Induction of Phytoalexin Formation in Suspension-cultured Rice Cells by N-Acetylchitoooligosaccharides

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Induction of phytoalexin formation in suspension-cultured rice cells by a series of N-acetylchitoooligosaccharides and chitoooligosaccharides was studied. N-acetylchitoooligosaccharides larger than hexaose induced the formation of momilactones A and B as well as oryzalexins A, B, and D at very low concentrations like 10^{-5} – 10^{-6} M (N-acetyldihexa-1,4-lactone). GlcNAc oligomers smaller than trimers had almost no activity and a series of deacetylated chitoooligosaccharides were also inactive. Strict requirement for the size and structure of GlcNAc oligomers as well as the sensitivity to them strongly indicates the presence of recognition systems specific for these compounds in rice cells. The level of momilactone A produced reached 100–500 μg/g of cultured cells, which appeared to be enough to prevent the growth of pathogenic fungi such as Pyricularia oryzae, thus indicating the importance of this phenomenon in the defense systems of rice plants. Suspension-cultured cells obtained only from a suitable period of cultivation, mainly those from lag phase, could respond to the elicitor and produce phytoalexins.

Higher plants have various defense reactions when they are attacked by pathogens such as fungi, bacteria, and viruses. These include the production of phytoalexins, enzymes such as chitinase and β-glucanase, proteinase inhibitors, hydroxyproline-rich glycoproteins, generation of active oxygen species, and lignification.1–3 Biotic elicitors that can activate these defense systems have been studied extensively and shown to be oligo-/polysaccharides in most cases. They could be derived from the cell surface of pathogenic microbes and also from plant cell wall. The information on the precise chemical structure of the active fragments is essential for the understanding of the recognition systems working in each plant and also for the development of potent pesticides.

Although the chemical structure of some active oligosaccharides from pathogens and plant cell walls have been elucidated, e.g., a heptasaccharide from β-glucan of Phytophthora megasperma4,5 and α-1,4-linked oligogalacturonides of specific size,6–8 most of these informations were obtained using dicot plants such as legumes, some vegetables, and fruts, including cultured cells derived from them. In contrast, only very limited information is available on the oligosaccharides that are active on monocots. Barber et al.9 found that N-acetylchitoooligosaccharides induce the lignification of wounded wheat leaves. Hirano et al.10,11 showed the induction of chitinase in rice seeds and callus by the derivatives of chitin and chitosan. Nishizawa and Hibii also reported the induction of mRNA coding a chitinase in cultured rice cells by glycol chitin. Very recently, Ren and West13 reported that chitin could induce diterpene hydrocarbon synthase, one of the enzymes proposed for the biosynthetic pathway of diterpenoid phytoalexins, in suspension-cultured rice cells. They did not show, however, the precise information on the size and structure of oligosaccharides required for the elicitation nor the formation of phytoalexin itself. We here show that very low concentrations of N-acetylchitoooligosaccharides of specific size elicit the production of phytoalexins in suspension-cultured rice cells and discuss its possible relationships with the defense systems of rice plants.

Materials and Methods

Cell culture. Callus of Oryza sativa L. cv. BL-1 was kindly supplied by Mr. Masahiro Kobayashi of Chugai Pharmaceutical Company and maintained on agar plates containing N6 medium with 1 mg/liter 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.8% agar. The callus was incubated at 25°C in the dark and transferred to fresh medium every 4 weeks. For suspension culture, approximately 4 g of the callus was transferred to a 500-ml Erlenmeyer flask containing 150 ml of N6 medium containing 1 mg/liter 2,4-D. The suspension culture was incubated on a rotary shaker at 25°C and 140 rpm in the dark. Cells were harvested every 2 weeks and filtered through a 20 mesh filter to make fine aggregates. A 10-ml sample of the loosely packed cells was transferred to a new flask containing 150 ml of the same medium and incubated further.

Elicitor. Purified chitoooligosaccharides (DP = 2–8) and N-acetylchitoooligosaccharides (DP = 2–6) were obtained from Seikagaku Corp. (Tokyo, Japan) and also from Yaizu Suisankagaku Industry Co., Ltd. (Shizuoka, Japan). N-acetyldihexa-1,4-lactone and octaose were prepared by the re-N-acetylation of corresponding chitoooligosaccharides.

Assay of elicitor activity. Samples to be tested were dissolved in 100 μl of distilled water and autoclaved at 121°C for 20 min. A 900-μl sample of the suspension-cultured cells (approximately 20–30 mg fresh cells) was added aseptically to this solution and incubated at 25°C with reciprocal shaking. The reaction mixture was centrifuged for 10 min at 3000 rpm and the cells were washed again with 1 ml of water. The amount of the cells used for each reaction was determined separately using this pellet. The supernatant and washings were combined and applied to a Bond Elute C-18 column (Analytichem International, CA, U.S.A.) equilibrated with water. The column was eluted successively with 10 ml of water and 1 ml of 80% methanol. The methanol fraction was concentrated until dryness at 40°C under vacuum, dissolved with 50 μl of methanol and used for the

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Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; DP, degree of polymerization; GC, gas chromatography; CG-MS, gas chromatography–mass spectrometry; FID, flame ionization detector.
gas-liquid chromatographic (GC) analysis of phytoalexins. Because of the variability of the amount of phytoalexins produced in each experiment, each set of experiments were done using the same batch of cultured cells. All the experiments were done in duplicate or triplicate for each experiment and treated statistically.

Analysis of phytoalexins. Phytoalexins were analyzed routinely using a Shimadzu GC 14A gas chromatograph with a moving needle sample injection system and flame ionization detector (FID). A fused silica glass capillary column coated with DB-5 (0.25 mm x 25 m, J & W Scientific, CA, U.S.A.) was used for the analysis. A Shimadzu QP-1000 gas chromatograph–mass spectrometer (GC-MS) was also used for the identification of each phytoalexin.

Results

Induction of phytoalexin formation by N-acetylchitooligosaccharides

For the assay of elicitor activity of each compound, we have established an assay system in which a sample of suspension-cultured rice cells (20–30 mg fresh cells) was incubated with the test compounds for a proper period, followed by the analysis of known phytoalexins by GC/GC-MS. This method enabled us to compare the elicitor activity of many samples at the same time using the cultured cells from a single flask in most cases, thus minimizing the effects of the difference between the flasks.

Figure 1 shows the formation of a major rice phytoalexin, momilactone A, by suspension-cultured rice cells that were treated with various amounts of N-acetylchitoxheptaose, (GlcNAc)₇. A detectable amount of momilactone A was induced even with 1 ng/ml (0.7 x 10⁻⁹ M) of N-acetylchitoxheptaose. The amount of induced momilactone A increased sharply with the increase of the added oligosaccharide up to 1 μg/ml (0.7 x 10⁻⁵ M). The maximum amount of momilactone A reached 100 to 500 μg/g fresh weight of the cultured cells, which varied depending on the conditions of the cell.

GC/GC-MS analysis of phytoalexins extracted from the medium showed the presence of momilactone A and B (Fig. 2). Formation of a small amount of oryzalexin A, B, and D were also detected by GC-MS (data not shown).

The amount of momilactone A was used as a measure of elicitor activity in the following experiments because of its abundance and also suitability for GC analysis.

Effects of the size of N-acetylchitooligosaccharides and chitooligosaccharides on the elicitor activity

The elicitor activity strictly depended on the size of N-acetylchitooligosaccharides (Fig. 3). N-Acetylchitooligosaccharides smaller than triose showed very low or negligible

Fig. 1. Effects of the Concentration of N-Acetylchitoxheptaose on the Formation of Momilactone A by Suspension-cultured Rice Cells.

A sample of suspension-cultured cells (20–30 mg fresh cells) was incubated with various amounts of N-acetylchitoxheptaose, (GlcNAc)₇, for 48 h at 25°C. Momilactone A in the incubation medium was analyzed as described in the text and expressed in terms of μg produced by one gram of fresh cells. Bar indicates the standard deviation.

Fig. 2. Gas Chromatographic Analysis of Phytoalexins Induced by N-Acetylchitoxheptaose.

Phytoalexins induced by the addition of 10 μg/ml (GlcNAc)₇ to the cultured cells were extracted after 48 h of incubation and analyzed as described in the text. GC analysis was done with the temperature program of 250–300°C (5°C/min). A and B indicate the peak of momilactone A and B, respectively. Left, with the elicitor; right, without the elicitor (control).

Fig. 3. Effects of the Size of N-Acetylchitooligosaccharides on the Elicitor Activity.

Each 100 μM N-acetylchitooligosaccharides (or deacetylated chitooligosaccharides) of different size was incubated with suspension-cultured rice cells for 48 h at 25°C. Momilactone A was analyzed as described in the text. Level of the momilactone A in the control experiment was shown at the DP of 0° for the convenience.
Fig. 4. Time Course of the Accumulation of Momilactone A.
Reaction conditions were the same as in Fig. 2. Phytoalexins were extracted every 24 h using two separate incubation mixtures and analyzed as described in the text.

<table>
<thead>
<tr>
<th>Incubation time (h)</th>
<th>Momilactone A (µg/g fresh cells used for incubation)</th>
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<tbody>
<tr>
<td></td>
<td>Medium</td>
</tr>
<tr>
<td>24</td>
<td>24</td>
</tr>
<tr>
<td>48</td>
<td>141</td>
</tr>
<tr>
<td>72</td>
<td>149</td>
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* Reaction conditions were the same as in Fig. 2.
+ Recovered cells were extracted with 2 ml of methanol for 10 min at 100°C. The methanol extract was diluted with 50 vol. of distilled water, filtered through a glass filter, and applied to a Bond Elute column. Momilactone A was recovered from the column and analyzed as described in the text.

Fig. 5. Responsiveness of Suspension cultured Rice Cells Harvested at the Different Periods of Cultivation
A 500-ml Erlenmeyer flask containing 180 ml of cell suspension was used for the cultivation. Samples of the suspension (0.9 ml x 5) were taken from this flask periodically and tested for the responsiveness to the elicitor, (GlcNAc)7. These cells were also used to get the information to draw the growth curve. Conditions for the elicitor assay were the same as in Fig. 2.

**Discussion**

The information about the biotic elicitors that can activate the defense systems of monocot plants is much poorer compared to the corresponding knowledge on dicots. Especially, little information is available on elicitors that can induce the formation of phytoalexins, one of the major constituents of plant defense systems, in monocots. Mayama et al.18) reported that a host-selective peptide toxin, victorin, induced the production of avenalumin in oat leaves. Yamaoka et al.19) also reported the elicitation of sorghum phytoalexins by the compounds obtained from Colletotrichum graminicola. These compounds mainly consisted of carbohydrate or protein but their structures were not known. The results described in this paper clearly demonstrated that the N-acetylchitooligosaccharides of certain size are highly potent biotic elicitors for rice cells and can elicit the production of phytoalexins of biologically significant level. Our results coincide with those obtained by Ren and West13) who showed that one of the enzymes proposed for the biosynthetic pathway of these phytoalexins could also be elicited by chinin. One of the most active oligosaccharides in our experiments, N-acetylchitohexaose, could elicit the production of a detectable amount of momilactone A from 10^{-9} to 10^{-6} M (1 ng—1 µg/ml). This value is comparable with those reported for the most active oligosaccharide elicitor to soybeans, a β-glucan heptamer isolated from the cell wall of Phytophthora megasperma.4)
and much lower (= higher activity), even $10^3$ times, compared to the value reported for chitin and its partial hydrolyzate by Ren and West.\(^{13}\) This is also true in comparison with the concentration reported for the elicitor activity of chitin and related carbohydrates in various dicot systems.\(^{20-25}\) One reason for the very high activity observed in our experiments may be the use of pure oligosaccharides. In the case of polymers such as chitin, they are supposed to be broken down into fragments that can penetrate the cell wall before the activation of plant cells. The efficiency of polymers can thus be much lower compared to the pure active oligomers as only a part of applied polymers can be converted into the corresponding active forms during the incubation. In the case of Ren and West,\(^{13}\) they used only GlcNAc oligomers smaller than tetramers as pure oligosaccharides, which are obviously insufficient for the elicitation at low concentrations. Differences in the sensitivity of each plant toward GlcNAc oligomers as well as the assay systems may have also caused the different results described above.

The fact that the deacetylated chitoooligosaccharides were inactive as elicitors indicates that rice cells require the N-acetyl group at the C-2 position of the hexopyranosyl residue (or the neutralization of positive charge) for recognition. These characteristics are similar to the one observed for the induction of lignification in wounded wheat leaves,\(^9\) although chitosan or its oligomers were reported to be active elicitors for the induction of phytoalexin,\(^{20}\) proteinase inhibitor,\(^{21}\) and callose\(^{22-24}\) formation in several dicots. With chitosan, a long chain carrying cationic residues was important for the activity and a possible interaction of these cationic groups with the anionic phospholipids on the plasma membrane was suggested.\(^{23}\) Thus, the mechanism of the interaction of the chitosan and related compounds with plant cells appears quite different from that of chitoooligosaccharides where a far lower concentration of electrically neutral oligosaccharides could deliver the information to the cell. The latter situation strongly suggests the presence of specific recognition systems on the cell surface rather than the non-specific ionic interactions postulated for chitosan.

Concerning the generation of these N-acetylchitoooligosaccharides, they could be derived from chitin, which is common in the cell walls of most pathogenic fungi, by chitinase produced by host plants during early steps of the invasion of these fungi. Presence of chitinase in rice seeds and cultured cells as well as the induction of the enzyme activity by chitin and related compounds were reported by several groups.\(^9\) Cloning of cDNA\(^{12}\) as well as genomic DNA\(^{26}\) coding rice chitinases was also reported.

The amount of the major phytoalexin produced, momilactone A, reached several hundred $\mu g$ of cultured cells in these experiments. Momilactones A and B were reported to inhibit the germ tube growth of \textit{Piricularia oryzae}, one of the most important pathogenic fungi for rice plant, to the degree of 50% at the concentration of 5 $\mu g$/ml or 1 $\mu g$/$ml$, respectively.\(^{14,27}\) Thus, the phytoalexin concentration induced by N-acetylchitoooligosaccharides in this study (100–500 $\mu g$/g cell) appears to be enough significant biologically, although these values cannot be compared directly.

All these results, the very low concentration of \(N\)-acetylchitoooligosaccharide of specific size which can be released from the cell walls of pathogenic fungi by the chitinase present in the corresponding plant eliciting the production of phytoalexins of biologically significant level, strongly suggest that these systems really constitute a part of defense systems of rice plant.

Suspension-cultured rice cells were not always responsive to these external signals to synthesize phytoalexins. As described in the results, only those cells harvested at the certain stage of cultivation can respond to the elicitor and produce phytoalexins. Moreover, successive cultivation in the artificial medium, especially in liquid medium, often resulted in non-reactive cells. These should reflects some physiological/genetic changes of cultured cells during the cultivation but the molecular basis for these phenomena are not clear.

It is possible that a single plant can use multiple signals for the activation of defense systems. We recently found that the rice cells can also recognize specific fragments derived from $\beta$-glucan isolated from the cell walls of \textit{P. oryzae}. Properties of these elicitors will be published elsewhere.

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References
Induction of phytoalexins in rice cells by GlcNAc oligomers