Transgenic Tobacco Plants Overexpressing Chitinases of Fungal Origin Show Enhanced Resistance to Biotic and Abiotic Stress Agents[1]

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Genes encoding defense-related proteins have been used to alter the resistance of plants to pathogens and other environmental challenges, but no single fungal gene overexpression has produced broad-spectrum stress resistance in transgenic lines. We have generated transgenic tobacco (*Nicotiana tabacum*) lines that overexpress the endochitinases CHIT33 and CHIT42 from the mycoparasitic fungus *Trichoderma harzianum* and have evaluated their tolerance to biotic and abiotic stress. Both CHIT33 and CHIT42, individually, conferred broad resistance to fungal and bacterial pathogens, salinity, and heavy metals. Such broad-range protective effects came off with no obvious detrimental effect on the growth of tobacco plants.

Plants respond to pathogen attacks by expressing a wide array of genes, most of them directly related to defensive molecular mechanisms. Both the hypersensitive response and the systemic acquired resistance that plants exhibit upon biological stress are complex processes in which a network of different signal cascades ends in modulation of the expression of different sets of genes (Ryals et al., 1996; Shirasu et al., 1996). Seemingly separated abiotic stress-signaling pathways also share common elements that are in some cases considered as possible cross-talk points (Ellis and Turner, 2001; Zhu, 2001; Singh et al., 2002).

Both pathogen attacks and abiotic stresses, such as salinity and drought, decrease crop yields worldwide. Many attempts have been made to confer resistance to pathogens and increase tolerance to abiotic stress to plants of agronomic interest. One of the most widely used strategies is to overexpress plant genes that are induced after biotic or abiotic stresses, such as chitinases and glucanases (Alexander et al., 1993; Hong and Hwang, 2006), vacuole and plasma membrane sodium transporters (Apse and Blumwald, 2002; Shi et al., 2003), mitogen-activated protein kinases (Piao et al., 2001; Zhang and Liu, 2001; Xiong and Yang, 2003), transcription factors (Park et al., 2001), peroxidases (Amaya et al., 1999), disease-related R genes, and ferritins (Deak et al., 1999). Alternatively or complementarily, efforts have been made to reinforce the plant array of responsive genes by introducing heterologous genes of well-known antipathogenic effect belonging to other phylla (Lorito et al., 1998; Bolar et al., 2001; Garg et al., 2002; Schutzendubel and Polle, 2002; Kunze et al., 2004). Chitinases are thought to play a dual role, both by inhibiting fungal growth by cell wall digestion and by releasing pathogen-borne elicitors that induce further defense reactions in the host. Transgenic plants overexpressing chitinases of several origins have been shown to exhibit enhanced levels of resistance to fungal infection and delayed disease symptoms when challenged with fungal pathogens (Jach et al., 1995; Lorito et al., 1998; Hong and Hwang, 2006).

The genome of mycoparasites, such as members of the genus Trichoderma, which have specifically evolved to attack other fungi, is a potential source of antipathogenic genes. Trichoderma species can inhibit the growth of other fungal species by means of antibiotics and cell wall-degrading enzymes: chitinases, proteases, glucanases, and mannanases, among others. Transgenic tobacco (*Nicotiana tabacum*) and potato (*Solanum tuberosum*) plants overexpressing an endochitinase (CHIT42) from *Trichoderma harzianum* have been shown to be highly tolerant to the foliar pathogens *Alternaria alternata*, *Alternaria solani*, and *Botrytis cinerea*, and also to the soil-borne pathogen *Rhizoctonia solani* (Lorito et al., 1998). Overexpression of another endochitinase from Trichoderma (CHIT33) considerably enhances the antifungal activity of *T. harzianum* strain CECT2413 in in vitro confrontation experiments against *R. solani* (Limón et al., 1995). The amino acid sequence of CHIT33 shows significant similarity to some pathogenic response-associated class III

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plant chitinases (Limón et al., 1995) and substantial biochemical differences with other Trichoderma chitinases (de la Cruz et al., 1992). Chitinases CHIT42 and CHIT33 exhibit synergistic in vitro hydrolytic properties when assayed against purified fungal cell walls (de la Cruz et al., 1992).

In this work, we report on the production of tobacco plants overexpressing the Trichoderma endochitinase-encoding gene *chit33*, alone or in combination with gene *chit42*, and on the evaluation of their tolerance to a broad range of stress agents. The overexpression of *chit33* in tobacco plants not only significantly enhances their tolerance to fungal and bacterial pathogens, but also their resistance to saline stress and high concentrations of heavy metals in the culture medium. We have confirmed previous reports of the enhanced tolerance of *chit42*-overexpressing plants to fungal pathogens and determined their tolerance to bacterial pathogens and abiotic stresses. Contrary to what has been reported in in vitro experiments with purified proteins, no synergistic effects of CHIT42 and CHIT33 have been observed in planta. The phenotype of the chitinase-overexpressing plants is morphologically indistinguishable from that of control lines with regard to biomass production, fertility, and seed viability. Although some reports exist regarding concomitant enhanced tolerance to biotic and abiotic stress in transgenic plants overexpressing stress response plant determinants, this report outlines broad range effects achieved by the overexpression of a single fungal gene in plants.

RESULTS

Ectopic Expression of the Trichoderma *chit33* Gene in Tobacco

To evaluate the defense potential of endochitinase CHIT33 from *T. harzianum* strain CECT2413, transgenic tobacco plants that expressed the *chit33* gene under the control of the cauliflower mosaic virus 35S promoter were produced. To mediate the export of the protein to the intercellular space, the nucleotide sequence of *chit33* encoding its signal peptide was substituted by that of the tomato (*Lycopersicon esculentum*) pathogenesis-related (PR) protein P1-p14 (Tornero et al., 1994). Nineteen independent tobacco lines harboring the transgene were obtained, and F3 homozygous plants from the five transgenic lines that showed the highest *chit33* mRNA accumulation (Fig. 1A) were selected for more extensive functional characterization. The CHIT33 protein was detected by western-blot analysis, and it proved to be identical in size to that produced by Trichoderma, which indicated correct posttranslational processing (Fig. 1B). To assess the functionality of the protein in the transgenic lines, chitinase activity of the total plant protein extracts was quantified by fluorescence assays, using the specific substrate 4-methylumbelliferyl-b-D-*N*,*N*′,*N*″,*N*‴-tetraacetylchitotetraoside [4-MU-(GINAc)4] (Fig. 1C). The chitinase activity of each transgenic line correlated with its levels of mRNA *chit33* accumulation and of CHIT33 production, whereas that of control plants was almost undetectable. Thus, it can be concluded that the chitinase activity detected in the transgenic *chit33* lines is a consequence of *chit33* overexpression and responds to its own lytic activity, either alone or in synergistic combination with plant chitinases. No visible phenotypic alteration was detected in transgenic *Pseudomonas syringae pschit33* plants grown in soil under greenhouse conditions (Fig. 1D).

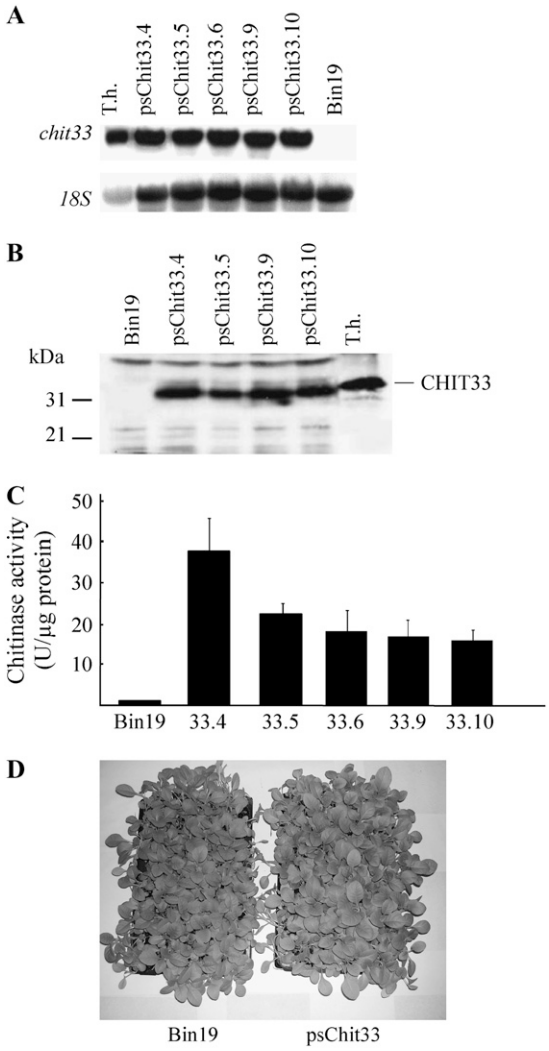

Figure 1. Molecular analysis of representative *pschit33* transgenic plants. A, Northern analysis of tobacco *pschit33* transgenic lines (33.4, 33.5, 33.6, 33.9, 33.10) and control line (*Bin19*) grown in standard conditions. As a positive control, total RNA of *T. harzianum* (T.h.) grown in *chit33*-inducing conditions was used (Dana et al., 2001). B, Western analysis of total proteins from leaf extracts of control and transgenic tobacco lines. As a positive control, culture supernatant of T.h. grown in *chit33*-inducing conditions was used. C, Specific chitinase activity in protein-soluble extracts of control and transgenic tobacco lines. 1 U = 1 nmol of 4-methylumbelliferone released per minute. D, *Bin19* and *pschit33* plants grown for 5 weeks.

Overproduction of *chit33* Promotes Disease Resistance of Transgenic Plants to Soil-Borne Pathogens

Transgenic *chit33* tobacco plants were tested for resistance to *R. solani*, an endemic soil-borne pathogen that causes camping-off, seedling blight, and root rot. In infection assays on agar-water plates, the survival rate of the transgenic *chit33* plants to Rhizoctonia reached 81%, whereas that of the control plants was 39.6%, a statistically significant difference according to one-way ANOVA (Table I). It has been reported that overexpression in tobacco transgenic lines of a *T. harzianum* gene (*chit42*) encoding the endochitinase CHIT42 also improves plant resistance to the same pathogen (Lorito et al., 1998), albeit to a lesser extent. Previous studies have shown that, in some cases, simultaneous expression of different proteins involved in mycoparasitism enhances the resistance of transgenic tobacco lines to fungal pathogens. Trichoderma *chit33* and *chit42* show synergistic chitinolytic activity in in vitro assays against purified fungal cell walls (de la Cruz et al., 1992). Hybrid lines *chit33* × *42* were obtained by crossing the transgenic *chit33* and *chit42* lines that showed the strongest constitutive expression of each gene; their level of resistance to *R. solani* was compared with that of the parental *chit33* and *chit42* lines. The level of resistance conferred by the combined expression of the two transgenes was slightly higher than that of the *chit42* parent, and considerably lower than that of *chit33* plants, exemplified by the *chit33* parental line (Table I).

The protective effect of CHIT33 against pathogenic bacteria was also investigated. *chit33*, *chit42*, and *chit33* × *42* transgenic plants were infiltrated with *P. syringae* pv *tabaci* 153, and necrosis was recorded 5 d after inoculation. The necrotic symptoms, defined by the size and density of necrotic lesions in the foliar tissue, were greatly reduced in the *pschit33* plants when compared with the controls. A similar reduction of pathogenic effects was observed in *pschit42* and *pschit33* × *42* transgenic plants (Fig. 2A). Proliferation of *P. syringae* in the infiltrated plants was determined as colony forming units (cfu)/cm^2 of infected leaves. Five days after treatment, the bacterial cell densities in control plants exceeded those in the *pschit* plants 2- to 10-fold. The *pschit33* plants were those that showed maximal inhibition of *P. syringae* growth in the foliar tissue (Fig. 2B). Thus, the reduction of disease symptoms seems to be associated with the inhibition of bacterial proliferation in the transgenic *pschit* plants. These results support the hypothesis that chitin hydrolysis is not the primary mechanism that causes the protective effect of *pschit33* and *pschit42* against plant pathogens.

Table I. *Resistance of pschit plants to R. solani*

Line	Survival Rate
	%
Bin19	39.62 ± 3.37
pschit33	81.00 ± 1.70
pschit42	56.77 ± 8.50
pschit33 × *42*	64.58 ± 4.33

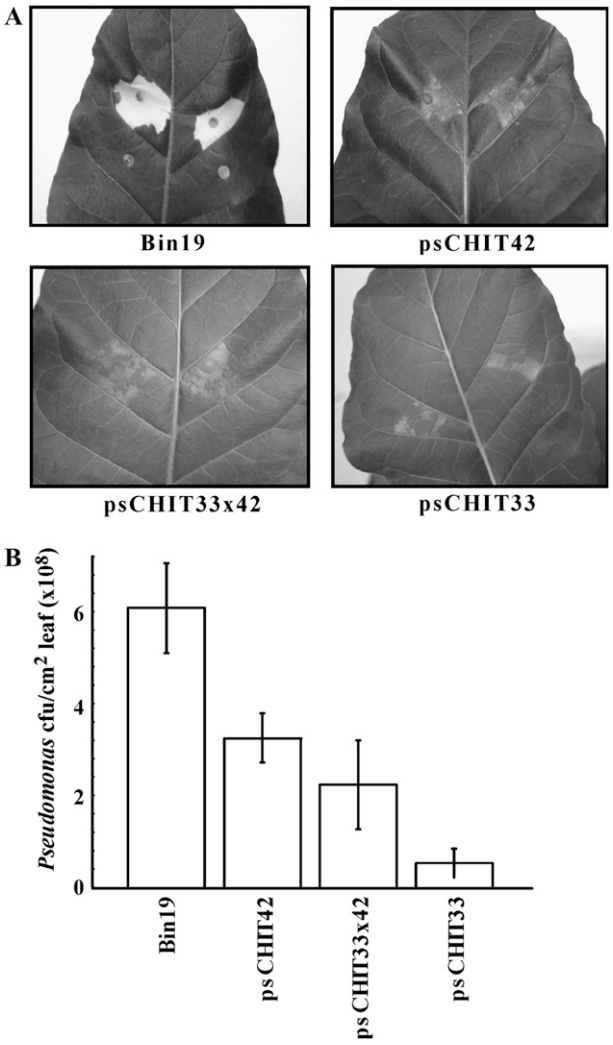

Figure 2. Disease resistance against the pathogenic bacteria *P. syringae* pv *tabaci* in *pschit* transgenic tobacco lines. A, Tobacco leaves showing disease symptoms (necrotic areas) 5 d after infection. B, Growth of Pseudomonas in transgenic *pschit* lines. Fully expanded leaves of 8-week-old tobacco plants were inoculated with 0.5 to 1 × 10^6 cfu/mL of *P. syringae*. Five days after inoculation, the infected leaves were collected and the bacterial populations determined. Each experiment was carried out with, respectively, two, three, three, and four independent *Bin19*, *pschit42*, *pschit33* × *42*, and *pschit33* lines. Values are the average results from two experiments, and three independent replicas were performed for each experiment.

Chitinase Transgenic Plants Show Increased Accumulation of Peroxidase Activity

Reactive oxygen species (ROS) are synthesized as signal molecules during the process of plant response to biotic and abiotic stresses (Chamnongpol et al., 1998; Mittler and Rizhsky, 2000; Fath et al., 2002; Neill

et al., 2002). To prevent the toxic effect of excess of ROS in plant cells, scavenging mechanisms exist, involving the enhanced enzymatic activity of superoxide dismutase, catalase, plasma membrane-bound NADP oxidases, cytoplasmic and cell wall-bound peroxidases, and amine oxidases in the apoplast (Wojtaszek, 1997; Yoshida et al., 2003). These enzymes produce reactive oxygen intermediates (ROIs), which can also act as signal molecules and play a central role in the defense of plants against pathogen attacks. Anionic peroxidases are involved in the production of ROIs; these act as precursors of lignin and suberin, thus playing a dual role—that of keeping the ROS concentrations at nontoxic levels and also contributing to the reinforcement of the plant cell wall. Previous studies showed that tobacco cell wall-bound anionic peroxidases are induced after pathogen attack and their activity is not inhibited by salicylic acid, as is the case for other ROS-scavenging enzymes, such as ascorbate peroxidase or catalase (Bi et al., 1995; Durner and Klessig, 1995; Takahashi et al., 1997; Mittler and Rizhsky, 2000). The anionic peroxidase activity of *pschit33*, *pschit42*, and *pschit33* × *42* transgenic plants was determined and found to be significantly higher than that of the control plants in standard growth conditions. As expected, 24 h after infection with Pseudomonas, the levels of peroxidase activity were higher in all lines tested, including control plants, but the differences among lines were maintained (Fig. 3). The enhanced basal and pathogen-induced peroxidase activity of *pschit* plants correlates with their improved resistance to fungal and bacterial pathogens. It is worth noting

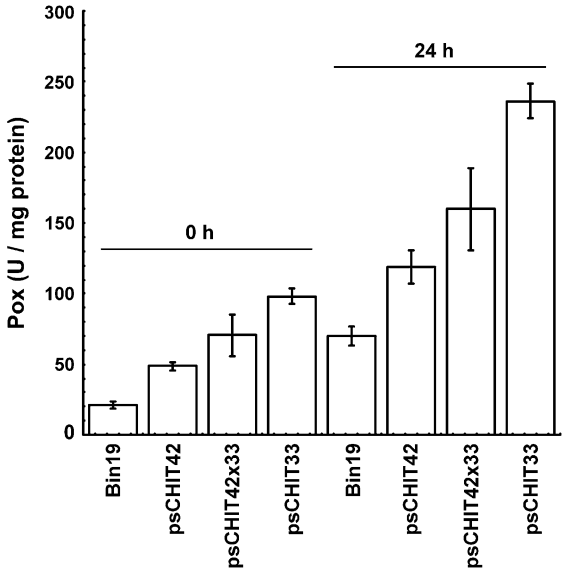

Figure 3. Peroxidase activity of *pschit* plants. Fully expanded leaves of 8-week-old tobacco plants were infiltrated with a suspension of *P. syringae* pv *tabaci* and collected 24 h after infection. The results shown are the mean of three independent experiments; and the activity measurements were made, respectively, on two, three, three, and four independent *Bin19*, *pschit42*, *pschit33* × *42*, and *pschit33* lines.

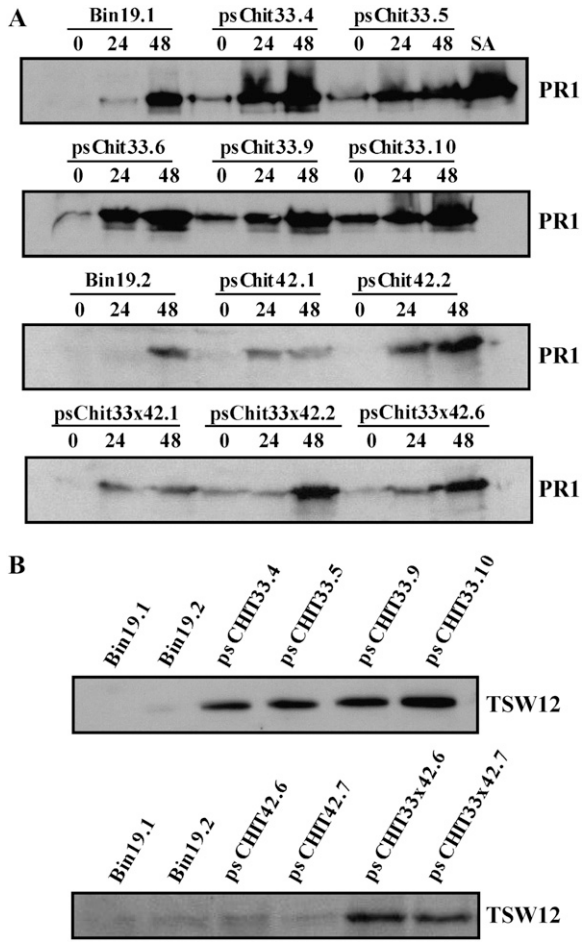

Figure 4. Enhanced defense-related protein expression in *pschit* transgenic lines. Western analysis of total proteins from leaf extracts of control and transgenic tobacco lines showing the expression levels of PR1 (A) and TSW12 (B). As a positive control, total proteins of salicylic acid-treated wild-type leaves (SA) were used.

that no undesirable effects on root growth and development were observed in the *pschit* lines, as was previously reported for transgenic tobacco lines overexpressing anionic peroxidase (Lagrimini et al., 1997).

Enhanced Expression of Pathogen-Response Proteins

One of the most utilized markers of plant response to pathogenic challenge is PR-1a, a protein of unknown function whose levels under standard conditions are very low and show a very substantial increase in infected plants (Kim et al., 2001). The levels of PR-1a in lines *pschit33*, *pschit42*, and *pschit33* × *42* were considerably higher than those of control plants, both in nonchallenging conditions and after infection with Pseudomonas, and correlated with those shown for peroxidase activity (Fig. 4A). Analysis of the expression of other PR proteins failed to show any increase in the levels of PR-2, PR-3, PR-4, and PR-5 with regard to those of control plants. Analysis of the expression of

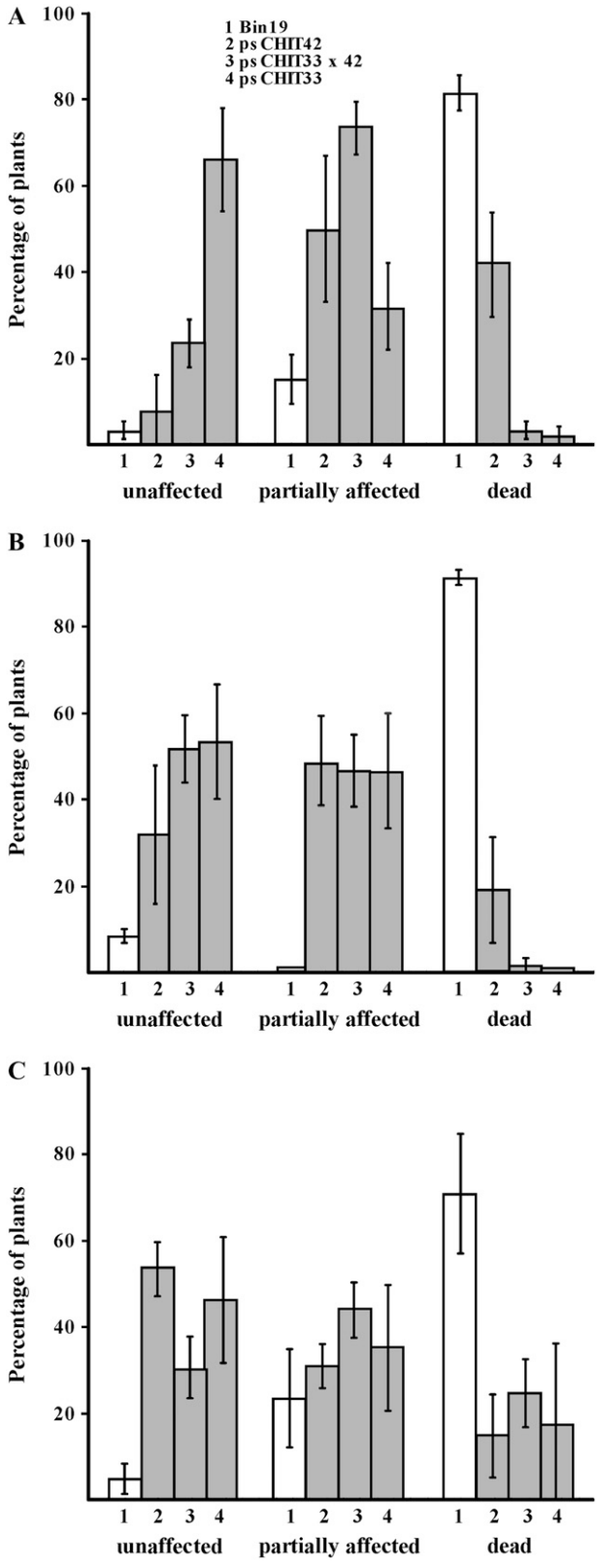

Figure 5. Abiotic stress tolerance of fungal chitinase-overexpressing plants. A, Salt stress tolerance. Seven-day-old seedlings grown in one-half-strength Murashige and Skoog basal salt medium were transferred to agar-water plates containing 256 mM NaCl. The effects of salt on plant growth and viability after 7 d at 25°C and continuous light allow the division of plant populations of each of the transgenic lines tested into three categories: unaffected plants (no visible effects, sustained growth, and viability), partially affected plants (deleterious effects, marked by growth arrest, and chlorosis symptoms), and dead plants. B and C, Cd and Cu tolerance. The assays were made as in A, substituting NaCl for 300 μM $CdSO_4$ (B) and 300 μM $CuSO_4$ (C) in the selective plates.

other abiotic stress-related defense proteins in basal conditions showed enhanced expression in plants *pschit33* of the protein TSW12, a nonspecific lipid transfer protein (Torres-Schumann et al., 1992; Fig. 4B). None of the *pschit* transgenic lines exhibited spontaneous necrotic lesions, which is a common phenotype for PR-overexpressing mutant or transgenic lines (Mittler and Rizhsky, 2000; Anand et al., 2003; Nishizawa et al., 2003).

Abiotic Stress Tolerance of Fungal Chitinase-Overexpressing Plants

The response of plants to different stresses constitutes a network of interconnected signaling pathways. This is partly due to the fact that the physiological disorders triggered by different stresses might require overlapping protective responses. For example, cold and drought stresses elicit a common array of dehydration response element-responsive genes, and a variety of scavenging enzymes that protect against oxidative damage are part of the plant response to different biological and abiotic stresses, such as fungal pathogen attack and salinity.

Among other deleterious effects within the plant cell, salt stress causes oxidative damage by enhanced production of ROS (Xiong and Zhu, 2002). Heavy metals such as copper (Cu), mercury (Hg), and cadmium (Cd) also induce the formation and accumulation of ROS in cells (Briat and Lebrun, 1999; Schutzendubel et al., 2001). The tolerance of *pschit33*, *pschit42*, and *pschit33* × *42* lines to toxic concentrations of salt, Cu, and Cd was evaluated in in vitro assays in conditions that allowed a low, but reproducible, survival rate of the control plants, thus establishing a quantifiable gradient from lethality to survival, and grouping the intermediate states as deleterious effects (Fig. 5). Among the lines tested, *pschit33* plants showed the highest survival rate and the lowest rate of stress symptoms and/or death when subjected to salt stress (Fig. 5A). Lines *pschit33* × *42* showed similarly low death rates, but, among the surviving plants, a majority presented some symptoms of stress in the form of growth arrest and/or chlorosis. Lines *pschit42* showed moderately higher death rates than *pschit33* and *pschit33* × *42*, albeit significantly lower than those of control plants. As is the case for *pschit33* × *42* plants, most of the surviving *pschit42* plants showed stress symptoms (Fig. 5A). Similar results were obtained when estimating the tolerance of the *pschit* lines to Cu (Fig. 5B) and Cd (Fig. 5C), although in these two

conditions the survival rates and incidence of stress symptoms were similar for all the transgenic *pschit* lines tested and significantly different from that of control plants. These results confirm that the expression of heterologous chitinases in tobacco plants causes not only a significant increase in resistance to fungal and bacterial pathogens, but also a concomitant tolerance to abiotic stress caused by high concentrations of salt and metal ions in the growth substrate. The differences in the degree of resistance to biotic stress exhibited by the different *pschit* lines can also be observed with regard to salt stress response. In all cases, the highest levels of salinity tolerance were obtained by overexpressing the Trichoderma gene *chit33*.

DISCUSSION

Chitinases belong to the repertoire of plant defense systems and are nontoxic to plants and higher vertebrates. Hence, the many reported attempts to enhance plant protection against pathogens by homologous and heterologous overexpression of plant chitinases. However, in most cases, the increase of resistance achieved by such a strategy has turned out to be effective within a narrow range of pathogens and is quantitatively modest, leading to the need for using gene combinations to achieve significant levels of plant tolerance.

Here we report the generation of transgenic tobacco plants overexpressing singly, or in combination, two endochitinases from the mycoparasitic fungus *T. harzianum*. Previous work in our laboratory had shown enhanced tolerance to a wide range of soil-borne and foliar fungal pathogens exhibited by tobacco *pschit42* plants overexpressing the Trichoderma endochitinase CHIT42 (Garcia et al., 1994; Lorito et al., 1998). To ensure its correct processing and secretion to the apoplast, the fungal hydrolase was modified by substituting its native signal peptide with a signal peptide of plant origin (Tornero et al., 1994). From the wide array of hydrolases produced by Trichoderma during mycoparasitic interactions, CHIT33 shows significant sequence homology with some defense-related class III plant chitinases. CHIT33 exhibits antifungal activity and synergistic lytic properties with CHIT42 in in vitro assays (de la Cruz et al., 1992; Dana et al., 2001) and its overexpression increases the mycoparasitic activity of transgenic *T. harzianum* strains (Limón et al., 1999). The *chit33* gene was modified in the same way described previously for *chit42* (Lorito et al., 1998) and transgenic *pschit33* tobacco lines were generated.

Five independent F3 homozygous *pschit33* lines were tested and showed significantly enhanced resistance to both fungal (*R. solani*) and bacterial (*P. syringae*) pathogens. Transgenic homozygous *pschit42* lines also exhibited improved resistance to both pathogens, although to a lesser degree, thus confirming previously reported data on their molecular and physiological characterization (Lorito et al., 1998) and providing new insights on how the defense mechanisms of the *pschit* plants are modulated by the presence of fungal chitinases. No synergistic effect was observed in the *pschit33* × *42* lines tested, their level of resistance being intermediate to that of their parental lines. The intermediate level of resistance achieved by the *pschit33* × *42* plants could be interpreted as the result of the competition and/or titration exerted by the less proficient chitinase on the activity of the more active one in any process related to its physiological effects in transgenic plants. Our results suggest that the improved tolerance against pathogens observed in the *pschit* plants is not the sole consequence of their enhanced chitinolytic activity and that it is very likely that other defense-related mechanisms are being triggered by the presence of either chitinase in the apoplast. Several lines of evidence support this hypothesis. The levels of PR-1a, but not those of PR-2, PR-3, PR-4, and PR-5, are significantly higher in the transgenic *pschit* lines, both in basal conditions and in plant-pathogen interaction assays. The systemic induction of PR proteins occurs upon stress, pathogen attack, and abiotic stimuli and it is usually accompanied by the development of local and/or systemic resistance to biotic or abiotic challenges. The PR protein expression profile helps to define different physiological responses to environmental challenges (Ryals et al., 1996; Thomma et al., 2001; Hammond-Kosack and Parker, 2003). One of the better-studied plant defense mechanisms is the salicylic acid-dependent pathway involved in systemic acquired resistance, which causes the concomitant activation of PR-1, PR-2, and PR-5. Induced systemic resistance responses dependent on the jasmonic acid pathway enhance expression of another set of PR proteins, PR-3, PR-4, and PR-12. The overexpression in transgenic plants of many components of the plant defense-signaling pathways, such as mitogen-activated protein kinases, transcription factors, and PR chitinases and glucanases, also leads to the activation of PR-1 expression in basal and induced conditions (Cao et al., 1998; Anand et al., 2003; Xiong and Yang, 2003; Park et al., 2004; Luo et al., 2005). Although the function of PR-1 is still unknown, its role in the plant mechanisms of defense against diseases is evident as its level of expression correlates with physiological states of plant-pathogen incompatible interactions, abiotic stress responses, and systemic resistance-like responses.

Cell wall-associated anionic peroxidase activity was considerably higher in the transgenic *pschit* lines than in control plants, both in standard growth conditions and after Pseudomonas infection. Peroxidase has often been used as an enzymatic marker in studies of defense-related processes (Young et al., 1995). Pathogen recognition by plant cells leads to the production of a variety of ROS that can act as second messengers and activate, in turn, various defense-related genes (Orozco-Cardenas et al., 2001; Bolwell et al., 2002), as

well as participating in the strengthening of the cell wall through callose deposition and wall-bound phenolics, suberification, and lignification. The enhanced peroxidase activity shown by the *pschit* plants can thus be linked to their increased pathogen resistance and to the significant diminution of necrotic symptoms that they exhibit after Pseudomonas infection. It can also account, at least partially, for the abiotic stress resistance observed in *pschit* plants by counteracting the oxidative stress resulting from salt and heavy metals, and also by favoring water retention in the cell walls, as reported by Amaya et al. (1999) for transgenic tomato lines overexpressing the peroxidase TPX2. Additionally, ROIs produced by the action of anionic peroxidase can trigger other abiotic stress-specific signaling pathways. It is worth noting that lines overexpressing *pschit33* and not those overexpressing *pschit42* also showed enhanced expression of TSW12, a member of the nonspecific lipid transfer protein family that is induced upon salt and abscisic acid treatment (Torres-Schumann et al., 1992; Molina and Garcia-Olmedo, 1997).

The enhanced tolerance of *pschit* plants to different stresses could be a result of the liberation of cell wall or apoplastic glycoprotein-derived oligomers due to the action of either of the chitinases, which would act as elicitors, triggering one or more defense-signaling pathways leading to a systemic acquired resistance-like state. It has been reported that apoplastic and cell wall-bound arabinogalactan proteins from carrot (*Daucus carota*) can contain detectable amounts of glucosamine and *N*-acetyl-glucosaminyl and are sensitive to endochitinase cleavage (van Hengel et al., 2001). Several oligosaccharines of plant origin also contain *N*-acetyl-glucosaminyl units and are thought to be involved in processes of biological relevance, such as modulation of flax (*Linum usitatissimum*) seedling growth and promotion of tomato fruit ripening. Although the biosynthetic origin of these oligomers is not well established, there is some evidence that they might derive from partial hydrolysis of apoplastic *N*-linked glycoproteins (Fry et al., 1993). The protein CHIT33, which shares 42% identity with plant chitinases (Limón et al., 1995), could additionally or alternatively act as an elicitor by itself, triggering at least one more mechanism of response exemplified by the overexpression of TSW12.

The results presented in this work identify *T. harzianum* endochitinases CHIT33 and CHIT42 as physiological determinants capable of generating innate defense responses and enhanced stress tolerance in tobacco transgenic plants without detectable morphological or physiological undesirable side effects.

MATERIALS AND METHODS

Constructs and Plant Transformation

The endochitinase-encoding full-length cDNA *chit33* from *Trichoderma harzianum* strain CECT2431 was modified by substituting its amino-terminal signal peptide (Limón et al., 1995) with the signal peptide of the tomato (*Lycopersicon esculentum*) PR protein P1-p14 (Tornero et al., 1994). The chimeric *ps*::*chit33* gene thus obtained was then placed under the control of the cauliflower mosaic virus 35S subunit and the nopaline synthase terminator in the pBIN19 vector. *Agrobacterium tumefaciens* strain LBA4404 containing the construction pBIN19::pschit33 was used to transform leaf discs of tobacco (*Nicotiana tabacum* var *Xhanti*) following standard protocols (Horsch et al., 1985). Nineteen independent kanamycin-resistant *pschit33* tobacco lines were obtained, and five of them were selected for further characterization. Transgenic F3 homozygous plants harboring single-copy integrations of the *chit33* or *chit42* transgenes were used for biotic and abiotic functional analysis. Transgenic *pschit42* × *chit33* plants, harboring *chit42* and *chit33* genes from *T. harzianum*, were obtained by crossing lines *pschit42* (Lorito et al., 1998) and *pschit33.5*. Line *pschit33.5* acted as the female parent. Four F1 hybrid lines, showing constitutive *chit42* and *chit33* expression, were selected for further analyses.

Molecular Analyses of Transgenic Tobacco Lines

Transgenic plants were propagated on Murashige and Skoog basal salt medium (Sigma) containing 3% Suc and 100 mg/L kanamycin. The presence of the transgenes was detected by PCR amplification using primers 5′-GCCATGCCTTCATTGACTGCTC-3′ (C35) and 5′-CCTCAAAGCATTGACAACCTG-3′ (C33) that amplified the entire open reading frame of the *chit33* gene, and primers 5′-GGTTATGCTTTCCATCGG-3′ (EC1) and 5′-CAAGGAGTCAGAGCCAGTCTT-3′ (BB2), which annealed, respectively, at positions 566 and 1,367 from the ATG of the *chit42* gene. Northern analyses were performed following standard procedures (Sambrook et al., 1989), using the complete open reading frames of genes *chit33*, *chit42*, and the *18S* RNA gene of carrot (*Daucus carota*) as probes on 10 μg of total plant RNA. Probes were labeled with the random primed DNA labeling kit (Amersham), following the manufacturer's instructions. Polyclonal rabbit antibodies raised against GST::CHIT33, GST::CHIT42, and PR-1a and TSW12 were used in western-blot assays of total protein extracts from transgenic plants (Torres-Schumann et al., 1992; Garcia et al., 1994; Limón et al., 1995). Protein transfer to nitrocellulose membranes was carried out in a trans-blot semidry transfer cell (Bio-Rad) following the manufacturer's instructions. Protein immunodetection was performed using a secondary antibody conjugated to horseradish peroxidase and the ECL western-blotting analysis system (Amersham).

Enzymatic Assays

Chitinase Activity Assay

Chitinase activity was determined by using the fluorescent-specific substrate [4-MU-(GlNAc)4] (Sigma) as described (Limón et al., 1995). Assay mixes (100 μL) containing 1 μg of total protein extract and 250 μM [4-MU-(GlNAc)4] in 100 mM sodium citrate buffer, pH 3.0, were incubated for 15 min at 30°C in the dark. The reactions were stopped with 2.9 mL of 0.5 M Gly-NaOH buffer, pH 10.4, and fluorescence was measured in a Hoefer TK0100 fluorimeter at 350-nm excitation and 440-nm emission wavelengths. The chitinase activity was expressed as picomoles of 4-methylumbelliferone liberated per minute and micrograms of protein.

Peroxidase Assay

For determination of peroxidase activity, 1 cm^2 of foliar tissue was homogenized in 50 mM phosphate buffer, pH 6.0 (1:6 [w/v]). The homogenate was centrifuged at 13,000*g* for 1 min and protein from the supernatant was used for the assays. Assay mixes (500 μL) contained 0.5 to 1.0 μg of protein, 6 mM guaiacol, and 6 mM hydrogen peroxide in 50 mM sodium acetate buffer, pH 4.5. The reactions were incubated for 10 min and OD_{470} was measured.

Bacterial Infection and Pseudomonas Resistance Assays

Fully expanded leaves of 8-week-old tobacco plants were inoculated with *Pseudomonas syringae* pv *tabaci* 153 according to Thilmony et al. (1995). Each leaf was infiltrated in six points symmetrical with regard to the central nerve with 50 μL of cold bacterial suspension (0.5–1 × 10^6 cfu/mL 10 mM $MgSO_4$). Mock-infected plants were infiltrated with the same volume of cold 10 mM $MgSO_4$ solution. Pathogen and mock-infected plants were kept at 25°C under 80% relative humidity and 16 h of diurnal light. Twenty-four hours after

infection, leaves were sampled and analyzed for peroxidase activity (Polle et al., 1994) and for expression of PR proteins. Necrotic areas were recorded 5 d after infection. To determine bacterial growth in planta, bacteria were extracted 5 d after infiltration from 1 cm^2 discs of leaf-infected areas and plated on King's B medium (Martin et al., 1993).

Rhizoctonia Resistance Assays

Mycelium of *Rhizoctonia solani* pv *tabaci* 153 was grown in potato (*Solanum tuberosum*) dextrose broth at 25°C, 150 rpm, for 4 to 5 d, and harvested, weighed, and homogenized in sterile water. Two-week-old tobacco seedlings, germinated on Murashige and Skoog medium, were transferred to water-agar plates (0.7% [w/v]) containing 0.75 g/L of Rhizoctonia mycelium. After 7 d at 25°C and continuous light conditions, survival rates of transgenic and control plants were estimated.

Abiotic Stress Assays

Transgenic tobacco lines were assayed for resistance against saline stress and heavy metals. Each assay was carried out with 2-week-old seedlings (60 per line and per assay; three independent replicas), germinated on Murashige and Skoog medium. Seedlings were transferred to water-agar plates (0.7% [w/v]) containing 256 mM NaCl (saline stress), 300 μM $CdSO_4$, or 300 μM $CuSO_4$. After 7 d at 25°C and continuous light conditions, the effect of each stress-producing agent on transgenic and control plants was estimated by classifying the plant populations of each of the transgenic lines tested into three categories: Unaffected plants (no visible effects, sustained growth, and viability), partially affected plants (deleterious effects, marked by growth arrest and chlorosis symptoms), and dead plants.

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