Involvement of lipid-protein complexes in plant-microorganism interactions

Oléagineux, Corps Gras, Lipides. Volume 9, Numéro 1, 31-6, Janvier - Février 2002, Dossier : Lipides des plantes

Auteur(s) : Jean-Pierre BLEIN, UMR 692, Laboratoire de phytopharmacie et de biochimie des interactions cellulaires, Inra, BP 86510, 21065 Dijon Cedex, France.

Summary : Increasing concerns about the environmental impact of modern agricultural have prompted research for alternate practices to pesticide treatments, notably using plant defense mechanisms. Thus, isolation and characterization of plant defense elicitors have been the main step of studies in many groups. Moreover, in the global concept of interactions between organisms and their environment, a major concern is to discriminate recognition between exogenous and endogenous signals, notably during pathogenic or allergenic interactions involving small proteins, such as elicitins or lipid transfer proteins (LTPs). Elicitins and lipid transfer proteins (LTP) are both able to load and transfer lipidic molecules and share some structural and functional properties. While elicitins are known as elicitors of plant defense mechanisms, the biological function of LTPs is still an enigma. They are ubiquitous plant proteins able to load and transfer hydrophobic molecules such as fatty acids or phospholipids. Among them, LTPs1 (type 1 lipid transfer proteins) constitute a multigenic family of secreted plant lipid binding proteins that are constitutively expressed in specific tissues and/or induced in response to biotic and abiotic stress (for reviews [1-4]). Their biological function is still unknown, even if some data provide arguments for a role of these proteins in the assembly of extracellular hydrophobic polymers (i.e., cutin and suberin) [2, 4] and/or in plant defense against fungal pathogens [1, 3]. Beside their involvement in plant defense, LTPs1 are also known to be pan-allergens of plant-derived foods [5]. Finally, the discovery of the sterol carrier-properties of elicitins has opened new perspectives dealing with the relationship between this function and the elicitor activity of these small cystein-rich proteins. Nevertheless, this elicitor activity is restrained to few plant species, and thus does not appear in accordance with a universal lipid transfer function. These considerations required a reassessment of the precise role of elicitins for both fungi and plants [6].

Résumé : La stimulation des mécanismes naturels de défense des plantes représente une stratégie alternative à l'utilisation de la lutte chimique en phytoprotection. Dans ce contexte, les élicitines, qui sont de petites protéines sécrétées par des Phytophthora et des Pythium, induisent une réaction de type hypersensible chez des plantes comme le tabac. Ces plantes deviennent résistantes à l'agression de leurs agents pathogènes. La récente découverte que les élicitines sont des protéines de transfert de stérols a apporté une vue nouvelle sur l'activité moléculaire des élicitines. Élicitines et protéines de transfert de lipides (LTP) sont capables de charger et de transporter des molécules lipidiques et partagent des caractères communs tant au plan structural que fonctionnel mais, tandis que les élicitines sont connues pour leur activité élicitrice de réaction de défense chez les plantes, l'activité biologique des LTP reste inconnue.

Cependant, les LTP se fixent sur le récepteur des élicitines et bloquent les réponses cellulaires résultant d'un traitement avec des élicitines. De plus, nous avons montré que la forme biologiquement active des élicitines est un complexe élicitine-stérol. Seules les élicitines chargées avec un stérol sont capables de se lier à leur récepteur membranaire qui est, par ailleurs, probablement un canal calcique. Ces résultats illustrent bien le rôle joué par les interactions lipides-protéines dans la signalisation cellulaire liée, par exemple, aux mécanismes de défense des plantes.

Mots-clés : élicitine, signalisation cellulaire, interaction protéine-lipide, LTP.

ARTICLE

Tobacco-*Phytophthora* interaction. Elicitins induce plant defense responses (for review see [6])

In tobacco fields, it was shown that necroses on plants were associated with the presence of *Phytophthora,* which are non-pathogenic [7]. Then, from culture filtrates of *P. cryptogea* and of *P. capsici* proteinaceous elicitors named elicitins (cryptogein and capsicein, respectively) were isolated [8]. These proteins stimulate natural defenses of tobacco against many pathogens, and this phenomenon is accompanied by the formation of restricted leaf necroses [9-11]. The development of this hypersensitive cell death involves the lipoxygenase-dependent production of fatty acid hydroperoxides [12]. Using cryptogein antibodies, it was shown that this elicitin can migrate through the plant and can be responsible for the systemic acquired resistance induced in tobacco [13-15]. A possible extension towards other plants could be offered with another family of the oomycete class, the *Pythium* which can induce protection of tomato against *Fusarium oxysporum* f. sp. *radicis-lycopersici* [16-19] and can also secrete elicitin-like proteins [18].

Responses of tobacco cells to elicitin treatment

When added in sub-lethal doses, cryptogein elicits a rapid (few minutes) and strong increase in pH and in conductivity of the extracellular medium, followed by a cytosolic acidification, without affecting the integrity of the plasma membrane [20-22]. These changes are accompanied by a transient production of AOS, like H_2O_2 [22-24]. Then, delayed cell responses are: ethylene production (120 min) [25], and 24-48hrs after treatment, induction of lipoxygenase activities [12, 23, 26] and of proteinase- inhibitor activities [24], phytoalexins accumulation [25]. During the same period of time, changes in total cell lipids have been reported [27]. Cryptogein-treated tobacco cells were also used to describe the early changes in gene expression [28-32]. All the responses described above are likely to depend on elicitin recognition by specific high affinity binding sites [20], and by protein phosphorylation events [33].

Cell signalling

Elicitin receptors

A specific binding of elicitin to high affinity sites was firstly described at the cell level [20]. Further experiments using either tobacco cell suspensions or plants showed that the cryptogein binding sites are located on the plasma membranes [34]. The binding is saturable, reversible, specific with an

apparent Kd of 2nM (well correlated with concentrations required for biological activities *in vivo*), and with a very low number of sites (about 100-200 fmoles/mg plasmalemma proteins), suggesting that these sites could be the biological receptors for elicitins [34].

These receptors are postulated to be glycoproteins since plasma membranes treated with proteases and N-glycosidase F are not able to bind cryptogein anymore [35, 36]. The molecular mass of the elicitin receptors has been tentatively approached by cross-link and radiation-inactivation experiments. They indicate a functional molecular mass of 193 ± 9kDa for the cryptogein binding sites [35, 36].

Phosphorylation events and calcium signalling

The earliest event of the elicitin signal transduction pathway is a protein phosphorylation/dephosphorylation cascade, since all biological effects are blocked by protein kinase inhibitors like staurosporine or K_{252a} [33]. This signalling involves SIP [37] and MAP kinases [38] probably at multiple steps of the signalling pathway. It leads to a huge Ca^{++} uptake [39], this cation reaches an apparent intracellular concentration of 200muM after 30 min, which could be responsible for the high cryptogein toxicity [38, 40, 41]. Obviously, the calcium entry triggers the other cryptogein-induced responses, since EGTA that chelates extracellular calcium, or lanthanum which blocks calcium entry, suppresses the downstream responses. However, the calcium amounts involved in the signal transduction have to be further reevaluated. Firstly, the calcium uptake kinetics is not transient and, the calcium accumulation is detected only 5 min after elicitin treatment and then increases during the following 90 min [39]. Secondly, changes in extracellular pH or in AOS production are observed almost just after elicitin addition [20, 21]. Therefore, 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 $^{45}Ca^{++}$ is not relevant for this purpose. On the contrary, using Ca^{++} specific electrodes, a rapid and transient calcium uptake (restoration of the original level over 2 min), involving very low concentrations, hads been reported in radish protoplasts treated with elicitins [42]. Finally, we recently observed that cryptogein induces a rapid and strong demethylation of cell wall pectins, which could result from the stimulation of apoplastic pectin-esterase activity *via* the alkalization of the extracellular medium [43]. Electronic microscopy observations of these cryptogein-treated tobacco cells, point out that calcium probes are mainly located in the cell wall, and that Ca^{++} ions are associated with demethylated pectins [43]. These results explain the dual role played by calcium during the elicitation of tobacco cells by elicitins: (i) strong second messenger with weak and transient uptake in the inner cell; (ii) high amounts sequestered in cell walls.

Ionic fluxes

Depending on the calcium signalling, other ion fluxes are also modified. Cryptogein induces a K^+ efflux (probably associated with a proton influx) [33] and an efflux of Cl^- [44], the latter triggering a large plasma membrane depolarization from -153±15mV to -36±21mV [21]. This depolarization occurs in less than 1 min, after a lag period of about 5min [44]. The plasma membrane depolarization could result from different additional causes: (i) the electron transfer through the plasma membrane, mediated by a NADPH oxidase; (ii) the Cl^- efflux; and most of all (iii) the inhibition of the plasma membrane H^+-ATPase. This 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 [21]. This is also supported by

cryptogein-effect reversion with fusicoccin, a well-known activator of H^+-ATPase [20, 22], according to similar observations reported from tomato cells treated with systemin [45]. At the same time, a strong and rapid alkalization of the extracellular medium and a concomitant acidification of the cytosol are observed [20, 22]. Few minutes later, a transient oxidative burst is noticed [22, 23]. Cryptogein elicits an extracellular production of O_2^- on tobacco cells which is then dismutated in H_2O_2 by extracellular superoxide dismutases. The extracellular production of O_2^- results from the activation of a NADPH oxidase which was cloned [46] and which seems to be regulated by small G protein-like Rac2, in a manner different from those of animal neutrophils [47, 48]. *Figure 1* presents the chronological steps of the elicitin induced cell responses.

Biological function of elicitins

Sterol carrier activity of elicitins

The interaction between elicitins and sterols has been investigated by fluorometry using dehydroergosterol (DHE). All the elicitins interact with DHE in the same way, but with some time-dependent differences. Elicitins have one binding site with a similar strong affinity for this sterol. Then, using a non-steroid hydrophobic fluorescent probe, we showed that phytosterols are similarly able bind to elicitins. Moreover, elicitins catalyze sterol transfer between phospholipidic artificial membranes [49, 50]. They are also able to trap sterols from biological membranes (plant cell suspensions or purified plasma membranes) and to transfer sterols from liposomes to isolated plasmalemma vesicles [51]. In addition, elicitins bind fatty acids and phospholipids. The stoichiometry of the complex is 1:1. Fatty acids and sterols compete for the same site, but elicitin affinity is lower for fatty acids than for sterols. We showed that C7 to C12 saturated and C16 to C22 unsaturated fatty acids are the best ligands. The presence of double bonds markedly increases the affinity of cryptogein for fatty acids [52]. These results afforded the first evidence for a molecular activity of elicitins, *i.e.* they are extracellular sterol carrier proteins. This property should contribute to understand the molecular mechanism involved in sterol uptake by *Phytophthora*. It opens new perspectives concerning the role of such proteins in plant-microorganism interactions, since elicitins trigger defense reactions in plants, as reported above.

The 3D structure of a cryptogein-ergosterol complex

The 3-dimensional structure of a K13H engineered cryptogein containing an ergosterol molecule in its hydrophobic core was obtained [53]. The presence of a sterol in the mutated cryptogein induces slight but important structural changes compared with the native form of the protein previously resolved as crystal [54] and solution [55] structures. These changes concern firstly some hydrophobic amino acids of the core which move to increase the cavity size, especially Tyr 87, that appears buried in the native structure and rotates to be solvent exposed when the sterol is present *(Figure 2)*. Secondly, a bending of helix alpha1 was also reported. Ergosterol seemsed to be stabilized in the cryptogein pocket by a hydrogen bond between the phenolic function of Tyr47 and the beta-hydroxyl of the sterol *(Figure 2)*, as well as with 28 Van der Waals interactions between the sterol rings and side-chain and 16 hydrophobic residues of the protein core [53]. This elicitin-ligand structure is in accordance with the biophysical demonstration of the sterol carrier activities of elicitins and the stoechiometry of the complex.

Relationship between sterol carrier and biological activities of elicitins

The link between the two functions of elicitins was assessed using a site-directed mutagenesis strategy. Tyrosine residues, previously suspected to be involved in protein-ligand complex [53], were replaced, and mutation effects were tested for sterol carrier properties, as well as for biological activities on tobacco cells and plants. These mutations result in the decrease of all the assayed activities. Moreover, strong correlations have been established between sterol affinity and biological activities, and between the rate of elicitins loading with sterols and their capability to bind specific high affinity proteins, located on plasmalemma [56]. The characteristics of the binding kinetics of the mutated elicitins to their putative receptors pointed out to a cooperative phenomenon. These results indicate that the formation of a sterol-elicitin complex is a prerequisite step before elicitin binds to high affinity proteins, which thus constitute their biological receptors. Consequently, this complex formation is the first event involved in the elicitin-plant cell interaction [56] and it. It leads to propose a receptor-organization model *(Figure 3)*.

Firstly, the elicitin receptor must reflect a multimeric organization (cooperative phenomenon), in which each monomer could be the 200kDa complex previously described [35, 36]. The elicitin binding to the receptor triggers an allosteric change of its subunits, probably associated with a phosphorylation event [56].

Secondly, the calcium signalling in tobacco cells treated with elicitins, shows the following characteristics: (i) transient Ca^{++} uptake can be induced by four sequential elicitin additions [42]; (ii) verapamil and nifedipine, which block voltage-dependent calcium channels in plant cells [57], had no effect on Ca^{++} influx, indicating that if calcium channels are involved in cryptogein-induced influx, they are not of voltage-gated type but probably of ligand-dependent type [39]; (iii) the mutated cryptogein (Tyr87-Phe) provokes a decrease in the spontaneous Ca^{++} exchanges in tobacco cells [56]; (iv) protein phosphorylation is required [39, 42].

Thirdly, a kinetic analysis of the elicitin binding curves, using the allosteric model of Monod [58], confirms that these receptors could be represented by an allosteric model corresponding to an oligomeric structure with four identical subunits [59]. Taking these results into account, we proposed that the elicitin receptor could be a ligand-dependent calcium channel constituted of a quadrimeric complex as shown in *Figure 3*, which summarizes the initial molecular events involving activation of elicitin by sterol loading that drive the elicitor function [56, 59].

Finally, all the elicitins tested are able to bind to the same sites (with a similar affinity), suggesting that they are recognized by the same receptors, although they induce differential cell and plant responses [40]. These apparently contradictory observations remained to be explained. The first elicitin-receptor interaction needs a sterol loading of elicitin from plant plasma membrane that induces a conformational change of the receptor subunits. This conformational modification allows the binding of other loaded/unloaded elicitin molecules to the receptor. But only loaded elicitins can trigger biological responses [56].

LTPs bind with the elicitin receptors

Cryptogein and LTP1 bind to high affinity specific sites located on the plasma membranes of tobacco and the saturation level of these sites is similar for both proteins [59]. Displacement experiments demonstrate that the specific binding sites for LTP1 and cryptogein are identical, and that the LTP1-interaction with the binding sites is reversible [59], as for cryptogein [34]. Thus, LTP1 binding sites exhibit all the characteristics of putative receptors. Finally, the effects of LTP1 on tobacco cells were analyzed. The addition of increasing concentrations of LTP1 reduced the production of active oxygen species, induced by a fixed cryptogein concentration [59]. This result indicates that the binding sites of LTP1 and cryptogein are their true biological receptors. The difference we observed in protein biological activity can be explained by their efficiency to induce the conformational changes of the receptor subunits. LTPs1 could also act as elicitin antagonists. However, although the formation of a sterol-elicitin complex is a requisite step in elicitin recognition by receptors [56], we still have no indication on the importance of lipid-LTP complexes formation for binding to specific sites and/or for triggering cell responses. Moreover, the nature of extracellular or membrane putative ligands for LTP1 remains to be elucidated, since these proteins do not capture phytosterols as elicitins do.

In addition, although the cryptogein binding curves could be analyzed as hyperbolic curves [34, 36, 40], that of wheat LTP1 presented a sigmoidal shape. This shape suggests that the LTP binding site is oligomeric, the molecular interaction involving positive cooperativity. This could correspond to an allosteric model with a transition from a conformer with little or no affinity for the ligand to a conformer exhibiting high affinity for the ligand. This model was used to describe the interaction of LTP1 and plasmalemma binding sites. In order to determine binding parameters, we tested the model set up by Monod *et al.* [58]. The model with four subunits fits well with the experimental values for both, cryptogein and LTP1 [59]. The apparent binding constants are very similar but the allosteric constants are very different, showing that cryptogein is more efficient than LTP in changing the binding protein conformation towards the active conformer.

Finally, these results address a major question about the structural motifs common to these protein families and involved in their recognition. Similarities in the topology of helices displayed by cryptogein and wheat LTP1 have been observed and could explain their similar affinity and competitiveness for the membrane receptor.

LTPs1 are ubiquitous in the plant kingdom. In the same way, LTP or elicitin receptors were found in all plants assayed [6, 36], although most of them do not develop a hypersensitive reaction after elicitin treatment [6]. It suggests that these receptors could be associated with a general mechanism involving LTP in a warning system able to detect exogenous organisms. Moreover, since elicitins trigger a hypersensitive reaction leading to the release of different mediators and molecules from cells, in a way comparable with that observed in severe allergy [60], it would be interesting to study if pan-allergen LTPs1 of plant-derived foods could interact with animal-specific receptors and if these receptors belong to the same family as that found in plants. Recognition by such receptors could be a first step in the cascade of metabolic pathways originating the allergenic response to plant LTPs1. It should stimulate further investigations towards the evolutionary relationships between the hypersensitive reactions in both allergy and plant defense responses.

Sterols in oomycete physiology

The dependence towards sterols among the oomycetes still remains debated. Some of these fungi can synthesize these molecules, for example *Achlya ambisexualis* uses them as precursors of sexual hormones involved in the formation of either oogonia (oogoniol) or antheridia (antheridiol) [61]. On the contrary, numerous oomycetes belonging to *Pythiaceae* and *Lagenidiales* are unable to use squalene for the biosynthesis of the steroid skeleton [62]. So, these fungi are completely devoid of sterol equipment. To what extent they really need these molecules is an open question. For several decades it has been considered that the pythiaceous *Pythium* and *Phytophthora* spp as well as the mosquito parasitizing *Lagenidium giganteum* require sterols for an efficient growth and for sexual or asexual reproduction [63-65]. This is 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 does not prevent the fungal growth of *P. cactorum*. Stimulation of reproduction-organ formation could be obtained by bringing phospholipids to *P. cactorum* [66, 67] or to *Pythium aphanidermatum* [68], even with synthetic compounds, which avoids traces of sterol contaminations as was proposed to explain phospholipids activity [69]. It was also reported that unsaturated fatty acids as well as their triglycerides are good inducers of reproduction in *P. cinnamomi* [70] and in both *P. cactorum* and *P. parasitica* [71]. In addition, other lipidic compounds like phytol, a degradation product of chlorophylls, were found to stimulate the reproduction of *P. cactorum*. Concerning the potent structural requirement for sterols in fungal membranes, it was suggested that these compounds could be replaced by triterpenoids [72] like phytophthorol [73] which are synthesized by these fungi and mimic sterol as far as structural and biochemical features are concerned.

As a conclusion, sterols constitute powerful signaling components for *Pythiaceae* and *Lagenidiales*, but are not necessarily required in the physiology of these fungi. According to this conclusion, one wonders 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 could not prefigure the reality during the parasitism of these fungi. In compatible interactions, elicitin genes expressed *in vitro* by certain tobacco isolates of *Phytophthora* are down-regulated, for example in potato during the early stages of *P. infestans* colonization [74] and during host pathogen confrontations, or in tobacco during *P. parasitica* invasion [75]. However, one elicitin-producing *P. parasitica* isolate that is pathogenic on tomato and avirulent on tobacco still expressed *parA1* (elicitin gene) during the compatible interaction [75]. This data illustrates the molecular dialogue between plants and *Phytophthora*, leading to the down-regulation or expression of elicitin genes.

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 or flattened between plant and fungal membranes in haustoria or other functionally-related structures during plant cell predation. In the latter case, elicitins cannot be viewed as random shuttles anymore. 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 fungal environment to gather foreign lipidic compounds that, by random return to the mycelium, inform the fungus about the presence and (or) abundance of

potential host. Are elicitins sensors for *Phytophthora*? In that way, a more general approach including other interactions, like the mycoparasitism of *Pythium oligandrum* towards *Fusarium oxysporum* pathogen on tomato [16, 18, 76], is in progress. This particular *Pythium* secretes an elicitin-like protein (oligandrin) able to carry sterols. Thus, this protein is presumed to pick up ergosterol from *F. oxysporum* (involvement in mycoparasitism?) and then, during hyperparasitism *in planta*, oligandrin could interact with the plant system devoted to ergosterol detection [77] as proposed above. As a matter of fact, the elicitins analyzed from the sterol point of view appear as components of the virulence of both *Phytophthora* and *Pythium*. So, the interaction between elicitins and tobacco is the exception in which a general virulence factor is recognized by the host cell and so perfectly illustrates host pathogen co-evolution [6].

Finally, the recent demonstration that elicitins and LTPs share the same biological receptors opens interesting speculations. The interactions between elicitins or LTPs and biotic or abiotic lipidic compounds should bring new surprising results which could be used in phytoprotection (see patents [78-80]).

CONCLUSION

Acknowledgements

This review is mainly a compilation of the following published papers [6, 56, 59]. The authors of these publications are thanked for their contribution.

REFERENCES

1. GARCIA-OLMEDO F, MOLINA A, SEGURA A, MORENO M (1995). The defensive role of nonspecific lipid-transfer proteins in plants. *Trends Microbiol*, 3: 72-4.

2. KADER JC (1996). Lipid-transfer proteins in plants. *Ann Rev Plant Physiol Plant Mol Biol*, 47: 627-54.

3. BROEKAERT WF, CAMMUE BPA, DE BOLLE MFC, THEVISSEN K, DE SAMBLANX GW, OSBORN RW (1997). Antimicrobial peptides in plants. *Crit Rev Plant Sci*, 16: 297-323.

4. DOULIEZ JP, MICHON T, ELMORJANI K, MARION D (2000). Structure, biological and technological functions of lipid transfer proteins and indolines, the major lipid binding proteins from cereal kernels. *J Cereal Sci*, 32: 1-20.

5. ASERO R, *et al.* (2000). Lipid transfer protein: a pan-allergen in plant-derived foods that is highly resistant to pepsin digestion. *Int Arch Allergy Immunol*, 122: 20-32.

6. PONCHET M, *et al.* (1999). Are elicitins cryptograms in plant-fungi communication? A review of the plant and cell responses to elicitin treatments and analysis of signaling involved. *Cell Mol Life Sci*, 56: 1020-47.

7. CSINOS A, HENDRIX JW (1977). Nonparasitic stunting of tobacco plants by *Phytophthora cryptogea. Can J Bot*, 55: 1156-62.

8. BONNET P, POUPET A, BRUNETEAU M (1985). Toxicité vis-à-vis du tabac des fractions purifiées d'un filtrat de culture de *Phytophthora cryptogea* Pethyb. & Laff. *Agronomie*, 5: 275-82.

9. BONNET P (1985). Réactions différentielles du tabac à 9 espèces de *Phytophthora. Agronomie*, 5: 801-8.

10. BONNET P (1988). Purification de divers filtrats de culture de *Phytophthora* et activités biologiques sur le tabac des différentes fractions. *Agronomie*, 8: 347-50.

11. RICCI P, *et al.* (1989). Structure and activity of proteins from pathogenic fungi *Phytophthora* eliciting necrosis and acquired resistance in tobacco. *Eur J Biochem*, 183: 555-63.

12. RUSTÉRUCCI C, *et al.* (1999). Involvement of lipoxygenase dependent production of fatty acid hydroperoxides in the development of the hypersensitive reaction induced by cryptogein on tobacco leaves. *J Biol Chem*, 274: 36446-55.

13. DEVERGNE JC, BONNET P, PANABIERES F, BLEIN JP, RICCI P (1992). Migration of the fungal protein cryptogein within tobacco plants. *Plant Physiol*, 99: 843-7.

14. ZANETTI A, BEAUVAIS F, HUET JC, PERNOLLET JC (1992). Movement of elicitins, necrosis-inducing proteins secreted by *Phytophthora* sp, in tobacco. *Planta*, 187: 163-70.

15. DEVERGNE JC, FORT MA, BONNET P, RICCI P, VERGNET C, DELAUNAY T, GROSCLAUDE J (1994). Immunodetection of elicitins from *Phytophthora* spp using monoclonal antibodies. *Plant Pathol*, 43: 885-96.

16. BENHAMOU N, REY P, CHERIF M, HOCKENHULL J, TIRILLY Y (1997). Treatment with the mycoparasite *Pythium oligandrum* triggers induction of defense-related reactions in tomato roots when challenged with *Fusarium oxysporum* f. sp. *radicis-lycopersici. Phytopathol*, 87: 108-22.

17. WULFF EG, PHAM ATH, CHERIF M, REY P, TIRILLY Y, HOCKENHULL J (1998). Inoculation of cucumber roots with zoospores of mycoparasitic and plant pathogenic *Pythium* species: differential zoospore accumulation, colonization ability and plant growth response. *Eur J Plant Pathol*, 104: 69-76.

18. PICARD K, PONCHET M, BLEIN JP, REY P, TIRILLY Y, BENHAMOU N (2000). Oligandrin, a proteinaceous molecule produced by the mycoparasite, *Pythium oligandrum*, induces resistance to *Phytophthora parasitica* infection in tomato plants. *Plant Physiol*, 124: 379-95.

19. BENHAMOU N, BELANGER RR, REY P, TIRILLY Y (2001). Oligandrin, the elicitin-like protein produced by the mycoparasite *Pythium oligandrum*, induces systemic resistance to Fusarium crown and root rot in tomato plants. *Plant Physiol Biochem*, 39: 681-96.

20. BLEIN JP, MILAT ML, RICCI P (1991). Responses of cultured tobacco cells to cryptogein, a proteinaceous elicitor from *Phytophthora cryptogea*: possible plasmalemma involvement. *Plant Physiol*, 95: 486-91.

21. PUGIN A, FRACHISSE JM, TAVERNIER E, BLIGNY R, GOUT E, DOUCE R, GUERN J (1997). Early events induced by the elicitor cryptogein in tobacco cells: involvement of a plasma membrane NADPH oxidase and activation of glycolysis and the pentose phosphate pathway. *Plant Cell*, 9: 2077-91.

22. SIMON-PLAS F, RUSTÉRUCCI C, MILAT ML, HUMBERT C, MONTILLET JL, BLEIN JP (1997). Active oxygen species production in tobacco cells elicited by cryptogein. *Plant Cell Environ*, 20: 1573-9.

23. RUSTÉRUCCI C, STALLAERT V, MILAT ML, PUGIN A, RICCI P, BLEIN JP (1996). Relationship between AOS, lipid peroxidation, necrosis and phytoalexin production induced by elicitins in *Nicotiana. Plant Physiol*, 111: 885-91.

24. BOTTIN A, VÉRONÉSI C, PONTIER D, ESQUERRÉ-TUGAYÉ MT, BLEIN JP, RUSTERUCCI C, RICCI P (1994). Differential responses of tobacco cells to elicitors from two *Phytophthora* species. *Plant Physiol Biochem*, 32: 373-8.

25. MILAT ML, RICCI P, BONNET P, BLEIN JP (1991). Capsidiol and ethylene production by tobacco cells in response to cryptogein, an elicitor from *Phytophthora cryptogea. Phytochemistry*, 30: 2171-3.

26. STALLAERT VM, DUCRUET JM, TAVERNIER E, BLEIN JP (1995). Lipid peroxidation in tobacco leaves treated with the elicitor cryptogein: evaluation by high-temperature thermoluminescence emission and chlorophyll fluorescence. *Biochim Biophys Acta*, 1229: 290-5.

27. TAVERNIER E, STALLAERT V, BLEIN JP, PUGIN A (1995). Changes in lipid composition in tobacco cells treated with cryptogein, an elicitor from *Phytophthora cryptogea. Plant Sci*, 104: 117-25.

28. SUTY L, BLEIN JP, RICCI P, PUGIN A (1995). Early changes in gene expression in tobacco cells elicited with cryptogein. *Mol Plant Microbe Interact*, 8: 644-51.

29. SUTY L, PETITOT AS, LECOURIEUX D, BLEIN JP, PUGIN A (1996). Isolation of partial length cDNAs corresponding to early differentially expressed genes during elicitation of tobacco cells by cryptogein: use of differential mRNA display. *Plant Physiol Biochem*, 34: 443-51.

30. PETITOT AS, BLEIN JP, PUGIN A, SUTY L (1997). Cloning of two plant cDNAs encoding a beta-type proteasome subunit and a SR-related protein: early induction of the corresponding genes in tobacco cells treated by cryptogein. *Plant Mol Biol*, 35: 261-9.

31. ETIENNE P, PETITOT AS, HOUOT V, BLEIN JP, SUTY L (2000). Induction of *tcI* 7, a gene encoding a beta-subunit of proteasome, in tobacco plants treated with elicitins, salicylic acid or hydrogen peroxide. *FEBS Lett*, 466: 213-8.

32. DAHAN J, ETIENNE P, PETITOT AS, HOUOT V, BLEIN JP, SUTY L (2001). Cryptogein affects expression of alpha3, alpha6 and beta1 proteasome subunits encoding genes in tobacco. *J Exp Bot*, 52: 1947-8.

33. VIARD MP, MARTIN F, PUGIN A, RICCI P, BLEIN JP (1994). Protein phosphorylation is induced in tobacco cells by the elicitor cryptogein. *Plant Physiol*, 104: 1245-9.

34. WENDEHENNE D, BINET MN, BLEIN JP, RICCI P, PUGIN A (1995). Evidence for specific, high-affinity binding sites for a proteinaceous elicitor in tobacco plasma membrane. *FEBS Lett*, 374: 203-7.

35. LEBRUN-GARCIA A, BOURQUE S, BINET MN, *et al.* (1999). Involvement of plasma membrane proteins in plant defense responses: analysis of the cryptogein signal transduction in tobacco. *Biochimie*, 81: 1-6.

36. BOURQUE S, BINET MN, PONCHET M, PUGIN A, LEBRUN-GARCIA A (1999). Characterization of the cryptogein binding sites on plant plasma membranes. *J Biol Chem*, 274: 34699-705.

37. ZHANG SQ, DU H, KLESSIG DF (1998). Activation of the tobacco SIP kinase by both a cell wall-derived carbohydrate elicitor and purified proteinaceous elicitins from *Phytophthora* spp. *Plant Cell*, 10: 435-49.

38. LEBRUN-GARCIA A, OUAKED F, CHILTZ A, PUGIN A (1998). Activation of MAPK homologues by elicitors in tobacco cells. *Plant J*, 15: 773-81.

39. TAVERNIER E, WENDEHENNE D, BLEIN J-P, PUGIN A (1995). Involvement of free calcium in action of cryptogein, a proteinaceous elicitor of hypersensive reaction in tobacco cells. *Plant Physiol*, 109: 1025-31.

40. BOURQUE S, PONCHET M, BINET MN, RICCI P, PUGIN A, LEBRUN-GARCIA A (1998). Comparison of binding properties and early biological effects of elicitins in tobacco cells. *Plant Physiol*, 118: 1317-26.

41. PUGIN A, GUERN J (1996). Mode of action of elicitors: involvement of plasma membrane functions. *C R Acad Sci [III]*, 319: 1055-61.

42. KEIZER DW, SCHUSTER B, GRANT BR, GAYLER KR (1998). Interactions between elicitins and radish *Raphanus sativus. Planta*, 204: 480-9.

43. KIEFFER F, LHERMINIER J, SIMON-PLAS F, *et al.* (2000). The fungal elicitor cryptogein induces cell wall modifications on tobacco cell suspension. *J Exp Bot*, 51: 1799-811.

44. ZIMMERMANN S, FRACHISSE JM, THOMINE S, BARBIERBRYGOO H, GUERN J (1998). Elicitor-induced chloride efflux and anion channels in tobacco cell suspensions. *Plant Physiol Biochem*, 36: 665-74.

45. SCHALLER A, OECKING C (1999). Modulation of plasma membrane H^+-ATPase activity differentially activates wound and pathogen defense responses in tomato plants. *Plant Cell*, 11: 263-72.

46. PLAS-SIMON F, ELMAYAN T, BLEIN JP (2001). The plasma membrane oxidase Ntrboh is responsible for AOS production in elicited tobacco cells. *Plant J*, submitted.

47. KIEFFER F, SIMON-PLAS F, MAUME B, BLEIN JP (1997). Tobacco cells contain a protein immunologically related to the neutrophil small G protein Rac2 and involved in elicitor-induced oxidative burst. *FEBS Lett*, 403: 149-53.

48. KIEFFER F, ELMAYAN T, SIMON-PLAS F, DAGHER MC, BLEIN JP (2000). A tobacco cDNA encoding a Rac-like protein cloned using the two-hybrid system in an heterologous screen. *Biochimie*, 82: 1099-105.

49. MIKES V, MILAT ML, PONCHET M, RICCI P, BLEIN JP (1997). The fungal elicitor cryptogein is a sterol carrier protein. *FEBS Lett*, 416: 190-2.

50. MIKES V, MILAT ML, PONCHET M, PANABIÈRES F, RICCI P, BLEIN JP (1998). Elicitins excreted by

Phytophthora are a new class of sterol carrier proteins. *Biochem Biophys Res Comm*, 245: 133-9.

51. VAUTHRIN S, MIKES V, MILAT ML, *et al.* (1999). Elicitins trap and transfer sterols from micelles, liposomes and plant plasma membranes. *Biochim Biophys Acta*, 1419: 335-42.

52. OSMAN H, *et al.* (2001). Fatty acids bind to the fungal elicitor cryptogein and compete with sterols. *FEBS Lett*, 489: 55-8.

53. BOISSY G, O'DONOHUE M, GAUDEMER O, PEREZ V, PERNOLLET JC, BRUNIE S (1999). The 2.1 angstrom structure of an elicitin-ergosterol complex: a recent addition to the sterol carrier protein family. *Protein Sci*, 8: 1191-9.

54. BOISSY G, DE LA FORTELLE E, KAHN R, *et al.* (1996). Crystal structure of a fungal elicitor secreted by *Phytophthora cryptogea*, a member of a novel class of plant necrotic proteins. *Structure*, 4: 1429-39.

55. FEFEU S, BOUAZIZ S, HUET JC, PERNOLLET JC, GUITTET E (1997). Three-dimensional solution structure of beta cryptogein, a beta elicitin secreted by a phytopathogenic fungus *Phytophthora cryptogea. Protein Sci*, 6: 2279-84.

56. OSMAN H, *et al.* (2001). Mediation of elicitin activity on tobacco is assumed by elicitin-sterol complexes. *Mol Biol Cell*, 12: 2825-34.

57. PINEIROS M, TESTER M (1995). Characterization of a voltage-dependent Ca^{2+}-selective channel from wheat roots. *Planta*, 195: 478-88.

58. MONOD J, WYMAN J, CHANGEUX JP (1965). On the nature of allosteric transitions: a plausible model. *J Mol Biol*, 12: 88-118.

59. BUHOT N, *et al.* (2001). A lipid transfer protein binds to a receptor involved in the control of plant defense responses. *FEBS Lett* (in press).

60. LEUNG D (1998). Molecular basis of allergic diseases. *Mol Genet Metab*, 63: 157-67.

61. RIEHL RM, TOFT DO (1985). Effect of culture medium composition on pheromone receptor levels in *Achlya ambisexualis. J Steroid Biochem*, 23: 483-9.

62. DOMNAS AJ, BISWAS SS, GALLAGHER PA (1994). Squalene metabolism in two species of *Lagenidium. Can J Microbiol*, 40: 523-31.

63. HENDRIX JW (1970). Sterols in growth and reproduction of fungi. *Ann Rev Phytopathol*, 8: 111-30.

64. DOMNAS AJ, SREBRO JP, HICKS BF (1977). Sterol requirement for zoospore formation in the mosquito parasitizing fungus *Lagenidium giganteum. Mycologia*, 69: 875-86.

65. KERWIN JL, WASHINO RK (1986). Regulation of oosporogenesis by *Lagenidium giganteum:* promotion of sexual reproduction by unsaturated fatty acids and sterol availability. *Can J Microbiol*, 32: 294-300.

66. KO WH (1985). Stimulation of sexual reproduction of *Phytophthora cactorum* by phospholipids. *J*

Gen Microbiol, 131: 2591-4.

67. JEE HJ, TANG CS, KO WH (1997). Stimulation of sexual reproduction in *Phytophthora cactorum* by phospholipids is not due to sterol contamination. *Microbiology*, 143: 1631-8.

68. KO WH (1986). Sexual reproduction of *Pythium alphanidermatum*: stimulation by phospholipids. *Phytopathol*, 76: 1159-60.

69. KERWIN JL, DUDDLES ND (1989). Reassessment of the role of phospholipids in sexual reproduction by sterol-auxotrophic fungi. *J Bacteriol*, 171: 3831-9.

70. ZAKI AI, ZENTMYER GA, SIMS JJ, KEEN NT (1982). Stimulation of sexual reproduction in the A2 mating type of *Phytophthora cinnamomi* by oleic acid and lipids from avocado roots. *Phytopathol*, 73: 199-203.

71. JEE HJ, KO WH (1997). Stimulation of sexual reproduction in *Phytophthora cactorum* and *P-parasitica* by fatty acids and related compounds. *Mycol Res*, 101: 1140-4.

72. NES WD, HEFTMANN E (1981). A comparison of triterpenoids with steroids as membrane components. *J Nat Prod*, 44: 377-400.

73. NES WD (1988). Phytophthorols-novel lipids produced by *Phytophthora cactorum. Lipids*, 23: 9-16.

74. KAMOUN S, VANWEST P, DEJONG AJ, DEGROOT KE, VLEESHOUWERS VGAA, GOVERS F (1997). A gene encoding a protein elicitor of *Phytophthora infestans* is down-regulated during infection of potato. *Mol Plant Microbe Interact*, 10: 13-20.

75. COLAS V, CONROD S, VENARD P, KELLER H, RICCI P, PANABIERES F (2001). Elicitin genes expressed *in vitro* by certain tobacco isolates of *Phytophthora parasitica* are down-regulated during compatible interactions. *Mol Plant Microbe Interaction*, 14: 326-35.

76. REY P, BENHAMOU N, HOCKENHULL J, TIRILLY Y (1997). Possible use of *Pythium oligandrum* in an integrated protection model against *Fusarium oxysporum* f. sp. *radicis-lycopersici* in hydroponic cultivation of tomatoes. *Cryptog Mycol*, 18: 145-6.

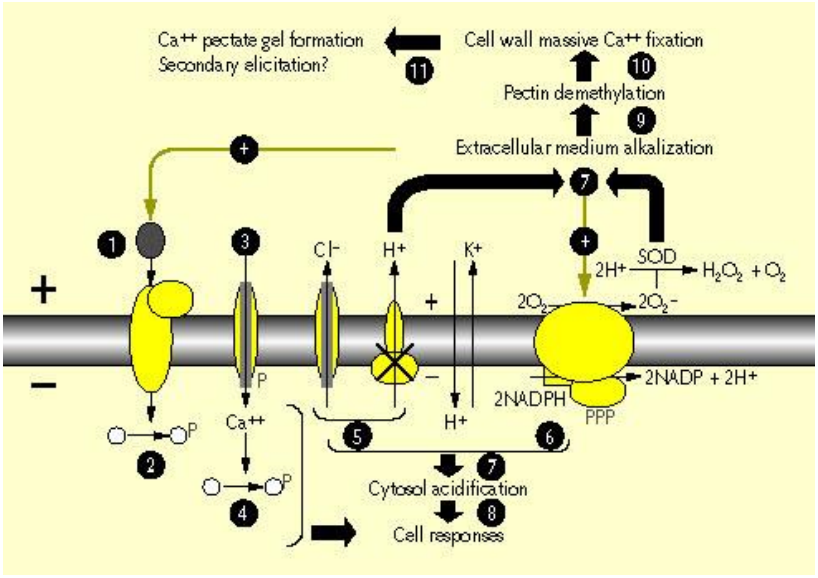
77. GRANADO J, FELIX G, BOLLER T (1995). Perception of fungal sterols in plants - subnanomolar concentrations of ergosterol elicit extracellular alkalinization in tomato cells. *Plant Physiol*, 107: 485-90.

78. MIKES V, BLEIN JP, MILAT ML, PONCHET M, RICCI P (1997). Utilisation d'élicitines pour le transport de lipides. 97 12876, 98 9949059.4 et 09/529,503.

79. BLEIN JP, BOUDON E, MIKES V, MILAT ML, PANABIÈRES F, PONCHET M, TIRILLY Y (1999). Utilisation d'un complexe élicitine-lipide pour la protection des plantes contre les pathogènes. 9916221 et 00403631.5.

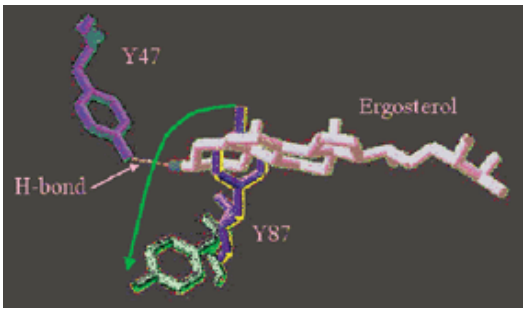
80. DEMIL C, BLEIN JP, SUTY L (2001). Utilisation en phytoprotection d'esters d'acide salicylique et de leurs associations avec des élicitines. 01 08611.

Illustrations



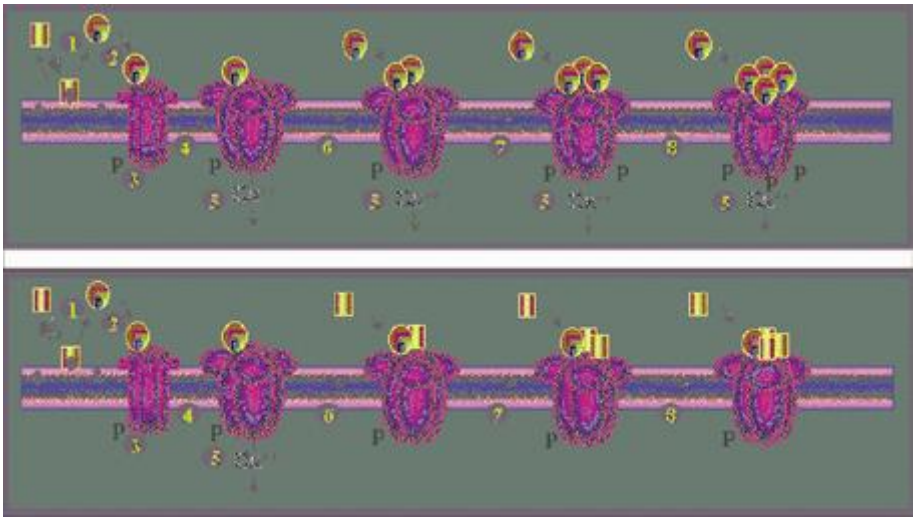
Elicitin is represented by the green ellipse. From the left, the different plasmalemma proteins involved are: the putative receptor (2 subunits, a 160kDa and a 50kDa protein), a calcium channel, a chloride channel, the H^+-ATPase (inhibited) and the NADPH oxidase. The signs + and - indicate the transmembrane potential. The protein phosphorylation steps are indicated by the blue "P". Orange arrows show the systems that create the changes in pH. The blue arrows indicate the positive feedback effects of the extracellular medium alkalization, and the numbers 1-11 indicate the events in their chronological order.

Figure 1. *Hypothetical signalling scheme that summarizes the pathways involved in the early responses of tobacco cells treated with elicitin (9 steps).*



From PDB coordinates of native and ergosterol-complexed cryptogein.

Figure 2. *Involvement of Y87 and Y47 during sterol intake in cryptogein.*



The receptor of elicitins, located on the plant plasma membranes, is presumed to be a calcium channel, constituted from four basal subunits (a 160kDa and a 50kDa protein), each of them able to specifically bind an elicitin molecule. The first elicitin-channel interaction needs an elicitin loaded from plant plasmamembrane sterols and triggers a conformational change of the channel, probably associated with the phosphorylation of the subunit bound to elicitin. This new state triggers the biological response and allows the conformational change of the other subunits (cooperative effect), which then binds either a loaded (upper scheme) or an unloaded (lower scheme) elicitin. This explains why all elicitins are able to saturate the receptor subunits, and why only loaded elicitins will trigger a new set of biological responses.

Figure 3. *First events in elicitin signalling.*