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Short Communication

Identification of an Elicitin Gene Cluster in *Phytophthora Cinnamomi*

JOANA DUCLOS[b], ALAIN FAUCONNIER[a], ANA-CRISTINA COELHO[d], ALEX BOLLEN[a], ALFREDO CRAVADOR[c] and EDMOND GODFROID[a*]

[a]*Service de Génétique Appliquée, Université Libre de Bruxelles, B 1400 Nivelles, Belgium,* [b]*Instituto de Tecnologia Quimica e Biologica, Quinta do Marquês, P-2780 Oeiras,* [c]*Unidade de Ciências e Tecnologias Agrárias (U.C.T.A.), Universidade do Algarve, Campus de Gambelas, P-8000 Faro and* [d]*Unidade de Ciências e Tecnologias Agrárias (U.C.T.A.), Universidade do Algarve, Campus de Gambelas, P-8000 Faro; 4 Escola Superior de Educação, Universidade do Algarve, P-8000 Faro, Portugal*

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Elicitins are a group of highly conserved proteins secreted by species of *Phytophthora* and a species of the related genus *Pythium*, *Pythium vexans*. Some of these proteins act as inducers of the necrotic hypersensitive-like response and the associated systemic acquired resistance phenomenon, in some species. We cloned and characterised the cinnamomin-β and -α genes and two related elicitin genes from *Phytophthora cinnamomi*. These four open reading frames (ORFs) are clustered in tandem pairs. Two out of these four genes present homologies with the basic and acidic elicitin groups; but the two others encode, if expressed, elicitin isoforms exhibiting homologies with the class II of highly acidic elicitins.

Keywords: Fungi, oomycete, transcription site promoter

The oomycetous fungus *Phytophthora cinnamomi* plays an important role in the necrotic activity observed on feeder roots of cork oaks (*Quercus suber* L.) and eucalyptus (*Eucalyptus marginata* Donn. Ex Sm.) trees, reducing the capacity of these hosts to absorb water and nutrients (Brasier *et al.*, 1993; Shearer and Tippett, 1989; Wills, 1993). *P. cinnamomi* is also involved in the extinction of chestnut trees (*Castanea sativa* L.) in Portugal and Spain (Pimentel, 1949).

In the relationships between plant and fungus, especially the genus of *Phytophthora*, elicitor proteins (elicitins), a family of highly conserved small secreted proteins (about 10 kDa), act as pathogenicity factors by inducing, in some plant species, a vigorous defense response, called the necrotic hypersensitive response (Grant *et al.*, 1996; Hahlbrock *et al.*, 1995). This phenomenon is generally associated to a systemic acquired resistance response in which the plant becomes resistant to the attack of other microorganisms such as fungal and bacterial pathogens.

The amino acid and nucleic acid sequences of a great number of elicitins have recently been determined. On the basis of their amino acid

* Corresponding author. Mailing address: Service de Génétique Appliquée, Université Libre de Bruxelles, 24, Rue de l'Industrie, B 1400 – Nivelles – Belgium. Phone: 32 67 88 94 63. Fax: 32 67 88 94 72. Electronic mail address: godfroid@sga.ulb.ac.be.

sequence, their biological activity, and their physico-chemical properties (circular dichroism, UV difference spectroscopy, and 3-dimensional nuclear magnetic resonance studies), elicitins were subdivided into at least five distinct classes (Bouaziz *et al.*,1994; Kamoun *et al.*, 1997a). β elicitins (basic - class IB) are characterised by a higher necrotic activity than α-elicitins and by the presence of a hydrophilic side-chain at position 13 in the mature protein, usually a lysyl residue (O. Donohue *et al.*, 1995). The less necrotic α-elicitins (acidic – class IA) have a hydrophobic valine at this position. α- and β-elicitins can be expressed simultaneously by the same species (Huet *et al.*, 1992; Le Berre *et al.*, 1994). Recently, southern blot analyses have indicated that elicitin genes occur as a multigene family (at least 2 to 10 copies) in *P. parasitica, P. capsici, P. citricola, P. citrophthora, P. cryptogea, P. drechsleri, P. megasperma, P. sojae* and *P. palmivora* (Kamoun *et al.*, 1993; Le Berre *et al.*, 1994; Mao and Tyler, 1996; Panabieres *et al.*, 1995). This hypothesis has been clearly demonstrated by cloning the sequences encoding cryptogein and three related elicitin isoforms from *P. cryptogea* (Panabieres *et al.*, 1995). These four sequences are clustered on a restricted genomic region and are organised in pairs of elicitins. In addition, two of these genes encode the third class (class II) of elicitins, also called highly acidic elicitins which possess a short hydrophilic C-terminal tail (Kamoun *et al.*, 1997a; Panabieres *et al.*, 1995). The fourth class (class Py) of elicitins comprises two proteins (Vex1 and Vex2) purified from *Pythium vexans* (Huet *et al.*, 1995; Kamoun *et al.*, 1997a). One of these novel elicitins (Vex2) exhibits a 100-residue sequence instead of 98 while the other (Vex1) has an N- glycosylation site, effectively glycosylated (Huet *et al.*, 1995). Finally, two novel elicitin-like proteins, inf2A and inf2B, were isolated from a cDNA library of *Phytophthora infestans* made from infected potato tissue and actually make up the fifth class (class III) (Kamoun *et al.*, 1997a).

To further investigate the presence of elicitin genes in the *P. cinnamomi* genome, we chose the vectorette PCR method (Arnold and Hogson, 1991). Restricted genomic DNA fragments were ligated to partial complementary oligonucleotides, called vectorette oligonucleotides. A pair of oligonucleotides within a PCR 327 bp DNA fragment, corresponding to an internal region of the cinnamomin gene, was used as initiating primers (Coelho *et al.*, 1997). On the basis of the results obtained in the test of specificity, 4 DNA fragments, called Cl6, Cl13, Cl16 and Cl17, were chosen, ligated in the pCRII™ vector and cloned in *E. coli*. The analysis of these DNA sequences allowed us to classify the selected DNA fragments into 2 groups. The first group is made up of the Cl6 and Cl16 clones. The comparison of the Cl6 DNA sequence to that of the 327 bp PCR DNA fragment showed 99.6 % homology. As expected, this DNA fragment (646 bp long) is composed of a part of the coding (256 bp) and the 3' non coding region (390 bp) of the cinnamomin gene. The amino acid sequence of the coding region perfectly matches with residues 15 to 98 of the published amino acid sequence of cinnamomin (Huet and Pernollet, 1989). The analysis of the Cl16 DNA sequence (449 bp long) showed that this DNA fragment displays in its 3' end a partial putative open reading frame (ORF) of 176 bp. The deduced amino acid sequence of this non-completed ORF exhibits higher homologies to acid elicitins, such as elicitinAl of *P. cryptogea* and paraA1 elicitin of *P. parasitica*, than to basic elicitins. These analyses suggest that we have not only isolated an important part of the cinnamomin gene, but also a part of an acidic elicitin gene. The second group comprises the Cl13 and Cl17 clones. Comparison of the DNA or deduced amino acid sequences of these ORFs to previously published or available sequences in the EMBL database, revealed that Cl13 and Cl17 ORFs present high homologies with the two highly acidic elicitin genes isolated in *P. cryptogea* (Panabieres *et al.*, 1995). These preliminary data put together strongly suggest that at least 4 different elicitin genes are present in the genome of *P. cinnamomi*.

In view of the aforementioned results, a genomic library of III-4 *P. cinnamomi* strain (Algarve, Portugal) was constructed in λ FIX® II and screened with the Cl6 DNA fragment. One positive phage was isolated and extensively studied by restriction enzyme (*Eco*RI, *Xba*I, *Hin*DIII, *Bam*HI, *Not*I, *Xho*I, and pairs of restriction enzymes) and southern hybridisation analyses (data not shown). The results of these experiments showed that several restriction DNA fragments of the insert hybridised to the Cl6 probe, confirming our previous results that several copies of the gene encoding cinnamomin or related elicitin genes are present in the genome of *P. cinnamomi*. The sequence analyses of the insert DNA showed that it exhibits 4 open reading frames that are identical to the sequences that we had previously isolated, namely Cl6, Cl13, Cl16 and Cl17 (EMBL accession number : AJ000071).

Restriction mapping of the λ phage genomic clone and sequence analysis revealed that these 4 ORFs are clustered in tandem pairs. Each pair contains an elicitin isoform gene followed by an highly acidic elicitin gene. This positioning is similar to that observed in *P. cryptogea* (Panabieres *et al.*, 1995), suggesting a conservation of the elicitin cluster structure through the *Phytophthora* species. Sequence analyses indicated that all of these ORFs, not interrupted by intervening sequences, possess a 60 bp region at their 5'-end (except for Cl17 ORF – 57 bp long), which may correspond to a signal peptide. Similar sequences were previously found in the *parA1* gene encoding parasiticein (Kamoun *et al.*, 1993), in the *soj2* gene of *P. sojae*, and in the cryptogein gene and its isoforms (Panabieres *et al.*, 1995). Cryptogein and cinnamomin – two basic elicitin – display an identical signal peptide (Fig. 2). Similarly, the paraA1 elicitin of *P. parasitica*, the elicitin A1 of *P. cryptogea*, the Soj2 elicitin of *P. sojae*, and the deduced amino acid sequence of the Cl16 ORF – four acidic elicitins – have the same signal peptide sequence signature. In the case of highly acidic elicitins (HAE20 and HAE26 of *P. cryptogea;* Cl13 and Cl17 of *P. cinnamomi*), although different, these putative signal peptide sequences present higher homologies among them than with the signal peptide sequence specific to acidic or basic elicitins. In conclusion, the comparison of these signal peptide amino acid sequences showed that the sequence signature is not species dependent, but is specific to a elicitin class (acidic, basic or highly acidic), which could mean that the elicitin isoforms may be secreted by distinct secretion pathways.

The Cl6 and Cl16 ORFs would encode proteins of 118 aa, including the signal peptide. The protein encoded by Cl6 ORF is identical to cinnamomin (Huet and Pernollet, 1989). The deduced amino acid sequence of Cl16 ORF is more closely related to the acidic elicitins (e.g., ParA1 elicitin of *P. parasitica* and the elicitin A1 of *P. cryptogea*) than to basic elicitins (e.g., cinnamomin and cryptogein). In addition, the region corresponding to the residues 1 to 40 perfectly matches (except for the serine residue at position 25 – Fig. 1) the amino terminal region of the acidic elicitin isoforms of elicitin A1 and paraA1 (Kamoun *et al.*, 1993; Le Berre *et al.*, 1994). The Cl13 and Cl17 ORFs would encode distinct proteins of 124 and 123 aa, respectively. Moreover, the deduced amino acid sequences of these two ORFs show higher homologies to the HAE proteins of *P. cryptogea* than the acidic and basic known elicitins. From their amino acid composition, these two putative proteins would be effectively more acidic (calculated pI_{Cl13} and pI_{Cl17} : 3.38 and 3.54, respectively) than the acidic (average calculated pI : 4.28) and basic (average calculated pI : 7.81) elicitins already described. We have also seen that the necrotic and active sites (see Fig. 1) experimentally determined in other elicitins, are present in the elicitin deduced amino acid sequences of *P. cinnamomi* (Huet *et al.*, 1994; Perez *et al.*, 1997).

Comparison of the deduced amino acid sequences of the mature form of the 4 elicitins of *P. cinnamomi* to other known elicitins (Fig. 2) indicated that cinnamomin belongs to the class

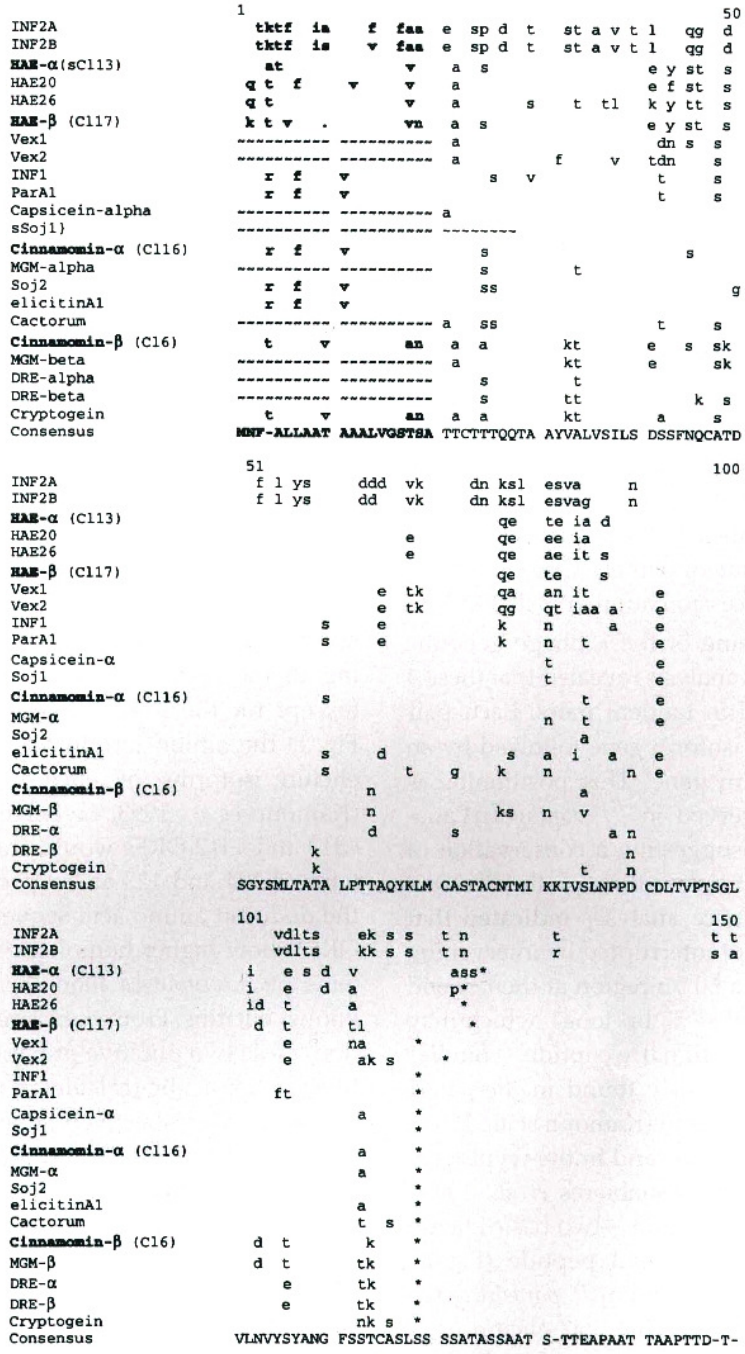

FIGURE 1 Alignment of the four elicitins of *P. cinnamomi* (cinnamomin-α and -β; and highly acidic elicitin-α and -β, in underlined bold characters), the four elicitin isoforms of *P. cryptogea* (HAE20, HAE26, elicitinA1 and cryptogein; (Panabieres *et al.*, 1995)), and the parA1 acidic elicitin of *P. parasitica* (Kamoun *et al.*,1993). Bold characters represent signal peptides

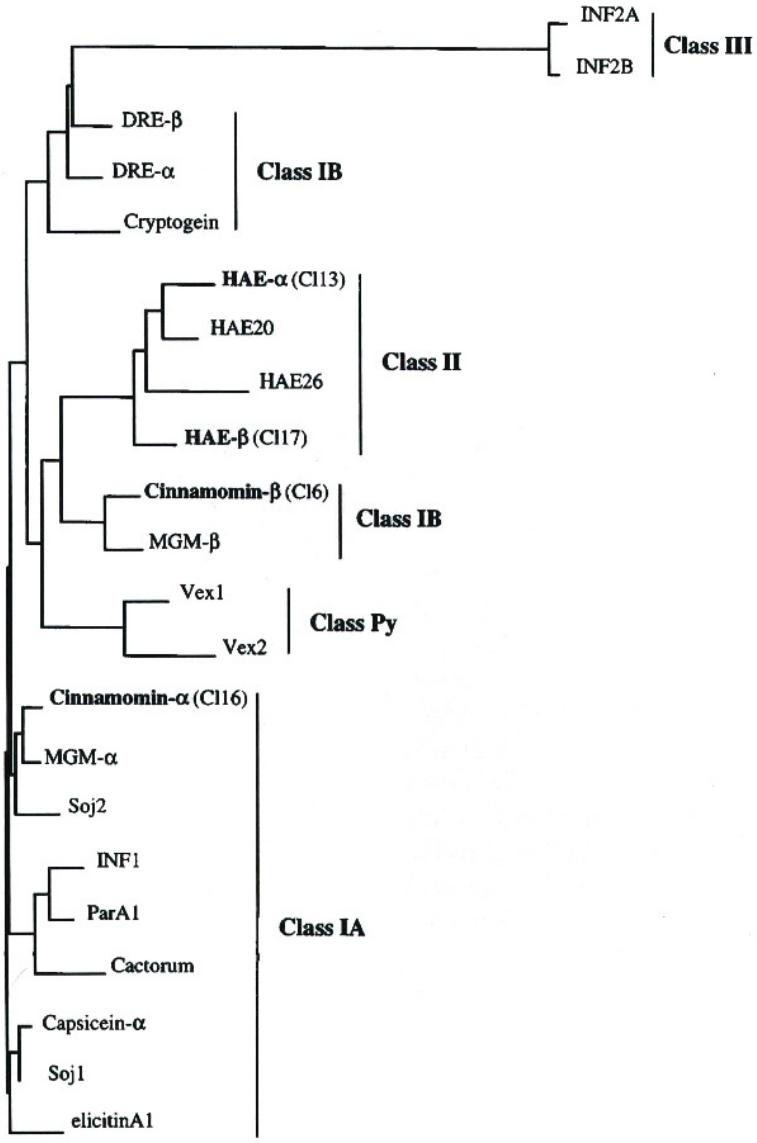

FIGURE 2 Phylogenetic tree of the elicitin family from *Phytophora* spp. and *Pythium vexans*. Underlined bold characters represent the elicitin isoforms of *P. cinnamomi*.The phylogenetic tree was constructed by the neighbor joining method (using the Pileup, Pretty, Distances, and Growtree facilities of the Genetics Computer Group sequence analysis software package – version 9.0, 1997; University Research Park, Madison, Wisconsin 53711, U.S.A.) based on the multiple alignment of elicitin sequences shown in Figure 1. The following parameter values were used : gap creation penalty = 12.0, gap extension penalty = 4.0. Cryptogein, elicitinA1, HAE20 and HAE26 correspond to the elicitin of *P. cryptogea* (Panabieres *et al.*, 1995). Vex and Vex2 are elicitins of *Pythium vexans* (Huet *et al.*, 1995). DRE-α and DRE-ß, and MGM-α and MGM-ß are the acidic and basic elicitins of *P. drechsleri* and *P. megasperma megasperma* (Huet *et al.*, 1992; Huet and Pernollet, 1993). Finally, INF1, ParA1, capsicein-α, and cactorum elicitin correspond to elicitins of *P. infestans, P. parasitica, P. capsici* and *P. cactorum*, respectively (Huet *et al.*,1993; Huet and Pernollet, 1989; Huet *et al.*, 1994; Kamoun *et al.*, 1993; Kamoun *et al.*, 1997b)

IB, corresponding to basic elicitins (Kamoun *et al.*, 1997a; Panabieres *et al.*, 1995; Pernollet *et al.*, 1993), Cl16 elicitin belongs to class IA (acidic elicitins), and finally, Cl13 and Cl17 elicitins belong to the class II, namely highly acidic elicitins (Kamoun *et al.*, 1997a; Panabieres *et al.*, 1995). Based on the classification used in the literature, we have opted to call Cl6 and Cl16 ORFs, cinnamomin-β and -α; and Cl13 and Cl17 ORFs, highly acidic elicitin-α and -β, respectively (Huet *et al.*, 1993; Huet *et al.*, 1992; Huet and Pernollet, 1989; Huet and Pernollet, 1993; Kamoun *et al.*, 1997a). Concerning the phylogenetic tree (Fig. 2), it remains to be determined if this theoretical classification corresponds to morphological, physiological and physico-chemical properties.

DNA sequence analyses enabled us to identify CAAT and TATA motifs 5' upstream to the coding region of each elicitin ORF. However, a 16 nucleotide sequence motif, matching the consensus sequence, GCTCATTYYNCAWTTT (where N=A or C or G or T; W=A or T; Y= C or T), was found (Pieterse *et al.*, 1994). This conserved sequence motif is located within the first 100 nucleotides upstream from the ATG start codon of each elicitin ORF and appears to be present around the transcription start point (*tsp*) of other oomycetous genes for which the *tsp* were experimentally determined (Kamoun *et al.*, 1993; Panabieres *et al.*, 1995; Pieterse *et al.*, 1994). This conserved motif sequence was not detected in genes of higher fungi, plants or animals, but only in the region surrounding the *tsp* of oomycetous genes, suggesting that this motif is important for transcription initiation in these organisms. We also observed an AC-rich region located between the *tsp* motif and the ATG codon, and a CAAG motif which precedes the translation start codon in three (cinnamomin-ß gene and the two highly acid elicitin genes) of the 4 ORFs. The functional significance of these two motifs is unclear, but it is considered that they could be involved in the binding of the mRNA to the ribosome. Finally, the 3' non coding region of these 4 elicitin ORFs did not reveal any putative polyadenylation signal (AATAAA).

In conclusion, we report the characterisation of four ORFs encoding elicitins of *P. cinnamomi*. Two out of these genes can be classified in the well known groups of basic and acidic elicitins (class IA and IB); but the two others encode elicitins which are more closely related to the highly acidic elicitins of *P. cryptogea* (class II). Further experiments would establish the expression level of these elicitin isoform genes and their involvement in plant-pathogen interactions.

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