MPMI Vol. 8, No. 2, 1995, pp. 302–310.

The Relationships Between the Toxicity and the Primary and Secondary Structures of Elicitinlike Protein Elicitors Secreted by the Phytopathogenic Fungus *Pythium vexans*

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Received 6 September 1994. Accepted 16 December 1994.

Elicitins are toxic and signaling proteins secreted by *Phytophthora* spp. responsible for the incompatible reaction and systemic hypersensitive-like necroses of diverse plant species leading to resistance against fungal or bacterial plant pathogens. Such proteins were observed in the culture filtrate of another species of the Oomycete genus, *Pythium vexans*. Two α elicitinlike proteins were purified and sequenced. One of these novel elicitins (Vex_2) exhibited a 100-residue sequence instead of 98 while the other (Vex_1) had an *N*-glycosylation site, effectively glycosylated (equivalent of 16 hexose residues). In addition to the point mutations already observed in *Phytophthora* species, we found several novel amino acid changes. Furthermore, circular dichroism revealed some differences in their structure in solution compared with the *Phytophthora* elicitins that were correlated with specific point mutations. These sequences permitted the establishment of a phylogenic tree, suggesting that *Pythium vexans* is a species close to the *Phytophthora* genus. The toxicity of the *Pythium vexans* elicitins to tobacco leaves was investigated and correlated with the occurrence of the carbohydrate moiety of one of the two isoforms, observed for the first time in an elicitin.

Additional keywords: amino acid sequence; *Nicotiana tabacum*.

The improvement of the natural active defense system against phytopathogenic fungi is an attractive goal that requires a detailed knowledge of the molecular biology and biochemistry of the host-microorganism interaction. One of the current working models of improved natural active defense systems features the fungal phytotoxins called elicitins, a novel class of protein elicitors and an original family of proteins (Huet and Pernollet 1989). With the exception of *Phytophthora nicotianae* (*parasitica*) var. *nicotianae* field isolates from tobacco, all the *Phytophthora* species so far studied are known to secrete in culture one or several isoforms of such molecules (Ricci *et al.* 1992; Pernollet *et al.* 1993; Kamoun *et al.* 1994). They are 10-kDa holoproteins causing remote leaf necrosis (hypersensitive-like response) and induction of systemic acquired resistance of some plants against pathogens, not only *Phytophthora* (Ricci *et al.* 1989) but also bacteria (Kamoun *et al.* 1993b). Elicitins, secreted into the external medium, penetrate the plant through the root system (Pernollet *et al.* 1993) and proceed to the leaf cell where they necrotize without requiring any *in planta* biochemical alteration for their activity to develop (Zanetti *et al.* 1992). These molecules do not undergo any posttranslational modification (Nespoulous *et al.* 1992) other than the removal of a signal peptide before secretion into the culture medium (Tercé-Laforgue *et al.* 1992; Kamoun *et al.* 1993a). Elicitins from different species of *Phytophthora*, when applied to tobacco petiole or stem at equal doses, exhibit different levels of toxicity in leaves but induce protection at the same level (Ricci *et al.* 1989). They are also toxic to plant species other than tobacco (Kamoun *et al.* 1993b; Pernollet *et al.* 1993; Huet *et al.* 1994). Elicitins are classified into two groups, α and β, based on their differing toxicity to tobacco (Nespoulous *et al.* 1992). β elicitins cause visible leaf necrosis when applied at approximately 100 pmoles per plant whereas 50- to 100-fold more α elicitin is required for the same reaction. This classification correlates with the sequence differences and the physico-chemical features of the elicitins. The complete sequences of 10 elicitins are known (Huet and Pernollet 1989; Huet and Pernollet 1993; Huet *et al.* 1992; Huet *et al.* 1993; Huet *et al.* 1994; Nespoulous *et al.* 1992). They all consist of 98 amino acids with homology greater than 66%. From sequence-function relationships, residue 13 was proposed as a key amino acid for toxicity, while remaining unrelated to resistance induction. Other residues involved in the control of the elicitin toxicity to tobacco have also been revealed in this way (Huet *et al.* 1992; Huet *et al.* 1993; Huet *et al.* 1994; Nespoulous and Pernollet 1994). The physicochemical properties of the elicitins offered the potential for the determination of their three-dimensional structure by multidimensional nuclear magnetic resonance (NMR). The resonance assignments were conducted on an α elicitin, capsicein, by three-dimensional homonuclear (Bouaziz *et al.* 1994a) and heteronuclear NMR (Bouaziz *et al.* 1994b), allowing the secondary structure location and the disulfide bridge pairing.

The search for new elicitins could reveal new information on the structure-function relationships of these molecules. Present among the Pythiaceae family, the fungal genera *Pythium* and *Phytophthora*, which are represented by about 67 and 100 species, respectively, are many economically important plant pathogens. The genus *Pythium* contains species that range from saprotrophic, facultative parasites with extensive host ranges to highly pathogenic species with limited host ranges. They cause disease in numerous crops, including rice and sugarcane (Hoy and Schneider 1988). In addition, these fungi cause diseases that are considered to be a serious threat to several greenhouse-grown crops, especially those grown in hydroponic culture with recirculating nutrient solutions (Stanghellini *et al.* 1988; Zinnen 1988). The Pythiaceae phylogeny is subject to controversy and has recently been revised using ribosomal RNA sequences (Briard *et al.* 1995), suggesting that some *Pythium* species are highly related to the *Phytophthora* genus.

We therefore looked for the presence of elicitin-like toxins secreted by *Pythium vexans*, one of the *Pythium* species closest to the genus *Phytophthora* known to be a parasite of *Vinca rosea,* a medicinal plant used for the production of alkaloids (Nef *et al.* 1991). We purified and sequenced these elicitin-like isoforms and investigated their toxicity to tobacco leaves in order to correlate the structural features, also determined by circular dichroism (CD), with biological activity. We also established a phylogenic tree based on the elicitin sequences that confirmed that *Pythium vexans* is very close to the *Phytophthora* genus.

RESULTS

Evidence of elicitin-like proteins secreted by *Pythium vexans*.

After 8 days of culture, the culture filtrate was submitted to analytical reversed phase–high performance liquid chromatography (RPLC) and purification. Figure 1 shows an RPLC profile of a crude *Pythium vexans* culture filtrate compared with the purified isoforms. Two major peaks were observed, eluting at 26 and 28% CH_3CN, designated *Pythium vexans* elicitins 1 (Vex_1) and 2 (Vex_2), respectively. The abundance of these isoforms was evaluated to be 22 and 24 mg ml^{-1} of an 8-day culture filtrate, respectively. These proteins were purified following the general procedure already described for α elicitins from the sterilized filtrate using preparative RPLC in a first step and desalting by Sephadex G50 exclusion-diffusion chromatography (Pernollet *et al.* 1993). A further RPLC step was necessary to separate the isoforms from each other. The proteins were purified to homogeneity as shown by analytical RPLC (Fig. 1) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 2). The electrophoretic pattern of the purified molecules revealed that, whereas Vex_2 elicitin, which only stained with R250 coomassie blue and exhibited an apparent relative molecular weight of 10,000, that of Vex_1 elicitin was found to be reactive with periodic acid-Schiff (PAS) reagent and exhibited a relative molecular weight of approximately 15,000. Electro-spray mass spectrometry (ES-MS) allowed a precise determination of the Vex_2 elicitin relative molecular weight (10,396 ± 1) while numerous ionized species were obtained with the Vex_1 elicitin characterized by a relative molecular weight ranging from 12,693 ± 2 to 13,260 ± 2. A Western blot showed that both molecules responded positively to antisera raised against a *Phytophthora* elicitin, β cryptogein (not shown). The amino acid composition (Table 1) revealed that both *Pythium vexans* elicitins are closely related to *Phytophthora* α elicitins. This classification is also supported by the acidic isoelectric points measured to be 3.1 ± 0.1 and 3.4 ± 0.1 for Vex_1 and Vex_2 elicitins, respectively, and the presence of a valyl residue at position 13 (Fig. 3).

Toxicity to tobacco detached leaves.

Both elicitin-like proteins induced necrosis on detached tobacco leaves quite comparable to that caused by *Phytophthora* elicitins. The extent of the induced necrosis was compared with the necrosis caused by an α elicitin secreted by *Phytophthora capsici*, capsicein. The symptoms were first observed about 24 h after treatment and reached their maximum level after about 2 days. Figure 4 shows that comparable necroses were obtained with equal amounts of Vex_2 elicitin and capsicein, the former was able to induce 15% necroses with 100 ng, but was slightly less toxic with 1 µg. Ten nanograms of Vex_1 elicitin was sufficient to induce a 33% pro-

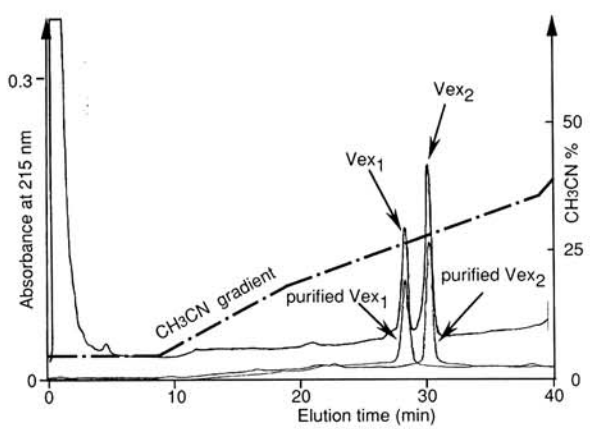

Fig. 1. Analytical RPLC profiles of *Pythium vexans* crude culture filtrates and of the corresponding elicitin-like purified proteins. Solid line: crude filtrate and purified proteins. Dotted line: acetonitrile gradient.

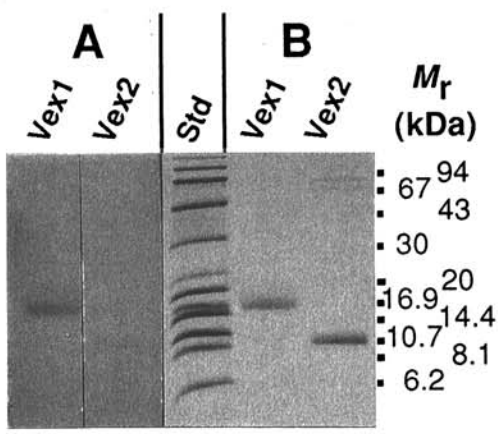

Fig. 2. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of elicitin-like proteins. **A,** Periodic acid-Schiff staining; **B,** Coomassie blue R250 staining; Std: M_r standards.

portion of necrotized leaf tissue. At low dose, this appeared even more toxic than β cryptogein, one of the most toxic *Phytophthora* elicitins. However, 100 ng of β cryptogein reproducibly provokes a 100% necrosis of the leaf (Pernollet *et al.* 1993) whereas the same dose of Vex_1 elicitin only caused a 40% necrosis. The reproducibility of the necrotized area was previously shown to be in a 5% range (Pernollet *et al.* 1993).

Amino acid sequences of *Pythium vexans* elicitins.

The sequences of the *N*-termini were determined with both native and 4-vinyl-pyridine reduced and alkylated proteins. Overlapping peptides were obtained either after digestion with CNBr, applied on reduced and alkylated proteins, or cleavage of the performic acid oxidized proteins with trypsin, chymotrypsin, and Glu-C endoproteinase. Only those peptides useful for the determination of the whole sequences are indicated in Figure 3. Peptides were separated by RPLC and numbered according to their increasing chromatographic retention times. For both molecules reduction and alkylation allowed the identification of Cys 3 and Cys 27. With an initial sequencing yield of 45%, the *N*-terminal end of the native Vex_1 elicitin was sequenced up to Ala 40 (repetitive yield 92%), whereas Vex_2 *N*-terminus was sequenced up to Ala 38 (initial yield of 61%; repetitive yield 92%).

The alignment of the Vex_1 peptides is shown in Figure 3A. After performic acid oxidation, trypsinolysis produced the V_1T peptide and chymotrypsin digestion the V_1C peptide. These peptides were aligned with the peptide V_1B_2 obtained after CNBr digestion, while another CNBr peptide, V_1B_1, allowed the alignment of the *N*-terminus with the tryptic peptide V_1T. The good agreement between the measured amino

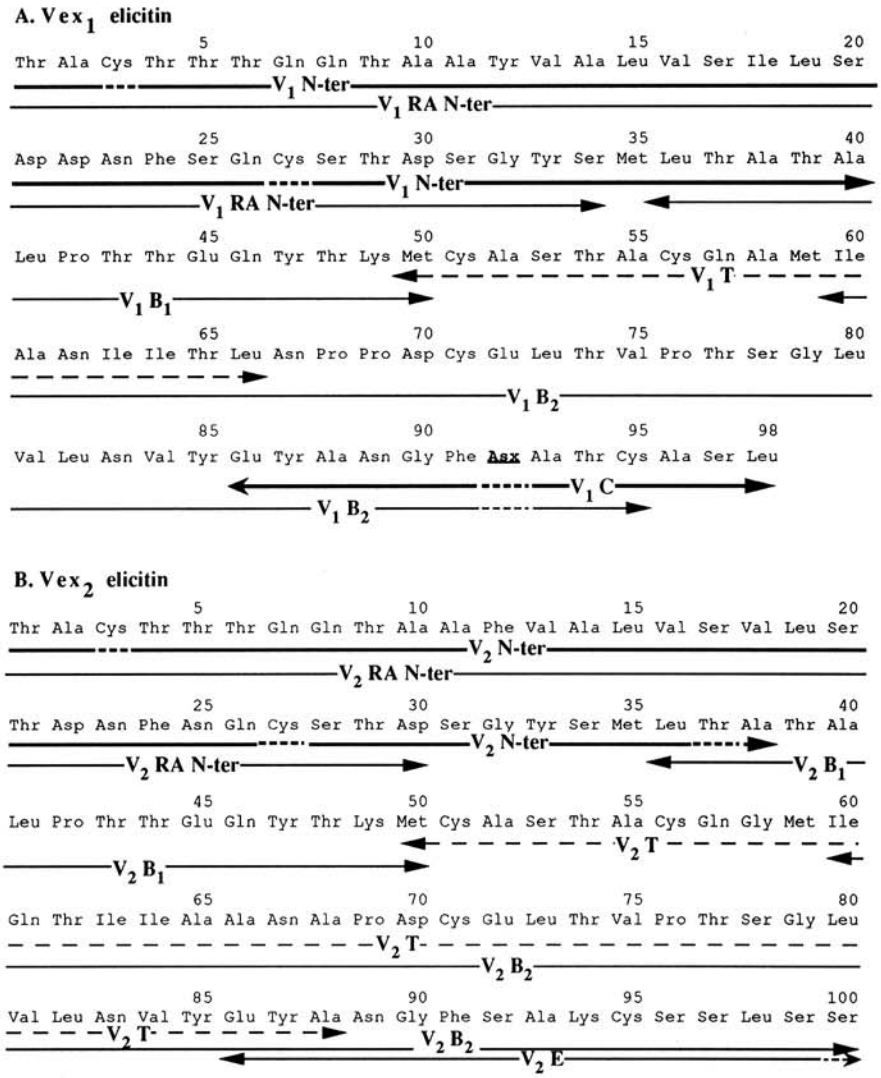

Fig. 3. Alignment of *Pythium vexans* elicitin-like sequences Peptides are numbered according to their elution times, V_1 and V_2 indicate peptides originating from Vex_1 (*Pythium vexans*) elicitin and Vex_2 elicitin, respectively. **A,** Vex_1 peptide alignment; V_1 N-ter: N-terminus of the native protein, V_1 RA N-ter: N-terminus of the reduced and alkylated Vex_1 elicitin; V_1B_1 and V_1B_2: CNBr-cleaved peptides; V_1T: peptide obtained after digestion with trypsin; V_1C: peptide obtained after digestion with chymotrypsin. **B,** Vex_2 peptide alignment; V_2 N-ter: N-terminus of the native protein, V_2 RA N-ter: N-terminus of the reduced and alkylated Vex_2 elicitin; V_2B_2 and V_2B_2: CNBr-cleaved peptide; V_2T: peptide obtained after digestion with trypsin; V_1E: peptide obtained after digestion with Glu-C endoproteinase. Dashed lines indicate residues not determined, underline Asx the N-glycosylation site of Vex_1 elicitin.

acid composition and that calculated from the amino acid sequence (Table 1) validates the sequence. Nevertheless, a large discrepancy was noted between 10,288, the relative molecular weight calculated from sequence, assuming the occurrence of 3 disulphide bridges as in other elicitins, and that measured by ES-MS (12,693–13,260). The amino acid phenylthiohydantoin derivatives were unambiguously assigned at all positions, with a normal sequencing yield, excluding the occurrence of any glycosylation except in the case of the V_1C peptide, a peptide arising from chymotrypsinolysis. No phenyl-thiohydantoyl derivative was found at the seventh position of this V_1C peptide (position 92 in the whole protein sequence). Its amino acid composition (Table 1) showed that the seventh residue was either asparagine or aspartic acid, with traces of *N*-glucosamine present, indicating that carbohydrates are linked to V_1C by *N*-glycosylation. Therefore, a glycosylated asparagine was assigned to position 92. The polypeptide relative molecular weight value was quite comparable to those of *Phytophthora* elicitins that vary from 10,161 to 10,373.

The Vex_2 peptides are aligned in Figure 3B. After performic acid oxidation, trypsinolysis produced the V_2T peptide and Glu-C endoproteinase digestion the V_2E peptide, which overlapped one with the other. The V_2T peptide is linked to the *N*-terminus via the V_2B_1 peptide that was obtained after CNBr digestion. The relative molecular weight of Vex_2 elicitin was calculated with the average isotopic composition being 10,306, but mass spectrometry allowed a direct relative molecular weight determination of 10,396 ± 1. To explain this discrepancy, the C-terminal end was investigated with another cyanogen bromide peptide, V_2B_2, obtained after reduction and alkylation with 4-vinyl-pyridine. V_2B_2 was purified by exclusion-diffusion chromatography, sequenced and characterized by mass spectrometry. The sequence of this 41 amino acid peptide began at Ile60 and was suspected to end with 2 Ser instead of 1. Its ES-MS measured mass (4,459.2 ± 0.4), in perfect agreement with the relative molecular weight (4,459) calculated with 2 C-terminal seryl residues, definitely assigned a serine at position 100. Vex_2 is therefore composed of 100 residues. Its calculated relative molecular weight (10,393) is in good agreement with that determined by ES-MS (10,396 ± 1), which confirmed the C-terminus and shows that Vex_2 is devoid of side chain posttranslational modification, in agreement with the SDS-PAGE data. Measured and calculated amino acid compositions are in agreement (Table 1). No micro heterogeneity was observed in the sequences of *Pythium vexans* elicitin-like toxins and sequencing did not reveal any unusual repetitive yield decrease characteristic of *O*-glycosylation at any clearly detectable Ser or Thr position.

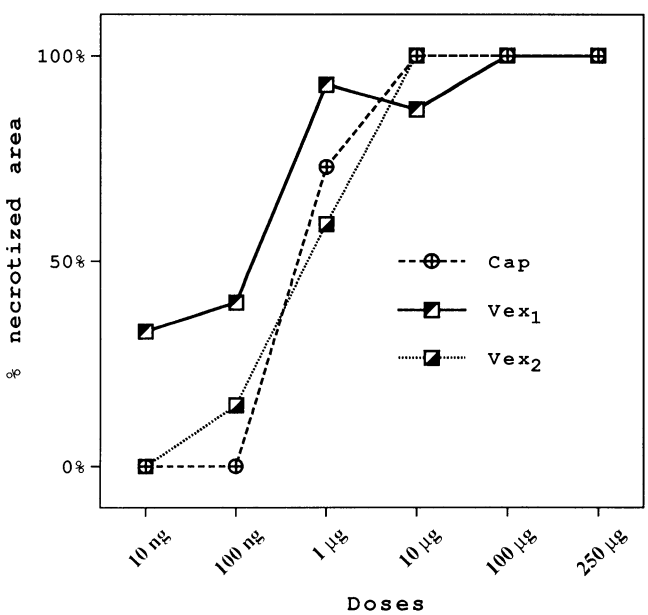

Fig. 4. Comparison of necrosis induction on tobacco detached leaves by Vex_1 and Vex_2 with capsicein, a *Phytophthora* typical α elicitin. Cap (dashed line), capsicein, *Phytophthora capsici* elicitin; Vex_1 (solid line) and Vex_2 (dotted line), *Pythium vexans* elicitins. The vertical ordinate indicates the proportion of necrotized area measured on four detached leaves. Proteins were tested on 60-day-old tobacco plants (cv. Xanthi) cultivated in a greenhouse. Six doses of each elicitin diluted in 10 µl of pure water were applied to detached leaves that were kept in nutrient solution at room temperature in the dark for 48 h.

Sequence homology of *Pythium vexans* with *Phytophthora* elicitins.

Figure 5 illustrates the sequence alignment of Vex_1 and Vex_2 with α elicitins secreted by some *Phytophthora* species.

Table 1. Amino acid compositions, M_r and isoelectric points of *Pythium vexans* elicitin compared with *Phytophthora* proteins[a]

Elicitin / peptide	Vex_1	Vex_2	Cap	V_1C
Amino acids				
Phe	2.2 (2)	3.1 (3)	2	0.9 (1)
Leu	9.6 (9)	8.1 (8)	10	0.9 (1)
Ile	2.8 (4)	2.8 (3)	3	0 (0)
Met	2.7 (3)	3.0 (3)	3	0 (0)
Val	5.0 (5)	6.0 (6)	6	0 (0)
Ser	9.7 (9)	11.2 (12)	12	1.0 (1)
Pro	4.3 (4)	3.2 (3)	4	0 (0)
Thr	17.5 (16)	15.4 (16)	16	1.1 (1)
Ala	13.6 (13)	12.9 (13)	13	2.9 (3)
Tyr	4.7 (5)	3.8 (4)	5	0.7 (1)
His	0.0 (0)	0.0 (0)	0	0 (0)
Lys	1.2 (1)	2.0 (2)	2	0 (0)
Asx (Asp + Asn)	8.2 (10)	8.1 (8)	8	2.0 (1)
Glx (Glu + Gln)	8.1 (8)	9.9 (9)	5	1.1 (1)
Cys	5.8 (6)	6.2 (6)	6	1.1 (1)
Trp[b]	0.0 (0)	0.0 (0)	0	0 (0)
Arg	0.0 (0)	0.0 (0)	0	0 (0)
Gly	4.5 (3)	4.4 (4)	3	1.1 (1)
(Leu + Ser + Thr + Ala)	50.4 (47)	47.6 (49)	51	5.9 (6)
M_r from sequence data[c]	10,288	10,393	10,161	
M_r from ES-MS[d]	12,693–13,260[e]	10,396 ± 1	10,165 ±1	
Measured pI	3.1 ± 0.1	3.4 ± 0.1	3.5 ± 0.1	
Calculated pI	3.88	4.26	4.54	

[a] Amino acids are indicated using the three-letter code. Vex_1 and Vex_2 indicate Vex_1 and Vex_2 *Pythium vexans* elicitins, respectively, Cap is capsicein, the α elicitin secreted by *Phytophthora capsici*. V_1C : the peptide obtained after digestion of Vex_1 with chymotrypsin. Values indicate the number of residue per molecule, those in parentheses are deduced from the amino acid sequence.

[b] Tryptophan amount determined by UV spectrometry.

[c] Polypepdide molecular mass derived from sequence data assuming an average isotopic mass and 3 disulfide bridges per molecule.

[d] Electrospray mass spectrometry.

[e] Glycoprotein molecular mass including both the polypeptide molecular mass and the carbohydrate moiety.

These eight sequences were homologous to each other and no deletion was necessary for aligning them. The percent match between this set of elicitins was 63% compared with the value of 77.6% corresponding to the six *Phytophthora* α elicitins alone. Amino acid mutations that have already been seen in *Phytophthora* β elicitins were observed at positions 2 and 65 in both *Pythium vexans* elicitins and Ser25 and Lys94 solely in Vex_1 and Vex_2, respectively. Other mutations at positions 21, 22, 57, 58, and 61 were frequently observed in *Phytophthora* elicitins and a Phe residue was found in a partial sequence of α cryptogein at position 12 (Pernollet *et al.* 1993). Moreover, positions 18, 23, 49, 62, 64, 66, and 92 were occupied by consensus amino acids in *Phytophthora* elicitins. Most of these novel mutations are grouped in two regions (18–25 and 57–66) and occur at the same positions for both *Pythium vexans* elicitins, with the exception of position 92 in Vex_1 and positions 18, 21, 66, 99, and 100 in Vex_2, which appears to be the most atypical elicitin so far known. A phylogeny inference from the α and β elicitin sequences obtained with the protein sequence parsimony method (Felsenstein 1988), in which any change of amino acid is consistent with the genetic code, is reported in Figure 6. It shows that the *Pythium vexans* elicitins are more closely related to the α elicitins of *Phytophthora parasitica, infestans,* and *cactorum* than to those of *Phytophthora capsici, megasperma megasperma,* and *drechsleri.* As expected from their physicochemical features, which obviously differ from those of α elicitins, β elicitins are not so closely related to the *Pythium vexans* elicitins.

Secondary structure.

The far UV CD spectra of the *Pythium vexans* elicitins are compared in Figure 7 and the deduced secondary structure proportions presented in Table 2. Although not strictly identical, these spectra were similar and show that α-helices account for the major secondary structure. The CD spectra and secondary structure content of Vex_1 and Vex_2 elicitins were very close to each other and slightly but significantly 2-nm blue shifted with respect to that of capsicein, indicating a lower proportion of periodic structure. The contribution of the different secondary structures, obtained after curve fitting, showed that both *Pythium vexans* elicitins were poorer in α helix than is capsicein (46 and 49 instead of 54%) and richer in aperiodic structure (33 instead of 25%).

DISCUSSION

Until now only fungi belonging to the *Phytophthora* genus were shown to secrete toxic proteins of the elicitin family. *Pythium vexans* is the first species of another Oomycete genus observed to abundantly secrete elicitin-like molecules. They consist of two isoforms that can be classified as mem-

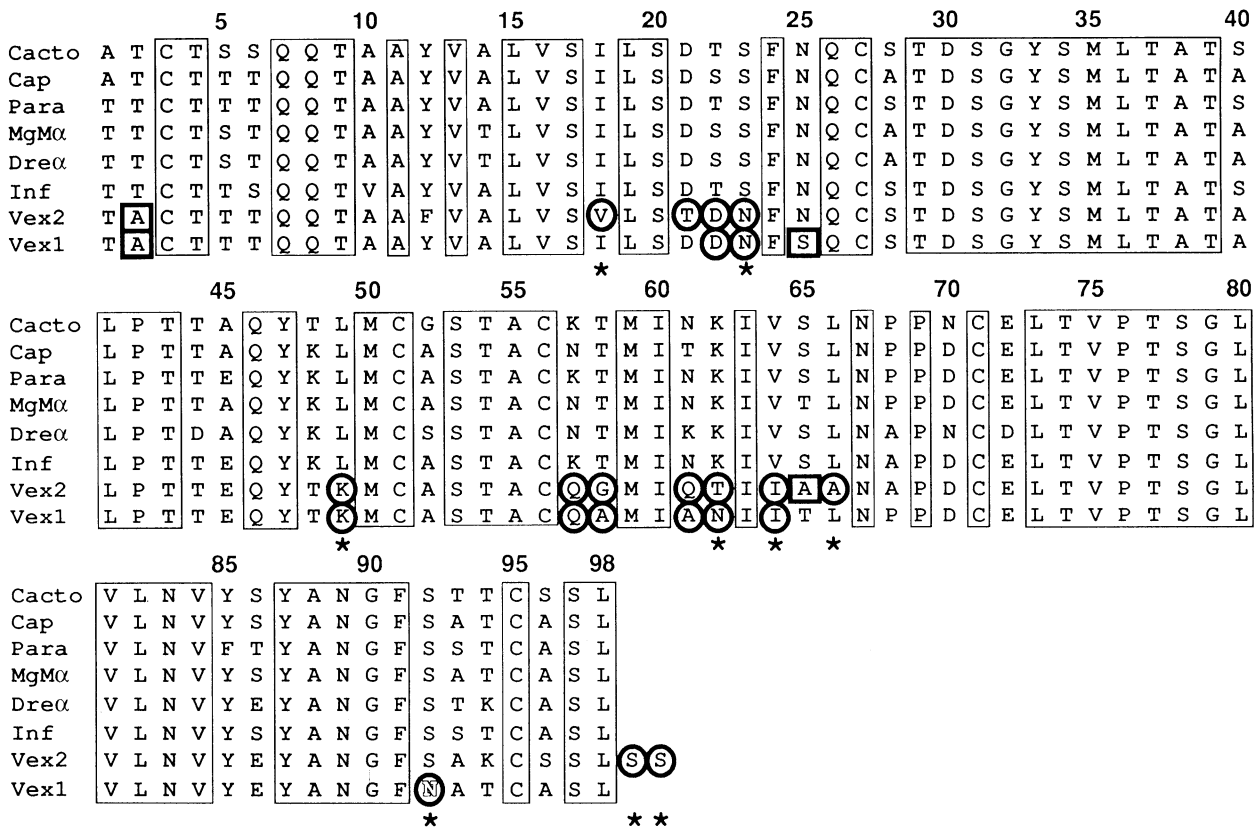

Fig. 5. Alignment of the *Pythium vexans* sequences with typical *Phytophthora* α elicitins Cacto, *Phytophthora cactorum* elicitin; Cap, capsicein, *Phytophthora capsici* elicitin; Dre α, *Phytophthora drechsleri* elicitin; Inf, *Phytophthora infestans* elicitin; MgM α, *Phytophthora megasperma megasperma* elicitin; Para, parasiticein, *Phytophthora parasitica* var. *parasitica* elicitin. The sequences are ordered from top to bottom according to increasing toxicity to tobacco detached leaves. Boxes show the conserved consensus regions. Bold circles indicate the residue typical of *Pythium vexans* elicitins, stars those that are strictly conserved in *Phytophthora* elicitins, and bold squares *Pythium vexans* residues, which are found in *Phytophthora* β elicitins.

bers of the α class of elicitins characterized by a valyl residue at position 13 and acidic isoelectric points (Nespoulous *et al.* 1992).

Like the other known ones, *Pythium vexans* elicitins exhibit 6 Cys and 3 Met and are devoid of 3 amino acids (Trp, His, Arg), while Leu, Ser, Thr, and Ala account for half of the residues. The absence of Trp was confirmed by the UV absorption spectrum (not shown). Although the *Pythium vexans* elicitin sequences are very similar to those of *Phytophthora*, they exhibit significant differences with respect to their sequences and posttranslational modifications. Some of the novel point mutations found, grouped in two regions of the sequences, were specific to *Pythium vexans* elicitins. With the exception of the two extra C-terminal seryl residues of Vex_2, only seven novel mutations occur at highly conserved positions of *Phytophthora* elicitins, including β ones (positions 18, 23, 49, 62, 64, 66, and 92). Among these mutations, Asn23 and 92 created putative *N*-glycosylation sites in the Vex_1 elicitin. Although abundant (26 ± 3% of the polypeptide mass, the equivalent of 15–18 hexose residues), the effective glycosylation of the Vex_1 elicitin only occurs at a single position (Asn92). Because of the presence of Thr94, this residue is likely to be an asparagine, forming an *N*-glycosylation site. This type of site was observed here for the first time in an elicitin. Contrary to Vex_1, Vex_2, like the *Phytophthora* elicitins, was totally devoid of *N*-glycosylation sites and of any side chain posttranslational modification. Both *Pythium vexans* elicitins exhibited isoelectric points slightly more acidic than those found in *Phytophthora* α elicitins (Pernollet *et al.* 1993). Like these elicitins, the measured isoelectric points were more acidic than those calculated from the sequences. For both *Pythium vexans* elicitins, a similar discrepancy of about 0.8 pH units was found, which strongly suggests that the carbohydrates linked to Vex_1 are likely to be neutral ones. The dispersion of the relative molecular weight found by ES-MS indicated that an important polymorphism is likely to occur in the carbohydrate chain length.

Like the other elicitins, the *Pythium vexans* proteins appeared as ordered proteins, exhibiting little or no β structure, with α helix accounting for half of the molecule and aperiodic structure representing about one-third of the protein. The amount of α helix determined through CD (54 ± 4%) is lower than that obtained with NMR spectroscopy, which reaches approximately 70% of the residues (Bouaziz *et al.* 1994a; Bouaziz *et al.* 1994b). This discrepancy might arise from the large difference in protein concentration used in these two methods. The very high concentration (4 mM) used in NMR experiments could favor interactions between polypeptide chains leading to more periodic structure, compared with that observed with CD, performed at 5 μM, i.e., 10^3 less concentrated. Nevertheless, the comparison of closely related proteins by CD spectroscopy is reliable enough to describe structural variations between them. The comparison of the CD spectra showed that the secondary structure of the *Pythium vexans* elicitins is only slightly affected by the point mutations with respect to *Phytophthora* elicitins (Nespoulous *et al.* 1992; Huet *et al.* 1993). Compared with capsicein (a reference *Phytophthora* α elicitin), both the blue shift of the CD spectra of *Pythium vexans* elicitins and their reduced α-helix content correlate with an increase in aperiodic structure. This may be assumed to result from the alteration of the α-helix D, which spans from Met55 to Asn67 in capsicein (Bouaziz *et al.* 1994a; Bouaziz *et al.* 1994b), because it is the only α helix in which a cluster of mutations (5 in Vex_2 and 7 in Vex_1) was observed in *Pythium vexans* elicitins. Mutations Lys 62 Asn in Vex_1 and Thr 58 Gly in Vex_2, involving helix-breaking residues (Chou and Fasman 1978), would be the major factors affecting the secondary structure. The very close structure in solution of Vex_1 and Vex_2 demonstrates that the carbohydrate moiety of Vex_1 has no effect on the global folding of the polypeptide chain.

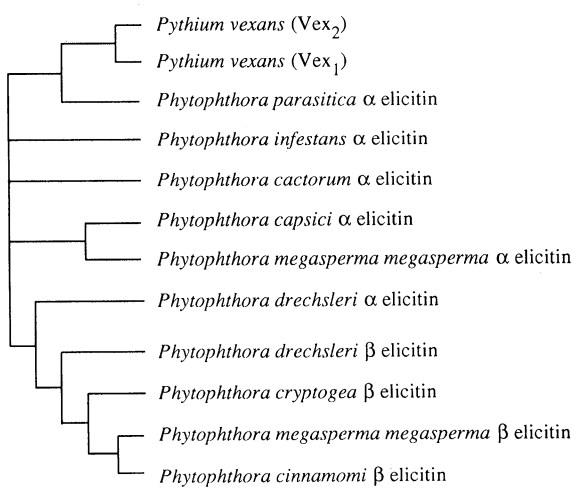

Fig. 6. Phylogeny inference from α and β elicitin sequences. Unrooted phylogeny established using the Protein Sequence Parsimony Method.

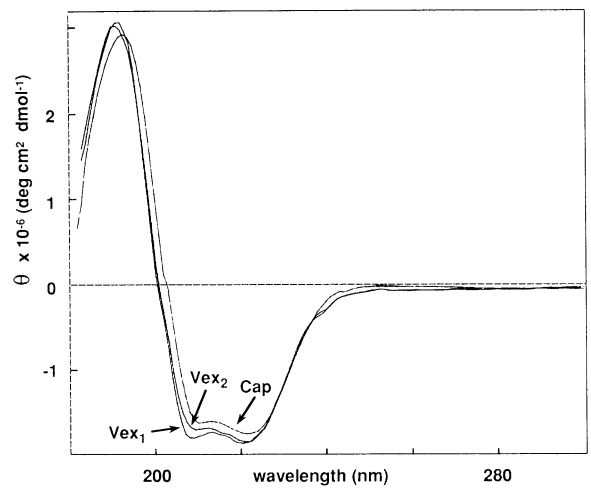

Fig. 7. Comparison of the CD spectra of capsicein, *Phytophthora capsici* α elicitin, with Vex_1 and Vex_2 elicitins. Cap, capsicein; Vex_1 and Vex_2, *Pythium vexans* elicitins.

Table 2. Secondary structure proportion of *Pythium vexans* elicitins[a]

Elicitin	α-helix (%)	β-sheet (%)	β-turn (%)	Aperiodic (%)
Vex 1	45.6 ± 2.1	0.5 ± 12.5	20.6 ± 4.0	33.3 ± 2.4
Vex 2	48.5 ± 2.6	0 ± 15.8	19.2 ± 5.0	32.4 ± 3.1
Capsicein	53.7 ± 3.9	0 ± 23.4	21.7 ± 7.4	24.6 ± 4.6

[a] Data obtained with circular dichroism measurements are given with their confidence limits ($P = 0.95$).

The toxicity of *Pythium vexans* elicitins to tobacco leaves was found to be higher than that of the other α elicitins. Whereas Vex_2 remained less toxic than β elicitins, Vex_1 was found to be highly toxic at low doses. Trying to correlate the sequence differences to the toxicity to tobacco, residues Ala2 (a typical β elicitin amino acid), Asp22 (an exposed residue), and Asn62 are likely to enhance the toxicity of *Pythium* α elicitins with respect to *Phytophthora* ones. Nevertheless, it is probable that the carbohydrate moiety plays a crucial role in the higher toxicity of Vex_1, complementary to that of the polypeptide structure. This is in agreement with the presence of carbohydrates in numerous fungal elicitors (Dixon and Lamb 1990).

Simple inspection of the aligned sequences suggests that individual members of the elicitin family diverged from a common ancestor during evolution. A more quantitative analysis using the method of Felsenstein (1988) supported this hypothesis and provided significant insights into the pathway by which they evolved. The phylogenetic relationships of the elicitin sequences reported in Figure 6 strongly suggests that *Pythium vexans* is closely related to a cluster of *Phytophthora* species that diverged from other ones, regardless of number of residues. This is in agreement with a phenogram derived from cluster analysis of the percent inhibition by hymexazol, a potent fungicide active against *Pythium* species, proposed by Kato *et al.* (1990). This analysis also shows that β elicitins have derived from α ones at an early stage and then evolved independently. As a consequence, these observations might contribute to the use of elicitins in classifying Oomycete fungi and to the understanding of their evolution.

The secretion of elicitins by a genus of pathogenic fungi, other than *Phytophthora*, supports the hypothesis that these molecules are primarily toxins that weaken the host to help the pathogen invade the plant tissues. Necrogenic activity was observed in a variety of plant species independent of any compatibility between the fungal species secreting the elicitin and the host plant (Pernollet *et al.* 1993). The elicitor function of elicitins leading to systemic acquired resistance is more rarely observed among plant species (Ricci *et al.* 1989; Kamoun *et al.* 1993b). In this case, elicitins can be considered to be avirulence factors. Whether or not the compatibility of *Pythium* species can be explained by the recognition of secreted elicitins has to be investigated and correlated with their host range. Nevertheless, whereas elicitins are ubiquitous among *Phytophthora*, the observation of elicitin-related proteins in other *Pythium* species is a prerequisite to answering such a question. This should also permit the identification of common structural features that will lead to a better understanding of the biological role of this protein family and to the elucidation of the molecular mechanisms involved in the elicitin signaling pathway.

MATERIALS AND METHODS

Culture of *Pythium vexans* mycelium.

Pythium vexans (isolate 11408 from the MUCL fungi library of the Catholic University of Louvain) was grown in Roux bottles on a medium deprived of yeast extract and peptone adapted from Hall *et al.* (1969) during 8 days at 26° C in the dark. Culture media were sterilized by filtration through a 0.22-μm membrane prior purification.

Analytical characterization of culture filtrate proteins.

Analytical RPLC was performed using a Spectra Physics chromatographic system consisting of an 8700 XR LC pump, an 8750 organizer, and an 8773 XR UV detector. Elicitins were chromatographed on an Aquapore (C8) RP 300 (Brownlee Labs) cartridge (4.6 × 30 mm). The compounds were eluted at room temperature (22° C) in a gradient of CH_3CN (Merck, Lichrosolv) / 95% CH_3COONH_4, 25 mM pH 7.2) as already reported (Pernollet *et al.* 1993). The solvent was applied at a flow rate of 0.5 ml min^{-1} and the eluate was monitored by measuring absorbance at 215 nm. The elicitin molecular mass was measured using a peptide SDS-PAGE system using the standards of the Sigma MW-SDS-17 calibration kit with corrected values (Sallantin *et al.* 1990) in a Bio-Rad Mini Protean II apparatus. Electrofocusing was performed using a Pharmacia FBE3000 apparatus according to the manufacturer's instructions using polyacrylamide gels (5%) with 3% cross-linkage containing 13.3% glycerol and 6.33% Pharmalyte. Elicitins were assayed over a 2.5–5.0 pH range and the pI determined using the Low pI Pharmacia IEF calibration Kit (pH 2.5–6.5). The gels were stained with Serva Blue R250 and periodic acid–Schiff reagent (Zacharius *et al.* 1969). Proteins were electroblotted onto nitrocellulose sheets using a Bio-Rad Transblot cell according to Sallantin *et al.* (1990) and immunodetection performed with antisera raised against β cryptogein (Tercé-Laforgue *et al.* 1992).

Protein purification.

Protein purification was performed according to the general procedure adapted to α elicitins (Pernollet *et al.* 1993). A further RPLC step was necessary to separate the isoforms from each other using another gradient of CH_3CN obtained with the same solvents. The gradient was programmed to run from 5.0 to 23.9% CH_3CN in 10 min, to 27.5% in 20 min and then to 50% in 5 min at a flow rate of 0.5 ml min^{-1}. Capsicein was obtained as already reported (Pernollet *et al.* 1993). The elicitin fractions were lyophilized and stored at –20° C before use.

Tests for necrotic activities on tobacco leaves.

Purified proteins were tested on detached leaves from approximately 60-day-old greenhouse-grown tobacco plants (cv. Xanthi) as described by Pernollet *et al.* (1993). Four replicates of each treatment were included in each experiment and all elicitins were assayed simultaneously to reduce variability. Symptoms reached their maximum extent after 2 days.

Amino acid analysis.

Elicitin isoforms were analyzed in triplicate in microgram quantities using an Applied Biosystems 420 H device following the methods of the manufacturer. The cystein content was determined as cysteic acid after performic oxidation according to Hirs (1967). Results were expressed as the proximal integer value, assuming a molecular mass of approximately 10 kDa. The molecular masses were calculated from the average isotopic composition.

Protein sequencing.

One hundred micrograms of protein was reduced with 2-mercaptoethanol and alkylated with 4-vinyl-pyridine according to Henschen (1986). The reduced and alkylated elicitins

was submitted to *N*-terminal Edman degradation and to peptide cleavage. The elicitin was digested with CNBr (Huet *et al.* 1992). In addition, 100 μg of *Pythium vexans* elicitins, previously oxidized with performic acid, was digested with sequencing grade modified trypsin (EC 3.4.21.4), obtained from Promega, according to Huet *et al.* (1993). Another enzymatic digestion was performed with sequencing grade chymotrypsin (EC 3.4.21.1) obtained from Boehringer. One hundred micrograms of protein, previously oxidized with performic acid, was incubated in 100 mM Tris HCl 10 mM $CaCl_2$, pH 7.8, for 18 h at 25° C (enzyme/protein ratio, 1:100). Sequencing grade Glu-C endoproteinase (EC 3.4.21.9) was purchased from Promega; 30 μg of protein, previously oxidized with performic acid, was incubated in 50 mM ammonium acetate pH 4.0 for 18 h at 37° C (enzyme/protein ratio 1:30). The digested peptides were separated by RPLC as described by Huet *et al.* (1992), either in CH_3COONH_4 or in CF_3CO_2H buffer using octadecyl or phenyl (100 × 4.6-mm) Aquapore Brownlee cartridges. Automated Edman degradation of the whole protein and of peptides was performed using an Applied Biosystems 475A sequencer and its on-line phenylthiohydantoin amino acid analyzer model 120A with reagents and methods of the manufacturer.

Atmospheric-pressure ionization mass spectrometry.

Molecular mass determination of proteins was performed on a Trio 2000 mass spectrometer using an electrospray ion source and a quadrupole mass analyzer with an upper mass limit of m/z = 3,000 Da (VG Biotech Manchester, UK). Ten-microliter samples containing 100 picomoles of protein were introduced into the source at a flow rate of 2 μl min^{-1} (carrier solvent: 50% CH_3CN, 49% water, 1% formic acid). Four thousand volts were applied on the capillary and 1,000 volts on the counter electrode. The quadrupole was calibrated using horse heart myoglobin. The calculated and experimental molecular masses are the average relative molecular weight and not the monoisotopic relative molecular weight.

Circular dichroism.

CD spectra were recorded at room temperature at 1 nm intervals over the wavelength range 180 to 300 nm, using a Mark V Jobin-Yvon dichrograph, calibrated using an isoandrosterone solution (1.3 mg ml^{-1} in dioxane). The optical rotation was checked with horse heart cytochrome C, chicken egg white lysozyme, and rabbit muscle L-lactate dehydrogenase. Elicitin concentrations were determined using UV spectroscopy employing an extinction coefficient of 7,685 M^{-1} cm^{-1} for Vex_1 and 6,235 M^{-1} cm^{-1} for Vex_2 at 277 nm (wavelength of the elicitin maximum). Protein samples (50 μg ml^{-1} in water) were placed in a 0.2-cm path-length cell; 5 spectra collected for each sample were averaged and the baseline obtained with pure water was subtracted. The CD data were analyzed following the method of Yang *et al.* (1986) for the determination of the contribution of the different secondary structures. The results were indicated with their confidence limits ($P = 0.95$) obtained after curve-fitting computation.

Phylogeny inference from protein sequences.

An unrooted phylogeny from elicitin sequences was established using the protein sequence parsimony method (PROTPARS) of Felsenstein (1988), algorithm version 3.4, run with the BISANCE server at CITI2 (Paris) (Dessen *et al.* 1990).

ACKNOWLEDGMENTS

We are grateful to Michael O'Donohue for his help in writing this manuscript, to Mathilde Briard for helpful discussion, and to Mauricette Sallé-Tourne, Monique Mansion, Marc Sallantin, and Christian Ouali for their skillful technical assistance.

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