Superoxide Anion Generation in Rice Blade Protoplasts with the Blast Fungus Proteoglucomannan Elicitor as Determined by CLA-phenyl Luminescence and Its Suppression by Treating the Elicitor with α-D-Mannosidase

Minoru Haga,† Yuniko Kohno,†† Michiaki Iwata,* and Yasuharu Sekizawa†††

Department of Agricultural Chemistry, Faculty of Agriculture, Tamagawa University, Machida-shi, Tokyo 194, Japan
* Pharmaceutical Research Center, Meiji Seika Kaisha Ltd., Kohoku-ku, Yokohama 222, Japan
Received December 15, 1993

A chemiluminescence probe specific for the superoxide anion, 2-methyl-6-phenyl-3,7-dihydroimidazo-[1,2-a]pyrazine-3-one, was used to directly demonstrate the $O_2^-$ generation in rice blade protoplasts with the purified blast-fungus elicitor (proteoglucomannan). A hyperbolic relationship between the relative photon emission and the amount of elicitor applied implied the presence of a putative receptor for the elicitor. Pretreatment of the elicitor with α-D-mannosidase completely crossed out the stimulatory activity to generate $O_2^-$. The presence of mannostatins, inhibitors of α-D-mannosidase, also inhibited $O_2^-$ generation. Plasma membrane turbulence was observed by the quenching of fluorescence from a membrane turbulence reporter, bis-(3-propyl-5-oxo-isoxazol-4-yl) pentamethine oxonol, when the protoplasts were stimulated with the elicitor. Although treating rice protoplasts with IAA alone did not stimulate $O_2^-$ generation, protoplasts pretreated with IAA markedly activated the $O_2^-$ generation capability when stimulated by the elicitor. The $O_2^-$ generation activated by stimulating the elicitor was abruptly blocked by the addition of IAA.

It has earlier been reported1,2) that the phospholipase C system is operative for transmembrane signalling in rice (Oryza sativa) blade cells stimulated by the proteoglucomannan elicitor from the blast fungus (Pyricularia oryzae), and that the signal-coupled formation of Ca$^{2+}$-MP by the intracellular signalling system is indispensable for activating $O_2^-$ generation and α-linolenate release, two biochemical events involved in the early phase of the defense mechanism induced in the rice blade tissue. It has recently been postulated that the IAA receptor residing on the plasma membrane of the outer epidermal cells of the maize (Zea mays) coleoptile is physiologically responsible for inducing elongation by growth,3) and that the application of IAA to a plasma membrane preparation of carrot (Daucus carota) suspension-cultured cells resulted in the operation of a phospholipase C system coupled with plant cell elongation.4) Auxin-binding proteins (BAP I and II) have been isolated from the cytosolic fraction from etiolated shoots of mung bean (Vigna radiata), and were shown to be involved in the initiation and promotion of transcription in nuclei.5-8) An explanation of why pretreating with auxins before the spray inoculation of a compatible blast fungus conidia makes rice seedlings temporarily resistant, has remained a long-standing mystery.9,10) As ACC synthase is generally induced in plant tissue by the application of IAA,11) it has been believed that ethylene generated in the rice blade tissue might be initiated by the induction of ACC synthase.12) Recent studies3,13,14) have revealed that the sequential induction of enzymes related to the operation of an inducible host defence mechanisms against rice blast disease depended on the enhanced generation of endogenous ethylene and that this ethylene generation depended on the generation of $O_2^-$, a cause of earlier hypersensitive resistance. In this study, we report further characterization of the blast fungus proteoglucomannan elicitor1) and the modulatory effects of IAA on $O_2^-$ generation in rice blade protoplasts stimulated by the elicitor, using a CLA-phenyl luminescent assay.15,16)

Materials and Methods

Reagents. CLA-phenyl15,16) was purchased from Tokyo Kasei Kogyo Co., (Tokyo, Japan). Oxonol VI17) was obtained from Molecular Probes (Junction City, Oregon, U.S.A.), and α-D-mannosidase (jack bean) and IAA (indole-3-acetic acid) were obtained from Sigma. Mannostatins18,19) was generously presented by Prof. Dr. Takaaki Aoyagi of the Institute of Microbial Chemistry (Tokyo, Japan). The other reagents used were of the highest grade commercially available.

Rice blade protoplasts and blast fungus elicitor. Rice blade protoplasts were prepared from undeveloped blades encapsulated in the leaf sheaths as previously described.1) The extraction and purification procedure for the native and most dominant blast-fungus proteoglucomannan elicitor (the 25-35 kDa fragment) from the mycelial mass was as previously described.1) The medium used for the submerged culture of blast fungus HOKU 373 consisted of 2.0% glucose and 0.5% yeast extract at pH 6.5. Both the seed culture and the submerged culture were conducted at 28°C for 5 days. The filtered mycelial mass (5.5 kg) obtained from 150 liters of the submerged culture was used to prepare the elicitor. We confirmed that the yeast extract used in this study did not have any elicitor activity to generate $O_2^-$ from rice blade disks. Determination of $O_2^-$ generation is stimulated rice blade protoplasts. It

---

1) Causal Analysis of Reaction Cascades in the Induced Defense Mechanisms of Rice Plants. XIV (see ref. 2).
2) To whom correspondence should be addressed.
3) Present address: Y. Kohno, Tokyo Research Institute, Seikagaku Corp., Higashiyamato-shi, Tatemachi 3-1253, Tokyo 190, Japan; Y. Sekizawa, Laboratory of Microbial Toxicology, The Institute of Physical and Chemical Research, Higashihara 2-1, Wako-shi, Saitama 351-01, Japan.
4) Abbreviations: IAA, indole-3-acetic acid; CLA-phenyl, 2-methyl-6-phenyl-3,7-dihydroimidazo[1,2-a]pyrazine-3-one; Oxonol VI, bis-(3-propyl-5-oxo-isoxazol-4-yl) pentamethine oxonol; Ca$^{2+}$-MP, Ca$^{2+}$ bound calcium-modulated proteins; ACC synthase, 1-aminocyclopropane-1-carboxylate synthase.
has been reported that the CLA-phenyl luminometric determination is specific to \( O_2^- \). The reaction mixture for measuring \( O_2^- \) generation from stimulated rice blade protoplasts consisted of 500 \( \mu \)l of the protoplast suspension containing \( 1 \times 10^6 \) cells/ml of 0.6 \( \mu \)l sorbitol, 30 \( \mu \)l of 10 \( \mu \)M CLA-phenyl, and 0.6 \( \mu \)l sorbitol containing 1% DMSO, and 100 \( \mu \)l of 0.6 \( \mu \)l sorbitol for applying the purified elicitor plus 100 \( \mu \)l of 0.6 \( \mu \)l sorbitol with or without chemicals. The total volume of the reaction mixture was 730 \( \mu \)l in a Bioluman LB 9505 cuvette. Photon counting was recorded at 27°C by a Bioluman LB 9505 (Berthold Japan, Tokyo) connected with a NEC PC-9801 Vm personal computer (Nihon Electric Corp., Tokyo) after stabilizing the 380 nm chemiluminescence emission for 5–10 min.

**Determination of membrane turbulence in stimulated rice blade protoplasts.** Oxonol VI is a fluorescence reporter (a membrane-associated potentiometric dye). The reaction mixture for measuring fluorescence quenching by the stimulated protoplasts consisted of 2 ml of the protoplast suspension containing \( 10^6 \) cells/ml in 0.6 \( \mu \)l sorbitol. This reaction mixture was placed in a 3.0 ml cuvette, and Oxonol VI was dissolved in an aqueous 10% acetone solution at 650 \( \mu \)g/ml. The dye solution was protected from light and stored frozen. The Oxonol VI solution (5 \( \mu \)l) was added to 2 ml of the protoplast suspension in the cuvette at 25°C under continuous stirring. After the fluorescence had stabilized, 100 \( \mu \)l of the blast fungus elicitor in 0.6 \( \mu \)l sorbitol was added to make concentration of 80 to 400 \( \mu \)g of elicitor per ml of the reaction mixture. A FP-550 spectrophotometer (Japan Spectroscopic Co., Tokyo) connected with a UP-6611A recorder (National Electric Corp., Tokyo) was used to continuously scan the fluorescence quenching just after stimulation with the elicitor. The fluorescence was measured at 645 nm with excitation at 609 nm.

**Results**

**Behavior of \( O_2^- \) generation in rice blade protoplasts stimulated by the purified blast-fungus proteglucosamman elicitor.**

The kinetics of \( O_2^- \) generation in rice blade protoplasts stimulated by the elicitor are shown in Fig. 1. After the chemiluminescence emitted from the reaction mixture in the absence of the elicitor had been stabilized, the elicitor was added to stimulate the protoplasts at the time shown by the arrow. The rice blade protoplasts generated \( O_2^- \) within approximately 2 min after being stimulated by the elicitor. This \( O_2^- \) generation (CLA-phenyl chemiluminescence counts/min) seems to indicate that the putative receptor (1) responsive to \( O_2^- \) generation might not have been fully saturated by the given dose of elicitor. The hyperbolic relationship shown in the inset to Fig. 1 between the relative photon emission per min at 12 min after stimulating the elicitor and the dose of elicitor applied, validates this hypothesis. The minor shock-wave-like pattern of signal-coupled \( O_2^- \) generation from the lowest dose of the elicitor (Fig. 1, curve E) about 6 min after stimulation is regarded as an earlier transitory wave. Using a datum for the dose of elicitor as 100 \( \mu \)g (Fig. 2, curve F) and the data in Fig. 1 under the same experimental conditions, a Scatchard plot of ligand-binding (20) showed a linear relationship, when relative \( O_2^- \) generation \( \cdot \) ml\(^{-1}\)cell\(^{-1}\) was plotted as a function of relative \( O_2^- \) generation \( \cdot \) ml\(^{-1}\)cell\(^{-1}\)/molar concentration of elicitor (plotted data not shown). The correlation coefficient (r) was −0.99, and the linear relationship again suggests the presence of putative receptor in the cells.

**Inactivation of the blast fungus elicitor by \( \alpha-\delta-\)mannosidase and the inhibition of \( O_2^- \) generation by mannostatin**

When 200 \( \mu \)g of the elicitor was hydrolyzed by 4 units of \( \alpha-\delta-\)mannosidase at 25°C for 1 h in 150 \( \mu \)l of a 50 mm phosphate buffer (pH 4.0) and the hydrolyzate (pH 6.5) was added to the reaction mixture, no stimulatory activity for generating \( O_2^- \) was apparent (Fig. 2, curve H). When 100 \( \mu \)g of mannostatin, potent inhibitors of \( \alpha-\delta-\)mannosidase, was added before stimulating the elicitor, the addition of the elicitor to the reaction mixture did not activate the \( O_2^- \) generation mechanism (Fig. 2, curve I).

**Fluorescence transition of Oxonol VI at the plasma membrane of rice blade protoplasts stimulated with the blast fungus elicitor.**

The quenching of fluorescence from Oxonol VI at the

![Fig. 1. Behavior and Kinetics of \( O_2^- \) Generation by Rice Blade Protoplasts Stimulated with the Purified Blast Fungus Elicitor.](image)

The doses of elicitor added to stimulate protoplasts in the reaction mixture (730 \( \mu \)l) at the time shown by the arrow were as follows: A, 50 \( \mu \)g; B, 25 \( \mu \)g; C, 10 \( \mu \)g; D, 1 \( \mu \)g; E, 0.1 \( \mu \)g. A resting culture of fresh protoplasts in 0.6 \( \mu \)l sorbitol for 3–5 h was indispensable for regaining the activity of \( O_2^- \) generation. Inset: Relative \( O_2^- \) generation at 12 min after stimulation by various concentrations of elicitor. See the results for selecting an adaptable time for plotting.

![Fig. 2. Hydrolytic Inactivation of Elicitor Activity by \( \alpha-\delta-\)Mannosidase and the Inhibition of \( O_2^- \) Generation by Rice Blade Protoplasts Pretreated with Mannostatin.](image)

The arrow shows the time of elicitor stimulation. F, addition of 100 \( \mu \)g of elicitor alone to the reaction mixture (730 \( \mu \)l); G, 4 units of \( \alpha-\delta-\)mannosidase alone; H, the blast fungus elicitor (200 \( \mu \)g/150 \( \mu \)l) was hydrolyzed by 2 units of \( \alpha-\delta-\)mannosidase (4 units) at pH 4.0 and 25°C for 1 h, and the whole hydrolyzate (200 \( \mu \)g at pH 6.5) was added to the reaction mixture; I, mannostatin (100 \( \mu \)g) was added 1 min prior to stimulation with the elicitor.
plasma membrane of rice blade protoplasts was stimulated by the blast fungus elicitor (Fig. 3), the arrow showing the time of elicitor stimulation. A negative correlation between the dose of elicitor applied and the relative fluorescent intensity at 35 min after stimulation was observed, as shown in the inset to Fig. 3, the correlation coefficient (r) being -0.99.

**Reversible effect on O$_3$ generation of IAA with post- and pre-stimulation by the elicitor**

The application of IAA alone did not activate the O$_3$ generation mechanism even at $7.7 \times 10^{-4}$ M (Fig. 4, curve C). Arrow 1 shows the time of IAA application, and arrow 2 shows the time of elicitor stimulation in run A and vice versa in run B in Fig. 4. The application of IAA with post-stimulation by the elicitor markedly enhanced O$_3$ generation, while the application of IAA with pre-stimulation by the elicitor abruptly blocks O$_3$ generation. No dose-dependent effect of IAA was apparent between $1.5 \times 10^{-6}$ and $7.7 \times 10^{-4}$ M. The O$_3$ generation depended on the dose of the elicitor as already shown in Fig. 1.

**Discussion**

Further characterization of the functions of the blast fungus proteoglucosaminan elicitor indicated the active moiety of the native and most dominant proteoglucosaminan elicitor (25 kDa; $ED_{50, pos} = 10 \mu g/\text{ml}$) to involve an x-d-mannosidic linkage, as hydrolysis with x-d-mannosidase completely cross out the stimulative activity to generate O$_3$ (Figs. 1 and 2). The precursor of this water-soluble native fragment of relatively small molecular mass is uncertain at present, but could be an immature fragment or a degraded fragment of mature proteoglucosaminan which forms the matrix of the outer cell wall of the blast fungus mycelium. The repeated preparative work on the native fragments have indicated the molecular mass of the native and most dominant elicitors to range between 25 and 35 kDa, depending on the culture conditions and blast fungus races used (unpublished data). The presence of a similar elicitor-active fraction in blast fungus conidia has been observed and the presence of mannosinibs, potent inhibitors of x-d-mannosidase, inhibited the elicitor-coupled O$_3$ generation (Fig. 2). This finding suggests two possibilities: (1) the binding site of the putative receptor might have a similar peptide domain to the substrate binding site of x-d-mannosidase, and (2) a stereo-specific type of mannosinibs-sensitive x-d-mannosidase at the host site might split the more active oligosaccharide chain with the x-d-mannosidic linkage. Relating to the latter, it has been elucidated that the native dominant $\beta$-d-glucan elicitor of Phytophthora megasperma f. sp. glycinea was released by the host $\beta$-1,3-endoglucanase.

It has been reported that the quenching of Oxonol VI fluorescence coincides to the membrane potential transition when cell cultures of cotton (Gossypium arboreum), tobacco (Nicotiana tabacum) or soybean (Glycine max) were stimulated with a pathogenic fungus (Verticillium dahliae) elicitor, and that the time lag after stimulation depended on the dose of stimulant applied. As shown in Figs. 1 and 3, the O$_3$ generation in rice protoplasts started 2 min after elicitor stimulation, requiring Ca$^{2+}$ to activate the O$_3$-forming redox system, and the fluorescence quenching of Oxonol VI in the plasma membrane started 15 min after elicitor stimulation. This time difference of ca. 13 min might
imply that degeneration of the plasma membrane by the hydroperoxidation of membrane-lipids due to $O_2^-$ generation caused a leakage of inorganic ions. Therefore, marked changes in the inorganic ion concentration outside and inside the membrane might cause the quenching of Oxonol VI in the membrane. Further study is required to confirm this hypothesis.

The reversible effect of IAA on $O_2^-$ generation in rice blade protoplasts, which was stimulated by the proteoglucomannan elicitor after or before IAA treatment, is an interesting phenomenon related to the regulatory mechanism of signal-coupled $O_2^-$ generation (Fig. 4). It has generally been accepted that increasing the affinity of the receptor for the ligand by a positive cooperative effect, which is either homotropic or heterotropic, might be involved in a positive regulatory mechanism, and that rapid internalization of the ligand-bound receptor might be involved in a down-regulatory mechanism. An amphiphilic compound like IAA might bind to any one of the signal-amplifying elements of the transduction system to activate an $O_2^-$-forming redox system. By another hypothetical view, two similar signal transduction system, which are linked with either the perception of the proteoglucomannan elicitor or of auxin, might have a cross-talk connection to exhibit the reversible regulation. Further studies are required to elucidate the regulatory mechanism concerned with the reversible effects of IAA on signal-coupled $O_2^-$ generation, as $O_2^-$ generation in the early phase of the defence mechanism caused the hypersensitive death of invaded cells and enhanced endogenous ethylene formation, which induced a set of enzymes related to resistance in rice plants.

The NADPH oxidoreductase of the plasma membrane vesicle from elongated hypocotyls of etiolated soybean (Glycine max) seedlings has been reported to be involved in numerous cellular processes in plants. A single application of auxins, including IAA, directly activated the solubilized NADPH oxidase activity. As indicated in this paper (Fig. 4), the application of IAA alone did not stimulate $O_2^-$ generation in rice blade protoplasts, therefore, the NADPH oxidase system of the rice blade, which directly transfers one electron to oxygen by elicitor stimulation, might be under a different regulatory mechanism. The feature of the NADPH oxidase system in rice blade cells has been observed to be similar to that of the flavocytochrome system in an animal cell (neutrophil).

At the beginning of our studies on elicitor-coupled $O_2^-$ generation in rice blade pieces, using the partially purified blast fungus proteoglucomannan elicitor, we used superoxide dismutase-sensitive cytochrome C reduction to detect the $O_2^-$ generated, but superoxide dismutase non-sensitive cytochrome C reduction was observed with some strains of rice (unpublished data). Subsequently, nitroblue tetrazolium reduction was successfully used for a routine assay of $O_2^-$ generation, and 85 to 90% of the reduction was inhibited in the presence of superoxide dismutase. However, this method was laborious and required skilled operation. These assays have been used to analyze the relationship between $O_2^-$ generation and a hypersensitive reaction in potato tuber slices stimulated with the hyphal wall component or spores of Phytophthora infestans. The luminometric determination of hydrogen peroxide or $O_2^-$ has been reported, using luminol in the absence or presence of superoxide dismutase, and has been successfully used to investigate the relationship between $O_2^-$ and the hypersensitive death of suspension-culture cells of tobacco (Nicotiana tabacum) after inoculation with Pseudomonas syringae pv. syringae. The luminol luminometric assay has also been applied to determine the generation of active oxygen species in suspension-culture cells of soybean (Glycine max) stimulated by the Phytophthora megasperma f. sp. glycinea elicitor.

The analysis of elicitor-coupled $O_2^-$ generation has been conducted with a crude or partially purified elicitor. In this present study, we used an isolated proteoglucomannan of relatively small molecular mass (25 kDa) with the characteristics of the most dominant water-soluble native fragment. This implies that the elicitor preparation used in this study was free from other species of elicitors such as $beta$-glucan and chitin of the blast fungus cell wall. A CL-A-phenyl luminometric assay of $O_2^-$ could automatically record $O_2^-$ generation and provided a micro-analytical procedure to obtain the kinetic data.

Acknowledgments. We thank Dr. K. Kaharao of Pharmaceutical Research Center, Meiji Seika Kaisha Ltd., for the use of a photon counter. This study was supported in part by a Grant-in-Aid for Scientific Research (No. 01480055) from the Ministry of Education, Science, and Culture of Japan.

References
22) M. Iwata, Thesis for Doctorate Degree, University of Tokyo, 1984, p. 166.


