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Pathogen-Induced Elicitin Production in Transgenic Tobacco Generates a Hypersensitive Response and Nonspecific Disease Resistance

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The rapid and effective activation of disease resistance responses is essential for plant defense against pathogen attack. These responses are initiated when pathogen-derived molecules (elicitors) are recognized by the host. We have developed a strategy for creating novel disease resistance traits whereby transgenic plants respond to infection by a virulent pathogen with the production of an elicitor. To this end, we generated transgenic tobacco plants harboring a fusion between the pathogen-inducible tobacco *hsr203J* gene promoter and a *Phytophthora cryptogea* gene encoding the highly active elicitor cryptogein. Under noninduced conditions, the transgene was silent, and no cryptogein could be detected in the transgenic plants. In contrast, infection by the virulent fungus *P. parasitica* var *nicotianae* stimulated cryptogein production that coincided with the fast induction of several defense genes at and around the infection sites. Induced elicitor production resulted in a localized necrosis that resembled a *P. cryptogea*–induced hypersensitive response and that restricted further growth of the pathogen. The transgenic plants displayed enhanced resistance to fungal pathogens that were unrelated to *Phytophthora* species, such as *Thielaviopsis basicola*, *Erysiphe cichoracearum*, and *Botrytis cinerea.* Thus, broad-spectrum disease resistance of a plant can be generated without the constitutive synthesis of a transgene product.

INTRODUCTION

As a result of host–pathogen coevolution, plants have developed sophisticated mechanisms to protect themselves from disease. Besides preformed physical and chemical barriers that hinder infection, a wide variety of defense responses is induced only after pathogen attack (Kombrink and Somssich, 1995). When these induced responses are triggered rapidly and coordinately during a given plant–pathogen interaction, the plant is resistant to disease. A susceptible plant responds more slowly with an onset of defense mechanisms after infection. Thus, the timely recognition of an invading microorganism and the rapid and effective induction of defense responses appear to make a key difference between resistant and susceptible plants (Yang et al., 1997).

The activation of resistance in plants is initiated by host recognition of molecules called elicitors, which are directly or indirectly released from an invading pathogen. Although elicitors vary widely in their chemical composition (Ebel and Cosio, 1994), and the mechanisms by which plants perceive them may differ (Nürnberger et al., 1994; Scofield et al., 1996; Tang et al., 1996), many pathogen elicitors appear to trigger a common network of signaling pathways that coordinate the overall defense responses of plants. The induced mechanisms frequently manifest themselves as a hypersensitive response (HR), which is characterized by necrotic lesions resulting from localized host cell death at the site of infection. The HR prevents growth and the spread of the pathogen into healthy tissues (Goodman and Novacky, 1996).

Exploitation of the HR by genetic means might be a major strategy for creating novel disease resistance traits. Constitutive induction of an HR has been achieved in mutants of Arabidopsis and barley and has been shown to coincide with elevated pathogen resistance (Dietrich et al., 1994; Greenberg et al., 1994; Büschges et al., 1997). However, the formation of an HR must be tightly regulated to avoid uncontrolled cell death throughout the plant. In this context, de Wit (1992) suggested generating transgenic plants that

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produce an elicitor in response to infection by a virulent pathogen. The strategy is predicted on the assumption that the elicitor is recognized by the plants' own perception system and that a virulent microorganism thus triggers an HR. Nevertheless, successful arrest of pathogen growth without damage to the transgenic plant requires that the transgene be expressed rapidly and locally. Because constitutive elicitor production can be lethal to a plant (Hammond-Kosack et al., 1994; Honée et al., 1995), transgene activation should occur only at the time of pathogen challenge and not under other circumstances. Thus, the strategy described above requires a gene encoding a highly active protein elicitor and a functional promoter that is specifically inducible by a virulent pathogen.

When this genetic engineering approach is applied to tobacco, a candidate elicitor is cryptogein. Cryptogein is a basic 10-kD holoprotein that is secreted by the hemibiotrophic oomycete plant pathogen *Phytophthora cryptogea* (Ricci et al., 1989). Cryptogein belongs to a family of homologous proteinaceous elicitors, termed elicitins, that are secreted by all *Phytophthora* species analyzed thus far (Ricci, 1997). In *P. parasitica*, elicitin production correlates with avirulence on tobacco, and only the specialized tobacco pathogen *P. parasitica* var *nicotianae*, causal agent of tobacco black shank disease, does not produce these proteins (Ricci et al., 1989). Therefore, elicitins are thought to be major determinants of the basic resistance of tobacco against *Phytophthora* spp (Yu, 1995). Recent experiments indicate that they may act as avirulence factors in the interaction between this fungus and plants from the genus *Nicotiana* (Kamoun et al., 1998). The application of nanomolar concentrations of purified cryptogein to tobacco leaves induces an array of responses that characterize the HR of this plant, including the transcriptional activation of defense-related genes, the synthesis of antimicrobial phytoalexins, and, eventually, hypersensitive cell death leading to tissue collapse (Ricci, 1997). The induction of the HR by cryptogein is mediated by protein recognition of specific, high-affinity binding sites that are associated with the plasma membrane of tobacco cells (Wendehenne et al., 1995).

Our goal was to generate transgenic tobacco plants that produce cryptogein upon challenge with virulent *P. p.* var *nicotianae.* The plant promoter that appeared to be the most suitable for this approach coordinates expression of the defense-related gene *hsr203J.* This gene was isolated from tobacco, and its putative product shows no homology with any known proteins (Marco et al., 1990). *hsr203J* expression occurs rapidly and locally when tobacco leaves are inoculated with the phytopathogenic bacterium *Ralstonia solanacearum* (Pontier et al., 1994). The gene promoter is neither developmentally nor tissue specifically regulated and does not respond to environmental stimuli or to endogenous plant signal molecules that are potentially pathogen induced (Pontier et al., 1998). Here, we show that the promoter is activated in tobacco during interaction with *P. p.* var *nicotianae*. Transgenic tobacco plants harboring a fusion between the *hsr203J* promoter and the cryptogein-encoding *X24* gene from *P. cryptogea* developed an HR in a normally compatible interaction with *P. p.* var *nicotianae.* This resistance response appeared to require timely and spatially coordinated activation of defense mechanisms. The resistance that we engineered was not organ specific, and transgenic plants showed enhanced protection to fungal pathogens that are unrelated to *Phytophthora* species. The possible applications of the presented strategy for breeding purposes are discussed.

RESULTS

The *hsr203J* Promoter Is Activated in Different Organs of Tobacco Plants Infected with *P. p.* var *nicotianae*

Under appropiate environmental conditions, disease initiation by *P. p.* var *nicotianae* can occur on the leaves or, most frequently, via the roots. Hyphae of the fungus can invade all tissues, growing both intercellularly and intracellularly, and also can penetrate the cells at any point. To analyze whether infection by this virulent fungus stimulates the *hsr203J* promoter in leaves and roots, we inoculated tobacco plants carrying a fusion between this promoter and the *uid*A reporter gene from *Escherichia coli* (Pontier et al., 1994) with suspensions of fungal zoospores. In response to leaf infiltration with 5000 spores per mL of *P. p.* var *nicotianae*, β-glucuronidase (GUS) activity inside the infiltrated areas started to increase 8 hr after inoculation and reached maximum activity 16 to 24 hr after inoculation (Figure 1A). The subsequent decrease in GUS activity was due to tissue collapse in the infiltrated area that resulted from heavy infestation with fungal mycelia. Root inoculation by the application of 20,000 spores per mL to the soil led to a continuous increase in GUS activity starting 16 to 24 hr after inoculation (Figure 1B). At this time point, the first symptoms of root penetration appeared. Later on, increasing GUS activity followed the spread of fungal mycelia in the roots.

The reporter gene was not significantly induced in leaves or roots when mock inoculated. Although the *hsr203J* promoter is strongly activated in tobacco by HR-inducing bacterial and viral pathogens as well as by cell death–inducing elicitors (Pontier et al., 1998), no significant induction was detected after inoculation with the avirulent, HR-inducing fungus *P. cryptogea.* The observed activation of the *hsr203J* promoter by *P. p.* var *nicotianae* was lower than the reported activation by the avirulent isolate GMI1000 of *R. solanacearum* (Pontier et al., 1994). However, we assumed that the responsiveness to *P. p.* var *nicotianae* infection would drive production of cryptogein in sufficient quantities to trigger the full defense response in transgenic tobacco.

For functional interaction with the membrane-bound binding sites within transgenic plants, cryptogein must be exported to the outside of the cell. We designed a chimeric

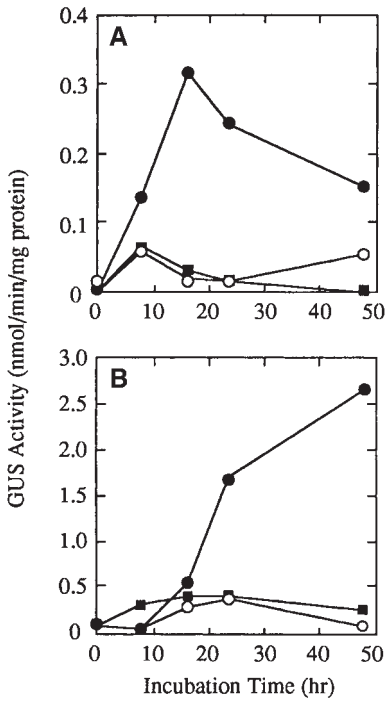

Figure 1. Induction of the *hsr203J* Gene Promoter in Response to Infection by *P. p.* var *nicotianae* and *P. cryptogea.*

Activity of the *uid*A reporter gene product (GUS) in protein extracts (Jefferson et al., 1987) was measured at different time points after mock inoculation (closed squares) of tobacco line pHG21-14A (Pontier et al., 1994) or after inoculation with virulent *P. p.* var *nicotianae* (closed circles) or avirulent *P. cryptogea* (open circles). *P. p.* var *nicotianae* is the causal agent of the tobacco black shank disease. Tobacco is a nonhost for *P. cryptogea*, and inoculations with this fungus generate an HR. The results are from a representative experiment among several repeats, which varied in absolute levels of GUS activities but gave the same trends for promoter activation.

(A) Leaves were infiltrated with water or a solution containing 5000 zoospores per mL, and GUS activity was determined inside the dissected, infiltrated area.

(B) Roots were inoculated by applying a solution of 20,000 zoospores per mL to the soil, and GUS activity was determined in whole-root protein extracts.

construct in which the region of the *P. cryptogea* gene *X24* (Panabières et al., 1995) encoding mature cryptogein was fused to the signal sequence of the extracellular pathogenesis-related PR-1a protein of tobacco (Payne et al., 1988). Similar fusions successfully target secretion of a transgene product in tobacco (Hammond-Kosack et al., 1994; Honée et al., 1995). Transcription of the chimeric gene was placed under the control of the *hsr203J* promoter and the nopaline synthase (*nos*) terminator (Figure 2). The final construct was transformed into tobacco by using Agrobacterium (Pontier et al., 1994). All regenerated plants displayed normal phenotypes compared with that of wild-type tobacco. We screened independently transformed plants for their ability to respond with necrosis formation to leaf infiltrations with suspensions containing 1000 zoospores per mL of *P. p.* var *nicotianae.* Of 35 independent transformants, 12 different lines were selected, the plants were allowed to self-pollinate for three generations, and lines homozygous for the selectable marker were then identified by screening for antibiotic resistance.

Transgenic Tobacco Plants Displayed an HR Phenotype When Leaves Were Inoculated with *P. p.* var *nicotianae*

The plants from four selected lines displayed clear necrotic lesions when leaves were inoculated with *P. p.* var *nicotianae.* This response was strongest for plants from line XPR24E8. For these plants, local infection of their leaves was restricted to the inoculation sites, which turned necrotic (Figures 3A and 3B). On leaves from tobacco lines XPR24E6 and b12, the fungus grew toward the central veins before a necrotic response arrested further growth. This response was more delayed in leaves from line XPR24E10. Here, resistance also was enhanced when compared with wild-type tobacco, but necrosis of the infected tissues started too late to stop fungal growth. The necrotic lesions that developed when leaves from line XPR24E8 were inoculated with the virulent pathogen resembled the HR of leaves undergoing an incompatible interaction with *P. cryptogea.* The lesions were also identical to those induced by local leaf infiltration with pure cryptogein (Figure 3B). These results indicate that the *X24* gene product is sufficient to induce an HR on tobacco and confirm that elicitins are avirulence factors during the tobacco–*Phytophthora* interaction. Full necrosis of tissues responding hypersensitively was observed within 3 to 4 days after infiltration with *P. p.* var *nicotianae* and thus appeared later than did the HR triggered by either the pure protein (within 24 hr) or *P. cryptogea* (within 2 days). Once established, neither further development nor spreading of the HR was observed.

The HR Phenotype Correlates with Stable Transgene Integration and Cryptogein Synthesis

The selected lines exhibited stable integration of the engineered DNA into the plant genome. Single-copy integration of the entire T-DNA fragment was sufficient for mediating the strong local necrotic response observed in line XPR24E8 (Figure 4A). All further analyses were performed with this transgenic line. To investigate whether necrosis formation correlated with cryptogein production, we analyzed protein extracts of *P. p.* var *nicotianae*–infected leaves from transgenic plants for the presence of the elicitor. Because cryptogein accumulated only in small quantities, the protein

extracts were concentrated (see Methods). Reinjection of the enriched protein solutions into leaves from untransformed tobacco (cv Xanthi-nc) plants allowed us to monitor the presence and quantity of cryptogein by its ability to induce necrosis. Protein that was extracted from wild-type plants after *P. p.* var *nicotianae* infection was unable at any time to induce a response from Xanthi plants. The extracts from line XPR24E8 induced an HR in leaves of this plant when they were prepared later than 2 days after infiltration with the zoospores (Figure 4B). Because cryptogein is stable in tobacco plants (Keller et al., 1996), increasing elicitin quantitities in the plant extracts reflect that the protein accumulated in the infected tissues. The necrosis-inducing activity of the protein preparations was maximal 3 to 4 days after infection, correlating with onset of the HR phenotype after infection of the transgenic plants.

Finally, a cryptogein-specific monoclonal antibody (Devergne et al., 1994) was used to verify whether the presence of the elictor was responsible for this necrosis-inducing activity in protein extracts from tobacco line XPR24E8. Immunoblot analysis with enriched extracts revealed no signal with protein from untransformed plants. In the transgenic line, cryptogein was produced after infection by *P. p.* var *nicotianae* but was not detectable in untreated plants and did not accumulate in response to mock inoculations (Figure 4C). Under native PAGE conditions, the detected protein comigrated with authentic cryptogein from *P. cryptogea*, indicating that no substantial modification of the transgene product occurred in the transgenic plants.

Restriction of Pathogen Growth Correlates with a Fast and Delocalized Accumulation of Defense Gene Transcripts in Cryptogein-Producing Plants

To monitor the growth of *P. p.* var *nicotianae*, we inoculated leaves from wild-type tobacco and from line XPR24E8 with zoospores of the fungus. At different time points after inoculation, the infected areas, as well as 1 cm of the surrounding uninfected leaf tissue, were dissected. RNA extracted from these samples was analyzed with gel blot hybridization experiments by using a 257-bp fragment corresponding to the extreme 3′ region of the *P. parasitica* 28S rDNA (Lacourt, 1994). Under high-stringency conditions, this fragment hybridizes specifically with *P. parasitica* 28S rRNA, whose abundance reflects fungal biomass (H. Keller, unpublished results). The rRNA of the pathogen accumulated to comparable quantities in the infected tissues of both wild-type and transgenic plants until 2 days after inoculation. The time course of rRNA accumulation indicated that the initiation of the infection process occurred similarly in both tobacco lines. Although in wild-type plants fungal rRNA levels continued to increase after 2 days, a decrease in 28S rRNA quantities was observed in plants from the transgenic line, reflecting the arrest of pathogen growth (Figure 5A).

Applications of elicitin to tobacco plants induce the transcriptional activation of defense-related genes encoding, among others, PR proteins (Keller et al., 1996) and the key enzyme in the sesquiterpenoid phytoalexin pathway, sesquiterpene cyclase (Yin et al., 1997; Keller et al., 1998). RNA gel blot hybridizations with probes corresponding to these genes revealed that substantial accumulations of PR gene transcripts were detectable at ~2 days after inoculation of untransformed tobacco with *P. p.* var *nicotianae*, whereas mRNA of the early and transiently expressed sesquiterpene cyclase gene accumulated 1 day after onset of the compatible interaction. Only weak and late (3 days after inoculation) mRNA inductions were detectable in tissues surrounding the infected areas of wild-type plants (Figure 5B). These late and local defense reactions in untransformed tobacco did not hinder fungal growth. Tobacco plants from line XPR24E8 reacted to *P. p.* var *nicotianae* inoculation with an overall stronger accumulation of defense gene mRNAs. Compared with induction in wild-type tobacco, this induction occurred earlier, and substantial sesquiterpene cyclase and PR gene transcripts accumulated 8 hr and 1 day after inoculation, respectively. In addition, all gene activations occurred early in tissues that surrounded the infected areas and were not invaded by fungal mycelia (cf. Figures 5A and 5B), indicating that cells in the vicinity of the infection site actively contribute to defense responses. These findings suggest that the early, delocalized, and coordinated onset of defense mechanisms is probably responsible for the arrest of fungal growth in necrotic areas of the transgenic plants.

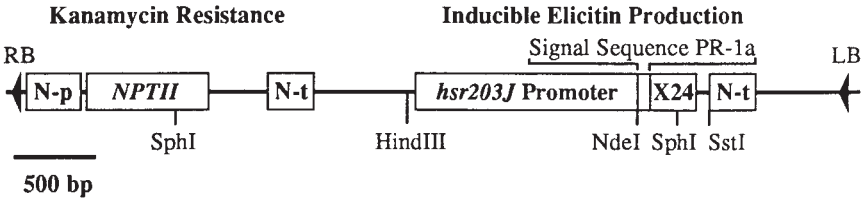

Figure 2. Schematic Representation and Partial Restriction Map of the Chimeric T-DNA in the Transformation Vector pBXPR24.

RB, right border; N-p, nopaline synthase (*nos*) promoter; *NPTII*, neomycin phosphotransferase II; N-t, *nos* terminating region; X24, coding region for mature cryptogein; LB, left border.

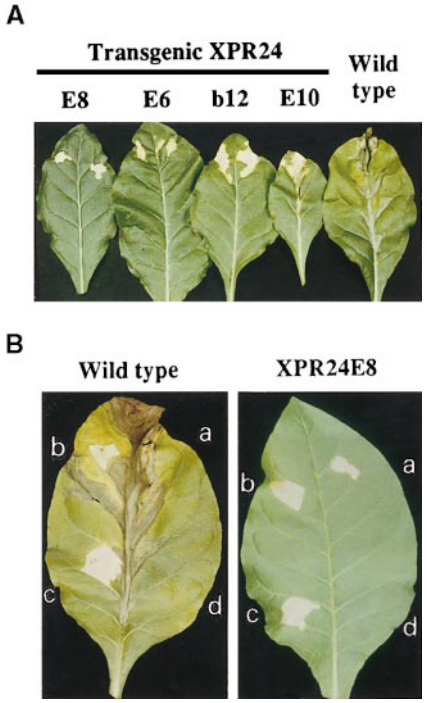

Figure 3. Induced Phenotypes of Selected, Transformed Tobacco Lines When Leaves Were Infiltrated with *P. p.* var *nicotianae.*

(A) Responses of leaves from wild-type plants and from four transgenic lines to local inoculation with 1000 zoospores per mL of *P. p.* var *nicotianae.* Whereas continuous invasion occurred in leaves of untransformed plants, transgenic plants developed necrotic lesions, which arrested (lines XPR24E8, E6, and b12) or accompanied (line XPR24E10) fungal growth.

(B) Local leaf necrosis in response to inoculation of line XPR24E8 with virulent *P. p.* var *nicotianae* (a) appeared identical to an HR induced by inoculation with avirulent *P. cryptogea* (b) or by treatment with pure cryptogein (c). The laminae of leaves were infiltrated with water (d), with zoospore suspensions adjusted to 1000 spores per mL, and with a 100 nM solution of cryptogein. Photographs were taken 10 days after inoculation.

The Stems and Roots of Plants Producing Cryptogein Are More Resistant to Infection

Commonly, the initiation of black shank disease occurs in roots. The primary inocula are zoospores in the soil that attach to the roots and germinate. The hyphae rapidly invade the epidermal tissues and the cortex and grow to the airborne parts of the plant. Infestation of the stem eventually kills the plant. To analyze whether the engineered resistance response to *P. p.* var *nicotianae* infection was restricted to leaves or was also functional in other organs, we inoculated untransformed plants and transgenic plants (line XPR24E8) on their stems and via the roots (Table 1). For stem inoculation, mycelium plugs were placed on the wound created by leaf excision. Although infection is facilitated by direct on-wound inoculation, invasion of the stem was reduced by >80% in the transgenic plants. This reduction of fungal growth was accompanied by necrosis of the infected tissues. Root inoculation was performed by infesting the soil with zoospores of the pathogen. Under these conditions, even some wild-type tobacco escaped invasion, but 32 of 48 inoculated untransformed plants (66%) were affected by the disease. In contrast, 35 of 48 transgenic plants (73%) were healthy (Table 1) and developed normally. In some roots of the healthy transgenic plants, growth of the fungus could be observed but was ultimately arrested at the hypocotyl and had no influence on the viability of the plants. Phenotypic symptoms were a browning of the infected roots and necrosis of the hypocotyls. Similar symptoms are observed during the resistance response of tobacco after root inoculation with avirulent *Phytophthora* species (Keller et al., 1996).

However, 10 plants from the transgenic line developed disease symptoms, indicating that the strategy did not confer full *P. p.* var *nicotianae* resistance. This could be explained by the fact that the engineered resistance is delayed in its onset compared with natural resistance, due to the time required for production of the transgenic elicitor. The successful invasion of some transgenic plants could reflect that fungal growth occasionally occurred faster than the induction of defense responses by the transgene product.

Cryptogein-Producing Plants Are More Resistant to Three Other Fungal Pathogens

We investigated whether the artificial resistance of tobacco extended to fungal pathogens that are unrelated to *P. p.* var *nicotianae.* A major tobacco pathogen under cold climatic conditions is *Thielaviopsis basicola*, which causes black root rot disease. By preference, this pathogen enters the roots of young seedlings from germinating spores. It penetrates the epidermis or enters through wounds and colonizes the cortex and the vascular system. This leads to root rot and destruction of the seedlings (Blancard, 1998). When plants from wild-type tobacco and from the transgenic line XPR24E8 were inoculated by soil infestation with conidia of *T. basicola*, the majority of untransformed plants were affected by the root disease, whereas three-quarters of the plants from the transgenic line were resistant to infection (Table 2). As already observed after root inoculations with *P. p.* var *nicotianae*, some transgenic plants were also infected by *T. basicola.* Thus, induced elicitin production strongly enhanced resistance to this pathogen but did not confer the same level of resistance as that observed for the genetically resistant cultivar ITB32 (Table 2).

In contrast to *P. p.* var *nicotianae* and *T. basicola*, which are hemibiotrophic plant pathogens, *Erysiphe cichoracearum* is a biotrophic pathogen that attacks tobacco leaves and provokes powdery mildew disease. Disease initiation occurs by germinating spores that form apressoria, and the fungus

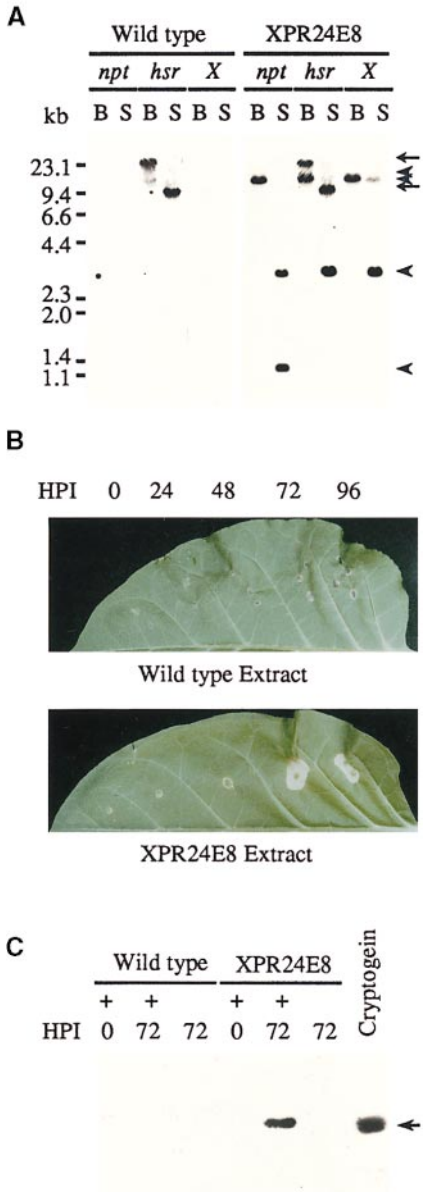

Figure 4. Line XPR24E8 Harbors a Single Transgene Copy and Produces Cryptogein When Infected with *P. p.* var *nicotianae.*

(A) Genomic DNA gel blot analyses with DNA from third (T_3) generation plants of the transgenic line XPR24E8. BamHI- (B; no site within the T-DNA) and SphI- (S; two sites) digested genomic DNA was probed successively with probes for the coding region of neomycin phosphotransferase II (*npt*), the *hsr203J* promoter *(hsr)*, and the *X24* coding region (*X*) under high-stringency conditions. Arrowheads indicate novel fragments; arrows indicate signals revealing the endogenous *hsr203J* promoter. Molecular length markers are indicated at left in kilobases.

(B) Bioassay for infection-responsive cryptogein production in the transgenic plants. Leaves from wild-type tobacco and line XPR24E8 were infiltrated with *P. p.* var *nicotianae* (1000 spores per mL). The infiltrated areas were dissected at different time points after inoculation (hours postinoculation [HPI]). Concentrated protein extracts from these tissues were injected into the laminae of leaves from untransformed tobacco (cv Xanthi-nc) to monitor necrosis-inducing activity.

(C) Immunoblot analysis for induced cryptogein production in the plant. Leaves of wild-type tobacco plants and of line XPR24E8 were inoculated with *P. p.* var *nicotianae* (+), as described above. Laminae of inoculated leaves were harvested immediately, and 72 hr after infiltration, as was done for water-infiltrated leaves (no symbol). Enriched protein extracts were analyzed using a cryptogein-specific monoclonal antibody (Devergne et al., 1994). The arrow indicates the position of pure, native cryptogein (10 pmol) included as a standard in the analysis.

feeds from single epidermal cells by the means of haustoria. Penetration of cells occurs occasionally, and hyphae grow mainly on the leaf surface or extracellularly in the epidermal layers. During infection, the fungus forms abundant conidia, which constitute the characteristic white powder (Blancard, 1998). Inoculation of untransformed tobacco plants with conidia of *E. cichoracearum* led to leaf invasions resulting in an almost complete white sporulation (Table 2). Transgenic plants that were inoculated in the same way showed a delay in invasion, which was in most cases not confluent but restricted to white patches. The level of resistance of tobacco line XPR24E8 to *E. cichoracearum* was lower than that observed after inoculation with *P. p.* var *nicotianae* and *T. basicola.* This may be related to the fact that *E. cichoracearum* is an ectoparasitic leaf pathogen that only occasionally enters the epidermal cells. Because the *hsr203J* gene promoter is expressed locally, we did not observe confluent necrotic lesions after *E. cichoracearum* inoculation, and elicitin production might be insufficient to trigger a strong response. Again, transgenic line XPR24E8 plants did not show the same level of resistance as those from cultivar TB22, which carries a dominant gene for powdery mildew resistance (Table 2).

Botrytis cinerea is a necrotrophic pathogen that attacks a wide range of host plants and causes gray mold disease of tobacco. Infection occurs mainly by germinating conidia on senescent leaves. The fungus enters the tissues preferentially through wounds but can also actively digest the cuticula and cell walls (Blancard, 1998). Until now, no genetic resistance of tobacco to this opportunistic parasite has been described. Although gray mold is not the major epidemic disease found in tobacco cultures, infections by this pathogen can be destructive and of agronomical importance when the fungus enters the stems. Thus, we estimated the resistance of transgenic plants to *B. cinerea*, with special emphasis on stem invasions. Inoculations were performed by spraying conidia of *B. cinerea* onto the surface of at least 30 plants each from the untransformed line and the transgenic line XPR24E8. Three weeks after inoculation, plants were sampled to rate disease symptoms and to score the

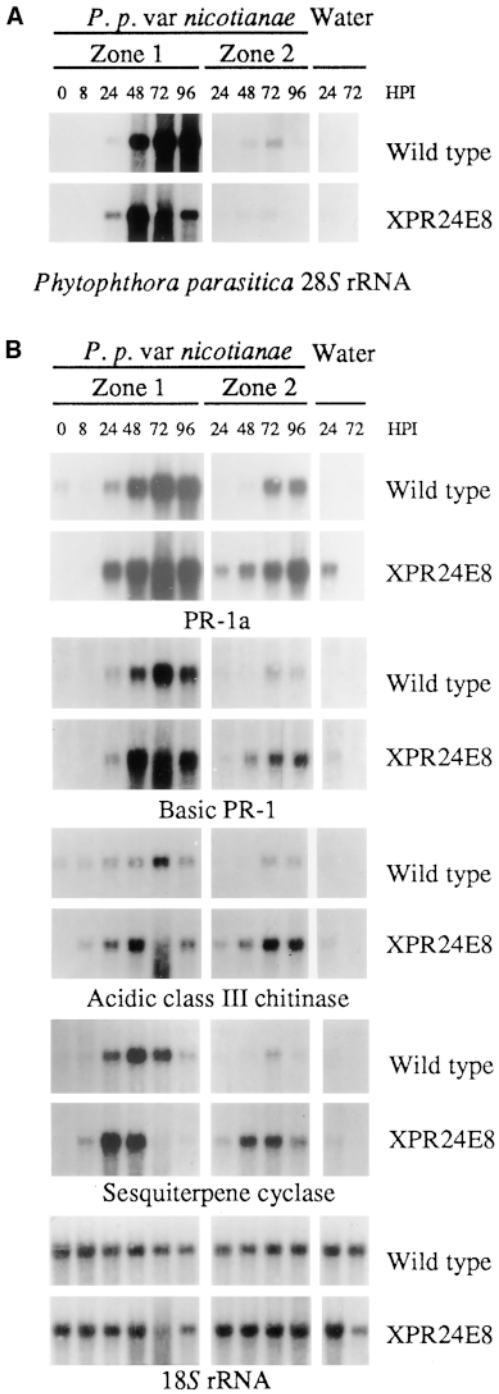

Figure 5. Decreased Pathogen Growth Rate in Line XPR24E8 Coincides with an Early and Delocalized Induction of Defense-Related Genes.

Leaves of untransformed tobacco and of line XPR24E8 were infiltrated with zoospore suspensions (1000 spores per mL) of *P. p.* var *nicotianae.* The infiltrated and infected areas (Zone 1) and 1 cm of the surrounding healthy leaf tissue (Zone 2) were dissected at different time points after inoculation (hours postinoculation [HPI]). Gel blots with total RNA extracted from the material from both zones were sequentially hybridized with different probes.

(A) Fungal growth was estimated by determining the amount of *P. p.* var *nicotianae* 28S rRNA in total RNA extracts from infected plant tissue. 28S rRNA abundance indicates a similar fungal development in both lines until 48 hr after inoculation. The decrease in 28S rRNA abundance at later time points indicates the arrest of fungal growth.

(B) Time course of the accumulation of defense-related gene transcripts in and around the infected leaf areas. The cDNA probes corresponding to genes encoding PR-1a, basic PR-1, and acidic class III chitinase have been described previously (Ward et al., 1991). As a probe for detecting sesquiterpene cyclase mRNA, the *str319* cDNA was used (Keller et al., 1998). Equal loading of the gel was verified with a 1618-bp fragment corresponding to the 18S rDNA from the root knot nematode *Meloidogyne incognita*, an organism unrelated to tobacco and *P. parasitica.* This fragment hybridizes equally with rRNA from both sources.

number of plants affected by these symptoms as a percentage of the total number of inoculated plants. Compared with 13% of the untransformed tobacco, 30% of the transgenic plants did not display disease symptoms. On 40% of the plants from line XPR24E8, maximal infections were restricted to the petioles or in stems to spots mostly <3 mm^2. Wild-type plants tended to show stronger rotting of the stem. Although the average surface of stem infection at the petiole insertion sites was 30 mm^2 in wild-type plants, this surface was reduced to 16 mm^2 in plants from line XPR24E8.

DISCUSSION

Genetic engineering has proved to be a powerful tool for controlling plant diseases and to be an alternative to economically costly and environmentally undesirable chemical control. Successful approaches for enhancing disease resistance of plants include constitutively overproducing toxins, such as α-thionin (Carmona et al., 1993) and the *Ustilago maydis* killer toxin (Park et al., 1996), and expressing genes encoding enzymes that are involved in the biosynthesis of antimicrobial compounds (Hain et al., 1993). Other approaches have been based on the overexpression of genes encoding proteins that are produced during the natural defense responses of a plant. These include PR-1a (Alexander et al., 1993), chitinase (Broglie et al., 1991), osmotin (Liu et al., 1994), and lipid transfer proteins (Molina and Garcia-Olmedo, 1997). An additional possibility involves the production of proteins that generate a signaling event and thus trigger the permanent onset of an array of defense responses. For this strategy, transgenic plants have been designed that constitutively produce cholera toxin (Beffa et al., 1995), a bacterial proton pump (Mittler et al., 1995), or H_2O_2-generating glucose oxidase (Wu et al., 1995). Recently,

tomato plants have been transformed with *Prf*, a gene that is required for the genetic resistance of tomato to the bacterial pathogen *Pseudomonas syringae* pv *tomato.* The transgenic plants constitutively express defense-related genes and display broad-spectrum disease resistance (Oldroyd and Staskawicz, 1998). Similar results were obtained on transgenic Arabidopsis plants that constitutively overexpress *NPR1*, a regulatory gene of the signaling cascade leading to systemic acquired resistance (Cao et al., 1998).

Most of the plants expressing a transgene constitutively do not display abnormal phenotypes. However, during the past few years, efforts have been made to generate transgenic plants that express the introduced gene under controlled conditions only. By overexpressing catalase genes with a resulting cosuppression event, or by overexpressing catalase antisense genes, catalase-deficient tobacco plants were generated that produced excessive H_2O_2, but only under high-light conditions (Chamnongpol et al., 1996, 1998). Light-induced H_2O_2 accumulation led to activation of defense mechanisms and enhanced tolerance to *P. syringae.* In transgenic tobacco plants carrying a fungal glucose oxidase gene under the control of the peroxidase promoter from a tropical pasture legume, expression of the transgene could be enhanced by supplying exogenous glucose. This led to increased accumulations of defense gene transcripts (Kazan et al., 1998). In another approach, a wound-inducible promoter was used to drive the expression of a barley ribosome-inactivating protein in transgenic tobacco. The transgene was expressed upon wounding, and plants showed enhanced resistance to *Rhizoctonia solani* through the toxicity of the produced protein (Logemann et al., 1992).

To assure the coordination of the entire signaling network of defense mechanisms in transgenic plants, our goal was to engineer in a controlled manner the earliest step in resistance, which is the recognition of an elicitor. Recently, a glucocorticoid-inducible promoter has been used to drive the expression of the avirulence gene *avrRpt2* from *P. syringae* in Arabidopsis plants carrying the corresponding *RPS2* resistance gene. Induction of elicitor production in transgenic plants led to the activation of defense mechanisms and resulted in hypersensitive cell death, demonstrating that induced and localized elicitor production in a reactive plant might be a powerful tool to artificially induce an HR (McNellis et al., 1998).

We designed experiments to produce an elicitor and to induce an HR upon infection by a virulent pathogen. Several promoters that drive gene expression in response to pathogen attack have been described previously; however, most of them exhibit organ specificities or respond to pathogen-induced signal molecules (Yin et al., 1997) and thus do not provide a rigorous control of transgene expression. We showed that the *hsr203J* promoter confers pathogen-inducible cryptogein production to transgenic tobacco without damaging the plant. Induction of the promoter by *P. p.* var *nicotianae* appeared weaker than the reported activation by biotic and abiotic inducers of hypersensitive-like necrosis (Pontier et al., 1998). Nevertheless, due to the strong biological activity of cryptogein, this induction was sufficient to trigger the defense responses that result in an HR and to confer enhanced broad-spectrum resistance. Aside from the advantages of its fast and localized activation, the promoter is normally silent and not activated by environmental or endogenous stimuli. However, it was important to know whether cryptogein production and necrosis formation might be damaging for the plant when the inducer is one of the necrotizing agents by which the *hsr203J* promoter is most specifically activated (Pontier et al., 1994, 1998). Therefore, we infiltrated leaves with the elicitor from *P. syringae* pv *syringae*, $Harpin_{Pss}$ (Huang et al., 1988), which is a potent activator of the promoter (Pontier et al., 1994). The leaves from both wild-type and transgenic plants responded to this elicitor with the same rapid and localized necrotic response, and we did not observe any uncontrolled spreading of necrotic spots (data not shown). We consider that the exogenous inducer of the HR triggers a full necrosis rapidly and that the endogenously produced cryptogein does not add considerably to this HR. One surprising finding of our study was that inoculations with the HR-inducing fungus *P.*

Table 1. Evaluation of Resistance to Stem and Root Inoculations with *P. p*. var *nicotianae* in Untransformed Tobacco and the Transgenic Line XPR24E8

		Root Inoculations		
	Stem Inoculations	Disease Rating[b]		
Line	Caulinar Invasion ±SD (mm)[a]	Diseased	Intermediate	Healthy
Wild type	119 ± 5	25	7	16
XPR24E8	19 ± 10	10	3	35

[a] Values in millimeters are the means ±SD based on triplicate assays. Invasion appeared as a discoloration of the stem tissue. Untransformed plants showed typical "disking" of the pith.

[b] Fourteen days after inoculation of 48 individuals per tobacco line, the number of healthy or diseased plants was determined. Diseased plants were entirely infested by fungal mycelia, whereas plants with an intermediate disease rating displayed symptoms on the roots, hypocotyl, and lower leaves. Healthy plants displayed no symptoms of disease.

Table 2. Disease Ratings after Inoculation of Wild-Type and XPR24E8 Transgenic Tobacco with *T. basicola* or *E. cichoracearum*

	T. basicola[b,c]			*E. cichoracearum*[b,d]		
Cultivar/Line[a]	Diseased	Intermediate	Healthy	Diseased	Intermediate	Healthy
Wild type	48	29	24	70	20	10
XPR24E8	26	0	74	40	40	20
ITB32	0	0	100	—[e]	—	—
TB22	—	—	—	8	8	84

[a] Other plants included in the analyses were from the cultivars ITB32 and TB22, which are genetically resistant to infections by *T. basicola* and *E. cichoracearum*, respectively.

[b] The number of healthy plants and diseased plants was determined and scored as a percentage of the total number of inoculated plants 2 weeks after inoculation with *E. cichoracearum* and 3 weeks after inoculation with *T. basicola*.

[c] Diseased plants displayed either complete root rot or a strong black discoloration of the entire root system, including the base of the stem. Plants with an intermediate disease rating had black lesions on all roots. Healthy plants displayed no or occasional minute spot lesions on only some of the roots.

[d] Diseased plants were coated by a white powder of spores on almost all leaves. Plants with an intermediate disease rating had circular white powdery patches on several leaves. Healthy plants displayed no symptoms or had occasional small patches on only some of the leaves.

[e] Dashes indicate analysis not performed.

cryptogea did not significantly stimulate the *hsr203J* promoter. These results were repeatedly observed and were also true for inoculations with *P. capsici*, which triggers an HR upon leaf infiltrations (data not shown). Therefore, nonhost *Phytophtora* species represent an exception to the finding that the *hsr203J* promoter is activated by inducers of HR.

In this study, we used the full-length *hsr203J* promoter because cryptogein is required in only trace amounts to trigger full responses in tobacco. However, the transcription rate of a transgene under the control of this promoter can be modulated. Excision of a silencer fragment leads to rates of transcriptional activation that are comparable to those of the 35S cauliflower mosaic virus promoter without a loss in the specificity of induction (D. Roby, unpublished results). Furthermore, a gene homologous to tobacco *hsr203J* was found in tomato (Pontier et al., 1998). The corresponding promoter might be used for driving the expression of a tomato-specific elicitor in response to pathogen attack. On the other hand, some crucifers, such as radish and oilseed rape, respond to an application of elicitins by HR and acquired immunity (Kamoun et al., 1993; Bonnet et al., 1996). Expression of an elicitin gene under the control of a pathogen-inducible promoter might considerably enhance resistance of these agronomically important plants. Thus, promoter/elicitor combinations adapted to a given host plant might be valuable tools for further approaches toward genetically engineered enhanced resistance.

The pattern of defense-related gene expression has been considered to be an important factor in the outcome of resistance or susceptibility. It has been suggested that the timing of gene expression might be essential, because in some cases expression may occur faster during an incompatible interaction than during a compatible one (Cuypers et al., 1988; Van Kan et al., 1992). A recent study suggests that a stronger rather than a faster induction of defense genes is responsible for the outcome of resistance (Cao et al., 1998). Here, we have shown that a novel disease resistance coincides with both stronger and faster expression of defense genes. In addition, local responses are accompanied by early accumulations of transcripts from these genes in tissues surrounding the infected areas. The activation of defense mechanisms in the vicinity of infection sites could be an additional factor for preventing the spread of the pathogen from a localized area. It might be interesting to analyze whether this pattern of gene expression is a characteristic feature of the novel resistance presented here or whether it also occurs during natural incompatible interactions.

A critical finding of our study was that resistance of genetically engineered plants to pathogens was enhanced in all cases but did not reach the same level as that observed during naturally occurring incompatible plant–pathogen interactions. A limiting factor might be the delay between pathogen attack and the onset of defense responses, which is most probably due to the time required for synthesis of the elicitor in the plant. In conclusion, we plan to apply our approach to resistance engineering as a complement to specific genetic resistance. In geographic regions with cultured plants threatened by a major disease, the addition of genetically engineered characteristics to a genotype conferring resistance to the specific disease might help to provide broad-spectrum resistance.

METHODS

Plant and Fungal Materials

Tobacco (*Nicotiana tabacum* cv Bottom Special) line pHG21-14A (Pontier et al., 1994) was used for analyzing promoter inducibility.

Wild-type tobacco cultivar Bottom Special was used for transformation with pBXPR24, and the biological activity of cryptogein produced in the plant was monitored in tobacco cultivar Xanthi-nc. Plants were inoculated with *Phytophthora parasitica* var *nicotianae* (strain 329) and *P. cryptogea* (strain 52) from the *Phytophthora* collection at INRA (Antibes, France) and with *Erysiphe cichoracearum* (strain ITB), *Thielaviopsis basicola* (strain 84.1), and *Botrytis cinerea* (strain B 91.3) from the tobacco pathogen collection of the Institut du Tabac (Bergerac, France).

β-Glucuronidase Assays

Leaves of aged tobacco plants were inoculated with a needleless syringe by infiltrating the lamina with either water or suspensions containing 5000 zoospores of *P. p.* var *nicotianae* per mL. Each leaf was infiltrated with 1 mL that was dispatched to 10 individual infiltration sites. For each treatment and time point, three leaves from three individual plants were cut, the infiltration areas precisely dissected and pooled, and the corresponding protein extracts analyzed for β-glucuronidase (GUS) activity (Jefferson et al., 1987). Root inoculations were performed with young tobacco plants (Keller et al., 1996) by pipetting 1 mL of zoospore suspensions (20,000 spores per mL) directly to the soil. For each time point and treatment, the roots and hypocotyls from three individual plants were washed, cut, pooled, and analyzed for GUS activity in the protein extracts.

Construction of the Transformation Vector

A fragment covering 1424 bp of the *hsr203J* promoter sequence of pHG21 (Pontier et al., 1994) with a NdeI restriction site at the transcription start site and an EcoRI site 11 nucleotides 3′ of the ATG was generated by polymerase chain reaction (PCR). EcoRI digest and subcloning into phagemid pBluescript SK− (Stratagene, La Jolla, CA) generated pHsr203.1KS. PCR with pBSPR-1a (Payne et al., 1988) allowed the amplification of a 117-bp fragment with EcoRI restriction sites at the extremities. The transcription start site and the sequence for signal peptide cleavage (93 bp 3′ of the ATG) were replaced by NdeI and PvuII restriction sites, respectively. This fragment was subcloned into EcoRI-digested pBluescript SK− to generate pPR1a.1KS. A 367-bp fragment was amplified from the BamHI subclone pB23 of the *P. cryptogea* genomic clone N1 (Panabières et al., 1995) carrying an EcoRV site covering the sequence of the signal peptide cleavage site 63 bp 3′ of the ATG and a SstI site 45 bp 3′ of the stop codon. This fragment was subcloned into EcoRV- and SstI-digested pBluescript SK− to generate plasmid pBXba23.1KS. The 144-bp fragment from ApaI- and PvuII-digested pPR1a.1KS was ligated into EcoRV- and ApaI-digested pBXba23.1KS to fuse the PR-1a signal sequence to *X24*, which encodes cryptogein. The NdeI-SstI insert from this plasmid was subcloned into NdeI- and SstI-digested pHsr203.1KS to generate pXPR24.2. In-frame cloning of all subclones was verified by sequence analyses.

Digestion of pXPR24.2 with HindIII and SstI liberated a 1873-bp fragment that was used to replace the *uid*A reporter gene from *Escherichia coli* between the HindIII and SstI restriction sites of the binary vector pBI101.2 (Jefferson et al., 1987) to fuse the entire construct with the nopaline synthase (*nos*) gene polyadenylation signal. Primers for generating the *hsr203J* promoter fragment were the reverse primer (Stratagene, La Jolla, CA) and primer 1 (5′-GAATTCAACCATATGGGCAAAGTTTGAAGTGTT-3′). PCR for obtaining the PR-1a signal sequence fragment was performed with primer 2 (5′-GAATTCATACATATGGGATTTGTTCTCTTTTCA-3′) and primer 3 (5′-GAATTCAGACAGCTGGGCACGGCAAGAGTGGGA-3′). The *X24* gene fragment was obtained with primer 4 (5′-GAATTCAACGATATCGCGTGCACCGCCACCCAG-3′) and primer 5 (5′-GAATTCAGACGCGAGCTCGACTCGAGGCTTACTACG-3′).

Plant Transformation and Selection of Transformants

The vector pBXPR24 was mobilized into *Agrobacterium tumefaciens* LBA4404. Transgenic tobacco plants were generated by using the leaf disc method, and transformants were selected as described previously (Pontier et al., 1994). Screening for a necrosis phenotype as a result of elicitin production was performed by infiltrating the leaves twice with 100 μL of a suspension containing 1000 spores per mL of *P. p.* var *nicotianae.*

DNA Gel Blot Analyses

Plants were kept in the dark for 5 days before the extraction of genomic DNA from the leaves (Dellaporta et al., 1983). Further purifications were performed by incubating 0.5 mL of DNA solution with 10 μL of a protein degrader (Sigma P-5700) at 50°C for 30 min followed by the addition of 50 μg of RNase and incubation at 37°C for 30 min. The solutions were successively extracted with phenol, chloroform, and diethylether and precipitated. Fifty micrograms of highly purified DNA was digested with 5 units per μg of BamHI and SphI, electrophoretically separated on 0.8% agarose gels, and blotted to membranes (HyBond N+; Amersham). Hybridizations were performed in 5 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate), 5 × Denhardt's solution (1 × Denhardt's solution is 0.02% Ficoll, 0.02% PVP, and 0.02% BSA), 0.1% SDS, and herring sperm DNA (100 μg per mL) at 65°C with ^{32}P-labeled (Multiprime labeling; Amersham) DNA fragments obtained from pHsr203.1KS by digestion with SmaI and NdeI, from pBI101.2 by PCR (primers 5′-AACAAGATGGATTGCACGCA-3′ and 5′-TCAGAAGAACTCGTCAAGAA-3′), and from pB23 by PCR (primers 5′-ACCCAGCAGACCGCTGCGTACAAGA-3′ and 5′-AGCTCGACTCGAGGCTTACTACGGA-3′). Before autoradiography, filters were washed with 0.2 × SSC containing 0.1% SDS at 65°C.

Analyses of Cryptogein Production

At different time points, the infiltrated areas (four per leaf) from three leaves of three individual plants were dissected, and proteins were extracted in 2 mL of water. The extracts were centrifuged for 15 min at 20,000*g*, the supernatants were lyophilized and resuspended in water, and protein concentrations were adjusted to 10 mg per mL. The protein solutions (50 μL) were then infiltrated into the leaves of wild-type tobacco (cv Xanthi-nc) plants to monitor the biological activity of cryptogein produced by the transgenic line XPR24E8. For immunoblot analysis, 60 areas of infiltration with either water or *P. p.* var *nicotianae* were dissected at different time points from leaves of wild-type and transgenic tobacco. Proteins from these tissues were extracted in 15 mL of extraction buffer (50 mM potassium phosphate, pH 7.0). The extracts were centrifuged for 15 min at 20,000*g*, and the supernatants were dialyzed against 20 mM sodium phosphate, pH 6.0, applied to a column containing 1 mL of Macro Prep Sulfopropyl HighS (Bio-Rad), and equilibrated to 20 mM sodium

phosphate, pH 6.0. Bound proteins were eluted with 2 mL of 20 mM sodium phosphate, pH 8.0, and concentrated by ultrafiltration. Proteins (40 μg per sample) as well as the cryptogein standard were separated by native PAGE on a 10% Mes–glycine gel (0.2 M Mes and 0.6 M glycine), pH 6.0, with 0.1 M citrate, pH 6.0, as running buffer. After electrophoresis, the gel was soaked for 20 min in Tris–glycine (0.25 and 1.9 M, respectively) with 1% SDS and electroblotted to nitrocellulose with the transfer buffer adjusted to pH 9.0. Staining with monoclonal antibody 177 (Devergne et al., 1994) was revealed by chemiluminescence (ECL Western blotting protocols; Amersham).

RNA Gel Blot Hybridization

At different time points after infiltration of the tobacco leaves with either water or a *P. p.* var *nicotianae* zoospore suspension (1000 spores per mL), the infiltrated and infected areas (four per leaf; three leaves per time point from three individual plants), as well as 1 cm of the surrounding uninfected leaf tissue, were precisely cut off. Extraction and separation of total RNA as well as the gel blot hybridizations of 10 μg of RNA per sample with α-^{32}P-dCTP–labeled DNA probes were performed as described previously (Keller et al., 1998). Fungal growth in the plant tissues was estimated by hybridization with a 257-bp fragment corresponding to the extreme 3′ region of the *P. parasitica* 28S rDNA (Lacourt, 1994). The cDNAs corresponding to the coding regions of the PR-1a, basic PR-1, and acidic class III chitinase genes (Ward et al., 1991) were kindly provided by J. Ryals (formerly at Novartis, Research Triangle Park, NC). Transcripts corresponding to sesquiterpene cyclase were detected with the labeled *str319* cDNA probe (Keller et al., 1998). Equal loading of the gel was controlled by hybridization with a 1618-bp fragment corresponding to the 18S rDNA from the root knot nematode *Meloidogyne incognita*, which was kindly provided by M.-N. Rosso (INRA, Laboratoire de Biologie des Invertebrés, Antibes, France). Before exposure, filters were washed with 0.2 × SSC and 0.1% SDS at 65°C, as described by Keller et al. (1998).

Plant Protection Assays

Plants were stem inoculated by placing mycelium plugs of *P. p.* var *nicotianae* on the petiole after excision of the third upper, fully expanded leaf of 60-day-old plants. Two weeks after inoculation, the stem was dissected, and the extent of stem invasion was measured. Root inoculations were performed on 30-day-old plants by pipetting 1 mL of a suspension containing 20,000 zoospores per mL into the soil of 48 individual plants per tobacco line. Root inoculations with *T. basicola* were performed on 6-week-old plants by pipetting 1 mL of a solution containing 10^6 conidia directly to the soil of at least 21 individual plants per tobacco line. For incubation, plants were kept under conditions favorable to fungal development (10 hr of light at 22°C; 14 hr of dark at 18°C; high soil moisture). For inoculations with *E. cichoracearum*, 50-day-old plants were placed in a top-open inoculation tower. Conidia were applied with an air stream from the surface of infested leaves of susceptible tobacco (cv Virgin D) onto the whole plants until a density of 3500 spores per cm^2 was reached, as counted at the bottom of the tower. At least 10 plants of each tobacco line were inoculated. After inoculation, plants were incubated at conditions favorable to growth and sporulation of the fungus (10 hr of light at 23°C; 14 hr of dark at 17°C). Inoculations of 6-week-old tobacco with *B. cinerea* were performed by spraying 1 mL per plant of a conidia suspension containing 5×10^5 conidia per mL onto the leaves of at least 30 plants per tobacco line. Plants were then covered with plastic bags to keep air humidity at saturation and incubated for 3 weeks (10 hr of light at 25°C; 14 hr of dark at 20°C).

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