Differences in Intensity and Specificity of Hypersensitive Response Induction in *Nicotiana* spp. by INF1, INF2A, and INF2B of *Phytophthora infestans*

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Elicitins form a family of structurally related proteins that induce the hypersensitive response (HR) in plants, particularly *Nicotiana* spp. The elicitin family is composed of several classes. Most species of the plant-pathogenic oomycete genus *Phytophthora* produce the well-characterized 10-kDa canonical elicitins (class I), such as INF1 of the potato and tomato pathogen *Phytophthora infestans*. Two genes, *inf2A* and *inf2B*, encoding a distinct class (class III) of elicitin-like proteins, also occur in *P. infestans*. Unlike secreted class I elicitins, class III elicitins are thought to be cell-surface-anchored polypeptides. Molecular characterization of the *inf2* genes indicated that they are widespread in *Phytophthora* spp. and occur as a small gene family. In addition, Southern blot and Northern blot hybridizations using gene-specific probes showed that *inf2A and inf2B* genes and transcripts can be detected in 17 different *P. infestans* isolates. Functional secreted expression in plant cells of the elicitin domain of the *inf1* and *inf2* genes was conducted using a binary *Potato virus X* (PVX) vector (agroinfection) and *Agrobacterium tumefaciens* transient transformation assays (agroinfiltration), and resulted in HR-like necrotic symptoms and induction of defense response genes in tobacco. However, comparative analyses of elicitor activity of INF1, INF2A, and INF2B revealed significant differences in intensity, specificity, and consistency of HR induction. Whereas INF1 induced the HR in *Nicotiana benthamiana*, INF2A induced weak symptoms and INF2B induced no symptoms on this plant. Nonetheless, similar to INF1, HR induction by INF2A in *N. benthamiana* required the ubiquitin ligase-associated protein SGT1. Overall, these results suggest that variation in the resistance of *Nicotiana* spp. to *P. infestans* is shadowed by variation in the response to INF elicitins. The ability of tobacco, but not *N. benthamiana*, to respond to INF2B could explain differences in resistance to *P. infestans* observed for these two species.

Specific recognition events are well established as the functional basis of numerous incompatible (resistance) interactions between plants and pathogens, particularly those occurring at the subspecific or varietal level (race- or cultivar-specific resistance). Recognition is defined by the direct or indirect perception of pathogen signal molecules by plant receptors (Dangl and Jones 2001; Staskawicz et al. 1995). The pathogen signal

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molecules commonly are referred to as elicitors, encoded by avirulence (*Avr*) genes, whereas the plant receptors are the resistance proteins encoded by *R* genes. Recognition results in the induction of signal transduction pathways leading to the expression of complex defense responses, including the hypersensitive response (HR), a form of programmed cell death often associated with disease resistance in plants (Dangl et al. 1996). Numerous examples of race- or cultivar-specific interactions follow the *Avr-R* gene model; however, the extent to which recognition events are involved in incompatible interactions occurring at the species or genus level (nonhost resistance) remains unclear (Heath 2000; Kamoun 2001; Kamoun et al. 1999c).

The oomycete plant pathogen *Phytophthora infestans* causes late blight, a devastating and re-emerging disease of potato and tomato (Birch and Whisson 2001; Fry and Goodwin 1997a,b; Schiermeier 2001; Shattock 2002; Smart and Fry 2001). In contrast to host plants, nonhosts, such as tobacco and other species of the genus *Nicotiana*, typically are resistant to *P. infestans*. Cytological analyses of leaves of several *Nicotiana* spp. inoculated with *P. infestans* showed that penetration of epidermal cells always occurred (Kamoun et al. 1998c). This was followed by the HR that varied between different *Nicotiana* spp. in timing, severity, and number of affected cells. In *Nicotiana tabacum* (tobacco), *P. infestans* was blocked early in the infection following penetration of epidermal cells, and secondary intercellular hyphae were not observed. In contrast, in *N. benthamiana*, secondary hyphae with haustoria were formed and some level of mesophyll colonization occurred. The plant response reached a climax 3 days post inoculation with clusters of HR cells engulfing the invading hyphae. These observations suggest that several layers of resistance to *P. infestans* occur with various degrees of effectiveness in the different *Nicotiana* species (Kamoun 2001; Kamoun et al. 1998b; Kamoun et al. 1999c).

P. infestans and other *Phytophthora* spp. express a family of structurally related extracellular proteins, known as elicitins, which induce the HR and other biochemical changes associated with defense responses in *Nicotiana* spp. but not in potato and tomato (Kamoun et al. 1993, 1997a; Ponchet et al. 1999; Ricci et al. 1989; Sasabe et al. 2000). *P. infestans* strains deficient in the elicitin INF1 induced disease lesions in *N. benthamiana*, suggesting that INF1 conditions resistance in this species (Kamoun et al. 1998b). In contrast, INF1-deficient strains remained unable to infect other *Nicotiana* spp., such as tobacco. In this case, tobacco was hypothesized to react to additional elicitors, perhaps other elicitin-like proteins (Kamoun

2001; Kamoun et al. 1998b, 1999c). Indeed, in *P. infestans*, a complex set of elicitin-like genes was isolated using polymerase chain reaction (PCR) amplification with degenerate primers, low stringency hybridizations, and random sequencing of cDNAs (Fabritius et al. 2002; Kamoun et al. 1997a, and b, 1999b). In total, eight elicitin and elicitin-like genes (termed *inf* genes) have been reported so far in *P. infestans*. All these genes encode putative extracellular proteins that share the 98-amino-acid (aa) elicitin domain corresponding to the mature class I elicitins, such as INF1. This domain is defined as the elicitin domain in many protein motif databases, such as pfam (PF00964) (Bateman et al. 2002) and InterPro (IPR002200) (Mulder et al. 2003). Six *inf* genes (*inf2A*, *inf2B*, *inf5*, *inf6*, *inf7*, and *M-25*) encode predicted proteins with a C-terminal domain in addition to the N-terminal elicitin domain. Sequence analysis of these C-terminal domains revealed a high frequency of serine, threonine, alanine, and proline. The amino-acid composition and the distribution of these four residues indicated the likely occurrence of clusters of *O*-linked glycosylation sites (Kamoun et al. 1997a). These proteins are likely to form a "lollipop on a stick" structure in which the *O*-glycosylated domain forms an extended rod that anchors the protein to the cell wall, leaving the extracellular N-terminal domain exposed

on the cell surface (Jentoft 1990). Therefore, these atypical INF proteins may be surface- or cell-wall-associated glycoproteins that could interact with plant cells during infection.

The intrinsic biological function of elicitins in *Phytophthora* spp. has long remained a mystery. Conclusive evidence finally emerged when it was demonstrated that class I elicitins bind sterols, such as ergosterol, and function as sterol-carrier proteins (Boissy et al. 1999; Mikes et al. 1997, 1998; Vauthrin et al. 1999). Consequently, elicitins were hypothesized as having a biological function of essential importance to *Phytophthora* spp. because they cannot synthesize sterols and must assimilate them from external sources (Hendrix 1970). In addition, phospholipase activity was assigned to elicitin-like proteins from *P. capsici* with significant similarity to INF5 and INF6 (Nespoulous et al. 1999), suggesting a general lipid binding or processing role for the various members of the elicitin family (Osman et al. 2001a). Other work by Osman and associates (2001b) using elicitin mutants altered in sterol binding revealed that sterol loading is important for specific-binding to a plasma membrane receptor and induction of the HR in tobacco. More recently, another gene with similarity to elicitins, *M-25*, was reported to be induced during mating in *P. infestans* (Fabritius et al. 2002).

In this article, we report the molecular and functional characterization of *P. infestans* genes encoding the class III elicitin-like INF2A and INF2B proteins (Kamoun et al. 1997a). We examined the occurrence of *inf2* sequences in *P. infestans* and other *Phytophthora* spp., the full genomic sequence of the *inf2A* gene, and the expression of the *inf2* genes in various isolates of *P. infestans* and during the *P. infestans*–tomato interaction. In addition, we compared INF2 proteins to the well-characterized INF1 elicitin for their elicitor activity using both the binary *Potato virus X* (PVX) expression system (agroinfection) and *Agrobacterium tumefaciens* transient transformation assays (agroinfiltration). Last, we characterized the defense responses induced by INF2 in *N. tabacum* and showed that, similar to INF1, necrosis induction by INF2A in *N. benthamiana* requires the ubiquitin ligase-associated protein SGT1. These experiments revealed significant differences in intensity, specificity, and consistency of HR induction among INF1, INF2A, and INF2B. We found that tobacco, but not *N. benthamiana*, responded to INF2B. This could explain differences in resistance to *P. infestans* observed for these two species.
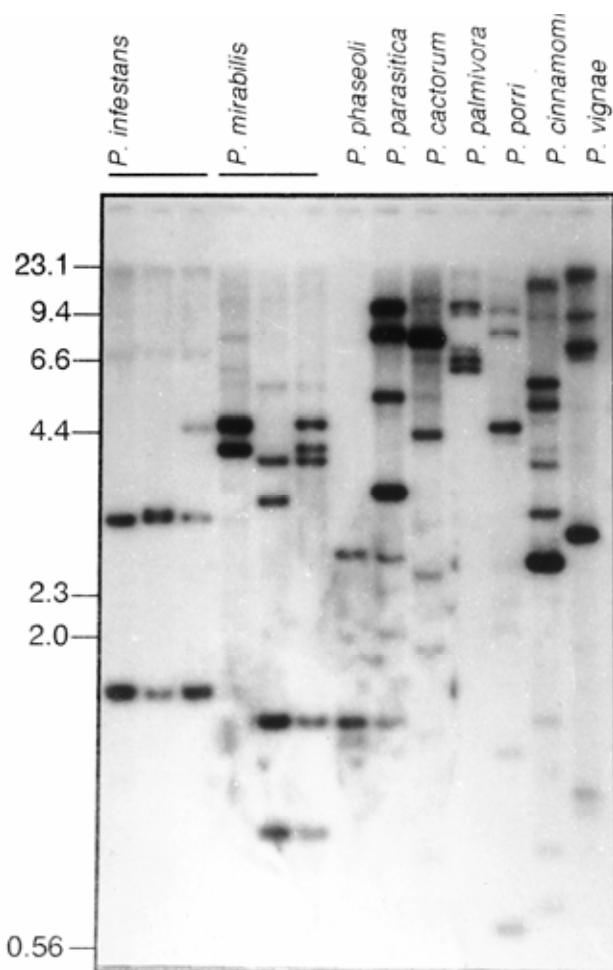
RESULTS

Occurrence of *inf2* sequences in *Phytophthora* spp.

The products of the *inf2A* and *inf2B* genes form a distinct class of elicitins previously designated class III (Kamoun et al. 1997a). In order to assess the occurrence and distribution of sequences similar to *inf2* across a range of *Phytophthora* spp., total DNA from 13 isolates representing nine *Phytophthora* spp. was hybridized at low stringency with a probe from the elicitin domain of *inf2* (Fig. 1). This *inf2* probe hybridized to both *inf2A* and *inf2B*; however, under similar hybridization conditions, no cross-hybridization between this probe and other *P. infestans inf* elicitin genes was observed. All tested isolates of the examined *Phytophthora* spp. appeared to contain from two to eight *Hin*dIII bands homologous to the *inf2* elicitin domain (Fig. 1). Similar hybridization experiments on total DNA from four additional oomycete species (*Pythium aphanidermatum*, isolate 28; *P. sylvaticum*, 933; *Aphanomyces leavis*, 465.64; and *Saprolegnia ferax*, G-1295) did not yield any detectable signals (data not shown). Therefore, it appears that *inf2*-like elicitin genes may occur as a small genus-specific gene family and are conserved in all tested species of *Phytophthora*.

Fig. 1. Occurrence of *inf2* sequences in *Phytophthora* spp. DNA blot containing 20 μm of *Hin*dIII-digested total DNA from 13 isolates representing nine *Phytophthora* spp. (i.e., *P. infestans* isolates 88069, 90128, and IPO-0; *P. mirabilis* CBS 678.85, CBS136.86, and CBS150.88; *P. phaseoli* CBS 556.88; *P. parasitica* 18; *P. cactorum* 436; *P. palmivora* 10; *P. porri* HH; *P. cinnamomi* 2; and *P. vignae* 20853) was hybridized with a probe from the elicitin domain of *inf2* which hybridizes to both *inf2A* and *inf2B*. Molecular marker sizes are shown on the left in kilobases.

Isolation and characterization of *inf2A* genomic region.

To determine the genomic structure of the *inf2A* gene, a λEMBL3 genomic library of *Phytophthora infestans* 88069 (Pieterse et al. 1993) was hybridized with the *inf2* probe. A total of five hybridizing clones were identified. DNA from these clones was digested with *Hin*dIII, blotted, and hybridized with the *inf2* probe (data not shown). Three of the clones contained a 1.7-kb *Hin*dIII hybridizing band that co-migrated with one of the bands revealed on the total DNA blot (Fig. 1, lane 1). The other two positive clones were not reconfirmed in subsequent hybridizations. The 1.7-kb *Hin*dIII fragment was subcloned into pBluescript SK– and fully sequenced using a primer walking approach. The nucleotide sequence revealed a 1,654-bp *Hin*dIII fragment (GenBank accession number AY693804) and was found to contain a 558-bp open reading frame (ORF) that perfectly matched the ORF in the *inf2A* cDNA sequence, suggesting that, similar to other elicitin genes from *Phytophthora* spp., the *inf2A* gene does not contain introns. Examination of the nucleotide sequence upstream of the ORF revealed, at position –50 relative to the ATG start codon, sequence TCTCATT CTACAATTT, similar to the oomycete transcriptional start site motif (Kamoun 2003; McLeod et al. 2004; Pieterse et al. 1993). Downstream of the ORF, the 51-bp sequence that corresponded to the 3′ untranslated region contained a potential polyadenylation signal ATTAAA, located 18 bp downstream of the TAA stop codon. No significant similarities between the noncoding sequences of the *inf2A* gene and the noncoding sequences of other elicitin genes were noted. In this screening, no genomic clone corresponding to *inf2B* was recovered from the genomic library.

Occurrence of *inf2A* and *inf2B* in *P. infestans*.

To determine whether the *inf2A* and *inf2B* genes are conserved in *P. infestans*, *Bam*HI-digested total DNA from a collection of 16 isolates of *P. infestans* (Kamoun et al. 1998a) was sequentially hybridized with gene-specific probes containing 3′ end portions of the *inf2A* and *inf2B* cDNAs as well as a specific 3′ end probe of the *inf1* cDNA (Kamoun et al. 1997a) (Fig. 2). All probes lack a *Bam*HI site. One to two genomic copies for each of the *inf2A* and *inf2B* genes could be detected in all 16 *P. infestans* isolates examined, whereas a single *inf1* band was revealed. In some isolates, both the *inf2A* and *inf2B* probes revealed bands with lower intensity. No cross-hybridization was noted between the *inf2* probes and other *inf* elicitin genes under the hybridization conditions used; therefore, we expected these bands to contain *inf2*-like sequences. However, we cannot conclude at this stage whether the faint bands correspond to additional alleles or gene copies of *inf2* or to pseudogene sequences. GE900083, an isolate from Germany, lacked the strongly hybridizing *inf2A* band observed in all other isolates.

inf2A and inf2B mRNAs are produced by all tested *P. infestans* isolates.

A small number of field isolates of *P. infestans* are deficient in mRNA of the elicitin gene *inf1* and in INF1 protein (Kamoun et al. 1998a). To determine whether these and other isolates show altered levels of *inf2A* and *inf2B* mRNA, total RNA from the 16 isolates of *P. infestans* examined in Figure 2, as well as *P. infestans* 88069, was sequentially hybridized with the *inf2A*, *inf2B*, and *inf1* gene-specific probes (Fig. 3). All tested isolates showed detectable levels of *inf2A* and *inf2B* mRNA, suggesting that *inf2* mRNAs are produced by all tested *P. infestans* isolates, including the two isolates DDR7602 and DDR7702 that previously were shown to lack *inf1* mRNA (Kamoun et al. 1998a). However, in this experiment, levels of *inf2* mRNA were variable between the examined isolates. GE900083, the isolate that

lacked the major *inf2A* band in the Southern blot analyses, also produced a signal for *inf2A* mRNA.

inf2A and inf2B are expressed during the *P. infestans*-tomato interaction.

We determined the expression profiles of *inf2A* and *inf2B* genes during a time course infection of tomato by *P. infestans* using semi-quantitative reverse transcription (RT)-PCR. Gene-specific primers for *inf2A*, *inf2B*, *inf1*, and the constitutive elongation factor 2-α (*ef2α*) gene were used. Expression of *inf2A* was detected 3 days after inoculation, whereas expression of *inf1* and *inf2B* was observed as early as 1 day after inoculation (Fig. 4). In contrast to *inf1*, which reached the highest levels of expression at the latest time point (day 4), *inf2A* expression peaked at day 3 and *inf2B* at days 3 and 4. These results show that both *inf2* genes are expressed during *P. infestans* colonization of tomato.

Heterologous expression of *inf2A* and *inf2B* in plants using agroinfection of PVX.

To express the *inf2* genes in plant cells and examine their elicitor activity, we first used the PVX system, which proved effective in assaying the HR-inducing activity of the *inf1* gene (Kamoun et al. 1999a; Qutob et al. 2002; Torto et al. 2003). A fusion between the signal sequence of the *PR-1a* gene of tobacco (Hammond-Kosack et al. 1995) and the sequence of the 98-aa elicitor domain of INF2A and INF2B (Kamoun et al. 1997a) was cloned into in the binary PVX vector pGR106 (Lu et al. 2003). Two recombinant plasmids, pGR106-INF2A and pGR106-INF2B, were confirmed to contain the correct inserts by DNA sequencing and subsequently were introduced into *Agrobacterium tumefaciens* to allow delivery of PVX in plants via agroinfection. *A. tumefaciens* strains carrying the pGR106-
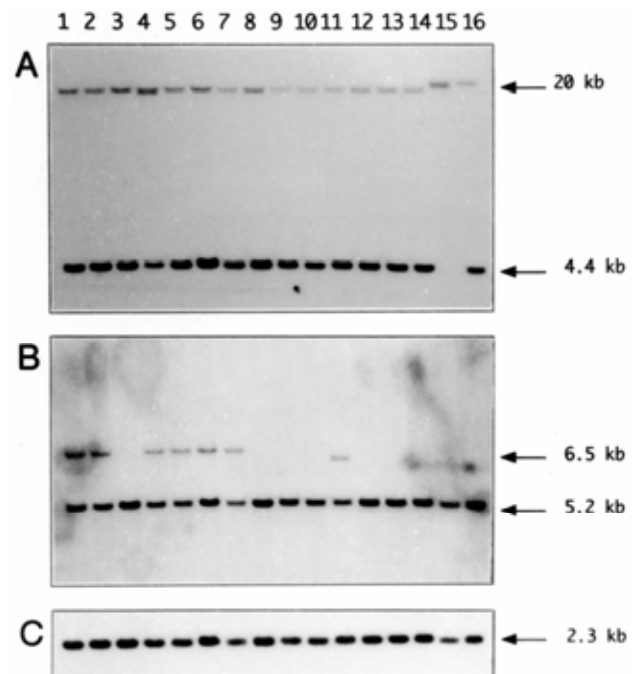

Fig. 2. Occurrence of the *inf2A, inf2B,* and *inf1* sequences in isolates of *Phytophthora infestans*. DNA blot containing *Bam*HI-digested total DNA from *P. infestans* isolates (1) DDR7601, (2) DDR7602, (3) DDR7702, (4) GER7401, (5) GER8451, (6) GER8501, (7) GER8601, (8) 46210, (9) 66006, (10) 68308, (11) 70001, (12) UK7225, (13) UK7818, (14) IT8001, (15) GE900083, and (16) GE900089 (Kamoun et al. 1998a) was hybridized with specific probes from the **A,** *inf2A,* **B,** *inf2B,* and **C,** *inf1* genes. The approximate sizes of the hybridizing bands are shown on the right in kilobases.

INF2 constructs were inoculated side-by-side on mature leaves of *N. tabacum* (tobacco, cv. Xanthi) and *N. benthamiana* and compared with a strain carrying a pGR106 derivative expressing the *PR1a*::*inf1* construct (pGR106-INF1) (Fig. 5). In tobacco, all three strains induced rapid symptoms consisting of localized HR-like necrotic lesions. In contrast, *N. benthamiana* leaves challenged with the pGR106-INF1 and pGR106-INF2A strains exhibited localized necrotic lesions, whereas the pGR106-INF2B strain failed to cause visible symptoms (Fig. 5).

In addition to these qualitative differences, there was significant variation in the efficiency and extent of elicitation of necrotic symptoms between the elicitins (Table 1). On responding plants, the pGR106-INF1 and pGR106-INF2B strains were very consistent, resulting in necrotic symptoms in at least 93% of the inoculation sites (Table 1). In contrast, the pGR106-INF2A strain was poorly efficient, resulting in necrotic symptoms in 36 and 70% of the inoculation sites on tobacco and *N. benthamiana*, respectively. Also, the pGR106-INF2A strain induced smaller necrotic lesions averaging 0.9 and 1.9 mm in tobacco and *N. benthamiana*, respectively, whereas the pGR106-INF1 and pGR106-INF2B strains induced lesions ranging from 3.2 to 5.8 mm in size on responding plants. Inoculations of all plants with mock and vector controls never resulted in necrotic symptoms.

To determine the significance of our observations, statistical analysis was performed on necrotic lesion size data. Analysis of variance using the generalized linear models procedure and subsequent *t* test comparisons indicated significant differences between the elicitors tested on both *N. benthamiana* and *N. tabacum* (Table 1).

To confirm the lack of response of *N. benthamiana* to INF2B, we repeated the agroinfection inoculations on individual young seedlings, an assay that is more sensitive than mature leaf inoculations because it allows systemic spread of the recombinant PVX and enhanced accumulation of the *inf* transcripts (Torto et al. 2003). Inoculation of *N. benthamiana* seedlings with the pGR106-INF2B strain consistently failed to result in any necrotic symptoms and always resulted in mosaic virus symptoms similar to those obtained with the empty vector strain. In contrast, both the pGR106-INF1 and pGR106-INF2A strains induced necrotic lesions starting 6 days after inoculation (data not shown).

Together, these results show that, unlike tobacco, *N. benthamiana* does not respond to INF2B, and suggest that INF2A may constitute a weaker HR elicitor than INF1 and INF2B.

Heterologous expression of *inf2A* and *inf2B* in plants using agroinfiltration.

To validate the elicitor activity of INF2A and INF2B and further compare it to INF1, we used agroinfiltration to express the three *inf* genes in plant cells. The *PR1a*::*inf* gene fusions were transferred to a *Cauliflower mosaic virus* (CaMV) 35S promoter and a potato proteinase-II terminator cassette in a T-DNA binary vector as described in the methods. *A. tumefaciens*
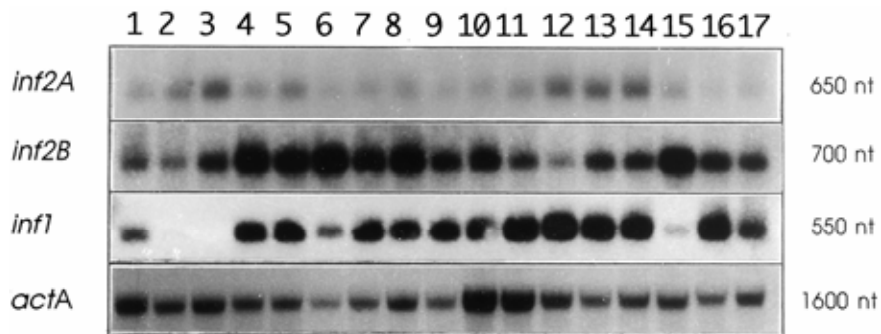

Fig. 3. Occurrence of the *inf2A, inf2B, and inf1* mRNA in isolates of *Phytophthora infestans*. RNA blots containing total RNA from DDR7601 (1), DDR7602 (2), DDR7702 (3), GER7401 (4), GER8451 (5), GER8501 (6), GER8601 (7), 46210 (8), 66006 (9), 68308 (10), 70001 (11), UK7225 (12), UK7818 (13), IT8001 (14), GE900083 (15), GE900089 (16) and 88069 (17) were hybridized with specific probes from the *inf2A, inf2B, inf1*, and *actA* genes. The approximate sizes of the *inf* and *actA* transcripts are shown on the right.
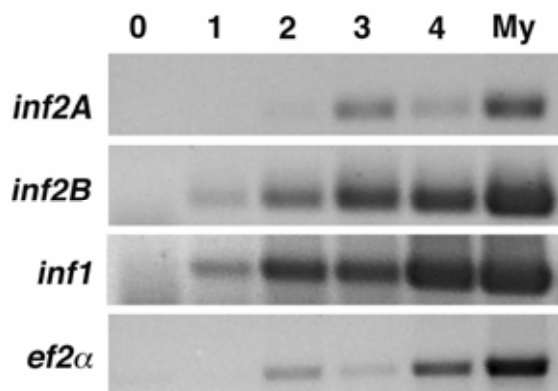

Fig. 4. Expression of *Phytophthora infestans inf2A*, *inf2B*, and *inf1* during infection of tomato. Total RNA from *P. infestans*-infected leaves of tomato 0, 1, 2, 3, and 4 days after inoculation, and *P. infestans* mycelium (My) grown in a synthetic medium were used in reverse-transcription polymerase chain reaction amplifications as described in the text. Amplification of the *P. infestans* elongation factor 2 (*ef2α*) was used as a control to determine the integrity of the RNA.
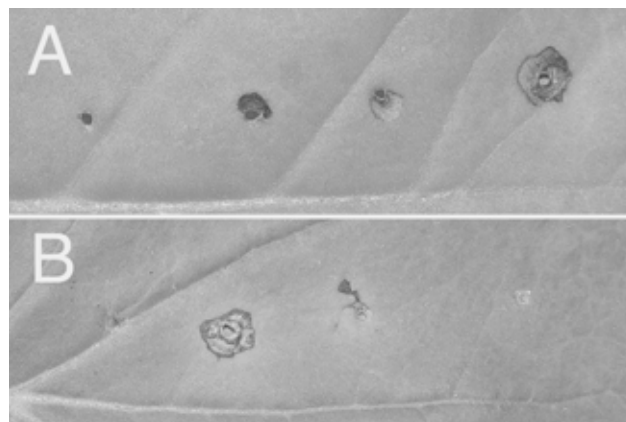

Fig. 5. Agroinfection assays. Symptoms observed on **A,** *Nicotiana tabacum* (tobacco) and **B,** *N. benthamiana* leaves after inoculation with *Agrobacterium tumefaciens* containing the binary *Potato virus X* (PVX) vector expressing *Phytophthora infestans inf* genes. Inoculated leaves were photographed 10 days after inoculation with *A. tumefaciens* containing, from left to right, the binary PVX vector pGR106, pGR106-INF1, pGR106-INF2A, and pGR106-INF2B.

strains carrying the various p35S-INF constructs were infiltrated into young and fully expanded leaves of N. tabacum (cv. Xanthi) and *N. benthamiana* (Fig. 6). As negative controls, *A. tumefaciens* carrying pGUSi, which contains a β-glucuronidase gene interrupted by an intron (Hood et al. 1993), as well as buffer solutions were used. In *N. tabacum*, confluent necrosis in the entire infiltrated areas appeared 2 days following infiltration of the p35S-INF2A-, p35S-INF2B-, and p35S-INF1-carrying strains. In contrast, in *N. benthamiana*, only the *A. tumefaciens* strain carrying p35S-INF1 consistently induced necrosis, generally starting at 3 to 4 days after infiltration. In repeated side-by-side infiltrations of *N. benthamiana* leaves with the p35S-INF1 and p35S-INF2 strains, the p35S-INF2B strain did not induce necrotic symptoms. However, occasionally, the p35S-INF2A construct caused necrosis in *N. benthamiana*. The negative control strain carrying pGUSi and the buffer solutions did not induce necrosis in both tobacco and *N. benthamiana*. These results confirm that *N. benthamiana* does not respond to INF2B and that INF2A may act as a weaker HR elicitor on this plant species.

INF2B induces *PR1a* and *Bgl2* expression in tobacco.

To assess whether the necrotic response elicited by the INF2 proteins is associated with the induction of plant defense response genes, we wound inoculated leaves of a transgenic tobacco line carrying the *GUS* reporter gene driven by the promoter of the pathogenesis-related genes *Bgl2* (*PR2*) (Livne et al. 1997) with *A. tumefaciens* strains carrying pGR106-INF1, pGR106-INF2A, and pGR106-INF2B (Fig. 7A). Negative controls consisted of the *A. tumefaciens* strain carrying the vector pGR106 and mock inoculations. GUS histochemical staining of inoculated leaves showed some blue staining in the pGR106 treatment, suggesting that the vector strain induces low levels of *Bgl2* expression. However, the pGR106-INF1 strain and, particularly, the pGR106-INF2B strain consistently induced stronger and larger areas of GUS staining than the vector control in 31 and 38% of the inoculation sites, respectively. The pGR106-INF2A strain did not consistently induce different GUS staining than the controls.

We also performed Northern blot analyses using RNA isolated from leaf discs surrounding inoculation sites of a non-

Table 1. Recombinant *Potato virus X* expressing *inf* elicitin genes induce variable levels of necrosis on *Nicotiana tabacum* and *N. benthamiana*[z]

	N. tabacum		N. benthamiana	
Construct	Inoculations with necrosis (%)	Necrotic lesion size (mm)	Inoculations with necrosis (%)	Necrotic lesion size (mm)
Mock	0	0	0	0
pGR106	0	0	0	0
pGR106-INF1	93	3.2 a	100	5.8 a
pGR106-INF2A	36	0.9 b	70	1.9 b
pGR106-INF2B	100	4.9 c	0	0 c

[z] Results were obtained from one representative experiment. A total of 14 inoculation sites were analyzed for *N. tabacum* and 20 for *N. benthamiana*. Lesion size data was used in *t* test comparisons of treatments (*P* = 0.01). Mean separations were performed within each species. Results followed by different letters are significantly different.
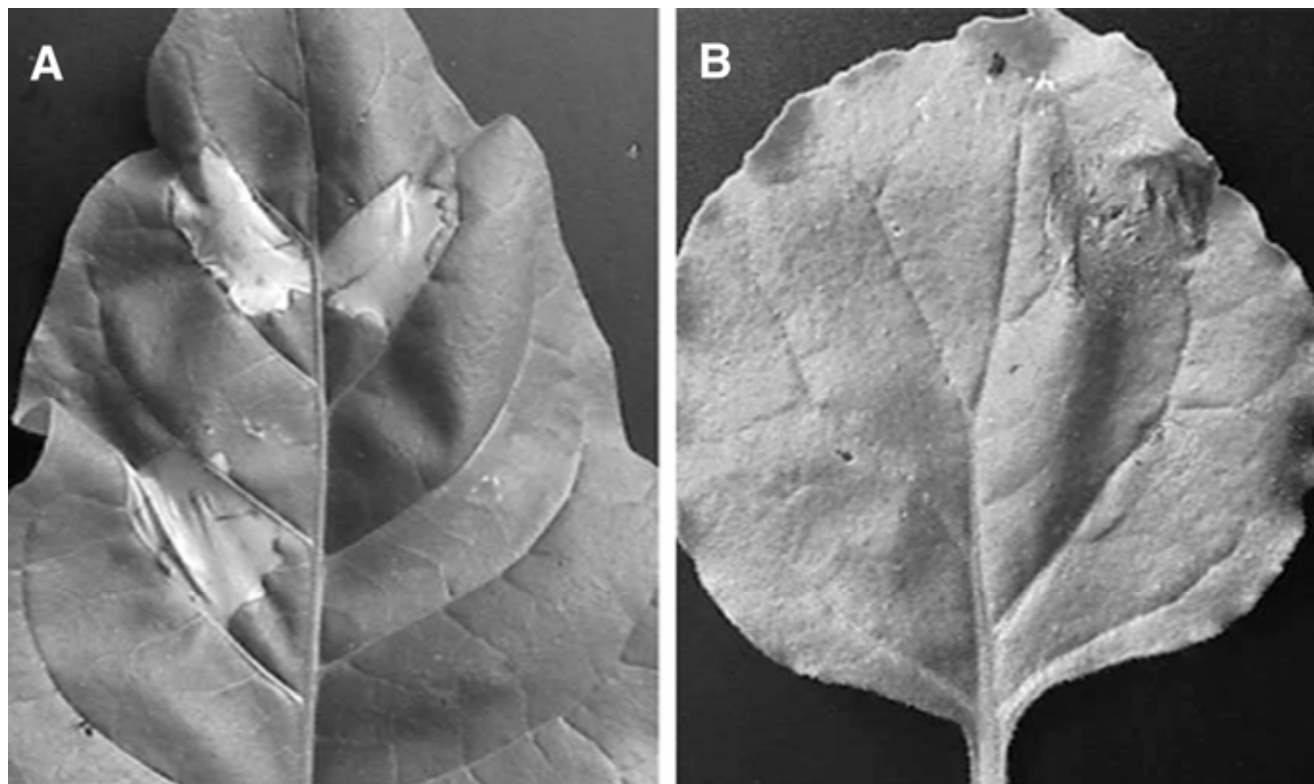

Fig. 6. Agroinfiltration assays. Symptoms observed on *Nicotiana tabacum* (tobacco) (left panel) and *N. benthamiana* (right panel) leaves after infiltration with *Agrobacterium tumefaciens* containing binary vectors expressing *Phytophthora infestans inf* genes. Inoculated leaves were photographed 6 days after inoculation with *A. tumefaciens* containing the binary vector p35S-INF2A (top left section of the leaves), p35S-INF2B (bottom left), p35S-INF1 (top right), and the negative control pGUSi (bottom right).

transgenic tobacco line using the same treatments as in the *Bgl2::GUS* experiment (Fig. 7B). Hybridization of the blots with probes of the pathogenesis-related gene *PR1a* and the constitutive gene α-tubulin revealed moderate induction of *PR1a* by the vector construct. Nevertheless, both pGR106-INF1 and pGR106-INF2B elicited increased levels of *PR1a* expression. These experiments suggest that, similar to INF1, INF2B induces the expression of the pathogenesis-related genes *PR1a* and *Bgl2* in tobacco. However, no significant induction by INF2A could be demonstrated under these experimental conditions.

SGT1 is required for HR elicitation by INF2A.

Peart and associates (2002) demonstrated that the ubiquitin ligase-associated protein SGT1 is required for HR induction by INF1 in *N. benthamiana*. To test whether response to INF2A also requires SGT1, we used *Tobacco rattle virus* (TRV) to silence *SGT1* in *N. benthamiana* (Huitema et al. 2004; Peart et al. 2002; Ratcliff et al. 2001). For this purpose, we infiltrated young *N. benthamiana* plants (five-leaf stage) with *A. tumefaciens* strains containing the binary vector pBintra6 (TRV RNA1) mixed with strains carrying the empty pTV00 vector (TRV RNA2) or pTV00:SGT1 (Peart et al. 2002). Three weeks after infiltration, we performed challenge inoculations using agroinfiltration (Fig. 8A) or agroinfection (Fig. 8B) as described above. In both experiments, INF1 consistently induced the HR on plants inoculated with the TRV vector but not on the TRV:SGT1 plants. Similarly, INF2A induced the HR in 20 to 50% of the inoculations in plants treated with the TRV vector but not on the TRV:SGT1 plants. As noted earlier, INF2B did not induce necrosis in *N. benthamiana*. These results suggest that, similar to INF1, the HR induced by INF2A in *N. benthamiana* is SGT1-dependent.
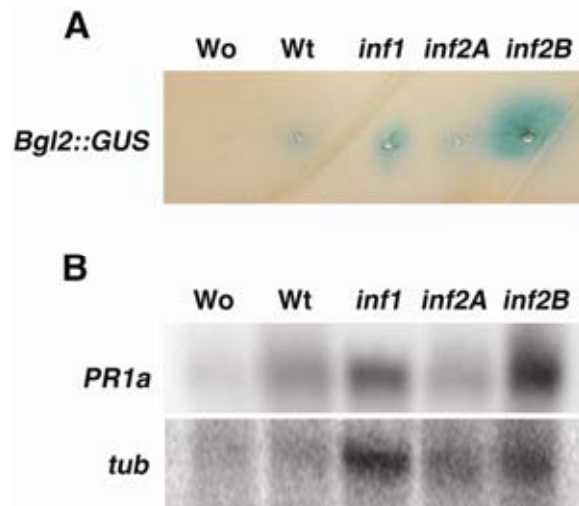

Fig. 7. Induction of defense response genes in tobacco by INF1 and INF2. **A,** Histochemical GUS assay of a leaf from transgenic tobacco line carrying a *Bgl2:GUS* construct. The leaf was wound-inoculated with a toothpick only (Wo) as well as with *Agrobacterium tumefaciens* strains carrying pGR106 (Wt), pGR106-INF1, pGR106-INF2A, and pGR106-INF2B. The picture illustrates a representative leaf stained 8 days after inoculation. B, Northern blot hybridization of RNA isolated from tobacco leaves (cv. Xanthi) that were wound-inoculated with a toothpick only (Wo) or toothpick inoculated with *A. tumefaciens* strains carrying pGR106 (Wt), pGR106-INF1, pGR106-INF2A, and pGR106-INF2B. The blot was hybridized with probes from the defense gene *PR1a* and the constitutive gene α-tubulin (*tub*). Total RNA was harvested from leaf discs surrounding the inoculation sites immediately after the onset of necrosis. Different leaves were used for the different treatments.

DISCUSSION

Elicitins form a ubiquitous family of structurally related proteins in *Phytophthora* spp. In *P. infestans*, eight elicitin and elicitin-like genes (*inf* genes) corresponding to distinct classes have been reported (Fabritius et al. 2002; Kamoun et al. 1997a, and b, 1999b). So far, most studies on *P. infestans* elicitins have focused on the 98-aa canonical elicitin, INF1 (class Ia) (Kamoun et al. 1997a; 1998a, and b, 1999b; Kanzaki et al. 2003; Sasabe et al. 2000; Sharma et al. 2003). In this article, we report the molecular and functional characterization of the *inf2* class (class III) of elicitin-like genes from *P. infestans* (Kamoun et al. 1997a). Our main finding is that variation in the resistance of *Nicotiana* spp. to *P. infestans* is shadowed by variation in the response to INF elicitins. The ability of tobacco, but not *N. benthamiana*, to respond to INF2B could explain differences in resistance to *P. infestans* observed for these two species.

Despite the rapid accumulation of sequence data from numerous organisms, elicitin-like genes and proteins have been identified only in the oomycete genera, *Phytophthora* and *Pythium*. In the genus *Phytophthora*, production of the 10-kDa class I elicitins is quasi-ubiquitous and has been attributed to more than 30 species so far (Kamoun et al. 1994; Ponchet et al. 1999). The Southern blot hybridizations illustrated in Figure
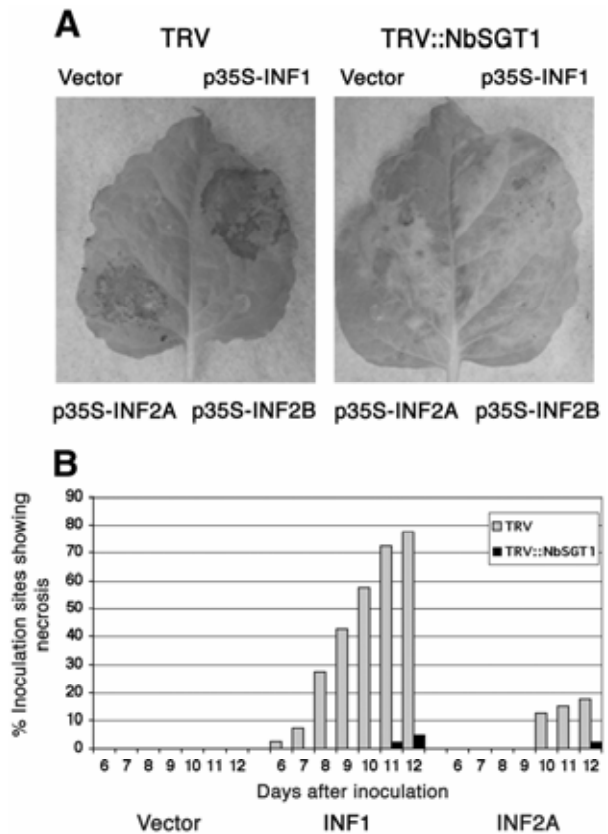

Fig. 8. Symptoms observed in *Nicotiana benthamiana* silenced for the ubiquitin ligase-associated gene *SGT1* following agroinfiltration and *Potato virus X* (PVX) agroinfection with *inf1* and *inf2* constructs. *N. benthamiana* plants were first inoculated with *Agrobacterium tumefaciens* carrying the *Tobacco rattle virus* vector (TRV) or a TRV:SGT1 construct (TRV:SGT1), and then challenged after 3 weeks with **A,** *A. tumefaciens* carrying vector (top left), p35S-INF1 (top right), p35S-INF2A (bottom left), and p35S-INF2B (bottom right) constructs, or **B,** *A. tumefaciens* carrying the binary PVX vector pGR106, pGR106-INF1, and pGR106-INF2A. Leaves in panel A were photographed 5 days after the secondary agroinfiltration. The bars in panel B correspond to the percentage of *A. tumefaciens* binary PVX inoculation sites showing the hypersensitive response over time (*n* = 40).

1 suggest that the *inf2* class of elicitin-like genes is similarly widespread because *inf2*-like sequences were detected in all nine *Phytophthora* spp. examined. In addition, we also identified sequences highly similar to *inf2* by searching the expressed sequence tag (EST) database of *P. sojae* (Qutob et al. 2000, 2003). These results indicate that the *inf2* class of elicitin-like genes occurs as a small conserved family in *Phytophthora* spp.

Using Southern blot analyses with gene-specific probes, one to two genomic copies of the *inf2A* and *inf2B* genes could be detected in 16 different *P. infestans* isolates. In addition, *inf2* mRNA was ubiquitously present in these *P. infestans* isolates, although at variable levels. The biological basis and significance of this variation remains unclear. The two *P. infestans* isolates previously described as naturally deficient in INF1 production (Kamoun et al. 1998a) were found to produce *inf2* mRNA. In addition, *inf1* sense and antisense transformants that showed no detectable levels of *inf1* mRNA (Kamoun et al. 1998b) were found to be unaltered in *inf2* mRNA using Northern blot hybridizations (van West et al. 1999). These results suggest that downregulation of *inf1* mRNA does not correlate with altered levels of *inf2* mRNA.

We monitored gene expression levels of both *inf2A* and *inf2B* during *P. infestans*–tomato interactions. Semiquantitative RT-PCR experiments revealed that both *inf2A* and *inf2B* are expressed during infection of tomato, indicating that these proteins are functionally relevant to *P. infestans* pathogenesis. Expression patterns of the *inf2* genes in planta were slightly different from those of *inf1*, which tends to peak late during infection (Kamoun et al. 1997b). However, more precise methods for measuring gene expression need to be applied to confirm these results prior to speculating on their biological implications.

Comparative analyses of elicitor activity of INF1, INF2A, and INF2B using PVX agroinfection and agroinfiltration revealed that, similar to INF1 and other elicitins, INF2A and INF2B induced HR-like symptoms on tobacco. However, using these assays, differences in HR induction were noted among the three elicitors in both tobacco and *N. benthamiana*. A significant difference in specificity of HR induction was obtained for INF2B, which, unlike INF1 and INF2A, failed to induce necrosis on *N. benthamiana* in both assays (Figs. 4 and 5; Table 1). On the other hand, INF2A only occasionally induced the HR in *N. benthamiana* using agroinfiltration, and induced small and inconsistent necrotic lesions using agroinfection (Table 1). These results suggest that expression of *inf2A* in *N. benthamiana* via these transient assays may not be efficient enough to consistently result in necrosis. Alternatively, unknown environ-

mental or host factors may affect the level of expression or response to INF2A in *N. benthamiana*, resulting in the inconsistent responses. In any case, these results support the view that INF2A is an overall weaker HR elicitor than INF1 and INF2B. This conclusion also is supported by the experiments described in Figure 7 that show that INF2B and INF1 but not INF2A induced the expression of the defense genes *PR1a* and *Bgl2* in tobacco. However, the extent to which these differences are significant to natural *P. infestans*–plant interactions remains to be determined.

We determined that the ubiquitin ligase-associated protein SGT1 is required for HR induction by INF2A, as previously shown for INF1 (Peart et al. 2002). SGT1 has emerged as a central player in *R* gene-mediated HR signaling in plants as diverse as barley, *Arabidopsis thaliana*, and *N. benthamiana* (Peart et al. 2002; Shirasu and Schulze-Lefert 2003; Tör et al. 2003). Using TRV-mediated gene silencing, Peart and associates (2002) found that, unlike abiotic inducers of cell death, all examined pathogen-derived elicitors required SGT1 for HR induction. Therefore, the result that INF2A-induced necrosis is SGT1 dependent suggests that this protein is likely to induce a typical HR similar to the one induced by the better-characterized INF1 protein and other HR elicitors. However, considering the differences highlighted above, the extent to which INF2 and INF1 induce similar cell death pathways in *Nicotiana* spp. remains to be determined.

The ability of tobacco, but not *N. benthamiana*, to respond to INF2B could explain differences in resistance to *P. infestans* observed for these two species (Kamoun 2001; Kamoun et al. 1998b). *P. infestans* strains engineered for INF1-deficiency by antisense gene silencing were found to reach significant levels of biomass and colonization in *N. benthamiana* but not in a number of other *Nicotiana* spp., including tobacco (Kamoun et al. 1998b). This led to the hypothesis that resistance to *P. infestans* in *N. benthamiana* is triggered mainly by INF1, whereas the resistance reaction observed in tobacco may involve additional elicitor or avirulence factors (Kamoun 2001; Kamoun et al. 1998b). An attractive hypothesis is that the inability of *N. benthamiana* to respond to INF2B, contributes to the difference in response to INF1-deficient strains between these two *Nicotiana* spp. Future experiments using *P. infestans* strains silenced for a combination of *inf* genes should help assess the contribution of the *inf2* genes to avirulence on *Nicotiana* spp.

The three-dimensional structure of cryptogein, the major basic elicitin (class Ib) of *P. cryptogea*, was determined both as a native protein and complexed with ergosterol (Boissy et al.
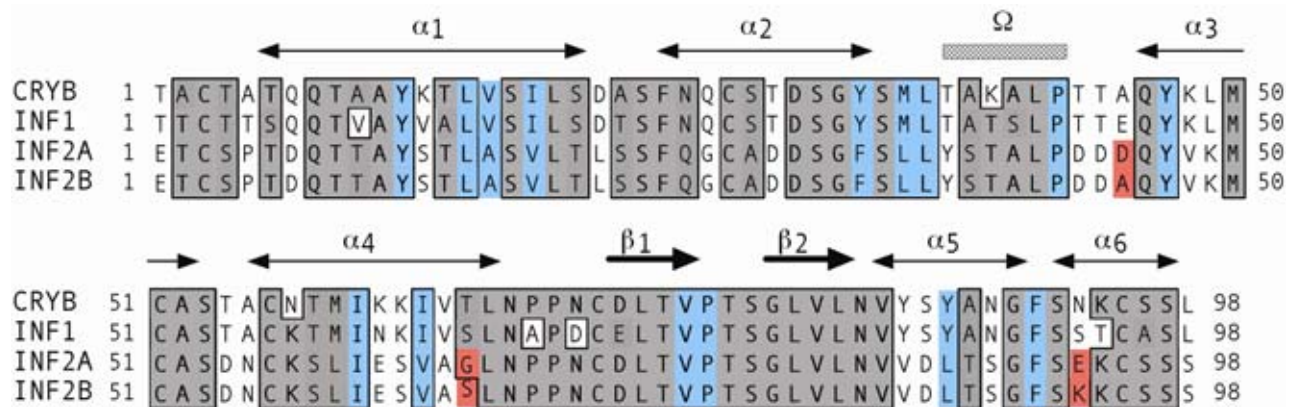

Fig. 9. Multiple alignment of the elicitin domain of selected *Phytophthora* elicitin and elicitin-like protein highlighting the major structural features. Multiple alignment of elicitin sequences from *Phytophthora cryptogea* cryptogein (CRY-B) and *P. infestans* (INF1, INF2A, and INF2B) was conducted using the program CLUSTAL-W (J. D. Thompson, EMBL, Heidelberg, Germany). Identical and similar amino acids are shaded in gray. Residue numbers flank the sequences. The secondary structure elements indicated above the sequences (six α helices, Ω loop, and two antiparallel β-sheets) correspond to CRY-B as described in Boissy and associates (1996). Residues in blue were shown by Boissy and associates (1999) to interact with an ergosterol substrate. Residues in red differ between INF2A and INF2B. Residue numbers flank the sequences.

1996, 1999; Fefeu et al. 1997; Gooley et al. 1998). The main features of the structure of cryptogein, three disulfide bridges, a beak-like motif formed by two antiparallel beta sheets, and an Ω-loop, are likely to be conserved among the *P. infestans* elicitins examined in this study (Fig. 9). Ergosterol binding to cryptogein occurs in a hydrophobic pocket and involves 15 aa residues in cryptogein (Boissy et al. 1999). All these residues are fully conserved among cryptogein, INF1, and other class I elicitins (Fig. 9) (Boissy et al. 1999). However, 6 of these 15 aa are replaced in INF2A and INF2B, including Tyr87 (replaced by Leu) which was shown experimentally to be important in sterol binding and HR induction in cryptogein (Osman et al. 2001b). This marked difference in amino acid composition of the hydrophobic pocket suggests that INF2 may bind different substrates from class I elicitins, perhaps lipid molecules other than sterols. Variation in substrate binding also could explain the difference in elicitor activity between INF2 and class I elicitins, because sterol loading is important for the ability to specifically bind a plasma membrane receptor and induce the HR in tobacco (Osman et al. 2001b).

The differences in HR-inducing activity observed for INF elicitins in *N. benthamiana* and tobacco and the availability of facile functional assays suggest that these genes are ideal for probing structure–function relationships in elicitor proteins. Differences in activity of INF2A and INF2B were observed even though their elicitin domains differ only by 3 aa (Fig. 9). INF2A appeared weaker than INF1 and INF2B in inducing the HR on tobacco, resulting in lower frequencies of necrosis induction and smaller necrotic lesions when delivered through PVX (Fig. 4; Table 1). On the other hand, INF2B consistently failed to induce the HR on *N. benthamiana* even though it functioned as a potent elicitor in tobacco (Figs. 4 and 5; Table 1). In INF2B, Ser65 is replaced by Gly and Glu93 is replaced by Lys. Both of these residues are located in the α helices and are not implicated in sterol binding. However, they are predicted to be surface exposed and are variable among class I elicitins (Fig. 9). Our results suggest that these residues are important for specific HR activity in *N. benthamiana* and the overall elicitor activity in tobacco. Future domain swapping and amino acid exchange experiments should help determine the role of these residues in elicitin activity.

The *Nicotiana* genes involved in recognition of elicitins have not yet been identified and, consequently, one can only speculate about the molecular basis of the differences between tobacco and *N. benthamiana* with respect to their response to INF2 elicitins. Elicitin recognition genes in *Nicotiana* spp. could be members of a variable *R* gene family, similar to those described in numerous plants to mediate HR induction by pathogen elicitors (Bent 1996; Meyers et al. 1999; Michelmore and Meyers 1998; Staskawicz et al. 1995). The difference observed between tobacco and *N. benthamiana* also indicates that, similar to the phenotypic expression of resistance (Kamoun et al. 1998b), the genetic basis of *Nicotiana* resistance to *P. infestans* could be diverse. Perhaps, recognition of species-specific elicitors, such as INF elicitins, by an arsenal of *R* genes forms the basis of resistance of *Nicotiana* spp. to *P. infestans* (Kamoun 2001). Considering this diversity, the *P. infestans*–*Nicotiana* spp. pathosystem appears ideal for the dissection and comparative analyses of the molecular basis of nonhost recognition in closely related species.

MATERIALS AND METHODS

Microbial strains and culture conditions.

The various *P. infestans* isolates used in this study were described previously (Kamoun et al. 1998a). *P. infestans* isolates were cultured routinely on rye agar medium supplemented with 2% sucrose (Caten and Jinks 1968) or in still cultures in the synthetic medium described by Kamoun and associates (1994).

Escherichia coli XL1-Blue and DH5α were used in most experiments and were routinely grown at 37°C in Luria-Bertani media (Sambrook et al. 1989). *A. tumefaciens* strains EHA105 (Hood et al. 1993) and GV3101 (Holsters et al. 1980) were used. All bacterial DNA transformations were conducted by electroporation.

DNA manipulations and plasmid constructions.

DNA manipulations and screening of the λEMBL3 library were conducted essentially as described elsewhere (Ausubel et al. 1987; Sambrook et al. 1989). Total DNA of *P. infestans* was isolated from mycelium grown in liquid culture as described previously (Pieterse et al. 1991). Alkaline DNA transfer to Hybond N+ (Amersham, Arlington Heights, IL, U.S.A.) and Southern hybridizations were performed at 65°C as described elsewhere (Ausubel et al. 1987; Sambrook et al. 1989). Filters were typically washed at 55°C in 0.5× SSC (75 mM NaCl and 7.5 mM sodium citrate) (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) except for the blot shown in Figure 1, which was washed at low stringency (room temperature in 2× SSC). Dideoxy chain-termination sequencing was carried out using an AmpliCycle sequencing kit (Perkin-Elmer, Foster City, CA, U.S.A.).

Plasmid pFB60 was obtained by subcloning a gel-purified 1.7-kb *Hin*dIII fragment from an *inf2A*-containing λEMBL3 clone into pBluescript SK- (Stratagene, San Diego, CA, U.S.A.). Sequencing of the full insert of pFB60 was conducted using vector primers as well as a series of internal sequencing primers.

Plasmids pGR106-INF2A and pGR106-INF2B were constructed by cloning PCR-amplified DNA fragments corresponding to a fusion between the signal sequence of the *PR-1a* gene of tobacco (Hammond-Kosack et al. 1995) and the sequence of the 98-aa elicitin domain of INF2A and INF2B (Kamoun et al. 1997) into the *Cla*I site of pGR106 (Lu et al. 2003) using the overlap extension strategy described by Kamoun and associates (1999a). The oligonucleotides used in the PCRs are PVX-F (5′-AATCAATCACAGTGTTGGCTTG C-3′) and PR-INF2A (5′-GGCGAGCACGTCTCGGCACGGC AAGAGTGGGATATTAC -3′); and PR-INF2B (5′-CTTGCCG TGCCGAGACGTGCTCGCCCACG-3′) and INF2-RSC (5′-G TG<u>GAGCTCATCGAT</u>CACGACGAGGAGCACTTCTTGGA-3′). *Sac*I and *Cla*I restriction sites were introduced in INF2-RSC and are underlined. The resulting recombinant plasmids, pGR106-INF2A and pGR106-INF2B, were confirmed by DNA sequencing to have a *PR1a*::*inf2* fusion inserted in the sense orientation with regard to the duplicated PVX coat protein promoter. The pGR106-INF1 plasmid was constructed by cloning the *PR1a*::*inf1* fusion sequence (Kamoun et al. 1999a) into the *Cla*I site of pGR106.

For agroinfiltration experiments, plasmids p35S-INF1 (previously named pInf1) was described earlier (Kamoun et al. 2003). p35S-INF2A and p35S-INF2B were constructed by cloning PCR-amplified DNA fragments corresponding to the *PR-1a*::*inf* fusions from the respective pGR106-INF constructs as *Nco*I and *Sac*I fragments into pAvr9 (Van der Hoorn et al. 2000). The oligonucleotides used in the PCRs are PR1-FNCO (5′-GCAT<u>CCATGG</u>GATTTGTTCTCTTTTCACAA-3′) and INF1-RSAC (5′-GGC<u>GAGCTC</u>TCATAGCGACGCACACGT AG-3′) for *PR1a*::*inf1* and INF2-RSC for *PR1a*::*inf2*. The introduced *Nco*I and *Sac*I restriction sites are underlined. The resulting p35S-INF plasmids were confirmed by DNA sequencing to contain intact PR1a::*inf* ORFs flanked by the CaMV 35S promoter and the Ω *Tobacco mosaic virus* (TMV)

leader on the 5′ side and the potato proteinase-II terminator region on the 3′ end.

RNA manipulations, Northern blot hybridizations, and RT-PCR analyses.

Total RNA was isolated from *P. infestans* mycelium using the guanidine hydrochloride extraction method (Logemann et al. 1987), and from *N. tabacum* using the Trizol RNA extraction protocol following the manufacturer's recommendations (Gibco-BRL, Bethesda, MD, U.S.A.). For Northern blot analyses, 10 to 15 μg of total RNA was denatured at 50°C in 1 M glyoxal, dimethyl sulfoxide, and 10 mM sodium phosphate, electrophoresed, and transferred to Hybond N+ membranes (Amersham) (Ausubel et al. 1987; Sambrook et al. 1989). Hybridizations were conducted at 65°C in 0.5 M sodium phosphate buffer, 7% sodium dodecyl sulfide, and 1 mM EDTA. Filters were washed at 55°C in 0.5× SSC for the *Phytophthora* blots or at 65°C in 0.5× SSC for the plant blots. For the RT-PCR experiments, cDNA derived from a *P. infestans*–tomato time-course experiment was generated as previously described (Tian et al. 2004). Equal amounts of cDNA were subjected to PCR amplification using the following primers: INF1TEV-F (5′-GGGAAATCGATACCAC GTGCACCACCTCGCA-3′), INF1TEV-R (5′-GGGAAATC GATTAGCGACGCACACGTAGACG-3′), INF2TEV-F (5′-G GGAAATCGATGAGACGTGCTCGCCCACGGAC-3′), and INF2A-Rnew (5′-CGCATAGCACTTAACAAGCCGCGGCG G-3′). Primers described by Torto and associates (2002) were used for amplification of *ef2α*.

Hybridization probes.

Probes from the *inf2* genes were obtained as gel-purified DNA fragments containing essentially the signal peptide and elicitin domain (amino acids 1 to 126) of the *inf2A and inf2B* cDNA inserts, generated by digestions of the original cDNA plasmids (Kamoun et al. 1997a,b) using appropriate restriction enzymes. A probe from the *actA* gene from pSTA31 (Unkles et al. 1991) was used as a loading control. The *PR1a* gene probe was generated through PCR amplification of tobacco genomic DNA using the gene-specific primers PR1-tob-F (5′-ATGGGATTTGTTCTCTTTTCACAA-3′) and PR1-tob-R (5′-GTATGGACTTTCGCCTCTATAATTAC-3′). A probe from the α-tubulin gene was amplified from an *N. otophora* cDNA clone using vector primers. Probes were radiolabeled with either α-32P-dATP or α-32P-dCTP using a random primer labeling kit (Gibco-BRL).

In order to obtain probes specific to the various genes, we used the primer extension strategy described by Kamoun and associates (1997b). Single-stranded, radiolabeled probe complementary to the 3′ end untranslated region of the *inf1* mRNA was generated by extending primer INF2-F1 (Kamoun et al. 1997b) from the gel-purified *inf1* insert from pFB7. Single-stranded, radiolabeled probes complementary to the 3′ end untranslated region of the *inf2A*, and *inf2B* mRNAs, were generated by extending primer INF2-F2 (5′-CCACCGCGGCTT GTTAAG-3′) from *Xho*I-digested pFB5 and pFB24, respectively. The sequence corresponding to the TAA stop codon of the *inf2A and inf2B* ORFs is underlined. The labeling reactions were performed as previously described (Kamoun et al. 1997b).

PVX agroinfection assays.

Tobacco (cv. Xanthi) and *N. benthamiana* plants with fully expanded leaves were used for the agroinfection assays. Plants were cultured and maintained in a greenhouse with an ambient temperature of 22 to 25°C and high light intensity. Inoculations were performed by dipping a wooden sterile toothpick in a recombinant *A. tumefaciens* GV3101 (pGR106-INF) colony grown on solid agar medium and wounding each leaf twice around the main vein. An excess of bacteria was used for the inoculations. Local necrotic symptoms were scored daily and typically started developing within 5 to 7 days after inoculation.

All constructs were re-evaluated on young *N. benthamiana* plants at approximately the three-to four-leaf stage (approximately 3 weeks old). Inoculations then were performed on two lower leaves by wounding each leaf twice around the main vein and near the base of the leaf with the *A. tumefaciens* strain. Mosaic, local, and systemic necrotic symptoms were scored daily and typically started developing within 5 to 7 days after inoculation.

Agroinfiltration assays.

Recombinant *A. tumefaciens* strains containing the various binary plasmids were prepared for agroinfiltration as described previously (Kapila et al. 1997; Van der Hoorn et al. 2000). Cultures were infiltrated into young and fully expanded leaves. Most p35S-INF1 and p35S-INF2 infiltrations were conducted side by side and repeated at least three times.

GUS assays.

We used a transgenic tobacco line (cv. Samsun NN) carrying a *Bgl2::GUS* reporter construct. The selected line (gglb-1233-3), generated by Livne et al. (1997) to express a chimeric gglb50 promoter (basic β-1,3-glucanase, GenBank accession number X53600) fused to the GUS reporter gene, contains the gglb50 promoter region between positions –1,233 and +19. Histochemical GUS staining was performed using 2 mM 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc) (Rose Scientific, Edmonton Alberta, Canada) as described previously by Huitema and associates (2003).

TRV-silencing experiments.

Agrobacterium strains carrying pBINTRA6 (RNA 1 vector), pTV00 (RNA 2 vector), and pTV00:SGT1 (Peart et al. 2002; Ratcliff et al. 2001) were prepared for agroinfiltration as described above, and mixed in a 2:1 RNA 1/RNA 2 ratio. The *SGT1* insert corresponds to the *NbSGT1.1* gene (GenBank accession number AF516180). Mixed cultures were incubated for at least 2 h before infiltration. The youngest fully expanded leaves of *N. benthamiana* plants (five-leaf stage) were infiltrated with the *Agrobacterium* suspensions using needleless syringes. Challenge inoculations using agroinfiltration or PVX agroinfection assays were started 3 weeks after TRV inoculation and performed as described above.

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AUTHOR-RECOMMENDED INTERNET RESOURCE

The Sainsbury Lab at the John Innes Center, D. Baulcomb's VIGS protocol webpage:
www.jic.bbsrc.ac.uk/Sainsbury-Lab/dcb/Services/vigsprotocol.htm