

Characterization of a Gene Cluster of *Phytophthora cryptogea* Which Codes for Elicitins, Proteins Inducing a Hypersensitive-Like Response in Tobacco

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Elicitins, proteinaceous elicitors secreted by *Phytophthora spp.*, act as inducers of a hypersensitive-like response in tobacco during incompatible interactions. We have isolated and cloned sequences encoding cryptogein and related isoforms from *P. cryptogea* that belong to the elicitin family. The isolation of a genomic clone led to the characterization of four clustered genes. Two of these genes encode distinct elicitins, and two genes would encode, if expressed, a class of highly acidic elicitins which had not been observed so far. Northern blots indicate that elicitin genes are expressed in the fungus grown in vitro, though at different levels. Southern hybridization revealed that elicitins are encoded by a multigene family in several other species of *Phytophthora*. Moreover, isolates of *Phytophthora parasitica* var. *nicotianae*, pathogenic to tobacco, which do not produce elicitins, possess several elicitin-encoding genes. Involvement of elicitins in plant-pathogen interactions is discussed.

Additional keywords: avirulence factors, elicitors, multigene family, *Phytophthora cryptogea*.

The interactions between a plant and a pathogenic microorganism can be roughly classified into three types. In most cases, there is no recognition between the plant and the microorganism (immunity). In the case of a susceptible host the development of a disease occurs (compatible interaction), whereas nonhost plants or resistant plants (incompatible interactions) may exhibit cell necrosis associated with the activation of defense mechanisms, which is known as the hypersensitive response (HR). Microbial signal molecules recognized by the plant and sometimes called elicitors of HR are

responsible for the triggering of this latter response. Elicitors have diverse chemical structures, but recently there has been increased interest in the role of proteins in HR elicitation (Ricci et al. 1993) since their involvement has been demonstrated in a wide range of interactions between plants and pathogens: viruses (Culver and Dawson 1991), bacteria (Willis et al. 1991; Wei et al. 1992) and fungi (De Wit et al. 1993; Ricci et al. 1993). As the recognition of such elicitors by the plant could be a basis for pathogen host specificity (Keen 1990) the characterization of the genes encoding proteinaceous elicitors is a significant step towards a precise understanding of the molecular basis of plant-pathogen interactions.

The involvement of a fungal proteinaceous elicitor in the induction of HR and acquired resistance was shown for the first time in the case of *Phytophthora*/tobacco interactions (Bonnet 1988). All *Phytophthora* species are incompatible with tobacco (Bonnet 1985), except isolates of *P. parasitica* called *P. parasitica* var. *nicotianae* (Ricci et al. 1992), strictly specialized on tobacco and causing the black shank disease (Tucker 1931). The interaction between tobacco and incompatible *Phytophthora* is associated with distal necrotic symptoms, resulting from the release by the fungus of 10-kDa proteins, called elicitins (for a review, see Ricci et al. 1993), which were shown to migrate within the plant (Devergne et al. 1992). This necrosis is accompanied by protection against subsequent infection with the isolates pathogenic to tobacco (Bonnet et al. 1986; Ricci et al. 1992). The compatible interaction between tobacco and *P. parasitica* var. *nicotianae* leads to the development of the disease, and no elicitin is produced by the pathogen (Bonnet et al. 1986). Purified elicitins are able, when applied to tobacco plants, to induce both the HR and acquired resistance against *P. parasitica* var. *nicotianae* (Ricci et al. 1989).

Several elicitins have been purified from various *Phytophthora* species (Ricci et al. 1993) and sequenced. They are highly conserved within the genus *Phytophthora* and exhibit limited amino acid replacements resulting in large differences in the protein net charge. They can be classified into basic elicitins produced by species such as *P. cryptogea* (Ricci et al. 1989) and acidic elicitins produced by species such as *P. capsici* (Ricci et al. 1989) or *P. parasitica* (Ricci et al. 1992).

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The nucleotide sequences reported appear in the GenBank database under the accession numbers Z34459 (X24), Z34462 (B14), Z34460 (B20), and Z34461 (B26).

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This classification, based on physicochemical properties, corresponds to differences in the ability to elicit HR-like symptoms, basic elicitors being 100 times more effective than acidic ones in causing leaf necrosis on tobacco plants. The situation has been complicated by the fact that both acidic and basic elicitors can be found concomitantly in the culture filtrate of a single isolate (Le Berre et al. 1994).

Over a large range of *P. parasitica* isolates, a high level of virulence to tobacco has been found associated with the lack of elicitor production (Bonnet et al. 1994). Elicitors have therefore been hypothesized to act as avirulence factors in the tobacco-*Phytophthora* interaction. However, unlike other fungal avirulence gene products (De Wit et al. 1993), elicitors seem to be constitutively expressed in high amounts in the absence of interaction with plants and during growth in complex culture media (Bonnet 1985; Le Berre et al. 1994), while most other proteinaceous elicitors are induced in planta or in particular media. They are also produced during compatible interactions such as *P. parasitica* and tomato, or *P. capsici* and pepper (Devergne et al. 1994), without induction of the HR. Finally, the tobacco-elicitors interaction is not restricted to a race-cultivar system, as in other pathosystems. All tobacco species are sensitive to the various elicitors produced by diverse species of *Phytophthora* (P. Bonnet, personal communication). Although a cultivar-specific resistance to *Xanthomonas campestris* pv. *armoraciae* was observed following the coinfiltration of elicitors with the pathogen into radish leaves (Kamoun et al. 1993a) the induction of HR following treatment of solanaceous plants with elicitors was only observed on plants of the genus *Nicotiana* (P. Bonnet, personal communication).

Recently, a gene coding for parasiticein was characterized in *P. parasitica* (Kamoun et al. 1993b). The use of this gene as a probe led to the identification of multiple copies of homologous sequences in various *Phytophthora* species, including aggressive isolates of *P. parasitica* var. *nicotianae*, confirming preliminary results (Ricci et al. 1993). In this paper we describe the characterization of several genes encoding elicitors from *P. cryptogea*. Two of these genes correspond to a class of elicitors previously purified (Billard et al. 1988, Le Berre et al. 1994). Two other genes would encode an unusual class of elicitors.

RESULTS

Characterization of elicitor clones.

Based on the comparison of several elicitor sequences (Ricci et al. 1989; Mouton-Perronet et al. 1995), we used two sets of degenerate oligonucleotides as sense and antisense primers in PCR experiments, using total DNA of *P. cryptogea* isolate 52 as a template (Fig. 1). Except for the possible presence of intervening sequences in the elicitor genes, the amplification of a fragment of 119 bp would be predicted. Such a fragment was obtained, purified from a polyacrylamide gel, ligated into *Sma*I-digested pUC19, and cloned into *E. coli*. Three recombinant clones, named pCry5, pCry19, and pCry38, were selected and sequenced. They all possessed three fragments of 119 bp, ligated together, whose deduced amino acid sequences corresponded to elicitors. The occurrence of several copies in each clone may be due to an artifact of cloning such short blunt end fragments. Based on their se-

quence, these nine fragments could be classified into three groups (Fig. 2).

The first group (six copies) comprises sequences that code for a polypeptide that matches exactly residues 24 to 63 of the published amino acid sequence of cryptogein B (Ricci et al. 1989). These fragments exhibit slight heterogeneity, located in the regions corresponding to the primers. As the primers were degenerate oligonucleotides, these differences may reflect the diversity of sequences used for priming the PCR.

The second group (two copies) comprises fragments identical to those of the first group, except for a lack of three adjacent nucleotides, leading to a peptide deleted of amino acid 38.

The third group (one sequence) would encode a peptide which is more closely related to parasiticein than to cryptogein (Mouton-Perronet et al. 1995), suggesting that PCR had amplified an acidic elicitor gene from *P. cryptogea*. The occurrence of such a gene was expected because of the previous isolation and purification of acidic elicitors (cryptogeins A1 and A2) from a culture filtrate of *P. cryptogea* 52 (Le Berre et al. 1994). The three types of amplified sequences confirm our previous deduction that the elicitors of *P. cryptogea* are encoded by several genes.

Cloning and characterization of elicitor genes.

Three recombinant phages, isolated from a genomic library of *P. cryptogea* 52, strongly hybridized to the insert of pCry38 under stringent conditions. One of the positive phages, called N1, was isolated and extensively studied by restriction and Southern hybridization analysis. Hybridization after a *Bam*HI digestion showed that several restriction fragments of the insert hybridized to the probe (not shown), suggesting that several copies of the gene coding for cryptogein

	24						30
CRY	F	N	Q	C	S	T	D
CIN	-	S	-	-	-	K	-
CAP	-	-	-	-	A	-	-
PAR	-	-	-	-	-	-	-
PRIMER 1							
	5' TTCAACCAATGCTCCACGGA >						
		G	T	G	A		
					T		
	56						63
CRY	C	N	T	M	I	K	K
CIN	-	-	-	-	-	-	-
CAP	-	-	-	-	-	T	-
PAR	-	K	-	-	-	N	-
PRIMER 2							
	< ACATTATGCTACTAATTCTTCTA 5'						
	G	G	G	G			

Fig. 1. PCR amplification of elicitor sequences: comparison of the sequences of cryptogein (CRY), cinnamomin (CIN), capsicein (CAP), and parasiticein (PAR) in the target regions of proteins used to prime PCR amplification of elicitor sequences, and deduced sequences of the degenerate oligonucleotides used as sense (primer 1) and antisense (primer 2) primers.

or related elicitors were present in this clone. Further analysis using several restriction enzymes showed that the region of the insert which hybridized to pCry38 spanned around 6 kb (Fig. 3). This region was sequenced, revealing four clustered open reading frames (ORF) (Fig. 4) which would encode elicitors exhibiting distinct physicochemical properties. Sequence analysis indicated that these ORFs are not interrupted by intervening sequences. Furthermore they all possess a 60-bp region at the 5' terminus of the ORF, which may correspond to a signal peptide. A similar sequence has been identified in the *parA1* gene encoding parasiticein (Kamoun et al. 1993b).

Two of the ORFs (X24 and B14) would encode proteins of 118 aa, including the signal peptide. The protein encoded by X24 would be strictly identical with cryptogin B (Ricci et al. 1989). The deduced amino acid sequence of B14 is more closely related to parasiticein than to cryptogin. Moreover the region corresponding to the residues 1 to 21 matches perfectly the N-terminal sequence of the acidic isoform of cryptogin A1 (Le Berre et al. 1994). The two other ORFs (B20 and B26) would encode distinct proteins of 123 aa, including the signal peptide, which might be processed into proteins of 103 aa, instead of 98 aa for elicitors. Multiple alignment revealed that the sequence of the four ORFs and the *parA1* gene (Kamoun et al. 1993b) are highly conserved, especially with regard to the position of the cysteine, methionine, and aromatic residues (Fig. 4B and C). The signal peptide sequences are also very conserved, and that of B14 and *parA1* are even identical. Based on these high sequence homologies, we assume that ORFs B20 and B26, if expressed, would encode elicitorlike proteins. From their amino acid composition, these proteins would be more acidic than the acidic elicitors described so far. We propose to call these genes *hae* (highly acidic elicitors) to distinguish them from the

genes encoding the previously characterized elicitors having 98 aa.

Sequence analysis enabled the identification of CAAT and TATA motifs 5' upstream of the coding region of B14, B20, and B26. Surprisingly, such sequences do not occur in the 5' noncoding region of X24 although related motifs (CAAC or CAAG and TAGA) are present. Between the TATA-like motifs and the ATG start codon of the four genes, a canonical sequence spanning 17 bp was found. This sequence, highly conserved among the elicitor genes (Fig. 5), was also observed in several other *Phytophthora* genes (Dudler 1990; Karlovsky and Prell 1991; Unkles et al. 1991; Pieterse et al. 1991; Kamoun et al. 1993b; Pieterse et al. 1994). This region was assigned as the transcription start site (TSS) of some of these genes (Pieterse et al. 1993). Primer extension experiments, using a primer specific of the B14 sequence, led to the characterization of the mRNA start site 56-bp upstream the ATG codon (data not shown), located exactly in the canonical sequence. Similar experiments performed on *P. parasitica* RNA indicated a quite similar location for the transcription start site of the *ParA1* mRNA (Kamoun et al. 1993b). Other motifs are highly conserved among the elicitor genes of *P. cryptogea* and the *ParA1* gene. An AC-rich region is present in the 5' untranslated region, close to the ATG codon. Moreover a CAAG motif precedes the translation initiation site in all four genes (data not shown). The functional significance of this highly conserved motif is unclear but these regions could be involved in the binding of the mRNA to the ribosome. Downstream of the stop codon, the 3' untranslated regions of the elicitor genes do not exhibit obvious conservation, except for short motifs of 4 to 6 bp in the two elicitor genes, and a longer motif (11 to 19 bp) in the two *hae* genes (data not shown). These conserved motifs surround putative polyadenylation sites (ATAAA) which were found in all cases.

A	FIRST GROUP	1	TTCAACCAGTGTTCACGGATTCTGGGCTACTCCATGCTGACGGCCAAGGCCCTCCCCACC	60
		1G.....	60
		1T.....	60
		1T.....	60
		1G.....	60
		1T.....	60
	SECOND GROUP	1G.....	57
		1	57
	THIRD GROUP	1A.....T.....C.....T.....G.....C.....C.T.G.....G	60
		1A.....T.....C.....T.....G.....C.....C.T.G.....G	60
	FIRST GROUP	61	ACGGCGCAGTACAAGCTCATGTGCGCGTCCACGGCATGTAACACCATGATCAAGAAGAT	119
		61C.....	119
		61C.....	119
		61C.....	119
		61C.....	119
		61C.....	119
	SECOND GROUP	58C.....	116
		58C.....	116
	THIRD GROUP	61AC.....G.C.....	119
		61AC.....G.C.....	119
B	24			
	F	N	Q	C
	S	T	D	S
	G	Y	S	M
	L	T	A	K
	A	L	P	T
	T	A	Q	Y
	K	L	M	C
	A	S	T	A
	C	N	T	M
	I	K	K	I
	F	N	Q	C
	S	T	D	S
	G	Y	S	M
	L	T	A	K
	A	L	P	T
	T	A	Q	Y
	K	L	M	C
	A	S	T	A
	C	N	T	M
	I	K	K	I

Fig. 2. Characterization of amplified elicitor sequences. A, Nucleotide sequence of nine inserts obtained by PCR. These sequences can be classified into three groups (see text). B, Deduced amino acid sequences of the three groups, corresponding to amino acids 24 to 63 of elicitors previously sequenced.

Restriction mapping of the genomic clone N1 and sequence analysis of overlapping subclones revealed that the genes encoding elicitors are organized in tandem pairs, each pair containing an elicitor gene followed by a *hae* gene (Fig. 3). The distance between the putative poly(A) site of X24 and the TSS of B20 is 566 bp, and the distance between the same motifs of B14 and B26 is 610 bp. The spacer between the two tandems is 1,764 bp long. Sequence analysis of these spacers revealed several ORFs, which may encode peptides ranging from 3.5 to 18 kDa, among which four ORFs exhibiting high coding probabilities (data not shown). However, comparison of DNA and deduced protein sequences of these ORFs with the nucleic acid and protein databases did not reveal homology to any known sequence. Further experiments are in progress to characterize these putative genes.

Clustering of elicitor genes was confirmed both by comparison of restriction patterns of the lambda clone and total genomic DNA, and by cloning different amplified intergenic regions. This structure is conserved among *P. cryptogea* isolates (F. Panabières, unpublished results). The organization of the elicitor gene cluster suggests at least two duplication events, the first duplication being followed by a speciation between the elicitor gene and the *hae* gene.

Expression of the elicitor and *hae* genes.

Poly(A)⁺ RNA from 4-day-old mycelium was isolated and analyzed by RNA blot experiments with probes corresponding to specific sequences, located in the putative 3' untranslated regions (UTR) of each of the four genes (Fig. 3, probes 1 to 4). The probes derived from the *hae* genes did not reveal any mRNA (Fig. 6, lanes 2 and 4), suggesting that B20 and B26, if functional, are not transcribed under standard culture conditions. The probes corresponding to B14 and X24 hybridized, under stringent conditions, to a single mRNA of approximately 600 bp (Fig. 6, lanes 1 and 3). This size is in good agreement with sequence data. It indicates that the poly(A) site located close to the stop codon and the TSS revealed by sequence analysis and primer extension may be functional. The intensity of hybridization was very different and probe 1 corresponding to X24 strongly hybridized to the mRNA. A probe corresponding to the complete coding sequence of X24 gave, in similar hybridization conditions, a signal which was much more intense than those observed with the other probes

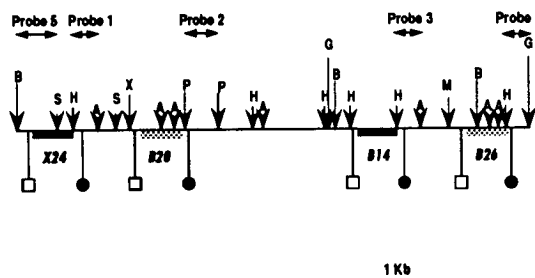


Fig. 3. Restriction mapping of the elicitor gene cluster. The insert of genomic clone N1 (see text) was digested with *AccI* (A), *ApaLI* (P), *BamHI* (B), *BglII* (G), *SmaI* (M), *SphI* (S), *XbaI* (X), and *XhoI* (H). Solid bars represent the coding region of elicitor genes (black bars) and *hae* genes (grey bars). Open squares (□) represent the putative transcription start sites, and black circles (●) represent the putative polyadenylation sites. Arrows represent the location of restriction fragments used as specific probes for Northern and Southern experiments.

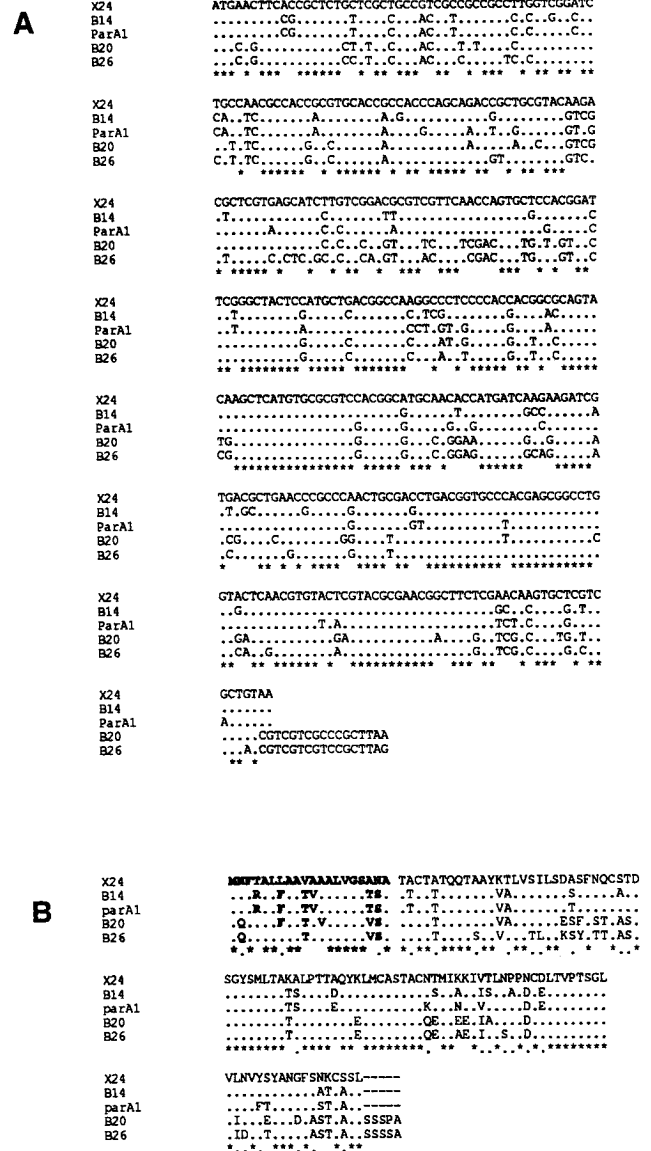


Fig. 4. Comparison of the coding regions of elicitor genes. A, alignment of the four elicitor-encoding ORFs of *Phytophthora cryptogea* (this work) and the parasiticein gene *parA1* of *P. parasitica* (Kamoun et al. 1993b). Identical residues are indicated by *. B, Comparison of deduced amino acid sequences. Bold characters represent signal peptides. C, percentage of homology between the different elicitor coding sequences (plain characters) and similarity between the deduced amino acid sequences (underlined characters).

(Fig. 6, lane 5), leading to the hypothesis that additional elicitor genes are expressed concomitantly.

To demonstrate that the faint signal observed in RNA blots with the B14-derived probe 3 was not due to a nonspecific hybridization to the mRNA detected by the X24-derived probe 1, the DNA from *P. cryptogea* 52 was digested with various restriction enzymes, transferred to nylon membranes, and hybridized with each of the probes. This DNA blot revealed several bands with each probe (Fig. 7), suggesting that at least two copies of these sequences occur in the genome. A probe containing the complete coding sequence of X24 revealed the same bands (not shown), indicating that they belong to elicitor genes. Finally, the patterns revealed by the X24 and B14-derived probes were different, confirming the specificity of the probes. Therefore we can conclude that X24 and B14 each belong to distinct classes of elicitor genes which are actually expressed.

Act	GGAGCTTGCTCGTCATTCCGCAATTGCTGCCAAGC
	△
Cal	TTTTGGATGGGATCATTGTTGGATTCCCTCGACAC
	△
B14	ACACCCCAACTGCCATTGTGCAATTGCTCTGTCAC
	▲
X24	CGCTCCTCTATCTCATTCTGCAATTGCCAGTCAC
B20	CCACAGTTGTGCTCATTCTGCAATTCCCTTGCCAA
B26	CCAACAGATTGCTCACTCCACAATTGCTTGCCAA
parA1	TGCCTCCAAGTGCATTGTGCAATTGCTCTCATCC
	△

Fig. 5. Comparison of sequences of the elicitor, *hae*, and other *Phytophthora* genes in the vicinity of transcription start site. Underlined nucleotides correspond to highly conserved regions. ▲ indicates the localization of transcription start site of B14 as determined by primer extension. △ indicates the transcription start sites of the actin gene (Act) of *P. megasperma*, the calmodulin gene (Cal) of *P. infestans* and the parasiticein gene (parA1) of *P. parasitica* (see text).

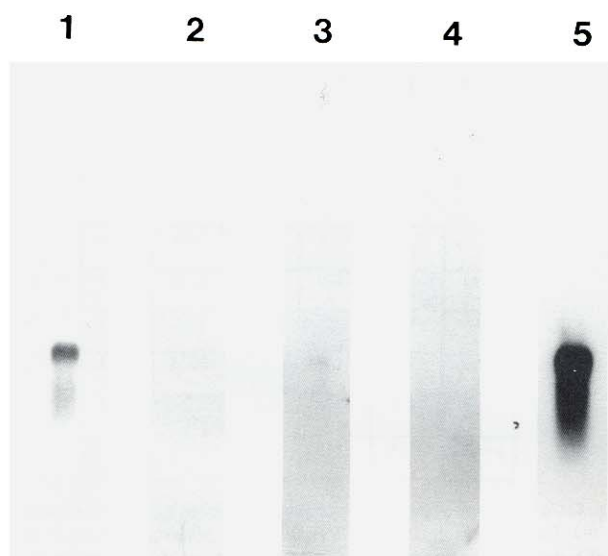


Fig. 6. Expression of elicitor and *hae* genes. One microgram of poly(A)⁺ RNA from *Phytophthora cryptogea* 52 was hybridized with specific probes (described in Fig. 3) corresponding to X24 (lane 1), B20 (lane 2), B14 (lane 3), B26 (lane 4), and a probe corresponding to conserved coding region (lane 5).

Diversity of elicitor genes among *Phytophthora* species.

Southern blots of *Msp*I-digested DNA of *P. cryptogea* 52, *P. capsici* 147, *P. parasitica* 44, and *P. parasitica* var. *nicotianae* 308 were performed, using mixed probes corresponding to the four elicitor and *hae* genes (Fig. 8). Numerous bands were detected in the pattern of *P. cryptogea* (lane B), showing that more than four elicitor and *hae* genes occur in the genome. Hybridization of the mixed probes to genomic DNA of *P. parasitica* and *P. parasitica* var. *nicotianae* revealed several bands (lanes A and D). This result confirms preliminary studies which indicated that multiple genes of elicitors occur in these *Phytophthora* species, even in *P. parasitica* var. *nicotianae* isolates (such as 308) which do not produce elicitor (Ricci et al. 1993). Whether elicitor genes are clustered in others *Phytophthora* species as occurs in *P. cryptogea* has still to be determined. An additional band is present in the pattern of the elicitor-producing isolate of *P. parasitica* (lane A), compared to that of the nonproducing isolate *P. parasitica* var. *nicotianae* 308. More isolates must be analyzed to determine if this polymorphism is related to elicitor production or only reflects individual variation.

DISCUSSION

Characterization of an elicitor multigene family in *P. cryptogea* and other species.

In this paper we report the characterization of four genes encoding elicitors of *P. cryptogea*, which can be classified into two groups: two genes encoding elicitors of 98 amino acid residues, such as those which were previously purified and sequenced (Ricci et al. 1993), and two genes whose deduced amino acid sequences are highly homologous to elicitors, but comprise 103 residues and would correspond to highly acidic proteins which have never been characterized so far. One of the two typical elicitor genes corresponds to cryptogein B, the other one to an acidic elicitor having the N-terminal sequence previously determined for cryptogein A1 (Le Berre et al. 1994). These four genes are members of a larger multigene family, as indicated by DNA blots, PCR experiments, and the previous purification of several elicitor isoforms (Ricci et al. 1993, Le Berre et al. 1994). These four genes are clustered in a region spanning around 6 kb. Sequences flanking the elicitor genes and the *hae* genes contain additional ORFs and RNA experiments using specific probes corresponding to these regions would be necessary to determine whether these putative ORFs correspond to functional genes.

A comparison of the sequences of the four genes presented here and the *parA1* gene encoding parasiticein (Kamoun et al. 1993b) reveals that homology is restricted to the coding sequences of the elicitor genes, and a small region of the putative 5' UTRs, whereas the 3' UTRs are largely divergent. Within the coding regions, the signal peptide of the acidic elicitor of *P. cryptogea* has the same sequence as that of parasiticein in *P. parasitica*, and exhibits some divergence with the signal peptide of the basic elicitor cryptogein B.

The occurrence of elicitor genes in the genome of a non-producing isolate (*P. parasitica* var. *nicotianae* 308) which was previously observed in preliminary experiments (Ricci et al. 1993), and recently confirmed (Kamoun et al. 1993b) raises major questions about the regulation of the expression

of elicitor genes. Unlike races of *C. fulvum* virulent on tomato Cf9 genotypes which lack the entire single copy avirulence gene *avr9* (Van Kan et al. 1991), two elicitor genes at least are detected in *P. parasitica* var. *nicotianae*. It needs to be analyzed now if these genes are functional and may be expressed in specific situations, although it was shown recently that no mRNA encoding parasiticein could be observed when non-producing strains are grown in vitro (Kamoun et al. 1993b), or if expression of elicitor genes is avoided by mutations, as observed in other systems (Kobayashi et al. 1990; Joosten et al. 1994). A comparison between the structure and the organization of the elicitor genes of producing isolates of *P. parasitica* and non-producing isolates of *P. parasitica* var. *nicotianae* is now necessary.

Expression of the elicitor genes.

The lack of homology between the 3' UTRs of the four genes allowed the construction of specific probes in order to study their expression separately. RNA blots revealed a differential expression when the fungus is grown in vitro.

The mRNAs hybridizing with the probe derived from X24 (encoding cryptogeyn B) were more abundant than those hybridizing with the probe derived from B14 (encoding an acidic elicitor). This observation is in good agreement with the respective production rates of cryptogeyn B and acidic elicitors in the culture filtrate of *P. cryptogea* 52 (Le Berre et al. 1994). The specificity of the two probes was confirmed by the fact that they hybridized with a different set of restriction fragments containing elicitor-related sequences in a DNA blot. In this experiment, each probe hybridized with more than one gene copy, demonstrating that X24 and B14 are members of distinct classes of elicitor genes. From the RNA blot experiment, it can be concluded that these classes (but not necessarily the individual cloned copies) are expressed. Furthermore, the level of mRNA detected with a probe including the coding sequence was much higher than that de-

tected by any of the specific probes, indicating that other classes of elicitor genes are also expressed. Elicitor production by the cultivated fungus is therefore the result of the expression of a complex set of genes, including the X24 and B14 classes.

In contrast, *hae* mRNA were not detectable. As no protein of 103 aa has been characterized so far, further experiments are necessary to conclude whether these so-called *hae* genes are nonfunctional, possess poor promoters, or are expressed only in particular culture conditions or during plant-pathogen interactions, as is the case for some *avr* genes, such as *avr9* from *C. fulvum* (De Wit et al. 1993).

Possible role of elicitors in plant-*Phytophthora* interactions.

Assays of pathogenicity to tobacco of a large set of *Phytophthora* isolates indicated that most tobacco isolates were characterized by a lack of elicitor production, in addition to high virulence. On the other hand, tobacco isolates which were shown to produce elicitor in vitro exhibited reduced virulence and induced leaf necrosis (Bonnet et al. 1994). This strong correlation between lack of virulence and production of elicitors in *Phytophthora*-tobacco interactions suggested that elicitors may be considered as avirulence factors (Kamoun et al. 1993b). Therefore we must assume the occurrence of corresponding resistance genes in tobacco species that are able to recognize a wide set of elicitors from various *Phytophthora* species. Previous experiments led to the hypothesis of an elicitor receptor (Blein et al. 1991).

It has to be noted that the high constitutive expression of elicitor genes among a wide set of *Phytophthora* species may suggest that they have a function independent of tobacco-*Phytophthora* interactions. However the absence of elicitor

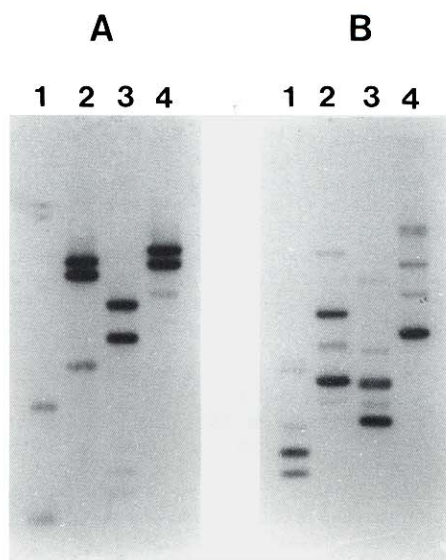


Fig. 7. Putative 3' UTR of X24 and B14 occur in several elicitor genes. Genomic DNA of *Phytophthora cryptogea* 52 was digested with *MspI* (lanes A1, B1), *BamHI* (lanes A2, B2), *XhoI* (lanes A3, B3), and *BglII* (lanes A4, B4), transferred, and hybridized in stringent conditions with probes corresponding to the putative 3' UTR of X24 (A) or B14 (B).



Fig. 8. Occurrence of multiple copies of elicitor and *hae* genes among various *Phytophthora* species. Five micrograms of genomic DNA of *Phytophthora parasitica* 44 (A), *P. cryptogea* 52 (B), *P. capsici* 147 (C), and *P. parasitica* var. *nicotianae* 308 (D) was digested with *MspI*, transferred, and hybridized in stringent conditions with probes corresponding to the complete ORF of the different genes described in this report.

production in tobacco isolates does not seem to affect their development (I. Lacourt, personal communication).

Most *Phytophthora* species have a wide host range, and *P. parasitica* is highly polyphagous, except for *P. parasitica* var. *nicotianae* isolates, which are strictly specialized on tobacco. In contrast, some tobacco isolates, which produce parasiticein, are pathogens on other plants, such as tomato and carnation (Bonnet et al. 1994). Moreover, a *P. parasitica* strain isolated from tomato, which lacks the parasiticein genes, was shown to be nonpathogenic on both tomato and tobacco (Kamoun et al. 1993b). Therefore a correlation between elicitor production and host range appears to exist. These observations suggest that elicitors, involved in the induction of HR on tobacco, might be pathogenicity determinants on other plants. If elicitor genes are involved both in HR induction on tobacco and pathogenicity on host plants, they would be the first *hrp*-like genes (Willis et al. 1991) characterized in fungi. As these genes are members of a highly conserved multigene family, they might be involved in interactions with a wide range of plant species. Transformation of tobacco isolates is necessary to confirm this hypothesis. In this case, inactivation of elicitor genes in elicitor-producing isolates could lead to a loss of pathogenicity, or a restriction of their host range.

MATERIALS AND METHODS

DNA analysis.

All DNA manipulations, except where specified, were performed according to standard protocols (Sambrook et al. 1989). Genomic DNA was isolated from 8-day-old mycelia from various *Phytophthora* isolates (see text) as previously described (Panabières et al. 1989). Sequencing was performed using the Sequenase Version 2.0 kit (U.S. Biochemical). Sequences were analyzed with MacMol 3.5.1 software (Soft gene GmbH). Sequence alignments were performed by using ClustalV (Higgins and Sharp 1988) software for Macintosh. Homologies with databases (EMBL, release 37.0; Genbank release 81.0) were searched using BLAST (Altschul et al. 1990) and FASTA (Pearson and Lipman 1988) programs.

Polymerase chain reaction and cloning.

PCR primers (Appligene, Inc.) were designed by deduction from the sequence of cryptogein already described, compared to other published sequences of elicitors. PCR reactions were carried out with a total volume of 100 µl containing 200 µM each of dATP, dCTP, dGTP, and dTTP, 5 µM each of the primers, 2.5 units of Taq DNA polymerase (Appligene, Inc.), and 0.5 to 1 µg of DNA template in the amplification buffer supplied with the enzyme by the manufacturer. The temperature cycling parameters were denaturation at 94°C for 5 min for the first cycle and 1 min for the subsequent cycles, primer annealing for 2 min at 47°C, and primer extension at 72°C for 3 min with a total of 35 cycles and a final extension step at 72°C for 10 min, using a Techne DNA thermal cycler (Techne Inc., Princeton, NJ). Fragments of interest were purified and subcloned as described (Sambrook et al. 1989).

Construction and screening of the genomic library.

A genomic library of *P. cryptogea* DNA was constructed in λ EMBL3 and screened with the insert of pCry38 (see text).

Prehybridization and hybridization were carried out at 68°C in 6× SSC (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate), 0.5% SDS, 5× Denhardt's solution (0.1% [w/v] BSA, 0.1% [w/v] polyvinylpyrrolidone, 0.1% [w/v] Ficoll), and 100 µg/ml denaturated herring sperm DNA. Blots were washed at 68°C in 0.2× SSC, 0.1% SDS for 30 min, and submitted to autoradiography.

Isolation of RNA and Northern blots.

Poly (A)⁺ RNA was purified from 4-day-old mycelia using a Quickprep micro mRNA purification kit (Pharmacia). Northern blots were performed as described (Sambrook et al. 1989). Hybridizations were performed at 42°C in 50% formamide, 5× SSC, 5× Denhardt's solution, 0.5% SDS, 50 mM NaH₂PO₄/Na₂HPO₄, pH 6.5, 10% dextran sulfate, and 100 µg/ml DNA from herring sperm. Various probes corresponding to the different elicitor genes were used for hybridization (see text). DNA probes were labeled by the random primer labeling technique (Feinberg and Vogelstein 1983). After hybridization the filters were washed in 2× SSC at room temperature, then in 0.2× SSC, 0.1% SDS at 60°C and exposed to Kodak X-Omat film.

Primer extension.

200 ng of the oligonucleotide (5'-TCCGTAGAACACTG-GTTGAA-3'), supplied by Appligene, Inc., complementary to the sequence at the position 130 to 149 relative to the ATG start codon of the B14 gene, was annealed to 2.5 µg of poly (A)⁺ RNA and extended as previously described (Panabières et al. 1984). Samples were analyzed on 6% sequencing gels and visualized by autoradiography.

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