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Citation

Eijsden, R. R. van, Diaz, C. L., Pater, B. S. de, & Kijne, J. W. (1995). Sugar binding activity of pea (pisum sativum) lectin is essential for heterologous infection of transgenic white clover hairy roots by Rhizobium leguminosarum bivar viciae. *Plant Molecular Biology*, *29*(3), 431-439. doi:10.1007/BF00020975

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Note: To cite this publication please use the final published version (if applicable).

Plant Molecular Biology **29**: 431–439, 1995.
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Sugar-binding activity of pea (*Pisum sativum*) lectin is essential for heterologous infection of transgenic white clover hairy roots by *Rhizobium leguminosarum* biovar viciae

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Received 26 January 1995; accepted in revised form 22 May 1995

Key words: heterologous infection, host specificity, nodulation, pea lectin, sugar-binding

Abstract

Legume lectin stimulates infection of roots in the symbiosis between leguminous plants and bacteria of the genus *Rhizobium*. Introduction of the *Pisum sativum* lectin gene (*psl*) into white clover hairy roots enables heterologous infection and nodulation by the pea symbiont *R. leguminosarum* biovar viciae (*R.l. viciae*). Legume lectins contain a specific sugar-binding site. Here, we show that inoculation of white clover hairy roots co-transformed with a *psl* mutant encoding a non-sugar-binding lectin (PSL N125D) with *R.l. viciae* yielded only background pseudo-nodule formation, in contrast to the situation after transformation with wild type *psl* or with a *psl* mutant encoding sugar-binding PSL (PSL A126V). For every construct tested, nodulation by the homologous symbiont *R.l. trifolii* was normal. These results strongly suggest that (1) sugar-binding activity of PSL is necessary for infection of white clover hairy roots by *R.l. viciae*, and (2) the rhizobial ligand of host lectin is a sugar residue rather than a lipid.

Introduction

Soil bacteria from the genera *Rhizobium*, *Bradyrhizobium* and *Azorhizobium* are able to induce formation of nodules on the roots of leguminous plants, in which they fix atmospheric nitrogen into ammonia. This process of nodulation is host-plant-specific; for instance, *R. leguminosarum* biovar viciae (*R.l. viciae*) nodulates *Pisum*, *Vicia*, *Lens* and *Lathyrus*, whereas *R. leguminosarum* biovar trifolii (*R.l. trifolii*) normally nodulates *Trifolium* [15, 30]. Host-plant specificity is determined at an early stage of the nodulation process. At this stage, both symbiotic partners exchange molecular signals, such as plant flavonoid inducers of bacterial nodulation genes (*nod* genes) [17] and Nod factors, products of bacterial *nod* genes [14].

Nod factors are lipo-oligosaccharides consisting of an oligomeric backbone of β-1,4-linked *N*-acetyl-D-glucosamine residues, varying in length between three and five sugar units, and a fatty acid chain attached to the amino group of the non-reducing end of the sugar backbone [42]. Dependent on the rhizobial species or biovar, other substituents such as sulfate or *O*-acetyl

groups can be present [42]. Nod factors act as determinants of the host-range of nodulation [14, 42, 44, 45]. The difference in host specificity of nodulation by the biovars *R.l. viciae* and *R.l. trifolii* is primarily determined by the *nodE* gene, the product of which is involved in production of the highly unsaturated fatty acid chain [44]. The sugar backbones of Nod factors from both biovars have an identical structure [43]. The effects of Nod factors on host plant roots include induction of nodule meristems, pre-infection thread formation, root hair deformations and expression of early nodulin genes (for a review, see [30]). Nod factors pave the way for actual infection of the host root by bacterial cells.

Nod factors appear not to be the only factors involved in determination of host-plant specificity during the nodulation process. In addition, the recognition mechanism of the symbionts has been reported to include legume lectins. Lectins are sugar-binding (glyco)proteins, which are not enzymes or antibodies, usually harbouring at least two sugar-binding sites per molecule [40]. Pea lectin (PSL) is a dimeric protein, composed of two identical monomers, possessing mannose/glucose-binding specificity. After synthesis, the lectin is processed but not glycosylated, yielding a protein with a total molecular mass of about 49 kDa, consisting of two small α-chains and two larger β-chains [21]. The lectin is encoded by one functional gene [16, 21, 27], is very abundant in pea seeds, and is produced and secreted in small amounts by pea roots [8, 48]. Díaz *et al.* have shown that transformation of white clover (*Trifolium repens*) hairy roots with the *psl* gene allows infection by *R.l. viciae* to proceed beyond root hair curling [10]. This indicated that root lectin contributes to a mechanism by which the plant selectively allows actual root hair infection and infection thread formation by *Rhizobium* bacteria.

In order to determine the role of PSL during nodulation, we focused our attention on the sugar-binding characteristics of the lectin. In previous reports, we showed that substitution of a single amino acid residue in the PSL monosaccharide-binding pocket resulted in a complete elimination of sugar-binding activity. These modified PSL molecules were neither (detectably) affected in formation of dimers nor in binding of metal ions [50, 51]. Here, we report on the introduction of one of these modified *psl* genes into white clover hairy roots, and its effect on nodulation by biovars *R.l. viciae* and *R.l. trifolii*.

Materials and methods

Constructs

For proper processing and transport of PSL, the *psl* constructs used for expression in *Escherichia coli* needed extension with the signal sequence. The sequence encoding the PSL signal peptide was amplified by standard PCR [51], using the genomic *psl* gene as a template [16] (Fig. 1A). During amplification a *Hin*dIII site was introduced 5′ of the coding region. In the sequence encoding the signal peptide an *Eco*RI site was introduced without changing the primary protein sequence, as described previously [51]. The amplified fragment was digested with *Hin*dIII and *Eco*RI and introduced into the multiple cloning site of the Bluescript SK$^+$ vector, yielding pFF 46. The *psl*-containing sequence was isolated from this construct as a *Sal*I/*Eco*RI fragment and introduced into the multiple cloning site of pIC 19H [33], yielding pFF 47. The 3′ part of *psl* was cloned into *Eco*RI/*Eco*RV-digested pFF 47, as an *Eco*RI/*Hin*dIII fragment from pMP 2809 [51], of which the *Hin*dIII site was filled in with the Klenow fragment of DNA polymerase I. From the resultant plasmid (pFF 48) the fragment containing the complete PSL-coding region was inserted into the *Hin*dIII site of pIC 19H, giving pFF 49 (Fig. 1A). This last step was necessary to create unique *Eco*RI and *Bam*HI restriction sites which were used to introduce the mutations N125D and A126V, located on an *Eco*RI/*Bam*HI fragment. Constructs were sequenced according to Sanger *et al.* [39]. For expression of *psl* in transformed tobacco and white clover, pAGS HB35S [49] and pBIN 19 [2] were used as binary vectors (yielding an efficiency of co-transformation of about 40% and 70%, respectively) [10]. From

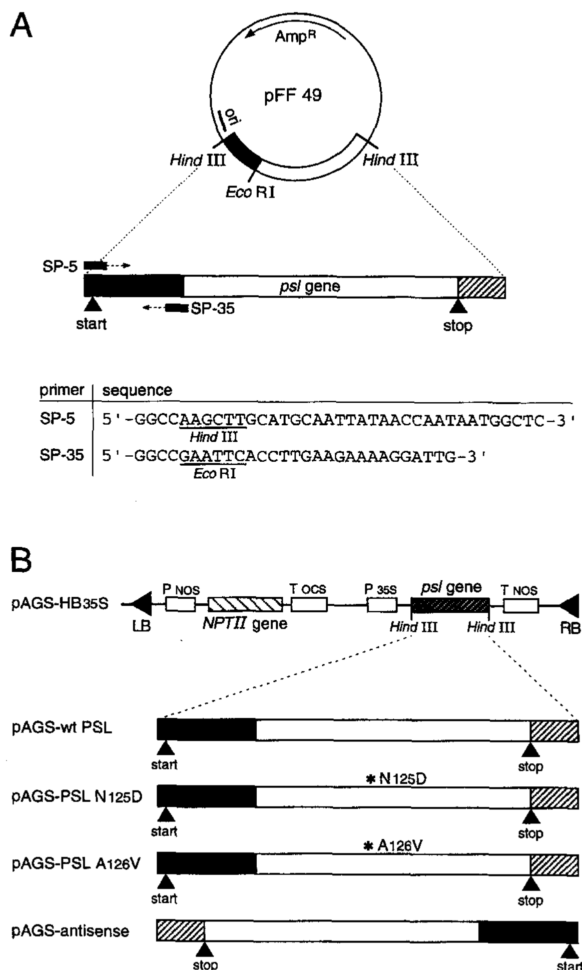

Fig. 1. Construction of *psl* genes used in transformation of clover roots. A. Before cloning of wild-type and modified *psl* cDNA into the binary expression vector pAGS HB35S, the complete sequence encoding the signal peptide was added. This sequence was amplified by PCR, using the genomic *psl* gene as template. Primers used were sp-5 and sp-35, and sites of annealing are indicated by black boxes. Sequences of the primers are given in the table which is included in this figure. The complete *psl* cDNA was introduced as a *Hin*dIII fragment into the multiple cloning site of pIC 19H, yielding pFF 49. Filled bars correspond with *psl* cDNA obtained after amplification of the sequence encoding the signal peptide, and open bars correspond with *psl* cDNA obtained from pMP 2809. The start of translation is indicated by start, and the translation termination point is indicated by stop. B. For cloning of wild-type and modified *psl* cDNA into pAGS HB35S, pFF 49 was used as the basic cloning construct. Mutations were introduced by substitution of corresponding fragments harbouring specific mismatches. Filled bars correspond with sequences encoding the signal peptide, hatched bars with the 3′-untranslated region and asterisks indicate location of specific mismatches. *Psl* genes were cloned into the unique *Hin*dIII site of pAGS HB35S in between the CAMV 35S promoter and the nopaline synthase polyadenylation signal.

pFF 49 and its derivatives the *psl* DNA was isolated as a *Hin*dIII fragment and introduced into the unique *Hin*dIII site of pAGS HB35S, in between the *CaMV 35S* promoter and the nopaline synthase (NOS) polyadenylation signal (Fig. 1B). The binary vector containing wild-type *psl* cDNA was termed pAGS-wt PSL, whereas vectors containing modified *psl* cDNA were termed pAGS-PSL N125D and pAGS-PSL A126V, respectively. As a negative control, a binary expression vector containing wild-type *psl* cDNA in antisense orientation, pAGS-antisense, was constructed. As a positive control, pBin-PSL was used. Here, the complete *psl* gene, under control of its own promoter, was cloned as an *Eco*RI/*Hin*dIII fragment in pBin 19 [2], which has been described in detail by Díaz *et al.* [10].

Binary vectors were transferred from *E. coli* DH5 cells [32] in a triparental conjugation, with *E. coli* containing plasmid pRK 2013, to *Agrobacterium tumefaciens* strain MOG 101 [22] for tobacco transformation or to *A. rhizogenes* strain LBA 1334 [34] for induction of hairy roots.

Transformation and nodulation

Tobacco (*Nicotiana tabacum* cv. Petit Havana SR1) was transformed with the MOG 101 derivatives by the leaf disc transformation method [23]. Expression of constructs pAGS-wt PSL, pAGS-PSL N125D and pAGS-PSL A126V in transformed tobacco was tested by western blot analysis of total leaf extract. Western blot analysis was performed as described previously by Van Eijsden *et al.* [51].

Trifolium repens L. (white clover) seeds (Kieft, Blokker, Netherlands) were surface-sterilized and allowed to germinate for 48 h on Jensen-0.75% agar medium [47], supplemented with 2 mM $Ca(NO_3)_2$ at 22 °C in the dark. Subsequently, growth of seedlings and induction of hairy roots were all performed as described [10].

Seven days after hairy root induction seedlings were transferred to fresh nutrient plates (one per plate), containing 0.75 mM $Ca(NO_3)_2$. Inoculation was carried out by spreading rhizobia, *Rhizobium leguminosarum* bv. viciae (*R.l.* viciae strain 248 [36] and *R. leguminosarum* bv. trifolii strain ANU843 (*R.l. trifolii*) [38] along the roots. Next, the plates were incubated for 24 h in the dark, allowing *Rhizobium* to infect the roots. Two weeks after inoculation, 2 to 2.5 ml liquid Jensen medium supplemented with 0.75 mM $Ca(NO_3)_2$ was added, to prevent shortage of nitrogen. The number of nodulated plants was scored 30 and 40 days after inoculation. Variability of the percentage of heterologous infection was less than 4% in all cases.

Re-isolation of rhizobia from the nodules of white clover

Nodules were surface-sterilized in 10% H_2O_2 for 1–5 min, the time depending on the size of the nodules, and rinsed with sterile 25 mM potassium phosphate buffer, pH 7.5 (3 times quickly and next 3 times for 3 min). Nodules were crushed in 150 μl of the same buffer, and a part of this suspension was plated on B^- medium [36]. The remaining part of the solution was used to inoculate *Vicia sativa* and *T. repens* roots. After incubation of the inoculated plants for 24 h in the dark, growth conditions were the same as described (before) [10].

Results

Expression of wild-type and modified PSL molecules

psl genes were introduced into the binary expression vector pAGS HB35S (see Fig. 1A + B), yielding binary vector constructs containing either the wild-type gene (pAGS-wt PSL), a non-sugar-binding mutant (pAGS-PSL N125D), a mutant possessing normal sugar-binding characteristics (pAGS-PSL A126V), or the wild-type gene in antisense orientation (pAGS-antisense). The constructs are shown in Fig. 1B. In addition, binary vectors pBin 19 and pBin-PSL were used as control constructs, which have been tested and described previously by Díaz *et al.* [10].

Because testing of expression of *psl* genes in white clover hairy roots is hampered by the presence of a cross-reactive protein with a molecular mass of about 20 kDa (possibly a Kunitz-type protease inhibitor [7]), tobacco was chosen to test expression of the most relevant *psl* constructs. Tobacco plants were transformed with pAGS-wt PSL, pAGS-PSL N125D or pAGS-PSL A126V, and expression was tested by immuno-analysis of total leaf extracts. Seed PSL, isolated from seeds of *Pisum sativum* cv. Finale [9], was loaded as a reference. Results are presented in Fig. 2. The western blots demonstrate proper expression and processing of the different PSL forms in tobacco, showing bands with a molecular mass of about 18 kDa corresponding with the PSL β-chains. Sugar-binding properties of the different PSL forms produced by tobacco corresponded with those of PSL (mutants) produced by *E. coli* [51] (results not shown).

From these results, it can be concluded that the different *psl* cDNA constructs were correctly expressed in plants.

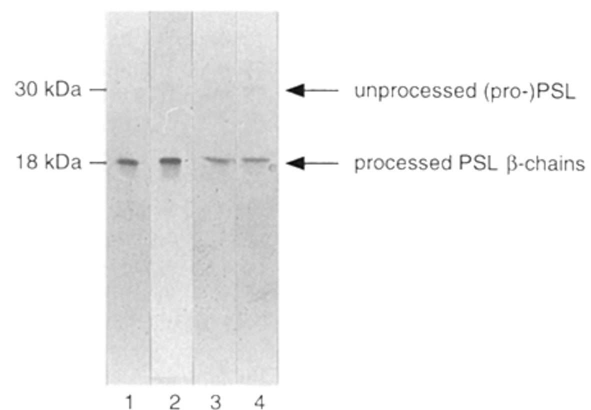

Fig. 2. Immunoblot of transgenic tobacco leaf extracts. Total leaf extracts of tobacco plants were tested for presence of PSL after transformation with pAGS-wt PSL (lane 2), pAGS-PSL N125D (lane 3) and pAGS-PSL A126V (lane 4), respectively. PSL isolated from pea seeds was loaded as a reference (lane 1). Arrows represent unprocessed PSL molecules and processed β-chains.

Nodulation of transgenic white clover hairy roots by Rhizobium

White Clover hairy roots, harbouring either pAGS-wt PSL, pAGS-PSL N125D, pAGS-PSL A126V, pAGS-antisense, pBin 19 or pBin-PSL, were inoculated with *R.l. viciae* and *R.l. trifolii*, respectively, and nodulation was scored 30 and 40 days after inoculation. Results of these nodulation experiments are listed in Table 1. Table 1A shows the nodulation percentage of transformed white clover hairy roots after inoculation with the homologous symbiont, *R.l. trifolii*. Forty days after inoculation, the nodulation percentage was more than 75% in all cases. This result demonstrated that conditions for nodulation were proper, and that co-transformation of clover hairy roots with wild-type or modified-PSL constructs did not (visibly) affect homologous nodulation. Table 1B shows the percentages of nodulation after inoculation of transformed roots with the heterologous symbiont, *R.l. viciae*. Plants co-transformed with wt-PSL, PSL A126V or pBin-PSL showed a nodulation percentage of about 60%, 40 days after inoculation. In comparison with homologous nodulation, nodulation by *R.l. viciae* was delayed and yielded a lower number of nodules. In contrast, plants co-transformed with PSL N125D, PSL-antisense or the binary expression vector pBin 19 showed much lower nodulation percentages of 15% or lower, which were close to background values obtained with hairy roots not containing a *psl* construct (Table 1B) or normal white clover roots (data not shown).

To check whether nodules of the transgenic white clover hairy roots contained the proper inoculant, *Rhizobium* bacteria were re-isolated from the nodules. Re-isolated rhizobia were used for inoculation of *V. sativa* and *T. repens* roots, respectively. Rhizobia could be re-isolated from nodules of *R.l. viciae*-inoculated roots, harbouring wt-PSL, PSL A126V or pBin-PSL, but not from those harbouring PSL N125D, PSL-antisense or pBin 19. Re-isolated rhizobia normally nodulated *Vicia* seedlings but only showed background nodulation on white clover roots,

Table 1. Nodulation of transgenic white clover hairy roots by *Rhizobium leguminosarum* bv. trifolii (A) or by *Rhizobium leguminosarum* bv. viciae.[1]

Hairy roots co-transformed with		Innoculation with *Rhizobium leguminosarum* bv.	% Nodulation (30 d.a.i.)	% Nodulation (40 d.a.i.)
A.	wt-PSL	*trifolii*	78	85
	PSL N125D	*trifolii*	75	81
	PSL A126V	*trifolii*	63	77
	PSL-antisense	*trifolii*	70	87
	pBin 19	*trifolii*	ND	79
	pBin-PSL	*trifolii*	ND	80
	–	*trifolii*	ND	97
B.	wt-PSL	*viciae*	32	63
	PSL N125D	*viciae*	10	16
	PSL A126V	*viciae*	21	59
	PSL-antisense	*viciae*	10	15
	pBin 19	*viciae*	ND	7
	pBin-PSL	*viciae*	ND	60
	–	*viciae*	ND	7

[1] Nodulation was scored (percentage of plants) 30 and 40 days after inoculation (d.a.i.), respectively. For transformation of each construct 30 plants were inoculated. Plants were scored as positive if nodules and/or emerging nodules were present. Since nodulation of plants transformed with pBin 19 and pBin-PSL, respectively, has been described previously [10], nodulation was not scored 30 days after inoculation (ND). As a control, hairy roots were induced by *Agrobacterium rhizogenes* strain LBA 1334 which did not contain any binary vectors (–). Percentages are given in rounded numbers.

which confirms their *R.l. viciae* identity. These results are consistent with results from Díaz *et al.* [10] and suggest that background nodulation of white clover by the heterologous symbiont *R.l. viciae* is due to the formation of empty pseudonodules. Rhizobia re-isolated from nodules induced by *R.l. trifolii* appeared to be unable to nodulate *V. sativa* roots and only infected and nodulated *T. repens* roots.

From these results it can be concluded that root infection of white clover by *R.l. viciae* is possible in the presence of a gene encoding sugar-binding PSL but not in the presence of a gene encoding a non-sugar-binding PSL mutant. This strongly suggests that the sugar-binding ability of PSL is essential for infection by *R.l. viciae* to proceed beyond root hair curling.

Discussion

Introduction of the *psl* gene in white clover hairy roots enables *R.l. viciae* to nodulate [10]. Here, we show that co-transformation with a mutant *psl* gene which is impaired in sugar-binding does not result in nodulation. These data strongly suggest that sugar-binding activity of PSL is essential for heterologous infection of *T. repens* by *R.l. viciae*. However, substantial background nodulation was found on plants transformed with non-sugar-binding PSL or on non-transformed plants, after inoculation with *R.l. viciae*. As known for some time, white clover (*T. repens*) allows background nodulation by the pea symbiont *R.l. viciae* [20]. Díaz *et al.* [10] have shown that such nodules are devoid of bacteria, do not contain infection threads, and should be categorized as pseudonodules. The present work corroborates these observations. Apparently, white clover recognizes and responds to a certain extent to Nod factors of *R.l. viciae* despite the presence of an inappropriate *nodE*-related fatty acid [44]. This permissiveness is also illustrated by the marked root hair curling induced by *R.l. viciae* in white clover roots [10, 53]. However, impaired infection points at the presence of an infection barrier at the level of direct bacterium-to-plant cell interactions in developing root hairs. In the presence of pea lectin (PSL) this barrier apparently can be overcome [10, this work]. Our results indicate that this action of PSL is dependent on its sugar-binding activity, since in the presence of a non-sugar-binding mutant of PSL, *R.l. viciae* is unable to pass the infection barrier.

It should be noted that we have not yet been able to conclusively demonstrate the presence of PSL in transgenic clover hairy roots. This may be due to a low level of PSL production in combination with the presence of harmful factors such as proteases in clover root extracts (Díaz, unpublished results). Immunocytological localization of PSL at clover root hair tips, as successfully performed for pea root hairs [11], failed due to the presence of a cross-reactive protein, possibly a 20 kDa Kunitz-type protease inhibitor [7]. Indirect evidence for the presence of functional PSL at the tip of transgenic clover roots has been presented by Díaz [12], who demonstrated that white clover hairy roots harbouring a *psl* gene construct bound a specific glycoprotein PSL-ligand, in contrast to control hairy roots. Since mutant PSL N125D has lost its ability to bind sugar, this method can not be used to check for the presence of PSL N125D. Possibly, this problem can be solved by tagging PSL N125D with a sequence encoding a heterologous epitope, for example, the human c-myc epitope [37]. Albeit indirect as well, our results on expression of PSL in tobacco and on the influence of PSL on nodulation by *R.l. viciae* are consistent with the hypothesis that PSL is properly produced and targeted in transgenic white clover hairy roots. Work aimed at optimization of the culture conditions and of extraction and detection of PSL from transgenic white clover roots is in progress.

Several observations indicate that the role of legume lectin in nodulation is based on direct interactions of the lectin with rhizobial cells. Bohlool and Schmidt [3] reported that 22 out of 25 *(Brady)rhizobium japonicum* strains capable of nodulating soybean were able to bind a crude preparation of soybean seed lectin (SBL), in contrast to other rhizobial species and biovars. Pretreatment of *B. japonicum* cells with SBL or with

the related soybean root lectin significantly enhanced infection and nodulation by low inocula of wild type bacteria or by normal inocula of a mutant strain exhibiting a delayed-nodulation phenotype [18, 19, 46]. Similarly, Brelles Mariño *et al.* [4] showed that pretreatment of *R. leguminosarum* biovar phaseoli cells with *Phaseolus vulgaris* seed lectin enhances the infectivity of the bacterial cells, strongly increasing the amount of infection threads per root. Clover lectin specifically binds to *R.l. trifolii* cells, whereas *R.l. viciae* cells do not bind this lectin [6]. Apparently, *R.l. viciae* cells lacking a proper 'lectin coat' at the root hair surface are noticed as heterologous bacteria, with a barrier for infection thread formation as a result.

The assumption that lectin binding by rhizobia facilitates root infection raises the question of the identity of the rhizobial lectin ligand. It has been suggested that PSL may interact with Nod factors [31]. Our results strongly suggest that in such a case PSL would bind to the oligo-chitin part of Nod factors rather than to the lipid part. The model of the sugar-binding site of PSL predicts that the lectin may bind to the non-reducing end of the oligo-chitin backbone [44, 51]. However, mitogenic Nod factors produced by *R.l. viciae* are *O*-acetylated at the C6-OH of the corresponding sugar residue. Van Wauwe *et al.* [52] already showed that the C6-OH of a PSL haptenic sugar must be unsubstituted for binding to occur, consistent with the model of the sugar-binding site. Computer-simulated docking experiments with Nod factors from *R.l. viciae* and various Viciae lectins, including PSL, performed by Fabre *et al.* [13], showed that 6-*O*-acetylation of the glucosamine residue at the non-reducing end creates a drastic steric hindrance, thereby inhibiting binding of this residue. From these data, it can be concluded that *R.l. viciae* Nod factors most probably will not be bound by PSL. Furthermore, the saccharide parts of Nod factors produced by *R.l. viciae* and *R.l. trifolii* are identical [43], which is inconsistent with the hypothesis that clover would need PSL to recognize *R.l. viciae* Nod factors. All rhizobial Nod factors identified until now contain an oligo-chitin backbone. Since legume lectins possess a considerable variation in sugar-binding specificity, putative rhizobial ligands more likely may show a corresponding variety in sugar composition. For *R.l. viciae*, crude lipopolysaccharide (LPS) preparations exhibit biovar-specific properties with regard to direct interactions with PSL [26, 28, 29]. Planqué and Kijne [35] isolated a glucan-type polysaccharide from a LPS-fraction of *R.l. viciae* strain A171, which appeared to be an appropriate PSL-ligand. In further research, presence of specific glucose/mannose-type capsular polysaccharides (CPS) in *R.l. viciae* should be tested. Likewise, it should be determined whether specific binding of clover lectin to LPS/CPS preparations of *R.l. trifolii*, as described by Dazzo and co-workers [1, 5, 24, 41], is due to the presence of specific lectin ligands.

In summary, sugar-binding activity of PSL seems to be essential for heterologous infection of transgenic white clover hairy roots by *R.l. viciae*. After secretion by root hairs, PSL binds a cell surface component of infective *R.l. viciae* cells, which probably is a LPS/CPS component [35]. As one possible working hypothesis, we suggest that this component if not covered by lectin prevents uptake of rhizobia in a root hair, in the same way that K-antigens prevent uptake of bacteria by lymphocytes [25].

Acknowledgements

We thank Dr A.A.N. van Brussel for providing us with *Vicia sativa*. This work was supported by the Foundation for Fundamental Biological Research (BION; nos SLW), which is subsidized by the Netherlands Organization for the Advancement of Research (NWO).

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