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Transfer of Lysozyme Gene into indica Parents of Hybrid Rice by Backcrossing

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Abstract: A lysozyme gene resistant to rice blast was transferred from the donor transgenic japonica rice Zhonghua 9 (D2-1-2) into a sterile line Pei'ai 64S(PA 64S) and restorer line 9311 of the two-line hybrid rice Liangyoupeijiu, and the restorer line Minghui 63 (MH 63) of three-line hybrid rice Shanyou 63 by successive backcrossing. The PCR analysis confirmed that foreign lysozyme gene was segregated at ratio of 1∶1 in backcross generations of $B_3$9311, B_3MH63 and B_2PA64S, and at ratio of 3∶1 in selfed generations of B_2F_2 9311, B_2F_2 MH63 and B_1F_2 PA64S, indicating that the foreign gene was stably inherited over successive generations as a dominant single copy gene. The resistance against rice blast in backcross or selfed generations and corresponding testcross combinations were investigated in 2003 and 2004. The results showed that the resistance of the transgenic rice to blast had a greater improvement than that of the corresponding recurrent parents or the corresponding check hybrid combinations. The resistance of the advanced backcross and selfed generations to rice blast is much stronger than that of the early generations. The study confirmed that transferring the lysozyme gene into hybrid parents by backcrossing was a simple and effective approach to develop new hybrid rice resistant to rice blast.

Key words: hybrid rice; lysozyme gene; rice blast; backcrossing; breeding

Rice blast (*Pyricularia oryzae* Cavara), one of the major fungal diseases in rice fields all over the world, is induced by the ascomycetous fungus *Magnaporthe grisea* (Hebert) Barr [Anamorph: *Pyricularia grisea* (Cooke) Sacc.] [1]. According to the statistical data from 1975 to 1990, the global losses in rice caused by rice blast was 1.57×10^8 tons with an annual average of more than 1.0×10^7 tons [2]; while in Japan the annual rice yield loss caused by rice blast accounted for 1.4–7.3% of the gross rice yield during 1953-1960, and 0.5–5.6% in 2001 [4]. In China, since 1990s rice blast has occurred over 3.8×10^6 ha of area and resulted in huge decrease in rice yield [5]. The above data shows the extensive distribution and serious harm of rice blast all over the world.

The development of biotechnology has pioneered a new approach to the breeding of rice variety resistant rice blast. After the development of first transgenic rice in 1988, a rapid progress has been noted in rice transgenic technology. The transgenic varieties of indica, japonica and javanica rice have been developed successfully up to date. Through transferring the foreign lysozyme gene (offered by Dr. Hain R. from the German Bayer Company) into the japonica rice Zhonghua 9 by microprojectile bombardment, the new transgenic line of T_7 generation D2-1-2 was developed by the Institute of Genetics and Developmental Biology, Chinese Academy of Sciences after five successive years field selection and disease-resistance experiment [7]. However, the application of D2-1-2 has some limitation because Zhonghua 9 is a japonica rice seldomly used in production.

In China, many elite super hybrid rice combinations have been planted on large area. However, some of them performed low resistance level to rice blast. Thus, it is important for improving the resistance of super hybrid rice. In this experiment, D2-1-2, as a foreign donor with resistance gene, was crossed and successively backcrossed with the parents of indica hybrid rice, and the resistance of transgenic generations to rice blast was evaluated. The objectives of the studies were to improve the resistance of elite hybrid rice combinations and to provide baseline for the selection

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of new indica hybrid rice parents and combinations with high resistance to rice blast.

MATERIALS AND METHODS

Materials

Rice seeds

The japonica rice Zhonghua 9 [ZH9(CK)] and D2-1-2 [ZH9(R)], a transgenic line with lysozyme gene were kindly provided by Prof. CHU Cheng-cai from the Institute of Genetics and Developmental Biology, Chinese Academy of Sciences; and restorer line Minghui 63 (MH63) of the indica hybrid rice combination Shanyou 63 and restorer line 9311 and sterile line Pei'ai 64S (PA64S) of the indica hybrid rice combination Liangyoupeijiu were provided by Prof. CHEN Li-yun, the Institute of Rice Research, Hunan Agricultural University.

Pathogen strains used to test the rice resistance to rice blast

The mixed pathogen strains, which were representative strains of rice blast in Hunan Province, were isolated from the blast diseased rice plants. The strains were provided by Prof. LI Ding-jun and Prof. LUO Kuan from the College of Bio-Safety Science and Technology, Hunan Agricultural University.

Reagents for molecular biological analysis

Reagents used in PCR analysis were 10 mmol/L dNTP Mix (Bebeco Company), 10×PCR buffer, 25 mmol/L $MgCl_2$ and *Taq* DNA Polymerase (Fermentase Company).

Methods

Crossing and backcrossing

The parents of hybrid rice MH63, 9311 and PA64S, were crossed with ZH9(R) to produce the F_1 generation; and then continuously backcrossed with the F_1 generation as the recurrent parents to get the BC_1F_1 generation, BC_2F_1 generation (the rest may be deduced by analogy). In order to prevent the loss of exogenous lysozyme gene during backcross, before flowering of segregated populations, PCR analysis and field selection were conducted in each backcross generation to select PCR-positive transgenic plants with similar phenotype to their parents for successive backcrossing, or selfing and testcrossing.

PCR analysis on the generations of transgenic rice

The SDS method was applied for the extraction of DNA [8]. A pair of PCR primers, i.e. L1: 5'-ATACGCGTCCCAAGTGTCAGTTCT-3' and L2: 5'-CGTATAGATGAACGTCTTAGAC-3', were designed according to the lysozyme gene sequence, and synthesized by Beijing AuGCT Biotechnology Co., Ltd. the length of PCR product was 462 bp. The conditions of PCR reaction were as follows: pre-denaturalizated for 2 min at 94℃; 1 min 40 s at 94℃, 2 min at 53℃, 2 min at 72℃, 40 cycles; and extended for 10 min at 72℃. The PCR product was observed by ultraviolet transmission device after separated by 1% of agarose gel electrophoresis and dyed by EB.

Identification of the resistance of transgenic rice to rice blast

In 2003 and 2004, the backcross generations and corresponding testcross combinations were planted in Daowushan Natural Disease Nursery, Liuyang City, Hunan Province and Tanjiawan Disease Nursery, Meishuidong Village, Gaoqiao Township, Taojiang County, Hunan Province, where the weather conditions were misty with less sunshine and dewy throughout the year so that rice blast pathogen propagates aptly. For identification of every plant line, 80 rice plants (single seedling per hill, 8 rows×10 seedlings and 13.3 cm×20.0 cm plant spacing) formed a small disease nursery and 5–8 small disease nurseries constituted a compartment. Peiliangyou 288 and Xiangzaoxian 7 used as the infection source of rice blast were planted around the field and beside the walkways of the compartments (the walkway between the compartments was 40 cm in width). The management of insects and weed control in the rice field was followed by conventional methods for high yielding cultivation, but no antiseptics were used. During the whole growing period the field was irrigated and fertilized with nitrogenous fertilizer according to the actual situation to create more favorable pathogenetic conditions.

Rice blast was induced by natural infection and spray inoculation. The backcross or selfed generations evaluated in 2003 were the progenies of 9311/ZH9(R), BC_3F_1 ($B_3$9311) and BC_2F_2 ($B_2F_2$9311); progenies of MH63/ZH9(R), BC_3F_1 (B_3MH63) and BC_2F_2 (B_2F_2MH63); progenies of PA64S/ZH9(R), BC_2F_1 (B_2PA64S) and BC_1F_2 (B_1F_2PA64S); and two testcross combinations, Zhenshan 97A/B_2MH63 and B_2PA64S/$B_2$9311; and the ones evaluated in 2004 were the generations by backcrossing and selfing in 2003, they are $B_4$9311, $B_3F_2$9311 and $B_2F_3$9311 from 9311/ZH9(R), B_4MH63, B_3F_2MH63 and B_2F_3MH63 from MH63/ZH9(R), B_3PA64S and B_1F_3PA64S from PA64S/ZH9(R), and testcross combinations Zhenshan 97A/ B_3MH63 and B_2PA64S/ $B_3$9311.

The mixed pathogenic strains of rice blast were cultured in the oatmeal culture medium containing 20 g/L of oatmeal (‘Shou’ Brand) [Enmai Foodstuff (Shenzhen) Co., Ltd.] and 15 g/L of agar. The 20 g of oatmeal plus appropriate distilled water was cooked into viscid porridge in a microwave oven; and then, the porridge was filtered by two layers of clean gauze. Later the filtered liquid was added into the dissolved agar. The mixture was dilute to volume of 1 liter with distilled water and then sterilized under high temperature and pressure (temperature: 121–126℃; pressure: 0.10–0.15 MPa). After cooled to 60℃, the sterilized culture medium was poured onto a plate on a super clean bench. The strains of rice blast were inoculated upon cooling of the plate. Afterwards, the plate was placed upside down in a dark environment of 26℃ for 10 days. After the culture dish was nearly filled with mycelia, the plate was placed up in a light culture case at the same temperature for 4 days in order to promote the growth of spores. The concentration of the strain solution used for inoculation was more than 30 spores per vision field under a 10×10 times microscope. The pathogenic strains were inoculated with a sprayer till all rice leaves were besprinkled with the spore solution at heading and full-heading stage. Since then, the rice plants were watered once or twice a day in a week.

Investigation methods and data processing

The rice blast can be classified into seedling blast, leaf blast, panicle blast, and the panicle blast is of the greatest harm to rice. The generations of transgenic rice suffered from seedling blast and leaf blast lightly, and over 80% of plants were at Grades 0-3, so the focus of present study was the identification and statistic of the resistance to panicle blast. At the yellow ripening stage, the occurrence of panicle blast of all plants was investigated. Panicles of all generations of each variety were sampled at random by the method of 5-site sampling for investigation: 5 hills per site, with 5 repetitions. The disease grade was recorded according to the international standard of panicle blast, while the disease index and the incidence of disease were calculated: disease index= 100×Σ(Representative value of each grade × The number of diseased panicles at each grade) / (Total of investigated panicles × Representative value of the highest grade), and incidence of disease=100× Σ(Incidence of disease at each grade × The number of diseased panicles at each grade)/ (Total of investigated panicles × Incidence of disease at the highest grade). In general, the resistance-susceptibility type was evaluated on the basis of the proportion of the diseased panicles at Grades 0-3 to total investigated panicles [9]: resistant (R), more than 80%; moderately resistant (MR), 60-79%; moderately susceptible (MS), 40-59%; and susceptible (S), less than 40%.

Data analysis

The statistical analysis was conducted via SPSS 10.0.

RESULTS

PCR analysis on transgenic generations

Following the extraction of DNA from transgenic backcross generations, $B_3$9311, B_3MH63 and B_2PA64S, and from selfed transgenic generations $B_2F_2$9311, B_2F_2MH63 and B_1F_2PA64S, the PCR identification was conducted with the restructured plasmid of lysozyme gene, ZH9(R), 9311/ZH9(R), MH63/ZH9(R) and F_1 generation hybrid of PA64S/ZH9(R) as the positive-PCR control, and ZH9 (CK) and the corresponding recurrent parents as the negative-PCR control.

The identification result is shown in Fig. 1 and

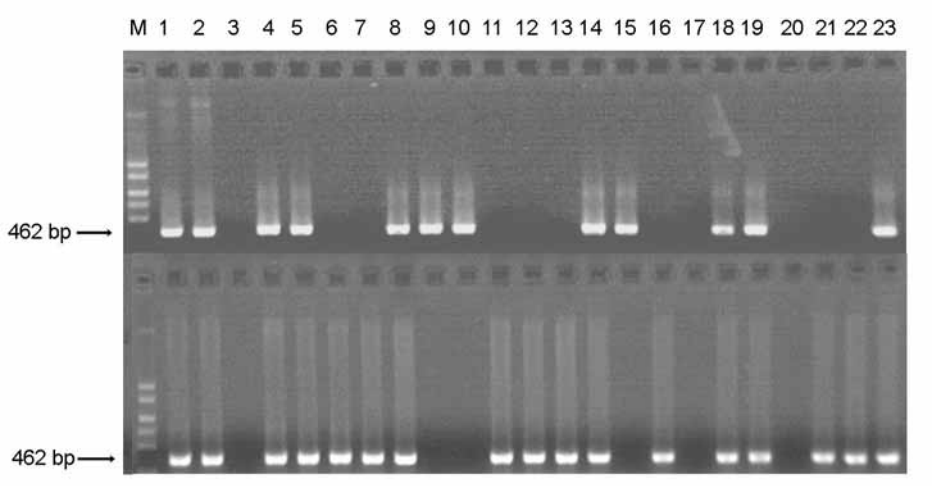

Fig. 1. PCR identification of foreign lysozyme gene in transgenic generations.

Above: Backcross generations. Lane 1, Plasmid; Lane 2, ZH9(R); Lane 3, ZH9 (CK); Lane 4, 9311/ZH9(R); Lane 5, MH63/ZH9(R); Lane 6, 9311; Lane 7, MH63; Lanes 8 to 21, Plants of backcross generation; Lane 22, PA64S; Lane 23, PA64S/ZH9(R).

Below: Selfed generations of the backcross generation. Lane 1, Plasmid; Lane 2, ZH9(R); Lane 3, 9311; Lanes 4 to 23, Plants of selfed generations of the backcross generations.

Table 1. The χ^2 test results showed that PCR-positive and negative plants were segregated at ratio of 1∶1 in backcross generations (BC_2F_1 and BC_3F_1) and at ratio of 3∶1 in selfed generations of the backcross generation (BC_1F_2 and BC_2F_2), which is in accordance with the Mendal Law of Segregation, indicating that the foreign gene was stably inherited over successive generations as a dominant single copy gene.

Identification of the resistance to rice blast

Identification of the resistance to rice blast in transgenic generations of ZH9(R) in 2003 and 2004

The resistance against rice blast of the transgenic generations of ZH9(R) was evaluated in 2003 and 2004. In order to accurately identify the resistance of transgenic rice against blast, disease nurseries were established at different places during these two years. The change in geographical position, climate and time resulted in different disease situation. Therefore, the parents ZH9 (CK) and ZH9(R) were planted as control in all nurseries in each year. In 2003, the incidence of disease on ZH9(CK) was 78.35%, the disease index was 87.12, and the diseased panicles at Grades 0–3 accounted for only 6.88% of total 1236 investigated panicles. Therefore, these generations were susceptible to disease (S) evidently. Regarding ZH9(R), the incidence of disease was 1.15%, and the disease index was 2.96, ranging from Grade 0 to Grade 3 in all 1192 investigated panicles, and they were resistant to the disease (R) obviously. Transgenic generations of 9311, MH63 and PA64S all suffered from the disease at different grades from 0 to 7. However, $B_3$9311, $B_2F_2$9311, B_3MH63 and B_2F_2MH63 presented moderate resistance (MR), and the diseased

Table 1. Segregation of foreign lysozyme gene in backcross and selfed generations after backcross.

Transgenic generation	No. of tested plants	No. of positive plants	No. of negative plants	χ^2	*P* value
$B_3$9311	166	80	86	0.151 (1:1)	0.50–0.75
B_3MH63	254	129	125	0.035 (1:1)	0.75–0.90
B_2PA64S	125	65	60	0.128 (1:1)	0.50–0.75
$B_2F_2$9311	381	284	97	0.022 (3:1)	0.75–0.90
B_2F_2MH63	241	183	58	0.068 (3:1)	0.75–0.90
B_1F_2PA64S	169	129	40	0.097 (3:1)	0.75–0.90
Total	1336	870	466		

Table 2. Occurrence of rice blast disease on backross or selfed generations derived from ZH9(R) in 2003 and 2004.

Variety or transgenic generation	No. of investigated panicles	No. of panicles at disease grades 0 to 3	Incidence of disease(%) (Mean±*SD*)	*t* value	Disease index (Mean±*SD*)	*t* value
Year 2003						
9311(CK)	1168	736	15.50±0.72	-	31.35±1.55	-
$B_3$9311	1070	746	14.11±0.96	1.940	29.44±1.75	1.374
$B_2F_2$9311	1264	882	13.89±0.57	3.230*	28.05±0.95	3.238*
MH63 (CK)	1293	818	16.17±0.87	-	33.48±1.49	-
B_3MH63	1281	834	14.23±0.79	4.355*	29.42±1.36	4.880**
B_2F_2MH63	1293	837	14.62±0.70	2.370	30.00±1.39	2.891*
PA64S (CK)	1306	630	22.26±0.90	-	43.73±1.14	-
B_2PA64S	1228	615	21.34±1.37	1.135	42.16±1.84	1.390
B_1F_2PA64S	1207	595	21.80±1.12	0.604	42.81±1.67	0.905
Year 2004						
9311(CK)	1329	390	33.41±3.58	-	57.38±2.29	-
$B_4$9311	1232	678	19.49±2.13	8.689**	45.81±2.32	9.928**
$B_3F_2$9311	1379	394	20.53±1.96	7.125**	48.43±2.82	5.201**
$B_2F_3$9311	1325	508	23.76±6.98	2.641	48.96±7.69	2.336
MH63 (CK)	1299	715	21.47±1.49	-	41.72±1.15	-
B_4MH63	1276	1026	10.37±0.62	13.719**	26.89±1.27	14.579**
B_3F_2MH63	1403	1071	13.18±1.45	9.163**	29.28±2.10	13.867**
B_2F_3MH63	1305	901	19.36±0.87	2.079	34.06±1.61	6.849**
PA64S(CK)	1171	233	45.31±3.69	-	67.58±2.89	-
B_3PA64S	1208	428	37.18±4.14	2.563	56.81±2.38	5.191**
B_1F_3PA64S	1163	390	44.29±2.93	0.386	60.65±1.82	3.617*

*,**show significant difference compared with CK at $P<0.05$ and $P<0.01$, respcetively.

panicles at Grades 0-3 accounted for 64.73-69.78%. The percentage of diseased panicles at Grades 0-3 in B_2PA64S and B_1F_2PA64S were 50.08% and 49.30% of total investigated panicles respectively, and these generations were classified as MS. From the Table 2, the incidence of disease and disease index of $B_3$9311 and $B_2F_2$9311 were lower than those of their recurrent parents 9311, but the *t* test results indicated the significant difference only existed between the $B_2F_2$9311 and 9311(CK). Similarly, the resistance against blast in transgenic generations of MH63 and PA64S was higher than that of their corresponding parents in incidence of disease and the disease index. The disease index and incidence of disease were significantly different between B_3MH63 and its parents. As to B_2F_2MH63, the incidence of disease showed no significant difference from that of its parents, while their disease index was significantly different. However, no significant differences were found between B_2PA64S, B_1F_2PA64S and their parents in the disease index and incidence of disease.

In 2004, with reference to ZH9 (CK), the incidence of disease was 86.15%, while the disease index was 91.96, and the diseased panicles at Grades 0-3 accounted for only 3.35% of total 1165 investigated panicles, suggesting that ZH9(CK) were susceptible to disease (S). For ZH9(R), the incidence of disease was 1.22%, and the disease index was 3.19, with a total of 1207 panicles investigated at Grades 0-3, which indicated it was resistant to blast (R) obviously. Transgenic generations of 9311, MH63 and PA64S suffered from blast at a greater degree. All $B_4$9311 plants were at Grades 3–7, while the diseased panicles at Grade 3 accounted for 55.03% of total investigated ones, which indicated it was moderately susceptible to disease (MS). Although a few $B_3F_2$9311 plants were not infected by rice blast, the diseased panicles at Grades 3–7 accounted for 98.19% of total

investigated ones, and diseased panicles at Grades 0-3 accounted for 28.57%. Except a few healthy panicles were noted in $B_2F_3$9311, most of the diseased panicles of $B_2F_3$9311 were at Grades 1-9, and the diseased panicles at Grades 0-3 accounted for 38.34% of total investigated ones. Therefore, $B_3F_2$9311 and $B_2F_3$9311 were susceptible to blast evidently (S). In B_3MH63, B_3F_2MH63 and B_2F_3MH63, the healthy panicles were quite more than in the transgenic generations of 9311. With 80.41% of the diseased panicles at Grades 0-3, B_4MH63 showed the obvious resistance to blast (R). B_3F_2MH63 and B_2F_3MH63 showed the moderate resistance to blast (MR). However, B_3PA64S and B_1F_3PA64S were both susceptible to blast (S). As listed in Table 2, all transgenic generations of 9311 belonged to MS or S level, their resistance to blast was enhanced greatly compared with their parents. All transgenic generations of MH63 and PA64S had shown a stronger resistance against blast than their parents. $B_4$9311 and $B_3F_2$9311 showed significant differences from their parents in disease index and incidence of disease, except for $B_2F_3$9311. As to the transgenic generations of MH63, the disease index and incidence of disease were significantly different comparing B_4MH63 and B_3F_2MH63 with their parents. And the disease index comparing B_2F_3MH63 with its parents was also significantly different. However, the resistance to blast of transgenic generations derived from PA64S was weaker, compared with transgenic generations from 9311 and MH63. The disease index was significant between B_3PA64S, B_3F_1PA64S and PA64S, but no significant differences were found in the incidence of disease.

As a whole, the rice blast was more serious in 2004 than in 2003. It might be due to high temperature at the heading stage of rice during first ten days of August, and no continued overcast and rainy days were occurred till full-heading of rice in 2003, while rice was grown under low temperature and high humidity conditions during heading in 2004, resulting in less infection by rice blast in 2003 than in 2004. The resistant effect of ZH9(R) was very evident. During two years all ZH9(R) plants suffered from blast at Grades 0-3, which manifested a high resistance to rice blast. Its panicles at Grade 0 (healthy) were 81.13% of total investigated panicles in 2003 and 80.20% in 2004. Furthermore, the incidence of disease was less than 2% and the disease index was less than 3.5 during two years. The difference between transgenic generation and its corresponding parents on the incidence of disease and disease index was less in early backcrossed and selfed generations in 2003 (Table 2), while the resistance against blast increased significantly in the advanced-generations of backcross in 2004.

Identification of the resistance to blast of testcross combinations

In 2003 and 2004, all diseased panicles of hybrid rice and testcross combinations were at Grades 0–9. The data in Table 3 showed that the disease was more serious in 2004 than in 2003, but diseased panicles at Grades 0-3 accounted for 40-59% of total investigated panicles so they were moderately susceptible to rice blast (MS). However, there was no significant

Table 3. Evaluation of rice blast disease for testcross combinations and their corresponding check hybrid combinations in 2003 and 2004.

Combination	No. of investigated panicles	No. of panicles at disease grades 0 to 3	Incidence of disease (%) (Mean±*SD*)	*t* value	Disease index (Mean±*SD*)	*t* value
Liangyoupeijiu	1263	653	22.97±1.07	-	37.25±1.05	-
B_2PA64S/$B_2$9311	1260	716	21.75±0.93	1.381	34.86±1.31	2.316
Shanyou 63（CK）	1196	637	21.39±1.37	-	36.23±2.19	-
Zhenshan 97A/ B_2MH 63	1237	709	19.89±0.80	1.983	34.13±1.28	1.495
Liangyoupeijiu (CK)	1297	570	26.60±1.89	-	45.19±2.09	-
B_2PA64S/$B_3$9311	1261	643	24.35±2.38	2.391	38.61±1.80	5.713**
Shanyou 63 (CK)	1326	609	26.13±0.92	-	41.89±1.81	-
Zhenshan 97A/B_3MH63	1381	736	21.61±2.67	3.189*	37.53±3.12	2.483

*, ** Show significant difference compared with CK at $P<0.05$ and $P<0.01$, respectively.

difference comparing B_2PA64S/$B_2$9311 and Zhenshan 97A/B_2MH63 with their respective control combinations Liangyoupeijiu and Shanyou 63 in the incidence of disease and disease index; and their diseased panicles at Grades 0-3 accounted for 56.83% and 57.32% of total investigated ones respectively in 2003. In 2004, the diseased panicles of B_2PA64S/$B_3$9311 at Grades 0-3 were 50.99% of total investigated ones and its disease index was significantly different from Liangyoupeijiu; and the diseased panicles at Grades 0-3 of Zhenshan 97A/B_3MH63 were 53.29% of total investigated ones with a significant difference in the incidence of disease than that of Shanyou 63.

DISCUSSION

Transferring of lysozyme gene by backcrossing

The resistant source is the basic material used in rice breeding to induce resistance against blast. Because of the complexity and variability of physiological races of *Magnaporthe grisea* and lack of resistance sources, it is difficult to breed some new rice varieties with broad-spectrum and high resistance to *Magnaporthe grisea* by conventional breeding methods. The plant gene engineering provides a good platform for resolving the problem of resistant sources against rice blast.

The lysozyme chitinase belongs to a broadly-distributed enzymatic family, which can decompose amylase ß-1,4-indican bonds in bacterial or fungal cell wall components to resist the invasion of pathogenic bacteria or fungi. Dùring et al [10] transferred the lysozyme gene from the T_4 bacteriophage into potatoes to improve the resistance against potatoes disease induced by *Erwina carotovora*. Nakajima et al [11] and Trudel et al [12] transferred the lysozyme gene from the egg white and the human body into tobaccos, which was resistant to both pathogenic bacteria and fungi. Xu et al [13] transferred lysozyme gene from the T_4 bacteriophage into the rice Nan 29. As a result, the preliminary experiments on inoculation of rice blast pathogens and the field experiment on selection and breeding showed that the transgenic plants are more resistant to blast than their controls. In this study, we used the transgenic rice Zhonghua 9 as the donor of the lysozyme gene and the restorer lines 9311 and Minghui 63 and the sterile line Pei'ai 64S as the recurrent parents, the lysozyme gene was transferred into the super-hybrid rice parents by successive backcrossing. The molecular identification approved that the foreign lysozyme gene had been transferred into partial progenies. This result indicated that it was an effective approach for breeding new rice varieties with transgenic target characters via transferring a target gene from the transgenic materials into other elite rice varieties by backcrossing. Thus, the combination of gene engineering technique and common breeding technique can accelerate breeding of new rice varieties with transgenic target characters enormously, which will help us to realize the merit of transgenic rice in breeding program.

Factors affecting the effect of identifying the resistance to rice blast

Rice blast can be harmful to rice at any growing stage. Affected by environmental conditions, pathogen pathogenicity, varieties distribution and another factors, population compositions of *Magnaporthe grisea* obviously varied with regions [14]. The new rice variety which will be planted on a large area, should be identified accurately and systematically during different growth periods and under distinct eco-environmental conditions for two or three years before extensive commercial use. Therefore, the inoculation time and method of microbial culture, the growing stage of rice, the microclimate in a field and the genotype of rice (homotype or heterotype) will have a great impact on identification. Rice blast prevails mainly in June, July and August in Hunan Province. An unexpected low temperature after long-term high temperature (lasting 5 or 7 days) along with high humidity caused a catastrophic outbreak of rice blast. Mao et al [15] found that 3-5 days of treatment by durative low temperature (≤20℃) benefited the occurrence of rice blast. In our experiment, the transgenic generations were planted in different disease nurseries in 2003 and 2004. In 2003, it was not cool and continuously rainy until the late heading period at the middle ten days of August. Therefore, the panicle blast happened later. At the heading stage to full-heading stage in 2004, there was low temperature and high

humidity, which resulted in severe blast.

During the identification of blast resistance, to inoculate a single strain needs too much time, especially when a large quantity of breeding parents or early generation materials are identified. He et al [16] suggested the inoculation by mixed strains for the resistance identification. The dominant pathogen strain with stronger pathogenicity in Hunan Province were mixed for inoculation in this study. Consequently, a visible effect was observed. Almost all identified plants suffered from rice blast and the transgenic generations and testcross showed lighter diseased symptom than the control parents and combinations. This indicated that the gene resistant to blast had been transferred into partial plants, whereas the effect of resistance to blast was various in different donor varieties. Though a part of transgenic generations exhibited a limited resistance in 2003, parents were set as controls in both 2003 and 2004 because of inadequate infection. The result showed that the resistance of all transgenic generations was stronger in 2004 than that in 2003. The transgenic generations of 9311 and MH63 had stronger resistance to blast than the transgenic generations of PA64S in both 2003 and 2004, which can be ascribed to higher resistance levels in backcross generations of 9311 and MH63 than that of PA64S. We have noted that the transgenic generations of MH63 had a stronger resistance to blast than the ones of 9311 in 2004, which may concern the resistance of parents. For example, MH63 possesses the lowest incidence of disease and disease index, compared with PA64S and 9311. There were also difference in different generations for the same variety in their resistance to blast, for example in transgenic generations of 9311, B_2F_2 generation had a stronger resistance than B_3 in 2003, while B_4 and B_3F_2 generations were stronger than B_2F_3 in 2004. In another example, the resistance of transgenic generations of MH63 and PA64S changed little in 2003, but the resistance of B_4 and B_3F_2 generations was obviously stronger than that of B_2F_3 and the resistance of B_3F_1PA64S was slightly stronger than that of B_1F_3PA64S in 2004. It is clear that the resistance of the advanced backcross and selfed generations to rice blast is much stronger than that of the early generations. For different backcross generations, the F_3 generation has a weaker resistance than the F_2 and F_1 generations. Therefore, further investigation is needed to find out whether different conclusions will be made to the same backcross generations. In this study, only backcross generations were used to further testcross. Compared with the contrastive hybrid combinations, the resistance of testcross combinations had been raised vastly; however, to confirm whether the testcross combinations of selfed generations will have a stronger resistance to the disease needs further research.

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