Plant Cell Physiol. 37(6): 894–898 (1996)
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Rapid Report

Localization and Binding Characteristics of a High-Affinity Binding Site for *N*-Acetylchitooligosaccharide Elicitor in the Plasma Membrane from Suspension-Cultured Rice Cells Suggest a Role as a Receptor for the Elicitor Signal at the Cell Surface

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A high-affinity binding site for *N*-acetylchitooligosaccharide elicitor was found to localize in the plasma membrane from suspension-cultured rice cells. Binding kinetics as well as the specificity of this binding site corresponded well with the behavior of the rice cells to the elicitor. These characteristics suggest that the binding site represents a functional receptor for *N*-acetylchitooligosaccharide elicitor in rice.

Key words: *N*-Acetylchitooligosaccharides — Elicitor — Host-pathogen interaction — Plasma membrane — Receptor — Rice suspension-culture.

Higher plants initiate various defense reactions when they are attacked by pathogens such as fungi, bacteria and viruses. These defense responses include the production of phytoalexins, proteinase inhibitors, hydrolases such as chitinase and β-glucanase, wall glycoproteins and lignin (Dixon and Harrison 1990, Ryan and Farmer 1991, Côté and Hahn 1994). These responses can be triggered by oligo-/polysaccharide elicitors derived from the cell surface of pathogenic microbes as well as host plants. Some of these elicitors could trigger these responses in plants at a very low concentration and their recognition by host plants seems to be very strict concerning to their structure (Cheong et al. 1991a, Côté and Hahn 1994). These results strongly indicate the presence of specific recognition systems in the host cells.

To understand the whole process of signal perception and transduction leading to the activation of specific sets of genes on molecular basis, it is critically important to know the detailed properties of the receptor molecules which perceive the elicitor signal. However, the knowledge on such receptor molecules is still very limited and none of their genes have been cloned. Only the presence of high-affinity binding sites, putative receptor molecules, for some elicitors have been reported in the membrane preparations from several plants. A high-affinity binding protein for the elicitor active hepta-β-glucoside has been detected in microsomal/plasma membrane preparation from soybean cotyledon (Yoshikawa et al. 1983, Cosio et al. 1988, 1990b, Cheong and Hahn 1991b) and solubilized (Cosio et al. 1990a, Cheong et al. 1993). Mithöfer et al. (1996) recently reported the purification of this binding protein to apparent homogeneity. The presence of high-affinity binding sites for a glycopeptide elicitor (Basse et al. 1993) and a peptide elicitor (Nürnberger et al. 1994) in the microsomal membrane preparation from suspension-cultured tomato cells and parsley cells respectively, were also reported.

Concerning to the putative receptor molecule for chitin fragment (*N*-acetylchitooligosaccharide) elicitor, another important elicitor molecule derived from the cell walls of pathogenic fungi, only two papers have been published so far (Shibuya et al. 1993, Baureithel et al. 1994). We reported the presence of a novel high-affinity binding site for *N*-acetylchitooligosaccharides in the microsomal membrane preparation from suspension-cultured rice cells (Shibuya et al. 1993), where the *N*-acetylchitooligosaccharides of certain size could induce various cellular responses including phytoalexin production (Ren and West 1992, Yamada et al. 1993), transient depolarization of membrane potential (Kuchitsu et al. 1993), ion flux (Kuchitsu, K. et al., unpublished), transient generation of reactive species of oxygen (Kuchitsu et al. 1995) and jasmonic acid (Nojiri et al. 1996), and transient expression of several unique "early responsive" genes (Minami et al. 1996) at a very low concentration such as nM. Baureithel et al. (1994) also reported the presence of a high-affinity bind-

Abbreviations: BSA, bovine serum albumin; DTT, dithiothreitol; EGTA, ethyleneglycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; $(GlcNAc)_8$-Tyr, tyramine conjugate of *N*-acetylchitooctaose; PEG, polyethyleneglycol; PMSF, phenylmethanesulfonyl fluoride; SHAM, salicylhydroxamic acid.

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ing site for *N*-acetylchitooligosaccharide elicitor in the microsomal membrane preparation from suspension-cultured tomato cells.

In this paper, we show that the binding site for this elicitor is mostly localized in the plasma membrane from rice cells and the detailed binding characteristics of this site correlate well with the behavior of rice cells with this elicitor. These results strongly indicate that this binding site represents a functional receptor for this elicitor. The results also indicate that the different specificity shown by rice and tomato cells concerning to the size of *N*-acetylchitooligosaccharides reflects the binding specificity of each binding site.

Materials and Methods—Suspension-cultured cells of *Oryza sativa* L. cv. Nipponbare were maintained using a modified N-6 medium as described previously (Kuchitsu et al. 1993). *N*-acetylchitoheptaose and *N*-acetylchitooctaose were prepared by re-*N*-acetylation of the corresponding chitosan oligomer (Yamada et al. 1993). Chitosan oligomers, $(GlcNH_2)_n$, were used as hydrochlorides. $Na^{125}I$ Solution was purchased from Amersham Japan (Tokyo, Japan). Protein was determined by the method of Bradford (1976). Hexosamine was determined by the modified method of Elson and Morgan (Roseman and Daffner 1956). Tyramine conjugate of *N*-acetylchitooctaose, $(GlcNAc)_8$-Tyr, was synthesized and radioiodinated with ^{125}I as described previously (Shibuya et al. 1993). Concentration of the $(GlcNAc)_8$-Tyr solution was determined from UV absorbance at 274 nm using the molar extinction coefficient, 1,420 $M^{-1}\,cm^{-1}$ (Cheong and Hahn 1991b). Specific activity of the ligand used for most experiments was 1.5-5.6 GBq μmol^{-1}.

The rice cells were harvested 7 days after the transfer to new medium, washed and then suspended in the homogenization buffer (50 mM MES-Tris (pH 7.6), 1 mM DTT, 5 mM EDTA, 5 mM EGTA, 20 mM NaF, 4 mM SHAM, 2 mM PMSF, 0.3 M sucrose and 0.5% BSA). They were homogenized with a mortar and a pestle and then with a glass homogenizer for 20-30 strokes. The homogenate was centrifuged at $10,000 \times g$ for 10 min at 4°C. The resulting supernatant was centrifuged at $100,000 \times g$ for 40 min at 4°C. The pelleted microsomal membrane preparation was resuspended in the microsome suspend buffer (10 mM sodium phosphate (pH 7.8) containing 0.25 M sucrose). The microsomal membrane was further fractionated by aqueous two-phase partitioning with the use of Dextran T-500 and PEG 3350 (Yoshida et al. 1983). The upper phase containing the plasma membrane was diluted with the plasma membrane suspend buffer (5 mM HEPES-bistrispropane (pH 7.0), 0.1 mM DTT and 0.25 M sucrose) and centrifuged at $150,000 \times g$ for 60 min at 4°C. Purified plasma membrane preparation was resuspended with the plasma membrane suspend buffer and stored at −80°C or in liquid nitrogen. Vanadate-sensitive Mg^{2+} ATPase, IDPase and NADPH-Cyt *c* reductase were chosen as the marker enzyme for plasma membrane, Golgi and endoplasmic reticulum, respectively and their activities were assayed as described (Kasai and Muto 1990).

An aliquot of the labeled ligand (2-10 pmol) was mixed with the plasma membrane preparation (typically 20 μg protein) and adjusted to a total volume of 300 μl with cold 25 mM Tris-HCl (pH 7.0) containing 1 M NaCl, 1 mM $MgCl_2$ and 2 mM DTT. For the inhibition analyses, appropriate amount of unlabeled ligand was added to this reaction mixture and incubated for 30 min prior to the addition of the labeled ligand. After the incubation for 1 h in an ice cold bath, the reaction mixture was divided into three aliquots (90 μl each). These aliquots were applied to the wells of Multiscreen-GV 96-well filtration plate (0.22 μm, Millipore Corp., Bedford, U.S.A.) and vacuum-filtered using a Multiscreen filtration plate assembly. After the wells were rinsed twice with 200 μl of cold binding buffer, membrane filters were punched out and the radioactivity of ^{125}I remained on the filter was directly analyzed using a gamma counter. Binding assay for the analysis of the time course of the binding was done using a conventional macro-scale filtration apparatus as described previously (Shibuya et al. 1993).

Results—Fig. 1 shows the binding of the ^{125}I-labeled $(GlcNAc)_8$-Tyr to the plasma membrane. The binding was saturable and the Scatchard plot analysis of the result showed the presence of a single class of binding site for this oligosaccharide in the plasma membrane preparation (Fig. 2). The dissociation constant, K_d, for the ligand was calculated as 29 nM. From its definition, K_d shows the concentration of the free ligand at which a half of the binding site was occupied with the ligand and the value obtained in this experi-

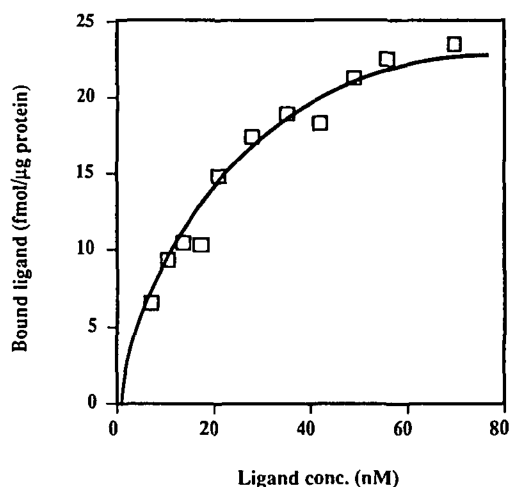

Fig. 1 Saturability of the binding of ^{125}I-$(GlcNAc)_8$-Tyr to plasma membrane. Varying amount of ^{125}I-$(GlcNAc)_8$-Tyr were reacted with plasma membrane preparation (20 μg protein). The difference between the bound ligand in the presence or the absence of 25 μM unlabeled $(GlcNAc)_7$ was taken as specific binding.

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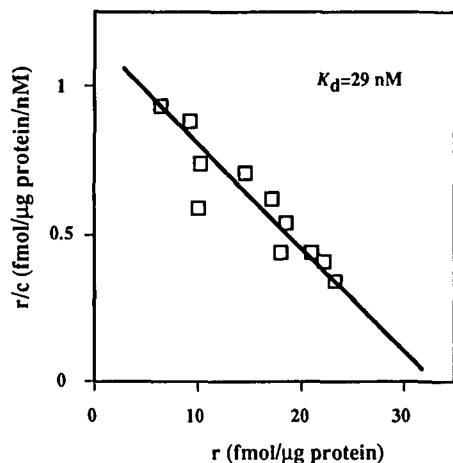

Fig. 2 Scatchard plot of the binding data. r=amount of bound ligand; c=concentration of free ligand.

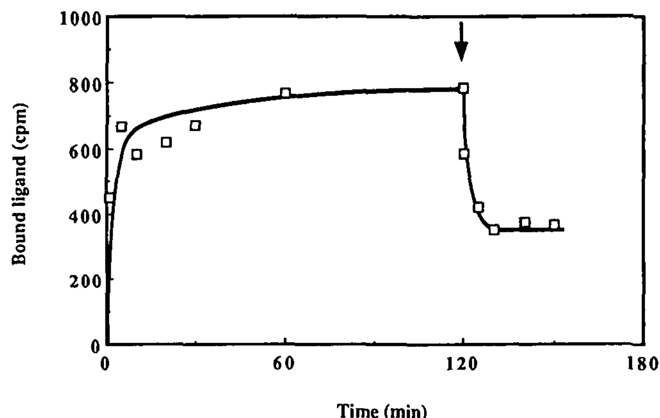

Fig. 3 Time course and reversibility of the binding. Plasma membrane preparation (20 μg protein) was incubated with 4 pmol of ^{125}I-(GlcNAc)$_8$-Tyr on ice and filtered and washed immediately after the indicated time interval. Glass fiber filters and a conventional macro-scale filtration apparatus (Shibuya et al. 1993) was used for this experiment because of quiker filtration and washing. To get specific binding, the amount of the bound ligand after 120 min incubation in the presence of 25 μM unlabeled (GlcNAc)$_7$ was subtracted from each value.

ment seems to correspond with the previous observation that the *N*-acetylchitoheptaose, the intact oligosaccharide portion of the labeled ligand, showed a half maximal elicitor activity at about 30–60 nM (Yamada et al. 1993). The K_d value obtained from this experiment was several times larger than the previous value obtained with the microsome preparation (Shibuya et al. 1993). Although the reason for the discrepancy between these results is not clear, it might reflect some changes of the affinity of the binding protein during plasma membrane preparation or the experimental error coming from the difficulty to determine the accurate specific activity of the small amount of labeled ligand.

The amount of this binding site in the plasma membrane preparation was also estimated from the Scatchard plot to be 33 pmol (mg protein)$^{-1}$, which corresponded to 110 fold enrichment compared to the value previously obtained for microsomal membrane preparation (Shibuya et al. 1993). On the other hand, the ratio of the activity of marker enzymes for plasma membrane (vanadate sensitive H^+-ATPase; 0.021 and 0.62 U (mg protein)$^{-1}$ for microsomal and plasma membrane preparation, respectively), endoplasmic reticulum (NADPH-Cyt *c* reductase; 0.071 and 0.037 U (mg protein)$^{-1}$ for microsomal and plasma membrane preparation, respectively) and Golgi body (IDPase; 0.091 and 0.029 U (mg protein)$^{-1}$ for microsomal and plasma membrane preparation, respectively) indicated 57–93 times enrichment of plasma membrane in this preparation compared to the microsomal membrane fraction. The fact that the enrichment of the binding site in the plasma membrane preparation roughly corresponded with that of the marker enzymes indicates that the binding site is mostly localized in the plasma membrane.

Fig. 3 shows the time course of the ligand binding. The binding of the labeled (GlcNAc)$_8$-Tyr to the membrane was a very rapid process even at an ice cold temperature and mostly completed within a minute, followed by the gradual increase of the bound ligand up to 60 min. Thus, all the binding experiments in this study were done with 60 min incubation on ice with the ligand. The addition of excess amount of unlabeled *N*-acetylchitohepta liberated a large part of the bound ligand from the membrane, indicating the reversible nature of the binding. However, over 40% of the bound ligand was still remained with the membrane. The reason for this is not clear at present. Similar incomplete displacement with unlabeled ligands were also observed by other groups (Cosio et al. 1988, Basse et al. 1993).

The ability of various oligosaccharides to inhibit the binding of the labeled (GlcNAc)$_8$-Tyr to the plasma membrane was analyzed to see the binding specificity of the binding site. Fig.4 shows the inhibition curves for each oligosaccharide, and the relative affinity obtained from the concentration required for 50% inhibition is also shown. *N*-Acetylchitooligosaccharides inhibited the binding of labeled (GlcNAc)$_8$-Tyr depending on their size. Larger oligosaccharides such as hexamer to octamer showed very high affinity to this binding site, whereas smaller *N*-acetylchitooligosaccharides showed much lower affinity. Also, deacetylated form of these oligosaccahrides, fragments of chitosan, did not inhibit the binding of the labeled ligand at the concentrations where the corresponding *N*-acetylated oligosaccharides inhibited the binding completely. Cellopentaose in which the *N*-acetyl groups of the *N*-acetylchitopentaose is replaced by hydroxyl groups also did not show affinity. The binding specificity obtained from

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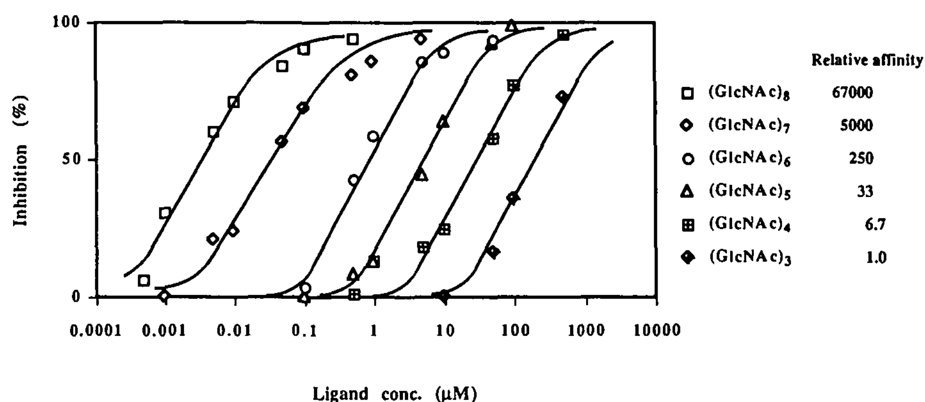

Fig 4 Inhibition of the binding of ^{125}I-(GlcNAc)$_8$-Tyr by unlabeled oligosaccharides. Plasma membrane preparation (20 μg protein) and 6 pmol of ^{125}I-(GlcNAc)$_8$-Tyr were reacted in the presence of varying amounts of unlabeled oligosaccharides. Following sugars did not show detectable inhibition at the indicated concentration. (GlcNH$_2$)$_3$, 10 mM; (GlcNH$_2$)$_4$, 1 mM; (GlcNH$_2$)$_5$ and (GlcNH$_2$)$_6$, 100 μM; (GlcNH$_2$)$_7$, 10 μM; (GlcNH$_2$)$_8$, 1 μM; cellopentaose, 1.2 mM.

these inhibition experiments corresponded well with the behavior of rice cells observed in previous experiments where only larger *N*-acetylchitooligosaccharides could induce various cellular responses (Yamada et al. 1993, Kuchitsu et al. 1993, 1995, Minami et al. 1996).

Discussion—The results obtained in this study showed that the high-affinity binding site for *N*-acetylchitooligosaccharide elicitor of rice cells mostly localized in the plasma membrane. Previous experiments showed that various cellular responses which are considered to take place at the level of plasma membrane were induced in rice cells by this elicitor molecule. They include the depolarization of membrane potential (Kuchitsu et al. 1993), ion flux (Kuchitsu, K. et al., unpublished) and generation of reactive oxygen (Kuchitsu et al. 1995). All these responses showed a very rapid and transient nature, suggesting their involvement in the early signal transduction cascade. Especially, in the case of the membrane depolarization, it started even within 30 s after the addition of the elicitor (Kikuyama, M., personal communication). The induction of such responses at the plasma membrane immediately after the perception of the elicitor signal indicates the presence of the receptor molecule also on the plasma membrane. Thus, the localization of the high-affinity binding site for the *N*-acetylchitooligosaccharide elicitor on the plasma membrane supports the possibility that it represents a true, functional receptor.

Binding specificity of this binding site also supported this possibility. In all the experiments so far established, any cellular responses of rice cells induced by the *N*-acetylchitooligosaccharide elicitor showed basically the same specificity concerning to the size and structure of the oligosaccharide. For example, the fact that the *N*-acetylchitoheptaose/octaose showed a very high elicitor activity even at a nM concentration while a smaller oligosaccharide such as trimer showed almost no activity at a much higher concentration (Yamada et al. 1993, Kuchitsu et al. 1993, 1995, Minami et al. 1996) corresponded well with the fact that the former oligosaccharides have 5,000–67,000 times higher affinity to this binding site. Also, deacetylated form of the elicitor-active oligosaccharides, chitosan fragments, showed neither the biological activity nor the affinity to this binding site. The situation was the same for cellooligosaccharides too. These results further support the possible role of this site as a functional receptor.

The *N*-acetylchitooligosaccharides were also reported to induce the alkalinization of the reaction medium and the phosphorylation of some proteins in suspension-cultured tomato cells (Felix et al. 1993). On the contrary to the rice cell system, this oligosaccharide signal did not induce later cellular responses such as the induction of ethylene biosynthesis or phenylalanine ammonia-lyase. In the case of tomato cells, the dependency of the activity on the size of *N*-acetylchitooligosaccharides was also significantly different from that of rice cells, that is, the oligosaccharides larger than trimer showed almost the same activity while much larger oligosaccharides such as heptamer or octamer showed higher elicitor activity in rice cells. As the binding site found in the membrane preparation of tomato cells showed a binding specificity corresponding to the cellular responses (Baureithel et al. 1994), different preference on the size of *N*-acetylchitooligosaccharides shown by these two plant cells seems to reflect different specificity of the binding proteins.

Although these results are well suggestive about the functionality of this binding site as the receptor for this elicitor, it is obviously necessary to identify and purify the binding protein itself and also clone the gene for that to prove this assumption. Several experiments toward this direction have been established and will be published elsewhere.

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The authors thank to Mrs. Tomoko Yoshimura for her help in preparing plasma membrane preparation. The authors are grateful to Yaizu Suisankagaku Industrial Co. for the supply of chitoheptaose and chitooctaose. This work was supported in part by a grant, Enhancement of Center-of-Excellence, the Special Coordination Funds for Promoting Science and Technology, from Science and Technology Agency, Japan.

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(Received July 29, 1996; Accepted August 8, 1996)

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