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Lipo-chitooligosaccharide Nodulation Signals from *Rhizobium meliloti* Induce Their Rapid Degradation by the Host Plant Alfalfa[1]

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Extracellular enzymes from alfalfa (*Medicago sativa* L.) involved in the degradation of nodulation (Nod) factors could be distinguished by their different cleavage specificities and were separated by lectin affinity chromatography. A particular glycoprotein was able to release an acylated lipo-disaccharide from all tested Nod factors having an oligosaccharide chain length of four or five residues. Structural modifications of the basic lipo-chitooligosaccharide did not affect the cleavage site and had only weak influence on the cleavage efficiency of Nod factors tested. The acylated lipo-trisaccharide was resistant to degradation. When alfalfa roots were preincubated with Nod factors at nanomolar concentrations, the activity of the dimer-forming enzyme was stimulated up to 6-fold within a few hours. The inducing activity of Nod factors decreased in the order NodRm-IV(C16:2,Ac,S) > NodRm-IV(C16:2,S) and NodRm-V(C16:2,Ac,S) > NodRm-V(C16:2,S) > NodRm-IV(C16:0,S) > NodRm-IV(C16:2). Pretreatment with NodRm-III(C16:2) as well as unmodified chitooligosaccharides did not stimulate the dimer-forming enzyme. Roots preincubated with *Rhizobium meliloti* showed similar stimulation of the dimer-forming activity. Mutant strains unable to produce Nod factors did not enhance the hydrolytic activity. These results indicate a rapid feedback inactivation of Nod signals after their perception by the host plant alfalfa.

Rhizobia are symbiotic soil bacteria of the genera *Rhizobium, Bradyrhizobium,* and *Azorhizobium* that fix nitrogen in legume nodules. *Rhizobium* infection and nodule development are the result of a complex communication cascade between the two partners (Caetano-Anollés and Gresshoff, 1991; Hirsch, 1992; Long and Staskawicz, 1993; Schultze et al., 1994a). Roots secrete specific flavonoids or isoflavonoids that induce the transcription of bacterial *nod* genes. Most of these *nod* genes are involved in the synthesis of Nod factors, a family of lipo-chitooligosaccharide signals that are β-1,4-linked oligomers of GlcNAc with an *N*-linked fatty acid moiety replacing the *N*-acetyl group on the nonreducing end (Dénarié and Cullimore, 1993; Carlson et al., 1994; Schultze et al., 1994a). Depending on the rhizobial strain, additional modifications are present on both ends of the sugar backbone. Nod factors of *Rhizobium meliloti* have a chitooligomer backbone of three to five GlcNAcs. They are *N*-acylated by unsaturated C16 fatty acids or by a series of C18 to C26 (ω-1)-hydroxylated fatty acids. Nod factors of *R. meliloti* have a sulfate group on the reducing end and may be *O*-acetylated on the nonreducing end (Lerouge et al., 1990; Roche et al., 1991; Truchet et al., 1991; Schultze et al., 1992; Demont et al., 1993).

The structural parameters of the Nod factors influence their biological activity on the host plant alfalfa (*Medicago sativa*). Although Nod factors are perceived by nonlegumes (De Jong et al., 1993; Staehelin et al., 1994a), it is generally thought that a given legume has specific receptors for specific Nod factors. Applied to alfalfa at pico- to nanomolar concentrations, sulfated Nod factors of *R. meliloti* stimulate a rapid membrane depolarization (Ehrhardt et al., 1992), induce cell cycle-related genes (Savouré et al., 1994; Yang et al., 1994) as well as early nodulin genes (Pichon et al., 1993; Bauer et al., 1994; Crespi et al., 1994; Journet et al., 1994), and cause morphological alterations such as root hair deformation and formation of nodule-like structures (Lerouge et al., 1990; Roche et al., 1991; Truchet al., 1991; Schultze et al., 1992).

Nod factors represent a new substrate class for plant chitinases (Schultze et al., 1993; Staehelin et al., 1994a, 1994b), enzymes extensively studied in the context of plant-pathogen interactions (Flach et al., 1992; Meins et al., 1992; Collinge et al., 1993; Stinzi et al., 1993), including hypersensitive reactions against rhizobia (Staehelin et al., 1992; Vasse et al., 1993; Parniske et al., 1994). It was shown that structural modifications in the Nod factors influence their stability against hydrolysis by purified chitinases of various plant species, and when they were applied to intact roots, the Nod factors were rapidly degraded and inactivated (Schultze et al., 1993, 1994b; Heidstra et al., 1994; Staehelin et al., 1994a, 1994b).

The observation that chitinases differentially inactivate Nod factors opened the possibility that stability and levels of active Nod factors might play a role in host-specific interactions (Schultze et al., 1993; Staehelin et al., 1994a,

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Abbreviation: Nod, nodulation.

1994b). Here we report that alfalfa roots respond to active Nod factors with an enhanced degradation activity, adding a new dimension of complexity to chemical communication between *Rhizobium* and its host plant.

MATERIALS AND METHODS

Plant Material and Bacterial Strains

Seeds of alfalfa (*Medicago sativa* L. cv Sitel) (Tourneur, Montauban, France) were surface sterilized and germinated on inverted agar plates as described (Staehelin et al., 1994b). Wild-type *Rhizobium meliloti* 1021, its Nod factor-overproducing derivative 1021(pNID6) (Kondorosi et al., 1989), and the Tn5 mutant derivatives of 1021(pNID6), ISV1502 (*nodA*::Tn5), and ISV1503 (*nodC*::Tn5) (Röhrig et al., 1994), were grown overnight at 28°C on a rotary shaker at 200 rpm in a modified GTS medium (Kiss et al., 1979) containing 1 g of monosodium glutamate per L as nitrogen source.

Nod Factors and Chitooligosaccharides

Purification of NodRm-IV(C16:2,S) and NodRm-V(C16:2,S), desulfation, and catalytic hydrogenation of NodRm-IV(C16:2,S) yielding NodRm-IV(C16:2) and the saturated NodRm-IV(C16:0,S), respectively, were described earlier (Schultze et al., 1992; Staehelin et al., 1994b). NodRm-III(C16:2) was prepared from NodRm-IV(C16:2) by alfalfa chitinase digestion followed by purification on reverse-phase HPLC (Staehelin et al., 1994b). NodRm-IV(C16:2,Ac,S) and NodRm-V(C16:2,Ac,S) were prepared by in vitro *O*-acetylation using NodL protein from *R. leguminosarum* bv *viciae* (kindly provided by G. Bloemberg and H.P. Spaink, University of Leiden, The Netherlands) (Bloemberg et al., 1994). Nod factors contained the unsaturated (C16:2) acyl chain except where specified otherwise. For all Nod factors, a stock solution of 1 mM in DMSO was prepared and stored at −20°C.

The chitooligosaccharides *N,N′,N″,N‴*-tetraacetylchitotetraose and *N,N′,N″,N‴,N⁗*-pentaacetylchitopentaose (pure grade) were obtained from Seikagaku Corporation (Tokyo, Japan).

Preparation of Root Exudates

One-day-old seedlings were placed horizontally on plastic Petri dishes, and 100 μL of deposit-free Jensen medium (Van Brussel et al., 1982) was added to each root. Plants were incubated at 24°C in the dark for 24 h. The seedlings were removed and the droplets of Jensen medium containing root exudates were collected.

Separation of Different Nod Factor Cleaving Enzymes

Lectin affinity chromatography was performed according to Müller et al. (1992) at 4°C with a flow rate of 1 mL min^{-1}. A 2-mL column of ConA insolubilized on 4% beaded agarose, type III-AS (Sigma), was equilibrated with 5 mM Mes (K^+), pH 6, containing 50 mM NaCl and 0.1 mM each $MgCl_2$, $MnCl_2$, and $CaCl_2$ (buffer A). Forty-five milliliters of root exudates mixed with 5 mL of buffer A, 10-fold concentrated, were loaded onto the lectin column. The flow-through was collected and loaded a second time. After washing the column with buffer A, glycoproteins binding on ConA were eluted with 10 mL of buffer A containing 0.2 M methyl-α-D-mannopyranoside.

Pretreatment of Seedlings

One-day-old germinated seedlings were placed on 1-mL sterile plastic syringes (Staehelin et al., 1994b) filled with 300 μL of Jensen medium containing 0.5% (v/v) DMSO and the indicated amounts of Nod factors or chitooligosaccharides. Plants were incubated for 19 h at 24°C in the dark.

For the 24-h time-course experiment, first the seedlings were placed on syringes filled with 0.5% (v/v) DMSO in Jensen medium, then the plants were transferred at various times to syringes containing 0.5% (v/v) DMSO in Jensen medium and 1 μM NodRm-IV(C16:2,S).

For the pretreatment of roots with rhizobia, the bacterial cultures were centrifuged and resuspended in modified GTS medium to yield an A_{700} of 1. Plants were incubated for 19 h in syringes containing 40 μL of bacterium suspension and 360 μL of Jensen medium. Syringes filled with 40 μL of modified GTS medium and 360 μL of Jensen medium were used for mock treatment.

Nod Factor Degradation Assays

Nod factors were incubated overnight with enzyme preparations from root exudates at 37°C in a mixture containing 20 mM acetate (Na^+), pH 5, 0.5% (v/v) DMSO, 10 μg mL^{-1} acetylated BSA (New England Biolabs), and 2.5 μM Nod factor.

For the in vivo assay with intact roots, seedlings were transferred to syringes filled with 200 μL of Jensen medium containing 0.5% (v/v) DMSO and 5 μM NodRm-IV(C16:2,S) or NodRm-V(C16:2,S). Plants of all pretreatment experiments were incubated for 2 h at room temperature. The seedlings were removed after incubation, and the contents of three syringes were pooled.

Samples were extracted with an equal volume of distilled 1-butanol and dried under reduced pressure. Nod factors and acylated cleavage products were fractionated on reverse-phase HPLC using 36% acetonitrile:water, 40 mM ammonium acetate as the mobile phase (Staehelin et al., 1994b). Neither Nod factors nor cleavage products were detected on control chromatograms when plants were pretreated with 1 μM Nod factor followed by an incubation in assay syringes without Nod factors.

RESULTS

Isolation and Substrate Specificity of a Dimer-Forming Nod Factor Hydrolase

We have shown recently that alfalfa roots hydrolyze the glycosidic bonds of the *R. meliloti* Nod factors with a rapid turnover (Staehelin et al., 1994b). Roots were incubated with purified Nod factors containing a (C16:2) fatty acid

moiety, and the seedlings were removed after incubation. The culture media, containing most of the Nod factors and their acylated cleavage products, were extracted with 1-butanol and fractionated on a reverse-phase HPLC column under isocratic conditions. Extracellular enzymes of alfalfa roots released the acylated cleavage products NodRm-III and NodRm-II from a mixture of purified Nod factors containing 5 μM each NodRm-V(S), NodRm-IV(S), and the desulfated NodRm-IV (Fig. 1a). Experiments using individual Nod factors as substrate demonstrated that NodRm-III was released only from NodRm-V(S) and NodRm-IV, whereas NodRm-II was formed from all tested Nod factors (Staehelin et al., 1994b). The purified cleavage product NodRm-III was strongly resistant to degradation by intact roots (Fig. 1b), indicating an enzyme activity that directly cleaves off NodRm-II from the Nod factors.

To test whether the formation of acylated tri- and disaccharides can be attributed to different enzymes, we collected root exudates and fractionated them by different chromatographic methods. Using NodRm-V(S) as substrate, the hydrolytic activity of the root exudates was tested (Fig. 1c). NodRm-V(S) was cleaved in the same way by the root exudates as by intact roots (Staehelin et al., 1994b), forming the acylated products NodRm-III and NodRm-II. Similarly, the cleavage pattern of NodRm-IV(S) or NodRm-IV obtained from root exudates was not different from that of intact roots (data not shown). Purification of the root exudates by lectin affinity chromatography resulted in the separation of two hydrolytic activities with different cleavage specificity as assayed on NodRm-V(S). Although the flow-through of a ConA-agarose column contained an activity releasing NodRm-III (Fig. 1d), a glycoprotein fraction eluted with methylmannopyranoside formed the dimer NodRm-II (Fig. 1e).

The glycoprotein, partially purified on the ConA column, was able to cleave all tested Nod factors containing at least four GlcNAc residues and released only the acylated dimers NodRm-II and NodRm-II(Ac) (Table I). The degradation of different substituted Nod factors was expressed in percent of the acylated dimer released from NodRm-IV(S) by the same amount of enzyme. Purified NodRm-III was not hydrolyzed by the dimer-forming enzyme. Moreover, the sulfate group on the reducing end of Nod factors barely affected their degradation. In contrast, O-acetylation of the nonreducing terminus significantly increased the Nod factor stability.

Stimulation of the Dimer-Forming Activity Is an Early Response after Perception of Nod Factors

We have reported previously that alfalfa roots rapidly cleave Nod factors, whereas root exudates had only a low degradation activity. Intact roots released high amounts of NodRm-II from Nod factors in situ, indicating a key role of the dimer-forming enzyme in Nod factor degradation. The nonlinear kinetics of this hydrolysis indicated an increase of the dimer-forming root enzyme (Staehelin et al., 1994b). To test whether Nod factors influence the Nod factor degradation activity of the host plant, alfalfa roots were preincubated with Nod factors or chitooligosaccharides at dif-

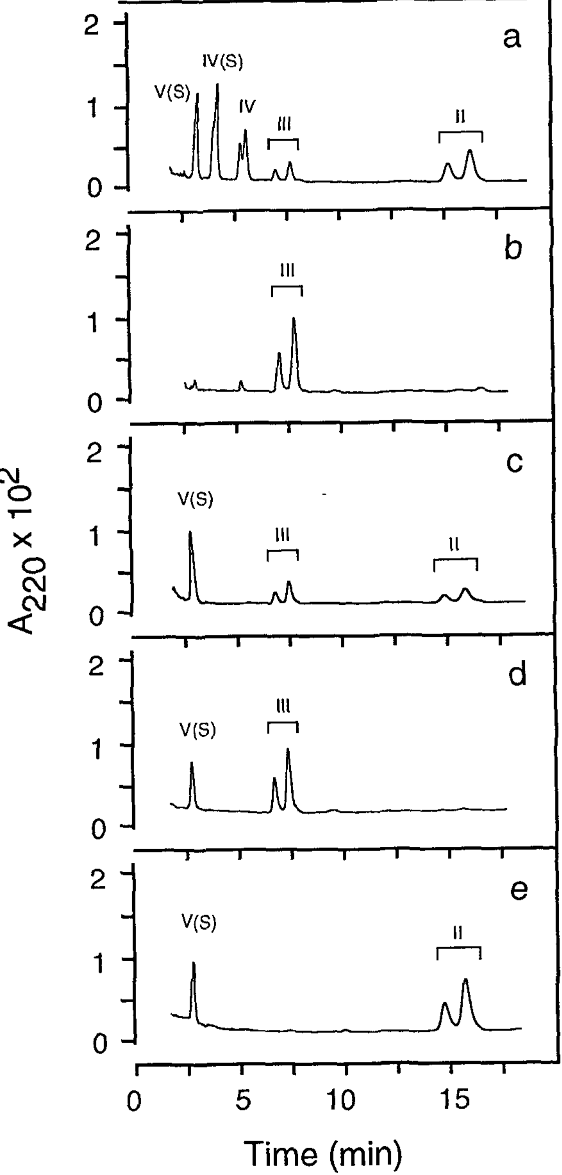

Figure 1. Reverse-phase HPLC of purified *R. meliloti* Nod factors and their acylated cleavage products after incubation with intact alfalfa roots (a and b) or with extracellular enzymes exuded by alfalfa roots (c–e). Nod factors and acylated products were extracted with 1-butanol and fractionated on reverse-phase HPLC under isocratic conditions with 36% acetonitrile:water, 40 mM ammonium acetate as the mobile phase. The length of the oligosaccharide chain (roman numerals) and the presence of a sulfate group are indicated. The acylated trimer and dimer were separated into their anomers (double peaks). Intact roots were incubated in a mixture containing 5 μM NodRm-V(S), 5 μM NodRm-IV(S), and 5 μM NodRm-IV for 6 h (a) or with 5 μM NodRm-III for 24 h (b). NodRm-V(S) as substrate was used to characterize the cleavage pattern of extracellular enzymes (c–e). Root exudates from alfalfa released NodRm-III and NodRm-II from NodRm-V(S) (c). Nod factor cleaving activities from root exudates were separated by lectin affinity chromatography. The flow-through of a ConA-agarose column contained a NodRm-III-forming activity (d), and a glycoprotein with affinity to ConA released the dimer NodRm-II from NodRm-V(S) (e).

Table I. *Substrate specificity of the dimer-forming Nod factor hydrolase*

The enzyme bound to ConA was eluted with 200 mM methyl α-D-mannopyranoside. Aliquots of the partially purified enzyme were incubated with 2.5 μM Nod factor and the amount of the acylated dimer formed was determined by HPLC. The production of the acylated dimer is expressed in percent of the enzyme activity obtained with NodRm-IV(S) as substrate. Arrows in the schematic presentation of Nod factor structures indicate the cleavage sites.

Substrate	Cleavage Site	Production of NodRm-II [percent of activity using NodRm-IV(S) as substrate][a]
NodRm-IV(S)	O-O↑O-O (R; OSO_3H)	100
NodRm-V(S)	O-O↑O-O-O (R; OSO_3H)	121 ± 18
NodRm-IV	O-O↑O-O (R)	72 ± 11
NodRm-III	O-O-O (R)	0[b]
NodRm-IV(Ac,S)	O-O↑O-O (O-Ac; R; OSO_3H)	23 ± 16
NodRm-V(Ac,S)	O-O↑O-O-O (O-Ac; R; OSO_3H)	64 ± 13

[a] Mean ± SD of three experiments. [b] NodRm-III was stable also after prolonged incubation.

ferent concentrations for 19 h. This treatment did not cause any alterations in root growth or in the number of root hairs. After preincubation, plants were assayed for their dimer-forming activity during the subsequent 2 h using 5 μM NodRm-IV(S) as substrate (Fig. 2). As a control, plants were pretreated with Nod factor at a concentration of 10^{-6} M and then incubated in assay solutions without NodRm-IV(S). Neither Nod factors nor cleavage products were detected on such control HPLC chromatograms (data not shown). A mock-treated alfalfa root, preincubated in Jensen medium containing 0.5% (v/v) DMSO, released about 30 pmol of NodRm-II from NodRm-IV(S) per hour.

Depending on the Nod factor concentrations during the pretreatment, roots responded with enhanced dimer-forming enzyme activity (Fig. 2, A–E and G). At pretreatment concentrations of 10^{-6} and 10^{-7} M NodRm-IV(S), the dimer-forming activity increased about 5-fold (Fig. 2A), whereas the pentamer NodRm-V(S) was a weaker stimulator (Fig. 2B). The *O*-acetyl modification of Nod factors on the nonreducing end conferred an increased stimulation of the dimer-forming activity. Pretreatment of roots with concentrations as low as 10^{-10} M NodRm-IV(Ac,S) exhibited a detectable enhancement (Fig. 2C). The *O*-acetylated pentamer NodRm-V(Ac,S) (Fig. 2D) was less active than the tetramer, with a threshold of about 10^{-8} M (Fig. 2D). The pretreatment of roots with the desulfated NodRm-IV stimulated the enzyme activity only slightly at 10^{-6} M (Fig. 2E). The lipotrisaccharide NodRm-III was completely inactive in the range of tested concentrations (Fig. 2F). NodRm-IV(C16:0,S), carrying the C16 saturated fatty acid, showed a weak stimulation effect (Fig. 2G). Pretreatment with the unmodified chitooligosaccharides *N,N′,N″,N‴*-tetraacetylchitotetraose or *N,N′,N″,N‴,N⁗*-pentaacetylchitopentaose did not influence the activity of the dimer-forming enzyme (Fig. 2, H and I). Similar results were obtained for pretreatment experiments when the substrate of the assay, NodRm-IV(S), was replaced by NodRm-V(S) (data not shown). These data indicate that alfalfa roots respond specifically to Nod factors with an increased activity of the dimer-forming enzyme. In contrast, the extracellular enzyme activity releasing NodRm-III from NodRm-V(S) or NodRm-IV was not affected by pretreatment with Nod factors (data not shown).

To measure the minimal time of pretreatment necessary for the stimulation of the dimer-forming enzyme, plants were preincubated for different times in 1 μM NodRm-IV(S). After this treatment, the dimer-forming activity was assayed using 5 μM NodRm-IV(S) as substrate. Whereas a pretreatment with NodRm-IV(S) for 1 h did not alter the dimer-forming activity, a preincubation for 2 h was sufficient for maximal stimulation of the enzyme activity. Longer preincubation times enhanced the dimer-forming activity to a similar extent (Fig. 3).

Stimulation of the Dimer-Forming Activity by *R. meliloti*

We also tested the capacity of alfalfa roots to hydrolyze Nod factors after pretreatment with its microsymbiont, *R. meliloti*. Pretreatment with all tested rhizobial strains did not increase the formation of the acylated trimer when NodRm-V(S) was used as substrate (data not shown). In contrast, roots incubated in bacterial suspensions of the wild-type strain *R. meliloti* 1021 or the Nod factor-overproducing strain 1021(pNID6) showed enhanced dimer formation from NodRm-IV(S) over the mock-treated control (Table II). Similar results were obtained when NodRm-V(S) was used as substrate (data not shown). However, dimer formation was low and not significantly different from the mock-treated control plants when seedlings were preincubated with *nodA*::Tn5 (strain ISV1502) or *nodC*::Tn5 (strain ISV1503), mutant derivatives of Rm1021(pNID6) that are unable to produce Nod factors (Table II). These data extend and confirm the results obtained from purified Nod factors to the *Rhizobium*-plant interaction, indicating that there are no other rhizobial factors that are able to increase the dimer-forming activity.

DISCUSSION

In this report, we have demonstrated that roots of alfalfa seedlings respond to Nod signals from *R. meliloti* with an increased Nod factor-degrading activity. We showed that Nod factor degradation releasing NodRm-II is due to an extracellular hydrolase that was characterized for its cleavage specificity using *R. meliloti* Nod factors with different substitutions as substrate. This dimer-forming enzyme hydrolyzed all tested Nod factors containing four or five GlcNAc residues. The cleavage site was always between the second and third GlcNAc residue from the nonreducing end, whereas the other β-glycosidic bonds were not accessible to this activity. The lipotrisaccharide NodRm-III, however, was not cleaved by the dimer-forming hydrolase (Table I). Based on its capacity to bind to ConA, the enzyme was characterized as a glycoprotein and separated from an

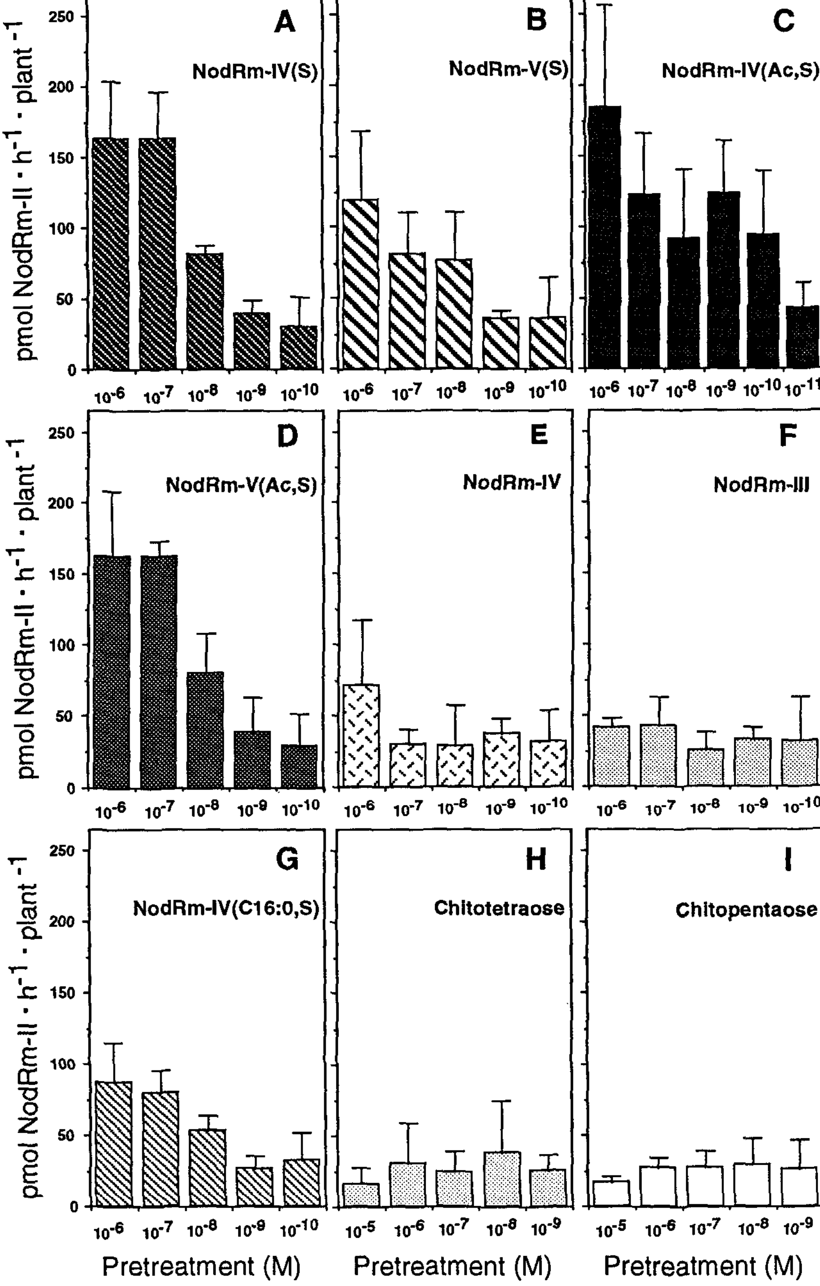

Figure 2. Activity of the dimer-forming enzyme after preincubation of intact roots with *R. meliloti* Nod factors (A–D), modified Nod factors (E–G), and chitooligosaccharides (H and I) at the indicated concentrations. For the enzyme assay, roots were incubated for 2 h in solutions containing 5 μM NodRm-IV(S), and the amount of the NodRm-II formed was determined by HPLC. A control root preincubated in Jensen medium released 30 ± 19 pmol NodRm-II from NodRm-IV(S) per hour. Note the different range of concentrations used for pretreatment with NodRm-IV(Ac,S) (C) and the chitooligosaccharides (H and I).

extracellular trimer-forming activity. The cleavage specificity of the dimer-forming enzyme differed from those of known chitinases, which apparently cleave the oligosaccharide backbone from the reducing end, whereby the cleavage site depends on the length of the oligosaccharide chain (Schultze et al., 1994b; Staehelin et al., 1994b).

In our assay, nonacylated chitooligosaccharides cannot be detected. It remains to be investigated to what extent the dimer-forming enzyme has the capacity to cleave unmodified chitooligosaccharides or the insoluble polymer chitin. Plant chitinases readily release chitooligosaccharides from chitin-containing organisms, and the generated chitin fragments often elicit defense responses including chitinase induction (Roby et al., 1987; Inui et al., 1991; Koga et al., 1992). The stimulation of the dimer-forming alfalfa enzyme, however, was restricted to Nod factors (Fig. 2) or to Nod factor-producing *R. meliloti* (Table II). Neither chitooligosaccharides nor any compounds secreted by mutant *R. meliloti* strains blocked in Nod factor synthesis enhanced the dimer-forming activity. These data indicate a specific response of the host plant alfalfa to rhizobial lipo-chitooligosaccharide signals, but not to chitooligosaccharides.

In our studies, the dimer-forming Nod factor hydrolase increased up to 6-fold upon application of active Nod

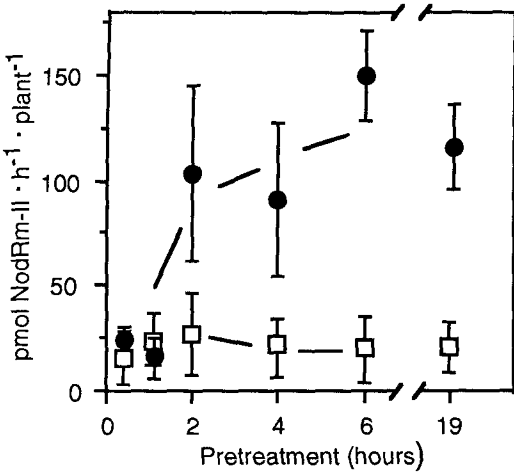

Figure 3. Time-dependent stimulation of the dimer-forming enzyme activity. Plants grown in Jensen medium were transferred to solutions with (●) or without (□) 1 μM NodRm-IV(S) and incubated for the indicated time periods. Plants were then assayed for their dimer-forming activity during the subsequent 2 h. The release of NodRm-II from 5 μM NodRm-IV(S) was measured as in the other experiments.

factors known to stimulate other plant responses such as root hair deformation or the induction of nodule meristems (Lerouge et al., 1990; Roche et al., 1991; Truchet al., 1991; Schultze et al., 1992). The most active Nod factor, NodRm-IV(Ac,S), stimulated the degradation activity at concentrations as low as 0.1 nM. This is higher than the minimal concentration necessary for root hair deformation (Roche et al., 1991; Schultze et al., 1992) or induction of the early nodulin gene *ENOD12* (Bauer et al., 1994; Journet et al., 1994), but lower than required for the stimulation of nodule-like structures (Roche et al., 1991; Truchet et al., 1991; Schultze et al., 1992). Based on the strong correlation found between the biological activity of *R. meliloti* Nod factors and their induced degradation by alfalfa, we suggest that Nod signal inactivation is a feedback mechanism after perception of the molecules by the host. Structural modifications of Nod factors, such as sulfation on the reducing end (Staehelin et al., 1994b) and *O*-acetylation on the nonreducing end (Schultze et al., 1994b), have been shown to contribute to their stability before perception of the molecules, and this paper demonstrates that the same modifications also influence the induction of a host response leading to Nod signal inactivation.

Pretreatment of roots with NodRm-IV(S) for 2 h was sufficient to induce Nod factor degradation, indicating a rapid plant response as early as root hair deformation or expression of the early nodulin gene *ENOD12* (Pichon et al., 1993; Bauer et al., 1994). It remains an open question whether the rapid stimulation of the dimer-forming activity is due to induced gene expression or to Nod factor-mediated posttranscriptional mechanisms, such as enhanced secretion of the dimer-forming enzyme to the extracellular space. We have never observed any alterations of root growth or of root hair number upon Nod factor application, and thus it is unlikely that an enlarged root surface might account for the stimulated dimer-forming activity. This conclusion is supported by the observation that another extracellular activity, characterized by acylated trimer formation from NodRm-V(S), was not affected by Nod factors (data not shown).

It is generally thought that the extent of nodulation is feedback regulated by autoregulatory mechanisms that suppress the establishment of symbiosis (Caetano-Anollés and Gresshoff, 1991; Vasse et al., 1993). It is interesting that Nod factors appear to act as morphogens only at a defined concentration range. Exceedingly high levels of Nod factors do not induce root hair deformation on alfalfa (Roche et al., 1991; Schultze et al., 1992). Our data indicate that perceived Nod factors are inactivated in the rhizosphere within a few hours. It is tempting to speculate that after perception of Nod factors their rapid inactivation might be an essential step of symbiotic signaling. During colonization of the root by rhizobia, the concentration of active Nod factors is determined by the balance between their continuous production and degradation. Inactivation of Nod signal molecules might be important to suppress continuous stimulation by active Nod factors. It would avoid inhibitory effects on plant development as well as a potential elicitation of defense reactions by high concentrations of Nod factors (Kondorosi et al., 1994). Thus, feedback-regulated inactivation of Nod signal molecules would contrib-

Table II. *Activity of the dimer-forming enzyme after preincubation of the roots with R. meliloti and its derivatives*

Plants were incubated for 19 h in suspensions containing the different *R. meliloti* strains. For the enzyme assay, plants were transferred to solutions containing NodRm-IV(S) and the cleavage product NodRm-II was determined by HPLC.

R. meliloti Strain	Characteristics	Formation of NodRm-II
		pmol h^{-1} *plant* $^{-1}$ [a]
None (mock treatment)		29.2 ± 14.2
1021	Wild-type	191.9 ± 31.9
1021 (pNID6)	Overproduction of Nod factors	171.5 ± 40.5
ISV1502	1021 (nodA::Tn5) (pNID6) no Nod factor production	39.8 ± 19.8
ISV1503	1021 (nodC::Tn5) (pNID6) no Nod factor production	54.1 ± 24.6

[a] Mean ± SD of six values (18 plants) from two independent experiments.

ute to a symbiosis-specific process that allows organogenesis but suppresses defense responses.

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