

Nod Factors Induce Nod Factor Cleaving Enzymes in Pea Roots. Genetic and Pharmacological Approaches Indicate Different Activation Mechanisms¹

Alexandra O. OvtSYna, Elena A. Dolgikh, Alexandra S. Kilanova, Viktor E. Tsyganov, Alexey Y. Borisov, Igor A. Tikhonovich, and Christian Staehelin*

All-Russia Research Institute for Agricultural Microbiology, 196608 St. Petersburg, Pushkin, Russia (A.O.O., E.A.D., V.E.T., A.Y.B., I.A.T.); St. Petersburg State University, 199034 St. Petersburg, Russia (A.S.K.); and State Key Laboratory of Biocontrol, School of Life Sciences, SunYat-Sen (Zhongshan) University, Guangzhou 510275, China (C.S.)

Establishment of symbiosis between legumes and rhizobia requires bacterial Nod factors (NFs). The concentration of these lipochitooligosaccharides in the rhizosphere is influenced by plant enzymes. NFs induce on pea (*Pisum sativum*) a particular extracellular NF hydrolase that releases lipodisaccharides from NFs from *Sinorhizobium meliloti*. Here, we investigated the ability of non-nodulating pea mutants to respond to NodRlv factors (NFs from *Rhizobium leguminosarum* bv *viciae*) with enhanced NF hydrolase activity. Mutants defective in the symbiotic genes *sym10*, *sym8*, *sym19*, and *sym9/sym30* did not exhibit any stimulation of the NF hydrolase, indicating that the enzyme is induced via an NF signal transduction pathway that includes calcium spiking (transient increases in intracellular Ca²⁺ levels). Interestingly, the NF hydrolase activity in these *sym* mutants was even lower than in wild-type peas, which were not pretreated with NodRlv factors. Activation of the NF hydrolase in wild-type plants was a specific response to NodRlv factors. The induction of the NF hydrolase was blocked by α -amanitin, cycloheximide, tunicamycin, EGTA, U73122, and calyculin A. Inhibitory effects, albeit weaker, were also found for brefeldin A, BHQ and ethephon. In addition to this NF hydrolase, NFs and stress-related signals (ethylene and salicylic acid) stimulated a pea chitinase that released lipotrisaccharides from pentameric NFs from *S. meliloti*. NodRlv factors failed to stimulate the chitinase in mutants defective in the *sym10* and *sym8* genes, whereas other mutants (e.g. mutated in the *sym19* gene) retained their ability to increase the chitinase activity. These findings indicate that calcium spiking is not implicated in stimulation of the chitinase. We suggest that downstream of *Sym8*, a stress-related signal transduction pathway branches off from the NF signal transduction pathway.

Establishment of symbiosis between legumes and nitrogen-fixing rhizobia results in the formation of a new plant organ, the root nodule. Rhizobia enter the host plant usually through infection of root hairs. Bacteria within root hairs induce the formation of an infection thread that grows toward the dividing cortical cells. In a later symbiotic stage, rhizobia are released from branched infection threads into the developing nodule tissue and differentiate into nitrogen-fixing bacteroids. Nodule formation is controlled by perception of rhizobial nodulation signals: the Nod factors (NFs). Flavonoids from the host plant, in conjugation with the rhizobial activator protein NodD, induce rhizobial nodulation genes (*nod*, *nol*, and *noe*) that are required for NF synthesis. NFs are modified lipochitooligosaccharides, i.e. chitin oligomers linked with a fatty acid replacing the *N*-acetyl group on their non-

reducing end (Perret et al., 2000; OvtSYna and Staehelin, 2003). On host plants, NFs induce various responses at picomolar to nanomolar concentrations. These rapid changes include root hair curling (Hac; e.g. Heidstra et al., 1994), depolarization of plasma membranes (Ehrhardt et al., 1992; Felle et al., 1995), changes in H⁺ and Ca²⁺ fluxes (e.g. Felle et al., 2000), and regular oscillation of intracellular Ca²⁺ concentrations, called calcium spiking (Ehrhardt et al., 1996; Walker et al., 2000; Engstrom et al., 2002). When applied to roots, NFs rapidly alter the expression of various genes of the host plant and induce symbiosis-specific early nodulin (*enod*) genes (e.g. Albrecht et al., 1998; Pingret et al., 1998; Charron et al., 2004; Mitra et al., 2004).

Using well-characterized plant mutants unable to form nodules (Nod⁻ phenotype), a number of plant genes controlling early symbiotic stages have been identified. These genes are part of a genetic network that controls perception and signal transduction of NFs. NF recognition seems to be mediated by the recently discovered NF receptors, i.e. Ser/Thr kinases with extracellular LysM domains (Limpens et al., 2003; Madsen et al., 2003; Radutoiu et al., 2003). The LysM domains of prokaryotic enzymes bind to bacterial peptidoglycan, which is structurally related to the oligosaccharide moiety of NFs. Pea (*Pisum sativum*) plants mutated in the symbiotic gene *sym10* lack

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* Corresponding author; e-mail lsdcs@zsu.edu.cn; fax 862084115516.

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a functional receptor kinase of the LysM type (Madsen et al., 2003) and thus do not show any responses when challenged with NFs from *Rhizobium leguminosarum* bv *viciae* (NodRlv factors; Walker et al., 2000). Other mutants exhibit a limited number of NF responses. For example, pea plants mutated in the genes *sym8* and *sym19* respond to NFs with membrane depolarization and ion fluxes but lack the calcium spiking response (Walker et al., 2000). It has been proposed that the *Sym8* gene of pea is orthologous to *DMI1* from *Medicago truncatula*. The *DMI1* gene encodes a putative membrane-spanning cation channel, which seems to play a role in NF signal transduction (Ané et al., 2004). In *Lotus japonicus*, two related proteins (CASTOR and POLLUX) are localized in the plastids of root cells (Imaizumi-Anraku et al., 2005). The *Sym19* gene from pea is orthologous to *SYMRK* from *L. japonicus*, *NORK* from alfalfa (*Medicago sativa*) and *DMI2* from *M. truncatula*. These genes encode for proteins belonging to the family of Leu-rich repeat (LRR) receptor kinases (Endre et al., 2002; Stracke et al., 2002). Another class of non-nodulating pea mutants (e.g. defective in the gene *sym9/sym30*) responds to NFs with calcium spiking but lacks downstream responses. The *Sym9/Sym30* gene from pea and the orthologous *DMI3* from *M. truncatula* are predicted to encode Ca^{2+} /calmodulin-dependent protein kinases. It has been proposed that these protein kinases are putative calcium-sensitive effector proteins that function downstream of calcium spiking (Lévy et al., 2004; Mitra et al., 2004). All mutants mentioned above are defective in root hair curling in response to NFs (Hac^- phenotype; Markwei and LaRue, 1992; Sagan et al., 1994). Other non-nodulating pea mutants display a Hac^+ phenotype and seem to be impaired in further steps of symbiotic signaling, which are required for bacterial infection and nodule development. Plants mutated in the gene *sym7* are affected at the stage of bacterial colonization of the curled root hair (Tsyganov et al., 2002). In the other mutants (i.e. plants with mutated *sym14*, *sym34*, *sym35*, and *sym36* genes), rhizobia enter root hairs but are blocked at various stages of infection thread development (Tsyganov et al., 2002). Table I gives an overview of the various pea mutants used in this study.

NFs are active at picomolar to nanomolar concentrations, and the actual concentration of these signals in the rhizosphere is influenced by hydrolytic enzymes of the host plant. Applied to roots, purified cleavage products were nearly inactive in inducing NF responses (Heidstra et al., 1994; Staehelin et al., 1994). Studies with alfalfa and pea plants indicated that NFs stimulate their own degradation. Roots pretreated with nanomolar concentrations of NFs exhibited an increased NF cleaving activity in the rhizosphere (Staehelin et al., 1995; Ovtsyna et al., 2000). The NF-inducible glycosyl hydrolase released lipodisaccharides from NFs from *Sinorhizobium meliloti* (NodSm factors; Staehelin et al., 1994, 1995; Ovtsyna et al., 2000). The partially purified enzyme from alfalfa roots did not exhibit activity toward the polymer chitin, but

cleaved the O-acetylated tetrameric NodSm-IV($C_{16:2}$, Ac, S) (Staehelin et al., 1995, 1997). The substrate and cleavage specificity of this novel NF hydrolase is therefore different from known plant chitinases that cannot release the lipodisaccharide from NodSm-IV($C_{16:2}$, Ac, S) (Staehelin et al., 1994; Minic et al., 1998; Schultze et al., 1998). Most chitinases are stress-induced enzymes involved in plant defense reactions, but certain isoenzymes appear to have a specific function in mutualistic symbioses. In the context of nodule symbioses, chitinase isoenzymes have been described to be induced at various stages of symbiosis in compatible and incompatible interactions (e.g. Staehelin et al., 1992; Vasse et al., 1993; Goormachtig et al., 1998; Xie et al., 1999; Salzer et al., 2004).

The work of this article was initiated in order to understand how NFs stimulate their own degradation. Using a combination of genetic and pharmacological approaches, we found that the lipodisaccharide-forming NF hydrolase is specifically activated via an NF signal transduction pathway that includes calcium spiking. In the course of these studies, we identified a chitinase isoenzyme, whose activity was enhanced after inoculation with rhizobia and application of purified NFs, as well as various stress conditions. NFs stimulated the chitinase activity in pea mutants that lack the calcium spiking response. These findings indicate that the NF signal transduction pathway may be linked with a stress-related signal transduction pathway.

RESULTS

NF Degradation in the Rhizosphere and in Vitro

We have shown in a previous study that nanomolar concentrations of NodRlv factors enhance an NF cleaving activity in the rhizosphere of pea plants. NodSm factors did not stimulate the hydrolytic activity and therefore served as an inert substrate for the enzyme assay (Ovtsyna et al., 2000). When incubated with roots from intact pea plants, NodSm factors and their acylated degradation products could be recovered from the incubation medium and subsequently analyzed by HPLC. As shown in Figure 1 (chromatogram A), the tetrameric NodSm-IV($C_{16:2}$, S) exhibited a high stability in the rhizosphere. When roots were pretreated with NodRlv factors for 20 h, considerable amounts of an acylated cleavage product, the lipodisaccharide NodSm-II($C_{16:2}$) (Staehelin et al., 1994), were formed (Fig. 1, chromatogram B). A similar induction of NodSm-II($C_{16:2}$) formation has been previously reported for pea plants pretreated with NodRlv factors and subsequently incubated with the pentameric substrate NodSm-V($C_{16:2}$, S) (Ovtsyna et al., 2000). Hence, NodRlv factors stimulate an extracellular NF hydrolase activity that releases lipodisaccharides either from tetrameric or from pentameric NodSm factors. In this study, we used both NodSm factors for the NF hydrolase assay and observed a similar activity of NodSm-II($C_{16:2}$) formation.

Table I. *Pea mutants used in this study*

Gene symbols for mutants from cv Finale and Frisson are given according to Borisov et al. (2000), based on personal communication with G. Duc and M. Sagan. Phenotypes of mutants marked with asterisks are inferred from analysis of allelic mutants with characterized phenotypes. Alleles of the *sym30* gene have been recently referred to a complementation group with the *sym9* gene (Schneider et al., 2002; Lévy et al., 2004; Mitra et al., 2004). Nod⁺, nodulation; Nod⁻, no nodulation; Inf⁻, no infection; Hac⁻, no root hair curling; Nod⁺⁺, hypernodulation; Crh⁻, blockage at the stage of bacterial colonization of the curled root hair; lti⁻, blockage at the stage of infection thread initiation; lth⁻, root hair with arrested infection thread; ltr⁻, root cortex with arrested infection threads. Initials of legume species preceding the gene names: *Lj*, *Lotus japonicus*; *Ms*, *Medicago sativa*; *Mt*, *Medicago truncatula*; *Gm*, *Glycine max*; *As*, *Astragalus sinicus*; *Sr*, *Sesbania rostrata*; *Ma*, *Melilotus albus*.

Genotype	Phenotype	Predicted Protein	Gene Localization and Sequence Information	Selected References	Related Genes in Other Legumes
Finale (wild type)	Nod ⁺			Engvild (1987)	
RisNod19 (<i>sym8</i>) and RisNod10 (<i>sym8</i>)	Nod ⁻ , Hac ⁻ , no Ca ²⁺ spiking*		Linkage group VI	Engvild (1987); Kneen et al. (1994)	
RisNod2 (<i>sym19</i>)	Nod ⁻ , Hac ⁻ , no Ca ²⁺ spiking*	LRR receptor kinase (Sym19)	Mutations sequenced in the allelic mutants P4 and P55	Engvild (1987); Endre et al. (2002); Stracke et al. (2002)	<i>LjSYM</i> RK (AF492655), <i>MsNORK</i> (AJ418368), <i>MtSYM</i> RK (AF491998), <i>MtDMI</i> 2 (AJ418371, AJ418373), <i>MaNORK</i> (AJ428991), <i>SrSYM</i> RK (AY751547), <i>AsNORK</i> (AY946203)
RisNod22 (<i>sym30</i>) (<i>sym30</i> = <i>sym9</i>)	Nod ⁻ , Hac ⁻ , Ca ²⁺ spiking*	Ca ²⁺ /calmodulin-dependent protein kinase (Sym9)	Mutation sequenced	Engvild (1987); Lévy et al. (2004)	<i>MtDMI</i> 3 (AY502066)
RisNod8 (<i>sym35</i>)	Nod ⁻ , Inf ⁻ (lti ⁻)	Transcriptional regulator (PsNin)	Mutation sequenced	Engvild (1987); Borisov et al. (2003)	<i>LjNin</i> (AJ239041)
RisNod26 (<i>sym36</i>)	Nod ⁻ , Inf ⁻ (lth ⁻)			Engvild (1987); Sagan et al. (1994)	
RisNod1 (<i>sym34</i>) and RisNod23 (<i>sym34</i>)	Nod ⁻ , Inf ⁻ (ltr ⁻)			Engvild (1987); Tsyganov et al. (2002)	
RisFixC	Nod ⁺⁺			Engvild (1987); Novak et al. (1997)	
Frisson (wild type)	Nod ⁺			Duc and Messenger (1989)	
P56 (<i>sym10</i>)	Nod ⁻ , Hac ⁻ , no Ca ²⁺ spiking	Receptor kinase of the LysM type (Sym10)	Mutation sequenced	Sagan et al. (1994); Walker et al. (2000); Madsen et al. (2003)	<i>LjNFR</i> 5 (AJ575255)
P6 (<i>sym19</i>)	Nod ⁻ , Hac ⁻ , no Ca ²⁺ spiking*	LRR receptor kinase (Sym19)	Mutations sequenced in the allelic mutants P4 and P55	Sagan et al. (1994); Walker et al. (2000); Endre et al. (2002); Stracke et al. (2002)	<i>LjSYM</i> RK (AF492655), <i>MsNORK</i> (AJ418368), <i>MtSYM</i> RK (AF491998), <i>MtDMI</i> 2 (AJ418371, AJ418373), <i>MaNORK</i> (AJ428991), <i>SrSYM</i> RK (AY751547), <i>AsNORK</i> (AY946203)

(Table continues on following page.)

Table I. (Continued from previous page.)

Genotype	Phenotype	Predicted Protein	Gene Localization and Sequence Information	Selected References	Related Genes in Other Legumes
P2 (<i>sym30</i>) (<i>sym30</i> = <i>sym9</i>)	Nod ⁻ , Hac ⁻ , Ca ²⁺ spiking*	Ca ²⁺ /calmodulin-dependent protein kinase (Sym9)	Mutation sequenced	Sagan et al. (1994); Walker et al. (2000); Lévy et al. (2004); Mitra et al. (2004)	<i>MtDMI3</i> (AY502066)
P54 (<i>sym9</i>)	Nod ⁻ , Hac ⁻ , Ca ²⁺ spiking*	Ca ²⁺ / calmodulin-dependent protein kinase (Sym9)	Mutation sequenced	Sagan et al. (1994); Walker et al. (2000); Lévy et al. (2004); Mitra et al. (2004)	<i>MtDMI3</i> (AY502066)
P64 (<i>sym28</i>) P88 (<i>sym29</i>)	Nod ⁺⁺ Nod ⁺⁺	LRR receptor-like kinase (Sym29)	Mutation sequenced	Sagan and Duc (1996) Sagan and Duc (1996); Krusell et al. (2002)	<i>LjHAR1</i> (AJ495844, AB092810), <i>GmNARK</i> (AY166655)
Sparkle (wild type) R72 (<i>sym9</i>)	Nod ⁺ Nod ⁻ , Hac ⁻ , Ca ²⁺ spiking*	Ca ²⁺ / calmodulin-dependent protein kinase (Sym9)	Mutation sequenced	Kneen and LaRue (1984) Markwei and LaRue (1992); Lévy et al. (2004); Mitra et al. (2004)	<i>MtDMI3</i> (AY502066)
E69 (<i>sym7</i>) E135N (<i>sym14</i>)	Nod ⁻ , Inf ⁻ (Crh ⁻)*, Ca ²⁺ spiking Nod ⁻ , Inf ⁻ (Iti ⁻)*		Linkage group III Linkage group II	Kneen et al. (1994); Walker et al. (2000) Kneen et al. (1990)	

The performed enzyme assay with roots from intact pea seedlings reflects the extracellular activity of the NF hydrolase. To measure the plant's total NF hydrolase activity in vitro, soluble proteins from root extracts were used for the hydrolytic assay with NodSm-IV(C_{16:2}, S). Compared to a mock treatment with Jensen medium, plants challenged with NodRlv factors exhibited a strong lipodisaccharide-forming degradation activity in their root extracts (Fig. 1, chromatograms C and D). A similar induction of NodSm-II(C_{16:2}) formation was also observed with the pentameric substrate NodSm-V(C_{16:2}, S) (data not shown). These results indicate that the activity of the lipodisaccharide-forming NF hydrolase can also be measured in vitro.

As only low activity was found in noninduced roots, NodRlv factors seem to activate synthesis of the NF hydrolase. To substantiate this possibility, a number of inhibitors were tested for their capacity to reduce the NF hydrolase activity. Studies were performed with intact plants, which were concomitantly treated with NodRlv factors and the indicated inhibitors (Table II). Roots were subsequently assayed for their extracellular activity to release lipodisaccharides from NodSm-V(C_{16:2}, S). Addition of the transcription inhibitor α -amanitin blocked the activation of the NF hydrolase. The translation inhibitor cycloheximide had a similar effect and completely abolished the enzyme activity. Induction of the enzyme was also suppressed by tunicamycin, an inhibitor of *N*-glycosylation. Brefeldin A, an inhibitor of protein trafficking between the endoplasmic reticulum and the Golgi apparatus, reduced the enzyme activity. These data indicate that activation of the extracellular NF hydrolase by NodRlv

factors includes transcription, de novo protein synthesis, *N*-glycosylation, and finally transport through the secretory pathway into the rhizosphere.

To determine the minimal time required for activation of the NF hydrolase, we pretreated pea plants with NodRlv factors for different times and then determined the extracellular NF hydrolase activity with NodSm factors. Stimulation of the enzyme activity already started after 2 h, and maximal induction was reached after treatment with NodRlv factors for 20 h. Longer treatments with NodRlv factors did not increase the hydrolytic activity (data not shown).

Activation of the NF hydrolase was also tested upon incubation with rhizobial cultures. As shown previously, a pretreatment with *R. leguminosarum* bv *viciae* CIAM 1026 induced the enzyme activity. Strains producing lower levels of NFs (e.g. strain TOM) were less active in enhancing the NF hydrolase (Ovtsyna et al., 2000). No stimulation of the enzyme was observed with *R. leguminosarum* bv *viciae* RBL5562, a strain with mutated *nodA* gene and, thus, defective in NF production (data not shown). Hence, NFs, but no other rhizobial stimuli, elicited the induction of the NF hydrolase activity.

Genes of the NF Signaling Pathway Required for Activation of the NF Hydrolase

Non-nodulating pea mutants of cultivars Frisson, Finale, and Sparkle (Table I) were used to characterize signaling events required for stimulation of the NF hydrolase. Plants were pretreated with 10⁻⁶ M NodRlv factors overnight and the extracellular NF hydrolase

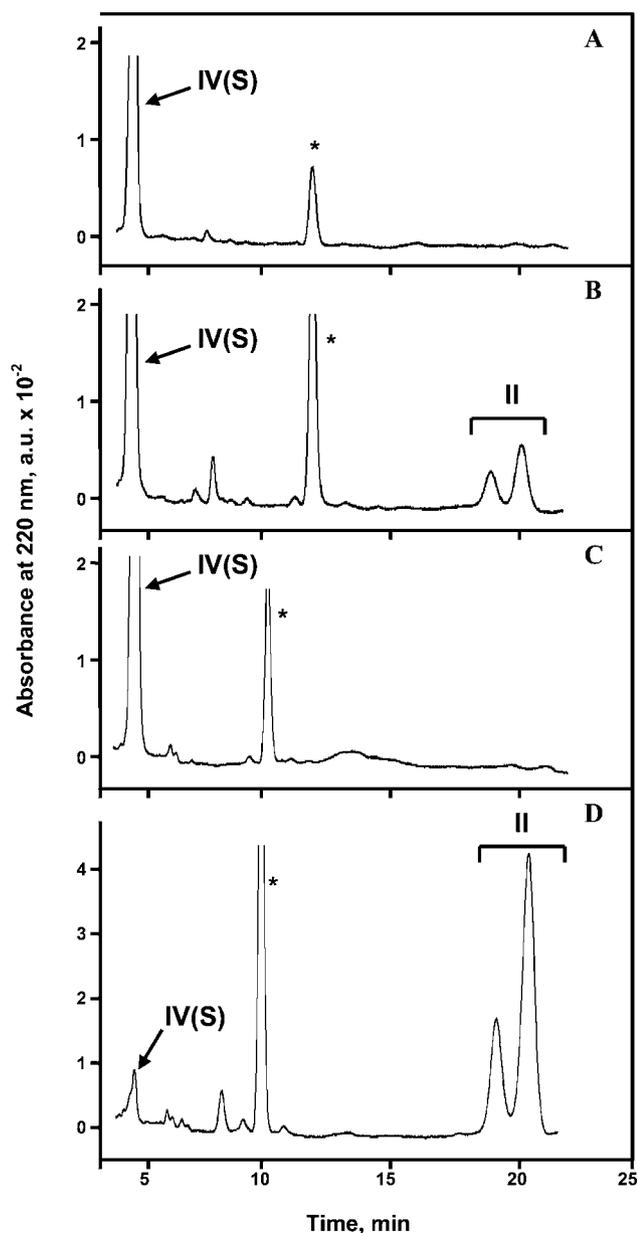


Figure 1. Reverse-phase HPLC of NodSm-IV(C_{16:2}, S) and its cleavage product NodSm-II(C_{16:2}). Roots (cv Frisson) were either pretreated with Jensen medium containing 10⁻⁶ M NodRlv factors [NodRlv-V(C_{18:4}, Ac) and NodRlv-IV(C_{18:4}, Ac)] for 20 h or mock treated with Jensen medium. The intact roots (A and B) and proteins from root extracts (C and D) were then assayed for their hydrolytic activity using NodSm-IV(C_{16:2}, S) as substrate. After extraction with *n*-butanol, the NF substrate and the acylated cleavage product NodSm-II(C_{16:2}) were fractionated on reverse-phase HPLC. a.u., Absorbance units. The length of the oligosaccharide chain (roman numerals) and the presence of a sulfate group are indicated. The NodSm-II(C_{16:2}) cleavage product is separated into its anomers (double peak). A nonidentified root compound is marked with an asterisk. A, Hydrolysis (25°C, 4 h) of 5 μM NodSm-IV(C_{16:2}, S) by the intact root after mock treatment with Jensen medium. B, Hydrolysis (25°C, 4 h) of 5 μM NodSm-IV(C_{16:2}, S) by the intact root after pretreatment with 10⁻⁶ M NodRlv factors. C, Hydrolysis (37°C, 4 h) of 40 μM NodSm-IV(C_{16:2}, S) by soluble proteins (30 μg) from root extracts after mock treatment with Jensen medium. D, Hydrolysis (37°C, 4 h) of 40 μM NodSm-IV(C_{16:2}, S) by proteins (30 μg) from root extracts after pretreatment with 10⁻⁶ M NodRlv factors.

activity assayed with the substrate NodSm-V(C_{16:2}, S). As shown in Figure 2 (chromatograms A and B), a pretreatment with NodRlv factors stimulated the NodSm-II(C_{16:2}) forming activity in wild-type plants. In the absence of NodRlv factors, plants from cv Sparkle exhibited a relatively high constitutive activity compared to cv Finale (Fig. 3). The NF activity of all three cultivars reached similar levels when plants were treated with NodRlv factors. All nodulation mutants with a Hac⁻ phenotype were unable to induce the NF hydrolase. When challenged with NodRlv factors, plants carrying mutations in the genes *sym10*, *sym8*, *sym19*, or *sym9/sym30* did not show any activation of the NF hydrolase (Fig. 2, chromatograms C–E, and Fig. 3). Interestingly, these mutants produced even lower amounts of NodSm-II(C_{16:2}) than mock-treated wild-type plants (Fig. 3). These data give genetic evidence that induction of the NF hydrolase requires a functional Nod signal transduction pathway.

Next, we extended our study on non-nodulating mutants, which are blocked at later symbiotic stages. The mutant E69 (*sym7*) has a Hac⁺ phenotype, and infection is blocked at the stage of bacterial colonization of curled root hairs. Induction experiments with this mutant showed that the NF hydrolase is enhanced to an extent comparable with that of wild-type plants (Fig. 3C). Thus, activation of the enzyme seems to be associated with rhizobial entry into root hairs.

Other mutants with a Hac⁺ phenotype and blocked at different stages of infection thread formation responded to NodRlv factors with increased activity of the NF hydrolase (mutants defective in the genes *sym34* and *sym36*; Fig. 3A). NodRlv factors also stimulated the NF hydrolase activity in the mutant E135N (*sym14*) and in RisNod8 (*sym35*) with mutated *nin* gene (Borisov et al., 2003). Due to reduced growth of seedlings, the NF hydrolase activity in these mutants did not allow direct comparison with wild-type plants (data not shown).

Finally, we tested the induction of the NF hydrolase in hypernodulating mutants that are affected in autoregulation of nodule formation. After pretreatment with NodRlv factors, we measured increased NF hydrolase activity in the rhizosphere of the hypernodulating mutants P64 (*sym28*), P88 (*sym29*) (Fig. 3B), and RisFixC (data not shown). NF hydrolase activities in these mutants were not significantly different from those in wild-type plants. Thus, genes involved in autoregulation seem not to be required for induction of the NF hydrolase.

Activation of the NF Hydrolase Is a Very Specific Response to NFs

Using a pharmacological approach, we further studied the effect of various chemicals on the NF hydrolase activity (Table III). Calyculin A is a phosphatase inhibitor that mimics elicitor action in plant cells (Felix et al., 1994). Calyculin A was not able to

Table II. Inhibitors of protein synthesis, *N*-glycosylation, and protein traffic negatively affect the extracellular NF hydrolase activity

Four-day-old seedlings (cv Frisson) were incubated for 20 h in 1-mL plastic syringes filled with 600 μ L of Jensen medium containing NodRlv factors (10^{-6} M) and the indicated inhibitor. For assaying the degradation of NFs in the rhizosphere, plants were transferred to syringes filled with Jensen medium containing 5 μ M NodSm-IV(C_{16;2}, S). Values of the NodSm-II(C_{16;2}) forming activity (relative units) indicate means \pm SE obtained from at least two independent experiments (three to four plants per each treatment).

Treatment	Mode of Action	Inhibitor Concentration	Lipodisaccharide Formation (%)
NodRlv factors	–	–	100
–	–	–	9.4 \pm 3.6
NodRlv factors + α -amanitin	Transcription inhibitor	100 μ M	4.4 \pm 2.0
NodRlv factors + cycloheximide	Translation inhibitor	10 μ M	0 \pm 0
NodRlv factors + tunicamycin	Inhibitor of <i>N</i> -glycosylation	10 μ g mL ⁻¹	2.8 \pm 1.6
NodRlv factors + brefeldin A	Inhibitor of protein translocation from endoplasmic reticulum to the Golgi apparatus	10 μ g mL ⁻¹	30.1 \pm 10.7

induce the NF hydrolase activity itself but efficiently inhibited enzyme activation by NodRlv factors. The calcium chelator EGTA blocked the activation of the NF hydrolase, indicating that Ca²⁺ is a prerequisite for the NF-induced increase in hydrolytic activity. The endomembrane Ca²⁺-ATPase inhibitor 2,5-di(*t*-butyl)-1,4-benzohydroquinone (BHQ) has been reported to interfere with NF signaling in *Medicago* sp. (Felle et al., 1999; Engstrom et al., 2002). In our study with pea plants, BHQ partially inhibited the activation of the NF hydrolase by NodRlv factors. The aminosteroid U73122, an inhibitor of phospholipase C activity, blocks calcium spiking (Engstrom et al., 2002) and NF-induced gene expression in *Medicago* plants (Pingret et al., 1998; Charron et al., 2004). NodRlv factors applied to pea roots did not induce the NF hydrolase in the presence of U73122, suggesting that heterotrimeric G-protein-mediated activation of phospholipase C is implicated in the activation of the NF hydrolase.

The phytohormone ethylene inhibits the NF signal transduction pathway and blocks the NF-inducible expression of early nodulin genes in *M. truncatula* (Oldroyd et al., 2001). In our study, the ethylene releasing compound ethephon partially inhibited the activation of the NF hydrolase. Inhibition of the plant's ethylene synthesis by 2-aminoethoxyvinylglycine (AVG) did not alter the enzyme activity of the NF hydrolase.

In the absence of NodRlv factors, the NF hydrolase was not induced by ethephon, the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC), and salicylic acid, which are known to induce pathogen-related proteins. Chitin oligomer elicitors (e.g. penta-*N*-acetylchitopentaose) were also completely inactive in inducing the NF hydrolase (Table III). Taken together, these data indicate that activation of the NF hydrolase is a very specific response to NFs, which cannot be mimicked by other molecules.

NFs also Stimulate a Lipotrisaccharide-Forming Chitinase

The extracellular NF hydrolase releases lipodisaccharides from tetrameric and pentameric NodSm factors (Ovtsyna et al., 2000; Fig. 1). When the pen-

tamer NodSm-V(C_{16;2}, S) was used as substrate for the assay, formation of the lipotrisaccharide NodSm-III(C_{16;2}) was observed, indicating the activity of an additional enzyme. A pretreatment of roots with NodRlv factors did not stimulate the lipotrisaccharide-forming activity in the rhizosphere (Fig. 2). In root protein extracts, however, inoculation with *R. leguminosarum* bv *viciae* CIAM 1026 (or preincubation with 10^{-6} M NodRlv factors) enhanced the lipotrisaccharide-forming activity in a reproducible manner (e.g. cv Finale: noninoculated plants, 1933 \pm 93 nmol h⁻¹ mg⁻¹ [protein]; inoculated plants, 2943 \pm 324 nmol h⁻¹ mg⁻¹ [protein]). These data suggest that NodRlv factors stimulate an intracellular enzyme. Interestingly, the lipotrisaccharide-forming activity was also enhanced in mutants that lack a functional NF signaling pathway. When inoculated with *R. leguminosarum* bv *viciae*, the mutant P6 (*sym19*) exhibited increased NodSm-III(C_{16;2}) formation (degree of induction 1.91 \pm 0.09; Fig. 4A). A similar stimulation was also found for the P54 (*sym9*) and P2 (*sym30*) mutants (degree of induction: P54, 1.79 \pm 0.24; P2, 2.21 \pm 0.11). The mutants P56 (*sym10*) and RisNod10 (*sym8*), however, did not significantly increase the lipotrisaccharide-forming activity when challenged with *R. leguminosarum* bv *viciae* (degree of induction: P56, 1.16 \pm 0.02; RisNod10, 1.10 \pm 0.03).

Previous studies on NF hydrolysis revealed that chitinases degrade NodSm-V(C_{16;2}, S) to NodSm-III(C_{16;2}) (Staehelin et al., 1994; Schultze et al., 1998; Ovtsyna et al., 2000). Based on these similarities, we expected that the lipotrisaccharide-forming enzyme is a chitinase that hydrolyzes glycol chitin. To visualize chitinase isoenzymes in pea roots, equal quantities of protein extracts were analyzed by electrophoresis on native polyacrylamide gels containing glycol chitin, followed by staining with a fluorescent brightener (Fig. 4, B–D). A typical chitinase isoenzyme profile from root extracts contained two bands. Leaf protein extracts and roots colonized with arbuscular mycorrhizal fungi showed additional bands in accordance with previously published data (Dumas-Gaudot et al., 1994; data not shown). The intensity of the top band varied under the conditions tested and was not further examined. Inoculation with *R. leguminosarum* bv *viciae*

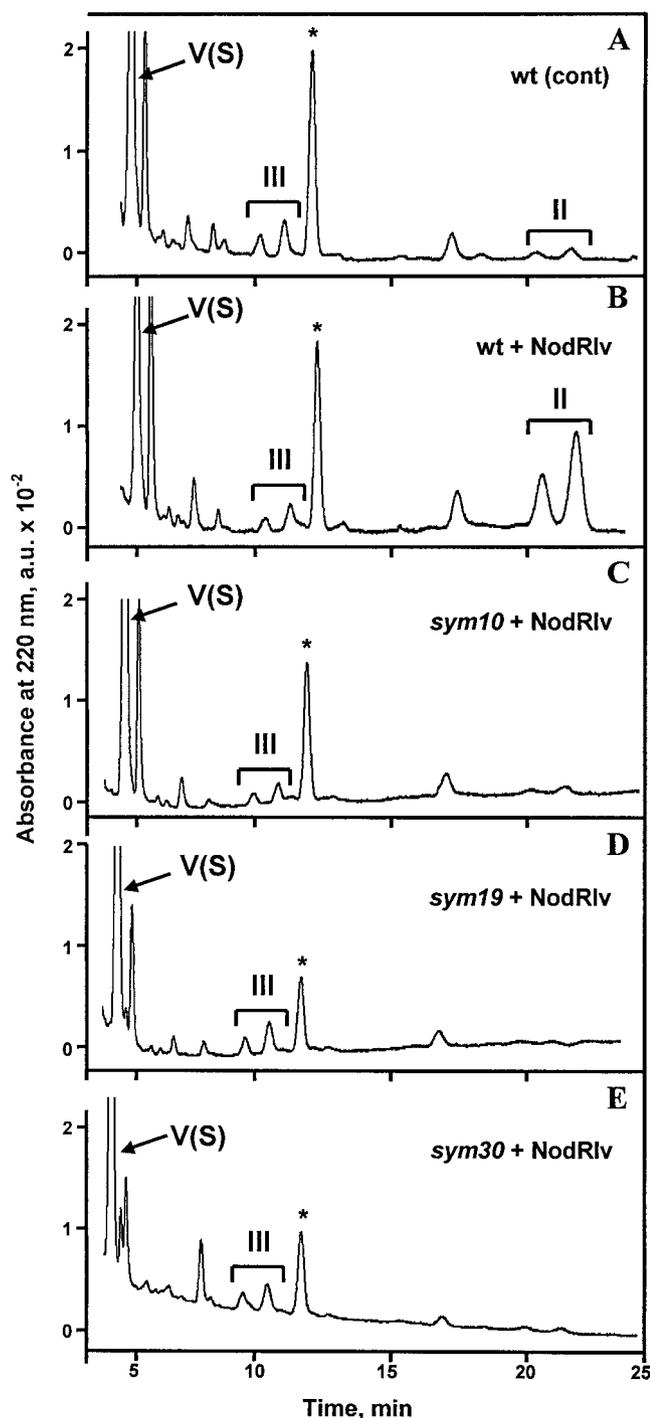


Figure 2. Hydrolysis of NodSm-V($C_{16:2}$, S) in the rhizosphere of pea cv Frisson and its non-nodulating mutants. Roots were either pretreated with 10^{-6} M NodRlv factors [NodRlv-V($C_{18:4}$, Ac) and NodRlv-IV($C_{18:4}$, Ac)] for 20 h or mock treated with Jensen medium. Intact roots were then incubated with NodSm-V($C_{16:2}$, S) for 4 h. After extraction with *n*-butanol, the NF substrate and the acylated cleavage products were fractionated on reverse-phase HPLC. a.u., Absorbance units. The length of the oligosaccharide chain (roman numerals) and the presence of a sulfate group are indicated. The cleavage products NodSm-III($C_{16:2}$) and NodSm-II($C_{16:2}$) are separated into anomers (double peak). An unidentified root compound is marked with an asterisk. wt(cont), Wild-type plants mock treated with Jensen medium; wt + NodRlv, wild-type

resulted in an increased intensity of the bottom band (marked with asterisk), indicating a *Rhizobium*-stimulated pea chitinase. In P56 (*sym10*) and RisNod10 (*sym8*) mutants, inoculation with *R. leguminosarum* bv *viciae* did not affect the intensity of the bottom band. All other pea mutants examined retained their capacity to increase the chitinase activity in response to *R. leguminosarum* bv *viciae* (Fig. 4, B and C). The intensity of the bottom band was also increased when NodRlv and NodSm factors were applied to the roots at micromolar concentrations (Fig. 4D). In contrast to the lipodisaccharide-forming NF hydrolase, which was activated by NodRlv factors at picomolar concentrations (Ovtsyna et al., 2000), NF concentrations $<10^{-6}$ M failed to enhance the chitinase activity. Inoculation with *R. leguminosarum* bv *viciae* RBL5562, which is deficient in NF synthesis, also did not result in enhanced chitinase activity (data not shown). Taken together, these data indicate a strong correlation between the glycol chitin cleaving activity and the lipotrisaccharide-forming activity with NodSm-V($C_{16:2}$, S) as substrate.

Further analysis revealed that NFs are not the only inducers of the identified chitinase. ACC (a precursor of the plant's ethylene biosynthesis), applied to roots at 10^{-4} M, stimulated the glycol chitin cleaving chitinase to a similar extent as NFs. An even stronger enzyme induction was seen when roots were treated with 10^{-3} M of salicylic acid. Moreover, a strong stimulation of the chitinase isoenzyme was observed at elevated plant growth temperature (30°C) as well as in response to excessive watering (flooding stress).

DISCUSSION

In the interaction between legumes and rhizobia, the bacterial NFs trigger multiple responses in the host plant. One of them is the rapid degradation of NFs to inactive cleavage products (Staehelin et al., 1995; Ovtsyna et al., 2000). In this work, we have analyzed NF cleaving pea enzymes using NodSm factors as substrates. When intact roots or protein extracts were incubated with pentameric NodSm-V($C_{16:2}$, S), two hydrolytic activities were observed. One activity released the lipodisaccharide NodSm-II($C_{16:2}$), whereas the other activity formed the lipotrisaccharide NodSm-III($C_{16:2}$). The tetrameric NodSm-IV($C_{16:2}$, S) was rapidly degraded by the lipodisaccharide-forming enzyme but was not a suitable substrate for measuring the lipotrisaccharide-forming activity. However, prolonged incubation of intact pea roots with NodSm-IV($C_{16:2}$, S) resulted in the release of low

plants pretreated with NodRlv factors; *sym10* + NodRlv, mutant P56 (*sym10*) pretreated with NodRlv factors; *sym19* + NodRlv, mutant P6 (*sym19*) pretreated with NodRlv factors; *sym30* + NodRlv, mutant P2 (*sym30*) pretreated with NodRlv factors.

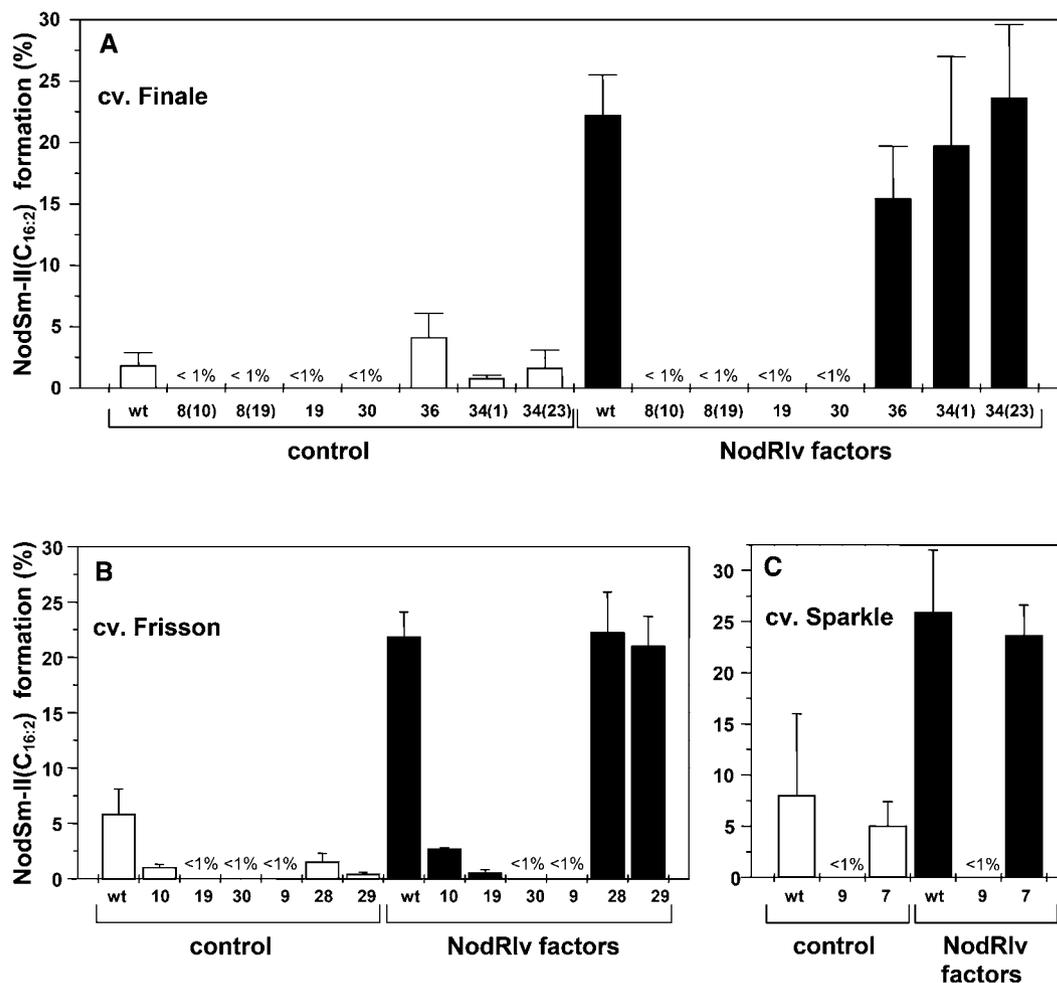


Figure 3. Activity of the lipodisaccharide-forming NF hydrolase in the rhizosphere. Roots were either pretreated with 10^{-6} M NodRlv factors [NodRlv-V(C_{18:4r} Ac) and NodRlv-IV(C_{18:4r} Ac)] for 20 h (treatment NodRlv factors, black columns) or mock treated with Jensen medium (treatment control, white columns). Intact roots were then incubated with NodSm-V(C_{16:2r} S) for 4 h and the released lipodisaccharide NodSm-II(C_{16:2}) determined by reverse-phase HPLC. Data (relative values) indicate means \pm SE from at least two independent experiments (three to four plants per each treatment). A, cv Finale wild type (wt) and its mutants: 8(10), mutant RisNod10 (*sym8*); 8(19), mutant RisNod19 (*sym8*); 19, mutant RisNod2 (*sym19*); 30, mutant RisNod22 (*sym30*); 36, mutant RisNod24 (*sym36*); 34(1), mutant RisNod1 (*sym34*); 34(23), mutant RisNod23 (*sym34*). B, cv Frisson wild type (wt) and its mutants: 10, mutant P56 (*sym10*); 19, mutant P6 (*sym19*); 30, mutant P2 (*sym30*); 9, mutant P54 (*sym9*); 28, mutant P64 (*sym28*); 29, mutant P88 (*sym29*). C, cv Sparkle wild type (wt) and its mutants: 9, mutant R72 (*sym9*); 7, mutant E69 (*sym7*).

amounts of NodSm-III(C_{16:2}) (data not shown). This is reminiscent to the lipodisaccharide-forming activity of a chitinase lysozyme in alfalfa roots (Minic et al., 1998). The lipodisaccharide-forming pea hydrolase was specifically induced by NodRlv factors (Ovtsyna et al., 2000; this work), whereas the lipodisaccharide-forming enzyme from alfalfa was induced by NodSm factors (Stahelin et al., 1995). In pea plants, the degradation of NodSm-V(C_{16:2r} S) to NodSm-III(C_{16:2}) correlated with a glycol chitin cleaving activity of a chitinase isoform. The chitinase was stimulated by high doses of NFs (NodRlv factors and NodSm factors) as well as stress-related signals (ethylene and salicylic acid).

Several reports indicate that NFs induce transcriptional changes on the host plant (e.g. Charron et al.,

2004; Mitra et al., 2004). Our experiments with the inhibitors α -amanitin and cycloheximide indicate de novo synthesis of the lipodisaccharide-forming NF hydrolase. Roots challenged with NodRlv factors up-regulated the enzyme activity within a few hours. NodSm factors (Ovtsyna et al., 2000) and a mutant strain unable to produce NodRlv factors were inactive in inducing the NF hydrolase. Hence, induction of the NF hydrolase is a highly specific response to active NFs, which could not be mimicked by other molecules (see Table III). As the activity of the NF hydrolase can be quantified for a single intact plant, hydrolysis of NFs in the rhizosphere is a noninvasive method to investigate whether the NF signal transduction pathway is functional. Using this assay, we found that activation of the NF hydrolase did not occur in a

Table III. Effect of chemicals on the NF hydrolase activity in the rhizosphere

Four-day-old seedlings (cv Frisson) were incubated for 20 h in 1-mL plastic syringes filled with 600 μL of Jensen medium containing the indicated chemicals. For assaying the NF hydrolase activity in the rhizosphere, plants were transferred to syringes filled with Jensen medium containing 5 μM NodSm-IV(S, C_{16,2}). Values of the NodSm-II(C_{16,2}) forming activity (relative units) indicate means \pm SE from at least two independent experiments (three to four plants per each treatment). Concentrations of chemicals were chosen according to the following publications: Calyculin A, Felix et al. (1994); EGTA and U73122, Pingret et al. (1998); BHQ, Felle et al. (1999); ethephon, Duodu et al. (1999) and He et al. (2002); AVG and ACC, Oldroyd et al. (2001); salicylic acid, Dassi et al. (1996); penta-*N*-acetylchitopentaose, Staehelin et al. (1995).

Chemicals	Mode of Action	Concentration	Lipodisaccharide Formation %
In the presence of 10 ⁻⁶ M NodRlv factors:			
–	–	–	100
Calyculin A	Protein phosphatase inhibitor	1 μM	12.1 \pm 1.8
EGTA	Calcium chelator	2 mM	7.1 \pm 0.9
BHQ	Endomembrane Ca ²⁺ -ATPase inhibitor	10 μM	68.4 \pm 10.6
U73122	Phospholipase C inhibitor	10 μM	3.8 \pm 3.8
Ethephon	Liberation of ethylene	150 μM	51.6 \pm 8.9
AVG	Inhibition of ethylene production	10 μM	99.6 \pm 18.7
In the absence of NodRlv factors:			
–	–	–	9.4 \pm 3.6
Calyculin A	Protein phosphatase inhibitor	1 μM	0 \pm 0
BHQ	Endomembrane Ca ²⁺ -ATPase inhibitor	10 μM	11.5 \pm 2.2
Ethephon	Liberation of ethylene	2 mM	7.4 \pm 1.4
ACC	Ethylene precursor	10 μM	5.4 \pm 1.3
Salicylic acid	Stimulation of plant defense reactions	1 mM	1.4 \pm 1.4
Penta- <i>N</i> -acetylchitopentaose	Stimulation of plant defense reactions	10 μM	11.9 \pm 2.7

number of non-nodulating *sym* mutants with a Hac⁻ phenotype, namely mutants defective in the genes *sym10* (LysM receptor kinase), *sym8* (putative ortholog of *DMI1* from *M. truncatula*, a membrane-spanning cation channel), *sym19* (LRR receptor kinase), and *sym9/sym30* (Ca²⁺/calmodulin-dependent protein kinase). We conclude from these data that all these genes are involved in activation of the NF hydrolase. Hence, an intact NF signal transduction pathway seems to be required for activation of the enzyme (see Fig. 5).

A constitutive NF hydrolase activity was measured for control plants, which were not pretreated with NodRlv factors. One possible explanation is that the NodSm factors used for the assay partially activated the enzyme in the wild-type peas. However, previous studies have shown that NodSm factors are inactive in inducing the enzyme (Ovtsyna et al., 2000). Interestingly, the constitutive NF hydrolase activity was significantly lower or not detectable in *sym* mutants that failed to induce the NF hydrolase (Fig. 3). Furthermore, a treatment with the protein phosphatase inhibitor calyculin A completely suppressed the constitutive NF hydrolase activity in the rhizosphere of wild-type plants (Table III). These data suggest that the NF signaling machinery in wild-type plants is partially active in the absence of NFs, especially in cv Sparkle. This is reminiscent of reports that certain legumes spontaneously induce nodule-like structures in the absence of rhizobia (Truchet et al., 1989; Blauenfeldt et al., 1994).

Calcium spiking (oscillation of intracellular calcium levels) is a rapid and specific response to NFs. It is

suggested that calcium spiking requires extracellular Ca²⁺ influx as well as mobilization of Ca²⁺ from internal stores (Ehrhardt et al., 1996; Walker et al., 2000; Engstrom et al., 2002). Omission of Ca²⁺ ions from the medium by addition of the calcium chelator EGTA totally blocked NF-induced expression of the *MtENOD12* promoter- β -glucuronidase fusion in alfalfa (Pingret et al., 1998). Our studies with the NF hydrolase show a similar requirement of Ca²⁺ in the growth medium. The endomembrane Ca²⁺-ATPase inhibitor BHQ mimicked NF responses of alfalfa, i.e. increased the cytosolic free Ca²⁺, triggered Cl⁻ efflux, depolarized the plasma membrane, and alkalized the root hair space (Felle et al., 1999), but blocked calcium spiking in *M. truncatula* root hairs (Engstrom et al., 2002). In our experiments, BHQ applied to pea roots partially inhibited the NF-dependent up-regulation of the NF hydrolase, suggesting that calcium spiking is causal to the subsequent induction of the NF hydrolase. This possibility is supported by the findings that pea mutants lacking calcium spiking (mutations in the genes *sym10*, *sym8*, and *sym19*; Walker et al., 2000) did not respond to NFs with increased NF hydrolase activity in our experiments.

Mounting evidence suggests that NFs induce phospholipid signaling via putative heterotrimeric G proteins (Pingret et al., 1998; den Hartog et al., 2001; Kelly and Irving, 2001, 2003; Charron et al., 2004). The aminosteroid U73122, an inhibitor of phospholipase C, inhibited NF-induced calcium spiking and early nodulin expression in *Medicago* sp. (Pingret et al., 1998; Engstrom et al., 2002; Charron et al., 2004). In our study, the stimulation of the NF hydrolase activity was

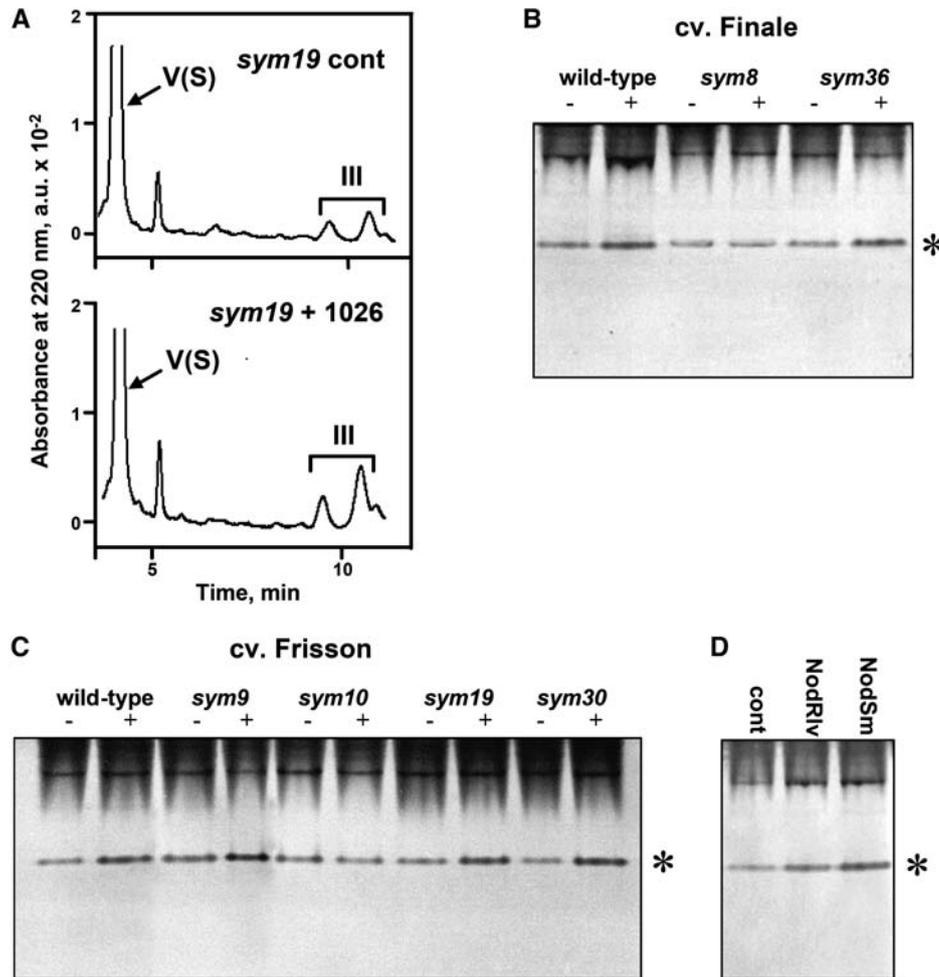


Figure 4. Characterization of a symbiosis-stimulated chitinase isoenzyme. A, Pea root extracts [e.g. from the mutant P6 (*sym19*)] contained a hydrolytic activity that released NodSm-III(C_{16:2}) from the pentameric substrate NodSm-V(C_{16:2}, S). Plants were either mock treated with Jensen medium (*sym19* cont) or inoculated with Jensen medium containing *R. leguminosarum* bv *viciae* CIAM 1026 (*sym19* + 1026). After this pretreatment, root extracts were assayed with NodSm-V(C_{16:2}, S) and the released cleavage product NodSm-III(C_{16:2}) determined by reverse-phase HPLC. a.u., Absorbance units. B to D, Detection of a symbiosis-stimulated chitinase isoform (marked with asterisk) after gel electrophoresis on polyacrylamide gels containing glycol chitin. Soluble proteins (30 μ g) from root extracts were separated under native conditions, stained with Calcofluor White M2R and visualized by UV illumination. B, cv Finale: wild-type plants and the mutants RisNod10 (*sym8*) and RisNod26 (*sym36*) either mock treated with Jensen medium (treatment –) or inoculated with *R. leguminosarum* bv *viciae* CIAM1026 (treatment +). C, cv Frisson: wild-type plants, P54 (*sym9*), P56 (*sym10*), P6 (*sym19*), and P2 (*sym30*) mutants after mock inoculation (–) or inoculation with strain CIAM1026 (+). D, Effect of purified NFs. Plants (cv Rondo) treated with Jensen medium (cont), 10^{-6} M NodRlv factors, or 10^{-6} M NodSm-IV(C_{16:2}, S).

blocked by U73122. Although toxic effects of U73122 on root hair viability cannot be excluded (Charron et al., 2004), these data provide pharmacological evidence that phospholipase C activity is essential for activation of the NF hydrolase in pea roots.

The phytohormone ethylene has many effects on plant development and activates proteins related to stress and plant defense, including the chitinase studied in this work. Ethylene appears to influence nodule formation at different symbiotic stages. Exogenously applied ethylene and ethephon inhibited nodulation of pea, whereas application of the ethylene synthesis inhibitor AVG increased the number of nodules

formed (e.g. Lee and LaRue, 1992; Guinel and Geil, 2002). In *M. truncatula*, ethylene inhibited the maintenance of calcium spiking and blocked NF responses, such as expression of the early nodulin genes *ENOD11* and *RIP1* (Oldroyd et al., 2001). In our study with pea plants, NFs stimulated the NF hydrolase in the presence of ethephon, albeit to a lower extent. AVG did not affect the enzyme activity, suggesting that the plant's ethylene synthesis does not influence the NF signal transduction pathway. Future experiments are required to assess the effect of ethylene on nodulation and the possible cross-talk between the NF and ethylene perception pathways.

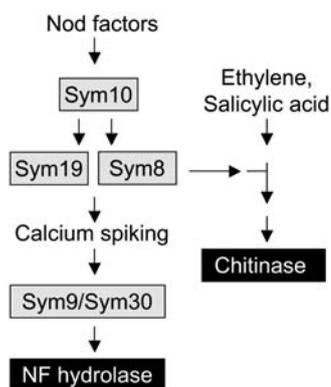


Figure 5. Activation of NF cleaving pea enzymes. Analysis of non-nodulating pea mutants suggests different activation mechanisms for the lipodisaccharide-forming NF hydrolase in the rhizosphere and a lipotrisaccharide-forming chitinase isoform in root protein extracts. Induction of the symbiosis-specific NF hydrolase depends on NodRlv factors and the indicated Sym proteins (Sym10, LysM receptor kinase; Sym19, LRR receptor kinase; Sym9/Sym30, Ca^{2+} /calmodulin-dependent protein kinase). Calcium spiking seems to be causal to the subsequent induction of the NF hydrolase. Elevated chitinase activity is likely a stress response to high doses of NFs (NodRlv factors or NodSm factors). Ethylene and salicylic acid also elicit increased chitinase activity, suggesting an induction via stress-related signal transduction pathways. Stimulation of the chitinase by NFs depends on Sym10 and Sym8, whereas the other indicated components of the Nod signal transduction pathway (Sym19, calcium spiking, and Sym9/Sym30) are not required. These findings suggest a signaling cross-talk between the NF pathway (downstream of Sym8) and stress-related signaling.

The number of nodules formed in a root system is restricted by a shoot-controlled autoregulatory mechanism of the host plant. Mutants lacking autoregulation display a hypernodulating phenotype. *Sym29* and related genes in other legumes encode CLAVATA1-like receptor kinases, which are required for nodule autoregulation (Krusell et al., 2002; Nishimura et al., 2002; Searle et al., 2003). Split-root experiments with purified NFs indicated that NF signaling induces the autoregulation circuit in *Vicia sativa* and alfalfa (van Brussel et al., 2002; Catford et al., 2003). In our study, the NF-induced hydrolase activity of P88 (*sym29*) and other pea mutants lacking autoregulation was increased when roots were treated with NodRlv factors. These data suggest that genes involved in autoregulation do not directly affect the NF hydrolase activity. Future split-root experiments are required to investigate whether the autoregulation circuit regulates the NF hydrolase activity in root regions that had not been in contact with NFs.

Chitinases stimulated during nodule symbiosis have been characterized in various host plants (e.g. Staehelin et al., 1992; Vasse et al., 1993; Goormachtig et al., 1998; Xie et al., 1999; Salzer et al., 2004). Here, we report that high doses of NFs and inoculation with *R. leguminosarum* bv *viciae* enhanced the activity of a specific chitinase isoenzyme in pea roots. Similar to various known plant chitinases, the identified isoform was activated by ethylene and salicylic acid, indicating

a stress-related enzyme. When inoculated with *R. leguminosarum* bv *viciae* CIAM 1026, peas with mutated *sym10* and *sym8* genes did not show elevated activity of the chitinase. Hence, chitinase stimulation seems to depend on Sym8 and the LysM receptor kinase Sym10, a component of the putative NF receptor complex. The chitinase was stimulated by high doses of NodRlv and NodSm factors. This suggests that the LysM receptor kinase Sym10 can recognize both types of NFs. In pea roots, NodSm factors were inactive in inducing host-specific responses, such as the induction of the NF hydrolase (Ovtsyna et al., 2000). Hence, perception of NodRlv factors resulting in symbiosis-specific responses seems to be more complex and might include an additional LysM receptor kinase. NF signaling in *L. japonicus* requires two LysM receptor kinases that possibly form a heterodimeric complex with NFs (Madsen et al., 2003; Radutoiu et al., 2003).

Pea mutants with defective *sym10* and *sym8* genes failed to respond to *Rhizobium* inoculation with stimulation of the chitinase. In all other non-nodulating mutants examined, NFs were active in stimulating the enzyme. In other words, a set of proteins involved in activation of the NF hydrolase, namely Sym19 and Sym9/Sym30, was not required for stimulation of the chitinase. We conclude from these findings that calcium spiking and putative downstream events are not implicated in stimulation of the chitinase. We suggest that the NF perception pathway downstream of Sym8 is connected with a stress-related signal transduction pathway. In this view, chitinase stimulation would be an NF response that dissects mutants with defective *sym8* and *sym19* genes. Differences between these two mutants have been also reported by Novak et al. (2004). These authors measured the *nod* gene-inducing activity of flavonoids from pea root exudates, which increased upon inoculation with *R. leguminosarum* bv *viciae*. This host plant response was observed in the RisNod25 (*sym8*) mutant, but not in RisNod20 (*sym19*). Taking these findings together, they suggest that *Sym19* is required for flavonoid accumulation in the rhizosphere, *Sym8* for stress responses (e.g. chitinase stimulation), and both genes together for calcium spiking and downstream events, such as induction of the NF hydrolase (see Fig. 5).

In conclusion, we show in this article that induction of an NF hydrolase in the pea rhizosphere requires a functional NF signal transduction pathway that includes calcium spiking and Sym9/Sym30 (Ca^{2+} /calmodulin-dependent protein kinase). Stimulation of a chitinase isoform in pea roots was less dependent on the NF signal transduction pathway and only required Sym10 and Sym8. Chitinase stimulation by high doses of NFs was most likely a stress reaction. The symbiosis-related NF hydrolase inactivated excess amounts of NodSm factors in the rhizosphere, perhaps in order to attenuate NF-induced stress effects. Future work will be required to elucidate the fate of NodRlv factors in the pea rhizosphere and to identify the symbiosis-specific NF hydrolase gene.

MATERIALS AND METHODS

Plant Material

Seeds of pea (*Pisum sativum* L.) cultivars Rondo, Frisson, Finale, and Sparkle and corresponding mutants (Table I) were kindly provided by Dr. E. Jacobsen (Agricultural University, Wageningen, The Netherlands), Dr. G. Duc (Institut National de la Recherche Agronomique, Dijon, France), Dr. K.J. Engvild (Riso National Laboratory, Roskilde, Denmark), and Dr. T.A. LaRue (Cornell University, Ithaca, NY) and further propagated in St. Petersburg.

Pea seeds were surface-sterilized with concentrated sulfuric acid for 5 to 10 min, washed several times with sterile water, and placed for germination on 1% water agar plates in the dark at 18°C for 3 to 4 d.

Bacterial Strains

Wild-type *Rhizobium leguminosarum* bv *viciae* strain CIAM 1026 (Safronova and Novikova, 1996) effectively nodulating pea was obtained from the collection of the All-Russia Research Institute for Agricultural Microbiology. *R. leguminosarum* bv *viciae* strain RBL 5562 ($\Delta nodA$; defective in synthesis of NFs; Wijffelman et al., 1985) and strain RBL 5799 (overproducing NFs; Spaink et al., 1991) were kindly provided by Dr. H.P. Spaink (Leiden University, Leiden, The Netherlands). *Sinorhizobium meliloti* strain 1021(pEK327) (Schultze et al., 1992) was kindly provided by Dr. E. Kondorosi (Institut des Sciences du Végétal, Centre National de la Recherche Scientifique, Gif-sur-Yvette, France).

Chemicals

Tunicamycin, the aminosteroid U73122, ethephon (2-chloroethyl-phosphonic acid), AVG, calyculin A, glycol chitosan, BHQ, and Calcofluor White M2R were obtained from Sigma-Aldrich. The other chemicals used were α -amanitin (Roche), ACC (Fluka), brefeldin A (Calbiochem, EMD Biosciences), cycloheximide and EGTA (Acros Organics), penta-*N*-acetylchitopentaoase (Seikagaku Corporation), and naringenin (Serva Feinbiochemica).

Purification of NFs

NodRlv factors were purified from *R. leguminosarum* bv *viciae* strain RBL 5799 grown at 28°C on a shaker in 2 liters of B⁻ medium (van Brussel et al., 1977) in the presence of 3 μ M naringenin. Supernatants of bacterial cultures were extracted with *n*-butanol and the butanol phase concentrated under reduced pressure. NodRlv factors were fractionated by reverse-phase HPLC (Ultrasphere ODS, 4.6 \times 250 mm or Waters, Nova Pak C₁₈, 3.9 \times 150 mm, particle size 4 μ m) using a linear gradient from 30% to 100% (v/v) acetonitrile/water. Pentameric NodRlv-V(C_{18,4r} Ac) and tetrameric NodRlv-IV(C_{18,4r} Ac) were detected by their absorption at 304 nm. Fractions containing these NodRlv factors were collected and concentrated under reduced pressure.

NodSm factors were purified from the overproducing strain *S. meliloti* 1021(pEK327). Supernatants of bacterial cultures were extracted with *n*-butanol, concentrated under reduced pressure, and fractionated by reverse-phase HPLC (Ultrasphere ODS, 4.6 \times 250 mm or Waters, Nova Pak C₁₈, 3.9 \times 150 mm, particle size 4 μ m) under isocratic conditions using 36% (v/v) acetonitrile/water containing 40 mM ammonium acetate as the mobile phase. Pentameric NodSm-V(C_{16,2r} S) and tetrameric NodSm-IV(C_{16,2r} S) (Schultze et al., 1992) were detected at 220 nm, collected separately, and dried under reduced pressure. The fractions containing purified NodSm factors were desalted as described by Staehelin et al. (2000).

Treatment of Roots with Bacteria, NFs, and Chemicals

Four or five days after sterilization, roots of germinated pea seedlings were either pretreated with a rhizobial culture ($A_{660} = 0.05$) or with NodRlv factors (10^{-6} M) dissolved in Jensen medium (van Brussel et al., 1982). Where indicated, roots were pretreated with chemicals dissolved in Jensen medium (Tables II and III). Control plants were mock treated with Jensen medium. For assaying the degradation of NFs in the rhizosphere, pretreatments were performed on individual plants placed on 1-mL plastic syringes (filled with 600 μ L Jensen medium with or without chemicals). Plants were incubated in a dark chamber at 18°C for 20 h.

For analysis of the NF cleaving activity in root extracts, 4-d-old seedlings were transferred into 200-mL plastic boxes containing sterile vermiculite.

Inoculation with *Rhizobium* was performed with overnight cultures (A_{660} approximately 1.0). Each plant was inoculated with at least 10^7 to 10^8 bacteria. To test the effect of NFs (in Jensen medium), a first treatment (at 21°C for 24 h) was performed with seedlings placed on plastic syringes. The plants were then transferred to vermiculite and treated with a new portion of NFs. Plants were incubated in a growth chamber (at 21°C, 60% relative humidity and a light phase of 16 h with a photon flux of 400 μ mol m⁻² s⁻¹). At the time of harvest, roots were washed in running water and weighed. Root tips were removed, and main roots from four to five plants from each treatment were combined and immediately frozen (-20°C).

Protein Extraction and Quantification

Plant material was ground with liquid nitrogen, and the powder was suspended in 100 mM MES-NaOH buffer (pH 6.8) containing 15% ethylene glycol, 100 mM Suc, 0.1 mM phenylmethylsulfonyl fluoride, and 1 mM MgCl₂. Crude homogenates were centrifuged at 14,000g for 30 min at 4°C. Contents of soluble proteins were determined by the method of Bradford (1976) using bovine serum albumin as a standard.

Hydrolysis of NodSm Factors by Intact Roots and Root Extracts

NodSm-IV(C_{16,2r} S) or NodSm-V(C_{16,2r} S) was used to determine the NF cleaving activity of intact roots and root extracts (Staehelin et al., 1995; Ovtsyna et al., 2000). Intact pea plants (pretreated with rhizobia, NFs, and chemicals) were individually analyzed. Each plant was placed on a 1-mL plastic syringe filled with 600 μ L Jensen medium containing 5 μ M NodSm factors. Plants were incubated at 25°C for 4 h and the medium extracted with 600 μ L *n*-butanol. Root extracts (corresponding to 30 μ g protein from four to five plants) were incubated in 20 mM sodium acetate buffer (pH 5.0) containing 40 μ M purified NFs at 37°C for 4 h. The reaction was stopped by addition of equal volumes of *n*-butanol.

The butanol phase from all samples was then dried in a speed-vac evaporator and finally dissolved in 1 μ L DMSO. NodSm factors and their acylated cleavage products were fractionated on a C₁₈ reverse-phase HPLC column at 220 nm under isocratic conditions using 35% or 36% acetonitrile/water containing 40 mM ammonium as the mobile phase. Hydrolytic rates of NFs were determined by the integration of peak areas of the substrate and the acylated cleavage products. Cleavage products were identified using the reference compounds NodSm-III(C_{16,2r}) and NodSm-II(C_{16,2r}), which have been purified and analyzed previously (Staehelin et al., 1994). The structure of NodSm-II(C_{16,2r}) was verified by mass spectroscopy (positive ionization mode). The obtained peaks (corresponding to the sodium adduct ion at a mass-to-charge ratio of 639 and the potassium adduct ion at a mass-to-charge ratio of 655) confirmed the mass spectroscopic measurements reported by Staehelin et al. (1994).

Separation and Detection of Chitinase Isoforms

Soluble protein extracts were analyzed by 12% (w/v) PAGE under native conditions according to Davis (1964). Glycol chitin, prepared from glycol chitosan by acetylation with acetic anhydride (Trudel and Asselin, 1989), was added to the gel [0.01% (w/v)] and served as a substrate for the chitinase activity (Michaud and Asselin, 1995). After electrophoresis, gels were incubated for 2 h at 37°C in 50 mM sodium acetate buffer (pH 5.0) with 1% Triton X-100. Gels were then washed with water and incubated for 10 min at 37°C in 100 mM Tris-HCl (pH 8.0) with 0.01% (w/v) fluorescent brightener (Calcofluor White M2R). Gels were left overnight in water at 28°C. The bands corresponding to chitinase activity were visualized under UV light. All PAGE analyses were repeated at least twice.

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