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Structural modifications in *Rhizobium meliloti* Nod factors influence their stability against hydrolysis by root chitinases

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Summary

Acylated chitooligosaccharide signals (Nod factors) trigger the development of root nodules on leguminous plants and play an important role in determining host specificity in the *Rhizobium*–plant symbiosis. Here, the ability of plant chitinases to hydrolyze different Nod factors and the potential significance of the structural modifications of Nod factors in stabilizing them against enzymatic inactivation were investigated. Incubation of the sulfated Nod factors of *Rhizobium meliloti*, NodRm-IV(S) and NodRm-V(S), as well as their desulfated derivatives NodRm-IV and NodRm-V, with purified chitinases from the roots of the host plant *Medicago* and the nonhost plant *Vicia* resulted in the release of the acylated lipotrisaccharide NodRm-III from NodRm-V, NodRm-IV and NodRm-V(S), whereas NodRm-IV(S) was completely resistant to digestion by both chitinases. Kinetic analysis showed that the structural parameters determining host specificity, the length of the oligosaccharide chain, the acylation at the nonreducing end and the sulfatation at the reducing end of the lipooligosaccharide, influence the stability of the molecule against degradation by chitinases. When the Nod factors were incubated in the presence of intact roots of *Medicago*, as well as of *Vicia*, the acylated lipotrisaccharide was similarly released *in vivo* from all Nod factors except NodRm-IV(S). In addition, a dimer-forming activity was observed in intact roots which also cleaved NodRm-IV(S). This activity was much greater in *Medicago* than in *Vicia* and increased upon incubation. The initial overall degradation rate of the Nod factors on *Medicago* was inversely correlated with their biological activities on *Medicago* roots. These results open the possibility that the activity of Nod factors on *Medicago* may partly be determined by the action of chitinases.

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Introduction

Soil bacteria of the genera *Rhizobium, Azorhizobium* and *Bradyrhizobium*, commonly referred to as rhizobia, interact with leguminous plants to form nitrogen-fixing root nodules. Generally, a given rhizobial strain or species is able to nodulate only a few host plant genera, e.g. *R. meliloti* nodulates *Medicago*, *Melilotus* and *Trigonella* while *R. leguminosarum* bv. *viciae* nodulates *Pisum*, *Vicia*, *Lathyrus* and *Lens* (Vincent, 1977). Conversely, one plant species accepts only a limited number of rhizobial strains as microsymbiotic partner. The high degree of host specificity is brought about by several consecutive communication steps between plant and bacterium.

First, the rhizobia recognize specific flavonoids or isoflavonoids exuded by legume roots as signal molecules (Firmin *et al.*, 1986; Peters *et al.*, 1986; Redmond *et al.*, 1986). Signal perception is mediated by the rhizobial regulatory NodD proteins (Mulligan and Long, 1985), several isoforms of which are present in some rhizobial species. Depending on the combination of flavonoid and type of NodD protein this interaction leads to the activation of the bacterial nodulation (*nod*) genes (Györgypal *et al.*, 1991; Horvath *et al.*, 1987; Spaink *et al.*, 1987).

The *nod* genes, in turn, are involved in the synthesis and excretion of the bacterial lipooligosaccharide signals (Nod factors). These molecules have been purified from *R. meliloti* (Lerouge *et al.*, 1990; Schultze *et al.*, 1992), *R. leguminosarum* bv. *viciae* (Spaink *et al.*, 1991), *B. japonicum* (Sanjuan *et al.*, 1992), *Rhizobium* NGR234 (Price *et al.*, 1992), *A. caulinodans* (Mergaert *et al.*, 1993) and *R. fredii* (Bec-Ferté *et al.*, 1993). They all are β-1,4-linked oligomers of three to five *N*-acetylglucosamine residues with an *N*-linked fatty acid moiety replacing the *N*-acetyl group on the nonreducing end. Different rhizobia produce different sets of Nod factors with specific modifications and these appear to determine host-specificity. A sulfate group at the *O*-6 position of the reducing end is present on the Nod factors produced by

wild-type strains of *R. meliloti* and the presence of this sulfate group is decisive for activity of the Nod factors on *Medicago* (Roche *et al.*, 1991b), at the same time, it strongly reduces the activity on the nonhost plant *V. sativa*. Conversely, nonsulfated Nod factors produced by *nodH* mutants of *R. meliloti* are active on *Vicia* but not on *Medicago*. Other structural variations influencing the biological activity of the Nod factor are the length of the oligosaccharide backbone and the length and degree of saturation of the lipid chain (Schultze *et al.*, 1992; Spaink *et al.*, 1991; Truchet *et al.*, 1991).

At pico- to nanomolar concentrations, the purified Nod factors are able to elicit various responses on host but not on nonhost plants. They induce the deformation of root hairs and the division of root cortical cells (Lerouge *et al.*, 1990; Schultze *et al.*, 1992; Spaink *et al.*, 1991; Truchet *et al.*, 1991). On *Medicago*, the host-specific Nod factors trigger even the formation of nodule-like structures (Truchet *et al.*, 1991), and on *Vicia* they induce cytoplasmic bridges, so-called pre-infection threads, in cells of the outer root cortex (Van Brussel *et al.*, 1992). Thus, the Nod factors constitute a new class of exogenous plant growth regulators, acting in a plant genus-specific manner. It is not known whether endogenous compounds of similar structure are produced by plants.

In analogy to compatible and incompatible interactions between pathogens and plants, a 'gene for gene' hypothesis (Flor, 1955) was also formulated for symbiotic interactions (Djordjevic *et al.*, 1987). While specific recognition is the basis for incompatibility in plant–pathogen interactions, it appears that specific recognition is necessary for compatibility in symbiosis. Failure to communicate will lead either to a complete lack of interaction or to abortion at various stages. This may include hypersensitive response-like reactions of nodule cells infected with certain ineffective *Rhizobium* mutants (Grosskopf *et al.*, 1993; Parniske *et al.*, 1990; Staehelin *et al.*, 1992; Werner *et al.*, 1985). Therefore, rhizobia may be regarded as 'refined pathogens' (Djordjevic *et al.*, 1987; Vance, 1983). The same or similar elements that are involved in the interaction of plants with pathogens appear to play a role in symbiotic interactions. Compounds derived from the phenylpropanoid pathway, the flavonoids, act as *nod* gene inducers, and precursors of the phytoalexin medicarpin can be converted to a *nod* gene-inducing isoflavonoid signal (Dakora *et al.*, 1993). Chitooligosaccharides have been reported to elicit various defense-related responses, like synthesis of phytoalexin precursors in cell suspensions (Ren and West, 1992), lignification of wounded leaves (Barber *et al.*, 1989) and the induction of chitinase gene expression (Koga *et al.*, 1992; Ren and West, 1992; Roby *et al.*, 1987, 1991). Moreover, the presence of a sensitive perception system for chitooligosaccharides has been demonstrated in tomato cells (Felix *et al.*, 1993). Conversely, the acylated derivatives of chito-oligosaccharides, the Nod factors, act as plant morphogens in the symbiotic interactions. Whether they would have the capacity to act as elicitors of defense responses, perhaps in nonhost plants, has not been investigated yet, although Nod factors are sensitively recognized in cells of a nonhost, tomato, by the perception system for chitooligosaccharides (Staehelin *et al.*, 1994). In all the cases mentioned, the analogy between pathogenic and symbiotic signaling systems seems to be very close and suggests the possibility that, during evolution, the symbiotic interactions of plants with microorganisms developed in close relationship with pathogenic interactions.

Plant-derived elements interacting with rhizobial Nod signals in legume roots have not yet been identified. The signals might be perceived by host plant-specific receptors. A signal transduction cascade, including plasma membrane depolarization (Ehrhardt *et al.*, 1992), may finally lead to the induction of specific early nodulin genes, like *Enod12* and *Enod40* (Franssen *et al.*, 1993; Pichon *et al.*, 1993).

Plant chitinases have been studied extensively in the context of plant–pathogen interactions (see Boller, 1988; Collinge *et al.*, 1993). These enzymes hydrolyze internal β-1,4-linkages of chitin, a major component of most fungal cell walls, acting as endochitinases and forming chitooligosaccharides. In addition, chitinases show also lysozyme activity, hydrolyzing the peptidoglycan of bacterial cell walls (Boller, 1987). Chitinase isoenzymes differ in their substrate specificity with respect to oligomeric substrates and need a chain length of at least three β-1,4-linked *N*-acetylglucosamine residues. Some chitinases release the *N*-acetylglucosamine monomer after digestion of chitin, others release chitobiose as the smallest end-product (Boller, 1988; De Jong *et al.*, 1992; Mauch *et al.*, 1988). It has been proposed that in root nodules, chitinases may protect the infected zone from external pathogens or protect the root system from pathogenic rhizobia (Staehelin *et al.*, 1992). Furthermore chitinases may have other functions apart from their role in defense mechanisms and may be involved in plant somatic embryo development (De Jong *et al.*, 1992).

The intriguing similarities between Nod factors and chitin prompted us to investigate whether legume chitinases would hydrolyze Nod factors as well as nonmodified chitooligosaccharides. In a preliminary study, it was indeed found that Nod factors are substrates for plant chitinases (Schultze *et al.*, 1993), a finding substantiated in a study on perception of Nod factors in tomato cells (Staehelin *et al.*, 1994). Here we show that Nod factors are more resistant to hydrolysis by chitinases than unmodified

chitooligosaccharides, and that different Nod factors show different degrees of stability against chitinase degradation *in vitro* and *in vivo*. This opens the possibility that chitinases, among other elements, may be involved in the recognition of the rhizobial nodulation signals. A possible role in autoregulation of nodulation is also suggested.

Results

The sulfate group at the reducing end protects Nod factors of R. meliloti *against hydrolysis by purified legume chitinases*

While different classes of chitinases have been characterized for a variety of plants (see Collinge *et al.*, 1993), purification of these enzymes has not been reported for *Medicago* and *Vicia*, the host- and nonhost plants, respectively, for *R. meliloti*. In order to obtain sufficient amounts of chitinase activity for purification, we treated *M. sativa* and *V. sativa* plants with 10 p.p.m. of ethylene. This treatment increased chitinase activity in the roots, as measured with ^{3}H-chitin as substrate, 10-fold and 13-fold, respectively (data not shown). The ethylene-induced chitinases were purified by ammonium sulfate precipitation, affinity chromatography on regenerated chitin, and gel filtration. Analysis of the purified enzymes by SDS–PAGE showed single bands for *Medicago* and for *Vicia* (Figure 1), and these bands reacted with an antiserum against bean chitinase (data not shown). The apparent molecular weights of 30 000 were similar to the one reported previously for the chitin-binding chitinase from bean leaves (Boller *et al.*, 1983). Overloaded gels revealed two minor bands of low molecular weight in samples of *Medicago*, but not of *Vicia* (data not shown).

Hydrolysis of Nod factors by the purified chitinases was followed by analysis of the butanol-extractable cleavage products, containing the (C16:2) fatty acid moiety, on reverse phase HPLC. The hydrophilic cleavage products arising from the reducing end of the oligosaccharide remained in the aqueous phase and were not analyzed. As shown in Figure 2(a) and (b), the nonsulfated NodRm-IV, but not the sulfated NodRm-IV(S), was cleaved by *Medicago* chitinase and a single acylated product, NodRm-III, with molecular mass of 819 was released. This result shows that the sulfate group at the reducing end prevents hydrolysis of the tetrasaccharide NodRm-IV(S), the cognate signal molecule of *R. meliloti*. In contrast, the sulfated pentasaccharide NodRm-V(S), whose biological activity on *Medicago* is 100-fold lower than that of NodRm-IV(S) (Schultze *et al.*, 1992), was cleaved (Figure 2c). Like NodRm-IV, it gave rise to the lipotrisaccharide NodRm-III. With the nonsulfated pentasaccharide, NodRm-V, two cleavage products, NodRm-IV and NodRm-III, were

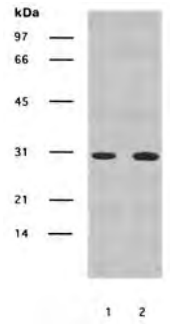

Figure 1. SDS–polyacrylamide gel electrophoresis and staining with Coomassie Brilliant Blue of chitinases from *M. sativa* (lane 1) and *V. sativa* (lane 2) after purification by affinity chromatography on regenerated chitin and gel filtration.
Five micrograms of protein were loaded. Positions of molecular weight markers are indicated.

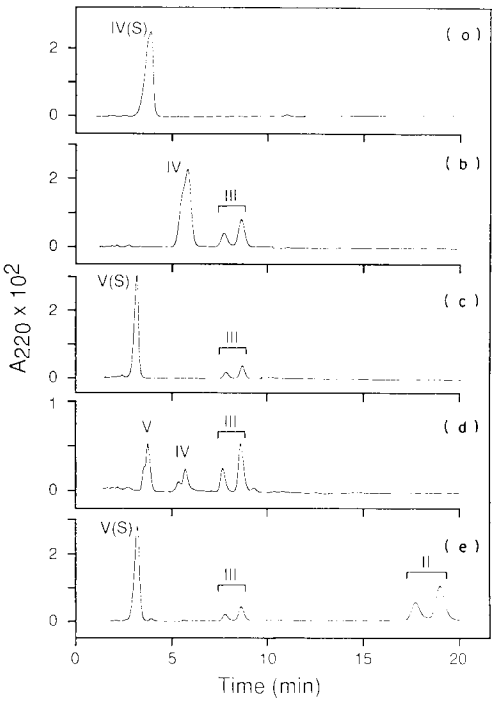

Figure 2. Reverse phase HPLC of different Nod factors and their acylated products after incubation with *Medicago* chitinase (a–d) or *Medicago* root (e).
Longer retention times led to the separation of anomers (double peaks). The length of the oligosaccharide chain (roman numerals) and presence of the sulfate group are indicated. Incubation conditions were as follows:
(a) 24 h, 5 µg NodRm-IV(S), 5 nkat chitinase in 0.1 ml;
(b) 12 h, 5 µg NodRm-IV, 0.5 nkat chitinase in 0.1 ml;
(c) 12 h, 5 µg NodRm-V(S), 0.5 nkat chitinase in 0.1 ml;
(d) 6 h, 2.5 µg NodRm-V, 0.1 nkat chitinase in 0.4 ml;
(e) 18 h, 25 µg NodRm-V(S) with root of 1-day-old seedling in 0.1 ml.
The loaded samples corresponded to 1/3 (a–d) or 1/6 (e) of the total amounts. The acylated cleavage product, NodRm-III, was not degraded further by purified chitinases (a–d).

detected (Figure 2d). Thus, by increasing the length of the oligosaccharide chain the number of cleavage sites was correspondingly increased. While NodRm-III was not

degraded further, NodRm-IV contained one, and NodRm-V two glycosidic bonds susceptible to hydrolysis by chitinase.

Using different concentrations of Nod factors in the enzyme assay, the affinity of the purified chitinases to the different Nod factors was quantified. For chitinases of both *Medicago* and *Vicia* the Michaelis–Menten constant was two orders of magnitude higher for NodRm-V(S) than for Nod-Rm-V, whereas Nod-Rm-IV showed a fivefold higher K_m value than NodRm-V (Table 1).

The chitinases were able to cleave off the terminal sugar residue from the reducing end of NodRm-IV and NodRm-V, but not from NodRm-IV(S) and NodRm-V(S). Thus, the release of the reducing end monomer was prevented by the sulfatation. Moreover, since NodRm-V(S) was more resistant to chitinase digestion than NodRm-IV (Table 1), the sulfatation acted also at a distance by partially protecting the β-glycosidic bond between the third and the fourth sugar residue.

We also tested the substrate specificity of the purified chitinases for the nonmodified chitooligosaccharides, chitobiose and chitotriose, by measuring the amount of released *N*-acetylglucosamine. As shown in Table 2, chitobiose, like NodRm-III, was not a substrate, as was expected for plant endochitinases (De Jong *et al.*, 1992; Mauch *et al.*, 1988). In contrast, chitotriose was hydrolyzed. This result shows that the acylated derivative of chitotriose is protected from degradation by the purified chitinases of *Medicago* and *Vicia* and that a minimum of three consecutive unmodified *N*-acetylglucosamine residues is required for hydrolysis.

Table 3 summarizes the cleavage sites (black arrowheads) and acylated products released from the Nod factors by purified chitinases. In conclusion, the length of the oligosaccharide chain, the acylation at the nonreducing end and the sulfatation at the reducing end, conferred stability towards digestion by chitinases. Interestingly, the striking differences in the resistance of different Nod factors to degradation by plant chitinases correlated with their biological activities on *Medicago* in the order NodRm-IV(S) > NodRm-V(S) > NodRm-IV.

Nod factors are hydrolyzed by two different hydrolytic activities in plant roots

In order to test whether Nod factors would be degraded *in vivo*, they were incubated with root exudates or intact roots of *Medicago*. While Nod factors were hydrolyzed very slowly by root exudates (data not shown), a rapid turnover was observed with intact roots, suggesting that the majority of the hydrolytic activity was retained on the surface of root cells. Subsequent assays were therefore carried out with roots instead of exudates.

Table 1. Michaelis–Menten constants of chitinases from *M. sativa* and *V. sativa* with Nod factors as substrate

	K_m (μM)[a]	
Nod factor	*Medicago*	*Vicia*
NodRm-V(S)	325 ± 221	199 ± 107
NodRm-V	4 ± 0.1	3 ± 0.6
NodRm-IV	21 ± 6	17 ± 3

[a]Mean and standard deviation of the mean of two independent experiments.

Table 2. Chitinases from *Medicago* and *Vicia* require a minimum of three *N*-acetylglucosamine residues for activity

	Enzyme activity (pkat mg^{-1} protein)[a]	
Substrate	*Medicago*	*Vicia*
N,N'-diacetyl-chitobiose[b]	0	0
N,N',N''-triacetyl-chitotriose[b]	140.0 ± 19.9	216.3 ± 14.9
NodRm-III[c]	0	0

[a] Mean and standard deviation of the mean ($n = 2$).
[b] Release of *N*-acetylglucosamine was measured.
[c] Stability was tested by HPLC analysis.

Table 3. Acylated cleavage products of Nod factors in the presence of intact roots or chitinases of *Medicago* and *Vicia*

		Acylated products	
Substrate[a]	(NodRm-)	Chitinases	Roots
	V	III, IV	II, III, IV
	V(S)	III	II, III
	IV	III	II, III
	IV(S)	–	II
	III	–	(II)[b]

[a]Schematic presentation of Nod factor structures and observed cleavage sites: (▽) intact roots; (▲) chitinase of *Medicago* or *Vicia*. The sizes of arrowheads indicate relative rates of hydrolysis. R and S indicate acylation and sulfatation, respectively.
[b]Hydrolysis of NodRm-III by plant roots was observed only after prolonged incubation.

Figure 2(e) shows the HPLC analysis of the acylated degradation products of NodRm-V(S). In contrast to the hydrolysis of NodRm-V(S) by purified chitinase (Figure 2c), after incubation with *Medicago* roots not only the

lipotrisaccharide, but an additional product was observed. Positive ion mass spectroscopy (FAB-MS) revealed a pseudomolecular ion at m/z = 617 and a fragment ion of m/z = 396 showing that the new product was the lipodisaccharide NodRm-II. Under the same incubation conditions, NodRm-II was produced not only from NodRm-V(S), NodRm-V and NodRm-IV, but the sulfated tetrasaccharide NodRm-IV(S) was also hydrolyzed to this product (Table 3) and the hydrolysis of all four Nod factors was complete after 22 h. After the same incubation time, however, only 10% of the purified trimer NodRm-III was hydrolyzed to the dimer (data not shown). These results suggest that the acylated dimer was cleaved off directly by hydrolysis of the second glycosidic bond from the nonreducing end of the Nod factors and not via NodRm-III as an intermediate. The appearence of a new cleavage product of Nod factors after incubation with roots indicates that the intact roots possess an additional hydrolytic enzyme, distinct from the purified chitinases, showing a different cleavage specificity.

In order to test whether the two hydrolytic activities observed *in vivo* would degrade different Nod factors at different rates, time course experiments were performed and the formation of the acylated trisaccharide and disaccharide, respectively, were recorded as shown in Figures 3 and 4. *Medicago* roots did not produce NodRm-III from NodRm-IV(S) while the rate of NodRm-III formation increased in the order NodRm-V(S) < NodRm-IV < NodRm-V (Figure 3a), much as it was observed with purified chitinase. Similarly, *Vicia* roots did not release NodRm-III from NodRm-IV(S) and degraded the non-sulfated pentasaccharide NodRm-V at the highest rate (Figure 3b). In contrast to *Medicago*, no significant difference in trimer formation was observed between NodRm-IV and NodRm-V(S). Thus, these results indicate that, with the exception of NodRm-V(S), the kinetics of NodRm-III production are similar for *Medicago* and *Vicia*.

In contrast, the release of lipodisaccharide from all four factors was strikingly different in the two plant species, and incubation with *Medicago* roots, but not *Vicia* roots, led to the release of high amounts of NodRm-II (Figure 4). As opposed to observations made with trimer formation, the dimer production was only little affected by the Nod factor structure, although during the first few hours the sulfated Nod factors seemed to be slightly more stable than the other factors. After 6 h, NodRm-IV(S) started to be degraded at an increased rate, suggesting an augmentation of the dimer-forming activity by NodRm-IV(S). It remains to be established to what extent the kinetics of NodRm-II production reflects induction of this enzymatic activity, versus continuous accumulation of the activity during root growth. Taken together, the different kinetics for the formation of the trisaccharide versus the disaccharide confirmed that these two reactions are catalyzed by two different hydrolases.

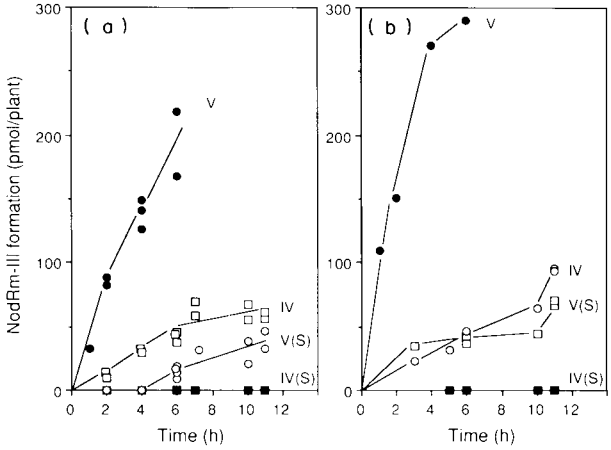

Figure 3. Production of the acylated trisaccharide NodRm-III from Nod factors by intact roots of (a) *Medicago* and (b) *Vicia*.
Each point represents the average of three seedlings.

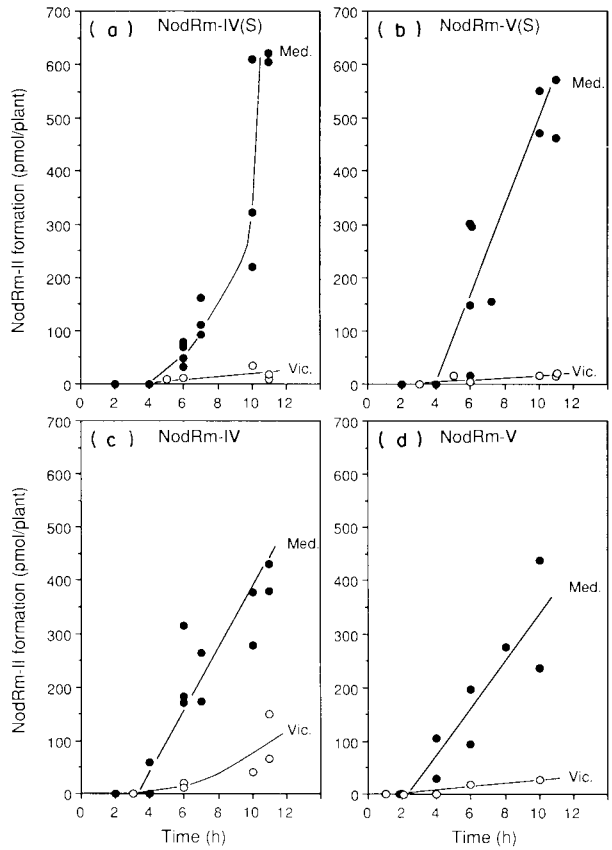

Figure 4. Production of the acylated disaccharide NodRm-II from Nod factors by intact roots of *Medicago* and *Vicia*.
Each point represents the average of three seedlings.

Figure 5 shows that the total hydrolysis of Nod factors in the presence of *Medicago* roots, i.e. the production of NodRm-II, III and IV, was different for the four Nod factors. While at 2 and 4 h, cleavage products were already visible with the nonsulfated NodRm-IV and NodRm-V factors, products released from the sulfated compounds were detectable by their UV absorption only after 6 h. Moreover, the tetrasaccharide factors were degraded more slowly than the corresponding sulfated and nonsulfated pentasaccharides. Unlike on *Medicago* roots, the total hydrolysis on *Vicia* roots was mainly dependent on the trimer-forming activity and, therefore, the kinetics followed roughly those seen in Figure 3(b) (data not shown). In conclusion, our data indicate that the sulfate modification as well as the length of the oligosaccharide chain both influence the differential stability of Nod factors in the rhizosphere, by influencing the number and accessibility of cleavage sites, as summarized in Table 3 (white arrowheads).

HPLC analysis after incubation of sulfated Nod factors with intact roots never detected any desulfatation of the compounds. In addition, the release of the reducing end monomer was completely prevented by the sulfate group (Table 3). This would not have been the case if a plant sulfatase activity had hydrolyzed the sulfate group before further degradation of the oligosaccharide backbone. We therefore suggest that sulfatases were not involved in the observed Nod factor degradation by plant roots.

For the quantitative analysis of Nod factor degradation by HPLC, about 2 nmol per analysis were required, which corresponded to a concentration of 6.6 μM in the incubation medium. This was far above the concentration needed for activity in bioassays, i.e. 10^{-11} M NodRm-IV(S) for eliciting root hair deformation and 10^{-8} M for the induction of nodule-like structures on alfalfa (Lerouge *et al.*, 1990; Schultze *et al.*, 1992; Truchet *et al.*, 1991). Therefore, it was necessary to verify that the pattern of Nod factor degradation observed *in vivo* was not artificially caused by excess amounts of Nod factors. For this purpose, a low concentration of radiolabeled Nod factor obtained by catalytic tritiation of the lipid chain was assayed for degradation. One-day-old seedlings of *M. sativa* were incubated in Jensen medium containing 10 nM ^{3}H-labeled NodRm-IV(C16:0,S). After 0, 5 and 10 h, respectively, the butanol extractable compounds in the medium were analyzed on reverse phase TLC. As shown in Figure 6a, most of the NodRm-IV(C16:0,S) had disappeared after 10 h giving rise to a new compound of much lower mobility, most likely the saturated lipodisaccharide NodRm-II(C16:0). The decrease in the total amount of radioactivity was probably due to absorption and uptake of the Nod factor and its degradation product by the plant root. At high concentration (6.6 μM), the non-

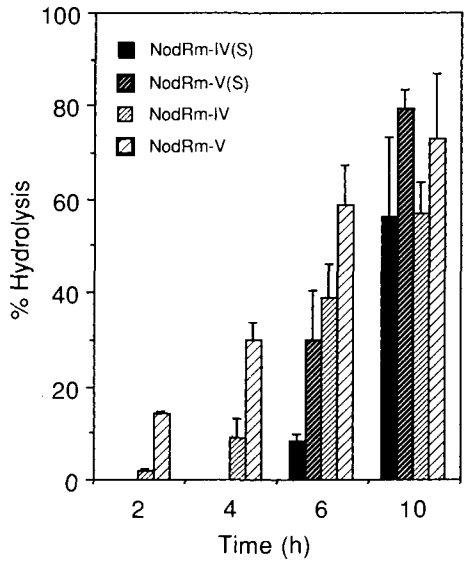

Figure 5. Total hydrolysis of Nod factors by intact roots of *Medicago*. Percentage of products NodRm-IV, III plus II from NodRm-V; NodRm-III plus II from NodRm-IV and NodRm-V(S); NodRm-II from NodRm-IV(S). Standard deviation of the mean (n = 2–4) is indicated.

labeled saturated NodRm-IV(C16:0,S) factor, obtained by catalytic hydrogenation, was degraded with similar kinetics within 10 h to a single product with the same mobility as that produced from ^{3}H-NodRm-IV(C16:0,S) (Figure 6b, lanes 1–3). This result indicates that degradation of the sulfated tetrasaccharide Nod factors was not significantly different at 10^{-8} M from that at 6.6×10^{-6} M. In comparison with the saturated NodRm-IV(C16:0,S), NodRm-IV(S) containing the nonsaturated C16:2 fatty acyl chain (= NodRm-IV(C16:2,S)) was degraded within a similar time period (compare lanes 1–3 with lanes 4–6). In this case, the product co-migrated, as expected, with purified NodRm-II(C16:2) (lane 7). Thus, the degree of saturation of the lipid chain does not seem to influence the stability of Nod factors.

Biological activity of the cleavage products

The purified hydrolytic products NodRM-II and NodRm-III were tested for biological activity in the root hair deformation assay. Activity was only observed at a concentration of 10 μM on both *Medicago* and *Vicia*, as shown in Table 4. In contrast, NodRm-IV(S) and NodRm-IV were active in the picomolar concentration range on *Medicago* and *Vicia*, respectively, and the presence of the sulfate group was necessary for activity on *Medicago* and its absence was necessary for activity on *Vicia*, in agreement with previous results (Roche *et al.*, 1991b). Thus, the acylated cleavage products are practically inactive compared with the cognate Nod factors. We have also assayed an equimolar

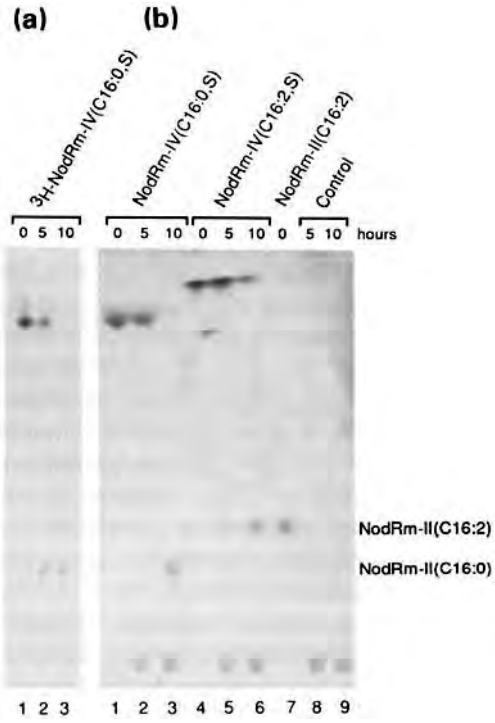

Figure 6. Degradation of sulfated tetrasaccharide Nod factors by *Medicago* roots.
(a) Separation on TLC and detection by autoradiography, of radiolabeled Nod factor and its lipophilic degradation product. For the assay, 2.5×10^5 c.p.m. per plant corresponding to a concentration of 10^{-8} M were used.
(b) TLC analysis and staining for sugars, of Nod factors and degradation products having saturated (C16:0) and unsaturated (C16:2) fatty acyl chains. Nod factors were incubated at a concentration of 6.6×10^{-6} M. Lane 7: the acylated hydrolysis product of NodRm-IV(C16:2,S), purified by HPLC and identified as NodRm-II(C16:2). Lanes 8 and 9: exudates from roots incubated in the absence of Nod factors.

mixture of NodRm-III and chitobiose, the cleavage products of NodRm-V, and found that the mixture was not more active than NodRm-III alone on *Vicia* (data not shown). This result shows that hydrolysis of the glycosidic bond is sufficient to inactivate the Nod factors.

Discussion

In this communication we have demonstrated the direct interaction of lipooligosaccharide Nod factors, a new class of plant growth regulators, with a class of host plant proteins, namely the chitinolytic enzymes. Purified plant chitinases have different affinities for different Nod factors which is indicated by the striking differences in the stability of Nod factors against hydrolysis by the chitinases. Three structural parameters, the length of the oligosaccharide chain, the acylation at the nonreducing end and the sulfatation at the reducing end, influenced the stability of Nod factors against degradation by chitinases purified from *Medicago* and *Vicia*.

Table 4. Root hair deformation assay

Treatment	Nod factor concentration (M)					
	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
Medicago sativa						
NodRm-IV(S)	+	+	+	+	+	+
NodRm-IV	+	+	–	–	–	–
NodRm-III	+	+/–	–	–	–	–
NodRm-II	+	–	–	–	–	–
Vicia sativa						
NodRm-IV(S)	+	+	–	–	–	–
NodRm-IV	+	+	+	+	+	+/–
NodRm-III	+	–	–	–	–	–
NodRm-II	+	–	–	–	–	–

The differential stability of Nod factors in the order NodRm-IV(S) > NodRm-V(S) > NodRm-IV > NodRm-V observed with purified chitinase was also seen when Nod factors were incubated with intact roots of *Medicago*. The stability of the Nod factors correlated with their biological activity on *Medicago*. The initial rate of hydrolysis was lowest for the cognate signal molecule of *R. meliloti*, NodRm-IV(S), followed by NodRm-V(S) and NodRm-IV. In the same order, the ability of the Nod factors to induce root hair deformation or the development of empty nodules decreases (Roche *et al.*, 1991b; Schultze *et al.*, 1992; Truchet *et al.*, 1991). Our finding that cleavage inactivates the Nod factors led us to hypothesize that plant chitinases may influence the biological activity of Nod signals by controlling their half-life time.

The finding that Nod factors are cleaved by chitinases at different rates raises the question of whether Nod factor stability could be one of the determinants of host specificity. Under certain growth conditions *Medicago* can be nodulated by *nodH* mutants of *R. meliloti* (Ogawa *et al.*, 1991), indicating that *Medicago* has the potential to recognize nonsulfated Nod factors also. The low biological activity of nonsulfated factors on *Medicago* in bioassays may therefore be at least partly due to their lower stability against hydrolysis by chitinase. The same parameters that conferred resistance of Nod factors against degradation by purified chitinase and roots of *Medicago*, also conferred resistance against hydrolysis by chitinase and roots of *Vicia*. Nod factor hydrolysis, therefore, cannot account for the specific recognition of nonsulfated Nod signals by *Vicia*. *Vicia* might contain a Nod signal receptor that is able to interact only with nonsulfated Nod factors. In contrast, a putative receptor on *Medicago* roots might be flexible enough to interact with sulfated as well as nonsulfated Nod factors. In this case, part of the differences in biological activity might be determined by chitinases whose activity is possibly reduced under certain growth conditions allowing *Medicago* plants to respond to nonsulfated Nod factors.

In both *Medicago* and *Vicia* roots, two Nod factor-cleaving activities were detected. One activity corresponded to the activity of the purified chitinases and produced an acylated trimer (NodRm-III) from all Nod factors excepting NodRm-IV(S), and another one which also cleaved NodRm-IV(S) and produced an acylated dimer (NodRm-II). This dimer-forming activity was the prevalent hydrolytic activity observed on *Medicago* roots and appeared to be inducible in the presence of high concentrations of NodRm-IV(S). We observed greater variations from one experiment to another for the activity of the dimer-releasing hydrolase than for the trimer-forming activity. This may possibly be due to temporal and spatial differences in the production of the enzyme during root development. It remains to be investigated which hydrolytic activity is present in different zones of the growing root and which enzymes accumulate at the onset of root hair infection by rhizobia.

Nod factors are highly active morphogenic and mitogenic signal molecules. It has been suggested that specific receptors exist in plant cells, which are able to distinguish between the various structural modifications of Nod factors produced by different rhizobial strains and species (see Hirsch, 1992). Nod signals are perceived rapidly by plant cells since they are able to induce membrane depolarization in alfalfa root hairs a few minutes after the addition of the Nod factors (Ehrhardt *et al.*, 1992). Possibly, the increased formation of NodRm-II in roots of *Medicago* is an early response after recognition of Nod signals leading to its inactivation. Such a process of autoregulation might be important to avoid continuous stimulation by active Nod factors as well as a potential elicitation of plant defense reactions by exceedingly high concentrations of Nod signal molecules. It would contribute to a mechanism which allows symbiotic bacteria to induce plant organogenesis but avoids defense reactions. While on plants like *Vicia* the trimer-forming chitinase is sufficient for inactivation of the cognate Nod signals, on *Medicago* only the dimer-forming enzyme can inactivate NodRm-IV(S), and this might explain the higher activity of this enzyme in *Medicago* roots.

In alfalfa, nodulation is under feedback control (Caetano-Anollés and Bauer, 1988) and this is exerted during the initiation of cortical cell divisions (Caetano-Anollés and Gresshoff, 1991) and the infection process (Vasse *et al.*, 1993). Interestingly, in the latter case a hypersensitive response, including the accumulation of chitinases, has been correlated with the abortion of infection threads, and Nod signals were proposed as possible targets for the chitinases. Our experiments showed that *Medicago* roots are indeed able to degrade efficiently the cognate Nod signal NodRm-IV(S). Our experimental set-up led probably to an underestimation of the hydrolytic activity present on the surface or within the root. In soil conditions it is not easy to calculate the actual turnover rate during the *Rhizobium*–plant interactions. This would depend on both the rate of synthesis and the velocity of degradation by plant enzymes. Whether the true degradation kinetics would be fast enough to interfere with the reception of Nod signals remains open.

Certain types of chitinases are inducible by the plant hormone ethylene (see Boller, 1988), such as those which we purified from *Medicago* and *Vicia*. Ethylene is able to inhibit nodulation (Goodlass and Smith, 1979; Grobbelaar *et al.*, 1971) while the ethylene inhibitor aminoethoxyvinylglycine (AVG) promotes nodule initiation (Peters and Crist-Estes, 1989; Zaat *et al.*, 1989). It is tempting to speculate that the control of nodulation by ethylene or by other factors, like light or nitrate, whose negative effects on nodulation are reported to be ethylene dependent (Lee and LaRue, 1992; Ligero *et al.*, 1991), may be mediated by Nod signal degradation.

With regard to the different stabilities of Nod factors, one may speculate about the evolutionary aspects of host-specific *Rhizobium*–plant interactions. Nod factors from *R. leguminosarum* bv. *viciae* usually do not have any specific modifications on the reducing end of their chito-oligosaccharide backbone. They seem to belong to a basic type of Nod factor which can easily be inactivated by chitinases of the host. Chitinases might have played an evolutionary role in providing a selective pressure which increased the stability of Nod factors through protective modifications, such as the sulfate group. The fucosyl or arabinosyl modification at the reducing end of Nod factors produced by other rhizobia might also contribute to the stabilization of the molecules against hydrolysis. Likewise, the possibility exists that *O*-acetylation at the nonreducing terminus, found in several rhizobial species including some strains of *R. meliloti* (Carlson *et al.*, 1993; Roche *et al.*, 1991a; Spaink *et al.*, 1991), confers increased stability. Interestingly, wild-type *R. meliloti* strains produce much lower quantities of Nod factors than *R. leguminosarum* bv. *viciae* (Spaink *et al.*, 1992). In strain TOM of *R. leguminosarum* bv. *viciae*, however, which produces Nod factors with an *O*-acetyl group at the reducing end, the production of Nod factors is substantially lower (Firmin *et al.*, 1993). It is tempting to speculate that in the rhizosphere a higher stability of Nod factors compensates for the lower amounts excreted by bacteria. The modified and more stable Nod factors have different chemical and physical properties, e.g. different hydrophobicity, with the result that in some cases they could not anymore be recognized by some of their former host plants. This might have been true for the sulfated Nod factors from *R. meliloti* which, at nanomolar concentrations, are inactive on *Vicia* (Roche *et al.*, 1991b). Conversely, *Medicago* seems to

have evolved new plant receptors that preferentially recognize Nod factors with a sulfate group.

Plant cells have a sensitive perception system for chitooligosaccharides leading to a rapid alkalinization of the culture medium in response to subnanomolar concentrations of the molecules (Felix *et al.*, 1993). Nod factors are recognized by the same perception system (Staehelin *et al.*, 1994). It is tempting to speculate that rhizobia exploited this system to develop a symbiotic signal perception.

Our data suggest that plant hydrolases are involved in controlling the biological activity of Nod factors by cleaving and inactivating them. These enzymes might therefore have the potential to control plant morphogenesis and cell division. This possibility is supported by recent observations which indicate that plant chitinases and other hydrolytic enyzmes, e.g. α-fucosidase (Augur *et al.*, 1993), may play a regulatory role in plant development of nonlegumes. It was reported that a glycosylated carrot chitinase is able to rescue a temperature-sensitive carrot cell line mutant deficient in embryogenesis (De Jong *et al.*, 1992). Interestingly, the same mutant can also be rescued for embryogenesis by Nod factors added to the culture medium, and it was postulated that Nod factors mimic an oligosaccharide signal generated by the carrot chitinase (De Jong *et al.*, 1993). Molecular genetic approaches are now required to test the hypothesis that hydrolytic enzymes, in addition to their role in plant defense, may be involved in the control of plant development. With respect to plant–microbe interactions, it would not be surprising that again plant defense related elements were exploited for symbiotic interactions.

Experimental procedures

Nod factors

Purification of NodRm-IV(C16:2,S) and NodRm-V(C16:2,S) has been described previously (Schultze *et al.*, 1992). Desulfated NodRm-IV(C16:2) and NodRm-V(C16:2) were prepared from the sulfated factors by mild acid hydrolysis in methanol–HCl (Truchet *et al.*, 1991) followed by purification on reverse phase HPLC. NodRm-IV(C16:0,S) and ^{3}H-NodRm-IV(C16:0,S) were obtained by catalytic reduction as described (Schultze *et al.*, 1992) using hydrogen and tritium gas, respectively.

Purification of plant chitinases

Chitinases were purified from 5-week-old roots of *Medicago sativa* cv. du Puits (TGCO, Montauban, France), and from 4-week-old roots of *Vicia sativa* L. cv. Hanka (Samen Schweizer, Thun, Switzerland) grown in modified sterilized Leonard Jars (Leonard, 1943) filled with perlite and vermiculite (ratio 1:1) and nutrient solution (Werner *et al.*, 1975) containing 20 mM KNO_3. Chitinase activity was induced by incubating the plants in closed plastic chambers containing 10 p.p.m. ethylene for 48 h (Boller *et al.*, 1983). Root material was ground in liquid nitrogen and extracted with 100 mM potassium phosphate buffer, pH 7 (1 ml g^{-1} fresh weight). After centrifugation (18 000 ***g***, 15 min) ammonium sulfate was added to the supernatant to 80% saturation. The redissolved precipitate was loaded on to a column of regenerated chitin as described by Mauch *et al.* (1988). Chitin-binding proteins were eluted with 100 mM acetic acid and immediately adjusted to pH 7 with Tris base. Samples were dialyzed, lyophilized and redissolved in 10 mM sodium phosphate buffer, pH 6 containing 140 mM NaCl. After filtration through a 0.22 μm Ultrafree-MC filter (Millipore) samples were loaded on to a FPLC Superose 12^{TM} column (Pharmacia) equilibrated with the same buffer. Fractions were collected and chitinase activity was measured radiometrically using ^{3}H-chitin as substrate (Boller *et al.*, 1983). The chitinases were separated on a 10% polyacrylamide gel (SDS–PAGE) according to Laemmli (1970) and were stained with Coomassie Brilliant Blue R 250. Protein was determined by the method of Bradford (1976).

Hydrolysis of Nod factors and chitooligosaccharides by chitinases

The reaction mixture (final volume 100 μl) for hydrolysis of Nod factors by chitinases contained 2.5 μg of Nod factors, 1% DMSO, 10 mM sodium phosphate buffer, pH 6, 10 mM NaCl, 0.02% sodium azide and chitinases. Samples of time zero served as controls. Incubation was at 37°C and reactions were stopped by freezing the samples. Finally 500 μl H_2O and 600 μl 1-butanol were added. Samples were shaken for 15 min and centrifuged. Aliquots of the 1-butanol phase were dried in a Speed-Vac evaporator and used for HPLC analysis. For kinetic studies 0.2 ng μl^{-1} chitinase were incubated with NodRm-V for 1 h , 0.2 ng μl^{-1} with NodRm-IV for 6 h, and 4 ng μl^{-1} with Nod-Rm-V(S) for 12 h. The volume of the reaction mixture with low Nod factor concentrations was increased in order to have sufficient amounts of cleavage products for analysis. After incubation, samples containing the same Nod factor were adjusted to the same volume with reaction mixture without Nod factors and chitinases, and extracted with an equal volume of butanol. Samples were dried and separated by HPLC. Acylated degradation products were quantified by integration of peak areas. K_m values were calculated from the double-reciprocal plot of the speed of release of acylated products (NodRm-III and NodRm-IV) against substrate concentration.

The reaction mixture of the chitinase assay with chitooligosaccharides as substrate contained 40 nmol chitobiose or chitotriose (pure grade, Seikagaku Corporation, Tokyo) instead of Nod factors. The formation of the monomeric *N*-acetylglucosamine was determined photometrically with 4-dimethylaminobenzaldehyde (Merck) according to the method of Reissig *et al.* (1955). Samples taken at time zero served as blanks.

Seed sterilization

Seeds of *M. sativa* cv. Sitel, used for *in vivo* hydrolysis and bioassays, were surface sterilized by treatment with 95% ethanol for 10 min followed by imbibition overnight in diluted commercial bleach (0.14% active chlorine). After thorough washing with sterile tap water, seeds were left to germinate on inverted 1.5% water agar plates in the dark at 24°C for 24 h. Seeds of *V. sativa* ssp. *nigra* were treated as described (Schultze *et al.*, 1992; Van Brussel *et al.*, 1982) using concentrated sulfuric acid and commercial bleach (12.5% active chlorine).

Hydrolysis of Nod factors by plant roots

The roots of 1-day-old seedlings of *Medicago* and *Vicia* were approximately the same size (1 cm) and root hairs started to emerge at the beginning of the experiment. Seedlings were placed on top of a 1 ml sterile plastic syringe filled with 0.1 ml deposit-free Jensen medium (Van Brussel *et al.*, 1982) containing 1% DMSO and 6.6 μM Nod factor, for HPLC analysis, or 10 nM radiolabeled Nod factor for TLC analysis. Syringes were capped with 1 ml pipette tips welded at the top and incubated for variable times at 24°C in the dark. The seedlings were removed and tested for contamination. The culture medium of three seedlings was pooled, diluted with water to 0.5 ml and extracted with 0.5 ml 1-butanol.

Analysis of cleavage products

Butanol extracts were dried under reduced pressure and the residue taken up in 1 μl DMSO and diluted in 40 μl 50% acetonitrile/water, 50 mM ammonium acetate. Twenty microliters were fractionated on a reverse phase HPLC column (Waters Nova Pak C_{18}, 3.9 × 150 mm, particle size 4 μm) under isocratic conditions with 35% acetonitrile/water, 50 mM ammonium acetate as the mobile phase. Substrates and products of the cleavage reaction were detected by their absorption at 220 nm. The structure of the products was confirmed by fast atom bombardment mass spectroscopy (FAB-MS), carried out on a Finnigan MAT90 in the positive ionization mode with a 8 keV xenon source and thioglycerol as matrix.

For TLC analysis of cleavage products, dried butanol extracts were redissolved in 2 μl butanol and fractionated on HPTLC plates (RP-18, 10 × 10 cm, Merck) using 50% acetonitrile/water as mobile phase. Radiolabeled compounds were detected by autoradiography of the dried plates for 10 days with X-Omat AR film (Kodak). Nonlabeled compounds were detected with the diphenylamine/aniline/orthophosphoric acid reagent (Walkley and Tillman, 1977).

Root hair deformation assay

Eight 1-day-old seedlings of *M. sativa* cv. Sitel or *V. sativa* ssp. *nigra* were transferred to 1.5% Jensen agar plates and preincubated in a vertical position at 24°C in the dark for 36 h. Nod factors were dissolved in DMSO and diluted in Jensen medium maintaining a final concentration of DMSO of 1%. Ten microliters of Nod factor solution were added to the root of each seedling. Plates were incubated vertically for several days in the greenhouse and root hair deformation and branching was scored as described (Faucher *et al.*, 1988; Zaat *et al.*, 1987). A positive score was given when at least five out of the eight plants showed strong root hair deformations of branching. The experiment was repeated two times.

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