Evidence for specific, high-affinity binding sites for a proteinaceous elicitor in tobacco plasma membrane

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Abstract Binding of cryptogein, a proteinaceous elicitor, was studied on tobacco plasma membrane. The binding of the [^{125}I]cryptogein was saturable, reversible and specific with an apparent K_d of 2 nM. A single class of cryptogein binding sites was found with a sharp optimum pH for binding at about pH 7.0. The high-affinity correlates with cryptogein concentrations required for biological activity in vivo.

Key words: Nicotiana tabacum; Cryptogein; Elicitor; Plasma membrane; Receptor

1. Introduction

Plants protect themselves from pathogen attacks by various defense responses such as production of antimicrobial phytoalexins and the reinforcement of the plant cell wall [1,2]. These inducible reactions can be triggered by various compounds, known as elicitors, isolated from pathogenic microorganisms as well as host plants. It is assumed that these elicitor molecules initially interact with membrane receptors required for the initiation of biochemical events leading to the expression of resistance in plant. However, mechanisms underlying elicitor perception on the plant cell surface and subsequent intracellular transmission of this signal are poorly understood. Evidence for the existence of elicitor high-affinity binding sites was initially obtained with carbohydrate elicitors [3,4,5,6]. High-affinity binding sites were also observed with glycoprotein elicitors, the carbohydrate moiety of which was the structure required for the interaction with the binding sites [7]. More recently, Nürnberger et al. [8] reported the existence of a novel class of elicitor binding sites which interact with a peptide sequence of a glycoprotein elicitor.

For the past few years, we have been studying the biological effects of elicitins, a novel class of protein elicitors which are produced by *Phytophthora* species. Elicitins constitute a family of 10 kDa holoproteins that cause leaf necrosis and induce systemic acquired resistance of tobacco plants against fungal and bacterial pathogens [9]. Our attention is particularly focussed on cryptogein, a basic elicitin produced by *P. cryptogea* that causes hypersensitive reaction-like necrosis in tobacco (*Nicotiana tabacum*) plants [10]. Cryptogein induces a series of metabolic events in tobacco cells such as accumulation of phytoalexins, ethylene production, fast changes in ion fluxes through the plasma membrane, and a rapid but transient production of active oxygen species [11,12,13]. Moreover, a phosphorylation/dephosphorylation process participates in the transduction of the cryptogein signal leading to a defense response in tobacco plants [14,15]. More recently, we showed that a fast and large influx of Ca^{2+}, which took place after protein phosphorylation and prior to the previously reported effects of cryptogein, was essential for the induction of the elicitor signal [15]. These effects were consistent with the presence of a cryptogein receptor-like binding factor on the plasma membrane. In the present study, we report the existence of specific, high-affinity binding sites for cryptogein in tobacco plasma membrane. Our results indicate that cryptogein specific binding sites show many of the characteristics expected for an elicitor receptor.

2. Materials and methods

2.1. Plant material and elicitor

Tobacco plants (*Nicotiana tabacum* cv. Xanthi nc) were grown in a greenhouse for 60 days (25°C, 16 h day-length). Cryptogein was purified according to Ricci et al. [9].

2.2. Plasma membrane preparation

Plasma membrane-enriched fractions were obtained following the aqueous partitioning procedure described by Widell et al. [16]. All the steps of isolation were performed at 4°C. Tobacco leaves (200 g) were homogenized in 400 ml of grinding medium (50 mM Tris-MES pH 8.0, 500 mM sucrose, 20 mM EDTA, 10 mM DTT, and 1 mM PMSF) using a Waring blender-homogenizer. The homogenate was filtered through a nylon screen (pore size, 75 μm) and then centrifuged at 16,000 × *g* for 20 min. After centrifugation, the supernatants were collected, filtered through two successive screens (pore size, 63 and 38 μm, respectively) and centrifuged at 96,000 × *g* for 35 min. The resulting supernatants were discarded and the pellets were suspended in the PSK buffer (5 mM phosphate buffer pH 7.8, 0.3 M sucrose, 3 mM KCl). Then, the microsomal fraction was added to an aqueous polymer two-phase system with final concentrations of 6.6% (w/w) dextran (500,000 MW), 6.6% (w/w) polyethylene glycol (3350 MW) in PSK buffer. The solution was mixed by 40 inversions of the tube and centrifuged in a swing out rotor at 1,200 × *g* for 5 min. The upper phase was carefully removed and washed two times with an equal volume of fresh lower phase. The last upper phase was diluted with 5 volumes of washing buffer (10 mM Tris-MES pH 7.3, 250 mM sucrose, 1 mM ATP, 1 mM EDTA, 1 mM DTT, and 1 mM PMSF) and centrifuged at 95,000 × *g* for 35 min. The pellet was suspended in the washing buffer and centrifuged at 120,000 × *g* for 40 min. The resulting pellet was resuspended in 10 mM Tris-MES pH 6.5, 250 mM sucrose, 1 mM PMSF, 2 mM $MgCl_2$, and 20% glycerol, and stored at −80°C. Protein content of the plasma membrane preparation was measured according to Lowry [17] using BSA as the standard and purity was determined using marker enzymes of other cell compartments as described by Hall [18]. The extent of contamination did not exceed 5%.

2.3. Radioiodination of cryptogein

Iodination of cryptogein was performed as described by Hulme [19]. Briefly, cryptogein (100 μg) was incubated 20 min at 20°C in 100 μl of 0.1 M phosphate buffer (pH 7.4) with 5 mCi Na^{125}I (Amersham) and iodogen as the catalyst. The ^{125}I-labeled cryptogein ([^{125}I]cryptogein)

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was purified by gel filtration on a G-25 Sephadex column (5 ml) equilibrated with 50 mM Tris-HCl buffer (pH 7.4). Fractions containing the radioactive peak were pooled and stored at −20°C.

2.4. Binding experiments

Plasma membrane preparations containing about 50 μg protein were suspended in a total volume of 100 μl of binding buffer (25 mM Tris-MES pH 7.0, 5 mM $MgCl_2$, 0.1 M sucrose and 0.1% BSA) and preincubated on ice for 30 min. Binding of [^{125}I]cryptogein was carried out for 90 min on ice after addition of an appropriate concentration of radiolabeled ligand. Non-specific binding was determined in presence of 10 μM unlabeled cryptogein. For competition experiments, an appropriate amount of unlabeled ligand was added to the reaction mixture. Binding assays were stopped by rapid filtration under vacuum through GF/B glass-fiber filters (Whatman) presoaked 60 min in 1% BSA. Then, the filters were immediately washed three times with 5 ml of ice-cold binding buffer. Radioactivity remaining on filters was measured directly with a gamma counter.

3. Results

Cryptogein was iodinated for binding studies. The specific radioactivity of the labeled ligand was about 250 Ci/mmol. This high activity was well suited for binding experiments. The labeling of cryptogein did not affect the previously described effects of the elicitor on tobacco cells (data not shown) and [^{125}I]cryptogein was stable over a period of one month at −20°C. The [^{125}I]cryptogein elicitor was tested for binding to purified tobacco plasma membrane using vacuum filtration to separate free from bound ligand. The filtration method reduces the loss of bound labeled ligand caused by rapid dissociation of the receptor–ligand complex [19]. Non-specific binding was determined in the presence of a 1000-fold molar excess of unlabeled elicitor (about 10 μM) and specific binding was obtained by subtracting non-specific binding from total binding.

Ligand saturation experiments were performed by incubating plasma membrane preparations with increasing concentrations (1–30 nM) of [^{125}I]cryptogein (Fig. 1). The saturation curve was hyperbolic, giving a half-maximal binding of cryptogein at about 2 nM. Saturability of specific binding sites was reached between 5 and 10 nM [^{125}I]cryptogein. No further increase in specifically bound ligand was observed at higher radioligand concentrations in the range of 50–1000 nM (data not shown). This demonstrated that the specific binding of the radioiodinated cryptogein was saturable. The non-specific binding, on the contrary, increased resulting in a rise of total binding (Fig. 1, inset). The non-specific binding represented about 50% of the total binding for ligand concentrations lower than 5 nM. The addition of 50 mM NaCl to the assay mixture reduced this non-specific binding but also abolished the specific binding of cryptogein to the plasma membrane (data not shown). A Scatchard plot of the binding data gave a straight line (Fig. 2A), indicating the presence of a single class of binding sites for cryptogein with a K_d value of 2 nM and an apparent

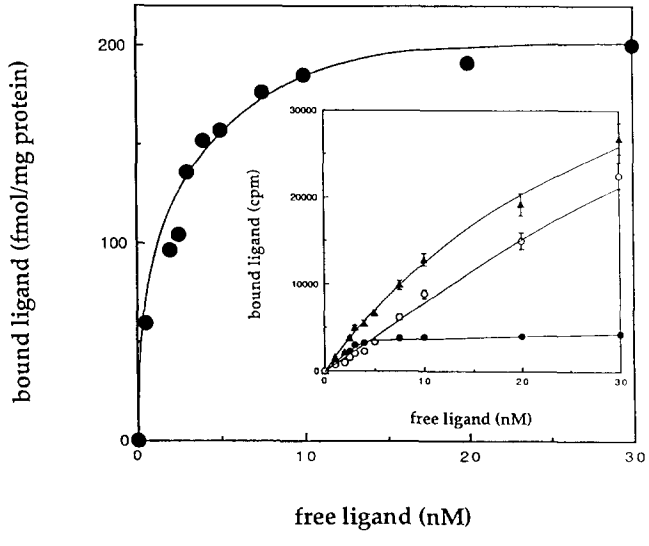

Fig. 1. Saturability of the binding of [^{125}I]cryptogein to plasma membrane. Specific binding was measured in a [^{125}I]cryptogein range of 1–30 nM, using 50 μg of plasma membrane protein per assay. Non-specific binding was measured in the presence of 10 μM unlabeled ligand. The inset shows the total (▲), non-specific (○) and specific binding (●) of cryptogein to plasma membrane. The experiment was repeated five times. The figure corresponds to a representative experiment and the results shown are the means ± S.D. for triplicate determination.

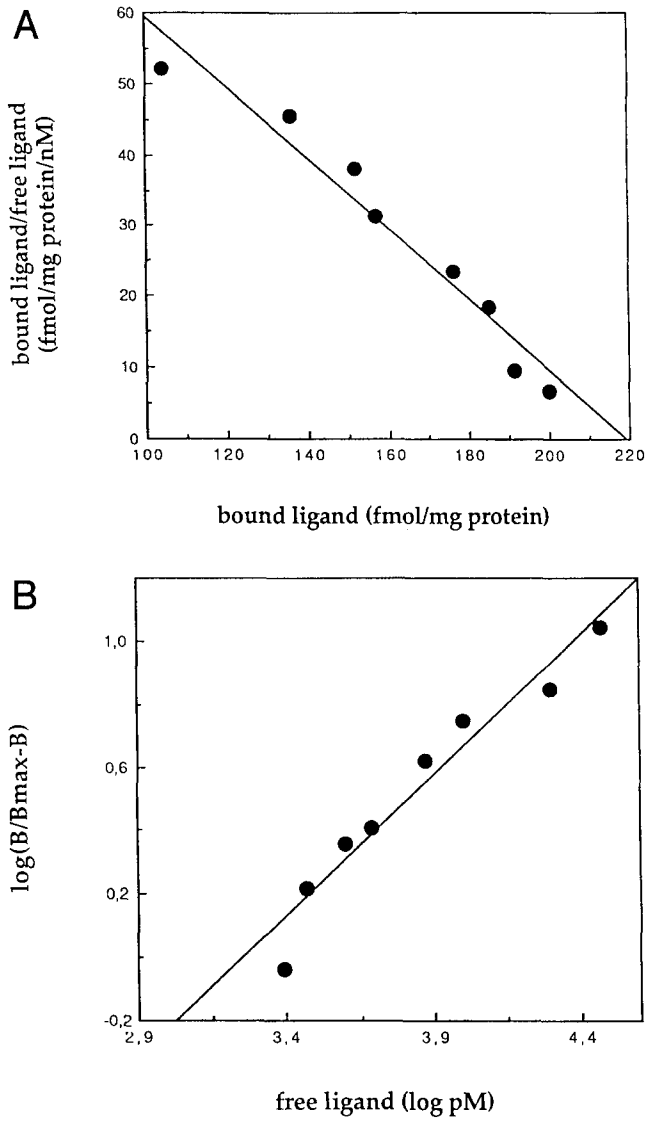

Fig. 2. Scatchard plot (A) and Hill plot (B) of the specific binding data (reported Fig. 1).

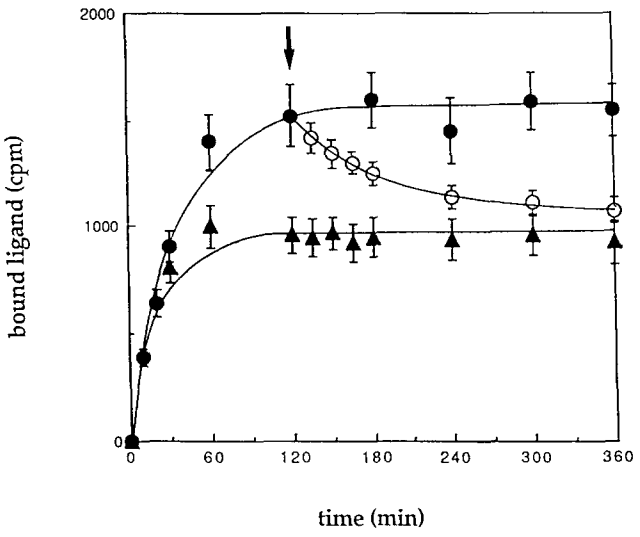

Fig. 3. Kinetics of specific association and dissociation of [^{125}I]cryptogein to plasma membrane. Plasma membrane (50 μg protein) was incubated with 2 nM [^{125}I]cryptogein for increasing periods of time to determine the association kinetics (●). Dissociation kinetics (○) were initiated 120 min later by the addition of 10 μM of unlabeled cryptogein (arrow). Specific binding was obtained by subtracting non-specific binding (▲) from total binding. The experiment was repeated five times. The figure corresponds to a representative experiment and the results shown are the means ± S.D. for triplicate determination.

concentration of binding sites of 220 fmol/mg plasma membrane proteins. The Hill plot (Fig. 2B) obtained from the binding data gave a Hill coefficient of 0.91, excluding cooperativity in binding of cryptogein. These results indicated the presence of binding sites with high-affinity for cryptogein but in relatively low abundance.

The kinetics of binding and dissociation of [^{125}I]cryptogein with plasma membrane binding sites are shown in Fig. 3. Association of cryptogein to its binding sites was faster than dissociation. At 4°C, maximum binding was attained within 60 min and half-maximum binding was obtained within 25 min. Non-specific binding remained almost constant throughout this period. Addition of an excess of unlabeled ligand 120 min after the addition of [^{125}I]cryptogein resulted in a dissociation of bound radioligand, demonstrating the reversibility of cryptogein binding. These experiments allowed calculation of the rate constants for association and dissociation to be 8.1×10^6 $M^{-1} \cdot min^{-1}$ and 1.38×10^{-2} min^{-1}, respectively. These results indicated that the K_d value given by the rate constant ratio (1.7 nM) was closely related to the K_d value calculated from saturation data (2 nM). Fig. 4 shows the effects of pH on [^{125}I]cryptogein binding to tobacco plasma membrane. Specific binding was found to be pH dependent with a sharp optimum binding at pH 7. The binding strongly decreased at pH values below 6 and above 7.5. Non-specific binding remained constant in the same range of pH values (data not shown).

Competition experiments were carried out by incubating plasma membrane preparations with increasing concentrations of unlabeled cryptogein in the presence of [^{125}I]cryptogein at 1, 2.5, 5 and 10 nM (Fig. 5A). These experiments could not be performed with [^{125}I]cryptogein concentrations below 1 nM because the sensitivity of detection was too weak for binding measures in these conditions. The competition curves shifted in a parallel fashion to the right on the concentration axis as the [^{125}I]cryptogein concentration increased, giving half-maximal effector concentration (IC_{50}) of 160, 230, 310 and 680 nM for [^{125}I]cryptogein concentration of 1, 2.5, 5 and 10 nM, respectively. The result of plotting IC_{50} values against [^{125}I]cryptogein concentrations revealed a linear relationship (Fig. 5B). The IC_{50} value corresponding to a minimum theoretical IC_{50}, or maximum binding affinity of the unlabeled ligand (K_a) was calculated to be 90 nM. The slope of the straight line (K_a/K_d) provided a K_d value of 1.51 nM in good agreement with the K_d value deduced from ligand saturation or kinetics experiments. The same experiments were carried out using unlabeled iodinated cryptogein instead of cryptogein to check the influence of iodination on affinity. Identical results were obtained (data not shown): both competitors inhibited the [^{125}I]cryptogein binding in the same manner indicating that both competitors had the same affinity for binding sites on plasma membrane. However, in our experimental conditions, the affinity of both competitors (unlabeled iodinated cryptogein and cryptogein) was lower than the theoretical prediction in which the K_a and K_d values are equal.

4. Discussion

The present study demonstrates the presence of high-affinity binding sites for cryptogein in tobacco plasma membrane. Cryptogein binding showed several properties expected from a ligand–receptor interaction [19] such as saturability, reversibility, high-affinity binding and pH dependence. In addition, the specific binding of [^{125}I]cryptogein was shown to be competable with unlabeled iodinated cryptogein or cryptogein, indicating that these compounds had complete access to all binding sites

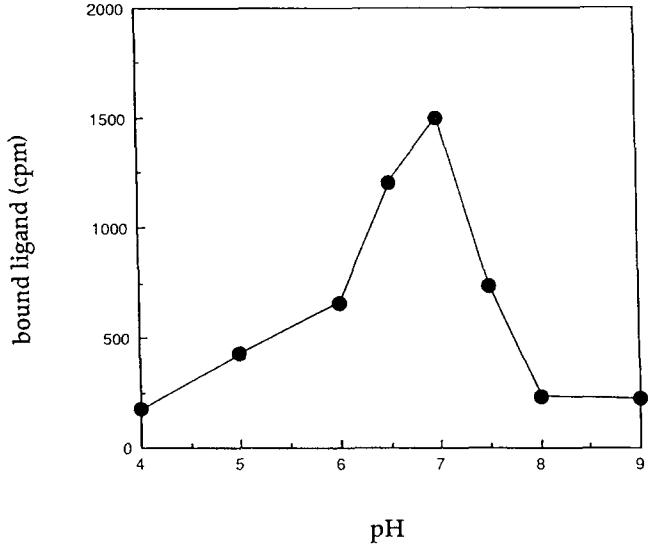

Fig. 4. Effect of pH on the specific binding of [^{125}I]cryptogein to plasma membrane. Plasma membrane (50 μg protein) was incubated with 2 nM [^{125}I]cryptogein. The following buffers were used: citrate (pH 4); Tris-MES (pH 5–8); Tris-HCl (pH > 8); buffer strength = 25 mM. Values for specific binding were calculated as in Fig. 1. The experiment was repeated three times. The figure corresponds to a representative experiment and each data point represents the average of three replicates.

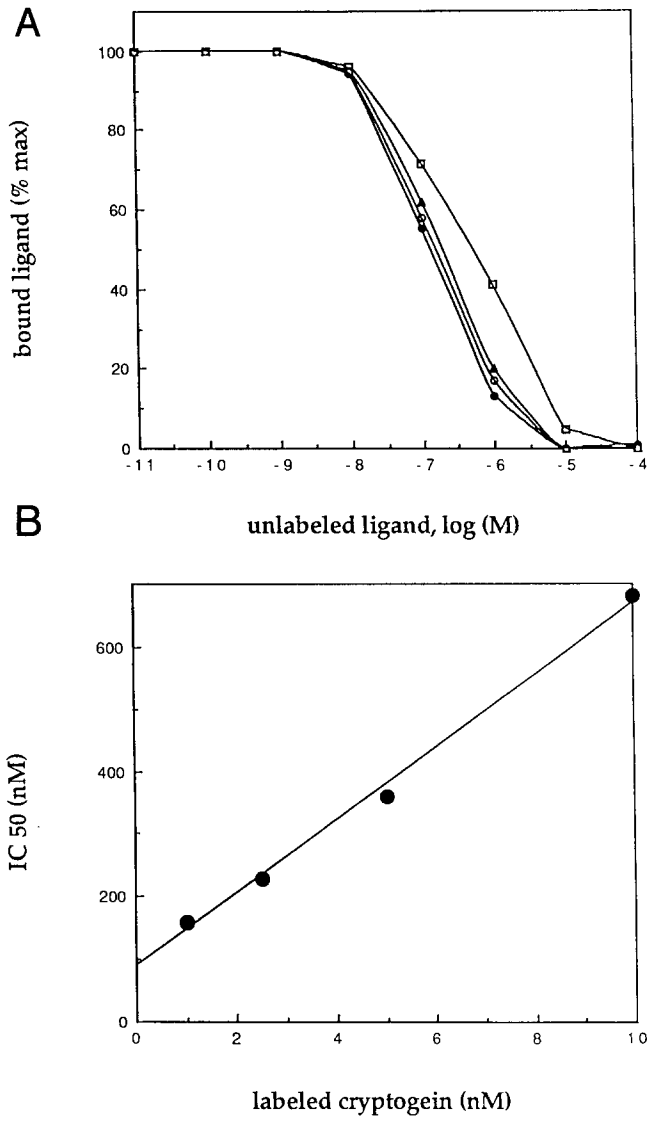

Fig. 5. (A) Inhibition of the binding of [^{125}I]cryptogein to plasma membrane by increasing concentrations of cryptogein. Plasma membrane (50 μg protein) was incubated with 1 (●), 2.5 (○), 5 (▲) and 10 nM (□) [^{125}I]cryptogein and increasing concentrations of unlabeled ligand. For normalization, all data for specific binding are expressed as a percentage of the specific binding of [^{125}I]cryptogein alone. The experiment was repeated three times. The figure corresponds to a representative experiment and data are the average of three replicates. (B) The IC_{50} against [^{125}I]cryptogein concentration plot of the data presented in Fig. 5A ($IC_{50} = K_a + K_a[L]/K_d$; K_a is the maximum binding affinity of the unlabeled ligand).

available to the labeled ligand. However, in our experimental conditions, competition results indicated that the cryptogein binding sites had a significantly lower affinity for the unlabeled iodinated cryptogein or cryptogein than for [^{125}I]cryptogein (90 nM in place of 2 nM). Although we cannot give a conclusive answer, this discrepancy could be attributed to the high non-specific binding which probably counteracted the inhibitory action of the competing ligand at low concentrations. Indeed, cryptogein may bind to non-specific acceptors or recognition sites with significant affinity as shown in Fig. 1 (inset), thus reducing the free unlabeled competing ligand concentration available for receptor binding. In this way, the overestimation of the true concentrations of the competing ligand in the assays could explain the underestimation of the unlabeled cryptogein affinity. Attempts to reduce the non-specific binding without affecting specific binding have met with limited success. In our experiments, the addition of 50 mM NaCl to the assay mixture abolished the specific binding of cryptogein to the plasma membrane (data not shown) suggesting that ionic bonds are involved in the association of cryptogein to its binding sites, as reported for some animal hormone receptors [20].

The K_d value is in good agreement with the concentrations of cryptogein required for biological activity [11,12,13,14,15]. In addition, a rapid transcriptional modification of various genes has been observed in cryptogein-treated tobacco cells at comparable concentrations [21]. This high-affinity is very similar to those found for carbohydrate elicitors [4,5,6], for glycopeptide elicitors, of which the carbohydrate moiety has been found to be sufficient for interaction between the ligand and its binding sites [7], and more recently for a fungal oligopeptide elicitor proteolytically released from a 42 kDa glycoprotein purified from the culture filtrate of *Phytophthora megasperma* f.sp.*glycinea* [8]. Moreover, such affinity is comparable to those found for numerous mammalian receptors [19].

In conclusion, this work reports the existence of cryptogein specific binding sites in tobacco plasma membrane with many of the characteristics expected for an elicitor receptor. We are now attempting to identify the cryptogein binding sites in order to elucidate the role of this molecule in signal reception and transduction pathways in plant defense response.

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References

[1] Dixon, R.A. and Lamb, C.J. (1990) Annu. Rev. Plant Physiol. Plant Mol. Biol. 41, 339–367.
[2] Ebel, J. (1986) Annu. Rev. Phytopathol. 24, 235–264.
[3] Yoshikawa, M., Keen, N.T. and Wang, M.C. (1983) Plant Physiol. 73, 497–506.
[4] Cosio, E.G., Frey, T., Verduyn, R., Boom, J. and Ebel, J. (1990) FEBS Lett. 271, 223–226.
[5] Cheong, J.J. and Hahn, M.G. (1991) Plant Cell 3, 137–147.
[6] Shibuya, N., Kaku, H., Kuchitsu, K. and Maliarik, M. (1993) FEBS Lett. 329, 75–78.
[7] Basse, C.W., Fath A. and Boller, T. (1993) J. Biol. Chem. 268, 14724–14731.
[8] Nürnberger, T., Nennstiel, D., Jabs, T., Sacks, W.R., Hahlbrock, K. and Scheel, D. (1994) Cell 78, 449–460.
[9] Ricci, P., Bonnet, P., Huet, J.C., Sallantin, M., Beauvais-Cante, F., Bruneteau, M., Billard, V., Michel, G. and Pernollet, J.C. (1989) Eur. J. Biochem. 183, 555–563.
[10] Devergne J.C., Bonnet P., Panabières F., Blein J.P. and Ricci, P. (1992) Plant Physiol. 99, 843–847.
[11] Milat, M.L., Ducruet, J.M., Ricci, P., Marty, F. and Blein, J.P. (1991) Phytopathology 81, 1364–1368.
[12] Blein, J.P., Milat, M.L. and Ricci, P. (1991) Plant Physiol. 95, 486–491.
[13] Bottin, A., Véronési, C., Pontier, D., Esquerré-Tugayé, M.T., Blein, J.P., Rusterucci, C. and Ricci, P. (1994) Plant Physiol. Biochem. 32, 373–378.
[14] Viard, M.P., Martin, F., Pugin, A., Ricci, P. and Blein, J.P. (1994) Plant Physiol. 104, 1245–1249.

[15] Tavernier, E., Wendehenne D., Blein J.P. and Pugin, A. (1995) Plant Physiol. (in press).
[16] Widell, S., Lundberg, T. and Larsson, C. (1982) Plant Physiol. 70, 1429–1435.
[17] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–272.
[18] Hall, J.L. (1983) in: Isolation of Membranes and Organelles from Plant Cells, Biological Techniques Series (Hall, J.L. and Moore, A.L. eds.) pp. 55–81, Academic Press, London.
[19] Hulme, E.C. and Birsdall, N.J.M. (1992) in: Receptor–Ligand Interactions: a Practical Approach, vol. 1 (E.C. Hulme ed.) pp. 63–176, Oxford University Press, Oxford.
[20] Cheng, K.W. (1975) Biochem. J. 148, 123–132.
[21] Suty, L., Blein, J.P., Ricci, P. and Pugin, A. (1995) Mol. Plant–Microbe Interact. (in press).