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Interaction of cryptogein with its binding sites in tobacco plasma membrane studied using the piezoelectric biosensor

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ABSTRACT

Elicitins are low-molecular-weight proteins representing the elicitor family secreted by many species of the oomycete *Phytophthora*. Elicitins induce a hypersensitive reaction in tobacco, a process that is triggered by binding of elicitin to the high-affinity site on the plasma membrane. Specific interaction of cryptogein with the binding sites on tobacco plasma membranes was studied using the piezoelectric biosensor in real time in a flow-through mode. Cryptogeins (wild-type and mutant forms) were covalently immobilized on the sensing surface, and membrane vesicles containing receptors were in solution. Kinetic characterization of the interaction provided values of kinetic rate association (k_a) = $5.74 \cdot 10^6\ M^1\ s^{-1}$ and kinetic rate dissociation (k_d) = $6.87\ 10^{-4}\ s^{-1}$ constants, respectively. The kinetic equilibrium dissociation constant was calculated as K_D = 12.0 nM. The piezoelectric biosensor appeared to be a convenient tool for studying interactions of receptors embedded in membrane vesicles.

Elicitins are a family of small proteins expressed as products of avirulent genes [1] of the *Phytophthora* species, widespread and highly damaging pathogenic fungi [2,3]. Elicitins are classified into two groups according to the net charge of the protein molecule: the acidic or α-elicitins (p*I* = 3–5) and the basic or β-elicitins (p*I* = 7–9). The difference in biological activity between the two groups is associated with structural properties; it might be correlated with one or several steps of the signal transduction pathway induced in the target cells. It is generally assumed that a putative receptor on the plasma membrane acts as the first component of the transduction pathway, and then secondary messengers, membrane proteins, or cytosolic proteins such as protein kinases transduce the signal to activate plant defense response [4]. The defense reactions in tobacco (*Nicotiana tabacum*) include a hypersensitive response and systemic resistance to further inoculation with fungi and bacterial pathogens [5,6].

Elicitins are proteinaceous elicitors produced by oomycetes of genus *Phytophthora,* exhibiting a high degree of homology (>60% sequence identity), with six invariant cysteine residues accounting for three disulfide bridges and containing a hydrophobic cavity that is able to bind sterols or fatty acids [7]. Among basic elicitins, the cryptogein produced by *Phytophthora cryptogea* has been studied extensively. It causes hypersensitive reaction-like necrosis in tobacco plants [8]. Cryptogein interacts with high-affinity binding sites on the tobacco plasma membrane, exhibiting properties consistent with receptor sites [9]. A few minutes after cryptogein treatment, the events induced on tobacco cell suspensions include influx of Ca^{2+} [10], modifications of ion fluxes (H^+, K^+, and Cl^-), depolarization of the plasma membrane [11], production of active oxygen species, phosphorylation [12], and acidification of cytosol [11,13]. Consequently, changes in gene expression [14,15], changes in lipid composition [16], and production of phytoalexin and ethylene have been measured [17].

To investigate the biological role of sterol binding in the hydrophobic cavity of cryptogein, a series of mutants with altered ability to bind sterols or fatty acids were constructed. The results showed that elicitins could activate two signal pathways that might not necessarily be connected. In the case of the first pathway leading to oxidative burst and pH changes, there is necessary conformation of the ω loop induced by the sterol binding. However, in the second pathway responsible for expression of pathogenesis-related proteins and cell necrosis, the overall structure of the proteins and charge distribution plays a major role [18]. Interaction of cryptogein with plasma membrane-based glycoprotein(s) seems to be saturable, reversible, and specific with an apparent K_D of 2 nM [19]. The high affinity correlates with the low concentrations of cryptogein required for biological activity in vivo. The optimum for binding occurred near pH 7.0, and the interaction became weak at pH values below 6.0 and above 7.5. Some 220 fmol binding sites/mg protein were detected by ^{125}I-labeled cryptogein on the tobacco cellular membrane. The crosslink using hydrophobic homobifunc-

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tional bis(sulfosuccinimidyl)suberate provided two groups of proteins with molecular masses of 50 and 162 kDa [19]. These polypeptides, which could represent subunits of a multimeric structure, were not joined by disulfide bridges. Therefore, cryptogein was probably bound to the 162-kDa *N*-glycoprotein subunit that is linked with the 50 kDa *N*-glycoprotein or was bound to both subunits.

Because radioactive labeling provides only information about the equilibrium binding parameters, real-time biosensor-based methods are currently considered for studies of bioligands with membrane receptors [20]. The membrane-bound receptors are usually investigated in a solubilized form because the presence of lipid membranes complicates measurements. The surface plasmon resonance (SPR)[1]-based Biacore system was used for investigation of the G-coupled receptors. To preserve activity, cell lysis and receptor solubilization were coupled on-line [21,22]. Piezoelectric systems were used for studies of receptors embedded in liposomes and membrane vesicles. Usually, the sensing surface was directly coated with membrane fragments containing the target receptor [23].

The biosensor based on piezoelectric quartz crystal resonators was adopted here. The signal-resonant frequency (f) depended on binding events occurring at the active surface modified with one of the binding partners (cryptogein covalently immobilized as ligand) and the other (native receptor in membrane vesicles) being free in solution.

Materials and methods

Biochemicals

Adenosine 5′-triphosphate disodium salt (ATP), bovine serum albumin (BSA), 1-amino-2-naphthol-4-sulfonic acid, ammonium molybdate, aprotinin, cystamine, dextran, dimethyl sulfoxide (DMSO), dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), fluorescein isothiocyanate (FITC), glutaraldehyde, glycerol, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (Hepes), leupeptin, 2-(*N*-morpholino)ethanesulfonic acid (Mes), sodium vanadate, phenylmethanesulfonyl fluoride (PMSF), poly(ethylene glycol) (PEG), potassium chloride, sodium borohydride, sodium dodecyl sulfate (SDS), Triton X-100, tris(hydroxymethyl) aminomethane (Tris), and organic solvents were obtained from Sigma. RCDC Protein Assay was obtained from Bio-Rad.

Preparation of cryptogeins

The wild-type cryptogein and individual mutants were expressed and purified as described by Lochman and coworkers [18] using the plasmid pPIC9 containing the X24 gene from *P. cryptogea*. Yeast transformation and protein purification were achieved by a technique described by Osman and coworkers [24].

Preparation of plasma membrane

Tobacco cell suspensions were cultivated in Chandler medium [25] on a rotary shaker (150 rpm, 25 °C) under continuous light (photon flux rate = 30–40 μmol m^{-2} s^{-1}). Cells were maintained in the exponential phase. Plasma membrane-enriched fractions were obtained following the aqueous partitioning procedure [26]. Tobacco cell suspensions (70 g) were homogenized in 400 ml of the grinding medium (50 mM Tris–Mes [pH 8.0], 500 mM sucrose, 20 mM EDTA, 10 mM DTT, and 1 mM PMSF) using a mixer (5 s) and a stake mixer (25,000 rpm, 10 s), with all steps being performed at 4 °C. The homogenate was filtered through a glass frit with gauze and then was centrifuged at 16,000 rpm for 20 min. The supernatants were collected, filtered again, and centrifuged at 35,000 rpm for 45 min (Beckman Coulter, 10 °C). The pellet with microsomal fraction was homogenized in PSK buffer (5 mM phosphate [pH 7.8], 300 mM sucrose, and 3 mM KCl).

The microsomal fraction (3 g) was placed over the polymeric phase (4.5 g of a mixture consisting of 4.5 g PEG, 4.5 g dextran, and 16 ml of PSK buffer). After rotational shaking (40×) and centrifugation (3000 rpm, 5 min, 10 °C), the upper phases were submitted to the same procedure once again. The product (upper phase) was diluted (1:5) with TSEDP buffer (10 mM Tris–Mes [pH 7.3], 250 mM sucrose, 1 mM EDTA [pH 8.0], 10 mM DTT, and 1 mM PMSF), and centrifuged (35,000 rpm, 45 min). The pellet was homogenized, diluted in 20 ml of TSEDP buffer, and centrifuged (38,000 rpm, 45 min). The final pellet was homogenized in 200 μl of the protective TSEDPG buffer (TSEDP containing 20% glycerol, 10 μg/ml aprotinin, and 10 μg/ml leupeptin).

Quantification of receptor sites in plasma membranes

Cryptogein was fluorescently labeled with FITC according to the standard procedure [27]. The efficiency of labeling was verified by high-performance liquid chromatography (HPLC) analysis of the produced cryptogein–FITC complex on C5 column with specific fluorescence and diode array detector (DAD) detection.

Plasma membrane preparations (50 μg of protein) were incubated 90 min on ice with 1 to 30 nM fluorescently labeled cryptogein in the presence of 10 mM Hepes (pH 7.0), 0.1 M sucrose, and 5 mM $MgCl_2$ (binding buffer). Nonspecific binding was determined in the presence of 10 μM unlabeled cryptogein. The bound ligand was separated by filtration through the GF/B filter (Whatman) presoaked in 1% BSA. After three washes with 5 ml of the cold binding buffer containing 0.1% BSA and one wash with 3 ml of 0.1% SDS, fluorescence released from the surface of the filter using 1% SDS (15 min incubation at 4 °C) was measured using a fluorimeter (Horiba Yvone, France). The content of the receptor was calculated using known standard amounts of the cryptogein–FITC conjugate placed on the filters. The calibration curve in the range from 1 ng to 1 μg was linear up to 1 μg of cryptogein, and the dependence of relative fluorescence (F) on the applied mass of cryptogein (m_{Cry}) was $F = (930 \pm 400) + (33.93 \pm 0.82)\ \text{ng}\cdot m_{Cry}$. The number of binding sites was calculated using the known molecular mass of cryptogein (M_{Cry} = 10 kDa).

Orientation of plasma membranes

The measurement of ATPase activity and its sensitivity on vanadate as described previously [28,29] was used. Plasma membrane (20–40 μg of protein) was introduced to 1 ml of the mixture containing 30 mM Tris–Mes (pH 6.5), 3 mM $MgSO_4$, 50 mM KCl, and 3 mM ATP and was incubated at 38 °C for 60 min. The reaction was stopped by the addition of SDS (10%), and the produced inorganic phosphate was detected from the absorbance at 660 nm using 0.9% ammonium molybdate in 1.45 M HCl and 0.125% 1-amino-2-naphthol-4-sulfonic acid after a 5-min preincubation. The parallel measurement in the presence of 0.1 mM sodium vanadate (noncompetitive inhibitor of ATPase) was carried out [29]. Total ATPase activity was obtained in the presence of 0.01% Triton X-

[1] *Abbreviations used:* SPR, surface plasmon resonance; ATP, adenosine 5′-triphosphate disodium salt; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FITC, fluorescein isothiocyanate; Hepes, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; PEG, poly(ethylene glycol); SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl) aminomethane; HPLC, high-performance liquid chromatography; DAD, diode array detector; ICM, International Crystal Manufacturing; RSI, right side in; RSO, right side out.

100, whereas the latent activity was given by the difference between the activities in the presence and absence of Triton.

Preparation of biosensors and measuring procedure

The piezoelectric quartz crystal resonators (10 MHz, optically polished surface, 5 mm gold electrodes) were obtained from International Crystal Manufacturing (ICM, USA). The crystal was washed in isopropanol/acetone (1:1) for 30 min. A self-assembled monolayer was prepared by spreading 30 µl of 20 mg/ml cysteamine solution in water over the electrode area for 2 h at room temperature. Thus, obtained amino groups were activated with 30 µl of glutaraldehyde (4% in water) for 2 h. The surface was washed and coated with 30 µl of the cryptogein solution (500 µg/ml, 7 h, 4 °C). The formed Schiff base was reduced for 1 h with 30 µl of $NaBH_4$ (5 mg/ml), and sensors were washed with water, dried, and stored in the refrigerator (Fig. 1A).

For measurements, the piezoelectric biosensor was mounted in a thin-layer flow-through cell with an internal volume of 10 µl. The peristaltic pump (Minipuls MP3, Gilson, France) provided a flow rate of 40 µl/min, and carrier buffer and sample solutions were changed manually. The piezoelectric crystal was driven by the Lever Oscillator (ICM), the counter UZ 2400 (Grundig, Germany) connected to a computer, and the program LabTools realized frequency data display and storage with a sampling interval of 1 s.

The initial background frequency (signal) was allowed to stabilize for 5 min. Next, the analyzed sample containing vesicles of the plasmatic membrane was pumped through the cell for 5 min, resulting in formation of the affinity complex (Fig. 1B). A zone of buffer followed to stabilize the response. As a signal for evaluation, the difference of frequency in buffer zones before and after sample was used. Regeneration of the sensing surface was achieved during a 2-min flow of 1% SDS (Fig. 1C).

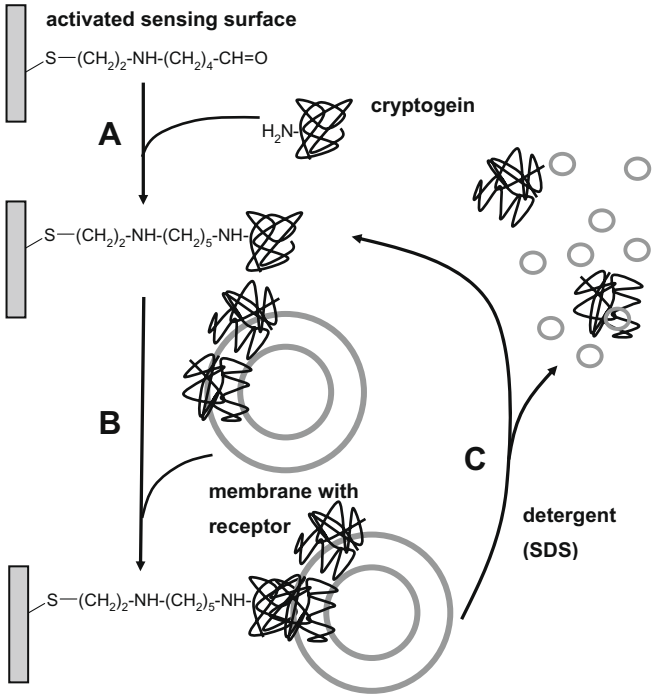

Fig. 1. Schematic view of the measuring procedure consisting of covalent immobilization of cryptogein on the activated surface of the piezoelectric quartz resonator (A), interaction with the membrane vesicles containing the cryptogein receptor (B), and regeneration of the sensing surface in the presence of detergent (SDS) (C).

The kinetic parameters were determined using the common approach [23], and the observed binding frequency (f) in dependence on time t measured by the piezoelectric detector was fitted to the following equation:

$$f = f_0 + f_{eq} \exp(-k_{obs} t), \quad (1)$$

where f_0 represents the initial frequency, f_{eq} represents frequency at equilibrium, and k_{obs} represents the apparent observed kinetic rate constant. The fitting procedure based on the nonlinear least squares method provided the values of parameters f_{eq} and k_{obs} best approximating the experimental traces. This calculation was carried out for each binding curve, resulting in the set of variable c_{rec} values (concentrations of receptor binding sites) and the corresponding k_{obs} parameters. Furthermore, the kinetic association (k_a) and kinetic dissociation (k_d) rate constants characterizing the interaction of cryptogein with its receptor were calculated using linear regression as slope and intercept, respectively, from the linear dependence of k_{obs} on the concentration of the receptor c_{rec}:

$$k_{obs} = k_a c_{rec} + k_d. \quad (2)$$

Finally, the values of the kinetic equilibrium association (K_a) and kinetic equilibrium dissociation (K_d) constants were calculated:

$$K_d = 1/K_a = k_d/k_a. \quad (3)$$

All calculations were carried out using the curve-fitting module from Origin (Microcal, USA).

Results and discussion

Characterization of plasma membranes containing receptor

For experiments involving membrane-based receptor, purification of the receptor in the native state and proper stabilization of its activity for the interaction are significant problems [21,22]. For this reason, the membrane vesicles containing the studied receptor were used here. The rather crude mixture used for measurements is not a problem; however, the concentration of (active) receptor binding sites should be exactly known for correct interpretation of the kinetic data. In addition, information on the orientation of the membrane vesicles (right side in/right side out [RSI/RSO]) is also important because the RSI vesicles are useless for the evaluation and only increase the background nonspecific signal. Using the measurement of ATPase activity, the RSO orientation of the plasma membrane vesicles was more than 90% for freshly isolated membranes. However, even a single freezing–thawing cycle changed this completely, providing nearly 100% RSI vesicles that were useless for bioaffinity measurements. Therefore, the biosensor experiments always involved freshly prepared membranes.

To calculate the rate constants, the concentration of cryptogein binding sites on tobacco plasma membrane should be known. Cryptogein was labeled by FITC, and it was verified that labeling did not influence its biological activity on the tobacco cell suspension. From the binding experiment of the cryptogein–FITC conjugate with plasma membranes, the number of specific binding sites was determined as 136 pmol/mg total plasma membrane proteins. This result is much higher than the previously reported value of 0.22 pmol/mg [9,19]. This substantial difference could be explained by different starting material used for plasma membrane preparation because previously the leaves were used and different procedures were used [9]. The chosen fluorescence-based method quantified only the active receptor binding sites, and nonspecific binding was carefully excluded. Furthermore, because the cryptogein–FITC cannot pass through the membrane, only the correctly oriented binding sites were assayed and the orientation of membrane vesicles was verified as well. Because the above given value

was obtained repeatedly, it was considered as correct and used for the following affinity calculations.

Biosensor interaction studies

Previously, the equilibrium binding constant for the interaction of cryptogein with its receptor was obtained using radioactive labeled iodinated cryptogein [9,19]. To obtain additional information for the interaction, including the kinetic rate constants, the piezoelectric sensor was adopted as a convenient tool for real-time biointeraction studies. In general, either the receptor or cryptogein should be immobilized on the sensing surface for such measurements. Here cryptogein was covalently attached to the surface using its amino groups (lysine residues or the N terminus). In this way, a robust sensing surface that was suitable for repeated use was obtained.

The binding of compounds to the piezoelectric sensor providing surface-bound affinity complexes results in a decrease of frequency; thus, this method allows monitoring the process of receptor–ligand binding in real time [23]. Fig. 1 presents the schematic arrangement of experiments, with cryptogein being covalently immobilized on the surface and plasma membrane free in solution. The binding measurements were carried out in the flow-through mode; a typical experimental trace is shown in Fig. 2. After the introduction of the plasma membrane-containing sample zone, a sharp and fast change of signal represented change of viscosity due to the presence of vesicles in the solution. This part of the signal was not considered for kinetic studies because it represents only physical change of the solution surrounding the sensing surface. Then a gradual decrease of frequency represented formation of affinity complexes between immobilized cryptogein and receptor binding sites from the membrane vesicles. This part (enclosed inside the rectangular area of Fig. 2) was used as the main binding trace and further served to generate the kinetic parameters. However, even the affinity interaction involving surface-bound cryptogein and the receptor embedded in membrane vesicles was rather complex because the association passed through a minimum frequency and then the frequency started rising even though the membrane vesicles were present. It is assumed that the surface-captured membrane vesicle (Fig. 1B) was gradually changing its configuration; more receptors in the same vesicle might form complexes with the surface molecules of cryptogein, thereby affecting the shape of the vesicle. Consequently, the modified surface viscoelasticity resulted in an opposite change of frequency. In addition, the surface-bound vesicle can be damaged by the flowing solution leaving only membrane fragments with receptors on the biosensing surface. This process can again result in an increase of frequency due to decreased surface mass.

When the buffer zone replaced the membrane vesicle-containing zone, the frequency increased sharply due to a bulk viscosity decrease in the absence of vesicles; however, a significant decrease of frequency versus the original baseline indicated tightly bound affinity complexes on the surface, dissociating rather slowly. The original baseline was renewed after dissolution of the bound receptor-containing membrane vesicles in the presence of a potent detergent (1% SDS). To confirm specific formation of the affinity complex, the carrier buffer containing 1 μM free cryptogein was introduced after the zone of membrane vesicles and complete release of the bound receptor-containing membranes was achieved in 2 min.

To study kinetics of interaction of cryptogein with membrane-containing receptor, freshly prepared membranes were used at different concentrations of total protein in the sample zone; in this way, different concentrations of the receptor (c_{rec}) were generated. Fitted zones of signals obtained in this way are overlaid in Fig. 3. Fitting of the measured frequency versus time values to Eq. (1) using nonlinear regression provided the values of k_{obs} parameters corresponding to each c_{rec} value. Finally, plotting of the k_{obs} values against molar concentrations of cryptogein binding receptor sites on the membrane (c_{rec}, determined independently using fluores-

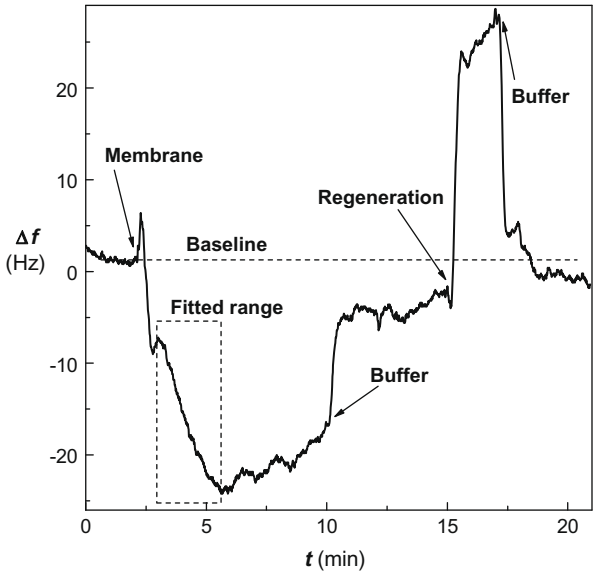

Fig. 2. Example of a real trace of frequency change in time for the typical bioaffinity experiment. The piezoelectric crystal with covalently attached cryptogein was allowed to stabilize the baseline signal, and the membrane diluted in the carrier buffer was introduced. After 30 s (due to a sharp change of frequency caused by viscosity of the solution containing membrane vesicles), the fitted range of signal started and the association data were collected for 2 min (indicated by the rectangular area). The membrane zone lasted for 8 min and was followed by buffer (washing out of the cell the solution containing membrane vesicles), regeneration (in the presence of SDS, the affinity complex is broken and membrane fragments and receptor molecules are washed away), and finally buffer again to recover the initial baseline signal.

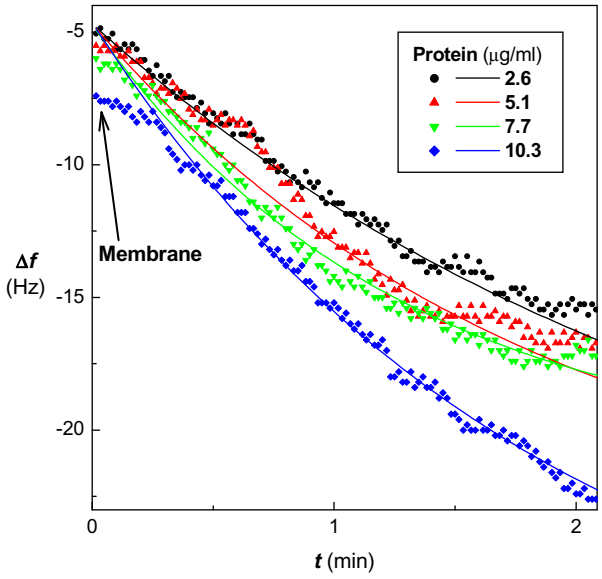

Fig. 3. Fitted parts of the association traces (similar to inside the rectangular area of Fig. 2) obtained for increasing concentrations of plasma membranes containing increasing amount of receptor molecules given by the c_{rec} values. The symbols represent measured values of frequency, and the lines indicate fitted data obtained using Eq. (1) and based on the optimized parameters k_{obs} and f_{eq} for each concentration of the receptor (c_{rec}).

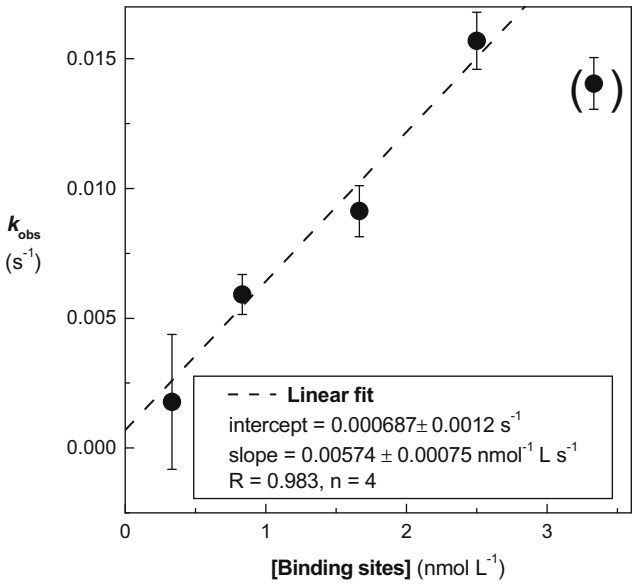

Fig. 4. Plot of the apparent association constants (k_{obs}, results of fitting the association traces to Eq. 1, as shown in Fig. 3) against the corresponding molar concentration of the cryptogein binding receptor sites (c_{rec}). The dashed line represents the linear fit of the values (with errors considered as weights), and parameters of the fit are indicated inside the graph. The experimental point obtained for the highest concentration (enclosed in parentheses) was evidently far away from the straight line and, thus, was not included in the linear regression.

cently labeled cryptogein) is presented in Fig. 4. The symbol enclosed in parentheses was not considered; for the highest concentration of receptors, the diffusion limitation started to negatively limit the binding kinetics. The values of slope and intercept indicated in Fig. 4 directly correspond to the kinetic rate association (k_a) = $5.74 \cdot 10^6$ M^{-1} s^{-1} and kinetic rate dissociation (k_d) = $6.87 \cdot 10^{-4}$ s^{-1} constants, respectively (Eq. 2). Thus, the values of kinetic equilibrium constants for the interaction were $K_A = 8.36 \cdot 10^9$ M^{-1} and K_D = 12.0 nM. The literature value of K_D = 2 nM [19] was six times lower than that found here. On the other hand, the obtained value of k_a was substantially higher than the corresponding value from the literature ($k_a = 1.35 \cdot 10^5$ M^{-1} s^{-1}, where the previous studies [9,19] used another approach based on radioactive labels. Here the exact concentration of binding sites was carefully determined, providing only the number of binding sites accessible on the outer surface of membrane vesicles. Furthermore, kinetic measurements were always realized using freshly prepared and correctly oriented membrane vesicles so as to avoid spontaneous reorientation due to storage in the frozen state.

Finally, the sensing surface of the piezoelectric transducer was modified with alternative ligands, namely, mutated forms of cryptogein described previously [18]. The sensing surfaces always contained a similar density of cryptogein molecules to allow comparison of binding rates. The k_{obs} constants were obtained (Eq. 1) using the same concentration of receptor binding sites for each biosensing surface and were used for comparison (Table 1). All mutant cryptogein variants provided significantly, but only slightly, lower rate of complex formation, with the only exception being the L19R cryptogein variant, where the binding rate was 18 times slower compared with the wild-type cryptogein. Thus, position 19 seems to be very critical for studied affinity interaction. This result is in very good agreement with previously obtained results, where the L19R mutations resulted in the loss of ability to bind sterols connected with no oxidative burst and pH changes. Also this result supports the importance of the ω loop conformation induced by the sterol binding for cryptogein receptor complex formation.

Table 1
Comparison of affinities of immobilized mutant cryptogeins for the membrane receptor in solution (protein concentration = 198 μg ml^{-1}).

Immobilized mutant cryptogein	k_{obs} (10^{-3} ± SD)
L19R	0.45 ± 0.17
M35F, M59W	3.48 ± 0.09
M35W, M59W	4.36 ± 0.19
X24	6.10 ± 0.27
Cryptogein (wild type)	7.92 ± 0.40

Note. The apparent rate binding constant (k_{obs}) describes the rate of formation of the cryptogein–receptor complex. SD indicates standard deviations (determined similarly as shown in Figs. 3 and 4, using piezoelectric sensors with immobilized mutant versions of cryptogein).

Conclusions

The interaction of the elicitor cryptogein with its membrane receptor was studied using the piezoelectric biosensor in real time. The receptor embedded in membrane vesicles was preserved in native conditions and in correctly oriented vesicles. The amount of freely accessible receptor binding sites was quantitatively determined using fluorescently labeled cryptogein; thus, the obtained concentration was applied for evaluation of the kinetic studies. The interaction of the cryptogein molecule immobilized covalently on the sensing surface with receptor in solution was characterized using kinetic rate and equilibrium constants. The chosen technology—piezoelectric quartz crystal resonators as transducer—was well compatible with the complex system of membrane vesicles containing receptor binding sites. The whole procedure was quite robust, and the biosensing surface was successfully used repeatedly.

Acknowledgments

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References

[1] M. Ponchet, F. Panabieres, M.L. Milat, V. Mikes, J.L. Montillet, L. Suty, C. Triantaphylides, Y. Tirilly, J.P. Blein, Are elicitins cryptograms in plant-oomycete communications?, Cell Mol. Life Sci. 56 (1999) 1020–1047.
[2] P. Ricci, P. Bonnet, J.C. Huet, M. Sallantin, F. Beauvaiscante, M. Bruneteau, V. Billard, G. Michel, J.C. Pernollet, Structure and activity of proteins from pathogenic fungi *Phytophthora* eliciting necrosis and acquired resistance in tobacco, Eur. J. Biochem. 183 (1989) 555–563.
[3] L.M. Yu, Elicitins from *Phytophthora* and basic resistance in tobacco, Proc. Natl. Acad. Sci. USA 92 (1995) 4088–4094.
[4] J.P. Blein, M.L. Milat, P. Ricci, Responses of cultured tobacco cells to cryptogein, a proteinaceous elicitor from *Phytophthora cryptogea:* Possible plasmalemma involvement, Plant Physiol. 95 (1991) 486–491.
[5] P. Bonnet, E. Bourdon, M. Ponchet, J.P. Blein, P. Ricci, Acquired resistance triggered by elicitins in tobacco and other plants, Eur. J. Plant. Pathol. 102 (1996) 181–192.
[6] H. Keller, J.P. Blein, P. Bonnet, P. Ricci, Physiological and molecular characteristics of elicitin-induced systemic acquired resistance in tobacco, Plant Physiol. 110 (1996) 365–376.
[7] G. Boissy, E. deLaFortelle, R. Kahn, J.C. Huet, G. Bricogne, J.C. Pernollet, S. Brunie, Crystal structure of a fungal elicitor secreted by *Phytophthora cryptogea*, a member of a novel class of plant necrotic proteins, Structure 4 (1996) 1429–1439.
[8] J.C. Devergne, P. Bonnet, F. Panabieres, J.P. Blein, P. Ricci, Migration of the fungal protein cryptogein within tobacco plants, Plant Physiol. 99 (1992) 843–847.
[9] D. Wendehenne, M.N. Binet, J.P. Blein, P. Ricci, A. Pugin, Evidence for specific, high-affinity binding sites for a proteinaceous elicitor in tobacco plasma membrane, FEBS Lett. 374 (1995) 203–207.
[10] E. Tavernier, D. Wendehenne, J.P. Blein, A. Pugin, Involvement of free calcium in action of cryptogein, a proteinaceous elicitor of hypersensitive reaction in tobacco cells, Plant Physiol. 109 (1995) 1025–1031.

[11] A. Pugin, J.M. Frachisse, E. Tavernier, R. Bligny, E. Gout, R. Douce, J. Guern, 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 (1997) 2077–2091.
[12] M.P. Viard, F. Martin, A. Pugin, P. Ricci, J.P. Blein, Protein phosphorylation is induced in tobacco cells by the elicitor cryptogein, Plant Physiol. 104 (1994) 1245–1249.
[13] H. Barbier-Brygoo, J. Joyard, A. Pugin, R. Ranjeva, Intracellular compartmentation and plant cell signaling, Trends Plant Sci. 2 (1997) 214–222.
[14] L. Suty, A.S. Petitot, D. Lecourieux, J.P. Blein, A. Pugin, Isolation of partial length cDNAs corresponding to early differentially expressed genes during elicitation of tobacco cells by cryptoge: Use of differential mRNA display, Plant Physiol. Biochem. 34 (1996) 443–451.
[15] A.S. Petitot, J.P. Blein, A. Pugin, L. Suty, Cloning of two plant cDNAs encoding a β-type proteasome subunit and a transformer-2-like SR-related prote: Early induction of the corresponding genes in tobacco cells treated with cryptogein, Plant Mol. Biol. 35 (1997) 261–269.
[16] E. Tavernier, V. Stallaert, J.P. Blein, A. Pugin, Changes in lipid composition in tobacco cells treated with cryptogein, an elicitor from *Phytophthora cryptogea*, Plant Sci. 104 (1995) 117–125.
[17] M.L. Milat, P. Ricci, P. Bonnet, J.P. Blein, Capsidiol and ethylene production by tobacco cells in response to cryptogein, an elicitor from *Phytophthora cryptogea*, Phytochemistry 30 (1991) 2171–2173.
[18] J. Lochman, T. Kasparovsky, J. Damborsky, H. Osman, A. Marais, R. Chaloupkova, M. Ponchet, J.P. Blein, V. Mikes, Construction of cryptogein mutants, a proteinaceous elicitor from *Phytophthora*, with altered abilities to induce a defense reaction in tobacco cells, Biochemistry 44 (2005) 6565–6572.
[19] S. Bourque, M.N. Binet, M. Ponchet, A. Pugin, A. Lebrun-Garcia, Characterization of the cryptogein binding sites on plant plasma membranes, J. Biol. Chem. 274 (1999) 34699–34705.
[20] M.A. Cooper, Advances in membrane receptor screening and analysis, J. Mol. Recognit. 17 (2004) 286–315.
[21] I. Navratilova, J. Sodroski, D.G. Myszka, Solubilization, stabilization, and purification of chemokine receptors using biosensor technology, Anal. Biochem. 339 (2005) 271–281.
[22] I. Navratilova, M. Pancera, R.T. Wyatt, D.G. Myszka, A biosensor-based approach toward purification and crystallization of G protein-coupled receptors, Anal. Biochem. 353 (2006) 278–283.
[23] P. Skladal, Piezoelectric quartz crystal sensors applied for bioanalytical assays and characterization of affinity interactions, J. Braz. Chem. Soc. 14 (2003) 491–502.
[24] H. Osman, S. Vauthrin, V. Mikes, M.L. Milat, F. Panabieres, A. Marais, S. Brunie, B. Maume, M. Ponchet, J.P. Blein, Mediation of elicitin activity on tobacco is assumed by elicitin–sterol complexes, Mol. Biol. Cell 12 (2001) 2825–2834.
[25] M.T. Chandler, N. Tandeau de Marsac, Y. Kouchkovsky, Photosynthetic growth of tobacco cells in liquid suspension, Can. J. Bot. 50 (1972) 2265–2270.
[26] S. Widell, T. Lundborg, C. Larsson, Plasma membranes from oats prepared by partition in an aqueous polymer 2-phase system: On the use of light-induced cytochrome B reduction as a marker for the plasma membrane, Plant Physiol. 70 (1982) 1429–1435.
[27] G.T. Hermanson, Bioconjugate Techniques, Academic Press, San Diego, 1996.
[28] G.L. Peterson, Simplified method for analysis of inorganic phosphate in presence of interfering substances, Anal. Biochem. 84 (1978) 164–172.
[29] S.R. Gallagher, R.T. Leonard, Effect of vanadate, molybdate, and azide on membrane-associated ATPase and soluble phosphatase activities of corn roots, Plant Physiol. 70 (1982) 1335–1340.