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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 (Osbourn, 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;

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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, digitlike 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 elicitins, 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).

Elicitins 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 elicitins (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 elicitins 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 elicitins 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 elicitins 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 elicitins in *Phytophthora*–plant interactions, we decided to exploit *P. infestans* for functional analysis of elicitins. 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.

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

Species/Cultivar	Response to *P. infestans*[a]	Interaction
N. alata		
cv Lime Green	No macroscopic response	Resistant
N. benthamiana	Necrotic spots	Resistant
N. clevelandii	No macroscopic response	Resistant
N. rustica		
var WAU	Necrotic spots	Resistant
var Americana	Necrotic spots	Resistant
N. tabacum		
cv Xanthi	No macroscopic response	Resistant
cv White Burley	No macroscopic response	Resistant
S. tuberosum		
cv Bintje	Extending lesions with sporulation	Susceptible
L. esculentum		
cv Moneymaker	Extending lesions with sporulation	Susceptible

[a] *P. infestans* isolates 88069, 90128, ME93-2A, and MEX580 were used on all genotypes and gave similar results.

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–trypan 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.

In tobacco, which showed no visible macroscopic response after inoculation with *P. infestans* (Table 2), cellular responses generally were limited to the penetrated epidermal cell and zero to three adjacent epidermal or mesophyll cells (Figure 1A). Granular cytoplasm, condensed nuclei, thickened cell walls, and increased trypan blue staining were observed in the responding cells, suggesting a typical HR

Table 1. *P. infestans* Strains Used in This Study

Strain	Description	Origin
88069	Wild type. Isolated in 1988 from tomato in the Netherlands. A1 mating type. INF1$^+$.	This laboratory[a]
90128	Wild type. Isolated in 1990 from potato in the Netherlands. A2 mating type. INF1$^+$.	This laboratory[a]
ME93-2A	Wild type. Isolated in 1993 from potato in the USA. A2 mating type. INF1$^+$. US-8 genotype.	W.E. Fry[b]
MEX580	Wild type. Isolated in the 1980s from potato in Mexico. A1 mating type. INF1$^+$.	W.E. Fry[b]
Transformants		
Y15	88069 transformed with G418 resistance plasmid pTH209. INF1$^+$.	This study
PY23	88069 cotransformed with pTH209 and *inf1* antisense construct pHIN26. INF1$^-$.	This study
PY37	88069 cotransformed with pTH209 and inf1 antisense construct pHIN26. INF1$^-$.	This study

[a] *Phytophthora* culture collection of the Department of Phytopathology, Wageningen Agricultural University.
[b] Cornell University, Ithaca, NY.

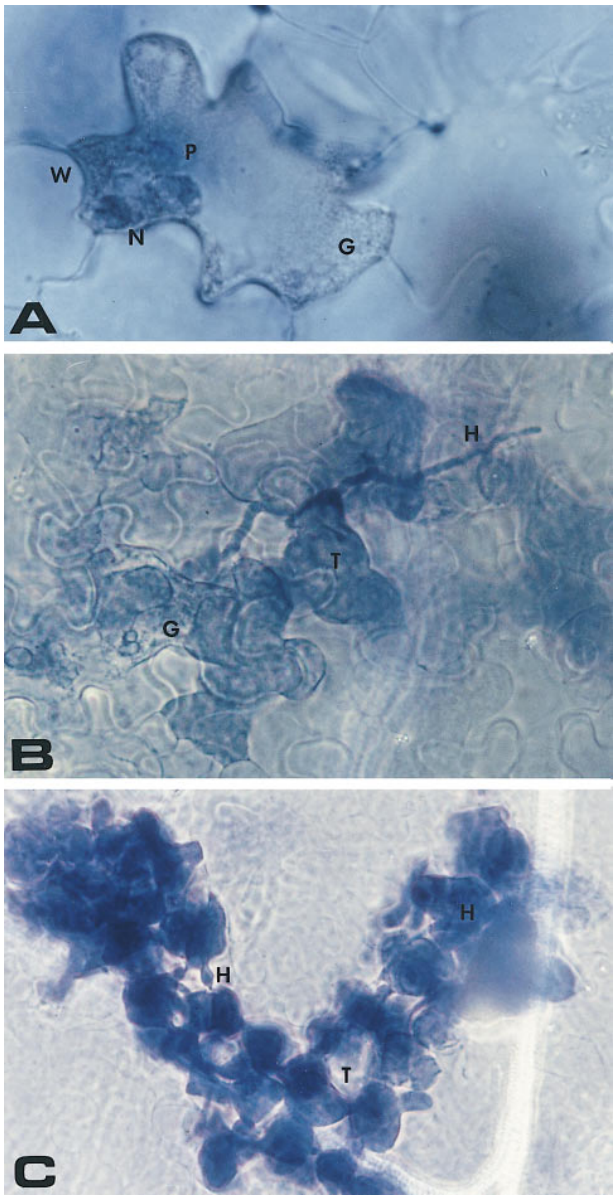

Figure 1. HR of *N. tabacum* and *N. benthamiana* Inoculated with Wild-Type Isolates of *P. infestans.*

(A) Trypan blue–stained leaf disc of tobacco 70 hr after inoculation with *P. infestans* wild-type isolate 88069 showing the response of one epidermal cell.

(B) and **(C)** Trypan blue–stained *N. benthamiana* leaf discs 46 hr **(B)** or 70 hr **(C)** after inoculation with *P. infestans* wild-type isolate 88069. Extensive blue staining in **(C)** is due to irreversible membrane damage.

G, granular cytoplasm; H, hyphae; N, condensed nucleus; P, penetration site; T, increased trypan blue staining; W, thickened cell wall.

response. Secondary infection hyphae were not visible, suggesting that the pathogen is restricted to the penetrated cell. In *N. benthamiana*, which showed macroscopic necrosis after inoculation with *P. infestans* (Table 2), cellular responses were more extensive than on *N. tabacum.* At 46 hr after inoculation (Figures 1B and 1C), cells displaying an HR were visible at some but not all infection spots. Secondary 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 elicitins 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 geneticin 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*,

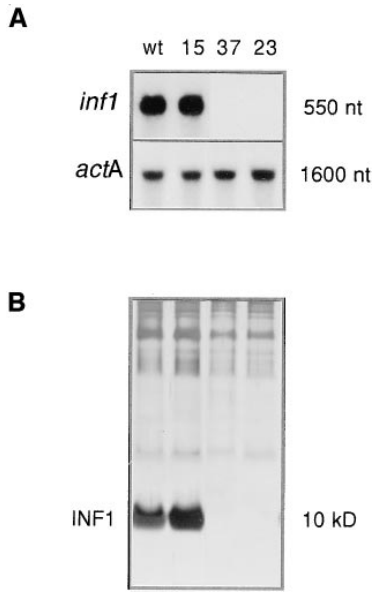

Figure 2. *P. infestans* Transformants Deficient in INF1 Production.

(A) Analysis of *inf1* mRNA production in the *P. infestans* wild-type recipient strain 88069 (wt), control G418 noncotransformed Y15 strain (15), and two independent silenced antisense transformants PY37 (37) and PY23 (23). Each lane of the gel contains 15 μg of total RNA isolated from mycelia grown in vitro for 10 days. The blot was sequentially hybridized with a probe from the *inf1* gene (*inf1*) and a probe from the actin gene (*actA*). Approximate transcript lengths are indicated at right in nucleotides (nt).

(B) Analysis of INF1 protein production in the same set of strains as given in **(A)**. Protein samples were obtained from filtrated culture medium of 7-day-old cultures, separated by Tricine–SDS-PAGE, and visualized by silver staining. The position of the 10-kD INF1 protein is indicated (INF1).

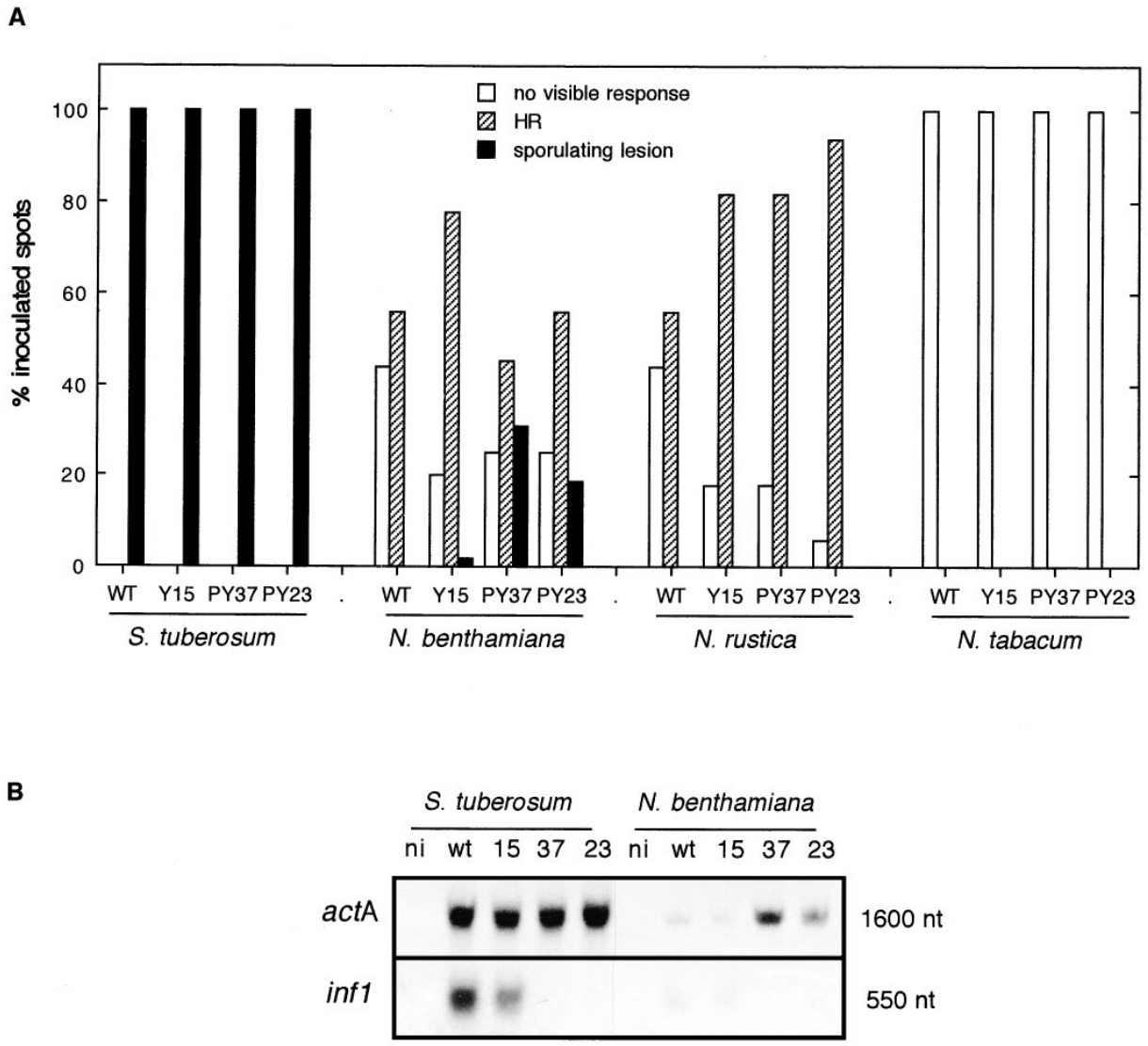

Figure 3. Virulence of *P. infestans* Wild-Type and INF1-Deficient Strains on Potato (*S. tuberosum*) and *Nicotiana* Species.

(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

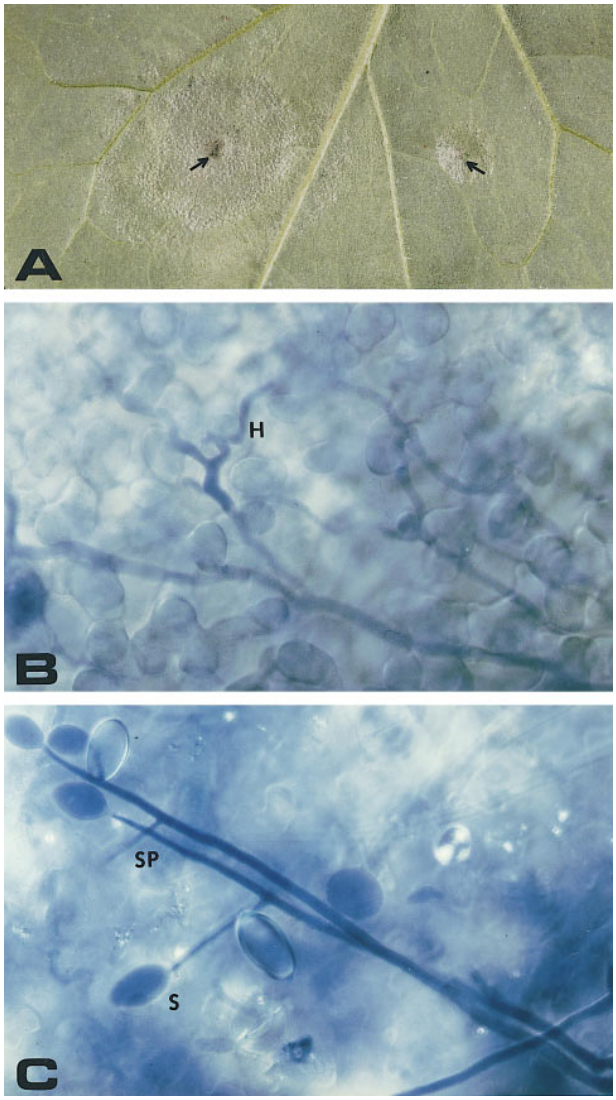

Figure 4. *N. benthamiana* Leaves Infected with an *inf1*-Silenced Strain.

(A) An *N. benthamiana* leaf 4 days after inoculation with the *P. infestans* recipient wild-type strain 88069 (right) and the INF1-deficient strain PY23 (left). Arrows indicate inoculation sites. Note the expanding lesion area caused by PY23 surrounding the inoculation spot with sporulation visible as a grayish white zone and the local necrosis (HR) caused by 88069. The dried inoculation droplet is visible as a mirroring area around the inoculation spot of 88069.
(B) and **(C)** Trypan blue–stained *N. benthamiana* leaf discs 70 hr after inoculation with *P. infestans inf1* mutant PY37 showing biotrophic growth and sporulation. Compare with Figure 1C. H, hyphae; S, sporangia; SP, sporangiophores.

(Figures 4B and 4C), extensive biotrophic colonization of the mesophyll by intercellularly growing hyphae with haustoria 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 interactions between *Phytophthora* and plants (Judelson, 1996, 1997). Elicitins, a family of host-specific elicitor proteins of *Phytophthora*, induce the HR on particular plant species, most notably tobacco and other *Nicotiana* species in the Solanaceae family. Using a single-step transformation procedure with an antisense construct of the *inf1* elicitin gene, we engineered stable *P. infestans* strains deficient in the production of the INF1 elicitin. Based on the observed increased virulence of these INF1-deficient strains on the plant species *N. benthamiana*, we conclude that the recognition of INF1 is a major determinant of the resistance response of *N. benthamiana* to *P. infestans.* These results directly demonstrate the role of a particular molecule in the host specificity of *Phytophthora* and suggest that elicitins are avirulence factors that condition resistance at the species level.

In this study, all of the interactions that we examined between *P. infestans* isolates and *Nicotiana* species resulted in resistance. On the five tested *Nicotiana* species, *P. infestans* zoospores encysted, germinated, and formed appressoria and penetration pegs. Resistance reactions occurred after penetration of an epidermal cell and were always associated with an HR of epidermal and mesophyll cells. This suggests that the interaction of *Nicotiana* species with *P. infestans* follows the classic model of pathogen elicitor recognition by a plant receptor and the subsequent activation of signal transduction pathways leading to an HR. A candidate elicitor is the INF1 elicitin protein (Kamoun et al., 1997a), which induces an HR on all of the examined *Nicotiana* species but not on the host plants potato and tomato and which supposedly interacts specifically with a high-affinity binding site in the tobacco plasma membrane, as was shown for the *P. cryptogea* elicitin, cryptogein (Wendehenne et al., 1995).

Even though the HR was always associated with the resistance response of *Nicotiana* to *P. infestans*, the timing, severity, and extent of the HR varied considerably, depending on the examined genotype. Similarly, wild-type *P. infestans* reached different levels of colonization on different *Nicotiana* plants. In tobacco, *P. infestans* was blocked early in the infection after penetration of the epidermal cell, and secondary intercellular hyphae were never observed. In contrast, in *N. benthamiana*, secondary hyphae with haustoria were formed, and some level of mesophyll colonization occurred. Plant response reached a climax 3 days after inoculation, and clusters of HR cells engulfing the invading hyphae were formed, correlating with the cessation of further *P. infestans* ingress. These observations suggest that several layers of resistance to *P. infestans* occur with various degrees of effectiveness in different *Nicotiana* species. Similar variation in the phenotype of plant resistance to pathogens was observed in interactions between Arabidopsis and the biotrophic oomycetes *Peronospora parasitica* and *Albugo candida* (Holub et al., 1995; Reignault et al., 1996). 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 (S. 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 (Baillieul 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 elicitins (discussed in Yu, 1995; Kamoun et al., 1997b). It has been suggested by others that elicitins may be nonspecific 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 nonhosts of *P. infestans.* Contrary to the assumption that nonhost 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 elicitins. 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

zoospores for plant inoculations, sporulating mycelia in rye sucrose 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.

Plasmid Construction and Transformation of *P. infestans*

Plasmid pHIN28, which contains the *inf1* coding sequence in antisense orientation fused to the *ham34* promoter of *Bremia lactucae* (Judelson and Michelmore, 1991), was constructed by generating a 390-bp polymerase chain reaction fragment of the *inf1* coding sequence with primers PIET28 (5′-TATCGGTACCCACTCCTCCTCACTC-3′) and PIET29 (5′-CGGCCCATGGACGCTGACTC-3′). The amplified fragment was digested with KpnI and NcoI and inserted into NcoI- and KpnI-digested pHAMT35G (Judelson and Michelmore, 1991). Stable cotransformation was conducted according to van West et al. (1998) by using EcoRI-linearized pH IN28 and the selection plasmid pTH209 (geneticin resistance) (Judelson et al., 1991). Polymerase chain reaction amplification of DNA from individual transformants by using the M13/pUC reverse primer (Gibco BRL) in combination with PIET28 was conducted to discriminate between cotransformants (amplification of a fragment of the expected size) and pTH209 transformants (no amplification product).

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 (Unkles et al., 1991), and the *ham34* promoter (Judelson and Michelmore, 1991) were used as probes and radiolabeled with α^{32}P-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 Merril 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 in 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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