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# Review

# Are elicitins cryptograms in plant-Oomycete communications?

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**Abstract.** Stimulation of plant natural defenses is an important challenge in phytoprotection prospects. In that context, elicitins, which are small proteins secreted by *Phytophthora* and *Pythium* species, have been shown to induce a hypersensitive-like reaction in tobacco plants. Moreover, these plants become resistant to their pathogens, and thus this interaction constitutes an excellent model to investigate the signaling pathways leading to plant resistance. However, most plants are not reactive to elicitins, although they possess the functional signaling pathways involved in tobacco responses to elicitin. The understanding of factors involved in this reactivity is needed to develop agronomic applications. In this review, it is proposed that elicitins could interact with regulating cell wall proteins before they reach the plasma membrane. Consequently, the plant reactivity or nonreactivity status could result from the equilibrium reached during this interaction. The possibility of overexpressing the elicitins directly from genomic DNA in *Pichia pastoris* allows site-directed mutagenesis experiments and structure/function studies. The recent discovery of the sterol carrier activity of elicitins brings a new insight on their molecular activity. This constitutes a crucial property, since the formation of a sterol-elicitin complex is required to trigger the biological responses of tobacco cells and plants. Only the elicitins loaded with a sterol are able to bind to their plasmalemma receptor, which is assumed to be an allosteric calcium channel. Moreover, *Phytophthora* and *Pythium* do not synthesize the sterols required for their growth and their fructification, and elicitins may act as shuttles trapping the sterols from the host plants. Sequence analysis of elicitin genes from several *Phytophthora* species sheds unexpected light on the phylogenetic relationships among the genus, and suggests that the expression of elicitins is under tight regulatory control. Finally, general involvement of these lipid transfer proteins in the biology of Pythiaceae, and in plant defense responses, is discussed. A possible scheme for the coevolution between *Phytophthora* and tobacco plants is approached.

**Key words.** Elicitin; cell death; hypersensitivity; LTP; *Phytophthora*; *Pythium*; resistance; SAR; SCP; signaling; tobacco.

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## Introduction

Crop protection constitutes a major challenge in both improving produce quality and preserving the environment. Interactions between microorganisms and plants have had major effects on the development of civilizations. Plant disease outbreaks have resulted in catastrophic crop failures and caused major social changes. However, disease is not the only outcome of plant-microbe interactions, and biological controls of plant disease are emerging, using molecules from pathogens able to induce defense mechanisms in plants (for a recent overview of plant-microbe interactions see [1]). In some situations, microorganism molecules could be recognized by plant cells, triggering many metabolic changes and leading to plant resistance. Such compounds have been named elicitors of plant defense and have been extensively studied [2–8].

The hypersensitive response (HR) is the most common feature associated with active plant resistance. It follows a primary pathogen attack. Activation of the HR leads to the death of cells at the site of infection, resulting in the restriction of the pathogen to small areas surrounding the initially infected cells (necrotic lesions). In plant resistance associated with HR, the knowledge of events which trigger cell death is fundamental. In some situations, the development of HR is followed by the induction of a systemic acquired resistance (SAR). The whole plant becomes resistant to further pathogen infection, wherever this infection occurs. SAR activation results in the development of broad-spectrum resistance. A strong body of evidence suggests that salicylic acid plays a key role in both SAR signaling and disease resistance [9–12].

In tobacco fields, it was shown that necroses on plants were associated with the presence of *Phytophthora*, which were nonpathogenic [13]. From culture filtrates of *P. cryptogea* and of *P. capsici*, proteinaceous elicitors named elicitins (cryptogein and capsicein, respectively) were subsequently isolated [14]. These proteins stimulate natural defenses of tobacco against many pathogens, accompanied by restricted leaf necrosis [15–17]. About 40 *Phytophthora* species were screened for the production of elicitins, and different modes of treatment were assayed for plant responses [18–20]. Using cryptogein antibodies, it was shown that this elicitin could migrate through the plant and could be responsible for the systemic acquired resistance induced in tobacco [21–23]. A possible extension towards other plants could be offered with another genus of the oomycete class, *Pythium* spp., which can induce protection of tomato against *Fusarium oxysporum* f. sp. *radicis-lycopersici* [24, 25] and can also secrete elicitin-like proteins.

This paper emphasizes recent results concerning elicitins and reflects on about their agronomic interest and biological functions. Recent contributions bring responses to some of the problems still unsolved after publication of the last reviews on that exciting model [26–28]. Until now, elicitins were viewed only as elicitors of plant defenses. The biological functions of these holoproteins remained unknown, although the genes coding elicitins, despite strong selection pressure, were highly conserved and seemed tightly regulated. The discovery of their sterol carrier properties has opened new perspectives dealing with the relationship between this function and the elicitor activity of these small cysteine-rich proteins. Nevertheless, this elicitor activity was restrained to few plant species, and thus did not appear to be in accordance with a universal lipid transfer function. These considerations required a reassessment of the precise role of elicitins for both *Phytophthora* and plants.

## Elicitins, a family of (not so?) well-characterized proteins

Elicitins can be merged within a single, highly conserved, family. Purified proteins as well as numerous sequences derived from complementary DNA (cDNA) clones are similar enough to fit this classification. On the other hand, elicitins do not resemble any other proteins, and previous attempts to compare them with other small, fungal proteins, such as hydrophobins, were poorly convincing and have been abandoned [26]. So what does allow the assignment of a given protein to the elicitin family from a structural point of view?

First of all, elicitins are restricted to the oomycete genus *Phytophthora* and a few *Pythium* species, which, on the basis of additional taxonomic features may be missing links between the two genera [29]. Oomycetes are no longer considered to be 'fungi', but have been phylogenetically grouped with the heterokont algae [30]. Moreover, the other families of Oomycetes appear to lack elicitin genes or equivalent sequences [30]. Thus, elicitins are located in a particular, unexplored, taxonomical niche. This may explain the paucity of comparative models available in plant (or animal) pathology.

Additional features that make a protein an elicitin are the sum of some characteristics: the size (98 amino acids in most cases); a biased amino acid composition, revealed by the lack of tryptophan, histidine and arginine residues and the significant abundance of a few amino acids, such as serine and threonine residues, that represent about 30% of the protein, and to a lesser extent alanine (more than 10%) and leucine (10%); the occurrence of six cysteine residues located on conserved positions and involved in three structurally determinant disulfide bridges, and an overall primary structure which represents a unique amino acid sequence that

enables definition of an 'elicitin signature' listed in the various pattern databases such as PRODOM [31] or PRINTS [32] and that spans the entire sequence (fig. 1). In addition, the lack of tryptophan implies that elicitins display typical, tyrosine-like ultraviolet (UV) spectra, giving a helpful signature for their characterization among other proteins. Finally, a protein would be also defined as an elicitin on the basis of its particular three-dimensional structure [33] and biophysical properties (see below).

To date, more than 30 *Phytophthora* species have actually been found to secrete elicitins. Based on their respective pI, all these proteins could be classified as either acidic ($\alpha$, pI $< 5$) or basic ($\beta$, pI $> 7.5$) elicitins. These two forms could be encountered within the same *Phytophthora* species, but an iterative rule could be pointed out: $\alpha$-elicitins were always produced, whereas $\beta$-elicitins were found to be secreted by a restricted range of species. The biological and taxonomical relevance of this situation will be discussed later.

A rapid comparison of $\alpha$- and $\beta$-elicitins led to identification of proper characteristics for each form. First, the evident difference proceeds from the global net charge due to differing composition in charged amino acids; the positive charge is provided by 6 Lys in $\beta$-elicitins, and only 2–4 in $\alpha$-elicitins. In contrast, the number of negatively charged Asp and Glu residues was almost constant within the proteins, ranging from 3 to 5, and was not correlated with resulting net charge. Second, structural differences were reported among the amino acid sequences that were not directly related to protein net charge. For example, in $\beta$-elicitins, residues 13 and

```
cinnamomin-ha1    TACTSTQQTAAYVALVSILSESYFSTCASDSGYSMLTATALPTTAQYKLMCASTACQEMI
hae1-cryptogein   TACTTTQQTAAYVALVSILSESFFSTCASDSGYSMLTATALPTTAQYELMCASTACQEMI
hae2-cryptogein   TACTTTQQTSAYVTLVTLLSKSYFTTCASDSGYSMLTATALPTTAQYELMCASTACQEMI
cinnamomin-ha2    TACTSTQQTAAYVALVSILSESYFSTCASDSGYSMLTATALPTTAQYKLMCASTACQEMI
ß-cryptogein      TACTATQQTAAYKTLVSILSDASFNQCSTDSGYSMLTAKALPTTAQYKLMCASTACNTMI
ß-cinnamomin      TACTATQQTAAYKTLVSILSESSFSQCSKDSGYSMLTATALPTNAQYKLMCASTACNTMI
parasiticeinA1    TTCTTTQQTAAYVALVSILSDTSFNQCSTDSGYSMLTATSLPTTEQYKLMCASTACKTMI
infestin1         TTCTTSQQTVAYVALVSILSDTSFNQCSTDSGYSMLTATSLPTTEQYKLMCASTACKTMI
α-cryptogein      TTCTTTQQTAAYVALVSILSDSSFNQCATDSGYSMLTATSLPTTDQYKLMCASTACNSMI
α-cinnamomin      TTCTSTQQTAAYVALVSILSDSSFSQCATDSGYSMLTATSLPTTAQYKLMCASTACNTMI
sojein2           TTCTSSQQTAAYVALVSILSDSSFNQCATGSGYSMLTATALPTTAQYKLMCASTACNTMI
infestin2A        ETCSPTDQTTAYSTLASVLTLSSFQGCADDSGFSLLYSTALPDDDQYVKMCASDNCKSLI
infestin2B        ETCSPTDQTTAYSTLASVLTLSSFQGCADDSGFSLLYSTALPDDAQYVKMCASDNCKSLI
                   :*:.::** ** :*.::*: : *  *: .**:*:* :.:**   **  **** *: :*

cinnamomin-ha1    TEIIALDPPDCDLTVPTSGLVINVYEYSNDFVSTCASLSSSASS----------------
hae1-cryptogein   EEIIALNPPDCDLTVPTSGLVINVYEYANDFASTCASLSSSPA----------------
hae2-cryptogein   AEIITLSPPDCDLTVPTSGLVIDVYTYANGFASTCASLSSSSA----------------
cinnamomin-ha2    TEIVSLSPPDCDLTVPTSGLVLDVYTYANGFTLTCASLSSSSA----------------
ß-cryptogein      KKIVTLNPPNCDLTVPTSGLVLNVYSYANGFSNKCSSL----------------------
ß-cinnamomin      KKIVALNPPDCDLTVPTSGLVLDVYTYANGFSSKCASL----------------------
parasiticeinA1    NKIVSLNPPDCELTVPTSGLVLNVFTYANGFSSTCASL----------------------
infestin1         NKIVSLNAPDCELTVPTSGLVLNVYSYANGFSSTCASL----------------------
α-cryptogein      AKIISLNAPDCELTVPTSGLVLNVYSYANGFSATCASL----------------------
α-cinnamomin      KKIVTLNPPDCELTVPTSGLVLNVYSYANGFSATCASL----------------------
sojein2           KKIVALNPPDCDLTVPTSGLVLNVYSYANGFSSTCASL----------------------
infestin2A        ESVASLNPPNCDLTVPTSGLVLNVVDLTSGFSEKCSSSSSTSNTASSAATSTTTEAPAAT
infestin2B        ESVAGLNPPNCDLTVPTSGLVLNVVDLTSGFSKKCSSSSSTSNTASSAATSRTTEAPAAT
                   .:  *..*:*:*********::*   :..*  .*:*

cinnamomin-ha1    -------------------------------------------------
hae1-cryptogein   -------------------------------------------------
hae2-cryptogein   -------------------------------------------------
cinnamomin-ha2    -------------------------------------------------
ß-cryptogein      -------------------------------------------------
ß-cinnamomin      -------------------------------------------------
parasiticeinA1    -------------------------------------------------
infestin1         -------------------------------------------------
α-cryptogein      -------------------------------------------------
α-cinnamomin      -------------------------------------------------
sojein2           -------------------------------------------------
infestin2A        TAAPTTDTTTASTATDAPAATPVATPATTTTNSTESVTQTS----AAAC
infestin2B        TAAPTTDSTATDAPAATPAVTPVATPAATSTNSTESVTQTSVTQTTAAC
```

Figure 1. Clustal W multiple sequence alignment of elicitins. (*) fully conserved residue and (:) conserved strong group; (.) conserved weaker group.

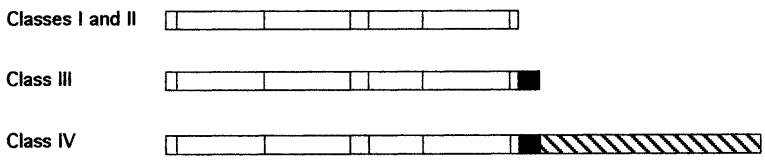


Figure 2. Schematic representation of the various elicitin classes. The 'elicitin' domain, as defined in the PRODOM [31] and PRINTS [32] databases, is represented by open rectangle. Vertical bars indicate the location of conserved cysteine residues. The carboxy-terminal extension shared by the genes encoding highly acidic elicitins (HAE) and elicitin-like proteins from *P. infestans* is represented by a black square, whereas the carboxy-terminal regions specific to class III are indicated by a dashed rectangle.

14 are polar amino acids (Lys, Thr), which constitute an interruption in the hydrophobic segment A(V)AYVA(T)LV, compared with $\alpha$-elicitin. It is noticeable that a chemical characteristic (pI) revealed structural coherences with evident links to gene structure, so that $\beta$-elicitins appeared as original proteins not only because of their presence in few species of *Phytophthora* but also according to their sequence.

### 3D structure of cryptogein

The three-dimensional (3D) structure of the $\beta$-elicitin cryptogein was determined by X-ray diffraction of the protein crystal [33] and by $^{1}H$ and $^{15}N$ nuclear magnetic resonance (NMR) of protein solution [34, 35]. These structures are closely related, and only slight differences occur. The crystal structure is probably more compact, whilst the solution structure highlighted the flexibility of the protein. Cryptogein is a globular protein containing five helices ($\alpha_1$–$\alpha_5$, from N- to C-term, respectively), a small antiparallel $\beta$-sheet and an Ω-loop, all these structures being linked together by disulfide and hydrogen bonds. The disulfide bridges were characterized as follows: Cys3–Cys71, Cys27–Cys56 and Cys51–Cys95 [33, 36, 37]. Roughly, the protein shows two opposite sides: on one side $\alpha$-helices making a smooth face with polar amino acids exposed to the solvent, and on the other side a protruding beaklike motif built from the proximity of the Ω-loop and the $\beta$-sheet (see fig. 10). A large hydrophobic cavity occurs between these two faces, mainly involving strictly or highly conserved hydrophobic residues (Met, Leu, Ile, Phe, Val, Tyr). From the solution structure, it appears that the Ω-loop is flexible. The tightening of Ω-loop and $\beta$-sheet is secured by hydrogen bondings and van der Waals interactions. The study of conformational changes, due to pH and elicitin concentration, demonstrates that cryptogein is able to dimerize [35].

### Distinct classes of elicitins

The further identification of numerous cDNA clones from various *Phytophthora* species [F. Panabières et al., unpublished results], as well as genomic clones containing elicitin-encoding sequences in *P. cryptogea* [38] *P. cinnamomi* [39] and border species of *Pythium* [29], revealed additional, unusual features, especially on elicitin genes, which appear not to be expressed [38]. Thus elicitin-encoding sequences can be separated (to date) into three broad classes as follows (figs 1 and 2):

- Class I encompasses all the proteins (or open reading frames, ORFs) corresponding to the elicitin type, exhibiting most of the features described above. In addition, the totally conserved residues are the 6 Cys, 3 Met, 2 Phe and 3 Gly. Residues Leu, Ile, Pro and Thr are always located on highly (or strictly) conserved positions.
- Among this class, one group (I′) corresponds to elicitins which slightly diverge from class I because, although derived from cDNAs, then expressed genes, they encode unusual proteins which have not been observed until now, and display new features, such as histidine residues, along with a flexible 98–101 amino acid size, and additional computer-deduced features, such as Asn glycosylation sites. Most of the elicitin-like sequences identified within the *Pythium* (or *Pythium*-like) species fit into this class [29].
- Class II contains HAE (hyperacidic elicitin) sequences, i.e. 103–104 amino acid-long ORFs from *P. cryptogea* [38] or *P. cinnamomi* [39] which would correspond, if expressed, to highly acidic proteins, exhibiting net charges ranging from $-6$ to $-10$. To date, no peptides or trancripts have been observed within the corresponding species, but cDNAs that encode highly acidic peptides have been identified in *P. insolita* [F. Panabières et al., unpublished results].
- Class III contains two elicitin-encoding sequences from *P. infestans* [40], which would encode 165–170 amino acid-long peptides that consist of the conserved 98-amino acid elicitin signature, followed by an $\sim$70-amino acid C-terminal domain. This region, which displays a highly biased amino acid composition (Ser, Thr and Ala residues comprise 75% of the region), could represent an O-glycosylated domain. From the combination of a putative extracellular domain (the elicitin signature) and a serine-threonine rich O-glycosylated

(or potentially glycosylated) domain, an analogy was proposed between the class III elicitins and cell surface- or cell wall-associated glycoproteins; therefore, a cellular location has been proposed for these proteins [40]. If elicitins (or elicitin domains) actually occur at two locations, as extracellular and cell wall proteins, novel hypotheses based upon dimerization events would be very attractive and would give new insights for this not so well characterized protein family.

## Structural domains

Apart from these intrinsic characteristics, elicitin sequences generally revealed other features which may be involved in their biological activity, and additional domains can be evidenced from a scanning of domain databases such as PROSITE (table 1) [41]. Thus, protein kinase C-dependent phosphorylation sites were found in the carboxy-terminal region (residues 92–94) of most of the basic, highly necrotizing elicitins, whereas the great majority of $\alpha$-elicitins lack them. Moreover $\beta$-cryptogein (isolated from *P. cryptogea*) and $\beta$-drechslerin (from the closely related species *P. drechsleri*) possess an additional PKC-dependent phosphorylation site, located within the Ω-loop (residues 37–39). Another domain found in elicitin sequences is a myristoylation site, also located in the carboxy-terminal part of the sequence (residues 79–84). Finally, a helix-loop-helix, dimerization domain signature was observed in all elicitin sequences, although it corresponds to a small region (residues 76–91). This domain may be constituted by two amphipathic helices linked by a region, which would form a loop. The signature observed in elicitins corresponds to one complete amphipathic helix. Interestingly, this domain completely overlaps the antiparallel two-stranded $\beta$-sheet revealed in the structure of cryptogein. If valid, this observation would suggest that several alternative structures might occur. Dimerization of elicitins is frequently observed in SDS-polyacrylamide gel electrophoresis (PAGE) and was recently reported [35], so that the exploration of this property may be relevant while dissecting the functions of elicitins.

It has to be noted that all these features proceed from computational analyses of elicitin sequence. Thus, the occurrence of posttranslational modifications still remains to be demonstrated, the removal of signal peptide excepted. Refined tools for structural analysis of elicitins, especially within the fungus, or when translocated *in planta*, must then be developed.

## Elicitin genes: basic bricks of a puzzling family

The high conservation of elicitins at the protein level suggests that either elicitins are young in evolutionary terms, since mutations have not altered the intrinsic nature of these proteins, or the selection pressure is high enough to maintain the cohesion of the family among the genus, whatever the species and the evolutionary events that affected the rest of the genome. So it is likely that elicitins are also conserved at the nucleotide level and share some structural features that may be typical

Table 1. Structural characteristics of the different elicitin classes.

| Class | pI range | PROSITE domains | Proteins |
|---|---|---|---|
| I$\alpha$ | 4–5 | myristyl<br>helix-loop-helix | cactorein, cinnamomin, cryptogein, citrophthorin, INF1, megaspermin, parasiticein |
| I$\beta$ | 7.5–8.5 | PKC phospho-site<br>myristyl<br>helix-loop-helix | cryptogein, drechslerin, megaspermin |
| I′ | 4.6 | N-glycosylation<br>myristyl<br>helix-loop-helix | œdochilin |
| II | 3.5 | CK2 phospho-site<br>myristyl<br>helix-loop-helix | cinnamomin-ha1, cinnamomin-ha2, cryptogein-ha1, cryptogein-ha2 |
| III | 3.5–4.0 | N-glycosylation<br>cAMP phospho-site<br>PKC phospho-site<br>CK2 phospho-site<br>leucine-zipper<br>myristyl<br>helix-loop-helix | infestin 2A, infestin 2B |

of the whole family. Despite the paucity of available data (only nine genes were characterized from *P. parasitica* [42], *P. cryptogea* [38] and *P. cinammomi* [39], and three additional messenger RNAs (mRNAs) from *P. infestans* [40, 43]), common features can be defined.

### Organization of elicitin genes

First, elicitins are encoded by small, multigene families. The number of genes varies from one species to another, as observed by Southern hybridizations [29, 38, 44], but does not appear to display intraspecific variation [F. Panabières et al., unpublished]. The multiplicity of elicitin genes does not totally overlap the complexity of elicitins found in the culture filtrates; thus, an elicitin isoform can be encoded by several copies, whilst some others appear to be unique. For example, $\beta$-cryptogein and parasiticein (from *P. parasitica*) are encoded by at least two identical copies [45, 46].

Four different elicitin genes were found to be clustered on a $\sim$5-kb region within *P. cryptogea* [38] as well as within *P. cinnamomi* [39]. All the genes share the same transcriptional orientation in these clusters. A refined analysis on a genomic clone of *P. cryptogea* revealed that at least 3 kb are duplicated in the vicinity, so that at least six elicitin genes (and maybe more) constitute this cluster [45]. Southern experiments and sequence data suggest that elicitin genes occur within a single cluster in *P. cryptogea*.

### Structural characteristics of the elicitin genes

The various elements that constitute the elicitin genes can been analyzed as follows.

**Coding regions.** Elicitin genes that have been characterized so far lack introns [29, 38, 39, 42]. However, splicing is likely to occur in *Phytophthora*, as deduced from the characterization of introns in some genes isolated from *P. parasitica* [47] and *P. infestans* [48]. Moreover, sequence analysis of the intron-exon boundaries indicates that splicing events that may occur in *Phytophthora* are similar to those of higher organisms [49].

**Signal peptide.** Elicitins, like other secreted proteins, are synthetized as preproteins that undergo posttranslational modifications through the removal of a signal peptide. This segment is typically 18–20 amino acids in elicitin precursors [29, 38, 40, 42, 43], and is highly conserved among all elicitins studied so far [29, 38], suggesting that elicitin precursors are probably processed and secreted following similar mechanisms.

**5′ untranslated regions (5′ UTR).** This segment of elicitin mRNA is generally short, in a 40–60-bp range. Despite its small size, this region has interesting features. First, it allows the characterization of the transcription start site (TSS) of elicitin genes. It has been experimentally identified in *parA1* [42] and in *B14*, the gene encoding $\alpha$-cryptogein, using the primer extension technique, then by sequence comparison on other elicitin genes [38]. This site encompasses a short (17-bp) sequence which occurs in several genes identified so far not only in *Phytophthora* [38] but also in other Oomycetes, such as *Bremia lactucae* [50]. From this, it is now considered an oomycete-specific transcription initiation consensus sequence [51]. Another feature of the 5′ UTR is that cytidine residues are particularly abundant and may be derived from a microsatellite sequence that would be based upon the (CCA)n motif. This motif, which is more or less conserved, is mainly located on the 3′ half moiety of the region, but may represent up to 60% of the 5′ UTR. This motif has not been observed to date in other *Phytophthora* genes, and could be a signature of elicitin genes. In addition, a CAAG motif has been found to precede the initiation codon in all elicitin genes from *P. cryptogea* and *P. cinnamomi*, regardless of the class of gene. In contrast, this consensus is not found upstream of the start codon of *parA1* or *Inf1*, or other genes from *Phytophthora*. As a result, it appears that regions overlapping the start codon are particularly well conserved among some elicitin genes, and are specific to them. This region corresponds to the ribosome binding site [52]. This sequence specificity, associated with strong conservation, suggests that specific mechanisms govern the translational control of elicitin gene expression. This speculation may be enforced by sequence comparisons. As already observed with signal peptide sequences, 5′ UTRs of equivalent elicitin genes are well conserved. Thus the $\beta$-elicitin mRNAs from *P. cryptogea* and *P. cinnamomi* possess 5′ UTRs that are 57 and 58 bp long, respectively, which share 84% similarity. The 5′ UTRs of acidic elicitins are 63 and 67 bp long, respectively; the similarity reaches 85%. In the case of the HAE genes, the similarity is not so high, and 'only' reaches $\sim$63%. Here again, the various elicitin genes display class-specific characteristics, which may be involved in the regulation of gene expression.

**3′ untranslated regions.** 3′ UTRs are generally a rich repository of cis-acting regulators of gene expression, which contain signals determining mRNA localization and controlling polyadenylation, mRNA stability and signals controlling translation initiation [53]. In addition, they have been shown in some cases to control gene expression at the transcriptional level [54]. However, the analysis of 60 different 3′ UTRs issued from 32 *Phytophthora* species revealed only few noticeable features or potential secondary structures [F. Panabières et al., unpublished results]. As a rule, the 3′ UTRs are highly variable, from 103 bp (in *P. heveae*) to 197 bp ($\beta$-cryptogein). The base composition varies from

37.6% GC (in *P. megakarya*) to 58.7% GC (α-elicitin from *P. megasperma*). Consequently, proper sequence alignment is quite impossible, although some sequences can be clustered. Interestingly, these regions, although largely diverging, are strictly conserved for a given gene at the species level, as observed for various elicitin genes from *P. cryptogea* [F. Panabières et al., unpublished results] or *P. cinnamomi* (F. Panabières et al., unpublished results [39]). Nevertheless, two canonical sequences could be defined. The first one is constituted by the stop codon, which is highly conserved. Thus the UAA codon is prevalent among all the sequences analyzed (about 87%). A notable exception is *inf1*, which possesses a UGA stop codon [43]. The first nucleotide located downstream of the stop codon is generally a G, even in the case of *inf1*, generally followed by a purine-pyrimidine doublet. More precise is the consensus sequence that corresponds to the polyadenylation signal site. This canonical sequence, which is generally AATAAA in most higher eukaryotes [55], is represented here by ATGAA, located 11–25 bp upstream from the 3′ end, which is itself frequently preceded by a AUAAA sequence, 11–15 bp upstream. This sequence is also encountered in other *Phytophthora* sequences, so that it may represent a signature at the generic level.

## Diversity of elicitins from an evolutionary point of view

Elicitins have been shown to represent a fertile, open field for research in plant pathology and mycology, since more than 100 papers devoted to these proteins have been published within the last 15 years. However, most of them used elicitins as tools for studying plant defense responses (for review, see [28]), if not lures that could mimic the incompatible interaction. On the other hand, elicitins have been considered avirulence factors on tobacco [42, 43, 56, 57], so that *parA1* and *inf1* were described as avirulence genes cloned from *Phytophthora* spp. [58]. As a consequence, the basic, if not intrinsic, role of the elicitins has been poorly investigated. The delay observed between the first description of elicitins [14] and the recent demonstration of a biological property, if not a function for elicitins [59], is rather demonstrative. Thus several black boxes and paradoxes remain to be explored, especially on the 'fungal side'. For instance, why are elicitins so diverse yet so conserved? This point has not been taken into account in the elicitin story, and remains an enigma. Is there a correlation between the diversity of elicitin genes within a given species and some genetic, biological, physiological or pathological traits of interest for *Phytophthora* that could help in defining of an ultimate function.

### Evolutionary relationships between elicitins: taxonomical consequences

The significance of the diversity of elicitin genes can been assessed at several levels. At a first level, this diversity (revealed by high performance liquid chromatography (HPLC) analysis or sequence data) can be examined as a phenotypic character, and a useful landmark of *Phytophthora* species. Hence, the extreme intraspecific conservation of elicitin sequences reflects the coherence of *Phytophthora* species, such as *P. parasitica* [46], whereas the nature and distribution of elicitins is quite variable among groupings formerly associated under the '*P. megasperma*' nomenclature [F. Panabières et al., unpublished results], and reassessed as several distinct species [60]. As another example, elicitins from *P. cryptogea* are easily distinguished from those of *P. drechsleri* [29, 38], although these two species are frequently merged or considered part of a continuous, polyphyletic complex [61, 62].

The 60 species described so far within the genus *Phytophthora* are typically classified into six groups according to the morphology of reproductive structures [63], in particular whether sporangia are papillate (groups I and II), semipapillate (groups III and IV) or nonpapillate (groups V and VI). This grouping was not 'intended to imply that this is a natural classification [64]'. Yet, this temporary classification was validated 30 years later by the ribosomal DNA (rDNA) analysis of the ITS I and ITS II [65, 66]. However, other criteria, such as the attachment of the antheridium to the oogonium (paragynous or amphigynous) that made it possible to distinguish groups I and II, III and IV, V and VI, respectively, were not supported by molecular data. In addition, the separation of semipapillate and papillate species was not possible from ITS analysis [66]. Thus sporangium morphology is a relevant basis for classification. It remains to be examined whether the diversity observed among elicitins follows the classification of species as a neutral character or whether it reflects another area of relationship within the genus.

To date, up to 30 species have been investigated for the complexity of their elicitin patterns ([28], F. Panabières et al., unpublished results), by Southern hybridization and HPLC experiments. Some species possess and express a single class of elicitins, whereas others display complex patterns [28]. The first group is mainly composed of papillate and semipapillate species, whereas nonpapillate species generally exhibit a complex pattern. As exceptions, *P. quercina*, a papillate species recently isolated from diseased oak in Europe possesses an elicitin pattern close to that of the nonpapillate *P. cryptogea*, whereas *P. gonapodyides*, which belongs to the morphological group VI, only secretes one type of elicitin [67]. A common trait between all *Phytophthora* species studied is that they all

possess genes encoding $\alpha$-elicitins, which are likely to be expressed [F. Panabières et al., unpublished results]. It must be noted that the elicitins identified within the *Pythium* species also belong to the class of acidic proteins [29]. Thus $\alpha$-elicitins represent the paradigm of the family. Conversely, $\beta$-elicitins or relative genes are present only in some species. They were first described in *P. cryptogea* [38, 68], *P. drechsleri* [69], *P. megasperma* [70] and *P. cinnamomi* [39, 71]. To date, $\beta$-elicitins or their genes have been identified in 17 species, among which 15 are nonpapillate species [28] [F. Panabières et al., unpublished results]. Ten among the 13 species that constitute group VI were analyzed and shown to possess $\beta$-elicitins [F. Panabières et al., unpublished results]. Thus three apparently independent characters (papilla type, ITS sequences and basic elicitins) appear to be of equal significance for classifying species.

At the amino acid level, a comparative analysis of available sequences confirms some phylogenetic data obtained from rDNA analysis and brings new information. Hence, the elicitins from group I species (i.e. cactorein, idaein, iranicin and pseudotsugaein) are tightly clustered, if not similar (fig. 3). Two other elicitins, PARA1 from *P. parasitica* and INF1 from *P. infestans* are linked to this group. Such a grouping has already been observed by rDNA analysis [66] and isozyme data [72]. Another group consists of elicitins isolated from *P. citrophthora* and *P. capsici*. Here again, the close relationship between these two species was previously noted using various criteria [66, 72, 73]. This analysis also confirms that *P. sojae* and *P. megasperma*, previously regarded as members of the *P. megasperma* species complex [74], represent distinct lineages, as indicated with rDNA analysis [65]. In contrast, some species previously clustered possess distant elicitins. Such is the case of *P. megakarya* and *P. palmivora*, both pathogenic on cocoa, and associated in molecular analyses [66, 73], or *P. citricola*, which is distant from the *P. capsici*/*P. citrophthora* group, on the basis of its elicitin, whereas it is closely related to them from isozyme data [75]. Finally, the clustering of the elicitins from the nonpapillate species does not fit the rDNA-based phylogeny, as basic and acidic elicitins broadly constitute distinct clades. It thus confirms that elicitins are sound tools for the classification, if not identification of species, but cannot be used in phylogenetic analyses as with ITS [29]. The comparative analysis can be extended to the signal peptides, as deduced from genomic clones [38, 39, 42] or full-length cDNAs [40, 43]. From this, it appears that signal peptides are characteristic of $\alpha$- and $\beta$-elicitins, respectively, whatever the species are (fig. 4). In addition, HAE genes possess distinct signal peptides, as the elicitin-like genes from *P. infestans*. From the comparison of phylogenetic inferences deduced from the alignment of elicitins, in their mature form, and their relative signal peptide, it is tempting to hypothesize that different selective forces act on signal peptides and mature proteins. It further supports the idea that the processing and further secretion of elicitins are important events for *Phytophthora*, and investigations in the secretion processes would be of prime value in unraveling elicitin functions.

**Diversity of elicitins: biological significance?**

The observation that elicitin diversity sometimes fits the *Phytophthora* phylogeny and sometimes not implies that additional correlations have to be searched with other traits that do not follow the rDNA- or papillation-based classification. However, the disconnected distribution of basic and acidic elicitins permits elimination of some candidate characteristics. Among them is obviously the antheridial attachment, as indicated above. Hence, both paragynous (group V) and amphigynous (group VI) species possess basic elicitins, and a close cluster contains elicitins from paragynous (*P. cactorum*, *P. idaei*) and amphigynous species (*P. parasitica*, *P. infestans*). Another trait is the type of sexual life cycle. *Phytophthora* species are either homothallic (self-fertile) or heterothallic (self-sterile). Here again, $\beta$-elicitins are observed in both homothallic (*P. megasperma*, *P. syringae*) and heterothallic species (*P. cryptogea*, *P. cinnamomi*). In addition, PARA1 and INF1, secreted by two heterothallic species, are close to elicitins from the homothallic species of the group I.

*Phytophthora* species are diverse in that some of them attack one or a few species of plants, whereas others have a very broad host range. The host specialization is not a phylogenetic criterion, and elicitin distribution is not related to the host range. For example, *P. pseudotsugae*, which is pathogenic only on Douglas fir [76], is closely related to the broad host range *P. cactorum* [66], following rDNA-based phylogeny. These two species possess and express nearly identical elicitin genes. Another example, *P. parasitica*, which is by far one of the most polyphagous species, exhibits phylogenetic affinities to *P. infestans*, which is restricted to solanaceous plants [76]. These affinities are also observed at the elicitin level. In addition, an unexpected cluster links the elicitins from low-temperature, host-specialized *P. fragariae* and the broad host range species *P. palmivora*, mainly located in tropical areas [76]. Isoform *soj2* from the host-specific *P. sojae* [77] is clustered with the $\beta$-elicitins from the broad host range species *P. cryptogea* or *P. cinnamomi*. In addition, as a consequence of the extreme intraspecific conservation of elicitin distribution, isolates of *P. parasitica* that are pathogenic and specialized on *Citrus* possess and express the same set of elicitin genes as those of broad host range isolates [V. Colas, personal communication]. Moreover, the broad

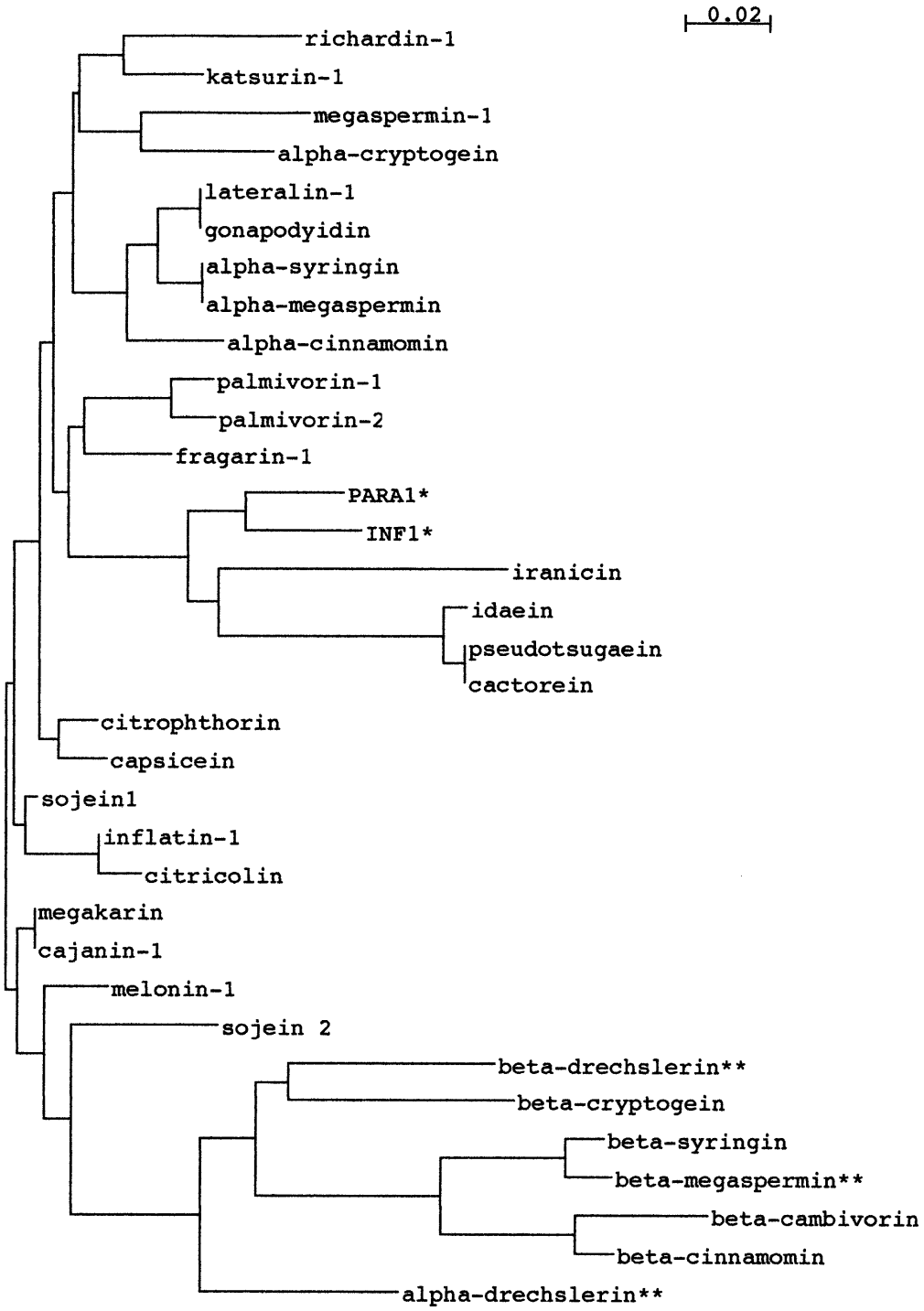


Figure 3. Phylogenetic relationships between elicitins, inferred from sequence alignment. Unless otherwise indicated, sequences were deduced from cDNA analysis. Additional sequences have been included, such as PARA1 and INF1 (*[42, 43]), $\alpha$-and $\beta$-cinnamomin [39], and data obtained from protein sequencing (**[69, 70]). Trees were constructed using the neighbour-joining method based on the multiple alignment of elicitin sequences performed with the Clustal W software [165]. Confidence limits (indicated in% at the nodes) were created in a bootstrap analysis using 10,000 replicates.

host range species *P. cryptogea* exhibits an elicitin pattern similar to those displayed by the host-specialized *P. quercina* [67] or the exceptional *P. undulata*, which has not been proven to be a pathogen of any plant [29, 76].

Some species, like *P. infestans*, infect aerian parts of the plants through the development of specialized infection structures named appressoria [78, 79], but *Phytophthora* species are generally soilborne root pathogens and do not develop such structures. Moreover, *P. infestans* is classically described as typical of hemibiotrophic pathogens, which developed intimate interactions with host plants, if not coevolved with their hosts [80]. Conversely, *P. parasitica* can be considered a typical broad host range, soilborne pathogen. However, the close affinity between PARA1 and INF1 impedes such a distinction on the sole basis of elicitin sequences.

From all these comparisons, it appears that the distribution of elicitins among the *Phytophthora* genus may fit some criteria of phylogenetic relevance, such as the papilla morphology, already supported by rDNA-based classification. Hence, these characteristics may have been submitted to equivalent evolutionary controls. In contrast, elicitin diversity cannot be linked to other sexual traits or pathological behaviors, and is unlikely to be involved as a determinant of host range, if not in pathogenesis, despite previous statements [26, 38, 43, 56, 58]. Moreover, accumulated evidence indicates that the different regions which constitute elicitin-encoding sequences are likely to be submitted to various, distinct evolutionary forces, which may be independent of other functions traditionally explored in the case of pathogenic fungi. Hence, alternative roles or functions must be proposed for elicitins, especially if we consider that this protein family is restricted to a narrow phylogenetic niche, as are the *Pythiaceae* [29, 81]. Moreover, their unique situation and their various properties described below suggest that if it occurs their role or function must be common for all the elicitins, and subsequently for all *Pythiaceae*. In this context, the most obvious unique feature of *Pythiaceae* among Oomycetes is that they are unable to synthesize sterols, but have been frequently described to require an exogenous source of $\beta$-hydroxy sterols for sporulation [76], despite debating hypotheses (M. Ponchet and P. Venard, unpublished results, [82]). The recent demonstration of a sterol-binding activity for elicitins [59, 83, 84] suggests that this track needs to be further explored. On the other hand, if elicitins share a common function, they are likely to be under the control of similar regulatory events. It is therefore worthwhile examining the regulation of elicitin gene expression.

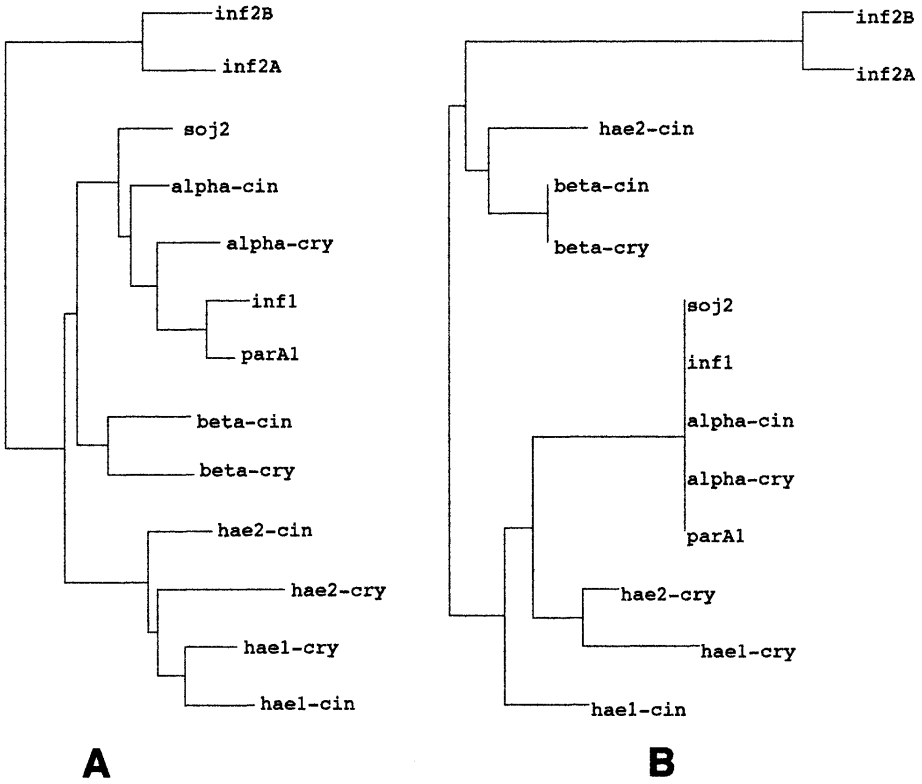


Figure 4. Phylogenetic inference of some elicitin genes deduced from the sequence alignment of the sequence of the mature peptides (*A*) or their corresponding signal peptides (*B*). Phylogenetic analyses were performed as described in figure 3.

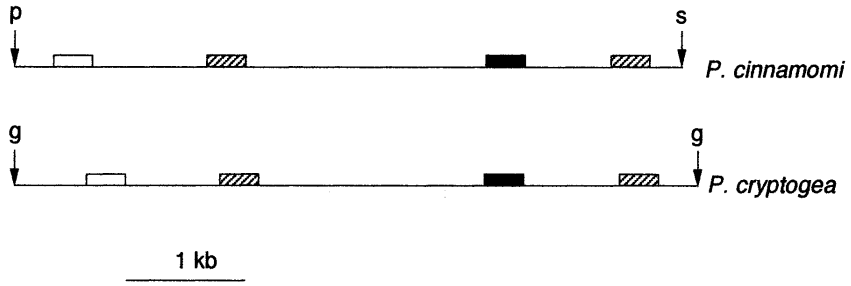


Figure 5. Schematic representation of the organization of the elicitin gene cluster, as determined in *P. cinnamomi* (upper, [39]) and in *P. cryptogea* (lower, [38]). The genes encoding $\beta$-elicitins are represented by white rectangles, and the genes encoding $\alpha$-elicitins by black solid rectangles. Dashed rectangles indicate the different genes encoding hyperacidic elicitins. The limits of the clusters are indicated by restriction sites for *Bgl*II (g), *Sph*I (s) and *Pvu*I (p), as described in the original papers [38, 39].

## Regulation of elicitin gene expression

**Is elicitin gene expression constitutive?** Elicitins have been generally described as the most abondant peptide in culture filtrates of *Phytophthora* spp. under various culture conditions [28, 44]. The tight correlation between the dosage of various isoforms of elicitins from *P. cryptogea* and their corresponding mRNAs in vitro may suggest that their expression would be constitutive [38]. However, the gene encoding the elicitin from *P. infestans*, called *Inf1*, was shown to be expressed only at the mycelial stage when observed in vitro [43]. In addition, isolates usually referred to as *P. parasitica* var. *nicotianae*, which are highly pathogenic on tobacco, generally fail to secrete elicitins, though they retain a set of elicitin genes [42, 44]. Thus, the regulation of elicitin gene expression remains an open question, and the consequences of this regulation are diverse. Are all elicitin genes regulated following identical pathways, subsequently suggesting that they share a common role or function? If not, do distinct regulation pathways occur for the various classes of elicitins (acidic, basic, hyperacidic)? Basically, is elicitin gene expression constitutive or is it regulated, i.e. inducible or repressible? Some preliminary elements can be provided by structural and functional analysis of elicitin genes and their putative promoter sequences. Available data concern the elicitin genes from *P. cryptogea* and *P. cinnamomi* [38, 39] and to a lesser extent *P. parasitica* [42]. First, elicitin genes of *P. cryptogea* and *P. cinnamomi* are tandemly clustered, as indicated above. The clusters of *P. cryptogea* and *P. cinnamomi* display a similar genomic organization (fig. 5). Genes belonging to the same class ($\beta$, $\alpha$ or HAE) occupy corresponding positions in the two clusters, which can be easily aligned. Moreover, the regions that separate elicitin genes are of similar length in both species. A similar interspecific conservation of cluster organization is observed in the case of *Hox* genes [85], globin genes [86] and in numerous multigene families whose expression is known to be developmentally regulated [87, 88].

It has been shown that elicitin genes, like other genes from *Phytophthora* and Oomycetes, share a consensus sequence that corresponds to the TSS [38, 51, 89]. However, an alignment of the relative TSSs of the various elicitin genes indicates a biased conservation between the TSSs of the $\alpha$- and the $\beta$-elicitins and to a lesser extent HAE genes (fig. 6). So perhaps interspecific, conserved, factors act as class-specific regulators of elicitin genes expression. The availability of sequences deduced from genomic clones allows the identification of putative promoter regions of the various elicitin genes. Sequence analysis was performed up to 150 bp upstream of the TSS of *parA1* and in a 0.4–1.8-kb region upstream of those of elicitins from *P. cinnamomi* and *P. cryptogea*, and failed to identify TATA

```
ß-CRY        CTCATTCTGCAATTTGC ccagtcac
ß-CIN        CTCATTCTGCAATTgGC tccgtcac

α-CRY        GCCATTGTGCAATTTGC tctgtcac
α-CIN        GGCATTGTGCAATTTGC tctgtcgc
α-PAR        GCCATTGTGCAATTTGC tctcatcc

HAE-CRY1     CTCATTCCTCAATTTcC cttgccaa
HAE-CIN1     CGCACTCCACAATTTGa aatttgcc
HAE-CRY2     CTCACTCCACAATTTGC cttgccaa
HAE-CIN2     TTCATTCTGCAATTTGC tttgccga
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Figure 6. Sequence alignment overlapping the TSS of elicitins. When experimentally defined, the nucleotide corresponding to the precise origin of transcription is indicated (underlined). The 'oomycete-specific transcription initiation consensus sequence' is indicated in uppercase, whereas the 5′ ends of the transcripts corresponding to different elicitins are represented by lowercase.

boxes as typically occurring 30–70 bp upstream of the TSSs of RNA pol II–transcribed genes [90]. However, sequences only slightly related to TATA boxes can be found 140–200 bp upstream of the TSSs of elicitin genes from the *P. cryptogea* cluster, whereas such features do not occur at all within the *P. cinnamomi* cluster. So, elicitin genes would be expressed through the control of TATA-less promoters. Such promoters are frequently observed in genes which are prone to undergo developmental [91], tissue-specific or cell cycle-specific regulation [92]. In summary, although additional elements are necessary for further hypotheses, the combination of an interspecific, conservative clustering of elicitin classes, a biased, class-specific sequence of the TSS and the likely 'TATA-less' structure of the promoters, as well as a stage-dependent expression of *inf1* likely suggest that elicitin gene expression is not constitutive but may undergo specific regulation events.

**Functional analysis of an elicitin promoter.** The hypotheses presented here, concerning potential class-specific modulations of elicitin gene expression, do not contradict the notion of a coarse, more or less common, regulation pathway for elicitin genes among the *Pythiaceae*. This is strengthened by the characterization of sterol-binding properties of various elicitins [83], along with their overall high sequence conservation. Thus if elicitins share a similar role among all *Phytophthora* species, the mechanisms that promote their expression and final secretion must be highly similar. In order to test this hypothesis, a gene encoding $\beta$-cryptogein from *P. cryptogea* was used for the transformation of *P. infestans*, resulting in the transcription and translation of this gene, and the efficient secretion of the corresponding peptide [93]. Structural analysis of the transformant, called H9, indicated that the introduced sequence had remained intact and was still flanked by $\sim$860 bp 5′ upstream of its TSS, and then was likely to retain its own putative promoter. The amount of cryptogein expressed in H9 and in *P. cryptogea* grown under identical conditions were similar. Consequently, the cryptogein gene is flanked by a region that is likely to contain all the elements that constitute its promoter. This promoter is functional and is expressed to the same extent within the two genomic environments. On another hand, the overall amounts of elicitins are similar in H9 and in a *P. infestans* isolate which was transformed with a gene that confers antibiotic resistance. Thus it appears that due to a defined, optimal amount of elicitin production, the expression of the alien cryptogein gene led to the lowered expression of the endogenous *inf1* sequence. As a result, the phenotype of H9 during interaction with tobacco plants was similar to that observed with the highly necrotizing *P. cryptogea*, whereas no apparent symptoms of hypersensitive necroses could be detected with the control *P. infestans* transformant. From this analysis, we can conclude that (i) the promoters of elicitin genes are functional, and may be regulated by identical factors in various species, (ii) the overall production of elicitins is constant within a given isolate, whatever the nature of the elicitin and (iii) elicitins from different classes may be substituted without any apparent modifications for the fungus (except the phenotypic behaviour on tobacco), and therefore may play a common role within *Phytophthora* [93].

## Elicitin gene expression in plant-*Phytophthora* interactions

Soon after their characterization, elicitins were compared with other small, cysteine-rich proteins that would play a role in plant-fungi interactions, such as avirulence gene products and hydrophobins [94]. The first observations indicated that isolates which produced elicitins were nonpathogenic on tobacco, whereas the strains virulent on tobacco did not produced parasiticein [18]. As elicitins are specifically active on *Nicotiana* spp. [18], it was likely that the absence of elicitin secretion was a basis for pathogenicity to tobacco, and elicitins, from their ability to induce both HR and SAR on tobacco, would therefore be considered virulence factors. Unlike other plant-fungus interactions which involve the recognition of race-specific elicitors [95], elicitins, if acting as avirulence factors, would behave like species-specific elicitors. However, virulent, nonproducing isolates were shown to retain elicitin genes [42, 44], which organization, similar to that occurring in nonpathogenic strains, impedes distinction between virulent and avirulent strains [44, 46]. Later, some *P. parasitica* isolates from Australia were shown to produce parasiticein, but were still virulent on tobacco, although to a lesser extent than nonproducing strains [20]. Analysis of a worldwide collection of *P. parasitica* isolates, mostly from tobacco growing areas, revealed the occurrence in South America as well as in Africa of some strains that were highly virulent on tobacco but still producing parasiticein, at least in vitro [96]. If elicitins actually act as avirulence factors on tobacco, the diverse situations imply that the development of virulence would be the outcome of several distinct mechanisms among *P. parasitica*, such as potential repression of elicitin gene expression among virulent, producing isolates. The expression of elicitin genes was thus evaluated in various types of interactions.

Incompatible interactions have been poorly studied because the lack of fungal development is a technical limitation for accurate transcriptional analysis. Nevertheless, elicitin production has been investigated by immunological detection of cryptogein in the leaves and stems of decapitated tobacco plants inoculated with a

mycelial plug of *P. cryptogea* [21]. However, it was not possible to determine whether it reflected actual transcription of the elicitin gene or the release of a previously synthesized peptide. The role of elicitins in incompatible interactions was also studied using an indirect approach. Indeed, histological analyses indicated that *P. infestans* induced symptoms suggesting an HR response when inoculated on *N. benthamiana*, a solanaceous species which is known to be deficient in displaying defense response to viral pathogens [56]. When inoculated by antisense transformants of *P. infestans* which no longer produced INF1, this particular *Nicotiana* species in some cases exhibited symptoms of disease, related to those that occur in the compatible interaction of *P. infestans* with potato. From these experiments, the authors concluded that 'the recognition of elicitin is a major determinant of the resistance response of *N. benthamiana* to *P. infestans*', and that 'elicitins are avirulence factors that condition resistance at the species level' [56]. It should be noted that *P. infestans* is restricted to solanaceous plants and mainly pathogenic on potato and tomato [76]. Thus we may speculate that *P. infestans* possesses additional virulence factors that are balanced by elicitins in interaction with atypical *N. benthamiana*, and opened out in INF 1-deficient transformants. Whatever the relevance of these results, they do not reflect the wide majority of interactions between *P. infestans* and other solanaceous plants, and therefore *Phytopthora* spp. and the diversity of host plants. Hence, the phenotype of INF 1-deficient strains is not modified when inoculated on other *Nicotiana* species, or on potato [56]. In the same context, *P. infestans* transformants that expressed $\beta$-cryptogein in addition to INF1 were not altered in their virulence against potato or tomato [P. Birch, personal communication]. Nevertheless, the results obtained in the INF 1-deficient strains-*N. benthamiana* interaction are relevant enough to offer a promising innovative field of research on the role of elicitins as determinants of host resistance.

Immunological methods have also been used to analyze elicitin production during various compatible interactions [23]. The correlation between the observation of symptoms and the detection of elicitins was rather variable, but clearly demonstrated in the cases of *P. parasitica*-tomato, *P. capsici*-tomato and *P. capsici*-pepper. There, elicitins could be detected 1 or 2 days following the inoculation of plants. Elicitin production was analyzed at the transcriptional level in the *P. infestans*-potato interaction [43]. In this system, expression of *inf1* did not follow the increase of fungal biomass during infection but only occurred in the late phase of fungal invasion, then reached a maximal level and decreased after 5 days following inoculation. This step corresponds to the transition between the hyphal growth and the phase of extensive sporulation. Conversely, expression of actin genes, presented to be constitutive, follows the development of invasion. Moreover, on the basis of the comparison of overall transcription of *inf1* in planta and in vitro, the authors concluded that expression of elicitin genes is downregulated during infection of potato [43]. As indicated below, *P. infestans* is a hemibiotroph, airborne pathogen, whereas most *Phytophthora* species are root, soilborne pathogens. However, several species are able to colonize aboveground tissues following rain splashings [76]. Therefore, such species must be analyzed for the time course of elicitin gene expression during infection in order to determine whether *P. infestans* offers a particular situation or whether it reflects a general regulation pathway for expression of elicitins. Actually, the response of a plant to elicitins, as exemplified by *N. tabacum*, represents an exception in the plant kingdom [97, 98]. It is difficult to consider downregulation of elicitins as an adaptation of *Phytophthora* spp. to evade plant defense responses that could have been triggered by elicitins; thus downregulation of elicitin gene expression may find its source in the events leading to various changes in cell types during infection, rather than direct interaction with the host plant. Finally, it remains to be seen whether downregulation occurs during infection of a plant typically responsive to elicitins, such as tobacco, by virulent strains of *P. parasitica*, which were shown to produce elicitin in vitro [20, 96], or whether specific virulence factors overcome the plant defense responses.

From these observations it may be concluded that elicitins play a special role in tobacco-*Phytophthora* interactions. This role cannot be restricted to a gene-for-gene model, since various situations are encountered, especially within solanaceous plants [20, 57, 96, 98]. Considering that the plant response (reactivity?) to elicitins is an exception in the huge diversity of plant families that are able to be infected by *Phytophthora*, the expression of elicitins during compatible as well as incompatible interactions must be further analyzed. Thus additional determinants involved in the wide diversity of interactions between plants and *Phytophthora* spp. remain to be identified.

## A first approach of the biological properties of elicitins

### Physiological and biochemical effects induced in plants leading to necrosis and SAR development

Cryptogein application on the petiole of excised tobacco leaves induces necroses that are correlated with histological alterations such as rapid chloroplast breakdown and the collapse of cells leading to disorganization of the parenchyma tissue [99]. In addition, treated leaves produce ethylene and accumulate phytoalexins

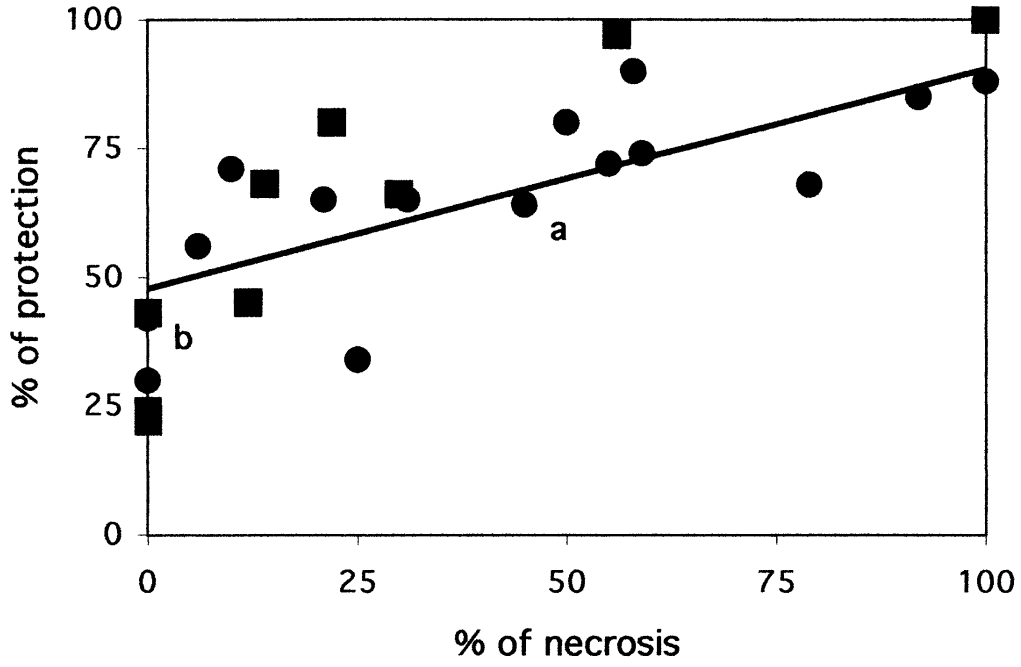


Figure 7. Relationship between necrosis and protection levels induced by mutated cryptogeins on tobacco plants. Each point represents a protein with a single or a multiple mutation. Circles and squares correspond to mutation on Lys or Tyr, respectively. 'a' represents Lys13Val and 'b' replacement of five Lys by polar and neutral amino acids.

such as capsidiol [99]. Elicitin application on the stem of decapitated plants is followed by rapid translocation of the protein in the plant [21, 22] and triggers necrosis development only when $\beta$-elicitins have been used [17]. Tobacco plants subsequently become resistant to further inoculation by pathogens. This protection depends on a complex signaling network, and its level results from the equilibrium between the intensity of plant defense and the rate of pathogen invasion. Better results are obtained when the pathogen inoculum is well quantified, as in *Phytophthora* zoospore infiltrations, than in direct contact of wounded tissues with mycelium. Elicitin-treated tobacco plants that express the bacterial *nahG* gene coding for salicylate hydroxylase do not exhibit SAR, but still respond to treatment with $\beta$-elicitins by intense leaf necrosis. Salicylic acid is clearly involved in SAR and in disease resistance to *Phytophthora*, but these results indicate that it does not mediate the hypersensitive-like necrosis response [100]. Moreover, the establishment of SAR seems to depend on the appearence of extracellular RNase activity, especially upon challenge infection [101], in the same way as in tobacco plants induced to SAR with tobacco mosaic virus [102]. This plant response could be fundamental in SAR, since RNA molecules may carry long-distance signals in plants [103]. In addition, active oxygen species (AOS) mediate a systemic signal network, and $H_2O_2$ orchestrates the plant hypersensitive disease resistance induction [104, 105]. In that way, two distinct sources (intra- and extracellular) of AOS in tobacco plants treated by cryptogein have been reported, and correlated with later cell death [106], although these cell responses could be associated with lipid peroxidation [107]. Cryptogein induces lipid peroxidation in tobacco leaves, evaluated by the accumulation of thiobarbituric acid-reactive substances as well as by high-temperature thermoluminescence emission, both indicating a progressive destabilization of the thylakoid membranes [108]. In addition, lipid peroxidation is closely correlated with the appearence of necrosis [109]. It has been recently demonstrated that the production of fatty acid hydroperoxides depends only on lipoxygenase activities [110]. Finally, expression of defense genes has been studied. Elicitins trigger the coordinate accumulation of transcripts from nine genes which have been previously shown to be expressed during establishment of SAR, $\beta$-elicitins like cryptogein inducing higher response than $\alpha$-ones, like capsicein. These SAR genes are expressed locally corresponding to necrosis formation, and systemically during induction of resistance [111]. Finally, elicitins were also shown to induce a new SAR gene, encoding a $\beta$-subunit of proteasome [L. Suty et al., unpublished data].

**Are necroses and protection related?**

Although all the elicitins put on the stem of decapitated tobacco plants induce a SAR, only $\beta$-elicitin treatment leads to the development of leaf restraint necroses [17]. Thus necrosis does not seem to be essential for the establishment of SAR. This observation has been confirmed by site-directed mutagenesis experiments. Systematic replacement of Lys or of Tyr clearly shows that necroses are not required for protection, but strongly enhance the protection level (fig. 7). The necrotic activity of engineered proteins produced in a bacterial $PT_{7\text{-}7}$ heterologous system and mutated on the six lysines of cryptogein, with cumulative permutations of these amino acids to uncharged residues, clearly demonstrated that (i) all the lysines and not only K13 are important to explain the toxicity of $\beta$-elicitins (fig. 7, spot a) and (ii) four to five lysines must be exchanged to obtain a typical $\alpha$-elicitin as capsicein in terms of necrosis and protection level (fig. 7, spot b) [I. Penot et al., unpublished data]. The single mutant K13V still remained strongly necrogenic, so that the reported results showing that this mutation gave a capsicein-like protein are irrelevant [112]. In fact, these authors assayed the necrotic properties of elicitins by foliar infiltration, a technique inappropriate to measure this activity. All the $\beta$- and $\alpha$-elicitins induce necroses after infiltration with the same subnecrotic threshold concentration ranging from 10 to 20 nM ([97], P. Bonnet unpublished results). Moreover, this mode of application is unable to lead to SAR [97]. As a final demonstration, a gene encoding the $\alpha$-cryptogein B14 [38] was mutated to give V13K and T94K proteins, overproduced in the same system as described above. These proteins were found poorly and highly necrogenic, respectively [A. Marais, unpublished data]. It is obvious that the 'toxicity' of $\beta$-elicitins could not be explained by highlighting single residues, but probably results from complex modifications within the structure of the molecule that lead to different behavior in planta rather than an oversimple protein-protein or protein-ligand complex involving unique sites. The net charge and tyrosine exchanges, which will be discussed further, are part of this multicomponent determinism. In addition, considerations of the importance of residues located in positions 2 (Ala), 13 (Lys), 44 (Thr) and 94 (Lys) resulting from sequence comparison [113, 114] must be used with caution, as these locations could result from a biological shift during evolutive processes. An interesting approach in mapping sites with synthetic peptides deduced from the sequences of capsicein and cryptogein was reported [115]. The experiments were carried out with saturated peptide solution reaching 1 mM with the foliar infiltration method discussed above. In these conditions, $10^4$–$10^5$ higher concentrations of peptides were necessary to mimic the elicitin effect, and when these peptides were infiltrated at lower concentrations, it resulted in a total loss of necrotic activity. Although such differences in mapping strategies were described as usual, the presented results need additional studies to become fully convincing, all the more because they do not clearly show how the different elicitin structural components could act to explain the signaling of the entire protein.

**Responses of tobacco cells to elicitin treatment**

When tobacco cells are treated with cryptogein, their growth is affected, and at 100–200 nM this treatment is lethal [116]. When added at sublethal doses, cryptogein elicits a rapid (few minutes) and strong increase in pH and conductivity of the extracellular medium, followed by cytosolic acidification, without affecting the integrity of the plasma membrane [116–118]. These changes are accompanied by a transient production of AOS, like $H_2O_2$ [109, 117, 119]. Capsicein requires 10-fold higher concentrations than those of cryptogein to induce similar AOS levels [109]. Delayed cell responses were ethylene production (120 min) [120] and, 24–48 h after treatment, induction of lipoxygenase and of proteinase inhibitor activities [119], and phytoalexin accumulation (capsidiol, phytuberin, phytuberol) [120]. This phytoalexin production depends neither on the presence nor on the intensity of the oxidative burst [109]. During the same period of time, changes in total cell lipids have been reported [121]. The most striking changes are an increase in acylated steryl glycosides and steryl esters levels, resulting in part from the glycosylation and/or esterification of free sterols, and in the other part from transient neosynthesis and an increase in the synthesis rate of phosphatidylethanolamine [121].

Cryptogein-treated tobacco cells were also used to describe the early changes in gene expression. The accumulation of mRNAs encoding several known plant proteins was examined by Northern and slot blot hybridizations. The results indicate (i) a significant transitory accumulation of mRNA encoding plasma membrane $H^+$-ATPase, (ii) a fast and strong accumulation of mRNA encoding 3-hydroxy-3-methylglutaryl coenzyme A reductase and (iii) a slow accumulation of mRNA encoding phenylalanine ammonia lyase and pathogenesis-related protein PRb1 [122]. Differential display of mRNA was used to isolate partial length cDNAs corresponding to genes differentially expressed early during elicitation of tobacco cells with cryptogein. These cDNAs were cloned and sequenced. The first hour of elicitation showed (i) a high accumulation of mRNAs hybridizable with cDNAs having sequence homologies with phenylalanine ammonia-lyase, $Ca^{2+}$-ATPase and lipoxygenase encoding genes, and (ii) a decrease of mRNA hybridizable with one cDNA having

sequence homology with another LOX-encoding gene. Five other differentially displayed cDNAs showed no significant homologies with known genes [123]. A combination of mRNA differential display (DDRT-PCR) and 5′-rapid amplification of cDNA ends (5′-RACE) allowed the isolation of full-length cDNAs corresponding to genes activated within 60 min. Cloning and sequencing two cDNAs led to the identification of open reading frames (ORFs) showing significant homologies with the coding sequence of $\beta$-type proteasome subunit and of a transformer-2-like serine/arginine-rich (SR) ribonucleoprotein [124]. The accumulation kinetics of mRNAs indicated transcriptional activation of the corresponding genes not only in cells but also in tobacco plants treated with cryptogein.

Proteasomes are multicatalytic complexes which catalyze the degradation of many rate-limiting enzymes, transcriptional regulators, critical regulatory proteins and highly abnormal proteins. They are involved in plant responses to environmental stresses (cold or high temperature, abscisic acid treatment, drought or salt stress), in cell death, in senescence or wounding processes. Whether the proteasome complex could play a role in the induction of oxidative burst, of cell death or of defense reactions triggered by cryptogein still remains to be studied. Using gene walking by PCR, the 5′ flanking region of the $\beta$-type proteasome subunit has been cloned and sequenced. Sequence analysis in the PLACE data bank allowed the characterization of regulatory sequences, especially mybcore and mybst1 boxes, that could be responsible for regulation by salicylic acid. Effectively, upregulation of the $\beta$-type proteasome subunit by salicylic acid in tobacco cells has been observed [L. Suty et al., unpublished data]. This upregulation was confirmed using *nahG* transgenic plants. $H_2O_2$ was also shown to upregulate the $\beta$-type proteasome subunit, and altogether these results suggest that this $\beta$-type proteasome subunit is a new marker of SAR [L. Suty et al., unpublished data].

SR proteins comprise a family of evolutionarily conserved pre-mRNA splicing factors. Transformer-2 like proteins play an important role in the alternative splicing of pre-mRNA, and SR proteins are among the first components that interact with pre-mRNA. Very little is known about SR protein functions in plants, but in comparison to those present in animals, the relative amount of each SR protein contributes to the regulation of gene expression. Identification of the target pre-mRNA could be useful to evaluate the importance of such SR proteins in elicitin signaling.

All the responses described above were likely to depend on elicitin recognition by specific high-affinity binding sites [116] and by protein phosphorylation events [125].

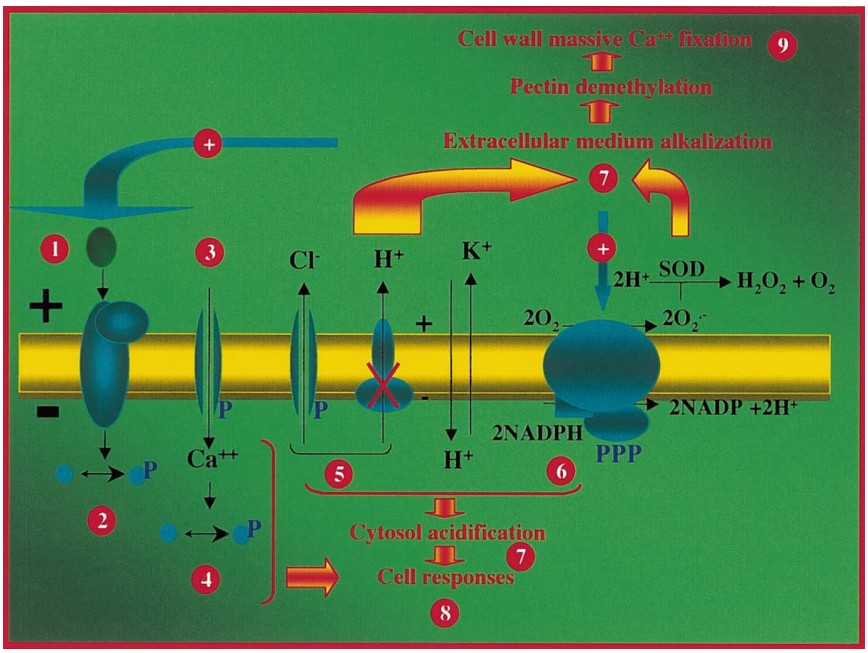


Figure 8. Elicitin signaling pathways (nine steps). Elicitin is represented by the green ellipse. From the left, the different plasmalemma proteins involved are the putative receptor (two subunits, a 160-kDa and a 50-kDa protein), a calcium channel, a chloride channel, $H^+$-ATPase (inhibited) and NADPH oxidase. The signs + and − indicate the transmembrane potential. The protein phosphorylation steps are indicated by the blue 'P'. Orange arrows show the systems which create the changes in pH. The blue arrows indicate the positive feedback effects of the extracellular medium alkalization, and the numbers 1–9 indicate the events in their chronological order.

**Signaling pathways involved in elicitin cell responses**

A specific binding of elicitin to high-affinity sites was first described at the cell level [116]. Further experiments showed that the cryptogein binding sites were located on the plasma membranes, which were purified from cell suspensions or tobacco plants [126]. The binding is saturable, reversible, specific with an apparent $K_d$ of 2 nM (well correlated with concentrations required for biological activities in vivo), and with a very low number of sites (about 100–200 fmol/mg plasmalemma proteins), suggesting that these sites could be the biological receptors for elicitins [126] (fig. 8). Early tobacco cell responses (changes in extracellular pH and in ionic fluxes, AOS production) have been used to investigate the possible desensitization of cells by successive cryptogein treatments. After a first treatment, tobacco cells still respond weakly to a second application of cryptogein or of other elicitors such as oligogalacturonides, suggesting that elicitins induce a desensitization process which corresponds to some forms of heterologous desensitization. It indicates that exposing tobacco cells to elicitins attenuates the response due to other signals operating through distinct receptors [127].

These receptors are postulated to be glycoproteins, since plasma membranes treated with proteases and N-glycosidase F are not able to bind cryptogein [128]. The molecular mass of the elicitin receptors has been tentatively approached by cross-linking experiments that indicate two possible complexes with molecular masses of $172 \pm 15$ kDa and $60 \pm 4$ kDa, respectively, and thus the molecular mass of the cross-linked glycoproteins would be about 160 and 50 kDa [128]. This is in good accordance with the functional molecular mass of the cryptogein binding sites ($193 \pm 9$ kDa) determined by radiation-inactivation experiments [128]. Finally, all the elicitins tested are able to bind to the same sites (with a similar affinity), suggesting that elicitins are recognized by the same receptors, although they induce differential cell and plant responses [129]. These apparently contradictory observations remain to be explained and will be discussed further.

The earliest event of the elicitin signal transduction pathway is a protein phosphorylation/dephosphorylation cascade, since all biological effects were blocked by protein kinase inhibitors, such as staurosporine or $K_{252a}$ [118, 125] (fig. 8). This signaling involves SIP [130] and MAP kinases [131], probably at multiple steps of the signaling pathway. It leads to a huge $Ca^{2+}$ uptake [132], since this cation reaches an apparent intracellular concentration of 200 μM after 30 min, which could be responsible for the high cryptogein toxicity [128, 129, 133]. Since EGTA, which chelates extracellular calcium, or lanthanum, which blocks calcium entry, suppresses the downstream responses, it is obvious that the calcium entry triggers the other cryptogein-induced responses; however, the calcium amounts involved in the signal transduction need to be precisely reevaluated.

First of all, calcium uptake is not transient, and calcium accumulation is detected only 5 min after elicitin treatment and increases during the following 90 min [132]. However, changes in extracellular pH or in AOS production are observed almost immediately after elicitin addition [116, 129]. Thus it must be concluded that the high calcium concentrations observed in these experiments do not correspond to a signal transduction phenomenon and that the use of $^{45}Ca^{2+}$ is not relevant for this purpose. On the contrary, using $Ca^{2+}$-specific electrodes, a rapid and transient calcium uptake (restoration of the original level over 2 min), involving very low concentrations, has been reported in radish protoplasts treated with elicitins [134]. Finally, we recently observed that cryptogein induces a rapid and strong demethylation of cell wall pectins, which could result from the activation of apoplastic pectin-esterase activity via the alkalization of the extracellular medium. Electronic microscopy observations of these cryptogein-treated tobacco cells reveal that calcium probes are mainly located in the cell wall and that $Ca^{2+}$ ions are associated with demethylated pectins [F. Kieffer et al., unpublished data] (fig. 8). These results explain the dual role played by calcium during the elicitation of tobacco cells by elicitins: (i) a strong second messenger with weak and transient uptake in the inner cell, and (ii) formation of a calcium-pectate gel leading to the reinforcement of the cell walls (fig. 8).

Depending on the calcium signaling, other ions fluxes are also modified. Cryptogein induces a $K^+$ efflux (probably associated with a proton influx) [125] and an efflux of $Cl^-$ [135], this later triggering a large plasma membrane depolarization from $-153 \pm 15$mV to $-36 \pm 21$ mV [118]. This depolarization occurs in less than 1 min, after a lag period of about 5 min [135]. The plasma membrane depolarization could be the result of different additional causes: (i) the electron transfer through the plasma membrane, mediated by an NADPH oxidase (see AOS production), (ii) the $Cl^-$ efflux and, above all, (iii) inhibition of the plasma membrane $H^+$-ATPase. This later evidence is supported by indirect observations. For example, plasma membrane depolarization and cytosolic acidification should activate the $H^+$-ATPase, leading to a rapid decrease in the intracellular ATP pool, which is not observed [118]. This is also supported by cryptogein effect reversion with fusicoccin, a well-known activator of $H^+$-ATPase [116, 117], according to similar observations reported for tomato cells treated with systemin [136]. At the same time, a strong and rapid alkalization

of the extracellular medium and a concomitant acidification of the cytosol are observed [116–118]. A few minutes later, a transient oxidative burst is noticed [109, 117]. The nature of AOS has been investigated. Cryptogein elicits an extracellular production of $O_2^{\cdot -}$ on tobacco cells which is then dismutated in $H_2O_2$ by extracellular superoxide dismutases [C. Rustérucci et al., unpublished data]. The extracellular production of $O_2^{\cdot -}$ results from the activation of an NADPH oxidase [118] which seems regulated by a small G protein such as Rac2, in a manner different from that of neutrophils [137]. In order to describe the molecular composition of the plant NADPH oxidase complex and to assess its regulatory mechanisms, we have developed an approach using a double hybrid method, first with the gp91 subunit we have recently cloned, and second with the neutrophil Rho-GDI factor which interacts with the regulator proteins Rac, as bait proteins [T. Elmayan et al. and F. Plas-Simon et al., unpublished data].

Another interesting question concerns the origin of the changes in extracellular and cytosolic pH reported [116–118]. It was suggested that these pH changes result from superoxide dismutase activity [118, 128, 133, 138]. In that case, inhibition of the production of $O_2^{\cdot -}$ using diphenyleneiodonium (DPI), which blocks the activity of the NADPH oxidase, or Tiron, which scavenges $O_2^{\cdot -}$, should restrict these changes in pH. But DPI and Tiron abolished AOS production without any effect on extracellular alkalization [117] according to other reported results, whatever the plant cell/elicitor interaction studied [139]. However, a precise observation of extracellular alkalization during the time course of the oxidative burst proves that the contribution of the superoxide anion dismutation to pH changes is very low (about 3% at its maximum level), whereas addition of exogenous superoxide dismutase only increases this response about 6% [Blein et al., unpublished data]. On the other hand, we previously reported different arguments leading to the conclusion that the depolarization of the plasma membrane mainly results from the $H^+$-ATPase inhibition [116–118, 128, 138]. Thus, it is obvious that the changes in pH mainly result from plasma membrane $H^+$-ATPase inhibition. Furthermore, changes in pH have been shown to modulate the intensity of AOS production by elicited cells, and the possible regulation of the NADPH oxidase activity of plant cells by modifications of pH has been proposed [117]. In this way, cytoplasmic acidification has been reported to be involved in the complex network of cell signaling leading to defense gene activation in tobacco [140], rice [141] and Californian poppy cells [142]. A hypothetical signaling scheme which summarizes the pathways involved in the early responses of tobacco cells treated with elicitin is proposed (fig. 8).

## Agronomic interest

### Durability and effectiveness of elicitin-induced SAR against plant pathogens

The SAR induced by three proteins (cryptogein and two $\alpha$-elicitins, capsicein and parasiticein) was reported to be quite efficient toward several *P. parasitica* aggressive strains on tobacco. High protection was obtained for at least 2 weeks after elicitin treatment [98]. Moreover, this resistance was not organ-specific, since it occurred in stems, leaves and roots, whatever the locus of elicitin application. Thus induced resistance triggered by these proteins was not transient and could be used in plant protection strategies. In addition, this SAR was demonstrated to be effective against other tobacco phytopathogenic fungi: *Sclerotinia sclerotium*, *Botrytis cinerea*, *Rhizoctonia solani*, *Erysiphe cichoracearum* and *Peronospora tabacina* [98, 143]. Concerning the polyphagic and highly pathogenic *S. sclerotium*, *R. solani* and *B. cinerea*, protection was observed on both stem and leaves. Some preliminary experiments showed that elicitins were also able to induce unambiguous resistance against the phytopathogenic bacteria *Pseudomonas cichorii* and *Erwinia chrysanthemi* in a tobacco stem challenge. Elicitins are therefore powerful elicitors of long term and aspecific protection of tobacco plants toward a wide variety of pathogens.

### Plant specificity in elicitin-induced HR and SAR

The range of botanical species able to react in exhibiting HR and (or) SAR after elicitin application was thoroughly investigated. HR and SAR were readily induced in all the species belonging to the three sections of *Nicotiana* genus. However, the intensity of induced responses was both cultivar- and species-dependent. In contrast, none of the other *Solanaceae* tested developed necrotic symptoms after elicitin infiltration or application on the petiole of detached leaves. Experiments achieved on bell pepper, tomato and *Petunia hybrida* with different amounts of elicitins suggested that neither HR nor SAR against *P. parasitica* could be put forward [97, 98]. Nevertheless, among plants belonging to more than 15 botanical families, including monocots and dicots, only some members of *Brassicaceae* were found to develop necroses in a cultivar-specific manner: some cultivars of *Raphanus* sativus and rape (*Brassica napus*) responded by foliar necrosis after elicitin treatment, often in a dose-dependent symptom intensity. The symptoms on detached leaves varied from yellowing at low elicitin quantities ($< 0.1$ nmol per leaf) to total leaf water soaking then turning brown with black punctuations at higher amounts ($> 1$ nmol per leaf) (Bonnet, unpublished data, [134]). There was a real differential response to elicitin among radish and rape cultivars

Table 2. $^{125}$I-cryptogein binding characteristics to plant plasma membranes [J.-P. Blein et al., unpublished data].

| | N fmol/mg protein | $K_d$ nM |
|---|---|---|
| | reactive plants | |
| *N. tabacum* | 101 ± 7 | 8.8 ± 1.9 |
| *B. napus* var: | | |
| yudal | 190 ± 15 | 5.7 ± 0.2 |
| liberator | 898 ± 107 | 13.5 ± 2.4 |
| lirabon | 251 ± 48 | 10.3 ± 2.1 |
| cobra | 292 ± 47 | 7.7 ± 1.4 |
| | unreactive plants | |
| *L. esculentum* | 148 ± 17 | 7.3 ± 1.3 |
| *A. thaliana* | 210 ± 23 | 10.2 ± 1.4 |
| *A. pseudoplatanus* | 203 ± 15 | 6.1 ± 1.5 |
| *B. napus* var: | | |
| darmor nain | 100 ± 3 | 3.1 ± 0.2 |
| jet 9 | 845 ± 18 | 22.5 ± 1.2 |
| bolko | 448 ± 29 | 4.0 ± 1.7 |
| shogun | 1012 ± 275 | 5.3 ± 1.7 |

which was never observed in *Nicotiana* spp. In addition, it was demonstrated that reactive radish varieties, when treated with elicitins, became resistant to the phytopathogenic bacteria *Xanthomonas campestris* pv *armoraciae* [97]. Some other cruciferous species were also reported to develop HR, with the exception of *Arabidopsis thaliana* ecotypes. A genetic approach of reactivity using rape cultivars is in progress in order to clarify the level of complexity of the mechanisms governing elicitin recognition and signaling. The results obtained on Brassicaceae, showing degrees in symptom severity ranging from null type to complete wilting and necrosis with intermediate behavior (yellowing or senescence), suggest that a continuum could exist in reactivity to elicitin. All the experiments achieved on different plants of various botanical origins were carried out with the attempt to describe HR. Whether this reaction is an obligatory mechanism in the general elicitin mode of action remains unknown. Thus it will be necessary to reassess plant reactivity not only with symptom description but also at the molecular level to evaluate stimulation of plant defense and stress pathways. However, the elicitin receptor, until now characterized as high-affinity binding sites, is present on all the plant plasma membranes assayed (table 2) and cannot be considered to be the specific support of plant reactivity; the cell wall has been proposed to play a role [83] (fig. 9). Elicitin effects on reactive and nonreactive plant cells (tobacco and tomato, respectively) have been compared. This plant cell wall/elicitin interaction is slight in tobacco and leads to an equilibrated distribution of elicitins between cells and their extracellular medium, whereas it is very strong in tomato cells. In the latter case, the extracellular applied elicitin rapidly becomes almost undetectable. Since elicitins could be desorbed from cell walls by salt buffers, these proteins are probably trapped in these structures through ionic bonds. The tomato cell walls behave as a filter which prevents elicitins from accessing the plasma membranes. However, high elicitin concentrations are able to saturate this barrier and trigger tomato cell responses, evidencing at least that the receptors involved in elicitin signaling pathways are functional in tomato. An approach showing that the cell wall components are involved in plant reactivity is being developed [M. L. Milat et al., unpublished data].

**Biotechnological implications**

The ability of elicitins to trigger plant protection toward phytopathogenic microorganisms could be used by introducing artificial resistance in plants of agronomic interest. But the high toxicity of such proteins combined with their apparent plant specificity (see above) could be limiting factors; however, the introduction of elicitin genes in reactive tobacco was achieved following two strategies. The first one, using uncontrolled expression of a synthetic gene encoding cryptogein under the strong 35S promoter led surprisingly to viable transgenic plants in both homo- and hemizygous states [144]. This could be due to the intracellular localization of the protein, which consequently was unable to interact with the outside specific site located on its plasmalemma putative receptor. Inoculation of transformed lines with *P. parasitica* aggressive strains led to a low level of resistance compared with the control. This resistance was not clear, since the inoculated plants were almost ruined by the pathogen, and the protection was estimated as the number of leaves on axillary shoots emerging from the low, still living stump of stems.

The second strategy [145] was to control foreign gene expression and to address the protein to the apoplastic space, which allowed the elicitin to bind to its putative receptor located on the outer plasma membrane [126]. Controlling gene expression is a guarantee for limiting cell death in the appropriate area where plant and pathogen interact. A promoter with a weak constitutive expression threshold but strongly inducible by pathogen attack was chosen to govern the expression of the natural gene encoding cryptogein [38]. The signal peptide of the extracellular PR1a was added to the construction between the promoter and the cryptogein gene to allow protein secretion in the apoplast. Transformed lines were obtained and screened for their ability to restrain *P. parasitica* spreading. After backcrosses, stabilized $F_2$ lines were used to evaluate their pathogenic phenotype. These transgenic lines reacted to zoospore foliar infiltration in an HR manner and were shown to be highly resistant. This resistance appeared in any organ of the plant and was efficient against several pathogens, including fungi, viruses and nematodes.

The biotechnological use of elicitor of resistance was improved. In the particular case of elicitins, a general use for crop protection still remains hazardous, since the mechanisms leading to reactivity and SAR or LAR (local acquired resistance) set up have to be elucidated in most plants of agronomic interest.

### What is the elicitin biological function?

Some phytopathogenic fungi within *Phytophthora* species are unable to synthesize sterols and therefore must pick them up from the membranes of their host plant, using an unknown mechanism. These pseudo-fungi secrete elicitins which are small hydrophilic cysteine-rich proteins, harboring a sterol carrier activity.

### Sterol carrier activity of elicitins

All these proteins interact with dehydroergosterol (DHE) in the same way, but with some time-dependent differences [59, 83]. Elicitins have one binding site with a similar strong affinity for DHE. Using a nonsteroid hydrophobic fluorescent probe, it was shown that phytosterols are able to similarly bind to elicitins. Moreover, elicitins catalyze sterol transfer between phospholipidic artificial membranes [59, 83]. In addition, these polypeptides are also able to trap sterols from biological membranes (plant cell suspensions or purified plasma membranes) and to transfer DHE from liposomes to isolated plasmalemma vesicles [84]. These results afford the first evidence for a molecular activity of elicitins, which appears to be an extracellular sterol carrier function. This property should contribute to an understanding of the molecular mechanism involved in sterol uptake by *Phytophthora*. It opens new perspectives concerning the role of such proteins in plant-microorganism interactions.

### The 3D structure of a cryptogein-ergosterol complex

Recently, the 3D structure of a K13H engineered cryptogein containing an ergosterol molecule in its hydro-

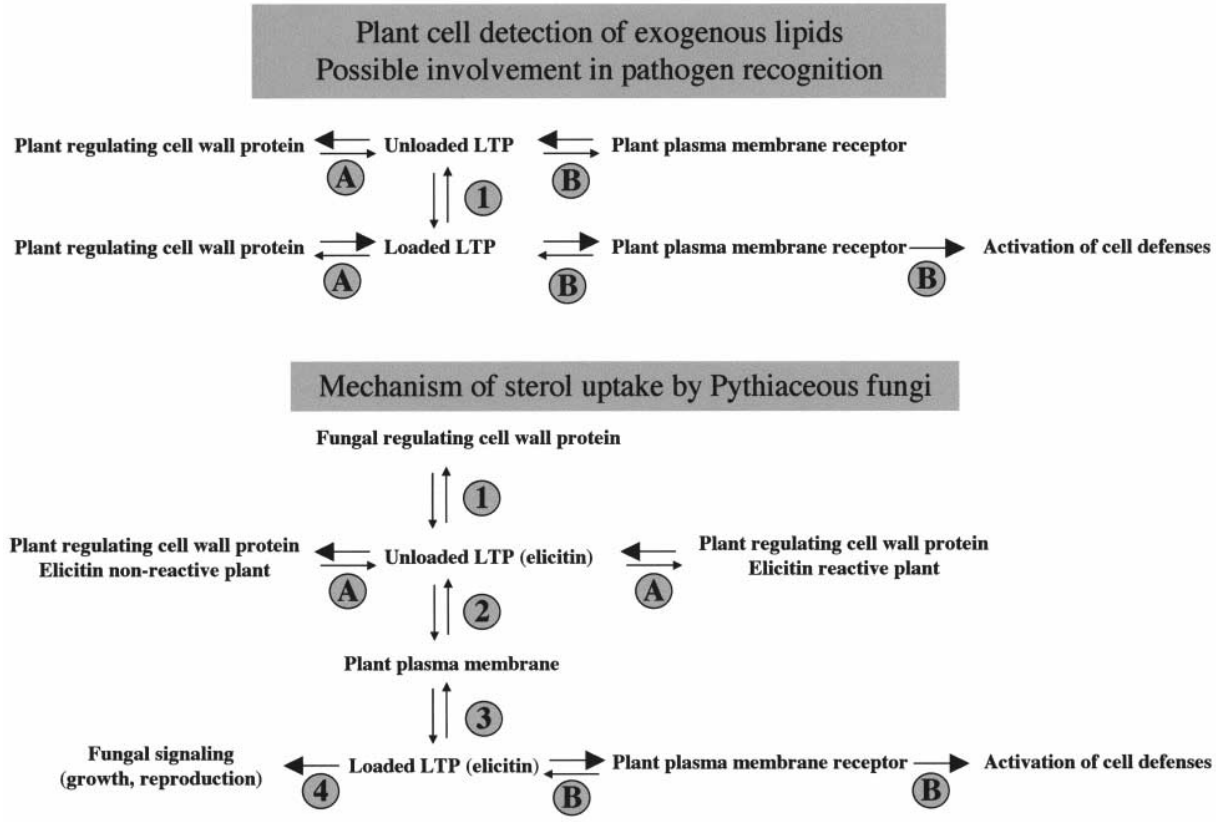


Figure 9. Hypothetical support of plant reactivity/nonreactivity to elicitins. The numbered steps (1–4) represent LTP activation through protein loading with sterols. *A* and *B* are relative to LTP interactions with cell wall-regulating proteins and plasma membrane receptors, respectively. Unloaded LTPs are presumed to exhibit a high or a low affinity for these two types of plant proteins, respectively, in contrast to loaded LTPs.

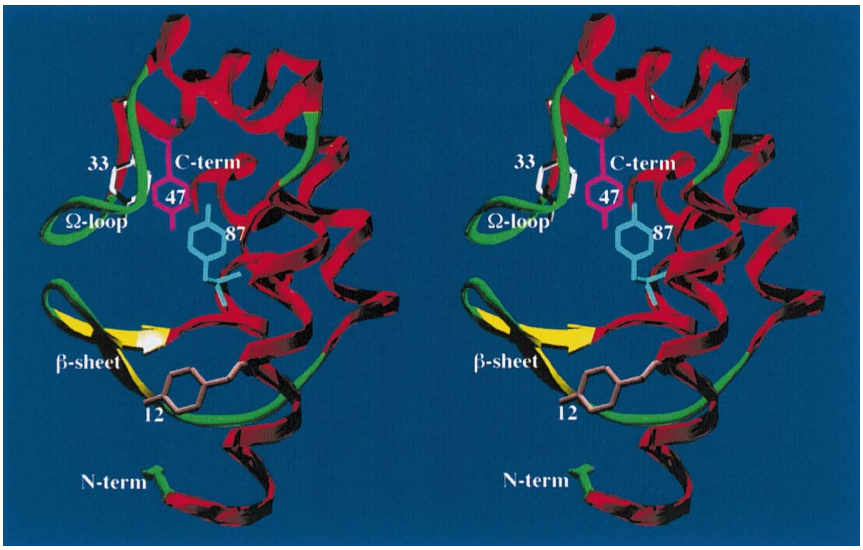


Figure 10. Ribbon diagram of the cryptogein unloaded/loaded with an ergosterol molecule, showing the location of the mutated Tyr residues. It represents the native cryptogein (PDB file 1beo) and the K13H (PDB file 1bxm). Figures were generated using the program Swiss-Pdb Viewer ver. 3.5b1 (http://www.expasy.ch/spdbv/mainpage.htm, and [166, 167]), the image renderer Quick Time ver. 3 (Apple Computer) and the ray tracer POV-Ray ver. 3.1 (http://www.povray.org/).

phobic core was presented [146]. This protein was obtained by overexpression of a synthetic gene encoding cryptogein [112] in the *Pichia pastoris* heterologous system [147]. The purified and crystallized mutated protein was found to contain a well-defined electron density in its cavity which was identified as ergosterol, the major sterol of yeasts [146]. The presence of a sterol in the mutated cryptogein resulted in slight but important structural changes compared with the native form of the protein previously resolved as crystal [33] and solution [34] structures. These changes concern first some hydrophobic amino acids of the core which were rejected to increase the cavity size, especially Tyr 87, which appears buried in native structure and rotates to be solvent exposed when the sterol is present (fig. 10). Second, a bending of helix $\alpha_1$ was also reported. Ergosterol seemed to be stabilized in the cryptogein pocket by a hydrogen bond between the phenolic function of Tyr47 and the $\beta$-hydroxyl of the sterol, as well as with 28 van der Waals interactions between the sterol rings and side chain and 16 hydrophobic residues of the protein core [146]. These results confirm the biophysical demonstration of the sterol carrier activities of elicitins [59, 83, 84].

### Relationship between sterol carrier and biological activities

The link between the two functions of elicitins was assessed using a site-directed mutagenesis strategy. Tyrosine residues (fig. 10), previously suspected to be involved in a protein-ligand complex [146], were methodically replaced, and mutation effects were tested for sterol carrier properties as well as for biological activities on tobacco cells and plants. These mutations resulted in a decrease of all the assayed activities. Moreover, strong correlations could be established between sterol carrier ability and biological functions, and between the rate of elicitins loaded with sterols and their capability to bind specific high-affinity proteins, located on the plasmalemma. These observations can be extended to all the natural elicitins assayed, indicating that the biological activity of these proteins depends on their ability to load sterols [J.-P. Blein et al., unpublished data]. These results indicate that the formation of a sterol-elicitin complex is a requisite step before elicitin binds to high-affinity proteins, which thus constitute their biological receptors. Consequently, this complex formation is the first event involved in elicitin-plant cell interactions [J.-P. Blein et al., unpublished data]. The characteristics of the binding curve kinetics highlighted a cooperative phenomenon during the interaction between elicitins and their putative receptors, which suggests a receptor organization model. First, the elicitin receptor must reflect a multimeric organization (allosteric regulation), in which each monomer could be the 200-kDa complex previously described [128]. The elicitin binding to the receptor triggers an allosteric change of its subunits, probably associated with a phosphorylation event [J.-P. Blein et al., unpublished data]. Second, the calcium signaling in tobacco cells treated

with elicitins shows the following characteristics: (i) protein phosphorylation is required [132, 134], (ii) verapamil and nifedipine, which block voltage-dependent calcium channels in plant cells [148], had no effect on $Ca^{2+}$ influx, indicating that if calcium channels are involved in cryptogein-induced influx, they are not of the voltage-gated type but probably of ligand-dependent type [132], (iii) transient $Ca^{2+}$ uptake can be induced by four sequential elicitin additions [134] and (iv) the mutated cryptogein (Tyr87-Phe) provokes a decrease of the spontaneous $Ca^{2+}$ exchanges in tobacco cells [J.-P. Blein et al., unpublished data]. Taking into account these results, we propose that the elicitin receptor could be a ligand-dependent calcium channel comprising a quadrimeric complex as shown in figure 11, which summarized the initial molecular events involving activation of elicitin by sterol loading that drive elicitor function.

## Sterols in oomycete physiology

The dependence toward sterol among the Oomycetes still remains debated. Some of them could synthesize these molecules, and *Achlya ambisexualis*, for example, uses them as precursors of sexual hormones involved in the formation of either oogonia (oogoniol) or antheridia (antheridiol) [149]. In contrast, numerous Oomycetes belonging to *Pythiaceae* and Lagenidiales are unable to use squalene for the biosynthesis of the steroid nucleus [150]. Thus these microorganisms are completely devoid of sterol equipment. To what extent they truly need these molecules is an open question. For several decades it was considered that the pythiaceous *Pythium* and *Phytophthora* spp. as well as the mosquito parasitizing *Lagenidium giganteum* require sterols for efficient growth and for sexual and asexual reproduction [151–153]. In fact, this is only partially true. It is obvious that sterols provided in artificial growing conditions trigger the formation of reproductive organs in both homo- and heterothallic mycetes. But a lack of sterol supply never affects the fungal growth of *P. cactorum* [M. Ponchet et al., unpublished data]. Stimulation of reproduction organ formation could be obtained by bringing phospholipids to *P. cactorum* [154, 155] or to *Pythium aphanidermatum* [156], even with synthetic compounds [M. Ponchet et al., unpublished data]. This later result excludes that the biological activity of phospholipids results from their contamination by sterols, in contrast to previous conclusions [157]. It was also reported that unsaturated fatty acids as well as their triglycerides were good inducers of reproduction in *P. cinnamomi* [158] and in both *P. cactorum* and *P. parasitica* [159]. In addition, other lipidic compounds such as phytol, a degradation product of chlorophylls, was found to stimulate the reproduction of *P. cactorum*. Concerning the potent structural requirement for sterols in pythiaceous membranes, it was suggested that these compounds could be replaced by triterpenoids [160] such as phytophthorol [161] which are synthesized by these microorganisms and mimic sterol as far as structural and biochemical features are concerned.

In conclusion, sterols constitute powerful signaling components for *Pythiaceae* and Lagenidiales, but are not necessarily required in the physiology of these Oomycetes. According to this conclusion, what is the interest for *Phytophthora* and *Pythium* to secrete high amounts (high energy cost) of different proteins (high genetic diversity) able to transport lipophilic compounds that are not essential for their spreading and dissemination? First of all, this argumentation is built from in vitro observations and cannot prefigure reality during the parasitism of these Oomycetes. For example, the level of elicitin biosynthesis in planta is unknown, even though it was reported that INF1 mRNA was downregulated in potato during the early stages of *P. infestans* colonization [43] and during host pathogen confrontations. Are these proteins free shuttles, as is suggested from biophysical experiments together with abundant secretion in liquid cultures? More probably, these elicitins are sequestered in plant cell walls (fig. 9) or flattened between plant and *Phytophthora* mem-

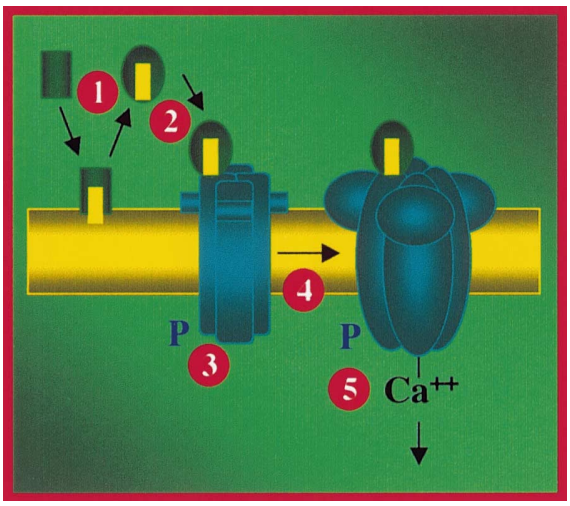


Figure 11. First events in elicitin signaling. The receptor of elicitins, located on the plant plasma membranes, is presumed to be a calcium channel, comprising four basal subunits (a 160-kDa and a 50-kDa protein), each of them able to specifically bind an elicitin molecule (as shown in fig. 8). The first elicitin-channel interaction needs a loaded elicitin from plant plasmamembrane sterols and triggers a conformational change of the channel, probably associated with the phosphorylation of the subunit bound to elicitin. This conformational modification allows the binding of other loaded/unloaded elicitin molecules to the receptor, and then to trigger biological responses only when this elicitin is loaded.

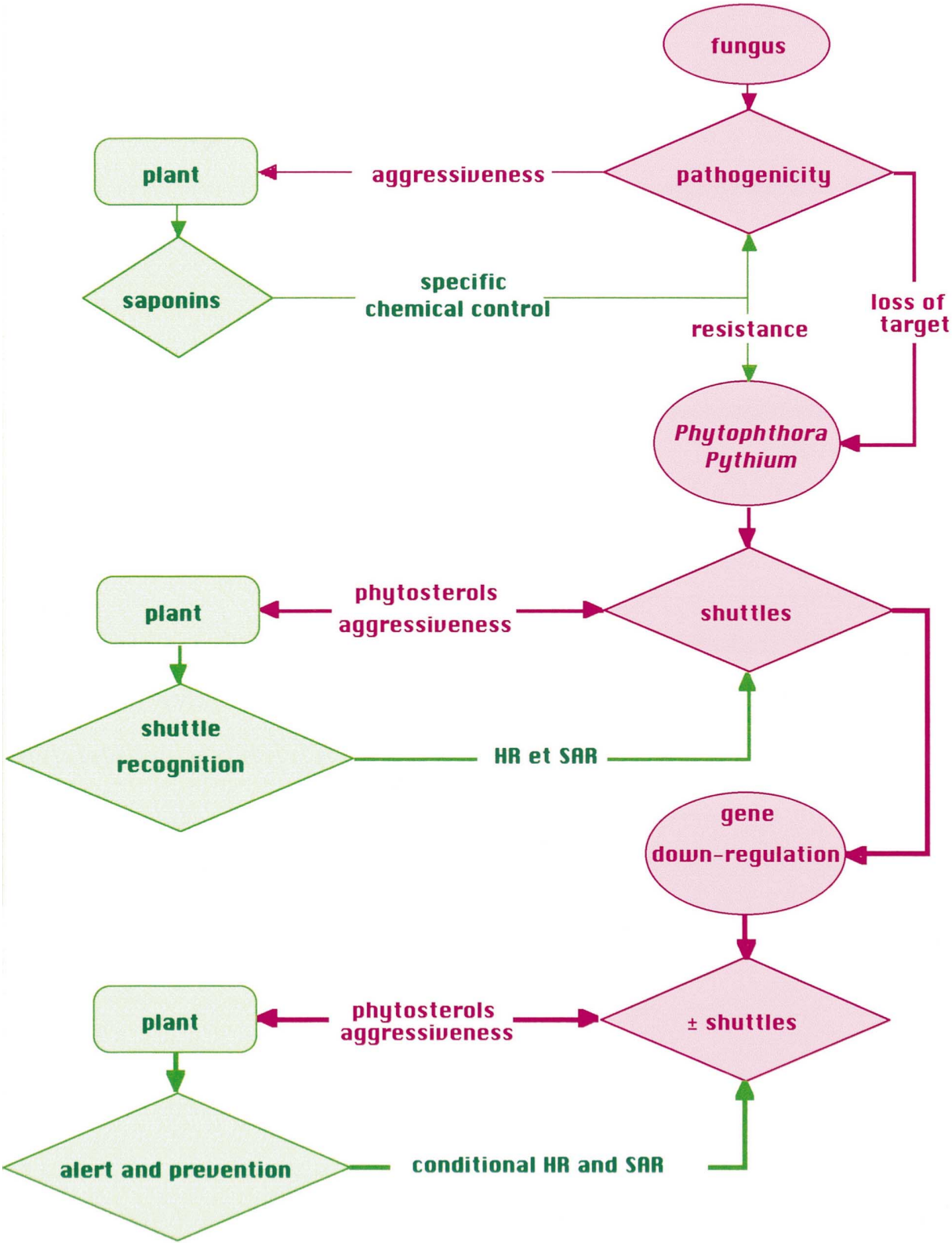


Figure 12. Possible coevolution scheme between plants and *Phytophthora*. Green and pink illustrations are relative to the hypothetical plant and fungal evolutions, respectively.

branes in haustoria or other functionally related structures during plant cell predation. In the latter case, elicitins cannot be viewed anymore as random shuttles. But in every scheme a question remains: why pick up sterols or other lipidic compounds that are not essential from a trophic point of view? An attractive hypothesis is that these proteins are distributed in the oomycete environment to gather foreign lipidic compounds that, by random return to the mycelium, inform the microorganism on the presence and (or) abundance of potential host. Are elicitins sensors for *Phytophthora*? In that way, a more general approach including other interactions, such as the mycoparasitism of *Pythium oligandrum* toward *Fusarium oxysporum* pathogen on tomato [24, 162] is in progress. This particular *Pythium* secretes an elicitin-like protein (oligandrin) able to carry sterols. Thus, this protein was presumed to pick up ergosterol from *F. oxysporum* (involvement in mycoparasitism?); then, during hyperparasitism in planta, oligandrin could interact with the plant system devoted to ergosterol detection [163], as proposed in figure 9.

As a matter of fact, the elicitins analyzed from the sterol point of view appear obviously as components of the virulence of both *Phytophthora* and *Pythium*. Thus the interaction between elicitins and tobacco is the exception in which a general virulence factor is recognized by the host cell and perfectly illustrates host pathogen coevolution.

Recent advances in the knowledge of tobacco/*Phytophthora* interactions reported in this review suggest a hypothetical coevolution scheme of this relationship (fig. 12). Plants have developed several resistance mechanisms, among them the saponin synthesis, which could play an essential role in plant-fungi interactions [164]. These compounds interact with the fungal sterol, and some Oomycetes such as *Phytophthora* and *Pythium* could have short cut this plant aggressiveness by repressing their sterol biosynthesis. Thus they became resistant to saponins and were again able to invade saponin-producing plants. However, they had to pick sterols up from their environment. Then they developed shuttle proteins like elicitins, and the plant invasion could continue. However, although it is now impossible to say which, plant or fungus, has mimicked the other, these elicitins should be homologous with plant proteins involved in in planta signaling, for example, in ergosterol detection, since this fungal sterol induces plant defense mechanisms (fig. 9 [163]). Consequently, after sterol loading, elicitins could be recognized by the warning system of the plant and then trigger a hypersensitive reaction associated with development of SAR. Moreover, although elicitin secretion in planta is poorly documented, downregulation of elicitin production has been reported [43]. Finally, both protagonists still have time to improve their own strategies, and the challenge can continue.

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