

FIG. 1. Cassettes containing *ThEn42* (genomic or cDNA) used for plant transformation. (A) CaMV35S-Pro = promoter; nopaline synthase (NOS) = transcription terminator; coding sequence for the fungal signal peptide (FSP), plant secretion peptide (PSP), fungal putative activation peptide (AP). (B) The 5' noncoding region of p35S-CHIT42 with PSP (pTE 28.1) and p35S-CHIT42 with FSP. Box indicates mRNA stabilization consensus sequence, and topline indicates A+T rich regions (20). Bold letters indicate consensus sequences for transcription in the ATG region compared with TAA ACA ATG GCT (33). (C) Elements reducing mRNA stability at the 3' noncoding region of the fungal gene (33) are boxed or underlined. In p35S-psCHIT42, the region downstream from restriction site *Ssp*I (arrowhead) was removed.

were constructed that contained *ThEn-42* and *chit42* under the control of the cauliflower mosaic virus 35S subunit (CaMV35S) promoter region and the *Agrobacterium* nopaline synthase terminator (Fig. 1A). A genomic copy of *ThEn-42*, which includes a 26-aa secretion peptide followed by a 12-aa putative activation peptide and a 424-aa coding sequence interrupted by three introns (19), was ligated into pBI121 to produce the plasmid p35S-ThEn42 (Fig. 1A). To express constitutively the *chit42* cDNA, two constructs were made in the plasmid pBin19:p35S-CHIT42 containing the *Trichoderma* signal and putative activation peptides and p35S-psCHIT42 containing a tomato pathogenesis-related protein (P1-p14) signal peptide from pTE 28.1 (20) (Fig. 1A and B). In the latter, the 3' noncoding region of the fungal gene was modified to improve transcription and mRNA stability (Fig. 1C) by removing a terminal sequence of ≈ 100 bp. The transformation procedure followed standard protocols (21): *Agrobacterium tumefaciens* strain LBA4404 containing vectors with the chimeric fungal endochitinase gene and corresponding empty vectors was used to transform leaf disks of *Nicotiana tabacum* cv. Samsun NN and cv. Xhantii and stem segments of *Solanum tuberosum* cv. Desiree (only p35S-ThEn42).

Screening and Molecular Analyses of Transgenic Lines. Kanamycin-resistant transgenic plants were propagated on Murashige-Skoog basal salt medium (Sigma) containing sucrose (3%) and kanamycin sulfate (100 mg/liter). Homozygous lines were selected by selfing F1 progeny. Plant and fungal DNA were obtained following published procedures (22, 23). The transgene in selected progeny was PCR-amplified with the primers GGTTATGCTTTCCATCGG (EC1), GTGTAGCCCGGAGCGTATT (Bio1N) and CAAGGAGTCA-GAGCCAGTCTT(BB2), which anneal at 566 bp (EC1), 813

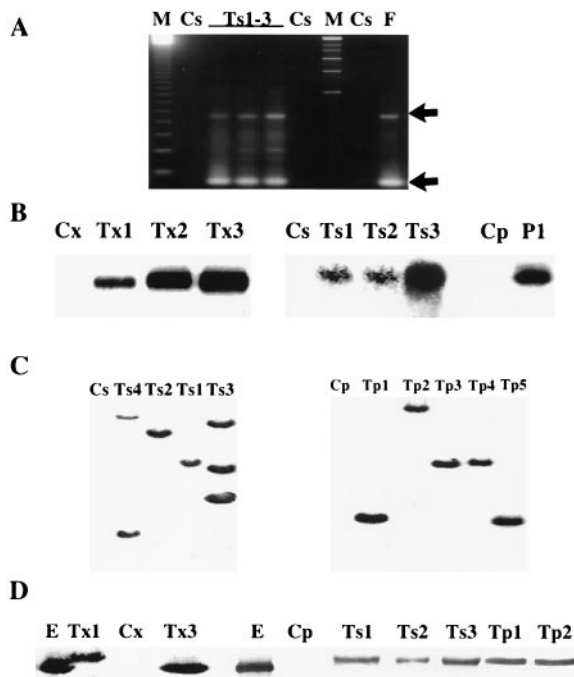


FIG. 2. Analyses of representative transgenic (T) tobacco and potato lines. C, control plants (nontransformed or empty-vector transformed); Cs, tobacco cv. Samsun NN; Cx, tobacco cv. Xhantii; Cp, potato cv. Desiree. Representative samples of transgenic lines are shown: Ts(no. of plant line) = tobacco cv. Samsun NN transformed with p35S-ThEn42; Tx (no. of plant line) = tobacco cv. Xhantii transformed with p35S-CHIT42 (Tx1) or with p35S-psCHIT42 (Tx2 and Tx3); Tp(no. of plant line) = potato cv. Desiree transformed with p35S-ThEn42; E = pure endochitinase from *T. harzianum* culture. (A) PCR amplification of the fungal transgene using transgenic, control plant DNA or control fungal DNA (F) with nested primer sets EC1-Bio1N and EC1-BB2 producing 250- and 800-bp bands, respectively (indicated by arrows) (18). (B) Northern analysis of plant tissues using the *ThEn-42* cDNA sequence as a probe and visualization of bands after 3–5 days of exposure. (C) Southern analysis of *Bam*HI-digested tobacco and potato DNA probed with an *Eco*RI 1.7-kb fragment of *ThEn-42*. (D) Western analysis of soluble leaf proteins of tobacco and potato separated by SDS/PAGE and visualized with anti-endochitinase antibodies and a standard alkaline phosphatase immunoassay.

bp (Bio1N), and 1,367 bp (BB2) from the 5' end of the gene (Fig. 2A). The copy number of the endochitinase gene was assessed by Southern analysis (24) of transformant DNA cleaved with *Bam*HI or *Eco*RI, followed by a test of segregation in the F1 generation. The DNA fragments used as probes were obtained from *Eco*RI digestion of p35S-ThEn42 and p35S-CHIT42 and were labeled (19, 25). For Northern analysis, 10 μ g of RNA were used following published methods (26, 27). ELISA tests and Western analysis with antibodies prepared against the purified endochitinase from *T. harzianum* (18, 28) were performed on protein extracts from leaves, stems, flowers, and roots of selected transgenic lines (29). The transgenic protein also was detected and quantified by using turbidity reduction and fluorescent assays with specific substrates (4-methylumbelliferyl- β -D-N,N',N''-triacetylchitotriose and nitrophenyl- β -D-N,N',N''-triacetylchitotriose) as described (30, 31). Localization of the transgenic protein in the plant tissues was performed by immunogold labeling following standard fixation and by electron microscopy observation.

Disease Resistance Assays. Transgenic tobacco and potato plants were tested for resistance to: *Alternaria alternata* isolate FC2600 and *A. solani* isolate 1191 from potato, airborne foliar pathogens that cause brown spot or leaf spot disease; *Botrytis cinerea* isolate 319 from tobacco, an airborne pathogen that

causes grey mold disease; and *Rhizoctonia solani* from tomato (isolate 1556), an endemic soilborne pathogen that causes damping-off, seedling blight, and root rot. All isolates were from the University of Naples culture collection.

Assays with *A. alternata*, *A. solani*, and *B. cinerea*. Transgenic and control tobacco were grown at 25°/15°C with 16 h of diurnal light in the greenhouse. Four leaves per plant from 10 plants/line of both cultivars were inoculated with 10 μ l of a spore suspension (10⁶/ml), obtained from *A. alternata* grown on potato dextrose agar (25°C with light), at four inoculation points between the leaf veins; and the plants were incubated at 20°C with 16 h of diurnal light and 90% relative humidity (RH). Observations were conducted daily (up to 11 days) for disease reaction (chlorosis, necrosis, or both) and severity (none, slight, strong, severe).

Ten potato plants/line were spray inoculated with *A. solani* conidia (10⁴/ml) obtained on potato dextrose agar (32), incubated at 100% RH for 16 h, and allowed to dry, and the procedure was repeated three times. Ten days after inoculation, the number of lesions/leaf was evaluated per plants/line and averaged.

Four leaves per plant from 10 plants/line of both tobacco cultivars were inoculated with *B. cinerea* agar plugs (0.5–1 cm²) from 1-week-old cultures grown on malt extract agar, and plants were incubated at 18°C with 100% RH. Lesion sizes were measured 10 and 14 days after inoculation, and results were combined and averaged for each plant line.

Assays with *R. solani*. For all *R. solani* assays, the fungus was grown on potato dextrose broth at 150 rpm, 25°C with light for 5 days, and the mycelium was vacuum harvested, weighed, and homogenized in distilled water. In one set of seedling assays, a water agar solution containing the fungal suspension (0.7% agar, 0–1.0 mg fungus/ml) was prepared in Petri plates. One-month-old tobacco seedlings (25 plants/line), germinated on Murashige–Skoog with 0.7% agar, were transferred to the fungus-containing or noncontaining plates. In the other seedlings assays, sterile soil (1 cm deep) was placed in containers (115 mm diameter \times 35 mm deep) and infested by evenly applying a 10-ml *R. solani* suspension (0.75 mg/ml) to the surface and then covered with a layer of noninoculated soil (1.5 cm deep). Seedlings grown to emergence of the second set of true leaves (20 plants/line) were transplanted into the surface layer, misted, and covered with a transparent lid. For both assays, plants were incubated at 20°C with 16 h of diurnal light at 90% RH. The number of dead or dying plants was counted every 3 days.

For assays with grown plants, tobacco (10 plants/line) was grown in peat moss pellets until the 5–6 leaf stage (2 months old) and then transplanted into boxes (20 \times 20 \times 5 cm³ or 35 \times 26 \times 9 cm³) containing 1 or 3 liters of sterile soil, respectively. A *R. solani* suspension (1–3 g/liter) was prepared and watered around the base of the plants, followed by incubation at 20°C with 16 h of diurnal light at 90% RH. Evaluations were conducted weekly, up to 1.5 months after transplant, for disease symptoms and effect on plant growth.

Potato plants were micropropagated on Murashige–Skoog media plus 0.3% sucrose with 0.7% agar, until root formation, then individually transplanted to 50-ml tubes containing sterile soil and climatized for 2 weeks. Each plant (10 plants/line) was inoculated with a 10-ml *R. solani* suspension (5 mg/ml) and then grown at 20°C with 16 h of diurnal light at 90% RH. Evaluations were conducted twice weekly for overall plant condition and measurement of plant height.

For all disease resistance assays, infected plant material was plated onto acidified potato dextrose agar to verify the presence of the test fungus. To maintain virulence, all pathogens were continuously re-isolated from infected plants. Each experiment contained several replicates and was repeated three or more times. Presented results consisted of combined data for each tobacco or potato line, with calculations of means and

SDs (indicated with bars in the figures). Data also were combined for all lines and analyzed by ANOVA.

RESULTS

Transgenic Expression of the *Trichoderma* Chitinase Gene in Tobacco and Potato. Fifty tobacco and 13 potato kanamycin-resistant transformants were screened for the presence and expression of the fungal endochitinase gene by PCR, Northern, Southern, and Western analyses (Fig. 2 A–D). Most transformed plants but not nontransformed and empty vector-transformed plants contained the transgene, the mRNA of the expected size, and a protein that reacted with antibodies raised against the *Trichoderma* endochitinase (18, 28) (Fig. 2 A–D). Southern analysis indicated that the copy number of the transgene was one to three and occasionally more, and there was no correlation between the number of copies and the level of transcription in transgenic lines (data not shown).

The transgenic protein accumulated mainly in leaves and stems, but also was found in roots and flowers. The protein encoded by the transgene was identical to the wild-type *Trichoderma* endochitinase in plants transformed with p35S-psCHIT42 but was slightly larger in plants transformed with p35S-ThEn42 and p35S-CHIT42 (Fig. 2D) because the 12-aa putative activation peptide at the 5' end of the gene (19) was not cleaved. However, both the *Trichoderma* and tomato secretion signal peptides were cleaved and induced extracellular accumulation of the fungal enzyme as shown by immunogold labeling (Fig. 3). Specific enzyme assays and protein band image analysis indicated that the fungal endochitinase was active in both transgenic tobacco and potato, and, depending on the lines tested, it represented 0.01–0.5% of total protein content. Specific enzyme activity, pH, and temperature optima and stability of the transgenic enzyme showed no significant changes in comparison to the purified *Trichoderma* endochitinase (data not shown).

In general, the highest endochitinase activity was found in tobacco and potato bearing the genomic copy (p35S-ThEn42) and in tobacco with the cDNA fused to the tomato secretion sequence (p35S-psCHIT42). Progenies reached 10- and 400-fold more endochitinase activity than control plants in roots and leaves, respectively, although intermediate, low, or non-detectable levels also were found. Tobacco and potato plants with high chitinase activity showed no obvious differences in germination, growth, or development when compared with the controls or wild types. In addition, up to 1 mg of purified endochitinase from *T. harzianum* administered by interveinal injection had no effect on tobacco and potato leaves (data not shown).

Disease Resistance of Transgenic Plants to Foliar Pathogens. Thirty-eight transgenic lines of tobacco and nine of

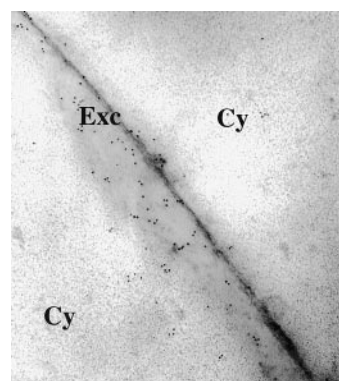


FIG. 3. Gold immunolabeling of transgenic tobacco cv. Samsun NN (Ts1) transformed with p35S-ThEn42. Cy, cytoplasm; Exc, extracellular space.

Table 1. Resistance of *Trichoderma* endochitinase tobacco plants to *A. alternata*

Plant lines	Disease symptoms			Disease severity			
	R	C	N	None	Slight	Strong	Severe
Ts2	20.5	18.1	15.7	79.5	20.5	0.0	0.0
Ts3	1.0	0.0	1.0	99.0	1.0	0.0	0.0
Ts4	44.3	15.7	37.1	55.7	44.3	0.0	0.0
Ts5	69.5	64.2	66.8	30.5	28.5	22.5	18.5
Tx1	17.5	17.5	0.0	82.5	17.5	0.0	0.0
Tx3	0.0	0.0	0.0	100.0	0.0	0.0	0.0
All trans.	33.6	29.3	25.9	66.4	33.6	0.0	0.0
Cs1	93.5	90.3	83.9	6.5	48.3	45.2	0.0
Cs2	100.0	100.0	83.3	0.0	0.0	0.0	100.0
Cs3	95.7	95.7	95.7	4.3	32.6	45.7	17.4
Cx1	100.0	100.0	66.7	0.0	0.0	0.0	100.0
Cx2	100.0	100.0	100.0	0.0	0.0	0.0	100.0
All contr.	95.8	94.7	89.5	4.2	31.6	36.8	27.4

Disease symptoms and severity (5 days after inoculation) of representative transgenic and control lines (see legend to Fig. 2) are indicated as percentage of inoculation points.

All trans., combined values of all transgenic lines (except line Ts5) tested; All contr., combined values of all control lines tested; R, any reaction (C, N, or C + N); C, chlorosis; N, necrosis.

potato were assayed. Tobacco controls showed typical symptoms of *A. alternata* infection at 96% of the inoculation sites (Table 1) whereas the overall value for all transgenics was 34%. Controls showed typical necrosis surrounded by a chlorotic halo and extensive sporulation of the pathogen whereas symptoms on transgenics, if present, were a faint chlorosis or a hypersensitive-like necrosis with little sporulation (data not shown). The level of disease resistance varied among transgenic lines, from no to complete resistance, but the average reaction to inoculation ranged from no (66%) to slight reaction

A



B

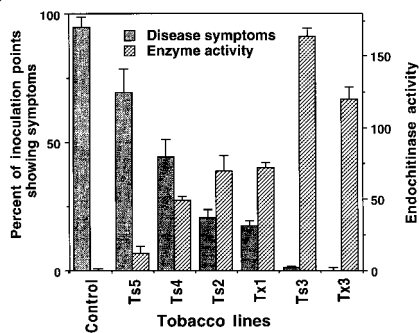


FIG. 4. Resistance of *Trichoderma* endochitinase tobacco plants to *A. alternata*. (A) Leaves of transgenic (line Ts3, on left) and control (on right) tobacco; dark dots indicate inoculation points. (B) Disease symptoms 11 days after inoculation and endochitinase activity in leaf protein extracts of representative transgenic lines (see legend to Fig. 2); control is an average of different lines of Cs and Cx; endochitinase activity was expressed as pmol of 4-methylumbelliferone released from 4-methylumbelliferyl- β -D-N-N'-N''-triacetylchitotriose/min/ μ g of protein (30–31).

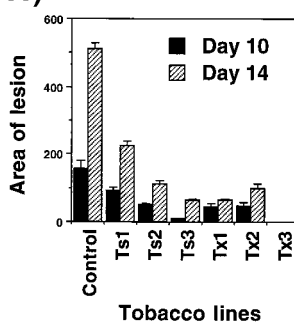
(34%) (Table 1). Only line Ts5 exhibited a strong or severe reaction like the controls, and it was not considered in the overall evaluation of transformants (Table 1). Approximately 5–10% of the transgenic lines were completely resistant to *A. alternata* (Fig. 4 A and B; Table 1; and data not shown) and never developed symptoms even if exposed to the pathogen repeatedly and for long periods. Generally, transgenic plants expressing higher amounts of *Trichoderma* endochitinase were more tolerant or completely resistant to *A. alternata* (Fig. 4B).

Assays with *B. cinerea* indicated that transgenic tobacco were highly tolerant and, in a few cases, nearly completely resistant to this pathogen. Generally, lesions were much larger in control plants than in transgenic lines expressing high levels of endochitinase activity. Selected lines, such as lines Tx3 and Ts3, that showed high levels of resistance to *A. alternata*, were also highly resistant to *B. cinerea* (Figs. 4B and 5A).

Potato control plants showed typical disease symptoms when infected by *A. solani*, whereas the level of resistance varied greatly among transgenic lines (Fig. 5B). One-third of the potato lines were highly resistant to *A. solani* (i.e., line Tp1 showed 85% disease reduction), even under assay conditions highly favorable to disease development (Fig. 5B). As in tobacco, potato lines producing more *Trichoderma* endochitinase (about 200-fold more than controls) were more resistant to *Alternaria* (data not shown).

Disease Resistance of Transgenic Plants Against a Soil-Borne Pathogen. In experiments on water agar containing tobacco seedlings and *R. solani*, the pathogen was highly virulent and killed most of the control plants (Fig. 6A). Instead, survival rates among transgenics ranged from 65 to 80%. In soil experiments with *Rhizoctonia* and tobacco seedlings, 10–15% of the transgenic lines consistently showed no disease symptoms whereas others had little or no improvement in disease resistance (data not shown). A strong difference between controls and transgenic tobacco also was observed when 2-month-old plants were tested (Figs. 6B and 7). Growth of controls in *R. solani*-infested soil was reduced \approx 90% in comparison to growth on noninfested soil. Several transgenic lines consistently performed better than controls and grew equally well as plants on noninfested soil, showing complete resistance (Fig. 7). Analysis of all of the transgenic lines combined indicated a significant increase in plant growth in comparison to the controls ($P < 0.05$) (data not shown). Surviving control plants grown in infested soil were noticeably stunted, whereas the *Trichoderma* endochitinase plants usually appeared larger and with more root regrowth (data not shown).

A)



B)

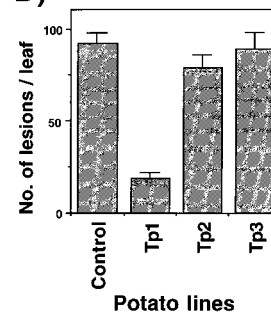


FIG. 5. Resistance of *Trichoderma* endochitinase tobacco plants to *B. cinerea* and of potato plants to *A. solani*. (A) Size of lesions (mm²) produced on leaves of different tobacco transgenic lines (see legend to Fig. 2) and controls (average of Cs and Cx lines) 10 and 14 days after inoculation with *B. cinerea* agar plugs. (B) Number of lesions observed on different potato transgenic lines and control (see legend to Fig. 2) 9 days after spray inoculation with *A. solani*.

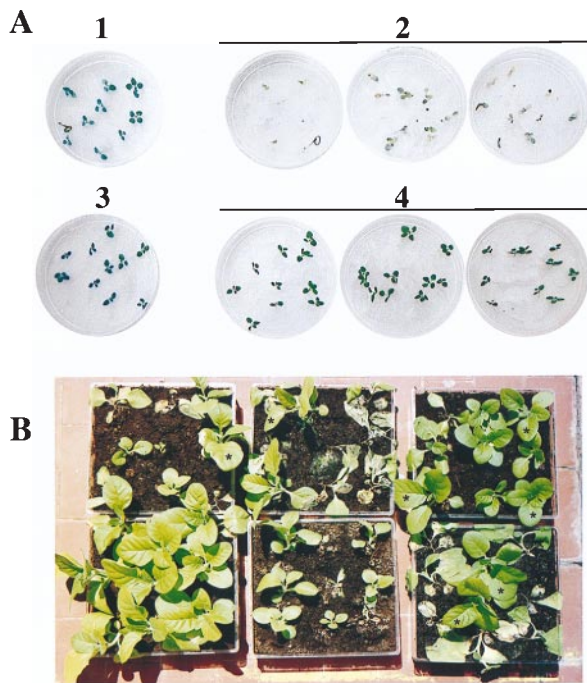


FIG. 6. Resistance of *Trichoderma* endochitinase tobacco plants to *R. solani*. (A) Seedlings grown 5 days on water agar containing *R. solani*: controls (Cs) without pathogen (1) or with pathogen (2); transgenics (Ts3) without pathogen (3) or with pathogen (4). (B) Transgenic and control plants grown on soil infested with *R. solani*; best standing plants are transgenic (boxes on right and asterisks) compared with plants grown on noninfested soil (left corner).

In disease tests of potato with *R. solani*, almost all of the control plants were killed after 23 days of incubation, and the few surviving plants appeared damaged and unhealthy. Most of the transgenic lines, five of nine tested, were considerably more resistant than controls, although a variation in disease resistance was observed (Fig. 8). A few potato lines, including Tp1, which was highly resistant to *A. solani*, consistently showed a complete or a nearly complete resistance to *R. solani* (Fig. 8).

DISCUSSION

The endochitinase gene from *T. harzianum* was expressed in tobacco and potato at high levels, and both secretion peptides

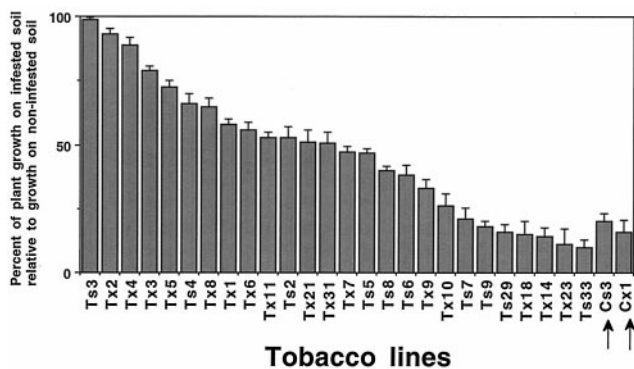


FIG. 7. Resistance of *Trichoderma* endochitinase tobacco plants to *R. solani*. Plant growth from 14 to 30 days on *R. solani*-infested soil relative to growth on noninfested soil (expressed as a percentage of plant height obtained on noninfested soil, which was taken as 100%). Control plants (indicated by arrows) and representative transgenic lines (see legend to Fig. 2); Tx (2 to 10 and 14) transformed with p35S-psCHIT42; Tx (1, 11, 18, 21, 23, and 31) transformed with p35S-CHIT42.

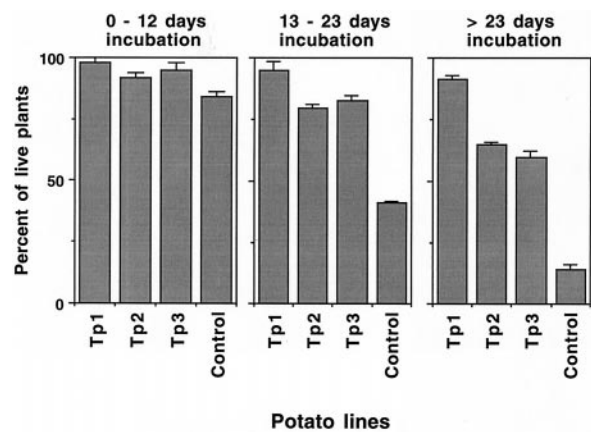


FIG. 8. Resistance of *Trichoderma* endochitinase potato plants to *R. solani*. Percent survival of control and representative transgenic lines (see legend to Fig. 2) grown on *R. solani*-infested soil measured at different incubation periods.

from fungus and tomato were correctly cleaved and able to drive the accumulation of the transgenic enzyme outside the plant cell, which indicated that genes and signal sequences from fungi may be used to secrete and accumulate fungal enzymes in plants or in media containing plant cell suspensions. The *Trichoderma* introns of the genomic copy (p35S-ThEn42) were spliced correctly in plant. The level of transcription obtained with the genomic copy was higher than with the original cDNA copy and as high as with the cDNA engineered to improve mRNA stability. This result suggests that the presence of introns and the absence of other noncoding fungal sequences may improve gene expression in plants (33, 34).

Many papers and reviews have discussed the advantages of using chitinases for plant protection because these enzymes are fungicidal, part of the plant defense system, and nontoxic to plants, animals, and higher vertebrates. Transgenic expression in plant of chitinase genes has been shown to improve disease resistance in various crops, and patents have been issued on related methods. However, the level and the spectrum of resistance obtained has not supported the development of this technology for the production of new disease-resistant varieties suitable for commercial agriculture. One of the main limitations has been the relatively low level of resistance obtained with a single chitinase gene, which has resulted in the need to use gene combinations. This strategy, however, may not be attractive because a higher degree of genetic modification of the plant may substantially increase the costs of product development, complicate and delay the registration process in some countries, and be in contrast with the public's feelings about accepting genetically modified crops. In addition, there are almost no reports of chitinase-transgenic plants showing resistance to several fungi, indicating that the genes and enzymes used so far have a narrow spectrum of antifungal activity. This spectrum may represent a major limitation for protecting crops that are susceptible to more than one pathogen and makes this transformation-based strategy less suitable to substitute or integrate the application of chemical fungicides that have a relatively broad spectrum of activity. The unsuccessful exploitation of chitinases for plant protection probably comes from the fact that the genes used so far, mostly from plants, code for enzymes that lack a strong inhibitory activity at low concentrations and the ability to efficiently degrade spores or other fungal preservation structures that serve as inoculum. The effectiveness of plant chitinases depends on the simultaneous action of other antifungal substances, and plant pathogens that have been exposed to these enzymes have developed mechanisms to overcome the defensive action of

these chitinases (35). In fact, *Cladosporium fulvum*, *Cercospora nicotiana*, *C. beticola*, *Fusarium solani*, and several other pathogenic fungi were not inhibited in the growth by treatment with chitinases obtained from plant (15, 17).

This work demonstrates a successful application of a single endochitinase-encoding gene from *T. harzianum* for controlling plant diseases. Expression of *ThEn-42* in tobacco and potato produced complete or nearly complete resistance to *R. solani*, *A. solani*, *B. cinerea*, and *A. alternata*, which was genetically stable and transferred to the progeny. This high level of resistance to both foliar and soilborne pathogens represents a major improvement in comparison to results from transgenic expression of other chitinase genes, i.e., from plants or bacteria. This improvement is in accordance with the finding that chitinases and other cell wall degrading enzymes from *Trichoderma* have a stronger and wider spectrum of antifungal activity in comparison to corresponding plant and bacterial enzymes (4). Conceivably, a number of useful genes may be found in these antagonistic fungi that developed a multi-component biochemical arsenal to survive in the highly competitive environment of the phyllosphere and rhizosphere. Therefore, this work discovers a new source of genes that can be used either singly or in synergistic combinations to provide protection against a wide range of fungal pathogens.

The level of disease resistance observed varied greatly among transgenic lines, but $\approx 5\text{--}10\%$ of the transformed tobacco and potato plants were highly resistant to all the pathogens tested. These results obtained with one transgene may be explained by the following points: (i) there was a very high level of chitinolytic activity in some transgenic lines; (ii) the endochitinase concentrated in the extracellular space had a strong antifungal activity; (iii) the *Trichoderma* enzyme is highly synergistic with antifungal pathogenesis-related proteins in plants, such as tobacco osmotin (8); and (iv) the *Trichoderma* endochitinase in plant tissues may release from the cell wall of invading fungi compounds that elicit the plant defense response. Ongoing experiments support this latter hypothesis because potent elicitors that produce resistance responses in wild-type tobacco can be obtained by treating the mycelium of *R. solani* and *Alternaria* spp. with purified *Trichoderma* chitinolytic enzymes (M.L. and S.L.W., unpublished work). Moreover, in some cases, *A. alternata* inoculated on transgenic leaves produced a hypersensitive-like reaction. However, the level of resistance to the three foliar pathogens corresponded to the level of chitinolytic activity of the plant, indicating that the antifungal/lytic activity of the *Trichoderma* enzymes had a major role in the observed resistance.

A full scale application of fungal biocontrol agents in commercial agriculture has been delayed because of the inconsistent results obtained by introducing these complex microorganisms into the ever-changing environment. We suggest a way to overcome the often troublesome and unpredictable application of the whole organism by transferring directly into the plant the microbial genes responsible for disease control.

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