Induction of Resistance against Rice Blast Fungus in Rice Plants Treated with a Potent Elicitor, N-Acetylchitoooligosaccharide

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The mode of action of a potent elicitor, N-acetylchitoooligosaccharide, in rice plants was examined. In intact seedlings, no significant uptake of the elicitor via the roots was observed within 3 h, whereas rapid uptake was observed in excised leaves. Rapid and transient expression of an elicitor-responsive gene, EL2, was induced in the leaves of intact seedlings sprayed with the elicitor or in the roots and leaves of intact seedlings by immersing roots in the elicitor solution. Histochemical analysis indicated that EL2 was expressed in cells exposed to the elicitor of root and leaves. In seedlings treated with the elicitor for 1 d or longer, hyphal growth of rice blast fungus was significantly delayed, and an accumulation of auto-fluorescence around the infection site was observed. Two defense-related genes, PR-1 and PR-10 (PBZ1), were induced in a systemic and local manner by elicitor treatment, in correlation with the induction of resistance against rice blast fungus. N-Acetylchitoheptaose did not inhibit the hyphal growth of the fungi. These results indicate the occurrence of systemic signal transmission from N-acetylchitoooligosaccharide in rice plants.

Key words: N-acetylchitoooligosaccharide; elicitor; rice; signal transduction

Higher plants can deploy a set of defense responses upon microbial attack, including a variety of reactions with different time courses. Extensive studies have been conducted to elucidate the molecular mechanisms by which plants recognize pathogens and mobilize signal transduction leading to the establishment of resistance. Among these, mechanisms of recognition by host resistance genes (R-genes) of pathogens carrying the corresponding avirulence genes (Avr-genes) have been a central interest in plant pathology.1) On the other hand, cellular components from pathogens or host plants are known to act as elicitors that condition resistance in plants.2) Elicitors have been a powerful tool to analyze resistance responses by host plants, and recently the biological significance of the plant-microbe interaction has been recognized as pathogen-associated molecular patterns (PAMPs).3) Furthermore, a proteinaceous fungal elicitor, INF1, has been shown to play critical roles in non-host resistance.4) Oligosaccharide fractions derived from fungal and plant cell walls include active elicitors, but, only a limited number of oligosaccharide elicitors has been chemically characterized, and large fraction of the molecular mechanisms of signaling from oligosaccharide elicitors remains to be elucidated.5) N-Acetylchitoooligosaccharide is a chemically characterized and potent elicitor inducing a set of defense responses such as changes in membrane electrochemical potential, ion fluxes, gene expression, and metabolic changes resulting in the production of reactive oxygen species and phytoalexins in rice,6–9) wheat,10–12) tomato,13) soybean,14) and arabidopsis,15) and in cell death in oats.16) Among these plants, the most extensive studies have been conducted on the molecular mechanisms of the recognition and signal transduction of the N-acetylchitoooligosaccharide elicitor using rice. A high-affinity binding site of N-acetylchitoooligosaccharide in the plasma membrane of suspension-cultured cells17,18) and seedlings of rice has been identified.19) It appears to act as a receptor for N-acetylchitoooligosaccharides. Because the signaling systems in intact plants in which cells are highly differentiated might be different from those in suspension-cultured cells, it is interesting to examine the mode of transduction of signals from the N-acetylchlo-
tooligosaccharide elicitor. As a first step to clarify the mode of elicitor-signaling in rice plants, we studied the responses of rice plants to treatment with N-acetylchito-
tooligosaccharide.

Materials and Methods

Plant and fungal materials. Two genotypes of rice (Oryza sativa cv. Nipponbare), “NP/++” and “NP/−”, compatible and incompatible with Magnaporthe grisea (strain P91-15B; race 001) respectively, were used as fungal and plant materials. Rice seedlings were grown by hydroponics in a nutrient solution containing 0.5 mM fungal and plant materials. Rice seedlings were grown (suspension of rice seedlings were excised, filled with a conidial as described previously.21) To test the effect of tooligosaccharide elicitor. As a first step to clarify the responses of rice plants to treatment with N-acetylchitoheptaose for 7 d at 30 °C.

Elicitor treatment. N-Acetylchitoheptaose, N-acetylchitoctaose, and 125I-labeled APEA (2-(4-aminophenyl)ethylamine)-conjugated N-acetylchitoctaose (125I-APEA-GN8) were prepared as described previously.6,18) All elicitors were dissolved in water.

In the experiments using excised leaves, the seedlings were cut at the base and the leaves were pre-incubated in H2O for 3 h to minimize the effect of wounding. Third leaves were used for further analysis. For intact seedlings of rice, elicitor treatment was carried out by immersing roots in or spraying leaves with N-acetylchitoheptaose at 10 μg/ml. For the assay of induced resistance by N-acetylchitoheptaose, roots of NP/++ and NP/− plants were immersed in water for 5, 4, 2, and 0 d, followed by treatment with N-acetylchitoheptaose for 0, 1, 3, and 5 d, respectively, so that all plants were pre-treated for 5 d in total with water followed by N-acetylchitoheptaose via roots before inoculation with M. grisea. As positive controls, NP/++ plants treated with 1,2-benzisothiazole-3(2H)-one 1,1-dioxide (BIT), an active form of chemical inducer of resistance, probenazole,23 via roots, or NP/− plants were inoculated with M. grisea.

For elicitor transport assay, the intact and excised plants were treated with 1 μg/ml 125I-APEA-GN8, as described above. A segment of 5 mm thickness was subjected to measurement of radioactivity with a γ-ray counter.

Inoculation of M. grisea on leaf sheath. Leaf sheaths of rice seedlings were excised, filled with a conidial suspension (1 × 107 conidia/ml), and incubated at 23 °C for 2 d.21) The epidermal cells were observed under light- and fluorescent-microscopy for hyphal growth and accumulation of fluorescent materials respectively.

By observation with light-microscopy at 2 d after inoculation, intact appressoria were counted and scored as “infected,” “one cell-infected,” or “non-infected,” corresponding to the appressoria that infected more than one cell beneath the appressorium, one cell, or no cell of rice respectively.

Other techniques. Tissue preparation and in situ hybridization were carried out as previously described.23) Tissue sections were fixed in 4% (w/v) paraform-aldehyde, 0.25% (w/v) glutaraldehyde, 50 mM sodium phosphate buffer (pH 7.2) for 12 h, dehydrated by passing them through a series of ethanol/1-butyl alcohol at 30 °C, and embedded in molten (about 65 °C) Para-plast Plus Tissue Embedding Medium (Oxford Labware, St. Louis, MO). Total RNAs from rice were isolated according to the phenol-SDS method. Northern blot hybridizations were carried out by the glyoxal method. For RT-PCR analysis, first-strand cDNA was synthesized from 1 μg of total RNA in a 20 μl RT reaction, and 1 μl aliquot of the products was added to 40 μl of PCR solution containing the gene-specific primers. The gene-specific primers used in this study were as follows: PR-1 (AK107926), 5'-ATACACACCGCGTACGTACTG-3' and 5'-ACTTGAGACATGACGCC-3'; PR-10 (PBZ1: AK071613), 5'-GAGGATATCTGCCTTCTATCC-3' and 5'-CTCTCAGGACTCAAACGCA-3'; UBQ (ubiquitin: AK121590), 5'-CCAGTAAGTCCTCCATGGAG-3' and 5'-GGACACAATGATTAGGGAATCAG-3'.

Results

Uptake of 125I-APEA-GN8 by rice plants
We first examined the uptake by excised leaves of 125I-APEA-GN8, which has been shown to retain sufficient activity as an elicitor.18) Cutting sites of the excised leaves were soaked in a solution of 125I-APEA-GN8 and incubated for up to 3 h. As shown in Fig. 1, significant radioactivity at 5 cm height was detected at 1 h and increased for up to 3 h after soaking. In the segment at 15 cm height, radioactivity was detected at 2 h and showed a drastic increase, indicating that 125I-APEA-GN8 can move inside leaves, likely via vascular bundles. On the contrary, no such elevation of radioactivity was detected in the attached leaves for the 3 h test period, indicating that N-acetylchitoheptaose is not incorporated by roots.

Expression of EL2 in response to N-acetylchitoheptaose
We further examined the response to N-acetylchito-heptaose of intact seedlings and excised leaves by analyzing the expression of an elicitor-responsive gene, EL2. In our previous study, EL2 was shown to be induced by N-acetylchitoheptaose at 100 ng/ml (about 70 nm) within 6 min in suspension-cultured rice cells.9) Therefore, EL2 was considered to be a suitable molecu-
lar marker for analysis of the elicitor response by rice plants. Rice seedlings were treated with N-acetylchitoheptaose and expression of EL2 was examined by northern blot hybridization (Fig. 2). When roots of intact seedlings were submerged in the elicitor solution, mRNA for EL2 accumulated in roots and attached leaves within 30 min. Similar accumulation of mRNA for EL2 was induced within 60 min in leaves sprayed with N-acetylchitoheptaose.

In experiments using excised leaves, sections were prepared at a height of 15 cm from the cutting site, which N-acetylchitoheptaose probably reached some time between 60 min and 120 min after soaking in the elicitor solution, as shown in Fig. 1. In situ hybridization analysis detected accumulation of EL2 mRNA in the parenchymal cells surrounding the vascular bundles at 120 min after elicitor treatment. Because the elicitor is considered to reach that point at 60 min or later after incubation (Fig. 1), this result indicates that EL2 was induced within 60 min in the parenchymal cells likely exposed to N-acetylchitoheptaose. In the intact roots, accumulation of EL2 mRNA was detected at 30 min in the exodermis and root cap, which were also probably exposed to N-acetylchitoheptaose, in addition to the stele, where the expression was detected in non-treated plants (Fig. 3).

**Induced resistance against rice blast fungus**

Because N-acetylchitoooligosaccharide can induce a variety of defense responses in suspension-cultured rice cells, it was thought likely to induce resistance in rice plants. We treated NP/++ for 0 to 5 d with N-acetylchitoheptaose at 10 μg/ml, followed by inoculation of M. grisea on the leaf sheath, and the extent of infection at 48 h was classified as “infected,” “one cell-infected,” or “non-infected,” and compared with NP/a or NP/++ pre-treated with BIT, an biologically active form of probenazole (Fig. 4).

In the control plants, 79.7% of appressoria were counted as “infected.” The ratio of “non-infected” appressoria increased as treatment with N-acetylchitoheptaose became longer. After elicitor treatment for 5 d, 54% of appressoria didn’t infect plants, although the extent of resistance was not as high as seen in NP/a plants (Fig. 4). Microscopic observation detected accumulation of fluorescent substances in the cells beneath appresorium in elicitor-treated NP/++, as observed in NP/a or BIT-treated NP/++ (Fig. 5).
Local and systemic induction of PR-1 and PR-10 by treatment with N-acetylchitoheptaose

The results shown in Fig. 5 indicate that rice plants acquired systemic resistance by treatment with N-acetylchitoheptaose. Because it is generally believed that systemic resistance involves the expression of Pathogenesis-related (PR) genes, we examined the activation of two PR genes, PR-1 and PR-10. A transient and continuous accumulation of mRNA for PR-10 within 6 h was observed in the roots and leaves of seedlings respectively, whose roots were immersed in the elicitor solution. On the contrary, no such rapid accumulation of mRNA was observed for PR-1 (Fig. 6A). By incubation longer than 2 d, the level of mRNA for both genes increased up to 5 d (Fig. 6B), in good correlation with the emergence of resistance against rice blast (Fig. 5).

Effect of N-acetylchitoheptaose on the growth of rice blast fungus

Although the results described above strongly indicate that N-acetylchitoheptaose induces systemic resistance in rice plants, it is difficult to exclude the possibility that trace amounts of N-acetylchitoheptaose were incorporated by plants and showed toxicity against M. grisea. To address this question, growth of M. grisea in the presence of N-acetylchitoheptaose was examined (Fig. 7). Compared with the control experiment, N-acetylchitoheptaose at 1, 10, and 20 μg/ml did not show any inhibitory effect on the growth of hyphae.

Discussion

N-Acetylchitooligosaccharides induce a set of defense responses in rice. A high-affinity binding site, probably working as a receptor, was detected in the plasma membrane of cultured cells, and thereafter in leaves and roots of rice. These results indicate that rice plants are equipped with mechanisms for the recognition of N-acetylchitooligosaccharides and signal transduction, as well as cultured cells, and they prompted us to examine the mode of action of N-acetylchitooligosaccharide elicitor in rice plants, focusing on elicitor signaling and disease resistance.

As shown in Fig. 2, EL2 was expressed in the distant leaves of intact seedlings within 30 min after roots were submerged in N-acetylchitoheptaose. The time course was comparable to that in leaves sprayed with N-acetylchitoheptaose. Because no significant level of radioactivity was detected in the attached leaves for up to 3 h after roots were submerged in 125I-APEA-GN8 (Fig. 1), the results strongly indicate that the elicitor signal was converted into a rapidly diffusible form in the roots, which induced EL2 in the leaves. This hypothesis is supported by the observation that application of elicitors, viz., CuCl2, jasmonic acid, UV, and chito-oligosaccharides, to detached leaves of rice induced emission of volatile compounds identified as (Z)-3-hexen-1-ol, monoterpens, sesquiterpens, and methyl salicylate, although the roles of these compounds in the defense responses have not been elucidated. It is tempting to speculate that systemic signals from the N-acetylchitooligosaccharide elicitor are carried by such second messengers, leading to expression of EL2.

At present, it is not clear whether the in planta signals activating early responses such as induction of EL2 are the same as those establishing resistance against infection by rice blast. A number of reports have de-
monstrated that elicitor treatment confers resistance to plants. For example, laminarin, a \( \beta \)-1,3-glucan from brown alga *Laminaria digitata*, protected grapevine from *Botrytis cinerea* and *Plasmopara viticola*. In these studies, no second messengers of elicitor signals were identified. On the other hand, exogenous treatment or endogenous expression of the gene for cryptogein, a proteinaceous elicitor secreted by *Phytophthora* species, induced systemic acquired resistance in tobacco, accompanied with activation of PR genes in a manner dependent on salicylic acid and ethylene. In this study, the systemic resistance in rice plants induced by N-acetylchitoheptaose was shown to be accompanied by the activation of PR-1 and PR-10 (Fig. 6), although the roles of salicylic acid and ethylene in the defense response of rice are not clear. Identification of second messengers of N-acetylchitoheptaose should give us critical clues to elucidate the mechanisms of induced resistance in rice plants, but it cannot be denied that traces amount of N-acetylchitoheptaose were incorporated and induced resistance against, not toxiciy to, rice blast fungus.

Chitin is one of the most abundant polysaccharides on the earth, comprising the exoskeletons of arthropods, etc., and it is recyclable through biodegradation by soil microbes. N-Acetylchitoheptaose at 10 mg/liter reduced

**Fig. 4.** Induction of Resistance in Rice Plants against *M. grisea* by Treatment with N-Acetylchitoheptaose.

A, Representative appresorium that infected one cell or more (a) (“infected”), one cell (b) (“one cell-infected”), and no cell (c) (“non-infected”) of leaf sheath. Arrowheads and arrows indicate appresorium and hypha respectively. A bar indicates 30 µm. B, Effect of pre-treatment with N-acetylchitoheptaose on the growth of hyphae of *M. grisea*. NP/++ was pre-treated with N-acetylchitoheptaose at 10 µg/ml for 0, 1, 3, and 5 d as indicated at left, and the leaf sheath was inoculated with *M. grisea* as described in “Materials and Methods,” followed by incubation for 2 d. At 2 d, the numbers of infected (a), one cell-infected (b), and non-infected (c) appresoria were counted, and ratio of each to the total number of appresoria counted (at right) is presented as a percentage, as indicated above the bars. As a positive control, NP/a (incompatible) was inoculated with *M. grisea*.

**Fig. 5.** Effects of N-Acetylchitoheptaose on the Hyphal Growth and Accumulation of Fluorescent Substance near the Infection Site.
NP/++ seedlings were treated for 5 d with N-acetylchitoheptaose at 10 µg/ml (panels 1, 2), BIT at 1 mM (panels 3, 4), or water (panels 5, 6), and inoculated with *M. grisea*. After incubation for 2 d, the infection sites were observed under light-(panels 1, 3, 5, 7) and fluorescent (panels 2, 4, 6, 8) microscopy. As a positive control, NP/a (incompatible) was inoculated with *M. grisea*, and hyphal growth and auto-fluorescence were observed (panels 7, 8). Arrowheads and arrows indicate appresorium and hyphae growing in the host cells surrounding the primarily infection site respectively. The bar indicates 30 µm.
hyphal development of *M. grisea* in rice. *N*-Acetylchitoooligosaccharide is recognized by a variety of plants. These features indicate that chitin might be a valuable source of new farm chemicals.

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