RESEARCH ARTICLE

Resistance of Nicotiana benthamiana to Phytophthora infestans Is Mediated by the Recognition of the Elicitor Protein INF1

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Phytophthora infestans, the agent of potato and tomato late blight disease, produces a 10-kD extracellular protein, INF1 elicitin. INF1 induces a hypersensitive response in a restricted number of plants, particularly those of the genus Nicotiana. In virulence assays with different P. infestans isolates, five Nicotiana species displayed resistance responses. In all of the interactions, after inoculation with P. infestans zoospores, penetration of an epidermal cell was observed, followed by localized necrosis typical of a hypersensitive response. To determine whether INF1 functions as an avirulence factor in these interactions, we adopted a gene-silencing strategy to inhibit INF1 production. Several transformants deficient in inf1 mRNA and INF1 protein were obtained. These strains remained pathogenic on host plants. However, in contrast to the wild-type and control transformant strains, INF1-deficient strains induced disease lesions when inoculated on N. benthamiana. These results demonstrate that the elicitin INF1 functions as an avirulence factor in the interaction between N. benthamiana and P. infestans.

INTRODUCTION

Microbial plant pathogens often exhibit high degrees of specialization and can only infect a limited number of plant species (Agrios, 1988). Pathogen specialization results when a complex set of preformed and induced mechanisms is put into motion to defend a plant against invading pathogens. In some interactions, preformed physical barriers and antimicrobial compounds in the plant help to ward off pathogens (Osborn, 1996a, 1996b). In other interactions, perception by the plant of signal molecules, namely, elicitors, produced by the avirulent pathogen leads to the induction of effective defense responses, including a programmed cell death response termed the hypersensitive response (HR) (Lamb et al., 1989; Dixon and Harrison, 1990; Ebel and Scheel, 1992; Baker et al., 1997; Morel and Dangl, 1997). This model has been genetically defined by Flor’s gene-for-gene hypothesis (Flor, 1956, 1971). According to this hypothesis, a resistance reaction is determined by the simultaneous expression of a pathogen avirulence (Avr) gene with the corresponding plant resistance (R) gene (Staskawicz et al., 1995).

In recent years, the gene-for-gene hypothesis has received tremendous experimental support through the identification and functional characterization of both Avr and R genes. A number of Avr genes from fungi, bacteria, and viruses were shown to encode specific elicitor proteins. This was demonstrated directly by infiltration of Avr proteins into plant leaves or indirectly by expression of Avr genes in plant cells containing the corresponding R gene (Culver and Dawson, 1991; de Wit, 1995; Alfano and Collmer, 1996; Knogge, 1996; Bonas and van den Ackerveken, 1997; van den Ackerveken and Bonas, 1997). Elicitor treatment or Avr gene expression triggers the HR and related defense responses in plants that mimic the response induced by avirulent pathogens (Hahlbrock et al., 1995; Hammond-Kosack and Jones, 1996). R genes, on the other hand, are thought to encode specific receptors that interact directly or indirectly with elicitors, thereby initiating signal transduction pathways that lead to the HR and expression of disease resistance response (Staskawicz et al., 1995; Bent, 1996;
Hammond-Kosack and Jones, 1996, 1997; Baker et al., 1997). One remarkable feature is the occurrence of similar structural domains in the products of R genes, suggesting conserved mechanisms of pathogen recognition and signaling of defense responses in the plant kingdom (Dangl, 1995; Staskawicz et al., 1995; Bent, 1996; Hammond-Kosack and Jones, 1997). It has now become apparent that mechanisms of pathogen-induced cellular defenses of plants share some analogies with the immune response of vertebrates and insects (Baker et al., 1997).

Most examples of pathogen-triggered resistance responses in plants have been examined at a subspecific or varietal level. However, it has been suggested that mechanisms of gene-for-gene recognition may also determine resistance at higher taxonomic levels, namely, species, genus, or family (Newton and Crute, 1989; Keen, 1990; Heath, 1991; Crute and Pink, 1996). Several bacterial and fungal pathogens contain avirulence genes or produce elicitors that condition avirulence toward a resistant species (Keen, 1990; Dangl et al., 1992; Kamoun et al., 1993, 1997b; Kang et al., 1995; Sweigard et al., 1995; Leach and White, 1996). Similarly, functional conservation of R genes in unrelated species has been noted, and it also contributes to a restriction of host range (Whalen et al., 1991; Dangl et al., 1992; Innes et al., 1993; Bent, 1996). These findings suggest that the traditional separation between “host” and “nonhost” resistance in plant-pathogen interactions may not reflect fundamentally different mechanisms of action. A complex overlay of gene-for-gene recognitions may therefore mediate interactions between pathogens and their nonhost plants. Durable and stable resistance responses may have evolved in nonhost plants through the accumulation of an arsenal of R genes governing the recognition of multiple and/or essential avirulence molecules in the pathogen (Heath, 1991; Crute and Pink, 1996).

Phytophthora infestans, a hemibiotrophic oomycete plant pathogen, causes late blight, an economically devastating disease of potato and tomato (Anonymous, 1996; Fry and Goodwin, 1997a, 1997b). The life cycle and infection process of P. infestans are well known (Pristou and Gallegly, 1954; Hohl and Suter, 1976; Coffey and Wilson, 1983; Judelson, 1997). Infection generally starts when motile zoospores that swim on the leaf surface encyst and germinate. Germ tubes form an appressorium and a penetration peg, which pierces the cuticle and penetrates an epidermal cell to form an infection vesicle. Branching hyphae with narrow, digitilike haustoria expand from the site of penetration to neighboring cells through the intercellular space. Later, infected tissue necrotizes, and the mycelium develops sporangiophores that emerge through the stomata to produce numerous asexual spores called sporangia. Penetration of an epidermal cell by P. infestans has been noted in all examined interactions, including those with plant species unrelated to the solanaceous hosts (Gross et al., 1993; Schmelzer et al., 1995; Naton et al., 1996; V.G.A.A. Vleeshouwers, F. Govers, and L. Colon, unpublished data). Fully resistant plants, such as some of the potato lines bearing R genes or the nonhosts Solanum nigrum and parsley, display a typical localized HR at all infection sites (Gees and Hohl, 1988; Colon et al., 1992; Gross et al., 1993; Freytag et al., 1994; Schmelzer et al., 1995; Naton et al., 1996), suggesting that the classic model of pathogen elicitor recognition by a plant receptor and the subsequent activation of signal transduction pathways leading to HR could mediate these interactions.

P. infestans is generally considered a specialized pathogen. Only sporadic reports of natural infection of plants outside of the genera Solanum and Lycopersicon have been provided (Erwin and Ribeiro, 1996). The molecular basis of host specificity of P. infestans is poorly understood (Judelson, 1996, 1997). To date, no late blight resistance gene of Solanum spp or race-specific avirulence gene of P. infestans has been isolated. However, in recent years, a family of extracellular protein elicitors, termed elicins, has been identified in P. infestans and other Phytophthora species. Evidence that these molecules play a role in delimiting the host range of Phytophthora is accumulating (Yu, 1995; Grant et al., 1996).

Elicins are highly conserved 10-kD proteins that are secreted by all tested Phytophthora and Pythium species (Kamoun et al., 1993; Pernollet et al., 1993; Huet et al., 1995). Elicitins induce defense responses, including an HR, on a restricted number of plants, specifically Nicotiana species within the Solanaceae family (Kamoun et al., 1993; Bonnet et al., 1996). In Phytophthora parasitica, the absence of elicitin production correlates with virulence on tobacco, a plant species that exhibits a strong response to elicins (Ricci et al., 1989; Kamoun et al., 1993). Moreover, in a sexual progeny of P. parasitica, elicitin production segregates with low virulence (Kamoun et al., 1994), suggesting that elicins function as avirulence factors in P. parasitica-tobacco interactions (Yu, 1995). Similarly, elicitin recognition has been proposed to be a component of nonhost resistance of Nicotiana species to P. infestans and other elicitin-producing Phytophthora species (Yu, 1995; Kamoun et al., 1997b). This recognition is thought to be determined by the interaction of elicins with a high-affinity binding site in the tobacco plasma membrane (Wendehenne et al., 1995; Yu, 1995). However, no direct assessment of the role of elicins as avirulence factors through genetic manipulation of elicitin production has been reported to date.

Molecular manipulations and stable DNA transformation of P. infestans are well-established techniques (Judelson and Michelmore, 1991; Judelson, 1996, 1997; van West et al., 1998). Because DNA transformation is a prerequisite for an unequivocal demonstration of the role of elicins in Phytophthora-plant interactions, we decided to exploit P. infestans for functional analysis of elicins. In this study, we examined in detail the response of five Nicotiana species to P. infestans and used transgenic P. infestans strains deficient in the production of INF1, the major elicitin of P. infestans, to determine whether INF1 elicitin acts as an avirulence factor that induces resistance in Nicotiana species to P. infestans.
RESULTS

Nicotiana Species Are Resistant to Wild-Type P. infestans Isolates

P. infestans is typically considered a host-specific pathogen with a host range limited to a few solanaceous hosts (Erwin and Ribeiro, 1996). To determine whether Nicotiana species are resistant to P. infestans, we examined the interaction between four isolates of P. infestans and five species of Nicotiana by using a well-defined virulence bioassay (see Methods). Zoospores from P. infestans strains 88069, 90128, and ME93-2A isolated from epidemics occurring in the Netherlands and United States and strain MEX580 isolated from Mexico (described in Table 1) were inoculated on leaves from potato, tomato, and seven tobacco cultivars representing the species N. alata, N. benthamiana, N. clevelandii, N. rustica, and N. tabacum. Inoculations of the host plants potato and tomato with all four isolates of P. infestans consistently yielded expanding disease lesions accompanied by sporulation. In contrast, resistance responses were observed after inoculations of tobacco plants. Such resistance responses consisted of either localized necrotic spots typical of the HR or no visible macroscopic response, as described in Table 2. These results indicate that the Nicotiana species used in this study are highly resistant to several P. infestans isolates of diverse origin.

The HR Occurs in Nicotiana Species Inoculated with P. infestans

To determine the cytological basis of the resistance of Nicotiana species to P. infestans, we examined several representative interactions microscopically by using lactophenol-trypsin blue-stained discs of inoculated leaves. As previously observed in other resistance interactions between plants and P. infestans (Gross et al., 1993; Schmelzer et al., 1995; Naton et al., 1996; V.G.A.A. Vleeshouwers, F. Govers, and L. Colon, unpublished results), penetration of an epidermal cell by a germinating cyst of P. infestans was noted in all combinations that were examined. This was followed by a necrotic HR response that varied between different Nicotiana species in severity and number of affected cells. The resistance responses of N. tabacum and N. benthamiana illustrate two typically different responses of Nicotiana species to P. infestans. Some examples from these interactions are shown in Figure 1.

Table 1. P. infestans Strains Used in This Study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>88069</td>
<td>Wild type. Isolated in 1988 from tomato in the Netherlands. A1 mating type. INF1&lt;sup&gt;a&lt;/sup&gt;.</td>
<td>This laboratory&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>90128</td>
<td>Wild type. Isolated in 1990 from potato in the Netherlands. A2 mating type. INF1&lt;sup&gt;a&lt;/sup&gt;.</td>
<td>This laboratory&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>ME93-2A</td>
<td>Wild type. Isolated in 1993 from potato in the USA. A2 mating type. INF1&lt;sup&gt;a&lt;/sup&gt;. US-8 genotype.</td>
<td>W.E. Fry&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>MEX580</td>
<td>Wild type. Isolated in the 1980s from potato in Mexico. A1 mating type. INF1&lt;sup&gt;a&lt;/sup&gt;.</td>
<td>W.E. Fry&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Transforms</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y15</td>
<td>88069 transformed with G418 resistance plasmid pH209. INF1&lt;sup&gt;a&lt;/sup&gt;.</td>
<td>This study</td>
</tr>
<tr>
<td>PY23</td>
<td>88069 cotransformed with pH209 and inf1 antisense construct pHIN26. INF1&lt;sup&gt;a&lt;/sup&gt;.</td>
<td>This study</td>
</tr>
<tr>
<td>PY37</td>
<td>88069 cotransformed with pH209 and inf1 antisense construct pHIN26. INF1&lt;sup&gt;a&lt;/sup&gt;.</td>
<td>This study</td>
</tr>
</tbody>
</table>

<sup>a</sup>Phytophthora culture collection of the Department of Phytopathology, Wageningen Agricultural University.

<sup>b</sup>Cornell University, Ithaca, NY.

Table 2. Response of Different Solanaceous Plants to Four Isolates of P. infestans

<table>
<thead>
<tr>
<th>Species/Cultivar</th>
<th>Response to P. infestans&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>N. alata cv Lime Green</td>
<td>No macroscopic response</td>
<td>Resistant</td>
</tr>
<tr>
<td>N. benthamiana</td>
<td>Necrotic spots</td>
<td>Resistant</td>
</tr>
<tr>
<td>N. clevelandii</td>
<td>No macroscopic response</td>
<td>Resistant</td>
</tr>
<tr>
<td>N. rustica var WAU</td>
<td>Necrotic spots</td>
<td>Resistant</td>
</tr>
<tr>
<td>var Americana</td>
<td>Necrotic spots</td>
<td>Resistant</td>
</tr>
<tr>
<td>N. tabacum cv Xanthi</td>
<td>No macroscopic response</td>
<td>Resistant</td>
</tr>
<tr>
<td>cv White Burley</td>
<td>No macroscopic response</td>
<td>Resistant</td>
</tr>
<tr>
<td>S. tuberosum cv Bintje</td>
<td>Extending lesions with sporulation</td>
<td>Susceptible</td>
</tr>
<tr>
<td>L. esculentum cv Moneymaker</td>
<td>Extending lesions with sporulation</td>
<td>Susceptible</td>
</tr>
</tbody>
</table>

<sup>a</sup>P. infestans isolates 88069, 90128, ME93-2A, and MEX580 were used on all genotypes and gave similar results.
Secondary infection hyphae were formed, and intercellular growth was noted. In most cases, secondary hyphae with protruding haustoria were found between a group of one to 10 spongy parenchyma cells displaying increased trypan blue staining (Figure 1B, data not shown). At 70 hr after inoculation, the invading hyphae did not appear to have spread much farther. They were surrounded by clusters of heavily stained mesophyll cells (Figure 1C). Apparently, the hyphae were restricted to these HR clusters and did not spread farther. The observed HR clusters corresponded to the necrotic spots observed macroscopically (Table 2).

Production of INF1 Elicitin by P. infestans and Response of Nicotiana Species to INF1

To determine whether the resistance and HR observed in tobacco after inoculation with P. infestans could involve the recognition of the protein elicitor INF1, we examined isolates of P. infestans for production of INF1. Culture filtrates from the four isolates of P. infestans used in virulence assays along with culture filtrates from 63 other isolates from recent epidemics in Europe and North America contained a 10-kD band that comigrated with authentic elicins in SDS-PAGE analyses (Table 1; data not shown). To determine whether our Nicotiana species would respond to INF1, we infiltrated a 100-nM solution of the Escherichia coli–produced FLAG-INF1 protein (Kamoun et al., 1997b) into leaves of the Nicotiana species listed in Table 2. Two days later, the leaves were inspected for signs of an HR. All infiltrated plants responded to INF1 with a typical necrotic HR (data not shown), suggesting that the resistance observed in these plants to P. infestans could involve the recognition of INF1. In contrast, and as previously shown (Kamoun et al., 1997b), potato and tomato did not respond to infiltrations of INF1 protein.

P. infestans Transformants Silenced in the inf1 Gene

A simple consequence of the elicitor–receptor model is that pathogen strains deficient in the production of a specific elicitor are predicted to be more virulent than elicitor-producing strains. To engineer P. infestans strains deficient in the production of INF1, we cotransformed strain 88069 with pHIN28, a construct containing inf1 in an antisense orientation, and the genetin resistance plasmid pTH209. All putative cotransformants were screened by polymerase chain reaction for presence of the transgenes. Culture filtrates
from 30 cotransformants and 26 control transformants, containing only the pTH209 plasmid, were screened for the absence of INF1 by using silver-stained polyacrylamide gels. Six of the 30 antisense cotransformants failed to produce INF1, whereas none of 26 control transformants was affected in INF1 production and produced INF1 in similar amounts, as did the wild-type recipient strain 88069.

To determine whether the absence of the INF1 protein in culture filtrates correlates with the absence of inf1 mRNA in the mycelium, we isolated total RNA from cultures grown in vitro and performed RNA gel blot analyses as shown in Figures 2A and 2B. High levels of inf1 mRNA were detected in the recipient strain 88069 and in a control transformant Y15. In contrast, no inf1 mRNA was detected in two independent antisense transformants, PY37 and PY23, that do not produce INF1 (Figure 2B). Hybridization with a probe of the constitutively expressed actin gene resulted in similar signals in all lanes, indicating that equal amounts of RNA were loaded. These results suggest that introduction of an antisense inf1 construct in P. infestans caused silencing of the inf1 gene.

Genomic DNA of the recipient strain 88069, the control transformant Y15, and the antisense transformants PY37 and PY23 was isolated and analyzed using DNA gel blot hybridization analyses (data not shown). Hybridizations with a probe of the inf1 gene showed that the endogenous inf1 gene remained intact, suggesting that the silencing of the inf1 gene obtained in PY37 and PY23 is not due to gene disruption or displacement.

Silencing of inf1 Is Mitotically Stable under Various Conditions

To determine whether the INF1 nonproducing phenotype of the antisense transformants PY37 and PY23 is mitotically stable and allows functional analyses, we cultured the transformants in different media and subjected them to various treatments. Silenced transformants were vegetatively cultured in vitro by transferring them monthly to fresh medium over a period of 8 months. Regularly, agar plugs containing sporulating mycelia were transferred to liquid media, and the culture filtrates were checked for INF1 production. Neither PY37 nor PY23 ever reverted to the wild-type state under these or other in vitro conditions. Furthermore, we investigated whether the silenced state of PY23 is maintained during growth in the plant. Potato tuber slices (1.0 cm thick) were inoculated on one side; after a week, when mycelia had grown through the tuber slice, young sporulating mycelia were transferred to fresh tuber slices. This procedure was repeated three times, after which mycelium was reisolated, transferred to liquid medium, and checked for INF1 production. No effect on silencing of inf1 in PY23 was observed after this treatment (data not shown). These results demonstrate that silencing of inf1 remains stable through vegetative growth over time in vitro and in the plant. Therefore, the INF1-deficient strains PY37 and PY23 are suitable for functional assays.

INF1-Deficient Strains Remain Virulent on Potato but Produce Disease Lesions on N. benthamiana

To determine whether deficiency in INF1 production alters virulence of P. infestans on host and nonhost plants, we inoculated potato and the seven Nicotiana lines previously examined (Table 2) with zoospore solutions from the INF1-producing strains 88069 and Y15 and inf1 mutants PY23 and PY37. As illustrated in Figure 3A, inoculated leaves were first examined for macroscopic symptoms of resistance (no response or HR) and susceptibility (disease lesions). Disease lesion formation followed by extensive production of sporangia (sporulation) was observed at all inoculation sites and with all strains of potato. Resistance responses were observed with 88069, Y15, PY23, and PY37 in N. alata.
Figure 3. Virulence of *P. infestans* Wild-Type and INF1-Deficient Strains on Potato (*S. tuberosum*) and Nicotiana Species.

(A) A histogram showing the percentage of resistance (no visible response or HR) and susceptible (sporulating lesion) responses observed after inoculation of potato (*S. tuberosum*), *N. benthamiana*, *N. rustica* (var Americana), and *N. tabacum* with the wild-type recipient strain 88069 (WT), a control transformant Y15, and two INF1-deficient strains PY37 and PY23. The number of inoculation spots examined per strain was 11 to 23 for potato, 38 to 65 for *N. benthamiana*, 9 to 17 for *N. rustica*, and 16 for *N. tabacum*. Note that *N. alata* and *N. clevelandii* gave results similar to those for *N. tabacum* (no visible response with all strains; data not shown).

(B) Analysis of actin and inf1 mRNA production in infected plant tissue. Each lane of the RNA gel blot contains 15 μg of total RNA isolated from infected potato or *N. benthamiana* leaves 6 days after inoculation with wild-type strain 88069 (wt), control transformant Y15 (15), and two INF1-deficient strains PY37 (37) and PY23 (23). The blot was sequentially hybridized with probes from the actin gene (actA) and the inf1 gene (inf1). The intensity of the *P. infestans* actin signals correlates with the extent of *P. infestans* colonization in the infected tissue. RNA extractions were conducted from pools of 10 leaf discs showing representative responses, as determined in Figure 4A. Approximate transcript lengths are indicated at right. ni, not inoculated control; nt, nucleotides.
N. clevelandii, N. rustica, and N. tabacum, indicating that INF1 is not a major determinant of these resistance responses. In contrast, 20 to 30% of inoculations of N. benthamiana with INF1-deficient strains PY23 and PY37 consistently resulted in disease lesion formation accompanied by sporulation; however, inoculations with 88069 always led to resistance reactions, and inoculations with Y15 led to disease lesions in <3% of the inoculations.

To assess P. infestans biomass in infected leaves, we isolated total RNA from leaves 6 days after inoculation, blotted it, and sequentially hybridized the blot with probes from the actin (actA) and inf1 genes. As shown in Figure 3B, high levels of actin RNA were detected in total RNA isolated from potato leaves infected by all four strains, whereas inf1 mRNA was only detected in the INF1-producing strains 88069 and Y15. This suggests that all strains can extensively colonize infected potato leaves independently of their ability to produce inf1 mRNA. In addition, this result also confirms that the inf1 gene remains silenced in PY23 and PY37 during growth in the plant. In total RNA samples isolated from N. benthamiana leaves inoculated with PY37 and PY23, significant levels of actin mRNA were detected, whereas inf1 smRNA was not detected. In contrast, trace amounts of actin and inf1 mRNA were detected in leaves inoculated with 88069 or Y15. These results confirm the macroscopic observations and indicate that the INF1-deficient strains PY37 and PY23 reach higher levels of colonization and biomass in leaves of N. benthamiana than do INF1-producing strains.

**Infection of N. benthamiana by INF1-Deficient Strains**

To explore in detail the infection of N. benthamiana by INF1-deficient strains, we carefully examined inoculated leaves at both the macroscopic and microscopic level, as illustrated in Figure 4. In numerous side-by-side inoculations of N. benthamiana leaves with INF1-producing and INF1-non-producing P. infestans strains, a dramatic difference in response was observed. In contrast to wild-type strains (Table 2 and Figure 3A), up to 30% of the sites inoculated with P. infestans INF1-deficient strains went through a full disease cycle. The first symptoms of colonization appeared within 2 or 3 days after inoculation, with a rapidly expanding water-soaking zone forming around the inoculation spot. As early as 3 days after inoculation, a grayish white sporulation zone became visible on the surface of the infected leaf, in contrast to the localized necrosis (HR) that was observed after inoculation with INF1-producing strains (Figure 4A). In contrast to infection of potato plants obtained under the same conditions, little browning or necrosis accompanied such sporulation.

Disease lesions expanded on N. benthamiana at a rate similar to potato and ultimately covered the entire leaf. In microscopic examinations of trypan blue-stained sections of N. benthamiana leaves infected by P. infestans mutant PY37...
(Figures 4B and 4C), extensive biotrophic colonization of the mesophyll by intercellularly growing hyphae withhaustoria was observed 70 hr after inoculation. In contrast to infections by the wild-type strains (Figure 1C), no response of the mesophyll cells surrounding these invading hyphae was observed (Figure 4B). On the surface of the infected leaf, sporangiophores emerging from the stomata and numerous sporangia were readily observed (Figure 4C).

**DISCUSSION**

Ever since the potato late blight epidemics of the mid-nineteenth century, members of the genus Phytophthora have emerged as major pathogens of numerous crops (Erwin and Ribeiro, 1996). Despite the importance of Phytophthora species as devastating plant pathogens, little is known about the molecular mechanisms that determine the outcome of these interactions. In these interactions, several classes of resistance reactions were defined on the basis of the strength and appearance of necrotic tissue. As observed in Nicotiana-P. infestans interactions, various levels of pathogen ingress correlated with the different necrotic responses (Reignault et al., 1996).

In contrast to the wild type and other P. infestans INF1-producing strains, the engineered INF1-deficient strains produced disease lesions with profuse sporulation on N. benthamiana. Furthermore, both RNA gel blot hybridizations with infected N. benthamiana tissue using a constitutive actin gene as a probe and cytological examinations of infected N. benthamiana tissue indicated that INF1-deficient strains achieve significant levels of biomass and colonization in N. benthamiana. In contrast, these mutants remained unable to infect other Nicotiana species, such as tobacco.

This disparity appears to reflect the differences observed by cytological examination of the resistance responses in the Nicotiana species. In N. benthamiana, wild-type INF1-producing P. infestans strains can penetrate the leaf as far as the mesophyll, whereas INF1-deficient strains can grow further and fully colonize leaf tissue. In tobacco, the first layer of response to infection by both INF1-producing and INF1-non-producing strains occurs immediately after penetration and can effectively stop further ingress by P. infestans. This indicates that resistance to P. infestans in N. benthamiana is mainly triggered by INF1, whereas the early resistance reaction observed in tobacco is not. Possibly, before recognition of INF1, tobacco responds to additional host-specific elicitors that are not detected by N. benthamiana. Putative candidates are the products of the inf2A and inf2B genes, both members of the P. infestans elicitin gene family (Kamoun et al., 1997a). These two genes are expressed in the plant during infection of potato and N. benthamiana, and their products induce an HR on tobacco (Kamoun, P. van West, and F. Govers, unpublished data). In addition, a 30-kD glycoprotein identified in several Phytophthora species is known to induce defense responses in tobacco (Baillieu et al., 1996). Whether these elicitors induce different responses on tobacco and N. benthamiana remains to be tested.
The suggestion that INF1 is not involved in the early resistance response of tobacco does not exclude the possibility that INF1 is effective as an avirulence factor on this plant at a later stage of the disease cycle. This hypothesis is supported by the increase in expression of the inf1 gene during the latest stages of infection of potato leaves by P. infestans (Kamoun et al., 1997b) and is in line with the observation that in N. benthamiana, INF1-producing strains are blocked at an advanced stage of colonization. Future experiments, such as constructing strains of P. infestans with multiple mutations, will help to test this hypothesis.

In relation to the observed difference between N. benthamiana and the other Nicotiana species, N. benthamiana is known to anomalously allow infection by numerous plant viruses and plant virus mutants, including some with restricted host range (van Dijk et al., 1987; Dawson and Hilf, 1992). Therefore, N. benthamiana may have a particular deficiency in its defense response, making it generally more susceptible to plant pathogens, including INF1-producing and INF1-non-producing P. infestans, than are other Nicotiana species.

Interactions between P. infestans and plants are notable for their quantitative nature and for the ambiguous response of the plant to infection by the pathogen. For example, susceptible and partially resistant potato plants display a mosaic pattern of responses to infecting spores: some sites are readily infected, whereas others respond by a typical localized HR, which effectively stops the pathogen at that particular site (Gees and Hohl, 1988; Freytag et al., 1994). Similar quantitative aspects were observed in this study in the interaction between N. benthamiana and P. infestans strains. INF1-deficient strains were able to produce disease lesions on N. benthamiana at only 20 to 30% of the inoculated sites. This is reminiscent of the infection efficiencies obtained after inoculation of partially resistant Solanum lines by P. infestans (Colon et al., 1995) and suggests that N. benthamiana may retain a low level of resistance against INF1-deficient P. infestans strains. Macroscopically, visible HR also varied quantitatively, because we did not observe lesions at all inoculation sites showing resistance (Figure 3A). No visible response was observed in 20 to 40% of the spots inoculated with both INF1-producing and INF1-non-producing P. infestans strains. Based on the cytological examinations of multiple infection sites (data not shown), we think that the absence of symptoms generally corresponds to aborted infections, which reflect the observed infection efficiency of P. infestans on N. benthamiana.

Conflicting results have appeared regarding the host specificity of elicitors (discussed in Yu, 1995; Kamoun et al., 1997b). It has been suggested by others that elicitors may be non-specific toxins that induce necrosis on all plant species, including hosts (Pernollet et al., 1993; Huet et al., 1994). In this study, we show unambiguously that INF1-deficient strains remain capable of infecting potato and tomato. No significant difference in disease severity or symptomology was noted between INF1-producing and INF1-non-producing isogenic strains. This indicates that the P. infestans elicitin INF1 is not required for pathogenicity on potato and tomato. It can then be ruled out that INF1 functions as a nonspecific toxin essential for virulence.

Based on traditional definitions (Heath, 1991), the Nicotiana species examined in this study can be considered as non-hosts of P. infestans. Contrary to the assumption that non-host resistance has multiple components and is genetically complex, our results show that resistance of N. benthamiana to P. infestans involves one major component, the recognition of the elicitor protein INF1. R genes are generally bred from resistant wild species into a cultivated species through conventional methods. Cultivars containing such R genes can then discriminate between genotypes of the pathogen (races). However, there is some evidence, such as the functional conservation of R genes between unrelated species (Whalen et al., 1991; Dangl et al., 1992; Innes et al., 1993; Bent, 1996), that higher taxa specificity may also be a reflection of gene-for-gene interactions. Experimental and particularly genetic characterization of such nonhost interactions is hampered by the absence of variation in plant resistance and in pathogen virulence. In addition, resistance identified in plants that are sexually incompatible with a given susceptible crop plant may not be dissected into discrete components, and R genes from such plants cannot be transferred into isogenic background for further study. Here, we demonstrate that pathogen protein elicitors that induce HR on nonhost plants can function as avirulence factors. Therefore, species-specific elicitors can be used as a tool to identify novel sources of resistance in germplasm unrelated to the host plant, to evaluate resistance levels, and to isolate R genes.

The postulate that elicitins are avirulence factors that restrict the host range of Phytophthora isolates points to a number of biotechnological applications. The ubiquitous occurrence of conserved structural features noted in R genes of diverse origin (Dangl, 1995; Staskawicz et al., 1995; Bent, 1996) suggests that a classic Nicotiana R gene could be involved in the recognition and response to INF1 and other elicitors. Further genetic and biochemical research should help to isolate Nicotiana R genes involved in the INF1 response. The results we present in this study further suggest that manipulation of potato and tomato to recognize and respond to elicitin molecules is predicted to yield plants with enhanced resistance to P. infestans.

**METHODS**

**Phytophthora infestans Strains and Culture Conditions**

The various P. infestans isolates used in this study are listed and described in Table 1. Strains were routinely cultured in the dark at 18°C on rye agar medium supplemented with 2% sucrose (Caten and Jinks, 1968). For INF1 elicitin production, culture filtrates were harvested after growth for 3 to 4 weeks at 18°C in still cultures in the synthetic medium described by Kamoun et al. (1994). To isolate...
Plasmid Construction and Transformation of *P. infestans*

Plasmid pHIN28, which contains the inf1 coding sequence in antisense orientation fused to the medium were flooded with water (10 mL per Petri dish) and incubated at 4°C for 2 hr. The zoospore solution was then gently poured out of the Petri dish and placed on ice until inoculation.

**DNA Manipulations**

Routine DNA manipulations were conducted essentially as described elsewhere (Ausubel et al., 1987; Sambrook et al., 1989). Total DNA of *P. infestans* was isolated from mycelia grown in liquid culture, as previously described (Pieterse et al., 1991). Alkaline DNA transfer to Hybond N+ membranes (Amersham, Arlington Heights, IL) and DNA gel blot hybridizations were performed at 65°C, as described elsewhere (Ausubel et al., 1987; Sambrook et al., 1989). Filters were washed at 55°C in 0.5× SSC (75 mM NaCl and 7.5 mM sodium citrate).

**RNA Manipulations**

Total RNA from *P. infestans* and infected plant tissue was isolated using the guanidine–hydrochloride extraction method (Logemann et al., 1987). For RNA gel blot analyses, 10 to 15 mg of total RNA was denatured at 50°C in 1 M glyoxal, 50% DMSO, and 10 mM sodium phosphate, electrophoresed, and transferred to Hybond N+ membranes (Ausubel et al., 1987; Sambrook et al., 1989). Hybridizations were conducted at 65°C in 0.5 M sodium phosphate buffer, 7% SDS, and 1 mM EDTA. Filters were washed at 65°C in 0.5× SSC.

**DNA and RNA Blot Hybridization Probes**

Gel-purified DNA fragments containing the full-length inf1 cDNA insert from pFB7 (Kamoun et al., 1997b), the actA gene from pSTA31 (Urkles et al., 1991), and the ham34 promoter (Judelson and Michelmore, 1991) were used as probes and radiolabeled with α32P-dATP using a random primer labeling kit (Gibco BRL).

**SDS-PAGE**

Culture filtrates were subjected to Tris-tricine-SDS-PAGE, as described elsewhere (Schagger and von Jagow, 1987; Sambrook et al., 1989). After electrophoresis, gels were silver stained according to the method of Merrill et al. (1981).

**Plant Assays**

The plant species and cultivars used in this study are listed in Table 2. Plants were grown in growth chambers or a greenhouse for 4 to 8 weeks, depending on the species. Infection assays with *P. infestans* were conducted as described by Turkensteen (1973). Resistance levels of solanaceous plants observed using this assay were shown to correlate with the resistance levels obtained with attached leaves in the field or in the greenhouse (V.G.A.A. Vleeshouwers, F. Govers, and L. Colon, manuscript submitted). In general, the third to sixth leaves from the top were detached from several plants, and the petioles were fitted into water-saturated florist foam (Oasis; V.L. Smithers A/S, Denmark). The leaves and the foam were then placed in plastic trays lined with wet filter paper and a plastic mesh to prevent direct contact between the leaves and the wet paper. Ten-microliter droplets containing ~500 zoospores were then applied to the underside of the leaves in the middle of a leaf panel. A total of two to 10 droplets were placed on each leaf, depending on its size. The trays were tightly fitted with a transparent plastic cover and placed under fluorescent light in a regulated growth chamber (15°C for a 16-hr photoperiod). Inoculation spots were examined for disease symptoms and necrosis daily for 7 days.

**Microscopic Observations and Trypan Blue Staining**

Leaf discs containing the inoculum were excised at various times after inoculation and examined by microscopy for plant response and growth of *P. infestans*. Lactophenol–trypan blue staining and destaining with chloral hydrate were performed as described earlier (Wilson and Coffey, 1980; Colon et al., 1992).

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Resistance of *Nicotiana benthamiana* to *Phytophthora infestans* Is Mediated by the Recognition of the Elicitor Protein INF1
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