Structural requirements of *Rhizobium* chitolipooligosaccharides for uptake and bioactivity in legume roots as revealed by synthetic analogs and fluorescent probes

Saleela Philip-Hollingsworth,* Frank B. Dazzo,* and Rawle I. Hollingsworth[1,†]

Department of Microbiology,* Departments of Biochemistry and Chemistry,† Michigan State University, East Lansing, MI 48824

Abstract *Rhizobium* chitolipooligosaccharides (CLOSs) are heterogeneous fatty acylated N-acetyl glucosamine oligomers with variations in both the polar (hydrophilic) oligosaccharide head group and the non-polar (hydrophobic) fatty acyl chain. They trigger root hair deformation and cortical cell divisions in legume roots during development of the nitrogen-fixing root-nodule symbiosis. It has been proposed that only certain unique molecular species of CLOSs made by a particular rhizobia can elicit these responses on the corresponding legume host, suggesting that receptor-mediated perception of CLOSs serves as a basis of symbiotic specificity. We evaluated the relative symbiotic importance of the hydrophilic and hydrophobic structural domains of CLOSs by comparing the biological activities of CLOSs from wild type *R. leguminosarum* bv. *trifolii* ANU843 with that of various synthetic analogs. These tests were performed in axenic bioassays on the compatible symbiotic host, white clover (*Trifolium repens*) and the incompatible non-host legume, alfalfa (*Medicago sativa*). Fluorochrome-tagged derivatives of the native CLOSs and the analogs were also prepared in order to evaluate the uptake and localization patterns of these molecules within host root cells. The results indicate a direct link between uptake and biological activities of *Rhizobium* CLOSs on legume roots. The smallest CLOS analog taken up and biologically active on white clover and alfalfa was a N-fatty acylglucosamine, without an essential requirement of oligomerization, fatty N-acyl unsaturation, or acetate/sulfate functionalization. This suggests that N-fattyacylglucosamine is the common minimum structure required and sufficient for uptake and biological activity of CLOS glycolipids in these legumes, and that the various specific modifications of its polar head group and hydrophobic tail modulate its inherent ability to further express these activities, thus influencing which legumes are capable of responding to CLOSs rather than dictating their biological activities per se.—**Philip-Hollingsworth, S., F. B. Dazzo, and R. I. Hollingsworth.** Structural requirements of *Rhizobium* chitolipooligosaccharides for uptake and bioactivity in legume roots as revealed by synthetic analogs and fluorescent probes. *J. Lipid Res.* 1997. **38:** 1229–1241.

Supplementary key words *Rhizobium leguminosarum* biovar *trifolii*–legume symbiosis • root hair • membrane glycolipid

Rhizobia are bacteria that produce chitolipooligosaccharides (CLOSs) that elicit root hair deformations (Had) and cortical cell divisions (Ccd) on roots of specific leguminous host plants during development of the root-nodule symbiosis (1–4 and references therein). These glycolipids are β-1,4-linked N-acetylglucosamine oligomers in which the nitrogen of the non-reducing terminus is acylated with a long chain fatty acid. Individual strains of rhizobia produce a diversity of CLOS glycolipids that differ in degree of N-acetylglucosamine oligomerization (d.p. 3, 4, 5) and other functionalizations (e. g., sulfate, acetyl, carbamoyl, fucosyl, N-methyl) in the oligosaccharide polar head group, and the structure of the N-fattyacyl component (degree of unsaturation, hydroxylation, chain length) (2). For instance, when grown with the appropriate flavone to activate expression of its symbiotic nodulation (*nod*) genes, wild-type *R. leguminosarum* bv. *trifolii* ANU843 (a rhizobial symbiont of clover) makes a diverse family of at least 22 related CLOS glycolipids consisting of chitotri-, tetra, and pentaoses bearing 0, 1, or 2 O-acetylations and a wide spectrum of saturated, unsaturated, and hydroxylated fatty acids ranging in chain length from C14 C22 (5, 6). The major fatty acid is *cis*-vaccenic acid (C18:1) with lesser amounts of C18:0, C16:0, C16:1, C18:2, C20:1, 3-hydroxy-C14:0, 3-hydroxy-C16:0, and 3-hydroxy-C18:0.

Several studies have reported that only certain unique molecular species of CLOSs made by a particu-

Abbreviations: CLOS, chitolipooligosaccharide; Ccd, cortical cell divisions; Had, root hair deformations; NBD, 7-nitrobenzo-2-oxa-1,3-diazole; NMR, nuclear magnetic resonance; NF, nitrogen-free plant growth medium.

[1]To whom correspondence should be addressed.

lar rhizobia can elicit the Had and Ccd responses on the corresponding legume host and that alterations in the *nod* genes controlling host range specify the diversity of their structural modifications (1–4 and references therein). These results have led to the general model suggesting that CLOSs serve as a basis of symbiotic specificity and that the elicitation of these host-selective responses involves specific recognition of their unique structural features by some receptor on the host plant (1, 4). However, the results of several studies do not support this general model in its entirety. For instance, four studies have shown that CLOSs can also be perceived by non-legume plants, and in three of these studies plant responses were induced (7–10). Another major study using synthetic CLOSs found that 2-O-methylfucosylation (once thought to be an important functionalization of the polar head group of CLOSs that control the host range for *Bradyrhizobium japonicum*) was not essential for the ability of tetrameric CLOSs made by this rhizobial symbiont to induce nodule initiation in the homologous host *Glycine soja* (11). Other studies showed that the diverse family of CLOSs made by *R. loti* and *R. etli* were identical despite their clearly non-overlapping host ranges (12,13). Finally, we have recently found that the diverse family of CLOS glycolipids isolated from the authentic wild type *R. leguminosarum* bv. *trifolii* strain ANU843 (no gene modifications) is fully capable of eliciting host-selective responses at 10^{-11} M (14), and is unchanged in an isogenic *nodE::*Tn*5* mutant derivative despite a major change in this mutant's legume host range (6). Considered collectively these studies raise questions on the importance of variations in CLOS structure in order to be "perceived" and be biologically active in eliciting symbiosis-related responses in specific legume hosts.

From the theoretical point of view, it is unclear how the same receptor molecule in vivo could simultaneously recognize structural features from such widely (spatially) separated parts of these amphiphilic molecules, such as its accessible oligosaccharide chain and/or functionalizations in the polar head group and the middle of its less accessible N-acyl chain in the nonpolar tail that would be integrated into lipid bilayer membranes, micelles, or vesicles. It is also difficult to reconcile the high degree of structural overlap between CLOSs of different rhizobia with the high degree of specificity that characterizes the *Rhizobium*–legume symbiosis. Certain subtle structural differences, such as an extra double bond in a fatty acid chain, would not be sufficient to give any appreciably higher degree of binding or interaction between one or the other CLOS species within the same family made by a given strain of rhizobia and any receptor system. This is because the binding energies involved with such structural features are only van der Waals in nature and are, therefore, very small. It is still possible, however, that there might be some conformational consequences to these modifications if the process is receptor-mediated. For instance, there is the possibility that the introduction of a *cis*-double bond in the 2,3-position of the fatty acyl chain would change the angle at which the oligosaccharide portion emerges from a membrane, micelle, or vesicle. However, the fact that the double bond in question in the *R. meliloti* CLOS has the *trans*- geometry negates this possibility as *trans* unsaturated double bonds and extended saturated alkyl chains have the same geometry. It is well established fact that a *trans* double bond does not alter the regular packing of stacked parallel saturated hydrocarbon chains in lipid bilayers and does little to the total interaction energy (15). The *trans* double bond in the *R. meliloti* CLOS is therefore conformationally and energetically insignificant.

As part of our studies to relate CLOS structure with their symbiotically important biological activity, we have addressed whether the wide spectrum of structural modifications in *Rhizobium* CLOSs is critical in their elicitation of host-selective symbiosis-related responses. We predicted that if the fine structural details of CLOSs are unimportant, then analogs that embrace the general chemical properties of these bacterial glycolipids should be able to elicit many of the symbiosis-related host responses of the plant to the bacteria. Such analogs could be simpler structures having the critical N-fatty-acyl chain and a polar carbohydrate head group containing only one glycosyl residue. Because foreign molecules affect different plant species differently, it is anticipated that the intensity of responses to such molecules would vary among different hosts, therefore appearing to exhibit some degree of plant selectivity. To test these hypotheses, we prepared two synthetic monoglycosyl analogs of CLOS, one having the same di-unsaturated C16:2 fatty acyl chain as found in the "host-specific" CLOS of the alfalfa symbiont, *R. meliloti*, and the other having a simple, saturated C16:0 alkyl chain. The C16:0-acylated monosaccharide analog was also further structurally disconnected (reduced) to an N-acylated 3-carbon fragment that was derived from 2-amino-1,3-dihydroxy-propane (serinol). The latter analog allowed us to test whether the N-acylated monosaccharide or a derivative of it with a smaller polar head group was the minimum structure that could induce the biological responses. It also allowed us to better define the hydrophilic/hydrophobic balance required for biological activity. Also, if host responses similar to those elicited by naturally occurring CLOSs are obtained with these analogs, it would be important to compare their uptake by plant cells. To determine this, we tagged the reducing glycosyl residue at the polar end of the native

CLOSs from wild type *R. leguminosarum* bv. *trifolii* ANU843 and the synthetic N-C16:0-acylGlcN analog with a relatively small, uncharged fluorescent probe to facilitate uptake studies by fluorescence microscopy. These studies have indicated a direct link between uptake and biological activities of *Rhizobium* CLOSs on legume roots, and identified N-fattyacylglucosamine as the minimal structure of CLOS analogs essential for biological activity and uptake on clover and alfalfa, without a requirement of oligomerization, fatty N-acyl unsaturation, or acetate/sulfate functionalization. Portions of this work were recently presented (16, 17).

MATERIALS AND METHODS

Synthesis of N-fatty acylglucosamine derivatives

To prepare N-C16:0-acylGlcN, glucosamine hydrochloride (2.17 g, 10 mmol) and palmitic acid (3.07 g, 12 mmol) were dissolved in 1:1 EtOH/H_2O (30 ml). To this was added 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (2.9 g, 15 mmol) and the reaction mixture was stirred at 25°C for 14 h. The mixture was then concentrated to dryness and treated with acetic anhydride (15 ml) and pyridine (30 ml) at 25°C for 14 h. It was then concentrated to a minimum volume, mixed with 30 ml of chloroform, and then extracted with water. The chloroform layer was concentrated to dryness and purified by silica gel column chromatography (eluant: petroleum ether–acetone 3:1) to yield the peracetylated derivative of N-C16:0-acylGlcN. The latter was deacetylated by stirring with 50 mM sodium hydroxide in methanol at 10°C for 20 min, acidified with acetic acid, concentrated to dryness, extracted with chloroform, and evaporated to dryness to yield the final product. The purity of the latter was established by TLC (solvent system: petroleum ether–acetone 3:2) on silica. The same method was used to prepare N-C16:2-acylGlcN. The C16:2$^{\Delta 2,9}$ fatty acid was prepared by a Wittig reaction between the triethyl phosphite adduct of ethylbromoacetate and the corresponding unsaturated aldehyde, *cis*-7-tetradecenal. This was accomplished by treating the unsaturated aldehyde (2 g, 4.75 mmol) in tetrahydrofuran (50 ml) with butyl lithium (0.37 g, 5.7 mmol) under nitrogen. To this was added carboxymethyltriethoxyphosphonium bromide ethyl ester (1.28 g, 5.7 mmol) and the reaction mixture was stirred for 15 min while kept cold in an acetone–dry ice mixture, followed by stirring at 25°C for 10 h. Excess butyl lithium was destroyed by adding acetic acid. Toluene (200 ml), water (100 ml), and petroleum ether (50 ml) were then added and the reaction mixture was stirred well. The organic layer was separated from the aqueous phase and the former was evaporated to dryness to yield the ethyl ester. This was converted to the free acid by stirring with 50 mM sodium hydroxide in *t*-butanol for 12 h, after which it was acidified with trifluoroacetic acid (4 ml), concentrated to dryness, and partitioned between chloroform and water. The chloroform layer was evaporated to dryness to yield the product which was purified by distillation under high vacuum (0.01 mm in a bulb-to-bulb apparatus).

Synthesis of N-C16:0-acylserinol

Palmitic acid (1 g) was converted to its methyl ester by refluxing with 5% HCl/methanol for 2 h and then evaporating the reaction mixture to dryness. The product was dissolved in diglyme (10 ml), serinol (0.75 g) added and heated at 130°C for 14 h. The reaction mixture was then poured into water and cooled by immersion in an ice bath. The solid that separated out was filtered at the pump and the excess serinol was washed away with cold water. The solid was dissolved in chloroform (10 ml) and the chloroform layer was washed with water, decolorized by refluxing with charcoal for 10 min, filtered, and evaporated to dryness. The purity of the product was verified by thin-layer chromatography (chloroform–methanol 9:1 on silica layers).

Synthesis of the fluorescent NBD-CLOS conjugates

Fluorescent conjugates of CLOSs and CLOS analogs were prepared by covalently attaching 7-nitrobenzo-2-oxa-1,3-diazole (NBD) to their reducing termini. To prepare the NBD-conjugate of the N-acylGlcN, 50 mg N-C16:0-acylGlcN peracetate, 0.1 ml 2-bromoethanol and 10 µl trifluoroacetic acid were mixed together and heated at 60°C for 2 h, then diluted with water and extracted with chloroform. The chloroform layer was evaporated to dryness, redissolved in 2 ml of concentrated ammonia solution, sonicated briefly, and kept at 25°C for 14 h. It was then heated at 50°C for 1 h, evaporated to dryness, and dissolved in 1 ml of 2 M sodium bicarbonate solution. To this was added 100 mg of NBD chloride (Aldrich Chemical Co., Milwaukee, WI). This reaction mixture was heated at 60°C for 30 min, then diluted with water (2 ml), extracted with chloroform (2 × 1 ml) and the chloroform layer was evaporated to dryness. The product was then purified by preparative layer chromatography using silica (solvent system: chloroform–acetone, 3:1). This TLC system resolved three fluorescent bands (excitation at 366 nm) at R_f 0.3, 0.5, and 0.75. Each band was scraped, eluted with chloroform–acetone–methanol 1:1:1, evaporated to dryness, redissolved in chloroform, and analyzed by ^{1}H-NMR spectroscopy. The fluorescent band at R_f 0.3 yielded the desired NBD-conjugated product while the one at R_f

0.75 gave free unconjugated NBD chloride. The resolved band with the R_f 0.5 was not analyzed further. Other NBD-conjugates were prepared by the same method, using 1 mg of chitotriose (Sigma Chemical Co., St. Louis, MO) and 3 mg of CLOSs isolated from *R. leguminosarum* bv. *trifolii* ANU843 (5), except that the conjugates were purified by adsorbing the entire reaction mixture onto a C-18 reverse phase cartridge in pure water and eluting stepwise with increasing 5 ml volumes of solvent (100% water; 20% acetonitrile/water; 50% acetonitrile/water; 80% acetonitrile/water; and 100% acetonitrile). Fractions were checked by ^{1}H-NMR spectroscopy and those containing the NBD-conjugate were purified further using the same scheme. The NBD-chitotriose and NBD-CLOS conjugates eluted in the 20% and 50% acetonitrile/water fractions, respectively, whereas the free NBD chloride eluted with 100% acetonitrile.

NMR analyses

NMR spectra were recorded on a Varian VXR300 (300 MHz for ^{1}H, 75 MHz for ^{13}C) or VXR500 (500 MHz) spectrometer at 25°C in either deuterated chloroform, deuterated methanol, or deuterated dimethyl sulfoxide. The chemical shifts were recorded relative to an external trimethylsilane standard.

Axenic seedling bioassays

Axenic seedlings of Dutch white clover (*T. repens*) and Gemini alfalfa (*M. sativa*) were prepared by surface-sterilization and germination of seeds on Fahraeus nitrogen-free plant growth medium (NF) as previously described (18). A working stock of CLOSs and CLOS analogs were prepared by suspension in NF medium at 10^{-3} M, treated at 100°C for 30 min, and verified as sterile by plating an aliquot on BIII and trypticase soy agar. This heat treatment did not alter the biological activity of ANU843 CLOS (G. Orgambide and F. B. Dazzo, unpublished observation). Sterile NF medium was used as the diluent and untreated control throughout. Seedling assays for induction of Had were performed on plates of NF medium solidified with 0.7% agarose with 2 days incubation in the plant growth chamber (19). Axenic seedling assays for induction of Ccd were performed on similar plates incubated for 2 weeks (14), or in enclosed tubes containing the diluted samples in 10 ml of sterile NF-agarose medium and incubated for 1 month (20). Purified synthetic CLOS analogs were assayed on both legumes at 10^{-7} and 10^{-9} M, representing concentrations of CLOSs that typically elicit Had and Ccd responses on legume hosts. Roots were then removed, cleared, stained, and scored microscopically (14, 19, 20). Each treatment was replicated with 9 plants incubated under microbiologically controlled conditions.

Uptake and internalization of fluorescent NBD-conjugates

Axenic seedlings were grown for 2 days on NF agarose plates, rinsed with NF liquid medium, transferred to microscope slides, and incubated at room temperature with 10 μL of the various NBD-conjugated CLOS derivatives, each at 10^{-6} M in NF. Seedlings were then rinsed and mounted in NF medium. To evaluate the internalization process, seedlings were examined by laser scanning microscopy in the epifluorescence confocal mode, using a Zeiss 210 Laser Scanning Microscope with 488 nm laser and 520 nm longpass barrier filter. For quantitative evaluations, four seedlings per treatment were examined by conventional epifluorescence microscopy using a Zeiss Research Photomicroscope I with a HBO-50 mercury illuminator, No. 11 dichroic filter set (band pass exciter combination BP450-500, dichroic beam splitter FT510, longwave pass LP528 barrier filter) and Neofluor objective.

RESULTS

Synthesis of CLOS analogs and NBD-conjugates

N-C16:0-acylGlcN was prepared by the scheme in **Fig. 1.** The product was peracetylated for easier isolation from the reaction mixture and its structure was confirmed by ^{1}H- and ^{13}C-NMR spectroscopy (**Figs. 2AB**). The ^{1}H-NMR spectrum (Fig. 2A) contained resonances due to the methyl and methylene protons of the fatty acyl component (0.8 and 1.2 ppm, respectively), O-acetyl groups (2.0–2.2 ppm), and the carbohydrate protons (H6,H6′ at 4.0 and 4.2 ppm, H2–H5 between 4.4–6.0 ppm and H1 at 6.15 ppm). The ^{13}C-NMR spectrum (Fig. 2B) supported the proton NMR data and clearly showed that the product contained all the resonances of the six carbons of the α-anomer of the carbohydrate moiety (50–100 ppm) in addition to six less intense peaks for the β-anomer. This spectrum also contained the expected resonances arising from the methyl and methylene carbons of the fatty acid (14–36 ppm) and carbonyl groups (168–174 ppm).

N-C16:2-acylGlcN was synthesized using the reactions outlined in Fig. 1 except that palmitic acid was replaced with the C16.2$^{\Delta 2,9}$ fatty acid. The ^{1}H-NMR spectrum of the ethyl ester of this diunsaturated fatty acid prepared by the Wittig-Horner reaction showed a multiplet at 5.3 ppm due to the C-9 and C-10 olefinic protons, a doublet (J = 15 Hz) at 5.8 ppm for the C-2 vinyl proton, and a doublet of triplets (J = 7 + 15 Hz) at 6.9 ppm for the vinyl C-3 proton. The chemical shifts and splitting pattern of these four protons are consistent with a *trans*

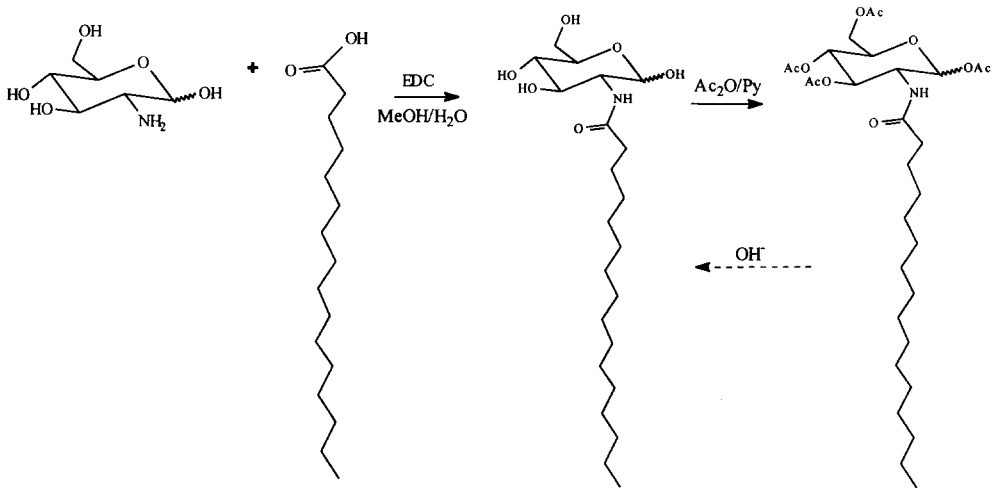

Fig. 1. Reaction scheme for synthesis of N-C16:0-acylGlcN from glucosamine and palmitic acid.

and *cis* configuration of the olefinic linkages at C-2 and C-9, respectively. The free fatty acid obtained after hydrolysis of the ethyl ester was then coupled to glucosamine as outlined earlier (Fig. 1).

N-C16:0-acylserinol was prepared by treating serinol with the methyl ester of palmitic acid. Its ^{1}H-NMR spectrum contained resonances for the fatty acid component at 0.8 (methyl protons), 1.25 (methylene protons), and 2.15 (methylene adjacent to carbonyl) ppm. In addition, there was a multiplet at 3.5 ppm arising from the methylene protons of the serinol moiety. This appeared as strong central peaks with low intensity wings characteristic of the AB component of an ABX spin system in which $\Delta\nu\nu \approx J_{AB}$. The methine proton of the serinol moiety appeared as a quintet at 3.81 ppm (J = 7 Hz). The spectrum contained minor peaks due to the tautomeric form of the amide.

Fluorescent NBD conjugates were prepared by converting CLOSs and CLOS analogs to aminoethyl glycosides and alkylating the amino groups with NBD. In this procedure, only one NBD moiety can attach per CLOS molecule, as it can only be linked at the reducing end. Verification of the desired conjugated product, after extensive purification, was established by ^{1}H-NMR spectroscopy. **Figures 3A and 3B** illustrate the scheme for synthesis of NBD-conjugates of CLOS analogs and the ^{1}H-NMR spectrum of the final purified product using the example of the NBD-N-C16:0-acylGlcN conjugate, respectively. The presence of the NBD group was indicated by the two aromatic proton signals which appear as a pair of AB doublets at 6.42 and 8.52 ppm. Also present were the resonances for the carbohydrate moiety (3–4 ppm) and the fatty acid chain (methyl at 0.9 ppm, methylene groups at 1.3 ppm, methylene group adjacent to carbonyl at 2.2 ppm). The NBD-CLOS and chitotriose conjugates were prepared by the same procedure and the presence of the AB doublet in the NMR spectrum verified the presence of the conjugated fluorochrome moiety.

Purity and identity of the synthesized CLOS analogs and its NBD-conjugates were established by a combination of TLC, reversed phase chromatography, and the above results of NMR spectroscopy. No residual unconjugated NBD was detected in any of the preparations by thin-layer chromatography analysis. Free NBD has chromatographic properties radically different from the conjugates. For instance, it cannot be eluted from a C-18 cartridge with the solvents used to purify the NBD-CLOS conjugates and runs way ahead of the conjugates on straight phase. The R_f difference between the conjugated N-C16:0-acylGlcN and the underivatized acylated glucosamine was also extremely large making it possible to obtain the conjugate without any contamination by the unlabeled molecule. The purity of the samples was also supported by the ^{1}H- and ^{13}C-NMR spectroscopy results.

Biological activities of ANU843 CLOSs and synthetic CLOS analogs

Table 1 summarizes the Had and Ccd biological activities of native CLOSs from wild type *R. leguminosarum* bv. *trifolii* ANU843 and various CLOS analogs on axenic seedlings of white clover and alfalfa. Native ANU843 CLOSs induced Had and Ccd in roots of the host legume, white clover, but not in the non-host legume, alfalfa, consistent with previous studies (5, 14). The synthetic analogs, N-C16:0-acylGlcN and N-C16:2-acylGlcN, induced weakly positive Had responses at frequencies above background (i.e., in 25–30% vs. 0–6% of the root hairs) in both legumes when assayed at 10^{-7}

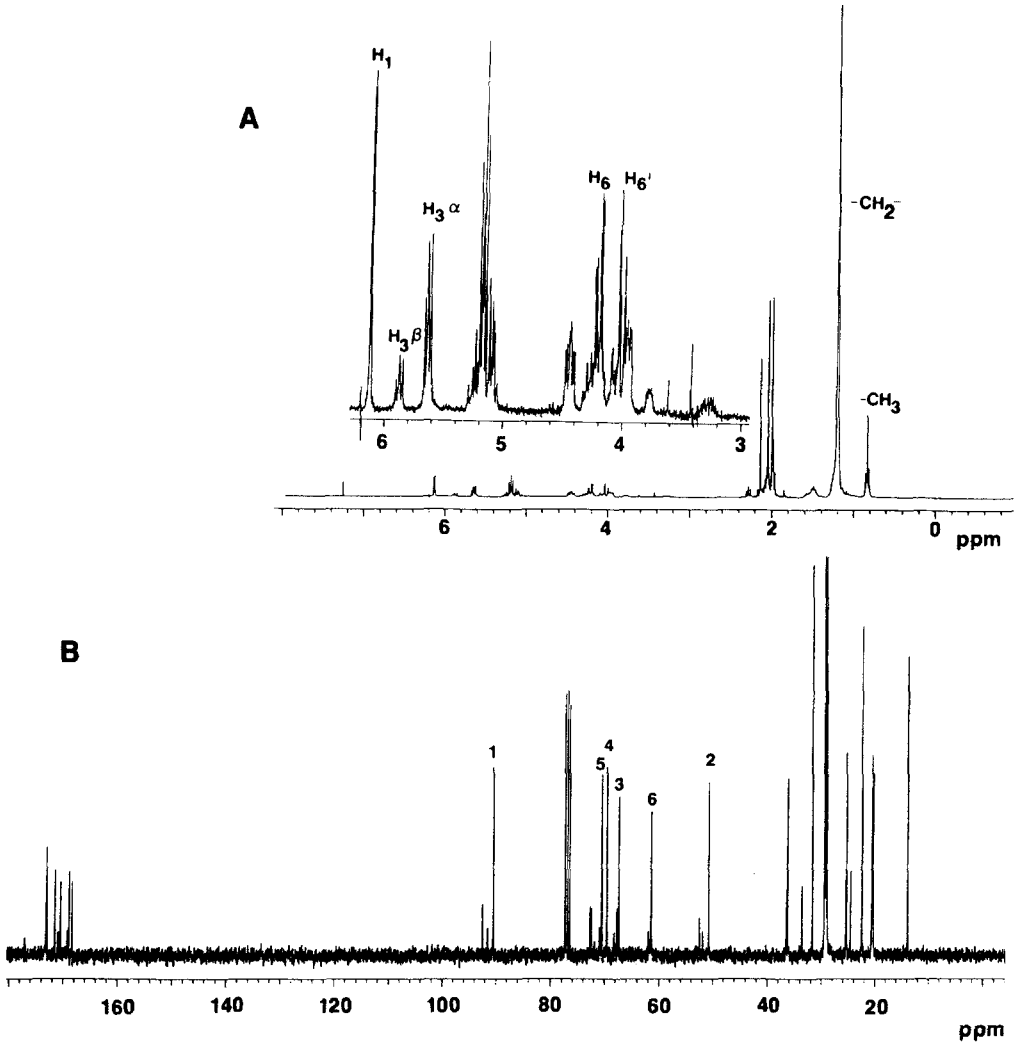

Fig. 2. (A) ^{1}H- and (B) ^{13}C-NMR spectra of N-C16:0-acylGlcN. The resonances labeled 1–6 represent the six carbon atoms of the glycosyl residue.

M. However, at 10^{-9}M, both analogs induced weakly positive Had responses only on white clover. Both synthetic analogs at 10^{-7} and 10^{-9} M induced intense Ccd responses significantly above the background frequency of spontaneous foci of Ccds on both legumes. These results indicate that sufficient structural information is provided by these two N-fattyacylglucosamine CLOS analogs to elicit Had and Ccd responses on both white clover and alfalfa, and that unsaturations in their N-fattyacyl moiety are not essential for induction of these symbiosis-related responses on these legumes. Further comparison of the frequency of these plant responses to the two CLOS analogs indicated that these unsaturations in the N-fattyacyl moiety enhanced the Ccd (but not Had) induction activity on alfalfa (as indicated at 10^{-9} M), but were without effect on either Had or Ccd activities on white clover. Hence, although not required, unsaturation of the N-fattyacyl moiety did influence the intensity of host Ccd responses to these CLOS analogs in certain legumes. In contrast to the above results, neither chitotriose nor the synthetic analog N-C16:0-acylserinol induced Had or Ccd responses on either plant in these axenic bioassays.

The morphological responses of root hairs and the root cortex to the synthetic N-acylGlcN CLOS analogs differed somewhat on the two legumes tested. Whereas native CLOSs of homologous rhizobia most commonly induced branching of alfalfa root hairs (21) and irregular distortions of white clover root hairs (14), the most common root hair deformations induced by the N-fattyacylglucosamine analogs of CLOS were corkscrews on alfalfa and balloon tip swellings on clover. All of these

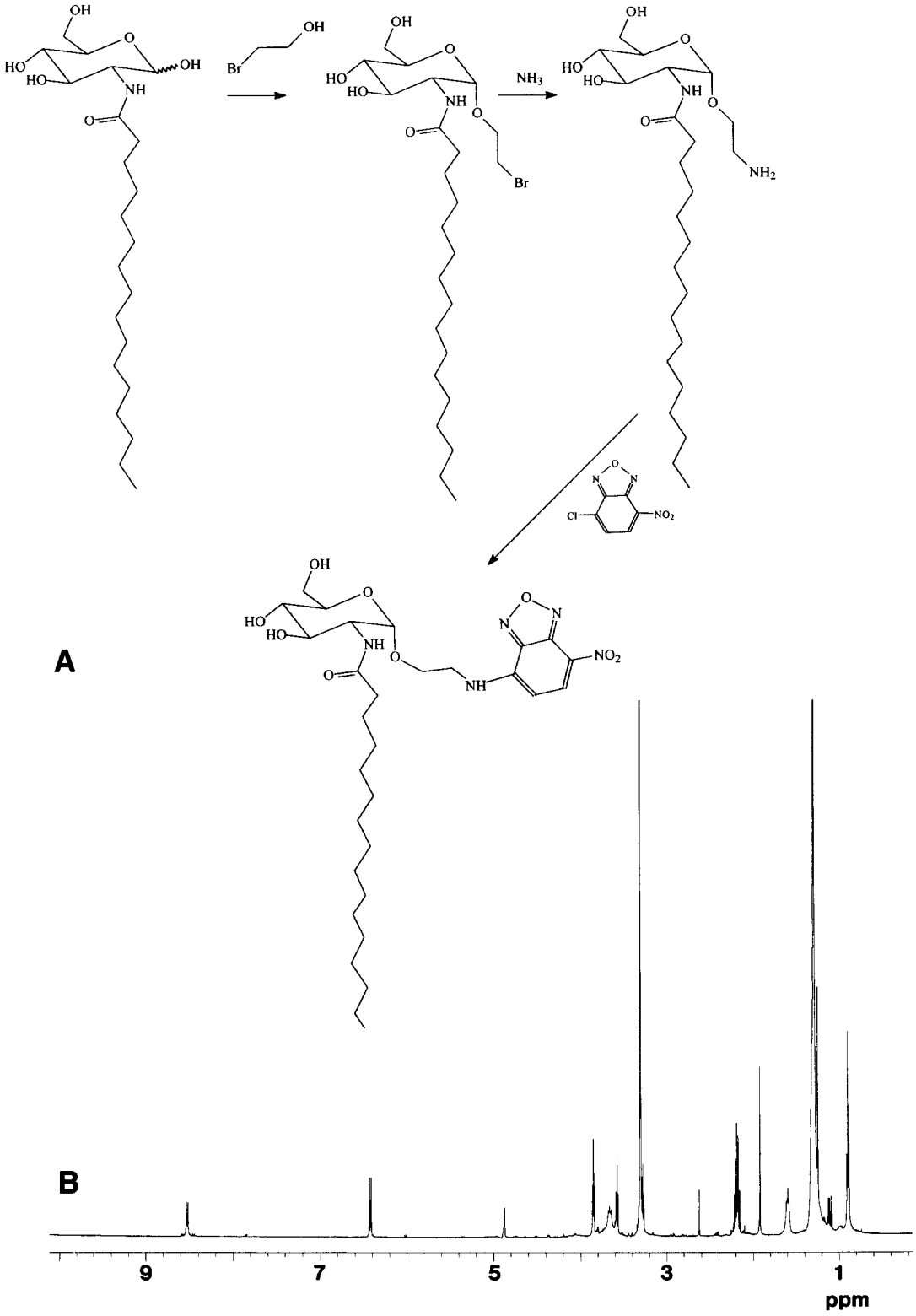

Fig. 3. (A) Reaction scheme for synthesis of the NBD-N-C16:0-acylGlcN conjugate and (B) ^{1}H-NMR spectrum of NBD-N-C16:0-acylGlcN conjugate. The doublets at 6.4 and 8.5 ppm are due to the aromatic protons of the NBD group. The narrow doublet at ~4.95 ppm is from the anomeric proton. The signals from the linker group and the carbohydrate ring appear between 3.2 and 4 ppm. Signals for the methylene groups adjacent to the carbonyl function appear at 2.2 ppm and those for the bulk methylene groups of the alkyl chain are at 1.3 ppm. The methyl group signals appear at 0.9 ppm.

TABLE 1. Induction of root hair deformation (Had) and foci of cortical cell divisions (Ccd) responses on Dutch white clover and Gemini alfalfa by native CLOSs and NBD-conjugates of CLOSs from wild type *R. leguminosarum* bv. *trifolii* ANU843, and by synthetic CLOS analogs

Test Plant	Treatment	Conc. (M)	Had Response	Ccd Response
Clover	NF Control	0	0.25 ± 0.17	0.20 ± 0.20
Clover	CLOSs	5×10^{-7}	3.39 ± 0.13	6.00 ± 0.70
Clover	CLOSs	5×10^{-9}	2.00 ± 0.19	3.40 ± 0.30
Clover	NBD-CLOSs	5×10^{-7}	3.39 ± 0.20	4.50 ± 0.40
Clover	NBD-CLOSs	5×10^{-9}	2.28 ± 0.19	5.30 ± 0.70
Clover	CLOSs	10^{-7}	2.20 ± 0.30	7.30 ± 0.90
Clover	CLOSs	10^{-9}	2.40 ± 0.20	5.70 ± 0.60
Clover	N-C16:0-acylGlcN	10^{-7}	1.33 ± 0.28	8.75 ± 1.60
Clover	N-C16:0-acylGlcN	10^{-9}	1.06 ± 0.26	6.25 ± 1.01
Clover	N-C16:2-acylGlcN	10^{-7}	0.94 ± 0.11	7.00 ± 1.61
Clover	N-C16:2-acylGlcN	10^{-9}	0.89 ± 0.16	7.63 ± 1.89
Clover	N-C16:0-acylserinol	10^{-7}	0.17 ± 0.17	0.14 ± 0.14
Clover	N-C16:0-acylserinol	10^{-9}	0.00 ± 0.00	0.17 ± 0.17
Clover	Chitotriose	10^{-7}	0.11 ± 0.07	0.33 ± 0.17
Clover	Chitotriose	10^{-9}	0.17 ± 0.08	0.22 ± 0.15
Alfalfa	NF Control	0	0.00 ± 0.00	1.33 ± 0.33
Alfalfa	CLOSs	10^{-7}	0.10 ± 0.10	1.20 ± 0.30
Alfalfa	CLOSs	10^{-9}	0.00 ± 0.00	0.80 ± 0.30
Alfalfa	N-C16:0-acylGlcN	10^{-7}	1.11 ± 0.23	9.71 ± 2.82
Alfalfa	N-C16:0-acylGlcN	10^{-9}	0.11 ± 0.07	4.88 ± 0.88
Alfalfa	N-C16:2-acylGlcN	10^{-7}	1.28 ± 0.22	7.38 ± 1.40
Alfalfa	N-C16:2-acylGlcN	10^{-9}	0.11 ± 0.07	9.13 ± 1.72
Alfalfa	N-C16:0-acylserinol	10^{-7}	0.00 ± 0.00	0.11 ± 0.11
Alfalfa	N-C16:0-acylserinol	10^{-9}	0.00 ± 0.00	0.13 ± 0.13
Alfalfa	Chitotriose	10^{-7}	0.11 ± 0.07	0.11 ± 0.11
Alfalfa	Chitotriose	10^{-9}	0.11 ± 0.07	0.25 ± 0.16

Values reported are the means ±SE for 9 plant replicates per treatment in axenic seedling bioassays.

types of root hair deformations are induced on the host legume by the homologous rhizobial symbiont. Various morphologies characterizing the foci of cortical cell divisions were recognized (compare **Figure 4A** control with Figs. 4B–F). These included a highly localized region of Ccd represented by just a few cells (Fig. 4B), a more extensively involved region spanning a wider distance along the root without a discrete meristem (Fig. 4C), and a discrete nodule-like primordium within the root cortex (Figs. 4D and 4F). Some foci of Ccd developed into emerged nodules on axenic alfalfa roots incubated with these CLOS analogs (Fig 4E) but not in the untreated controls. In contrast, the Ccd response in clover developed up to the stage of a nodule primordium (Fig. 4F), but none fully emerged from the root.

Uptake and internalization of NBD-CLOS conjugates in root cells

NBD-conjugates of ANU843 CLOSs retained the ability to induce both root hair deformations and foci of cortical cell divisions in white clover roots (Table 1), providing evidence that the NBD-fluorophore itself did not impose a confounding effect on the biology of the system. Laser scanning confocal microscopy of white clover roots after short-term incubation with the NBD-CLOS conjugate showed rapid uptake of these fluorescent molecular probes into root hairs and other epidermal cells (Fig. 4G). More detailed examinations of roots after brief incubation with the fluorescent conjugate provided evidence that CLOSs rapidly traversed the root hair wall, were inserted within the plasma membrane, and were internalized with a portion remaining within the nucleus and other portions migrating to the base of the root hair and disseminating into underlying cortical cells (Figs. 4H–K). Root hairs on roots exposed to the NBD-CLOS conjugate for 24 h were deformed and still fluorescent (Fig. 4L). These same fluorescence optics revealed no autofluorescence of root hairs on untreated plants or plants treated with unconjugated ANU843 CLOSs.

To further investigate internalization of the fluorescent conjugate within the intact clover root, we examined longitudinal optisections that transect the epidermal cell layer and several underlying cell layers within the root cortex, above the pericycle. Figures 4M–N are typical confocal optisections that sample the cortex at different depths below the root surface. This procedure detected fluorescence within some epidermal cells, at the periphery of other epidermal cells, and also within some underlying cortical cells. The root cortex of white clover consists of 4–5 subepidermal cell layers, and evidence for uptake of the fluorescent conjugate into in-

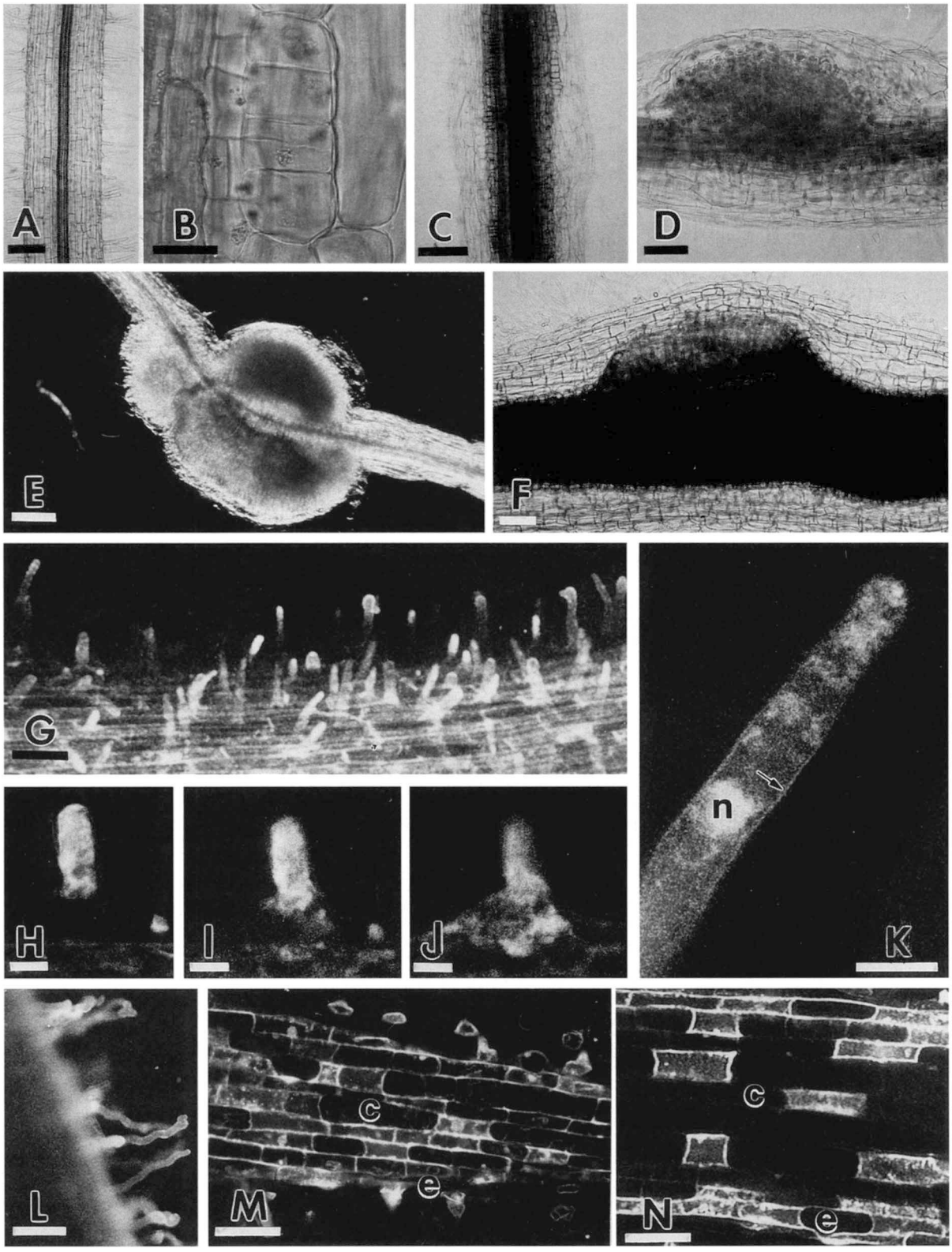

Fig. 4. (A–F) Induction of mitogenic responses in the cortex of legume roots by the synthetic CLOS analog, N-C16:2-acylglucosamine. (A) Untreated alfalfa control (same as white clover control). (B–E) alfalfa and (F) white clover treated with N-C16:2-acylGlcN. (G–N) Laser scanning confocal fluorescence micrographs of white clover roots after incubation with NBD-labeled CLOSs from *R. leguminosarum* bv. *trifolii* ANU843. (G) General view of the root after 1 h incubation. (H–K) Real-time series longitudinal optisections of a root hair after incubation for 15 min (H), 20 min (I), 25 min (J), and 60 min (K); n, nucleus; arrowhead, protoplast membrane. (L) Fluorescent deformed root hairs (24 h). (M–N) Fluorescent epidermal (e) and cortical (c) cells (1 h). Control roots were not fluorescent when incubated with unlabeled CLOSs. Bar scales are 125 μm for A; 25 μm for B; 200 μm for C and E; 100 μm for D, F, G and L; and 10 μm for H–K.

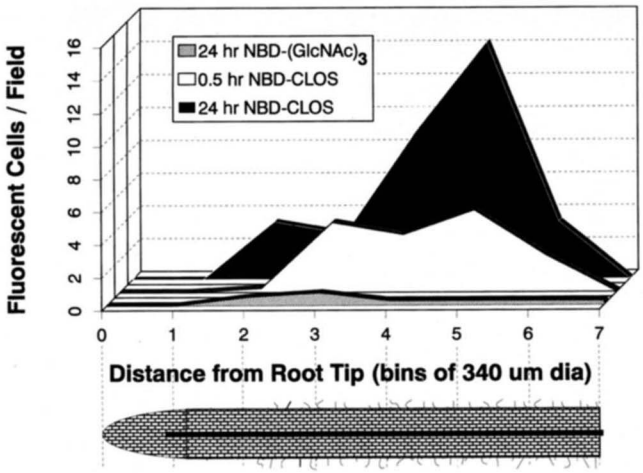

Fig. 5. Location and distribution of white clover root cortical cells that have internalized NBD-conjugates of ANU843 CLOSs and chitotriose relative to the seedling root tip. Periods of incubation with the NBD-conjugates are indicated.

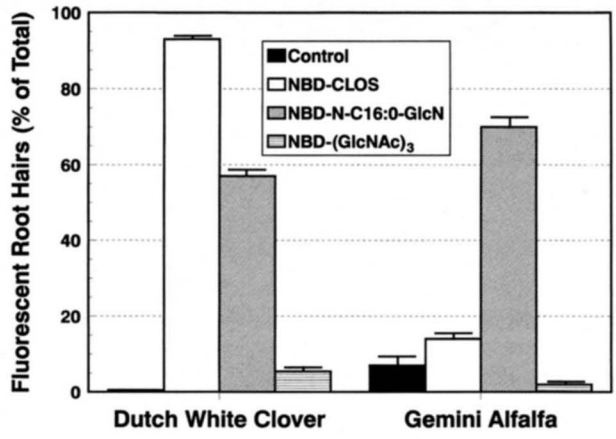

Fig. 6. Proportion of root hairs that internalize NBD-conjugates of ANU843 CLOSs and CLOS analogs after 1 h incubation.

terspersed cells within four cell layers of the root cortex was found (Fig. 4M–N). Examination along the entire length of intact roots after 30 min incubation with the NBD-CLOS conjugate indicated that this molecular probe had entered cortical cells located primarily beneath the intact root hair region (**Fig. 5**). Strikingly, after more extended incubation, most fluorescent cortical cells were located in a zone within the root hair region between 1.4 and 2.0 mm from the root tip (Fig. 5).

To further evaluate the uptake of the NBD-CLOS conjugate into plant cells, we determined the proportion of fluorescent root hairs along the optical median plane of roots after 1 h incubation with this molecular probe. This quantitative assay provided evidence that ANU843 CLOSs were internalized by a significantly higher proportion of root hairs of the compatible host white clover than of the incompatible non-host legume, alfalfa (**Fig. 6**). In contrast to the responses of the white clover host, ANU843 CLOSs did not induce root hair deformations or foci of cortical cell divisions in the non-host legume alfalfa (Table 1). Thus, these two legumes differed significantly in ability to internalize ANU843 CLOSs, and a link between uptake and induction of symbiosis-related responses by wild-type rhizobial CLOSs was indicated.

We also used NBD-conjugates to assess the structural requirements for uptake of CLOS analogs into root cells of white clover and alfalfa. Root hairs and underlying cortical cells exhibited only background marginal fluorescence after roots of both legume plants were incubated for even extended periods with the NBD-conjugate of the CLOS analog, chitotriose (lacks the N-acyl fatty acid) (Figs. 5 and 6). Interestingly, 50–70% of the root hairs of both white clover and alfalfa were fluorescent after 1 h incubation with the NBD conjugate of N-C16:0-acylGlcN. Fewer white clover root hairs displayed internal fluorescence after incubation with NBD-N-C16:0-acylGlcN than with NBD-CLOSs (Fig. 6). This was likely due to lower availability of labeled analog because of solubility problems of the N-C16:0-acylGlcN conjugate in the highly aqueous medium in which the experiments were carried out.

DISCUSSION

Recent studies with synthetic CLOSs clearly indicate a degree of structural flexibility in functionalization of CLOS oligomers displaying nodule initiating activity in the legume *Glycine soja* (11). Therefore, if induction of biological activity is mediated through a specific receptor pathway, then there would have to be several different Nod signal perception systems operative in the same plant, rather than just one. In our study, we have synthesized smaller analogs of CLOSs to address whether the oligomerization of the carbohydrate chain, the functionalization (e. g., O-acetylation, sulfation), and the fatty acyl unsaturations of CLOSs applied ex planta are necessary for elicitation of host-selective symbiosis-related responses in the two test legumes, white clover and alfalfa. Our results indicate that N-fattyacylglucosamine is the smallest sub-structure of CLOSs that is both necessary and sufficient to elicit Had and Ccd responses on both test plants, without an absolute requirement of oligomerization or functionalization of the polar head group, or unsaturation of the hydrophobic N-fattyacyl tail. Because the CLOS analogs N-C16:0-acylGlcN and

N-C16:2-acylGlcN (unlike the native CLOSs from the corresponding homologous rhizobia) were active on both hosts, oligomerization and functionalization of the polar head group of CLOSs are likely to influence the range and extent to which plants respond to CLOSs. Although fully supported by the data, this interpretation opposes the conventional model that these structural modifications of the polar head group of CLOSs act as positive effectors of host receptor recognition necessary for activation of host responses. We further modified native wild-type ANU843 CLOSs and various analogs by introducing the fluorochrome NBD at the reducing end. Bioassays indicated that this modification of ANU843 CLOSs did not alter their cognate biological activities. These fluorescent molecular probes were used to investigate the temporal/spatial relationships and structural requirements for intracellular uptake of CLOSs in situ during early interactions with active host root cells. These studies showed that only the probes with structures enabling internalization within root hairs were able to elicit Had and Ccd activities on these two legume hosts, indicating strong evidence for a link between their intracellular uptake and symbiotic biological activities.

We envision three possible mechanisms by which CLOSs could be taken up by legume root hairs. One is by a specific receptor-mediated event that relies on specific recognition of the unique structural features of each active CLOS glycolipid by homologous plant cell surface receptor molecules. The second is by a general receptor system that recognizes some common substructure of CLOSs, with further events being potentiated by specific modifications on the individual CLOS molecules. Recent in vitro studies supporting both of these proposed mechanisms suggest that indeed legume roots do have multiple, constituitive binding sites varying in affinity for CLOS molecules (10, 22, 23). The third is by a diffusive route that relies on partitioning of the amphiphilic CLOS glycolipids into the plant cell through its protoplast membrane without the involvement of a specific receptor molecule. The degree to which this mechanism would contribute to uptake of different CLOS glycolipids would be modulated on the bacterial side by a combination of their gross features such as relative abundance, molecular size, charge, and hydrophobicity/hydrophilicity balance, and on the plant side by the lateral fluid mobility of the host membrane controlled by the diversity and relative abundance of its lipid components. The ability of the synthetic CLOS analog N-C16:0-acylGlcN to be taken up and elicit Had and Ccd responses on both tested legumes does not support the first mechanism proposed above, as this active analog lacks the structural features of both the polar head group and hydrophobic tail that distinguish the families of CLOSs made by wild type *R. leguminosarum* bv. *trifolii* and *R. meliloti* (5, 6, 24). On the other hand, all of the results of this study (especially the unresponsiveness of either host to the synthetic CLOS analog N-C16:0-acylserinol) are consistent with the second general mechanism in which a putative host receptor recognizes a fatty N-acylated glucosamine as the minimum common substructure of CLOSs sufficient to trigger uptake and Had/Ccd responses on white clover and alfalfa. These same data are also fully consistent with the third proposed mechanism which relies on a critical hydrophilic/hydrophobic balance for uptake of the amphiphilic effector molecule via its spontaneous fusion into the lipid bilayer cell membrane, followed by lateral fluid mobility of the effector molecule through the endomembrane system into the host cell. In this case, the oligosaccharide chain and its functionalizations could possibly constitute significant barriers to uptake of the effector molecule. This third model would explain the clear distinction between the apparent **host-selective** uptake of native ANU843 CLOSs and the lack thereof for the N-fattyacylglucosamine analogs in the two test plants as a "leveling" effect of removing the oligosaccharide chains and their functionalizations. The structure of cell surface components, e.g., extracellular slime, cell wall, membrane composition of lipids and proteins, would be expected to differ somewhat in these two different test plants, and therefore the extent to which these structural features of the polar head group constitute a diffusion barrier to uptake of CLOSs would differ. It is obvious that a small hydrophobic molecule such as the N-acylmonoglycosyl analogs that do not interact with these barriers would pass through without entrainment but more polar molecules could have a problem breaching them, being either attracted and sequestered by the matrix if they have the opposite charge or repelled away if they have the same charge. Non-polar molecules are not influenced by such barriers. We propose that a common minimum structure is required and sufficient for uptake and biological activity, and that further expression of its inherent ability to elicit these host responses is modulated by the various specific CLOS modifications. Modifications that mask this minimum required structure might lead to reduction of uptake and/or loss of activity, hence explaining the unresponsiveness of the non-host legume to CLOSs from heterologous rhizobia. The extent that incorporation of CLOSs may change or perturb host membrane structure will differ among plant species. Therefore, the intensity of responses to such molecules is likely to vary among different hosts, thus appearing to exhibit some degree of plant selectivity. The change or extent of perturbation of host membrane structure resulting from incorporation of CLOSs that trigger cellular processes

would also be expected to differ between plant species for the same reasons cited above.

The retention of symbiosis-related biological activities of NBD-CLOS conjugates and their stable fluorescence properties make these fluorescent molecular probes ideal for studies of *Rhizobium*–host root hair interactions at the cellular and molecular level. Localization experiments using these probes indicated that the protoplast membrane (rather than the cell wall) of the root hair was the first resting place for ANU843 CLOSs applied to clover roots. This was predicted from earlier studies on the preferred accumulation of CLOSs in rhizobial membranes (5). Partitioning into membrane would be favored by a high hydrophobicity/hydrophilicity balance and would definitely be facilitated by the hydrophobic chain. Hence (as was observed) the labeled chitotriose should not be internalized significantly by plant cells. This lack of intracellular or intercellular fluorescence within roots incubated with NBD-chitotriose also served as an important negative control that addressed several potential problems in our use of NBD-conjugated fluorescent molecular probes to study the internalization of CLOSs into roots. First, it ruled out an ability of the NBD moiety itself to potentiate uptake of GlcN-containing glycoconjugates that are otherwise excluded from root hairs or for the tag itself to create an artifact interaction with host components, implying that the interactions of the bioactive CLOS derivatives with the test plants do exhibit some specificity. A second issue addressed by this control was the extent to which excreted plant chitinases (14, 25, 26) may have partially depolymerized the NBD-conjugated native ANU843 CLOSs into NBD-$GlcN_x$ + N-acyl$GlcN_x$ fragments that might be internalized. The very low level of intracellular fluorescence (hence NBD) in clover roots even after 24 h incubation with NBD-chitotriose suggests that this possible source of error in interpretation of the fluorescence localization results was minimal to nonexistent. The alternate possibility that coupling of NBD to CLOSs may even hinder their degradation would increase their usefulness as fluorescent molecular probes to follow internalization of CLOSs. This remains to be tested. Finally, the result of this negative control ruled out passive entry of the fluorescent probes into the root by simple diffusion through small open crevices in the root system.

Our studies using NBD-conjugates of wild type ANU843 CLOSs also revealed that these molecular probes rapidly communicate with the nucleus of intact clover root hairs and are also internalized within this organelle. Detection of this novel result was made possible through the use of laser scanning microscopy in the epifluorescence confocal mode. Of major importance here is the ability of this microscopical imaging tool to immediately acquire, image process, and serially reconstruct digital optisections in order to examine the in situ distribution of the fluorescent conjugates at subcellular resolution within host cells that are still physiologically active. Further use of these powerful tools will undoubtedly help to further elucidate the role of these bioactive rhizobial glycolipids during early cell–cell interactions as a prelude to development of the nitrogen-fixing *Rhizobium*–legume symbiosis.

This work was supported by the Department of Energy Grant DE-FG02-89ER14029 (to R.I.H.), United States Department of Agriculture NRICGP Grant 94-03537 (to F.B.D. and R.I.H.), and by the Michigan Agricultural Experiment Station. The NMR data were obtained on instrumentation purchased in part with funds from National Institutes of Health 1-S10-RR04750, National Science Foundation (NSF) Grant CHE-88-00770, and NSF Grant CHE-92-13241. We thank J. Whallon for technical assistance.

Manuscript received 3 October 1996 and in revised form 3 March 1997.

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