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The NodC protein of *Azorhizobium caulinodans* is an *N*-acetylglucosaminyltransferase

(chitin synthase/nodulation/*Rhizobium*/*Sesbania rostrata*)

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ABSTRACT **Nod factors are signal molecules produced by *Azorhizobium*, *Bradyrhizobium*, and *Rhizobium* species that trigger nodule formation in leguminous host plants. The backbone of Nod factors consists of a β-1,4-*N*-acetylglucosamine oligosaccharide from which the *N*-acetyl group at the nonreducing end is replaced by a fatty acid. The *nodABC* gene products are necessary for backbone biosynthesis. By incubation of cell extracts from *Azorhizobium caulinodans* with radioactive uridine diphosphate-*N*-acetylglucosamine, Nod factor precursors were identified and characterized as β-1,4-*N*-acetylglucosamine oligosaccharides. By analysis of different *nod* gene mutants and by expression of *nodC* in *Escherichia coli*, the *N*-acetylglucosaminyltransferase activity was ascribed to the NodC protein. The results suggest that the first step in biosynthesis of Nod factors is the assembly of the oligosaccharide chain.**

The symbiosis between *Azorhizobium*, *Bradyrhizobium*, or *Rhizobium* species and legumes results in nitrogen-fixing nodules. The first stage of this interaction leads to the activation of bacterial nodulation (*nod*) genes by plant flavonoids. The *nod* genes are involved in the production of Nod factors (1–6), which act as host-specific signals to trigger nodule formation (1, 2, 5, 7, 8). The backbone of all Nod factors consists of an *N*-acetylglucosamine oligosaccharide (three to five sugar residues) from which the acetyl group at the nonreducing end is replaced by an acyl chain. The specificity of the Nod factor molecules resides in the nature of the acyl group and in substitutions on the oligosaccharide chain (2, 9). The *nodABC* genes, common to all rhizobia, are essential for the synthesis of the lipooligosaccharide backbone (2). Only for NodB has a function been shown; it is an enzyme that removes the *N*-acetyl moiety from the nonreducing end of *N*-acetylglucosamine oligosaccharides (10). The role of NodC is controversial, because it was reported to share sequence homology with chitin synthases (11–13) and to be an outer membrane protein with receptor structure (14–16). Finally, NodA has been proposed to be an acyltransferase (17).

Azorhizobium caulinodans elicits N_2-fixing nodules on roots and at stem-located root primordia of the tropical legume *Sesbania rostrata*. The *nodABC* genes are located in a large operon, Nod locus *1*, that comprises the genes *nodABCSUIJ* (18, 19) (Fig. 1*A*). Induced expression of these genes leads to the formation of *A. caulinodans* Nod factors (NodARc) (5, 19). NodARc are chitotetra- or chitopentasaccharide molecules, at the nonreducing end N-acylated with vaccenic or stearic acid and N-methylated. All chitotetrasaccharides and some of the chitopentasaccharides have an *O*-carbamoyl substitution on C-6 of the nonreducing end. On C-6 of the reducing end, a fraction of the chitopentasaccharides are branched with D-arabinose. The latter substitution is not found in the chitotetrasaccharides (5).

This paper reports on *in vitro* biosynthesis of the oligosaccharide moiety of Nod factors. The *nodC* gene of *A. caulinodans* is shown to code for the *N*-acetylglucosaminyltransferase activity responsible for its polymerization.

MATERIALS AND METHODS

Preparation of Cell Extracts. *A. caulinodans* ORS571 and its derivatives (Table 1) were grown at 37°C in YEB medium (0.5% beef extract/0.1% yeast extract/0.5% peptone/0.5% sucrose/2 mM $MgSO_4$) and *E. coli* were grown in LB medium (23) without glucose, supplemented with the appropriate antibiotics. Overnight cultures were diluted with prewarmed medium to an OD_{600} of 0.2. Sixty minutes after dilution, *nod* genes were induced, when needed, in *A. caulinodans* with 10 μM naringenin and in *E. coli* with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG). After 4 hr of further incubation, cells were collected by centrifugation, washed once with 50 mM Tris·HCl, pH 7.4/10 μM 2-mercaptoethanol, resuspended in the same buffer (1 ml/100 ml of culture), and disrupted with a French pressure cell (20,000 psi; 1 psi = 6.89 kPa). Unbroken cells were removed by centrifugation at 5000 × *g* for 15 min. Membranes were sedimented at 105,000 × *g* and resuspended in 50 mM Tris·HCl, pH 7.4/10 μM 2-mercaptoethanol at a concentration of 10 μg of protein per μl. The protein extracts were stored at −70°C. Protein concentrations were determined with the Bio-Rad *DC* protein assay kit using bovine immunoglobulin as the standard.

For preparation of permeabilized cells, cultures were grown as above and cells were collected by centrifugation. The cell pellet was washed once with 30 mM Tris·HCl/10 mM EDTA, pH 8, and resuspended in the same buffer (1 ml/100 ml of culture). These suspensions were frozen and thawed three times and then stored at −70°C until use.

Enzymatic Assay. Incubations were carried out for 60 min at 10°C in 50 μl of a solution containing 50 mM Tris·HCl (pH 7.4), 5 mM *N*-acetylglucosamine, 10 mM $MgCl_2$, 1 mM pyrophosphate, cell extract (200 μg of protein or 30 μl of permeabilized cell suspension), and 0.1 μCi of uridine diphosphate-*N*-acetyl-D-[U^{14}C]glucosamine (UDP-*N*-acetyl-[^{14}C]glucosamine, 298 mCi/mmol, Amersham; 1 μCi = 37 kBq). The reaction was stopped by addition of 200 μl of

Abbreviations: IPTG, isopropyl β-D-thiogalactopyranoside; GlcNAc, *N*-acetylglucosamine; $GlcNAc_2$, *N*,*N*′-diacetylchitobiose; $GlcNAc_3$, *N*,*N*′,*N*″-triacetylchitotriose; $GlcNAc_4$, *N*,*N*′,*N*″,*N*‴-tetraacetylchitotetraose; $GlcNAc_5$, *N*,*N*′,*N*″,*N*‴,*N*⁗-pentaacetylchitopentaose.

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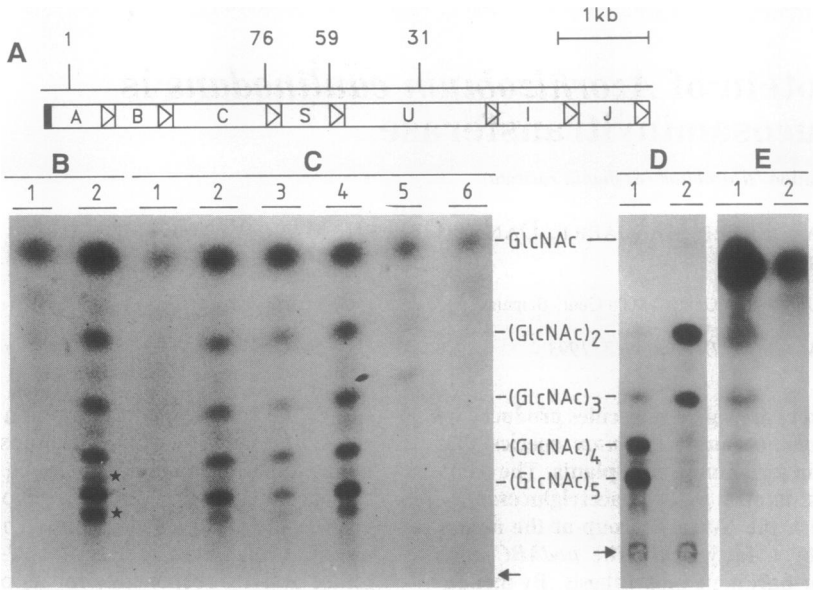

FIG. 1. (*A*) Genomic organization of *A. caulinodans* ORS571 Nod locus *1* containing the genes *nodABCSUIJ* (19). The positions of the Tn*5* insertions used in this study are indicated by their number above the gene in which they are inserted. The corresponding mutant strains are listed in Table 1. The *nod* promoter is presented as a black box. (*B*) Production *in vitro* of *nod*-related *N*-acetyl-D-glucosamine oligosaccharides. TLC of radioactive products, produced by French-press extracts of ORS571(pRG70) cells uninduced (lane 1) or induced (lane 2) with naringenin. Reaction products of 100 μg of protein were loaded. Bars indicate the position of GlcNAc, $GlcNAc_2$, $GlcNAc_3$, and $GlcNAc_4$ standards and the calculated position of $GlcNAc_5$. Arrows indicate the origin of the TLC, and stars indicate the positions of deacetylated products. (*C*) Identification of the *N*-acetylglucosaminyltransferase gene. Reaction products produced by French-press extracts from *A. caulinodans* ORS571 uninduced (lane 1), ORS571 induced (lane 2), ORS571-1.31U ($NodU^-$) induced (lane 3), ORS571-1.59S ($NodS^-$) induced (lane 4), ORS571-1.76C ($NodC^-$) induced (lane 5), and ORS571-V.44 ($NodA^-$) induced (lane 6). Reaction products of 100 μg of protein were loaded. (*D*) Chitinase digestion of oligosaccharides produced *in vitro*. $GlcNAc_4$ and $GlcNAc_5$ oligosaccharides purified with Bio-Gel P-2 (lane 1) were incubated with *Trichoderma harzianum* chitinase (CHIT37; lane 2). This enzyme hydrolyzes chitin and chitooligosaccharides to $GlcNAc_3$, $GlcNAc_2$, and GlcNAc (20). (*E*) Subcellular location of the *N*-acetylglucosaminyltransferase. Membrane fraction (lane 1) and cytosolic fraction (lane 2) from *A. caulinodans* ORS571(pRG70) were assayed for synthesis of the oligosaccharides.

water and boiling for 3 min. After centrifugation, the supernatant was loaded on a Sephadex A-25 anion-exchange column (gel volume, 300–500 μl) equilibrated with water. The column was washed with 2 ml of water. The unbound fraction (flowthrough and wash) was lyophilized. NodC activity in *A. caulinodans* and *E. coli* could be demonstrated with both French-press protein extracts and permeabilized cells.

Thin-Layer Chromatography. Samples were spotted onto a TLC plate (precoated TLC plates, silica gel 60; Merck). Reaction products of 100 μg of protein were loaded. The chromatogram was developed twice with 1-propanol/water/concentrated ammonium hydroxide (70:30:1.5, vol/vol). For each TLC run, a lane with reference compounds [*N*-acetylglucosamine (GlcNAc), *N,N'*-diacetylchitobiose ($GlcNAc_2$), *N,N',N"*-triacetylchitotriose ($GlcNAc_3$), and *N,N',N",N"'*-tetraacetylchitotetraose ($GlcNAc_4$), 20 μg of each; Sigma] was included. Standards were stained by spraying with 0.2% orcinol in 20% H_2SO_4 and heating. Radioactive spots were detected by fluorography as described (19). Kodak X-Omat films were exposed for 2–10 days.

Bio-Gel P-2 Chromatography. Radioactive products purified by Sephadex A-25 were subjected to gel filtration on a Bio-Gel P-2 column (90 × 1.5 cm) equilibrated with 0.1 M pyridine acetate (pH 6) at a flow rate of 0.3 ml/min. Fractions of 2 ml were collected, and radioactivity was measured by liquid scintillation counting.

Affinity Chromatography. *N*-Acetylglucosamine oligosaccharides purified by anion exchange were subjected to affinity chromatography on a column of wheat germ lectin-Sepharose 6MB (gel volume, 500 μl; Pharmacia) using 20 mM potassium phosphate, pH 7.2/150 mM NaCl for binding and washing. Bound radioactivity was eluted with the same buffer supplemented with 0.5 M GlcNAc.

Enzymatic and Chemical Treatments. Chitinase digestion was by overnight incubation with 0.1 μg of CHIT37 (20) in water at 37°C. CHIT37 was used because it degrades low molecular weight chitin derivatives to GlcNAc, $GlcNAc_2$ and $GlcNAc_3$ (20). N-acetylation was according to Molano *et al.* (24).

Cloning of the *A. caulinodans nodC* Gene. *Eco*RV and *Sph* I sites flanking *nodC* were engineered by PCR using the

Table 1. Strains

Strain	Relevant characteristics	Ref(s).
A. caulinodans		
ORS571	Wild-type strain, nodulates root and stem of *Sesbania rostrata*	21, 22
ORS571(pRG70)	Contains supplementary copies of Nod locus 1 on a pLAFR1 plasmid	5
ORS571-V.44	Chromosomal Tn*5* insertion in *nodA* (Tn*5* insertion 1; Fig. 1)	18
ORS571-1.76C	Chromosomal Tn*5* insertion in *nodC* (Tn*5* insertion 76; Fig. 1)	19
ORS571-1.59S	Chromosomal Tn*5* insertion in *nodS* (Tn*5* insertion 59; Fig. 1)	19
ORS571-1.31U	Chromosomal Tn*5* insertion in *nodU* (Tn*5* insertion 31; Fig. 1)	19
E. coli		
DH5α(pUCNC)	*E. coli* DH5α containing *nodC* of ORS571 cloned in pUC18	This work

oligodeoxynucleotide primers 5'-TTGATATCCAGCCGTTGCCATGAG-3' (upper primer) and 5'-TCGCGCGTCGAGATCCTTGCATGC-3' (lower primer). These restriction sites were used to clone the gene in the *Sma* I and *Sph* I sites of plasmid pUC18, so that the transcription of *nodC* was controlled by the *lac* promoter. This construct (pUCNC) was electroporated into *E. coli* DH5α.

RESULTS

Identification and Characterization of Nod Factor Precursors. Biosynthesis of Nod factors was studied by incubating bacterial extracts (French-press protein extracts or permeabilized cells) with UDP[^{14}C]*N*-acetylglucosamine, a putative sugar donor for β-1,4-*N*-acetylglucosamine oligosaccharide synthesis. The *A. caulinodans* ORS571(pRG70) strain containing extra copies of Nod locus 1 was used to increase sensitivity (5). To distinguish products related to *nod* genes, enzyme was prepared from cells cultured with or without the flavonoid naringenin, which induces azorhizobial *nod* gene expression (18). Radioactive products synthesized *in vitro* were separated from unreacted UDP[^{14}C]*N*-acetylglucosamine by anion-exchange chromatography and analyzed by TLC. Extracts from naringenin-induced cells produced radioactive products that comigrated with *N*-acetylglucosamine and β-1,4-*N*-acetylglucosamine oligosaccharide standards with polymerization degree 2–5. Extracts from uninduced cells, however, produced only *N*-acetylglucosamine, suggesting that the oligomers were Nod metabolites (Fig. 1*B*, lanes 1 and 2). When the membrane and cytosolic fraction of cell extracts from induced ORS571(pRG70) were assayed separately, it was found that the enzymatic activity for synthesis of the oligosaccharides was located in the membrane (Fig. 1*E*).

To obtain additional evidence for the proposed identity of these Nod metabolites, reaction products were subjected to chitinase digestion. An incubation mixture was chromatographed on a Bio-Gel P-2 column and fractions containing the putative β-1,4-*N*-acetylglucosamine tetra- and pentasaccharide were pooled and concentrated. Thus purified products were treated with a chitinase from *Trichoderma harzianum* that cleaves chitooligosaccharides to $GlcNAc_3$, $GlcNAc_2$, and GlcNAc (20). Analysis by TLC of the hydrolysis products showed degradation of the substrates to products comigrating with $GlcNAc_2$ and $GlcNAc_3$ standards (Fig. 1*D*, lanes 1 and 2).

The *in vitro* synthesized oligomers were analyzed by affinity chromatography on a column of wheat germ lectin-Sepharose 6MB, which binds β-1,4-*N*-acetylglucosamine oligosaccharides. The radioactive molecules bound to the column were eluted with *N*-acetylglucosamine and characterized as di-, tri-, and tetrasaccharides by Bio-Gel P-2 chromatography (Fig. 2).

In conclusion, the behavior on TLC and Bio-Gel P-2 chromatography, the wheat germ lectin binding, and the susceptibility to chitinase digestion indicate that the *in vitro* produced Nod metabolites are chitooligosaccharides with two to five GlcNAc residues.

To analyze the possibility that the observed oligomers arose by degradation of larger precursors, the occurrence of chitinase activity in *A. caulinodans* was investigated. Cell extracts from induced and uninduced cultures were incubated with the fluorogenic methylumbelliferyl β-D-glycoside of the mono-, di-, and trisaccharides as chitinase substrates. No significant increase in fluorescence was observed (data not shown), indicating that at least the di- and trisaccharide Nod metabolites did not arise as a consequence of endogenous chitinase activity.

The TLC pattern of some incubations showed satellite bands accompanying the oligosaccharides (Fig. 1*B*, lane 2, stars). When incubations were prolonged, these bands became predominant while the di-, tri-, tetra-, and pentasaccharides disappeared (data not shown). This suggests that the oligosaccharides were further metabolized to these slower migrating products. N-acetylation caused a shift of these bands to the mobilities of the original oligosaccharides. Therefore, the satellite bands most probably represent N-deacetylated forms of the oligomers and might arise due to the action of NodB (10).

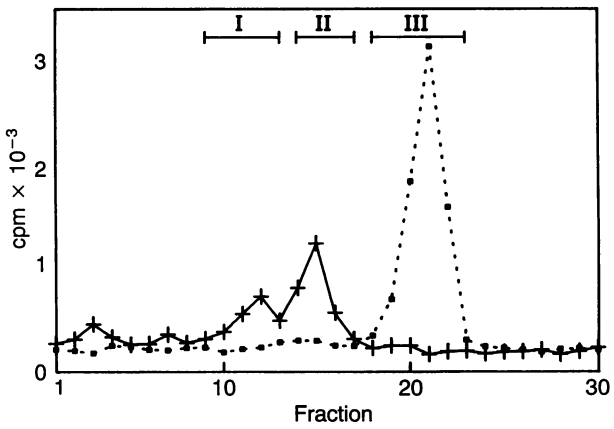

FIG. 2. Bio-Gel P-2 chromatography of oligosaccharides separated by affinity chromatography on a wheat germ agglutinin column. Radioactive oligosaccharides purified by DEAE chromatography were dried, redissolved in phosphate buffer, and applied to an affinity chromatography column. Bound products (full line) and unbound products (dotted line) were fractionated by Bio-Gel P-2 chromatography. Radioactivity was pooled and analyzed by TLC; peak III contains GlcNAc, peak II $GlcNAc_2$, and peak I a mixture of $GlcNAc_3$ and $GlcNAc_4$.

Identification of the Gene Encoding the *N*-Acetylglucosaminyltransferase. The gene coding for the *N*-acetylglucosaminyltransferase was identified by using extracts from the wild-type strain and derivatives with Tn*5* insertions in Nod locus *1* genes (19) (Fig. 1*A*; Table 1). The oligosaccharides were produced by cell extracts from the naringenin-induced wild-type strain and both the *nodS* and *nodU* mutant strains ORS571-1.59S and ORS571-1.31U (Fig. 1*C*, lanes 2–4); they were not produced by extracts from the uninduced wild-type strain and the induced *nodA* and *nodC* mutant strains ORS571-V.44 and ORS571-1.76C (Fig. 1*C*, lanes 1, 5, and 6). Even with longer exposure times or overloading of the lanes from the *nodA* and *nodC* mutants, the oligosaccharides could not be detected (data not shown). The *nodA* Tn*5* insertion in ORS571-V.44 exerts a polar effect on *nodC* expression: a double mutant containing this Tn*5* insertion and a *nodC–lacZ* fusion showed no induced β-galactosidase activity (M.H., unpublished results). Therefore, *nodC* is the most likely candidate to encode the *N*-acetylglucosaminyltransferase.

As a direct proof, the *nodC* gene was cloned under the control of the *lac* promoter, for expression in *E. coli*. Cell extracts from *E. coli* containing *nodC* on the plasmid pUCNC produced the same oligosaccharides as ORS571 extracts, whereas no production could be detected by the control extracts from *E. coli* (pUC18) (Fig. 3*A*). These products were degraded by chitinase (Fig. 3*B*, lane 2) and they were bound to wheat germ lectin (data not shown).

DISCUSSION

Nod factors are lipooligosaccharides that contain a chitooligosaccharide chain with three to five GlcNAc residues. We have demonstrated that cell extracts from *A. caulinodans* can produce, from UDP[^{14}C]*N*-acetylglucosamine, chitooligosaccharides with a polymerization degree from 2 up to 5.

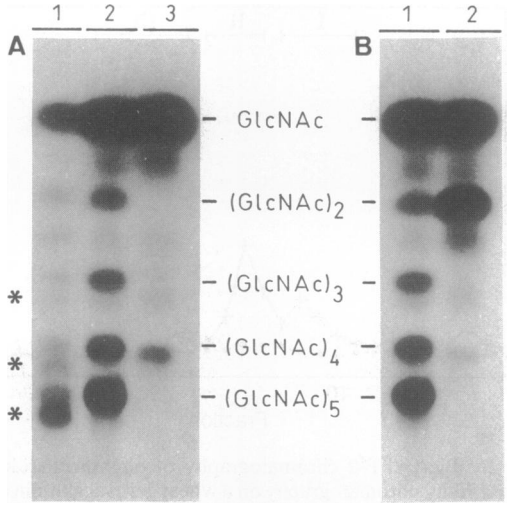

FIG. 3. (*A*) Synthesis of β-1,4-*N*-acetylglucosamine oligosaccharides by extracts from an *E. coli* strain harboring *nodC* under the control of the *lac* promoter. Oligosaccharides produced *in vitro* by cell extracts from *A. caulinodans* ORS571(pRG70) induced with naringenin (lane 1), *E. coli*(pUCNC) induced with IPTG (lane 2), and *E. coli*(pUC18) induced with IPTG were isolated and analyzed by TLC. Reaction products of 15 μl of permeabilized cells were loaded. The spot in lane 3 migrating slightly more slowly than $GlcNAc_4$ is not related to chitooligosaccharides, as it is not degraded by chitinase (data not shown). (*B*) Chitinase treatment of the *E. coli*(pUCNC) reaction products. Oligosaccharides produced by *E. coli*(pUCNC) (lane 1) were treated with chitinase (CHIT37; lane 2). Bars indicate the positions of GlcNAc, $GlcNAc_2$, $GlcNAc_3$, $GlcNAc_4$, and $GlcNAc_5$.

The identity of these products was confirmed by comparison of their chromatographic behavior with authentic standards, by their binding to wheat germ lectin, and by chitinase digestion. As we could not detect chitinase activity in *A. caulinodans* and since no oligo- or polysaccharides with more than five GlcNAc residues were formed, it is very unlikely that the oligosaccharides arose by degradation of larger precursors.

Only cell extracts from *A. caulinodans* cultures grown in the presence of the *nod* gene inducer naringenin produced the chitooligosaccharides, suggesting that they were Nod metabolites and that their production depended on a Nod protein. Analysis of different *nod* gene mutants and cloning for expression in *E. coli* showed that *nodC* encoded the *N*-acetylglucosaminyltransferase needed for the synthesis of the chitooligosaccharides. The localization in the membrane of the *N*-acetylglucosaminyltransferase activity is in agreement with the reported subcellular localization of NodC (14–16, 25). Our results also confirm the reported similarity of NodC with chitin synthases (11–13), as both enzymes catalyze the formation of β-1,4-linked GlcNAc chains. However, in contrast to chitin synthases, NodC produces only oligosaccharides with a polymerization degree up to 5 and no polysaccharides.

At least three steps are needed to synthesize the Nod factor backbone: synthesis of the oligosaccharide chain, deacetylation, and transfer of an acyl moiety. The chitooligosaccharide chain is produced by NodC. Recently, it has been reported that NodB is a deacetylase that removes the *N*-acetyl group of the nonreducing end in chitooligosaccharides (10). Since NodB cannot use the GlcNAc monosaccharide as a substrate, we propose that sugar oligomerization by NodC precedes the action of NodB and is the first step in Nod factor biosynthesis. This is supported by the observation that the NodC reaction products are further metabolized by *A. caulinodans* cell extracts, probably to a deacetylated form resulting from NodB action. *E. coli* extracts containing NodC produce chitooligosaccharides but no putative deacetylated forms, in agreement with the absence of NodB in these extracts. N-acylation on the deacetylated nonreducing end of chitooligosaccarides is by consequence the last step to form the lipooligosaccharide backbone of Nod factors and is hypothesized to be carried out by NodA (17). The biosynthesis in *E. coli* of lipid A, a lipooligosaccharide present in the envelope of Gram-negative bacteria, includes also a N-deacetylation and N-acylation step on *N*-acetylglucosamine derivatives. However, here the sugar nucleotide UDP-*N*-acetylglucosamine is the substrate (26).

Nod factors are signal molecules involved in the development of nodules in leguminous plants. However, the biological activity of Nod factors seems not to be restricted to nodulation: in carrot a somatic embryo mutant could be rescued with Nod factors (27), and expression of *nodA* and/or *nodB* of *Rhizobium meliloti* in transgenic tobacco alters growth and development of the plant (28). The presence of Nod factor-related molecules in plants has been hypothesized (29, 30), and the assay described here may be used to study the occurrence in plants of *N*-acetylglucosaminyltransferases with similar activities as NodC.

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