Family 19 Chitinase of *Streptomyces griseus* HUT6037 Increases Plant Resistance to the Fungal Disease

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Chitinase C (ChiC) is the first bacterial family 19 chitinase discovered in *Streptomyces griseus* HUT6037. In vitro, ChiC clearly inhibited hyphal extension of *Trichoderma reesei* but a rice family 19 chitinase did not. In order to investigate the effects of ChiC as an increase of plant resistance to fungal diseases, the chiC gene was introduced into rice plants under the control of the increased CaMV 35S promoter and a signal sequence from the rice chitinase gene. Transgenic plants were morphologically normal. Resistance to leaf blast disease caused by *Magnaporthe grisea* was evaluated in R1 and R2 generations using a spray method. Ninety percent of transgenic rice plants expressing ChiC had higher resistance than non-transgenic plants. Disease resistance of sibling plants within the same line was correlated with the ChiC expression levels. ChiC produced in rice plants accumulated intercellularly and had the hydrolyzing activity against glycol chitin.

Key words: *Streptomyces griseus*; family 19 chitinase; antifungal activity; transgenic rice; disease resistance

Chitin is a linear β-1, 4-linked polymer of N-acetylglucosamine and is a major component of the cuticles of insects, the shells of crustaceans, and the cell walls of many fungi. Chitinase (EC 3.2.1.14) is the enzyme which catalyzes the hydrolysis of chitin and this enzyme occurs in a wide range of organisms, including viruses, bacteria, fungi, insects, higher plants, and animals. On the basis of sequence criteria, chitinases are classified into families 18 and 19 glycosyl hydrolases.1,2) These family members have different three-dimensional structures and use different catalytic mechanisms.2,3) The family 18 chitinases are ubiquitous, but the family 19 enzymes have been identified mostly in plants9) and are thought to constitute a part of the defense mechanism against fungal pathogens.8,10) The assumption that chitinases are involved in the plant defence reaction was deduced from the following observations; 1) chitin is the major component of the cell wall of plant pathogens, 2) chitinase is one of the pathogenesis-related proteins, and 3) some plant chitinases have antifungal activity in vitro. This idea has been supported by the observations that transgenic plants with over-expression of the plant chitinase gene showed increased resistance to fungal diseases. For example, constitutive expression of CHIT2, a class I chitinase of family 19 from rice, in strawberry, grapevine, cucumber, and rice increased their resistance to fungal diseases.11,17)

On the other hand, bacterial chitinases are thought to be less promising in such attempts due to the lack of antifungal activity.

However, antifungal activity has been recently demonstrated in a new group of bacterial chitinases that belong to family 19. Chitinase C (ChiC) from *Streptomyces griseus* HUT6037 was discovered as the first family 19 chitinase in an organism other than higher plants.18) It shares significant sequence similarity with the plant family 19 chitinases in the catalytic domain, while its N-terminal chitin-binding domain (ChBD hac) differs from those of plant family 19 chitinases, and has similarity to substrate-binding domains of some bacterial family 18 chitinases and...
cellulases. ChiC inhibited hyphal extension of Trichoderma reesei, in contrast to no antifungal activity of other bacterial chitinases that belong to family 18. Experiments using the purified CHBD_{ChiC} and the purified catalytic domain of ChiC (CatD_{ChiC}) demonstrated that CHBD_{ChiC} plays a vital role in expression of the antifungal function of this chitinase. In addition, it was shown that CHBD_{ChiC} is required for the full hydrolytic activity of ChiC against both insoluble and soluble chitin polymers, but not against the chitin oligomer.

In this study, we first compared antifungal activity of ChiC with one of the rice family 19 chitinases, CHIT3. Then, transgenic rice plants were constructed by introducing the chiC gene, and the effects of the ChiC expression on the resistance to blast disease were studied.

Materials and Methods

Antifungal activity. ChiC was produced by Escherichia coli BL21(DE3) cells harboring plasmid pGCO1 bearing the chiC gene of S. griseus HUT6037, and purified to homogeneity in SDS-PAGE by hydroxyapatite column chromatography as described by Watanabe et al.\(^{20}\) Purified CHIT3, a rice class I chitinase encoded by the Chit-3 gene,\(^{21}\) was provided by Dr. Y. Itoh (NFRI, Japan). The CHIT3 with a histidine-tag was expressed in Pichia pastoris and purified by a Ni\(^{2+}\)-chelate affinity column.\(^{22}\) Chitinase A1 from Bacillus circulans WL-12 was produced in E. coli HB101 cells carrying pHT012 and purified by chitin column chromatography as described previously.\(^{23,24}\)

Antifungal activity was assayed using the hyphal extension-inhibition assay essentially described by Itoh et al.\(^{19}\) The concentration of T. reesei conidia was standardized to 2.5–5 × 10\(^{5}\) conidia/ml and a paper disk placed on the center of a potato dextrose agar plate was soaked with 40 μl of the conidia suspension. After a 24-h incubation at 25°C, new paper disks were placed around at the edge of the T. reesei colony and 40 μl solutions containing various chitinases were added to these disks.

Construction of binary vector. A DNA fragment containing a signal sequence of the rice chitinase gene was obtained by PCR using the following primers: 5'-CACGGATCCCGATGTCGACGCCGAGAGC-3' and 5'-AGGAGCTCCAGGCCGTGGCGC-3'. The amplified fragment was then digested with BamHI and SacI. Another DNA fragment containing the chiC gene without its signal sequence region was prepared from pGC01\(^{19}\) by digestion with SacI and KpnI. These two DNA fragments were ligated together into the BamHI and KpnI sites of pUC19 to generate pUC-RC2ss/ChiC.

RCC2 in the binary vector pBI333-EN4-RC2c2 described by Nishizawa et al.\(^{17}\) was replaced with the DNA fragment containing the chimeric chiC gene from pUC-RC2ss/ChiC to complete construction of a binary vector designated pBI333-EN4-RC2ss/ChiC (Fig.1).

Transformation of rice plants. Agrobacterium-mediated transformation of rice was done essentially according to the protocol patented by Tanaka et al.\(^{25}\) Briefly, the surfaces of husked seeds of rice (Oryza sativa L. japonica) cv. Nipponbare were sterilized, and the seeds put on N6D medium,\(^{29}\) and incubated at 28°C for 5 days. The germinating seeds were co-cultivated with Agrobacterium tumefaciens strain EHA101\(^{27}\) harboring pBI333-EN4-RC2ss/ChiC at 28°C for 3 days on 2N6-AS medium.\(^{20}\) Germinated seeds were washed with sterile water containing 500 mg/l carbenicillin and incubated at 28°C on N6D medium containing 500 mg/l carbenicillin and 50 mg/l hygromycin. About 2 weeks later, hygromycin-resistant calli were transferred onto the regeneration medium\(^{26}\) and then onto the MS
hormone-free medium to generate transformed rice plants.

Disease resistance analysis. At the five-leaf stage, rice seedlings were sprayed with a conidia suspension of the blast fungus (Magnaporthe grisea) strain Lna 86-137 (race 007.0). The concentration of conidia was standardized to $5 \times 10^6$ conidia/ml and Tween 20 or Triton X-100 was added to 0.05% just before spraying. After inoculation, the seedlings were kept in a dark chamber at 25°C and 100% relative humidity for 24 h and then transferred to a greenhouse at 28°C (day)/23°C (night). About 7 days after inoculation when typical lesions appeared on the leaves of non-transgenic plants, disease resistance was scored. The scoring system was based on the total area of lesions per inoculated leaf area to give a disease severity index (DSI). 0–5 and DSI was converted to% lesion area according to the reference diagram shown by the Furuta laboratory, Chugoku Agricultural Experimental Station, MAFP.

Preparation of extracellular proteins. Proteins were extracted according to the previously outlined procedure.\(^{17}\) Culm and leaf-sheath parts of the transgenic plants were cut into 4-cm length pieces and rinsed with water. The pieces were then vacuum-infiltrated for 20 min in the following buffer: a mixture of 0.1 M citrate and 0.2 M sodium phosphate, pH 6.0, and 0.2 mM phenylmethylsulfonyl fluoride (PMSF). The pieces were gently blotted dry and put into syringes. The syringes were placed in centrifuge tubes and centrifuged at 1,500 × g for 10 min. The eluent expected to contain extracellular proteins at the bottom of the tubes was collected and used immediately for western blotting. For comparison, proteins in the cells were extracted with the same buffer from the tissues after the extracellular proteins were removed by centrifugation.

Western blotting. Fresh leaves, or leaves stored at −80°C, were ground in liquid nitrogen with a mortar and pestle, then homogenized in extraction buffer [50 mM citrate-phosphate buffer (pH 6.0), 1 mM EDTA, and 0.2 mM PMSF] and centrifuged at 4°C for 10 min at 14,000 rpm. The supernatant was kept on ice for 1 h or longer and centrifuged again. The protein concentration was measured by using the Bio-Rad Protein dye reagent. Bovine serum albumin was used as the standard.

Proteins were separated on SDS-polyacrylamide gels and transferred to Immobilon membranes (Millipore Corp., Bedford, U.S.) by using a semi-dry blotter (Nihon Eido Co., Ltd., Tokyo, Japan). The chiC gene product (ChiC) was detected by means of rabbit polyclonal antibodies raised against ChiC, which was overexpressed in E. coli carrying the pGCO2 and purified by hydroxypatite column.\(^{20}\) Rabbit antibodies against the large subunit of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) of rice was provided by Dr. M. Miyao-Tokutomi (NIAS, Japan).

Enzyme assays. Glycol chitin used as a substrate for the chitinase assay was prepared by the method of Yamada and Imoto.\(^{29}\) The reaction mixture (total 750 µl) for the chitinase assay contained 0.1 M sodium phosphate buffer (pH 6.0), the glycol chitin (1 mg of dry weight), and prepared 40 µg of proteins. The reaction was done at 37°C for 10 min. The amount of reducing sugar generated was measured by the modified method of Schales,\(^{30}\) and one unit of chitinase activity was defined as the amount of enzyme that produces 1 µmol of reducing sugar per minute. Since soluble protein fractions extracted from fresh leaves contain some reducing sugar, the extracts were desalted to remove reducing sugar by using Microcon YM-10 (Millipore Corp., Bedford, U.S.) before the activity assay.

Results

Antifungal activity

ChiC has an antifungal activity as demonstrated previously by the inhibitory effect on the growth of T. reesei in a and ChBDchic at its N-terminus is important in the antifungal activity.\(^{19}\) To assess the possibility of ChiC as a genetic tool to increase disease resistance of plants, antifungal activity of ChiC was compared with that of CHIT3, a rice family 19 chitinase, by hyphal extension-inhibition assay using T. reesei. It was reported that overexpression of CHIT3 in rice resulted in the increase of disease resistance to rice blast.\(^{17}\) As shown in Fig. 2, only ChiC inhibited hyphal extension of T. reesei and CHIT3 as well as ChiA1 from B. circulans, a bacterial family 18 chitinase, did not show any effect on fungal growth under these assay conditions. Since CHIT3 (named RCG3 in ref. 22) had an inhibitory effect on the growth of T. reesei as reported previously,\(^{22}\) the inhibition assay under the conditions used in this study appeared to be not sensitive enough to detect antifungal activity of CHIT3. However, this observation suggested that ChiC is a promising candidate to elevate the fungal disease resistance of plants by introducing its gene. To verify this possibility, we produced transgenic rice plants expressing the chiC gene and assessed their resistance to blast disease.

Production of transformed rice plants

In order to over-express the chiC gene and secrete its products properly in rice plants, the chimeric chiC gene, which encodes a signal sequence from the rice gene and the chitin-binding and catalytic domains of ChiC, was introduced with the improved 35S
promoter by the *Agrobacterium*-mediated transformation method. Transformants were selected by hygromycin-resistance and finally 47 independent lines of transformants were grown in a greenhouse for further analysis.

Production of ChiC protein in the leaves of the 47 regenerated plants (R₀ generation) was analyzed by western blotting. Intensity of the protein bands corresponding to ChiC and its derivatives indicated that 40 out of 47 lines expressed the *chiC* gene as much as 0.05–0.5% of total soluble protein (data not shown). Three lines showed extremely low levels of the expression and four lines showed no expression. Self-pollinated seeds were collected from the transformant lines over-expressing the *chiC* gene and planted on agar plates containing 35 μg/ml hygromycin after they were husked and sterilized. As a result, 29 out of 35 lines tested showed that the hygromycin-resistance was inherited by the R₁ generation. Transgenic rice plants constitutively expressing ChiC were morphologically normal, and fertility and germination percentage of the transgenic rice plants were indistinguishable to those of non-transgenic rice plants.

**Evaluation of disease resistance**

Ten to twenty hygromycin-resistant plants from each of 29 transgenic lines in the R₁ generation were tested for resistance to blast disease. Control plants were non-transformants or transformants with an empty vector. The levels of blast resistance of 26 lines were higher than controls (data not shown). In an effort to reveal the relationship between the expression level of ChiC and disease resistance, the amount of ChiC in two plants selected from each of several lines was analyzed by western blotting after the disease resistance of every plant was scored. One of the two plants was scored as the most resistant and the other scored the most susceptible in a line. As shown

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**Fig. 2.** Comparison of Antifungal Activity of Different Chitinases.

A paper disk placed on the center of a potato dextrose agar plate was soaked with a suspension of conidia of *T. reesei*. After 24 h of incubation at 25°C, paper disks with various chitinases were placed around the *T. reesei* colony, and the plate was incubated another 24 h at 25°C. Disk 1, no chitinase control; 2, 20 μg *S. griseus* ChiC; 3, 20 μg rice class I chitinase, CHIT3; 4, 20 μg *B. circulans* ChiA1.

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**Fig. 3.** Relationship between the Quantity of ChiC and Disease Resistance in R₁ Generation.

The quantity of ChiC expressed in transformants was estimated by western blotting. Disease severity index (DSI) was scored 7 days after inoculation. Each DSI corresponds to the total area of susceptible-type lesions per inoculated leaf (0 = 0%; 0.5 = 0.4%; 3 = 12.5%; 4 = 41.5%). Lane 1, molecular mass standards; lane 2, 100 μg recombinant ChiC purified from *E. coli*; lanes 3 to 12, 40 μg proteins from leaves of the R₁ transgenic rice plants. Arrows (a), (b), and (c) indicate predicted glycosylated ChiC, intact ChiC, and CatDchic, respectively.
in Fig. 3, in the comparison between the two plants in the same line, the plant showing higher resistance always expressed more ChiC. This relationship, however, was not necessarily observed for individuals across the lines (e.g. cf. Fig. 3 lane 8 and 9).

To clarify the effect of the chiC expression on the disease resistance, we selected 6 promising lines (N-ERC-1, 8, 31, 42, 60, 61) based on their DSI, ChiC expression level, and the percentage of hygromycin resistant R1 plants, and further analyzed them in the R2 generation. About 50 plants from each of the 6 lines were grown without a hygromycin selection and scored for disease resistance. After the scoring, proteins were extracted from every individual and analyzed by western blotting. About 50 R1 plants from each of two lines (N-ERC-23 and N-ERC-38), which showed hygromycin resistance without expressing ChiC, and non-transformants were tested as controls. Figure 4 shows the averages of % lesion area of groups of plants expressing the chiC and groups of plants without ChiC expression. There were no plants without expression of ChiC in N-ERC-60–10 and N-ERC-61–8. All groups of plants expressing chiC were more resistant than controls. In comparison within the same line, resistance of groups of plants expressing the chiC was also always higher than that of groups of plants without ChiC expression although the resistance levels were varied.

**Accumulation site of ChiC**

To discover the subcellular localization of the expressed ChiC, proteins were fractionated from internodes consisting of culm and leaf-sheath. Figure 5 shows the results of western blotting of different fractions: total soluble proteins (T), extracellular proteins (E), and intracellular proteins (I). Protein (40 µg) from T fraction or I fraction and extracellular proteins from the corresponding amount of internode were put on SDS-PAGE.

As shown in Fig. 5A, the immunostained bands with anti-RuBisCO antibodies were detected in T and I fractions but not in E fraction. Because RuBisCO is the major protein of chloroplasts within the cell, contamination of the E fraction with intracellular proteins should be negligible. The immunostained bands with anti-ChiC antibodies were detected in all the three fractions (Fig. 5B), indicating that chiC product is targeted extracellularly, as expected from the addition of a signal sequence of the rice chitinase. Intensities and migration positions of immunostained bands detected in fraction I were comparable to those in fraction E. This observation suggests that extraction of intracellular fluid was incomplete. Significant amounts of ChiC and its derivatives remained unextracted and recovered in fraction I together with ChiC in the cells which is either in the process or before the process of secretion.

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**Fig. 4. Relationship between the Production of ChiC and Disease Resistance in R2 Generation.**

Two transgenic R1 plants without expression of ChiC (N-ERC-23 and N-ERC-38) and non-transformants (NT) were used as controls. Open bars indicate average of % lesion area of controls and filled bars indicate that of transgenic R1 plants. (+) indicates the expression of ChiC and (−) indicates no expression of ChiC measured by western blotting. Error bars represent standard errors. Numerals in parentheses are the number of the tested plants.
Chitinase activity of ChiC produced in rice plant

To examine whether ChiC produced in transgenic rice plants has chitinase activity, the specific hydrolytic activity in leaf extracts was measured using glycol chitin, which is the most suitable substrate to detect catalytic activity of ChiC.\(^{19,20}\) Specific activities of transgenic plants harboring the chiC gene were compared with that of a rice plant transformed with plasmid pBI333, which has the 3S5 promoter and \(\beta\)-glucuronidase gene instead of EN4-RC2ss/ChiC (Fig. 6A). Furthermore, the quantity of ChiC in the extracts was assessed blotting analysis (Fig. 6B). There were significant correlations between the specific activities and the quantity of ChiC, suggesting that the increase of the chitinase activity in transformants was due to the expression of the chiC gene.

Discussion

Under our assay conditions for antifungal activity, ChiC clearly inhibited hyphal extension of \(T.\) reesei but CHIT3, one of the family 19 chitinases of rice, did not. Of course, this does not mean that antifungal activity of the class I chitinases is generally weaker than that of \(S.\) griseus ChiC. However, since the constitutive over-expression of CHIT3 was able to control rice blast disease to some extent,\(^{17}\) higher antifungal activity of ChiC than CHIT3 suggests that ChiC is a promising candidate as a tool to improve fungal disease resistance of plants. Gene resources to improve plant resistance to fungal diseases are still insufficient and an effort to seek new gene for this purpose is apparently desirable. Therefore, in order to investigate the effects of ChiC on the fungal growth in planta, the chiC gene was introduced into

Fig. 5. Western Blotting to Identify Accumulation Site of ChiC in the Transformant.

Proteins were prepared from internodes and analyzed with antibodies against RubisCO (A) and ChiC (B). Lane 1, molecular mass standards; 2, total soluble proteins; 3, extracellular proteins; 4, intracellular proteins. The amount of protein put on in each lane is described in the text.

Fig. 6. Ratio of Specific Hydrolytic Activities Measured against Glycol Chitin (A) and Western Blotting of ChiC Produced in Transgenic Rice Plants (B).

Lane numbers in (A) correspond to those in (B). Lanes: 1, soluble protein prepared from leaves transformed with an empty vector, pBI333 as a control; 2 to 11, soluble proteins prepared from leaves of different lines of transformants with the chiC gene; M, molecular mass standards. Amount of protein used for the activity was 40 \(\mu\)g and 25 \(\mu\)g protein was put on in each lane in Western blotting.
rice plants as a model system and disease resistance to leaf blast was evaluated in this model system. Since it is possible that stress during the tissue culture induces defense reactions, and causes the increased resistance in the R₀ generation, we tested the disease resistance of R₁ and R₂ progeny as well. Transgenic rice plants expressing ChiC showed higher disease resistance than plants that produced no ChiC (Fig. 4). It indicates that the increased resistance is due to the expression of the chiC gene. There is a highly significant correlation between the expression level of ChiC and disease resistance within the same line, while this relationship was not observed between the plants from the different lines. For example, N-ERC-31-12(−) had almost the same level of disease resistance as N-ERC-1-4(+), despite no expression of ChiC. The reason of this exception is unknown at this stage, but alteration of genomic DNA during the Agrobacterium-mediated transformation might be involved.

Chitinase activity against glycol chitin in the transformants increased in parallel with the quantity of expressed ChiC (Fig. 6). Therefore, the increased chitinase activity is most likely to be due to the production of ChiC. It indicates that the increase of the disease resistance observed in transgenic rice plants resulted from the increment in chitinase activity.

Transgenic rice plants constitutively expressing ChiC were morphologically normal and fertile. However, chitinase-like proteins have long been proposed to play roles in growth and development of normal plants. Mutation of a chitinase-like gene caused ectopic deposition of lignin and aberrant shapes of cells with incomplete cell walls in the pith of inflorescence stems in Arabidopsis. Therefore, detailed study to clarify whether introduction of chiC affect growth and development of plants needs to be carried out in the near future.

In Western blotting of ChiC expressed in plants, several bands were detected, as shown in Fig. 3. ChiC has three predicted N-glycosylation sites (Asn-X-Ser/Thr): one site (Asn54-Thr56) in ChiBDchic and the other two sites (Asn143-Ser145 and Asn291-Ser293) in CatDBchic. By comparison of the molecular weight with recombinant ChiC purified from E. coli, we speculate that band (a) and (b) in Fig. 3 are N-glycosylated ChiC and intact ChiC, respectively. The major chitinase in the culture medium of S. griseus HUT6037 grown with colloidal chitin as a sole carbon source is detected in the form of CatDBchic. Some chitinases with modular structure are known to be easily cleaved in the linker regions. Together with the consideration of the molecular weight, we estimate that band (c) in Fig. 3 is CatDMchic.

Chimeric ChiC carrying a signal sequence of CHIT2 (RC2ss) was targeted extracellularly as expected (Fig. 5). CHIT2 has a putative vacuole target-


31) Zhong, R., Kays, S. J., Schroeder, B. P., and Ye, Z. H., Mutation of a chitinase-like gene causes ectopic deposition of lignin, aberrant cell shapes, and over-
Transgenic Rice Plants Expressing S. griseus Chitinase C


