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EPR evidence for generation of hydroxyl radical triggered by *N*-acetylchitooligosaccharide elicitor and a protein phosphatase inhibitor in suspension-cultured rice cells

Rapid communication

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Summary. *N*-acetylchitooligosaccharides, fragments of the backbone of fungal cell wall, trigger rapid membrane responses such as transient depolarization, and elicit defense reactions including phytoalexin production in suspension-cultured rice cells. The generation of reactive oxygen species triggered by the oligosaccharide signal was analyzed with EPR spectroscopy using a spin trapping system, 4-pyridyl 1-oxide *N-tert*-butyl nitrone (4-POBN) and ethanol. $^{\cdot}OH$ generation was detected as the α-hydroxyethyl adduct of 4-POBN after elicitation. Superoxide dismutase, catalase or diethylenetriamine pentaacetic acid, a metal chelator, inhibited $^{\cdot}O$ generation, proposing the following reaction sequence: generation of $^{\cdot}O_2^-$ in response to the oligosaccharide elicitor, followed by dismutation to H_2O_2, then generation of $^{\cdot}OH$ by the reaction of H_2O_2 with Fe^{2+} that is generated by the reduction of Fe^{3+} by $^{\cdot}O_2^-$. Generation of the same reactive oxygen species was also triggered by calyculin A, a protein phosphatase inhibitor, alone, suggesting the involvement of protein phosphorylation in its regulation during the oligosaccharide signal transduction.

Keywords: Chitin fragments; Elicitor; Electron paramagnetic resonance; Hydroxyl radical; Reactive oxygen species; Rice suspension culture.

Abbreviations: DMPO 5,5-dimethyl-1-pyrroline N-oxide; DTPA diethylenetriamine pentaacetic acid; 4-POBN 4-pyridyl 1-oxide *N-tert*-butylnitrone; SOD superoxide dismutase; 4-hydroxy-TEMPO 2,2,6,6-tetramethyl-4-hydroxypiperidine-1-oxyl.

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Introduction

Plant cells recognize specific oligosaccharides, so-called elicitors, derived from the cell surface of pathogenic microorganisms and initiate defense responses including gene expression and production of antimicrobial substances such as phytoalexins (Ryan and Farmer 1991). In spite of the importance of the use of purified elicitors for the molecular elucidation of signal transduction network, crude mixtures of cell wall components have been used in most studies, and little is known about mechanisms for initial responses and signal transduction triggered by purified oligosaccharides.

We have shown that *N*-acetylchitooligosaccharides, fragments of the backbone polymer of fungal cell wall, act as elicitors for the production of phytoalexins at nM concentrations in suspension-cultured rice cells (Amada et al. 1993). Requirements for the size and structure of the oligosaccharides were very strict. The oligosaccharide elicitor also triggered rapid and transient membrane depolarization with similar specificity and sensitivity, which might be an initial membrane response for signal transduction just after the signal recognition (Kuchitsu et al. 1993). The presence of a novel high-affinity binding site for the oligosaccharide was detected in the microsomal

membrane fraction, and thought to be a putative receptor for the elicitor (Shibuya et al. 1993). Thus this system could be an excellent model for a study of the early signal transduction network triggered by elicitors in plants.

The generation of superoxide anion or hydrogen peroxide by plants after infection of pathogenic fungi or treatment with their crude preparations was suggested using methods such as cytochrome *c* reduction or fluorescence quenching (Doke 1983; reviewed in Sutherland 1991, Mehdy 1994). However, molecular identification and generating mechanisms of reactive oxygen species are still controversial. Deighton et al. (1992) tried to analyze free radicals produced in potato tubers inoculated with *Erwinia carotovora* by EPR spectroscopy, but could not identify the molecular species.

In the present study, we show evidence for the generation of hydroxyl radical as well as superoxide anion and hydrogen peroxide triggered by the purified elicitor in rice cells with a sensitive spin trapping method using 4-pyridyl 1-oxide *N-tert*-butyl nitrone (4-POBN) in conjunction with ethanol. In this system, hydroxyl radical production was detected as the α-hydroxyethyl adduct of 4-POBN. Moreover, we also found that calyculin A, a protein phosphatase inhibitor, triggered generation of the same reactive oxygen species.

Materials and methods

Plant material and chemicals

Suspension-cultured cells of *Oryza sativa* L. cv. Nipponbare were grown in the dark at 25 °C as described previously (Kuchitsu et al. 1993). Cells were transferred to a fresh medium every week, and filtered through a 20 mesh screen every two weeks to make fine aggregates. Purified chitooligosaccharides were obtained from Seikagaku Corp. (Tokyo, Japan) and Yaizu Suisankagaku Industry Co., Ltd. (Shizuoka, Japan). *N*-Acetylchitoheptaose and *N*-acetylchitooctaose were prepared by re-*N*-acetylation of the corresponding chitooligosaccharide (Yamada et al. 1993). 4-Pyridyl 1-oxide *N-tert*-butylnitrone (4-POBN) and 2,2,6,6-tetramethyl-4-hydroxypiperidine-1-oxyl (4-hydroxy-TEMPO) were purchased from Aldrich Chemical Co., catalase from Boehringer-Mannheim, superoxide dismutase (SOD) from Sigma, 5,5-dimethyl-1pyrroline *N*-oxide (DMPO) from Dojindo Laboratories, calyculin A from Wako (Japan). *N*-Acetylchitooligosaccharides and calyculin A were dissolved in water and dimethyl sulfoxide, respectively.

Preparation of cells and spin trapping

Cells were harvested by filtration on filter paper, then washed and suspended in a medium A that contained the following components: KNO_3 (28 mM); $(NH_4)_2SO_4$ (3.5 mM); $CaCl_2$ (1.13 mM); $MgSO_4$ (0.91 mM); KH_2PO_4 (2.94 mM); sucrose (3%, w/v) (pH was adjusted to 5.8 with KOH). Cells (fresh weight 500 mg) suspended in 1 ml of the medium A in a plastic tube were shaken vigorously to aerate the cells in a reciprocal shaker at 25 °C, unless otherwise stated, and 4-POBN (10 mM) and ethanol (170 mM) were added just before the elicitation.

EPR measurements

EPR detection of spin adducts was performed with a Bruker ESP300 EPR spectrometer. Extracellular medium was collected and transferred to a quartz flat cell, which was in turn placed in the cavity of the EPR spectrometer. Sequential scans were then recorded at 20 °C. Spectrometer settings were as follows: incident microwave power, 20 mW; modulation frequency, 100 kHz; modulation amplitude, 1.0 G; response time, 0.7 s; scan width, 100 gauss and scan time, 2.8 min. The spin concentrations were determined by double integration of the EPR signal using a 4-hydroxy-TEMPO solution as a standard. 4-Hydroxy-TEMPO was weighed, and the concentration was optically determined using $\varepsilon_{240} = 1440/M/cm$ (Kosaka et al. 1992, Morrisett 1976).

Results and discussion

EPR detection of spin adducts of hydroxyl radical

We tried to identify the possible reactive oxygen species generated after elicitation with *N*-acetylchitooligosaccharide elicitor using a spin trapping method. First we used the spin trapping reagent, DMPO that traps both $^{\cdot}O_2^-$ and $^{\cdot}OH$, but no spin adducts were detected after elicitation with *N*-acetylchitooctaose (data not shown). This may be because the amount of generated reactive oxygen species was too small to trap with DMPO, or the DMPO adduct was degraded during the reaction.

For the sensitive detection of $^{\cdot}OH$, we used the 4-POBN/ethanol system. In this system, $^{\cdot}OH$ reacts with excess ethanol to yield α-hydroxyethyl radical, which is rapidly trapped by 4-POBN (Ramos et al. 1992), leading to higher sensitivity than the spin trapping by 4-POBN without ethanol. Elicitation of the rice cells with *N*-acetylchitooctaose in the presence of 4-POBN and ethanol resulted in the detection of a nitroxide, a spin adduct, with hyperfine splitting constants, $A_N = 15.80 \pm 0.10$ G (n = 8), $A_H = 2.61 \pm 0.08$ G (n = 6) (Fig. 1 B), consistent with those reported for the α-hydroxyethyl adduct of 4-POBN, 4-POBN-$CH(CH_3)OH$ (Ramos et al. 1992), though the splitting constants for lipid peroxyl are also similar (Buettner 1987). The spin adduct was not detected in the absence of *N*-acetylchitooligosaccharides (Fig. 1 A).

The baselines of the spectra were not flat because the signals for this spin adduct were on the broad signal derived from Mn. This is supported by the result that the spectrum in the presence of diethylenetriamine

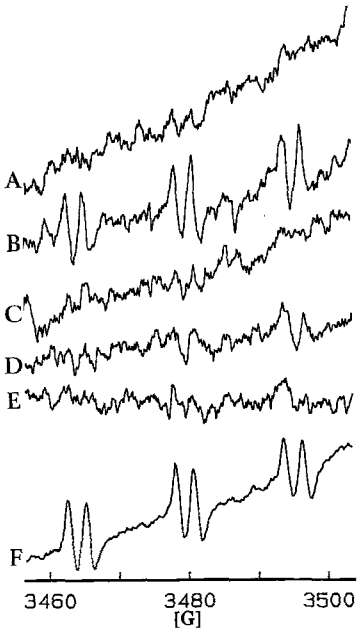

Fig. 1. EPR detection of the generation of hydroxyl radical from suspension-cultured rice cells elicited with *N*-acetylchitooligosaccharides. Spin trapping reagents, 4-POBN (10 mM) and ethanol (170 mM) as well as SOD (50 U/ml; **C**), catalase (500 U/ml; **D**) or DTPA (0.1 mM; **E**) were added to the cell suspension just before the elicitation. The extracellular medium of each sample was collected 32 min after the elicitation with *N*-acetylchitooctaose (5 μM) (**B–E**), *N*-acetylchitoheptaose (1 μM) (**F**), and EPR spectra were taken. **A** Control (without elicitors). Spectrum **F** was obtained from an average of ten scans

pentaacetic acid (DTPA; 0.1 mM), a chelator for metal ions, showed a flat baseline (Fig. 1 E).

Addition of *N*-acetylchitoheptaose (Fig. 1 F) or simultaneous addition of *N*-acetyloctaose and *N*-acetylchitoheptaose also resulted in the detection of a similar amount of the nitroxide.

Effects of SOD, catalase and DTPA on ·OH generation

Addition of superoxide dismutase (SOD; 50 U/ml) or catalase (500 U/ml) to the reaction mixture inhibited the formation of 4-POBN-CH(CH_3)OH (Fig. 1 C, D), suggesting the involvement of both $\cdot O_2^-$ and H_2O_2 in the formation of ·OH. Addition of DTPA (0.1 mM) to the reaction mixture inhibited the formation of the spin adduct completely (Fig. 1 E). This result suggests the involvement of iron in the ·OH generation. These inhibitory effects of SOD, catalase and DTPA also support the conclusion that the trapped spin adduct in the presence of 4-POBN and excess amount of ethanol was derived from ·OH.

Reaction mechanism for ·OH generation

From these results, we propose the following reaction mechanism for the generation of ·OH triggered by the oligosaccharide elicitor. $\cdot O_2^-$ was first generated from the elicited cells and dismutated spontaneously to H_2O_2 [2]. Ferric iron, which is probably abundant in the apoplastic fluid, was reduced to ferrous iron by the reaction with a part of $\cdot O_2^-$ [1]. The ferrous iron then reacts with H_2O_2 to form ·OH [3; Fenton reaction], which reacts with ethanol to form ·CH(CH_3)OH [4].

$$\cdot O_2^- + Fe^{3+} \rightarrow O_2 + Fe^{2+} \quad [1]$$

$$\cdot O_2^- + \cdot O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad [2]$$

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \cdot OH + OH^- \quad [3]$$

$$\cdot OH + CH_2(CH_3)OH \text{ (excess)} \rightarrow H_2O + \cdot CH(CH_3)OH \quad [4]$$

$$\text{4-POBN} + \cdot CH(CH_3)OH \rightarrow \text{4-POBN-}CH(CH_3)OH \quad [5]$$

Generation of reactive oxygen species triggered by a protein phosphatase inhibitor, calyculin A

Possible involvement of protein phosphorylation in elicitor signal transduction was suggested in several plants (Ryan and Farmer 1991), and Felix et al. (1994) recently reported that calyculin A, a protein

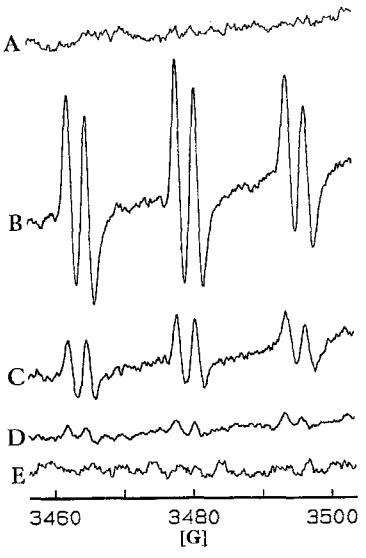

Fig. 2. EPR detection of the generation of hydroxyl radical from suspension-cultured rice cells elicited with calyculin A. Spin trapping reagents, 4-POBN (10 mM) and ethanol (170 mM) as well as SOD (50 U/ml; **C**), catalase (500 U/ml; **D**) or DTPA (0.1 mM; **E**) were added to the cell suspension just before the elicitation. The extracellular medium of each sample was collected 79 min after the elicitation with calyculin A (1 μM) (**B–E**), and EPR spectra were taken. **A** Control (without calyculin A). The number of scan was 1

phosphatase inhibitor, mimicked elicitor action such as medium alkalinization. Our preliminary studies by chemiluminescence methods also suggested the possible involvement of protein phosphorylation in the reactive oxygen generation triggered by *N*-acetylchitooligosaccharides (Kuchitsu and Shibuya 1994).

Addition of calyculin A to the cell suspension resulted in marked formation of 4-POBN-CH(CH_3)OH (Fig. 2 B). The time courses of the reactive oxygen generation triggered by *N*-acetylchitooligosaccharides and cylyculin A were significantly different. In the case of calyculin A treatment, the reaction prolonged much longer compared to that induced by the oligosaccharide, and the amount of the spin adduct accumulated in the extracellular medium after 79 min reached 1.34 μM. The formation of the spin adduct with calyculin A was also inhibited by SOD, catalase and DTPA (Fig. 2 C–E), indicating that the mechanisms for generation of reactive oxygen species triggered by calyculin A are identical to those by *N*-acetylchitooligosaccharides. The generation of $^{\cdot}O_2^-$ with *N*-acetylchitooligosaccharides must therefore be regulated by phosphorylation and dephosphorylation of regulatory protein(s). Experiments to confirm this hypothesis are now under way.

Possible physiological significance of generated reactive oxygen species

There are several possibilities for the physiological significance of the reactive oxygen generation induced by the oligosaccharide elicitor. Reactive oxygen species may act as intracellular and/or intercellular signal molecule(s) in the defense reaction (Dempsey and Klessig 1994). Hydroxyl radicals induced by the oligosaccharide could react with membrane components such as unsaturated fatty acids and generate hydroperoxides, which might play some roles as signal molecules. This agrees with the observation with soybean cotyledons that scavengers of the hydroxyl radical inhibited the accumulation of phytoalexins (Epperlein et al. 1986). If the hydroxyl radical plays some roles in plant defense responses, the Fe^{2+} ion, which is necessary for $^{\cdot}OH$ generation and is abundant in apoplastic intracellular space of plants, may have an important role in preventing infection. Hydrogen peroxide might also be involved in the cross-linking of cell wall components, which leads to the toughening of the plant cell wall (Bradley et al. 1992) and prevents the fungal infection. It is also possible that reactive oxygen species or their derivatives directly attack fungal cells, or plant cells themselves for the hypersensitive reaction (Levine et al. 1994). Further studies using this simple model system with a purified oligosaccharide elicitor and suspension-cultured cells would reveal the role of active oxygen species in the initial step of plant-microbe interaction.

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