A Gene Encoding a Protein Elicitor of *Phytophthora infestans* Is Down-Regulated During Infection of Potato

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Most species of the genus *Phytophthora* produce 10-kDa extracellular protein elicitors, collectively termed elicitins. Elicitins induce hypersensitive response in a restricted number of plants, particularly in the genus *Nicotiana* within the Solanaceae family. A cDNA encoding INF1, the major secreted elicitin of *Phytophthora infestans*, a pathogen of solanaceous plants, was isolated and characterized. The expression of the corresponding *inf1* gene during the disease cycle of *P. infestans* was analyzed. *inf1* was shown to be expressed in mycelium grown in various culture media, whereas it was not expressed in sporangiospores, zoospores, cysts, and germinating cysts. In planta, during infection of potato, particularly during the biotrophic stage, expression of *inf1* was down-regulated compared to in vitro. The highest levels of expression of *inf1* were observed in in vitro grown mycelium and in late stages of infection when profuse sporulation and leaf necrosis occur. The potential role of INF1 as an elicitor in interactions between *P. infestans* and *Solanum* species was investigated. Nineteen lines, representing nine solanaceous species with various levels of resistance to *P. infestans*, were tested for response to an *Escherichia coli* expressed INF1. Within the genus *Solanum*, resistance to *P. infestans* did not appear to be mediated by a defense response elicited by INF1. However, INF1 recognition could be a component of nonhost resistance of tobacco to *P. infestans*.

Interactions between plants and pathogens can be either compatible, leading to successful infection by the pathogen (plant is susceptible), or incompatible, leading often to localized cell death through a hypersensitive response (HR) of the plant (plant is resistant). Both types of interactions are believed to involve an exchange of molecular signals between the plant and the pathogen, and the nature of such signals would lead to either disease or resistance (de Wit 1992; Dixon

and Lamb 1990; Ebel and Scheel 1992; Lamb et al. 1989; Staskawicz et al. 1995). A simple and well-characterized illustration of this model lies in incompatible interactions mediated by specific elicitor molecules. In such interactions, elicitors produced directly or indirectly by avirulence genes of the pathogen induce a defense response only in plants that contain the corresponding *R* gene. These *R* genes are thought to encode receptors that bind to the elicitors and activate an array of plant genes leading to HR and inhibition of pathogen growth.

In this paper, we describe studies on the role of an elicitor protein in the *Phytophthora infestans*-Solanaceae pathosystem. *P. infestans*, an oomycete pathogen, causes late blight, economically the most important disease of potato worldwide. The oomycetes have traditionally been included in the kingdom Fungi. However, based on biochemical characteristics, oomycetes are now classified along with golden-brown algae (chrysophytes) in the kingdom Protoctista (Protista) (Corliss 1984; Dick 1990; Margulis 1996). Among the notable oomycete pathogens, members of the genus *Phytophthora* (order Peronosporales) cause destructive diseases on thousands of plant species (Erwin et al. 1983).

The molecular basis of host specificity of *Phytophthora* is not known (Judelson 1996). However, in recent years, a family of extracellular elicitor proteins, termed elicitins, has been identified and evidence has accumulated for a role of these molecules in delimiting the host range of *Phytophthora* species (Yu 1995). Elicitins are highly conserved 10-kDa proteins that are secreted by all tested *Phytophthora* and *Pythium* species (Huet et al. 1995; Kamoun et al. 1994; Pernollet et al. 1993). These proteins induce defense responses including HR on a restricted number of plant species, specifically species in the Solanaceae and Cruciferae families (Kamoun et al. 1993b; Ricci et al. 1989). The basic hypothesis behind studies on elicitins is that responsive plants display higher levels of resistance to elicitin-producing isolates of *Phytophthora* than to elicitin-deficient ones. The role of elicitins as avirulence factors that trigger plant defense responses leading to resistance has been examined in detail in the *Phytophthora parasitica-Nicotiana* pathosystem. In *P. parasitica*, the absence of elicitin production correlates with high virulence on tobacco, a plant species that strongly responds to elicitins. In contrast, isolates that produce elicitins either cause a mild brown rot disease or are completely avirulent on tobacco (Bonnet et al.

1994; Kamoun et al. 1994). The response of tobacco to elicitins also leads to acquired resistance against otherwise virulent *P. parasitica* isolates (Kamoun et al. 1993b; Keller et al. 1996; Ricci et al. 1989), and in a sexual progeny of *P. parasitica*, elicitin production segregates with low virulence on tobacco (Kamoun et al. 1994), suggesting that elicitins indeed can function as avirulence factors. However, despite the availability of cloned elicitin genes (Kamoun et al. 1993a; Panabières et al. 1995), direct demonstration of the role of elicitins through transformation of elicitin deficient strains has not been achieved due to difficulties in the genetic manipulation of *P. parasitica*.

The late blight pathosystem is an attractive model system to investigate the role of elicitins in *Phytophthora*-plant interactions. *P. infestans* produces elicitins (Huet et al. 1994; Kamoun et al. 1994) and displays a complex range of interactions with solanaceous plants. A great deal of information on the life cycle, the infection process, and the cytology of both compatible and incompatible interactions is available (Coffey and Gees 1991; Coffey and Wilson 1983; Freytag et al. 1994; Gees and Hohl 1988), and methods for measurements of gene expression in planta have been developed (Pieterse et al. 1991, 1994). Moreover, molecular manipulation as well as stable DNA transformation is well established for *P. infestans*

(Judelson et al. 1991; Judelson 1996). For these reasons and because DNA transformation is a prerequisite for unequivocal demonstration of the role of elicitins in *Phytophthora*-plant interactions, we decided to exploit *P. infestans* for functional analyses of elicitins.

We present here the cloning and characterization of cDNAs from *P. infestans* encoding the elicitin INF1. The expression of the corresponding elicitin gene (*inf1*) in various developmental stages of *P. infestans* and during interaction of *P. infestans* with potato was investigated. Finally, *inf1* was expressed in *Escherichia coli* and the elicitor activity of the recombinant protein was tested in several solanaceous plants which differ in levels of resistance to *P. infestans*.

RESULTS

Cloning and molecular analysis of cDNAs encoding *P. infestans* elicitin.

A cDNA library constructed in the phagemid vector, lambda-ZAP, from RNA isolated from leaves of potato cultivar Ajax 3 days after inoculation with *P. infestans* 88069 (Pieterse et al. 1994b) was hybridized with a 0.3-kb fragment internal to the open reading frame (ORF) of the *parA1* gene of *P. parasitica* (Kamoun et al. 1993a). A total of 19 positive

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   1                                                      M
 -36   TGTTAT CCCCACCCAC TCCTCCTCAC TCCGTCCACG ATG

   2    N   F   R   A   L   F   A   A   T   V   A
   4   AAC TTT CGT GCT CTG TTC GCT GCC ACC GTT GCT

  13    A   L   V   G   S   T   S   A   T   T   C
  37   GCC CTC GTC GGC TCC ACT TCC GCC ACC ACG TGC

  24    T   T   S   Q   Q   T   V   A   Y   V   A
  70   ACC ACC TCG CAG CAG ACC GTA GCG TAC GTG GCG

  35    L   V   S   I   L   S   D   T   S   F   N
 103   CTC GTA AGC ATC CTC TCG GAC ACG TCG TTT AAT

  46    Q   C   S   T   D   S   G   Y   S   M   L
 136   CAG TGC TCG ACG GAC TCC GGC TAC TCG ATG CTG

  57    T   A   T   S   L   P   T   T   E   Q   Y
 169   ACG GCC ACC TCG CTG CCC ACG ACG GAG CAG TAC

  68    K   L   M   C   A   S   T   A   C   K   T
 202   AAG CTC ATG TGC GCG TCG ACG GCG TGC AAG ACG

  79    M   I   N   K   I   V   S   L   N   A   P
 235   ATG ATC AAC AAG ATC GTG TCG CTC AAC GCT CCC

  90    D   C   E   L   T   V   P   T   S   G   L
 268   GAC TGC GAG CTG ACG GTG CCA ACT AGT GGC CTG

 101    V   L   N   V   Y   S   Y   A   N   G   F
 301   GTA CTC AAC GTG TAC TCG TAC GCG AAC GGG TTC

 112    S   S   T   C   A   S   L   *
 334   TCG TCT ACG TGT GCG TCG CTA TGA GTGGACTTGA

 368   CTCAGCGCGC TCAGCCGCTG AGTCAGCGTC CATTGGCCGT

 408   ATCATCTTGT CTTTCATCGA CGATTAGGCT TTGTTCTCCG

 448   CGTCTGTTGC TATTACTGTA ATAGTGTAGC AGCTGCGGTA

 488   TAAAGCTATT TGAATAAAAA TTCTTGTATG CCAT
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Fig. 1. Nucleotide sequence of the *inf1* cDNA clone pFB7 from *Phytophthora infestans* isolate 88069 (GenBank accession number U50844), and the deduced amino acid sequence of the pre-INF1 protein. The mature secreted INF1 elicitin is shown in bold. The putative polyadenylation sequence AATAAA is underlined. The internal *Sal*I site is located at nucleotides 216-221.
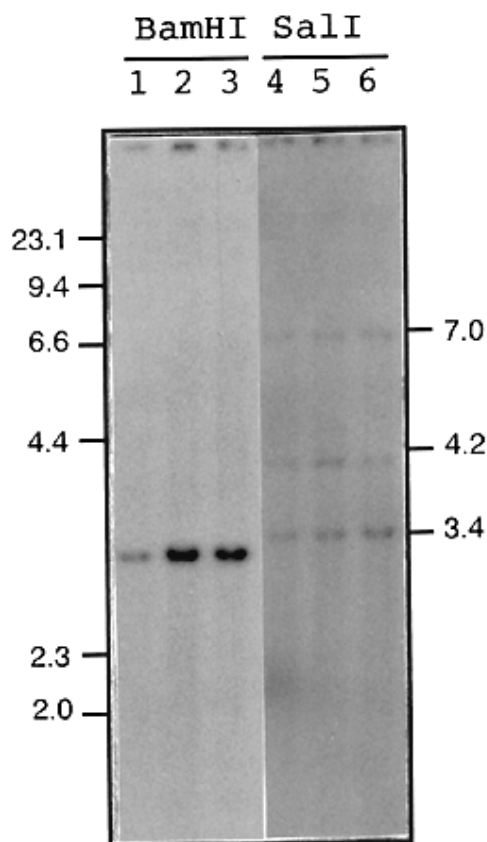

Fig. 2. Occurrence of *inf1* sequences in *Phytophthora infestans.* Total DNA (20 µg) of *P. infestans* isolates 88069 (lanes 1 and 4), IPO-r0 (lanes 2 and 5), and 90128 (lanes 3 and 6) was digested with *Bam*HI (lanes 1-3) or *Sal*I (lanes 4 to 6) and hybridized with a probe containing the *inf1* cDNA. The numbers on the left indicate the sizes in kb of the molecular size marker fragments. On the right, the approximate sizes in kb of the three hybridizing *Sal*I fragments are indicated.

clones were isolated, and plasmids were rescued by in vivo excision. Based on restriction enzyme mapping and Southern blot hybridization, two classes of clones were identified. One class consisted of six clones of which the inserts hybridized weakly to the *parA1* probe. DNA sequencing revealed that these clones contain cDNA sequences of two novel elicitin-like genes that will be described elsewhere. The remaining 13 clones hybridized strongly to the *parA1* probe and appeared to contain sequences highly homologous to the *parA1* gene. These cDNA clones were further characterized. DNA sequence analysis revealed no sequence polymorphism between the overlapping regions of the 13 cDNA clones indicating that they all encode the same protein. A 354-bp ORF was found (Fig. 1) which encodes a protein composed of a 20-amino acid signal peptide, followed by a 98-amino acid sequence that is identical to the amino acid sequence of a *P. infestans* elicitin published by Huet et al. (1994). We have designated the *P. infestans* elicitin INF1 and consequently the cDNA was named *inf1* cDNA.

In all 13 *inf1* cDNA clones, a 164-bp 3′ untranslated region and a stretch of poly(dA) followed the ORF. This suggests that transcriptional termination occurs at one site which is probably governed by the putative polyadenylation signal AATAAA (underlined in Fig. 1). The longest cDNA clone obtained, pFB7, possesses a 36-bp untranslated 5′ region, which is 20 bp shorter than the 5′ untranslated region of *parA1* (Kamoun et al. 1993a). The size of the cDNA insert in pFB7 (554 bp) corresponds to the estimated size of the *inf1* mRNA (550 nt) as described below.

Occurrence of *inf1* sequences in *P. infestans* genome.

To determine the number of copies of the cloned *inf1* sequences that occur in the *P. infestans* genome, Southern blot

Fig. 3. Expression of *inf1* in various developmental stages of *Phytophthora infestans.* Total RNA from *P. infestans* 88069 zoospores (1), cysts (2), cysts germinating for 2.5 h in water (3), cysts germinating for 2.5 h in Lima bean medium (4), sporangiospores (5), and mycelium obtained from sporangiospores germinating in Lima bean medium for 20 h (6) was sequentially hybridized with probes from the *inf1* and *actA* genes. The approximate sizes of the *inf1* and *actA* transcripts are shown on the right.

hybridizations were performed. *Bam*HI and *Sal*I digested DNA of *P. infestans* isolate 88069 was hybridized with the entire insert of *inf1* cDNA. One *Bam*HI fragment of approximately 3.2 kb and three *Sal*I fragments of 7.0, 4.2, and 3.4 kb were detected (Fig. 2). Since there is one *Sal*I site and no *Bam*HI site in the cloned *inf1* cDNA (Fig. 1), this result indicates that at least two copies of the *inf1* gene occur in the *P. infestans* genome. These two copies could correspond to two *inf1* alleles polymorphic for a flanking *Sal*I site, or could correspond to conserved repeated sequences of *inf1*. Hybridization of the same blot with a probe containing the 3′ noncoding region of the *inf1* cDNA (see Materials and Methods) yielded a slightly different result. Although the same 3.2-kb *Bam*HI fragment and the 7.0-kb *Sal*I fragment were detected, the 4.2- and 3.4-kb *Sal*I fragments were not detected (data not shown), suggesting that the 4.2- and 3.4-kb *Sal*I fragments contain the 5′ half of the *inf1* gene. Southern blot analyses with two other *P. infestans* isolates, IPO-r0 and 90128, revealed the same hybridization pattern as *P. infestans* 88069 with both the entire cDNA probe (Fig. 2) and the 3′ end probe (data not shown) even though these isolates are polymorphic at other loci (data not shown). This suggest that *inf1* sequences are conserved between the three tested *P. infestans* isolates. No cross-hybridization with other elicitin-like genes was observed under the hybridization conditions used in these experiments.

Expression of *inf1* during various developmental stages of *P. infestans*.

To determine the patterns of expression of the *inf1* gene, Northern blot analyses were performed, and accumulation of *inf1* mRNA in various developmental stages of *P. infestans* 88069 was determined. No *inf1* mRNA was detected in RNA extracts of zoospores, cysts, germinating cysts (2.5 h in either water or Lima bean medium), and sporangiospores. However, *inf1* mRNA was detected in extracts of mycelium obtained 20 h after germination of sporangiospores in Lima bean medium (Fig. 3). Additionally, *inf1* mRNA was consistently detected at high levels in extracts of mycelium cultured in rye-sucrose or other standard media (data not shown, see also Fig. 4) suggesting that the *inf1* gene is exclusively expressed in mycelium. Control hybridizations with a probe of the constitutively expressed *actA* gene (Unkles et al. 1991) showed that all lanes contained similar amounts of total RNA (Fig. 3).

Expression of *inf1* in planta.

To analyze the expression of *inf1* during the interaction of *P. infestans* with its host plant potato, total RNA was isolated from leaves of potato cv. Bintje 1, 2, 3, 4, 5, 6, 7, and 8 days after inoculation with *P. infestans* 88069 and from *P. infestans*
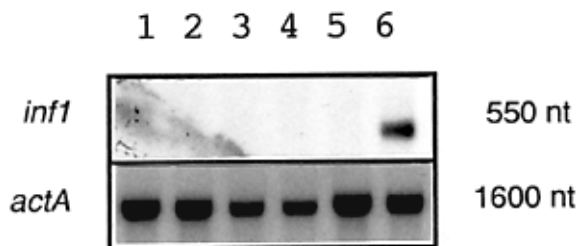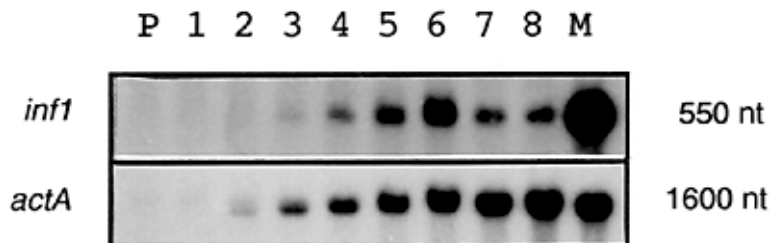

Fig. 4. Time course of expression of *inf1* and *actA* of *Phytophthora infestans* during infection of potato cv. Bintje. Total RNA isolated from infected leaves of potato, 1, 2, 3, 4, 5, 6, 7, or 8 days after inoculation, from noninfected leaves (P), and from *P. infestans* mycelium grown in rye-sucrose medium (M) was sequentially hybridized with probes from the *inf1* and *actA* genes. The approximate sizes of the transcripts are shown on the right.

mycelium grown in rye-sucrose medium. A Northern blot containing these samples was hybridized with probes of the *P. infestans inf1* and *actA* genes (Fig. 4). Because the total RNA extracted from infected leaves consists of a mixture of fungal and plant RNA, the signals obtained on Northern blots with probes of differentially expressed genes should be normalized to actual *Phytophthora* RNA levels as determined by the signals obtained with a probe of a constitutively expressed gene. Consistent with increases in fungal biomass during the infection, the mRNA of the constitutively expressed *actA* gene was first detected at day 2 and its level increased in the following days until reaching a maximal level at days 6, 7, and 8. In contrast, *inf1* mRNA was first detected at day 3 after inoculation and reached the highest level at days 5 and 6, when extensive sporulation of *P. infestans* and leaf necrosis occurred. Subsequently, at days 7 and 8 when little additional sporulation occurred, the level of *inf1* mRNA decreased. In contrast to the *actA* mRNA, the levels of the *inf1* mRNA observed in planta were always lower than the levels observed in vitro, particularly during the early biotrophic stages of infection

Fig. 5. Bacterial expression of the cloned *inf1* cDNA. **A,** Western blot immunodetection of recombinant proteins in SDS-PAGE of culture supernatants (lanes 1 and 2) and soluble cellular fractions (lanes 3 and 4) of *Escherichia coli* strain pFB52 (FLAG-INF1 *S3*, lanes 1 and 3) and strain pFB53 (FLAG-INF1, lanes 2 and 4). FLAG M2 monoclonal antibody was used for detection. Molecular weight standards are shown on the left. **B,** Leaf infiltration of tobacco cv. Xanthi with 100 nM solutions of purified FLAG-INF1*S3* (left side of the leaf) and FLAG-INF1 (right). The photograph was taken 5 days after infiltration, but the response was already visible 24 h after infiltration.

(days 2 to 4) and the late postsporulation stages (days 7 and 8). These results suggest that expression of *inf1* is down-regulated during infection of potato. Similar results were obtained when the Northern blot was hybridized with the 3´ end *inf1* probe instead of the full cDNA probe.

Bacterial expression of *inf1* cDNA.

To determine whether the protein encoded by the *inf1* cDNA is biologically active as an elicitor molecule and to determine the host-specificity of INF1, the *inf1* cDNA was subcloned into pFLAG-ATS, a vector which allows isopropyl-β-D-thiogalactopyranoside (IPTG) induced expression of the inserted gene in *E. coli*. The product is secreted by *E. coli* as a fusion protein containing a signal peptide derived from the *ompA* gene followed by the epitope tag, FLAG, at the amino-terminus. Two plasmids were constructed in which the coding sequence of the processed form of the INF1 protein is inserted downstream of the epitope tag. pFB53 contains a wild-type *inf1* sequence (FLAG-INF1), whereas pFB52 contains a mutated *inf1* sequence, in which the cysteine codon at position 23 (or position 3 of the processed protein) is mutated into a serine (FLAG-INF1*S3*). The mutant is expected to serve as a negative control, since it should lack one of the three disulfide bridges and is predicted to have reduced elicitor activity (Ricci et al. 1989). Western blots containing soluble cellular extracts and culture supernatants of IPTG-induced *E. coli* strains bearing pFB52 or pFB53 were incubated with FLAG monoclonal antibodies (Fig. 5A). Two bands of approximately 12 and 14 kDa reacted with the FLAG antibody in the cellular fraction, whereas only the lower band was present in the supernatant fractions, suggesting that secretion of the fusion proteins is accompanied by the proper removal of the OMPA signal peptide. Following affinity chromatography purification of the fusion proteins from *E. coli* supernatant fractions, a single band corresponding to either FLAG-INF1 or FLAG-INF1*S3* was detected on silver stained SDS-PAGE gels or on Western blot incubated with FLAG antibodies (data not shown). Infiltration of tobacco (cv. Xanthi) leaves with 100 nM of purified FLAG-INF1 consistently induced a hypersensitive response, whereas infiltration with FLAG-INF1*S3* did not induce any visible response (Fig. 5B). Infiltration with the various buffers and culture supernatant from *E. coli* bearing the cloning vector induced no visible response. These results demonstrate that the cloned *inf1* cDNA encodes an active elicitor protein.
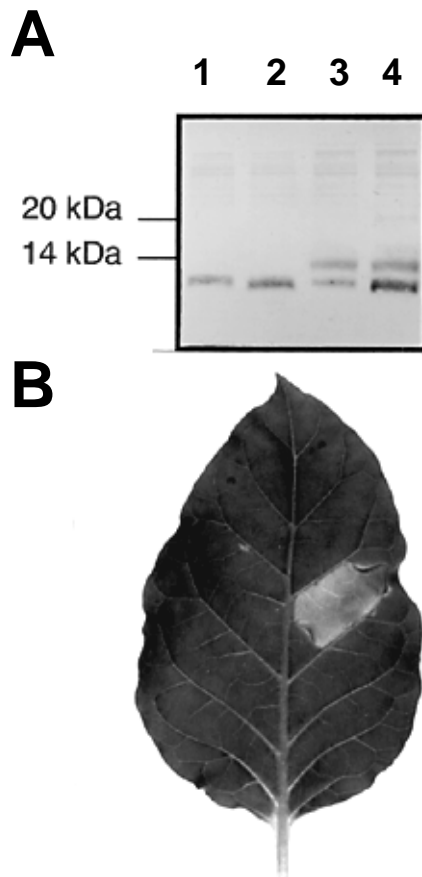
Response of *Solanum* plants to INF1 and relation to late blight resistance.

To determine whether INF1 elicitin plays a role as an elicitor in the interaction between solanaceous plants and *P. infestans*, the reponse to INF1 of 19 lines representing nine solanaceous species was examined (Table 1). Besides tobacco, all plants tested failed to respond to infiltration with 100 nM solutions of purified FLAG-INF1 and none of the tested plants responded to FLAG-INF1*S3*. Previously, the response of these lines to INF1-producing strains of *P. infestans* was determined in both field and growth chamber experiments (Colon and Budding 1988; Colon 1992; Colon et al. 1994, 1995) and ranged from full resistance to susceptible (Table 1). These results confirm the narrow specificity of elicitins (Kamoun et al. 1993b) and indicate that resistance to *P. infestans* within the

genus *Solanum* is not determined by the recognition and response to INF1 elicitin.

DISCUSSION

Molecular structure of the elicitor cDNA *inf1* of *P. infestans*.

In this paper, we report the molecular cloning of a *P. infestans* cDNA, *inf1*, encoding a host-specific elicitor protein of the elicitin family. The DNA sequence of the *inf1* cDNA revealed a 354-bp ORF encoding a pre-INF1 protein of 118 amino acids. Processing of the 20 amino acid N-terminal signal peptide results in the mature 98 amino acid INF1 protein (Fig. 1). The deduced amino acid sequence of INF1 is identical to the sequence of an acidic elicitin protein purified from another isolate of *P. infestans* (Huet et al. 1994). Sequence comparisons indicate a high degree of homology between the coding sequence of *inf1* of *P. infestans* and those of other characterized elicitin genes, *parA1* of *P. parasitica* (Kamoun et al. 1993a), and cryptogein A1-B14, cryptogein B-X24, *hae*-B20 and *hae*-B26 of *P. cryptogea* (Panabieres et al. 1995). *inf1* is more similar to the genes encoding acidic elicitins, *parA1* (92% identity at the DNA level over the coding region) and cryptogein A1-B24 (85%), than to the basic elicitin gene cryptogein B-X24 (81%).

Using Southern blot analyses, at least two genomic copies of the *inf1* gene could be detected in three different *P. infestans* isolates. These two copies are both contained in a conserved 3.2-kb *Bam*HI fragment and could correspond to two alleles of *inf1*. Even though additional elicitin-like sequences were isolated from *P. infestans* (unpublished data), no cross-hybridization was noted between these genes and the *inf1* probes under the Southern and Northern blot hybridization conditions used in this study. This indicates that the *inf1* transcript detected in the Northern blot analyses must be derived from one or both genomic copies of the *inf1* gene.

The *inf1* gene is down-regulated during infection of potato.

The pattern of expression of the *inf1* gene was followed throughout the disease cycle and in various stages of the life cycle of *P. infestans*. During the early stages of infection, expression of *inf1* does not occur prior to the penetration of plant cells. This contrasts to the expression pattern of the in planta-induced genes *ipiO* and *ipiB* of *P. infestans* (Pieterse et al. 1994a), which are already expressed at high levels in germinating cysts. Expression of *inf1* occurs later during infection although at a reduced level. The highest levels of *inf1* expression occur at later stages concurrent with the onset of extended leaf necrosis, saprophytic growth and profuse sporulation. The expression of *inf1* is then repressed in sporangiospores and zoospores until a new infection cycle is initiated. The *inf1* gene reaches maximal levels of expression in sporulating mycelium whether growing in vitro or in planta. Comparison of the expression patterns of *inf1* and *actA* indicate that *inf1* is down-regulated during infection of potato.

Based on their abundant secretion and primary amino acid structure, elicitins have been proposed to function as structural proteins in *Phytophthora* (Templeton et al. 1994; Yu 1995). The high expression of *inf1* during the onset of the saprophytic stage of infection and in mycelium growing in vitro

supports this hypothesis. Both stages are characterized by abundant sporulation. Increased synthesis of structural proteins might be required in sporulating mycelium, particularly in sporangiophores, in order to sustain the additional amounts required for spore formation. Additional experiments, including cellular localization of elicitins in *Phytophthora* should help unravel the biological function of these proteins.

The products of elicitor or avirulence genes trigger defense responses in plants that are ultimately deleterious to the pathogen. Therefore these genes are under selective pressure to mutate to inactive forms leading the pathogen into an evolutionary race with the plant (Staskawicz et al. 1995). The down-regulation of the expression of the *inf1* gene during infection of potato, noted in this study, could therefore be an adaptation of *P. infestans* to evade plant defense responses. However, since no known host plant of *P. infestans* has been shown to respond to elicitins (Table 1), the expression pattern of *inf1* might simply reflect the potential role of elicitins as structural proteins in *Phytophthora*.

Host specificity of elicitins.

Purified elicitin proteins from several *Phytophthora* species were shown to induce defense responses in two plant families. In the Solanaceae, response to elicitins appears to be restricted to the genus *Nicotiana*, whereas in the Cruciferae, cultivar-specific response was noted for radish, turnip, and rape

Table 1. Specificity of hypersensitive response induced by INF1 elicitin in the Solanaceae

Species (cv. or accession no.)	Resistance level[a]	Response to[b]	
		INF1	INF1 *S3*
Nicotiana tabacum			
Xanthi	Fully resistant	+	–
Solanum berthaultii			
BGRC 10.063-9	Fully resistant	–	–
BGRC 10.063-11	Partially resistant	–	–
S. circaeifolium ssp. *circaeifolium*			
BGRC 27.057	Partially resistant	–	–
S. microdontum			
BGRC 24.981-167	Partially resistant	–	–
BGRC 24.981-178	Partially resistant	–	–
S. microdontum ssp. *gigantophyllum*			
BGRC 18.570-265	Susceptible	–	–
S. nigrum			
SN18	Fully resistant	–	–
S. sucrense			
BGRC 27.370-23	Partially resistant	–	–
BGRC 27.370-71	Partially resistant	–	–
S. tuberosum			
Bintje	Susceptible	–	–
Desiree	Susceptible	–	–
Ehud	Susceptible, *R*-1	–	–
Estima	Susceptible, *R*-10	–	–
Premiere	Susceptible, *R*-10	–	–
Surprise	Partially resistant	–	–
Robijn	Partially resistant	–	–
S. vernei			
BGRC 24.733-530	Partially resistant	–	–
Lycopersicon esculentum			
Moneymaker	Susceptible	–	–

[a] The resistance data were obtained from Colon and Budding 1988; Colon 1992; Colon et al. 1994, 1995.
[b] 100 nM of purified fusion proteins FLAG-INF1 and FLAG-INF1*S3* were infiltrated in attached leaves of the listed plants: + indicates a confluent hypersensitive response and – indicates no visible response.

(Bonnet et al. 1996; Kamoun et al. 1993b). The results obtained in this study with INF1 confirm the narrow specificity of elicitins. In the Solanaceae, no INF1-responsive plant was identified outside the genus *Nicotiana* (Table 1).

It should be noted that conflicting results have been published on host-specificity of elicitins (discussed by Yu 1995). Pernollet and co-workers (1993) described elicitins as toxic molecules capable of inducing necrosis on all tested plants. In their studies, solanaceous plants including tomato, pepper, and potato responded similarly to tobacco after elicitin treatment (Huet et al. 1994; Pernollet et al. 1993). These results are particularly intriguing considering that systemic necrosis should then be observed during compatible interactions between *Phytophthora* and plants. This is generally not the case, for example in pepper plants infected by *Phytophthora capsici*, elicitins were detected systemically but no corresponding necrosis was observed (Devergne et al. 1994). In studies by various laboratories, specificity in the response to elicitins has been reported not only for whole plants, but also for cell cultures (Blein et al. 1991; Yu 1995). Additionally, specificity was observed after treatment with elicitins purified by various biochemical methods from either *Phytophthora* or *E. coli*, and applied to plants by either leaf infiltration or a number of other uptake methods (Bonnet et al. 1996; Kamoun et al. 1993b) (this study). The conclusion that elicitins are non-specific toxins involved in pathogenesis of *Phytophthora* and *Pythium* species (Huet et al. 1994; Pernollet et al. 1993) is therefore inconsistent with reports from several laboratories, and will not be further considered in this paper.

Role of INF1 elicitin in the interaction between *P. infestans* and solanaceous plants.

Similar to other elicitors, elicitins induce a number of plant responses that can be attributed to defense mechanisms, indicating that these proteins function as classical elicitor molecules that trigger defense responses in a restricted range of plant genotypes (Kamoun et al. 1993; Keller et al. 1994; Milat et al. 1991a, 1991b). In this paper, we show that the INF1 elicitin protein of *P. infestans* does not induce a HR on potato and tomato, two major host plants. Expression of the *inf1* gene during infection of potato occurs, albeit at a down-regulated level. Therefore, even though INF1 is produced during infection, it is not detected by compatible host plants and does not appear to play a role in restricting the disease process. Several *Solanum* species, with either partial or full resistance to *P. infestans*, also failed to respond to INF1, indicating that the observed resistance involves other molecular mechanisms than elicitin recognition. On the other hand, resistance of tobacco to *P. infestans* could involve to some degree the response to elicitins. Future experiments using *P. infestans* transformants altered in INF1 production should help determine whether elicitin recognition is a significant component of the resistance of tobacco to *P. infestans*. Additionally, biotechnological manipulation of potato to acquire recognition and response to elicitin molecules is predicted to yield plants with enhanced resistance to *P. infestans*.

METHODS

Phytophthora strains and culture conditions.

P. infestans 88069 (A1 mating type, race 1.3.4.7), a tomato isolate from Bennekom, NL was used throughout this study. *P. infestans* 90128 (A2 mating type, race 1.3.4.7.8.9.10.11, potato isolate, NL) and IPO-r0 (A1 mating type, race 0, unknown origin) were also used in Southern blot analyses. *P. infestans* strains were routinely grown on rye agar medium supplemented with 2% sucrose (Caten and Jinks 1968) or modified Plich medium (Kamoun et al. 1993b). To isolate sporangiospores, sporulating mycelium in rye-sucrose medium was flooded with water (10 ml per petri dish) and gently rubbed with a sterile glass rod. Sporangiospores were then pipetted with the water. Zoospores were obtained after incubation of similarly flooded plates at 4°C for 2 h. After pipetting the solution, the zoospores were separated from the remaining sporangiospores by filtration through a 10-µm nylon mesh. Encystment was induced by continuous shaking for 2 min. Germinating sporangiospores and cysts were obtained by incubation for various times in water or media at 18°C. All three *P. infestans* isolates used in this study produce INF1 abundantly when grown in liquid Plich medium.

Bacterial strains and plasmids.

E. coli XL1-Blue and DH5α were used in most experiments and were routinely grown at 37°C in Luria-Bertani (LB) media (Sambrook et al. 1989). Phage manipulations were conducted according to the protocols provided by Stratagene (La Jolla, CA). Helper phage VCS-M13 was used in in vivo excision experiments (Stratagene, La Jolla, CA).

Plasmids pFB52 and pFB53 were constructed by cloning polymerase chain reaction (PCR) amplified DNA fragments corresponding to the *inf1* ORF into the *Hind*III site of pFLAG-ATS (IBI-Eastman Kodak, New Haven, CT). The oligonucleotides used in the PCR are SK-F1 (5´-GCGAAGCTTACCACGTGCACCACCTCG-3´) and SK-R1 (5´-GCGAAGCTTATAGCGA*C*GCACACGTAGA-3´) for the fragment cloned in pFB53, and SK-F1S (5´-GCGAAGCTTACCACGAGCACCACCTCG-3´) and SK-R1 for the fragment cloned in pFB52. The introduced *Hind*III restriction sites are underlined and the T to A mutation in SK-F1S is shown in bold. Partial nucleotide sequencing of the cloned fragments (including the mutated site) in pFB52 and pFB53 fully matched the predicted sequence. The N-terminal sequence of the processed recombinant FLAG-INF1 protein of pFB53 is "DYKDDDDK DKVKI **TT*C*TTSQQTV**...". The FLAG antibody binding site is underlined, and the first 10 amino acids of mature INF1 are shown in bold with the mutated cysteine of pFB52 shown in bold italics.

DNA manipulations.

DNA manipulations 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 previously described (Pieterse et al. 1991). Alkaline DNA transfer to Hybond N+ (Amersham, Arlington Heights, IL) and Southern 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). Dideoxy chain-termination sequencing was carried out using an AmpliCycle sequencing kit (Perkin Elmer, Foster City, CA). Sequence analysis was made using software DNA Strider 1.0 (C. Marck, Institut de Recherche Fondamentale, France). Database

homology searches were conducted using the BLAST software package (Altschul et al. 1990) as available through the Internet.

Screening of the cDNA library.

The screened cDNA library was constructed in the phagemid vector, lambda-ZAP (Stratagene, La Jolla, CA), from leaves of potato cultivar Ajax 3 days after inoculation with sporangiospores of *P. infestans* (Pieterse et al. 1994b). A total of 30,000 plaques was screened with the 0.3-kb insert fragment of a subclone of pELC100 containing nucleotides 61 to 358 of the *parA1* gene of *P. parasitica* (Kamoun et al. 1993a). Positive plaques were purified, subjected to in vivo excision of pBluescript SK– (Stratagene, La Jolla, Calif.), and characterized by restriction enzyme digestion, Southern blot hybridization, and DNA sequencing.

RNA manipulations.

Total RNA from *P. infestans* and from infected potato leaves was isolated using the guanidine hydrochloride extraction method (Logemann et al. 1987). For Northern blot analyses, 10 to 15 µg of total RNA was denatured at 50°C in 1 M glyoxal, DMSO, and 10 mM sodium phosphate, electrophoresed, and transferred to Hybond N+ membranes (Amersham, Arlington Heights, IL) (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 55°C in 0.5× SSC (75 mM NaCl and 7.5 mM sodium citrate).

Southern and Northern blot hybridization probes.

Gel purified DNA fragments containing the full *inf1* cDNA insert from pFB7, or the *actA* gene from pSTA31 (Unkles et al. 1991) were used as probes and radiolabeled with α-^{32}P-dATP using a random primer labeling kit (Gibco-BRL, Bethesda, MD). To obtain a probe specific to the *inf1* sequence, single stranded, radiolabeled probe complementary to the 3´ end untranslated region of the *inf1* mRNA was generated by extending a single primer, INF2-F1, from the gel purified *inf1* insert from pFB7. The primer INF1-F1 (5´-CTAT<u>TGA</u>GTGGACTTGACTC-3´) binds to the region surrounding the TGA stop codon (underlined) of the *inf1* open reading frame. The probe was generated by incubating in a final volume of 20 µl, 0.2 µg of primer INF2-F1, 10 ng of *inf1* cDNA template, 0.0166 mM each of dCTP, dGTP, and dTTP, 50 µCi of α-^{32}P-dATP, 1× Taq PCR buffer, and 3 units of Taq polymerase (as supplied by Perkin Elmer) in a Perkin Elmer 9600 thermocycler for 2 min at 95°C followed by 25 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C.

Expression of *inf1* in *E. coli*.

Expression of *inf1* in pFLAG-ATS and immuno-affinity purification of FLAG fusion proteins were conducted following the protocols provided by the manufacturer (IBI-Eastman Kodak, New Haven, CT). Overnight cultures of *E. coli* DH5α containing either pFB52 or pFB53 were diluted (1:100) in LB medium containing ampicillin (50 µg/ml) and incubated at 37°C. When the OD_{600} of the cultures reached 0.6, IPTG was added to a final concentration of 0.4 mM. The cultures were further incubated for 3 to 4 h before processing. Immuno-affinity purification was performed using a FLAG M2 antibody affinity gel. Elution of fusion proteins from the affinity column was obtained after treatment with 0.1 M glycine (pH 3.0). Proteins were then diluted in water and protein concentration determined by a Bradford assay (Bradford 1976).

SDS-PAGE and Western blot analyses.

Proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as previously described (Sambrook et al. 1989; Schagger and von Jagow 1987). Following electrophoresis, gels were silver stained following the method of Merril et al. (1981) or the proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Immobilon, Millipore Corporation, Bedford, MA) using a Mini Trans-Blot apparatus (Bio-Rad Laboratories, Richmond, CA). Detection of antigen-antibody complexes was carried out with a Western blot alkaline phosphatase kit (Bio-Rad Laboratories, Richmond, CA).

Plant assays.

Inoculation of potato with *P. infestans* in time course experiments was conducted by placing 10-µl water droplets containing approximately 1,000 zoospores on the underside of detached potato leaves. The leaves were placed in petri dishes containing water agar (15 g/liter) to maintain high humidity. Late blight symptoms were scored daily, and leaf disks of similar sizes were dissected around the inoculated area and used for RNA extractions. Induction of hypersensitivity was determined by infiltration of elicitin solutions into attached leaves as described previously (Kamoun et al. 1993b). Virulence of *P. infestans* on the examined collection of solanaceous plants was determined in field tests, in growth chamber assays, and in detached leaf assays (Colon and Budding 1988; Colon 1994; Colon et al. 1992, 1995) (data not shown). Plants were routinely grown in greenhouses or in regulated growth chambers (16-h photoperiod, 18°C). The infiltrations described in Table 1 were conducted on plants cultivated in the growth chamber.

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