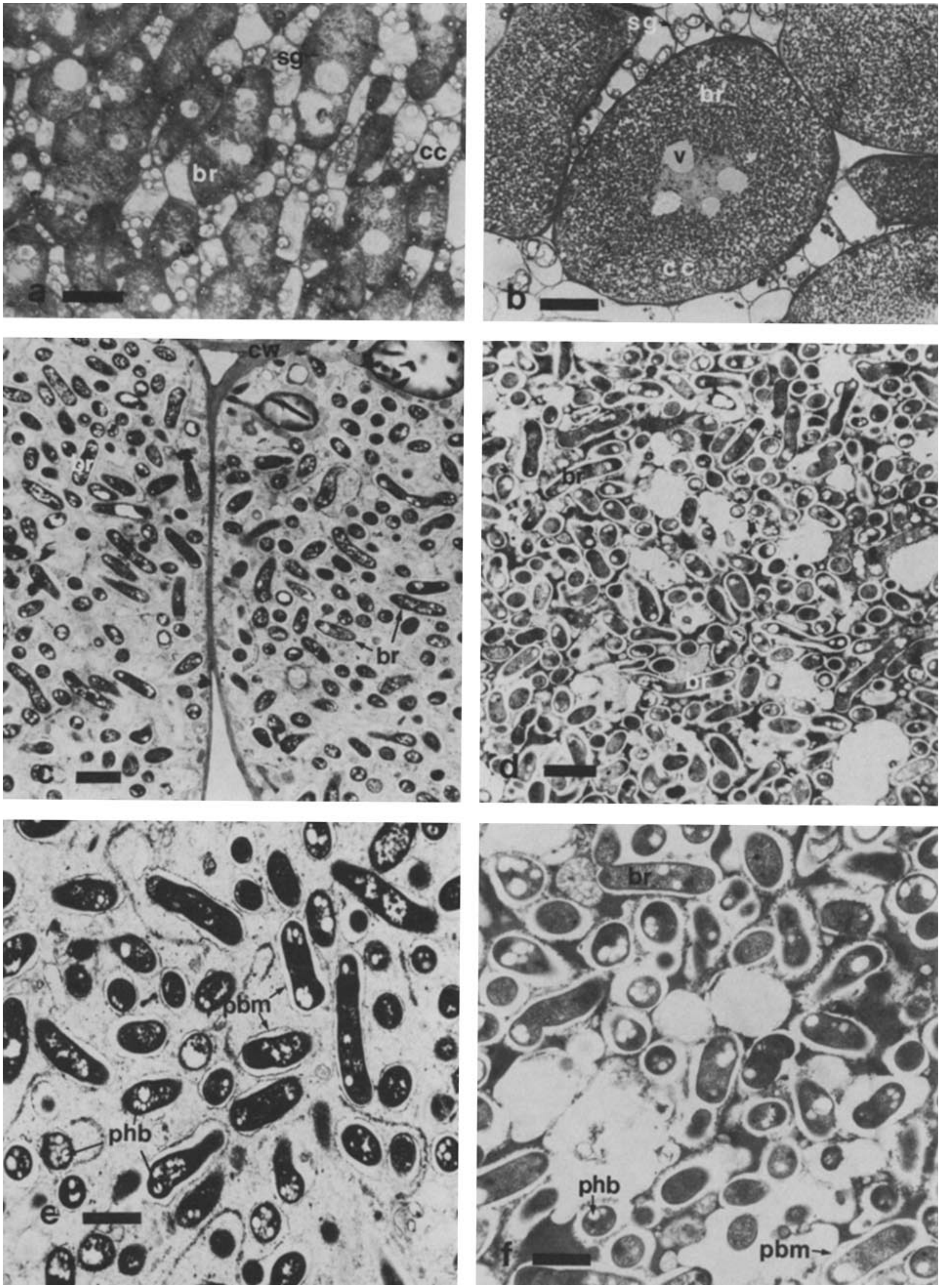

Figure 3. Light and electron micrographs of nodule tissues produced by *R. meliloti* AK1188(pMPIK3030*a*) transconjugants on *V. unguiculata* (*a*, *c*, and *e*) and *P. tetragonolobus* (*b*, *d*, and *f*). (*a* and *b*) Light micrographs of median longitudinal sections of 4–5-wk-old nodules showing cortical cells filled with bacteroids (*br*). Surrounding uninfected cortical cells (*cc*) of effective nodules contain starch grains (*sg*). Surrounding uninfected cortical cells (*cc*) of ineffective nodules contain no starch grains. Bar, 20 μm. (*c–f*) Electron micrographs of parts of infected nodules showing the cortical cell wall (*cw*), bacteroids (*br*), and the peribacteroid membrane (*pbm*). Polyhydroxybutyrate granules (*phb*) are recognizable within the bacteroids. (*c* and *d*) Bar, 2 μm. (*e* and *f*) Bar, 1 μm.

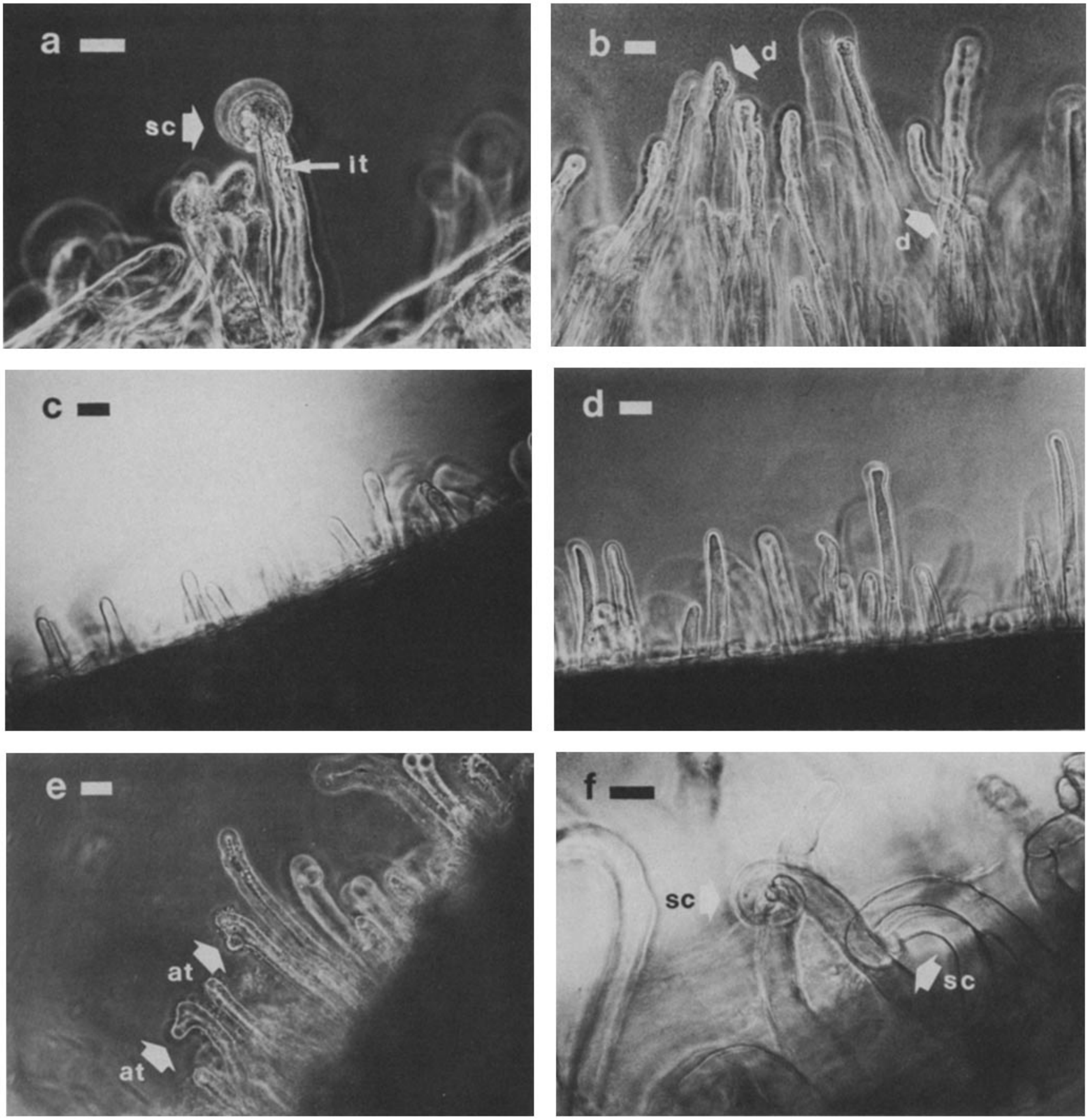

Figure 4. Interaction between various rhizobia and the root hairs of *M. atropurpureum* examined in Fåhraeus slides under dark-field illumination. (*a*) Root-hair curling induced by wild-type *Rhizobium* sp. MPIK3030 showing the typical shepherd's crook curling of the tip (*sc*) and infection threads (*it*). Bar, 10 μm. (*b*) Root hairs exposed to *R. loti* NZP4010 showing some deformation but no curling. Bar, 100 μm. (*c*) Root-hairs exposed to slow-growing *Rhizobium* sp CB376 display a complete lack of interaction. Bar, 100 μm. (*d*) No interaction was apparent between *R. meliloti*, L5.30, and the root hairs. Bar, 100 μm. (*e*) CB376(pWA110) transconjugants induce deformation and an atypical type of curling (*at*) on the root-hairs. Bar, 100 μm. (*f*) L5.30(pWA110) transconjugants produce typical shepherd's crook type curling on *Macroptilium* root hairs. Bar, 10 μm.

nod genes but which otherwise could not react with *Psophocarpus*, *Vigna*, etc. in order to screen for *hsn* genes. We chose *R. loti* strain NZP4010, the wild-type *R. meliloti* strain L5.30, and a slow-growing, extremely narrow host-range isolate CB376. Each of these three strains could not nodulate the hosts listed above or the small-seeded legume *Macroptilium atropurpureum*, which we used to screen for root-hair curling (Fig. 4). Wild-type MPIK3030 readily nodulates *M. atropurpureum*, and the early manifestations of nodulation (root-hair curling and infection thread formation) are apparent (Fig. 4*a*) in this plant. NZP4010 has the advantage of being free of plasmids, but it is intrinsically resistant to ampicillin and reacts weakly with the root hairs of *Macroptilium* (Fig. 4*b*). Neither curled root-hairs nor infection threads were observed, but the deformations seen in Fig. 4*b* may represent some form of weak recognition. Both CB376 and L5.30 were com-

Table III. Transfer of Various pMPIK3030a Cosmids: :Tn5-Mob Co-integrates to Different Rhizobia and the Effect of the Transconjugants on Nodulation of V. unguiculata

Donor	Recipient	Frequency of transconjugants per recipient	Nodulation of *V. unguiculata**	Nodulation of	
				L. pedunculatus	*M. sativa*
	Rhizobium sp				
pWA58 (*hsn*II)	CB376	—	2/21, I		
pWA110 (*hsn*I)		—	0/32		
	R. meliloti				
pWA58 (*hsn*II)	L5.30	1×10^{-3}–1×10^{-4}	1/21, I		
pWA76 (*hsn*I)			31/37, I, PE		+
pWA88 (*hsn*I)			20/38, I, PE		+
pWA110 (*hsn*I)			1/24, I		
	R. loti				
pWA54 (*hsn*III)	NZP4010	1×10^{-3}–1×10^{-4}	1/5, I		
pWA58 (*hsn*II)			2/8, I, PE		
pWA76 (*hsn*I)			5/14, I, PE	+	
pWA88 (*hsn*I)			10/15, PE	+	
pWA110 (*hsn*I)			4/4, PE		

* No. of Leonard jar assemblies (three plants per jar) nodulated/no. of transconjugants tested. PE, partially effective nodules; I, ineffective nodules. +, nodules visible on plants grown on B & D agar.

*Table IV. Homology, Extent of Overlap, Insert Size, etc., of pMPIK3030a Cosmids That Hybridize with Each Other and with nif and nod Regions of R. meliloti**

Plasmid (insert size in kb)	Extent of overlap (kb) with							
	pWA45	pWA46	pWA54	pWA58	pWA59	pWA76	pWA88	pWA90/110
pWA45 (43.2)	x	6	—	14	11	—	—	—
pWA46 (37.4)	6	x	12	—	—	—	—	—
pWA54 (36.3)	—	12	x	—	—	—	—	—
pWA58 (38.2)	14	—	—	x	—	—	—	—
pWA59 (42.2)	11	—	—	—	x	—	—	—
pWA76 (35.1)	—	—	—	—	—	x	20	28
pWA88 (41.3)	—	—	—	—	—	20	x	14
pWA90/110 (39.9)	—	—	—	—	—	28	14	x
Hybridization with*	*nif,nod*	*nod*	*nod*	—	—	*nod*	*nod*	*nod*
hsn group	—	—	III	II	—	I	I	I

* Hybridization with *hif* KDH or *had* DABC of *R. meliloti* (2, 23).
kb, kilobase.
x, represents the homologous situation.

pletely nonreactive (Fig. 4, *c* and *d*), although CB376 is a poor recipient for plasmids as it is resistant to most antibiotics. Selection was only possible by plating the mating mixture on rhizobial minimal medium.

Although NZP4010 reacted slightly with *M. atropurpureum* it was the best recipient to use for general screening purposes. Furthermore, when the Tn5-Mob transposon was used to mobilize the plasmids, it was easy to select transconjugants by their resistance to neomycin. Accordingly, a number of cosmid-clones of pMPIK3030*a* (proof that these are indeed clones of pMPIK3030*a* is shown in Fig. 1*b*; pWA88 hybridizes to pMPIK3030*a*) were individually mobilized (using Tn5-Mob and RP4) into NZP4010. Transconjugants were screened first by checking for the presence of the introduced plasmid::RP4 co-integrates using Eckhardt electrophoresis and afterwards by testing their abilities to nodulate *Vigna*. A number of transconjugants failed to nodulate but three independent groups of cosmids were identified that conferred upon NZP4010 the ability to nodulate *Vigna* (Table III and Fig. 1). Since the central portions of these sets of cosmids showed no overlap with each other (Fig. 1*c*, Table IV), we have named the regions *hsn*I, *hsn*II, and *hsn*III. NZP4010 (*hsn*I, *hsn*II, or *hsn*III) transconjugants were ineffective or partially effective, stable, and produced nodules that resembled those produced by the wild-type MPIK3030 (Fig. 5*a*). Numbers of nodules were reduced from 200 or so to 6–20, but re-isolates from the nodules still possessed the cosmid::RP4 co-integrate and could re-nodulate *Vigna* as well as *Psophocarpus*. Within the nodules, the proportion of bacteroid-containing cells was reduced below that found in *R. meliloti* AK1188(pMPIK3030*a*) transconjugants (cf Fig. 2*a* and Fig. 5*b*), but apart from the fact that several bacteroids were enclosed in one peribacteroid membrane, the infected cells appeared completely normal.

As NZP4010 reacts weakly with *M. atropurpureum* (and may therefore contain some *hsn* genes for *Vigna*, etc.), it was necessary to repeat these preliminary findings using completely nonreactive rhizobia as the recipients. Only

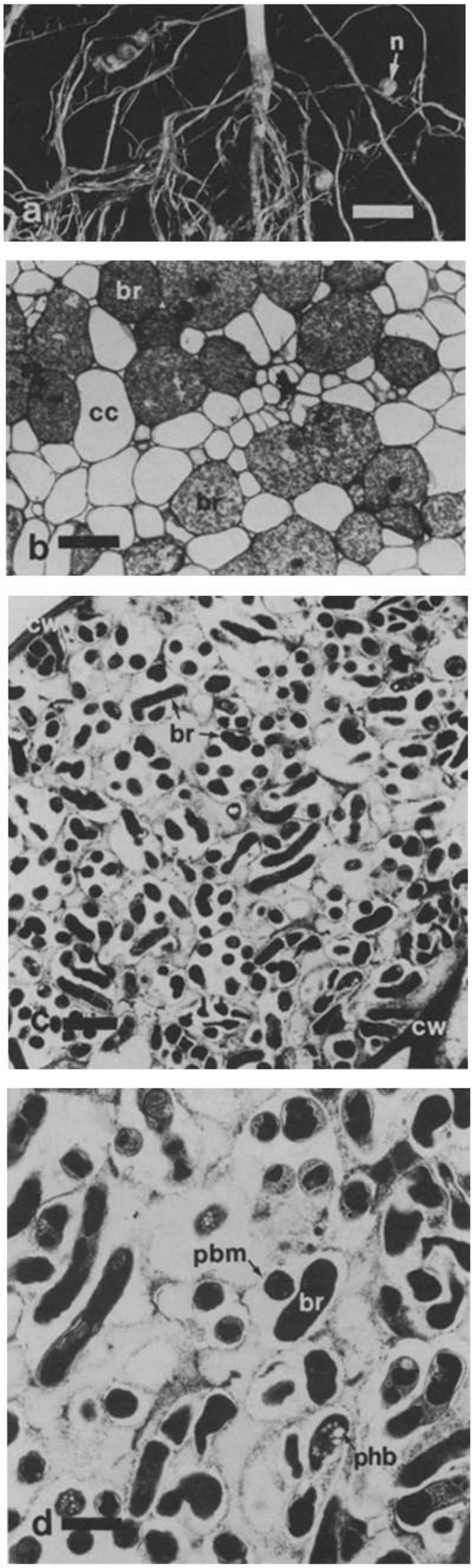

Figure 5. Light and electron micrographs of nodule tissue produced by *R. loti* NZP4010(pWA58-*hsn*II) transconjugants on *V. unguiculata.* (*a*) A photograph of the root system showing reduced nodulation (nodule, *n*). Bar, 1 cm. (*b*) A light micrograph of a mean longitudinal section of a 4–5-wk-old nodule showing cortical cells filled with bacteroids (*br*) and empty, uninfected cortical cells (*cc*). Bar, 20 μm. (*c* and *d*) Electron micrographs of parts of the nodules shown above. Plant cell wall (*cw*), bacteroids (*br*), the peribacteroid membrane (*pbm*), and polyhydroxybutyrate (*phb*) within the bacteroids are shown. (*c*) Bar, 2 μm. (*d*) Bar, 1 μm.

those cosmids that had produced positive nodulation responses when introduced into NZP4010 were mobilized, and as expected, CB376(pWA58) transconjugants as well as L5.30(pWA58) and L5.30(pWA110) transconjugants could nodulate *Vigna* (Table III). Neither CB376 nor L5.30 reacts with the root hairs of *M. atropurpureum* (Fig. 4, *c* and *d*). Introduction of a *hsn*-containing cosmid (pWA110) into either strain using the Tn5-Mob system caused them to deform root hairs, however. Classical "shepherd's crook" type curling was observed with L5.30(pWA110) transconjugants (Fig. 4*f*). CB376(pWA110) transconjugants also caused extensive deformation of the root tip although the tip seemed to bend at right angles rather than curl (Fig. 4*e*). All the transconjugants tested could also nodulate *Lotus pedunculatus* (NZP4010 transconjugants) or *Medicago sativa* (L5.30 transconjugants) (Table III).

Discussion

A pre-requisite for the later experiments described in this paper was that *sym* genes would function in heterologous bacterial and plant hosts. As mentioned in the introduction, this certainly is the case when *nod* genes are transferred between *R. leguminosarum* and *R. meliloti.* As MPIK3030 belongs to another species, however, it was necessary to demonstrate that the *nod* genes of pMPIK3030*a* could also function in foreign chromosomal backgrounds. This was adequately shown by the ability of pMPIK3030*a* to complement Nod⁻ deletions in *R. leguminosarum* and *R. meliloti.* Furthermore, when pMPIK3030*a* was transferred to *A. tumefaciens*, the resulting transconjugants could also nodulate *P. tetragonolobus.* Morrison et al. (29) made similar observations with *M. atropurpureum* by transferring pNGR234*a* into other rhizobia (and into *A. tumefaciens*).

Hooykaas et al. (16) first used *A. tumefaciens* as a recipient for *Rhizobium* plasmids. A number of other workers have followed their example (see, e.g., 20, 42). Usually the results have been the same: nodule-like structures composed of meristematic cells devoid of bacteroids (e.g., 29, 45). Only in Hooykaas et al. (16), Schofield et al. (36), and in the present paper was bacteroid release in nodules induced by *Agrobacterium* (rhizobial plasmid) transconjugants observed. Since the same transconjugants produced bacteroid-containing nodules on *Psophocarpus* but not on *Vigna*, we can assume that legume host genes exerted an influence on the expression of *Rhizobium* plasmids contained in *Agrobacterium.* Similarly, host-functions influence the expression of pMPIK3030*a* in different rhizobial hosts (cf *R. leguminosarum* 6015 [pMPIK3030*a*] transconjugants on *M. atropurpureum* [29] and on *V. unguiculata* [8; this paper]). Since *Agrobacterium* (rhizobial plasmid) transconjugants do not produce effective nodules, while *Rhizobium* (rhizobial plasmid) transconjugants can, we may assume that expression of introduced plasmids is dependent on both the macro- and micro-symbiont as well as the plasmid itself.

In our search for *hsn* genes we turned these experiments around so that the conserved *nod* genes of the recipient rhizobia (apart from those cases when *nod* of pMPIK3030*a* was co-introduced) were the only ones available for nodulation. Putative *hsn* genes were then introduced into these recipients using the Tn5-Mob system. As transconjugants arose at a sufficiently high frequency we can assume that co-integrate formation with RP4-4 occurs efficiently as neither the cosmid replicon (pJB8) nor the Tn5-Mob vector (pSUP5011) can replicate in *Rhizobium*. Demonstration of these co-integrates in re-isolates from the nodules means that they can also be stably maintained. Only one caveat remains in this connection—size heterogeneity of the introduced cosmid::RP4-4 co-integrates was sometimes seen in Eckhardt gels of the re-isolates. This may explain why both effective and ineffective nodules were sometimes found on plants inoculated with the same transconjugants. Complex co-integrate formation with RP4-4 and Tn5-Mob would also explain the instability of some transconjugants that seem to be resolved by passage of the isolate through the nodules (data not shown). Nevertheless, a number of these transconjugants could nodulate *Psophocarpus* and *Vigna*. Nodules were formed when a cosmid from one of the three *hsn* groups was introduced into CB376, L5.30, or NZP4010, thus demonstrating that heterologous *nod* and *hsn* genes can function in organisms as different as slow-growing rhizobia, *R. loti*, and *R. meliloti*.

We were surprised to find three separate groups of *hsn* genes but the data of Fig. 1*c* and Table IV clearly show that the three groups do not overlap. Although *nod* DABC of *R. meliloti* hybridizes to DNA in two of the *hsn* groups, it does not hybridize with pWA58 (*hsn*II), which demonstrates that *nod* genes of the introduced cosmids are neither an essential part of the *hsn* response nor necessary for further nodule development. Tests are underway to see if the three regions represent *hsn* genes for different plants. A map is also being constructed of this region but it is complicated by the lack of linkage between the three *hsn* groups as well as multiple copies of *nod* genes as observed in other, similar rhizobia (34).

In conclusion, these data show that *hsn* genes can be identified by mobilizing individual cosmids into heterologous wild-type rhizobia. Furthermore, when *R. loti* strain NZP4010 and *R. meliloti* strain L5.30 were used as recipients, the transconjugants had truly broadened host-ranges: they could nodulate their original hosts as well as those of MPIK3030.

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