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Genomic Requirements of *Rhizobium* for Nodulation of White Clover Hairy Roots Transformed with the Pea Lectin Gene

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Soil bacteria from the genera *Rhizobium*, *Bradyrhizobium*, or *Azorhizobium* induce formation of nitrogen-fixing nodules in legume roots in a host plant-specific way. For instance, *R. leguminosarum* bv. *viciae* (*R. l.* bv. *viciae*) nodulates pea but not white clover roots. Previously, we have shown that introduction of the pea lectin gene (*psl*) into white clover hairy roots enabled nodulation of such roots by *R. l.* bv. *viciae* (C. L. Díaz, L. S. Melchers, P. J. J. Hooykaas, B. J. J. Lugtenberg, and J. W. Kijne, Nature 338:579-581, 1989). To establish which nodulation (*nod*) genes of *R. l.* bv. *viciae* are required for this heterologous nodulation, we inoculated *psl*-transformed hairy roots with a set of derivatives of *R. l.* bv. *viciae* strain 248, each carrying a transposon insertion in one *nod* gene as well as with a set of strain 248-derivatives containing a curtailed Sym plasmid pRL1JI carrying *nod FDABCIJ* and in addition harboring different *nod* genes cloned in IncP plasmids. The results show that together with the *nodD* and the *nodABCIJ* operons, the complete *nodFEL* operon of *R. l.* bv. *viciae* is required for nodulation of white clover hairy roots transformed with the *psl* gene. The nodulation frequencies and number of nodules observed are comparable to those obtained with strains containing the complete Sym plasmid pRL1JI, regardless of the chromosomal background. Inoculation with the more distantly related *R. meliloti* and *R. etli* species did not result in nodule induction. These results indicate that nodulation of *psl*-transformed white clover hairy roots by *R. l.* bv. *viciae* does not represent an aberrant type of nodulation, and involves recognition by white clover of mitogenic *R. l.* bv. *viciae*–Nod signals. Interestingly, the homologous biovar *R. l.* bv. *trifolii* consistently induced formation of more nodules on *psl*-transformed white clover hairy roots, which suggests that pea lectin recognizes *R. l.* bv. *viciae* as well as its close relative *R. l.* bv. *trifolii*.

Additional keywords: pea root lectin (root PSL), *Rhizobium leguminosarum* bv. *trifolii* (*R. l.* bv. *trifolii*), specificity, (*Trifolium repens*) hairy roots.

In nitrogen-poor soils, the roots of leguminous plants can be nodulated by bacteria from the genera *Rhizobium*, *Bradyrhizobium*, or *Azorhizobium* (reviewed by Kijne 1992).

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Nodulation is characterized by host-plant specificity: a bacterial species or biovar effectively induces nodule formation on the roots of only a selected group of host plants. For instance, white clover is nodulated by *R. leguminosarum* bv. *trifolii* (*R. l.* bv. *trifolii*) but not by *R. leguminosarum* bv. *viciae* (*R. l.* bv. *viciae*), which nodulates pea and common vetch. This host-specific process results from an exchange of signals between bacteria and plants.

Transcription of bacterial genes involved in nodulation (*nod* genes) is triggered by flavonoid inducers secreted by the roots of the homologous host plant (reviewed by Schlaman et al. 1992). This results in the production and secretion of specific rhizobial signals, lipo-chitin oligosaccharides, that induce formation of nodule primordia in the roots of leguminous plants in a host-specific way. The so-called common *nodA*, *nodB*, and *nodC* genes code for enzymes involved in the synthesis and processing of the β-1,4-linked *N*-acetyl glucosamine oligosaccharide backbone, which has an *N*-acyl substitution on the nonreducing sugar moiety and is found in all rhizobial Nod signals. The sugar backbone can consist of three to five residues. The length of the acyl chain and its degree of unsaturation as well as substitutions on the terminal sugar residues are characteristic for each *Rhizobium* and determine the host-specific activity of the Nod signals (reviewed by Dénarié and Cullimore 1993; Spaink et al. 1993). For instance, the biological activity of *R. l.* bv. *viciae* signals depends both on the presence of four double bonds in the 18-carbon acyl chain and on the presence of an *O*-acetyl group attached to the carbon-6 of the nonreducing sugar (Spaink et al. 1991; Van Brussel et al. 1992). Proteins involved in the production of the unsaturated fatty acid are encoded by *nodF* and *nodE* genes, which most likely function as an acyl carrier protein and a β-ketoacylsynthase, respectively (reviewed by Spaink 1992; Spaink et al. 1993). Nodulation experiments have shown that the *nodE* genes from *R. l.* bv. *viciae* and from *R. l.* bv. *trifolii* are not interchangeable and determine the host-specific nodulation by both biovars (Spaink et al. 1989). The protein encoded by *nodL* is an *O*-acetyl transferase that adds the *O*-acetyl moiety to the nonreducing terminal sugar of *R. l.* bv. *viciae* Nod signals (Spaink et al. 1991; Bloemberg et al. 1994). The Nod signals secreted by *R. l.* bv. *trifolii* are similarly acetylated and differ from those of *R. l.* bv. *viciae* only in the number of carbon atoms and in the position of double bonds of the acyl chain (Spaink et al. 1995).

Lectin from the host plant root is another factor that has been shown to play a role in host-specificity of nodulation. Lectins are nonenzymatic sugar-binding proteins that are characterized by their specificity of binding a ligand. Four observations strongly suggest an involvement of pea lectin in nodulation: (i) root lectin is localized at the tips of growing root hairs, where *Rhizobium* attaches and penetrates the host root by means of an infection thread (reviewed by Kijne et al. 1992); (ii) the pattern of localization of pea lectin on the root surface entirely corresponds with the pattern of susceptibility to rhizobial infection (Díaz et al. 1986); (iii) introduction of the pea lectin gene (*psl*) into white clover hairy roots breaks an infection barrier in these roots and allows nodulation by *R. l.* bv. *viciae*, which normally does not nodulate white clover (Díaz et al. 1989); and (iv) lectins isolated from host plants of the cross-inoculation group nodulated by *R. l.* bv. *viciae* have similar sugar-binding specificity. In this paper, we describe how we used *psl*-transgenic white clover hairy roots to establish which *R. l.* bv. *viciae nod* genes are required for heterologous nodulation of white clover. To this end, we inoculated *psl*-transformed hairy roots with a set of *R. l.* bv. *viciae* strains carrying *nod* genes mutated by transposon insertions, or with another set of *R. l.* bv. *viciae* derivatives carrying a curtailed symbiotic (Sym) plasmid and different combinations of cloned *nod* genes. We show that the combination of *nod* genes required for nodulation of *psl*-transformed white clover hairy roots is identical to that required for nodulation of *R. l.* bv. *viciae* host plants, which demonstrates that heterologous nodulation of white clover is not an abnormal form of nodulation and which provides additional evidence for a role of pea root lectin (root PSL) in nodulation.

RESULTS

Nodulation characteristics of white clover hairy roots.

As specified in Materials and Methods, small variations of growth conditions of white clover plants with hairy roots do not seem to affect the reproducibility of nodulation experiments. However, factors such as incorrect light intensity and nutrient deficiency (especially nitrate) result in growth arrest, overproduction of lateral roots, and "browning" or "greening" of root tissues. We have observed that hairy roots that do not elongate are not able to nodulate. White clover hairy roots are also quite sensitive to desiccation and become easily deformed when root meristems get into contact with surfaces that they can not penetrate. Induction of hairy roots on white clover stems does not seem to affect already formed leaves or meristematic activity. The differences in growth rate and number of leaves of transformed plants are similar to those of nontransformed plants and are quite commonly observed in seedlings originating from a random seed population. However, because the main root of white clover seedlings is cut following transformation (Díaz et al. 1989), approximately 10% of the transformed plants may present some reduction of growth resulting from desiccation of emerging hairy roots or damage caused by manipulations at the time of transformation, or during transfer to fresh plates. For nodulation experiments, we selected healthy-looking plants with a few hairy roots (two to four per plant) growing vigorously on filter paper.

On a single plant, the hairy roots of cv. Dutch clover may show different phenotypes: some roots are thicker than others; some have a tendency to produce more lateral roots and all of them seem capable of growing at different rates; some will become brownish or greenish; and some will remain white or translucent. In some cases, emerging lateral roots may be as thick as the parent root, possess a rounded tip, and grow out very slowly, making it difficult to distinguish these from nodule meristems. However, at a later stage of development, this type of lateral root could be distinguished from nodule meristems and emerging nodules because these roots are usually longer than root nodules, do not have a narrowed zone at the site of emergence from the root, and do not carry deformed and curled root hairs at their tips. It has been reported that the normal roots of white clover show localized swellings, occasionally covered by curled root hairs (McIver et al. 1993). We observed that such swellings are rare in white clover cv. Dutch clover, and that they also rarely appear in hairy roots. However, to avoid misinterpretations of such structures, putative nodule meristems that remained flat and did not develop into the rounded form that could be easily identified as an emerging nodule were not scored as such.

Nodulation by wild-type *Rhizobium* strains.

To test the susceptibility of white clover hairy roots transformed with the *psl* gene to nodulation by different rhizobial species, these roots were inoculated with various wild-type *Rhizobium* strains. The results of these experiments are summarized in Table 1. Inoculation with the homologous microsymbiont *R. l* bv. *trifolii* ANU843 resulted in up to 95% nodulated plants, regardless of the introduction of the *psl* gene. As previously described (Díaz et al. 1989), nodules could already be observed 4 to 6 days after inoculation, which is a similar period of time for nodule emergence on the roots of nontransformed white clover plants. Most of the plants with hairy roots are nodulated by *Rhizobium* 10 to 15 days after inoculation, and, exceptionally, plants presented nodules only later. Interestingly, 40 days after inoculation, an average of 150 nodules per 30 plants was observed on hairy roots induced by LBA1334 carrying Bin19, whereas 230 nodules were noticed on hairy roots induced by LBA1334 carrying pBin19 *psl* (Fig. 1). This means that formation of approximately 50% more nodules was induced by *R. l.* bv. *trifolii* on white clover hairy roots transformed with the *psl* gene. This increase in nodule formation was already evident 20 days after inoculation.

Table 1. Nodulation of white clover plants with hairy roots inoculated with wild type *Rhizobium* strains

Strains	Relevant characteristics	Root type	Nodulated plants	
			30 dai[a]	40 dai[a]
248	*wt* bv *viciae*	Bin19[b]	0/30	0/30
		Bin19 *psl*[c]	10/30	15/30
ANU843	*wt* bv *trifolii*	Bin19	17/20	19/20
		Bin19 *psl*	17/20	19/20
R. meliloti 2011	*wt*	Bin19	0/30	0/30
		Bin19 *psl*	0/30	0/30
R. etli	*wt*	Bin19	0/30	0/30
		Bin19 *psl*	0/30	0/30
No *Rhizobium* applied		Bin19	0/20	0/20
		Bin19 *psl*	0/20	0/20

[a] Days after inoculation with *Rhizobium*.
[b] Hairy roots induced by *A. rhizogenes* LBA1334 carrying pBin19.
[c] Hairy roots induced by LBA1334 pBin19 containing the *psl* gene.

Outgrowths that look like emerging, young, or fully grown nodules (nodulelike structures) could be observed following inoculation of white clover hairy roots induced without the *psl* gene with the heterologous microsymbiont *R. l.* bv. *viciae* strain 248. These structures appeared within the marginal frequencies previously observed for wild-type white clover roots (Hepper 1978) and for white clover hairy roots (Díaz et al. 1989). In contrast, transformation with the *psl* gene yielded nodulation frequencies varying from 50 to 70% following inoculation with *R. l.* bv. *viciae* (see Tables 2 and 3; Díaz et al. 1989).

Nodules were not observed following inoculation with *R. meliloti* strain 2011 and *R. etli*. Careful microscopic observations at 20, 30, and 40 days after inoculation revealed neither root hair deformations such as branching, swelling and curling, nor swellings of the roots that could be taken for (putative) nodule meristems. These results show that introduction of the *psl* gene into white clover roots only breaks a host-specificity barrier toward nodulation by *R. l.* bv. *viciae*, which is closely related to the homologous microsymbiont *R. l.* bv. *trifolii*. This facilitation of nodulation is not extended to more distantly related *Rhizobium* species.

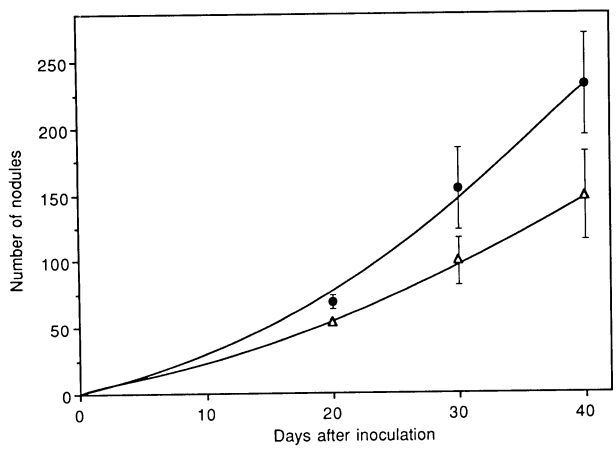

Fig. 1. Number of nodules induced on hairy roots of 30 white clover plants following inoculation with *Rl trifolii* ANU843. Average of two independent transformation and nodulation experiments. Nodules induced on roots transformed with pBin19 carrying the *psl* gene (bulleted line) and, (line with open triangles), on roots transformed with pBin19. The bars represent standard deviations.

Table 2. Nodulation of white clover plants with hairy roots inoculated with *Rl viciae* 248-derivatives carrying transposon insertions in *nod* genes, 40 days after inoculation

Strains	Relevant characteristics	Plants with hairy roots	
		With *psl*[a]	Without *psl*[b]
RBL1409	*nodA*⁻	0/30	0/30
RBL1242	*nodL*⁻	0/30	0/30
RBL1401	*nodE*⁻	4/30	2/30
RBL1387[c]	cured	0/30	0/30
248[c]	*wt* bv *viciae*	15/30	2/30
ANU843[c]	*wt* bv *trifolii*	19/20	19/20

[a] Hairy roots on white clover were induced by LBA1334 pBin19 carrying the *psl* gene.
[b] Hairy roots were induced by LBA1334 pBin19 without the *psl* gene.
[c] Inoculation with these strains was included as a control for proper transformation and nodulation conditions.

Nodulation by *nod* mutants.

We investigated which *nod* genes are involved in heterologous nodulation of *psl*-transgenic white clover roots by *R. l.* bv. *viciae*. Nodulelike structures were not observed on white clover hairy roots following inoculation with *R. l.* bv. *viciae* strain 248-derivatives carrying transposon insertions in either *nodL* or *nodA*, (strains RBL1242 and RBL1409, respectively) as shown in Table 2. Strain RBL1401 (*nodE*::Tn5) induced formation of only a few small nodulelike structures on the roots of pBin19-transformed plants, and of similar structures on the roots of pBin19 *psl*-transformed plants, 30 to 40 days after inoculation. *Rhizobium* bacteria could not be reisolated from such structures.

Extensive root hair curling on Bin19- and Bin19 *psl*-induced hairy roots could be observed as early as 2 to 4 days after inoculation with the wild-type 248, as well as with strains carrying transposon insertions in *nodL* and *nodE*. Root hair curling was not observed when hairy roots were inoculated with RBL1409, in which the *nodABC* operon is inactivated by a polar Tn5 insertion in *nodA* (Zaat et al. 1989). Also, strain 1387, which is a Sym plasmid-cured 248 strain, did not induce any response (neither nodulelike structures nor root hair deformations) in white clover hairy roots. These results show that the responses of *psl*-transformed white clover hairy roots to *R. l.* bv. *viciae* strains carrying transposon insertions in *nod* genes do not differ from those of homologous host plants (Wijffelman et al. 1985; Zaat et al. 1989; Canter Cremers et al. 1989), in that elimination of *nodABC*

Table 3. Nodulation of white clover plants, 40 days after inoculation with *Rhizobium* strains carrying cloned *nod* genes of *Rl viciae*.

Strains	*nod* genes present	Root type		
		Hairy roots-*psl*[a]	Hairy roots[b]	Normal roots[c]
248 chromosomal background				
248	All, *viciae*	18/30[d]	2/30[d]	2/30
RBL1420 pMP424	*FDABCIJ FEL*	14/30	2/30	2/30
RBL1420 pMP258	*FDABCIJ E*	3/30	0/30	0/30
RBL1420 pMP1060	*FDABCIJ L*	3/30	2/30	0/30
RBL1420	*FDABCIJ*	10/30	0/30	ND[e]
RBL1387	None	0/30	0/30	ND
LPR5045 chromosomal background				
RBL5601	All, *viciae*	21/30	8/30	4/30
RBL5580 pMP424	*FDABCIJ FEL*	17/30	6/30	5/30
RBL5580 pMP258	*FDABCIJ E*	8/30	2/30	3/30
RBL5580 pMP1060	*FDABCIJ L*	8/30	3/30	ND
RBL5580	*FDABCIJ*	0/30	1/30	0/30
LPR5045	None	0/30	0/30	0/30
ANU843[f]	All, *trifolii*	26/30	26/30	29/30

[a] Hairy roots induced by LBA1334 pBin19 carrying the *psl* gene.
[b] Hairy roots induced by LBA1334 pBin19.
[c] normal roots: non-transformed roots of white clover seedlings.
[d] Average of 4 sets of independent experiments.
[e] Nodulation test not done.
[f] Nodulation by *Rl trifolii* was included as a control for proper transformation and nodulation conditions. Average of 2 sets of independent experiments.

disables nodule induction and root hair deformation whereas improperly decorated Nod signals as produced by *nodE* and *nodL* mutants still induce root hair deformation but do not allow for root infection.

Nodulation by strains containing cloned *nod* genes.

Nodulation experiments using *Rhizobium* strains containing a combination of a derivative of Sym plasmid pRL1JI carrying only *nod* genes *FDABCIJ* and an Inc P plasmid containing either *nodL*, *nodE*, or *nodFEL* were performed to test which combination of *R. l.* bv. *viciae nod* genes is necessary for nodule induction in white clover hairy roots transformed with the *psl* gene. Cloned *nod* genes were introduced in strain RBL1387, which is a Sym plasmid-cured *R. l.* bv. *viciae* strain 248. The results of the nodulation experiments are summarized in Table 3. Inoculation with strain RBL1420 pMP424, which carries *nodFDABCIJ* and *nodFEL*, resulted in 14 nodulated plants out of 30, whereas strain 248 nodulated on average 18 out of 30 plants. In both cases, nodulation was poor during the first 20 days after inoculation, with most of the nodules emerging or being formed in the following 20 days. Both strains induced a number of nodules in the same order of magnitude (Fig. 2). *Rhizobium* could be isolated from nodules appearing on transgenic roots, and was identified as strain RBL1420 pMP424 or as strain 248 as judged from antibiotic resistance and from host-specificity of nodulation. The percentages of nodulated *Vicia sativa* ssp. *nigra* plants and the timing of nodule appearance after inoculation with the reisolated strains (about 100% for 248 with nodules emerging within 10 days after inoculation, and 60% for RBL1420 pMP424 with nodules emerging 10 days after inoculation) did not differ from those with the original strains on the same host plant.

The results (Table 3) also show that *Rhizobium* carrying the *R. l.* bv. *viciae nod* genes *FDABCIJ* (strain RBL1420) or *nodFDABCIJ* supplemented with a *nodE* or *nodL* clone (strains RBL1420 pMP258 and RBL1420 pMP1060, respectively) induced formation of small nodulelike structures on white clover hairy roots in only a few cases. These structures appeared 30 to 40 days after inoculation. *Rhizobium* bacteria could not be reisolated from such structures, in contrast to sucessful reisolation of bacteria from structures of similar size induced by *R. l* bv. *trifolii*.

Taken together, these results show that, in order to obtain frequencies of nodulation and a number of true nodules comparable to those observed following inoculation with wild-type *R. l.* bv. *viciae*, white clover hairy roots transformed with the *psl* gene require inoculation with a *R. l.* bv. *viciae* strain containing *nod* genes *DABCIJ* and the complete *nodFEL* operon.

Effect of the chromosomal background.

With low frequencies, varying from 7 to 10%, wild-type *R. l.* bv. *viciae* strain 248 induces formation of nodulelike structures in nontransformed white clover roots and in hairy roots transformed with LBA1334 carring pBin19 (see Table 3 and Fig. 2; Díaz et al. 1989). Such low frequencies of incidental heterologous nodulation have been previously reported (Hepper 1978). In order to study the effect of the chromosomal background, we crossed *R. l.* bv. *viciae nod* genes into the *R. l.* bv. *trifolii* Sym plasmid-cured strain LPR5045 and used these derivatives to inoculate white clover roots. Results are summarized in Table 3. The frequency of incidental heterologous nodulation increased to 27% when plants were inoculated with the derivative carrying the complete pRL1JI Sym plasmid (strain RBL5601). Most of these nodulelike structures were small; however, *Rhizobium* bacteria could be reisolated in 50% of the cases only if the surface disinfection period was shortened compared with the situation with nodules of the same size induced by *R. l.* bv. *trifolii* ANU843. Also, the number of colonies appearing on B⁻ plates was significantly lower (less than 20%), compared with reisolations from nodules of the same size induced on *psl*-transformed roots by strains 248 and RBL5601 or by ANU843. These results suggest that, although with low efficiency, the nodulation program of normal white clover roots can be triggered to a certain extent by signals encoded by *R. l.* bv. *viciae nod* genes and that the degree of success (as judged from the fre-

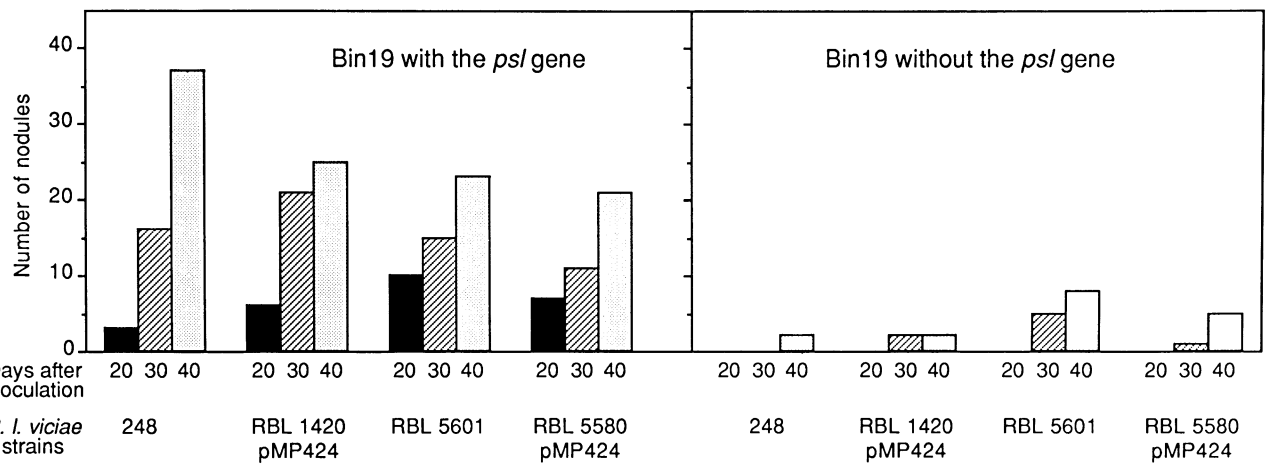

Fig. 2. Average number of nodules induced on hairy roots of 30 white clover plants following inoculation with *Rl viciae* carrying pRL1JI (strains 248 and RBL5601) and *Rl viciae* carrying *nodFDABCIJ* complemented with *nodFEL* clones (strains RBL1420 pMP424 and RBL5580 pMP424). Inoculations with strains carrying cloned *nod* genes were performed twice. Data for strain 248 are the average of five independent transformation and nodulation experiments. Standard deviations varied between 3 and 6 for the number of nodules that emerged on pBin19 *psl*-induced roots, and 1 to 2 for nodules that emerged on pBin19-induced roots.

quency of nodulation) also depends on chromosomally encoded genes.

The effect of the presence of the *psl* gene on nodulation of white clover hairy roots by *Rhizobium* strains carrying *R. l.* bv. *viciae nod* genes can be seen when incidental heterologous nodulation is subtracted from nodulation on *psl*-transformed roots. This correction shows an increase in nodulation frequencies of roughly 50% following inoculation with strains 248 and RBL5601 (both carrying the complete Sym plasmid) and of 40% following inoculation with RBL1429 pMP424 and RBL5580 pMP424 (carrying *nodFDABCIJ* complemented with *nodFEL* clones). In each case, the timing of nodule appearance and the number of nodules that emerged were very similar, as shown in Figure 2.

Results of reisolation and identification of *Rhizobium* from nodules induced by strains RBL5601 and RBL5580 pMP424 on *psl*-transformed white clover roots were identical to those obtained from reisolations from nodules induced following inoculation with strains 248 and RBL1420 pMP424.

We also observed that the roots of plants inoculated with strains containing *R. l.* bv. *viciae nodFDABCIJ* complemented with *nodE* (RBL5580 pMP258), with *nodL* (RBL5580 pMP1060) or *nodFDABCIJ* (RBL5580) in a LPR5045 chromosomal background presented small nodulelike structures at higher frequencies than plants inoculated with strains carrying the same *nod* gene combinations in a cured 248 chromosomal background (Table 3). These structures were apparent 30 to 40 days after inoculation. Note that in contrast with strains carrying pMP258 plasmids, strain RBL1242 (which carries a TN5phoA insertion in *nodL*) did not induce nodulelike structures. This might be due to the difference in copy numbers of *nodE* (6 to 10) in pMP258-containing strains compared with the single copy of *nodE* present in the Sym plasmid pRL1JI. With the exception of one case resulting from inoculation of *psl*-transformed roots with RBL5580 pMP258, reisolations of *Rhizobium* from these structures always failed. These results confirm that the combination of *nodFDABCIJ* and *nodFEL* is indeed required for effective nodule induction independently of the chromosomal background used.

DISCUSSION

We have previously shown that white clover hairy roots transformed with the *psl* gene can be nodulated by wild-type *R. l.* bv. *viciae* strain 248, which is a microsymbiont specifically nodulating the roots of pea and vetch, but not those of white clover (Díaz et al. 1989). It could be argued that this effect represents an aberrant form of nodulation, due to introduction of a foreign gene. To establish which *R. l.* bv. *viciae nod* genes are required for this heterologous nodulation, we inoculated *psl*-transformed hairy roots with a set of *R. l.* bv. *viciae* strains, each carrying mutations in one *nod* gene (Table 2), as well as with a second set of strains containing combinations of cloned *nod* genes (Table 3 and Fig. 2). Only after inoculation with strains carrying *nodD*, the *nodABCIJ* operon as well as the complete *nodFEL* operon, nodulation frequencies, number of nodules, and timing of nodule appearance were similar to those observed following inoculation with wild-type *R. l.* bv. *viciae* strains. These sets of genes are also required for nodulation of normal host plants, such as pea (at a frequency of 83%; Downie and Surin 1990) and *Vicia sativa* ssp. *nigra* (at a frequency of 60%; this paper). These results show that nodulation of *psl*-transformed white clover hairy roots requires the same set of *R. l.* bv. *viciae nod* genes as does nodulation of homologous host plants of the Vicieae-cross inoculation group, and, most probably, does not represent an aberrant type of nodulation.

The main difference between lipo-chitin oligosaccharide Nod factors from *R. l.* bv. *viciae* and *R. l.* bv. *trifolii* resides in the composition of the acyl chain, as determined by the *nodE* gene (Spaink et al. 1995). Apparently, the nodulation program of white clover roots allows to a certain extent for the acceptance of an aberrant acyl chain in Nod factors. For instance, white clover responds with low frequencies of nodulation (25%) to inoculation with strain K11, a *R. l.* bv. *trifolii* ANU843 derivative with a transposon insertion in *nodE*. In contrast, *Trifolium pratense* (red clover) does not nodulate in the presence of this strain (Spaink et al. 1989). Therefore, it seems that red clover is more restrictive than white clover in its perception of the acyl chain of appropriate Nod factors. It is questionable, however, if all nodulelike structures incidentally induced by *R. l.* bv. *viciae* in white clover roots are real nodules (Hepper 1978; this paper). In some cases, white clover hairy roots form outgrowths that look like nodules but do not contain infection threads (Díaz et al. 1989). In this paper, we report that *R. l.* bv. *viciae* can be reisolated at most in 50% of the cases from nodulelike structures appearing on white clover hairy roots. Compared with reisolation from nodules of the same size induced by *R. l.* bv. *trifolii* or by *R. l.* bv. *viciae* in *psl*-transgenic white clover hairy roots, reisolations yielded less cfu and succeeded only if surface disinfection times were shortened. These structures may be similar to arrested emerging nodules that sometimes can be induced by *R. l.* bv. *viciae* on hairy roots of plants transformed with the *psl* gene and that show degenerated meristems and aborted infection threads (Díaz et al. 1989). These data suggest that, incidentally, white clover roots and white clover hairy roots respond to *R. l.* bv. *viciae* Nod factors by forming nodule meristems and that aborted infection threads can also be formed.

The percentage of white clover roots and hairy roots presenting nodules increased from 3–10% to 13–27% following inoculation with strains based on a Sym plasmid-cured *R. l.* bv. *trifolii* containing *R. l.* bv. *viciae nod* genes (the complete Sym plasmid or a combination of *nodDABCIJ* and *nodFEL* clones). These results suggest that incidental heterologous nodulation of white clover appears to be affected by the chromosomal background of the bacteria. Incidental heterologous nodulation of white clover by *R. l.* bv. *viciae* has not been observed by other authors (Yao and Vincent 1969, Djordjevic et al. 1986; Huang et al. 1993), which may be explained by the use of other nodulation conditions, rhizobial strains, or white clover cultivars.

After subtraction of incidental heterologous nodulation, we found that introduction of the *psl* gene into white clover hairy roots yielded an increase in nodulation frequency of about 40% for strains containing at least the *R. l.* bv. *viciae nodD, nodABCIJ,* and *nodFEL* operons, independently of the chromosomal background (*R. l.* bv. *viciae* or *R. l.* bv. *trifolii).* Similar to nodulation induced by *R. l.* bv. *viciae* strains carrying the complete Sym plasmid, this nodulation is delayed and poor. The *psl* gene is functionally expressed at the time of

inoculation with *Rhizobium* (Díaz et al., unpublished). Root hair deformations, branching and curling were observed within 48 h after inoculation with strains carrying the complete pRL1IJ plasmid, pRL1IJ with mutations in *nodL* or in *nodE*, or carrying *R. l.* bv. *viciae nodDABCIJ*, alone or combined with *nodL*, *nodE*, and *nodFEL* clones. This suggests that *R. l.* bv. *viciae nod* genes are induced by flavonoids secreted by white clover hairy roots, which is similar to the situation with normal clover roots (Spaink et al. 1987). In white clover roots, *R. l.* bv. *viciae* strain 300 induces limited cortical cell divisions 72 h after inoculation, which do not proceed to form nodule meristems (Huang et al. 1993). This observation suggests that in these roots nodulation is controlled at the level of nodule initiation, as has been proposed for *Medicago sativa* (Caetano-Anollés and Gresshoff 1991). Delayed nodulation of *psl*-transformed white clover hairy roots could be controlled by the same autoregulatory mechanism. Nodules induced by *R. l.* bv. *viciae* on *psl*-transgenic white clover hairy roots began emerging 20 days after inoculation. However, nodules that emerged were normal in the sense that reisolation of rhizobia was possible for all nodules tested, yielding a significant amount of cfu. Electron microscopy of such nodules has revealed no differences with nodules induced by *R. l.* bv. *viciae* and *R. l.* bv. *trifolii* in their respective host plants (Díaz et al. 1989). Inoculation with *R. meliloti* or *R. etli* did not elicit observable root responses such as root hair deformations, nodule primordium formation, and nodulation. Most probably, Nod factors secreted by these strains are not recognized by white clover hairy roots, and introduction of the *psl* gene does not change this situation.

PSL, a glucose/mannose binding protein (Díaz et al. 1990), is encoded by a single gene yielding identical proteins in seed and root (Hoedemaeker et al. 1994). We recently obtained evidence that conservation of the sugar-binding site is required for PSL to stimulate nodulation. A PSL mutant lacking sugar-binding activity was obtained by a single amino acid substitution in the sugar-binding pocket of the protein (Van Eijsden et al. 1992). The mutant lectin, N125D, was introduced into white clover roots and nodulation tests were performed as described in this paper. *R. l.* bv. *viciae* strain 248 failed to nodulate, in contrast to the situation after transformation with wild-type *psl* or with a control *psl* mutant encoding sugar-binding PSL (Van Eijsden et al. 1993). Our data strongly support previous observations that suggest the presence of a step in the rhizobial infection process in which binding of host root lectin to a rhizobial saccharide ligand affects the efficiency of nodulation. Binding of acidic capsular polysaccharide and lipopolysaccharide fractions obtained from *R. l.* bv. *trifolii* to white clover lectin have been shown to be hapten-reversible and to be related to the age of the bacterial culture (Dazzo et al. 1979; Habrak et al. 1981; Dazzo et al. 1982; Sherwood et al. 1984). Stimulation of infection thread formation on white clover roots was observed following simultaneous application of lectin-binding oligosaccharide fragments derived from *R. l.* bv. *trifolii* capsular and extracellular polysaccharides and *R. l.* bv. *trifolii* cells (Abe et al. 1984). Pretreatment of a *B. japonicum* mutant with soybean seed or root lectin corrected the inability of this mutant to promptly initiate infection thread formation and nodulation (Halverson and Stacey 1986). After use of a similar approach, Brelles Mariño et al. (1993) reported stimulation of infection thread formation by *Rl phaseoli* after pretreatment with *Phaseolus vulgaris* seed lectin. Somatic ligands of *R. l.* bv. *viciae* that show specific binding to PSL have been reported (Wolpert and Albersheim 1976; Planqué and Kijne 1977; Kamberger 1979, Kato et al. 1980). However, a role for these ligands in nodulation awaits further investigation.

It is unlikely that the putative rhizobial PSL ligand is a Nod factor. PSL binds sugars such as glucose and mannose that have unsubstituted C-4 and C-6 hydroxyls (Kijne et al. 1980). The backbone of the Nod factor secreted by *R. l.* bv. *viciae* is composed of β-1,4-linked N-acetyl-D-glucosamine (Spaink et al. 1991). As a monosaccharide, this sugar binds well to root and seed PSL (Díaz et al. 1990). Polymers of this sugar may bind to the lectin with their terminal nonreducing sugar residue. However, in mitogenic Nod factors this residue is 6-*O*-acetylated, due to the action of the *nodL* gene (Bloemberg et al. 1994). This modification theoretically precludes binding to PSL. In the present paper, we have shown that *R. l.* bv. *viciae nodL* and *nodE* genes are necessary for *Rhizobium* to nodulate *psl*-transformed white clover roots. Lack of direct binding of PSL to *R. l.* bv. *viciae* Nod factors implies that the heterologous nodulation we describe in this paper might be due to the combined action of two different mechanisms that together lead to nodule formation: efficiency of induction of nodule meristems and preinfection thread structures following perception of correctly decorated Nod signals, and involvement of root PSL in a complementary step, such as infection tread formation.

Nodules were continuously induced by *R. l* bv. *trifolii* on hairy roots of white clover during the test period. Significantly, introduction of the *psl* gene into white clover hairy roots yielded an increase in the number of nodules induced by the homologous symbiont *R. l.* bv. *trifolii*, which was already apparent 20 days after inoculation. When nodules were counted 40 days after inoculation, 50% more nodules had emerged on these hairy roots than had emerged on hairy roots induced in the absence of the *psl* gene (Fig. 1). One possible explanation for this increase would be if the *psl* gene is continuously expressed in transgenic hairy roots and if *R. l.* bv. *viciae* and *R. l.* bv. *trifolii* share chromosomal genes encoding ligands capable of bindind to PSL. Functional PSL has been isolated from transgenic white clover roots 23 days after transformation, and probably the lectin continues to be expressed through the life cycle of the plant in emerging and growing root hairs, which are the cellular targets for rhizobial infection (Díaz et al., unpublished). Up to now, *R. l.* bv. *trifolii* and other rhizobia have not been systematically analyzed for the presence of PSL ligands, either secreted or somatic. Such an analysis is necessary in order to test the possibility that PSL stimulates root infection and nodulation by rhizobia other than *R. l.* bv. *viciae*, provided that appropriate Nod factors are produced.

MATERIALS AND METHODS

Plant material and induction of hairy roots.

Hairy roots were induced on *Trifolium repens* L. seedlings (white clover cv. Dutch clover, Kieft, Blokker, The Netherlands) as described previously (Díaz et al. 1989), using *Agrobacterium rhizogenes* LBA1334 harboring the complete pea lectin gene in the binary vector pBin19 (called pBin19 *psl*).

As a control, hairy roots were induced by LBA1334 carrying pBin19. To improve growth and nodulation of hairy roots, the following conditions were implemented: Jensen medium (Van Brussel et al. 1982) was supplemented with 0.75 mM $Ca(NO_3)_2$ and solidified with 0.75% pronarose (Hispanagar, S.A., Burgos, Spain). Furthermore, plants received 2.5 ml Jensen-0.75 mM $Ca(NO_3)_2$ per plate, 20 days after inoculation with *Rhizobium*. Plants were grown at 20 ± 2° C in 12 h light and 12 h darkness, with roots growing on filter paper, as previously described (Díaz et al. 1989). Plants grew and nodulated well with illumination by Philips TLD36W/33 or Philips TLD36W/84 fluorescent tubes with an irradiance of 72 or 96 μE m^{-2} s^{-1}.

Bacterial strains and plasmids.

Rhizobium strains and plasmids used in this study are listed in Table 4, and can be grouped into four sets. The first set consists of strains derived from wild-type strain *R. l.* bv. *viciae* 248 that carry transposon insertions in the *nod* gene region of Sym plasmid pRL1J1. The second set is based on the 248-derivative RBL1420, which harbors pRL1JI with a deletion covering *nodELMNTO*. In this strain, plasmids with cloned *nod* genes were introduced. To test the influence of the chromosomal background, the third set was generated by crossing the nodulation gene combinations of the second set into the Sym plasmid-cured *R. l.* bv. *trifolii* strain LPR5045. The fourth set includes wild-type *Rl* biovars, *R. meliloti*, and *R. etli*.

Table 4. *Rhizobium* strains and plasmids used in this work

Strain	Relevant characteristics	Source
248	Wild-type *Rl viciae* containing pSym pRL1JI	Josey et al. (1979)
RBL1387	248 Sym plasmid-cured	Priem and Wijffelman (1984)
RBL1409	248 pRL1JI *nodA*::Tn5[a]	Zaat et al. (1987)
RBL1242	248 pRL1JI *nodL*::Tn5phoA[b]	Canter Cremers et al. (1989), this work
RBL1401	248 pRl1JI *nodE*::Tn5	Wijffelman et al. (1985), this work
RBL1420	248 pRL1JI::Tn1831(1)[c], *nodELMNTO* absent	Canter Cremers et al. (1989)
LPR5045	Sym plasmid-cured derivative of *Rl trifolii* RCR5, Rif^R	Hooykaas et al. (1981)
RBL5601	5045 pRL1JI *mep2*::Tn5	Wijffelman et al. (1985)
RBL5580	5045 pRL1JI::Tn1831(1), *nodELMNTO* absent	Canter Cremers et al. (1989)
ANU843	Wild-type *Rl trifolii* containing pSym 843	Rolfe et al. (1980)
Rhizobium meliloti 2011	Wild type, Rif^R	Faucher et al. (1988)
Rhizobium etli	Wild type	Noel et al. (1986)
Plasmids		
pMP92	Inc P cloning vector conferring Tc^R	Spaink et al. (1987)
pMP258	*nodE* genes of pRL1JI cloned in pMP92, Tc^R	Spaink et al. (1989)
pMP424	*nodFEL* genes of pRL1JI (*Bam*HI- *Sal*I fragment) cloned in pMP92, Tc^R	This work
pMP1060	*nodL* gene of pRL1JI cloned behind the *nodA* promoter of *Rl viciae* (*Bgl*II-*Sph*I fragment) cloned in pMP92 Tc^R	This work

[a,b] Tn5 and Tn5phoA encode Km^R
[c] Tn1831 encodes Spc^R, Sm^R and Hg^R

Nodulation experiments.

White clover hairy roots were inoculated with *Rhizobium* 8 to 9 days after transformation, as described previously (Díaz et al. 1989). Bacteria were grown for 2 to 3 days on solid YMB medium (Rolfe et al. 1980) containing 2 μg tetracycline in the case of Inc P plasmid-harboring strains, with addition of appropiate antibiotics depending on resistances encoded by chromosomal or Sym plasmid-located genes. Roots were examined with use of a Wild M3Z binocular microscope (Heerbrugg, Switzerland) 20, 30 and 40 days after inoculation with *Rhizobium*. Three types of structures were scored: (i) emerging nodules (rounded nodule meristems clearly protruding from the root cortex and showing deformed root hairs on the outermost cell layer); (ii) young nodules (small, round structures about 1 mm in diameter); and (iii) nodules (the typical elongated and oval indeterminate legume root nodule, 2 to 3 mm in length). To facilitate reading, these structures are collectively refered to as nodules or nodulelike structures.

Bacteria were reisolated from nodules and identified by determination of antibiotic resistance and nodule induction ability on homologous or heterologous host plants. Nodules were surface sterilized by submersion in 10% H_2O_2 for 1 to 5 min, depending on their size. For comparison of cfu, nodules of similar size induced by heterologous strains and by ANU843 were submitted to the same period of surface sterilization and, as a control, nodules of similar size induced by ANU843 were submitted to a twice-as-long surface sterilization period. Nodules were rinsed by three consecutive changes of sterilized 25 mM K_2HPO4-KH_2PO_4 buffer, pH 7.5, followed by another three changes at 1 min intervals. Nodules were crushed in 100 to 200 μl of phosphate buffer, and bacteria were directly transferred onto the roots of *Vicia sativa* ssp *nigra* and of *Trifolium repens* as well as to plates with B^- medium (Van Brussel et al. 1977). Colonies appearing on B^- plates were tested for resistance against antibiotics and were then used to inoculate the roots of *Vicia sativa* and *Trifolium repens* plants as described by Van Brussel et al. (1982), with the plants growing under conditions specified above. Nodulation was scored 10, 20, and 30 days after inoculation.

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