Hydrogen peroxide mediates defence responses induced by chitosans of different molecular weights in rice

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Summary

To investigate the mechanisms whereby treatment with chitosan (CHN) is observed to increase the capacity of plants to resist pathogens, CHNs of different molecular weights (MWs) prepared by enzyme hydrolysis were used to treat rice cells in suspension culture and also rice seedlings. The results obtained with cultured cells showed that in this material CHN treatment could trigger a set of defence responses, including the production of hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}), increases in the activities of phenylalanine ammonialyase (PAL; EC 4.3.1.5) and chitinase (CHI; EC 3.2.1.14), increases in transcription of defence-related genes \textbeta\textsubscript{-}1,3-glucanase (\textit{glu}) and chitinase (\textit{chi}) and accumulation of pathogen-related protein (PR1). Furthermore, CHNs of different MWs were observed to have different capacities to induce defence responses. CHNs of low MWs were more effective at inducing the described defence responses than those of higher MWs. Enhanced defence against rice blast pathogen \textit{Magnaporthe grisea} 97-23-2D1 was observed in rice seedlings treated with low MW CHNs compared to seedlings treated with higher MW CHNs. In all cases, suppressing the production of H\textsubscript{2}O\textsubscript{2} by adding scavengers dimethylthiourea (DMTU), 2,5-dihydroxycinnamic acid methyl ester (DHC), catalase (Cat) or ascorbate (As) blocked...

KEYWORDS

CHN; Defence response; H\textsubscript{2}O\textsubscript{2}; Rice

Abbreviations: As, ascorbate; Cat, catalase; \textit{Chi}, chitinase gene; CHI, chitinase; CHN, chitosan; 2, 4-D, dichlorophenoxyacetic acid; DHC, 2, 5-dihydroxycinnamic acid methyl ester; DMTU, Dimethylthiourea; DP, degree of polymerization; \textit{glu}, \textbeta\textsubscript{-}1,3-glucanase gene; JA, jasmonic acid; MW, molecular weight; PAL, phenylalanine ammonialyase; Phe, phenylalanine; PR1, pathogen-related protein

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Introduction

The early phase of plant response to either pathogen- or plant-derived elicitors is usually accompanied by the production of hydrogen peroxide, H\textsubscript{2}O\textsubscript{2} (Levine et al. 1994; Lamb and Dixon 1997; Alvarez et al. 1998). On the one hand, H\textsubscript{2}O\textsubscript{2} can kill pathogenic bacteria directly and restrain its propagation and pore formation. On the other hand, H\textsubscript{2}O\textsubscript{2} production can reinforce the cell wall by inducing associated protein cross-linking. H\textsubscript{2}O\textsubscript{2} can also act as a messenger to induce or increase the transcription of genes related to the plant immune system. For example, the elicitor from the cell wall of phthoramycin can induce the production of H\textsubscript{2}O\textsubscript{2} in soybean suspension cells, H\textsubscript{2}O\textsubscript{2} in turn mediating increases in the transcription of the defence-related genes pal and gst (Levine et al. 1994). Suppression of the production of elicitor-induced H\textsubscript{2}O\textsubscript{2} leads to the suppression of transcription of the genes (Levine et al. 1994; Orozco-Cardenas and Ryan 1999).

Chitosan (CHN) is the deacetylated or partially deacetylated product of chitin (Vander et al. 1998). CHNs of different MWs can be prepared by enzyme hydrolysis, acid hydrolysis or other chemical degradations (Pantaleone et al. 1992; Muraki et al. 1993; Yalpani and Pantaleone 1994). The biological functions of CHN have come under increasing scrutiny recently and it has been shown that CHN treatment can increase crop yield, induce plant defence responses and enhance the capacity to tolerate stress in plants (Chang et al. 1992; Lee et al. 1999; Orozco-Cardenas and Ryan 1999; Roller and Covill 1999). However, the molecular mechanism of defence responses induced by CHN is not wholly understood.

As part of on-going investigations into the mechanism of CHN-induced defence responses, we prepared CHNs of different MWs and used them to treat rice cells in suspension culture. Our results show that CHNs can induce the production of H\textsubscript{2}O\textsubscript{2}, enhance transcriptions of defence-related genes and increase activities of defence-related proteins. Furthermore, the MW of the CHNs affected the capacity to induce the production of H\textsubscript{2}O\textsubscript{2} and defence responses. The role of H\textsubscript{2}O\textsubscript{2} induced by CHNs of varying MWs in the defence responses of rice cells was studied.

Materials and methods

Rice suspension cells

Rice (Oryza sativa L. spp. japonica cv. Xiushui) cell suspension cultures were induced from young embryos as reported previously (Yamada et al. 1993). Briefly, immature rice embryos were used to inoculate N\textsubscript{6} medium supplemented with additional dichlorophenoxyacetic acid (2,4-D, 2 mg L\textsuperscript{-1}). Visible rice calli were harvested for 3 days and used to inoculate fresh N\textsubscript{6} medium (containing 1 mg L\textsuperscript{-1} 2, 4-D). After 1-month cultivation, rice calli were transferred to liquid N\textsubscript{6} medium (containing 1 mg L\textsuperscript{-1} 2, 4-D) to form rice suspension cells maintained at 25°C and 120 R min\textsuperscript{-1} in a rotary incubator under darkness. The suspension cultures were subcultured every three weeks into fresh N\textsubscript{6} medium in a 1:10 ratio. Three-day-old rice cells in suspension were used as the experimental material.

Preparation of CHNs of different MWs

CHNs of different MWs were prepared as reported previously (Pantaleone et al. 1992; Yalpani and Pantaleone 1994). Briefly, 40 g crude CHN (60% deacetylation, Weikang Biology Co., Shanghai, China) was dissolved in 1 L 1% acetic acid solution, then 200 mg a-amylase (4000 U mg\textsuperscript{-1}, Sigma, St Louis, USA) was added. CHN was hydrolyzed at 50°C for 0.5h, 1h, 1.5h, 2h, 2.5h, 3.5h, 5.5h, 7.5h, 9h, 9.5h, and 10 h, respectively, and enzyme activity was eliminated by heating at 100°C. Following filtration on 0.22\textmu m membranes (Millipore, Bedford, USA), the filtrate was passed through a D315 ion exchange Column (Sigma, St Louis, USA) and then subjected to ultrafiltration membrane (MW cut-off 10,000 Da). The filtrate was vacuum concentrated and freeze-dried. The MW and distribution (MW/Mn) of purified CHN were determined by gel permeation chromatography using an HPLC (Beckman Instruments, Brea, CA) equipped with TSKG3000SW (1000–100,000) columns and RI150 refractive index detector. Mobile phase was acetic acid buffer. The standards were chitotriose, chitopenteose, chitohexaose and pullulans.
Measurement of H₂O₂ production

The concentration of H₂O₂ in the culture medium was measured by luminol chemiluminescence (Murphy and Huerta 1990). At indicated times after the addition of CHNs to rice cell suspensions, 0.2 mL sample was taken from each culture flask. The sample was mixed immediately with 0.6 mL potassium phosphate buffer (50 mmol L⁻¹, pH 8.0) and 0.1 mL luminol (1.1 mol L⁻¹ in phosphate buffer) in a cuvette, followed by the addition of 0.1 mL, 14 mmol L⁻¹ K₃Fe(CN)₆. Following a delay of 5 s, the luminescence counts were read every 15 s over a period of 1 min in a luminometer. The maximum counts of duplicate treatments were averaged and taken as the result. The concentration of H₂O₂ in the sample was determined with a calibration curve drawn up with a standard H₂O₂ solution.

Assay of PAL and CHI activity

Rice cells were recovered by centrifugation (1000g, 5 min). The resulting cell pellets were immediately homogenized in extraction buffer (0.5 mg L⁻¹) containing 50 mmol L⁻¹ Tris–Cl pH 8.0, 10 mmol L⁻¹ 2-mercaptoethanol, 40 mmol L⁻¹ EDTA and 10 μmol L⁻¹ leupeptin. The homogenate was centrifuged at 1000g to obtain the supernatant, which was used for the PAL assay. PAL activity was determined as reported previously (Tang and Smith 2001) and calculated in nmol cinnamic acid mg⁻¹ protein. CHI activity was determined as reported (Schlumbaum et al. 1986) and calculated in U g FW⁻¹.

RNA gel blotting

Rice cells subjected to various treatments were recovered via vacuum filtration. Total RNA was extracted using guanidine-thiocyanate, phenol and chloroform as described previously (Sambrook and Russell 2001). Electrophoresis and blotting were performed as described by Sambrook and Russell (2001), equal RNA loading being assessed by use of an act probe. Labeling and detection of hybridization to act, glu and chi were performed using the digoxigenin-dUTP system (Dig-system, Roche, Cat No. 1093 657, Germany).

Protein gel blotting

Rice cells exposed to different treatments were recovered by centrifugation (1000g, 5 min). After rinsing twice with extraction buffer (10 mmol L⁻¹ Tris–Cl, pH 8.0, 1 mmol L⁻¹ DTT, 1 mmol L⁻¹ PMSF), the resulting cell pellets were ground in the same buffer. Proteins were separated on SDS-PAGE gels and transferred to a PVDF membrane (Amersham Biosciences, UK) using a Multiphor II semi-dry blotting apparatus (Amersham Biosciences) according to the manufacturer’s instructions. Protein gel blotting was carried out as described (Sambrook and Russell 2001). The antibody used was PR1 antibody in rice at a dilution of 1:1000. Hybridization was detected using the ECL Chemiluminescence system (Roche, Mannheim, Germany).

Analysis of degree of infection to rice leaves treated with CHNs

Thirty-day seedlings were applied with 50 g L⁻¹ CHNs of different MW. P. grisea was inoculated by the spraying method after 6 h of CHN treatment. The daytime temperature during the infection period was around 28 °C at 85% relative humidity and the night temperature was 25 °C and 90% relative humidity (maintained by artificial sprinklers). In addition, during the night the beds were covered with polythene paper pre-wetted with water. The severity of the disease was scored 10 days after the treatment with the SES scale (Standard Evaluation Scale) that is based on the length and type of lesion, percentage of disease spread and overall performance of the seedlings (Gandikota et al. 2001). The disease response is classified into the following types: resistant plants show no lesions or, if present, lesions are restricted and non-spreading with a pin head to a small round size and receive a score of 0–2; moderately resistant plants, where the number of small round lesions are localized to the upper leaves receive a score of 3; plants with score 4 begin to show typical blast lesions with less than 4% of the infected area; plants with score 5 have 4–10% of the leaf area infected; moderately susceptible plants with around 25–50% of leaf area infected are scored at 6–7; plants classed as sensitive show severe necrotic lesions with 51–71% of leaf area infected and are given a score of 8–10.

Results

Preparation of CHNs of different MWs

More than 30 enzymes of varying specificities have been reported to hydrolyze CHN. Following the screening of many kinds of enzymes (data not shown), we found α-amylase to be the most
effective. Initially, the speed of hydrolysis was very fast and the CHN segments with MWs of about 5000 Da were generated in 1.5 h. CHN segments with MWs of about 1335 Da were produced following 9–10 h of hydrolysis. The MW distribution index (MW/Mn) of CHN segments of different MWs also showed that the MW distribution was narrow (Table 1).

**CHN-induced generation of H2O2 in rice cells in suspension culture**

A dose response was observed during CHN-induced production of H2O2 in rice cells in culture (Fig. 1A). Using CHN of MW 1335 Da, the production of H2O2 could be detected when the concentration of CHN was 15 µg mL⁻¹, reached a maximum at 60 µg mL⁻¹ and decreased at higher CHN concentrations. A time response was also observed. For rice cells treated with 60 µg mL⁻¹ CHN (MW 1335 Da), the generation of H2O2 reached a maximum after 50 min, then decreased over the next 2 h (Fig. 1B). At constant duration of treatment and all concentrations tested the capacity of CHN to induce H2O2 production was a function of MW at the three CHN sizes tested, being highest in the presence of 1335 Da and lowest with 50,000 Da CHN (Fig. 1C). Treatment with all the H2O2 scavengers tested, Cat, DMTU, DHC and As, suppressed the CHN (MW 1335 Da)-induced production of H2O2 in rice cells in culture (Fig. 1D).

**CHN-induced increases in PAL and CHI activity**

CHN induced substantial increases in PAL and CHI activity in rice cells in suspension culture. (Table 1).

Table 1. Hydrolysis of CHN by commercial α-amylase (4000 U mg⁻¹ final, Sigma, St Louis, USA), one α-amylase is the micrograms starch hydrolyzed per 30 min under the assay buffer containing 10 mmol L⁻¹ Tris–Cl at pH 8.0

<table>
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<th>Reaction time (h)</th>
<th>MW (Da)</th>
<th>MW/Mn</th>
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<tr>
<td>0.0</td>
<td>50000</td>
<td>1.15</td>
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<tr>
<td>0.5</td>
<td>9400</td>
<td>1.17</td>
</tr>
<tr>
<td>1.0</td>
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1335 Da, 50 µg mL⁻¹) treatment increased PAL activity to 229 nmol cinnamic acid, mg protein⁻¹, h⁻¹ in 3 days (Fig. 2A) and, as observed for H2O2 production, the increase in PAL activity decreased as a function of CHN MW, being lowest following treatment with 50,000 Da CHN. Cat, DMTU, DHC and As all suppressed the increase of PAL activity (CHN of MW 1335 Da) (Fig. 2B). Treatment with CHN also induced increases of CHI activity (Fig. 3A) and the increases were a function of CHN MW as observed for PAL. Cat, DMTU, DHC and As all suppressed the increase of CHI activity induced by CHN (Fig. 3B).

**CHN-induced transcription of defence-related genes glu and chi and accumulation of PR1**

CHN treatment induced strong and transient transcription of glu and chi — two major defence-related genes. RNA gel blotting analysis showed that the capacity of induction was also a function of CHN MW as observed for enzyme activities, levels of specific transcripts being highest when 1335 and lowest when 50,000 Da CHN was applied (Fig. 4A). Treatments with Cat, DMTU, DHC and As all inhibited the CHN-dependent increase in specific transcripts of glu and chi (Fig. 4B). Furthermore, CHN (MW 1335 Da) induced the accumulation of PR1 — one of the pathogen-related proteins (Fig. 5A). Treatment with H2O2 (1 mmol L⁻¹) also induced the accumulation of PR1. Cat, DMTU, DHC and As inhibited the CHN-dependent accumulation of PR1 (Fig. 5B).

**The effect of CHN pretreatment on the infection severity of rice blast**

Rice seedlings that have been pretreated with CHN (1335 Da) prior to inoculation with rice blast lineage, Magnaporthe grisea 97-23-2D, demonstrated distinct reduction of the infection on their leaves compared to controls. Treatment with CHN of 1335 Da reduced the infection to a greater extent than treatment with CHN of 3350 Da or 50,000 Da. The addition of either DMTU, DHC or As increased the CHN (1335 Da)-reduced infection of rice leaves (Table 2).

**Discussion**

CHNs of different MWs were prepared by non-specific enzyme hydrolysis. Analysis of the MW distribution showed that CHNs of all three MWs
purified had similar distribution indices. CHNs of the lowest MW (1335 Da), consisting of 6–9 amino-glucoses, were formed after 9–10 h of hydrolysis. This data confirmed that only the effect of MW needed to be taken into account when interpreting the results and that any effect of differing purity could be excluded.

Both of the enzymes investigated in this study are known to implicate in plant disease resistance. PAL is a key enzyme of the Phe pathway in plants that plays an important role in determining the content of phytoalexins and many secondary phenolic metabolites. CHI can degrade cell walls of pathogenic fungi. Increasing the activities of these two enzymes would therefore be expected to enhance the plant’s defence capacity. It has been shown previously that CHN can induce PAL activity in maize and grape (Vander et al. 1998). CHN treatment can also induce rapid accumulation of JA (Inui et al. 1997), which is one of the most important signal molecules in plants. It has been suggested that the accumulation of JA is the reason for the increase of disease resistance following treatment with CHN (Rakwal et al. 2002). Our results show that CHNs of different MWs have varying capacity to induce the activities of PAL and CHI, with the increase being inversely related to the CHN MW at the three sizes tested. Furthermore, the effect of CHN MW on these enzyme activities was in accordance with the effect of varying CHN MW on plants infected with rice blast. The lowest MW CHN (MW 1335 Da) distinctly reduced the severity of infection compared to controls, while CHNs of the two higher MWs were less effective. In general, it has been found that CHN can induce biological responses, such as the production and accumulation of phytoalexins, when the DP is greater than 4 (Daye Sun et al. 2000). Yamada et al. (1993) showed CHN (DP 6–8, or between 800 and 1100 Da) could induce antitoxin, a phytoalexin in rice, when the concentration was at the nanomolar level – although CHN of DP 2–3 (about 300 Da) had no detectable effect. Similar reports have been presented for soybean cells (Day et al. 2001).

**Figure 1.** Induction of H$_2$O$_2$ in rice cells in suspension culture. (A) Dose dependent production of H$_2$O$_2$ in rice cell suspension cultures treated with different concentrations of CHN (MW 1335 Da). Data was taken after treatment for 60 min. (B) Time dependent accumulation of H$_2$O$_2$ in rice cell suspension cultures treated with 60 µg mL$^{-1}$ CHN (final) (MW 1335 Da). (C) Production of H$_2$O$_2$ in rice cell suspension cultures treated with CHNs of different MWs. CHN (MW 1335 Da) (●), CHN (MW 2770 Da) (○), CHN (MW 50,000 Da) (▼). Data was taken after treatment with 50 µg mL$^{-1}$ (final) CHNs for 60 min. (D) Effect of scavengers on accumulation of H$_2$O$_2$ in rice cell suspension cultures treated with 50 µg mL$^{-1}$ (final) CHN (MW 1335 Da). After treating with scavengers 10 mmol L$^{-1}$ (final) DMTU, 10 mmol L$^{-1}$ (final) DHC, 5 U mL$^{-1}$ (final) Cat and 5 mmol L$^{-1}$ (final) As, respectively, for 60 min, 50 µg mL$^{-1}$ (final) CHN (MW 1335 Da) was added. Data was recorded following treatment with CHN (MW 1335 Da) for 60 min. Bars = SE (n = 5).
lowest CHN MW used in the present paper was 1335 Da, which corresponds to the DP of the most effective CHN for the stimulation of biological response cited in the work by Yamada et al. It has been suggested that this molecular size is the most effective at binding to the specific receptors on the plasma membrane (Daye Sun et al. 2000; Day et al. 2001). CHN receptors have been reported in soybean and tomato (Felix et al. 1998; Bradley 2001). These authors suggest that the binding of CHN to its receptor on the plasma membrane is indispensable for CHN-induced plant defence responses. It can be deduced from the present results that CHN-dependent H$_2$O$_2$ production is mediated by a CHN-specific receptor in rice. However, the relationship to CHN size needs to be investigated further for rice by extending the biological response tests to even lower DPs/MWs than used in the present work.

H$_2$O$_2$ is known to be a local (and potentially mobile) signal mediating the induction of plant defence responses (Orozco-Cardenas and Ryan 1999). The early processes of defence responses induced by pathogen are usually accompanied by the production of H$_2$O$_2$. Our results show that the CHN-dependent production of H$_2$O$_2$ is dose dependent within a limited CHN concentration range up to 60 $\mu$g mL$^{-1}$, above which H$_2$O$_2$ production decreases. The fact that the suppression of the production of H$_2$O$_2$ by scavengers inhibits transcription of defence-related genes and decreases the activity of defence-related enzymes and also that
direct application of H2O can induce accumulation of PR1, points to a second messenger role for H2O2 following its induction by CHN. Our findings that lower MW CHN is more effective at inducing the defence responses that were measured than the higher MWs may be explained by the higher production of H2O2 induced by this CHN MW. These suppositions are supported by the observations on intact plants that removal of H2O2 by scavengers in CHN-treated plants had the effect of reducing the inhibition of pathogen infection compared to controls.

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References


