The establishment of the legume-rhizobia symbiosis between Medicago spp. and Sinorhizobium meliloti is dependent on the production of sulfated lipo-chitooligosaccharidic nodulation (Nod) factors by the bacterial partner. In this article, using a biochemical approach to characterize putative Nod factor receptors in the plant host, we describe a high-affinity binding site \((K_d = 0.45 \text{ nM})\) for the major Nod factor produced by S. meliloti. This site is termed Nod factor-binding site 3 (NFBS3). NFBS3 is associated to a high-density fraction prepared from roots of Medicago truncatula and shows binding specificity for lipo-chitooligosaccharidic structures. As for the previously characterized binding sites (NFBS1 and NFBS2), NFBS3 does not recognize the sulfate group on the chitooligosaccharidic structures. As for the previously characterized binding sites (NFBS1 and NFBS2), NFBS3 does not recognize the sulfate group on the S. meliloti Nod factor. Studies of Nod factor binding in root extracts of early symbiotic mutants of M. truncatula reveals that the new site is present in Nod factor perception and does not make infections 3 (dmi3) mutants but is absent in dmi1 and dmi2 mutants. Roots and cell cultures of all these mutants still contain sites similar to NFBS1 and NFBS2, respectively. These results suggest that NFBS3 is different from NFBS2 and NFBS1 and is dependent on the common symbiotic genes DMI1 and DMI2 required for establishment of symbioses with both rhizobia and arbuscular mycorrhizal fungi. The potential role of this site in the establishment of root endosymbioses is discussed.

The legume-rhizobia symbiosis is characterized by the formation of a novel plant organ, the root nodule, in which rhizobia bacteria fix atmospheric di-nitrogen in exchange for nutrients provided by the host plant. The establishment of the symbiosis is dependent on a signal exchange between the two partners, leading to the production of lipo-chitooligosaccharidic (LCO) bacterial signals called nodulation (Nod) factors. Nod factors consist of an N-acetyl glucosamine backbone which is \(N\)-acylated on the terminal nonreducing sugar. Various substitutions on the oigosaccharide backbone and variation in the acyl chain are important for the species partner specificity shown by the symbionts (for review, see Dénarié et al., 1996).

Nod factors are able to induce many responses in root hairs of host plants, including root hair deformation, alkalinisation of the cytosol, depolarization of the plasma membrane, calcium influx, periodic oscillations referred to as calcium spiking, and induction of expression of specific genes (Journet et al., 1994; Felle et al., 1995, 1998; Ehrhardt et al., 1996; Shaw and Long, 2003). Nod factors also induce gene expression in the cortex and pericycle (Vijn et al., 1995; D‘Haeze and Holsters, 2002) and initiate cortical cell divisions, which in some species can lead to nodule organogenesis (Truchet et al., 1991). The ability of Nod factors to elicit symbiotic responses within host plants is dependent on the chemical structure of the Nod factor. For example, the major Nod factor produced by Sinorhizobium meliloti, the symbiotic partner of Medicago spp., is \(N\)-acylated with a C16:2 fatty acid, \(O\)-acylated on the nonreducing sugar, and sulfated on the reducing sugar, and is referred to as S. meliloti Nod factor \([\text{NodSm-IV(Ac, S, C16:2)}]\) (Lerouge et al., 1990; Roche et al., 1991b). S. meliloti mutants that are unable to sulfate their Nod factor are symbiotically defective, and their Nod factors have a dramatically reduced biological activity in all Medicago bioassays (Roche et al., 1991a; Journet et al., 1994). The lack of the
O-acetate group or changes to the structure of the fatty acid reduce the activity of the Nod factor in certain bioassays (Schultz et al., 1992; Demont-Cauluet et al., 1994). The requirement for precise structures and the fact that Nod factors are active at subnanomolar concentrations suggest that they are perceived by high-affinity receptors (Cullimore et al., 2001; Geurts and Bisseling, 2002).

Using a genetic approach, plant mutants unable to nodulate and affected in early responses to Nod factors have been identified in several legumes including Medicago truncatula (Catoira et al., 2000; Ben Amor et al., 2003), Pisum sativum (Walker et al., 2000), and Lotus japonicus (Madsen et al., 2003; Radutoiu et al., 2003). Some of these mutants are unable to establish a symbiosis with both their rhizobial partner and also with arbuscular endomycorrhizal (AM) fungi, and these have been called does not make infections (DMI) or common symbiotic (SYM) mutants. In M. truncatula, dmi1 and dmi2 mutants are blocked in most Nod factor responses but still exhibit rapid calcium influx and root-hair deformation after Nod factor addition, whereas a dmi3 mutant shows in addition the calcium-spiking response (Shaw and Long, 2003). The mutant Nod factor perception (nfp) of M. truncatula (Ben Amor et al., 2003), like sym10 mutants of P. sativum (Walker et al., 2000) and nfr5 and nfr1 mutants of L. japonicus (Radutoiu et al., 2003), are completely unresponsive to Nod factors but are still capable of establishing a symbiotic interaction with AM fungi. Based on these observations, it was proposed that DMI1, DMI2, and DMI3 are involved in a common signaling pathway (called the common SYM pathway) implicated in the establishment of both the mycorrhizal and bacterial endosymbioses (Catoira et al., 2000), whereas SYM10 (in P. sativum), NFR5, NFR1 (in L. japonicus), and NFP (in M. truncatula), acting upstream of the common SYM pathway, would be specifically involved in rhizobial Nod factor perception (Walker et al., 2000; Ben Amor et al., 2003; Radutoiu et al., 2003).

A major breakthrough has been achieved in the cloning of these genes, providing key information for understanding symbiotic signaling pathways. The first genes to be cloned were the orthologs of DMI2, part of the common SYM pathway. Cloned simultaneously in M. sativa, M. truncatula (Endre et al., 2002), and in L. japonicus (Stracke et al., 2002), these genes encode proteins belonging to the receptor-like kinase (RLK) family, with extracellular domains containing three Leu-rich repeats. Recently, DMI1 and the L. japonicus homologs have been shown to encode membrane-spanning ion channel-like proteins (Ané et al., 2004; Imaizumi-Anraku et al., 2005). DMI3 encodes a putative Ca2+ calmodulin-dependent protein kinase and could play a role in interpreting calcium signatures elicited in response to rhizobia and perhaps AM fungi (Levy et al., 2004; Mitra et al., 2004).

The cloning of NFR1 and NFR5 from L. japonicus and SYM10 from P. sativum has identified these genes as encoding Lysin motif (LysM)-RLKs that are membrane receptor-like kinases containing an extra-cellular region with two or three LysM domains (Madsen et al., 2003; Radutoiu et al., 2003). In addition, Limpens et al. (2003) have identified a cluster of M. truncatula LysM-RLK genes (the LKY genes), of which the LKY3 gene has been shown to be involved in the Nod factor structural dependency of infection thread formation. NFP has also been cloned and is the probable ortholog of L. japonicus NFR5 and P. sativum SYM10 (J.-F. Arrighi and C. Gough, personal communication). The symbiotic phenotype of the LysM-RLK mutants and the fact that LysM domains have been shown to interact with glycan structures (Steen et al., 2003) and to be present in certain chitinases (Ponting et al., 1999), suggest that symbiotic LysM-RLKs are likely to be Nod factor receptors. However, direct evidence for a physical interaction with Nod factors still needs to be provided.

By using a biochemical approach, based on equilibrium binding studies performed with radioactive Nod factors, two types of Nod factor-binding sites (NFBSs) have been characterized. The first binding site, NFBS1, characterized using a tritiated Nod factor, is associated to a particulate fraction of M. truncatula roots, and exhibits a moderate affinity (Kd = 86 nM) for the Nod factor of its symbiotic partner Sinorhizobium meliloti (Bono et al., 1995). NFBS1 does not discriminate the different substitutions on the chitin backbone important for establishing the symbiosis, and a similar site is present in roots of the nonlegume tomato (Lycopersicon esculentum), suggesting it does not play an exclusive role in the symbiosis with rhizobia. NodSm, labeled with 35S ([35S]Nod factor), with a specific radioactivity 13-fold higher than that of the tritiated compound, enabled binding sites with higher affinity and lower abundance to be detected. NFBS2 was characterized in the microsomal fraction of Medicago varia cell suspension cultures and exhibits a Kd = 4 nM for the NodSm factor (Niebel et al., 1997). NFBS2 shows selectivity toward the O-acetyl group, the length and saturation of the acyl chain, and the length of the chitin backbone of the NodSm factor (Gressent et al., 1999). In addition, an NFBS2-like site, differing in its selectivity, has been characterized in Phaseolus vulgaris (Gressent et al., 2002). A binding site exhibiting a high affinity for the [35S]Nod factor was also detected in the high-density root fraction of M. truncatula (Gressent, 1999). Unfortunately its characterization was difficult because of its low abundance and its interference by NFBS1, present in the same fraction. In an attempt to link the genetic dissection of Nod factor signaling with biochemical data, we have undertaken a renewed investigation of this site in roots of wild type and early Nod factor-signaling mutants of M. truncatula (namely nfp, dmi1, dmi2, and dmi3). In this paper we present the characterization of this high-affinity NFBS in roots of M. truncatula and show that mutations in the DMI1 and DMI2, but not the NFP or DMI3 genes, affect Nod factor binding to this site.
RESULTS
A High-Affinity NFBS, NFBS3, Is Present in a High-Density Particulate Fraction of M. truncatula Roots

Different aeroponic culture conditions were tested to optimize the plant material used to characterize the potential high-affinity NFBS previously detected in roots of M. truncatula (Gressent, 1999). We found that a shorter cultivation time, 16 d instead of 21 d, resulted in an increase in the abundance of binding sites and that, as indicated in previous experiments, these sites were mainly in the high-density root fraction (sedimenting at 3,000 g), which also contains the low-affinity binding site, NFBS1 (Bono et al., 1995). Microscopy analysis of this fraction revealed mainly the presence of cell wall fragments and plastids, whereas western-blot analysis detected low levels of H^+--ATPase compared to the high-affinity binding site of M. truncatula. The presence of some plasma membrane (data not shown).

Two types of experiments were used to analyze binding to the high-density root fraction using the major Nod factor of S. meliloti [NodSm-IV(Ac, 35S, C16:2)] radiolabeled to a high-specific activity of 800 Ci/mmol (the [35S]NodSm factor). Figure 1 represents a Scatchard plot analysis of a saturation experiment performed at a fixed concentration of [35S]NodSm factor (0.86 nM) and increasing concentrations of the corresponding nonlabeled factor ranging from 0.2 nM to 2 μM (cold saturation). This type of analysis covers a large range of ligand concentrations and thus can analyze sites with different affinities. It can be used also as a basis for comparing other potential ligands through competition experiments (see next paragraph). The plot, analyzed using the RADLIG software, reveals two classes of binding sites. In addition to the previously identified low-affinity binding site, NFBS1 (K_d = 86 nM, maximal binding capacity [B_max] = 2 pmol mg protein^{-1}), a binding site exhibiting a higher affinity for the [35S]Nod factor exists in the 3,000 g fraction. To obtain a better estimation of the thermodynamical parameters of this site, a saturation experiment was carried out with increasing concentrations of [35S]Nod factor ranging from 0.2 nM to 3.2 nM (hot saturation). At these ligand concentrations, the results obtained (Fig. 1, inset) reveal only the high-affinity site and allowed the estimation of an equilibrium-binding constant (K_d) of 0.45 nM ± 0.06 and a B_max of 60 fmol mg protein^{-1}. Similar experiments performed on the particulate fractions sedimenting at either 10,000 g or 45,000 g (the microsomal fraction) prepared from roots, showed only low, specific Nod factor binding, suggesting that the high-affinity binding site is enriched in the high-density root fraction. Moreover, binding experiments performed on Arabidopsis (Arabidopsis thaliana) root extracts using 0.6 nM NodSm factor failed to detect any specific binding in the 3,000 g, 10,000 g, or 45,000 g fractions, suggesting that high-affinity NFBSs do not occur in roots of this nonlegume. The high-affinity binding site of M. truncatula has been termed NFBS3.

NFBS3 Does Not Show Selectivity toward the Specific Decorations of the NodSm Factor But Is Specific for the LCO Structure

The selectivity of NFBS3 toward the sulfate group, the O-acetate group, and the structure of the acyl chain was investigated, as these features are important for Nod factor activities. The former is essential for all biological activities of the Nod factor, and the others are known to affect the ability of the Nod factor to induce certain responses in the plant. The effect of the presence of the O-acetate chain on the affinity for NFBS3 was determined by performing a saturation experiment with increasing concentrations of the [35S]NodSm factor and the corresponding non-O-acetylated 35S-labeled compound [LCO-IV(S, C16:2Δ2,9)], ranging from 0.2 nM to 3.2 nM (hot saturation). The Scatchard plot revealed that the affinity of the non-O-acetylated LCO (K_d = 0.34 nM ± 0.04), was very similar to that of the corresponding O-acetylated [35S]NodSm factor, determined in the same manner. Thus, NFBS3 does not show selectivity toward the O-acetate group (Fig. 2).

The selectivity toward the sulfate group was determined by performing a competition experiment using a fixed concentration of the [35S]NodSm factor and increasing concentrations of non-O-acetylated and nonsulfated chemically synthesized LCO [LCO-IV(C16:2Δ2,9)], ranging from 0.25 nM to 2 μM. The same experiment was carried out with the sulfated
NodSm-IV(Ac, S, C16:2 Δ2,9) used as reference. The competition curves corresponding to these experiments are shown in Figure 3. Analysis of the binding data was performed with the RADLIG software and gave the best curve fitting for a two-sites model. The affinity ($K_d$) of the nonsulfated compound for the high-affinity binding site was estimated at $0.25 \pm 0.14$ nM whereas that of the sulfated Nod factor was estimated at $0.14 \pm 0.16$, in the same type of experiment, using the same data analysis. The values obtained by this method are not as precise as those deduced from a Scatchard plot because of the difficulties in eliminating interference by NFBS1 but nevertheless provide strong evidence that NFBS3 does not exhibit selectivity toward the sulfate group.

A competition experiment performed with LCO-IV(S, C16:1 Δ9) did not reveal a significant difference with LCO-IV(C16:2 Δ2,9), suggesting that the presence of two double bonds in the acyl chain, characteristic of the NodSm factor, is not discriminated by NFBS3 (Fig. 3). In contrast, a nonacylated chitoooligosaccharide (penta-N-acetyl-chitopentaose) exhibited only a weak competition for Nod factor binding to NFBS3 ($K_i = 130 \text{ nM}$) demonstrating that the site is specific for the LCO structure.

Mutations in $DMI1$ and $DMI2$, But Not in $NFP$ or $DMI3$, Influence Binding of the NodSm Factor to NFBS3

Genetic dissection of Nod factor signaling in $M. truncatula$ has identified four genes ($DMI1$, $DMI2$, $DMI3$, and $NFP$) controlling the earliest steps of Nod factor signaling. We took advantage of the availability of these genetic tools and examined whether mutations in these four genes affect the binding of the NodSm factor to NFBS3. The mutants used are presented in Table I. Figure 4A shows Scatchard plot analyses of the different saturation experiments performed on the high-density root fraction prepared from wild-type or mutant plants: the concentrations of $[^{35}S]$NodSm factor ranged from 0.2 nM to 3.2 nM (hot saturation). In comparison to the wild type, the Scatchard plots obtained in the mutants Y6 ($DMI1$) and P1 ($DMI2$), defective respectively in a potential transmembrane channel protein and a Leu-rich repeat-RLK, reveal that the high-affinity site, NFBS3, is undetectable. Similar plots (data not shown) were observed for other mutant alleles of the $DMI1$ and $DMI2$ genes: B129 ($DMI1$), C71 ($DMI1$), and TR25 ($DMI2$). The binding capacity of the mutant C31, affected in the $NFP$ gene encoding a LysM-RLK, is about the same as the wild type ($K_i = 0.35 \pm 0.08$). In addition, the $DMI3$ mutant TRV25, affected in the gene encoding the calcium- and calmodulin-dependent protein kinase, still contains the high-affinity binding site, which exhibits a $K_i$ close to that of the wild type but with a lower abundance ($B_{max} = 28 \text{ fmol mg protein}^{-1}$). Since for both $NFP$ and $DMI3$ only one mutant allele was available for these experiments at this time (Catoira et al., 2000; Ben Amor et al., 2003), three independent experiments were performed with each mutant and gave similar results. Experiments performed with $DMI1$ and $DMI2$ mutants were repeated twice. In all of these mutants ($nfp$, $DMI1$, $DMI2$, and $DMI3$) the low-affinity binding site NFBS1 was shown to be present by performing a saturation experiment (cold saturation) as described previously for the wild type (Fig. 4B compares the plots obtained for $DMI2$ and wild type).
These experiments thus show that the high-affinity NFBS in roots, NFBS3, is dependent on two genes (DMI1 and DMI2), which are required for establishing symbioses with both rhizobia and AM fungi. NFBS3 is not dependent on NFP or DMI3. In contrast, the lower-affinity binding site in roots, NFBS1, is not dependent on any of the four genes tested.

A High-Affinity NFBS Associated to the Microsomal Fractions of M. truncatula Cell Suspension Cultures Is Not Affected in Early Symbiotic Mutants

In the next experiments we examined the relationship between NFBS3 and the site NFBS2, which had previously been characterized in cell suspension cultures of M. varia and P. vulgaris (Gressent et al., 1999, 2002). As a first step toward this comparison we established cell cultures of M. truncatula and measured Nod factor binding to different fractions. The results revealed two classes of binding sites in the microosomal fraction of M. truncatula cell suspension cultures (Fig. 5). The high-affinity binding site has a $K_d$ of 3.5 nM for NodSm-IV(Ac, S, C16:2) which is similar to that of NFBS2 from M. varia ($K_d \approx 4$ nM). Thus, a NFBS2-like site is present in M. truncatula cell suspension cultures. The $B_{\text{max}}$ was however lower, around 60 fmol mg protein$^{-1}$ in contrast to 1.5 pmol mg protein$^{-1}$ observed for the sites of M. varia or P. vulgaris. In addition

<table>
<thead>
<tr>
<th>Line or Mutant</th>
<th>Name of Line or Mutant</th>
<th>Characteristics of the Mutations and Prediction of the Proteins</th>
<th>Reference</th>
</tr>
</thead>
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<tr>
<td>Wild type</td>
<td>A17</td>
<td>DM1 (883 amino acids)</td>
<td>Ané et al. (2004)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DM2 (925 amino acids)</td>
<td>Endre et al. (2002)</td>
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<tr>
<td></td>
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<td>DM3 (523 amino acids)</td>
<td>Lévy et al. (2004)</td>
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<tr>
<td>dmi1</td>
<td>Y6</td>
<td>Point mutation leading to a predicted truncated protein of 305 amino acids</td>
<td>Ané et al. (2004)</td>
</tr>
<tr>
<td>dmi1</td>
<td>C71</td>
<td>Point mutation that causes a missplicing event</td>
<td>Ané et al. (2004)</td>
</tr>
<tr>
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<tr>
<td>dmi2</td>
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<td>dmi3</td>
<td>TRV25</td>
<td>Fourteen-base pair deletion leading to predicted truncated protein of 209 amino acids</td>
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<td>Ben Amor et al. (2003)</td>
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Figure 4. Analysis of NFBS3 in roots of symbiotic mutants. A, Scatchard plot of saturation experiment (hot saturation) using increasing concentrations of $^{35}$S-NodSm factor [NodSm-IV(Ac, $^{35}$S, C16:2$\Delta$2, 9)] ranging from 0.2 nM to 3.2 nM in high-density root fractions of M. truncatula wild-type line (▲) and mutants Y6 (dmi1; ◦), P1 (dmi2; ⊡), TRV25 (dmi3; ▼), and C31 (nfp; ●). B, Saturation experiment using 0.86 nM $^{35}$S-NodSm factor and different concentrations of unlabeled NodSm-IV(Ac, S, C16:2$\Delta$2, 9) in the high-density root fraction of M. truncatula wild-type line (▲) and the dmi2 mutant line (P1; ⊡).
Figure 5. Analysis of NFBS2 in cell suspension cultures of symbiotic mutants. Scatchard plot of a saturation experiment using 0.2 nM of \[^{35}S\]NodSm factor [NodSm-IV(Ac, \[^{35}S\], C16:2\(\Delta_2\), 9)] and increasing concentrations of purified, unlabeled NodSm-IV(Ac, S, C16:2\(\Delta_2\), 9) ranging from 0.25 nM to 2 \(\mu\)M (cold saturation) in microsomal extracts of M. truncatula cell suspension cultures of wild-type line (▲) and the dmi1 mutant line Y6 (○).

there is a low-affinity binding site similar to NFBS1, as observed also in M. varia cell cultures (Gressent et al., 1999).

To evaluate a putative relationship between NFBS3 and this NFBS2-like binding site, we examined whether the latter was present in cell cultures generated from roots of four Nod factor-signaling mutants of M. truncatula (nfp, dmi1, dmi2, and dmi3). Saturation experiments, using 0.2 nM \[^{35}S\]NodSm factor and increasing concentrations of unlabeled NodSm-IV(Ac, S, C16:2) ranging from 0.25 nM to 2 \(\mu\)M (cold saturation), were performed on microsomal fractions from cell suspension cultures generated from roots of the mutants Y6 (dmi1), TR25 (dmi2), TRV25 (dmi3), and C31 (nfp). Analysis of the binding data of each of these mutants was consistent with a two-site model giving affinities for NodSm-IV(Ac, S, C16:2) similar to those of the wild type (data for the dmi1 mutant are shown in Fig. 5 inset), thus suggesting the presence of NFBS1- and NFBS2-like sites in all of these cell lines. The \(K_{\text{d}}\) corresponding to the NFBS2-like site ranged from 60 fmol mg protein\(^{-1}\) for the wild type to 250 fmol mg protein\(^{-1}\) for TRV25 (dmi3).

These results show that NFBS2 and NFBS3 differ not only in their affinities for Nod factors, but also in their dependence on DMI1 and DMI2.

DISCUSSION

Nod factors are able to induce symbiotic responses within the host plant at concentrations in the pico- to nano molar range. This result, together with the fact that most of these responses depend on structural features of the Nod factor, indicates the involvement of high-affinity Nod factor receptor(s) in the establishment of the symbiosis with rhizobia (Cullimore et al., 2001; Geurts and Bisseling, 2002). Recent models for Nod factor perception and subsequent downstream signaling, based on genetic studies, propose that Nod factors are initially perceived by LysM-type receptor-like kinases (Cullimore and Dénaire, 2003; Radutoiu et al., 2003). The involvement of LysM domains in glycan binding in other systems is compatible with this hypothesis (Amon et al., 1998; Steen et al., 2003). In M. truncatula, NFP is required for all known Nod factor responses, including Ca\(^{2+}\) uptake and root hair swelling, Ca\(^{2+}\) spiking, and induction of early nodulin genes. By contrast, mutants altered in DMI1 and DMI2 genes retain the Ca\(^{2+}\) uptake and hair-swelling responses, but fail to elicit calcium spiking and early nodulin gene expression upon Nod factor treatment. (Catoira et al., 2000). As DMI2 is also a receptor-like kinase (Endre et al., 2002), it is possible that a second Nod factor perception step is involved in the induction of these other responses (Cullimore and Dénaire, 2003; Radutoiu et al., 2003). Moreover, as the DMI genes are also required for establishment of symbioses with AM fungi, it is speculated that they may also be involved in the perception and transduction of a hypothetical AM fungal factor, termed the Myc factor (Geurts et al., 2005). This hypothesis has recently been reinforced by the finding that a diffusible factor from AM fungi can stimulate lateral root formation via the DMI1/DMI2 signaling pathway (Oláh et al., 2005).

In this paper we have characterized a Nod factor-binding site, NFBS3, in roots of M. truncatula, which has a very high affinity (\(K_{\text{d}} = 0.45\) nM) for the major Nod factor of S. meliloti. Such a site was not found in roots of a nonlegume, Arabidopsis. The affinity of this site for this NodSm factor is about 200-fold higher than that displayed by a second site found in roots, NFBS1 (\(K_{\text{d}} = 86\) nM), and almost 10-fold higher than that of NFBS2 (\(K_{\text{d}} = 4\) nM), the site previously characterized in cell suspension cultures (Gressent et al., 1999). Together with its presence in roots, this affinity may suggest that NFBS3 is able to perceive Nod factors at physiological concentrations. In this article we have investigated whether the proteins encoded by early symbiotic genes of M. truncatula could be necessary for the formation of NFBS3 or the two other previously characterized NFBSs. Our results show that NFBS3 is present in roots of the nfp mutant that is defective in a symbiotic LysM-RLK, necessary for early Nod factor perception. Moreover, roots and cell suspension cultures of this nfp mutant contain sites characteristic of NFBS1 and NFBS2, respectively. As the mutation in this nfp mutant is predicted not to result in the production of an NFP protein with a functional ligand-binding domain (J.-F. Arrighi and C. Gough, personal communication), this result clearly indicates that NFP is not necessary for the formation of NFBS3 or the...
other two sites. However, we cannot conclude that NFP is not a Nod factor-binding protein, as it is possible that NFBSs dependent on this protein are of too low abundance to be detected in our experimental conditions.

In contrast, NFBS3 was not detected in roots of dmi1 and dmi2 mutants, but roots of these mutants still contained NFBS1. This result was found using at least two nonallelic mutants for each gene, some of the alleles predicted to drastically affect the protein structure (Table I). Thus the presence of functional DMI1 and DMI2 proteins are required for detecting the high-affinity binding site, NFBS3, in roots.

The question now is what is the role of DMI1 and DMI2 in NFBS3? Could they be the binding proteins, or do they play an essential but indirect role in NFBS3? As DMI2 is a RLK, it is a good candidate to bind and transduce an extracellular ligand. The extracellular region of this receptor-like protein contains a domain of unknown function and three Leu-rich repeats that are required for its activity (Endre et al., 2002), but this structure has not provided any clues concerning a potential ligand. DMI1 is a potential transmembrane channel protein encoded by a single gene in M. truncatula (Ané et al., 2004). Recent work suggests that the two genes are induced together in the pre-infection zone of nodules (Bersoult et al., 2005; Limpens et al., 2005), and they are also expressed in roots (Ané et al., 2004; Bersoult et al., 2005). At the subcellular level, studies with a green fluorescent protein fusion have shown that DMI2 is located in the plasma membrane and also the membrane surrounding infection threads in nodules (Limpens et al., 2005). Although the subcellular localization of DMI1 has not so far been established, recent work on transiently expressed green fluorescent protein fusions suggest surprisingly that its two L. japonicus symbiotic homologs (CASTOR and POLLUX) may be located in the plastids (Imaizumi-Anraku et al., 2005). Clearly the localization of the M. truncatula DMI1 protein needs to be determined prior to evaluating its possible interaction with DMI2 and the role of these two proteins in NFBS3.

However, one possibility is that either DMI1 or DMI2 may be a direct Nod factor-binding protein and that the other is required either as an accessory protein in NFBS3 or as an essential element for the production and/or stability of this binding site. A second possibility is that DMI1 and DMI2 are not the binding proteins in NFBS3 but facilitate Nod factor binding to another protein. The fact that mutants of dmi1 and dmi2 still contained an NFBS2-like site in cell suspension cultures suggests that there must be additional proteins encoded in the M. truncatula genome that are directly involved in high-affinity Nod factor binding. Although NFP is not the binding protein in any of these sites, other LysM-RLKs, such as the LYK3 and LYK4 proteins (Limpens et al., 2003), would be clear candidates for this role. The availability of null mutants in these genes is required to test this hypothesis.

A third possibility is that DMI1 and DMI2 have no specific role in NFBS3, but that the lack of this site in dmi1 and dmi2 mutants is due to some pleiotropic effect of these mutations. However as dmi1 and dmi2 mutants are clearly defective in nod factor signaling it would be surprising if the lack of a NFBS in these mutants is not related to the symbiotic phenotype.

A second important question concerns the physiological role of NFBS3. This site is not selective toward the sulfate group, a decoration that is important for the biological activity of Nod factors in Medicago spp. (Roche et al., 1991a; Journet et al., 1994; Demont-Caulet et al., 1999). Moreover, as NFBS3 requires DMI1 and DMI2 and not NFP we can hypothesize that this site is not involved in the initial perception of Nod factors, leading to calcium uptake and root hair swelling, which requires NFP but is independent of DMI1/DMI2. However, the fact that this site is dependent on two genes (DMI1/DMI2) required for establishing root endosymbioses, strongly suggests that NFBS3 plays a symbiotic role. As it has a high affinity for the major Nod factor of S. meliloti, this role may be to participate in the Nod factor-signaling pathway leading to ENOD11 expression, calcium spiking, cortical cell division, and infection, initiated by rhizobia. Alternatively, since DMI1 and DMI2 are also required for establishing symbioses with AM fungi, it could be hypothesized that the role of NFBS3 is not in Nod factor perception but rather in the perception of AM fungal signals exhibiting some structural similarities with Nod factors. In this respect it is noteworthy that Olah et al. (2005) have recently found that DMI1 and DMI2, but not DMI3 or NFP, are required for a diffusible factor from AM fungi to stimulate lateral root formation. However, although the same plant genes are required for both NFBS3 and for this AM fungal response, it appears that the two may not be related as the fungal factor response is not mimicked by nonsulfated Nod factors. Clearly further analysis is required to unravel the roles of NFBS3 and the other two NFBSs in M. truncatula. The presence of these different binding sites suggests that LCO signaling is more complicated than suggested by current symbiotic signaling models.

**MATERIALS AND METHODS**

**Plant Material and Growth Conditions**

Seeds of Medicago truncatula Jemalong, wild-type line A17, and the mutant lines (Table I) were grown aeroponically under axenic conditions as previously described (Lullien et al., 1987). Plants were grown for 11 d in a high nitrogen culture medium (5 mM NH4NO3). After this period, the culture medium was replaced with one lacking nitrogen. Following 5 d of nitrogen starvation, roots were harvested, frozen in liquid nitrogen, and kept at −80°C until use.

Arabidopsis (Arabidopsis thaliana L. Heynh. ecotype Columbia) was grown as described in Centin-Aubay et al. (2003). Ten-day-old seedlings were used and the roots were harvested and processed as for M. truncatula.

M. truncatula wild-type and mutant cell suspension cultures were grown as described by (Niebel et al., 1997). Cells having reached stationary phase were harvested by filtration on a scinttered glass filter (size 1) and washed with...
1 volume of 20 mM KCl. Cells were then frozen in liquid nitrogen and stored at −80°C until use.

Root Extract Fractionation

Approximately 25 g of frozen material was ground with a pestle in mortar and extracted as described (Bono et al., 1995). The fraction sedimenting at 3,000 g was resuspended in extraction buffer and then purified on a discontinuous percoll gradient. The resulting pellet was then washed twice with Tris-HCl-binding buffer (25 mM Tris-HCl buffer pH 7.0, 250 mM Suc, 1 mM CaCl₂, 1 mM MgCl₂, 0.1 mM phenylmethyisulfonyl fluoride, 2 μM leupeptin) and resuspended in binding buffer. This fraction was termed the high-density root fraction. Glycerol was then added to give a final concentration of 10% (v/v). The fraction was then frozen in liquid nitrogen and stored at −80°C.

Preparation of Microsomal Fractions from Cell Suspension Cultures

The microsomal fraction was obtained as previously described (Gressent et al., 1999) and corresponds to the fraction sedimenting between 10,000 g and 45,000 g. This fraction was suspended in Na-cacodylate-binding buffer (25 mM Na-cacodylate buffer pH 6.0, 250 mM Suc, 1 mM CaCl₂, 1 mM MgCl₂, 0.1 mM phenylmethyisulfonyl fluoride, 2 μM leupeptin) and an equal volume of glycerol added to give a final concentration of 50% (v/v). This fraction was then frozen in liquid nitrogen and stored at −80°C until use.

Radiolabeling of LCOs and NodSm Factors

A biologically produced nonsulfated Nod factor [NodSm-IV( Ac, C16:2,9)] or the chemically synthesized analog LCO-IV(C16:2,2,9), synthesized as described in Rasmussen et al. (2004), was labeled with Na₂³⁵SO₄ according to the protocol established by Bourdineaud et al. (1995) and modified by Gressent et al. (2004). Following synthesis, the radiolabeled factors designated as [³⁵S]NodSm factor, or [³⁵S]LCO respectively, were purified by HPLC on a reverse-phase C₁₈ column. Their specific radioactivity was determined as described in Bono et al. (1995).

Binding Assays

For the binding assays performed on root extracts, aliquots containing 40 to 60 μg of protein were incubated in Tris-HCl-binding buffer in the presence of [³⁵S]Nod factor or [³⁵S]LCO at the concentration indicated for each experiment. For the competition experiments unlabeled LCOs (from 0.25 mM to 1 μM) were simultaneously added to the incubation mixture. Binding assays performed on the microsomal fractions were carried out in a similar way, however aliquots containing 100 μg of protein were used and the Tris-HCl binding buffer was replaced with the Na-cacodylate-binding buffer. All the incubations were carried out in 96-well microtiter plates at 0°C for 1 h. Nonspecific binding was determined by addition of 2 μM NodSm-IV(Ac, S, C16:2,2,9) to the incubation mixtures. Following incubation, samples were filtered through 1.5 μm sieve filter mats with a Skatron Cell Harvester. For the studies on root extracts each filter disc was washed with 25 mM Tris-HCl buffer pH 7.0 containing 1 mM CaCl₂ and 1 mM MgCl₂. For the microsomal fractions, filter discs were washed with 12.5 mM Bis-Tris buffer pH 6.0, containing 1 mM CaCl₂ and 1 mM MgCl₂. Filter discs were then dried, transferred to scintillation vials, and the radioactivity measured by scintillation spectrometry. Each binding point was done in triplicate. Radioligand binding data were analyzed by RADLIG software, version 4.5 (Biosoft).

Protein Determination

Protein was measured by the bichinchoninic acid procedure (Pierce) with bovine serum albumin as the reference.

ACKNOWLEDGMENTS

We thank Martin Rasmussen and Hugues Dirgeuz of Centre de Recherches sur les Macromolécules Végétales, Grenoble, for supplying the LCOs; Clare Gough at Laboratoire des Interactions Plantes Micromicroorganismes, Institut National de la Recherche Agronomique (INRA)-Centre National de la Recherche Scientifique (CNRS) 441/2594 for growing the plant mutants; and Jean-Marie Prosperi at INRA Montpellier, France, for supplying the A17 seeds. We gratefully acknowledge the work of Sylvie Camut at INRA-CNRS 441/2594 for growing the mutants and establishing the cell cultures. We also acknowledge Frederic Gressent for his pioneering work on NFBS3. We thank Claire Gough, Lonneke Mulder, and Jean Dénario for critically reading the manuscript.

Received July 27, 2005; revised November 14, 2005; accepted November 15, 2005; published December 23, 2005.

LITERATURE CITED


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