High-Affinity Nod Factor Binding Site from *Phaseolus vulgaris* Cell Suspension Cultures

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The lipo-chitoooligosaccharidic Nod factors produced by rhizobia are key molecules in the establishment of symbiosis with legumes and probably are recognized by the host plant via specific receptors. Here, we report on the presence of a binding site in cell cultures of *Phaseolus vulgaris* displaying a high affinity for Nod factors from *Rhizobium tropici* (NodRt-V) (Me, S, C18:1), a symbiont of this legume. The binding site shares common properties with NFBS2, a Nod-factor binding site previously characterised in *Medicago varia*, in terms of affinity, preferential plasma-membrane location, and sensitivity to proteases and lysine reactive reagents. However, the bean site poorly recognizes the Nod factors produced by *Sinorhizobium meliloti*, the symbiont of *Medicago*. The study of selectivity toward the Nod factors reveals that the length and degree of unsaturation of the acyl chain and the length of the oligosaccharidic moiety are important determinants of high affinity binding to the bean site; whereas, the N-methyl and O-sulfuryl groups play a minor role. Thus, the common characteristics of *P. vulgaris* and *M. varia* Nod-factor binding sites suggest that they probably correspond to structurally related proteins, but their different selectivity suggests that they may be involved in a differential perception system for Nod factors in legumes.

Additional keyword: lipo-chitoooligosaccharides.

The symbiosis between bacteria termed rhizobia (*Sinorhizobium*, *Mesorhizobium*, *Bradyrhizobium*, *Azorhizobium*, and *Rhizobium*) and leguminous plants leads to the formation of a new organ, the nodule, on the legume roots. In the nodule, the rhizobia are able to fix dinitrogen for the benefit of the plant. The establishment of the symbiosis is characterized by its partner specificity, and broad and narrow host-range bacteria can be distinguished. *Rhizobium* sp. NGR234 is able to nodulate more than 70 legume genera, whereas *R. leguminosarum* bv. *trifolii* nodulates only the genus *Trifolium* (Dénarié et al. 1996).

The nodulation process depends on the expression of the bacterial nodulation genes (*nod* genes), which are induced by the host plant. The *nod* genes encode mainly enzymes respon-

sible for the synthesis of excreted signalling molecules, the nodulation factors (Nod factors). These factors are lipo-chito-oligosaccharides (LCOs), consisting of an N-acetylglucosamine backbone, N-acylated on the terminal nonreducing sugar, and bearing different substitutions on the oligosaccharidic backbone. Each bacterial strain produces a characteristic set of Nod factors: for example, those of *R. tropici* (Rt) are pentameric (V), with a cis-vaccenic fatty acyl chain (C18:1) and an N-methyl group (Me) on C₂ of the nonreducing sugar. Approximately 50% of these Nod factors are sulfated (S) on C₆ of the reducing sugar (Poupot et al. 1993). Using the nomenclature defined by Roche and associates (1991), these factors are written as NodRt-V(Me, S, C18:1). The major Nod factor produced by *Sinorhizobium meliloti* (NodSm-IV(Ac, S, C16:2)) is tetrameric (IV), with a C16:2 fatty acyl chain, an acetyl group (Ac) on C₆ of the nonreducing end, and a sulfate group (S) on the reducing sugar.

Purified Nod factors provoke early symbiotic responses in the plant and, in certain species, they can induce the morphogenesis of bacteria-free nodules (Cohn et al. 1998; Dénarié et al. 1996; Long 1996). The fact that many of the biological responses require a very low concentration of Nod factor with a precise structure strongly suggests that Nod factors may be perceived by specific receptors on the roots of the host plants. Indeed, studies suggest that there may be multiple perception mechanisms with different stringencies for the Nod factor decorations (Cullimore et al. 2001).

A search for putative Nod factor receptors in *Medicago* spp. has led to the identification and the characterization of two Nod-factor binding sites from plant extracts, termed NFBS1 and NFBS2. NFBS1, characterized from *M. truncatula* roots, does not discriminate the different substitutions of the NodSm factors, has a relative low affinity ($K_d = 86$ nM), and is present in tomato, a nonlegume (Bono et al. 1995). By contrast, NFBS2, a higher-affinity binding site ($K_d = 4$ nM) associated to a plasma membrane-enriched fraction of *M. varia* cell suspension cultures, discriminates the presence of the O-acetyl group, the nature of the fatty acid chain, and the length of the oligochitin backbone (Gressent et al. 1999). However, the binding protein NFBS2 is not selective toward the sulfate group, which is the major determinant of the *Medicago* spp.–*S. meliloti* interaction (Roche et al. 1991).

In order to evaluate whether binding sites with similar characteristics are widespread in legumes, we examined *Phaseolus vulgaris*, a phylogenetically distinct legume with symbiotic characteristics very different from those of *Medicago* spp. For these studies, we used cell suspension cultures; first, in order to

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have a direct comparison with *M. varia* and, second, because cell suspensions have been used by many authors as a simplified system to study the perception of signalling molecules (Baureithel et al. 1994; Nühse et al. 2000; Shibuya et al. 1996). In contrast to *Medicago* spp., *P. vulgaris* is a tropical legume that makes nodules of the determinate rather than indeterminate type. Moreover, it is promiscuous, forming nitrogen-fixing nodules with several rhizobial species (*R. tropici*, *R. etli*, *Rhizobium* sp NGR234, *Rhizobium* GRH2), and nonfixing nodules with several others (e.g., *Azorhizobium caulinodans*) (Laeremans and Vanderleyden 1998), whereas *Medicago* spp. have a strict specificity for *S. meliloti*. Although the symbionts of *P. vulgaris* produce a variety of Nod factors with different decorations (N-methyl, fucose, carbamate, sulfate groups), all these LCOs possess five N-acetylglucosamine residues and are acylated with cis-vaccenic acid. In contrast, *S. meliloti* produces mainly tetrameric LCOs with a C16:2 fatty acyl chain. In this work, we have characterized a high-affinity Nod-factor binding site from *P. vulgaris* cell suspension cultures. Its selectivity for the different structural features of the Nod factors has been established and compared with that of NFBS2 from *M. varia*. On this basis, we propose a model for the recognition of LCOs by NFBS2.

RESULTS

A high-affinity ^{35}S -NodRt factor binding site is present in a microsomal fraction prepared from cell culture extracts of *P. vulgaris* but not of carrot.

A nonsulfated Nod factor of *R. tropici*, NodRt-V(Me, C18:1), was sulfated and labeled to a high specific activity with ^{35}S , and referred to as the (^{35}S)-NodRt factor. The incubation of ^{35}S -NodRt factor to particulate fractions obtained by differential centrifugation of extracts of *P. vulgaris* cell cultures revealed that the fraction sedimenting at $45,000 \times g$ (the microsomal fraction) contained 72% of the binding activity ($73 \text{ dpm } \mu\text{g protein}^{-1}$), with the remaining binding activity being present in the $10,000 \times g$ fraction. In contrast, the particulate fractions obtained from carrot cell culture extracts ($10,000$ and $45,000 \times g$ fractions) did not show any specific binding activity when incubated with either ^{35}S -NodSm or ^{35}S -NodRt factors (data not shown).

The association and dissociation curves of the binding of ^{35}S -NodRt to the microsomal fraction showed that binding

Fig. 1. Association and dissociation kinetics of NodRt-V(Me, ^{35}S , C18:1 $_{\Delta 11}$) to the microsomal fraction of *Phaseolus vulgaris* cell culture extracts. Experiments were performed using $50 \mu\text{g}$ of proteins and 0.4 nM ^{35}S -NodRt. The dissociation was initiated (arrow) by the addition of $1 \mu\text{M}$ of unlabeled NodRt factor. Specific binding during association (■) and dissociation (●).

reached an apparent equilibrium after 40 min of incubation at 0°C (Fig. 1). After the equilibrium had been reached, addition of $1 \mu\text{M}$ of nonlabeled NodRt-V(S, C18:1) resulted in the dissociation of the ligand. The dissociation was essentially complete after 15 min, demonstrating the reversibility of the specific binding. A saturation experiment performed with 0.4 nM of labeled ligand in the presence of increasing concentrations of unlabeled ligand (NodRt-V(Me, S, C18:1)), ranging from 5×10^{-10} to $2 \times 10^{-7} \text{ M}$, is presented in Figure 2. The saturation plot showed that the specific binding was saturable, and the deduced Scatchard plot revealed an equilibrium dissociation constant (K_d) of 15 nM and a maximal binding capacity (B_{max}) of 1.5 pmol per milligram of microsomal protein. Furthermore, the Hill number was 0.94 , suggesting the presence of a single class of binding sites. Thus, the *P. vulgaris* microsomal fraction binding to the ^{35}S -NodRt factor is saturable, reversible, and displays a high affinity. The binding site is specific for the LCO structure because neither chitopentaoase with a K_i of $11 \mu\text{M}$, nor $1 \mu\text{M}$ cis-vaccenic acid (concentration corresponding to its limit of solubility) could compete efficiently with the ^{35}S -NodRt binding (data not shown).

The *P. vulgaris* binding site is proteinaceous, involves a lysine residue in ligand binding, and is preferentially located in plasmalemma-enriched fractions.

The proteinaceous nature of the binding site was investigated by studying its sensitivity to proteinase K. Binding activity was almost completely abolished after treatment with a high concentration of the protease (Table 1). Inactivation of the protease either by heat denaturation or by addition of antiproteases (leupeptin and Phenylmethylsulfonyl fluoride [PMSF]) largely prevented this loss of binding activity. Furthermore, incubation of the microsomal fraction with 0.1 or 1 mM 4-4'-diisothiocyanato-2-2'-stilbenedisulfonic acid (DIDS), a compound reacting with lysine residues, resulted in a loss of binding activity of 30 and 53% , respectively, compared with the control. Taken together, these data suggest that the binding site is most likely a protein containing a lysine residue or residues involved in ligand binding. However, it is noteworthy that, with the lower concentration of protease used, even in the absence of antiproteases in the extraction buffer, 49% of the binding activity still remained; whereas, theoretically, the amount of added protease should have been able to digest all the proteins. This result could suggest that the binding site is either not easily accessible or is recalcitrant to the protease.

Fig. 2. Saturation plot and deduced Scatchard plot (inset) of the binding of NodRt-V(Me, ^{35}S , C18:1 $_{\Delta 11}$) to the microsomal fraction of *Phaseolus vulgaris* cell culture extracts. Experiments were performed using $50 \mu\text{g}$ of proteins and 0.4 nM ^{35}S -NodRt, with increasing concentrations of nonlabeled NodRt-V(Me, S, C18:1 $_{\Delta 11}$).

The subcellular location of the *P. vulgaris* binding site was investigated by separating different membranes using the free-flow electrophoresis (FFE) technique. The microsomal fraction of *P. vulgaris* was separated into four fractions, representing 16, 42, 30, and 12% (fractions I, II, III, and IV, respectively) of the total proteins recuperated. The specific binding activity was the highest in fraction I (119 dpm $\mu\text{g protein}^{-1}$) and decreased in the other fractions (fraction II: 81 dpm $\mu\text{g protein}^{-1}$; fraction III: 69 dpm $\mu\text{g protein}^{-1}$; fraction IV: 52 dpm $\mu\text{g protein}^{-1}$) (Fig. 3). The distribution of the binding activity corresponds well with the distribution of the plasmalemma marker (H^+ -ATPase), whereas no such correlation was observed with markers for the endomembranes (BiP) or the tonoplast (V-ATPase). However, fraction II, corresponding to endomembrane-enriched fraction, contains a large proportion of the binding activity (41%); therefore, a dual location of the binding site in the plasma membrane and in the endomembranes cannot be excluded.

The NodRt and NodSm factors bind to the microsomal fraction prepared from *M. varia* and *P. vulgaris* with different affinities.

Next, we tested the ability of the major Nod factor of *S. meliloti* (NodSm-IV(Ac, S, C16:2): the NodSm factor) to bind to the *P. vulgaris* microsomal fraction. The results showed that, under the same experimental conditions, the radiolabeled NodSm factor binds weakly to the microsomal fraction from *P. vulgaris* (6 dpm $\mu\text{g protein}^{-1}$) in comparison with the NodRt factor (75 dpm $\mu\text{g protein}^{-1}$). This was confirmed by the low affinity of the NodSm factor for the binding site ($K_i = 413$ nM), as determined by a competition experiment in which the specific ^{35}S -NodRt binding to the *P. vulgaris* microsomal fraction was inhibited by increasing concentrations of unlabeled NodSm-IV(Ac, S, C16:2). Thus, the *P. vulgaris* binding site is able to bind NodRt but not NodSm factors with high affinity (Table 2). In contrast, the microsomal fraction prepared from *M. varia* binds both the NodRt and the NodSm factors with a high affinity (0.7 and 4 nM, respectively). In both species, the abundance of the binding sites was approximately the same. In the next experiments, the selectivity of the Nod factor binding site of *P. vulgaris*, which seems to be different from that of *M. varia*, was examined further.

The structure of the acyl chain greatly influences the recognition of LCOs by the *P. vulgaris* binding site.

The influence of the structure of the acyl chain on the recognition of the Nod factor by the binding site was assessed by performing competition experiments with synthetic tetrameric LCOs harboring fatty acyl chains varying either in

Table 1. Effect of protease and lysine-reactive reagents on the *Phaseolus vulgaris* high-affinity binding activity

Treatment ^a	Binding activity (%)
Control ^b	100
Proteinase K (5 μg)	49
Proteinase K (50 μg)	8
Denaturated proteinase K (5 μg) ^c	90
Proteinase K (5 μg) + antiproteases ^d	82
DIDS 0.1 mM	53
DIDS 1 mM	30

^a Microsomal fraction aliquots (50 μg of proteins) were preincubated in the different conditions for 10 min at 37°C, then assayed at 0°C for binding activity according to the standard procedure in the presence of 0.4 nM of NodRt-V(Me, ^{35}S , C18:1). DIDS = 4,4'-diisothiocyanato-2,2'-stilbenedisulfonic acid.

^b Incubation in buffer only.

^c Proteases were denaturated by boiling for 10 min.

^d 1 mM Phenylmethylsulfonyl fluoride and 4 μM leupeptin.

length or in the number and the position of the double bonds. The mono-unsaturated LCO-IV(S, C16:1_{Δ2}) and LCO-IV(S, C16:1_{Δ9}) have a similar K_i (55 and 51 nM, respectively) (Table 3A); that is, one order of magnitude lower than that of the bi-unsaturated NodSm-IV(S, C16:2_{Δ2,9}) (412 nM). Thus, the presence of the two double bonds on the fatty acyl chain greatly decreased the affinity of the LCOs for the binding site compared with either of the LCOs with one double bond. Moreover, the affinity increased with the number of carbons in the fatty acyl chain: the LCO with the shortest chain (12 carbons) exhibited the lowest affinity, whereas the LCO with a C18 acyl chain had the highest affinity, 10-fold higher than the LCO with a C16 acyl chain. The affinity ($K_i = 70$ nM) of the LCO-IV(S, C18:2), when compared with that of the corresponding LCOs with a C18:1 fatty acid ($K_i = 5$ nM) or a C16:2 fatty acid ($K_i = 412$ nM), respectively, confirms the negative effect of two double bonds and the positive effect of the length of the fatty acid on ligand binding. Thus, the number of double bonds and the length of the acyl chain both play a major role in Nod factor recognition by the *P. vulgaris* binding site.

The affinity of the LCOs also depends on the oligosaccharidic moiety.

About half of the *R. tropici* Nod factors bear a sulfate group on C₆ of the reducing sugar. The role of this decoration was examined by using different sulfated or nonsulfated LCOs. The nonsulfated NodRt-V(Me, C18:1) presented an affinity ($K_i = 2.5$ nM) which is approximately six times higher than the affinity of the corresponding sulfated compounds ($K_i = 15$ nM) (Table 3B). Thus, the presence of the sulfate group on the reducing end of pentameric LCOs decreases their affinity for the binding protein. In contrast, the affinity of the sulfated tetrameric compound (LCO-IV(S, C16:1)) was at least seven-fold higher than the nonsulfated one (LCO-IV(C16:1)), suggesting a positive effect of the sulfate group when the oligosaccharidic backbone is tetrameric.

The possible role of the length of the sugar backbone in binding of LCOs to the *P. vulgaris* site was further evaluated using LCOs acylated with a C16:1 fatty acyl chain and differing in the number of N-acetylglucosamine residues. The high affinity of LCOs for the binding protein depends largely on the

Fig. 3. Characteristics of membrane fractions separated by free-flow electrophoresis as determined by immunological detection of marker proteins: H^+ -ATPase (white bars), BiP (hatched bars), V-ATPase (black bars), and ^{35}S -NodRt binding activity (■).

presence of the fifth sugar (Table 3C). Indeed, the tetrameric and trimeric LCOs have over 100-fold lower affinities for the site compared with the pentameric compound, and the dimeric LCO is a very poor ligand. However, the affinity of NodSm-V(S, C16:2) was only fivefold higher than the NodSm-IV(S, C16:2) ($K_i = 51$ and 412 nM, respectively). This probably is due to the effect of the sulfate group, as described above, that decreases the affinity of the pentameric LCOs and increases that of the tetrameric LCOs.

The importance of the nonreducing end on the recognition of the LCOs by the *P. vulgaris* binding site was evaluated by studying the effect of the presence of the O-acetyl and N-methyl groups on the affinity. The NodSm-IV(Ac, S, C16:2) and its non-O-acetylated homologue have similar K_i of approximately 410 nM (Table 3D). The non-N-methylated LCO homologous to the NodRt factor was not available. However, pentameric and non-N-methylated LCOs, harboring long and mono-unsaturated acyl chain, display affinities in the nanomolar range similar to that of the N-methylated NodRt factors (Table 3B and C). Thus, the N-methyl group does not seem to be a major determinant of the ligand binding.

DISCUSSION

In this article, we have characterized a high-affinity Nod factor binding site from cell cultures of *P. vulgaris*. This binding site shows a high affinity for the NodRt-V(Me, C18:1) factor from *R. tropici* ($K_d = 2.5$ nM), whereas it poorly recognizes the Nod factors from *S. meliloti*, thus suggesting that it may preferentially recognize Nod factors from bean-symbiotic bacteria. This binding site shows many similarities to the high-affinity binding site (NFBS2) described from cell cultures of *M. varia*: it is specific for LCOs because it poorly recognizes either the lipid or oligosaccharide moieties alone, it is preferentially located in the plasma membrane, and it is sensitive to proteases and lysine-reactive reagents. Both sites can be efficiently solubilized from the membranes using detergents and retain binding activity (F. Gressent and J. J. Bono, unpublished data). Thus, we speculate that these binding sites are based on structurally related membrane proteins and we propose to rename these high-affinity binding sites MvNFBS2 and PvNFBS2, for the sites from *M. varia* and *P. vulgaris*, respectively.

Characterizing the ligand selectivity of PvNFBS2 using the sulfated *R. tropici* Nod factor (NodRt-V(Me, S, C18:1)) has revealed that the site has a strong preference for pentameric factors and that high-affinity binding is highly dependent on the structure of the acyl chain. The presence of the O-sulfuryl group on the *R. tropici* factors—approximately 50% of the factors isolated from this species are O-sulfated (Poupot et al. 1993)—appears to have a negative effect on binding, but can partly compensate for the absence of a fifth sugar on tetrameric factors. Although we have not been able to isolate or synthesize factors varying in only the presence or absence of the N-methyl group, characterization of the affinity of two N-methylated factors with their nearest nonmethylated counterparts suggest that this substitution is not essential for high-affinity binding. Interestingly,

PvNFBS2 poorly recognizes the NodSm factor. However, the NodRt factor has a higher affinity for MvNFBS2 than the NodSm factor, which agrees with previous results (Gressent et al. 1999) showing that binding to this site depends mainly on the hydrophobicity of the acyl chain and the length of the oligosaccharidic backbone. Thus, the NodRt factor, consisting of a C₁₈ acyl chain and a pentameric sugar moiety, displays a higher affinity than the NodSm factor (a tetrameric oligosaccharide acylated with a C₁₆ fatty acid with two double bonds) for the *Medicago* site.

Comparisons between the selectivity of PvNFBS2 and MvNFBS2 show several similarities but also some notable differences, which are illustrated in the model shown in Figure 4. It is likely that recognition of the LCOs occurs primarily from the nonreducing end of the molecules (Fig. 4). Binding of the acyl chain probably involves a hydrophobic pocket which, in PvNFBS2, shows an enhanced specificity for interacting with long and mono-unsaturated acyl chains, whereas the MvNFBS2 protein also is able to accommodate doubly unsaturated acyl chains and specifically recognizes the O-acetyl group of the LCOs produced by *S. meliloti*. Both proteins recognize primarily the third and fifth N-acetylglucosamine residues of the oligosaccharidic moiety. The sulfate group on the reducing sugars is not specifically recognized but, on tetrameric factors, it may partially compensate for the absence of the fifth sugar for binding to PvNFBS2.

The presence of NFBS2 in *Medicago* and *Phaseolus* spp. which belong to different tribes of the *Papilionoideae* (the largest *Leguminosae* subfamily) may suggest that NFBS2 is widespread in legumes. Whether it is restricted to this family, as suggested by its absence in carrot cell cultures, needs to be investigated further by examining a wider range of plant species. Moreover, at the present stage, the biological role of these binding sites characterized in cell suspension cultures remains

Table 3. Affinities of the *Phaseolus vulgaris* high-affinity Nod factor binding site towards differently substituted lipo-chitooligosaccharides (LCOs)^a

Compounds ^b	Affinity ± SEM (nM)
A. Fatty acyl chain	
LCO-IV(S, C16:1 _{Δ2})	55 ± 4.5
LCO-IV(S, C16:1 _{Δ9})	51 ± 6.9
NodSm-IV(S, C16:2 _{Δ2,9})	412 ± 5
LCO-IV(S, C12:1 _{Δ2})	308 ± 31
LCO-IV(S, C16:1 _{Δ2})	55 ± 8.1
LCO-IV(S, C18:1 _{Δ2})	5 ± 1.1
LCO-IV(S, C18:2 _{Δ2,9})	70 ± 3.5
B. O-sulfate group	
Pentameric compounds	
NodRt-V(Me, C18:1 _{Δ11})	2.5 ± 0.4
NodRt-V(Me, S, C18:1 _{Δ11})	15 ± 1.4
Tetrameric compounds	
LCO-IV(C16:1 _{Δ2})	373 ± 24.8
LCO-IV(S, C16:1 _{Δ2})	51 ± 6.9
C. Sugar backbone length	
LCO-II(C16:1 _{Δ2})	15 000
LCO-III(C16:1 _{Δ2})	425 ± 16.2
LCO-IV(C16:1 _{Δ2})	373 ± 24.8
LCO-V(C16:1 _{Δ2})	3 ± 0.6
NodSm-IV(S, C16:2 _{Δ2,9})	412 ± 5
NodSm-V(S, C16:2 _{Δ2,9})	51 ± 6.1
D. O-acetate group	
NodSm-IV(Ac, S, C16:2 _{Δ2,9})	413 ± 21
LCO-IV(S, C16:2 _{Δ2,9})	412 ± 5

^a The affinities were measured by competition assays using the NodRt-V(Me, ³⁵S, C18:1) ligand, are expressed as $K_i \pm$ SEM and are the mean of three experiments.

^b A, Effect of the nature of the fatty acyl chain; B, effect of the O-sulfate group on pentameric or tetrameric compounds; C, effect of the sugar backbone length; D, effect of the O-acetate group.

Table 2. Comparison of the high-affinity Nod factor binding sites from *Medicago varia* and *Phaseolus vulgaris*

Affinity and abundance	<i>M. varia</i>	<i>P. vulgaris</i>
Affinity (K_d) for NodRt-V(Me, S, C18:1)	0.7 nM	15 nM
Affinity (K_d) for NodSm-IV(Ac, S, C16:2)	4 nM ^a	413 nM
Relative abundance (B_{max}) (pmol mg protein ⁻¹)	1.5 ^a	1.5

^a Gressent et al. (1999).

to be understood, and it is not obvious that cell cultures respond to Nod factors with the same degree of specificity that is observed in the symbiotic root tissues. Thus, at present, it is difficult to assign a symbiotic role to these sites; on the other hand, there is no other clear role for Nod factor-like LCO binding sites in legumes.

Concerning PvNFBS2, the structural requirements of the LCOs for the recognition by the binding site correlate with the common characteristics of the Nod factors produced by all *P. vulgaris* nodulating bacteria: a pentameric backbone acylated with a mono-unsaturated fatty acid. The bacteria that nodulate *P. vulgaris* produce Nod factors with various decorations on the reducing (O-sulfuryl, O-fucosyl, O-methyl fucosyl, O-arabinosyl groups) or terminal nonreducing (O-acetyl, carbamoyl) sugars of these various factors (Laeremans et al. 1999; Laeremans and Vanderleyden 1998; Perret et al. 2000). However, it appears that none of these decorations are essential for nodule initiation because (i) none of them are common to the Nod factors produced by all the bean-nodulating bacteria and (ii) mutants affected in genes specifying these decorations (e.g., the O-sulfuryl and N-methyl groups) nodulate this species, albeit in some cases with lower efficiency (Laeremans and Vanderleyden 1998). Thus, *P. vulgaris* appears to require only the basic Nod factor structure (pentameric, acylated with vaccenic acid) for triggering nodulation. In this way, PvNFBS2 could be proposed to play a role in the broad specificity of *P. vulgaris* because it has a high affinity for this basic Nod factor structure which is not severely affected by the presence of decorations either on the reducing sugar (O-sulfuryl group on C₆) or the terminal nonreducing sugar (N-methyl group on C₂ and O-acetyl group on C₆).

For MvNFBS2, the lack of recognition of the O-sulfuryl group of the *S. meliloti* factors suggests that, in *Medicago* spp., the symbiotic recognition of sulfated Nod factors must involve an additional component (Gressent et al. 1999). Either this component could be part of a multicomponent perception system involving NFBS2 or there may be other Nod factor perception systems, expressed in the symbiotic tissues that may recognize specifically this particular substitution in this species.

In conclusion, this work shows that a high-affinity Nod factor binding site exists in *P. vulgaris* which has physicochemical properties compatible with recognition of Nod factors from various bean-symbiotic rhizobia. The similarities between this site and MvNFBS2 from *M. varia* suggest that the corresponding binding proteins may be structurally related. The purification of the NFBS2 binding proteins and the cloning of their

corresponding gene or genes are needed in order to solve the question of their putative role as Nod factor receptors in a symbiotic context.

MATERIALS AND METHODS

Plant material.

P. vulgaris cv. *immuna* cell suspension cultures were provided by Prof. M. T. Esquerré-Tugayé (UMR CNRS-UPS 5546, Toulouse, France). They were grown in a Schenk and Hildebrandt medium as modified by Dixon and Fuller (1976), at 24°C in the dark and with agitation (120 rpm). Cells reaching the stationary growth phase (14 days) were harvested by filtration on a scintered glass filter (size 1), and the cells were washed with one volume of 20 mM KCl and stored at -80°C until use. *Daucus carota* cell suspension cultures were grown according to Thuleau and associates (1993) and harvested in the same way.

Extraction, fractionation, and immunodetection of proteins.

P. vulgaris or *D. carota* cells were homogenized as previously described (Gressent et al. 1999). The resulting fractions, sedimenting at 10,000 or 45,000 × g, were resuspended in binding buffer (20 mM Tris buffer, pH 7.0, containing 0.25 M sucrose, 1 mM MgCl₂, 1 mM CaCl₂, 0.1 mM PMSF, 2 μM leupeptin) with 50% glycerol and stored at -80°C. The separation of the different membranes of the 45,000 × g fraction was performed by free-flow electrophoresis using the protocol described by Gressent and associates (1999). The identification and enrichment of the various fractions was checked by immunodetection of marker proteins. Samples (10 μg of protein) were separated on 8% polyacrylamide resolving gels, and the resolved polypeptides were electrotransferred to Hybond-C membranes (Amersham, Little Chalfont, U.K.). H⁺-ATPase (98 kDa) for the plasma membrane, the endoplasmic reticulum resident chaperone BiP (78 kDa) for the endomembranes, and vacuolar (V)-ATPase (60 kDa) for the tonoplast fractions were detected with the corresponding antibodies using an ECL technique as described by Gressent and associates (1999). Quantification was performed using the NIH Image 1.62 software (Wayne Rasband, NIH, Bethesda, MD, U.S.A.).

Synthesis and purification of Nod factors and LCOs.

According to the nomenclature defined by Roche and associates (1991), the term Nod factors will be devoted to com-

Fig. 4. Models of Nod factors binding to **A**, PvNFBS2 and **B**, MvNFBS2 putative binding proteins. The Nod factor components are represented by ovals (N-acetylglucosamine residues), rectangles (fatty acyl chain and double bonds), squares (O-sulfate group), triangles (O-acetate group), and a star (N-methyl group). Black-colored elements are those influencing the affinity of lipo-chitooligosaccharides for the binding sites.

pounds of biological origin, whereas LCOs will correspond to chemically synthesized compounds, even if both are lipo-chitooligosaccharides. The tetrameric sulfated LCOs were chemically synthesized (Demont-Caulet et al. 1999). Nonsulfated LCOs were synthesized according to Gressent and associates (1999). Biologically produced Nod factors from *R. tropici* were provided by J. Dénarié (UMR CNRS-INRA 215, Toulouse, France). NodRt-V(Me, S, C18:1) and NodRt-V(Me, C18:1) were separated and purified on a reverse phase C₁₈ high-performance liquid chromatography column, eluted at 1 ml min⁻¹ with a linear gradient of water/acetonitrile (80:20 to 40:60 in 40 min). NodSm-IV(Ac, S, C16:2) and NodSm-V(Ac, S, C16:2) were purified from *S. meliloti* culture supernatants as described by Truchet and associates (1991). LCOs were detected by their absorbance between 200 and 230 nm (UV 3000, ThermoQuest, Les Ullis, France) and quantified by the measurement of the peak area, by reference to known quantities of pure chemically-synthesized LCOs used as standards: LCO-IV(S, C16:2_{Δ2,9}) for the LCOs containing a bi-unsaturated fatty acid, or LCO-IV(S, C16:1_{Δ9}) for the mono-unsaturated compounds.

Radiolabeling of Nod factors.

Biologically produced nonsulfated factors from *R. tropici* were labeled with ³⁵S according to Bourdineaud and associates (1995) with modification in the synthesis of 3'-phosphoadenosine 5'-phosphosulfate according to Gressent and associates (1999). At the end of the synthesis, the labeled ligands, termed the ³⁵S-NodRt factor [NodRt-V(Me, ³⁵S, C18:1_{Δ9})] or the ³⁵S-NodSm factor [NodSm-IV(Ac, ³⁵S, C16:2_{Δ2,9})], have a specific radioactivity of 800 Ci mmol⁻¹.

Binding assays.

50 to 70 µg of microsomal fraction protein were incubated in the presence of 0.3 nM ³⁵S-NodRt factor in binding buffer in a total volume of 200 µl. The nonspecific binding component was determined in the presence of 1 µM LCO-V(S, C18:1). Incubations were performed for 1 h at 0°C in 96-well microtiter plates (Nunc, Roskilde, Denmark) and the following steps were as previously described (Gressent et al. 1999), except that the washing buffer was 20 mM Tris, pH 7, containing 20% ethanol. Binding data were analyzed by the RADLIG software (ver. 4; BIOSOFT, Cambridge).

Protein determination.

Protein was measured by the bicinchoninic acid procedure developed by PIERCE (Rockford, IL, U.S.A.) with bovine serum albumin as the reference.

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