Nod Factor Requirements for Efficient Stem and Root Nodulation of the Tropical Legume Sesbania rostrata*

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Azorhizobium caulinodans ORS571 synthesizes mainly pentameric Nod factors with a household fatty acid, an N-methyl, and a 6-O-carbamoyl group at the nonreducing-terminal residue and with a D-arabinosyl, an L-fucosyl group, or both at the reducing-terminal residue. Nodulation on Sesbania rostrata was carried out with a set of bacterial mutants that produce well characterized Nod factor populations. Purified Nod factors were tested for their capacity to induce root hair formation and for their stability in an in vitro degradation assay with extracts of uninfected adventitious rootlets. The glycosylations increased synergistically the nodulation efficiency and the capacity to induce root hairs, and they protected the Nod factor against degradation. The D-arabinosyl group was more important than the L-fucosyl group for nodulation efficiency. Replacement of the 6-O-L-fucosyl group by a 6-O-sulfate ester did not affect Nod factor stability, but reduced nodulation efficiency, indicating that the L-fucosyl group may play a role in recognition. The 6-O-carbamoyl group contributes to nodulation efficiency, biological activity, and protection, but could be replaced by a 6-O-acetyl group for root nodulation. The results demonstrate that none of the studied substitutions is strictly required for triggering normal nodule formation. However, the nodulation efficiency was greatly determined by the synergistic presence of substitutions. Within the range tested, fluctuations of Nod factor amounts had little impact on the symbiotic phenotype.

Bacteria of the genera Allorhizobium (1), Azorhizobium, Bradyrhizobium, Mesorhizobium, Rhizobium, and Sinorhizobium, can establish a symbiosis with specific leguminous host plants. A compatible interaction leads to the development of nitrogen-fixing nodules as the result of a fine-tuned signal exchange (2).

Early plant responses for nodule development and invasion are triggered by bacterial Nod factors (3–5). Nod factors consist of a chitooligosaccharide backbone. An acyl group replaces the N-acetyl group at the nonreducing-terminal residue, and strain-specific substitutions modify the two terminal residues. NodA, NodB, and NodC proteins are required for the synthesis of the acylated Nod factor backbone, whereas most other Nod proteins are required for specific modifications (6, 7). NodL of Rhizobium leguminosarum is an acetyltransferase (8), and nodZ encodes an α,1,6-fucosyltransferase (9–11). Sulfation of Nod factors produced by Sinorhizobium meliloti is carried out by the sulfotransferase NodH that uses 3′-phosphoadenosine 5′-phosphosulfate, synthesized by the NodPQ complex (12, 13).

Nod factors are indispensable for nodulation and cause multiple effects. Plateau-like increases in intracellular free calcium (14), plasma membrane potential changes (15), and actin cytoskeleton disorganizations, which are a prelude for root hair deformation and curling (16), occur within seconds to minutes after Nod factor application. Nod factors also induce the formation of root hairs and preinfection threads, the division of cortical cells, and the expression of plant genes involved in nodule development (17).

In a few interactions, structural requirements for Nod factor-triggered responses have been studied. Stokkermans et al. (18), for example, showed that pentameric Nod factors produced by Bradyrhizobium japonicum require a 2-O-methylfucosyl group at the reducing-terminal residue to induce root hair deformation and the formation of nodule primordia on Glycine soja. A nodL and a nodFE mutant of S. meliloti, which synthesize nonacylated Nod factors and Nod factors with altered acyl chains, respectively, have a reduced capacity to induce infection threads in alfalfa (3). Nonsulfated Nod factors produced by a S. meliloti nodH mutant were biologically inactive on alfalfa (19).

Other studies have dealt with Nod factor stability. Plant chitinases produced during nodule development can degrade Nod factors (20–24). However, particular decorations contribute to Nod factor stability. Sulfated Nod factors of S. meliloti are more resistant to degradation than the corresponding nonsulfated molecules (21).

Here, the influence of Nod factor amounts and substitutions on nodulation efficiency was investigated in the symbiosis between Azorhizobium caulinodans ORS571 and the tropical legume Sesbania rostrata. S. rostrata can be nodulated on the stem and on the root at adventitious rootlets and at the bases of lateral roots, respectively. The Nod factors of A. caulinodans are mainly pentamers that are vaccenoylated (C18:1), palmitoylated (C16:0), or stearoylated (C18:0). The nonreducing-terminal residue carries an N-methyl and a 6-O-carbamoyl group, and the reducing-terminal residue may be substituted with a
d-arabinosyl group, an L-fucosyl group, or both (25, 26) (Fig. 1). The latter product with a vaccenoyl chain for example, will be referred to as NodARc-V(Carb,3\text{Me},C18:1,Ara,Fuc) (17).

Azorhizobial nodulation genes for the synthesis and secretion of Nod factors are clustered in the nodABCDSUIJZnoca operon and the nolK operon (Fig. 1). A collection of bacterial mutants producing well characterized altered Nod factors was used in stem and root nodulation assays. Additionally, purified Nod factors were assayed for biological activity and for stability against degradation. Together, these approaches provide new information about the relative importance of Nod factor modifications and substitutions for the overall effectiveness of the signaling molecules in nodulation at adventitious and lateral root bases of S. rostrata.

**EXPERIMENTAL PROCEDURES**

**Features of Bacterial Strains and Plasmids**—Bacterial strains and plasmids are listed in Table I. A. caulinodans ORS571 and derivatives were grown at 37 °C in YEB medium (27). For Nod factor preparations, nod gene expression was induced with 20 \(\mu\text{M}\) naringenin (26). *Escherichia coli* strains were grown at 37 °C in Luria Bertani medium (28). Antibiotics were applied in the following concentrations: 200 \(\mu\text{g}\text{ml}^{-1}\) carbenicillin (Cb), 10 \(\mu\text{g}\text{ml}^{-1}\) kanamycin (Km), 100 \(\mu\text{g}\text{ml}^{-1}\) spectinomycin (Sp), 20 \(\mu\text{g}\text{ml}^{-1}\) streptomycin (Sm), 10 \(\mu\text{g}\text{ml}^{-1}\) tetracycline (Tc), and 12.5 \(\mu\text{g}\text{ml}^{-1}\) chloramphenicol (Cm).

**TABLE I**

**Bacterial strains and plasmids**

| Strains/plasmids | Relevant characteristics |
|------------------|--------------------------|
| **A. caulinodans strain** |
| ORS571         | A. caulinodans wild-type strain (Cb)⁶ |
| ORS571(pBBNK)  | ORS571 containing pBBNK (Cb, Km) |
| ORS571–1.59S    | ORS571 derivative carrying a Tn5 insertion in nodS (Cb, Km) |
| ORS571–1.31U    | ORS571 derivative carrying a Tn5 insertion in nodU (Cb, Km) |
| ORS571–1.31I(pBBRNU) | ORS571–1.31I containing pBBRNU (Cb, Km, Cm) |
| ORS571–1.39J    | ORS571 derivative carrying a Tn5 insertion in nodJ (Cb, Km) |
| ORS571–1.11Z    | ORS571 derivative carrying a Tn5 insertion in nodZ (Cb, Km) |
| ORS571–1.2      | ORS571 derivative carrying a Tn5 insertion in noeC (Cb, Km) |
| ORS571–4.2K     | ORS571 derivative carrying a Tn5 insertion in noeK (Cb, Km) |
| ORS571–1.31U–0K | ORS571–1.31U derivative carrying an 0-cassette in noeK (Cb, Km, Sp, Sm) |
| ORS571–1.31U–0K(pBBRNU) | ORS571–1.31U–0K containing pBBRNU (Cb, Km, Sp, Sm, Cm) |
| ORS571–1.31U–0K(pMP1060) | ORS571–1.31U–0K containing pMP1060 (Cb, Km, Sp, Sm, Tc) |
| ORS571–1.11Z–0K | ORS571–1.11Z derivative carrying an 0-cassette in noeK (Cb, Km, Sp, Sm) |
| ORS571–1.11Z–0K(pRTHPQ) | ORS571–1.11Z–0K containing pRTHPQ (Cb, Km, Sp, Sm, Tc) |

| Plasmid        | Relevant characteristics |
|----------------|--------------------------|
| pRK2013        | Helper plasmid used in triparental conjugations |
| pBBRNU         | pBBR1MCS containing a 1850-bp Xhol-PshAI fragment carrying nodU |
| pMP1060        | Derivative of pMP1070 harboring a 1000-bp fragment containing nodL of R. leguminosarum |
| pRTHPQ         | plAFR3 containing a 4000-bp polymerase chain reaction fragment carrying the nodHPQ genes of *R. tropici* |
| pRGG901ΩB      | pBR325 containing a 8200-bp EcoRI fragment in which an 0-cassette is cloned in the noeK gene |
| pBBNK          | pBBR1MCS-2 containing a 2300-bp fragment carrying noeK |

⁶ Antibiotic resistance(s) of the respective strains. For concentrations of antibiotics, see “Experimental Procedures.”
Collision-induced dissociation (CID) spectra were recorded from the first field-free region by scanning in the constant B/E mode. Helium was used as collision gas.

In Vivo Radioactive Labeling and Analysis of Nod Factors—Nod factors were labeled with 2-[^14]acetate and analyzed by reversed-phase thin-layer chromatography (RP-TLC) (29). The Nod factor spots were quantified using a PhosphorImager and the ImageQuant™ software.


table ii

| Azorhizobium derivative | Me | Carb | Fuc | Ara | Reference |
|-------------------------|----|------|-----|-----|-----------|
| ORS571                  | +  | +    | +   | +   | 26        |
| ORS571-1.31U            | +  | -    | -   | -   | 35        |
| ORS571-1.31U(pBBRNU)    | +  | -    | +   | +   | 35        |
| ORS571-1.39J           | +  | +    | +   | +   | 60        |
| ORS571-1.11Z           | +  | +    | +   | +   | 10        |
| ORS571-1.31U-1.2K      | +  | +    | +   | +   | 10        |

*Derivatives of A. caulinodans ORS571 are described in Table I. + Nod factor substitutions correspond to those mentioned in Fig. 1B. The presence of a substitution on Nod factors synthesized by A. caulinodans derivatives is indicated as follows: +, present as in the wild-type strain; −, only a minor fraction of the Nod factors contained this modification; −−, Nod factors carrying this modification could not be detected.

RESULTS

Bacterial Strains with Altered Nod Factor Production—To complete the set of available A. caulinodans mutants that are affected in Nod factor synthesis (Table II), several additional strains were constructed and characterized and the Nod factor population of the previously described nodS mutant ORS571–1.59S (Table I) was analyzed (Fig. 1). The azorhizobial nodS gene encodes a methyltransferase that is involved in Nod factor methylation (29, 34). Nod factors were prepared from 50 liters of ORS571–1.59S culture. The vaccenoylated and stearylated Nod factors were separated on RP-HPLC and analyzed by LSIMS associated with CID (Table III). Because protonated NodArc-VCarb(C18:0,Fuc) and NodArc-V(Carb,Me,C18:0,Ara) were isobaric at $m/z = 1447$, CID-MS was performed on the parental ion with $m/z = 1447$ (Table III, Fig. 2A). Two series of four characteristic fragment was observed. A loss of D-arabinose and GlcNAc-OH led to a fragment with $m/z = 1094$ and three other consecutive losses of GlcNAc to fragments with $m/z = 891, 688,$ and 485 (Fig. 2A). The latter fragment (m/z = 485) corresponded to the mass of a nonreducing-terminal residue substituted with stearic acid, an N-methyl, and a 6-O-carbamoyl group (25, 26). The second series, with $m/z = 1080, 877, 674,$ and 471, differed from the first series in that the $m/z$ value of all the fragments was 14 units lower (Fig. 2A), demonstrating that this Nod factor was fucosylated and nonmethymethylated. Thus, the nodS mutation affected, but did not completely abolish, Nod factor methylation. Moreover, the mutation was nonpolar and half of the 2% Nod factors produced by the nodS mutant (Table III) were "wild-type" Nod factors (data not shown).

The mutant strains ORS571–1.31U and ORS571–1.11Z contained a polar Tn5 insertion in nodU and in nodZ, respectively (Fig. 1). Although the expression of the nodZ gene was knocked out in both strains, a fraction of Nod factors was still fucosylated (Table II), impeding conclusions about Nod factor glycosylation and nodulation. Knock out of the nodK gene, on the other hand, led to a complete absence of fucosylation (10) (Table II). Therefore, an Ω-cassette was introduced into the nodK gene of ORS571–1.31U and ORS571–1.11Z (see “Experimental Procedures”). The LSIMS spectra of the vaccenoylated and palmitoylated Nod factors produced by ORS571–1.31U-1.11Z.
ORS571–1.11Z–K are presented in Fig. 2 (B and C). Mass spectra of stearylated Nod factors were similar to those of vaccenoylated Nod factors, except that all masses were 2 units higher because of the saturation of the fatty acid (data not shown). The mass spectra of Nod factors synthesized by ORS571–1.31U–K showed two products with m/z 1270 and 1244, corresponding to N-methylated Nod factors (Fig. 2B) that carried a vaccenoyl and a palmitoyl group, respectively (35). None of the Nod factors produced by ORS571–1.31U–K were vaccenoylated. No fucosylated, arabinosylated, or fucosylated carbamoylated Nod factors could be detected (absence of products with m/z values that may represent two different Nod factors. When no CID-MS analysis was performed, both products might be present.

From RP-TLC analysis, Nod factors of ORS571–1.31U– K(pRTHPQ) (Table I), which expressed the nodL gene of R. leguminosarum, were found to be more hydrophobic than those of ORS571–1.31U– K(pBBRNU) (data not shown), but production and secretion levels were similar to those of the parental strain ORS571–1.31U–K (Table IV). ORS571–1.31U– K(pRTHPQ) and ORS571–1.11Z–K(pRTHPQ) (Table I), which expressed the nodHPq genes of Rhizobium tropici, produced Nod factors that were more hydrophilic than those of the respective parental strains (data not shown), but production and secretion levels were the same as those of strains ORS571–1.31U–K and ORS571–1.11Z–K (Table IV). LSIMS analysis had previously shown that introduction of pRTHPQ into ORS571 led to Nod factors with a 6-O-sulfate ester at the reducing-terminal residue (36).

Nod factor concentrations may influence plant gene expression (37) and the relative importance of specific modifications for biological activity (38, 39). Therefore, Nod factor secretion and production (the total amount of secreted plus bacteria-associated products) were determined for all A. caulinodans derivatives used in this study (see “Experimental Procedures”) (Table IV). The presence of the NodIJ secretion system had only a minor influence on Nod factor secretion (Table IV), which might be due to the overnight growth of cultures prior to Nod factor preparation (40). Mutations in nodS and nodU strongly diminished the total Nod factor production, whereas introduction of pBBRNU into nodU mutants led to a 2-fold higher production compared with that of the wild type (Table IV).

Symbiotic Properties of nod Gene Mutants—Stem and root nodulation experiments were performed with the mutant strains and their complemented derivatives listed in Table I. Two weeks after inoculation, mature nodules were counted. Their relative average number per plant is presented in Fig. 3. Regarding the root nodulation efficiencies, three major groups of strains can be distinguished. The first group (a) of most

### TABLE III

| Fatty acid | Substitution at the nonreducing-terminal residue | Substitution at the reducing-terminal residue | DP | m/z of the nonreducing-terminal residue |
|-----------|-------------------------------------------------|---------------------------------------------|----|----------------------------------------|
| C18:0     | Carbamoyl                                       |                                             |    |                                        |
| C18:1     | Methyl, carbamoyl                               |                                             |    |                                        |
| 1096      | Carbamoyl                                       |                                             |    |                                        |
| 1242      | Carbamoyl                                       |                                             |    |                                        |
| 1299      | Methyl, carbamoyl                               |                                             |    |                                        |
| 1402      | Carbamoyl                                       |                                             |    |                                        |
| 1444      | Carbamoyl                                       |                                             |    |                                        |
| 1591      | Carbamoyl                                       |                                             |    |                                        |
| 1244      | Carbamoyl                                       |                                             |    |                                        |
| 1250      | Carbamoyl                                       |                                             |    |                                        |
| 1301      | Carbamoyl                                       |                                             |    |                                        |
| 1315      | Carbamoyl                                       |                                             |    |                                        |
| 1404      | Carbamoyl                                       |                                             |    |                                        |
| 1445      | Carbamoyl                                       |                                             |    |                                        |
| 1461      | Carbamoyl                                       |                                             |    |                                        |
| 1593      | Carbamoyl                                       |                                             |    |                                        |
efficient strains contained the wild type, the nonpolar nodS mutant ORS571–1.59S, which was affected in degree of Nod factor methylation as well as in yield (22% total yield of the wild type), but still produced a fraction (half of this population) wild-type Nod factors, and the strain ORS571–4.2K, a mutant that cannot synthesize fucosylated Nod factors. The second group of somewhat less efficient nodulators (60–70% (b) and 50–60% (c) of the wild type), basically consisted of strains that produced nonarabinosylated Nod factors with normal (ORS571–1.2), reduced (ORS571–1.39J, ORS571–1.11Z), or no (ORS571–1.11Z-VK) fucosylation (Fig. 3). No major effects of Nod factor levels on nodulation efficiency were noticed (Fig. 3). For example, no difference in nodulation efficiency could be observed among ORS571–1.39J, ORS571–1.2, and ORS571–1.11Z, although the two former strains produced approximately 3-fold more Nod factors than the latter strain (Fig. 3). Fig. 3 illustrates as well that the absence of a D-arabinosyl group has a more severe influence than the absence of an L-fucosyl group. The third group, with a more strongly reduced number of root nodules (d; less than 20%) (Fig. 3) contained ORS571–1.31U and ORS571–1.31U-VK strains, which do not produce carbamoylated Nod factors and which are affected in the synthesis of glycosylated Nod factors (Fig. 3). Introduction of pBBRNU into the nodU mutants partially restored the root nodulation efficiency (Fig. 3) (see “Discussion”).

The phenotypes on the stem were more pronounced. A CID-MS spectrum of Nod factors produced by ORS571–1.59S. The parental ion is at m/z = 1447 and represents two molecules. The m/z = 350–1150 mass scale is presented. A symbolic representation of both Nod factors, including the fragmentation scheme, is shown above the mass spectrum. The m/z values of characteristic fragments are indicated. Fragments due to the loss of fucose or arabinose (indicated by stars) are not abundant enough to be detected above the background. B and C, LSIMS analysis of Nod factors produced by two double mutants. The Nod factors were purified from ORS571–1.31U-VK (B) and ORS571–1.11Z-VK (C). A mass spectrum of the fraction containing vaccenoylated (C18:1) and palmitoylated (C16:0) Nod factors is shown. The spectra are preceded by a symbolic representation with the fragmentation scheme of the analyzed Nod factors. The m/z values of molecular and fragment ions are indicated. The absence of arabinosylated, fucosylated, or arabinosylated and fucosylated Nod factors is demonstrated by the absence of signals due to protonated molecules at m/z = 1402, 1416, and 1548 (B), and 1445, 1459, and 1591 (C). Ions seen at m/z higher than 1270 (B) and 1313 (C) correspond to alkali ion adducts or are due to impurities in the sample.
greater impact of the absence of an L-fucosyl group and, in general, a stronger requirement of the glycosylations and the carbamoylation (i.e., for all modifications studied) for efficient nodulation were noticed. Replacement of the 6-O-carbamoyl group by a 6-O-acetyl group as in strain ORS571–1.31U-\(\text{Ki}\)(pBBRNU) did not affect root nodulation, whereas stem nodulation was reduced compared with ORS571–1.31U-\(\text{Ki}\). Introduction of \(\text{pRTYPQ}\) into ORS571–1.31U-\(\text{Ki}\) did not affect root nodulation, whereas stem nodulation was 2-fold higher than wild type (Fig. 3). These results are similar to reports on decreases in Nod factor production of a mutant of \(\text{Rhizobium}\) sp. NGR234 (43), and nodL mutants of \(\text{S. meliloti}\) (3) and \(\text{R. leguminosarum}\) (44, 45). Introduction of \(\text{pBBRNU}\) in the mutant strains ORS571–1.31U-\(\text{Ki}\) or ORS571–1.11Z-\(\text{Ki}\) did not alter stem or root nodulation efficiency (Fig. 3).

**Biological Activity of Purified Nod Factors**—Purified Nod factors were assayed for their capacity to induce root hair formation at the bases of lateral roots of \(\text{S. rostrata}\) (25, 26). Five different pentameric Nod factors (Fig. 4) were applied at concentrations of \(10^{-8}\), \(10^{-7}\), \(10^{-6}\), \(10^{-5}\) M. The average number of dense groups of root hairs per plant is presented in Fig. 4. Because no differences in biological activity had been observed previously between vaccenoylated and stearamylated Nod factors (25), only the former type was tested. \(\text{NodARc-V(Carb,Me,C18:1,Ara,Fuc)}\), \(\text{NodARc-V(Me,C18:1,Fuc)}\), and \(\text{NodARc-V(Me,C18:1,Ara,Fuc)}\) showed the highest activity (Fig. 4). Whereas a small decrease in activity was found in stearamylated Nod factors with one glycosyl group (\(\text{NodARc-V(Carb,Me,C18:1,Ara,Fuc)}\), \(\text{NodARc-V(Me,C18:1,Fuc)}\), and \(\text{NodARc-V(Me,C18:1,Ara,Fuc)}\)) and \(\text{NodARc-V(Carb,Me,C18:1)}\), which lacks the carbamoyl group and both glycosylations, was the least active molecule. \(\text{Nod Factor Substitutions and Stability against Degradation—An in vitro assay was used to investigate the role of substitutions in protection against Nod factor-degrading enzyme activities of S. rostrata. Purified }^{14}\text{C-labeled pentameric vaccenoylated Nod factors were incubated with crude plant extracts and assayed by semiquantitative RP-TLC (see “Experimental Procedures”). The degradation assays were carried out at pH 5 to monitor the activity of extracellular hydrolases with a low pH optimum (24), and to mimic the in vivo extracellular pH (41, 42). When extracts of uninoculated adventitious rootlets were used, the RP-TLC pattern contained four different spots (data not shown), corresponding to pentameric Nod factors and tetra-, tri-, and dimer acylated degradation products as described (23). \(\text{NodARc-V(Carb,Me,C18:1,Ara,Fuc)}\) was the most stable molecule with a half-life time of more than 20 h (Fig. 5A), whereas \(\text{NodARc-V(Carb,Me,C18:1,Fuc)}\), and \(\text{NodARc-V(Carb,C18:1,Me,Me,C18:1,S)}\), \(\text{NodARc-V(Carb,C18:1,Me,Me,C18:1,S)}\), and \(\text{NodARc-V(Carb,Me,C18:1,S)}\) of approximately 15 h (Fig. 5A), and \(\text{NodARc-V(Me,C18:1,Fuc)}\), which had one of approximately 20 min (Fig. 5B). The Nod factors were all degraded to yield dimeric acylated degradation products (data not shown). When Nod factors were incubated for 24 h in the absence of extracts, no degradation products could be detected (data not shown).

**Discussion**

The Importance of Nod Factor Levels for Nodulation Efficiency—Loss of the nodS or the nodU gene activity diminished severely Nod factor production (1.59S, 1.31U, and 1.31U-\(\text{Ki}\) in Fig. 3). These results are similar to reports on decreases in Nod factor production of a nodSU mutant of \(\text{Rhizobium}\) sp. NGR234 (43), and nodL mutants of \(\text{S. meliloti}\) (3) and \(\text{R. leguminosarum}\) (44, 45). Introduction of \(\text{pBBRNU}\) in the nodU mutant strains ORS571–1.31U-\(\text{Ki}\) or ORS571–1.31U-\(\text{Ki}\), not only restored Nod factor carbamoylation but led to a Nod factor production that was 2-fold higher than wild type (Fig. 3). This phenomenon was not observed when the nodL gene from \(\text{R. leguminosarum}\) was expressed in the nodU mutants. Acetylated Nod factors were produced but still at reduced levels. NodU may act relatively early in the Nod factor biosynthesis pathway. NodS is known to methylate deacetylated chitopentameric substrates (34). Enzymes downstream in the pathway, such as the acyltransferase NodA (46, 47) of \(\text{A. caulinitodans}\) may be specific for methylated

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\(^{2}\text{M. Schultze, personal communication.}\)
the absence of glycosylations the relative average nodule number dropped to a mere 20%. The positive role of the glycosylations for efficient nodulation of *S. rostrata* is supported by the observation that *Sinorhizobium saheli* ORS611 and *Sinorhizobium teranga* bv. *sesbaniae* ORS604, two other symbionts of *S. rostrata* (49), produce Nod factors identical to those of *A. caulino-dans* (50). Replacement of the 6-O-α-fucosyl group by a 6-O-sulfate ester interfered with nodulation efficiency without changing the Nod factor stability. The presence of the 6-O-sulfate ester did not actively hinder Nod factor function, because introduction of the nod*HPQ* genes did not decrease severely the nodulation efficiency compared with that of the parental mutant strain (Fig. 3). This situation is different from that in *R. leguminosarum* bv. *viciae* where the nodulation of the host plant *Vicia sativa* was dramatically reduced upon expression of the *S. meliloti* nod*HPQ* genes (51). A bio-assay led to very analogous conclusions concerning the impact of Nod factor modifications. The influence of substitutions at the reducing-terminal residue on root hair induction capacity and on nodulation efficiency may be partly explained by the greater stability of glycosylated Nod factors against degradation by hydrolytic enzymes of *S. rostrata*. Nod factor-degrading enzymes are present in uninfected, stem-located, adventitious rootlets and in developing nodules (23). The degradation kinetics showed an inverse relationship between the number of glycosylations at the reducing-terminal residue and the degradation rate when Nod factors were incubated with extracts of uninfected adventitious rootlets. Tetrameric Nod factors of *S. meliloti* were more stable against degradation by root hydrolytic enzymes when they carried a sulfate ester at the reducing-terminal residue (21). Recently, Ovsyanya et al. (52) proposed that the 6-O-acetyl group at the reducing-terminal residue of Nod factors of *R. leguminosarum* bv. *viciae* may play a role in increasing the stability toward Afghan pea chitinases, but not in a specific receptor-ligand interaction, because a fucosyl group could functionally replace the structurally different 6-O-acetyl group. However, in the *A. caulino-dans*–*S. rostrata* interaction, modifications at the reducing-terminal residue seem to have a dual function: protection of the Nod factor against degradation as well as recognition. Indeed, a 6-O-α-fucosyl group can be replaced by a 6-O-sulfate ester for protection, but not for nodulation (compare ORS571–1.2 and ORS571–1.11Z-OH[KpRTHPQ] in Fig. 3), whereas the α-arabinosyl group protects the Nod factor against degradation to the same extent as the 6-O-α-fucosyl group, but is more important for nodulation (compare ORS571–4.2K to ORS571–1.2 in Fig. 3). When extracts of developing nodules were used, all nod factors tested were quickly degraded, suggesting that genes encoding hydrolytic enzymes are up-regulated. Indeed, Goormachtig et al. (23) showed that a Nod factor-degrading chitinase is produced during stem nodule development on *S. rostrata*. These findings resemble those of Staelin et al. (22), who demonstrated that Nod factors of *S. meliloti* induced the production of *Medicago sativa* chitinases that rapidly degrade *S. meliloti* Nod factors. The biochemical characterization of the azorhizobial chitinase and its substrate specificity are subject of a parallel study in our laboratory.

**Modifications at the Nonreducing-terminal Residue**—The presence of the carbamoyl group contributes to nodulation efficiency, carbamoylated Nod factors have a higher biological activity than their noncarbamoylated counterparts, and the carbamoyl group enhances Nod factor stability in the *in vitro* Nod factor degradation assay with extracts of uninfected adventitious rootlets (Figs. 3–5). Staelin et al. (22) showed that a 6-O-acetyl group at the nonreducing-terminal residue of *S. meliloti* Nod factors increased their stability against degrada-

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3 W. D’Haeze and M. Holsters, unpublished data.
tion by an N-acyl chitobiase-forming Nod factor hydrolase. The impact of the carbamoyl group in Nod factor protection is comparable to that of the fucosyl group at the reducing-terminal residue (Fig. 5); however, strains that are affected in carbamoylation are more severely altered in their nodulation efficiency (Fig. 3), suggesting an additional role of the carbamoyl group in recognition. Nevertheless, the carbamoyl group can be replaced by an acetyl group for root nodulation and partially also for stem nodulation. Perhaps the keto function, which is common for both groups, or a blocked C-6-hydroxy group at the nonreducing-terminal residue contribute equally to recognition. As discussed above, the reduced Nod factor production by nodU mutant strains is not the major cause of the reduced nodulation efficiencies. Similar to the azorhizobial nodU mutation. As discussed above, the reduced Nod factor production by nodU mutant strains is not the major cause of the reduced nodulation efficiencies. Similar to the azorhizobial nodU mutation. As discussed above, the reduced Nod factor production by nodU mutant strains is not the major cause of the reduced nodulation efficiencies. Similar to the azorhizobial nodU mutation. As discussed above, the reduced Nod factor production by nodU mutant strains is not the major cause of the reduced nodulation efficiencies. Similar to the azorhizobial nodU mutation. As discussed above, the reduced Nod factor production by nodU mutant strains is not the major cause of the reduced nodulation efficiencies. Similar to the azorhizobial nodU mutation. As discussed above, the reduced Nod factor production by nodU mutant strains is not the major cause of the reduced nodulation efficiencies. Similar to the azorhizobial nodU mutation. As discussed above, the reduced Nod factor production by nodU mutant strains is not the major cause of the reduced nodulation efficiencies. Similar to the azorhizobial nodU mutation. As discussed above, the reduced Nod factor production by nodU mutant strains is not the major cause of the reduced nodulation efficiencies. Similar to the azorhizobial nodU mutation. As discussed above, the reduced Nod factor production by nodU mutant strains is not the major cause of the reduced nodulation efficiencies. Similar to the azorhizobial nodU mutation. As discussed above, the reduced Nod factor production by nodU mutant strains is not the major cause of the reduced nodulation efficiencies. Similar to the azorhizobial nodU mutation. As discussed above, the reduced Nod factor production by nodU mutant strains is not the major cause of the reduced nodulation efficiencies. Similar to the azorhizobial nodU mutation. As discussed above, the reduced Nod factor production by nodU mutant strains is not the major cause of the reduced nodulation efficiencies. Similar to the azorhizobial nodU mutation. As discussed above, the reduced Nod factor production by nodU mutant strains is not the major cause of the reduced nodulation efficiencies. Similar to the azorhizobial nodU mutation. As discussed above, the reduced Nod factor production by nodU mutant strains is not the major cause of the reduced nodulation efficiencies. Similar to the azorhizobial nodU mutation. As discussed above, the reduced Nod factor production by nodU mutant strains is not the major cause of the reduced nodulation efficiencies. Similar to the azorhizobial nodU mutation. As discussed above, the reduced Nod factor production by nodU mutant strains is not the major cause of the reduced nodulation efficiencies. Similar to the azorhizobial nodU mutation. As discussed above, the reduced Nod factor production by nodU mutant strains is not the major cause of the reduced nodulation efficiencies. Similar to the azorhizobial nodU mutation. As discussed above, the reduced Nod factor production by nodU mutant strains is not the major cause of the reduced nodulation efficiencies. Similar to the azorhizobial nodU mutation.

As for methylation, we cannot reach conclusions about the necessity of the methyl group for nodulation as strain ORS571–1.59S still produced 10% wild-type Nod factors. A lack of polarity of a Tn5 insertion has also been observed, for instance, for certain chromosomal Tn5 insertion mutants in the E. coli lac operon (55). The fact that a mutation in nodS did not completely abolish methylation was seen in Rhizobium sp. NGR234 as well (43). The residual methyltransferase activity may be due to a yet unknown Nod protein or to a household methyltransferase. The location of the insertion at the carboxyl-terminal part of the nodS gene does probably not cause a partial inactivation of the nodS protein because the Rhizobium sp. NGR234 nodS mutant did carry an ω-cassette in the middle of the nodS gene and still produced a fraction of methylated Nod factors (43).

Nod Factor Requirements for Stem and Root Nodulation—Although the overall tendencies regarding Nod factor requirements were quite similar for both stem and root nodulation, the structural requirements of Nod factors for root nodulation were overall less stringent than for stem nodulation. Root nodulation takes place in an aqueous environment; stem nodulation occurs under aerial conditions that are probably more restrictive, regarding inoculation and bacterial growth conditions, perhaps explaining the lower efficiency of several mutants on the stem.

No macroscopic differences were seen between nodules induced by the wild-type strain and those induced by any of the mutant strains, either on the stem or on the root (data not shown), neither were other phenotypes, such as the formation of pseudo-nodules, observed. Furthermore, with none of the A. cauliformans mutants that were studied in the Sesbania interaction, an uncoupling of epidermal and cortical responses took place, as described by Ardourel et al. (3) in the S. meliloti-alalfa interaction. These results are thus more in line with observations by Stokkermans et al. (18), who showed that in C. soja natural and synthetic Nod factors that were biologically active induced both root hair formations and nodule initiations.

Nod factor decorations are major contributors to host specificity by interaction with a specific receptor (3–5). This hypothesis is supported by recent indications that heterotrimeric GTP-binding regulator proteins may mediate Nod factor signal transduction mechanisms in the S. meliloti-Medicago interaction (54). In addition, the Nod factor substitutions may allow efficient nodulation by protecting against degradation (21). Perhaps Nod factor modifications evolved to protect an elementary lipochitooligosaccharide from degradation by plant-de-
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