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IDENTIFICATION OF AN ELICITIN GENE CLUSTER IN *PHYTOPHTHORA CINNAMOMI* AND ANALYSIS OF THE NECROTIC ACTIVITY OF A PURIFIED RECOMBINANT ß-CINNAMOMIN

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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. *P. cinnamomi* is also involved in the extinction of chestnut trees (*Castanea sativa* L.) in Portugal and Spain.

In the case of the relationship between plant and fungus, especially the genus of *Phytophthora*, elicitor proteins (elicitins), a family of highly conserved small secreted proteins, act as pathogenicity factors by inducing, in some plant species, a vigorous defense response, called the necrotic hypersensitive response. 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.

Elicitins were subdivided into at least five distinct classes on the basis of their amino acid sequence, their biological activity, and their physico-chemical properties. 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. 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. Recently, southern blot analyses have indicated that elicitin genes occur as a multigene family (at least 2 to 10 copies) in numerous *Phytophthora* species. These results have been confirmed by cloning the sequences encoding cryptogein and three related elicitin isoforms from *Phytophthora cryptogea*. 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. The fourth class (class Py) of elicitins comprises two proteins (Vex1 and Vex2) purified from *Pythium vexans*. One of these new elicitins (Vex2) exhibits a 100-residue sequence instead of 98 while the other (Vex1) has an N- glycosylation site, effectively glycosylated. Finally,

two novel elicitin-like proteins, inf2A and inf2B, were characterised from a cDNA library of *Phytophthora infestans* isolated from infected potato tissue, and actually make up the fifth class (class III).

In this study, we investigate the presence of elicitin genes in the *P. cinnamomi* genome by using the vectorette PCR method. This methodology allowed us to isolate 4 DNA fragments : Cl6, Cl13, Cl16 and Cl17. We then compared the DNA sequences of these DNA fragments to the DNA sequence of a PCR fragment, previously isolated in our laboratory, corresponding to a part of the cinnamomin gene. The comparison of the Cl6 DNA sequence to that of the PCR DNA fragment showed a 99.6 % identity. The analysis of this DNA fragment indicated that it is composed of a part of the coding and the 3' non coding region 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. The analysis of the Cl16 DNA sequence 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 identities to acid elicitins than to basic elicitins. Comparison of the DNA or deduced amino acid sequences of the Cl13 and Cl17 DNA fragments to those, previously published or available in the EMBL database sequences, revealed that these sequences present high identities with the two highly acidic elicitin genes isolated in *P. cryptogea*. These preliminary data 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 and southern hybridisation analyses. The results of these experiments showed that several restriction DNA fragments of the positive phage 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.

These 4 genes 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*, suggesting a conservation of the elicitin cluster structure through the *Phytophthora* species. Sequence analyses also indicated that almost all of these genes possess a 60 bp region at their 5'-end, which may correspond to a signal peptide. Similar sequences were previously found in the *parA1* gene encoding parasiticein of *Phytophthora parasitica*, in the *soj2* gene of *Phytophthora sojae*, and in the cryptogein gene and its isoforms. Cryptogein and cinnamomin – two basic elicitin - display an identical signal peptide. 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 gene – 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 summary, 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).

The Cl6 and Cl16 genes may encode proteins of 118 aa, including the signal peptide. The protein encoded by the Cl6 gene is identical to cinnamomin. The deduced amino acid sequence of Cl16 ORF is more closely related to the acidic elicitins than to basic elicitins. In addition, the region corresponding to the residues 1 to 40 perfectly matches (excluding the serine residue at position 25) the amino terminal region of the acidic elicitin isoforms of elicitin A1 and paraA1. The Cl13 and Cl17 genes would encode distinct proteins of 124 and 123 aa, respectively. Moreover, the deduced amino acid sequences of these two genes show higher identities 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.

Comparison of the deduced amino acid sequences of the mature form of the 4 elicitins of *P. cinnamomi* to other known elicitins indicated that cinnamomin belongs to the class IB, corresponding to basic elicitins, Cl16 elicitin belongs to class IA (acidic elicitins), and finally, Cl13 and Cl17 elicitins belong to the class II, namely highly acidic elicitins. Based on the classification used in the literature, we have opted to call Cl6 and Cl16 genes, cinnamomin-β and -α; and Cl13 and Cl17 genes, highly acidic elicitin-α and -β, respectively.

DNA sequence analyses did not allowed us to identify CAAT and TATA motifs 5' upstream to the coding region of each elicitin gene. 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. This conserved sequence motif is located within the first 100 nucleotides upstream from the ATG start codon of each elicitin gene and appears to be present around the transcription start point (*tsp*) of other oomycetous genes for which the *tsp* were experimentally determined. 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-β gene and the two highly acid elicitin genes) of the 4 genes. 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 genes did not reveal any putative polyadenylation signal (AATAAA).

Furthermore, we evaluated by RT-PCR the expression of these four elicitin isoforms. The results indicated that all of these elicitin isoforms are expressed in *in vitro* culture of *P. cinnamomi*. In addition, to study the role of these elicitins in the plant-fungi interaction, we purified a recombinant β-cinnamomin produced by the methylotrophic yeast *Pischia pastoris*. Based on the calculated isoelectric point of the protein (pI 7.55), the purification of this recombinant β-cinnamomin was performed using a cation exchange resin in sodium acetate buffer at pH 4.0. The yield of this purification protocol was about 16 mg of purified protein per litre of culture. As antibodies against β-cinnamomin were unavailable, we used antibodies against β-cryptogein (87.8% homologous to β-cinnamomin) to characterise the recombinant product. This recombinant β-cinnamomin has the same size as the natural protein on a polyacrylamide gel. In order to test the putative necrotic activity of the recombinant β-cinnamomin, the petiole of detached tobacco leaves was soaked in 20 ml of distilled water containing 0.1 to 1 µg of purified recombinant or natural β-cinnamomin. The results showed that the leaves incubated with both types of β-cinnamomin were rapidly necrotised in contrast to the control leaf, incubated in water only. We also replaced in the β-cinnamomin protein the lysyl residues located at position 13 and 94 with a valine and a threonine residue, respectively. These two amino acid residues are found in the same position in the α-cinnamomin isoform that is known to have a lower necrotic activity. The necrotic activity of these two mutants was compared to those of the recombinant β-cinnamomin. The results indicated that the mutants were less active than the wild type isoform emphasising the importance of these amino acid residues in the necrotic properties of basic elicitins.

In conclusion, we report the characterisation of four genes 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. We also report the expression of a biologically active form of recombinant β-cinnamomin in the *P. pastoris* system. It confirms the efficiency of methylotrophic yeasts for producing bio-active elicitin isoforms. The recombinant β-cinnamomin isoform constitutes a very useful tool to understanding the structural and functional properties of its natural counterpart. The same methodology can be applied to other elicitin isoforms (acidic and highly acidic elicitins) which are probably secreted by *P. cinnamomi*. It is expected that these experiments will lead to a clearer definition of the precise role of each elicitin isoform during interaction between fungi and plants, and will allow the production of elicitin-like molecules devoid of their necrotic properties, but which are able to elicit the plant defence response. The protection of cork oak plantations against *P. cinnamomi* is of fundamental importance to the Portuguese economy.

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