Science in China Ser C: Life Sciences

© 2007 Science in China Press

Springer-Verlag

Enhancing disease resistances of Super Hybrid Rice with four antifungal genes

ZHU HuaChen[1], XU XinPing[1], XIAO GuoYing[2,3], YUAN LongPing[2†] & LI BaoJian[1†]

[1] Biotechnology Research Center, Key Laboratory of Gene Engineering of Ministry of Education, Sun Yat-sen University, Guangzhou 510275, China;

[2] China National Hybrid Rice R&D Center, Hunan Hybrid Rice Research Center, Changsha 410125, China;

[3] Institute of Subtropical Agriculture, the Chinese Academy of Sciences, Changsha 410125, China

A plant expression vector harboring four antifungal genes was delivered into the embryogenic calli of '9311', an indica restorer line of Super Hybrid Rice, via modified biolistic particle bombardment. Southern blot analysis indicated that in the regenerated hygromycin-resistant plants, all the four antifungal genes, including *RCH10*, *RAC22*, *β-Glu* and *B-RIP*, were integrated into the genome of '9311', co-transmitted altogether with the marker gene *hpt* in a Mendelian pattern. Some transgenic R_1 and R_2 progenies, with all transgenes displaying a normal expression level in the Northern blot analysis, showed high resistance to *Magnaporthe grisea* when tested in the typical blast nurseries located in Yanxi and Sanya respectively. Furthermore, transgenic F_1 plants, resulting from a cross of R_2 homozygous lines with high resistance to rice blast with the non-transgenic male sterile line Peiai 64S, showed not only high resistance to *M. grisea* but also enhanced resistance to rice false smut (a disease caused by *Ustilaginoidea virens*) and rice kernel smut (another disease caused by *Tilletia barclayana*).

Super Hybrid Rice, multi-gene transformation, rice blast, rice false smut, rice kernel smut, anti-fungi, disease resistance

In recent years, China has made remarkable achievements and new breakthroughs in breeding new types of hybrid rice. The development of a series of elite hybrid combinations with a two-line approach has attracted global attention，putting China on the cutting edge of the world's Super Hybrid Rice Plan (SHRP). Among these most promising two-line hybrid varieties, Peiai 64S/9311 (also known as Liang-you-pei 9 or LYP9) and P88S/0293 ('0293' is a non-arista line derived from '9311', which could be an alternative of '9311' in practice) have reached a super high-yield of 10500 kg and 12000 kg per hectare respectively, fulfilling the first- and second-stage goal set by the 'China Super Hybrid Rice Project'. Subsequent to the success in three-line hybrid rice breeding, this progress was reputed as a new version of the 'Great Green Myth' by the international rice research community[1−3].

Although the Super Hybrid Rice had such great yield potential, and some varieties had been cultivated in large scale in south China, their quality and yield were often compromised by the infestation of plant pathogens and insect pests. As an example, rice false smut (caused by *Ustilaginoidea virens*), rice kernel smut (caused by *Tilletia barclayana*), rice sheath blight (caused by *Rhizoctonia solani*) and rice panicle blast (caused by *Magnaporthe grisea*) were the most prevalent diseases in fields planted with LYP9[4−6].

As a novel and powerful tool, genetic modification of crops provides a viable strategy to enhance plant resistances to insects and diseases. Compared with the conventional methods, this technology can endow crops with more comprehensive, more specific and more durative

Received April 5, 2006; accepted June 9, 2006
doi: 10.1007/s11427-007-0001-9

†Corresponding authors (email: lsslbj@zsu.edu.cn or lpyuan@public.cs.hn.cn)

Supported by the National High-Tech Research and Development Project (863) (Grant No. 101-01-02-02), National Specific Program for Research and Industrialization of Transgenic Plant (Grant Nos. J00-A-009 and J99-B-012), and Science & Technology Project of Guangdong Province (Grant No. B201)

tolerance with wider spectrum, which results in a more environmentally friendly protection and more convenience in use. In plant genetic engineering for antifungal breeding, it was well documented that β-1,3-glucanases, chitinases and ribosome inactivating proteins (RIPs) had a synergistic protective interaction and could coordinately inhibit the growth of fungi[7−10].

In this research, we applied the Multi-gene Transformation Strategy (MTS) to enhance disease resistance of the elite indica restorer line '9311' of the Super Hybrid Rice. Four antifungal genes (*RCH10*, *RAC22*, *β-Glu* and *B-RIP*) carried on a plant expression vector[11] were co-introduced into the genome of '9311' by the biolistic method. A batch of transgenic plants and progenies with improved resistance to the main fungal diseases of rice were selected by molecular detection and field tests. By hybridizing with other suitable male sterile lines, we are expecting to obtain novel types of genetically modified Super Hybrid Rice with high, multiple resistances to different diseases.

1 Materials and methods

1.1 Materials

Mature seeds of indica (*Oryza sativa* L.subsp. indica) rice variety '9311', a restorer line of Super Hybrid Rice, were kindly provided by Prof. Zhu Yingguo from Wuhan University. Vectors with a single antifungal gene including pAAG89, pRC24/B-RIP, pARAC2 and pARBC6 were gifts from Prof. Xu Yao of the Salk Institute for Biological Studies, Vanderbilt University, harboring the alfalfa glucanase gene (*β-Glu*), barley ribosome-inactivating protein gene (*B-RIP*), rice acidic chitinase gene (*RAC22*) and the rice basic chitinase gene (*RCH10*), respectively[8−11]. *Agrobacterium tumefaciens* binary vector pCAMBIA1300[11] was provided by Dr. Richard Jefferson of CAMBIA Center, Australia. Plant multi-gene expression vector pRAS1300 was constructed by our colleagues, Li Ming et al.[11] (Figure 1), harboring four antifungal genes (*RCH10*, *RAC22*, *B-RIP*, *β-Glu*) and the hygromycin phosphotransferase marker gene (*hpt*). *B-RIP* gene was driven by a rice inducible promoter, pRC24, *hpt* by CaMV35S, and the other 3 genes were all under the control of rice actin1 promoter (Act1).

1.2 Generation of rice transformants

Bio-Rad Biolistic® PDS1000/He Particle Delivery System was used to transfer plasmid pRAS1300 into the '9311' embryogenic calli. The rice tissue culture process and the method for biolistic transformation was as described in ref. [12] with some modifications: Using NB medium as the basic culture medium for '9311', 5 mg/L ABA and 2 mg/L 2,4-D were added into the calli-induction medium as the additive phytohormones; bombardments were conducted using helium as a carrier gas at 1100 psi (1 psi=6.895 kPa) in a vacuum of 25—27 inHg (1 inHg=3.386 kPa); per 3 mg gold particles were coated with 100 μg purified pRAS1300 plasmid DNA. After the DNA-coated microcarriers were fully suspended with plenty of ethanol, pipet them onto the center of each macrocarrier till the gold particles reached 0.5 mg/macrocarriers. Each sample was bombarded at a distance of 6 cm only once. The selection medium, pre-regeneration medium, regeneration medium and rooting medium should contain 20—25, 30, 30—40 and 50 mg/L Hygromicin B (Roche) respectively. Plantlets with vigorous roots of about 6—8 cm in height were suitable to sow in pots and grow in the greenhouse.

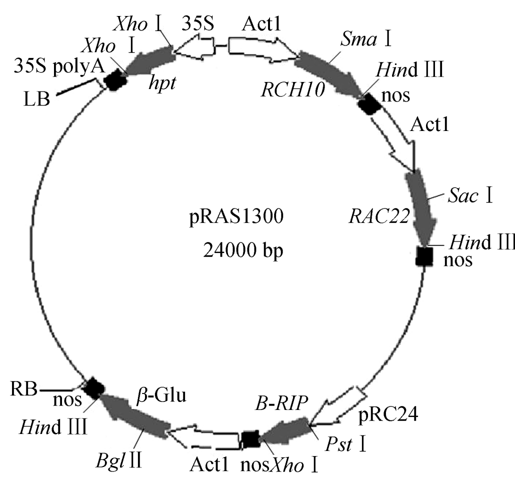


Figure 1 Plasmid map of the plant multigenic expression vector, pRAS1300[11]. LB: T-DNA left border; RB: T-DNA right border; 35S: cauliflower mosaic virus (CaMV) 35S promoter; 35S polyA: terminator of CaMV35S; Act1: promoter of rice actin1 gene; pRC24: promoter of rice chitinase gene *RC24*; nos: promoter of the nopaline synthase gene; *hpt*: hygromycin phosphotransferase gene; *RCH10*: rice basic chitinase gene; *RAC22*: rice acidic chitinase gene; *B-RIP*: barley ribosome-inactivating protein gene; *β-Glu*: alfalfa glucanase gene.

1.3 Southern blot analysis of the transgenic plants

DNA samples were extracted from the fresh rice leaf tissue using the CTAB method[13]. Probes were labeled using TAKARA Random Primer DNA Labeling Kit Ver. 2, and [α-^{32}P]dCTP was purchased from Yahui Biomedical Engineering Corp of Beijing. Labeled *Hin*d

III/*Sma* I fragment (about 0.7 kb) from plasmid pARBC6 was used to probe *RCH10* gene; pARAC2/ *Hin*d III+*Sac* I fragment (about 0.8 kb) for *RAC22* gene; pRC24/B-RIP/ *Pst* I+*Xho* I fragment (about 0.9kb) for *B-RIP* gene; pAAG89/ *Hind* III+*Bgl* II fragment (about 1.1 kb) for*β-Glu* gene; and a pCAMBIA1300/ *Xho* I fragment of 1.1 kb for *hpt* gene[9,10]. Details of Southern blot analysis were as described in ref. [14]. Plasmid harboring the corresponding target gene was used as a positive control, while the untransformed '9311' plant as a negative control.

1.4 Northern blot analysis of the transgenic plants

Rice total RNA was extracted from the fresh leaves according to the user's manual of the Trizol™ reagent (Invitrogen). As for detection of the *B-RIP* gene which was driven by the pRC24 inducible promoter, a wound treatment was needed beforehand: making small cuts along both edges of the leaf blade at an interval of 1.5 cm without damaging the middle vein. Each cut might be around 1.5 mm in depth. After 20 h, this wounded leaf was excised and total RNA was extracted from it. Northern blot analysis was carried out as in a previous report[10], and the preparation and labeling of probes were as described in sec. 1.3.

1.5 Disease resistance field tests of the rice transformants

Blast resistance appraisal of the transgenic '9311' R_1 plants from 16 lines was performed in Yanxi Blast Nursery, Hunan Province during September to October, 2002. Yanxi Nursery is located in a moist valley with little sunshine throughout a year, where the hereditary constitution of the local *Magnaporthe grisea* strains is in great diversity. As it was reported, the dominant isolates there were ZB and ZC groups, and the extremely virulent ZA group showed up frequently as well, thus making this region a very typical mountain ecosystem for blast [15].

Evaluation of '9311' R_2 plants' resistance to blast was conducted in Sanya Nursery, Hainan Province from March to April in 2004. Sanya Nursery was in a subtropical indica rice region and was a blast assessment base of the National Hybrid Rice R&D Center. All the R_2 plants to be tested derived from 191 R_1 plants. For each code of R_1 plants, 30—50 seeds were sown. The scales of the panicle blast (PB) and the leaf blast (Bl) were evaluated and scored by every single plant [15, 16].

Later on, with the evidence from the molecular detection and the field test, 12 homozygous lines with high resistance to rice blast were chosen accordingly to mate with non-transgenic Peiai 64S, giving rise to the transgenic F_1 generation.

In October 2004, field tolerance of the transgenic F_1 hybrid rice was tested against rice blast, rice false smut and kernel smut by the National Hybrid Rice R&D Center in Changsha, Hunan Province. Non-transgenic '9311' and LYP9 were used as a control when biological resistance characterization was carried out. For every hybrid combination, 30—50 seeds were sown. The incidence of fungal disease was recorded by each individual plant according to the Standard Evaluation System for Rice (SES) of IRRI [16].

As a supplement to the natural induction of rice disease, artificial induction and inoculation with infected leaves were necessary as well. Besides, all seeds for test were grown without any treatments beforehand. No disinfectants were applied to the tested plants throughout their whole life cycle. Excessive nitrogenous fertilizer was used to favor the blast. Sensitive rice varieties (Xiang'aizao7) were planted closely around the objective rice sample in 2 lines per region. Irrigation or density of plants was the same to common field management.

2 Results and analysis

2.1 Generation of the multi-gene transformed rice plants

Plasmid pRAS1300 was introduced into the '9311' embryogenic calli with a modified biolistic process as described in sec. 1.2. In total, 62 hygromycin-resistant regenerated plants of 18 independent transgenic lines were generated in this research. Most of the transgenic plants were normal in morphology, except the sterile D1 line and the less-reproductive E2 line.

2.2 Southern blot analysis of the transgenic R_0 plants of '9311'

Monogenic vectors including pCAMBIA1300, pAAG89, pRC24/B-RIP, pARAC2 and pARBC6 were digested with proper restriction enzymes (see sec. 1.3) and subjected to electrophoresis to isolate the corresponding target gene fragments. Then these fragments were labeled as gene-specific probes for the Southern blot analysis of all the transgenic R_0 plants. The results showed that among the 62 hygromycin-resistant R_0 plants, the introduction frequency of *hpt* gene was 100%. Moreover, the

co-integration frequencies of *hpt* gene and the other 4 antifungal genes lined up in pRAS1300 vector were also 100%, which meant that all these foreign genes were successfully co-transferred into the '9311' regenerated plants.

The Southern blot analysis of '9311' R_0 plants is shown in Figure 2. It was clear that all the undigested DNA samples of the transgenic plants gave a hybridization signal in the high-molecular-weight (MW) region, while the corresponding digested ones gave bands in the low-MW region. Therefore, it was concluded that the foreign transgenes had integrated into the genome of the recipient plants.

As shown in Figure 2(a) and (b), when the nylon membrane was probed by labeled *RAC22* and *RCH10* fragments in turn, transgenic samples upon digestion (lanes 6, 8, 10, 12) gave hybridization bands in consistence with both the positive control in lane 1 and the negative control in lane 4. Similar phenomenon occurred in Figure 2(c) when *RAC22* probe was used to detect another membrane, indicating that the two foreign chitinase genes both had been integrated into the genome of '9311' after the pRAS1300 plasmid was bombarded into the target calli. Besides, all the '9311' plants, including the transformants and the untransgenic samples, carried endogenous sequences homologous to the foreign *RCH10* and *RAC22* genes so that there were background hybridization bands in both pictures. Since there were many different rice chitinase genes with high homology to each other, and the *RCH10* and *RAC22* genes shared high similarity [9,10, 17], more than one bands appeared in the Southern blot assay of the chitinase genes.

In Figure 2(d) for a *B-RIP* gene assay, transgenic samples upon digestion (lanes 5, 7, 9, 11) gave hybridization bands not only at about 0.9 kb just as the plasmid control did, but also in the other regions with different sizes, whereas the negative control showed no hybridization signal at all. Similar circumstance could be found in Figure 2(e) for a *β-Glu* gene assay, where digested DNA sample in lane 6 gave several extra bands when compared with positive control in lane 1 and lane 2. It

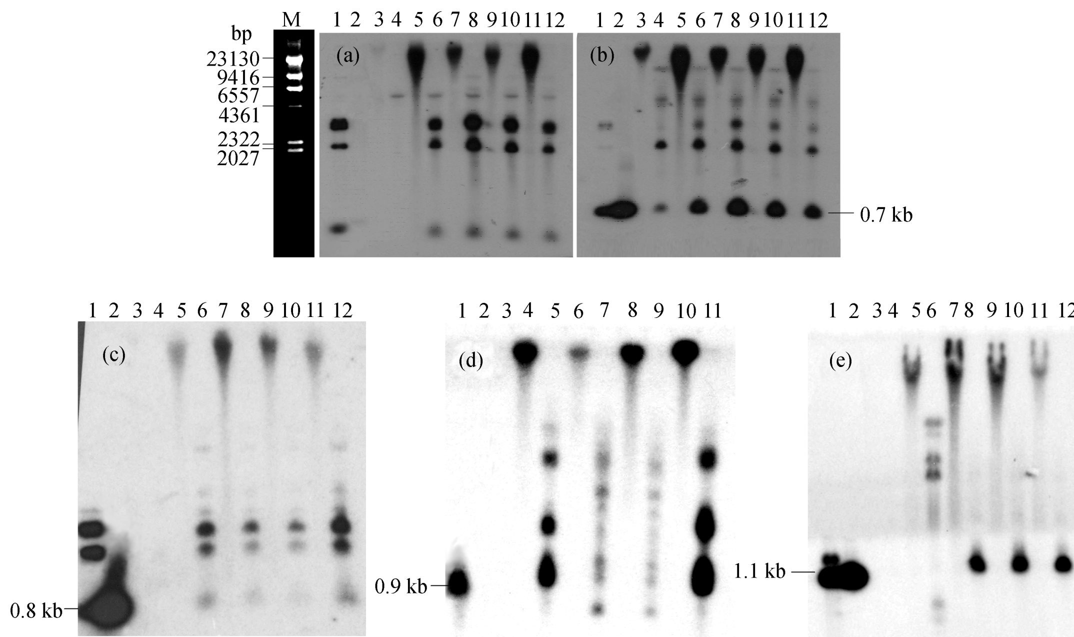


Figure 2 Southern blot analysis in part of the transgenic '9311' R_0 plants. M: *λ*/*Hin*d III. In (a) and (b), the same nylon membrane was hybridized with different probes in turn. (a) A labeled *Hin*d III+*Sac* I fragment from plasmid pARAC2 (0.8 kb) was used as the *RAC22* gene probe. 1, pRAS1300/ *Hin*d III+*Sma* I; 2, pARBC6/ *Hin*d III+*Sma* I; 3, '9311' non-transformed control (NT); 4, '9311' NT/ *Hin*d III+*Sma* I; 5, A4; 6, A4/ *Hin*d III+*Sma* I; 7, A5; 8, A5/ *Hin*d III+*Sma* I; 9, A1; 10, A1/ *Hin*d III+*Sma* I; 11, A2; 12, A2/ *Hin*d III+*Sma* I. (b) A labeled pARBC6/*Hin*d III+*Sma* I fragment of about 0.7 kb was used as the *RCH10* gene probe to hybridize with the same membrane as in (a). (c) A labeled pARAC2/ *Hin*d III+*Sac* I fragment of 0.8 kb was used as the *RAC22* gene probe. 1, pRAS1300/ *Hin*d III+*Sac* I; 2, pARAC6/ *Hin*d III+*Sac* I; 3, '9311' NT; 4, '9311' NT / *Hin*d III+*Sac* I; 5, A1; 6, A1/ *Hin*d III+*Sac* I; 7, A4; 8, A4/ *Hin*d III+*Sac* I; 9, A5; 10, A5/ *Hin*d III+*Sac* I; 11, A2; 12, A2/ *Hin*d III+*Sac* I. (d) A labeled pRC24-B-RIP/ *Pst* I+*Xho* I fragment of 0.9 kb was used as the *B-RIP* gene probe. 1, pRAS1300/ *Pst* I+*Xho* I; 2, '9311' NT; 3, '9311' NT / *Pst* I+*Xho* I; 4, B1; 5, B1/ *Pst* I+*Xh o*I; 6, B3; 7, B3/ *Pst* I+*Xho* I; 8, B3(2); 9, B3(2)*Pst* I+*Xho* I; 10, E1; 11, E1/ *Pst* I+*Xho* I. (e) A labeled pAAG89 / *Hin*d III+*Bgl* II fragment of 1.1 kb was used as the *β-Glu* gene probe. 1, pRAS1300/ *Hin*d III+*Bgl* II; 2, pAAG89/ *Hin*d III+*Bgl* II; 3, '9311' NT; 4, '9311' NT / *Hin*d III+*Bgl* II; 5, A9; 6, A9/ *Hin*d III+ *Bgl* II; 7, A1; 8, A1/ *Hin*d III+ *Bgl* II; 9, A4; 10, A4/ *Hin*d III+ *Bgl* II; 11, A3; 12, A3/ *Hin*d III+ *Bgl* II.

was speculated that bands not corresponding to the plasmid control might be due to multicopy integration and rearrangements of the target genes.

According to the Southern blot analysis of the *hpt*, *B-RIP* and *β-Glu* genes, all the transgenic lines showed a multicopy integration and rearrangement mode at least in one of these 3 genes. They gave more than one hybridization bands in these assays. It was concluded that transgenic plants obtained by the above-mentioned method had high frequency in multicopy integration and rearrangement of the transgenes

2.3 Genetic and molecular analysis of the transgenic R_1 plants of '9311'

Seeds set by the R_0 transgenic plants were sown and grown up as the R_1 plants. Total DNA was then prepared from the fresh leaves of the R_1 plants, and without digestion by any restriction enzymes, Southern blots of these uncut DNA were performed using *β-Glu* and *B-RIP* genes as probes in turn (Figures are not shown). Among the tested R_1 population, *β-Glu* and *B-RIP* genes were always co-transmitted, suggesting that they were genetically linked. As shown in Table 1, segregation of these 2 genes in each of the 17 transgenic lines conforms to Mendal's 3:1 ratio, but in some of these lines (such as A3, A6, A7, A9 and E2), the possibility of a 15:1 segregation ratio could not be completely excluded. This result indicated that the multiple copies of the foreign genes might be inserted at the same or adjacent chromosome site, or integrated into 2 separate chromosomes.

2.4 Disease resistance tests of the transgenic '9311' R_1, R_2 plants in the blast nursery

Disease resistance field test of the '9311' R_1 transformants was carried out in Yanxi Blast Nursery, Hunan Province. According to the calculations, 45.1% of the 102 R_1 plants showed high resistance level to rice blast, though this percentage differed from lines to lines.

Seeds from the resistant R_1 plants were collected and the R_2 plants were grown in Sanya Blast Nursery, Hainan Province. Among the 69 R_1 plants tested, 19 gave R_2 offspring with no symptom of either panicle blast or leave blast. Another 23 R_1 plants had R_2 progenies showing high resistance to panicle blast and slight infection symptom of leave blast.

Seeds from 122 R_1 plants which had not yet been tested for blast tolerance were planted in Sanya as well. Among them, 54 produced offspring plants without any panicle infections, and 13 out of these 54 even gave rise to R_2 plants with no symptom of blast.

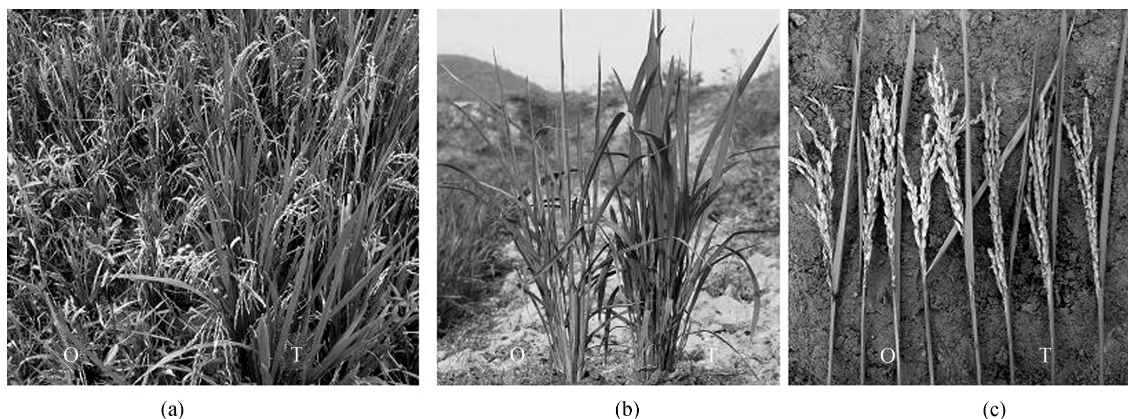
Table 1 Genetic analysis of the transgenic R_1 plants of '9311'

R_0 transgenic line	No. of Southern blot positive plant	No. of Southern blot negative plant	Chi-squared (χ_i^2) of the expected 3:1 segregation ratio	χ_i^2 of the expected 15:1 segregation ratio
A1	12	5	0.020	14.592
A2	14	5	0.018	12.214
A3	11	1	1.000	0.022
A4	6	4	0.533	18.027
A5	15	5	0.067	11.213
A6	15	3	0.296	2.904
A7	14	3	0.176	3.298
A8	14	5	0.018	12.214
A9	18	1	2.965	0.004
B1	14	7	0.397	25.146
B2	8	6	1.524	30.476
B3	12	6	0.296	21.393
B4	6	3	0.037	10.141
B5	15	4	0.018	6.488
B6	12	7	0.860	29.056
E1	28	12	0.300	37.500
E2	4	1	0.000	1.080

Note: $\chi^2_{0.05}$=3.841. If $\chi_i^2<\chi^2_{0.05}$, the expected segregation ratio was accepted.

In total, there were 96 R_1 plants out of the 191 samples tested in Sanya showing a potential to produce offspring with high resistance to panicle blast. Among them, there were 32 R_1 plants whose R_2 progenies had exhibited a persistently high resistance to blast throughout their whole growth periods.

In particular, it was worthy of notice that of all the transgenic plants tested for blast resistance, there were 4 R_1 plants, whose R_2 progenies had a panicle blast incidence lower than 10%. Impressively enough, the most supereminent line was an R_1 plant coded as A2-10. Only 2.00% of its R_2 plants showed panicle blast lesions and 9.09% showed small necrotic spots on the leaves. As for A2-9, the corresponding percentage came to 9.88% and 11.11%,; and for A9-10, 9.90% and 10.00%, respectively. Except this small percentage, the progenies of these lines were immune from rice blast, while the untransgenic '9311' and some lines of the transformants revealed an incidence of either panicle blast or leaf blast of 100%, scale 7—9 (highly sensitive), without a single grain for harvest (Figure 3).



(a) (b) (c)

Figure 3 Disease resistance of the transgenic offspring of '9311' in the blast nurseries. O: Untransgenic '9311'(highly sensitive); T: transgenic '9311'(highly resistant). (a) '9311' transgenic R_1 plants in the Yanxi Blast Nursery, Hunan Province; (b) '9311' transgenic R_2 plants in the Sanya Blast Nursery, Hainan Province; (c) panicles of the '9311' transgenic R_2 plants in the Sanya Blast Nursery, Hainan Province.

2.5 Northern blot analysis of the '9311' transgenic plants

In order to detect the expression of the multiple transgenes at mRNA level, Northern blot was performed on some of the high resistance R_2 plants. 20 h before the RNA was extracted, the plants were wounded according to the method described in sec. 1.4. Then *RCH10*, *β-Glu* and *B-RIP* gene probes were hybridized with the RNA on the same membrane in turn (Figure 4). Results indicated that most of the foreign genes could be expressed at high levels in these high resistance plants, but the 3 genes might not be expressed at a consistent level. Chitinase gene seemed to have a higher expression level in general, whereas *β-Glu* and *B-RIP* genes appeared to vary in its mRNA level. For example, all the 3 genes detected in A2-10 (lane 1) and A9-16 (lane 7) revealed a high expression level, whereas A2-4 (lane 3), A9-1(lane 5), A9-5 (lane 6) showed a much lower level in the expression of *β-Glu* and *B-RIP* genes. Such differences in the mRNA expression levels between plant lines correlated with that in the resistance levels in the fields.

2.6 Disease resistance of the F_1 hybrid rice plants produced by the transgenic 9311/Peiai 64S

Southern blot was performed in the transgenic R_2 populations to screen for the homozygous lines. Each of the 12 presumptive pure lines with high resistance to blast was then crossed with the non-transgenic male sterile line Peiai 64S, and the F_1 hybrid rice plants were grown in an isolated field in Changsha, Hunan.

During the late-season in 2004, all the 12 transgenic F_1 lines showed a high resistance against rice blast (scale 0—2), whereas the untransgenic F_1 of 9311/Peiai 64S (LYP9) had an intermediate resistance or intermediate sensitive level in the fields (scale 4—5).

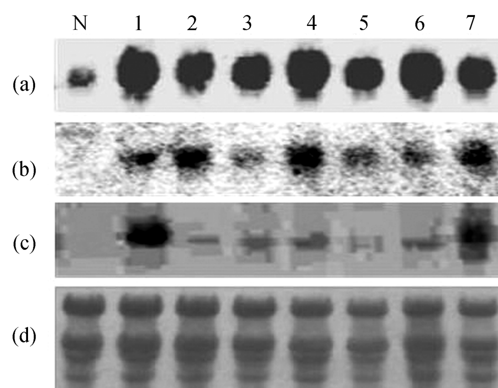


Figure 4 Northern blot analysis of the '9311'R_2 plants with high resistance to blast. N, Untransgenic '9311'; 1, A2-10; 2, A2-13; 3, A2-4; 4, A5-7; 5, A9-2; 6, A9-5; 7, A9-16. (a) Hybridized with an *RCH10* gene probe; (b) hybridized with a *β-Glu* gene probe; (c) hybridized with a *B-RIP* gene probe; (d) RNA stained in the denaturing gel.

When 12 F_1 populations of the transgenic 9311/Peiai 64S combinations were tested for rice false smut, 10 of them appeared to have no infected symptom. The other two lines of the transgenic F_1 population (coded as 04CF069 and 04CF071) each had one panicle affected, thus Line 04CF069 gave an incidence of infected panicles of 1.2%, incidence of infected plants of 5.9%, while Line 04CF071 gave the corresponding incidence of 2.8% and 14.3%. As a control, untransgenic 9311/Peiai 64S hybrid rice showed a panicle infection incidence of about 7.7% (Figure 5(a)).

From each F_1 population, 6—17 plants were randomly selected to calculate the number of filled grains and the

amount of infected seeds with kernel smut. As listed in Table 2, transgenic F_1 population with a code of 04CF077 exhibited no visible lesions (Figure 5(b)), and the other lines of transgenic hybrid F_1 showed a much lower incidence of kernel smut, compared with the untransgenic ones.

Table 2 Incidence of rice kernel smut in F_1 hybrids of the transgenic '9311' R_2 plants/Peiai 64S

Line code of the hybrids	Average incidence (No. of seeds with kernel smut/ total No. of filled grains ×100%)	Average No. of infected seeds per panicle
04CF064	1.87	1.38
04CF065	2.23	1.75
04CF066	5.01	2.92
04CF067	0.81	1.00
04CF068	0.41	0.45
04CF069	5.60	5.29
04CF070	0.60	0.50
04CF071	1.14	1.57
04CF074	5.05	2.29
04CF076	7.10	3.57
04CF077	0.00	0.00
04CF078	6.75	3.25
Untransgenic 9311/Peiai 64S	17.12	14.50

According to the above data, transgenic F_1 plants of 9311/Peiai 64S have showed a more comprehensive resistance to multiple fungal diseases in the fields, with significantly enhanced resistances to *M. grisea*, *Ustilaginoidea virens* and *Tilletia barclayana*, when compared with the untransformed LYP9 hybrid rice.

3 Discussion and conclusion

As most biological traits and physiological functions in an organism are manifested through the cooperative expression of multiple genes, the inherited characteristics may be changed and modified to a greater extent if Multi-gene Transformation Strategy (MTS) is adopted. It is foreseeable that MTS will certainly become the mainstream in the future research of gene engineering, either in basic theoretical research or practical application area[18,19]. With regards to plant genetic manipulation for crop protection, multiple transgenes with different resistant mechanisms could broaden the resistance spectrum of transgenic crops, improve their resistance level, delay the development of tolerance of the pathogens and insects, and prolong the usefulness of the existing cultivars[18−22]. Hence, applying multiple genes to simultaneously improve various kinds of biotic or abiotic stress resistance in rice and other crops has already become a new trend for developing genetically modified crops[8−10, 18,19].

In this research, 4 antifungal genes from rice, barley and alfalfa were co-delivered into the genome of '9311'. Up to date, we have obtained a batch of transgenic plants and progenies with a significant increase in their resistance to rice blast. Subsequent research proved that these high resistance transgenic '9311' plants could serve as a paternal line in the breeding of LYP9, conferring simultaneous resistances against rice false smut, kernel smut and blast to their F_1 progenies. On the basis of the pre-

Figure 5 Field resistance of the F_1 hybrids of the transgenic '9311' R_2 plants/Peiai 64S against rice false smut and kernel smut. O: Untransgenic control (F_1 of the non-transformed 9311/Peiai 64S); T: F_1 of the transgenic 9311/Peiai 64S. (a) Rice false smut; (b) rice kernel smut.

vious reports[7−11, 17, 20−24], we inferred that the possible synergistic mechanism of the 4 anti-fungal transgenes in the resistant rice plants might be as follows: when pathogens invaded the transgenic plants, they first encountered the constitutively expressed acidic chitinase RAC22 (driven by the Act1 promoter) in the intercellular space. Chitinase could lyse the cell wall of most fungi, and the lysate would serve as a signaling molecule to further induce the systemic defense mechanism against the intruders. Meanwhile, the invasion of pathogens might trigger the expression of *B-RIP* gene as it was under the control of an inducible promoter, pRC24. Once the remainder of the pathogens entered the cell, the β-1, 3-glucanase and the basic chitinase of RCH10 released from the vacuoles would align themselves to act on their substrates — the glucans and chitins, which have been interlaced in the fungal cell wall in a filamentous form. This joint action might result in a thorough disorganization or lysis of the cell wall, which would facilitate the entry of RIP into the cytoplasm as well. As a consequence, the protein synthesis and propagation of the fungi would eventually be completely inhibited.

Since the 4 antifungal genes and their promoters (rice Act1 promoter and rice pRC24 promoter) used in this research were all derived from the edible crops[8−11], including rice, barley and alfalfa, which had served as the staple food for human or livestock, and had been widely planted and spread over thousands of years, they were supposed to present less problems in the food and environment safety evaluation. On the other hand, when the action mechanisms of these genes were concerned, there were no active substrates for β-1,3-glucanase and chitinase in the mammalian cell[24], and the B-RIP was a type-I RIP which was avirulent to intact tissues or cells[24]. Therefore, all these genes expressed in a genetically modified plant were theoretically safe to domestic animals and human beings, but deleterious enough to eliminate the fungal pathogens.

As an elite indica restorer line involved frequently in the breeding of Super Hybrid Rice, '9311' itself could be used as a high-quality and high-yielding conventional variety in agricultural practice as well. By now, multiple hybrid combinations such as Liangyoupeijiu (LYP9), Guangliangyou 6, Yueyou 938, P88S/9311, 58S/9311 and 38S/9311 have been popularized in country-wide indica rice-growing region, using '9311' and its derivatives as the restorer line[25]. For this reason, we are convinced that the transgenic '9311' plants attained in this research will provide a useful germplasm resource for the breeding of super high-yielding rice with high resistance to multiple fungal diseases. By integrating the conventional breeding methods into the succeeding cultivation, it is highly possible that new types of multigene-transgenic Super Hybrid Rice with good agricultural traits and comprehensive resistances will be widely recognized in the near future.

We thank Prof. Y. G., Zhu Prof. Y. Xu and Dr. R. Jefferson for their kind gifts of rice seeds and plasmids. We are also grateful to Dr. Qiuyun Liu and Dr. Zengfu Xu for helpful discussions in the preparation of this manuscript.

1 Yuan L P, Xin Y Y. Light of hope: Super Hybrid Rice. World Agr (in Chinese), 2001, 10(10): 46
2 Yang C X, Xiang B H, Zhang Q M, et al. Cultural techniques of achieving a yield of 12.26 t/hm^2 in the demonstrative production of two-line Super Hybrid Rice under an area over 6.7 hectares. Hybrid Rice (in Chinese), 2003, 18(2): 42−44
3 Wang Q, Lu C M, Zhang Q D, et al. Characterization of photosynthesis, photoinhibition and the activities of C_4 pathway enzymes in a superhigh-yield rice, Liangyoupeijiu, Sci China Ser C-Life Sci, 2002, 45(5): 468−476
4 Lu K Y, Wu H M, Lu B L. Identification of the elite medium rice variety Liangyoupeijiu and Yangdao 6. Hubei Agr Sci (in Chinese), 2001, (3): 9−12
5 Dong X H, Tian X Z, Wang L, et al. The occurrence rule and its IPM technique of the diseases and pests of two-line hybrid medium indica rice- Liangyou Peijiu. J Anhui Agr Sci (in Chinese), 2001, 29(3): 342−344
6 Ma Z, He S L, Jin K M, et al. The reason of the occurrence of rice kernel smut in the hybrid seed production of Liangyou Peijiu and its prevention and cure. J Xinyang Agr College (in Chinese), 2001, 11(2): 13−15
7 Jach G, Gornhardt B, Mundy J, et al. Enhanced quantitative resistance against fungal disease by combinatorial expression of different barley antifungal proteins in transgenic tobacco. Plant J, 1995, 8(1): 97−109
8 Feng D R, Wei J W, Xu X P, et al. Introduction of multiple antifungal protein genes into rice and preliminary study on resistance to *Pyricularia oryzae* of transgenic rice. Acta Sci Nat Univ Sunyatseni (in Chinese), 1999, 38(4): 62−66
9 Feng D R, Xu X P, Fan Q, et al. Rice plants of multiple transgenes for resistance to rice blast and sheath blight diseases. Acta Agron Sin (in Chinese), 2001, 27(3): 293−301
10 Feng D R, Xu X P, Qiu G H, et al. Inheritance and expression of multiple disease and insect resistance genes in transgenic rice. Chin Sci Bull, 2001, 46: 101−106
11 Li M, Qiu G H, Xu X P, et al. The construction of plant expression vector containing multi-antifungal genes. Acta Sci Nat Univ Sunyatseni (in Chinese), 1999, 38(5): 67−71
12 Xu X P, Wei J W, Fan Y L, et al. Fertile transgenic indica rice from microprojectile bombardment of embryogenic callus. Acta Genet Sin (in Chinese), 1999, 26(3): 219−227

13 Doyle J J, Doyle J L. Isolation of plant DNA from fresh tissue. Focus, 1990, 12: 13－15
14 Zhu H C, Xu X P, Li B J. A simple, rapid method of Southern blot analysis. Acta Sci Nat Univ Sunyatseni (in Chinese), 2004, 43(4): 128－130
15 Xiao F H, Luo H R, He Z N, et al. Identification and application of the multiple durable resistance of the dryland rice. Hunan Agr Sci (in Chinese), 2001, (5): 39－41
16 International Rice Research Institute. Standard Evaluation System for Rice (SES). Los Banos: IRRI, 2002, 14－18
17 Zhu Q, Lamb C J. Isolation and characterization of a rice gene encoding a basic chitinase. Mol Gen Genet, 1991, 226: 289－296
18 Li B J, Zhu H C. On the prospects of applying the Multi-gene Transformation Strategy to modify the inheritance of organisms I. The gene resources and technical platform for multi-gene transformation. Acta Sci Nat Univ Sunyatseni (in Chinese), 2004, 43(6): 11－15
19 Li B J, Zhu H C. On the prospects of applying the Multi-gene Transformation Strategy (MTS) to modify the inheritance of organisms II. General principles, possible problems and prospects of the MTS. Acta Sci Nat Univ Sunyatseni (in Chinese), 2005, 44(4): 79－83
20 McDowell J M, Woffenden B J. Plant disease resistance genes: recent insights and potential applications. Trends in biotechnol, 2003, 21(4): 178－183
21 Shah D M. Genetic engineering for fungal and bacterial diseases. Curr Opin Biotechnol, 1997, 8(2): 208－214
22 Punja Z K. Genetic engineering of plants to enhance resistance to fungal pathogens—A review of progress and future prospects. Can J Plant Pathol, 2001, 23: 216－235
23 Ming X T, Wang L J, An C C, et al. Resistance to rice blast (*Pyricularia oryzae*) caused by the expression of trichosanthin gene in transgenic rice plants transferred through agrobacterium method. Chin Sci Bull, 2000, 19: 1774—1778
24 Shan L B, Jia X. Ribosome inactivating protein and its application in plant anti-fungal disease genetic engineering. Progr Biotechnol (in Chinese), 2000, 20(6): 74－78
25 Bai H S, Zhan C Y, Wang B H, et al. Medium indica rice variety Yangdao 6 and its application in hybrid rice breeding. Hybrid Rice (in Chinese), 2001,16 (6): 13－15