Theor Appl Genet (1999) 99:383–390 © Springer-Verlag 1999

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Enhanced resistance to blast (*Magnaporthe grisea*) in transgenic Japonica rice by constitutive expression of rice chitinase

Received: 16 November 1998 / Accepted: 30 January 1999

Abstract Rice blast is the most devastating plant disease in Japan. Our goal is to create new rice varieties which show enhanced resistance against blast, regardless of the race of blast. By an *Agrobacterium*-mediated transformation method, we reintroduced a rice class-I chitinase gene, *Cht-2* or *Cht-3*, under the control of the enhanced CaMV 35S promoter and a hygromycin phosphotransferase gene, as a selection marker into the Japonica rice varieties Nipponbare and Koshihikari, which have retained the best popularity over a long period in Japan. In regenerated plants (R_0), the *Cht-2* product was found to accumulate intracellularly whereas the *Cht-3* product was found to be targeted extracellularly. The transgenic rice plants which constitutively expressed either chitinase gene showed significantly higher resistance against the rice blast pathogen *Magnaporthe grisea* races 007.0 and 333. Both high-level expression of the chitinase and blast-resistance were stably inherited by the next generation in several lines.

Key words Blast disease-resistance · Chitinase · Non-race-specific resistance · Overexpression · Transgenic rice

Introduction

Rice blast disease caused by *Magnaporthe grisea* occurs worldwide and results in severe damage, particularly where rice is irrigated or receives high amounts of rainfall and high levels of nitrogen fertilizer (Zeigler et al. 1994). Thus, blast resistance in rice plants is one of the most important traits to have been pursued in breeding programs over several decades. Breeders have adopted three methods to achieve this challenging objective (Kushibuchi 1997). First, they made use of the field resistance to blast disease carried by native Japanese varieties. Second, they introduced genes from foreign varieties having true resistance which was previously non-existent in Japanese rice. As a result, several new varieties with true resistance were developed. However, shortly after their release, they became seriously susceptible to blast disease in many areas because of the appearance of new races of the blast fungus. This breakdown of disease resistance made it clear that the problem would not be solved merely by the introduction of true resistance genes. Thus, as a third approach, emphasis was placed on the development of high-level field resistance. Breeders have developed many new varieties showing a high level of field resistance.

Although a considerable number of new varieties have been released in Japan over the past 25 years only three of these, Nipponbare, Sasanishiki and Koshihikari, have been consistently in the top ten varieties grown in Japan (Nakagahra et al. 1997). Especially, Koshihikari, which was planted in 33.6% of the rice fields in Japan in 1998, and other varieties of the same family, have strong name recognition by Japanese consumers because of their excellent quality, especially their palatability. These varieties have retained their popularity over a long period, despite the fact that Koshihikari has some drawbacks such as susceptibility to blast disease and lodging (Kushibuchi 1997). Because palatability sometimes shows a negative correlation with blast field resistance and because screening for field resistance during breeding is technically difficult, research aimed to combine palatability and blast resistance has not advanced.

With the recent development of transformation technology for several important crops including rice (Chan et al. 1992; Hayakawa et al. 1992; Hiei et al. 1994; Christou 1997), genetic engineering has emerged as an alternative approach to create blast-resistant rice. In ad-

Communicated by G. Wenzel

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dition to the shortening of breeding time, since the transgenic plants acquire a single character from the introduced gene without disturbance of the genetic background, this new approach seems to be advantageous for introducing a disease resistance trait into high grain-quality varieties like Koshihikari.

In view of the above, we attempted to enhance field resistance by manipulating a chitinase gene. Chitinase, which hydrolyzes the polymer chitin, is one of the pathogenesis-related (PR) proteins and shows antifungal activity in vitro (Schlumbaum et al. 1986). Thus, it is speculated that chitinase may be involved in field resistance. Transgenic plants with increased disease resistance due to constitutive expression of a chitinase gene were first reported in the case of tobacco plants (Broglie et al. 1991). Since then, there have been several reports of transgenic plants whose resistance against fungal disease was enhanced by the introduction of a chitinase gene, although most were tobacco plants (Zhu et al. 1994; Jach et al. 1995). Lin et al. (1995) introduced a rice chitinase gene back into Indica rice and succeeded in producing transgenic rice plants with increased resistance to infection by the sheath blight pathogen *Rhizoctonia solani*. This seems to be a promising strategy also for the development of blast-resistant rice.

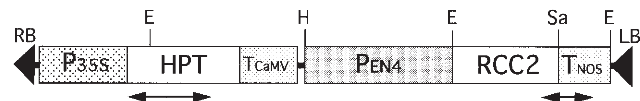
We previously cloned and characterized three class-I chitinase genes from rice (Nishizawa and Hibi 1991; Nishizawa et al. 1993). We have shown that one of them, *Cht-2*, is useful for increasing the resistance to powdery mildew of strawberry (Asao et al. 1997) and tobacco (Akutsu et al. submitted) as well as to gray mold of cucumber (Tabei et al. 1998). In the present study, a number of morphologically normal and fertile transgenic Japonica rice plants (cv Nipponbare and Koshihikari), which constitutively expressed the rice chitinase gene, were produced by an *Agrobacterium*-mediated transformation method. These plants exhibited enhanced resistance to rice blast, probably in a non-race-specific manner.

Materials and methods

Binary vector construction and transformation of rice plants

A 1.1-kb DNA fragment encoding *Cht-2* was obtained from a rice chitinase cDNA clone (RCC2) and a similar fragment encoding *Cht-3* was obtained from a genomic clone (RCG3). The enhanced cauliflower mosaic virus (CaMV) 35S promoter (EN4), which possesses four tandemly repeated enhancer regions (–290 to –90) of its own, was kindly supplied by Dr. Hirochika (NIAR, Japan). The binary vector plasmids pBI333-EN4-RCC2 and pBI333-EN4-RCG3 were constructed by replacing the β-glucuronidase gene and the CaMV 35S promoter in the binary Ti plasmid pBI333 (kindly provided by Prof. Kamata, Tsukuba University) with the 1.1-kb DNA fragment described above, under the control of the enhanced 35 S promoter (Fig. 1). These vectors were transferred into *Agrobacterium tumefaciens* strain EHA101 (Hood et al. 1986) and used for *Agrobacterium*-mediated transformation of *Oryza sativa* L. *japonica* cv Nipponbare (*Pi-a*) and Koshihikari (++) by following the protocol reported by Toki (1997).

<< pBI333-EN4-RCC2 >>



<< pBI333-EN4-RCG3 >>

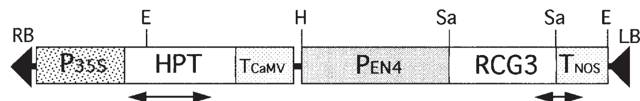


Fig. 1 Diagram of the T-DNA regions of the binary vectors used for rice transformation. *RB* and *LB* are the right and left border sequences of the T-DNA region, respectively. *Arrows* indicate the PCR-amplified regions, which were used to confirm the existence of each gene in the regenerated plants. *P35S* CaMV 35S promoter, *TCaMV* CaMV terminator, *PEN4* enhanced CaMV 35S promoter, *TNOS* terminator of the nopaline synthase gene, *HPT* hygromycin phosphotransferase gene, *RCC2* coding region of the *Cht-2* gene, and *RCG3* coding region of the *Cht-3* gene. *E Eco*RI, *H Hin*dIII, *Sa Sac*I

DNA and RNA analyses

DNA extraction

Total DNA was extracted from one 8–mm-diameter leaf disk for each line. The leaf disk was homogenized in 3 µl of 100×TES [1 M Tris-HCl (pH 8.0), 0.1 M EDTA (pH 8.0), 1% SDS] and 3 µl of 10 mg/ml of proteinase K and incubated for 10 min at 65°C. After equilibration to room temperature, the extract was vortexed with 100 µl of solution I [50 mM glucose, 25 mM Tris-HCl (pH 8.0), 10 mM EDTA) and 100 µl of water-saturated phenol, then centrifuged at room temperature for 5 min. To precipitate the DNA, a 1/10 vol of 3 M sodium acetate (pH 5.2) and 2 vol of isopropanol were added and the sample was left at room temperature for 1 h. The precipitate was washed twice with 70% ethanol and dried in vacuo briefly, then dissolved in 50 µl of distilled water.

PCR amplification

Five microliters of the resulting DNA solution were used for each PCR. The reaction conditions employed were those described by Asao et al. (1997). The primer pairs employed to detect the introduced genes were as follows: 5'-ATGAAAAAGCCTGAACTCACCGCGA-3'/5'-TCCATCACAGTTTGCCAGTGATACA-3' (for the HPT gene), 5'-T*GGATCC*AGCGGCTCGTCGGTTG-3' (with an artificial *Bam*HI site added; underlined)/5'-GTATAATTGCGGGACTCTAATC-3' (for the RCC2-NOS terminator chimera region), and 5'-AAGCATGCCCTACCCGCCTTCCTAGTTG-3'/5'-GTATAATTGCGGGACTCTAATC-3' (for the RCG3-NOS terminator chimera region).

Northern-blot analysis

Total RNA was isolated by the method of Chomczynski and Sacchi (1987) with slight modifications as reported by Nishizawa et al. (1999). For Northern blotting, 10 µg of total RNA was separated on 1.5% (w/v) agarose gels with 0.66 M formaldehyde, 20 mM MOPS, 5 mM sodium acetate and 1 mM EDTA, and transferred to positively charged nylon membranes (Boehringer Mannheim) by standard procedures (Sambrook et al. 1989). Hybridization was performed as described previously (Nishizawa et al. 1993) with DIG-labelled DNA probes, which were generated using the PCR DIG Probe Synthesis Kit (Boehringer Mannheim). These probes, corresponding to the 3' non-coding regions of *Cht-2* and *Cht-3* (see Fig. 1), were employed to detect the gene-specific transcripts.

Protein analysis

Western-blot analysis

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, 0.2 mM phenylmethylsulfonyl fluoride (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 in the resulting soluble protein extract from the leaves was determined using the Bio-Rad Protein dye reagent. Proteins (40 μg) were separated on SDS-polyacrylamide gels and transferred to Immobilon membranes (Millipore) using a semi-dry blotter (Nihon Eido Co., Ltd., Tokyo, Japan). The *Cht-2* gene product (CHIT2) and the *Cht-3* gene product (CHIT3) were detected by means of polyclonal antibodies raised against a histidine-tagged CHIT2 fusion protein or a histidine-tagged CHIT3 fusion protein, respectively, which were expressed in *Escherichia coli* using the pRSETA vector (Invitrogen).

Extraction of proteins in extracellular washing fluids (EF)

Proteins were extracted by the method of Parent and Asselin (1984) with the following modifications. Culm and leaf-sheath parts of the transgenic or control rice plants were cut into 4 cm-length pieces and washed in water. The pieces were then vacuum-infiltrated for 20 min with the following buffer: a mixture of 0.1 M citrate and 0.2 M sodium phosphate, pH 6.0, and 0.2 mM PMSF. The pieces were gently blotted dry and put into syringes. The syringes were placed in centrifuge tubes and centrifuged at 1500 g for 10 min. The EF at the bottom of the tube was collected and used immediately or frozen at –20°C. For comparison, proteins expected to be in the cells were extracted with the same buffer from the tissues after the EF was removed by centrifugation.

Disease resistance analysis

At the four- to five-leaf stage, rice seedlings were transferred into an inoculation chamber and each pot was inoculated with approximately 2.5 ml of a suspension of conidia of the blast fungus strain Ina 86-137 (race 007.0; kindly provided by Dr. Naito, NARC, Japan) or 0528-2 (race 333; supplied by NIAR Gene Bank, Japan) by spraying. The concentration of conidia was standardized to 5×10^5 conidia/ml and Tween 20 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 moist vinyl tunnel in a greenhouse at 25–28°C. Disease resistance was scored based on the total area of lesions per inoculated leaf [disease severity index (DSI), 0–5] about 7 days after inoculation, when typical lesions appeared on the leaves of susceptible plants (non-transgenic Nipponbare or Koshihikari).

Results

Transformation of rice

The T-DNA regions of the transformation vectors are shown in Fig. 1. Two different class-I chitinase genes from rice were used. The *Cht-2* gene (clone name: RCC2) is predicted to encode a vacuole-type chitinase whereas the *Cht-3* gene (clone name: RCG3) product is predicted to be secreted (Nishizawa et al. 1993). The DNA sequences of the *Cht-2* and *Cht-3* genes in the vectors are present in the EMBL Nucleotide Sequence Database under the accession number X56787 (from nucleotide position number 1 to 1115) and in the DDBJ Database under the accession number D16223 (from position 1830 to 2808), respectively.

Agrobacterium-mediated transformation of two elite Japonica rice varieties (Nipponbare and Koshihikari) was performed by the rapid and efficient method reported by Toki (1997). Using this method, Nipponbare calli suitable for transformation were obtained within 2–3 weeks; however, Koshihikari calli had to be cultured for around 5 weeks because the growth rate of Koshihikari calli is slower than those of Nipponbare. The longer culture periods resulted in a reduction of the regeneration rate. Another point to be noted with respect to Koshihikari is that, after co-cultivation, the calli were pre-cultured in medium containing carbenicillin but without hygromycin for 1 week. The transformation efficiency (the number of regenerated plant lines/the number of calli co-cultured with *Agrobacterium*×100) was about 33% for Nipponbare and 3.8% for Koshihikari. As a result of the transformation experiments, 65 regenerated plant lines were obtained for *Cht-2* (designated as N-ER2- lines) and 133 lines were obtained for *Cht-3* (designated as N-ER3- lines) in the case of Nipponbare. For further characterization, in total 93 hygromycin-resistant plants, which were considered to have resulted from independent transformation events, were grown in a greenhouse. In the case of Koshihikari, 23 hygromycin-resistant

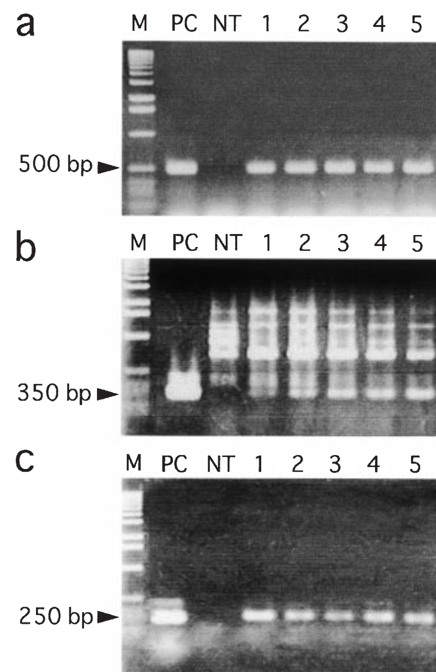


Fig. 2 a–c Detection of the transgenes from the R_0 regenerated plants (cv Nipponbare) by PCR. The regions described in Fig. 1 were amplified by PCR using primers for the HPT gene (**a**), the RCC2-NOS terminator region (**b**), and the RCG3-NOS terminator region (**c**). *M* 1-kb ladder molecular-size marker, *PC* positive control (transformation vector), *NT* non-transformant, *lanes 1–5* regenerated plants

plants (designated as K-ER2- or K-ER3- plants) were obtained from 17 callus lines in all and were further characterized.

Molecular analysis of transgenic plants

The existence of the hygromycin phosphotransferase gene (HPT) and the transferred chitinase gene in the regenerated plants (R_0 generation) was proved by PCR using specific primers (partial data shown in Fig. 2). Out of 57 N-ER2-plants, 55 were found to possess both HPT and *Cht-2* genes, one plant possessed only the HPT gene, and one plant had *Cht-2* only. As for the N-ER3-plants, 23 out of 34 plants were found to possess both genes, but 11 plants had only the HPT gene. In the case of Koshihikari, only the existence of the transferred chitinase gene was tested. All of the 13 K-ER2- plants and the 10 K-ER3-plants were found to possess *Cht-2* (K-ER2) and *Cht-3* (K-ER3), respectively.

Expression of the chitinase gene in the leaves of R_0 plants was analyzed by Northern blotting and Western blotting (partial data shown in Fig. 3). The results obtained from these two analyses were correlated; that is, in the case of lines expressing a high level of the chitinase mRNA the immunoreactive bands detected showed a high intensity signal, whereas in the case of lines which scarcely expressed the chitinase mRNA the immunoreactive bands were very faint (data not shown). Thirty four out of 57 transgenic N-ER2-plants (plants positive by PCR) were found to express the *Cht-2* gene and 15 out of 21 transgenic N-ER3-plants were found to express the *Cht-3* gene, while chitinase mRNA and protein were not detected in the non-transgenic control plants. Nine out of ten transgenic K-ER2-plants and nine out of ten transgenic K-ER3-plants were confirmed to express the chitinase genes. Both the ER2-plants and the ER3-plants accumulated the chitinase protein in the leaf, leaf-sheath, culm, root and flower (data not shown.) Non-transformed Nipponbare and Koshihikari did not accumulate chitinase even 10 days after infection under the assay conditions employed. One-to-three fast-migrating bands were seen in Western-blot analysis. Some of these additional bands were due to protein degradation during the boiling process in the course of sample preparation (data not shown) while others presumably were derived from the 32-kDa chitinase by proteolytic processing.

Subcellular localization of the introduced chitinases

In an effort to determine the subcellular localization of the expressed chitinases, proteins were extracted from internodes consisting of culm and leaf-sheath, and the extracts were analyzed for the presence of specific proteins. Figure 4 shows the results of a Western-blot analysis of different extracts: the extracellular washing fluid (EF), the protein fraction from the internodes after removal of the EF (-EF), and the total internode protein fraction (T). For analysis of T or -EF (extracts from about a 1-cm internode), 60 µg of protein was loaded in lanes 1, 2, 4 and 5, and the EF from the corresponding

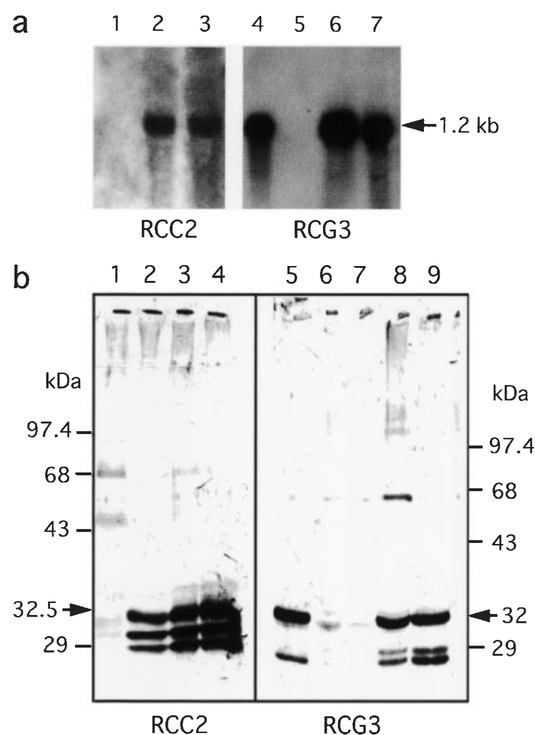


Fig. 3 Northern-blot analysis with total RNA (**a**) and Western-blot analysis of total soluble proteins (**b**) from the leaf of the R_0 transgenic rice. **a** *Lanes 1 and 5* non-transformants, *lanes 2 and 3* N-ER2-plants, *lane 4* RNA from elicitor-treated suspension-cultured rice cells as a positive control, *lanes 6 and 7* N-ER3-plants. **b** *Lane 1* non-transformant, *lanes 2–4* N-ER2-plants, *lanes 5–9* N-ER2-plants

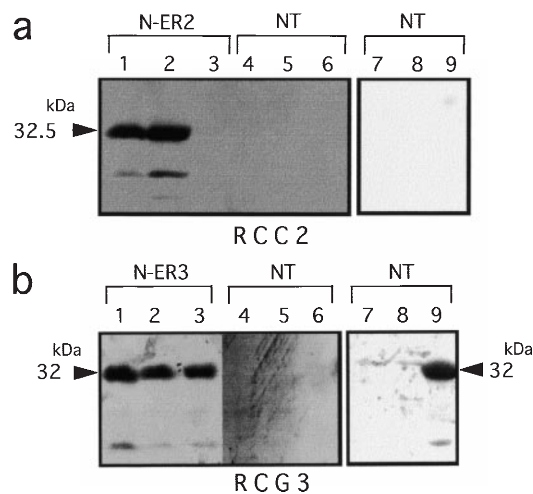


Fig. 4a, b Comparison of subcellular expression patterns of two chitinase genes in transgenic rice plants (cv Nipponbare). Total soluble protein fraction (*lanes 1, 4 and 7*), materials remaining after removal of the extracellular washing fluid (*lanes 2, 5 and 8*), and extracellular washing fluid (*lanes 3, 6 and 9*), were isolated from internode parts of non-transformant (*NT*), an *N-ER2*-plant (**a**), and an *N-ER3*-plant (**b**). The amount of protein loaded in each lane is described in the text

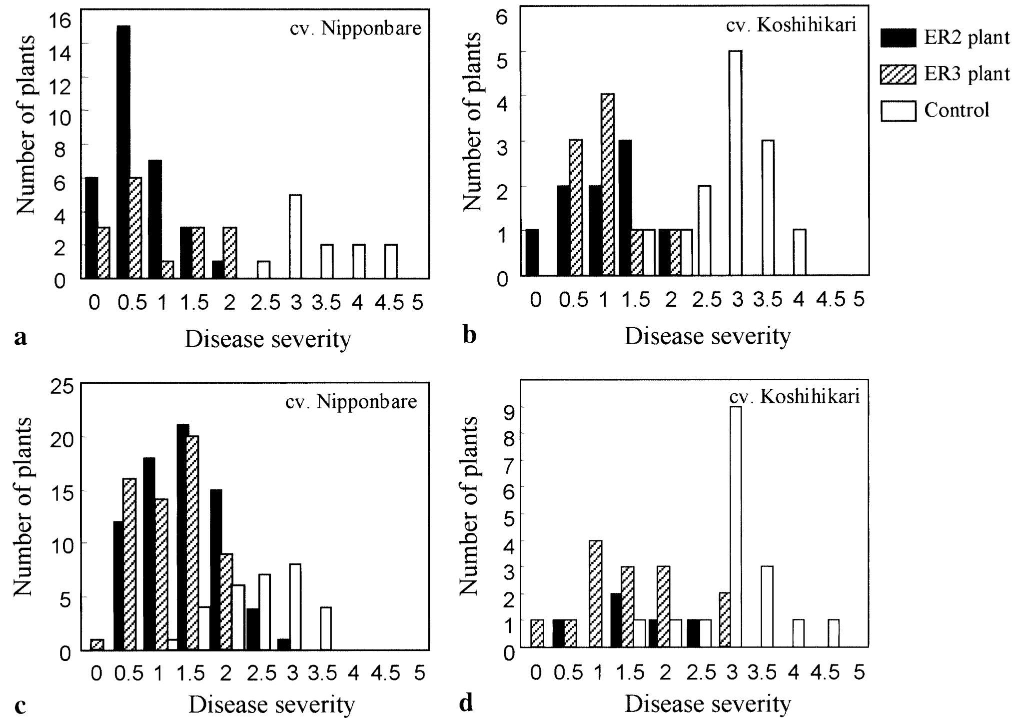


Fig. 5a–d Distribution of disease severity in control (non-transformants) and transgenic rice plants infected by *M. grisea* (race 007.0). **a** Nipponbare R_0 plants, **b** Koshihikari R_0 plants, **c** Nipponbare R_1 plants, **d** Koshihikari R_1 plants

amount of internode was loaded in lanes 3 and 6. In the case of transgenic plants expressing the *Cht-2* gene, immunostained-bands specific for the transgenic plants were found only in the lanes containing the -EF and T fractions. However, in the case of transgenic plants expressing the *Cht-3* gene, the immunostained-bands were detected in the case of all three fractions. The small amounts of protein present in the -EF fraction were probably residual protein not removed in the course of preparation of the EF fraction. When a large amount of EF protein (40 μg per lane) was applied, endogenous CHIT3 was detected in the EF fraction of a non-transformant (Fig. 4b, lane 9). These observations indicate that the *Cht-2* product is expressed intracellularly whereas the *Cht-3* product is targeted extracellularly, as expected based on their primary amino-acid sequences (Nishizawa et al. 1993). Furthermore, the level of expression of the *Cht-2* product was below the detection level in the culm and leaf-sheath, as well as the leaf of the non-transformed plant.

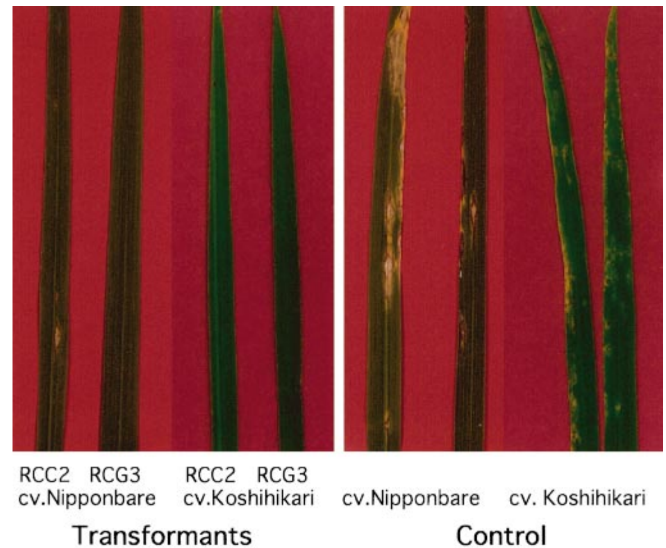


Fig. 6 Symptoms on the leaves of control and transgenic rice plants infected by *M. grisea* (race 333) 7 days after infection

Evaluation of disease resistance

The reaction to leaf blast of the transgenic R_0 plants, which were constitutively expressing the *Cht-2* or *Cht-3* gene, was evaluated by inoculation with race 007.0 or 333 of *M. grisea.* Since Nipponbare has the *Pi-a* gene and Koshihikari has no true resistance genes against blast, both races are virulent in Nipponbare and Koshihikari. The expected resistance is not a true resistance-type reaction. Consequently, it is ideal to assess several clonal or sibling plants at the same time. However, because there was only one regenerated plant for each line in the R_0 generation, we evaluated the increase in blast resistance through a comparison of the distribution of disease severity between control plants and a number of transgenic lines. As shown in Fig. 5a and b, 37 out of 42 Nipponbare transformants and 16 out of 18 Koshihikari transformants exhibited enhanced resistance to rice blast. Transgenic plants which expressed chitinase at a high level (12-fold over control) tended to exhibit higher resistance, although the level of the chitinase activity was not always proportional to the resistance level (data not shown). Lesions appeared within 4–5 days after in-

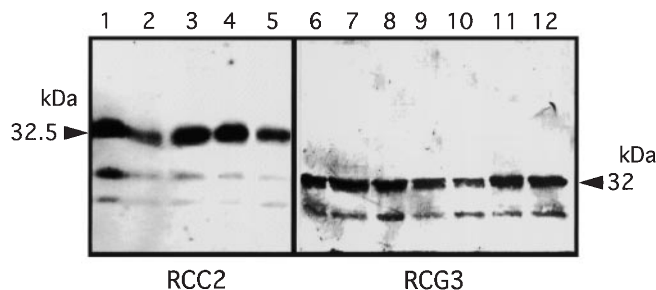


Fig. 7 Western-blot analysis of total soluble proteins from the leaf of R_1 transgenic rice. *Lane 1* N-ER2-63 (R_0), *lanes 2–5* N-ER2-63-1–4 (R_1), *lane 6* K-ER3-24 (R_0), *lanes 7–12*: K-ER3-24-1–6 (R_1)

oculation of non-transgenic control plants or transformants with an empty vector (pBI333 R_1 plants) and the disease severity was finally scored as DSI 3–5 at 7-days post-inoculation. However, in the case of transformants with overexpression of the chitinase, the development of symptoms was retarded, the number of lesions was significantly fewer, and the lesions were significantly smaller compared to the controls (Fig. 6). The disease severity in these plants was scored as DSI 0–2 at 7-days post-inoculation and they never died-back even when culturing was continued in the greenhouse.

Inheritance of the transgene

Transgenic lines which overexpressed the chitinase gene and showed high-level blast resistance were selected. For such lines, the heredity of the transgene from the R_0 to the R_1 generation was demonstrated by PCR and Western-blot analysis. The heredity of disease resistance was assessed for hygromycin-resistant R_1 plants. Expression of the transgene was detected in almost all selected R_1 plants (partial data shown in Fig. 7), it had no effect on plant growth or development, and the distribution of disease severity was significantly different between R_1 plants and non-transgenic control plants (Fig. 5c and d). However, disease resistance shown in the offspring of some lines was not as strong as that of their parents. Through the entire analyses, there was no pronounced difference in disease resistance between the transgenic rice transformed with the *Cht-2* and *Cht-3* genes.

Discussion

A few reports of transgenic rice with enhanced disease resistance have been published. Transgenic rice plants with enhanced resistance against sheath blight were generated by polyethyleneglycol-mediated protoplast transformation (Lin et al. 1995), while plants with enhanced resistance against bacterial blight were created by particle bombardment (Song et al. 1995; Tu et al. 1998). It has been pointed out that these methods have low transformation efficiency and cause deformity or low fertility in regenerated plants. The *Agrobacterium*-mediated transformation system developed for rice has only recently been used to create a number of stable transgenic rice plants with agronomically important genes (Cheng et al. 1998; Yoshimura et al. 1998).

In the present study, fertile rice plants constitutively expressing the rice chitinase gene were produced using two elite Japonica varieties, Nipponbare and Koshihikari, by the *Agrobacterium*-mediated transformation procedure, although there seems to be room for improvement in the transformation protocol for Koshihikari, as better efficiency is needed. The most palatable and consequently the most popular variety in Japan, Koshihikari, has the drawback of susceptibility to blast disease in cultivation. As shown in the present study, genetic engineering has opened the door to introducing disease resistance into such an elite variety in a short time without changing the original excellent traits.

Almost all of the regenerated plants except for the N-ER3-lines were shown to possess both HPT and the transferred chitinase genes. The reason why several N-ER3-lines were lacking the chitinase gene is not clear, but such a problem may be solved by using a binary vector with a selection marker in the left border side.

About 10–40% of the R_0 plants (Nipponbare and Koshihikari) did not overexpress the chitinase gene despite the presence of its 3'-end region as confirmed by PCR. This might be due to a "position effect", which is one of the models explaining variation in transgene expression (Meyer 1995). On the other hand, it has been reported that roughly 20–60% of homozygous, sibling *Nicotiana sylvestris* plants transformed with a tobacco class-I chitinase gene exhibited a silent phenotype: levels of expression of both the transgene-encoded chitinase and the host-encoded chitinase were greatly reduced (Hart et al. 1992). In this context, we think that a larger number of progeny should be obtained in order to select good homozygous lines with a consistently high level of resistance to rice blast, although gene silencing was not found in the R1 rice plants analyzed in the present study.

We had assumed that distinct effects would be observed using genes for two differentially localized chitinases. Through the entire analyses, however, there was no remarkable difference between the transgenic plants which accumulated the chitinase intracellularly (EN4-RCC2 plants) and those which accumulated the chitinase extracellularly (EN4-RCG3 plants). The reason for this may be that when the blast fungus penetrates into the host cell wall, the infection hyphae might meet the extracellular chitinase first, whereas the hyphae subsequent might be influenced by the intracellular chitinase when they grow in the cells, and then be influenced by the extracellular chitinase again when they grow through the intercellular spaces. It is assumed that overexpressed chitinases degrade the chitin of hyphae and thereby retard their growth, and that the consequent release of chitin oligomers causes the elicitation of surrounding cells. A delay in the appearance of symptoms, and decreases in lesion size and number, were observed in the trans-

formed rice plants. Similar observations were reported in transgenic oilseed rape expressing a tomato chitinase gene (Grison et al. 1996), suggesting that the mechanism of protection in transgenic rice and oilseed rape is the same.

Although most transgenic R_1 plants showed higher blast resistance that the non-transformed control plants, overall disease resistance of most transgenic lines tended to diminish in the R_1 generation compared with the R_0 generation. This is probably due to some differences in the physiological conditions of plants between the R_0 and R_1 generations. For instance, the leaf stage of inoculated plants was not exactly the same between R_0 and R_1; 4–5 leaf-stage seedlings of R_0 give rise to regenerated plants with 4–5 leaves which newly developed after the transplant and therefore differ from those of R_1 plants grown from seed. Furthermore, in preliminary experiments, it was observed that some R_0 rice plants which regenerated from non-transformed calli also exhibited higher blast resistance than that of non-transformed control plants normally grown from seed. It might be possible that the acquired resistance was induced by stress during the tissue-culture period and persisted throughout the R_0 generation, although such a phenomenon has not been reported previously for other plant species. Therefore, the significantly higher resistance demonstrated in transgenic R_0 rice plants could be caused synergistically by both the above-mentioned acquired resistance and the overexpression of the chitinase transgene. However, the acquired resistance shown in the non-transformed R_0 rice plants was completely lost in the R_1 generation. On the other hand, most of the transgenic R_1 plants maintained a rather higher resistance and the offspring of several transgenic lines showed enhanced resistance as high as that of their parents. This enhanced resistance in the R_1 generation is assumed to depend merely on overexpression of the chitinase transgene. However, the reason for the difference in the level of disease resistance observed among R_1 transgenic lines is not clear and is currently under investigation.

A final point worth mentioning is that, unlike the outcome upon the introduction of a true resistance gene, the plants obtained in this study showed enhanced resistance to blast in a non-race-specific manner. This suggests that the resistance gained would last for a long period regardless of the appearance of new races of the blast fungus. Furthermore, it is possible that these plants may display increased resistance against types of other fungal disease in addition to blast since Lin et al. (1995) reported that transgenic rice plants which overexpressed a rice chitinase gene became more tolerant to sheat blight.

According to the biosafety guidelines of Japan, the biosafety of the transgenic lines obtained in the present study has to be assessed prior to any field testing. We hope to conduct further experiments on a larger scale in the field to test whether these transgenic rice plants display enhanced field resistance and to determine the extent to which the application of fungicides can be reduced.

Acknowledgements We are grateful to H. Hirochika of the National Institute of Agrobiological Resources, Tsukuba, Japan, for the gift of the enhanced CaMV 35S promoter, H. Kamata of the University of Tsukuba, Tsukuba, Japan, for providing the plasmid pBI333, E.E. Hood of ProdiGene, Inc., Texas, USA, for providing EHA101, and H. Naito of the National Agriculture Research Center, Tsukuba, Japan, for supplying *M. grisea* race 007.0. We also thank M. Shiraishi of DuPont K.K. for his kind advice on bioassay, S. Hirose, Y. Nishizawa, K. Ono, H. Onodera and A. Tagiri for teaching us rice transformation, and K. Iijima-Tokano and K. Iwasaki for technical assistance. This work was supported by a grant from the Ministry of Agriculture, Forestry and Fisheries of Japan, and a grant-in-aid for scientific research from the Ministry of Education, Science, Sports and Culture of Japan.

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