Plant Biotechnology 23, 349–356 (2006)

Original Paper

Effectiveness of genotype-based selection in the production of marker-free and genetically fixed transgenic lineages: ectopic expression of a pistil chitinase gene increases leaf-chitinase activity in transgenic rice plants without hygromycin-resistance gene

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Received January 6, 2006; accepted April 27, 2006 (Edited by Y. Ohashi)

Abstract An extra copy of a rice pistil chitinase gene, termed *chip*, which is expressed predominantly in floral organs just before anthesis, was introduced into an economically important cultivar of rice (*Oryza sativa* L. cv. Koshihikari) together with the hygromycin-resistance gene (*hph*) as a marker gene by co-transformation. Over 100 regenerated rice plants (R_0) and their self-pollinated first and second generations (R_1 and R_2) were genotypically characterized by PCR-based genomic DNA analysis of the two transgenes to obtain stable transgenic lines, in which *chip* was inserted into a single locus separately from *hph*. We obtained homozygous *chip*-transgenic R_1 lines free of *hph* that stably expressed the *chip* mRNA and protein in rice leaf tissue. Chitin-hydrolytic activity in the leaves of these transgenic lines was higher than in those of non-transgenic controls. The resistance of the transgenic lines to rice blast disease fungus (*Magnaporthe grisea*) was examined by smearing water agar mixed with overcrowded conidia (race 007.0) on mechanically injured leaves, showing tendency to blast-disease resistance. Thus, the genotype-based selection was demonstrated to be effective at obtaining genetically stable transgenic plant lines without marker genes.

Key words: Genetically modified crops, marker free, one-factor inheritance, *Oryza sativa* cv. Koshihikari, pathogenesis-related protein.

Chitinases belong to a family of pathogenesis-related proteins, and their expression is induced in plant tissues by a series of elicitors after infection by fungi and viruses. In contrast with this pathogen-induced expression, a novel class-I chitinase gene is expressed predominantly in rice pistil in a developmentally regulated manner just before anthesis (Takei et al. 2000, Takakura et al. 2000), but not in vegetative organs by several chemicals, UV, or wounding (Takakura et al. 2000). The deduced amino acid sequence of this pistil-predominant chitinase consists of 340 amino acid residues and contains an N-terminal Cys-rich domain and a C-terminal extension (which functions as a vacuolar targeting signal). On the basis of these structural characteristics and sequence identity, this protein was assigned as a class I chitinase, although its sequence similarity is low (54%–61%; and 67%–69% in DNA sequence). Flowers are rarely infected by pathogens, despite having no physically or mechanically protective structures on their tissue surfaces, so some genes related to host defense against pathogen infection are expected to be active in their tissues (Neale et al. 1990). Such defense-related genes are candidates for introduction into crops and other commercially important plants for the improvement of host-defense potential. For example, blast disease resistance of rice was enhanced by the introduction and constitutive expression of a pathogen-induced type of class-I chitinase gene (Nishizawa et al.

Abbreviations: CaMV, cauliflower mosaic virus; CBB, Coomassie Brilliant Blue; *chip,* pistil chitinase gene; EDTA, ethylenediamine tetraacetate; GM, genetically modified; *hph*, hygromicin phosphotransferase gene (hygromycin-resistance gene); PR, pathogenesis-related; RT-PCR, reverse transcriptase polymerase chain reaction; SDS, sodium dodecylsulfate.

This article can be found at http://www.jspcmb.jp/

1999).

Koshihikari, a cultivar of *japonica* rice, is one of the most popular rice cultivars in Japan because of its palatability, and is grown in most of Japan and in some other countries, including the United States, Australia and China. Despite its excellent characteristics, it is seriously susceptible to blast disease, caused by *Magnaporthe grisea*, the most devastating fungal disease of rice. Some rice cultivars with resistance to blast disease have been developed by classical breeding methods, but it takes a long time and much labor to breed a new cultivar possessing both desirable qualities and disease resistance. Moreover, new cultivars can lose their resistance with the appearance of new variant races of blast fungi. Koshihikari had another flaw in that its transformation efficiency was too low to produce enough transformants until a modified culture method was recently developed: Hashizume et al. (1999) achieved efficient *Agrobacterium*-mediated transformation of Koshihikari by modifying the culture conditions under which scutellum callus was induced for inoculation with *Agrobacterium tumefaciens*.

Genetically modified (GM) crops are now being cultivated in several countries, such as the United States, Argentina, and China, and the production of GM foods is increasing. These GM crops are exported to other countries where GM crops are not grown commercially. Many consumers in such countries express fears of GM crops and food. In particular, opponents object to retained genes for drug resistance (used as markers in breeding) and their products, regardless of safety evaluations, such as the kanamycin-resistance gene, which is widely used in breeding of GM crops, even though its safety has been adequately evaluated (Fuchs et al. 1993). However, the safety of *hph*, which confers resistance to hygromycin and is the most commonly used selection marker in transgenic rice, has not yet been adequately evaluated.

The alternative is to remove the selection maker gene from transgenic plants after stable transgenic lines have been established. The aim of the present study was to produce genetically stable transgenic rice lines carrying a novel introduced gene for pistil-predominant chitinase without a selection maker gene and to evaluate the gene expression, enzymatic activity, and blast-disease resistance of transgenic lines. We regenerated over 100 transgenic rice plants from calluses, and selected lines on the basis of genotype analysis of regenerated rice plants and their self-pollinated progeny, in which the introduced target gene was inherited alone, without the drug-resistance gene. The rice lines we selected indeed reproduced stable progeny carrying the expected and genetically fixed phenotype: the increased chitinase protein and chitin hydrolytic activity.

Materials and methods

Construction of vectors for co-transformation

The original plasmids, pSB4 and pSB24, used for co-transformation (Komari et al. 1996), were kindly provided by Dr. Komari (JT Inc.). The plasmid pBE7131-GUS (Mitsuhara et al. 1996), containing the enhanced CaMV 35S promoter region (E7ΩIn) used for high expression of foreign genes, was kindly donated by Dr. Ugaki (University of Tokyo).

A 2.2-kb fragment containing the enhanced CaMV 35S promoter region was transferred from pBE7131-GUS to pSB24 by cleaving at *Hin*dIII and *Bam*HI sites, resulting in the replacement of the promoter region, to generate pSB25. A 1.3-kb DNA fragment encoding rice pistil-predominant chitinase was cut out by *Sma*I and *Eco*RI digestion from pBSChi411 (Takei et al. 2000), treated with the Klenow fragment, and inserted at the blunt end of pSB25 which had been digested with *Bam*HI and *Sac*I followed by the Klenow-fragment treatment. The direction of *chip* was confirmed by the size of an *Sph*I-fragment (the sizes of *Sph*I-fragment inserted in forward and backward directions are expected to be 2.6 kb and 2.2 kb, respectively). The resultant plasmid containing the enhanced CaMV 35S promoter: *chip* fusion gene (termed pSB25Chi) was integrated into pSB4 by the tri-parental mating method (Ditta et al. 1980) using three kinds of bacteria, *Bacillus* LE392 containing pSB25Chi, *Agrobacterium tumefaciens* LBA4404 containing pSB4, and *Bacillus* HB101 containing a helper plasmid pRK2013. The finally obtained plasmid, LBA4404/pSB425Chi (Figure 1), was used for the transformation of rice calluses as described below.

Transformation of rice plants

Rice plants (*O. sativa* L. ssp. *japonica* cv. Koshihikari) were transformed with the *Agrobacterium* clone LBA4404/pSB425Chi as described previously (Hashizume et al. 1999). The regenerated plantlets (R_0) were planted individually in pots (nitrogen 0.27 g, phosphoric acid 0.40 g, and potassium 0.33 g kg^{-1} · soil), and grown in a greenhouse. Four to five months later, mature seeds of the R_1 generation were harvested individually.

DNA preparation from rice tissue

An SDS-extract buffer (200 mM Tris · HCl, pH 7.5, containing 250 mM NaCl, 25 mM EDTA, 0.5% SDS, and 200 μg ml^{-1} protease K) was added to a test tube containing a plantlet blade (120 μl per 5-cm length of blade), and incubated at 55°C for 30 min. The extract was separated from the tissue debris by centrifugation at 19 000×*g* for 10 min, and mixed with 100 μl isopropanol to precipitate DNA. After centrifugation at 19 000×*g* for 5 min, the precipitate was washed with 70% ethanol at room temperature, and then incubated with 30 μl ribonuclease (20 μg ml^{-1} of 0.1×TE) at 55°C for 15 min. The obtained solution was used as a genomic DNA preparation in the following experiments.

Genomic PCR and RT-PCR analyses

The genomic DNA preparation (5–50 ng DNA in 1 μl per tube) was used as a template for PCR for the amplification of specific regions of transgenes. The primer pairs used for the PCR were

as follows. For the 280-bp DNA fragment of *chip*: forward, 5′-TTGAACGCCACCGTCGGGTCCGTT-3′; reverse, 5′-GACACCCGCAAGCGTGA-3′. For the 500-bp DNA fragment of *hph*: forward, 5′-TCCATCACAGTTTGCCAGTGATACA-3′; reverse, 5′-ATGAAAAAGCCTGAACTCACCGCGA-3′. The amplification reaction was done in a 10-μl aliquot by using Ex-Taq DNA polymerase (Takara Bio) according to the attached manual, except that the $MgCl_2$ concentration was adjusted to 1.0 mM. The DNA was denatured at 94°C for 20 s and the primers were annealed at 55°C for 1 min, and then the enzyme reaction was conducted at 72°C for 2 min. This reaction cycle was repeated 35 times in a PCR Thermal Cycler MP (Takara Bio). The resultant PCR products were analyzed by electrophoresis in a 3% agarose gel containing 0.5 μg ml^{-1} ethidium bromide.

mRNA was isolated from leaf tissues by using a Quick Prep Micro mRNA Purification Kit (Amersham Biosciences). RT-PCR was performed by using a SuperScript One-step RT-PCR System (Life Technologies). The mRNA (5–10 ng mRNA in 1 μl per tube) with the *chip*-specific primers described above was used for RT-PCR. cDNA was synthesized at 50°C for 30 min and pre-denatured before PCR at 94°C for 2 min. PCR amplification was then performed in 25 cycles of 94°C for 15 s, 55°C for 30 s, and 68°C for 2 min. The resultant PCR products were analyzed by electrophoresis in a 3% agarose gel containing 0.5 μg ml^{-1} ethidium bromide.

Genotypic selection of transgenic lines

Leaves of the regenerated rice plants (R_0) were gathered from each plant. DNA was extracted from them and analyzed by PCR as above. Seeds (R_1) harvested from each R_0 plant were sown in multi-hole pots (15–20 seeds per line), and the plantlet blades were gathered one week later. DNA extraction and PCR analysis were repeated as described above. Several promising R_0 lines were selected, and a larger number (30–90) of R_1 seeds from each line was germinated and again subjected to DNA analysis. Some R_0 lines were selected on the basis of the genotype of their progeny (R_1), i.e., one-factor inheritance of *chip* and unlinked inheritance between *chip* and *hph*.

Two lines of the selected R_0 plants were analyzed by Southern blot hybridization to estimate the copy number of the transgene. Genomic DNA was isolated from three R_0 plants by using a Nucleon Phytopure Plant DNA Extraction Kit (Amersham Biosciences), followed by digestion with *Hin*dIII, a single site in the T-DNA region of the plasmid vector (Figure 1). The DNA digests were subjected to an agarose gel electrophoresis (0.8% gel), transferred to nylon membrane (Roche Diagnostics), and hybridized at 68°C for 14 h with the *chip* or *hph* gene probe labeled with digoxygenin by using DIG DNA Labeling Kit (Roche Diagnostics). The hybridized signal was detected by using DIG Luminescent Detection Kit (Roche Diagnostics). Each of the R_1 plants derived from the selected R_0 lines was grown as a new line, and their self-pollinated seeds (R_2) were harvested.

The R_2 seeds (30–60 seeds per line) derived from the selected R_1 lines with *chip* but without *hph* were subjected to the DNA analysis as above, and genetically fixed R_1 lines were finally selected from among them on the basis of the inheritance in the R_2 generation.

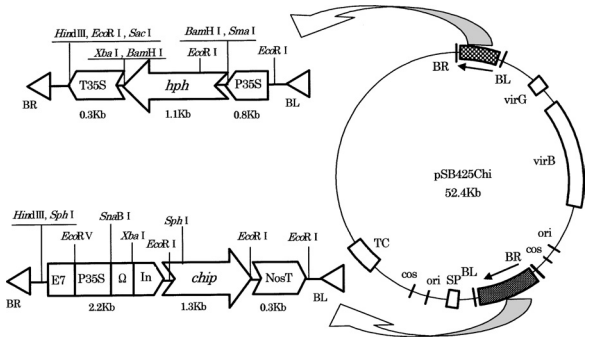

Figure 1. Structure of pSB425Chi and the T-DNA regions of the introduced genes. The two T-DNA regions containing *chip* and *hph* genes, respectively, are shown in detail with the restriction enzyme sites. BR, right border; BL, left border; T35S, cauliflower mosaic virus 35S terminator; *hph*, hygromycin phosphotransferase gene (hygromycin-resistance gene); P35S, cauliflower mosaic virus 35S promoter; E7, 5′-up-(−290 to (−90)×7; Ω, 5′-untranslated sequence of tomato mosaic virus; In, first intron of gene for phaseolin; *chip*, rice chitinase cDNA; NosT, nopaline synthase terminator; virG, *vir*G gene from pTiBo542; virB, *vir*B gene from pTiBo542; ori, origin of replication of ColE1; cos, cos site of phage λ; SP, spectinomycin-resistance gene; TC, tetracycline-resistance gene.

SDS-PAGE and immunoblot analysis

To extract proteins, including chitinase, from rice plants, the leaf tissue samples were homogenized in cold 0.5 M NaCl solution (300 μl per 50 mg tissue) and centrifuged at 4°C at 19 000×g for 20 min. The supernatant (8 μg protein from each sample) was mixed with sample buffer (2% SDS, 20 mM Tris·HCl [pH 6.8], 6% glycerol, 0.012% bromophenol blue, and 1% 2-mercaptoethanol) and boiled for 3 min. The proteins were separated by SDS-polyacrylamide gel electrophoresis (Laemmli 1970) and stained with Coomassie Brilliant Blue (CBB) and/or transferred onto Clear Blot Membrane-p (Atto). The blotted membrane was reacted with the rabbit antiserum raised against recombinant ChiP protein (Takakura et al. 2000), which was kindly given to us by Dr. Komari of JT Inc. After being incubated with peroxidase-labeled anti-rabbit IgG, the immuno-reactive bands were detected with an ECL Western blot detection system (Amersham Biosciences).

Chitinase activity measurement

The 0.5 M NaCl-extracted protein (1.0 mg ml^{-1}) was spotted (10 μl each) onto an agarose plate containing 0.05% ethylene glycol chitin as a soluble substrate and incubated at 37°C for 24 h. Then the plate was stained with 0.01% Congo red solution and the diameters of non-stained areas (hollows), in which chitin was decomposed by chitinase, were measured.

Disease resistance assay

Two lines of homozygous R_2 progeny were planted in pots (10 plants per line), and the resistance to rice blast disease fungus (*Magnaporthe grisea*) was assayed at the four- to five-leaf stage by the method of Asaga (1981). Holes (3 holes per reaf) were made with a needle on leaves (2 leaves per plant, total 20 leaves per line) of several surviving stems which had escaped from the fungus infection, and the leaves were inoculated in these areas by smearing water agar overcrowded with conidia (race 007.0). One week later, lesions on leaves were observed, and the extent

Table 1. DNA analysis of R_0 plants by PCR.

	Number of R_0 plants				
	Total	*chip*+ *hph*+	*chip*+ *hph*−	*chip*− *hph*+	*chip*− *hph*−
Regenerated plants	119 (100)	102 (85.7)	0 (0)	17 (14.3)	0 (0)
Fertile plants	88 (73.9)	78 (65.5)	0 (0)	10 (8.4)	0 (0)

The values in parentheses represent the proportion (%) of each plant group against the total regenerated plants. *chip*+, *chip*-positive plant; *chip*−, *chip*-negative plant; *hph*+, *hph*-positive plant; *hph*−, *hph*-negative plant.

of disease resistance was expressed as the proportion (%) of the total area of lesions per inoculated leaf (disease severity index, 0–10) (Asaga 1981).

Results

Establishment of stable, marker-free transgenic lines by genotypic analysis of regenerated plants and their progeny

A total of 119 lines of regenerated rice plants (R_0), which showed hygromycin resistance, were obtained from 502 infected calluses. Of these, 88 lines were fertile, and self-pollinated mature seeds (R_1) were harvested individually from each line. The genotype of the 119 regenerated plants was determined by PCR analysis (Table 1). All of the hygromycin-resistant rice plants indeed possessed the transgenic *hph* gene, whereas some (17/119) did not contain the transgenic *chip* gene. Two PCR products of 280 and 500 bp were clearly detected on the gels of the transgenic lines but not of the non-transgenic controls (see Figure 2A), indicating that the genomic PCR analysis worked well.

The DNA analysis of the two transgenes in a large number of R_1 plants (30–90 lines) revealed some segregation patterns of the two genes in the R_1 generation. A typical example of PCR analysis of a selected R_0 line, T56-6, and its R1 progeny is shown in Figure 2. Some R_1 plants showed two bands for both *chip* (280 bp) and *hph* (500 bp) genes, while others showed only one (either *chip* or *hph*) or none (Figure 2A). The segregation pattern of the two genes in 47 R_1 plants from T56-6 showed that *chip* and *hph* genes clearly behaved as two independent Mendelian loci, indicating that T56-6 contained each gene as a single locus.

On the basis of the gene segregation pattern, we estimated the number of loci of each gene in the rice genome in 12 R_0 lines (Table 2). Seven R_0 lines in five line groups showed one-factor inheritance of *chip* and a separate selection marker. Integration of *chip* and *hph* genes in the genomic DNA was confirmed for three selected R_0 lines by Southern blot hybridization (data not shown), and the copy number of the transgenes was estimated to be one or two (Table 2).

To look for R_1 plants homozygous for *chip*, we selected seven R_1 lines (one from each of 7 R_0 lines) with *chip* and without *hph*, and germinated their self-pollinated seeds (R_2). DNA analysis of the R_2 plants (30–60 per R_1 line) from the 7 R_1 lines demonstrated that three lines in three line groups were homozygous for the introduced *chip* gene (Table 3). In all of the selected R_1 lines, the *hph* gene was confirmed to have segregated effectively. Some typical PCR profiles for a homozygous R_1 line, T56-6-1, and for a heterozygous R_1 line, T56-6-38, are shown in Figures 2B and C.

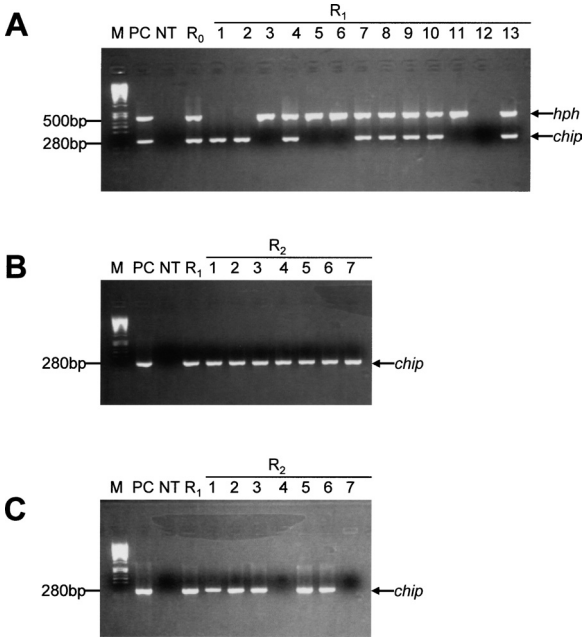

Figure 2. PCR analysis of selected R_0 plant T56-6 and its progeny. (A) T56-6 (R_0, one-factor inheritance and separate selection marker). Lane numbers 1 to 13 belong to each R_1 plant derived from the T56-6 R_0 line. (B) T56-6-1 (R_1, homozygous). Lane numbers 1 to 7 belong to each R_2 plant derived from the T56-6-1 R_1 line. (C) T56-6-38 (R_1, heterozygous). Lane numbers 1 to 7 belong to each R_2 plant derived from the T56-6-38 R_1 line. M, molecular size maker (λDNA digested with *Hin*cII and *Hin*dIII); PC, positive control (plasmid DNA); NT, non-transgenic rice.

Stable expression of the introduced gene and chitinase activity in the genetically fixed R1 lines and their progeny

To confirm the expression of the *chip* gene at the mRNA level, RT-PCR analysis was done on leaf tissues of several transgenic lines. A typical example of RT-PCR analysis of an R_0 line, T52-62, and its R_1 and R_2 progeny is shown in Figure 3A. The 280-bp RT-PCR product was clearly detected on the gel of the *chip*-positive transgenic

Table 2. Segregation of the introduced genes in the R_1 progeny and estimation of the number of loci in the R_0 lines.

Line no. (R_0)	Copy number (R_0)		Number of R_1 plants				Number of loci in R_0[a]			Selected plant[b]
	chip	*hph*	*chip*+ *hph*+	*chip*+ *hph*−	*chip*− *hph*+	*chip*− *hph*−	Unlinked *chip*	Unlinked *hph*	Linked *chip*− *hph*	
T52-35	2	1	39	14	0	2	1	0	1	○
T52-46			40	1	14	0	1	2	0	○
T52-62	1	1	64	16	23	7	1	1	0	○
T52-65			27	7	0	2	1	0	1	○
T55- 3			72	18	0	1	2	0	1	
T55- 6			45	3	0	2	1	0	2	○
T55- 7			37	0	3	0	0	2	1	
T55- 8			39	0	0	9	0	0	1	
T56- 2			54	4	0	1	1	0	2	○
T56- 3			34	0	0	7	0	0	1	
T56- 4			35	0	0	11	0	0	1	
T56- 6	1	1	22	9	12	4	1	1	0	○

chip+, *chip*-positive plant; *chip*−, *chip*-negative plant; *hph*+, *hph*-positive plant; *hph*−, *hph*-negative plant. [a] The number of loci, which gave the smallest chi-square value was adopted. [b] Type of inheritance in one factor and separating the selection marker was selected.

Table 3. Segregation of the introduced genes in the R2 progeny and estimation of the number of the genotype of R1 lines.

Line no. (R_1)[a]	Number of R_2 plants				R_1 genotype	
	chip+ *hph*+	*chip*+ *hph*−	*chip*− *hph*+	*chip*− *hph*−	*chip*	*hph*
T52-35-8	0	33	0	0	*chip*/*chip*	−/−
T52-46-12	0	22	0	4	*chip*/−	−/−
T52-62-112	0	61	0	0	*chip*/*chip*	−/−
T55- 6-23	0	37	0	8	*chip*/−	−/−
T55- 6-26	0	29	0	9	*chip*/−	−/−
T56- 6- 1	0	21	0	0	*chip*/*chip*	−/−
T56- 6-38	0	36	0	8	*chip*/−	−/−

chip+, *chip*-positive plant; *chip*−, *chip*-negative plant; *hph*+, *hph*-positive plant; *hph*−, *hph*-negative plant. [a] Selected R_1 plants having *chip* gene and but no *hph* gene.

R_1 line T52-62-112 and all its R_2 progeny, but not in a *chip*-null R_1 line, T52-62-42, or the non-transgenic control. An almost identical level of the RT-PCR product was detected among the R_2 lines from the *chip*-positive homozygous R_1 plants T52-62-112, indicating that the introduced *chip* gene was genetically fixed and stably inherited in the progeny.

The expression of *chip* at the protein level was analyzed by SDS-PAGE/immunoblot analysis using anti-chitinase antibody. Figures 3B and C show typical SDS-PAGE/immunoblot profiles on the stable transgenic line T52-62-112 and its progeny. No large difference was observed in the CBB-stained profile among the transgenic and non-transgenic rice lines (Figure 3C). By immunoblot analysis, on the other hand, a 32-kDa band corresponding to ChiP was observed in the transgenic R_1 line and its progeny more strongly than in the non-transgenic controls, though more weakly than in pistil of the non-transgenic one (Figure 3B). A faint band of endogenous chitinase(s) with a similar molecular mass (32 kDa) was detected in the non-transgenic controls by limited cross-reactivity of the anti-ChiP antibody with the pathogen-induced type of class I chitinase(s), which is expressed constitutively in leaves at a low level (Nishizawa et al. 1999).

The chitinase activity of leaf tissue from the transgenic plants was assayed by using ethylene glycol chitin as a synthetic substrate. A representative result is shown in Figure 4. Congo red stains chitin polymers with high molecular mass, but not chitin oligomers. Thus, the area of non-stained hollows in the gel containing chitin polymers reflects chitinase activity in the sample (Figure 4B). The sizes (square of diameter) of hollows made by leaf extracts from the *chip*-positive homozygous R_1 plant T52-62-112 and its progeny were close to each other and significantly larger than those of the non-transgenic control (Figure 4A).

Preliminary evaluation of blast disease resistance of the transgenic rice plants

Two R_1 lines, T52-35-8 and T56-62-112, among the three finally obtained homozygous lines were assayed for blast disease resistance. Seven days after fungal inoculation of injured leaves, the blast disease symptoms on the transgenic and control lines were observed and ranked in order of the disease severity level (from zero to

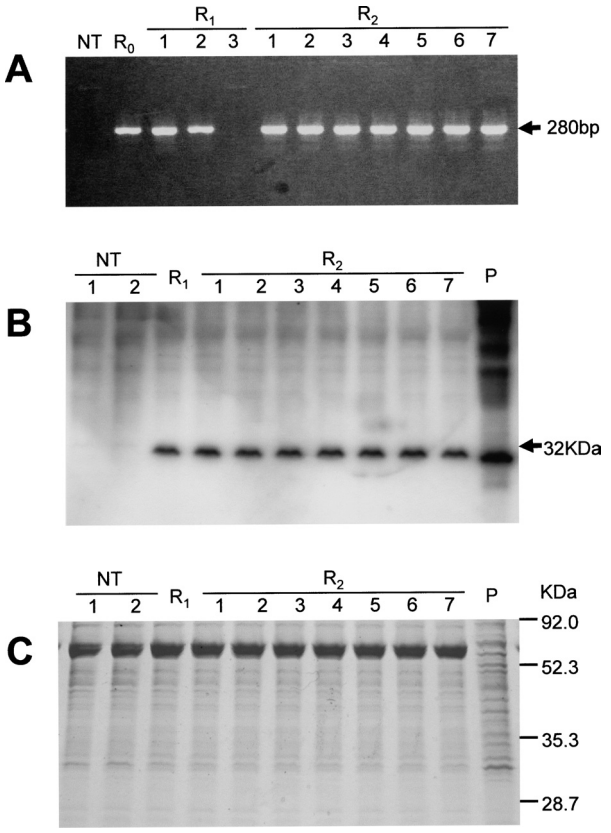

Figure 3. Stable expression of the introduced *chip* at mRNA and protein levels. mRNA of R_0 plant T52-62 and its progeny were used as template for RT-PCR with a *chip*-specific primers (A). The 0.5 M NaCl-extracted proteins from the leaves of homozygous R_1 plant T52-62-112 and its progeny were analyzed by immunoblotting using anti-ChiP antiserum (B), and by SDS-polyacrylamide gel electrophoresis (C). R_0, T52-62 (one-factor inheritance and separate selection marker); R_1, (1) T52-62-112 (homozygous); (2) T52-62-77 (heterozygous); (3) T52-62-110 (*chip-null*); R_2, lane numbers 1 to 7 belong to each R_2 plant derived from the T52-62-112 R_1 line; NT, non-transgenic rice, P, pistil of non-transgenic rice.

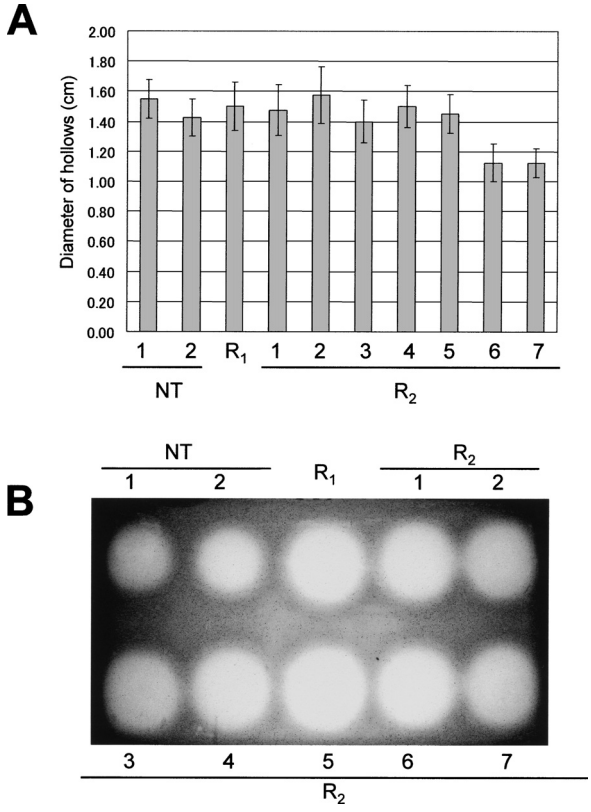

Figure 4. Chitinase activity assay of homozygous R_1 plant T52-62-112 and its progeny. (A) Diameter of hollow in agarose containing ethylene glycol chitin. (B) The agarose containing ethylene glycol chitin was stained with Congo red to visualize enzymatic degradation of the ethylene glycol chitin. The values are the means±S.D. of 6 replicates. All transgenic lines were significantly different from the wild type by *t*-test ($P=0.05$). R_1, T52-62-112; R_2, lane numbers 1 to 7 belong to each R_2 plant derived from the T52-62-112 R_1 line; NT, non-transgenic rice.

10). As shown in Figure 5, 13 of the 20 inoculated leaves (65%) of the control non-transgenic plants showed higher severity index (6 or more), whereas only one or two leaves (5–10%) did so in the case of the both transgenic lines, T52-35-8 and T52-62-112. A month after inoculation, most of the non-transgenic control plants had withered, whereas some plants of the transgenic lines survived.

Discussion

So far, to the best of our knowledge, there are not many transgenic rice plants in which the excellent traits conferred by introduced foreign genes are stably inherited in self-pollinated posterity. Generally, in the first screening step, attention is paid only to observed phenotypes of target traits, and promising plant lines are often selected according to the phenotypes instead of genotypes. For instance, even if a transgenic R_0 plant exhibited strong disease resistance, a population with genetically fixed traits cannot easily be established when the transgene is not inherited in one factor. Consequently, genotypic analyses such as the PCR-oriented genome analysis used in the present study should be used, by which genetically fixed lines can be selected preferentially and a large descendent population can be analyzed. By using the improved transformation method of Hashizume et al. (1999), we obtained over 100 regenerated rice plants and, moreover, selected several promising lineages by genotypic analyses of both regenerated plants (R_0) and their progeny (R_1 and R_2).

By DNA analysis of the R_1 generation, we selected R_0 plants with the genotype "one-factor inheritance and separated selection marker", in which an independent target gene exists at one locus, and an independent selection maker or a linked target-selection maker gene exists at another locus. This genotype is essential to obtaining genetically stable, marker-free transgenic lines by subsequent self-breeding. In the DNA analysis of the R_2 generation, we confirmed that all R_1 lines selected

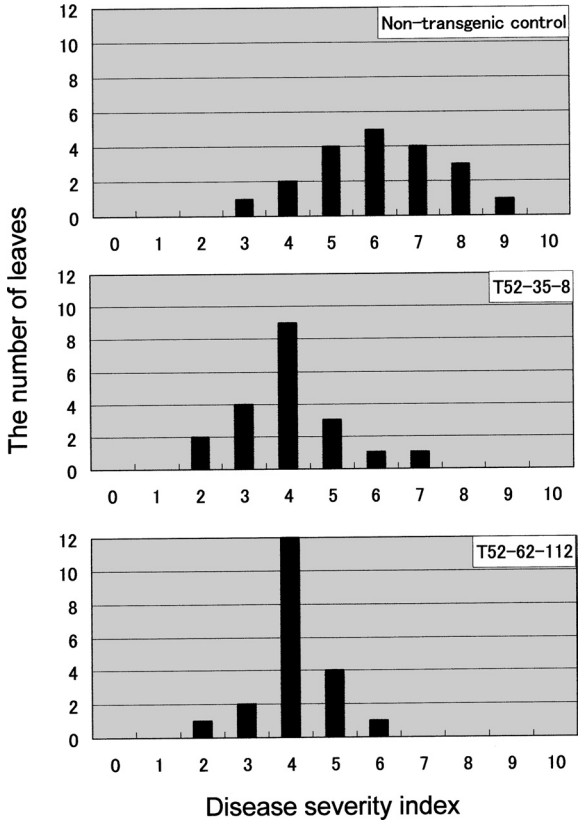

Figure 5. Disease resistance assay in the homozygous R_2 progeny of T52-35-8 and T52-62-112 with blast disease fungus. The leaves (20 leaves per line) of non-transgenic control and transgenic lines were inoculated by smearing water agar overcrowded with conidia (race 007.0). One week later, lesions on leaves were observed, and the extent of disease symptom was expressed as disease severity index, 0–10 (Asaga 1981). The ratio (%) of lesion area against the total area of inoculated leaves for each severity index is as follows. Index 0; 0%, index 2; 1–2%, index 3; 2–5%, index 4; 5–10%, index 5; 10–20%, 1ndex 6; 20–40%, index 7; 40–60%, index 8; 60–80%, index 9; 80–90%, index 10; 90–100%.

were free of the selection marker and contained only the target *chip* gene. Theoretically, if R_0 plants having an independent target gene and an independent selection marker gene, the next generation, R_1, would contain only the target gene at a probability of 3/16 and, moreover, 1/3 of these R_1 plants would be homozygous. Our results agreed well with this theoretical inheritance (see Table 2). Thus, the genotypic selection method was efficient because removal of the marker gene and one-factor inheritance in offspring could be examined at the same time by the genomic DNA analysis.

The effectiveness of genotypic selection was demonstrated not only in the genotype but also in the phenotype of the obtained transgenic lines, in which distinct and stable expression of the *chip* transgene was observed at the mRNA, protein, and enzyme-activity levels. The genotypic selection described here would be applicable to the efficient introduction of any useful gene into another plant species, especially for the development of economically important crop cultivars, in consideration of safety and public acceptance.

Four kinds of chitinase gene have been identified in rice: Class I (*CHT-1* and *CHT-2*) (Nishizawa et al. 1993), Class II (Kim et al. 1998), and Class IV (Nakazaki et al. 1997, Nakazaki and Ikehashi 1998). The amino acid sequence of the pistil-predominant chitinase (ChiP) is similar to these known rice chitinases, especially to Class I chitinases (54%–61% identity), in that all have an additional cysteine-rich chitin-binding domain and a hinge region at the N-terminus (Collinge et al. 1993). In the catalytic domain, on the other hand, ChiP has two short deletions of seven and two residues, and one residue insertion. In spite of these differences in the primary structure, the recombinant ChiP indeed showed hydrolytic activity against chitin oligomer (Takakura et al. 2000) and against ethylene glycol chitin (this study). These results suggest that the pistil chitinase, ChiP, may play some roles in the biochemical defense of rice gametes against fungal pathogens.

One of the most important traits in plant breeding is resistance to fungal disease. Several reports on transgenic tobacco plants have shown that their resistance to fungal disease was enhanced by constitutive expression of chitinase genes (Broglie et al. 1991, Zhu et al. 1994, Jach et al. 1995). As for rice, introduction of rice chitinase genes back into *indica* rice (Lin et al. 1995) and *japonica* rice (Nishizawa et al. 1999) have successfully produced transgenic rice plants with increased resistance to the sheath blight pathogen *Rhizoctonia solani* and the rice blast *Magnaporthe grisea*. In the present study, a possibility of the increased resistance to blast-disease of the *chip*-transgenic rice plants was shown in an experimental condition. Only by this limited experiment, however, it could not clearly be concluded that the introduction of *chip* gene to rice truly enhanced the resistance to blast-disease. Further studies would be required with detailed analyses using some other evaluation methods for blast-disease resistant.

Acknowledgements

This work was partly supported by a Grant-in-Aid for the 21st Century COE Program (14COEA12) from the Ministry of Education, Science, Sports and Culture of Japan (T. M.). The authors are grateful to Dr. T. Komari (JT Inc.) for providing the pSB vectors and the anti-chitinase-antibody, and Dr. M. Ugaki (University of Tokyo) for supplying plasmid pBE7131-GUS.

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