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Role of the Differentiation of Root Epidermal Cells in Nod Factor (from *Rhizobium meliloti*)-Induced Root-Hair Depolarization of *Medicago sativa*[1]

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The stage of differentiation of epidermal cells and the development of root hairs was found to be important for the induction of depolarization in root hairs of *Medicago sativa* by Nod factor [NodRm-IV(S)] isolated from the bacterium *Rhizobium meliloti*. The electrical membrane response was concentration dependent, having its major effect (amplitude of the depolarization and number of root hairs that responded) at 10^{-8} and 10^{-7} M Nod factor. This response was correlated with a morphological effect of Nod factor in the root-hair-deformation bioassay at similar concentrations. The effect of Nod factor on depolarization and root-hair deformation showed specificity with respect to the structure, since unsulfated Nod molecules were inactive, as was the synthetic *N,N′,N″,N‴*-tetraacetylchitotetraose. The Nod factor that is *O*-acetylated at the nonreducing sugar was as efficient in root-hair deformation and membrane depolarization as the sulfated Nod factor.

The symbiosis between the bacterium *Rhizobium meliloti* and the plant *Medicago* results in the formation of nitrogen-fixing nodules on the root of the host. Flavonoids excreted by the plant roots act as signal molecules that, in conjunction with the rhizobial NodD regulatory proteins, can activate the transcription of structural *nod* genes in the bacteria (for reviews, see Long, 1989; Dénarié et al., 1992; Hirsch, 1992). Common bacterial nodulation genes (*nod ABC*) are also involved in the production of signal molecules that play a crucial role in root-hair deformation and nodule organogenesis (Bauer, 1981; Rolfe and Gresshoff, 1988; Banfalvi and Kondorosi, 1989; Long, 1989; Fisher and Long, 1992; Kondorosi, 1992). In addition, other genes are implicated in the specificity of plant/bacteria recognition (see Dénarié and Cullimore [1993] for a review).

One of the signals produced by the bacteria has been purified from *Rhizobium* cultures and identified as a sulfated and acylated glucosamine oligosaccharide [NodRm-IV(S)] (Nod factor). This factor is able to elicit root-hair deformation and the formation of ineffective nodules in alfalfa (Lerouge et al., 1990; Truchet et al., 1991; Dénarié and Cullimore, 1993). In addition to this tetrasaccharide, a family of Nod factors having different degrees of specificity have been isolated (Roche et al., 1992; Schultze et al., 1992). Among them, the pentasaccharide shows the same activity (in the Had bioassay) on the host plants (*Medicago sativa*) as the tetrasaccharide. In addition, the pentasaccharide induces also a Had response on nonhost plants (*Vicia sativa*), indicating that this molecule has a broader host specificity than the tetrasaccharide (Schultze et al., 1992).

An early cell response was shown for the first time by Ehrhardt et al. (1992) using a *Rhizobium*-free culture filtrate "Nod$^+$ extract" as well as a purified Nod factor at low concentration (10^{-9} M). Both were able to induce a membrane depolarization of root hairs of *Medicago*. It appeared from previous studies that the stage of development of root hairs was important for the obtention of efficient infection threads and subsequent nodule organogenesis, short emerging root hairs being the most sensitive (Bhuvaneswari et al., 1980; Turgeon and Bauer, 1982; Wood and Newcomb, 1989). Thus, to understand the possible role of ΔE_m by the Nod factor in the signal transduction, the possible relationship between the sensitivity of *Medicago* root hairs to membrane depolarization and root-hair deformation was studied with regard to root-hair development and differentiation. The structural specificity of the Nod factor molecule was investigated using an unsulfated Nod molecule that is not active in the Had bioassay in alfalfa (Roche et al., 1991). The effect on the root-hair E_m of a synthetic tetrasaccharide (TACT) that induces elicitor-like responses in tomato cell-suspension cultures (Felix et al., 1993) was also studied. Finally, the role of the *O*-acetylation at the nonreducing end of the Nod factor molecule was investigated because *O*-acetylated factors [NodRm-IV(Ac,S)] might be more active than a sulfated molecule in eliciting root-hair deformations in *Medicago* (Roche et al., 1991; Truchet et al., 1991).

This report concerning the early response of the *Medicago* plant to a signal synthesized by the bacterium *Rhizobium* demonstrates the specificity of interaction between rhizobial Nod factor and a legume and suggests a role for this Nod factor molecule in signal transduction during symbiosis.

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Abbreviations: ΔE_m, change in electrical membrane potential; E_m, electrical membrane potential; Had bioassay, root-hair deformation bioassay; TACT, *N-N′-N″-N‴*-tetraacetylchitotetraose.

MATERIALS AND METHODS

Plant Material

Alfalfa seeds (*Medicago sativa* cv Sitel) were sterilized according to a technique described before (Schultze et al., 1992). The seeds were then deposited on Mes (5 mM) buffered to pH 7.0 with Tris (5 mM) and solidified with Bacto agar (Difco, Detroit, MI) (10 g L^{-1}) containing KCl, NaCl, and $CaCl_2$ (100 μM each). They were incubated (with the plates upside down) for 24 h in the dark at 22°C for germination. At this stage, the plantlets were about 1 cm long and had no root-hair development. They were placed (four per plate, three plates per concentration of factor tested) flat on the same buffer solution as before and the plates were positioned vertically. The differentiation of epidermal cells into root hairs was initiated when the root came into contact with the solid buffer solution, and root hairs were allowed to develop for 5 h.

Had Bioassay

NodRm-IV(S) factor (Schultze et al., 1992) was tested at different concentrations ranging from 10^{-6} to 10^{-13} M to determine the concentration that is optimal for obtaining typical root-hair deformations such as branching and twisting. A 70-μL volume of Nod factor (from a stock solution of 1 mM in sterile, double-distilled H_2O kept frozen at −20°C) diluted in buffer just before use was delivered under a spherical sterile 1-cm-diameter coverslip deposited on each root. The same experimental protocol was used for dissolving NodRm-IV(Ac,S) factors and for testing their ability to induce root-hair deformations on *Medicago* plantlets. This factor consisted of a mixture of sulfated molecules and *O*-acetylated molecules (60/40) (Truchet et al., 1991).

The unsulfated Nod molecule, NodRm-IV, was first dissolved in DMSO at 10^{-3} M (stock solution) and then diluted in buffer solution. The highest final concentration of DMSO (0.01%) neither affected the membrane potential nor prevented the depolarizing effect of Nod factor. Plates were sealed with Parafilm and incubated in a growth chamber (16 h of light, 8 h of dark) for 3 d. The control consisted of plantlets inoculated with the same volume of buffer solution. At d 3, the plates were observed under an inverted microscope. In accordance with the bioassay developed by Wood and Newcomb (1989), who used the bacterium *Rhizobium* as an inducer of the formation of infection threads and nodules on *M. sativa* plantlets, we considered that "branching" and "twisting" are typical deformations when using the Nod factor. The number of plantlets showing these types of root-hair deformations was determined for each concentration of Nod factor tested. For the measurement of root-hair length, plantlets were used 2 d after application of buffer solution or Nod factor (10^{-8} M). The size of 600 root hairs (100 per plant along one root axis) belonging to six different plantlets was measured on paper prints using an eyepiece micrometer (for straight hairs) or a curvimeter (for deformed hairs).

Electrophysiology

Two different conditions were used: root hairs were either allowed to grow for 5 to 7 h at 24°C or for 24 h at 22°C. Unless otherwise specified, the E_m of young, growing hairs (10–50 μm long) initiated for 5 to 7 h were measured because these hairs were the most sensitive to the infection with *Rhizobium* (Bhuvaneswari et al., 1980; Turgeon and Bauer, 1982; Wood and Newcomb, 1989). Whole plantlets were mounted in a Petri dish (3-cm diameter) and constantly perfused with the buffer solution at a rate of 2.5 mL min^{-1} using two peristaltic pumps (Pharmacia). The cytoplasmic streaming in root hairs generally did not stop upon impalement with a microelectrode except in a small number of short hairs (10–20 μm long) in which the movements decreased and sometimes stopped but then recovered a few minutes after the impalement.

Because of the low noise in the recordings, variations in the E_m higher than ±4 mV were considered significant. The results were expressed as means ± SD for n of cells.

The size of long root hairs were precisely measured using an eyepiece micrometer just after the E_m was measured.

RESULTS

Had Bioassay using NodRm-IV(S) as an Effector

To address the question of a possible relationship between the electrical membrane response and the morphological modifications of root hairs after treatment with purified Nod factor, the optimal conditions (age of root hairs, concentration of Nod factor) that were determined in the Had bioassay were used for the electrophysiological studies. The results of the experiments reported in Table I concern plantlets (24 h old) having developed their root

Table I. *Had bioassay on alfalfa*

Twenty-four-hour-old plantlets with root hairs grown for an additional 5 h were used. The Had bioassay indicated the presence of typical deformations such as branching and twisting. N, Total number of plantlets tested; N_{Had} and percent Had are, respectively, the number and the percentage of plantlets showing root-hair deformations. Data were collected from two (a), three (b), four (c), or five (d) independent experiments.

Value	Concentration of NodRm - IV (S) (M)								
	0	10^{-13}	10^{-12}	10^{-11}	10^{-10}	10^{-9}	10^{-8}	10^{-7}	10^{-6}
N	72[d]	56[b]	54[b]	51[a]	56[b]	55[c]	56[c]	55[c]	55[c]
N_{Had}	0	17	17	34	46	50	53	48	51
Percent Had	0	30.3	31.5	66.7	82.1	90.9	94.6	87.3	92.7

hairs for 5 h (29-h total duration of plantlet growth). Control plantlets (no addition of Nod factor) showed only straight root hairs with no deformations (Fig. 1A). Addition of the purified Nod factor [NodRm-IV(S)] at 10^{-8} M was optimal for root-hair deformation, since 95% of the plantlets assayed ($n = 56$) showed branching and twisting, i.e. typical deformation phenomena, as well as some waving and curling (Fig. 1, C and D). Root hairs from these plantlets were elongated compared with those of control plantlets. The mean ± SD length of control plantlet root hairs was 270 ± 47 μm ($n = 600$); the application of Nod factor (10^{-8} M) induced an increase in root-hair length to 580 ± 134 μm ($n = 600$). These morphological changes, i.e. elongation and increased number of root hairs observed (data not shown), have already been described as characteristics of the Nod factor effect (Roche et al., 1991). At 10^{-9} and 10^{-10} M still a high percentage (91 and 82%, respectively) of root hairs were elongated and showed waving, curling, and swelling (Fig. 1B). The number of plantlets showing branching was only slightly decreased. At concentrations higher than 10^{-8} M, the plantlets also showed a high number of typical deformed hairs. However, the number of hairs that grew on the roots decreased, indicating that at high concentrations Nod factor had an inhibitory effect on root-hair development (data not shown).

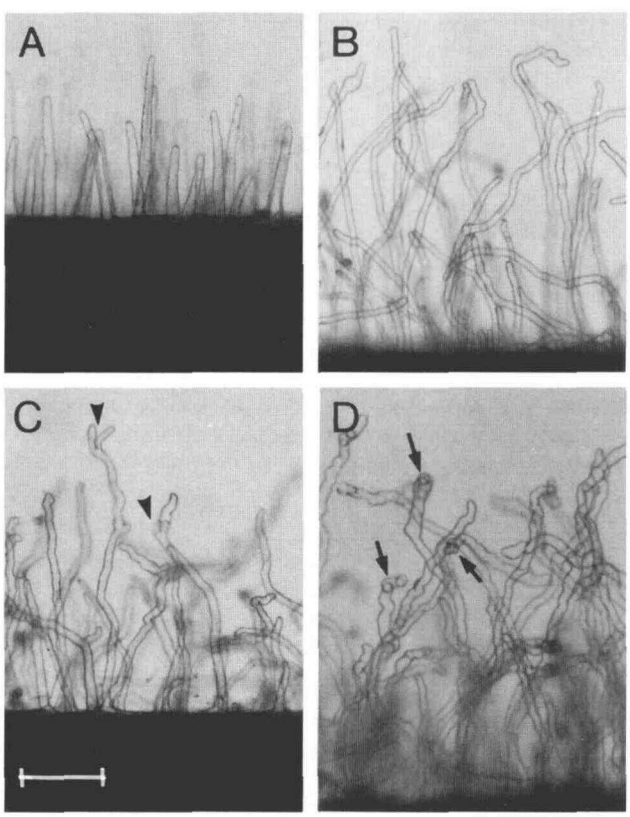

Figure 1. Root-hair deformations of *M. sativa* induced by NodRm-IV(S). The experiments were performed according to the protocol described in "Materials and Methods" for the Had bioassay. Light micrographs were taken 3 d after application of buffer solution (control) or Nod factor. A, Control plantlets showing straight root hairs. Plantlets treated with NodRm-IV(S) at 10^{-10} M (B) or 10^{-8} M (C and D) are shown. Root hairs are elongated and show various types of deformations such as branching (C, arrowheads) or twisting (D, arrows). Bar scale is 150 μm.

Similar results in terms of sensitivity to Nod factor in the Had bioassay were obtained for plantlets with root hairs grown for 3 or 7 h. NodRm-IV(Ac,S) tested at 10^{-8} M was as active as the purified Nod factor in inducing root-hair deformations (data not shown).

Membrane Depolarization of Root Hairs Induced by NodRm-IV(S)

The experimental conditions determined as optimal for the Had bioassay (24-h-old plantlets, root hairs grown for an additional 5–7 h) were used for electrophysiological studies. Unless otherwise indicated, the measurements were performed on young, growing root hairs (up to about 50 μm long).

In these conditions, the value of the E_m of root hairs (using the standard buffer solution, pH 7.0, containing KCl, NaCl, and $CaCl_2$ at 100 μM each) was −170.8 ± 14.3 mV ($n = 200$) and ranged from −130 to −200 mV. The values more positive than about −150 mV certainly corresponded to measurements recorded while the electrode was located in the vacuole; in this case, the sum of the transtonoplast and the transplasmalemma E_m values was measured. These cells were also used because they also reacted to Nod factor in depolarizing their membrane. A typical example of the E_m recording of a root hair treated with Nod factor (10^{-8} M) is shown in Figure 2A. The temporary depolarization occurred rapidly and lasted several minutes, thus probably drastically changing the ionic balance in the cells. The membrane repolarized in the absence as well as in the presence of the factor. Mostly, the final value of E_m was higher than the one measured at time zero. This phenomenon, called "overshoot," has been reported when a stimulus inducing the inhibition of the H^+ pump ATPase is removed (Felle et al., 1991).

To determine which concentration of Nod factor induced the maximal depolarization, a dose-response study was performed. The results reported in the frequency distribution histograms of the values of depolarization (Fig. 3) indicate that at 10^{-9} M (Fig. 3A) the extent of depolarization was small, ranging from +5 to +20 mV, and a low number of root hairs responded (24 of the 45 that were tested). At 10^{-8} M (Fig. 3B), all of the root hairs except 1 (of 45) responded to Nod factor by a significant depolarization of E_m (between +5 and +45 mV); these two concentrations of Nod factor were optimal for the Had bioassay using the conditions described in this paper. At 10^{-7} M, at which a high number of plantlets still show root-hair deformations (Table I), the number of root hairs that responded (43 of 45) and the range of the amplitude of the depolarization were about the same as the ones described for 10^{-8} M (Fig. 3C). However, at this high Nod factor concentration (10^{-7} M), the number of root hairs that were initiated was much lower, indicating that the differentiation of epidermal cells into root hairs was partly inhibited. Root hairs treated with Nod factor at 10^{-10} M did not depolarize ($n = 28$). The mean values of the depolarizations induced for the various

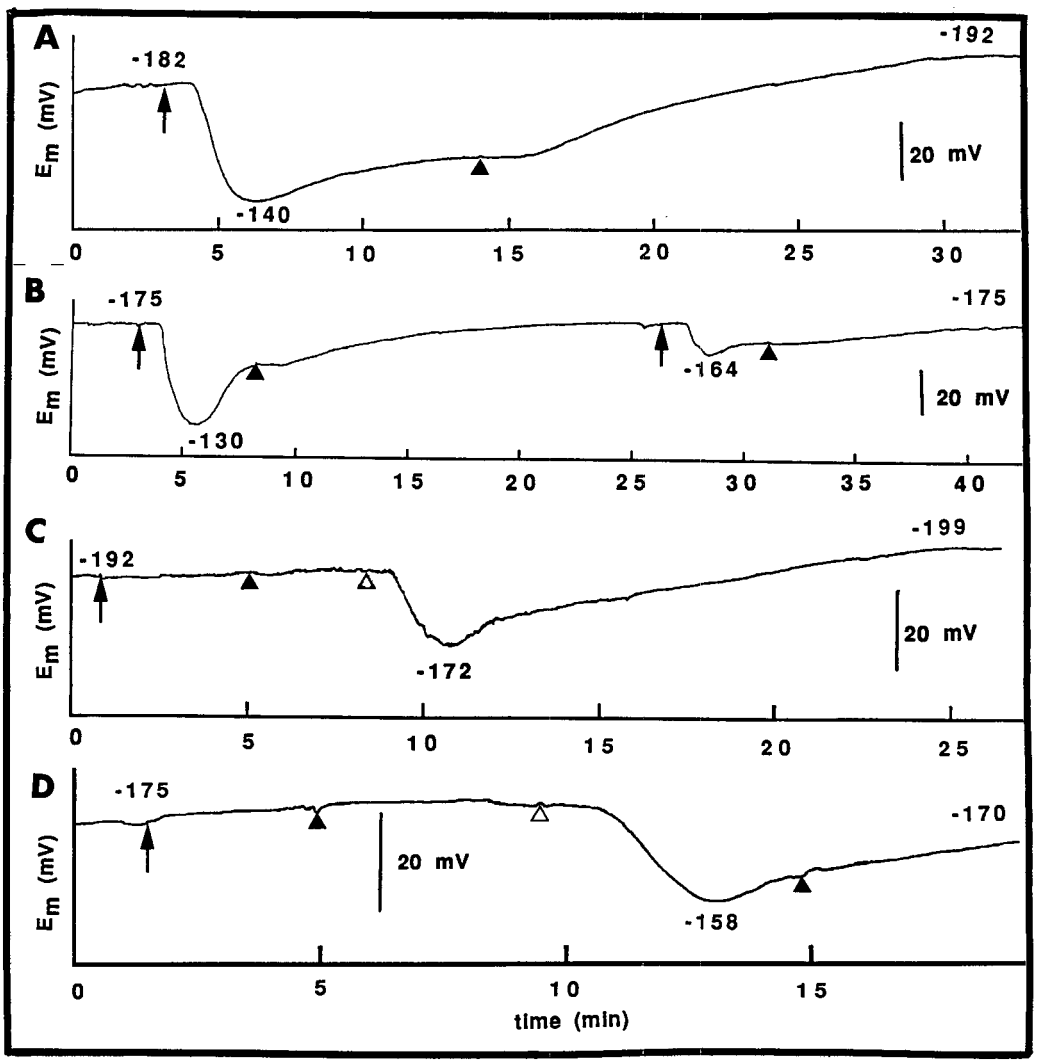

Figure 2. A, Recording of the E_m of a root hair treated with NodRm-IV(S). Whole plantlets (29 h old) were deposited in a cuvette and constantly perfused with buffer solution. The E_m of young, growing root hairs (10–50 μm long) was measured with a microelectrode. At the time indicated Nod factor (10^{-8} M) was perfused (arrow); buffer solution was perfused at the time indicated by the arrowhead. The Nod factor induced a reversible depolarization of the E_m starting 30 to 60 s after the first drop of Nod factor had reached the bath solution. The time (starting from the beginning of the depolarization) needed to reach the maximal response was 111.0 ± 22.0 s (n = 28) at 10^{-8} M Nod factor. This time was not concentration dependent as indicated by the similar values obtained with 10^{-7} and 10^{-9} M (99 ± 20.1 s, n = 26 and 113.1 ± 22 s, n = 16), respectively. The repolarization proceeded in two steps. The first one was fast (30–60 s) and allowed the E_m to recover about half the value of the total depolarization; this value was quite stable for a few minutes. The second step of the repolarization was usually slower with a duration that was variable from one hair to another (mean ± SD = 15.5 ± 3.2 min [n = 22] for 10^{-8} M; 16.9 ± 4.4 min [n = 15] for 10^{-7} M). B, Recording of the E_m of a root hair treated twice with NodRm-IV(S): desensitization. Whole plantlets (29 h old) were deposited in a cuvette and constantly perfused with buffer solution. The E_m of young, growing root hairs (10–50 μm long) was measured with a microelectrode. Nod factor (10^{-7} M) (arrow) perfused a first time induced a reversible membrane depolarization; buffer solution (arrowhead) was perfused to remove the effector. When the E_m recovered its initial value, Nod factor (arrow) at the same concentration as before was perfused. The induced depolarization displays a smaller amplitude (30.4%). C, Recording of the E_m of root hairs treated with TACT. Whole plantlets (29 h old) were deposited in a cuvette and constantly perfused with buffer solution. The E_m of young, growing root hairs (10–50 μm long) was measured with a microelectrode. TACT (10^{-7} M) (arrow) was perfused for a few minutes, as was the buffer solution (solid arrowhead) to remove the TACT; NodRm-IV(S) at 10^{-7} M (open arrowhead) induced a depolarization. D, Recording of the E_m of a root hair treated with the unsulfated factor NodRm-IV. Whole plantlets (29 h old) were deposited in a cuvette and constantly perfused with buffer solution. The E_m of young, growing root hairs (10–50 μm long) was measured with a microelectrode. NodRm-IV (10^{-7} M) (arrow) was perfused for a few minutes, as was the buffer solution (solid arrowhead) to remove the factor; NodRm-IV(S) (10^{-8} M) (open arrowhead) induced a depolarization.

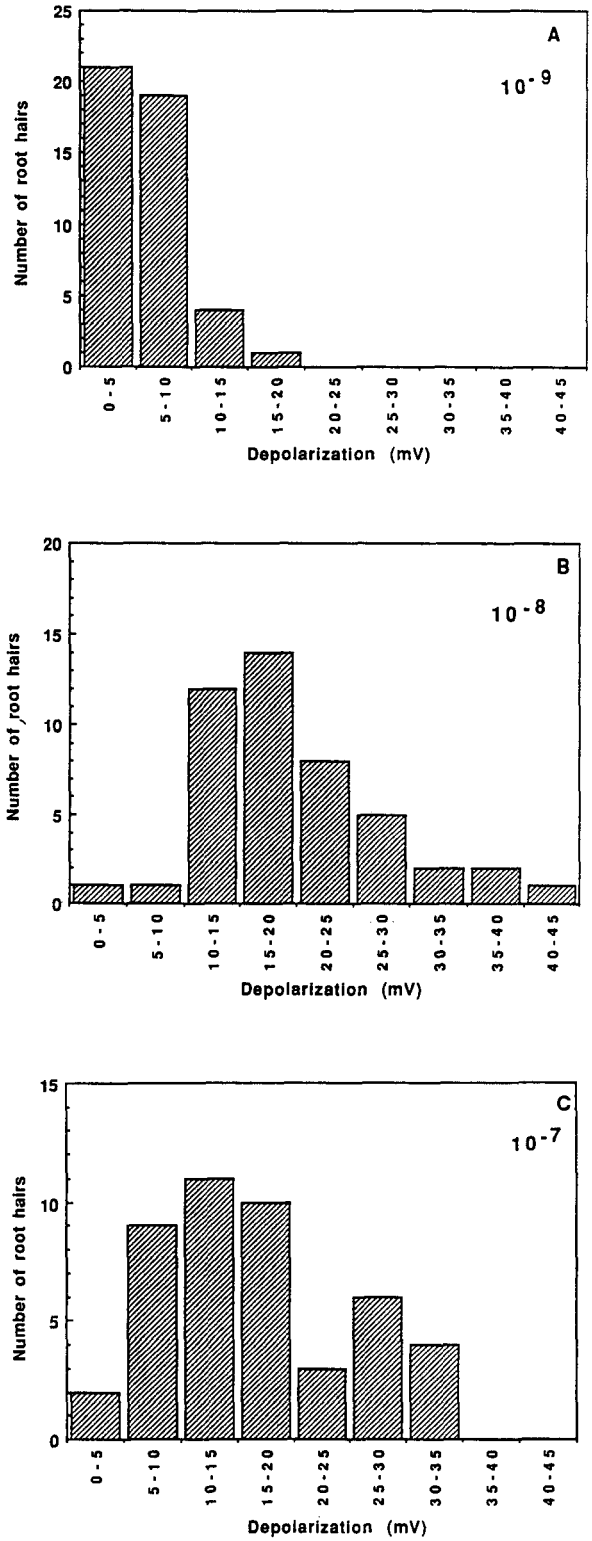

Figure 3. Dose-response effect of NodRm-IV(S) on the E_m of *Medicago* root hairs. The experimental conditions were the ones described for Figure 2. At equilibrium E_m, Nod factor at 10^{-9} M (A), 10^{-8} M (B), or 10^{-7} M (C) was perfused. The amplitude of the depolarization (mV) was calculated for each root hair and the values were reported in the distribution histograms. The E_m of 45 root hairs was measured for each of the concentrations tested.

concentrations of Nod factor tested are reported in Table II and indicate that the electrical response was dose dependent and saturated at 10^{-8} M Nod factor.

To test the sensitivity of root hairs after the first treatment with Nod factor, two successive treatments of root hairs with NodRm-IV(S) at increasing concentrations or at the same concentration were performed. The results of these experiments show that when root hairs were successively treated with 10^{-10} and 10^{-8} M Nod factor they responded by a second depolarization, which was of smaller amplitude (mean ± SD ΔE_m = +10.5 ± 3.3, n = 13) than the one observed after a single Nod factor treatment at 10^{-8} M (+18.2 ± 8.4, n = 45) even if the first concentration used did not induce any electrical response. When root hairs were treated successively with the same Nod factor concentration (10^{-8} M), the amplitude of the depolarization during the second treatment with Nod factor (Fig. 2B) decreased even more, and it became zero for the third treatment (data not shown). The percentage of the amplitudes of depolarizations induced by the second perfusion of Nod factor (10^{-8} M) represented about 25% (25.2 ± 7.1%, n = 10) of the amplitude reached after the first perfusion (Fig. 2B). The decreasing reactivity of root hairs to Nod factor could not be explained by the fact that their physiological state deteriorates with time because (a) the steady-state value of the E_m after the first and second treatment remained identical, and (b) the cytoplasmic streaming continued during the whole experiment.

Sensitivity to Nod Factor Is Dependent on the Developmental Stage of the Root Hair

The reactivity of epidermal cells was tested using two batches of plantlets. The first one consisted of 24-h-old plantlets with no root hairs. None of the epidermal cells of these plantlets differentiated into hair cells, probably because they did not get enough humidity (because of the position of the root growing for the first 24 h out of the agar and because of the upside-down position of the plate); this environmentally dependent development of the root-hair zone was previously described by Cormack (1962). The epidermal cells that were measured were the ones just above the elongation zone (corresponding to the zone where root-hair growth is currently initiated). The E_m of these cells was −183.4 ± 11.9 mV (n = 29). The Nod factor used at 10^{-8} M (n = 14) or 10^{-7} M (n = 15) had no effect.

The second batch consisted of 24-h-old plantlets transferred to the solid, buffered medium in plates kept in a vertical position for an additional 5 h to allow root hairs to grow (standard conditions). Root hairs (less than 10 μm long) just bulging out were sensitive (mean ± SD ΔE_m = +16.3 ± 7.8 mV, n = 12), whereas epidermal cells that were not yet differentiated into root hairs did not respond to Nod factor (no depolarization in any of the 20 cells tested), indicating that epidermal cells have to reach a certain degree of development to become competent and responsive to Nod factor.

Finally, the sensitivity of very long root hairs (mean ± SD length 183.3 ± 44.0 μm, n = 11; belonging to 24-h-old plantlets and grown for an additional 24 h) to Nod factor

Table II. *Depolarization of the membrane of root hairs induced by NodRm-IV(S) depends on the concentration of Nod factor*

Medicago seeds were germinated for 24 h and root hairs were allowed to grow for an additional 5 h before the E_m was measured. The plantlets were 1 to 1.5 cm long. The length of the root hairs at the time of the measurement was between 10 and 50 μm. ΔE_m, Amplitude of the depolarization (mV). The results were expressed as mean values ± SD.

Value	Concentration of NodRm-IV(S) (M)			
	10^{-10}	10^{-9}	10^{-8}	10^{-7}
Mean ± SD ΔE_m (mV)	0	+10.0 ± 4.9	+18.2 ± 8.4	+19.5 ± 9.6
Number[a]	0/28	24/45	44/45	43/45

[a] The number of root hairs that responded over the total number of root hairs measured.

was weak. Only 2 root hairs of 11 responded to Nod factor at 10^{-8} M (mean ± SD ΔE_m = +13 ± 9.9 mV, n = 11).

Structural Requirement of the Nod Factor Molecule

To investigate whether the electrical membrane response described for the "active" Nod factor shows specificity with respect to the structure of the molecule, the effect of the *O*-acetylated Nod factors and one of the two molecules structurally related to NodRm-IV(S) was studied. NodRm-IV(Ac,S) tested at 10^{-8} M was active in inducing membrane depolarization of 12 of 14 root hairs with an amplitude of +10 to +32 mV (mean ± SD ΔE_m = +17.9 ± 8.3 mV). The TACT represents the backbone of the Nod factor molecule. The other molecule was the unsulfated Nod molecule, NodRm-IV, which differed from the active Nod factor only by the lack of the sulfate group at the reducing end of the molecule. Neither TACT (data not shown) nor NodRm-IV (Roche et al., 1991) induced root-hair deformations on *Medicago*.

TACT tested at 10^{-6} M (n = 26) or 10^{-7} M (n = 6) had no effect. The successive perfusion of the TACT (10^{-6} or 10^{-7} M) and active Nod factor (10^{-7} M) induced a depolarization of the E_m (mean ± SD ΔE_m = +15.5 ± 6.9, n = 13) only after the addition of the Nod factor (Fig. 2C), and this depolarization was not different from the one obtained when the Nod factor was perfused alone as indicated before, thus excluding any competition between the TACT and the active Nod factor signal.

The unsulfated factor, NodRm-IV, perfused at 10^{-8} M (n = 11) or 10^{-7} M (n = 9) also had no effect. When the perfusion with the unsulfated factor was followed by one with the active molecule at 10^{-8} M, the E_m depolarized with a typical amplitude of +6 to +24 mV (mean ± SD ΔE_m = +17.1 ± 6.9, n = 9; Fig. 2D).

DISCUSSION

The results presented in this study demonstrate the specific action of Nod factor in terms of the age and development of root hairs and in terms of the chemical structure of the Nod factor molecule in the electrical membrane response.

The state of differentiation of root hairs was also found to be important for their sensitivity to Nod factor in the Had bioassay. Young root hairs (emerged for a few hours) belonging to intact plantlets (29 h old) were the most sensitive, indicating that epidermal cells have to reach a certain degree of development to become competent and to respond to Nod factor. The competence could correspond to metabolic changes, modifications of membrane permeability, or the setting up of receptors at the plasma membrane surface. Until now, there has been no direct evidence for the presence of receptors at the root-hair plasma membrane. However, the fact that a fast electrical membrane response is the earliest known signaling event during the interaction of Nod factor and root hairs favors this hypothesis.

According to our results, the epidermal cells that are not yet differentiated into root hairs do not depolarize. This appears to be contradictory to another response of root hairs to Nod factor, i.e. the transcription of the early nodulin gene *MSEnod 12*, which occurs in young root hairs as well as in epidermal cells before they are differentiated into root hairs (Pichon et al., 1992; Allison et al., 1993; Bauer et al., 1994; Journet et al., 1994). The difference in reaction of epidermal cells to Nod factor suggests that different signal cascades may be involved in the two different types of response.

Root-hair deformation and the electrical membrane response were dose dependent. A small shift was observed between the concentrations of Nod factor that were the most efficient for depolarization and the ones that were optimal for root-hair deformation. In spite of the shift (which is most probably due to differences in the experimental procedures used for electrophysiology and the Had bioassay) a good correlation between the two responses could be shown.

A question raised by our results concerns the origin of the depolarization induced by Nod factor. Several reports have indicated that effectors such as light and various elicitors also induce a depolarization of the E_m (Pélissier et al., 1986; Thain et al., 1990; Mathieu et al., 1991; Kuchitsu et al., 1993; Nishizaki, 1994). These responses have been shown to correspond either to an inhibition of the plasma membrane proton pump ATPase or to a modification of the fluxes of ions other than protons. The depolarization induced by Nod factor could be the result of the effect of one or both of these mechanisms (ion channels and pump) even though it is too early to favor one or the other of these hypotheses.

The smaller amplitude of the depolarization of root hairs treated a second time with the same stimulus can be interpreted as a "desensitization" (Horn et al., 1989). This phenomenon of desensitization has previously been described for root hairs of *Medicago* treated successively with high concentrations (10^{-3} and 10^{-2} M) of *Rhizobium* "Nod^+ culture filtrate extract" (Ehrhardt et al., 1992), for K^+ efflux from tobacco cells treated with oligogalacturonides (Mathieu et al., 1991), and for alkalinization of the medium of suspension-cultured tomato cells treated with chitin fragments (Felix et al., 1993). That pretreatment even with low concentrations of the Nod factor (10^{-10} M) caused desensitization in this study suggests that a putative "receptor" is already occupied, thus shifting the sensitivity for the next treatment even if this concentration does not induce a detectable electrical membrane response.

NodRm-IV(Ac,S) factors have been previously shown to be active in root-hair deformation (Roche et al., 1991) and in the formation of empty nodules (Truchet et al., 1991). These factors are also active in inducing membrane depolarization. This is not surprising because these factors consist mainly of sulfated Nod factor molecules with only two-thirds *O*-acetylated molecules, which differ from the active Nod factor by the addition of an acetylated group. A Nod factor molecule with no sulfate group at the reducing end does not induce root-hair deformation on alfalfa (Roche et al., 1991). We have shown that the sulfate group is also important for the induction of the electrical membrane response in *M. sativa* root hairs. Our results also indicate that the unsulfated Nod factor molecule does not reduce the effect of the active Nod factor. Another molecule (TACT) also containing four GlcNAc residues did not modify the E_m of root hairs even at high concentration (up to 1.0 μM). Furthermore, neither molecule competed with the active Nod factor signal. TACT has been shown, however, to induce other early responses such as fast and transient alkalinization of the medium of suspension-cultured cells (Felix et al., 1993), which is a typical signaling response in the interaction between elicitors and plant cells (see refs. in Guern et al., 1992).

In addition to the depolarizing effect of purified active Nod factor on root hairs from *Medicago*, which has previously been reported (Ehrhardt et al., 1992), we have shown here that the electrical membrane response is highly specific for the chemical structure of the Nod factor molecule. Finally, the structural specificity of the Nod factor molecule was correlated with its biological activity (Had bioassay). At this stage, further studies using more sophisticated techniques such as patch-clamp and the quantitative measurement of the concentration of ions are needed to clarify the signaling cascade involved in the Rhizobia-legume symbiosis.

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