Construction of Cryptogein Mutants, a Proteinaceous Elicitor from *Phytophthora*, with Altered Abilities To Induce a Defense Reaction in Tobacco Cells†

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ABSTRACT: We prepared a series of cryptogein mutants, an elicitor from *Phytophthora cryptogea*, with altered abilities to bind sterols and fatty acids. The induction of the early events, i.e., synthesis of active oxygen species and pH changes, in suspension tobacco cells by these mutated proteins was proportional to their ability to bind sterols but not fatty acids. Although the cryptogein−sterol complex was suggested to be a form triggering a defense reaction in tobacco, some proteins unable to bind sterols induced the synthesis of active oxygen species and pH changes. The modeling experiments showed that conformational changes after the introduction of bulky residues into the ω loop of cryptogein resemble those induced by sterol binding. These changes may be necessary for the ability to trigger the early events by elicitins. However, the ability to stimulate necrosis in suspension tobacco cells and the expression of defense proteins in tobacco plants were linked neither to the lipid binding capacity nor to the capacity to provoke the early events. On the basis of these experiments and previous results, we propose that elicitins could stimulate two signal pathways. The first one induces necroses and the expression of pathogen-related proteins, includes tyrosine protein kinases and mitogen-activated protein kinases, and depends on the overall structure and charge distribution. The second type of interaction is mediated by phospholipase C and protein kinase C. It triggers the synthesis of active oxygen species and pH changes. This interaction depends on the ability of elicitins to bind sterols.

Plants have a variety of strategies to protect themselves against pathogens. One of the generally accepted concepts acknowledges that compounds originating from these pathogens (elicitors) are recognized by specific receptors and this primary interaction triggers a defense reaction. One of the plant−pathogen models is the interaction of tobacco with *Phytophthora*, a group of widespread and highly pathogenic fungi. Elicitins are a family of 10 kDa proteins with a high degree of homology excreted by all species of *Phytophthora* (*1*−*3*). These proteins did not exhibit any protease, β-glucanase, or phospholipase activity, and no other enzymatic activity has been reported so far.

The mode of interaction of cryptogein, a very efficient basic elicitin from *Phytophthora cryptogea*, with plant cells was investigated in detail. When added to a suspension of tobacco cells, it triggered depolarization of the plasma membrane, protein phosphorylation, alkalinization of extracellular medium, concomitant potassium and chloride efflux, fast and large influx of calcium and transient production of AOS,[1] and cell wall modifications (*4*−*8*). These effects could be prevented by the treatment of cells with calcium channel inhibitors or by the inhibition of protein kinases by staurosporin (*9*). Treated plants produce ethylene and accumulate phytoalexins such as capsidiol (*10*). Elicitins may also induce a hypersensitive reaction including cell necroses. The expression of PR proteins and the induction of local acquired resistance were observed after the treatment of leaves with cryptogein (*11*−*13*). Distribution on the stem of decapitated plants is followed by rapid translocation of cryptogein, and the plant becomes resistant to further inoculation by pathogens (*14*, *15*). Other cultivated *Solanaceous* genera (petunia, pepper, and tomato) did not develop any leaf necrosis and protection in response to cryptogein (*16*).

Specific binding of cryptogein to high-affinity binding sites on the tobacco plasma membrane, a putative cryptogein receptor, has been previously reported (*5*, *17*, *18*). This plasma membrane component is a heterodimeric *N*-glycoprotein with subunits of 162 and 50 kDa. The signal pathway of cryptogein that controls the synthesis of AOS and pH changes of the intracellular medium includes calcium channels, Ser/Thr protein kinases, phospholipase C, and protein

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[1] Abbreviations: AOS, active oxygen species; au, arbitrary units; CPA, *cis*-parinaric acid; DHE, dehydroergosterol ($\Delta^{5,7,9(11),22}$-ergostatetraen-3β-ol); DIG, digoxigenin; MAP-kinase, mitogen-activated protein kinase; PAL, phenylalanine ammonia lyase; PR proteins, pathogen-related proteins.

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kinase C but not Tyr protein kinases (*19*−*21*). Otherwise, cryptogein activates the signal pathway including Tyr-specific MAP-kinases in tobacco cells not linked to the production of AOS (*22*).

The three-dimensional structures of cryptogein from *P. cryptogea* and cinnamomin from *Phytophthora cinnamomi* were determined by crystallography and NMR (*23*−*25*). The structures of elicitins are composed of five α-helices and one *β*-sheet arranged in a unique protein fold. A hydrophobic cavity is located in the protein core and connected with the protein surface by a tunnel.

Previous studies show that elicitins are a new class of sterol carrier proteins able to bind and transport sterols between biological membranes (*26*, *27*). The biological role of elicitins has been suggested to be the storage and the transport of sterols used by *Phytophthora* spp. Additionally, elicitins are known to bind fatty acids although this affinity is significantly lower (*28*).

Some attempts were made to find a relationship between the structure and biological effects of elicitins (*29*−*31*). Osman et al. (*31*) suggested a link between sterol binding ability and the activation of hypersensitive reactions in tobacco cells using site-directed mutagenesis of Tyr47 and Tyr87. Therefore, sterol loading may be a prerequisite step of the fixation of elicitins to the receptor, and the sterol−elicitin complex could be the active form that triggers the defense reaction.

In this paper, we carried out site-directed mutagenesis on cryptogein to modify residues mainly in the hydrophobic cavity. The ability of the mutants to bind sterols and fatty acids was investigated using fluorescent lipids. A link between the ability of lipid binding and stimulation of the early events in tobacco cells, their ability to induce necrotic effects, and the expression of PR proteins was also examined.

MATERIALS AND METHODS

Chemicals. *cis*-Parinaric acid (CPA) was obtained from Molecular Probes. Other chemicals were obtained from Sigma-Aldrich. Cryptogein mutants were dissolved in water, and CPA and dehydroergosterol (DHE) were dissolved in ethanol. All stock solutions were stored at −20 °C.

Plant Material. Tobacco suspension cells (*Nicotiana tabacum* L. cv. *Xanthi*) were grown in medium described by Chandler (*32*) on a rotatory shaker (150 rpm, 25 °C) under continuous light and used during the exponential phase. Tobacco seeds (*N. tabacum* L. cv. *Xanthi*) were sown into peat soil, and plants were grown in controlled conditions (22 °C, 16 h light, 6000 lux, 80% hygrometry).

Isolation of Recombinant Proteins. The wild type of cryptogein was expressed and purified as described by Panabieres et al. (*33*) using the plasmid pBG38 containing the X24 gene from *P. cryptogea*, isolate 52. Site-directed mutagenesis was conducted using a QuikChange kit (Stratagene, France) and a couple of specific forward−reverse oligonucleotides (Eurogentec, France) introducing the mutation to the targeted codon. Primers used for mutagenesis are listed in Table 1. Multiple mutants were obtained by introducing each mutation individually. Each mutant coding sequence (even sequences used only as intermediary templates for multiple mutations) was controlled by DNA sequencing (Genome Express, France) before *Pichia pastoris* transformation. Yeast transformation as well as protein purification was achieved by a technique described previously (*31*).

Table 1: Sequences of the Oligonucleotides Used for Mutagenesis of Cryptogein

mutation	primers[a]
L15W	F: GCG TAC AAG ACG **TGG** GTG AGC ATC
	R: GAT GCT CAC **CCA** CGT CTT GTA CGC
L19R	F: GTG AGC ATC **CGA** TCG GAC GCG TCG
	R: CGA CGC GTC CGA **TCG** GAT GCT CAC
M35F	F: TCG GGC TAC TCC **TTC** CTG ACG GCC A
	R: T GGC CGT CAG **GAA** GGA GTA GCC CGA
M35W	F: TCG GGC TAC TCC **TGG** CTG ACG GCC A
	R: T GGC CGT CAG **CCA** GGA GTA GCC CGA
L36F	F: GGC TAC TCC ATG **TTC** ACG GCC AAG
	R: CTT GGC CGT **GAA** CAT GGA GTA GCC
M59F	F: GCA TGC AAC ACC **TTC** ATC AAG AAG ATC G
	R: C GAT CTT CTT GAT **GAA** GGT GTT GCA TGC
M59W	F: GCA TGC AAC ACC **TGG** ATC AAG AAG ATC G
	R: C GAT CTT CTT GAT **CCA** GGT GTT GCA TGC
I63F	F: ATG ATC AAG AAG **TTC** GTG ACG CTG
	R: CAG CGT CAC **GAA** CTT CTT GAT CAT

[a] F is the forward primer and R is the reverse primer, respectively. The target codon is underlined, and changed nucleotides are in bold.

Fluorescence Measurements. The sterol and fatty acid binding of proteins was measured using a Shimadzu RF 5001 PC spectrofluorometer in a stirred cuvette according to a previously described method (*27*) by the titration of proteins with DHE and CPA in elicitation buffer [2 mM MES (pH 5.75), 175 mM mannitol, 0.5 mM K_2SO_4, and 0.5 mM $CaCl_2$]. Dissociation constants of the lipid−protein complexes were determined by linear plots of $1/C_b$ vs $1/C_f$ using the equation $1/C_b = (K_d/A)(1/C_f) + 1/A$, where C_b, C_f, and A are concentrations of bound lipid, free lipid, and maximal binding capacity, respectively. The concentration of the bound lipid C_b was calculated using the equation $C_b = (F - F_0)/(Q_b - Q_0)$, where F is the fluorescence [arbitrary units (au)] of the lipid−protein mixture at concentration C of the lipid, F_0 is the fluorescence of the lipid concentration C without protein, and Q_b and Q_0 are fluorescence quantum yields of bound and free lipids (au μM^{-1}), respectively. The excitation and emission wavelengths were 325 and 370 nm for DHE and 305 and 420 nm for CPA. The values were read after equilibration.

Synthesis of AOS and pH Changes Induced by Cryptogeins. The synthesis of active oxygen species induced by cryptogein in tobacco cell cultures was measured by a luminometric method in elicitation buffer. The concentrations of H_2O_2 were monitored every 5 min in 250 μL aliquots. The pH changes were registered every 10 min after the addition of the elicitor with a pH electrode (*21*).

Measurements of Plant Cell Death. Plant cell death was induced by cryptogein added to the tobacco cells (0.1 g fresh weight mL^{-1}) suspended in a fresh Chandler medium (*32*). The elicited suspensions were incubated for 24 h. The viability of the cells was tested using fluorescein diacetate and propidium iodide as described earlier (*34*, *20*). The percentage of living cells was calculated on the basis of green fluorescence of fluorescein. Four hundred cells were observed in each preparation, and numbers were expressed as a percentage of total cells.

Expression of PR Proteins. Upper, middle, and lower leaves, each from a different tobacco plant, were treated with 100 nM cryptogeins. At different times after infiltration, the leaves were detached, the infiltrated areas of three leaves were cut out, mixed, and stored at −76 °C. Total RNA was extracted by Tri-Reagent (Sigma-Aldrich) according to manufacturer's instruction. Isolated RNA was dissolved in 10 μL of deionized formamide, quantified by spectrophotometry, and stored at −20 °C. For the Northern blot analysis, 15 μg of total RNA was separated by 1.2% agarose gel electrophoresis under denaturing conditions, blotted onto positively charged nylon membranes (Hybond N^+; Amersham), and UV cross-linked. 18S RNA was used as a loading control.

Probe Labeling and Hybridization. The specific probes were labeled by incorporation of DIG-11-dUTP (Roche Molecular Biochemicals) by PCR. The primer sequences of specific genes were as follows: PAL (5′ATGGAACACATTTTGGATGGTAG3′, 5′TAGTCACTGGATTTGCCAAGAAT3′), NADPH oxidase (5′GCTTCAGTCTCTTCACCATGC3′, 5′CAAAAATGATGACAGCCACCT3′), PR1a (5′CCTCGTACATTCTCATGGTCAAT3′, 5′CCATTGTTACACTGAACCCTAGC3′), PR1b (5′GTTGCTTGTTTCATTACCTTTGC3′, 5′TTCTCATCGACCCACATTTTTAC3′). The amplicons were purified with an UltraClean PCR cleanup kit (Mobio), characterized, and quantified by IE-HPLC on a TSK-gel NPR column as published previously (*35*). Hybridization was performed at 55 °C for 12 h in DIG-Easy Hyb buffer (Roche Molecular Biochemicals). The DIG-labeled DNA probes were used at concentration levels of 15 ng mL^{-1} of hybridization buffer. After hybridization, filters were washed twice for 5 min with 2 × SSC (Serva) at room temperature and twice for 20 min with 0.2 × SSC at 65 °C. Detection was performed with anti-DIG antibodies conjugated with alkaline phosphatase and BCIP-NBT as a color substrate (Roche Molecular Biochemicals).

Modeling of the Protein−Ligand Interactions. The 3D structures of protein mutants were modeled starting from the crystal structures of free cryptogein (PDB ID 1BEO). Substitutions were introduced from the library of rotatamers by Swiss-PDB-Viewer v3.7 in a way to maximize their overlap with the wild-type side chains and minimize a number of bumps with the surrounding residues. The structures were minimized by the steepest descent algorithm, followed by the conjugate gradient algorithm until the energy change was less than 10E−10 kcal mol^{-1}. The AMBER force field (*36*) was used for all molecular mechanics calculations.

RESULTS AND DISCUSSION

Computer modeling and quantitative structure−activity relationship analysis conducted in a previous study (*37*) resulted in design of cryptogein variants with high selectivity toward binding of specific ligands. Construction and biochemical characterization of these mutants should provide information about the role of ligand binding for triggering of the defense reaction. We have prepared five new recombinant proteins including multiple mutations of the residues L15, L19, M35, L36, M59, and I63 (Table 2) directed mainly into the hydrophobic cavity (Figure 1). To reinforce the effects, we carried out double or triple mutations. The proteins expressed in *P. pastoris* were isolated and lyophilized, and their structures were tested by far-UV−CD spectrometry. The near-UV−CD spectrometry could not be used because of the presence of new Trp groups in mutated proteins. The far-UV−CD spectra of all mutants were similar to the spectrum of the wild type so that the mutations did not perturb markedly their structure. However, the spectrum of the triple mutant M35W/M59W/I63F showed a slightly diminished ratio of α-helix/β-structure when compared with the other proteins (data not shown). The L15 residue is positioned near the mouth opening, and it rotates upon the binding of DHE (*24*). The substitution for Trp may prevent this rotation. The L19 residue is positioned at the mouth opening, and it makes hydrophobic contact with L36. The mutation will change the electrostatics and may influence the kinetics of binding specifically for the ligands with large dipole moments. The substitution of L19 for the more hydrophilic Arg will increase the p*I* of the protein. The L36 residue is positioned near the mouth of the opening, and it causes a slight conformational change upon the binding of DHE that makes hydrophobic contact with L36. Residues

Table 2: Dissociation Constants of Complexes of CPA and DHE with Mutated Cryptogeins and Biological Effects of Mutants[a]

mutation	K_d (μM) DHE	K_d (μM) CPA	AOS synthesis	pH change
wild type	0.050 ± 0.002	0.19 ± 0.01	+++	+++
M35F/M59W	0.21 ± 0.01	0.132 ± 0.007	++	+++
M35W/M59W/I63F	0.068 ± 0.005	0.09 ± 0.01	+++	+++
M35W/M59W	no binding	0.08 ± 0.01	+	++
L19R	no binding	0.12 ± 0.02	no effect	no effect
L15W/L36F	no binding	no binding	+	++

[a] 1 μM protein was titrated with 0.6−3 μM lipid in elicitation buffer, and fluorescence values of bound lipids were recorded. The dissociation constants with standard deviations were obtained from these values by double reciprocal plots of $1/C_b$ vs $1/C_f$ as described in the Materials and Methods section. The biological efficiency of mutated proteins in suspension tobacco cells was taken from Figure 3 as the ability of 25 nM cryptogeins to induce the synthesis of AOS (after 40 min) and pH changes (after 20−30 min).

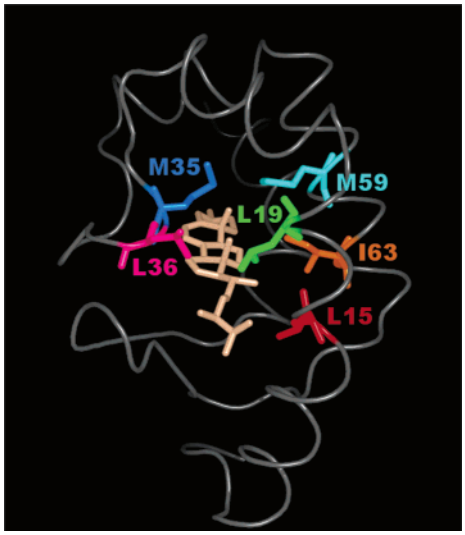

FIGURE 1: Three-dimensional model of cryptogein with ergosterol (in brown) bound in the hydrophobic cavity. The model shows the position of the mutated residues affecting the binding of lipids and biological effects of cryptogein. The model is based on the structure of the cryptogein−ergosterol complex determined by protein crystallography (PDB ID 1BXM).

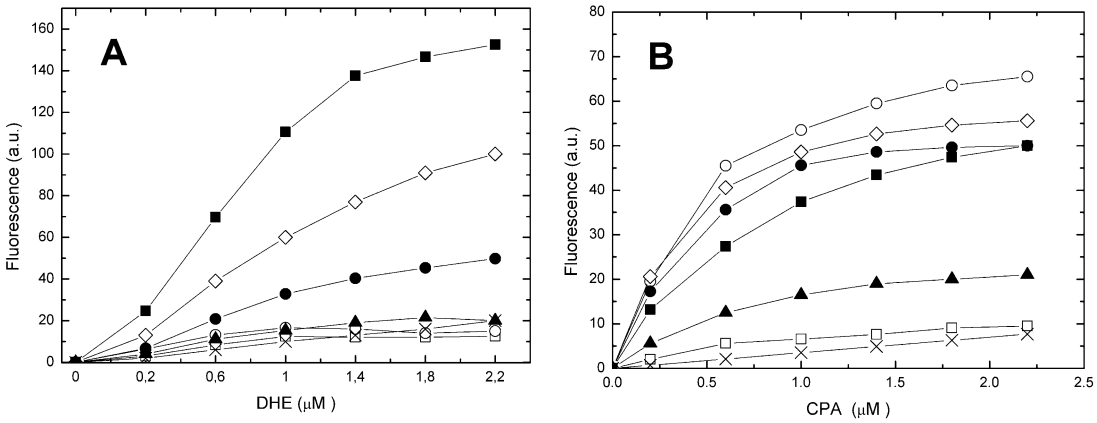

FIGURE 2: Fluorescence titration curves of mutated cryptogeins with fluorescent lipids. 1 μM proteins were titrated in elicitation buffer with 0.2−3 μM lipids, and the resulting fluorescence was read after equilibration at the wavelengths specified in the Materials and Methods section. Key: (■) wild type; (◇) M35F/M59W; (●) M35W/M59W/I63F; (○) M35W/M59W; (▲) L19R; (□) L15W/L36F; (×) DHE or CPA. (A) Fluorescence binding curve of DHE. (B) Fluorescence binding curve of CPA.

M35 and M59 rotate upon the binding of DHE and determine the size of the cavity by stacking if both residues are aromatic. The I63 residue is absolutely conserved among all currently known elicitins. It forms a part of the hydrophobic core of the protein and interacts with sterols.

Interaction of Mutated Cryptogeins with Lipids. All recombinant cryptogeins were tested for their ability to bind DHE and CPA. Both lipids only slightly fluoresce in water due to self-quenching of the fluorescent molecules in lipid micelles. The mutated proteins were titrated with CPA and DHE. After the lipids bind into the central cavity, their fluorescence markedly increases (*27*). The fluorescence binding curves are shown in Figure 2. The resulting fluorescence intensities depend on the concentrations of bound lipids and on their fluorescence quantum yields. The latter value is influenced by the polarity and microviscosity of the protein cavity or by the presence of quenching amino acids, namely, Trp. The fluorescence of the mixture of DHE with the mutants L19R, L15W/L36F, and M35W/M59W could not be distinguished from the base fluorescence of free DHE. So it seemed that these mutants did not bind sterols. The lower DHE fluorescence bound in the mutants M35F/M59W and M35W/M59W/I63F could be explained by the presence of Trp in the central cavity.

As for the interaction with fatty acids, L15W/L36F was the only protein unable to bind CPA because the fluorescence of the CPA−protein mixture could not be distinguished from the base fluorescence of free CPA. The other mutations did not change markedly in the affinity of proteins to fatty acids. The dissociation constants of the complexes were determined on the basis of these titration curves and fluorescent quantum yields of the bound lipids. The values of dissociation constants of CPA and DHE are presented in Table 2.

M35F/M59W and M35W/M59W are potentially more stable proteins with a reduced cavity. The results showed that M35F/M59W was able to bind sterols and fatty acids, whereas the protein M35W/M59W did no bind sterols. As for the triple mutation M35W/M59W/I63F, this cryptogein has steric hindrance resulting in disruption of protein structure. The modeling experiments proved that this protein has an enlarged pocket due to van der Waals repulsion of F63 with V84. This repulsion probably caused a reorganization of the cavity and reconstitution of the binding ability lacking in M35W/M59W. The mutation L15W/L36F results in a partial close up of the entrance tunnel and prevented rotation of W15, resulting in the lack of binding. This double mutated protein binds neither sterols nor fatty acids. As for cryptogein L19R, the mutation changes long-range electrostatic interactions that are important for binding of ligands to the cavity and the p*I* value (estimated 8.8 vs 8.6 of cryptogein). This protein did not bind sterols, but it could bind fatty acids.

Induction of Early Events by Mutated Cryptogeins in Suspension Tobacco Cells. We measured the effects of site-directed mutagenesis on the synthesis of AOS and changes of extracellular pH in suspension tobacco cells induced by cryptogeins. Synthesis of AOS could be demonstrated on the leaves of whole plants. However, suspension cultures enable the exact parallel evaluation of the early events in time, which proved to be important because of the small differences of eliciting activities of some mutated proteins. These effects were elicited with 10, 25, and 50 nM cryptogeins (Figure 3). It has been shown that the alkalinization of extracellular medium due to cryptogein interaction could be a result of the combination of several events, the most important being the inhibition of plasma membrane H^+-ATPase (*7*). Cryptogein L19R stimulated neither the synthesis of AOS nor pH changes. The differences of the elicitation abilities of other proteins clearly increased at lower concentrations. Mutated protein M35W/M59W/I63F was as efficient as the wild type. The production of AOS stimulated by a 50 nM concentration of cryptogein M35F/M59W was delayed by 20 min. In fact, we observed that the rate of the loading of this protein by DHE was half that of the wild type and M35W/M59W/I63F (data not shown), which could explain the lower biological efficiency of M35F/M59W. Proteins L15W/L36F and M35W/M59W (50 nM), although unable to bind sterols, were still able to induce the synthesis of AOS but showed only slight effects at 10 nM concentration. The actual concentration of AOS is a result of the synthesis vs degradation so that the absence of AOS in the medium after the elicitation does not necessarily mean that the synthesis was not triggered at all. The pH changes induced by mutated proteins gave a similar pattern. The effects of 10 nM cryptogeins L15W/L36F and M35W/M59W stimulated the alkalinization of the external medium but with

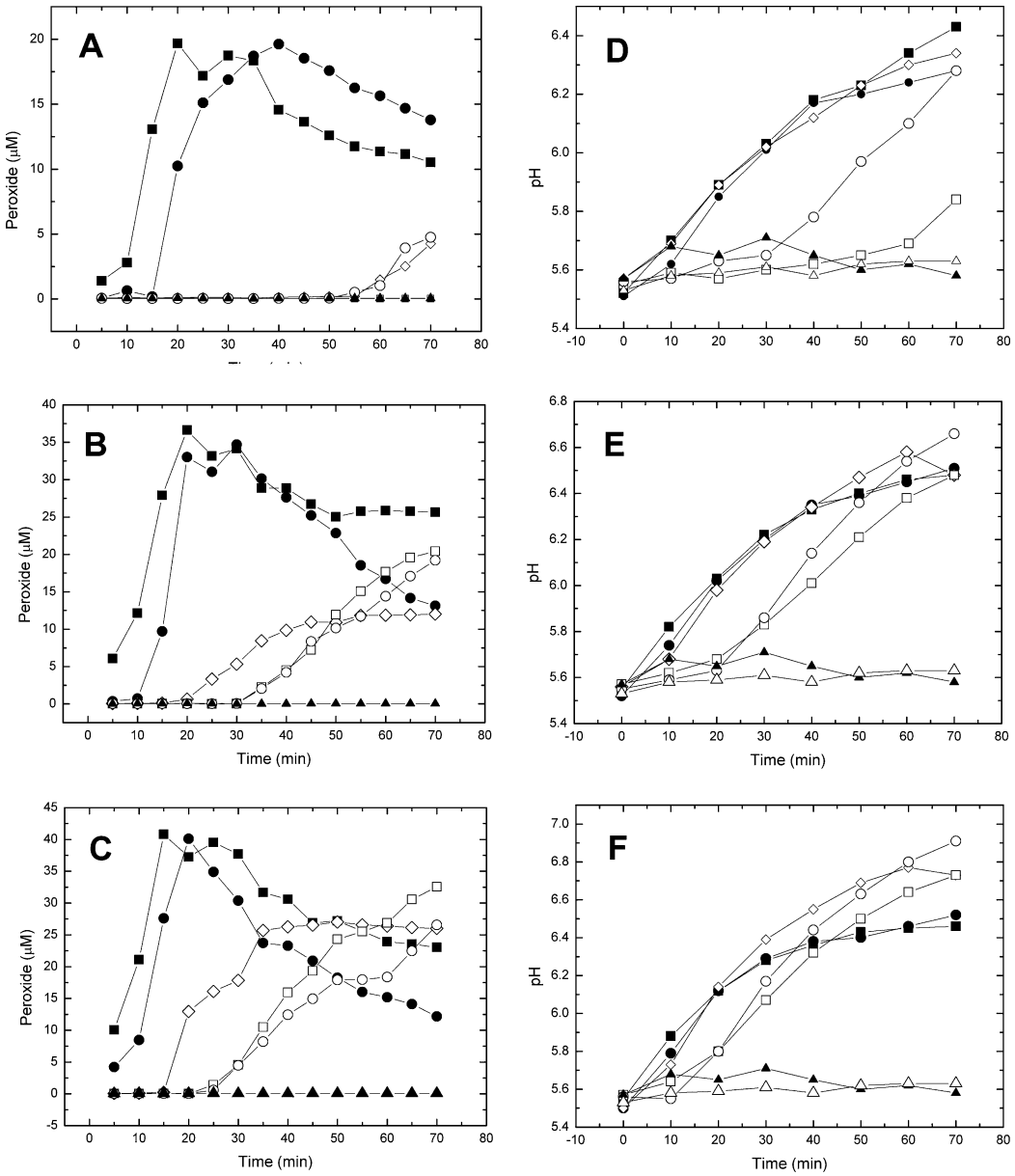

FIGURE 3: AOS synthesis and pH changes in tobacco suspension cells stimulated by cryptogein mutants. Cells were equilibrated for 3 h in elicitation buffer, and cryptogeins were added to the suspension at time zero. The concentrations of H_2O_2 were monitored every 5 min in 250 μL aliquots by a luminol method. The pH changes were registered every 10 min after the addition of the elicitor with a pH electrode. Key: (■) wild type; (◇) M35F/M59W; (●) M35W/M59W/I63F; (○) M35W/M59W; (▲) L19R; (□) L15W/L36F; (△) control. AOS synthesis was elicited by 10 (A), 25 (B), and 50 nM (C) cryptogeins; pH changes were elicited by 10 (D), 25 (E), and 50 nM (F) cryptogeins.

potency markedly lower than that of the other mutants. The relationship between the sterol binding of mutated proteins and their ability to trigger the early events is summarized in Table 2. These results showed that the ability to induce the synthesis of AOS and pH changes is linked to the ability to bind sterols and not fatty acids. The computer modeling showed that DHE binding initiates conformation changes of the ω loop and consequently overall protein structures (Figure 4A). Fatty acids did not stimulate such changes. They could accommodate the shape of the cavity because of their flexibility (*37*). The ω loop is very flexible and highly conserved. Its conservative structure suggests an important function.

Proteins L15W/L36F and M35W/M59W did not bind sterols but remained efficient to induce the early events. It seems to be contradictory to our hypothesis that the formation of the sterol−cryptogein complex is a prerequisite step necessary to induce a plant defense response (*31*). It could be argued that proteins L15W/L36F and M35W/M59W can also bind sterols but their loading rates could be too small to be detected by fluorescence. However, the modeling experiments showed that the binding of sterols by these mutated proteins, even to a small extent, is very improbable. Both proteins contain bulky residues in the ω loop (W35, F36). Such a big residue directed inside the structure must be compensated by conformation changes in the ω loop that are very similar to those induced by the binding of sterols (Figure 4B). Our results suggest that the conformation of the ω loop induced by sterol binding or by the presence of bulky residues could be necessary for the ability to trigger the early events caused by cryptogeins.

Necroses of Suspension Tobacco Cells Induced by Cryptogeins. Suspension tobacco cells harvested at the exponential phase and resuspended in fresh cultivation medium were

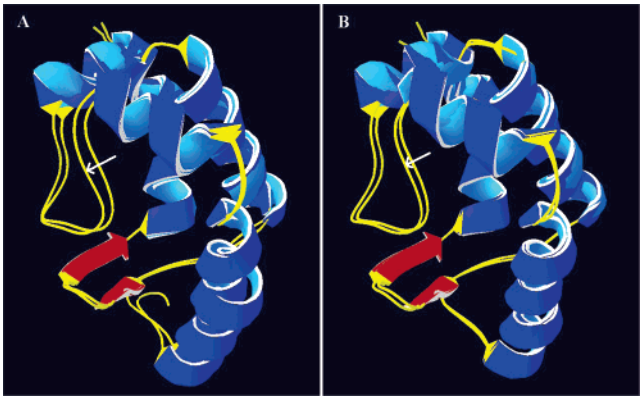

FIGURE 4: Superposition of the free wild type, dehydroergosterol-bound wild type, and mutant structures of cryptogein. (A) Binding of dehydroergosterol to the cavity of cryptogein wild type induces a conformational change in the ω loop (arrow). (B) Superposition of the structures of wild type and the M35W/M59W mutant (arrow) of cryptogein. Changes induced by the mutation are similar to those induced by sterol binding. Proteins are represented as ribbons; α-helices are in blue, β-strands in red, and loops in yellow.

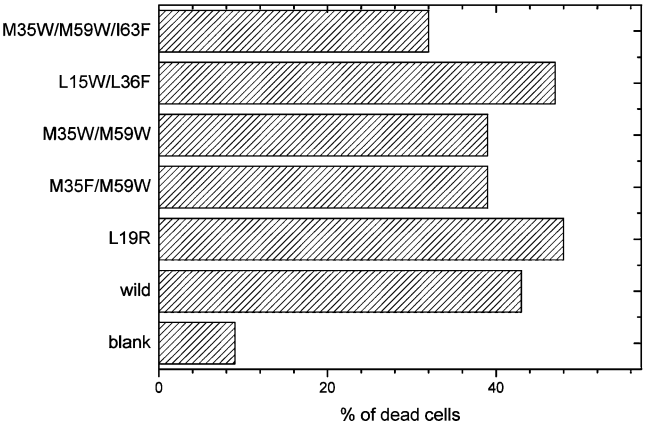

FIGURE 5: Cell death induced by mutated cryptogeins. The viability of at least 400 cells was examined using propidium iodide and fluorescein diacetate after 24 h following the addition. The cells with green fluorescence were taken as live cells. The data represent the mean values of two independent experiments. The mean relative standard deviation of the measurements was ±8%.

treated with mutated cryptogeins. After 24 h following the addition of the mutants, the viability of the cells was tested by fluorescence microscopy using fluorescein diacetate and propidium iodide. Fluorescein diacetate penetrates into the living cells and is then hydrolyzed by esterases, giving rise to a highly fluorescent product. The red fluorescence, due to penetration of propidium iodide through the damaged plasma and nuclear membranes, indicates the presence of dead cells.

The results showed that all mutated cryptogeins were able to stimulate cell necrosis (Figure 5). Surprisingly, protein L19R that neither was able to bind sterols nor induce the early events was very efficient to induce cell death. Cells incubated with L19R died after 24 h, but their color remained unchanged (green as the control cells) whereas those incubated with the other cryptogeins had a brownish color probably due to the synthesis of AOS.

Expression of Defense Proteins Induced by Cryptogeins on Tobacco Plants. The abilities of mutated proteins to induce the expression of PAL, NADPH oxidase, and PR proteins 1a and 1b were tested on whole tobacco plants. The use of suspension cells for this purpose is not possible because the natural level of these proteins in control suspension cells is too high. PAL is a key enzyme of the phenylpropanoid pathway that is activated by stress and pathogen attack (*38*). Although their mode of action is unknown, PR1 is a dominant group of pathogen-related proteins induced by pathogen in tobacco and is commonly used as a marker for systemic acquired resistance (*39*). Cryptogeins were injected into leaves, and the samples of the tissue were taken up in the surroundings of the infiltration zone after 3, 10, and 24 h following injection. The mRNA concentration was determined by hybridization with specific probes as described in the Materials and Method section (Figure 6). The results show that PAL was expressed after 3 h, and its high mRNA concentration remained constant during the 24 h period. The highest expression of NADPH oxidase was observed after 10 h following the addition of cryptogeins, and then the concentration diminished. The highest concentrations of PR1a,b were expressed after 24 h (Figure 6). The mechanisms leading to the cell death and the gene expression in cell suspension or in whole plants may not be the same so that the results cannot be easily compared. However, the abilities of all cryptogein mutants to provoke necroses in leaves and in cell suspensions were identical (data not shown). These results showed that all mutated proteins were able to express the defense proteins, including those that did not bind sterols (L15W/L36F and M35W/M59W) or were unable to stimulate the synthesis of AOS and pH changes (L19R). Natural elicitins interact with sterols showing close binding characteristics, i.e., 1:1 sterol: protein stoichiometry and similar dissociation constants. However, rates of sterol loading are much higher for basic cryptogein than for acidic capsicein (*26*). The loading rates correlate with their biological efficiency in tobacco; i.e., basic elicitins proved to be more efficient to induce the synthesis of AOS depending on the rate of complex formation. Similarly, leaf necroses and plant protection in tobacco are markedly higher for basic cryptogein than for acidic capsicein (*1*, *16*).

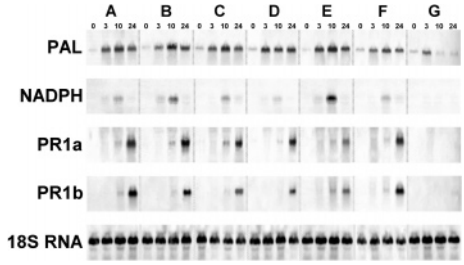

FIGURE 6: Concentration of mRNA of the proteins induced by mutated cryptogeins in tobacco leaves measured by hybridization probes. Upper, middle, and lower leaves, each from different tobacco plants, were treated with 100 nM cryptogeins. At different times after infiltration (3, 10, and 24 h), the infiltrated areas of three leaves treated with each cryptogein were cut out, mixed, and used for mRNA isolation. RNA was separated by agarose gel electrophoresis, and the gels were blotted. Hybridization was carried out with DIG-labeled DNA probes and detected with anti-DIG antibodies and a color substrate. 18S RNA was used as a loading control. Lanes A−G were loaded with the following proteins: (A) wild type; (B) L19R; (C) M35W/M59W; (D) M35W/M59W/I63F; (E) L15W/L36F; (F) M35F/M59W; (G) water.

Our results suggest lack of relationships between the ability of elicitins to induce the production of PR proteins together

with tissue necrosis and the synthesis of AOS or ΔpH changes. This hypothesis could be supported by the fact that cryptogein interaction with tobacco cells activated Tyr MAP-kinases that were not linked to the production of AOS (*19*) whereas the signal pathway from the receptor to the production of AOS and to the pH changes did not include Tyr protein kinases (*20*).

Moreover, the ability to induce acquired systemic resistance may not to be linked to the necrotic symptoms. Oligandrin, an elicitin-like protein from *Pythium oligandrum*, stimulates the plant defense machinery but fails to provoke the hypersensitive response-associated necrotic effects in tomato (*40*, *41*). No general relationship appeared between the extent of necrosis and the level of acquired resistance (*16*). The protecting activity should depend on the high number of basic residues contributing to their charge (*42*).

The results further suggest that elicitins could activate two signal pathways that may not be necessarily connected. The ability to induce the early events could be conditioned by a necessary conformation of the ω loop induced by the sterol binding or by a mutation in the ω loop whereas the ability to express PR proteins and to induce cell necroses is by overall structure of the proteins and charge distribution. It is not obvious which forms trigger these two signal pathways. The cross-linking studies of the interaction of cryptogein with tobacco plasma membrane revealed that this elicitin binds to two protein subunits: 162 and 50 kDa (*18*). They can constitute one or two receptors. Lipid transfer protein from wheat endosperm and tobacco binds to high-affinity sites of tobacco plasmalemma (*43*, *44*) previously identified as an elicitin receptor (*18*) so that elicitins and lipid transfer proteins compete for the same high-affinity binding sites. It has been reported that cryptogein in aqueous solution in the millimolar range forms a discrete homodimer where the N-terminal helices of each monomer form an interface (*45*). Conformation changes upon the sterol binding could influence the dimer−monomer equilibrium. Local concentration of positively charged cryptogein bound in the plasma membrane could be considerably higher than in solution so that molecules would be in tight contact, consequently inducing cryptogein dimerization. However, the concentration in solution exceeds by six orders the biologically efficient concentrations that are in the nanomolar range. Therefore, a study of interactions of the cryptogein mutants with binding proteins of tobacco plasma membrane should be performed.

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