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Flavone-Enhanced Accumulation and Symbiosis-Related Biological Activity of a Diglycosyl Diacylglycerol Membrane Glycolipid from *Rhizobium leguminosarum* Biovar trifolii

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Rhizobium leguminosarum **bv. trifolii is the bacterial symbiont which induces nitrogen-fixing root nodules on the leguminous host, white clover (*Trifolium repens* L.). In this plant-microbe interaction, the host plant excretes a flavone, 4′,7-dihydroxyflavone (DHF), which activates expression of nodulation genes, enabling the bacterial symbiont to elicit various symbiosis-related morphological changes in its roots. We have investigated the accumulation of a diglycosyl diacylglycerol (BF-7) in wild-type *R. leguminosarum* bv. trifolii ANU843 when grown with DHF and the biological activities of this glycolipid bacterial factor on host and nonhost legumes. In vivo labeling studies indicated that wild-type ANU843 cells accumulate BF-7 in response to DHF, and this flavone-enhanced alteration in membrane glycolipid composition was suppressed in isogenic *nodA*::Tn*5* and *nodD*::Tn*5* mutant derivatives. Seedling bioassays performed under microbiologically controlled conditions indicated that subnanomolar concentrations of purified BF-7 elicit various symbiosis-related morphological responses on white clover roots, including thick short roots, root hair deformation, and foci of cortical cell divisions. Roots of the nonhost legumes alfalfa and vetch were much less responsive to BF-7 at these low concentrations. A structurally distinct diglycosyl diacylglycerol did not induce these responses on white clover, indicating structural constraints in the biological activity of BF-7 on this legume host. In bioassays using aminoethoxyvinylglycine to suppress plant production of ethylene, BF-7 elicited a meristematic rather than collaroid type of mitogenic response in the root cortex of white clover. These results indicate an involvement of flavone-activated *nod* expression in membrane accumulation of BF-7 and a potent ability of this diglycosyl diacylglycerol glycolipid to perform as a bacterial factor enabling *R. leguminosarum* bv. trifolii to activate segments of its host's symbiotic program during early development of the root nodule symbiosis.**

At early stages in development of the *Rhizobium*-legume symbiosis, the bacterial symbiont expresses several nodulation (*nod*) genes located on its symbiotic plasmid (pSym) (12, 27). The action of certain pSym *nod* genes is correlated with the production of specific molecules capable of acting as signals to elicit various symbiosis-related morphological responses in the plant root, including root hair deformations (Had), induction of foci of cortical cell divisions (Ccd), and a thick, short root (Tsr) (reviewed in references 2, 3, and 16). The functions of Nod proteins are necessary for bacterium-plant signaling that enables rhizobia to successfully infect and nodulate the host legume, leading to establishment of a nitrogen-fixing symbiosis (10, 48).

Recent studies have described a family of *Rhizobium nod*-dependent chitolipooligosaccharides, first reported by Lerouge et al. (26), that are capable of eliciting Had and Ccd responses on legume roots (5, 40, 44, 49, 51). These molecules are reported as excreted metabolites. However, from the viewpoints of membrane biophysics and microbial physiology, it is more likely that these glycolipids reside primarily in cell membranes and are shed in small quantities into external environments as the bacteria grow and divide. This fate of membrane glycolipids has already been documented with *Rhizobium* lipopolysaccharides, which are released from the bacteria into the external root environment and modulate primary infection of host root hairs (9). In general, the physiological process leading to extracellular accumulation of lipopolysaccharides in gram-negative bacteria involves shedding of excess outer membrane micelles at the division site (new cell poles) prior to daughter cell separation. The large degree of heterogeneity in fatty acid chain length and degree of unsaturation in lipooligosaccharide Nod metabolites (44, 49) also suggests that these molecules might be membrane components. As is the case for other amphiphilic molecules, the very polar head group and the long hydrophobic alkyl tail of lipooligosaccharides would force them to form aggregates and micelles in aqueous environments which will fuse with rather than easily be transported across any similar membranous system. In support of the proposal that rhizobial membranes might accumulate *nod*-dependent metabolites, recent studies have reported that approximately 70% of the *nod*-dependent labeled lipid originating from [^{14}C]acetate is butanol extractable from cells grown with *nod*-inducing flavones (29).

This reasoning led us to investigate membrane-associated lipophilic glycoconjugates whose accumulation is affected by *nod* gene expression. In a recent study (33), we isolated a novel membrane glycolipid (BF-7) from the bacterial symbiont of white clover, *Rhizobium leguminosarum* bv. trifolii wild-type strain ANU843, and characterized it as 1,2-di-*O*-acyl-3-*O*-[α-D-glucopyranosyl-(1→3)-*O*-α-D-mannopyranosyl)-glycerol, with iso-hexadecanoic and anteiso-heptadecanoic acids as the major fatty acids esterifying the glycerol moiety (Fig. 1A). BF-7

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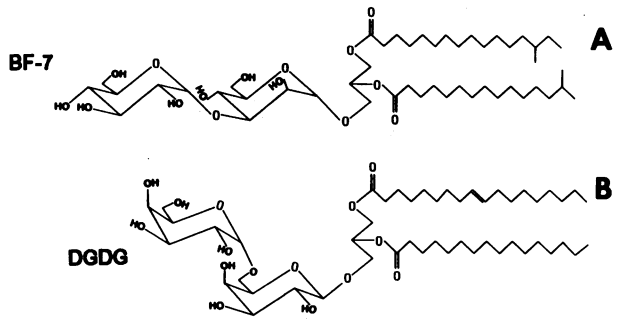

FIG. 1. Diglycosyl diacylglycerols used in this study. (A) The diglycosyl diacylglycerol BF-7 isolated from cells of wild-type *R. leguminosarum* bv. trifolii ANU843 [1,2-diacyl-3-*O*-(α-D-glucopyranosyl-(1→3)-*O*-α-D-mannopyranosyl)-glycerol]. (B) DGDG from wheat [1,2-diacyl-3-*O*-(α-D-galactopyranosyl-(1→6)-*O*-β-D-galactopyranosyl)-D-glycerol].

is a member of a family of diverse glycosyl diacylglycerols which are widespread membrane glycolipids of plants (22, 23, 43), animals (30, 47), and gram-positive bacteria (22, 24, 43), but their occurrence in gram-negative bacteria has been reported for only a few species of the genera *Pseudomonas*, *Bacteroides*, *Spirochaetes*, and *Mycoplasma* and photosynthetic bacteria (22, 24). In this study, we report on the results of the screen which led to identification of BF-7 as a glycolipid whose accumulation in *R. leguminosarum* bv. trifolii ANU843 is influenced by the *nod*-activating flavone, 4',7-dihydroxyflavone (DHF), as well as Tn*5* inactivation of *nodA* and the positive regulatory gene *nodD*, and on the ability of this glycolipid to elicit symbiosis-related morphological responses on host and nonhost legume roots. Preliminary reports of this work have been presented elsewhere (7, 31, 32).

MATERIALS AND METHODS

Bacterial strains and cultures. Strains of *R. leguminosarum* bv. trifolii used in this study included wild-type ANU843, a *nodA*::Tn*5* mutant derivative ANU252, and a *nodD*::Tn*5* mutant derivative ANU851 (11, 42), each obtained from B. Rolfe (Australian National University, Canberra, Australia). Batch cultures were grown at 30°C in defined BIII medium (6) (with 30 μg of kanamycin per ml for strains ANU252 and ANU851), with or without 4 μM DHF to activate pSym *nod* gene expression (41). Studies using *lacZ* fusions indicate that expression of pSym *nodA* in ANU843 was enhanced at least 10-fold in BIII medium when supplemented with 4 μM DHF (36). Exponentially growing cells at a population density of 8×10^8 cells per ml were harvested by centrifugation before cell lysis occurred, i.e., prior to detection of the cytoplasmic enzyme marker NADP-dependent glucose-6-phosphate dehydrogenase (19) in the culture supernatant.

Influence of DHF and a Tn*5* insertion in *nodA* on the accumulation of BF-7 in ANU843. Lyophilized cells (100 mg) were extracted with chloroform-methanol (1:1) as previously described (33). The extracts were fractionated on a silica gel column (Silicar; Mallinckrodt), using stepwise elutions with 5, 10, 15, 20, 30, and 50% methanol in chloroform. Equivalent fractions obtained from cell extracts of ANU843 (with or without DHF) and ANU252*nodA*::Tn*5* (with DHF) were analyzed next to a purified sample of BF-7 by silica thin-layer chromatography (TLC) with a chloroform-methanol-ammonium hydroxide (4:2:0.5) solvent. The carbohydrate-containing components were located by spraying the plate with a methanol-sulfuric acid-orcinol (75:25:0.5) reagent and heating at 120°C.

In vivo labeling of BF-7. Cultures were grown in 10 ml of BIII medium modified by substituting mannitol with glucose (2 g/liter) as the carbon source with or without 4 μM DHF and with kanamycin (30 μg/ml) for strain ANU252. Cultures were continuously shaken and harvested at a density of 2×10^8 cells per ml by centrifugation under sterile conditions. Each pellet was resuspended in 4 ml of fresh sterile culture medium except that unlabeled glucose was replaced with 50 μCi of uniformly labeled [^{14}C]glucose as the carbon source. Cells were further incubated for 4.5 h at 30°C and were then centrifuged and lyophilized. Dried cells were extracted with 3 ml of chloroform-methanol (1:1) for 7 h at 60°C, and the cell extracts were filtered through 0.22-μm-pore-size Millipore GVWP04700 membranes. Filtrates were dried under nitrogen, redissolved in chloroform-methanol (95:5), and fractionated through silica Sep-Pak cartridges, using stepwise elutions of 5, 10, 15, and 20% methanol in chloroform, and eluted with pure methanol. Equivalent fractions from the three in vivo-labeled cultures (ANU843 with DHF, ANU843 without DHF, and ANU252 with DHF) were spotted on reverse-phase C18 TLC plates next to a purified sample of BF-7 and chromatographed with methanol-chloroform-water (4:3:1) as the solvent. The TLC plate was then analyzed by autoradiography using Kodak X-Omat AR5 film. The chromatographic position of ^{14}C-labeled BF-7 on the TLC plate was located by comparing the labeled spots of an overlay of the X-ray autoradiogram to the R_f of the pure BF-7 sample (recognized by cutting off the lane of unlabeled BF-7 and staining it with orcinol). The radioactive spot matching the R_f of BF-7 was scraped off the TLC plate, eluted from the solid phase by chloroform-methanol (2:1), and counted for radioactivity in a scintillation counter, with correction for background activity within the same lane.

Influence of the positive regulatory gene, *nodD*, on accumulation of BF-7 in ANU843. Lyophilized cells (1 g) of ANU843 and ANU851 *nodD*::Tn*5* (each grown with 4 μM DHF) were extracted with 400 ml of chloroform–methanol–*n*-butanol–water (4:2:1:1) with constant stirring for 16 h at 30°C and then centrifuged. The lower chloroform layer was collected, filtered through a 0.22-μm-pore-size Millipore GVWP04700 membrane, and dried by evaporation under partial vacuum. The residues were dissolved in 5% methanol in chloroform, fractionated on a silica gel column, and analyzed by silica TLC, using the solvent system chloroform-methanol-ammonium hydroxide (8:2:1).

Axenic seedling bioassays. Purified BF-7 was dried, resuspended in nitrogen-free (NF) plant growth medium (13), and sterilized by autoclaving at 121°C for 10 min. Sterility and stability of the autoclaved sample were verified by plating aliquots on BIII medium and Trypticase soy agar (Difco) and by adsorption and reverse-phase TLC in comparison with an unautoclaved sample. Quantitative assays for Had and Ccd were performed on white clover (*Trifolium repens* L. cv. Dutch), alfalfa (*Medicago sativa* cv. Gemini), and hairy vetch (*Viciae hirsuta*) seedlings under microbiologically controlled conditions in a plant growth chamber as described earlier (17, 39). Evaluations of the Tsr response were made by stereomicroscopy of root thickness and quantitation of root length by image analysis (8). Sterile NF medium was used as the diluent and untreated control throughout.

Variations of the standard bioassay included addition of filter-sterilized aminoethoxyvinylglycine (AVG) solution (Sigma Chemical Co., St. Louis, Mo.) to NF medium, examination of cortical cell divisions by laser scanning microscopy (633-nm neon laser, transmitted brightfield with edge enhancement

digital image processing) of cleared roots, and by combined brightfield microscopy and transmission electron microscopy of thin cross sections of fixed and Epon-embedded roots (6). The standard of wheat digalactosyl diacylglycerol (DGDG) (Fig. 1B) was obtained from Serdary Research Laboratories Inc. (London, Ontario, Canada).

RESULTS AND DISCUSSION

Screen for *nod*-influenced lipophilic glycoconjugates in wild-type *R. leguminosarum* bv. trifolii ANU843. Extracts of cells grown with or without DHF were fractionated by adsorption chromatography, and fractions were analyzed by silica TLC. Target compounds of interest had the four following characteristics: (i) extracted with an organic solvent system known to favor bacterial glycolipids (45), (ii) detected with orcinol as a carbohydrate stain, (iii) increased when wild-type cells are grown with DHF, and (iv) reduced in the isogenic ANU252 *nodA*::Tn*5* mutant derivative. The latter criterion was included since expression of *nodA*, a rhizobial common *nod* gene, is enhanced in response to specific flavonoid compounds and Tn*5* insertions in this gene block rhizobial induction of Had, Tsr, and Noi phenotypes on host legumes (12, 54). Interestingly, several orcinol-positive components in different fractions from the silica column chromatography were shown by TLC to fulfill the criteria specified above. Of these, the most prominent component (BF-7) eluted from the column in the 10% methanol in the chloroform fraction. Figure 2A shows the TLC pattern of this fraction obtained from wild-type ANU843 grown with and without DHF and from the *nodA*::Tn*5* mutant grown identically with DHF. The orcinol-positive spot at the R_f position of BF-7 was detected only in the fraction from ANU843 grown with DHF.

To verify and quantitate these results, we modified the experiment by using cells grown with [^{14}C]glucose prior to extraction, fractionation, and TLC analysis of the same lipophilic fractions. Autoradiography of the TLC plate, followed by scintillation counting of an extract from the TLC matrix at the R_f position of BF-7, indicated that accumulation of in vivo ^{14}C-labeled BF-7 increased in ANU843 by more than threefold when grown with DHF (Fig. 2B). In contrast, the radioactive level of ^{14}C-labeled product extracted from the TLC matrix at the R_f position of BF-7 in the corresponding sample from the *nodA*::Tn*5* mutant was approximately 25-fold lower than the level of [^{14}C]BF-7 obtained from an equivalent amount of ANU843 cells grown identically with DHF (Fig. 2B). These results indicate that the minor membrane glycolipid BF-7 increased in cells of *R. leguminosarum* bv. trifolii ANU843 in response to DHF, and this alteration in membrane composition is suppressed in a *nodA*::Tn*5* mutant.

Influence of NodD on accumulation of BF-7 by ANU843. The current model for the function of the NodD protein is that it serves as a positive regulator for *nod* expression by binding to the promoter region referred to as the *nod* box and upon activation by specific flavonoid compounds enables transcription of downstream *nod* genes (10, 12, 27, 41). Wild-type ANU843 has a single copy of *nodD* (12), and thus Tn*5* inactivation of this positive regulatory gene should completely shut down the *nod* regulon. To further investigate the possible direct roles of flavone and the *nod* regulon in BF-7 accumulation, we compared the level of BF-7 in cell extracts of wild-type ANU843 and the isogenic *nodD*::Tn*5* mutant derivative, both grown with DHF. Cells were exhaustively extracted with a modified solvent system to include both BF-7 and other, more hydrophilic membrane glycolipids. Silica TLC analyses of the extracts fractionated by adsorption chromatography detected an orcinol-positive component in the 10% methanol in chloroform fraction from the ANU843 extract which had the same R_f as purified BF-7, but this component was not detected in the corresponding fraction from the *nodD*::Tn*5* mutant derivative (Fig. 2C). These results indicate that *nodD*, in addition to DHF and *nodA*, is involved in elevated accumulation of BF-7 in cells of wild-type ANU843. In addition, we again noted minor quantities of several other orcinol-stained components present in the ANU843 cell extract but not in the corresponding cell extract from the *nodD*::Tn*5* mutant (Fig. 2C).

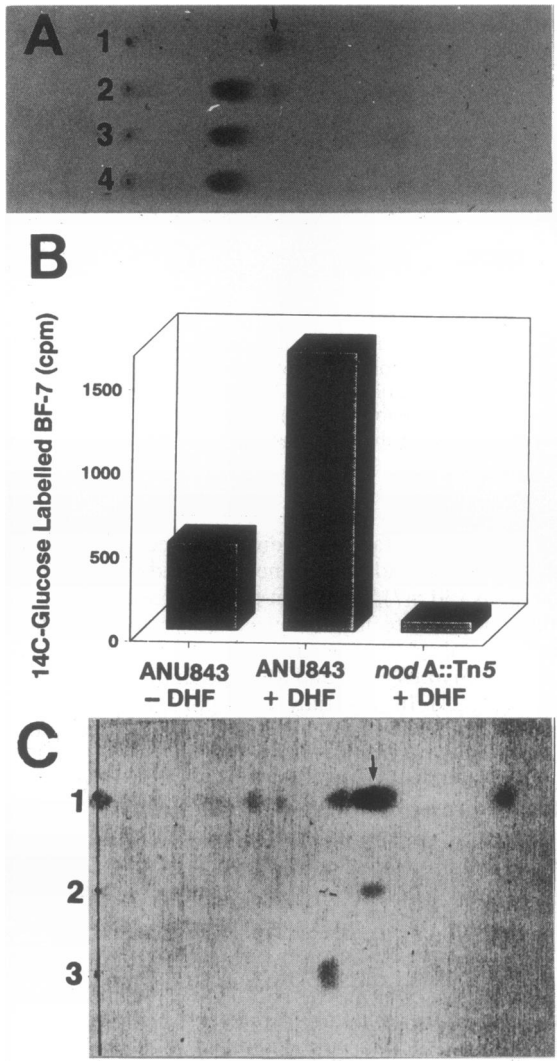

FIG. 2. TLC evaluation of BF-7 accumulation in cells of *R. leguminosarum* bv. trifolii ANU843 as affected by the flavone DHF and Tn*5* inactivation of pSym *nod* genes. (A) Orcinol-stained chromatogram of purified BF-7 (lane 1) and equivalent fractions from ANU843 grown with 4 μM DHF (lane 2), ANU843 grown without DHF (lane 3), and ANU252*nodA*::Tn*5* grown with 4 μM DHF (lane 4). (B) Liquid scintillation counts of in vivo-labeled BF-7 isolated by TLC from equivalent fractions of ANU843 grown without (−) and with (+) DHF and the corresponding R_f region in the lane of the TLC plate spotted with the sample from the ANU252*nodA*::Tn*5* mutant derivative. (C) Orcinol-stained chromatogram of purified BF-7 (lane 2) and equivalent fractions from cells of ANU843 (lane 1) and the ANU851 *nodD*::Tn*5* mutant derivative (lane 3), each grown with 4 μM DHF.

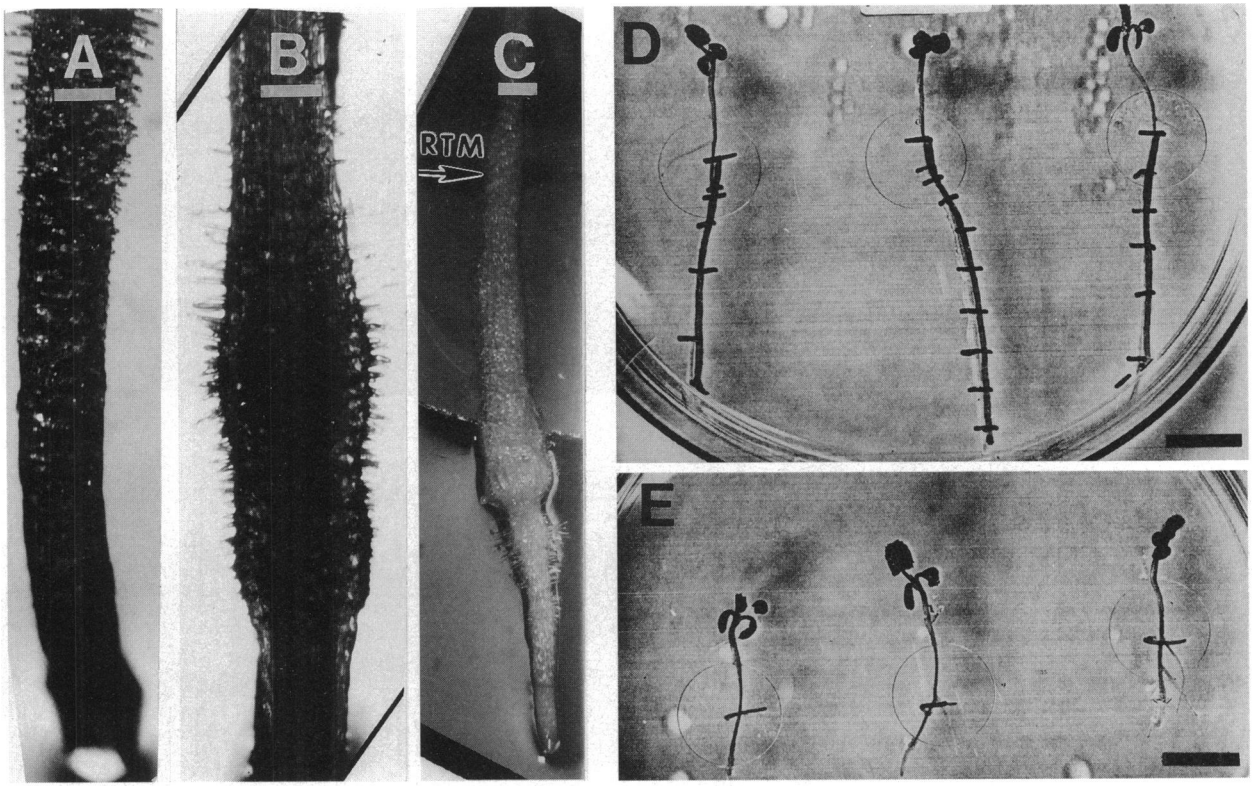

FIG. 3. Development of thick short roots (Tsr) on axenic white clover seedlings treated with BF-7. (A and D) Control seedling roots grown in NF medium only. (B, C, and E) Seedling roots grown in NF medium supplemented with purified BF-7 (10^{-9} to 10^{-10} M). Roots produced localized thickenings below the root tip mark (RTM; arrow in panel C) made at the time of purified BF-7 application and were shorter than control roots. Seedlings were 3 days old in panels A to C and 10 days old in panels D and E. Marks in panel D indicate the daily increment of the traced line of root growth under NF control conditions. Bar scales are 200 μm in panels A and B, 500 μm in panel C, and 1 cm in panels D and E.

Purification of BF-7 for bioassays. BF-7 was extracted from ANU843 grown with DHF and exhaustively purified to homogeneity by a sequence of alternate adsorption and reverse-phase chromatographies as previously described (33). The yield of this minor membrane glycolipid was 0.8 mg/g of lyophilized cells. For comparison, at least 100 mg of membrane phospholipids would be obtained from the same amount of cells (34). Contamination of the purified BF-7 sample with the more hydrophilic chitolipooligosaccharides was unlikely because of the extensive purification sequence used (33). This lack of chitolipooligosaccharide contamination was confirmed by chemical analyses of the purified BF-7 sample (33), indicating the absence of glucosamine (flame ionization gas-liquid chromatography and combined gas chromatography-mass spectrometry of alditol acetates), no methyl proton resonances for *N*-acetyl groups (500-MHz ^{1}H nuclear magnetic resonance spectroscopy), and no diagnostic ion fragmentation pattern compatible with oligomers of *N*-acetylglucosamine (positive-mode fast atom bombardment mass spectrometry).

BF-7 elicits various symbiosis-related morphological responses on white clover. Plant bioassays indicated that very low concentrations of purified BF-7 applied to axenic roots will elicit the phenotypes of thick short roots (Fig. 3), root hair deformations (especially of the corkscrew type) indicative of alterations in root hair wall architecture (Fig. 4), and various cytological changes in the root cortex (Fig. 5 and 6). Elicitation of root thickening by BF-7 was more pronounced just below rather than above the root tip mark (Fig. 3C), implicating a requirement of active root growth in this response. The gross morphologies of the shoot, cotyledonary, and unifoliate leaves were not affected by BF-7 in this seedling bioassay (Fig. 3E). Foci of Ccd made in response to the mitotic stimulus of BF-7 were occasionally recognized as isolated rows of divided cells (Fig. 5B) but more frequently involved a localized collar of the entire cortex encircling the pericycle without developing mer-

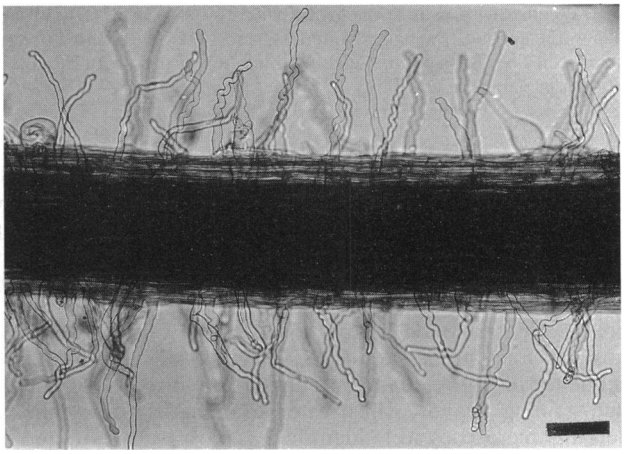

FIG. 4. Root hair deformations (Had) on a white clover root treated with 10^{-9} M BF-7. Note the predominance of corkscrew deformations. Bar scale is 50 μm.

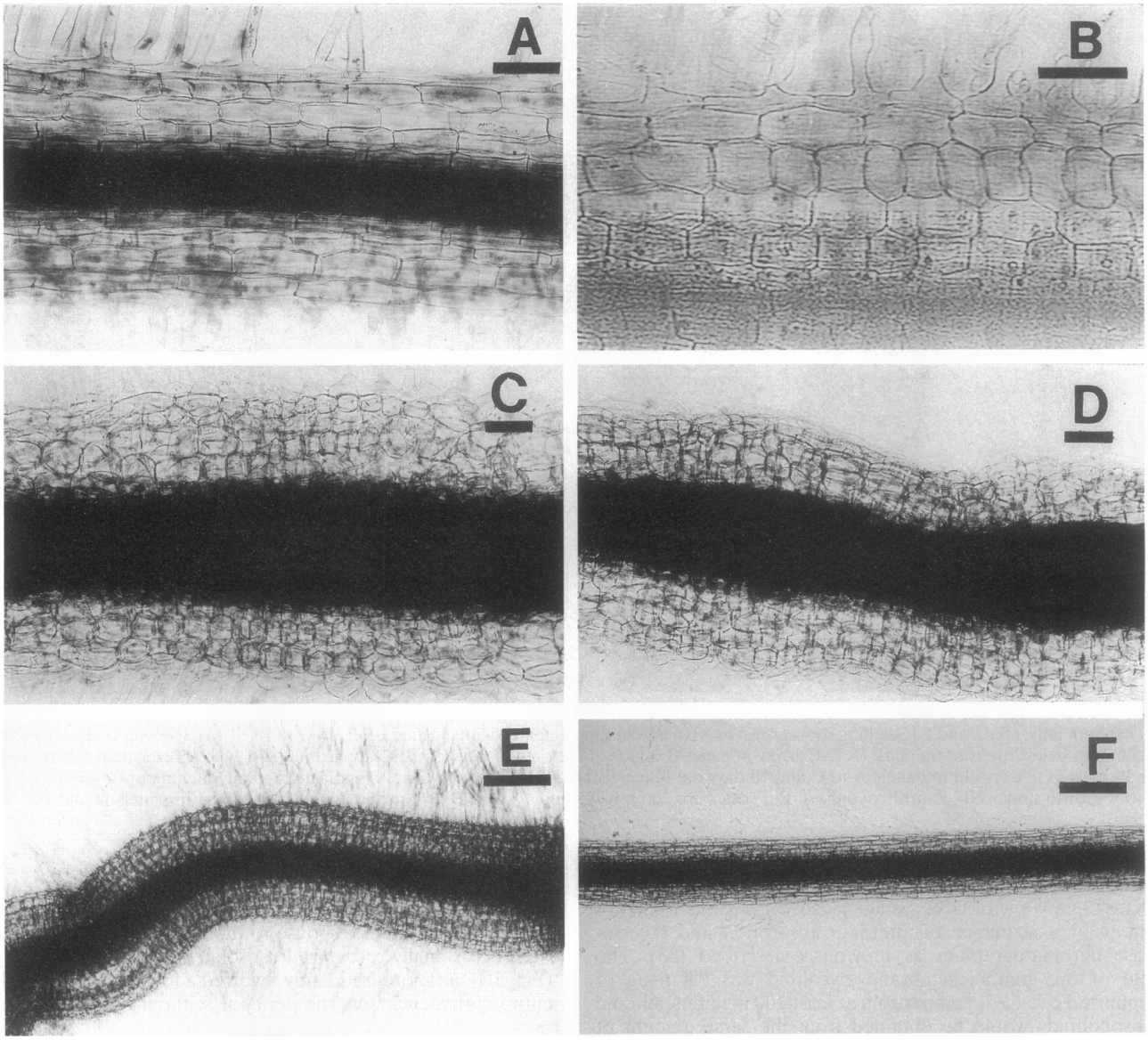

FIG. 5. Alterations in the root cortex of axenic white clover seedlings treated with BF-7. Roots have been cleared and stained, and are viewed in longitudinal plane. (A) Control root grown in NF medium developed a cortex containing approximately four layers of cells. (B to D) Foci of cortical cells divisions in roots grown with 10^{-9} M BF-7. (B) Edge-enhanced laser scanning micrograph showing a localized area where cortical cells in the second row have divided. (C and D) Collaroid foci of cortical cell divisions completely encircling the endodermis. Occasionally, these foci of cell divisions spanned extended lengths of the root (E); compare with the NF control (F). Bar scales equal 100 μm in panels A to D and 380 μm in panels E and F.

istems, hereafter referred to as collaroid foci of Ccd (Fig. 5C and D). In some roots, the regions exhibiting this collaroid mitotic response to BF-7 extended considerable distances, up to several millimeters in length (Fig. 5E and F).

The cytological responses to BF-7 were examined also by comparing cross sections of control root segments and segments which exhibited BF-7-induced foci of Ccd (Fig. 6). The most obvious influence of BF-7 action was an increased frequency of both clinally and anticlinally oriented, toluidine blue-staining strands, which when examined by transmission electron microscopy were identified as cross walls bordered by cytoplasm of smaller, divided cortical cells.

Dose response and host range of BF-7 action. Quantitation of the dose responses on axenic white clover seedlings indicated that BF-7 induced Had in the concentration range of 10^{-7} to 10^{-12} M and Ccd in the concentration range of 10^{-5} to 10^{-11} M (Fig. 7). BF-7 action was optimal at 10^{-9} M for induction of both of these morphological responses on the host legume, Dutch white clover. Since the bioassay uses only 10-μl samples, a threshold of 10^{-16} to 10^{-17} mol of BF-7 was sufficient to trigger these morphological responses on individual white clover seedlings. In contrast, BF-7 did not induce Had above background levels on the heterologous nonhost legumes, Gemini alfalfa and hairy vetch (Fig. 7A). The incidence of foci of collaroid Ccd increased in hairy vetch treated with BF-7, albeit to a lesser extent than in Dutch white clover and with an optimum range shifted to a higher concentration (Fig. 7B). The frequency of Ccd in Gemini alfalfa was not

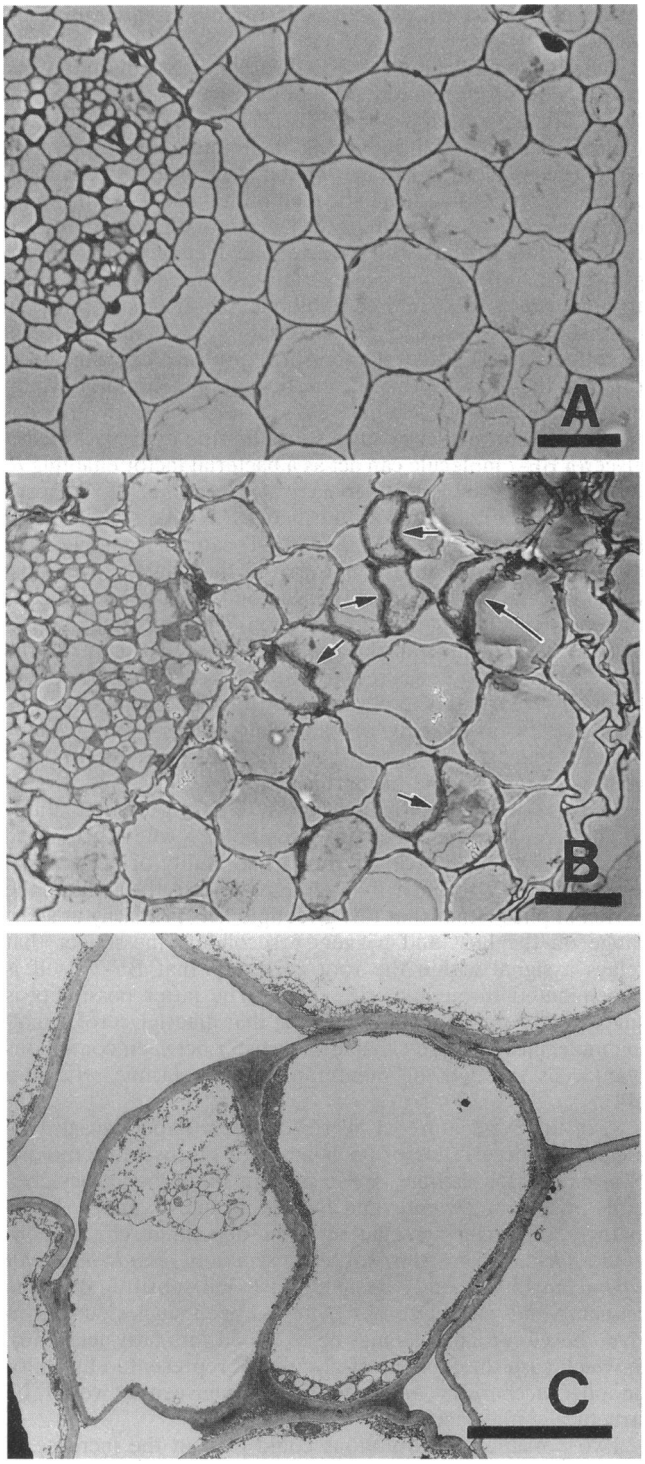

FIG. 6. Alterations in the root cortex of axenic white clover seedlings treated with BF-7. Shown are cross sections of roots grown in NF medium only (A) and in 10^{-11} M BF-7 (B and C). Bar scales are 30 μm in panels A and B and 5 μm in panel C. Arrowheads in panel B correspond to cross walls of dividing cells.

significantly affected by BF-7 action in these bioassays. Therefore, plant perception of subnanomolar concentrations of BF-7 capable of eliciting a morphological response is more effective in the host legume white clover than in the nonhost legumes alfalfa and vetch.

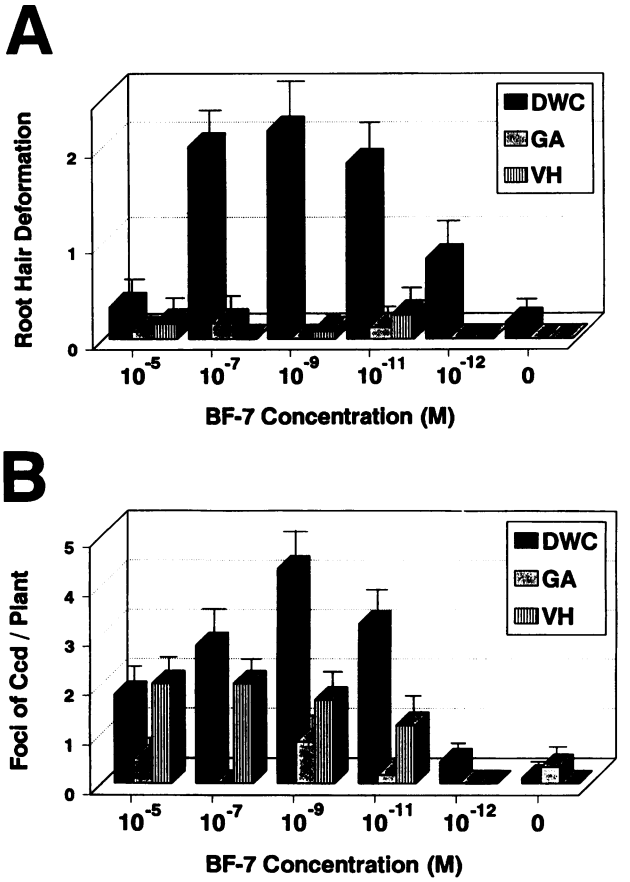

FIG. 7. Dose response and host range of BF-7 action evaluated by axenic plant bioassays and quantitative microscopy. (A) Root hair deformation; (B) foci of cortical cell divisions. Plants tested were Dutch white clover (DWC), Gemini alfalfa (GA), and hairy vetch (VH).

Action of a different diglycosyl diacylglycerol on white clover roots. To determine if diglycosyl diacylglycerols in general are able to elicit the morphological responses described above in axenic white clover seedlings, we compared the activities of BF-7 and a different, commercially available diglycosyl diacylglycerol from wheat (DGDG) (Fig. 1A and B). The results clearly showed that the biological activity of BF-7 resulting in Had and Tsr (as measured by root length) could not be duplicated by DGDG in the same concentration range (Fig. 8). Also, no increased frequency of Ccd was detected in roots of Dutch white clover treated with DGDG and examined after clearing with hypochlorite or in cross sections of embedded roots (data not shown). Thus, there are structural constraints on the ability of glycosyl diacylglycerols to elicit these morphological responses in white clover.

Modulation of BF-7 action on white clover roots by the ethylene synthesis inhibitor AVG. The endogenous plant hormone ethylene has been shown to suppress development of leguminous root nodules incited by rhizobia (reference 21 and references cited therein). To determine whether plant-produced ethylene influences the action of BF-7, we evaluated the BF-7-induced responses of Tsr and Ccd in white clover grown in the presence of the ethylene biosynthesis inhibitor AVG (1). Nontoxic concentrations of AVG (10 μg/liter) abolished the BF-7-induced Tsr response, as measured by root length at 10

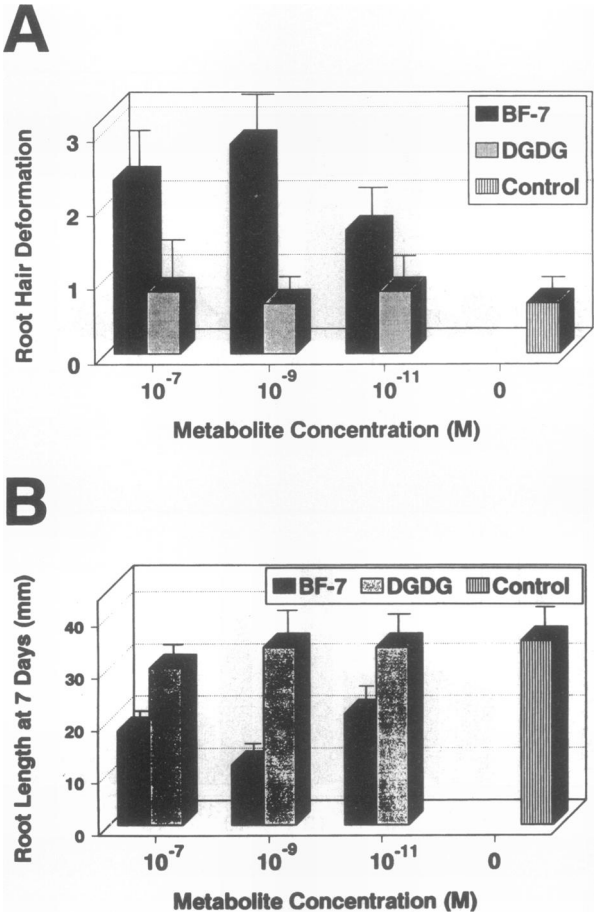

FIG. 8. Comparison of action of different diglycosyl diacylglycerols (BF-7 and wheat DGDG) on axenic white clover seedings. (A) Root hair deformations; (B) Tsr as measured by root length at 7 days.

days (Fig. 9A), and also greatly affected the type of BF-7-induced mitogenic response manifested in the root cortex (Fig. 9B to D). The nonmeristematic collaroid-type foci of cortical cell divisions produced in response to BF-7 (Fig. 5) were suppressed in the presence of AVG (Fig. 9B); instead, meristematic-type foci of cell divisions developed more frequently (Fig. 9C and D). Like nodule primordia, these meristems were ellipsoid rather than cone shaped, with the longest dimension lying parallel to the longitudinal root axis. However, like lateral root primordia, there was a clear boundary between the meristem and the surrounding nondividing cells. Recent studies have shown that this same type of wide-based meristematic structure is induced in white clover roots inoculated with rhizobia, and it has been interpreted as an intermediate or hybrid structure with ontogeny characteristics from both nodules and lateral roots in white clover, which have been proposed to be physiologically homologous (28). These results indicate that suppression of plant-produced ethylene significantly affects the meristematic organization of mitogenic responses to BF-7 in the root cortex of white clover, with a suggestion that BF-7 has the potential of initiating very early stages of nodule formation. These results obtained with purified BF-7 are also consistent with a previous study (53) in which AVG suppressed the Tsr response and restored the development of nodule primordia on the primary root of *Viciae sativa* inoculated with viable cells of *R. leguminosarum* bv. viciae.

Possible significance of BF-7 for *R. leguminosarum* bv. trifolii. Several roles for glycosyl diacylglycerol-type glycolipids in bacteria have been proposed (22, 24, 43, 46), including their main role as integral stabilizing membrane lipids providing structural adaptation to environmental changes, specific activation or inhibition of catalytic membrane-associated proteins, compensation of cell viability when peptidoglycan rigidity is reduced, interactions with extracellular lectins, adaptation to growth on surfaces, and aggregation to form membrane pores through which ions and hydrophilic metabolites may pass. All of these roles assigned to bacterial glycosyl diacylglycerol glycolipids are of potential relevance to the ecological niche of rhizobia that enables them to participate in a complex symbiotic interaction with legumes. In addition, this study provides several lines of evidence suggesting that the diglycosyl diacylglycerol BF-7 molecule can act as a bacterial factor enabling *R. leguminosarum* bv. trifolii to activate segments of its host's symbiotic program. Indeed, purified BF-7 (like several other factors from ANU843 [17, 35, 37, 39]) can elicit many of the morphological responses on roots of the white clover host which normally result from inoculation with the homologous rhizobial symbiont. This information adds to the complex network of signal transduction pathways that control the expression of host genes required for cortical cell division and helps to reveal how the development of a plant organ can be regulated by a diversity of exogenous factors produced by bacteria that have acquired the ability to trigger the organogenesis of root nodule primordia (52).

Because of its amphiphilic property, BF-7 would predictably be incorporated into cellular membranes when presented exogenously to white clover roots. The ability of an external source of BF-7 to elicit mitogenic responses throughout the cortex implies either that this glycolipid acts primarily at short range on the host and triggers a secondary messenger that relays a signal within the root cortex or that BF-7 itself is disseminated throughout this tissue. The latter possibility is suggested by a recent study showing that diacylglycerol signaling molecules (15) are capable of transfer between contiguous plant cells through the membranous endoplasmic reticulum (14).

A distinctive feature of all rhizobial chitolipooligosaccharides is the occurrence of an N-acylated glucosamine moiety. N-acylated glucosamine is also present in glycosyl diacylglycerols of certain *Thermus* and *Bacillus* isolates (25, 38). Interestingly, the reducing sugar of a *nod*-dependent chitolipooligosaccharide from the soybean symbiont *Bradyrhizobium japonicum* has recently been found to be substituted at the reducing end with glycerol (5). It has been suggested that in vivo, this glycerol moiety may be esterified with fatty acids (50), in which case the families of glycolipids represented by chitolipooligosaccharides and glycosyl diacylglycerols would be structurally interfaced.

Two possible interpretations could explain the increase in BF-7 in membranes of ANU843 cells when grown with DHF. The first is that DHF may activate a gene or genes directly involved in the synthesis of this glycolipid. Of potential relevance to this proposal is the early report that chromosomal genes are responsible for production of Had^+ and Tsr^+ factors from *R. leguminosarum* bv. trifolii, but expression of its pSym *nod* genes (e.g., *nodDABC*) in the clover root environment enhances the production of these undefined bacterial factors which modify root growth and development (4).

The alterations in proportion of cellular membrane components is a typical and very general adaptive response to

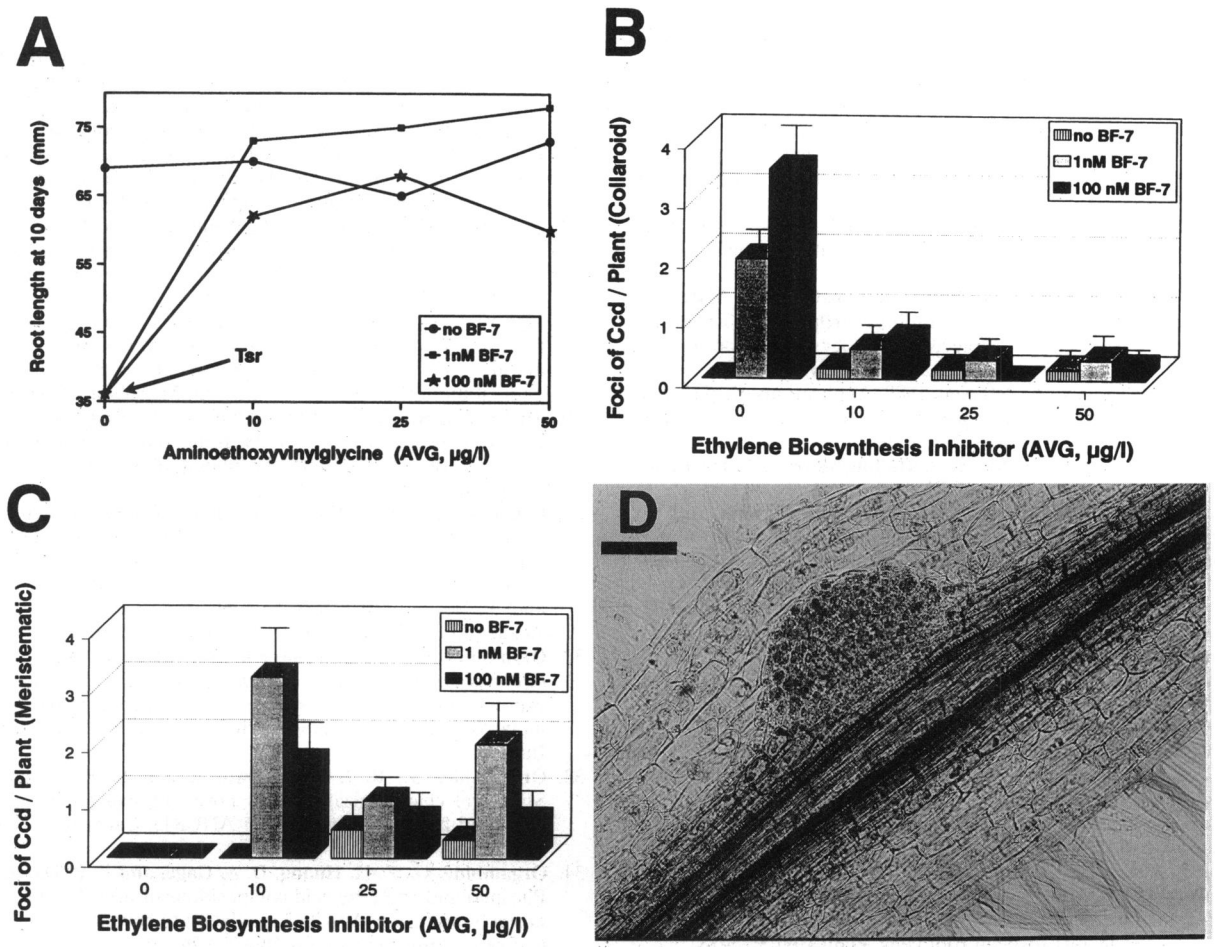

FIG. 9. Modulation of BF-7 action on axenic white clover roots by nontoxic levels of the ethylene biosynthesis inhibitor AVG (A) Abolishment of BF-7-induced Tsr response, as measured by root length at 10 days; (B and C) alterations in frequency of collaroid (B) and meristematic (C) foci of Ccd; (D) ellipsoid meristematic foci of cell divisions induced by 10^{-9} M BF-7 in the presence of AVG. Bar scale equals 100 µm.

environmental stresses. This study and several other lines of evidence (18, 34, 35, 37) indicate several changes in membrane glycolipid composition when rhizobia are grown with flavone to activate *nod* expression. Thus, a second possible explanation for the effect of DHF on synthesis of BF-7 is that it reflects a bacterial adaptive response to the accumulation of deleterious foreign substances (e.g., flavonoid compounds themselves) in its membranes. Of relevance to this proposal are recent studies showing that accumulation of the *nod*-activating flavone, luteolin, in the inner membrane of *R. meliloti* leads to a rapid inhibition of NADH oxidase activity which must eventually be restored in order for this bacterial respiratory enzyme to function in the host root environment (20). The suppressive effect of the *nodA* mutation on BF-7 accumulation would then suggest that this gene (and/or downstream *nod* genes polarly affected by the Tn*5* insertion in *nodA*) is involved in the synthesis of another membrane component, the proportion of which (relative to other phospholipids and glycolipids) affects the levels of BF-7 which can be synthesized. This alternative *nodA* effect then would be indirect. Regardless of whether the mechanism is direct or indirect, the results with the *nodD*::Tn*5* mutant provide further support for the involvement of the *nod* regulon in membrane accumulation of BF-7 and may even suggest a more specific involvement of *nod*-activating flavonoids in inducing its synthesis.

In summary, the influence of DHF and mutations in *nodA* and *nodD* on production of BF-7 by wild-type ANU843, and the mitogenic action of subnanomolar concentrations of purified BF-7 on the white clover host, indicate that this minor membrane glycolipid fits the criteria that one would expect of an important molecular component involved in the development of the *R. leguminosarum* bv. trifolii-white clover symbiosis.

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