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Expression of *Rhizobium galegae* Common *nod* Clones in Various Backgrounds

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The cosmid clone pRg30, carrying common nodulation genes of *Rhizobium galegae* HAMBI 1174, and pRg33, a subclone of pRg30 that contains a 5.7-kb *Cla*I insert carrying *nodDABC* were conjugated into various *Rhizobium nod*$^-$ mutant strains and into a Ti plasmid-cured *Agrobacterium tumefaciens*. Complementation and expression of the *nodABC* genes of *R. galegae* were studied by following microscopically the infection process and the nodulation on different test plants. The *nodABC* genes of *R. galegae* complemented the *nod*$^-$ strains of other *Rhizobium* species. The presence of extra copies of common *nod* genes in the homologous *R. galegae nodABC*$^-$ strain induced an increased nodulation on *Galega orientalis*. However, the inserts of *R. galegae* in pRg30 and pRg33 do not carry sufficient genetic information for normal nodulation of test plants in an *Agrobacterium* background, because the *Agrobacterium* transconjugants induced root hair deformation on *Galega* plants, but no infection threads were detected and nodulelike structures developed only at low frequency. The *Agrobacterium* carrying the *nodDABC* of *R. galegae* did not cause the root hairs of *Medigo sativa* to deform.

Additional keyword: symbiosis.

The development of symbiosis between *Rhizobium* bacteria and leguminous plants is a complex process. At least 15 nodulation genes of *Rhizobium* are known to participate in the signal exchange between the symbionts. The *nodDABC* genes of *Rhizobium* bacteria, which are often located in so called symbiotic plasmids, are required for the earliest stages of nodule formation. *nodA*, *nodB*, and *nodC* gene products are needed for the bacteria to cause deformation of root hairs and for divison of plant cells. *nodABC* genes are designated as common (conserved) nodulation genes, because each of these genes can functionally complement mutations in the *nodABC* genes of other *Rhizobium* species (see review of Long 1989). Two additional *nod* genes, *nodI* and *nodJ*, are transcribed together with *nodABC* in many fast-growing rhizobia (Evans and Downie 1986; Canter-Cremers *et al.* 1988). The *nodI* and *nodJ* proteins are suggested to be involved in nodulation efficiency and normal development of infection threads (Schlaman *et al.* 1990). Exudates of host plants together with the constitutive *nodD* gene product induce the expression of the *nodABC* genes (Rossen *et al.* 1985). According to present theory, the induced common *nodABC* genes together with host-specific genes produce extracellular factor(s), which interact with the host plant and trigger the root hair deformation, making it possible for the bacteria to invade the plant. (Faucher *et al.* 1988, 1989; Banfalvi and Kondorosi 1989). Lerouge *et al.* (1990) have determined the chemical structure of the major *Medigo sativa* L.-specific signal NodRm-1.

Rhizobium galegae K. Lindström is a new *Rhizobium* species that is only distantly related to other fast-growing rhizobia. It nodulates *Galegae* sp., *Galega officinalis* L., and *Galega orientalis* Lam. in a very host-specific manner. *Rhizobium* strains isolated from *G. orientalis* plants only form nitrogen-fixing (effective) nodules on *G. orientalis* but ineffective nodules on *G. officinalis*. For strains isolated from *G. officinalis* plants, the situation is reversed. *R. galegae* strains did not infect other leguminous plants tested. Plants of *Galegae* sp. are only occasionally infected by other rhizobia, and then the nodules are ineffective (Lindström 1989). *R. galegae* infects *Galega* sp. by causing deformation of root hairs and penetrating the root cortex cells via infection threads, but it does not induce the formation of shepherd's crooks. *R. galegae* causes deformation in root hairs of *G. orientalis* and *G. officinalis* plants to cauliflower-like structures. Infection threads are usually found in very short root hairs or starting from cauliflower-like structures of long root hairs (Lipsanen and Lindström 1988).

G. orientalis is a potential perennial pasture legume for northern temperate conditions. To be able to optimize its nodulation and nitrogen fixation at low temperature, we initiated a study of the nodulation genes of *R. galegae*.

A pLAFR1 cosmid clone, pRg30, carrying the common nodulation genes of *R. galegae* has been isolated from a gene library of *R. galegae* strain HAMBI 1174 by complementation of the *nodC*$^-$ mutant *Rhizobium meliloti* Dangeard Rm1126. The common nodulation genes have been located in the 26-kb insert of pRg30. pRg33, a subclone of pRg30 in the vector pWB5a, carries *nodABC*, *nodD*, and one *nod* box sequence in a 5.7-kb *Cla*I fragment (Fig. 1) (L. Suominen, unpublished). *R. galegae* strain HAMBI 1174 forms effective nodules on *G. orientalis* but ineffective nodules on *G. officinalis* (Lipsanen and Lindström 1988).

The purpose of this work was to study if the functions of *R. galegae* common nodulation genes are identical with those of common nodulation genes of other *Rhizobium* species. For this study, the cosmid clone pRg30, which carries common nodulation genes of *R. galegae* HAMBI

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1174 and pRg33, a subclone of pRg30, were conjugated into *Rhizobium leguminosarum* bv. *viceae* Jordan RBL5515 strains, which had transposons inserted in their *nodA*, *nodB*, or *nodC* genes (Wijffelman *et al.* 1985); into *R. l.* bv. *viciae* strains LPR5045, which had transposons in their *nodI* or *nodJ* genes (Evans and Downie 1986); into *R. meliloti* strain Rm1126, which carries an endogenous insertion sequence in *nodC* (Meade *et al.* 1982); and into *R. galegae* strain HAMBI 1587, which had a transposon in *nodABC* (L. Suominen, unpublished). The complementation of *nod*$^-$ strains was studied by following the nodulation and the infection process in root hairs of test plants. Expression of the cloned common *nod* genes in the Ti plasmid-cured *Agrobacterium tumefaciens* (Smith and Townsend) Conn C58C1 was also studied.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are shown in Table 1. *Rhizobium* strains were grown on yeast extract mannitol (YEM) agar with Congo red (Lindström *et al.* 1985) at 28° C. *Escherichia coli* strains were grown in LB media (Maniatis *et al.* 1982). The strains were stored on agar plates at +4° C and in 20% (wt/vol) glycerol at −80° C.

Conjugations. Plasmids were transferred into recipients by a triparental conjugation technique with helper plasmid pRK2013 (Ditta *et al.* 1980). Conjugants were selected on defined medium (Lindström and Lehtomäki 1988). Selective media were supplemented with streptomycin, 500–1,000 μg/ml; spectinomycin, 500 μg/ml; chloramphenicol, 50 μg/ml; kanamycin, 20–150 μg/ml; tetracycline, 5–10 μg/ml; and trimethoprim, 500 μg/ml alone or in combination. The presence of plasmids in the recipient strains was verified by isolating plasmid DNA with the alkaline lysis method (Maniatis *et al.* 1982), by digesting the plasmids with *Eco*RI or *Cla*I restriction enzymes and then by running them in agarose gel electrophoresis (Kaijalainen and Lindström 1989).

Plant material. *G. orientalis* (goat's rue) unbred seeds were from Viikki Experimental Farm, Helsinki, Finland. *G. officinalis* seeds were collected from wild plants and were a gift from Paul Buckley, Massey University, Palmerston North, New Zealand. *M. sativa* cv. Iroquis seeds were a gift from Fred Ausubel, Massachusetts General Hospital, Boston. *Vicia villosa* Roth seeds were a gift from Petri Leinonen, The Center for Rural Development, Juva, Finland.

Plant tests. Plant tests were done according to Lipsanen and Lindström (1988) with some modifications. *M. sativa* and *Galegae* seeds were first rinsed for 30 s with 70% ethanol and then with sterile water 3 × 10 min. Then, they were sterilized with 0.1% $HgCl_2$ for 5 min and rinsed with sterile water 6 × 10 min. *V. villosa* seeds were sterilized with 6% H_2O_2 for 45 min and then rinsed with sterile water three times. Sterilized *V. villosa* and *Galega* seeds were germinated on YEM-Congo red plates for two days and *M. sativa* seeds for one day in the dark at room temperature until the roots were about 1 cm long. The seedlings were transferred onto Jensen agar slants (Vincent 1970), two *M. sativa* plants, one *V. villosa* and one *Galega* plant per test tube (2 cm diameter × 15 cm high). Plants were inoculated at the same time. Before inoculation, the *Rhizobium* strains were grown for 2 days on YEM-Congo red plates at 28° C. Bacteria were suspended in sterile water to a final concentration of 10^8/ml. Portions (0.5 ml) of this suspension were added onto each slant, the roots were flushed five times with the suspension by using a Pasteur pipette, and the suspension was removed. Inoculated plants were grown in a growth chamber at 18–22° C with a 16-hr light and 8-hr dark period, and a 400 W Na-lamp (Airam, Finland) as a light source. In each plant experiment, the wild type strain, the transconjugants, and the parent strain were used. Uninoculated plants and those inoculated with sterile water served as negative controls.

Microscopy of root hairs. Two to three plants were examined in each case about 5, 10, 15, and 20 days after inoculation. At least two parallel plant experiments were done for microscopic study. Root hairs were rinsed with tap water and stained according to Vasse and Truchet (1984) with a 0.01% methylene blue solution, and observed under bright field microscopy.

Nodulation. For nodulation tests, at least 10 plant tubes for each test combination were used, except that in two parallel experiment plants inoculated with *A. tumefaciens* C58C1 and with the same strain carrying pRg30 130 plants

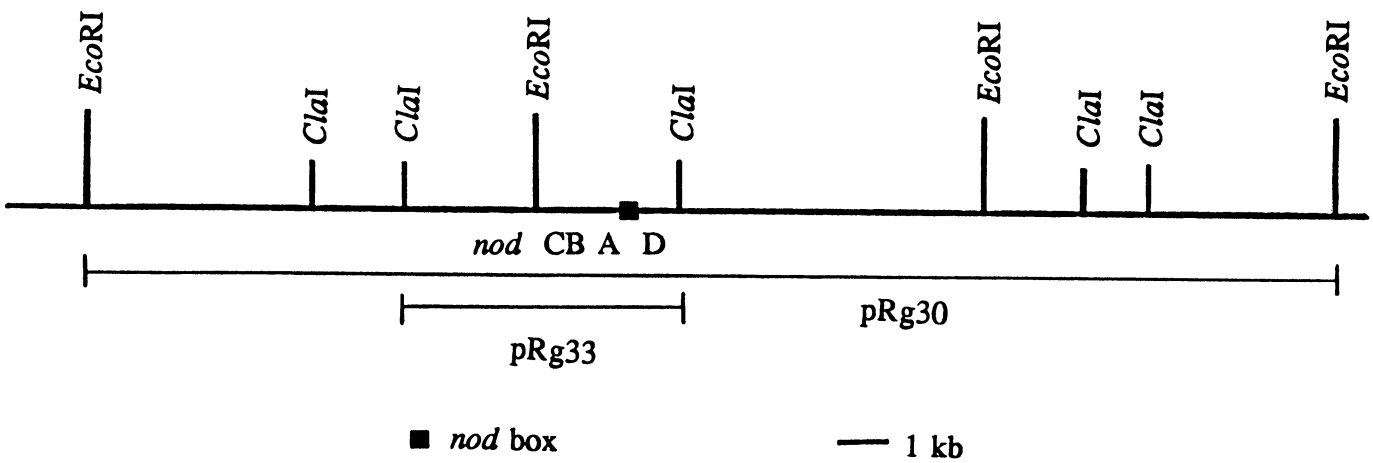

Fig. 1. pRg30 and pRg33, the cosmid clones that carry the common nodulation genes of *Rhizobium galegae* HAMBI 1174.

were tested. The plant tests for nodulation were usually done three times. Nodules were counted 2, 3, 4, 6, and 7 wk after inoculation. Nitrogenase activity of nodules was determined 4 wk (*V. villosa* and *M. sativa*) or 6 wk (*G. orientalis*) after inoculation by the acetylene reduction method (Lindström 1984a,b).

Table 1. Bacterial strains and plasmids used in this study

Strain	Description	Source or reference
Rhizobium leguminosarum bv. *viceae*		
HAMBI[a] 499	Wild type, Nod$^+$, Fix$^+$	This study
nodA$^-$ (HAMBI 1594)	RBL5515 pRL1JI*nodA*10::Tn*5* Rif^r, Nod$^-$	Wijffelman *et al.* 1985
nodB$^-$ (HAMBI 1595)	RBL5515 pRL1JI*nodB*11::Tn*5* Rif^r, Nod$^-$	Wijffelman *et al.* 1985
nodC$^-$ (HAMBI 1599)	RBL5515 pRL1JI*nodC*13::Tn*5* Rif^r, Nod$^-$	Wijffelman *et al.* 1985
nodI$^-$ (HAMBI 1597)	LPR5045 pRL1JI*nod*82::Tn*5* Rif^r, Nod$^+$	Downie *et al.* 1985
nodJ$^-$ (HAMBI 1598)	LPR5045 pRL1JI*nod*29::Tn*5* Rif^r, Nod$^+$	Downie *et al.* 1985
Rhizobium meliloti		
Rm1021 (HAMBI 1463)	Wild type, Str^r Nod$^+$, Fix$^+$	Meade *et al.* 1982
Rm1126 (HAMBI 1213)	Rm1021::Tn*5* in *nodC* Str^r, Nod$^-$	Meade *et al.* 1982
Rhizobium galegae		
HAMBI 1174 (1261R)	Wild type, *G. orientalis* Sm^r, Spc^r, Nod$^+$, Fix$^+$	Lindström *et al.* 1985
HAMBI 1209 (B7is)	Wild type, *G. officinalis* Sm^r, Nod$^+$, Fix$^+$	This study
HAMBI 1587 (1261T1)	HAMBI 1174::Tn*5* in *nodABC*, Sm^r, Tp^r, Km^r, Nod$^-$	L. Suominen, University of Helsinki, Finland
Agrobacterium tumefaciens		
C58C1 (HAMBI 1218)	Derivative of C58, cured of Ti-plasmid, Cm^r	F. M. Ausubel, Massachusetts General Hospital
Escherichia coli		
HB101	supE44*has*S20(r_B^-, m_B^-), *recA13, ara14, proA2 lacY1, galK2, rpsL20 (sm^r), xyl-5, mtl-1*	F. M. Ausubel
DK1	*(lac)x74-del, galU, galK rpsL(sm^r), (Srl-recA)306-del*	F. M. Ausubel
Plasmids		
pRg30	pLAFR1 carrying 26-kb common *nod* insert of *Rg*1174, Tc^r	L. Suominen
pRg33	5.7-kb *ClaI* fragment of pRg30 carrying common *nod* genes subcloned in pWB5a, Tc^r	L. Suominen
pRK2013	*repcolE1*, Km^r, Nm^r	Ditta *et al.* 1980

[a] HAMBI = the culture collection of the Department of Microbiology, University of Helsinki.

Microscopy of nodules. Nodules of *G. officinalis* and *G. orientalis* plants induced by their wild type strains, HAMBI 1209 and HAMBI 1587, and nodulelike structures induced by *A. tumefaciens* C58C1 carrying pRg30 were prepared for light and electron microscopy according to a modification of the method of Truchet *et al.* (1984) (Lipsanen and Lindström 1988).

RESULTS

Root hair deformation and infection threads. The results of the effects of the tested bacterial strains on the root hair deformation and on the development of infection threads are shown in Tables 2 and 3. Root hairs of test plants inoculated with *nod*$^-$ mutant *Rhizobium* strains and with the Ti plasmid-cured *A. tumefaciens* C58C1 were straight and long, except for the root hairs of *V. villosa* induced by *R. l.* bv. *viceae nodB*$^-$ strain, which were branched and sometimes strongly deformed (Table 2).

R. l. bv. *viceae* strain 5515 carrying Tn*5* in *nod* genes *A*, *B*, or *C* conjugated with pRg30 or pRg33 caused deformation of the root hairs of *V. villosa* (Table 2), but the deformation differed to some degree from that caused by the wild type strain *R. l.* bv. *viceae* HAMBI 499. Root hairs of *V. villosa* inoculated with the wild type strain were mostly short and thick, whereas deformed root hairs induced by the transconjugants were mostly long and unshaped. The frequency of appearance of the infection threads was lower for the transconjugants than for the wild type. The infection threads of the wild type were mostly in short root hairs (Fig. 2A), whereas infection threads induced by the transconjugants usually appeared in longer root hairs (Fig. 2B,C). Often the deformation induced by the transconjugants was stronger than that induced by the wild type (Fig. 2E). Later, there were nodes at the top of the long root hairs induced by the transconjugants (Fig. 2F).

The deformation of the *M. sativa* root hairs caused by *R. meliloti nodC*$^-$ mutant strain Rm1126 carrying pRg30 or pRg33 was like the wild type strain *R. meliloti* Rm1021. The difference was that in the root hairs of *M. sativa* inoculated with transconjugants there were very few infection threads, or those were difficult to detect, possibly because the infection threads might develop into very short root hairs (Fig. 3C).

R. galegae strain HAMBI 1587 carrying Tn*5* in *nodABC*, into which pRg30 or pRg33 had been transferred, deformed the root hairs and induced the infection thread development on its own host plant *G. orientalis* (Table 2). The infection threads mostly occurred in short root hairs (Fig. 4C). The deformation induced by the transconjugants was to a certain extent stronger and there were more unshaped root hairs (Fig. 4D), but otherwise the deformations were similar to the positive controls.

A. tumefaciens C58C1, conjugated with pRg30, induced deformation of root hairs on *G. officinalis* and *G. orientalis* plants (Table 3; Fig. 5C,D); root hairs were mostly long, misshapen, and screw-formed. Sometimes they were shaped like root hairs deformed by the wild type *R. galegae*. *A.*

tumefaciens containing pRg33 also caused deformation of root hairs on *G. officinalis* (Fig. 5E), but caused deformation of root hairs of *G. orientalis* only slightly (Fig. 5F). *A. tumefaciens* transconjugants did not cause the root hairs of *M. sativa* to deform at all (Fig. 5G,H).

Nodulation. The results of the nodulation tests are shown in Tables 2 and 3. The *nodA*, *nodB*, and *nodC* mutants of *R. l.* bv. *viceae* 5515, conjugated with pRg30 or pRg33, nodulated *Vicia sativa* L. plants (Table 2). However, the nodulation was about 1 wk delayed and the amounts of nodulated plants were smaller than for the wild type. The nodulation mutants carrying pRg30 caused more nodulated plants than the mutants carrying pRg33, and *nodA*$^-$ and *nodB*$^-$ mutants with pRg30 caused about twice as many as those with pRg33. The nodules induced by the transconjugants were formed at the upper parts of the main

Table 2. The effect of *Rhizobium* transconjugants on root hair deformation, development of infection threads, and nodulation

Strain	Introduced plasmid	Test plant	Root hair deformation[a]	Infection threads detected	Nodulated plants[b] (%)	Standard deviation	Number of nodulation experiments
Rhizobium leguminosarum bv. *viceae*							
Rl5515 *nodA*$^-$	None	*Vicia villosa*	−	−	0	0	3
	pRg30	*V. villosa*	+	+	41	10	3
	pRg33	*V. villosa*	+	+	19	17	3
Rl5515 nodB$^-$	None	*V. villosa*	+	−	0	0	3
	pRg30	*V. villosa*	+	+	55	17	3
	pRg33	*V. villosa*	+	+	31	20	3
Rl5515 nodC$^-$	None	*V. villosa*	−	−	0	0	3
	pRg30	*V. villosa*	+	+	46	16	3
	pRg33	*V. villosa*	+	+	38	23	3
Rl499 Wild type	None	*V. villosa*	+	+	100	4	3
Rl5045 *nodI*$^-$	None	*V. villosa*	ND	ND	100	21	3
	pRg30	*V. villosa*	ND	ND	76	11	3
	pRg33	*V. villosa*	ND	ND	96		1
	None	*V. villosa*	ND	ND	100	4	3
Rl5045 nodJ$^-$	pRg30	*V. villosa*	ND	ND	73	40	3
	pRg33	*V. villosa*	ND	ND	116		1
Rhizobium meliloti							
Rm1126 nodC$^-$	None	*Medicago sativa*	−	−	3	5	4
	pRg30	*M. sativa*	+	+	58	25	4
	pRg33	*M. sativa*	+	+	74	24	4
Rm1021 Wild type	None	*M. sativa*	+	+	100	8	4
Rhizobium galegae							
*Rg*1587 *nodABC*$^-$	None	*Galega orientalis*	−	−	0	0	3
	pRg30	*G. orientalis*	+	+	108	43	3
	pRg33	*G. orientalis*	+	+	122	24	3
Rm1174 Wild type	None	*G. orientalis*	+	+	100	10	3

[a] + = Reaction, − = no reaction, ND = not done.
[b] The results of nodulated plants are expressed as percentages of the positive controls or of the nodulation induced by *R. leguminosarum nodI*$^-$ or *nodJ*$^-$ strains. The wild type nodulation or that induced by *nodI*$^-$ and *nodJ*$^-$ strains is considered as 100%. The nodules of test plants, which were inoculated with the *R. galegae* transconjugants and with the wild type of *R. galegae* were counted 42 days after inoculation, the others 30 days after inoculation.

Table 3. The effect of *Agrobacterium* transconjugants on root hair deformation, development of infection threads, and nodulation compared with *Rhizobium* controls

Strain[a]	Introduced plasmid	Test plant	Root hair deformation[b]	Infection threads detected	Nodulated plants (%)	Standard deviation	Number of experiments[c]
Agrobacterium tumefaciens C58C1	None	*Galega officinalis*	−	−	0.5	0.9	3*
	pRg30	*G. officinalis*	+	−	7	3	3*
	pRg33	*G. officinalis*	+	−	11		1
Rg1209 Wild type	None	*G. officinalis*	+	+	97	5	3
Agrobacterium tumefaciens C58C1	None	*G. orientalis*	−	−	0	0	4*
	pRg30	*G. orientalis*	+	−	3	5	4*
	pRg33	*G. orientalis*	·	−	0	0	2
Rg1174 Wild type	None	*G. orientalis*	+	+	88	18	4
Agrobacterium tumefaciens C58C1	None	*Medicago sativa*	−	−	1	2	5*
	pRg30	*M. sativa*	−	−	4	4	5*
	pRg33	*M. sativa*	−	−	10	10	3
Rm1021 Wild type	None	*M. sativa*	+	+	97	5	5

[a] Rg = *Rhizobium galegae* and Rm = *Rhizobium meliloti.*
[b] + = Reaction, − = no reaction, · = root hairs were slightly deformed.
[c] At least 10 plant tubes were used in each experiment with the exception of those marked with an asterisk where 130 plant tubes were used in two parallel experiments. The nodules of test plants were counted 6 wk after inoculation.

rootlike nodules induced by the wild type bacteria, and they were able to fix nitrogen. pRg30 and pRg33 were also transferred into *R. l.* bv. *viceae* 5045 mutant strains carrying Tn*5* in *nod* genes *I* and *J*. Because these transposon mutants nodulate test plants (delayed nodulation), the nodulation patterns of the transconjugants were compared with that of the nodulation of *V. villosa* plants induced by the parent *nodI*$^-$ and *nodJ*$^-$ strains (Table 2).

R. meliloti nodC$^-$ mutant strain Rm1126, conjugated with pRg30 or pRg33, nodulated *M. sativa* plants (Table 2). The nodulation was about 1 wk delayed compared with that of the wild type. The nodules were able to fix nitrogen.

R. galegae nodABC$^-$ mutant strain HAMBI 1587, conjugated with pRg30 or pRg33, nodulated *G. orientalis* plants (Table 2). After 6 wk incubation with the transconjugants, a higher frequency of nodulated plants was observed compared with plants inoculated with the wild type. The nodules were able to fix nitrogen.

Within 4 wk, *A. tumefaciens* C58C1 containing pRg30 or pRg33 produced white nodulelike structures on some *G. officinalis* plants, but not on *G. orientalis* plants. After 6 wk incubation, there were white nodulelike structures also on a few *G. orientalis* plants inoculated with *A. tumefaciens* containing pRg30 (Table 3). The light and electron microscopic analyses of these structures showed that an apical meristem and vascular bundles, the typical features of a nodule, were missing (data not shown). The *Agrobacterium* transconjugants induced small, white nodulelike structures on *M. sativa* plants after 6 wk at low frequency. The parent *A. tumefaciens* strain also induced nodulelike structures on *G. officinalis* and on *M. sativa* plants but at still lower frequency than the transconjugants (Table 3).

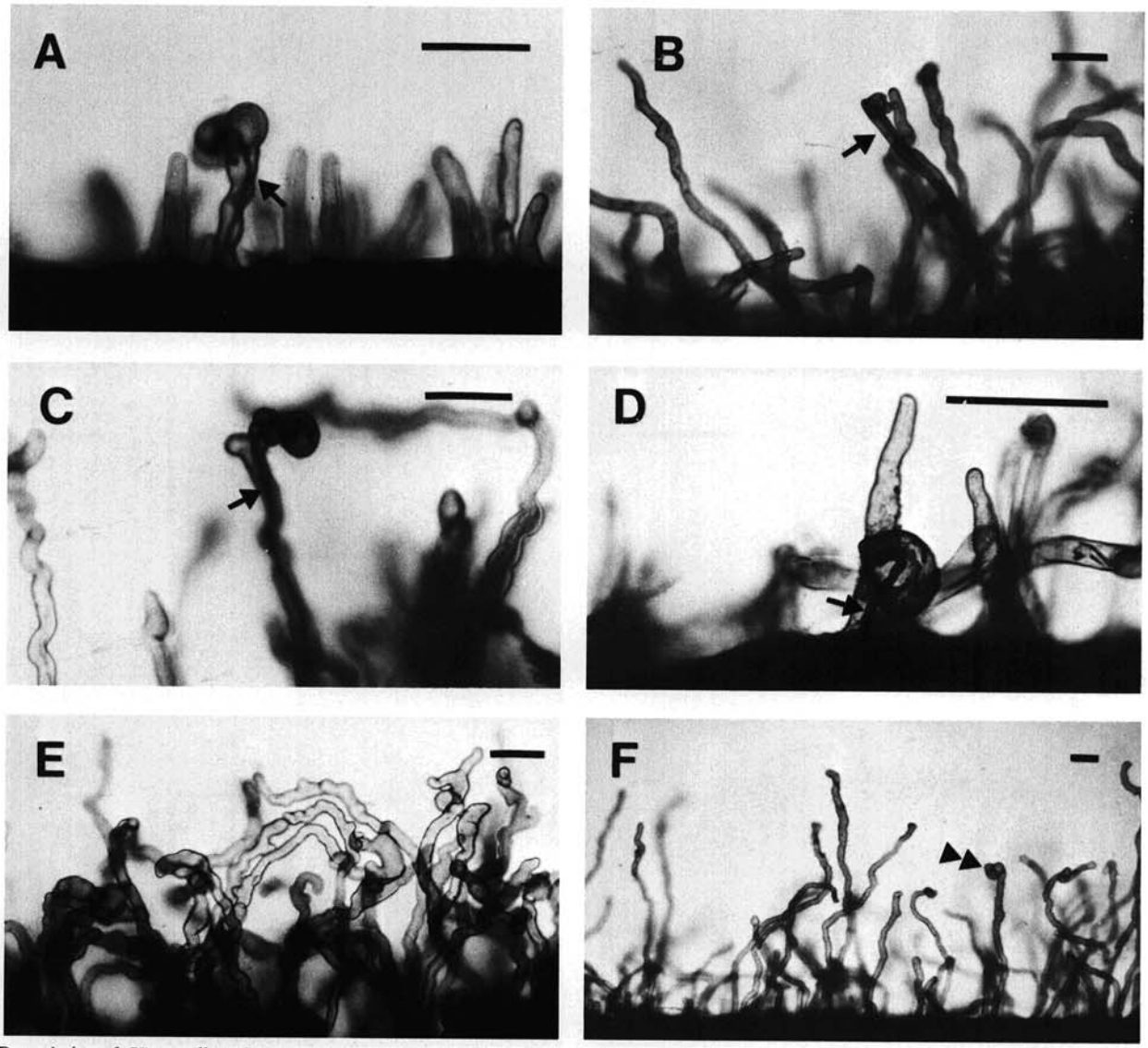

Fig. 2. Root hairs of *Vicia villosa* inoculated with **A,** *Rhizobium leguminosarum* bv. *viceae* HAMBI 499 (positive control); **B,** *R. l.* bv. *viceae* 5515 *nodA*$^-$ carrying pRg30; **C,** *R. l.* bv. *viceae* 5515 *nodB*$^-$ carrying pRg33; **D,** *R. l.* bv. *viceae* 5515 *nodC*$^-$ carrying pRg30; **E,** *R. l.* bv. *viceae* 5515 *nodC*$^-$ carrying pRg33; **F,** *R. l.* bv. *viceae* 5515 *nodC*$^-$ carrying pRg30. The arrow points at an infection thread. Double arrow points at a node. Bars = 50 μm.

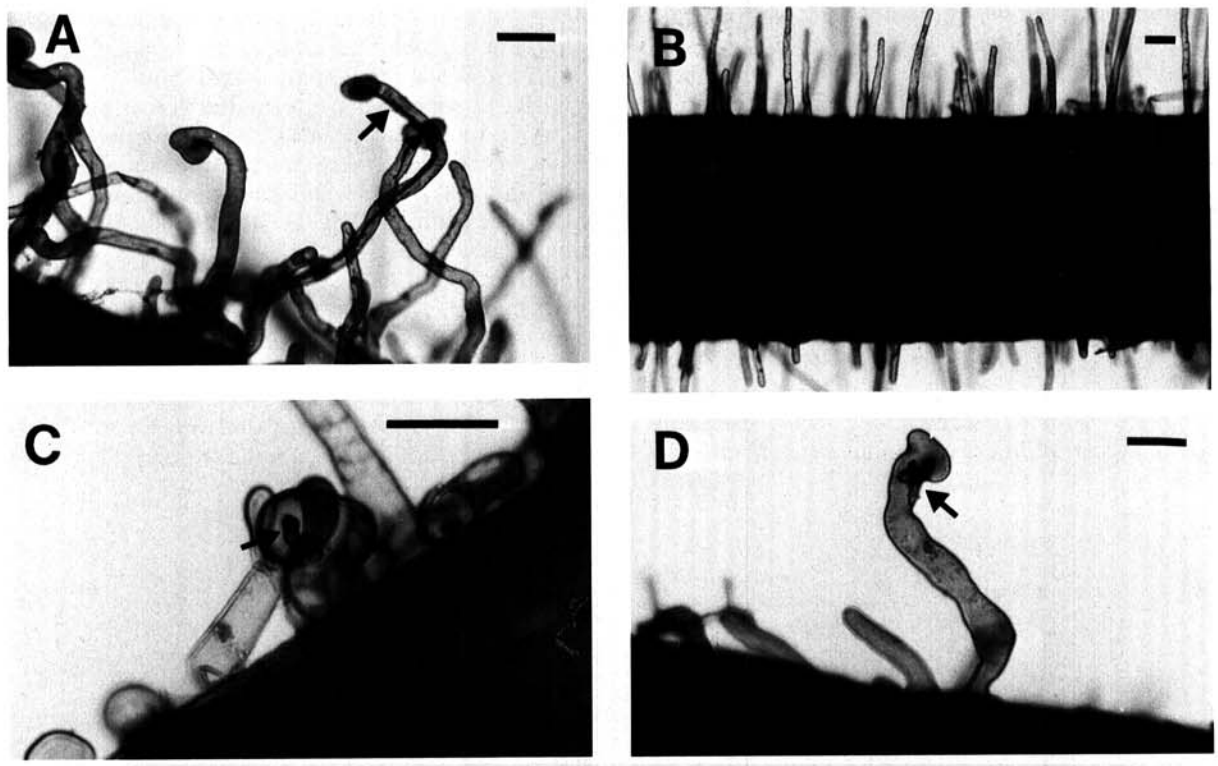

Fig. 3. Root hairs of *Medicago sativa* inoculated with **A,** *Rhizobium meliloti* Rm1021 (positive control); **B,** *R. meliloti* Rm1126 *nodC*$^-$ (negative control); **C,** *R. meliloti* Rm1126 *nodC*$^-$ carrying pRg30; **D,** *R. meliloti* Rm1126 *nodC*$^-$ carrying pRg33. The arrow points at an infection thread. Bars = 50 μm.

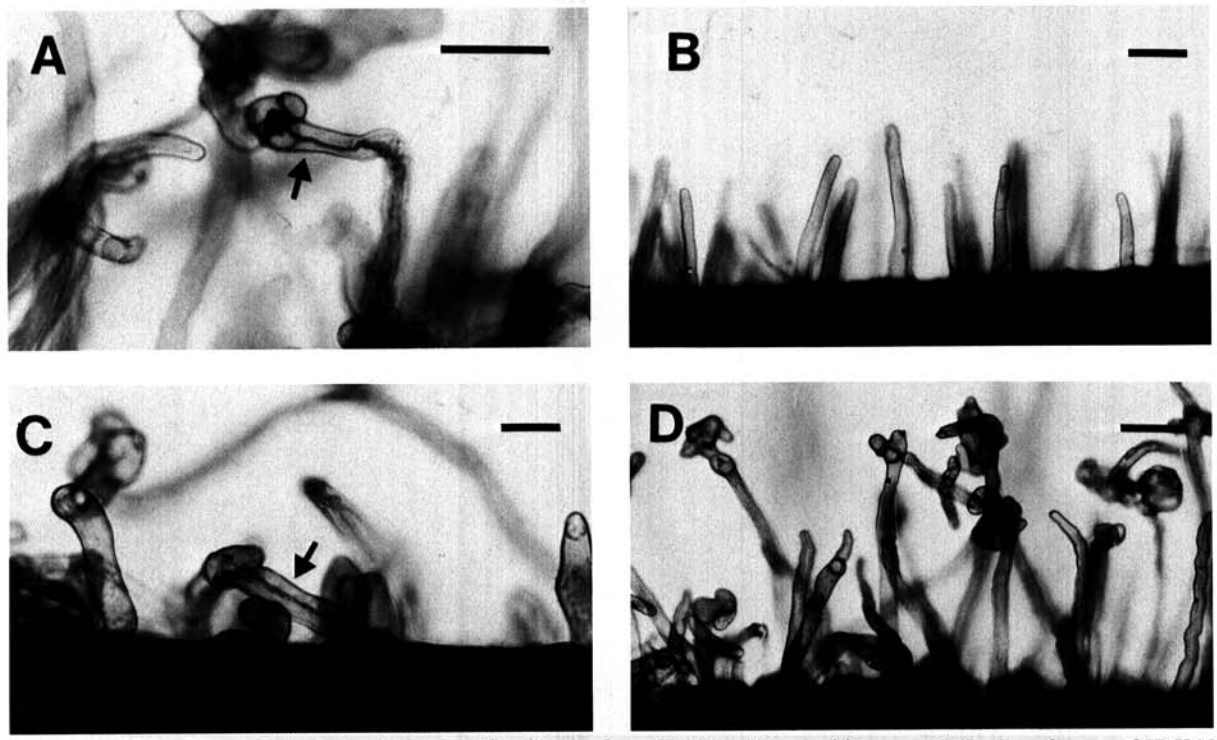

Fig. 4. Root hairs of *Galega orientalis* inoculated with **A,** *Rhizobium galegae* HAMBI 1174 (positive control); **B,** *R. galegae nodC*$^-$ HAMBI 1587 (negative control); **C,** *R. galegae nodABC*$^-$ carrying pRg30; **D,** *R. galegae nodABC*$^-$ carrying pRg33. The arrow points at an infection thread. Bars = 50 μm.

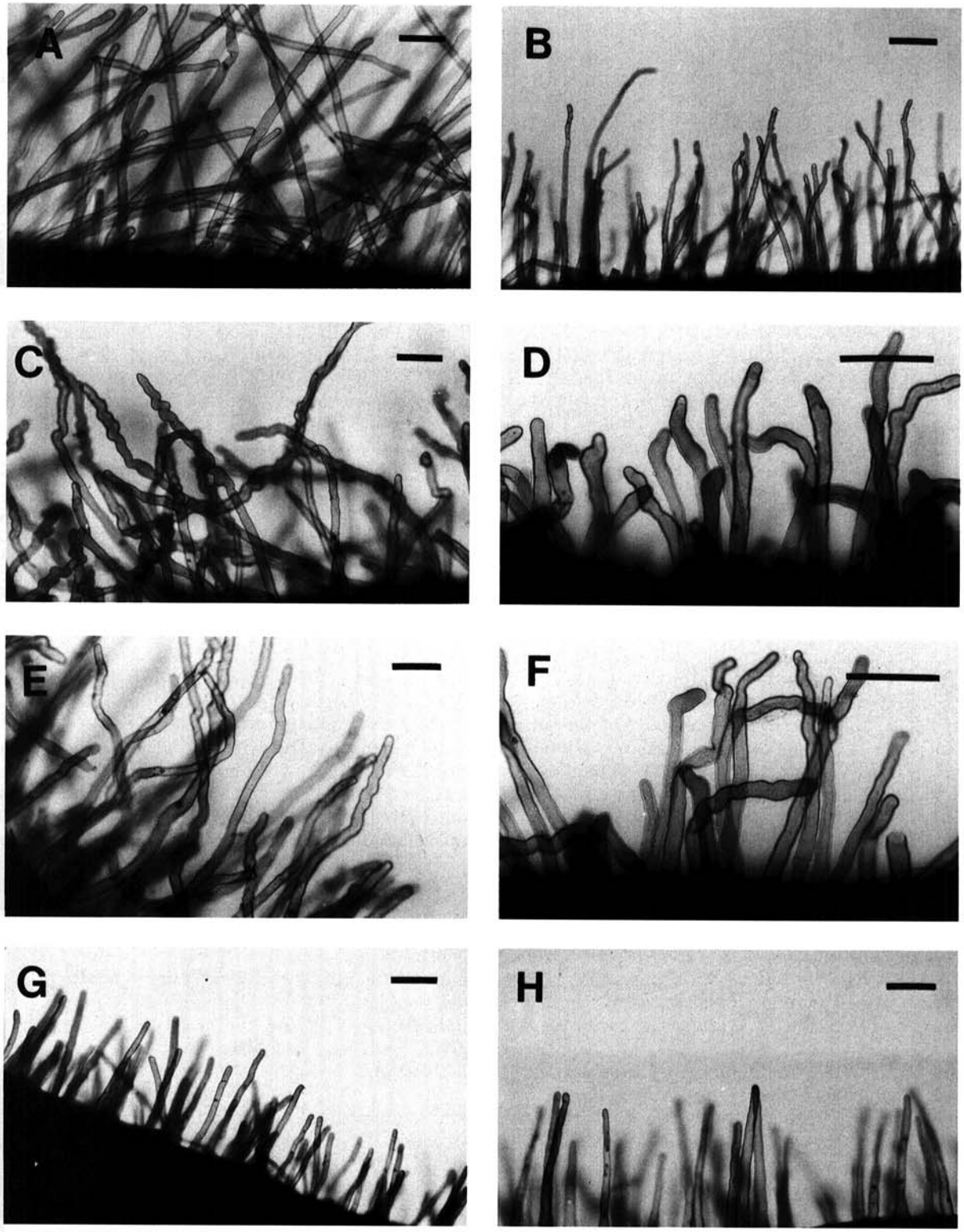

Fig. 5. Root hairs of **A,** *Galega officinalis* and **B,** *Galega orientalis* inoculated with *Agrobacterium tumefaciens* C58C1 (negative controls). Root hairs of **C,** *G. officinalis* and **D,** *G. orientalis* inoculated with *A. tumefaciens* carrying pRg30. Root hairs of **E,** *G. officinalis* and **F,** *G. orientalis* inoculated with *A. tumefaciens* carrying pRg33. Root hairs of *Medicago sativa* inoculated with *A. tumefaciens* C58C1 carrying **(G)** pRg30 and **(H)** pRg33. Bars = 100 μm.

DISCUSSION

Complementation of *nodABC*. The plant tests showed that the common nodulation genes of *R. galegae* HAMBI 1174 complement the nodulation genes of other *Rhizobium* species. However, depending on the *Rhizobium* species, the complemented strains behaved in different ways. The *nod* genes of pRg30 and pRg33, when introduced into heterologous *Rhizobium nod*$^-$ strains, nodulated fewer plants than the wild type strains. After conjugation, the homologous *nodABC*$^-$ strain *R. galegae* HAMBI 1587 nodulated more *G. orientalis* plants than the wild type in similar conditions.

Other research groups have shown that mutations in common *nod* genes of fast-growing *Rhizobium* can be complemented by nodulation genes from both other fast-growing and from slow-growing *Rhizobium* species so that deformation of root hairs, the development of infection threads, and the nodules formed by complemented *nod*$^-$ mutants appeared morphologically and functionally the same as those induced by the wild type strains (Kondorosi *et al.* 1984; Fischer *et al.* 1985; Marvel *et al.* 1985; Russel *et al.* 1985; Debelle *et al.* 1986). The nodulation genes of *R. meliloti* and *R. l.* bv. *viceae* have been sequenced and a comparison of the nucleotide and predicted amino acid sequences revealed about 70% homology. A region of highest amino acid homology (about 95%) was found in the *nodC* product (Török *et al.* 1984; Rossen *et al.* 1984).

In our experiment, slight differences in the root hair deformation (sometimes strong and untypical), in the occurrence of infection threads, and in the nodulation (lower frequency) between the transconjugants and the wild type strains might be caused by the fact that the transconjugants carry extra copies of nodulation genes of *R. galegae*, which then disturb the normal expression of nodulation genes of heterologous bacteria. On the other hand, extra copies of nodulation genes in the homologous *R. galegae nodABC*$^-$ strain enhanced the nodulation of its host plant *G. orientalis*. Both vector plasmids, pLAFR1 (pRg30) and pWB5a (pRg33), are low copy number plasmids, derived from pRK 290. Thus differences in the functions between the two different *nod* clones are not due to different amounts of insert. The *nodC*$^-$ mutant *R. meliloti* Rm1126 carrying pRg30 or pRg33 nodulated its host plant *M. sativa* better than did the *R l.* bv. *viceae* transconjugants their host plants. The *nodA*, *nodB*, and *nodC* mutants of *R. l.* bv. *viceae* 5515 that carry pRg30 induced about 50% of the amount of nodules formed by the wild type strain on *V. villosa* plants, and with pRg33 even less.

***nodI* and *nodJ*.** Besides the *nodABC* genes, many fast-growing *Rhizobium* bacteria carry the *nodI* and *nodJ* genes (Young and Johnston 1989). Transposons in *nodI* and *nodJ* genes of *R. l.* bv. *viceae* cause slightly delayed nodulation on *V. sativa* and *V. hirsuta* (L.) S. F. Gray plants (Canter-Cremers *et al.* 1988). The *nodI*$^-$ and *nodJ*$^-$ of *R. l.* bv. *viciae* LPR5045, after conjugation with the *nod* clones of *R. galegae*, behaved in a different way. It seems that the big 26-kb insert of pRg30 disturbed the nodulation process, and that the small 5.7-kb insert of pRg33 did not have any influence or slightly enhanced nodulation compared with the effects of the parent *nodI*$^-$ and *nodJ*$^-$ strains. On the basis of these plant tests, it is not possible to conclude whether there are *nodI* and *nodJ* genes in pRg30 and in pRg33 or not. DNA hybridization experiments have shown that pRg30 and pRg33 share no homology with *nodI* and *nodJ* genes of *R. leguminosarum* (L. Suominen, unpublished). However, in the complementation tests of *R. l.* bv. *viceae nod*$^-$ mutants carrying pRg30 nodulated *V. villosa* plants better than those carrying pRg33, indicating that the 26-kb insert of pRg30 possibly carries *nodIJ* genes or other genes, which behave like *nodIJ*, whereas the 5.7-kb insert of pRg33 may carry only partly *nodIJ*- or *nodIJ*-like genes.

***Agrobacterium* background.** In several earlier investigations the function of Sym plasmids has been studied by transferring the whole Sym plasmid of different *Rhizobium* species into *A. tumefaciens* or into Sym plasmid-cured *Rhizobium* strains (*R. l.* bv. *trifolii*, Hooykaas *et al.* 1981; Schofield, *et al.* 1984; *R. l.* bv. *viceae*, van Brussel *et al.* 1982; *R. meliloti*, Wong *et al.* 1983; Truchet *et al.* 1984; Hynes *et al.* 1986; *R. l.* bv. *phaseoli*, Martínez *et al.* 1987). It has been observed that these recipients that harbor the rhizobial Sym plasmid induce nodules on homologous host plants. Although the nodules were small, white, and mostly not capable of nitrogen fixation, they had a real nodule structure. Also a relatively small region of the *R. meliloti* Sym plasmid, if it includes nodulation genes, elicits a significant nodulation response (Hirsch *et al.* 1984, 1985; Truchet *et al.* 1985; Putnoky and Kondorosi 1986; Ramakrishnan *et al.* 1986; Rodríguez-Quiñones et. al. 1989). However, it seems that the inserted region should include both the common nodulation genes and at least some host range genes before it can induce significant nodulation in *Agrobacterium* or in a Sym plasmid cured *Rhizobium* background (Putnoky and Kondorosi 1986; Rodríguez-Quiñones *et al.* 1989).

In our study, pRg30 and pRg33, which carry the *nodDABC* genes of *R. galegae*, caused root hair deformation on *Galega* plants in the *Agrobacterium* background, but they did not carry sufficient genetic information for normal nodulation. This result was expected, because both pRg30 and pRg33 are devoid of the host-specific genes of *R. galegae* (Suominen *et al.* 1990), and obviously both the common *nod* genes and host-specific genes are required for the infection process and for the nodulation of *G. officinalis* and *G. orientalis* plants. In the *Agrobacterium* background pRg30 expressed better than pRg33 and caused deformation of the root hairs of *Galega* more clearly. This indicates that the 26-kb insert of pRg30 carries some additional genes required for the early functions of nodulation.

The *A. tumefaciens* transconjugants did not induce root hair deformation on *M. sativa* plants, but nodulelike structures were formed 6 wk after inoculation at very low frequency. Truchet *et al.* (1989) have observed that certain *M. sativa* plants can develop non-nitrogen-fixing nodules, even when grown under strictly axenic conditions. This indicates that the host plant possesses the genetic programme for nodule morphogenesis, and the role of *R. meliloti* is to switch on this programme (Truchet *et al.* 1989; Lerouge *et al.* 1990). In our case, it is also probably

more a question of spontaneous nodulation caused by the genes of the *M. sativa* plant itself or of other unspecific induction caused by the rhizobia than of nodulation caused by the function of the *nod* genes of *R. galegae*. This assumption is supported by the fact that the parent *Agrobacterium* strain induces nodulelike structures on *M. sativa* and also on *G. officinalis*, although at lower frequency than the *Agrobacterium* strain carrying pRg30 or pRg33.

Why did the *Agrobacterium* transconjugants not cause the root hairs of *M. sativa* to deform? One explanation might be that the flavonoids exudated by *M. sativa* do not induce the *nodD* of *R. galegae*. Another explanation could be that the induced *R. galegae nodABC* genes produce an extracellular factor(s), which can not recognize the receptors of the root hairs of *M. sativa* without modification by host-specific genes of *R. meliloti*. Faucher *et al.* (1989) have proposed that *nodABC* gene products of *R. meliloti* are involved in the synthesis of a root hair deformation factor effective on vetch, and that conversion of this to an *M. sativa*-specific deformation factor requires the action of *nodH* and *nodQ*, the host-specific genes of *R. meliloti*.

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