Theor Appl Genet (2002) 105:809–814
DOI 10.1007/s00122-001-0817-9

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Overexpression of the wasabi defensin gene confers enhanced resistance to blast fungus (*Magnaporthe grisea*) in transgenic rice

Received: 23 January 2001 / Accepted: 18 April 2001 / Published online: 14 September 2002

Abstract Transgenic rice (*Oryza sativa* cv. Sasanishiki) overexpressing the wasabi defensin gene, a plant defensin effective against the rice blast fungus, was generated by *Agrobacterium tumefaciens*-mediated transformation. Twenty-two T2 homozygous lines harboring the wasabi defensin gene were challenged by the blast fungus. Transformants exhibited resistance to rice blast at various levels. The inheritance of the resistance over generations was investigated. T3 plants derived from two highly blast-resistant T2 lines (WT14-5 and WT43-5) were challenged with the blast fungus using the press-injured spots method. The average size of disease lesions of the transgenic line WT43-5 was reduced to about half of that of non-transgenic plants. The 5-kDa peptide, corresponding to the processed form of the wasabi defensin, was detected in the total protein fraction extracted from the T3 progeny. Transgenic rice plants overproducing wasabi defensin are expected to possess a durable and wide-spectrum resistance (i.e. field resistance) against various rice blast races.

Keywords Rice · Defensin · Rice blast fungus · *Agrobacterium tumefaciens*

Introduction

Rice blast disease caused by *Magnaporthe grisea* occurs worldwide and results in severe damage of the plants and a reduced yield. Therefore, breeding rice cultivars resistant to the blast disease has been a major goal over several decades. Recently, genetic engineering has emerged as an alternative approach to create blast-resistant rice. This approach is advantageous for introducing disease resistance into elite rice cultivars, since transgenic plants can acquire a single desired trait without any alteration of the original genetic background.

Many antimicrobial proteins have been identified and their antimicrobial activity tested against fungi or bacteria. In some plant species, transgenic plants overexpressing foreign antimicrobial protein genes actually acquired resistance to pathogens. In rice, three corresponding experiments have been reported to date. Stark-Lorenzen et al. (1997) showed that rice plants overexpressing the stilven synthase gene acquired resistance to rice blast. Recently, Datta et al. (1999) reported that rice plants overexpressing a gene for a thaumatin-like protein belonging to the PR-5 class exhibited enhanced resistance to *Rhizoctonia solani*, a causal agent of sheath blight disease. In addition, Nishizawa et al. (1999) reported transgenic rice plants harboring a rice chitinase gene that conferred enhanced resistance to the rice blast fungus. These three experiments all demonstated an overexpression of antifungal protein-enhanced resistance to pathogens in transgenic rice. However, other antimicrobial protein-encoding genes have not been explored.

Defensins are low-molecular-weight (5 kDa) proteins occurring in seeds, stems, roots and leaves of a number of plant species that are toxic to bacteria, fungi and yeast in vitro. Defensins have been shown to cause permeabilization of fungal membranes, leading to the inhibition of fungal growth (Commue et al. 1992; Florack et al. 1994; Broekaert et al. 1995; Thevissen et al. 1999).

We have previously cloned and characterized a cDNA encoding the defensin gene from wasabi (*Wasabia japonica*) plants (Genbank accession no. AB012871). Transgenic tobacco plants overexpressing the wasabi defensin gene were observed to inhibit the growth of *Botrytis cinerea* on their leaves (Nishihara, personal communication). Wasabi defensin protein was produced in *Nicotiana benthamiana* plants using potato virus X

Communicated by G. Wenzel

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(PVX) vector harboring wasabi defensin cDNA (Saitoh et al. 2001).

The presence of the defensin protein at a concentration of 5 μg per milliliter inhibited the growth of rice blast fungal hyphae in vitro. In the present article, we report the generation of transgenic Japonica rice plants (*Oryza sativa*. cv. Sasanishiki) constitutively expressing the wasabi defensin gene. These plants exhibited enhanced resistance against rice blast.

Materials and methods

Construction of a binary vector and transformation of rice plants

The binary plasmid vector pEKHSubWT, containing the 414-bp coding region of the wasabi defensin gene driven by the maize ubiquitin-1 promoter (Christensen et al. 1992) together with the hygromycin phosphotransferase gene (*Hpt*) driven by the CaMV 35S promoter, was transferred into *Agrobacterium tumefaciens* strain EHA105. The transformation and regeneration of rice plants (*Oryza sativa* L. cv. Sasanishiki) was carried out according to Hiei et al. (1994) with a slight modification. Hygromycin (50 mg/l) was used for the selection of transformants during in vitro culture. After acclimatization, the transformants were grown in soil in a greenhouse. Hygromycin-resistant transformants (T0) were self-pollinated, and 25 seeds of each T1 line were used for segregation analysis of hygromycin resistance. Twenty-two selected homozygous T2 lines were further studied for blast resistance.

Polymerase chain reaction (PCR) amplification

Total DNA was extracted from leaf tissues of T0 plants and non-transformed control plants using a modified method of Shure et al. (1983). The PCR reaction conditions were those described by Edwards (1991). The primer pairs for the detection of the introduced wasabi defensin gene region were: 5′-TGTTTCTTTTG-TCGATGCTCACCCTGTTGTTTGGT-3′/5′-GATTGAATCCTGT-TGCCGGTCTTGCGATGATTATC-3′.

Western blot analysis

Total protein was extracted from 0.05 g of leaf tissues of each progeny plant by homogenization in extraction buffer (250 m*M* Tris-HCl, pH 7.5, 2.5 m*M* EDTA, 0.1% ascorbic acid, 1 m*M* PMSF). The supernatant obtained after centrifugation of the extract at 12,000 rpm for 10 min was boiled for 10 min in sample buffer (extraction buffer containing 0.2% β-mercaptoethanol, 2% SDS), separated by SDS-PAGE and electroblotted onto a polyvinylidine difluoride (PVDF) membrane. Immunodetection was performed essentially according to the method of Matsudaira (1987). Antiserum was raised against a synthetic peptide corresponding to part of the wasabi defensin protein (LEGARHGSCNYIFPYHRC-ICYFPC). Detection of the 5-kDa wasabi defensin protein was carried out with anti-rabbit IgG immunoglobulin (Boehringer, Germany) as the secondary antibody and an HRP color reagent.

Blast resistance analysis

At the four- to five-leaf stage (30 days after sowing), rice seedlings were transferred into an inoculation chamber, and each pot was inoculated by spraying 250 μl of a conidial suspension (containing 0.05% Tween20) of the rice blast fungus (race 007.0). Disease severity was scored on a scale of 0 to 4 with respect to grades in lesion size among 20 plants of each rice transgenic line (T2 progeny). In addition, the number of lesions were counted. As an alternative inoculation method, 40 μl of a conidial solution was applied to press-injured spots (2 mm in diameter) on leaves (three spots per leaf) made by a pressing machine (Fujiwara, Osaka, Japan) according to Kawasaki et al. (1999). The conidia concentration was adjusted to 5×10^5/ml, and Tween 20 was added to a concentration of 0.05% just before inoculation. After inoculation, plants were kept in a closed chamber at 25 °C and 100% relative humidity for 20 h, and then transferred to a moist incubator at 25 °C. Disease severity was inferred from the lesion size developed from inoculated spots on the leaves 10 and 14 days after inoculation, respectively.

Results

Production of transgenic rice containing the wasabi defensin cDNA

The 23-kbp plasmid EKHSubWT harboring the 414-bp wasabi defensin cDNA under the control of maize ubiquitin-1 promoter (Christensen et al. 1992) was used for rice transformation (Fig. 1). After *Agrobacterium tumefaciens*-mediated transformation, transformants were selected for hygromycin resistance (50 mg hygromycin/l). For further characterization, 84 plants (T0 generation), originating from independent transformation events were grown in a greenhouse. Integration of the wasabi defensin gene into the genome was confirmed by PCR with wasabi defensin-specific primers and total DNA as template (Fig. 2). Seeds obtained from 84 T0 self-pollinated plants were analyzed for segregation of hygromycin resistance, and 22 lines were selected as homozygous lines of the T2 generation (Fig. 3).

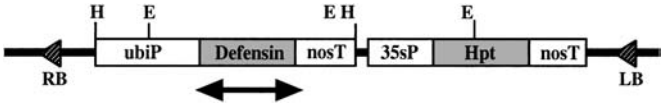

Fig. 1 T-DNA region of the binary vector pEKHSubWT used for rice transformation. *RB* and *LB* Right and left border sequences of the T-DNA region, respectively, *double arrow* PCR-amplified region, which was used to confirm the existence of the defensin gene in the regenerated plants. *ubiP* Maize ubiquitin1 promoter, *35Sp* CaMV 35S promoter, *Defensin* wasabi defensin cDNA, *nosT* terminator of the nopaline synthase gene, *Hpt* hygromycin phosphotransferase gene, *E Eco*RI, *H Hin*dIII

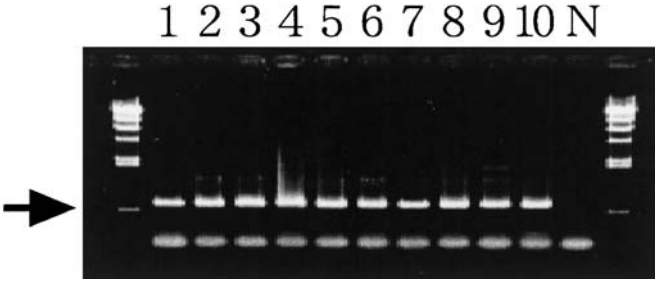

Fig. 2 Detection of transgenes in T0 regenerated rice plants (cv. Sasanishiki) by PCR. The region described in Fig. 1 was amplified using primers complementary to the wasabi defensin gene. *N* Non-transgenic plants, *lanes 1–10* transgenic plants

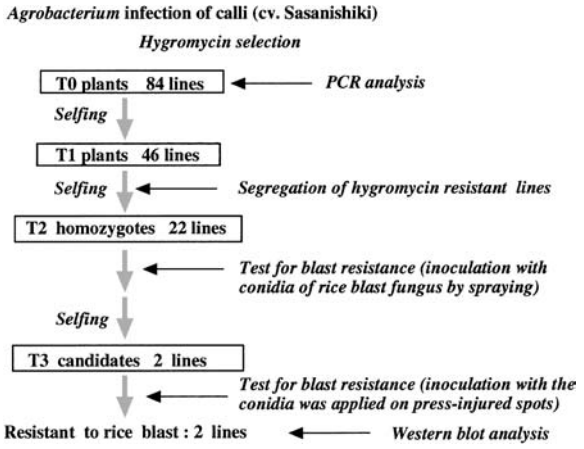

Fig. 3 Selection scheme for blast-resistant transgenic rice lines

Evaluation of disease resistance in transgenic rice plants

Cultivar Sasanishiki does not possess the resistance gene *Pi-k* against the rice blast fungus race 007.0. Therefore, 22 lines of T2 homozygotes harboring the wasabi defensin gene were studied for disease resistance against race 007.0 by comparing their disease severity with the non-transgenic control plants. Cultivar Tsuyuake, with the true resistance gene *Pi-k* against race 007.0, was used as the resistant control. Transgenic and control plants were inoculated with conidia, and the sizes of the disease lesions measured. As shown in Fig. 4A, transformants exhibited susceptibility to rice blast at various levels. Whereas the line WT43-5 was almost as resistant as resistant cv. Tsuyuake, line WT21-3 was susceptible to blast at the same level as cv. Sasanishiki. In addition to

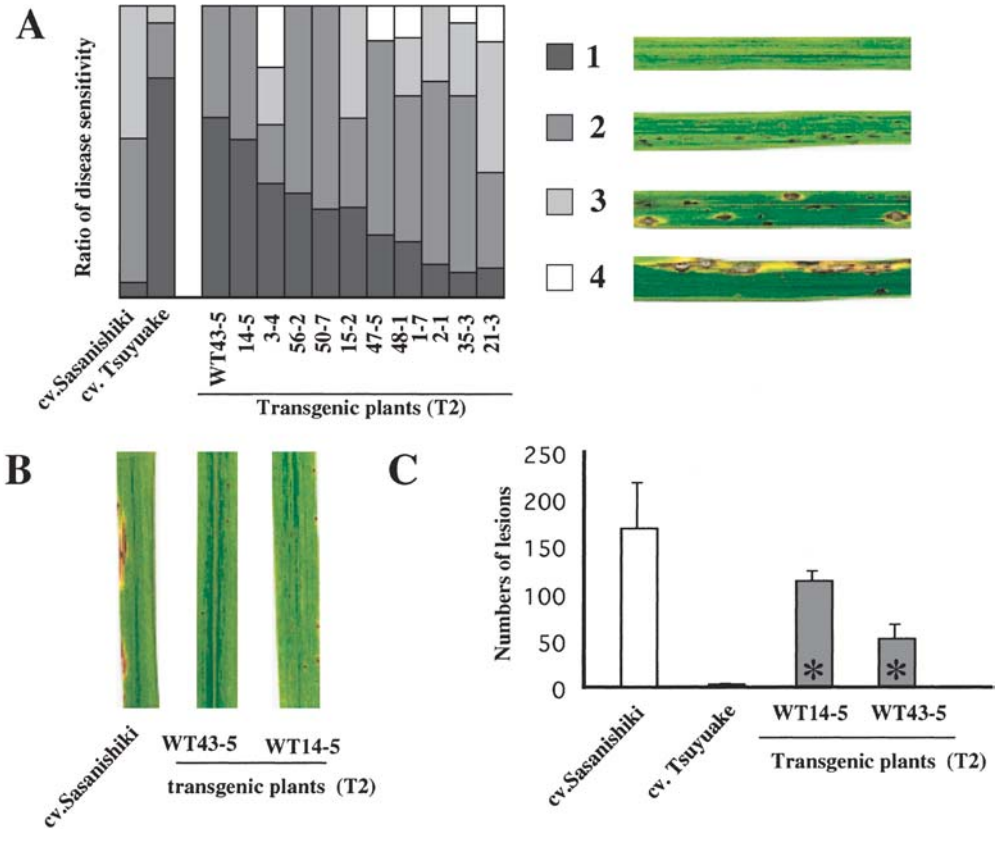

Fig. 4A–C Blast disease lesions on leaves of control and T2 transgenic rice plants 10 days after inoculation with the rice blast fungus (*Magnaporthe grisea* race 007.0). At the four- to five-leaf stage, rice seedlings were transferred into an inoculation chamber, and each pot was inoculated with approximately 2.5 ml of a suspension of blast conidia by spraying. **A** Disease severity expressed as the percentage of disease lesions at each level (0 to approximately 4 gradations) in 20 plants of each transgenic line. **B** Reduced lesions in two resistant lines. **C** Numbers of spots on the leaves of control cv. Sasanishiki (without R gene against race 007.0) and cv. Tsuyuake (with R gene against race 007.0) and on the leaves of transgenic rice plants infected by race 007.0, 7 days after inoculation (bars ± SE, $*P < 0.01$)

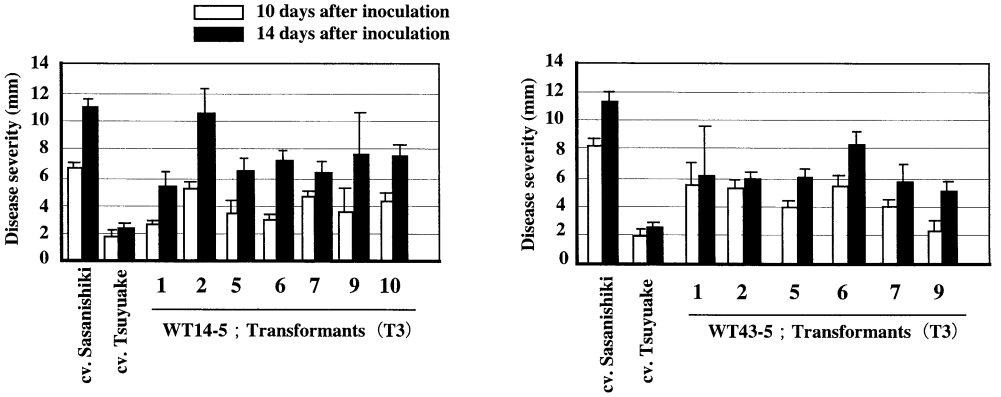

Fig. 5 Disease severity as inferred from the mean lesion sizes on punch-injured leaves in control (non-transformants) and T3 transgenic rice plants after inoculation of fungal spores (race 007.0). Buffer-suspended spores of *M. grisea* were applied onto press-injured spots made by a pressing machine, and lesion sizes were measured 10 and 14 days after inoculation (bars ± SE)

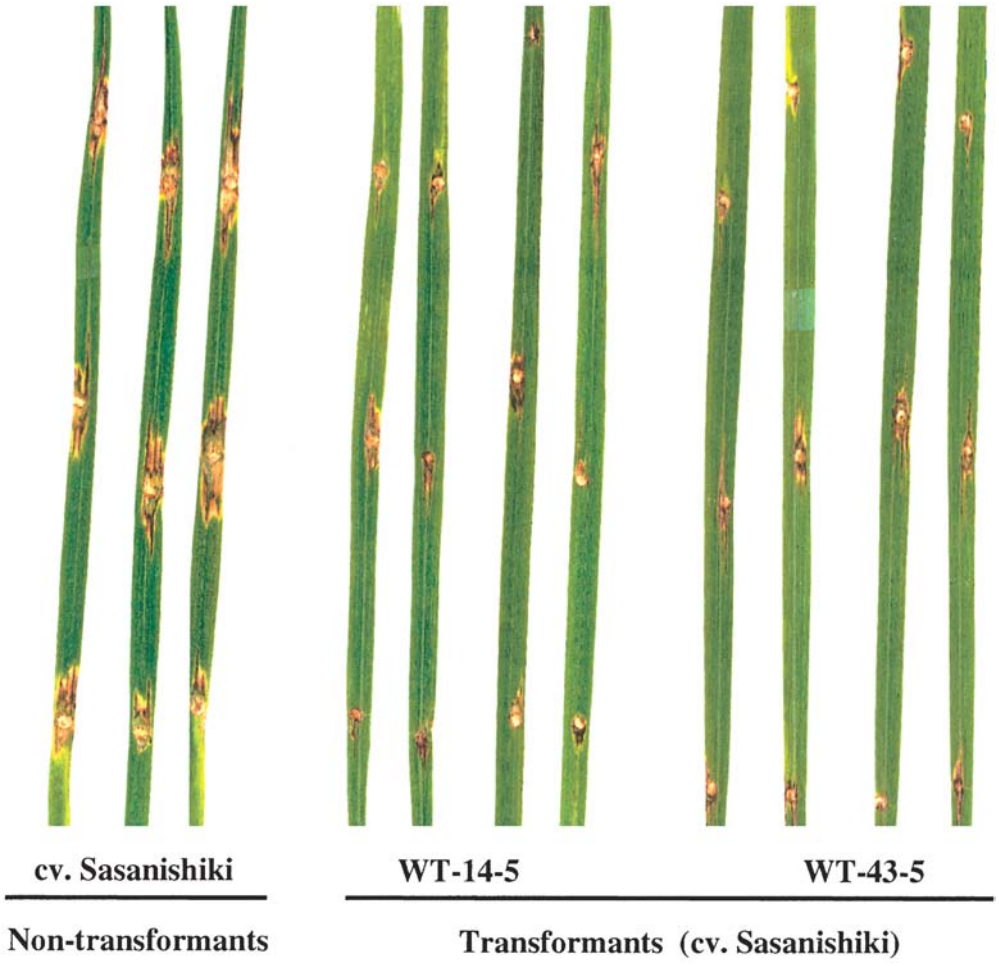

Fig. 6 Typical lesions on leaves of control and transgenic T3 plants inoculated with *M. grisea* (race 007.0), 14 days after inoculation

lesion sizes, disease resistance was also inferred by comparing the number of lesion spots between cv. Sasanishiki and transgenic lines WT14-5 and WT43-5, the latter two had about two-thirds (WT14-5) and one third (WT43-5) the number of lesion spots of non-transgenic cv. Sasanishiki (statistical significance: $P < 0.05$; Fig. 4C).

To investigate whether resistance to rice blast was inherited further to the T3 generation, T3 plants derived from the T2-resistant lines WT14-5 and WT43-5 were challenged with the blast fungus using press-injured spot methods. While the average size of the lesions of cv. Sasanishiki was 8.1 mm 10 days post-inoculation (dpi), those of transgenic plants were 4.1 mm for line WT14-5 and 4.0 mm for the line WT43-5 (Figs. 5, 6; Table 1). The average size of the lesions on transgenic line WT43-5 was about half of that of non-transgenic plants, although not as reduced as the lesion size of cv. Tsuyuake.

Molecular analysis of transgenic plants

Protein extracts of leaves from T3 transformants and non-transgenic controls were electrophoresed on SDS-PAGE, transferred to a PVDF membrane, and incubated with antiserum raised against wasabi defensin. The 5-

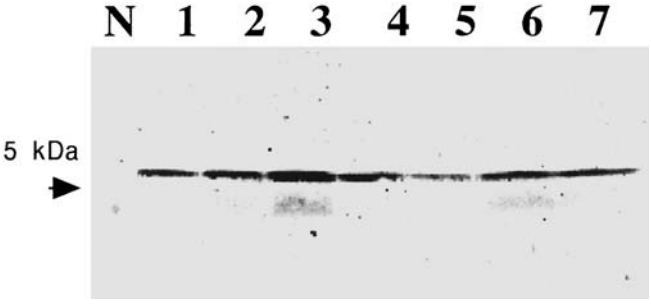

Fig. 7 Western blot analysis of defensin in leaves of T3 transgenic rice. *N* Non-transgenic rice plants, *lanes 1–3* T3 transgenic rice (WT14-5), *lanes 4–7* T3 transgenic rice (WT43-5)

Table 1 Mean lesion sizes[a] in control and transgenic rice plants (WT) after punch-injured infection with *M. grisea*, race 007.0 (*dpi* days post-inoculation)

Cultivar	Number of lesion spots	10 dpi	15 dpi
Sasanishiki	101	8.1 ± 0.4	11.1 ± 0.6
Tsuyuake	9	1.9 ± 0.3	2.7 ± 0.3
WT14-5	219	4.1 ± 0.2*	6.7 ± 0.3*
WT43-5	206	4.0 ± 0.2*	5.9 ± 0.3*

*$P < 0.001$

[a] Sizes expressed as a mean value ± standard error, in millimeters

kDa peptide, corresponding to the processed form of the wasabi defensin, was detected in the total protein fraction extracted from T3 progeny (Fig. 7). The levels of wasabi defensin expression were variable in the different plants.

Discussion

Plants have developed various defense mechanisms against environmental stresses like infection by foreign pathogens. Upon the perception of pathogens, a signal is transmitted downstream, and a number of antimicrobial proteins are expressed to fight off pathogens. Defensin, a protein belonging to the defensin family, is one of just such antimicrobial proteins. Low concentrations of wasabi defensin actually inhibited the growth of the rice blast fungus in vitro (Saitoh et al. 2001). Based on the SYTOX Green uptake assay, defensins seem to increase the permeability of fungal membranes (Thevissen et al. 1999). We used the maize ubiquitin 1 promoter to drive the expression of the defensin gene. This promoter is extremely strong in monocots (Christensen et al. 1992) and is expected to constitutively produce wasabi defensin in transgenic rice plants. In these plants, wasabi defensin should also inhibit the growth of fungal hyphae after their invasion of the epidermis cells.

Rice blast inoculation experiments revealed various levels of disease resistance in transgenic plants harboring the wasabi defensin gene (Fig. 4A). Different levels of blast resistance in T2 transformants may be caused by two factors. First, transcriptional or post-transcriptional silencing of the transgenes could have affected the level of disease resistance. The silencing of transgenes has been reported for many plant species. For transgenic rice plants harboring a chitinase gene, silencing of the transgene was reported in T2 and T3 progeny (Hart et al. 1992; Chareonporwattana et al. 1999). Second, a position effect in the genome may cause differences in transgene expression (Meyer et al. 1995).

The disease resistance of transgenic plant T3 progeny was enhanced about twofold relative to that of non-transgenic plants (Table 1). These transgenic plants were overproducing wasabi defensin proteins (Fig. 7). It is obvious that the overproduction of wasabi defensin was the causual factor for the enhancement of disease resistance, which had been transmitted to the T3 progeny.

Cultivar Tsuyuake with a resistance gene against race 007.0 is strongly resistant against this race (Figs. 4, 5, 6; Table 1). Two resistance genes, *Pi-b* against *M. grisea* (Wang et al. 1999) and *Xa21* against bacterial leaf blight disease (Song et al. 1995), have already been isolated from rice. Since pathogens can overcome true resistance in the field by mutations in the cognate avirulence genes, any transgenic resistance based on true resistance genes may easily break down. On the other hand, this problem probably does not occur in trangenic plants overexpressing antifungal genes. Therefore, the overproduction of the wasabi defensin peptide with antifungal activity is expected to confer durable resistance (i.e. field resistance) against a wide variety of rice blast races containing race 007.0. In addition, transgenic rice harboring the wasabi defensin gene may additionally have enhanced resistance against diseases caused by other microbes. It is already known that transgenic rice plants overexpressing chitinase have enhanced resistance against both sheath blight disease (Lin et al. 1995) as well as rice blast (Nishizawa et al. 1999).

Disease resistance of wasabi defensin transgenic rice progeny did not reach the disease resistance level of cv. Tsuyuake (carrying a true resistance gene to rice blast race 007.0). Since true resistance genes are located upstream of the signal transduction cascades of the defense system against pathogen infection, many defense genes are supposed to be activated in response to attacks by the rice blast in cv. Tsuyuake. To obtain a high level of resistance as observed in the R-gene mediated resistance, overexpression of multiple antifungal proteins with different functions may be necessary. The performance of tobacco plants co-expressing the barley transgenes, a class-II chitinase, a class-II β-1,3-glucanase and a type-I ribosome-inactivating protein, in a *Rhizoctonia solani* infection assay was reported to reveal significantly enhanced protection against fungal attack when compared with the protection levels obtained with corresponding isogenic lines expressing a single barley transgenes at a similar level (Jach et al. 1995). Transgenic plants harboring either other antimicrobial gene or multiple ones as well as the wasabi defensin gene, recombined by crossing of different transgenic plants, will inhibit the growth of *M. grisea* at an even higher level and with increased durability.

Acknowledgements We thank Dr. H. Uchimiya (Institute of Molecular Cellular Bioscience, University of Tokyo) for excellent advice on the research and Dr. G. Kahl (Biocentre, University of Frankfurt) for improving the manuscript. Thanks are extended to Ms. M. Fujiwara and Ms. E. Norita (Iwate Agricultural Research Center) for assistance in producing transgenic rice plants. We are grateful to Dr. S. Koizumi (Tohoku Agricultural Research Center, MAFF) for kindly supplying rice blast fungal races. We express our cordial gratitude to the late Mr. T. Tada (Iwate Agricultural Research Center), to whom this research owes much.

References

Broekaert WF, Terras FRG, Cammue BPA, Osborn RW (1995) Plant defensins: novel antimicrobial peptides as components of the host defense system. Plant Physiol 108:1353–1358

Chareonporwattana S, Thara KV, Wang L, Datta SK, Panbangred W, Muthukrishnan S (1999) Inheritance, expression, and silencing of a chitinase transgene in rice. Theor Appl Genet 98:371–378

Christensen AH, Sharrock RA, Quail PH (1992) Maize polyubiquitin genes: structure, thermal perturbation of expression and transcript splicing, and promoter activity following transfer to protoplasts by electroporation. Plant Mol Biol 18:675–689

Commue BPA, De Bolle MFC, Terras FRG, Proost P, Van Damme J, Rees SB, Vanderleyden J, Broekeart WF (1992) Analysis of two novel classes of plant antifungal proteins from radish (*Raphanus sativa* L.) seeds. J Biol Chem 267:2228–2233

Datta K, Velazhahan R, Oliva N, Ona I, Mew T, Khush GS, Muthukrishnan S, Datta SK (1999) Over-expression of the cloned rice thaumatin-like protein (PR-5) gene in transgenic rice plants enhances enviromental friendly resistance to *Rhizoctonia sonali* causing sheath blight disease. Theor Appl Genet 98:1138–1145

Edwards K, Johnstone C, Thompson C (1991) A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. Nucleic Acids Res 19:1349

Florack DEA, Stiekema WJ (1994) Thionins: properties, possible biological roles and mechanisms of action. Plant Mol Biol 26: 25–37

Hart CM, Fischer B, Neuhaus JM, Meins F Jr (1992) Regulated inactivation of homologous gene expression in transgenic *Nicotiana sylvestris* plants containing a defense-related tobacco chitinase gene. Mol Gen Genet 235:179–88

Hiei Y, Ohta S, Komari T, Kumashiro T (1994) Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. Plant J 6:271–82

Jach G, Görndhardt B, Mundy J, Logemann J, Pinsdorf E, Leah R, Schell J, Maas C (1995) Enhanced quantitative resistance against fungal disease by combinatorial expression of different barley antifungal proteins in transgenic tobacco. Plant J 8:97–109

Kawasaki T, Henmi K, Ono E, Htakeyama S, Iwano M, Satoh H, Shimamoto K (1999) The small GTP-binding protein Rac is a regulator of cell death in plants. Proc Natl Acad Sci USA 96:10,922–10,926

Lin W, Anuratha CS, Datta K, Potrykus I, Muthukrishnan S, Datta K (1995) Genetic engineering of rice for resistance to sheath bright. Biotechnology 13:686–691

Matsudaira P (1987) Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. J Biol Chem 262:10,035–10,038

Meyer P (1995) Understanding and controlling transgene expression. Trends Biotechnol 13:332–337

Nishizawa Y, Nishio Z, Nakazawa K, Soma M, Nakazawa E, Ugaki M, Hibi T (1999) Enhanced resistance to blast (*Magnaporthe grisea*) in transgenic Japonica rice by constitutive expression of rice chitinase. Theor Appl Genet 99:383–390

Saitoh H, Kiba A, Nishihara M, Yamamura Y, Suzuki K, Terauchi R (2001) Production of antimicrobial defensin in *Nicotiana benthamiana* with a potato virus X vector. MPMI 14:111–115

Song WY, Wang GL, Chen LL, Kim HS, Pi LY, Holsten T, Gardner J, Wang B, Zhai WX, Zhu LH (1995) A receptor kinase-like protein encoded by the rice disease resistance gene, *Xa21*. Science 270:1804–6

Stark-Lorenzen P, Nelke B, HanBler G, Muhlbach HP, Thomzik JE (1997) Transfer of a grapevine stilbene synthase gene to rice (*Oryza sativa* L). Plant Cell Rep 16:668–673

Shure M, Wessler S, Fedoroff N (1983) Molecular identification and isolation of the *waxy* locus in maize. Cell 35:225–233

Thevissen K, Terras FRG, Broekaert WF (1999) Permeabilization of fungal membranes by plant defensins inhibits fungal growth. Appl Environ Microbiol 65:5451–5458

Wang ZX, Yano M, Yamanouchi U, Iwamoto M, Monna L, Hayasaka H, Katayose Y, Sasaki T (1999) The *Pib* gene for rice blast resistance belongs to the nucleotide binding and leucine-rich repeat class of plant disease resistance genes. Plant J 19:55–64