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Modulation of Development, Growth Dynamics, Wall Crystallinity, and Infection Sites in White Clover Root Hairs by Membrane Chitolipooligosaccharides from *Rhizobium leguminosarum* biovar trifolii†

FRANK B. DAZZO,[1]* GUY G. ORGAMBIDE,[1] SALEELA PHILIP-HOLLINGSWORTH,[1] RAWLE I. HOLLINGSWORTH,[2] KENNETH O. NINKE,[1] AND JANET L. SALZWEDEL[3]

Department of Microbiology[1] and Departments of Biochemistry and Chemistry,[2] Michigan State University, East Lansing, Michigan 48824, and Department of Biology, Adrian College, Adrian, Michigan 49221[3]

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We used bright-field, time-lapse video, cross-polarized, phase-contrast, and fluorescence microscopies to examine the influence of isolated chitolipooligosaccharides (CLOSs) from wild-type *Rhizobium leguminosarum* bv. trifolii on development of white clover root hairs, and the role of these bioactive glycolipids in primary host infection. CLOS action caused a threefold increase in the differentiation of root epidermal cells into root hairs. At maturity, root hairs were significantly longer because of an extended period of active elongation without a change in the elongation rate itself. Time-series image analysis showed that the morphological basis of CLOS-induced root hair deformation is a redirection of tip growth displaced from the medial axis as previously predicted. Further studies showed several newly described infection-related root hair responses to CLOSs, including the localized disruption of the normal crystallinity in cell wall architecture and the induction of new infection sites. The application of CLOS also enabled a NodC$^-$ mutant of *R. leguminosarum* bv. trifolii to progress further in the infection process by inducing bright refractile spot modifications of the deformed root hair walls. However, CLOSs did not rescue the ability of the NodC$^-$ mutant to induce marked curlings or infection threads within root hairs. These results indicate that CLOS Nod factors elicit several host responses that modulate the growth dynamics and symbiont infectibility of white clover root hairs but that CLOSs alone are not sufficient to permit successful entry of the bacteria into root hairs during primary host infection in the *Rhizobium*-clover symbiosis.

Rhizobium is a genus of soil bacteria which specifically infect and nodulate legume roots, forming a nitrogen-fixing root nodule symbiosis of major importance to agriculture. The development of this bacterium-plant symbiosis involves the exchange of molecules that activate the symbiotic program encoded in the genomes of both symbiotic partners. For instance, the plant root secretes flavonoid compounds that activate the expression of nodulation (*nod*) genes of the microsymbiont, ultimately resulting in the production of various bioactive Nod metabolites which, in turn, activate the host symbiotic program involved in development of a functional root nodule (41).

The most thoroughly examined class of Nod metabolites is represented by a family of chitolipooligosaccharides (CLOSs), first discovered in *Rhizobium meliloti* by Lerouge et al. (24) and now documented for many rhizobia (reference 4 and references therein). At very low concentrations, rhizobial CLOSs can elicit certain developmental responses in the host legume that mimic the symbiosis with the bacteria. Molecular host responses include the induction of nodulin gene expression and increased enzyme activities (2, 22, 41). Physiological host responses include membrane depolarization, alterations in Ca^{2+} and H^+ fluxes, and relocation of actin filaments in host root hairs (1, 14). Morphological host responses include induction of root hair deformations, radially aligned transvacuolar cytoplasmic bridges within cortical cells, foci of cortical cell divisions, and formation of cortical meristems that develop into nodule primordia (19, 31, 36, 43, 45).

Most CLOSs described to date have been isolated from the extracellular milieu of *nod*-induced *Rhizobium* cultures. However, we recently found that CLOSs accumulate primarily in membranes of wild-type *Rhizobium leguminosarum* bv. trifolii ANU843 and *R. meliloti* 2011 (5, 32, 34). Accumulation of membrane CLOSs in ANU843 requires a functional *nodC* and *nod*-activating flavone (32), and these glycolipids are fully capable of eliciting root hair deformation and foci of cortical cell divisions in white clover in the same concentration range as that found for extracellular CLOSs of other rhizobia on their corresponding legume hosts (31).

In view of the amphiphilic properties of CLOS glycolipids and their accumulation within rhizobial membranes, we predicted that under natural conditions, CLOS action should primarily be short range and highly localized during rhizobial infection of host root hairs (32). Bacterial induction of a shepherd crook deformation (Hac) and an infection thread at the site of bacterial entry within the root hair (Inf) are the best known examples of short-range host-symbiont interactions during root hair infection, since they require close proximity of living cells of the homologous bacterial symbiont (25, 44, 47, 48). Also, in situ expression of common *nod* genes required for primary host infection is optimal for *R. leguminosarum* bv. trifolii in the highly localized, symbiont-specific pattern of bac-

* Corresponding author. Phone: (517) 353-8649. Fax: (517) 353-8953. Electronic mail address: 23249mgr@msu.edu.

† This work is dedicated in memory of David H. Hubbell, who died unexpectedly on 26 August 1995. His studies on the infection process in *Rhizobium*-legume root nodule symbiosis were inspirational and continue to be a bright beacon light to a better understanding of plant-microbe interactions.

terial attachment at the root hair tip of the white clover host (9). We therefore conducted the present study to define the short-range effects of membrane CLOSs from wild-type *R. leguminosarum* bv. trifolii on the development, growth dynamics, and wall biogenesis of white clover root hairs. The aim was to gain a more thorough understanding of the mechanism(s) of action and role(s) of these bioactive bacterial glycolipids during early *Rhizobium*-host root hair interactions before infection thread formation. The determination of host specificity is expressed at or before this developmental stage of primary host infection in the *Rhizobium*-clover symbiosis (25). To accomplish this goal, we used phase-contrast light microscopy, time-lapse video microscopy, polarized light microscopy, and fluorescence microscopy to reveal newly described, CLOS-induced alterations in physiologically active root hairs at single-cell resolution.

(Portions of this work were presented at the 15th North American Symbiotic Nitrogen Fixation Conference, 13 to 17 August 1995, Raleigh, N.C. [35].)

MATERIALS AND METHODS

Bacterial and plant cultures. *R. leguminosarum* bv. trifolii wild-type strain ANU843 and the isogenic *nodC*::Tn*5* mutant derivative ANU277 (13) were obtained from M. Djordjevic and B. Rolfe (Australian National University, Canberra). Cultures were grown on BIII agar (7), with 30 μg of kanamycin per ml for strain ANU277. Seeds of white clover (*Trifolium repens* L. cv. Dutch) and alfalfa (*Medicago sativa* cv. Gemini) were surface sterilized by treatment with 70% ethanol followed by acidified $HgCl_2$ solution and germinated at 20°C in the dark on agar plates of nitrogen-free (NF) Fahraeus medium. Plants were grown in a growth chamber programmed for a 16-h photoperiod, 22°C (day)-20°C (night) cycle, and 70% relative humidity.

Preparation of ANU843 membrane CLOSs. The extraction, purification, and structural characterization of membrane CLOSs from ANU843 have been described elsewhere (32, 34). A 10^{-5} M stock of the purified CLOS sample (32) was prepared in NF medium, assuming a nominal molecular mass of 1,000 Da. The CLOS stock was steamed for 30 min and verified as sterile by plating aliquots on BIII medium and trypticase soy agar (Difco, Detroit, Mich.).

Axenic seedling bioassays of ANU843 CLOSs. Seedlings were germinated in humid air for 2 days, transferred to NF agarose plates, irrigated with NF medium, grown vertically for 1 day, treated at the root tip with 15 μl of a sterile 5×10^{-9} M CLOS sample, covered with a 12-mm-diameter circular coverslip, sealed with Nescofilm (Karlan Chemical Corp., Santa Rosa, Calif.), and further incubated vertically in a growth chamber under microbiologically controlled conditions (21). For all experiments, sterile NF medium was used as the diluent and untreated control throughout.

To assay for induction of epidermal differentiation into root hairs, seedlings were incubated with CLOSs for 2 days and then examined along the optical median plane of the root under the coverslip. The density and mature lengths of root hairs were measured by computer-assisted image analysis (11) of video micrographs with Bioquant software (R & M Biometrics, Nashville, Tenn.).

Influence of ANU843 CLOSs on growth dynamics of white clover root hairs. The development of root hairs on seedlings grown axenically and geotropically on sterile NF agarose plates was recorded by time-lapse video microscopy with a specially constructed horizontal video workstation (8, 11). Recordings in the region of emerging root hairs commenced immediately after the CLOS sample and coverslip were applied to the root tip and continued at room temperature for ca. 20 h. Elongation of root hairs was evaluated by tracking the tips in focus during playback, followed by time-series image analysis (11). The distance was calibrated with a slide micrometer, and the time-date generator imprinted the time lapsed to a precision of 4 s at a time compression of 240:1. Representative video micrographs were made by photographing still-frame pictures on the playback video monitor.

CLOS-induced alterations in crystallinity of root hair wall. After growth in the presence of ANU843 CLOSs or an inoculum of viable bacteria, seedling roots were examined by cross-polarized light microscopy (23, 29). The polarized light microscope system utilized a bright quartz-halogen illuminator, a fixed-polar analyzer above the objective lens, a polarizer filter rotated to the fully crossed position underneath the condenser, and the specimen placed on a rotating stage.

Infection-related biological activity of ANU843 CLOSs. Root tips of germinated white clover seedlings were treated with a 15-μl sample of 5×10^{-9} M CLOSs on NF agarose plates, covered with a coverslip, and incubated vertically for 9 h in the growth chamber. Seedlings were then rinsed with NF medium; inoculated with 15 μl of a standardized, washed suspension of ANU843 cells; covered with a new coverslip; and incubated in the growth chamber for 4 days. The bacterial inoculum was prepared from a plate culture grown for 5 days at 30°C, suspended in NF medium, centrifuged at 2,000 × *g* for 15 min, and resuspended to 10^8 cells per ml of NF medium. Afterwards, root segments under the coverslips were excised, mounted in NF medium, and examined by phase-contrast microscopy to count the markedly curled (Hac) and infected root hairs (Inf). Detection of infection-related biological activity was based on the ability of CLOS pretreatment to create new infection sites, recognized as an increased frequency of infected root hairs (i.e., containing refractile infection threads [25]) per unit length of inoculated root (12).

To investigate whether ANU843 CLOSs can rescue the defective Hac and Inf phenotypes of a $NodC^-$ mutant, seedlings were planted in Jensen tubes containing NF medium plus 5×10^{-9} M CLOSs, inoculated with mutant ANU277 *nodC*::Tn*5* (10^7 cells per plant), and incubated in a growth chamber. Afterwards, plant roots were removed and examined directly for infection structures by phase-contrast microscopy with the 546-nm-wavelength interference contrast filter and epifluorescence microscopy with the Zeiss no. 11 fluorescence filter set (band pass exciter combination BP450-500, dichroic beam splitter FT510, and longwave pass LP528 barrier filters).

FIG. 1. Uncovered portion of a white clover root in the standard Had plate bioassay. Note that root hairs exposed to the CLOS-impregnated agarose matrix are deformed (arrows) whereas neighboring root hairs (slightly out of focus) that grew in humid air are straight. Root hairs on control, untreated plants remain undeformed. Bar, 50 μm.

RESULTS AND DISCUSSION

ANU843 membrane CLOSs elicit developmental responses in white clover root hairs. The most obvious response of root hairs to CLOSs is their development of intense deformations (Had). Quantitative bioassays indicate that the Had responses are induced in axenic white clover seedlings by ANU843 CLOSs at an optimal concentration of 5×10^{-9} M and a threshold concentration of 10^{-11} to 10^{-13} M (31, 35). These results are based on the intensity of the Had response on a portion of the root exposed to a range of CLOS concentrations beneath the coverslip in the standard plate assay. Examination of the younger, uncovered portion of the root revealed that only the root hairs in direct contact with the CLOS-impregnated agarose matrix underwent deformation, whereas the neighboring root hairs growing in humid air remained straight and undeformed (Fig. 1). This result indicates that Had induction is a short-range, highly localized (nonsystemic) response of root hair development to CLOS action.

It has long been known that *Rhizobium* spp. will stimulate the production of root hair growth on legume roots (42). This trait is referred to as Inh, for induction of hairs, and in *R. leguminosarum* bv. trifolii is influenced by its resident symbiotic plasmid (3). Here we show that the Inh activity of ANU843 CLOSs caused a threefold increase in the density of white clover root hairs but no significant change in the density of alfalfa root hairs (Table 1). These results indicate that these glycolipids promote the differentiation of root epidermal cells into root hairs in the white clover host.

Membrane CLOSs modulate growth dynamics of root hairs. The average lengths of mature root hairs following 2 days of growth without and with CLOSs were 239 and 338 μm, respectively, for white clover, and 198 and 112 μm, respectively, for

TABLE 1. Induction of root hair differentiation by membrane CLOSs from *R. leguminosarum* bv. trifolii ANU843

Test plant	Root hair density (mean ± SD)[a]	
	Without CLOS	With CLOS (5×10^{-9} M)
Dutch white clover	31 ± 10	98 ± 15
Gemini alfalfa	39 ± 8	44 ± 6

[a] Density is expressed as the number of root hairs per millimeter of root optical median plane under the coverslip.

alfalfa. Statistical analyses indicated that these CLOS-elicited changes in root hair development were significant at the 99.9% level (for clover, t of 13 with 934 df; for alfalfa, t of 14 with 611 df). The mature lengths of white clover root hairs had similar distributions which centered on a larger mean when grown in the presence of CLOSs (Fig. 2A). In contrast, alfalfa root hairs grown in the presence of ANU843 CLOSs had a narrower distribution in root hair length that was centered on a smaller mean (Fig. 2B). These results indicate that ANU843 CLOSs affect root hair growth on both clover and alfalfa but that the response is opposite for these host and nonhost legumes.

We envisioned three possibilities to explain how CLOS action increases the mature length of host root hairs: by extending the period of growth elongation, increasing the rate of elongation, or both. Time-lapse video microscopy and digital image analysis provided an unambiguous test for these hypotheses. Time-series analysis of periodic incremental growth clearly indicated that root hairs elongated at the same constant rate (ca. 19 μm/h) during growth extension in the presence or absence of ANU843 CLOSs (data not shown). This result implies that CLOS action prolongs the period of active elongation and delays maturation of root hairs without affecting the elongation rate per se. The calculated duration of dynamic elongation for an average root hair to reach its mature length is increased from 12.6 h for growth without CLOSs to a period of 17.8 h for growth with CLOSs. We postulate that this additional 5.2 h of elongation of the average root hair reflects an ability of CLOS action to extend its window of infectibility before the cessation of growth and that this newly described developmental response to CLOSs is central to the dynamic process of primary host infection in the *Rhizobium*-legume symbiosis.

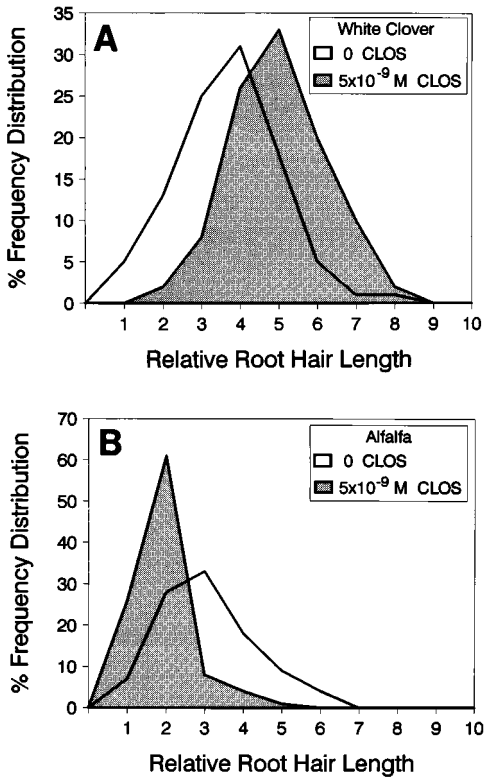

FIG. 2. Percent frequency distribution of root hair lengths on axenic seedlings of white clover (A) and alfalfa (B) grown in the presence or absence of ANU843 CLOSs. Each increment of the relative root hair length (abscissa) is 75 μm.

Time-lapse video microscopy and image analysis revealed further insight into the nature and mechanism of CLOS-induced Had under axenic conditions (Fig. 3). Time-series analysis of six independent experiments at 4-s resolution indicated that the earliest discernible root hair deformations occurred after 2.12 ± 0.65 h of exposure to 5×10^{-9} M ANU843 CLOSs. Image analysis of entire video sequences showed that the morphological basis of the dominant type of CLOS-induced Had is a redirection of tip growth that deviates from the medial axis of the root hair cylinder, consistent with previously proposed hypothetical models (10, 44). These video sequences also revealed that all older deformed portions of the root hair remain morphologically unchanged during subsequent tip growth, indicating that most of the deformed root hair wall is rigid. Thus, this type of deformation is due to short-range alterations in polar extension of the root hair tip rather than distortion of the preelongated root hair wall.

Membrane CLOSs disrupt the ordered crystalline architecture of white clover root hair walls. In general, plant cell expansion requires adequate turgor pressure as the driving force; wall rearrangement of the cellulose microfibrils interwoven with networks of hemicellulose, pectin, and structural proteins to allow the intercalation of new wall material; and de novo synthesis of wall polymers followed by their deposition at the correct domain at the plasma membrane (38). During normal root hair growth, the wall will experience multiaxial tension from turgor but will be modified only at the tip intersection of the medial axis to permit the cell to expand unidirectionally during elongation, forming a straight cylinder (28). Since clover root hairs continue to grow at a constant elongating rate in the presence or absence of CLOSs, it appears unlikely that alterations in either turgor pressure or synthesis of wall polymers are the primary mechanism(s) of CLOS-induced Had. As an alternative explanation, we hypothesized that these bacterial glycolipids may cause short-range alterations in the architecture of wall polymers responsible for wall stiffening, e.g., the ordered crystallinity of cellulose microfibrils and associated wall polymers aligned along a preferred orientation relative to the cell axis. Such localized changes in wall plasticity would explain the increase in differentiation and outgrowth of root hairs, the redirection of tip growth causing deformation of root hairs when the site of plasticity is displaced from the tip intersection of the medial axis, and the prolongation of growth extension resulting in delayed maturation of root hairs.

To test this hypothesis, we used crossed polarized light microscopy to assess the influence of ANU843 CLOSs on the crystalline architecture of growing root hair walls. This method of microscopy permits direct detection of alterations in molecular alignments of the ordered, crystalline components of cells within resolvable units less than 1 μm (23). Polarized light microscopy of control root hairs showed that all of the walls except the growing tips had optically anisotropic molecules

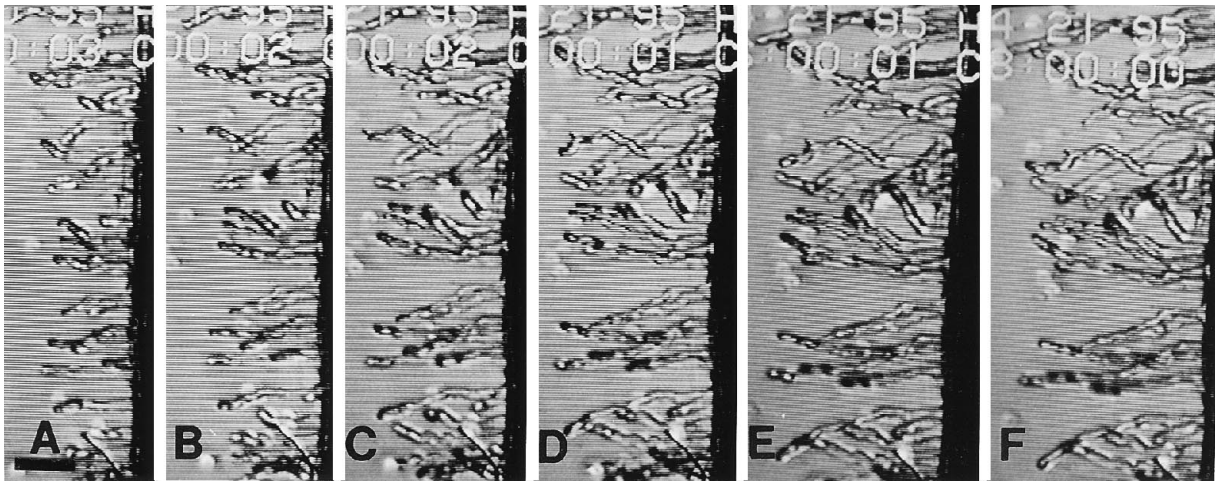

FIG. 3. Time-lapse video microscopy showing the spatial and temporal aspects of dynamic root hair growth and deformation on an axenic white clover seedling treated with ANU843 CLOSs. Video images are separated by 2-h intervals. Bar, 50 μm.

oriented in a crystalline array (Fig. 4a and b). In contrast, the walls of root hairs grown in the presence of ANU843 CLOSs contained multiple, localized, isotropic domains that lack an oriented or crystalline molecular architecture between regions of crystalline wall structures (Fig. 4c to g). These isotropic alterations in wall architecture can be detected as early as 2 to 3 h after the exposure of seedling roots to CLOSs (Fig. 4c) and are recognized as a displacement in the distribution of the isotropic domain at the growing root hair tip before it deforms. Later, the distribution of isotropic domains in root hairs is more extensive (Fig. 4d to g). Image analysis of complete, horizontal, optical sections through 11 root hairs grown with CLOSs for 2 days indicated that the proportion of the total extended length of the wall displaying these localized, isotropic domains varied widely, with a range of 5 to 51% and an average of 24%.

For comparison, we also evaluated the degree of alteration in the ordered crystalline architecture of root hair walls on seedlings inoculated with ANU843. Root hairs that had been grown in association with the bacteria exhibited multiple isotropic domains of altered wall architecture (Fig. 4h to j). Most noteworthy were the isotropic domains of disoriented wall architecture near the tip of deformed root hairs where the bacteria had attached (Fig. 4h and i) and where root hair tips had undergone marked curlings (so-called shepherd crooks) and contained infection threads as evidence of bacterial infection (Fig. 4j). We also noted isotropic, noncrystalline infection threads within root hairs (Fig. 4j). The above-described results provide direct evidence that ANU843 membrane CLOSs disrupt the normal process of wall crystallization and maturation in elongating root hairs of the white clover host and such wall alterations occur normally during primary root hair infection in the *Rhizobium*-clover symbiosis.

We envision three hypothetical mechanisms to account for these CLOS-induced alterations in the architecture of root hair walls which still allow biosynthesis of cellulose and self-assembly of the extracellular matrix components into a functioning cell wall. The simplest model predicts that insertion of CLOSs into the root hair plasma membrane (33, 35) creates localized point defects in its two-dimensional, ordered domains. These defects would affect the spatial integrity of the cellulose synthase complex, which is thought to be directed by cytoskeletal microtubules aligned in parallel beneath the plasma membrane during cellulose microfibril synthesis (15, 18, 30, 37). The result would be disarray of the cellulose microfibrils in localized regions of the wall. According to this model, differences in the partitioning of CLOSs from the external medium into root membranes of different plants (33, 35) would account for quantitative differences in the dose response of CLOS-induced Had among various legumes. A second mechanism to account for these CLOS-induced wall alterations is the possibility that these bacterial glycolipids disrupt the function of another protein(s) that participates in the ordered assembly of the wall architecture during normal cell growth and elongation, e.g., xyloglucan endotransglycosylase (17), expansins (6), and possibly wall-associated cellulases, pectinases, and hemicellulases (16). A third hypothetical mechanism proposes that CLOSs activate a signal transduction pathway in the specific host plant which leads to the production of another active molecule(s) (20) that, in turn, causes the disruption of the crystalline architecture in the root hair wall.

ANU843 membrane CLOSs exhibit infection-related biological activity that promotes root hair infectibility. We predicted that the localized rearrangements of the wall architecture which reduce its degree of crystalline order as described above would affect the mechanical strength of the root hair wall and make it more prone to successful attack by wall-degrading enzymes, thus promoting the ability of rhizobia to penetrate it during primary host infection (10, 26, 27). To examine the role of CLOSs in primary host infection, we determined their infection-related biological activity in white clover coinoculated with rhizobia. Quantitative microscopy indicated that CLOS pretreatment was unable to induce marked curlings or infection threads in root hairs of axenic white clover seedlings but significantly increased the frequency of root hairs which made these structures when seedlings were inoculated with live cells of wild-type ANU843 (Fig. 5). These CLOS-induced differences were statistically significant at the 99.9% level (for Hac, t of 10.0 at 22 df; for Inf, t of 8.5 at 10 df). A unique feature found only in roots pretreated with CLOSs before inoculation with ANU843 was the occurrence of infected root hairs containing multiple infection threads (Fig. 6A). Together, these results support the idea that CLOS-induced alterations in growth dynamics and wall architecture change the plasticity of the root hair walls and promote their infectibility by rhizobia as predicted but that additional bacterial functions are also re-

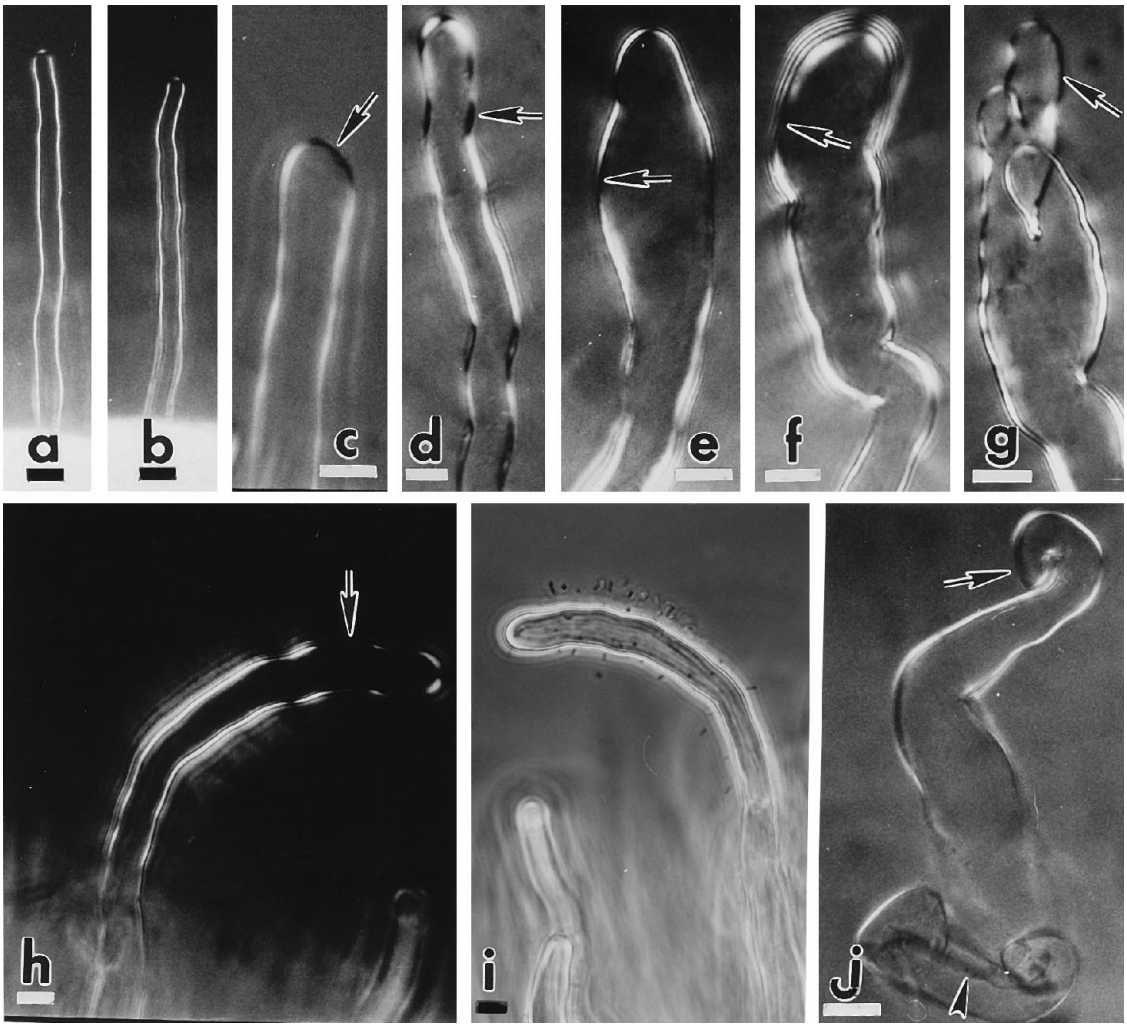

FIG. 4. Influence of rhizobia on crystalline wall architecture of white clover root hairs, as shown by cross-polarized light microscopy. Regions of the wall with ordered crystalline architecture are bright (anisotropic) against a dark background when rotated to maximum extinction and viewed with crossed polars. Unordered or noncrystalline domains in walls appear as dark (isotropic) patches when viewed with the same optics at all extinction rotations. (A and B) Uniform crystallinity of the wall along all but the tip of control root hairs; (C to G) discrete domains of isotropic unoriented patches (arrows) interspersed between anisotropic, ordered crystalline walls of axenic root hairs grown with ANU843 CLOSs and exhibiting various types of deformation; (H to J) deformed and infected root hairs on root inoculated with ANU843 cells. The isotropic disorder of the root hair wall (H) occurs near the tip where bacteria have attached. (I) Inverted phase-contrast micrograph. (J) Isotropic unordered domains in walls of a marked curled root hair with a shepherd crook and an infected root hair. Note that most of the cell wall and all of the intracellular infection thread (arrowhead) within the infected root hair are isotropic. Bars, 20 μm (A and B) and 10 μm (C to J).

quired to trigger all the necessary host responses to express the Hac and Inf phenotypes during primary infection.

Next, we determined if ANU843 CLOSs can rescue any defective phenotypes in primary root hair infection by an isogenic Hac^- Inf^- *nodC*::Tn*5* mutant derivative (13) that is blocked in CLOS production (32). A clue that links CLOSs with primary host infection has been reported for the *Rhizobium* sp. NGR234-siratro symbiosis, where it has been found that a CLOS preparation will induce Hac^+ root hair deformations and rescue the ability of a $NodABC^-$ mutant derivative to induce Fix^+ nodules on this host plant (36). Our studies indicated that inoculated clover plants grown without ANU843 CLOSs developed root hairs that remained straight and undeformed, with no structures indicative of infection (Fig. 6B), consistent with the Had^- Hac^- Inf^- phenotype of $NodC^-$ mutants (13). However, root hairs on plants inoculated with the $NodC^-$ mutant and grown with ANU843 CLOSs developed various deformations (but not shepherd crooks [Hac^-]) and refractive bright spots (Fig. 6C and D) representing localized alterations in the refractive index of the root hair wall that occur prior to penetration by rhizobia early in the infection process (10). These localized alterations in the root hair wall resemble the bright refractive spots induced by rhizobia at the center of the marked curvature of the root hair tip at the origin of the infection thread (46). Further examination of the same seedlings indicated that the refractile spots on root hair walls were distinctly autofluorescent when viewed by epifluorescence microscopy (Fig. 6E and F), indicating the accumulation of fluorescent substances at these sites within the root hair wall, as would result from a localized host defense response. No infection threads were found within root hairs of plants inoculated with the $NodC^-$ mutant and grown with exogenously added ANU843 CLOSs. These results indicate that ANU843 CLOSs alone are sufficient to rescue the ability of a $NodC^-$ mutant to alter the root hair wall and form refractile bright spots as an early integral step of the infection process,

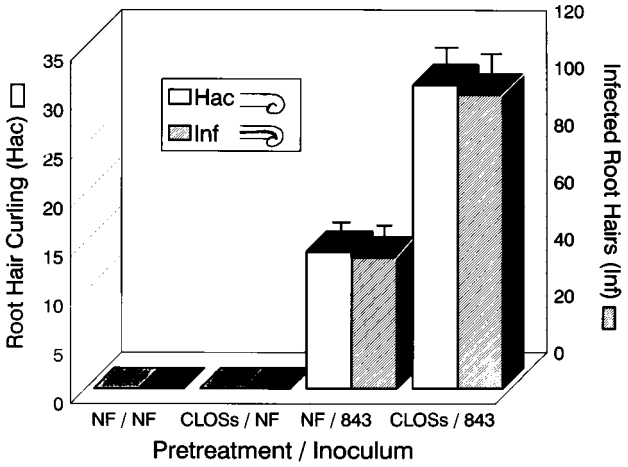

FIG. 5. ANU843 CLOSs promote primary root hair infection in the *Rhizobium*-white clover symbiosis. Seedlings were pretreated with 5×10^{-9} M CLOSs or NF medium before inoculation with cells of ANU843 or NF medium. After 4 days of incubation, root hairs were evaluated for shepherd crook deformations (Hac) along the optical median planes and infected root hairs (Inf) above the root tip mark. Error bars indicate standard deviations of the means.

but this process aborts before formation of the infection thread within the root hair.

In summary, this study shows that wild-type rhizobial membrane CLOSs elicit highly localized, short-range responses that modulate the differentiation, dynamics of growth extension, and crystalline wall architecture of host root hairs. Each of these responses is likely to increase root hair infectibility. Exogenously added CLOSs can also promote the development of new infection sites for wild-type rhizobia but cannot fully replace the NodC function during primary host infection. The latter finding suggests that CLOSs are only one of the contributing factors required for successful entry of the bacteria into the root hair. Other bacterial components, e.g., those involved in symbiont recognition (8–10, 12), further localized degradation of the root hair wall (10, 26, 27), transient suppression or tolerance of host defense responses (12, 39, 40), and sustained development of infection threads (12, 39), would also be needed to accomplish primary host infection in the nitrogen-fixing *Rhizobium*-clover symbiosis.

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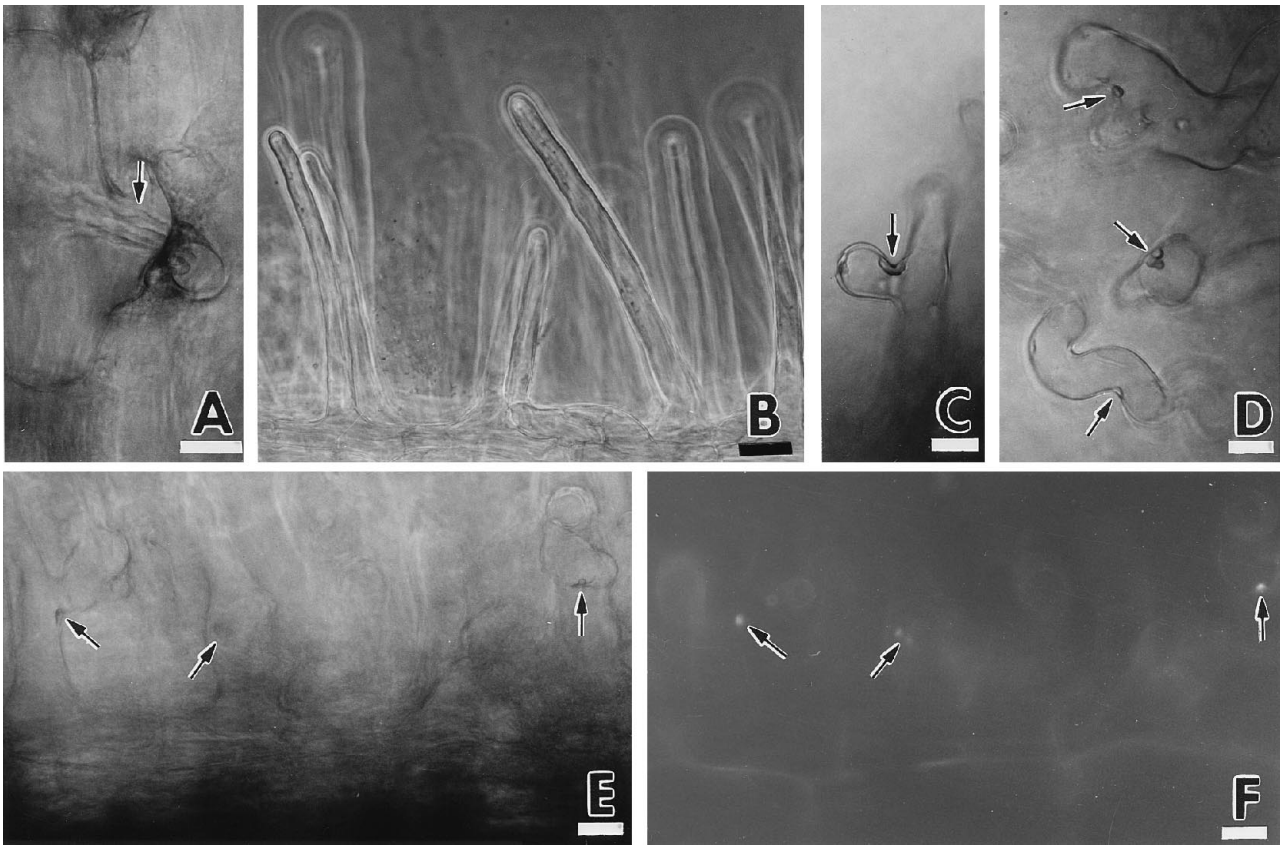

FIG. 6. Infection-related biological activity of ANU843 CLOSs on white clover root hairs. (A) Multiple infection threads (arrows) within an infected root hair on a seedling pretreated with CLOSs and then inoculated with ANU843 cells. (B to F) ANU277*nodC*::Tn*5* inoculum. Root hairs are Had$^-$ Hac$^-$ Inf$^-$ without refractile bright spots without pretreatment with CLOSs (B) but are deformed and develop bright refractile spots (arrows) with pretreatment with CLOSs before inoculation (C to E). These localized refractile spots did not develop into infection threads and were autofluorescent (F, arrows), indicating that root hair infection aborted at this stage and was accompanied by a localized host defense response. Bars, 10 μm (A and C to F) and 20 μm (B).

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