The Role of Nod Factor Substituents in Actin Cytoskeleton Rearrangements in *Phaseolus vulgaris*

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Submitted 15 June 2002. Accepted 25 November 2002.

In order to define the symbiotic role of some of the chemical substituents in the *Rhizobium etli* Nod factors (NFs), we purified Nod metabolites secreted by the SM25 strain, which carries most of the nodulation genes, and SM17 with an insertion in *nodS*. These NFs were analyzed for their capabilities to induce root hair curling and cytoskeletal rearrangements. The NFs secreted by strain SM17 lack the carbamoyl and methyl substituents on the nonreducing terminal residue and an acetyl moiety on the fucosyl residue on the reducing-terminal residue as determined by mass spectrometry. We have reported previously that the root hair cell actin cytoskeleton from bean responds with a rapid fragmentation of the actin bundles within 5 min of NF exposure, and also is accompanied by increases in the apical influxes and intracellular calcium levels. In this article, we report that methyl-bearing NFs are more active in inducing root hair curling and actin cytoskeleton rearrangements than nonmethylated NFs. However, the carbamoyl residue on the nonreducing terminal residue and the acetyl group at the fucosyl residue on the reducing terminal residue do not seem to have any effect on root hair curling induction or in actin cytoskeleton rearrangement.

Additional keywords: FITC-phalloidin, lipochitin-oligosaccharide, microinjection.

Species of the genera *Rhizobium*, *Sinorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, and *Azorhizobium* are gram-negative soil bacteria that elicit the development of root (and sometimes stem) nodules on plant hosts, which the bacteria inhabit as nitrogen-fixing endosymbionts. The plant roots secrete signals, usually flavonoids, which activate transcription of a set of rhizobial *nod* genes. These are then responsible for the synthesis of lipochitin-oligosaccharides, the Nod factors (NFs), which are morphogenetic compounds responsible in part for the specificity of the symbiotic relationship between a bacte-

rium and its host during the development of nitrogen-fixing nodules in leguminous plants (Schultze and Kondorosi 1998; Spaink 2000). These molecules, in turn, act as signals that activate early nodule development in the host plant. In response to NFs, the root hair cells of susceptible plants undergo deformations that lead to the entrapment of and the invasion by the bacterium. Although isolated NFs are unable to induce infection thread formation, they are capable of stimulating most of the early steps of the symbiosis (e.g., depolarization of root hair cells due to changes in ion fluxes as H⁺, Cl⁻, K⁺, and Ca²⁺) (Cárdenas et al. 1999, 2000; Ehrhardt et al. 1992; Felle et al. 1995; Kurkdjian 1995), of which changes in intracellular Ca²⁺ ion concentration have been proposed to be part of the signal transduction process (Cárdenas et al. 1999; Ehrhardt et al. 1996; Felle et al. 1998, 1999; Gehring et al. 1997; Pingret et al. 1998). Thus, NFs permit the experimental analysis of the initial events in signal transduction leading to nodule formation.

It has been reported that chemical substituents on the NFs are important for their biological activity, either for host specificity or enhanced activity; in alfalfa, for example, sulfation of the reducing terminal GlcNAc residue of the NFs synthesized by *Sinorhizobium meliloti* is essential for their biological activity (Felle et al. 1995; Schultze et al. 1992). It has subsequently been demonstrated that the inactive, nonsulfated NFs from *S. meliloti* are able to induce cytoplasmic alkalization to an extent even greater than the active sulfated NFs on alfalfa (Felle et al. 1996). This observation demonstrates that the plant is capable of recognizing NFs with a range of different decorations, and may reflect the existence of a putative ancient receptor in addition to that recognizing the active NFs. Thus, although there is compelling information on the effects of the highly active NFs, little work has been aimed at studying the role of several substituents on the less active NFs on the plant symbiotic response. In order to define the role of some of the chemical substituents of the *Rhizobium etli* NFs in the actin cytoskeleton rearrangements, we analyzed the ability to induce root hair curling with several NFs lacking the methyl and carbamoyl groups produced and secreted by genetically modified strains. We previously have isolated a symbiotic region from *R. etli* strain CE3 carrying essential genes for nodulation and several insertional mutations were generated in these regions (Cevallos et al. 1989).

In this article, we report that NFs produced by some of these mutants were purified and shown, using mass spectrometry, to lack the methyl or carbamoyl substituents on the nonreducing terminal residue and an acetyl group on the fucosyl residue on

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the reducing terminal GlcNAc residue. Purified NFs secreted by a mutant lacking the methyl group on the nonreducing terminal residue were shown to be able to induce root hair deformation and actin cytoskeleton rearrangements, albeit yielding an attenuated response in comparison to that induced by the methylated NFs.

RESULTS

Nonmethylated Nod factors.

In *R. etli* strain CE3, a pSym region harboring *nod* genes called pSM991.25 was previously isolated and partially characterized. This region was mutagenized with a miniMu transposon (Cevallos et al. 1989) resulting in construct pSM17.1 carrying an insertion within a *nodS* gene (Vázquez et al. 1991). Cosmids pSM991.25 and pSM17.1 were transferred independently to CFN 2001 (pSym cured strain) and the resulting strains were termed SM25 and SM17, respectively. The resulting transconjugants were grown in the presence of *L*-[methyl-¹⁴C]-methionine in order to label NFs, and they were analyzed by thin layer chromatography (TLC). Extracts from strains SM25 and CE3 yield a series of radiolabeled spots (Fig. 1, lanes 2 and 3, arrows), while those from CFN 2001 (pSym cured strain) and SM17 (*nodS* mutant) do not show similar labeled spots (Fig. 1, lanes 1 and 4). It was clear that the *nodS* mutant was unable to produce labeled NFs, suggesting the role of this gene in the methylation step. From the three different bands shown in Figure 1, only one seems to be more intense. It is interesting that CE3 and SM25 show similar radiolabeled spots, suggesting that the different NFs from both strains probably could not be differentiated by TLC analysis.

When the growth medium was supplemented with labeled *D*-[1-¹⁴C]-glucosamine and NFs produced by strains SM17 was analyzed again by TLC, it became evident that the *nodS* mutant is able to produce labeled spots corresponding to non-methylated NFs (data not shown).

Structural determination of purified NFs.

In order to obtain large quantities of NFs to allow chemical analysis and biological assays, 1-liter flasks with SM25 and SM17 strains were grown. High performance liquid chromatography (HPLC) separation of SM25 and SM17 extracts after the reverse phase prepurification step gave one major peak (Fig. 2). It should be noted that the peaks in the HPLC chromatogram obtained from the NFs from strain SM25 (upper chromatogram, fraction 18-20) are relatively more intense than those corresponding to the same elution time in the chromatogram obtained from strain SM17 (lower chromatogram, fraction 18-20). This is consistent with our hypothesis that the *nodS* insertion could have a polar effect on the *nodII* genes, which have been demonstrated to have an important role in NF secretion (Cárdenas et al. 1996; Fernández-López et al. 1996; Spaink et al. 1995). In order to demonstrate that the major peaks correspond to the spots observed by TLC, we mixed the purified NFs from SM25 or SM17 with a small amount of *D*-[1-¹⁴C]-glucosamine-labeled crude extracts and then separated it by HPLC. The major peaks from the HPLC analysis were collected and analyzed on reversed-phase silica TLC. These results suggested that the large HPLC peaks isolated from SM25 (fraction 18-20) or SM17 (fraction 16-18) correspond to the major spots observed by TLC (data not shown). It is worth mentioning that even when labeled spots corresponding to the NFs from SM25 and SM17 were similar on TLC analysis (Fig. 1, arrows), the HPLC chromatogram showed a shift between the major peaks (Fig. 2).

For the structural determination, all the fractions corresponding to each individual peak of UV absorbance were pooled and

the resulting peak fractions were pooled, dried, and redissolved in 25% acetonitrile for further analysis by mass spectrometry. Samples from strain SM25 corresponding to pooled fractions 16-17 and 18-20 and single fractions 21-25, and samples from strain SM17 corresponding to pooled fractions 16-18 and 19-21, were collected and submitted to fast atom bombardment mass spectrometric analysis (Table 1). The samples corresponding to fractions 16-18 and 19-21 from strain SM17 yielded intense NF-derived signals. The mass spectrum obtained from SM17 fraction 16-18 yielded two sets of pseudomolecular ions that can be assigned as arising from nonmethylated NFs as follows: V (18:1, Fuc), ions were observed at *m/z* values of 1402 ([M+H]⁺), 1424 ([M+Na]⁺), 1510 (thioglycerol adduct [Fukuda et al. 1985] of [M+H]⁺) and 1532 (thioglycerol adduct [Fukuda et al. 1985] of [M+Na]⁺); and IV (16:0, Fuc), ions were observed at *m/z* values of 1173 ([M+H]⁺) and 1195 ([M+Na]⁺). In addition, due to the large quantities of NFs in the sample, fragment ions derived from each of the NFs present also were observed in the spectrum. These confirm the structural assignments and demonstrate that the fucosyl residue is located on the reducing-terminal GlcNAc residue in each of the NFs. From the pentamer, these fragment ions were observed at *m/z* 426 (B₁), 534 (B₁ + thioglycerol), 629 (B₂), 737 (B₂ + thioglycerol), 832 (B₃), 940 (B₃ + thioglycerol), 1035 (B₄), and 1143 (B₄ + thioglycerol). From the tetramer, fragment ions were observed at *m/z* values of 400 (B₁), 603 (B₂), and 806 (B₃). However, the most intense ion in the spectrum was for pentamers.

The spectrum obtained from SM17 fraction 19-21 contained even more intense pseudomolecular ions corresponding to a

Fig. 1. Thin layer chromatography of labeled Nod factors (NFs) from strains CFN2001 (pSym cured strain) in lane 1, SM25 (cosmid carrying most of the *nod* region) in lane 2, *Rhizobium etli* (wild type) in lane 3, and SM17 (*nodS* mutant) in lane 4. Arrows indicate labeled NFs resulting from incorporation of *L*-[methyl-¹⁴C]-methionine; the arrow in the middle indicates the major peak.

nonmethylated NFs species, at m/z values of 1199 ($[M+H]^+$ for IV [18:1, Fuc]) and 1307 (its thioglycerol adduct). Fragment ions that confirm the structural assignment and allow the site of the fucosyl residue on the reducing-terminal GlcNAc residue to be assigned were observed at m/z 426 (B_1), 534 (B_1 + thioglycerol), 629 (B_2), 737 (B_2 + thioglycerol), 832 (B_3), and 940 (B_3 + thioglycerol).

Intense mass spectrometric signals for ions corresponding to methylated NFs were obtained from all fractions from strain SM25 and, in addition, fragment ions (B-ions) were observed that confirm the structural assignments. These are summarized in Table 1. All NFs from strain SM25 bear methyl groups attached to the nonreducing terminal glucosamine residue and vary principally in the length and degree of unsaturation of the fatty acyl chain. These NFs also lack the acetyl group on the fucose residue and the carbamoyl group on the nonreducing terminal residue. This is consistent with the demonstration that SM25 lacks the *nolL* and *nolO* genes. These genes are responsible for the acetylation and carbamoylation of the NFs (Corvera et al. 1999; Jabbouri et al.

1998). We summarize the characteristics of the NFs isolated from each strain and indicate which were used for further analysis in Table 1. In general, our results demonstrate that the most intense ion in the spectrum of fraction 18-20 from SM25 is that at m/z 1438 for V (18:1, Me, Fuc) and the most intense ion in the spectrum of SM17 fraction 16-18 is that at m/z 1402 for V (18:1, Fuc).

Biological activity.

Purified NFs from strain SM25 (fraction 18-20), which are mainly pentameric (18:1, Me, Fuc), were able to induce root hair deformation to the same extent as NFs from the wild-type *R. etli* strain CE3; the latter are also pentameric (18:1, 18:0, *O*-acetyl-fucose, Met, Cb) (Cárdenas et al. 1995). The NFs and the control chitin oligomer (pentamer) concentration were at 10^{-8} M (Cárdenas et al. 1999). The root hair cells responded to the NFs from strain SM25 with swelling at the tip and, later on, with root hair deformation (data not shown) even when the NFs are noncarbamoylated and acetylated. These results resemble the biological response induced by the wild-type NFs

Fig. 2. High performance liquid chromatography (HPLC) analysis of Nod factor (NF) extracts from strain SM25 and SM17. *n*-Butanol extracts previously were prepurified with a reversed-phase extraction column. Then, prepurified samples were purified with an HPLC reversed-phase C_{18} column using the indicated acetonitrile/water gradient and fractions were collected at indicated times. The major peak in each chromatogram is labeled with dotted line at the base and the main NFs composition at the top is indicated.

(Cárdenas et al. 1995, 2000) at the same concentration. Neither of these responses was observed on exposure to the chitin pentamer as a control, even at a higher concentration (10^{-7} M) (data not shown).

NFs from strain SM17 (fraction 16-18) which are composed mainly of pentameric species (18:1, Fuc) also contained some traces of tetrameric NFs with a different fatty acid (16:0), but all components of the mixture lack the methyl group on the nonreducing terminal residue. These mixtures of NFs were able to induce root hair deformation, but this response is delayed by approximately 4 h when compared with that induced by the methylated NFs (data not shown). When we tested an increased concentration of SM17 NFs (10^{-7} M), this did not result in faster responses, indicating that the delayed response is most likely related to the lack of the methyl group. Thereafter, we selected 10^{-8} M as the standard concentration for the future experiments. In all examples, the root hairs responding to the NFs were located in region II, the region where they are rapidly growing (data not shown). This observation stands in contrast to that in the literature (Heidstra et al. 1994), which reports that the NF-responsive root hairs are the old ones and had almost stopped growing.

Actin cytoskeleton responses to methylated NFs.

In order to determine the role of the isolated NFs on actin cytoskeleton rearrangement, we treated the bean root hair cells with the different NFs and analyzed the actin cytoskeleton by microinjecting fluorescent phalloidin at different times using living root hair cells and no chemical fixation procedure. We had 20% success with the microinjection approach, selecting those cells with cytoplasmic streaming, growing, and enough phalloidin to stain the actin cytoskeleton. In 80% of the cases, the needle went into the vacuole or the cell stopped streaming. These were discarded for further studies.

The actin cytoskeleton in root hair cells exposed to the chitin oligomers (GlcNAc₃) as a control is presented in Figure 3 (A, B, and C). The actin organization is observed as large actin bundles (Fig. 3, arrow heads) running the length of the cells until the proximity of the apical dome where F-actin staining becomes diffuse (Fig. 3, asterisks). In these hair cells, the nucleus is covered by actin microfilaments as determined by the cortical sectioning and always anchored to the actin bundles (Fig. 3, arrows); these bundles are also the tracks for the transvacuolar strands. This organization is similar if we remove the chitin oligomers, indicating that they do not alter the actin architecture. Two root hair cells, after 5 min of being exposed to the methylated NFs isolated from strain SM25, are shown in Figure 4A. These cells show a marked increase in diffuse staining, which is interpreted as

fragmentation of the actin cytoskeleton (Fig. 4A, arrow heads). This response could be faster than 5 min, but our set up was limited to this time, mainly because we had to add the Nod factor, then do the microinjection and acquire the images. Interestingly, these effects are similar to those previously described for the wild-type *R. etli* NFs, which are *O*-acetyl fucosylated, carbamoylated, and methylated (Cárdenas et al. 1995, 1998). We observe that, after approximately 30 min (average 30.7 min, standard deviation 2.89 min) of continued exposure to NFs from SM25, long microfilament cables start to recover (Fig. 4B). It can be observed that, although the reorganization of the actin bundles is evident, fragmented actin filaments remain at the apical dome (Fig. 4B, asterisk). These results are consistent in approximately 70% of the examples. In the next 30% of the cases, the root hairs did not show any responses or simply responded with fragmentation but never recovered into bundles. Whenever we tried to observe actin organization after 5 to 25 min, all the cells had no recovered actin bundles at this time.

Then we selected root hair cells that, after 70 min of NF exposure, had restarted growth after swelling. Actin accumulation observable in a longitudinal optical section across the nucleus is presented in Figure 4B (arrow). Actin organization in responding hair cells after 70 min of NF treatment, when the root hairs reinitiate growth and the curling response begins is shown in Figure 4C. In all the cases, it can be observed that an area with a high concentration of fragmented actin precedes the site of reinitiation (Fig. 4C-b, asterisks), which is not observed in a control root hair cell (Fig. 3).

Actin cytoskeleton reorganization in response to nonmethylated NFs.

Root hair cells were exposed to the nonmethylated NFs isolated from SM17 (V, 18:1, Fuc, and some IV, 16:0, Fuc) and, after 5 min, we microinjected fluorescein phalloidin. We found that root hair cells responded with fragmentation of actin bundles (Fig. 5A), albeit to a different extent than the ones induced by the methylated NFs (Fig. 4A). In all instances, we observed an accumulation of diffuse actin staining at the apical dome (Fig. 5A, asterisk). Actin bundles recovered after 20 min (average 20.9 min, standard deviation 2.37), which is faster than those induced by the methylated NFs (Fig. 5B-a, arrowhead). Root hair cells also accumulated actin fragments in the apical region after 3 h, which seem to precede the new tip growth (Fig. 5C-a, asterisk), even when the root hair is not totally swollen. Again, the site with the higher concentration of fragmented actin indicates the site where redirection of growth will resume. It should be noted, from the optical sectioning of the nucleus in Figure 5C-b, that it is not as bright as observed in Figure 4B-b.

Table 1. Summary of mass spectrometric data obtained from high performance liquid chromatography fractions containing nod factors (NFs) from strain SM17 and strain SM25

NF structure	<i>m/z</i> of pseudomolecular ion ^a	Found in fraction number	<i>m/z</i> values of fragment ions (B-ions)
V (18:1, Fuc) ^a , fraction used	1402/1510 (+TG), 1424 [M+Na] ⁺ /1532 (+TG)	SM17, 16-18	426, 629, 832, 1035
IV (16:0, Fuc) ^a , fraction used	1173, 1195 [M+Na] ⁺	SM17, 16-18	400, 603, 806
IV (18:1, Fuc)	1199/1307 (+TG)	SM17, 19-21	426, 629, 832
V (16:0, Me, Fuc)	1390	SM25, 16-17	414, 617, 820, 1023
V (18:1, Me, Fuc) ^a , fraction used	1416/1524 (+TG), 1438 [M+Na] ⁺ /1546 (+TG)	SM25, 16-17, 21, 18-20	440, 643, 846, 1049
IV (18:1, Me, Fuc)	1213/1321 (+TG)	SM25, 21, 22, 23	440, 643, 846
V (18:1, Me)	1270/1378 (+TG)	SM25, 21	440, 643, 846, 1049
V (18:0, Me, Fuc)	1418	SM25, 24, 25	442, 645, 848, 1051
IV (18:0, Me, Fuc)	1215	SM25, 25	442, 645, 848
V (18:0, Me)	1272	SM25, 25	442, 645, 848, 1051

^a [M+H]⁺ unless otherwise stated: +TG = thioglycerol adduct.

DISCUSSION

We have found that methylated *R. etli* NFs are more active in inducing *Phaseolus vulgaris* root hair deformation and actin cytoskeleton rearrangement than nonmethylated NFs. However, nonacetylation and noncarbamoylation of the NFs do not seem to have a strong effect on these root hair cells. In this regard, root hair cells responding to the methylated, nonacetylated, and noncarbamoylated NFs produced by SM25 are functionally equivalent to the NFs from wild-type *R. etli* strain CE3. Furthermore, SM25 nodulates Negro Jamapa bean plants (data not shown) in a way similar to *R. etli* strain CE3, indicating that acetylation and carbamoylation of NFs do not play an important role in nodule formation, at least in Negro Jamapa bean. The above statement also is supported by the fact that *R. etli* mutated in *nolL*, the gene encoding the protein responsible for *O*-acetylation of the fucose on the reducing terminal residue of the NFs, has an insignificant effect on *P. vulgaris* (Negro Jamapa) nodulation (Corvera et al. 1999). Thus, the similarity between the responses in root hair deformation and actin cytoskeleton reorganization with acetylated or nonacetylated *R. etli* NFs supports the idea that these chemical substituents are not essential for bean nodulation. However, this does not exclude a possible role for acetylation or carbamoylation in nodulating other cultivars, possibly by increasing nodulation efficiency (Corvera et al. 1999) or preventing NF degradation by plant chitinases as shown in *S. meliloti* (Schultze et al. 1998; Staehelin et al. 1994; Stokkermans et al. 1995). Mass spectrometry indicated that the NFs from SM17 are not methylated and that the presence of *nodS* in SM25 results in the production of NFs decorated with the methyl substituent. It strongly suggests the role of *nodS* in the methylation step for the NFs in *R. etli*, and confirms the phenotypic role of NodS as an *S*-adenosyl-L-methionine-dependent methyltransferase (Geelen et al. 1995; Jabbouri et al. 1995; Lewin et al. 1990; Villalobos et al. 1994; Waelkens et al. 1995). On the other hand, *nodU* involved

in 6-*O*-carbamoylation of *Rhizobium* sp. NGR234 NFs (Jabbouri et al. 1995) is truncated in *R. etli* (*unpublished data*). However, *nolO* reported as being implied in 3-*O*-carbamoylation of NGR234 NFs (Jabbouri et al. 1998), although present in *R. etli* pSym, is not located within SM25 and SM17 strains (*unpublished data*), resulting in a noncarbamoylated NF in these strains.

We have found that the more active the NFs are in eliciting root hair deformation (methylated NFs), the faster actin cytoskeleton rearrangements occur. This supports our previous hypothesis concerning the role of actin cytoskeleton in nodulation and growth polarity (Cárdenas et al. 1998), probably through the activation of actin-severing proteins such as villin, which is calcium regulated (Gungabissoon et al. 1998; Vidali et al. 1999), and could be important in modulating reorganization prior to infection thread formation. In addition, calmodulins also could play a role as demonstrated for primordia development (Camas et al. 2002). In this regard, the study of Felle and associates (1998) showing that Ca^{2+} ionophore A-23187 mimics NF-induced events in alfalfa root hair cells, lends support to the idea that Ca^{2+} influx is a primary step in the signal transduction cascade. Furthermore, we have described apical Ca^{2+} increases in bean root hair cells soon after NF application (Cárdenas et al. 1999) and correlated it with actin rearrangements at the root hair tip zone, suggesting that both processes are closely related. On the other hand, the role of nuclear calcium spiking in *Medicago truncatula* root hairs after NF treatment also has been reported, but *DMI1* and *DMI2* mutants are defective in this response and in root hair deformation (Ehrhardt et al. 1996; Wais et al. 2000; Walker et al. 2000). Interestingly, *DMI3*, a mutant that is able to induce root hair deformation, maintains the capability for calcium spiking. These observations suggest that calcium spiking, which occurs after calcium influxes at the tip, could be responsible for the activation of the genes necessary for the infection and nodulation process.

Fig. 3. Actin cytoskeleton arrangements in control root hair cells exposed to the chitin-oligomer (pentamer). **A**, **B**, and **C** show the actin cytoskeleton as bundles running the length of the cell (see arrowhead) and tip dome showing a diffuse F-actin staining (see asterisk). Note actin bundles, which appear to be bound to actin filaments surrounding the nucleus in cell **C** (see arrow).

What is the physiological role for each chemical modification on the NFs, and why do *Rhizobium* spp. produce a whole mixture of them? These are questions that have been only partially answered (Schultze et al. 1992). It is interesting that the nonmethylated NFs induced major actin rearrangements only in the apical region, whereas the rest of the cell responded with a weak fragmentation. The current model suggests that NF modifications could have a particular effect on plant recognition. It has been reported that, in *S. meliloti*, three structural parameters, including the length of the oligosaccharide chain, the acylation of the nonreducing terminal residue, and the sulfation of the reducing terminal residue, influence the stability of NFs to degradation by root chitinases from *Medicago* spp. (Staehelin et al. 1994). Interestingly, sulfated NFs, which are active in inducing deformation, resulted in increased resistance to hydrolysis by chitinases, suggesting specific roles for the chemical substituents. In a more recent study, Staehelin et al. (2000) reported that *N*-deacetylation of *S. meliloti* NFs increases their stability in the rhizosphere but decreases their biological activity.

Our results suggest that methylated NFs are more active in inducing severing of large actin bundles than nonmethylated NFs. However, the nonmethylated NFs have a similar effect in inducing actin fragmentation in the tip region without much fragmentation in the rest of the cell. This is the first time that a differential effect on actin cytoskeleton rearrangement has been described in response to specific NFs. Furthermore, after 3 h, the nonmethylated NFs induce a high localized region of fragmented actin prior to growth reinitiation. This is not a result of NF concentration, as demonstrated by increasing the concentration from 10^{-8} to 10^{-7} M, which did not result in an increased actin breakdown. This result shows that nonmethylated NFs could be more specific in inducing changes at the tip region, which could allow the bacteria to modulate rearrangements by producing diverse specific NFs when needed. However, actin reorganization in the rest of the cell seems to be very important for a faster response. This is not the only example of differential activities in NF signaling; in alfalfa root hair cells, the sulfated NFs, which are active in root hair curling, are able to induce cytoplasmic alkalization with membrane depolarization; however, the unsulfated NFs, which are not able to induce root hair deformation, can induce cytoplasmic alkalization without membrane depolarization (Felle et al. 1996). This supports the idea of a specific role for every chemical modification in the NFs. It will be interesting to analyze the effect on Ca^{2+} changes for every NF modification and to correlate them with localized changes in actin cytoskeleton.

MATERIALS AND METHODS

Bacterial strains, cosmids, and growth conditions.

R. etli strain CE3 is the corresponding wild type (Noel et al. 1984). CFN2001 is the pSym cured strain derived from strain CE3 (Palacios et al. 1983). SM25 and SM17 are strain CFN2001 carrying the cosmid pSM991.25 or pSM17.1, respectively, which will be further described. Nodulation genes from *R. etli* have been isolated in a cosmid harboring most of the nodulation genes, pSM991.25 (Cevallos et al. 1989; Vázquez et al. 1991). This cosmid lacks *nolL* and *nolO* genes (*unpublished data*). Insertional mutations on pSM991.25 were carried out with a minitransposon (miniMu) (Vázquez et al. 1991). Heterologous Southern blot analysis (data not shown) and nucleotide sequencing identified an insertion in *nodS*, and we named it pSM17.1. Cosmids pSM991.25 and pSM17.1 were mobilized independently by triparental mating to the non-nodulating CFN 2001 (pSym cured strain). The resulting strains were named SM25 (Tc^r , Rif^r) and SM17 (Km^r , Tc^r , Rif^r), respectively.

Growth conditions were as previously reported (Cárdenas et al. 1995, 1996).

Isolation

of pSM17.1 and pSM991.25 NFs and analysis by TLC.

To determine the lack of methylation activity, we used *L*-[methyl- ^{14}C]-methionine or *D*-[1- ^{14}C]-glucosamine as a labeled

Fig. 4. Actin cytoskeleton rearrangements in bean root hair cells responding to methylated Nod factors (NFs) produced by strain SM25 (V, 18:1, Me, Fuc). **A**, Large actin bundles break down after 5 min of NF exposure (see arrowhead); fragmented actin accumulates at the tip dome (see asterisk). **B**, Actin bundles restart to form (see arrowhead) after 30 min of NF exposure; the tip still remains with fragmented actin (see asterisk). The nucleus shows a particular actin accumulation in this region (see arrow). **C**, Swollen root hair after 4 h with NFs; actin fragments remain at the tip and they seem to precede the redirection of growth (see asterisk).

precursor for NFs synthesis and the labeled products were analyzed by TLC as described (Cárdenas et al. 1995).

Purification by HPLC.

In order to obtain sufficiently large quantities of NFs, 1-liter cultures of strains SM25 and SM17 were grown as previously reported (Cárdenas et al. 1995). After growth, 500 ml of *n*-

butanol was added to extract the NFs from the culture; then, the recovered extracts were brought to dryness under vacuum. The concentrates containing the NFs were redissolved in 20 ml of 50% aqueous acetonitrile solution with vigorous shaking for 15 h, then concentrated on an octadecyl extraction column (J. T. Baker) as a prepurification step. The column was eluted stepwise with at 20, 40, 60 and 80% aqueous acetonitrile, but only the 60% fraction was collected because this was expected to contain the NFs. This fraction was purified using reversed-phase HPLC with a Nova-Pak C18 column (Water, Milford, MA) and an isocratic gradient of at 20, 30, 40, and 60% acetonitrile:water for 5, 30, 30, and 15 min, respectively. Then we used a linear gradient over 10 min from 60 to 100% acetonitrile. The HPLC elution was performed at a flow rate of 0.7 ml/min, and the eluent was monitored at 206 nm. The samples were analyzed using fast atom bombardment mass spectrometry.

Fast atom bombardment mass spectrometric analysis of Nod factors.

Positive ion fast atom bombardment mass spectra were obtained from the HPLC fractions using MS1 of a JEOL JMS-SX/SX102A tandem mass spectrometer operated at 10 kV accelerating voltage. The Fab gun was operated at 6 kV accelerating voltage with an emission current of 10 mAmps and using Xenon as the bombarding gas. The NFs in the dried HPLC fractions were redissolved in 20 μ l of dimethyl sulfoxide and 1 μ l of solution was loaded into approximately 2 μ l of a matrix of monothioglycerol. Spectra were scanned at a speed of 30 s for the full mass range specified by the accelerating voltage used, and were recorded and averaged on a Hewlett Packard HP9000 data system running JEOL COMPLEMENT software.

Plant growth conditions and treatment.

Seed from *P. vulgaris* cv. Negro Jamapa were germinated in absorbent towel paper for 2 days as reported (Cárdenas et al. 1999). Germinated seed then were transferred to liquid Fåhræus medium and maintained for 24 h. At this time, the roots became approximately 3 cm in length and rich with growing root hair cells, which were used to assay responses to the NFs.

NFs from SM25 and SM17 strains were purified by HPLC, then resuspended in 1% (wt/vol) of a nondenaturing zwitterionic detergent CHAPS (3-[3-cholamidopropyl]-dimethylammonia)-1-propane-sulfonate). NFs were mixed with 0.5 ml of the Fåhræus medium and then added gently to the growing root hairs to replace the NFs free medium. It contained NFs at 10^{-8} M and CHAPS to 0.001% final concentration. Penta-*N*-acetylchitopentaose (Seikagaku America, Inc., Tokyo) at 10^{-8} M, dissolved in CHAPS under the same condition as NFs, was used as a control.

Mounting living root hairs.

Intact seedlings containing the growing root hairs were mounted in chambers constructed on glass cover slips forming a well and visualized under the microscope (Diaphot 300, Nikon) with a $\times 40$ water immersion lens, N.A 0.75 (Zeiss, Thornwood, NY, U.S.A.). No mounting substance was needed and the well was filled with approximately 0.5 ml of the Fåhræus medium.

Microinjection of fluorescent phalloidin.

Microneedles were pulled in a vertical pipette puller (Kopf model 700D, David Kopf Instruments, Tujunga, CA, U.S.A.) from filamented capillaries (World Precision Instruments, Sarasota, FL, U.S.A.). FITC-phalloidin (Molecular Probes, Eugene, OR, U.S.A.) was prepared as a 20- μ M stock solution in methanol at 10%. Before use, these were sonicated and spun

Fig. 5. Actin cytoskeleton responses in bean root hair cells responding to nonmethylated Nod factors (NFs) isolated from strain SM17 (V, 18:1, Fuc and IV, 16:0, Fuc). **A**, Root hairs after 5 min of NF addition; fragmented actin accumulate more to the tip region (see asterisk), and there is not a dramatic breakdown of the actin bundles; however, long actin bundles still persist. **B**, After 20 min, actin bundles appear well recovered (see arrowhead); accumulated fragments of actin at the tip remain. **C**, Root hair after 3 h of NF treatment; large actin bundles are evident, as well as a rich region of fragmented actin preceding the redirection of growth (see asterisk).

at $5,000 \times g$ to remove particles, which can clog the needle. The microneedle was back-filled with 1 μ l of the indicator dye and microinjected into root hairs by pressure. The living root hairs, mainly those in the region corresponding to zone II (Heidstra et al. 1994), were selected and treated with NFs or the control chitin-oligomer prior to loading them with FITC-phalloidin as previously described (Cárdenas et al. 1998). Microinjections were carried out along the shank of the root hair, never at the extreme apex. Once the cells were loaded, the actin cytoskeleton was visualized under the confocal microscope.

ACKNOWLEDGMENTS

This work was supported by grants from Dirección General de Asuntos del Personal Académico/Universidad Nacional Autónoma de México (DGAPA/UNAM; no. IN 212298, IN 212298, IN 200800) and Consejo Nacional de Ciencia y Tecnología (CONACYT), México (no. N-33056 to C.Q.), and the United States National Science Foundation (no. MCB-0077599 to PKH). L. Cárdenas was supported by a scholarship from CONACYT and an installation grant for young scientists (no. I 29972-N). We thank H. P. Spaink for providing support for NF purification, Marga Harteveld for maintaining the HPLC system, and Olivia Santana for technical support.

LITERATURE CITED

- Camas, A., Cárdenas, L., Quinto, C., and Lara, M. 2002. Expression of different calmodulin genes in bean (*Phaseolus vulgaris* L.): Role of Nod factor on calmodulin gene regulation. *Mol. Plant-Microbe Interact.* 15:428-436.
- Cárdenas, L., Domínguez, J., Quinto, C., López-Lara, I. M., Lugtenberg, B., Spaink, H., Rademarker, G. H., Haverkamp, J., and Thomas-Oates, J. E. 1995. Isolation, chemical structure and biological activity of the lipo-chitin oligosaccharide nodulation signal from *Rhizobium etli*. *Plant Mol. Biol.* 29:453-464.
- Cárdenas, L., Domínguez, J., Santana, O., and Quinto, C. 1996. The role of *nodI* and *nodJ* genes in the transport of Nod metabolites in *Rhizobium etli*. *Gene* 173:183-187.
- Cárdenas, L., Feijó, J. A., Kunkel, J. G., Sánchez, F., Holdaway-Clarke, T. L., Hepler, P. K., and Quinto, C. 1999. *Rhizobium* Nod factors induce increases in intracellular free calcium and extracellular calcium influxes in bean root hairs. *Plant J.* 19:347-352.
- Cárdenas, L., Holdaway-Clarke, T., Sánchez, F., Quinto, C., Feijó, J. A., Kunkel, J., and Hepler, P. K. 2000. Ion changes in legume root hairs responding to Nod factors. *Plant Physiol.* 123:1-9.
- Cárdenas, L., Vidali, L., Domínguez, J., Pérez, H., Sánchez, F., Hepler, P. K., and Quinto, C. 1998. Rearrangement of actin microfilaments in plant root hairs responding to *Rhizobium* nodulation signals. *Plant Physiol.* 116:871-877.
- Cevallos, M., Vázquez, M., Dávalos, A., Espín, G., Sepúlveda, J., and Quinto, C. 1989. Characterization of *Rhizobium phaseoli* Sym plasmid regions involved in nodule morphogenesis and host range specificity. *Mol. Microbiol.* 3:879-889.
- Corvera, A., Promé, D., Promé, J.-C., Martínez-Romero, E., and Romero, D. 1999. The *nolL* gene from *Rhizobium etli* determines nodulation efficiency by mediating the acetylation of the fucosyl residue in the nodulation factor. *Mol. Plant-Microbe Interact.* 12:236-246.
- Ehrhardt, D. W., Atkinson, E. M., and Long, S. R. 1992. Depolarization of alfalfa root hair membrane potential by *Rhizobium meliloti* Nod factors. *Science* 256:998-1000.
- Ehrhardt, D. W., Wais, R., and Long, S. R. 1996. Calcium spiking in plant root hairs responding to *Rhizobium* nodulation signals. *Cell* 85:673-681.
- Felle, H. H., Kondorosi, E., Kondorosi, A., and Schultze, M. 1995. Nod signal-induced plasma membrane potential changes in alfalfa root hairs are differently sensitive to structural modifications of the lipochitooligosaccharide. *Plant J.* 7:939-947.
- Felle, H. H., Kondorosi, E., Kondorosi, A., and Schultze, M. 1996. Rapid alkalization in alfalfa root hairs in response to rhizobial lipochitooligosaccharide signals. *Plant J.* 10:295-301.
- Felle, H. H., Kondorosi, E., Kondorosi, A., and Schultze, M. 1998. The role of ion fluxes in Nod factor signaling in *Medicago sativa*. *Plant J.* 13:455-463.
- Felle, H. H., Kondorosi, E., Kondorosi, A., and Schultze, M. 1999. Elevation of the cytosolic free $[Ca^{2+}]$ is indispensable for the transduction of the Nod factor signal in alfalfa. *Plant Physiol.* 121:273-279.
- Fernández-López, M., D'Haese, W., Mergaert, P., Verplancke, C., Prome, J. C., Van Montagu, M., and Holsters, M. 1996. Role of *nodI* and *nodJ* in lipo-chitooligosaccharide secretion in *Azorhizobium caulinodans* and *Escherichia coli*. *Mol. Microbiol.* 20(5):993-1000.
- Fukuda, M. N., Dell, A., Oates, J. E., Wu, P., Klock, J. C., and Fukuda, M. 1985. Structure of glycosphingolipids isolated from human granulocytes. The presence of a series of linear poly-*N*-acetylactosaminyl-ceramide and its significance in glycolipids of whole blood cells. *J. Biol. Chem.* 260:1067-1082.
- Geelen, D., Leyman, B., Mergaert, P., Klarskov, K., Van Montagu, M., Geremia, R., and Holsters, M. 1995. *NodS* is an S-adenosyl-L-methionine-dependent methyltransferase that methylates chitooligosaccharides deacetylated at the non-reducing end. *Mol. Microbiol.* 17(2):387-397.
- Gehring, C. A., Irving, H. R., Kabbara, A. A., Parish, R. W., Boukli, N. M., and Broughton, W. J. 1997. Rapid, plateau-like increases in intracellular free calcium are associated with Nod-factor-induced root-hair deformation. *Mol. Plant-Microbe Interact.* 7:791-802.
- Gungabissoon, R. A., Jiang, C. J., Drobak, B. K., Maciver, S. K., and Hussey, P. J. 1998. Interaction of maize actin-depolymerising factor with actin and phosphoinositides and its inhibition of plant phospholipase C. *Plant J.* 16:689-696.
- Heidstra, R., Geurts, R., Franssen, H., Spaink, H. P., van Kammen, A., and Bisseling, T. 1994. Root hair deformation activity of nodulation factors and their fate on *Vicia sativa*. *Plant Physiol.* 105:787-797.
- Jabbouri, S., Fellay, R., Talmont, F., Kamalaprija, P., Burger, U., Relic, B., Prome, J. C., and Broughton, W. J. 1995. Involvement of *nodS* in *N*-methylation and *nodU* in 6-O-carbamoylation of *Rhizobium* sp. NGR234 nod factors. *J. Biol. Chem.* 270(39):22968-22973.
- Jabbouri, S., Relic, B., Hanin, M., Kamalaprija, P., Burger, U., Prome, D., Prome, J. C., and Broughton, W. J. 1998. *nolO* and *noelI* (HsnIII) of *Rhizobium* sp. NGR234 are involved in 3-O-carbamoylation and 2-O-methylation of Nod factors. *J. Biol. Chem.* 273(20):12047-12055.
- Kurkdjian, A. C. 1995. Role of the differentiation of root epidermal cells in Nod factor from *Rhizobium meliloti*-induced root-hair depolarization of *Medicago sativa*. *Plant Physiol.* 107:783-790.
- Lewin, A., Cervantes, E., Chee-Hoong, W., and Broughton, W. J. 1990. *nodSU*, two new nod genes of the broad host range *Rhizobium* strain NGR234 encode host-specific nodulation of the tropical tree *Leucaena leucocephala*. *Mol. Plant-Microbe Interact.* 3(5):317-326.
- Noel, K. D., Sánchez, A., Fernández, L., Leemans, J., and Cevallos, M. A. 1984. *Rhizobium phaseoli* symbiotic mutants with transposon Tn5. *J. Bacteriol.* 158:148-155.
- Palacios, R., Quinto, C., De la Vega, H., Flores, M., Fernández, L., Hernández, M., Ballado, T., and Soberon, G. 1983. General organization of nitrogen fixation genes in *Rhizobium phaseoli*. Pages 164-168 in: *Molecular Genetics of Bacteria-Plant Interaction*. A. Pühler, ed. Springer Verlag, Berlin.
- Pingret, J. L., Journet, E. P., and Barker, D. G. 1998. *Rhizobium* Nod factor signaling: evidence for a G protein-mediated transduction mechanism. *Plant Cell* 10:659-671.
- Schultze, M., and Kondorosi, A. 1998. Regulation of symbiotic root nodule development. *Annu. Rev. Genet.* 32:33-57.
- Schultze, M., Quiclet-Sire, B., Kondorosi, E., Virelizier, H., Glushka, J. N., Endre, G., Gero, S. D., and Kondorosi, A. 1992. *Rhizobium meliloti* produces a family of sulphated lipo-oligosaccharides exhibiting different degrees of the plant host specificity. *Proc. Natl. Acad. Sci. U.S.A.* 89:192-196.
- Schultze, M., Staehelin, C., Brunner, F., Genetet, I., Legrand, M., Fritig, B., Kondorosi, E., and Kondorosi, A. 1998. Plant chitinase/lysozyme isoforms show distinct substrate specificity and cleavage site preference towards lipochitooligosaccharide Nod signals. *Plant J.* 16:571-580.
- Spaink, H. P. 2000. Root nodulation and infection factors produced by rhizobial bacteria. *Annu. Rev. Microbiol.* 54:257-88.
- Spaink, H. P., Wijffjes A. H., and Lugtenberg, B. J. 1995. *Rhizobium* NodI and NodJ proteins play a role in the efficiency of secretion of lipochitin oligosaccharides. *J. Bacteriol.* 177 (21):6276-81.
- Staehelin, C., Schultze, M., Kondorosi, E., Mellor, R., Boller, T., and Kondorosi, A. 1994. Structural modifications in *Rhizobium meliloti* Nod factors influence their stability against hydrolysis by root chitinases. *Plant J.* 5:319-330.
- Staehelin, C., Schultze, M., Tocuyaso, K., Poinot, V., Prome, J.-C., Kondorosi, E., and Kondorosi, A. 2000. *N*-deacetylation of *Sinorhizobium meliloti* Nod factors increases their stability in the *Medicago sativa* rhizosphere and decreases their biological activity. *Mol. Plant-Microbe Interact.* 13:72-79.
- Stokkermans, T. J., Ikeshita, S., Cohn, J., Carlson, R. W., Stacey, G., Ogawa, T., and Peters, N. K. 1995. Structural requirements of synthetic and natural product lipo-chitin oligosaccharides for induction of nodule primordia on *Glycine soja*. *Plant Physiol.* 108:1587-1595.

- Vázquez, M., Dávalos, A., de las Peñas, A., Sánchez, F., and Quinto, C. 1991. Novel organization of the common nodulation genes in *Rhizobium leguminosarum* bv. *phaseoli* strains. J. Bacteriol. 173:1250-1258.
- Vidali, L., Yokota, E., Cheung, A. Y., Shimmen, T., and Hepler, P. K. 1999. The P-135-ABP from *Lilium longiflorum* pollen is the plant homologue of villin. Protoplasma 209:283-291.
- Villalobos, M. A., Nava, N., Vázquez, M., and Quinto C. 1994. Nucleotide sequence of the *Rhizobium etli* *nodS* gene. Gene 150:201-202.
- Waelkens, F., Voets, T., Vlassak, K., Vanderleyden, J., and van Rhijn, P. 1995. The *nodS* gene of *Rhizobium tropici* strain CIAT899 is necessary for nodulation on *Phaseolus vulgaris* and on *Leucaena leucocephala*. Mol. Plant-Microbe Interact. 8(1):147-54.
- Wais, R. J., Galera, C., Oldroyd, G., Catoira, R., Penmetsa, R. V., Cook, D., Gough, C., Denarie, J., and Long, S. R. 2000. Genetic analysis of calcium spiking responses in nodulation mutants of *Medicago truncatula*. Proc. Natl. Acad. Sci. U.S.A. 97(24):13407-13412.
- Walker, S. A., Viprey, V., and Downie, J. A. 2000. Dissection of nodulation signaling using pea mutants defective for calcium spiking induced by Nod factors and chitin oligomers. Proc. Natl. Acad. Sci. U.S.A. 97(24):13413-13418.