A Gene Encoding a Host-Specific Elicitor Protein of *Phytophthora parasitica*

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Extracellular elicitor proteins (elicitins) from *Phytophthora* species induce local and distal defense responses specifically in plants of the Solanaceae and Cruciferae. Based on elicitin amino acid sequences, elicitin-coding sequences from *P. parasitica* were amplified by the polymerase chain reaction. A genomic clone containing a complete elicitin gene, *parA1*, was isolated and sequenced. Elicitin was confirmed to be encoded as a precursor protein containing a 20–amino acid signal peptide that is processed before secretion. Bacterial expression of the cloned elicitin gene as a translational fusion protein containing glutathione S-transferase yielded a biologically active protein capable of inducing a hypersensitive response in tobacco, suggesting that fungus-specific postranslational modifications of elicitin are not required for its activity. Southern blot analysis indicated that elicitin genes occur as a multigene family (at least two to 10 copies) in *P. parasitica*, *P. capsici*, *P. citricola*, *P. citrophthora*, *P. cryptogea*, *P. drechsleri*, *P. megasperma*, and *P. palmivora*. Some isolates of *P. parasitica* that did not produce elicitins still contained elicitin-coding sequences but did not accumulate elicitin mRNA.

Most plant pathogens are host-specific, being specialized for infection of particular host plants. Some pathogens have relatively wide host ranges, able to infect 100–1,000 plant species, while others may be restricted to single species. The molecular mechanisms controlling host range are poorly understood.

Pathogens of the oomycete class, especially in the order Peronosporales, cause destructive diseases of a wide range of plants, many of them of economic importance. Plant-pathogenic oomycetes include downy mildews, white rusts, and numerous species of *Pythium* and *Phytophthora*. The oomycetes have classically been included with the fungi by taxonomists. However, several biochemical characters, such as cell wall composition, the pathway for lysine biosynthesis, and, most conclusively, the sequence of the 17S rRNA (Förster *et al.* 1990), have demonstrated that the oomycetes are more closely related to algae in the protoctista (Dick 1990), in particular to chrysophytes and diatoms, than to true fungi or to higher plants. Thus oomycetes constitute a distinct group of eukaryotic plant pathogens with an independent evolutionary history. They may have distinct biochemical mechanisms for interacting with plants and distinct genetic mechanisms for regulating those interactions and creating variation. Recent advances in the molecular genetics of oomycetes, particularly the transformation of *Phytophthora* species (Judelson *et al.* 1991, in press), have provided the opportunity to examine the mechanisms of host specificity within this group at a molecular level.

P. parasitica Dastur causes root, stem, and fruit rot on more than 90 plant species, including conifers, citrus, tomato, and tobacco (Ribeiro 1978). Although *P. parasitica* as a species has a wide host range, individual strains usually have very narrow host ranges (Bonnet 1985; Hine and Aragaki 1963; Matheron and Matjeka 1990). For example, tobacco isolates (sometimes referred to as *P. parasitica* var. *nicotianae*) usually can infect only that host (Ho 1981). A family of small extracellular proteins produced by many *Phytophthora* species (including *P. parasitica*), which may contribute to the host-specificity of these pathogens, has been identified (Ricci *et al.* 1989). These proteins, collectively termed *elicitins*, induce a vigorous defense response—the hypersensitive response (HR)—locally and distally in certain plants of the Solanaceae and the Cruciferae (Ricci *et al.* 1992; Kamoun *et al.* 1993). In the Solanaceae, the response to elicitins is genus-specific, being restricted to *Nicotiana* species. In the Cruciferae, the response is cultivar-specific, being restricted to certain radish and turnip cultivars (Kamoun *et al.* 1993). The response to elicitins provides protection against subsequent infection by *P. parasitica* (in tobacco) (Ricci *et al.* 1989, 1992) and by the bacterial pathogen *Xanthomonas campestris* pv. *armoraciae* (in radish) (Kamoun *et al.* 1993). Isolates of *P. parasitica* that are highly virulent in tobacco do not produce elicitins, while nearly all isolates of *P. parasitica* and other *Phytophthora* species that are nonpathogenic to tobacco secrete large amounts of elicitins (Ricci *et al.* 1989, 1992; Kamoun, unpublished). Therefore, it has been proposed that elicitins act as avirulence factors in the *Phytophthora*-tobacco interaction. That is, they block or slow infection

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A

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PRIMER 1. aa 3 -> 11

Cry   C   T   A   T   Q   Q   T   A   A   Y
Cin   -   -   -   -   -   -   -   -   -   -
Cap   -   -   T   -   -   -   -   -   -   -

   5' TGC ACA ACA ACA CAA CAA ACA GCA GCA TA 3' ->
        T   G G G   G   G   G   G   G   G
            C   C   C               C   C   C
            T   T   T               T   T   T

PRIMER 2. aa 50 <- 60

Cry   M   C   A   S   T   A   C   N   T   M   I
Cin   -   -   -   -   -   -   -   -   -   -   -
Cap   -   -   -   -   -   -   -   -   -   -   -

<- 3' TAC ACA CGA AGA TGA CGA ACA TTA TGA TAC TA 5'
            G   G TCG   G   G   G   G   G
                C   C   C   C               C
                T   T   T   T               T

PRIMER 3. aa 83 <- 91

Cry   N   V   Y   S   Y   A   N   G   F
Cin   -   -   -   T   -   -   -   -   -
Cap   -   -   -   -   -   -   -   -   -

<- 3' TTA CAA ATA AGA ATA CGA TTA CCA AA 5'
        G   G   G TCG   G   G   G   G
            C       C       C       C
            T       T       T       T
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B

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                1           11          21
Cry         TACTATQQTA AYKTLVSILS DASFNQCSTD
Cin         ---------- ---------- ES--S---K-
Cap         AT--T----- --VA------ -S-----A--
Para        -T--T----- --VA------ -T--------
P3118-1                  VA------ -T--------
P3118-2                  VA------ ET--TT-A--

                31          41          51
Cry         SGYSMLTAKA LPTTAQYKLM CASTACNTMI
Cin         --------T- ---N------ ----------
Cap         --------T- ---------- ----------
Para        --------TS ----E----- ------K---
P3118-1     --------TS ----E----- ------K---
P3118-2     --------T- -------

                61          71          81
Cry         KKIVTLNPPN CDLTVPTSGL VLNVYSYANG
Cin         ----A----D ---------- --D--T----
Cap         T---S----D -E-------- ----------
Para        N---S----D -E-------- ----FT----
P3118-1     N--------D -E-------- --
P3118-2

                91      98
Cry         FSNKCSSL
Cin         --S--A--
Cap         --AT-A--
Para        --ST-A--
P3118-1
P3118-2
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Fig. 1. Amplification of elicitin-coding sequences. **A,** Design of degenerate oligonucleotides for the amplification of elicitin-coding sequences. The amino acid sequence of the selected conserved regions of cryptogein (Cry), cinnamomin (Cin), and capsicein (Cap) is shown along with the sequence of the three degenerate primers used. **B,** Comparison of published amino acid sequences of cryptogein (Cry), cinnamomin (Cin), capsicein (Cap), and parasiticein (Para) and deduced amino acid sequences of the two fragments of *Phytophthora parasitica* isolate P3118 amplified by polymerase chain reaction. The P3118-1 sequence was obtained from plasmid pELC38.7; the P3118-2 sequence was from pELC32.3.

by the pathogen by triggering a defense response in the host (Kamoun *et al.* 1993; Ricci *et al.* 1989, 1992).

The amino acid sequences of several *Phytophthora* elicitins have been determined, and isoforms of elicitins have been classified in two groups based on both structural properties and biological activities (Nespoulous *et al.* 1992). The α-elicitin group includes acidic proteins produced by *P. parasitica* and *P. capsici*, whereas β-elicitins are basic, more hydrophilic proteins produced by *P. cryptogea* and *P. cinnamomi* (Bonnet 1985; Nespoulous *et al.* 1992). Recently, an isolate of *P. drechsleri* was shown to produce both α- and β-elicitin isoforms (Huet *et al.* 1992). β-Elicitins induce more severe necrosis than α-elicitins in distal assays. However, both isoforms move equally well in the tobacco vascular system and induce similar necrosis in leaf infiltration assays (Kamoun *et al.* 1993; Nespoulous *et al.* 1992; Devergne *et al.* 1992; Zanetti *et al.* 1992).

In this paper, we investigate the molecular basis of elicitin production in *P. parasitica*. Elicitin-coding sequences were amplified by the polymerase chain reaction (PCR) using redundant oligonucleotides derived from amino acid sequences. Genomic clones containing elicitin genes were obtained and sequenced. Sequences coding for elicitins were found in both elicitin-producing and non-elicitin-producing isolates of *P. parasitica*. Northern blot analyses indicated the absence of elicitin mRNA in the nonproducing isolates. The molecular cloning of elicitin genes should allow a direct evaluation of the role of these proteins as avirulence factors in the *P. parasitica*–tobacco interaction.

RESULTS

Cloning of elicitin-coding sequences.

Elicitin gene sequences were amplified from genomic DNA of *P. parasitica* P3118 by PCR. As shown in Figure 1A, three degenerate primers for the amplification were designed, based on conserved regions of the amino acid sequences of elicitins from *P. cryptogea* (cryptogein), *P. cinnamomi* (cinnamomin), and *P. capsici* (capsicein) (Huet and Pernollet 1989; Ricci *et al.* 1989). Under appropriate reaction conditions, primers 1 and 2 yielded an amplified product of about 175 bp, while primers 1 and 3 yielded a product of about 270 bp. The sizes of these fragments matched the sizes predicted from the amino acid sequences, namely, 174 and 266 bp, respectively, suggesting that elicitin sequences had been successfully amplified.

To confirm the identity of the fragments, the two PCR products were cloned into pTZ18R (Mead *et al.* 1985). Two clones, pELC38.7 and pELC32.3, were further characterized by DNA sequencing. pELC38.7 contained two copies of the 266-bp fragment, ligated together in the same orientation. The two cloned copies had identical amplified sequences (210 bp each), whose deduced amino acid sequences showed strong homology to the capsicein amino acid sequence and matched closely (in 69 out of 70 residues) the recently published sequence of the *P. parasitica* elicitin (parasiticein), as shown in Figure 1B

(Nespoulous *et al.* 1992). pELC32.3 contained two 174-bp fragments ligated together as an inverted repeat. One copy matched the sequence of the 266-bp fragments exactly. The amplified sequence of the second copy (108 bp) was different from the other parasiticein sequences but nevertheless 79% homologous at the DNA sequence and 81% homologous at the inferred amino acid sequence. Therefore, two distinct elicitin sequences were amplified and cloned, one corresponding to a parasiticein amino acid sequence and the other corresponding to a previously unknown elicitin. Since both sequences were obtained from the genome of P3118, it appears that *P. parasitica* may contain genes encoding at least two distinct elicitins.

Multiple elicitin genes in *P. parasitica*.

Southern blot hybridizations of total DNA with the amplified 266-bp fragment internal to the elicitin open reading frame (ORF) were conducted on nine representative isolates of *P. parasitica*. Figure 2 shows that multiple bands (at least three) containing sequences homologous to the elicitin probe were detected in both *Hin*dIII and *Bam*HI digests of eight of the tested isolates (P1955, P582, P1751, P1979, P3118, P3550, P3442, and P3441), suggesting that multiple copies of elicitin-coding sequences are present in the *P. parasitica* genome. It is notable that two isolates from tobacco, P1955 and P582, which do not produce elicitins, still contained elicitin-coding sequences. On the other hand, one isolate from tomato, P3461, which is completely nonpathogenic, did not bear any detectable elicitin-coding sequences.

Multiple elicitin genes in various *Phytophthora* species.

Southern blot hybridizations with the elicitin probe were also conducted on total DNA of a collection of *Phytophthora* species, including *P. capsici*, *P. citricola*, *P. citrophthora*, *P. cryptogea*, *P. drechsleri*, *P. megasperma*, and *P. palmivora*. (Fig. 3). All tested isolates of these species appeared to contain from two to 10 *Hin*dIII bands homologous to the cloned elicitin gene. It therefore appears that elicitin genes occur as a small gene family and are conserved in all tested *Phytophthora* species.

Molecular cloning and analysis of a complete elicitin gene.

In order to obtain a complete elicitin gene, a 2.1-kb *Hin*dIII elicitin fragment from *P. parasitica* P1979, which secretes elicitin, was cloned by ligation of size-fractionated fragments into the phagemid vector lambda-ZAPII, followed by plaque hybridization with the insert of pELC38.7. Following in vivo excision, Southern blot analyses of the cloned plasmid (pELC100) localized the elicitin gene on a 0.6-kb *Sal*I-*Cla*I fragment. As shown in Figure 4, DNA sequence analysis of this fragment revealed a 354-bp ORF, corresponding to a 20–amino acid signal peptide, followed by a 98–amino acid elicitin sequence that is almost identical to the published parasiticein sequence (Nespoulous *et al.* 1992). This gene was designated *parA1*. Primer extension analysis was used to locate the transcriptional start point 56 bp upstream of the start codon (data not shown). No putative eukaryotic promoter sequence or polyadenylation signal was identified in the sequence of the 0.6-kb *Sal*I-*Cla*I fragment.

Bacterial expression of cloned elicitin gene.

In order to demonstrate that the product of the cloned elicitin gene is biologically active, the coding sequence for the processed form of elicitin was inserted into a vector, which allowed it to be expressed in *Escherichia coli*

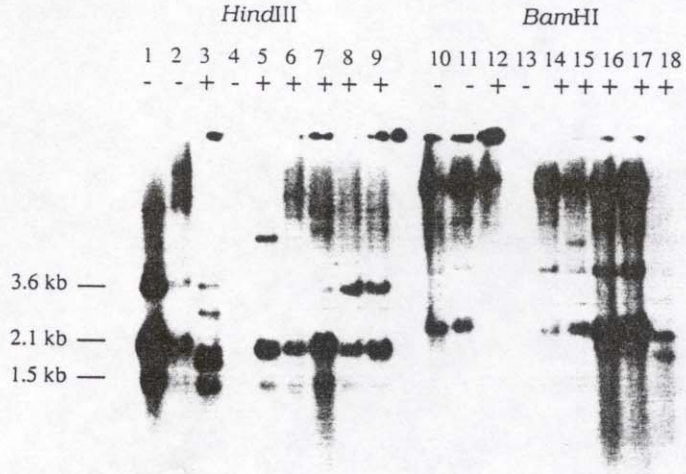

Fig. 2. Multiple elicitin-coding sequences are present in *Phytophthora parasitica*. Total DNA (5 µg) of *P. parasitica* isolates P1955 (lanes 1 and 10), P582 (2 and 11), P1751 (3 and 12), P3461 (4 and 13), P1979 (5 and 14), P3118 (6 and 15), P3550 (7 and 16), P3442 (8 and 17), P3441 (9 and 18) was digested with *Hin*dIII (lanes 1–9) or *Bam*HI (lanes 10–18) and probed at low stringency with a 270-bp fragment internal to the elicitin open reading frame. Plus and minus signs under the lane numbers indicate whether the isolate produces or does not produce elicitin. The numbers on the left indicate the sizes of the three major *Hin*dIII fragments.

as a fusion protein containing glutathione S-transferase (GST) at the amino terminus. Protein extracts from IPTG (isopropyl-β-D-thiogalactopyranoside)–induced *E. coli* transformed with pGEX2TK (GST) or pELC105 (GST-PARA1) were incubated with glutathione-agarose, the columns were washed, and bound proteins were eluted with free glutathione. Affinity-purified proteins were electrophoresed on a sodium dodecyl sulfate (SDS)–polyacrylamide gel, as shown in Figure 5A. In the GST preparation, two major proteins were detected, with masses of approximately 28 kDa, the expected size of the recombinant protein (lanes 1 and 2). The predominant protein in the affinity-purified GST-PARA1 preparation has a mass of approximately 38 kDa, as predicted for the full-length fusion protein (arrow, lane 3). The smaller proteins co-purifying with the full-length fusion protein are likely to be GST-PARA1 degradation products still able to bind to the glutathione-agarose column. Proteins of similar size are also present at a low level in the GST preparation (lanes 1 and 2). Infiltration of the affinity-purified GST-PARA1 preparation into tobacco mesophyll induced a necrotic HR of the infiltrated tissue identical to the response induced by 100 nM parasiticein purified from the culture filtrate of *P. parasitica* (Fig. 5B). In contrast, various amounts of affinity-purified preparations of GST, buffer, or H_2O induced no visible response after infiltration. These results suggest that the cloned elicitin gene encodes an active elicitor protein, and that fungus-specific posttranslational modifications of elicitin are not required for its activity.

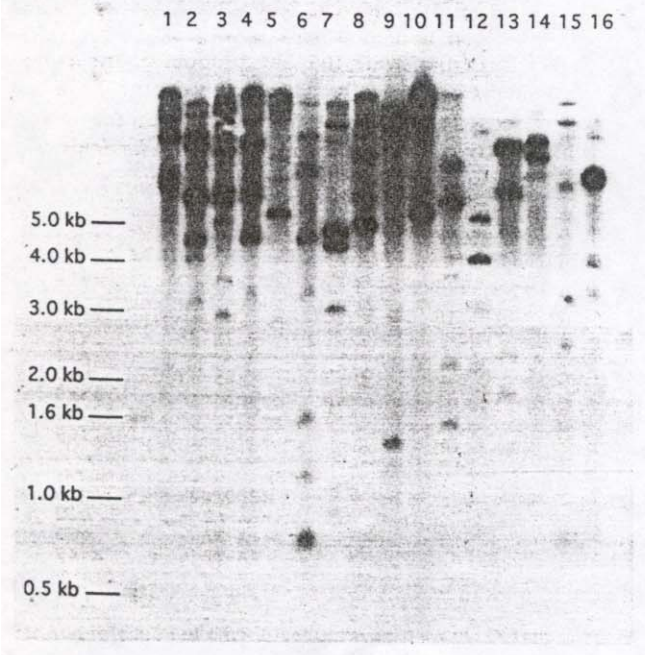

Fig. 3. Multiple elicitin-coding sequences are present in various *Phytophthora* species. Total DNA (5 μg) of *P. cryptogea–P. drechsleri* isolates P1087, P3700, P1810, P3145, P3850, P3105, and P3197 (lanes 1–7, respectively); *P. megasperma* P6667, P3112, P7029, and P3114 (lanes 8–11); *P. citricola* P1823 (lane 12); *P. citrophthora* P1200 (lane 13); *P. capsici* P412 (lane 14); and *P. palmivora* P1182 and P1787 (lanes 15 and 16) was digested with *Hin*dIII and probed at low stringency with a 0.36-kb fragment containing the complete elicitin open reading frame. The numbers on the left indicate the sizes of the molecular markers.

Elicitin mRNA in *P. parasitica* isolates.

As noted above, a number of the *P. parasitica* strains analyzed by Southern analysis contained elicitin-coding sequences even though they did not produce elicitin (Fig. 2). In order to determine if elicitin production is correlated with the expression of elicitin mRNA, total RNA from elicitin-producing and non-elicitin-producing *P. parasitica* strains was isolated and analyzed by Northern blot (Fig. 6). The three elicitin-producing strains tested, P1751, P1979, and P3118, were all found to express a 550-nucleotide transcript when hybridized with an elicitin-specific probe (pELC38.7), whereas the non-elicitin-producing strains, P1955 and P3461, did not express detectable levels of elicitin mRNA. All strains tested contained equivalent amounts of actin mRNA. P3461 (lane 3) has no detectable elicitin genes and is

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-206 GTCGACGAAA GCCGAAGTGC GTGGCAGATC TTGCCGTTCG
      SalI
-166 AATGCTACGC GCCACGGCAA AACCTACACG GTACAACAGC

-126 TTCAAATAAA CCTGCAAGCG AGCCGCCAGC CCAACTCCAG

 -86 CTAGTCAAGC CTAGTTTGCC TCCAACTGCC ATTGTGCAAT

 -46 TTGCTCTCAT CCACACCCAC CCCACTTCTC CCCCACCTCA

   1          M  N  F  R  A  L  F  A  A
  -6 TCCGAA  ATG AAC TTC CGC GCT CTG TTC GCC GCC

  10  T  V  A  A  L  V  G  S  T  S  A
  28 ACC GTC GCC GCC CTC GTC GGC TCC ACC TCC GCC

  21  T  T  C  T  T  T  Q  Q  T  A  A
  61 ACC ACG TGC ACC ACC ACG CAG CAA ACT GCG GCG

  32  Y  V  A  L  V  S  I  L  S  D  T
  94 TAC GTG GCG CTC GTA AGC ATC CTC TCG GAC ACG

  43  S  F  N  Q  C  S  T  D  S  G  Y
 127 TCG TTC AAC CAG TGC TCG ACG GAC TCT GGC TAC

  54  S  M  L  T  A  T  S  L  P  T  T
 160 TCA ATG CTG ACG GCC ACC TCG TTG CCC ACG ACG

  65  E  Q  Y  K  L  M  C  A  S  T  A
 193 GAG CAG TAC AAG CTC ATG TGC GCG TCG ACG GCG

  76  C  K  T  M  I  N  K  I  V  T  L
 226 TGC AAG ACG ATG ATC AAC AAG ATC GTG ACG CTG

  87  N  P  P  D  C  E  L  T  V  P  T
 259 AAC CCG CCC GAC TGC GAG TTG ACG GTG CCT ACG

  98  S  G  L  V  L  N  V  F  T  Y  A
 292 AGC GGC CTG GTA CTC AAC GTG TTC ACG TAC GCG

 109  N  G  F  S  S  T  C  A  S  L  *
 325 AAC GGG TTC TCG TCT ACG TGC GCG TCA CTG TAA

 358 GCGGGTTTGA TCTCTGCGTC CAGAATCGAT
                           ClaI
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Fig. 4. Sequence analysis of the *parA1* gene from *Phytophthora parasitica* isolate P1979. The sequence of the 593-bp *Sal*I-*Cla*I fragment was determined and found to contain a 354-bp open reading frame encoding pre-elicitin. The amino acid sequence of the 20-residue signal peptide is shown in bold. The primary transcriptional start site was determined by primer extension and was located 56 bp upstream from the start codon (indicated by the arrow).

therefore not expected to contain elicitin mRNA. However, P1955 (lane 1) does contain elicitin-coding sequences. Therefore, these results show that, at least in P1955, elicitin expression is regulated at the mRNA level.

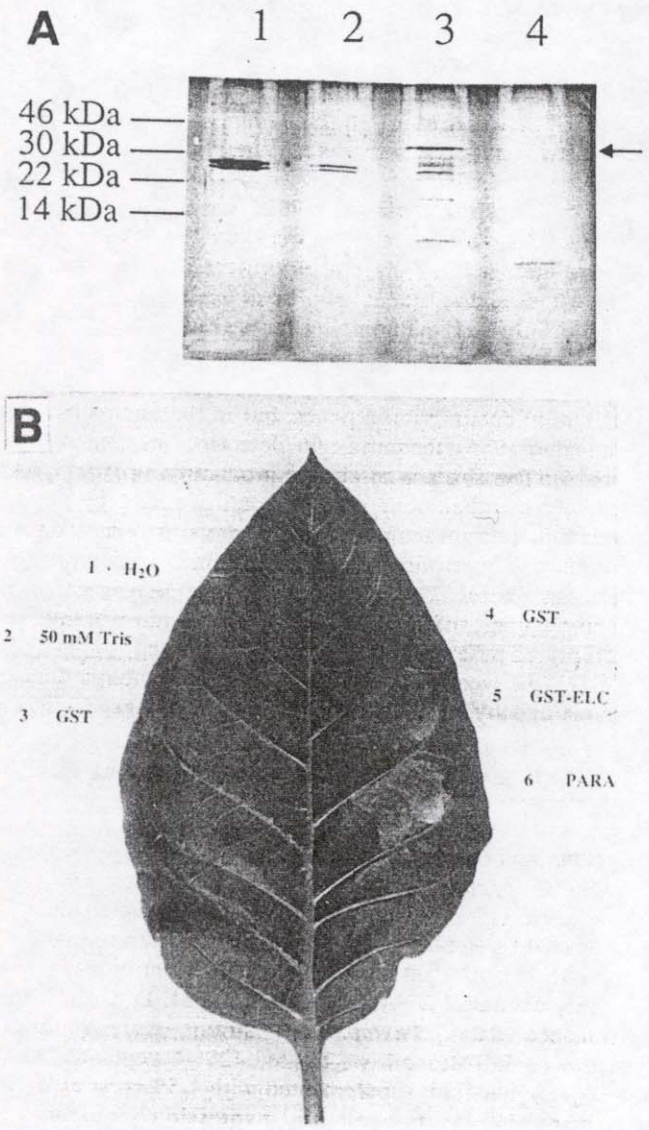

Fig. 5. Bacterial expression of cloned elicitin gene. **A,** Polyacrylamide gel electrophoresis of purified GST-PARA1. Expression of GST (a fusion protein containing glutathione S-transferase) or GST-PARA1 was induced with isopropyl-β-D-thiogalactopyranoside in *Escherichia coli* containing pGEX2TK or pELC105, respectively. Recombinant proteins were affinity-purified on glutathione-agarose columns, and 10-µl samples of eluted GST (2.59 µg) (lane 1), GST (133 ng) (lane 2), GST-PARA1 (255 ng) (lane 3), and purified parasiticein (10 ng, from *Phytophthora parasitica* 6H-11A) (lane 4) were electrophoresed through a 15% polyacrylamide gel. Molecular weight standards are shown on the left. The arrow indicates full-length GST-PARA1 fusion protein (lane 3). The protein concentration of recombinant proteins was determined by Bradford assay. **B,** Tobacco leaf infiltrations with purified GST-PARA1 fusion protein. Purified proteins (0.5-ml fractions) shown in Figure 4A were infiltrated into tobacco mesophyll. Infiltrated samples were (1) H_2O, (2) 50 mM Tris (pH 8), (3) GST (10 µM), (4) GST (500 nM), (5) GST-PARA1 (1 µM, GST-PARA1), and (6) purified parasiticein 6H-11A (100 nM). The photograph was taken 48 hr postinfiltration.

DISCUSSION

Genetic structure of a host-specific elicitor gene.

We report here the molecular cloning of a *P. parasitica* gene, *parA1*, encoding a host-specific elicitor, parasiticein. The DNA sequence of *parA1* revealed a 354-bp ORF encoding a protein of 118 amino acids (pre-elicitin) (Fig. 4). Pre-parasiticein contains a 20–amino acid N-terminal signal peptide that would be processed to yield the 98–amino acid mature extracellular protein. Terce-Laforgue *et al.* (1992) estimated that pre-cryptogein also has a 20–amino acid leader sequence. The signal peptide is typical; it is hydrophobic, with a positively charged arginine residue at position four. The deduced amino acid sequence of the mature elicitin is 99% homologous (having one mismatch at position 65) to the sequence of a purified active parasiticein from a carnation isolate of *P. parasitica* (Nespoulous *et al.* 1992).

Expression of the cloned elicitin sequence as a GST-PARA1 fusion protein in *E. coli* yielded a biologically active protein capable of inducing necrosis in tobacco, similar to that caused by purified parasiticein (Fig. 5). The complete elicitin gene we cloned is therefore likely to encode an active elicitin. Ricci *et al.* (1989) proposed that elicitins are holoproteins and do not undergo any detect-

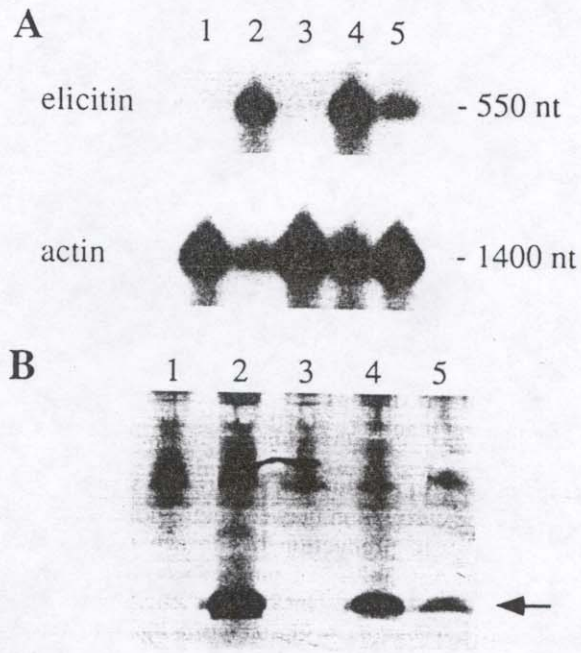

Fig. 6. Elicitin mRNA is absent from non-elicitin-producing isolates of *Phytophthora parasitica*. **A,** Total RNA (5 µg) from mycelia of *P. parasitica* isolates P1955, P1751, P3461, P1979, and P3118 (lanes 1–5, respectively) were probed with a 270-bp fragment internal to the elicitin open reading frame (top panel). The number on the right indicates the approximate size of the elicitin mRNA. Northern blot analysis of the same isolates using an actin probe is shown in the bottom panel. The size of the actin mRNA is indicated on the right. **B,** Polyacrylamide gel electrophoresis of 10 µl of undiluted culture filtrates of *P. parasitica* isolates P1955, P1751, P3461, P1979, and P3118 (lanes 1–5, respectively) indicating the presence or absence of the 10-kDa elicitin protein (arrow). The filtrates were obtained from the mycelial cultures used for RNA extraction.

able posttranslational modifications. The ability to express active elicitins in *E. coli* conclusively confirms that fungus-specific posttranslational modifications of elicitins are not required for their biological activity. With appropriate engineering, it should be possible to directly express significant amounts of active elicitins in bacteria.

Elicitins are encoded by a multigene family.

Southern blot analyses using a probe internal to the elicitin ORF identified multiple copies (two to 10) of elicitin-coding sequences in the genomes of *P. parasitica* (Fig. 2), *P. capsici*, *P. citricola*, *P. citrophthora*, *P. cryptogea*, *P. drechsleri*, *P. megasperma*, and *P. palmivora* (Fig. 3). Additionally, PCR amplification with the degenerate primers yielded multiple elicitin sequences in both *P. parasitica* (Fig. 1B) and *P. megasperma* f. sp. *glycinea* (Y. Mao, personal communication), confirming the occurrence of multiple elicitin genes in these species. Preliminary DNA sequencing indicate that three additional elicitin copies in *P. parasitica* correspond to intact ORFs (K. Klucher, unpublished). The occurrence of an elicitin multigene family raises interesting questions as to the expression of the additional copies, their cellular localization, and their function.

Although many avirulence genes cloned to date from fungi and bacteria occur in single copy (Keen 1990; van Kan *et al.* 1991), multiple genes encoding elicitin occur in *P. parasitica*. Multiple homologs to the avirulence gene *avrBs3* of the bacterial pathogen *X. campestris* pv. *vesicatoria* were identified in several other *Xanthomonas* pathovars (Bonas *et al.* 1989; Knoop *et al.* 1991). Multiple genes related to *avrBs3* were found to control cultivar specificity in *X. c.* pv. *malvacearum* and *X. oryzae* (De Feyter and Gabriel 1991; Hopkins *et al.* 1992). In addition, another member of the family, the *pthA* gene of *X. citri* was recently shown to be required for pathogenicity on citrus along with host specificity on bean and cotton (Swarup *et al.* 1992). Future studies should determine whether the different members of the elicitin gene family have different specificities and whether they are required for virulence on plants.

Absence of elicitin production in virulent strains.

Based on the correlation between high virulence and the absence of elicitin production in tobacco isolates of *P. parasitica*, and the ability of purified elicitins to induce resistance to *P. parasitica* in tobacco, elicitins appear to act as avirulence factors in the *P. parasitica*–tobacco interaction (Ricci *et al.* 1989, 1992; Kamoun *et al.*, unpublished). With the cloning of a parasiticein gene from *P. parasitica*, it will be possible to test this hypothesis directly by introducing the cloned gene into a virulent non-elicitin-producing strain, using a constitutive promoter if necessary.

We examined three virulent tobacco pathogens that do not produce elicitin for the presence of elicitin-coding sequences (Fig. 2, data not shown). All virulent isolates retained a set of elicitin genes, which appeared similar to those in elicitin-producing strains, as judged by Southern hybridization analyses. Elicitin genes were completely absent from one strain originally isolated from tomato (P3461). However, this strain is now completely nonpathogenic to both tobacco and tomato.

Commonly, avirulence genes are not expressed in virulent races of bacterial and fungal pathogens. This is often associated with the absence of DNA homologous to the avirulence gene in the virulent races (Keen 1990). For example, in the fungus *Fulvia fulva*, the avirulence gene *avr9* encodes a 28–amino acid peptide elicitor that is specific for the resistance gene *Cf9* (van Kan *et al.* 1991). *F. fulva* races that do not produce the elicitor and that are virulent on *Cf9*-containing cultivars lack the *avr9* gene (van den Ackerveken *et al.* 1992; van Kan *et al.* 1991). Alternatively, a nonfunctional recessive allele of the avirulence gene may be present in virulent races. Such is the case of the *avrD* gene of *Pseudomonas syringae* pv. *tomato*, in which the homolog from virulent *P. s.* pv. *glycinea* encodes an altered gene product (Kobayashi *et al.* 1990). In *P. parasitica*, the virulent, non-elicitin-producing strains retain elicitin genes, but in the one isolate examined (P1955) there was no detectable elicitin mRNA (Fig. 6). The absence of elicitin production in P1955, and possibly in other nonproducing strains, could be due to mutations in transacting regulatory proteins, a mechanism suitable for controlling a multigene family. Alternatively, there may be *cis*-acting mutations in the elicitin genes of nonproducing strains that affect transcription or mRNA stability. These possibilities will be tested by genetic crosses between elicitin-producing and nonproducing strains and by studying the expression of tagged elicitin genes in the various genetic backgrounds.

METHODS

Strains and culture conditions.

The *Phytophthora* strains used in this study are listed and described in Table 1. Isolate P3461, which did not hybridize to elicitin sequences, was confirmed to be a *P. parasitica* isolate on the basis of the restriction pattern of its mitochondrial DNA (H. Förster and M. D. Coffey, unpublished data). *Phytophthora* strains were routinely grown in full-strength or cleared (20%) vegetable juice (V8 juice) medium supplemented with 1.5% agar at 24° C (Ribeiro 1978). For elicitin expression experiments,

Table 1. *Phytophthora parasitica* strains used in this study[a]

Strain	Characteristics
P1955	*Nicotiana tabacum* (South Africa), A1, ELC–, race 0
P582	*N. tabacum* (Kentucky), A2, ELC–
P1751	*N. tabacum* (Australia), A1, ELC+
P3461	*Lycopersicon esculentum* (Great Britain), ELC–
P1979	*L. esculentum*, ELC+
P3118	*L. esculentum* (Australia), A2, ELC+
P3550	*Dianthus* sp. (France), ELC+
P3442	*Fragaria vesca* (Japan)
P3441	Soil (Taiwan), A2

[a] A1 and A2 refer to the compatibility type. ELC+ and ELC– indicate elicitin producers and nonproducers, respectively. The host species or source and place of origin are also given. All strains were provided by M. D. Coffey, University of California, Riverside.

strains were grown for 10 days at 25° C in still culture in a medium containing (per liter) 0.5 g of KH_2PO_4, 0.25 g of $MgSO_4 \cdot 7H_2O$, 1 g of asparagine, 1 mg of thiamine, 0.5 g of yeast extract, and 25 g of glucose (modified from Bonnet *et al.* 1985). Occasionally, a mixture of antibiotics (pimaricin, 60 µg/ml; ampicillin, 375 µg/ml; rifampicin, 30 µg/ml; and pentachloronitrobenzoic acid, 375 µg/ml) was added to the medium.

E. coli XL1-Blue (Stratagene, La Jolla, CA) was used in most experiments and was 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.

DNA manipulations.

DNA manipulations were conducted essentially as described elsewhere (Ausubel *et al.* 1987; Sambrook *et al.* 1989). DNA fragments were isolated from agarose gels, by means of a Geneclean kit (Bio101, La Jolla, CA). Total DNA of *Phytophthora* was isolated from mycelium grown in liquid culture, following the procedure of Raeder and Broda (1985) with the modifications described by Mao and Tyler (1991). Alkaline DNA transfer to Nytran Hybond N+ (Amersham, Arlington Heights, IL) and Southern hybridizations were performed at 65° C as previously described (Ausubel *et al.* 1987; Kamoun *et al.* 1992). All filters were washed at low stringency at room temperature in 1% SDS plus 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate).

Sequencing plasmids were constructed by digesting pELC100 with *Sal*I and subcloning fragments into *Sal*I-digested pTZ18R (Mead *et al.* 1985). DNA sequencing was carried out with a Sequenase 2.0 kit (United States Biochemicals, Cleveland, OH) as suggested by the manufacturer. Primers were obtained from United States Biochemicals and Operon Technologies (Alameda, CA). Sequence analysis and comparisons were made with Mac Vector software (International Biotechnologies, New Haven, CT).

RNA manipulations.

Total RNA from *P. parasitica* was isolated by a modification of the procedure described by Frederick and Kinsey (1990). Briefly, mycelium from 5-day-old *P. parasitica* grown in liquid culture was frozen in liquid nitrogen and ground to a fine powder. The powder was resuspended in a solution containing 85 mM Tris (pH 7.5), 85 mM LiCl, 17 mM dithiothreitol (DTT), and 1.5% SDS and extracted with phenol/$CHCl_3$ (50:50) for 20 min at room temperature. Following centrifugation, the aqueous phase was reextracted with phenol/$CHCl_3$ (50:50) and then again with $CHCl_3$. RNA was precipitated with 2M LiCl and reprecipitated with ethanol.

Total RNA (5 µg) was electrophoresed through a 1.2% agarose (w/v) and 6.6% formaldehyde (w/v) gel as described by Irminger *et al.* (1987). Following electrophoresis, RNA was transferred to Hybond N (Amersham) in 10× SSC and then baked at 80° C for 2 hr in a vacuum oven. The baked filter was prehybridized at 68° C in 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 7.7]), 5× Denhardt's reagent, 0.5% SDS, and denatured salmon sperm DNA (100 µg/ml). After 1 hr, radiolabeled *P. parasitica* elicitin DNA or *P. infestans* actin DNA (Unkles *et al.* 1991) was added, and hybridization was continued for 17–20 hr. Filters were washed in 2× SSC plus 1.0% SDS at room temperature, and then autoradiography was conducted at –70° C.

Primer extension analysis.

For primer extension studies an oligonucleotide (5'-AACAGAGCGCGGAAGTTCAT, Operon Technologies) specifically corresponding to *parA1* coding sequence (from nucleotides 1 to 20 relative to ATG) was end-labeled using T4 kinase as previously described (Sambrook *et al.* 1989). Labeled primer (0.03 pmol) was added to 5 µg of total RNA from *P. parasitica* strain P1979 (an elicitin producer) or P1955 (a nonproducer), and the mixture was precipitated with ethanol. The pellets were dissolved in 0.3 M NaOAc (150 µl) and reprecipitated with ethanol. These pellets were washed in 70% ethanol (v/v), dried in a SpeedVac concentrator, and dissolved in 2 mM Tris (pH 7.4), 0.25 M KCl, and 2 mM EDTA (10 µl). Samples were heated at 75° C for 1.5 min and then at 57° C for 4 hr to allow hybridization of the primer to the RNA. The annealed primer-RNAs were precipitated with ethanol, and the pellets were washed in 70% ethanol (v/v), dried in a SpeedVac concentrator, and dissolved in a solution of 50 mM Tris (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, actinomycin D (125 µg/ml), and (0.35 mM each) deoxynucleoside triphosphate (dNTP), 40 U of RNasin ribonuclease inhibitor (Promega), and 200 U of mouse mammary leukemia virus (MMLV) reverse transcriptase (Bio-Rad Laboratories, Richmond, CA) (50 µl). The reverse transcription was carried out at 37° C for 1 hr and was terminated by adding 300 µl of ethanol and precipitating. The pellets were washed in 70% ethanol (v/v), dried in a SpeedVac concentrator, and dissolved in loading dye (a 2:1 mixture of formamide loading dye and 0.1 M NaOH). Samples were heated to 95° C for 5 min before being loaded on a 6% acrylamide–8 M urea sequencing gel. Dideoxy sequencing reactions using the labeled primer and a plasmid containing the elicitin gene were run to determine the precise length of the extended product. The gels were dried and subjected to autoradiography at –70° C.

PCR amplifications.

Reaction conditions to specifically amplify elicitin sequences were 50 mM KCl, 10 mM Tris-HCl (pH 8.2), 2.5 mM $MgCl_2$, and (200 µM each) dNTP, gelatin (200 µg/ml), *P. parasitica* DNA (5–10 µg/ml), degenerate primer (1.5 µg/ml), and *Taq* polymerase (40 units per milliliter). The reaction was incubated in a Perkin-Elmer Cetus thermocycler at 94° C for 2 min and then was subjected to 35 cycles of 94° C for 1 min, 25° C for 1 min, and 72° C for 2 min. Finally, the reaction was incubated at 72° C for 5 min.

Targeted cloning of an elicitin gene.

By Southern blot analysis with the elicitin probe, *P. parasitica* P1979 was shown to contain bands with elicitin

sequences, including a 2.1-kb *Hin*dIII fragment (Fig. 2). To directly clone this fragment, 25 μg of P1979 DNA was digested with *Hin*dIII and electrophoresed through a 0.7% agarose gel. Six sequential pools of DNA fragments ranging from approximately 1.6 to 2.5 kb were gel-purified, and subsequent Southern analysis identified one fraction highly enriched in the 2.1-kb *Hin*dIII elicitin fragment. DNA of this fraction (250 ng) was partially filled (A and G) and ligated with partially filled (C and T) *Spe*I-digested lambda-ZAPII phagemid vector (Stratagene). After packaging (GigaPack, Stratagene) and transfection in LE392, approximately 300 plaques were hybridized to the elicitin probe, and one positive clone was identified. In vivo excision of the pSK-based recombinant plasmid was obtained by coinoculating overnight *E. coli* XL1-Blue with the positive clone and helper phage M13KO7 (Vieira and Messing 1987) and plating on LB plates supplemented with ampicillin. One colony was selected, and the excised plasmid (pELC100) was checked by restriction enzyme digestions and Southern blot analysis, and the presence of elicitin-coding sequences was confirmed.

Expression of elicitin in *E. coli*.

The GST-elicitin fusion protein (GST-PARA1) expression plasmid, pELC105, was constructed by digesting pELC100 with *Dra*III and *Cla*I and isolating the 320-bp fragment containing elicitin-coding sequences from amino acids 23 to 118. This fragment was blunt-ended using the Klenow fragment of DNA polymerase I and subcloned into *Sma*I-digested pTZ18R, creating pELC106. pELC106 was digested with *Bam*HI and *Eco*RI (sites flanking the elicitin gene), and the resulting 326-bp fragment was isolated and ligated with the GST expression vector, pGEX2TK (Kaelin *et al.* 1992), which had been digested with *Bam*HI and *Eco*RI. Correct clones were determined by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) of *E. coli* proteins and DNA sequence analysis and designated pELC105.

Overnight bacterial cultures containing the GST-PARA1 fusion plasmid were diluted 1:10 in LB containing ampicillin (50 μg/ml) and grown for 2 hr at 37° C. IPTG was added to a final concentration of 0.1 mM, and cultures were harvested 3 hr later. Cultures were centrifuged at 4000 × *g* for 10 min, and bacterial pellets were suspended in 50 mM Tris (pH 7.5) and 10 mM EDTA. This solution was frozen in a dry ice–ethanol bath and then thawed at room temperature. Phenylmethylsulfonyl fluoride (PMSF) (1 mM) and lysozyme (1 mg/ml, or 10–15 ml/g wet pellet) were added, and the solution was incubated on ice for 30 min; Triton X-100 was then added to 1%, and the solution was further incubated on ice for 30 min. KCl was added to 200 mM, and the reaction was centrifuged at 105,000 × *g* for 30 min. Triton X-100 was added to the supernatant to a final concentration of 2%. GST-PARA1 was affinity-purified on a glutathione-agarose column and eluted with 10 mM reduced glutathione in 50 mM Tris-HCl (pH 8). Eluted protein was dialyzed in 50 mM Tris-HCl (pH 8) to remove glutathione. Dialyzed proteins were frozen in a dry ice-ethanol bath and stored at –80° C. Total protein concentration of recombinant proteins was determined by a Bradford assay (Bradford 1976). Parasiticein 6H-11A was purified and quantitated as previously described (Kamoun *et al.* 1993). Biological activity of recombinant and native elicitins was determined by leaf infiltrations (Kamoun *et al.* 1993).

SDS-PAGE.

Proteins were subjected to SDS-PAGE as previously described (Sambrook *et al.* 1989). SDS-polyacrylamide gels consisted of a 5% stacking gel and a 15% resolving gel. Prior to electrophoresis, protein samples were suspended in SDS-PAGE sample buffer and heated at 90° C for 3 min. Following electrophoresis, gels were silver-stained as described by the manufacturer (Bio-Rad). Molecular weights were estimated by comparison with known molecular weight standards (Amersham).

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