NopL, an Effector Protein of *Rhizobium* sp. NGR234, Thwarts Activation of Plant Defense Reactions[1]

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Bacterial effector proteins delivered into eukaryotic cells via bacterial type III secretion systems are important virulence factors in plant-pathogen interactions. Type III secretion systems have been found in *Rhizobium* species that form symbiotic, nitrogen-fixing associations with legumes. One such bacterium, *Rhizobium* sp. NGR234, secretes a number of type III effectors, including nodulation outer protein L (NopL, formerly y4xL). Here, we show that expression of *nopL* in tobacco (*Nicotiana tabacum*) prevents full induction of pathogenesis-related (PR) defense proteins. Transgenic tobacco plants that express *nopL* and were infected with potato virus Y (necrotic strain 605) exhibited only very low levels of chitinase (class I) and β-1,3-glucanase (classes I and III) proteins. Northern-blot analysis indicated that expression of *nopL* in plant cells suppresses transcription of PR genes. Treatment with ethylene counteracted the effect of NopL on chitinase (class I). Transgenic *Lotus japonicus* plants that expressed *nopL* exhibited delayed development and low chitinase levels. In vitro experiments showed that NopL is a substrate for plant protein kinases. Together, these data suggest that NopL, when delivered into the plant cell, modulates the activity of signal transduction pathways that culminate in activation of PR proteins.

Plants have evolved many defenses against invading pathogens. Among them are preformed antimicrobial compounds, as well as inducible defense proteins, the so-called pathogenesis-related (PR) proteins (van Loon, 1997). It has been shown, for example, that pathogen-induced chitinases (EC 3.2.1.14) and β-1,3-glucanases (EC 3.2.1.39) synergistically lyse fungal cell walls (Mauch et al., 1988). Specific recognition of pathogens often induces a hypersensitive response, which is characterized by an oxidative burst and localized death of the host cells. In most cases, the induction of a hypersensitive response arrests invasion by the pathogen (Dangl and Jones, 2001).

Virulence in gram-negative bacteria often depends on proteins injected into eukaryotic cells. Some bacteria elaborate a specialized protein secretion apparatus, the type III secretion system (TTSS). The TTSS exports a set of proteins from the bacteria, some of which are delivered directly into the eukaryotic cells—a process called translocation (Cornelis and van Gijsegem, 2000; Plano et al., 2001). Most translocated type III effectors act on the cytoskeleton or interfere with intracellular signaling cascades of the host cell. *Yersinia pestis*, for example, injects at least six type III effectors into host cells, where they thwart the signaling machinery of the immune system (Cornelis, 2002). Plant pathogens such as *Pseudomonas syringae* and *Xanthomonas campestris* also use TTSSs (Kjemtrup et al., 2000; Lahaye and Bonas, 2001). Mounting evidence suggests that type III effectors translocated into plant cells (Casper-Lindley et al., 2002; Szurek et al., 2002) act as virulence factors in susceptible hosts. One likely function of type III effectors is the suppression of inducible defense reactions. In plants that carry a corresponding resistance gene, recognition of bacterial effector proteins occurs, resulting in a hypersensitive response and arrest of invasion by the pathogen (Dangl and Jones, 2001; Lahaye and Bonas, 2001; Staskawicz et al., 2001).

TTSSs were once thought to be confined to pathogenic bacteria, but they have also been identified in rhizobia, symbiotic soil bacteria of the family *Rhizo-*

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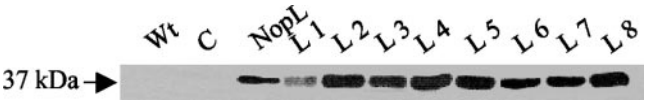

Figure 1. Detection of NopL protein ($M_r \approx 37$ kDa) in different transgenic tobacco lines that express *nopL (Nt*NopL lines). Total soluble proteins (10 μg) from individual plants were analyzed by immunoblotting using antibodies raised against purified NopL protein. Purified NopL served as a control. Wt, Wild-type plants; C, control plants transformed with the vector pPZP112; NopL, NopL protein (1 μg) purified from *Escherichia coli* pPROEX-1*nopL* from which the 6xHis tag was removed (apparent $M_r \approx 37$ kDa). L1 through L8, *Nt*NopL lines 1 through 8 (tobacco lines expressing *nopL*).

biaceae (Marie et al., 2001). Rhizobia enter legume roots via root hairs where they induce the formation of infection threads and new organs called nodules. Infection threads and the symbiosome membranes that develop from them insulate the invading bacteria from the plant cytoplasm. Once the infection thread has penetrated cortical cells of the root, the bacteria differentiate into bacteroids that reduce atmospheric nitrogen to ammonia (Broughton et al., 2000; Perret et al., 2000). Depending on the host plant, mutants of NGR234 without a functional secretion apparatus exhibit different nodulation phenotypes (Viprey et al., 1998). NGR234 secretes a number of proteins, among them nodulation outer protein L (NopL, formerly called y4xL) via its TTSS (Freiberg et al., 1997; Viprey et al., 1998). In comparison with wild-type bacteria, mutants lacking *nopL* induce fewer nodules on *Flemingia congesta* (Marie et al., 2003), indicating that NopL is a rhizobial "virulence factor" for this plant.

To test the effect of NopL within plant cells, tobacco (*Nicotiana tabacum*) as well as the NGR234 host *Lotus japonicus* were transformed with *nopL*. Ectopic expression of *nopL* inhibited both plants' ability to accumulate PR proteins. As NopL is a substrate for plant protein kinases (Bartsev et al., 2003), it probably suppresses defense responses by thwarting intracellular signaling cascades required for activation of PR genes.

RESULTS

Tobacco Plants That Express *nopL* Are More Susceptible to Pathogen Attack

Several independently transformed tobacco lines in which expression of the *nopL* gene was placed under control of the cauliflower mosaic virus (CaMV) 35S promoter were obtained. Most plants from this T_0 generation exhibited reduced apical dominance, altered leaf morphology, early leaf senescence, delayed flowering, and reduced seed production (control plants transformed with the vector pPZP112 were normal). This altered phenotype is reminiscent of disease symptoms induced by pathogens. Healthy progeny of these plants [the T_1 generation-independent lines were called *Nt*NopL(1), *Nt*-NopL(2), etc.] stably inherited the trans-gene, and plants from most lines developed normally. Immunoblot analyses of individual plants using antibodies raised against the purified NopL protein indicated that these lines accumulated NopL in the soluble fraction (Fig. 1).

To test whether the T_1 generation had an altered susceptibility to pathogens and modulated defense reactions, we infected plants from five independent *Nt*NopL lines with potato virus Y N605 that induces a broad range of well-defined plant defense responses in tobacco plants. Symptom development and virus accumulation were monitored on the neighboring younger leaf. Virus accumulation in plant tissue was determined by a quantitative ELISA-based assay, using antibodies raised against the virus

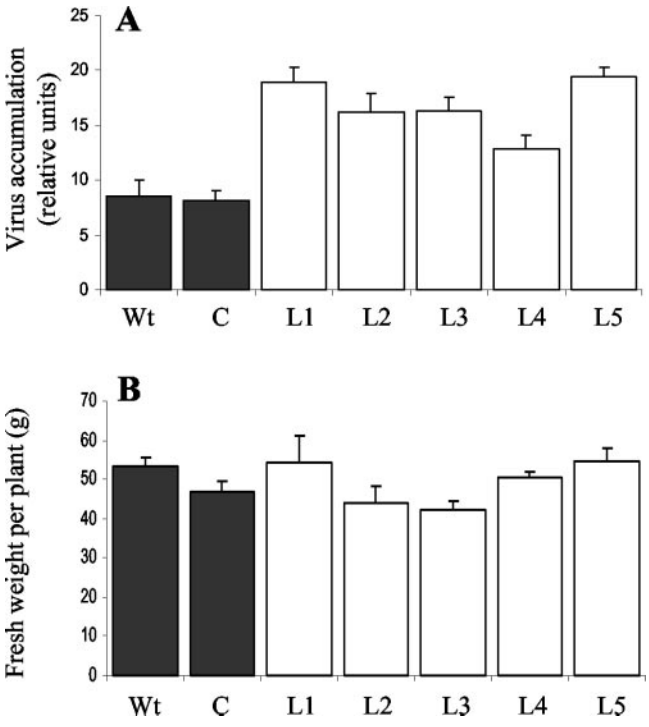

Figure 2. Tobacco plants that express *nopL* have a heightened susceptibility to potato virus Y N605. A, Virus accumulation in tobacco plants inoculated with potato virus Y N605 2 weeks after inoculation (six plants per line expressing *nopL*, 15 control plants transformed with the vector pPZP112, and 15 wild-type plants; all plants were grown individually in pots). An ELISA was used to quantify the degree of infection using antibodies directed against the virus coat protein that was conjugated to alkaline phosphatase. Values are means ± SE of the signal expressed in relative units. Compared with wild-type plants, virus accumulation was significantly increased in plants that express *nopL* (L1, $n = 21$, $\chi^2_{[1]} = 8.31$, $P = 0.004$; L2, $n = 21$, $\chi^2_{[1]} = 8.31$, $P = 0.004$; L3, $n = 21$, $\chi^2_{[1]} = 8.31$, $P = 0.004$; L4, $n = 19$, $\chi^2_{[1]} = 5.4$, $P = 0.02$; L5, $n = 21$, $\chi^2_{[1]} = 8.31$, $P = 0.004$). B, Fresh weight (aerial parts) of virus-infected plants 3 weeks after inoculation. Data show similar values, indicating normal growth of the *Nt*NopL lines tested. Wt, Wild-type plants; C, control plants with the vector pPZP112; L1 through L5, *Nt*NopL lines 1 through 5 (tobacco lines that express *nopL*).

coat protein. Two weeks after inoculation, accelerated symptom development and statistically significant increases in virus accumulation were observed in leaf discs taken from all tested *Nt*NopL lines (Fig. 2A). In contrast, control plants transformed with the vector pPZP112 only accumulated wild-type levels of the virus. At the end of the experiment, 3 weeks after inoculation, plants were harvested and the fresh weight of the aerial parts was determined. Similar values were obtained for all virus-infected plants, indicating that plant growth and development of the tested *Nt*NopL lines (T_1 generation) were probably not affected by expression of *nopL* (Fig. 2B). Thus, ectopic expression of *nopL* in tobacco plants appears to weaken plant defense without otherwise affecting plant development.

Expression of *nopL* Inhibits Induction of PR Proteins in Tobacco

The activity of PR proteins, particularly chitinases and β-1,3-glucanases, increases when plants are challenged by pathogens. These hydrolases are strongly induced in tobacco plants after infection by pathogens, including viruses (Beffa et al., 1993; Collinge and Slusarenko, 1987; Kang et al., 1998). To investigate the accumulation of these hydrolases in plants expressing *nopL*, leaf discs were harvested from plants infected with the potato virus Y N605. Proteins from this material were extracted and probed using antibodies directed against tobacco chitinase (class I) and β-1,3-glucanases (two related enzymes belonging to classes I and III). Two weeks post-inoculation (p.i.), PR proteins were strongly induced in virus-infected, nontransformed plants or plants transformed with the vector pPZP112. However, the transgenic *Nt*NopL lines exhibited approximately 10-fold lower levels of these PR proteins when challenged with the virus (Fig. 3, A and B). Similar profiles of PR protein accumulation were obtained with leaf discs harvested 1 week p.i.. Noninfected control plants from *Nt*NopL lines did not accumulate chitinase or glucanase (data not shown). When total chitinase activity was measured enzymatically using [^{3}H]chitin as the substrate, similar suppressive effects were observed in virus-infected *Nt*NopL lines (not shown). These data show that expression of *nopL* in plants inhibits the accumulation of virus-inducible PR proteins.

Infection of tobacco plants with potato virus Y N605 results in necrotic lesions that correlate with an oxidative burst (Fodor et al., 2001) and induction of peroxidase activity. In our experiments, peroxidase activities were about 7-fold higher in plants inoculated with potato virus Y N605 than in noninfected control plants. Virus infection of plants expressing *nopL* had similar, stimulatory effects (Fig. 3C). Thus, NopL does not significantly suppress the peroxidase activity that is associated with an oxidative burst.

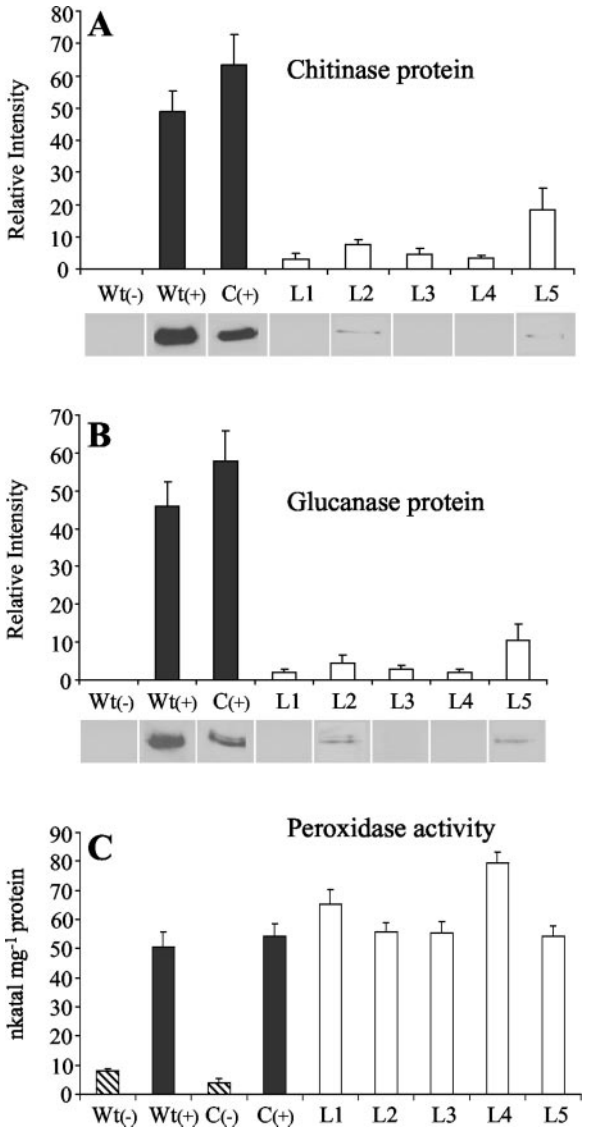

Figure 3. Defense reactions of tobacco plants that express *nopL*. Plants were infected with potato virus Y N605. Two weeks later, plant defense reactions were measured in virus-infected leaf discs harvested from the neighboring leaf. A, Chitinase (class I) protein. Bottom, Extracts (10 μg of protein) from leaves were subjected to SDS-PAGE, blotted onto polyvinylidene difluoride (PVDF) membranes, and immunodetected using a rabbit serum raised against tobacco chitinase (class I; $M_r \approx 32$ kDa). Top, For each plant extract tested, the intensity of the band that correlated with the amount of immunolabeled protein was quantified using the Gene Genius Bioimaging system. Data (relative values) indicate means ± SE ($n = 6$ for each *Nt*NopL line, $n = 15$ for wild-type plants, and $n = 15$ for control plants with the vector pPZP112). B, β-1,3-Glucanase proteins. Bottom, Immunoblotting of protein extracts from *Nt*NopL lines using an antiserum raised against tobacco β-1,3-glucanase class I ($M_r \approx 33$ kD), which crossreacts with β-1,3-glucanase class III ($M_r \approx 35$ kD), resulting in a double band. Top, Intensity of the β-1,3-glucanase spots quantified with the Gene Genius Bioimaging system. C, Peroxidase activity, as measured using guaiacol as the substrate. Values indicate means ± SE of enzyme activity per total soluble protein content in the extract ($n = 6$). Wt, Wild-type plants; C, control plants containing the vector pPZP112; L1 through L5, *Nt*NopL lines 1 through 5 (tobacco lines expressing *nopL*); (+), infected with potato virus Y N605; (−), noninoculated control.

Expression of *nopL* in Tobacco Also Inhibits Activation of PR Gene Transcription

Northern-blot analysis was used to investigate whether the low levels of PR proteins in *Nt*NopL lines are due to reduced amounts of transcripts produced. RNA from virus-infected plants was isolated and hybridized with a tobacco chitinase (class I) cDNA (clone CHN50; Shinshi et al., 1987). As seen in Figure 4, transcripts of chitinase (class I) were strongly induced in virus-infected control plants, resulting in an intense hybridization signal. However, chitinase transcripts were barely detectable in virus-infected *Nt*NopL lines. The lowest transcript levels were seen in the lines *Nt*NopL(1), *Nt*NopL(3), and *Nt*NopL(4), which matched the reduced accumulation of chitinase protein (Figs. 3A and Fig. 4). These findings suggest that NopL, once accumulated in the cytoplasm, can block signal transduction pathways resulting in expression of PR genes.

Ethylene Activates PR Proteins in Tobacco Plants That Express *nopL*

Ethylene activates distinct sets of PR genes, including chitinase (class I) and β-1,3-glucanase (class I; Boller et al., 1983; Ecker and Davis, 1987; Vögeli-Lange et al., 1994; Stepanova and Ecker, 2000). As activation of these enzymes is inhibited in *Nt*NopL plants, we asked whether NopL affects the ethylene signal transduction pathway. To test this hypothesis, plants were treated with excess amounts of ethylene, and the levels of chitinase (class I) protein were assayed by immunoblotting. Nontransformed plants responded strongly to treatment with ethylene and they synthesized considerable amounts of chitinase (class I) protein (Fig. 5A). Treatment of *Nt*NopL plants with ethylene also induced chitinase synthesis (Fig. 5B). Similar results were obtained for β-1,3 glucanase (class I) when the antiserum against β-1,3 glucanases was used. However, the β-1,3 glucanase (class III) band was not induced by ethylene (data not shown). These results indicate that ethylene counteracts the suppressive effects of *nopL*, suggesting that the ethylene signal transduction pathway remains functional in plants that express *nopL*.

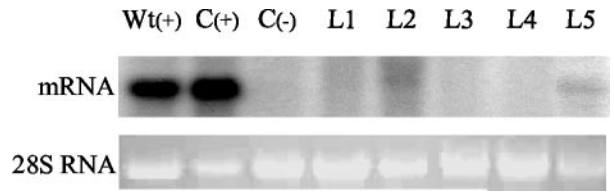

Figure 4. Expression of *nopL* in tobacco inhibits transcription of class I chitinase. RNA isolated from virus-infected leaf discs (2 weeks p.i.) was hybridized against DNA of clone CHN50 that encodes tobacco class I chitinase. Ethidium bromide staining of the agarose gel confirmed equal loading of RNA. Wt, Wild-type plants; C, control plants containing the vector pPZP112; L1 through L5, *Nt*NopL lines 1 through 5 (tobacco lines expressing *nopL*); (+), infected with potato virus Y N605; (−), noninoculated control.

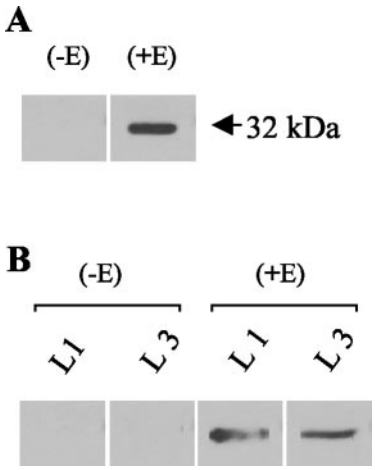

Figure 5. Effect of ethylene on induction of class I chitinase in tobacco. Tobacco plants were incubated in a closed chamber containing 100 μL L^{-1} ethylene for 48 h, whereas control plants were incubated for the same period in another chamber without ethylene. After treatment, induction of chitinase (class I) was analyzed on immunoblots. A, Wild-type plants. B, Plants that express *nopL*. L1, Line *Nt*NopL(1); L3, line *Nt*NopL(3); (+E), treated with ethylene; (−E), control without ethylene.

Expression of *nopL* in *L. japonicus*

L. japonicus is a legume that forms nitrogen-fixing nodules with NGR234. The mutant strains NGR234Ω*rhcN* and NGR234Ω*nopL*, which are unable to export NopL (Viprey et al., 1998), formed similar numbers of nodules as the parent strain NGR234 (20 ± 2 for NGR234Ω*rhcN*; 22 ± 2 for NGR234Ω*nopL*; and 17 ± 1 for NGR234) on *L. japonicus*. Thus, this plant belongs to the group of hosts of NGR234 that establish symbioses independently of NopL and the TTSS, perhaps because cell-wall components act as barriers to the injection of effector proteins into the host cell. To investigate how NopL acts within the cytoplasm of a legume, we introduced *nopL* into *L. japonicus*. Several independently transformed *L. japonicus* lines that expressed *nopL* under control of the CaMV 35S promoter were obtained. The time-to-flower in some of these lines was delayed and seed production was poor. Nevertheless, a number of regenerated lines [T_1 generation–independent lines *Lj*NopL(1), *Lj*NopL(2), etc.] in which the trans-genes were stably inherited were obtained. Immunoblot analyses showed that the NopL protein is present in the *Lj*NopL lines (Fig. 6A). However, development of the *Lj*NopL lines was retarded in comparison with wild-type plants as well as control plants carrying the vector pPZP112. As shown in Table I, the biomass of the *Lj*NopL lines was also reduced when plants were inoculated with NGR234. Nevertheless, when expressed per unit of biomass, the *Lj*NopL lines formed more nodules than the wild-type plants. Per-

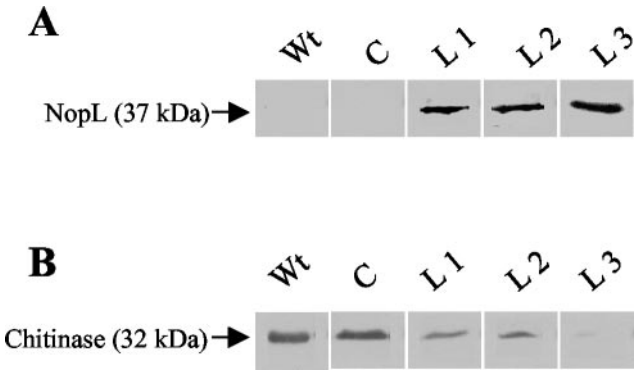

Figure 6. Detection of NopL and chitinase in different transgenic *L. japonicus* lines that express *nopL* (*Lj*NopL lines). A, Protein extracts (10 μg) from 4-week-old *L. japonicus* plants were used for immunodetection with an antiserum directed against the NopL protein ($M_r \approx$ 37 kD). B, A chitinase ($M_r \approx$ 32 kD) of *L. japonicus* nodulated with NGR234 crossreacted with the antiserum raised against tobacco chitinase (class I). Wt, Wild-type plants; C, control plants with the pPZP112 vector; L1 through L3, *Lj*NopL lines 1 through 3 (*L. japonicus* lines expressing *nopL*).

haps these differences are the result of slower development of the *Lj*NopL lines, because the number of nodules of an individual plant was not increased in the *Lj*NopL lines (Table I).

Extracts from nodulated *L. japonicus* plants contained basal levels of a 32-kD chitinase that crossreacted with the antiserum raised against the tobacco class I chitinase. This band was also seen in protein extracts of *Lj*NopL lines. Nevertheless, the signals were attenuated and barely detectable in the line *Lj*NopL(3) (Fig. 6B), indicating that *nopL* negatively affects basal levels of chitinase expression in *L. japonicus* plants.

NopL Is a Substrate for Protein Kinases

YopJ, a type III effector of *Yersinia pestis*, inhibits the innate immune response of mammalian cells by modulating signal transduction pathways (Orth et al., 1999, 2000; Juris et al., 2000). Our data indicate that NopL, when delivered to plants, inhibits the induction of chitinase and β-1,3-glucanase, suggesting that NopL blocks signal transduction pathways involved in activation of PR genes. Therefore, we asked whether NopL could interfere with plant protein kinases. To answer this question, in vitro [γ-^{33}P]-labeling experiments using purified NopL protein were performed. Reaction mixtures containing soluble protein fractions from tobacco leaves, [γ-^{33}P] ATP, and recombinant NopL produced radioactively labeled proteins. SDS-PAGE analysis showed that the radioactivity was mostly incorporated into an $\approx$40-kD protein that corresponds to the molecular mass of NopL carrying the 6x-His tag. Substitution of NopL with the control fraction obtained from *E. coli* pPROEX-1 abolished this band (Fig. 7A). Proteolytic removal of the 6x-His tag reduced the size of the radioactive band to $\approx$37 kD (the apparent molecular mass of NopL). Finally, antibodies directed against NopL were able to precipitate phosphorylated NopL. Separation of the precipitated protein by SDS-PAGE revealed two radioactively labeled bands that are probably proteolytic degradation products of NopL. Addition of NopL was required for the appearance of the bands, and replacement of the anti-NopL antibody by a control rabbit serum was without effect (Fig. 7B).

DISCUSSION

Ectopic expression of type III effectors in plant cells is a powerful way of investigating their function. In susceptible host plants, expression of type III effectors may induce disease-like symptoms (Kjemtrup et al., 2000). In other plants that carry appropriate resistance genes, expression of type III effectors elicits hypersensitive responses (Gopalan et al., 1996; Leister et al., 1996; van den Ackerveken et al., 1996; Shao et al., 2002). Here, we report that constitutive expression of the *nopL* gene in planta blocks inducible plant defense responses. Levels of chitinases and glucanases remained low in virus-infected tobacco plants, suggesting that a link between low PR protein expression and heightened susceptibility to potato virus Y N605 exists. As strong inhibitory effects of PR proteins on virus accumulation have not been reported (Murphy et al., 2001), it is possible that ex-

Table I. *Plant growth and nodule formation of L. japonicus plants expressing nopL*

Wild-type and plants that express *nopL* (T_1 generation of the lines *Lj*NopL(1), *Lj*NopL(2), and *Lj*NopL(3) were cultivated in Magenta jars (four plants per jar). Plants were harvested six weeks p.i. with NGR234. Plant growth, as quantified by measuring the above-ground biomass at the time of harvest, and the number of nodules were determined for each Magenta jar. Data indicate mean values ± SE (number of Magenta jars: wild-type, n = 14; *Lj*NopL(1), n = 11; *Lj*NopL(2), n = 9; *Lj*NopL(3), n = 16).

Plant Line	Biomass Fresh Weight	Number of Nodules per Plant	Number of Nodules per gram of Fresh Weight
	(milligrams) per plant		
Wild-type	112.9 ± 11.6	15.0 ± 1.9	135 ± 13
*Lj*NopL(1)	56.9 ± 9.5	9.6 ± 1.3	193 ± 27
*Lj*NopL(2)	76.3 ± 5.1	14.7 ± 1.3	200 ± 22
*Lj*NopL(3)	84.1 ± 2.7	13.2 ± 1.1	163 ± 15

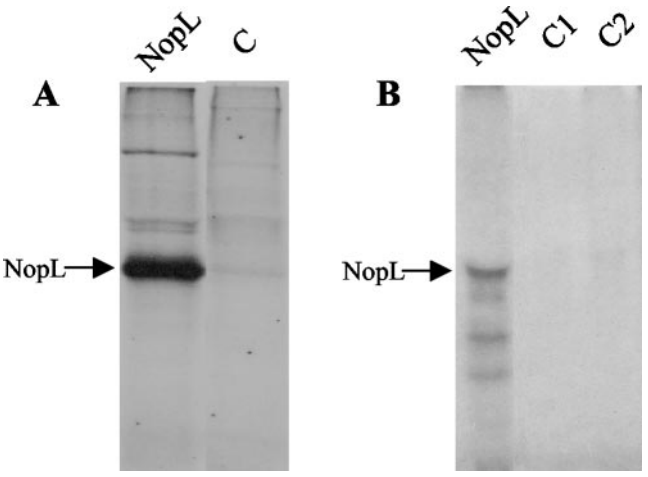

Figure 7. NopL is a substrate for plant protein kinases. A, In vitro phosphorylation assay. Recombinant NopL (1 μg) purified from *E. coli* pPROEX-1*nopL* (carrying a 6x-His tag; apparent size of ≈40 kD) was incubated with a tobacco extract (10 μg of soluble protein) in the presence of [γ-^{33}P]ATP (see "Materials and Methods"). The reaction mixture was separated by SDS-PAGE and the dried gel was exposed to an x-ray-sensitive film. C, Control reaction containing the corresponding fraction purified from *E. coli* pPROEX-1 (without *nopL*). B, Immunoprecipitation of phosphorylated NopL. Recombinant NopL protein was radioactively labeled with the protein kinase assay and was then incubated with the antiserum directed against NopL. Immunocomplexes were precipitated with protein-A agarose beads. The precipitated NopL protein was analyzed by SDS-PAGE followed by autoradiography. NopL, Reaction with NopL protein and antiserum against NopL; C1, control reaction without NopL protein (using the protein fraction from *E. coli* pPROEX-1); C2, reaction with control rabbit serum.

pression of *nopL* renders plants even more susceptible to bacterial and fungal pathogens.

Our data show that NopL blocks transcription of PR genes and can serve as a substrate for protein kinases. We suggest that NopL modulates signaling pathways that culminate in the activation of PR genes. One possibility is that NopL perturbs the expression of genes that are activated by mitogen-activated protein kinase pathways (Bartsev et al., 2003). Alternatively, protein kinases could activate NopL. The phosphorylated NopL protein would then interact with another host-plant protein. Candidates for such protein-protein interactions are regulators of plant defense, such as RIN4 from *Arabidopsis* (Mackey et al., 2002, 2003; Axtell and Staskawicz, 2003).

Virus infection of tobacco plants results in activation of various PR proteins (including class III β-1,3-glucanase), whereas ethylene stimulates only a subset of these PR proteins (e.g. class I chitinase and class I β-1,3-glucanase). The observation that ethylene treatment considerably enhances PR protein levels in plants that express *nopL* suggests that NopL has little effect on the ethylene perception pathway (Stepanova and Ecker, 2000). Nevertheless, NopL seems to interfere with the basal pathway that mediates induction of PR proteins after infection with potato virus Y N605 (Beffa et al., 1993). Moreover, it is possible that NopL interferes in other signal transduction events that are required for normal plant development because tobacco (T_0 generation) and *L. japonicus* lines that strongly express *nopL* displayed changed growth patterns. Activation of PR proteins is developmentally regulated in many plants, and various PR proteins, such as β-1,3-glucanases, modulate plant growth during development (Leubner-Metzger and Meins, 2001). Thus, it is possible that the observed developmental abnormalities of plants that express *nopL* could be the result of changes in the levels of PR proteins.

Rhizobial stimulation of transient or localized plant-defense responses has been reported for various legumes, especially during ineffective symbioses (e.g. Staehelin et al., 1992; Vasse et al., 1993; Mithöfer et al., 1996; Goormachtig et al., 1998; Salzer et al., 2000). The demonstration that NopL is a repressor of plant defense reactions suggests that invading rhizobia can manipulate metabolic pathways of their hosts using mechanisms that are common to pathogens. Data presented here show inhibition of *L. japonicus* growth. Yet, because type-three secretion by NGR234 has little effect on nodulation of *L. japonicus*, it is not an optimal plant to assess the function of NopL. Future work will focus on the interaction between *F. congesta* and NGR234, in which *nopL* has been shown to promote nodule formation (Marie et al., 2003). It will be interesting to see whether induced systemic resistance and/or systemic acquired resistance interfere with the establishment of mutualistic symbioses.

MATERIALS AND METHODS

Biological Materials and Vector Construction

Agrobacterium tumefaciens strains LBA4404 and AGL1 were used for transformation of tobacco (*Nicotiana tabacum* cv Xanthi) and *Lotus japonicus* (Regel) K. Larsen ecotype "Gifu" B-129-S9, respectively. Tobacco plants were inoculated with potato virus Y necrotic strain 605 (PVY N605; Jakab et al., 1997). The virus was maintained on tobacco by manual transmission, and infectious leaf material was stored at −70°C. Where appropriate, *L. japonicus* plants were inoculated with *Rhizobium* sp. NGR234. *Escherichia coli* strain DH5α was used for cloning and for production of recombinant NopL protein.

PCR-based techniques were used to amplify *nopL* (accession no. NP–444148; formerly called y4xL; Freiberg et al., 1997) from the cosmid pXB110 of NGR234 (Perret et al., 1991). Two primers, A (5′-GACTCCATGGATATCAATTCAACCAGC-3′) and B (5′-TATCTAGATCAAATGTCAAAATCCACCGA-3′) were used and the amplified fragment was verified by sequencing. The fragment containing *nopL* was then subcloned into pRT104 (Töpfer et al., 1987), which contains the CaMV 35S promoter and a poly-(A) signal. This expression cassette was subcloned into the binary vector pPZP112 (Hajdukiewicz et al., 1994; yielding pPZP112*nopL*) and was transferred to *A. tumefaciens* by triparental matings using the helper plasmid pRK2013 (Figurski and Helinski, 1979). To produce recombinant protein, *nopL* was cloned into the pPROEX-1 vector (Invitrogen, Carlsbad, CA) to give pPROEX-1*nopL* (Bartsev et al., 2003; Marie et al., 2003). This vector added an in-frame hexa-His tag (6x-His) to the N terminus of NopL. Purified recombinant NopL (Bartsev et al., 2003) was used to raise a polyclonal antibody (Marie et al., 2003).

Transformation of Plants

A. tumefaciens strain LBA4404 carrying pPZP112*nopL* or pPZP112 was cultivated in yeast tryptone medium (Sambrook et al., 1989) at 28°C and used to transform tobacco plants by the leaf-disc transformation procedure (Horsch et al., 1985). Regenerated shoots were selected on 0.8% (w/v) agar plates containing Murashige and Skoog medium (Murashige and Skoog, 1962) as well as 30 g L^{-1} Suc, 1 mg L^{-1} benzylaminopurine, 0.2 mg L^{-1} naphtaleneacetic acid, 100 mg L^{-1} meso-inositol, 0.5 mg L^{-1} thiamine hydrochloride, 0.5 g L^{-1} pyridoxine hydrochloride, 0.5 g L^{-1} nicotinic acid, 0.5 g L^{-1} cefotaxim, and 100 μg L^{-1} kanamycin. Regenerated plantlets (from independent explants) that possessed well-developed roots were transferred to pots containing autoclaved soil and were grown under greenhouse conditions (25°C day, 20°C night). Plants were watered with a Murashige and Skoog-water medium (ratio of 1:1). PCR amplification and immunoblot analysis confirmed the transgenic nature of the plants. Many of the transformants that strongly expressed *nopL* showed delayed development and were excluded from further analysis. Plants expressing *nopL*, as well as plants transformed with the empty vector pPZP112, were allowed to self-fertilize, yielding independent lines carrying pPZP112*nopL* and pPZP112, respectively.

Hypocotyl explants of *L. japonicus* were transformed with *A. tumefaciens* strain AGL1 bearing pPZP112*nopL* or pPZP112 and were regenerated as described (Handberg and Stougaard, 1992; Stiller et al., 1997). Plantlets regenerated from independent transformation events were propagated under greenhouse conditions and were allowed to self-fertilize, yielding seeds of lines carrying pPZP112*nopL* and pPZP112, respectively.

Protein Extraction and Immunoblotting

Plant material was homogenized in a mortar and pestle with three volumes (w/v) of 100 mM Tris-HCl (pH 8.1) containing 400 mM sucrose, 10% (v/v) glycerol, 10 mM EDTA, 10 mM KCl, and the protease inhibitors phenylmethanesulfonyl fluoride (1 mM), E64 (0.5 μg mL^{-1}), leupeptin (0.5 μg mL^{-1}), aprotinin (2 μg mL^{-1}), and pepstatin (1 μg mL^{-1}). After centrifugation (at 13,000*g* for 30 min), the supernatants were separated by SDS-PAGE and transferred onto PVDF membranes (Millipore, Bedford, MA). Staining with Ponceau S (Sigma-Aldrich, St. Louis) allowed visualization of the amount of protein loaded. To detect the NopL protein, the membranes were incubated overnight with monospecific NopL antiserum (1:2,500 dilution). Chitinase class I, as well as β-1,3-glucanase proteins, were detected using antiserum (1:2,000 dilution) raised against basic chitinase (class I; Shinshi et al., 1987) and basic β-1,3-glucanase (class I; Felix and Meins, 1985), respectively (kindly provided by Frederick Meins, Friedrich Miescher Institute, Basel). After incubation with the antiserum, the PVDF membranes were reincubated with a goat anti-rabbit antibody coupled to horseradish peroxidase and were developed using the enhanced chemiluminescence western-blotting analysis system (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK). When necessary, the signals were quantified by the Gene Genius Bio-Imaging system (Gene Tools; Syngene, Cambridge, UK).

Inoculation of Plants and Treatment with Ethylene

Tobacco seeds were surface sterilized, germinated on agar plates Murashige and Skoog medium for 10 d, and grown in pots filled with autoclaved soil under greenhouse conditions for 14 d. One leaf per plant was manually inoculated with infectious leaf material containing PVY N605. Virus accumulation in infected tobacco plants was monitored by ELISA using anti-PVY N605 antibodies conjugated to alkaline phosphatase (Voller et al., 1976). Where indicated, 4-week-old tobacco plants were treated with 100 μL L^{-1} ethylene in a closed chamber for 48 h. Control plants were incubated in a similar chamber without ethylene. *L. japonicus* seeds were surface sterilized by treatment with 70% (v/v) ethanol and 3% (v/v) hydrogen peroxide and were left to germinate on sterilized humid filter paper. Plantlets were then transferred to Magenta jars in which the lower assembly was filled with B&D nutrient solution (Broughton and Dilworth, 1971). NGR234 was cultivated in RMM medium (Broughton et al., 1986) on a rotary shaker (150 rpm) at 27°C before inoculation. Five-day-old *L. japonicus* plants were inoculated with $\approx 10^9$ NGR234 cells per plant and were grown at a daytime temperature of 26°C, a night temperature of 20°C, and a light phase of 16 h (including a 1-h stepped "sunrise" and a 1-h stepped "sunset"; maximum intensity of illumination was 350 μmol m^{-2} s^{-1} PAR). Two weeks later, the lower Magenta vessels were replaced with ones containing B&D medium supplemented with 1 mM KNO_3.

Northern-Blot Analysis

Total RNA (15 μg) was isolated from tissues (Ausubel et al., 1990), separated on formaldehyde containing agarose gels, and blotted onto nylon membranes. To check that equal amounts of RNA were loaded, the gels were stained with ethidium bromide. A basic tobacco chitinase class I gene (cDNA clone CHN50) was used as the hybridization probe (Shinshi et al., 1987).

Enzyme Assays and Protein

Plant material was extracted with a mortar and pestle in cold phosphate buffer (100 mM, pH 7.0) containing protease inhibitors as described above. After centrifugation (at 13,000*g* for 30 min), peroxidase activity was measured photometrically by following the rate of increase in A_{470} of a solution containing 15 mM guaiacol ($\epsilon = 26.6$ mM^{-1} cm^{-1}) and 5 mM H_2O_2 in 100 mM phosphate buffer (pH 5.8). Chitinase activity was assayed radioactively using [^{3}H]-labeled chitin as the substrate (Boller et al., 1983). Protein concentration was determined using a protein assay kit (Bio-Rad, Hercules, CA).

Phosphorylation and Immunoprecipitation of NopL

NopL was purified from *E. coli* pPROEX-1*nopL* (see above). Control fractions were obtained from *E. coli* pPROEX-1 cultures using the same purification protocol. A typical reaction mixture (20 μL) of the in vitro protein kinase assay contained 40 mM HEPES buffer (pH 7.4), 1 μg of purified NopL, 5 μCi of [γ-^{33}P]ATP (or [γ-^{32}P]ATP), 10 mM $MgCl_2$, 3 mM $MnCl_2$, 100 μM Na_3VO_4, protease inhibitors (as indicated above), and 10 μg of freshly extracted soluble proteins from tobacco leaves. Phosphorylation reactions were carried out at 30°C for 20 min. The reaction mixtures were separated by SDS-PAGE and the dried gels were exposed to an x-ray-sensitive film.

After in vitro phosphorylation (24 μg of NopL, 40 μCi of [γ-^{32}P]ATP, and 100 μg of soluble tobacco protein), an aliquot (80 μL) of the reaction mixture was incubated with 100 μg of anti-NopL protein (at 4°C for 2 h). Another 80-μL aliquot was used as a control and was incubated with 100 μg of nonimmune serum. After incubation, 10 μL of packed Protein-A-agarose beads was added and the samples were incubated for an additional 2 h (at 4°C). The beads were recovered by centrifugation and were extensively washed with phosphate-buffered saline-Tween buffer (80 mM Na_2HPO_4, 20 mM NaH_2PO_4, 100 mM NaCl, and 0.1% [v/v] Tween 20, pH 7.5). Finally, the beads were mixed with 50 μL of loading buffer (Laemmli, 1970), heated to 100°C (5 min), and the proteins were separated by SDS-PAGE. Dried-gels were exposed to x-ray-sensitive films.

Statistical Analysis

Statistical analysis was performed using the S-PLUS 6 program from MathSoft (Bagshot, Surrey, UK). Data were analyzed by the nonparametric Kruskal-Wallis rank sum test, which is suitable for unequal replications. Kruskal-Wallis statistics are approximately distributed as a $\chi^2_{(a-1)}$, with "*a*" being the number of treatments. $P \leq 0.05$ was considered as representing a significant difference in this study. All data are means ± the SE.

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