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Transgenic *indica* rice expressing a bitter melon (*Momordica charantia*) class I chitinase gene (*McCHIT1*) confers enhanced resistance to *Magnaporthe grisea* and *Rhizoctonia solani*

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Abstract McCHIT1 chitinase (DQ407723), a class I secretory endochitinase from bitter melon (*Momordica charantia),* had been demonstrated to enhance resistance against *Phytophthora nicotianae* and *Verticillium* wilt in transgenic tobacco and cotton. In order to obtain disease-resistant transgenic rice, *McCHIT1* was transformed into a restorer line JinHui35 (*Oryza sativa* subsp. *indica*) by using the herbicide-resistance gene *Bar* as the selection marker. Transgenic rice lines and their progenies overexpressing the *McCHIT1* gene showed enhanced resistance to *Magnaporthe grisea* (rice blast) and *Rhizoctonia solani* (sheath blight), two major fungal pathogens of rice. *McCHIT1*-transgenic rice confirmed the inheritance of the transgene and disease resistance to the subsequent generation. The T_2 transformants exhibited significantly increased tolerance to *M. grisea*, with a 30.0 to 85.7 reduction in disease index, and *R. solani*, with a 25.0 to 43.0 reduction in disease index, based on that of the control as 100. These results indicated that over-expression of the *McCHIT1* gene could lead to partial disease reduction against these two important pathogens in transgenic rice.

Keywords Disease resistance · *Indica* · *McCHIT1* gene · Blast · Sheath blight

Abbreviations

AS	acetosyringone
CaMV35S	*Cauliflower mosaic virus* 35S promoter
CIAP	calf intestinal alkaline phosphatase
GlcNAc	N-acetylglucosamine
GLU	glucanase
GUS	β-glucuronidase
NPR1	non-expressor of pathogenesis-related genes 1
PAL	phenylalanine ammonia lyase
PMSF	pheylmethylsulfonyl fluoride
PPT	phosphinothricin
PR	pathogenesis-related
SAR	systemic acquired resistance

Introduction

Blast and sheath blight, seriously affecting yield and quality of rice (*Oryza sativa*) worldwide, are caused by

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the fungal pathogens *Magnaporthe grisea* and *Rhizoctonia solani* respectively. Usually the control of these fungal pathogens mainly involves three strategies: culture technique, the application of agrochemicals and breeding of resistant cultivars. Incidence of plant diseases has been controlled by crop rotation and excellent culture technique to some extent. However, Campbell et al. (2002) reported that planting and harvesting a field planted with diverse germplasm are not always practical in some crops, and the application of agrochemicals not only poses many potential risks that include harmful effects on the ecosystem and human health, but their abuse can reduce the efficiency of fungicides due to the evolution of tolerant pathogens. Conventional breeding of resistant cultivars is a major method to control fungal disease, but it is time-consuming and not effective enough for taxonomically-related species which have no effective sources of disease resistance. Genetic engineering can contribute to the agronomic improvement of crops in terms of disease resistance as a supplement to traditional breeding methods, and will break fertility barriers by inserting exogenous antimicrobial genes from different species to engineer increased disease resistance.

Genetic engineering using *R* genes is an economical approach to increase disease resistance (Campbell et al. 2002), and most breeders have utilised *R* genes into all improved lines in rice. Recently >70 major blast resistance genes have been identified, and rice blast *R* genes *Pi-b*, *Pita*, *Pi2, Piz-t, Pi36*, *Pi9* and *Pi-d2* (previously named *Pi-d(t)2*) have been isolated (Chen et al. 2006; Liu et al. 2007). However, cloning of *R* genes for sheath blight is lagging behind, because genetic variability for high levels of disease resistance against sheath blight is lacking in both cultivated rice and wild relatives (Song and Goodman 2001). So far, only one locus has been mapped on chromosome 5 of rice (Che et al. 2003). An alternate promising choice for engineering broad-spectrum resistance is to introduce various pathogenesis-related (PR) protein genes in plants. There have been numerous reports that over-expression of PR genes in transgenic plants showed increased disease resistance (Gurr and Rushton 2005). Various PR genes containing PR-2 (β-1, 3-glucanases), PR-3 (chitinases), PR-5 (thaumatin-like proteins) and PR-6 (protease inhibitors) have been demonstrated to confer resistance against fungal pathogens in rice (Muthukrishnan et al. 2001). Especially, there are a number of reports showing that constitutive expression of chitinase genes gave a significantly improved disease resistance in rice defence response (Datta et al. 2000, 2001; Itoh et al. 2003; Kim et al. 2003; Kumar et al. 2003; Lin et al. 1995; Nishizawa et al. 1999). However, the protective effects are not limited to rice chitinase genes, and over-expression of heterologous chitinase genes has also conferred disease resistance in transgenic rice (Ghareyazie et al. 2001; Itoh et al. 2003). Honée (1999) demonstrated that tolerant plants with over-expression of genes from organisms other than the plant itself, usually rely on the activation of a whole array of defence responses by activating the HR response. Therefore, the heterologous chitinases with broad-spectrum antifungal activity may be more promising candidates in rice disease-resistant genetic engineering.

Bitter melon (*Momordica charantia*), which has high chitinolytic activities in leaves, is highly tolerant to various pathogens. The overexpression of the *McCHIT1* chitinase gene from bitter melon in tobacco and cotton enhanced resistance against *Phytophthora nicotianae* and *Verticillium* wilt, respectively (Pei et al. 1993; Xiao et al. 2007). With the aim of producing resistant transgenic rice, we transformed *McCHIT1* into a restorer line JinHui35 (*O. sativa* subsp. *indica*) via *Agrobacterium tumefaciens* strain EHA105, and disease resistance to blast and sheath blight in *McCHIT1*- transgenic rice plants were assessed.

Materials and methods

Vector construction and rice transformation

Binary vector pCAMBIA1305.1 was used as the backbone to construct the expression vector of *McCHIT1* coding the region driven by the maize *ubiquitin1* promoter. The vector also harbours expression cassettes of the *Bar* gene as a selectable marker and the *β-glucuronidase* (*GUS*) gene as the transformation reporter. The *Bar* (550-bp) gene restriction fragment took the place of the *hygromycin* gene of pCAMBIA1305.1 plasmid to obtain pCAM-2x35S-*Bar*. The 1500-bp *ubiquitin1* promoter *Hin*dIII/*Nco*I fragment was inserted into the corresponding sites of the pCAM-2x35S-*Bar* to obtain the new plasmid pCAM-*Bar*-*Gus*, while the *McCHIT1 Sal*I/*Sac*I fragment (942-bp cDNA) was constructed to form the expression cassettes UbiPro-*McCHIT1*-Nos. Then

about 2.7-kb of the *McCHIT1* expression cassette (*Hin*dIII/*Eco*RI) was inserted into pCAM-*Bar*-*Gus* to form the recombinant vector pCAM-*McCHIT1*. pCAM-*McCHIT1* was then transformed into *A. tumefaciens* strain EHA105 by electroporation for rice transformation.

The seeds of cv. JinHui35 from an immature panicle, 10–20 days after anthesis, were dehulled, sterilised with 75% ethanol for 1–2 min and 2.5% NaOC1 for 30 min (or 0.1% $HgCl_2$ for 20–25 min), and washed with sterile deionised water five times. NMB medium, which contained N6 macronutrient salts (Chu et al. 1975), B_5 vitamins (Gamborg et al. 1968), MS micronutrient components (Murashige and Skoog 1962), 3% sucrose, 500 mg l^{-1} casein hydrolysate, and 3 g l^{-1} gelrite, were used as the basal medium. Immature embryos were excised aseptically, placed on NMB supplemented with 500 mg l^{-1} glutamine, 500 mg l^{-1} proline, 2 mg l^{-1} 2,4-D, pH 5.8, and cultured at 25°C under 16 h light/8 h dark. The induced calli with shiny, nodular, compact structures were subcultured on the same medium for 4 days before *Agrobacterium* inoculation. The transformation and differentiation of the calli were carried out as described by Hiei et al. (1994) with minor modifications. The EHA105 engineering strain was cultured in YEB liquid medium supplemented with 50 mg l^{-1} kanamycin on a shaker at 200 rpm and 28ºC until OD_{600}=0.8–1.0.

The bacterial suspension was prepared by centrifuging at 1000×*g* for 10 min, and the pellet was resuspended in AAM medium (Toriyama and Hinata 1985) supplemented with 200 μM AS and diluted to OD_{600}=0.5–0.6. After 30 min of incubation for infection, the liquid was removed by pipettes, and the calli blot-dried. The infected calli were transferred to co-cultivation medium (NMB basal medium supplemented with 10 g l^{-1} glucose, 2 mg l^{-1} 2,4-D and 200 μM AS, pH 5.3, layered with Whatman No. 1 filter paper), and incubated at 26°C in the dark for 3 days. Subsequently, the calli were washed in sterile water supplemented with 500 mg l^{-1} cefotaxime, and transferred to a selection medium (NMB supplemented with 2 mg l^{-1} 2,4-D, 10–20 mg l^{-1} PPT and 500 mg l^{-1} cefotaxime) every 14–20 days for about 2 months. PPT-resistant calli were transferred to pre-regeneration medium (MSB medium: MS salts, B5 vitamins, 40 g l^{-1} sorbitol, 3 mg l^{-1} 6-BA, 1.0 mg l^{-1} KT, 1.0 mg l^{-1} NAA, 0.2 mg l^{-1} IAA, 0.5–2 mg l^{-1} PPT and 200 mg l^{-1} cefotaxime) for about 7–14 days, and the calli were transferred to regeneration medium which was the same as the pre-regeneration medium but sorbitol-deprived. Shoot regeneration was observed after 2–4 weeks. For rooting, regenerated shoots were transferred to 1/2 MSB medium, which contained 1/2 MS salts, 1/2 B5 vitamins, 3% sucrose, 0.5 mg l^{-1} NAA or 0.5 mg l^{-1} IBA, 3 g l^{-1} gelrite. After acclimatisation, the plantlets were transplanted into pots in a greenhouse.

Assay for β-glucuronidase (GUS) activity

The histochemical assay for *GUS* gene expression was performed according to the method of Jefferson (1987), using 5-bromo-4-chloro-3-indolyl glucuronide (X-gluc) as the substrate. An incubation temperature of 37°C was used.

Analysis of polymerase chain reaction (PCR)

Genomic DNA was isolated from leaves of rice plants by a modified CTAB method (Dellaporta et al. 1983). The T_2 plants were determined further by PCR amplification of a 300-bp *McCHIT1* fragment using upstream primer 5'-GACGTTGGCAGGATCATCAC-3' and downstream primer 5'-GCCATTGTTGGTTGGGTGA-3'. The 25-μl PCR mixture contained 1 μl DNA template (about 50 ng), 1× PCR buffer, 1.5 mM $MgCl_2$, 200 μM dNTPs, 0.2 μM of each primer and 1U *Taq* DNA polymerase. The reactions were carried out on a PTC-100 Peltier® Thermal cycler (BIO-RAD) with the following protocol: pre-incubation at 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, 58°C for 40 s and 72°C for 1 min, and at 72°C for 10 min. PCR products were analysed on 1.5% agarose gels by electrophoresis.

Sheath blight bioassay

Highly virulent *R. solani* strain RH-9 was a gift of Prof. Xuebiao Pan, Yangzhou University, China. RH-9 was cultured on potato dextrose agar (PDA) at 28°C for about 3 days, and mycelial-PDA discs of 5 mm diam were used for inoculation.

T_0 transformants were used for preliminary identification. Leaves from T_0 transgenic lines and control plants were cut into strips of about 20 cm in length, and placed on wet filters at 28°C. Three mycelial-

PDA discs were put onto each leaf. The percentages of infected leaf area were investigated 3 days later (Kalpana et al. 2006).

T_1 seeds from T_0 transformants were de-husked, sterilised and placed on solid 1/2 MSB medium without any hormone. About 2 weeks later, GUS-positive seedlings were transplanted into $1 \times 0.5 \times 0.2$ m^3 plastic boxes for growing for about 5–6 weeks. GUS-negative plants and non-transgenic plants were used as controls. The plants were inoculated with RH-9 using the method reported earlier (Kalpana et al. 2006; Kumar et al. 2003) with slight modifications. The mycelial-PDA disc was fixed on the healthy leaf sheath with a toothpick, and the infected plants were kept at 90% relative humidity (RH). After 14 days of inoculation, disease development was estimated on the basis of a five-class disease severity scale (Jach et al. 1995), and the relative disease intensity was expressed as a relative disease index (DI), i.e., $DI=\sum (N \times R) / (M \times T) \times 100$ (N: number of tillers showing the same grade of infection; R: relative disease grade; M: maximum grade; T: total number of tillers inoculated) (Inger 1996).

Rice blast bioassay

The *M. grisea* strain Y-6-2-1 for bioassay of T_1 transgenic plants was provided by Daihua Lu, Sichuan Academy of Agricultural Sciences, China. *Magnaporthe grisea* races for identification of T_2 transgenic plants were provided by Rong Xie, Rice Research Institute, Luzhou, China. Each strain was grown on rice bran agar plates (20 g l^{-1} rice bran and 15 g l^{-1} agar) at 28°C for 5–7 days, and inoculated on to sterile sorghum seeds until the seed surface was covered with the mycelia. After washing off excessive mycelia, sorghum seeds were laid on an enamel tray, covered with wet gauze, and maintained at 22–25°C in the dark for 3 days. Fungal spores were washed off the sorghum seeds, and debris discarded by filtering the suspension. The spore concentration was calculated by a Bürker counting chamber after microscopic examination and adjusted to 3×10^5 spores ml^{-1}, supplemented with 0.05% Tween 80 and 2 g l^{-1} gelatin.

T_0 transformants were used for preliminary identification through resistance of detached leaves against *M. grisea* according to Kanzaki et al. (2002) with minor modifications. Bioassay for *M. grisea* resistance was carried out as described by Schaffrath et al. (2000) with some modifications. Transgenic, non-transgenic and GUS-negative plants at the 3–4 leaf stage were sprayed with *M. grisea* conidial suspension until the leaf surface was covered with water droplets. The plants were maintained at 25–28°C with a RH of 90% for 10–14 days, and the symptoms investigated. The lesions were scored based on a lesion size scale of 0–9 grades described by the International Rice Research Institute (IRRI, Manila, Philippines) (Inger 1996). The relative DI was calculated as described above.

Transcription levels of *McCHIT1* in transgenic plants

Total RNA was isolated from leaves of two month-old T_1 progenies inoculated with *R. solani* by the method of Chomczynski and Sacchi (1987). The *McCHIT1* transcription levels were determined by RT-PCR analysis using the same primer pair as described above. The 147-bp *ACTIN* fragment of rice was amplified as the internal control using suitable primers (upstream primer: 5'-TATGG TCAAGGCTGGGTTCG-3'; downstream primer: 5'-CCATGCTCGATGGGGTACTT-3').

Chitinase activity in *McCHIT1*-transgenic plants

Leaves of six T_1 GUS-positive transgenic lines and control plants were sampled for measuring total chitinase activity. Crude protein extracts were prepared by grinding 1 g of leaves in liquid nitrogen and extracting the fine powder with 2 ml of buffer (0.05 M NaAc, pH 5.0, 100 μM PMSF) for 1 h (Neuhaus et al. 1991). After centrifugation at 15,000 *g* for 15 min, the supernatant was collected for enzyme assays. The concentration of total soluble protein was determined by employing the method of Bradford (1976) with bovine serum albumin (BSA) as a standard.

The chitinase activity of crude protein was analysed by the method of Mauch et al. (1984) using colloid chitin as the substrate. The colloid chitin was prepared as described by Shimahara and Takiguchi (1988). A unit of chitinase activity was defined as the amount of enzyme required for releasing 1 μM N-acetylglucosamine (GlcNAc) in 1 h using a wavelength of 540 nm.

Results

Rice transformation and identification of transgene

The construction of plasmid vector pCAM-*McCHIT1* is shown in Fig. 1. Embryogenic calli were induced from immature seeds of *indica* rice cv. JinHui35. A total of 180 clumps of scutellum-derived calli were transformed. About 2 months later, PPT-resistant calli were transferred to PPT-free medium for regeneration. Low concentrations of PPT, even at the 0.5 mg l^{-1} level, would seriously influence the regeneration of PPT-resistant calli (data not shown). The 500 regenerated T_0 plantlets were analysed by histochemical GUS staining, and 45 independent GUS-positive plantlets were transplanted to a greenhouse. All the plantlets were fertile, and their fertility rates were lower than those of the non-transgenic plants in the greenhouse; however, the seed setting rate of most T_1 progenies showed no difference from the control in field cultivations. In addition, a total of 14 tested T_0 transformants was consistent with the 3:1 segregation ratio in the T_1 generation by the x^2 test according to GUS staining. Data from the PCR analysis of the *McCHIT1* gene completely agreed with the GUS enzyme assay data (data not shown), which indicated that the *McCHIT1* gene in the selected 14 transgenes was inherited as a single-copy Mendelian trait.

Enhanced resistance to *R. solani* in *McCHIT1*-transgenic plants

The 45 T_0 independent transformants (GUS^+) were preliminarily screened for resistance by inoculating excised leaves with *R. solani*, and symptoms were recorded after 3 days. Twenty of the 45 transgenic lines showed reductions in disease severity against *R. solani*, compared to the control (GUS^-). However, the reductions were not seen after 5 days. T_1 progenies from six T_0 lines with single-copy insertion were selected for further bioassay of sheath blight; 45 day-old T_1 progenies in the growth chamber were inoculated with *R. solani* (10 GUS-positive seedlings per line, 10 GUS-negative and 10 non-transgenic plants were controls, with three replicates). Two weeks later, the percentages of infected sheath area were recorded, and disease indices were calculated. As shown in Fig. 2, GUS-negative plants showed no significant difference in disease index from the non-transgenic plants. All six lines showed fewer numbers and smaller sizes of infection cushions than the controls in the infection assay, respectively. Disease indices of six transformants ranged from 66.0 to 76.9 relative to the control as 100. T_1 progenies from a transformant showed segregation in disease resistance, exhibiting different disease resistance levels. T_2 progenies from six promising T_1 lines were challenged with *R. solani* for further identification. A reduced blighting level was observed in six tested T_2 transformants inoculated with *RH-7* compared to that in the control plants (Fig. 3). T_2 *McCHIT1*-transgenic rice plants demonstrated increased resistance to *R. solani* with a reduction of 25.0–43.0 in disease indices as compared with the control plants. And C22 and C24 with disease indices of 40.0 and 43.0 respectively showed promising resistance among transgenes (Fig. 2). Although variations in resistance

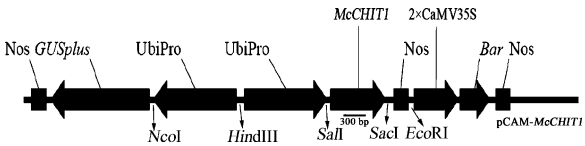

Fig. 1 Structure of transfer DNA (T-DNA) region of the transformation plasmid. The transverse line indicated the PCR-amplified regions (300 bp), used to confirm the existence of the McCHIT1 gene in the progenies from transgenes. GUSplus: β-glucuronidase gene from pCambia1305.1. McCHIT1: coding region of McCHIT1 chitinase gene (945 bp). UbiPro: the maize ubiqutin1 promoter. Nos: terminator of the nopaline synthase gene

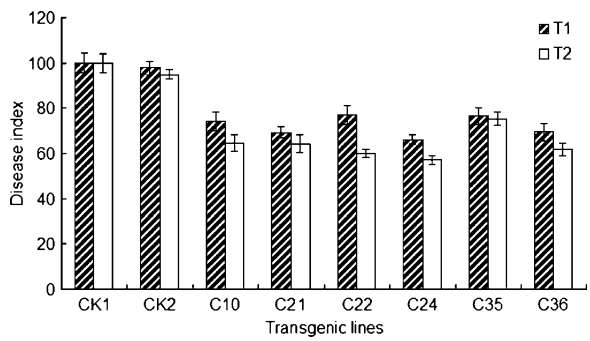

Fig. 2 Increased sheath blight resistance in McCHIT1-transgenic rice plants. All experiments were carried out at different times in the same greenhouse. Development of symptoms was observed 14 days after infection with Rhizoctonia solani. Data shown represent mean ± standard error of three independent experiments. T1: Disease indices of T1 transgenic lines *in planta* inoculation assays. T2: Disease indices of T2 transgenic lines *in planta* inoculation assays. CK1: non-transgenic plants; CK2: GUS-negative plants

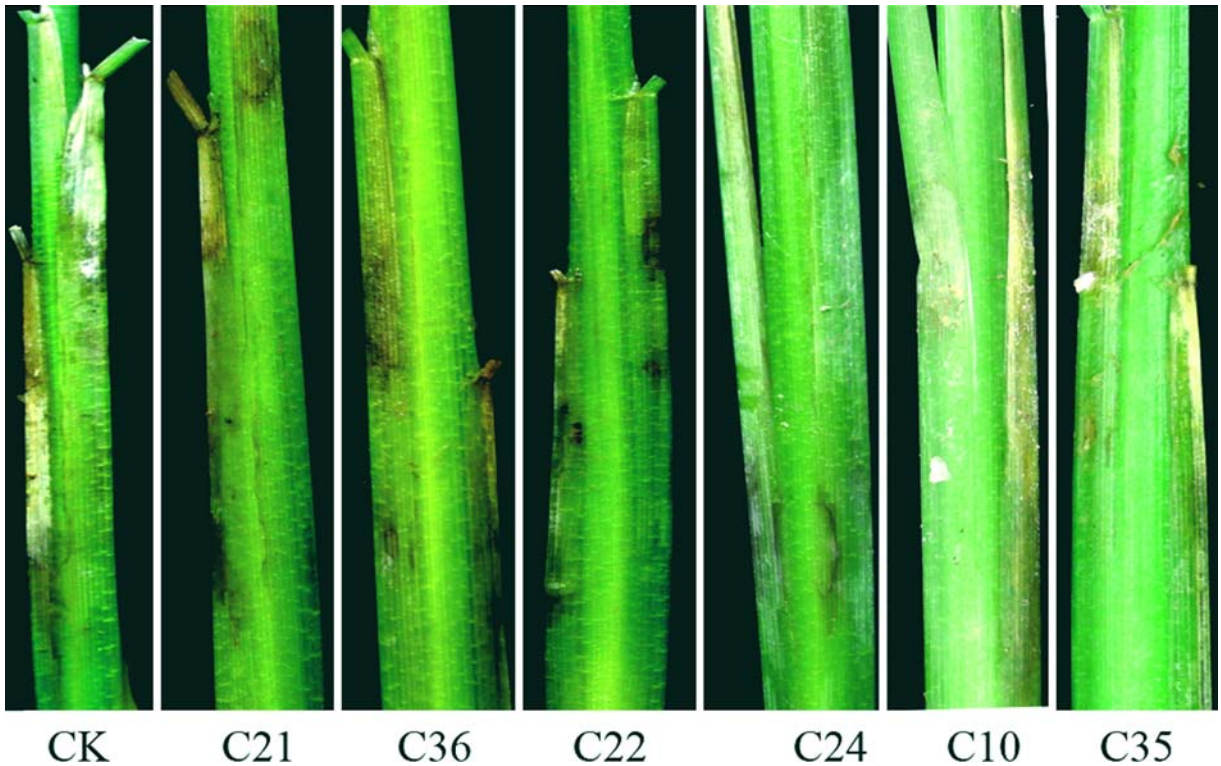

Fig. 3 Bioassay of T2 transgenic individual plants for leaf sheath resistance 14 days after infection with Rhizoctonia solani. CK: untransformed plant

existed among individual transformants in both T_1 and T_2 bioassays, disease resistance of all tested transgenic lines was significantly higher than the control plants. These results indicated to some extent that *McCHIT1*- transgenic rice resulted in apparent increased tolerance to *R. solani*.

Enhanced resistance to *M. grisea* in *McCHIT1*-transgenic plants

Detached leaves of T_0 transformants were inoculated with *M. grisea* to evaluate their resistance to rice blast. The same six T_0 lines also showed reduced susceptibility to blast caused by *M. grisea*, and the six promising T_0 transformants were propagated for further bioassay in T_1 and T_2 generations challenged with *M. grisea*. Similarly, 10 T_1 GUS-positive plants of each transformant were inoculated (GUS-negative and non-transgenic plants as controls, three independent replications) with *M. grisea*. All tested T_1 transgenes resulted in reductions in disease severity compared with the control plants, and most transgenic plants showed type 3–6 lesions in the infection assay (Fig. 4), while the disease severity of control plants developed type 7–9. Disease indices of six promising T_1 lines (C10, C21, C22, C24, C35 and C36) were reduced by 34.4, 36.5, 27.8, 29.0, 69.0 and 40.0, respectively compared with the control plants. In contrast, no difference in susceptibility of GUS-negative plants compared to wild-type plants was observed after inoculation with the pathogen *M.*

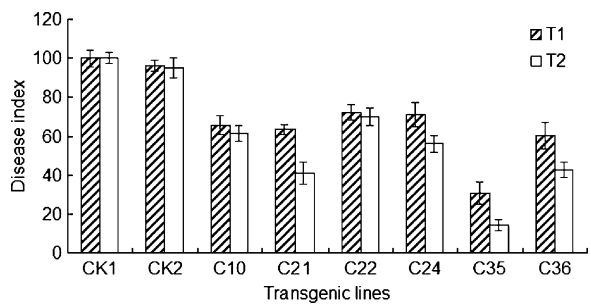

Fig. 4 Enhanced rice blast resistance in McCHIT1-transgenic rice plants. Development of symptoms was scored 14 days after infection with Magnaporthe grisea. Data shown represent mean ± standard error of three independent experiments. CK1: non-transgenic plants; CK2: GUS-negative plants

grisea. T_2 progenies, from six promising individual T_1 lines in the bioassay of blast resistance, were subjected to a bioassay of blast resistance challenged with mixed races of *M. grisea* spores. The control plants showed typical susceptible-type lesions after 10 days, whereas most transgenic plants showed lesions of only type 1–5, which were smaller than those of the control and surrounded by a conspicuous necrotic circle (Fig. 5). Six tested transformants consistently showed enhanced resistance to more than one *M. grisea* race, and exhibited lower disease indices compared to control plants (Fig. 4). Different disease severities were also shown among different transgenes. Moreover, in the bioassay, the lines C36, C21 and C35 showed high levels of resistance against mixed races of *M. grisea* spores. The line C35 had a disease grade of 1 and a disease index of 14.3. Lines C36 and C21 also decreased by 57.0 and 59.0 in the disease index, compared with the control plants. For both the T_1 and T_2 infection assays, the tested transformants showed higher resistance than the control plants. These observations indicated over-expression of *McCHIT1* in transgenic rice-improved resistance to *M. grisea*.

In addition, to obtain genetically homogeneous material, seeds from six individual T_1 lines were inoculated with *M. grisea*. Scored 10 days later, the results showed C36 and C35 T_1 were homozygous transgenic lines due to no segregation of disease resistance among 20 T_2 progenies tested. PCR assay was carried out on genomic DNA of tested T_2 disease-resistant plants after inoculation of *M. grisea* using the *McCHIT1*-specific primers, and the expected 300-bp *McCHIT1* PCR product was obtained in 95% of the disease-resistant T_2 plants. The results also suggested both the *McCHIT1* gene and disease resistance were stably inherited from T_0 to T_2 generations.

McCHIT1 transcription levels and chitinase activity in transgenic rice

Six T_1 promising individual inoculated transgenes were chosen to determine the *McCHIT1* transcription expression levels by RT-PCR analysis. As shown in Fig. 6, the results showed expression of the *McCHIT1* gene in six selected plants, and differences of *McCHIT1* expression levels existed among various transgenes. The individual plants C22, C24 and C36 showing the higher expression level exhibited significantly reduced disease areas, as compared to those with much lower *McCHIT1* expression levels (C10, C21 and C35) in the T_1 generation. Their higher disease resistance (C22, C24 and C36) to sheath blight was stably transmitted to T_2 generations. However, line C22 had the highest level of gene expression, but not the highest resistance to *R. solani*; line C24 with a moderately *McCHIT1* expression level had the highest disease resistance.

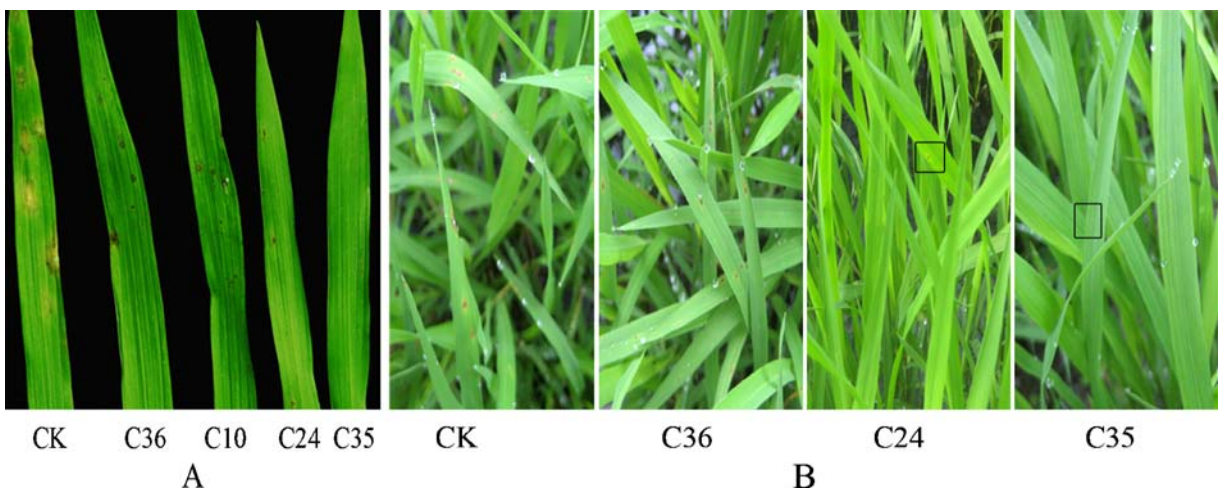

Fig. 5 Bioassay of T2 McCHIT1-transgenic lines for rice blast resistance 14 days after inoculation with Magnaporthe grisea. CK: untransformed plant. A: symptoms on the detached leaves of control and transgenic rice plants inoculated with M. grisea. B: symptoms of control and transgenic lines inoculated with M. grisea *in planta* inoculation assays. The square inserted in photograph C24 and C35 shows the disease spot of rice blast in C24 and C35 transgenic lines

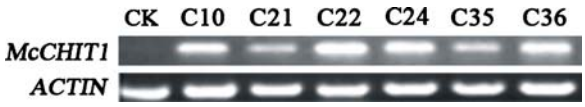

Fig. 6 RT-PCR analysis of the McCHIT1 gene in T1 McCHIT1-transgenic individual plants challenged with *Rhizoctonia solani*

To determine the relationship between the *McCHIT1* expression levels and disease resistance, total chitinase activity of six promising uninoculated and inoculated T_1 individual transgenic plants inoculated with *R. solani* was evaluated indirectly. Disease resistance against *R. solani* was determined after 14 days by relative diseased leaf area (the percentage of the diseased leaf sheath area on transgenic plants relative to that on the control plant). As shown in Fig. 7, the level of chitinase activities significantly increased in various uninoculated transgenic lines, as compared with the uninoculated control plant. Moreover, differences in chitinase activities existed among various transgenic lines. In contrast, the chitinase activities of the GUS-negative plant were not significantly different from the non-transgenic plant. On the other hand, the chitinase enzyme activities in all tested plants were elevated after infection with *R. solani* compared to the healthy plants. There was no significant difference in chitinase activity of healthy and infected transgenic lines ($P>0.05$). Similarly, in all infected plants, the chitinase enzyme activities in all transgenes were higher than that in control plants. Chitinase activity values of most tested transgenic lines were 2–4 fold those of the control plant, and the relative diseased leaf sheath area and chitinase activity were negatively correlated ($R^2=0.934$) (Fig. 8), indicating that chitinase activities were correlated with disease resistance to leaf sheath blight within a certain range.

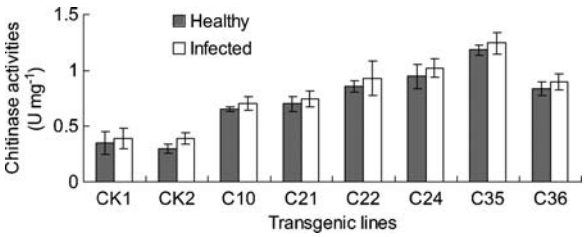

Fig. 7 Chitinase activities of uninoculated and inoculated transgenic plants challenged with *Rhizoctonia solani* in three independent experiments. The chitinase activities of the transgenic lines shown were all significantly different from the control ($P<0.05$). There was no significant difference in chitinase activity of healthy and infected transgenic lines ($P>0.05$). CK1: untransformed rice; CK2: GUS-negative plants

Discussion

Rice is one of the world's most important cereal crops, providing food for more than a half of the global population. One hundred and fifty-seven million tons of rice have been lost to rice blast disease from 1975 to 1990, a figure that equals 11 to 30% of global rice production (Baker et al. 1997). The yield losses have ranged from 8 to 50% due to sheath blight, particularly when the infection is well distributed and severe in the field (Savary and Mew 1996). Therefore, it is significantly important to produce disease-tolerant rice cultivars. One alternative to increased resistance is the insertion and expression of antifungal genes.

It is well known that plant chitinases are important PR proteins, which have been shown to confer broad-spectrum disease resistance in plant genetic engineering. Previous studies showed that transgenic rice plants over-expressing Class I *chitinase* gene conferred resistance to both sheath blight (Lin et al. 1995) and rice blast (Nishizawa et al. 1999). Lin et al. (1995) reported that the development of the symptoms was considerably slower in *Chi-11* transgenic rice, and the number and

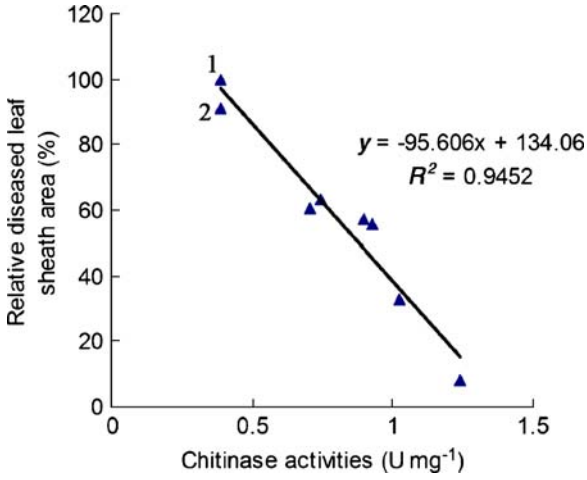

Fig. 8 Correlation between chitinase activities and relative diseased leaf sheath area. (Relative diseased leaf area: the percentage of diseased leaf sheath area on transgenic plants relative to that on non-transgenic plant.) 1: untransformed rice; 2: GUS-negative plants. The chitinase activity values shown represent those of inoculated transgenic plants

size of lesions and infected areas of the leaf sheath were smaller than those of the control. Nishizawa et al. (1999) found that transgenic rice plants expressing *Cht-2* and *Cht-3* showed significantly higher resistance to *M. grisea* than non-transgenic plants. Datta et al. (2001) also showed that the IR72 *indica* rice expressing rice chitinase gene *RC7* exhibited various levels of enhanced resistance to *R. solani*. Indeed, the over-expression of *McCHIT1* was proven to confer resistance to *Phytophthora nicotianae* and *Verticillium* wilt in transgenic tobacco and cotton (Xiao et al. 2007). In the present study, The *McCHIT1*-transgenic rice exhibited an increased resistance to *R. solani* and *M. grisea* in both T_1 and T_2 generations. T_2 *McCHIT1*-transgenic rice plants demonstrated resistance to *R. solani* with a reduction of 25.0–43.0 in disease indices compared with the controls (Fig. 2), while all the tested transformants also exhibited increased resistance to *M. grisea* with a significantly lower number of blasts and smaller blast size than non-transgenic plants (Fig. 5). The tested transgenes exhibited resistance to rice blast with a reduction of 30.0–85.7 in disease indices, compared to that of the controls (Fig. 4). In addition, the resistance conferred by *McCHIT1* was not specific to a particular race of rice blast fungus in our study. These results indicated that *McCHIT1* conferred a broad-spectrum fungal resistance in plants.

As presented in this research, the levels of *McCHIT1* expression were found to contribute to increased fungal resistance to a certain extent. However, not all *McCHIT1*-transgenic plants exhibited the desired enhanced disease resistance, and it was concluded that either the transcriptional and post-transcriptional silencing of transformants might affect the levels of disease resistance (Anand et al. 2003) or the insertion of a transgene may result in suppression (Yang et al. 2008) Moreover, the line C35 showed promising resistance to blast with a disease index reduction of 85.7 in T_2 bioassay, but it did not show desirable resistance to sheath blight. Wally et al. (2009) showed the amount of chitinase produced and the proportion of chitin present in different fungal cell walls may account for the different disease reduction values. On the other hand, the line C22 with the highest *McCHIT1* transcript level of any of the other lines had the third highest chitinase activities, which showed *McCHIT1* transcription levels were inconsistent with the level of chitinase enzyme found in a few lines. There was also some variation between the activity levels of the *McCHIT1* chitinase enzyme and the degree of fungal resistance in a few particular lines. The line C35 with the highest chitinase enzyme activities did not exhibit desirable disease resistance. The amount of transgenic protein may account for this phenomenon. Shrestha et al. (2007) demonstrated that chitinase activity is associated with moderate resistance of rice cultivars against sheath blight. In addition, the reason might be the position effect of target gene insertion that resulted in different expression levels of *McCHIT1* and other antifungal proteins (Meyer 1995). Another reason may be the SAR-related components induced by the over-expression of McCHIT1 and the pathogen components in the plant that can be recognised by plants, which would lead to the activation of defence response sufficient against potential pathogen attack. Furthermore, considering the complicated signal transduction cascades of the defence system against pathogen inoculation, it is necessary to further estimate the degree of resistance to different races of *R. solani* and *M. grisea* in future work. It will also be very important to investigate whether T_3 and subsequent generations still retain high *McCHIT1* expression levels and enhanced disease resistance.

Obviously, *McCHIT1* contributed apparent resistance against rice important pathogens, but the defence was insufficient to protect plants completely. Disease resistance is a complex trait controlled by several groups of genes, so it was expected that epigenetic expression of a single PR-protein gene like *McCHIT1* could not be expected to confer sufficient levels of disease resistance in transgenic rice (Anand et al. 2003). Furthermore, in order to obtain long-term disease-resistant plants, the prime alternative means is to apply to over-expressing stacked antimicrobial genes with different functions. Another strategy is to constitutively express some transcription factor genes, such as *NPR1*, *WRKY*, *MYB* and *TGA*, which play an important role in stimulating many downstream defence genes of the SAR signalling pathway (Campbell et al. 2002; Gurr and Rushton 2005). Meanwhile, in order to reduce toxicological and allergenic risks, the application of the tissue-specific promoter and the defence-response-specific expression are also important in the transgene (Collinge et al. 2008). In addition, to broaden the spectrum of resistance, fine tuning of engineered gene expression, the establishment of optimal expression levels

and insertion sites of the target gene are also necessary in future research (Punja 2001).

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