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RESEARCH ARTICLE

Identification of molecular markers linked to rice bacterial blight resistance genes from *Oryza meyeriana*

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Abstract Y73 is a progeny of asymmetric somatic hybridization between *Oryza sativa* cv. Dalixiang and the wild rice species *Oryza meyeriana*. Inoculation with a range of strains of *Xanthomonas oryzae* pv. *oryzae* showed that Y73 had inherited a high level of resistance to rice bacterial blight (BB) from its wild parent. An F_2 population of 7125 individuals was constructed from the cross between Y73 and a BB-susceptible cultivar IR24. After testing 615 SSR and STS markers covering the 12 rice chromosomes, 186 markers were selected that showed polymorphism between Y73 and IR24. Molecular markers linked to the BB resistance genes in Y73 were scanned using the F_2 population and the polymorphic markers. The SSR marker RM128 on chromosome 1, the STS marker R03D159 on chromosome 3 and the STS marker R05D104 on chromosome 5 were found to be linked to the rice BB resistance genes in Y73.

Keywords *Oryza meyeriana*, bacterial blight resistance gene, genetic population, linkage molecular marker

1 Introduction

Bacterial blight (BB) of rice, caused by *Xanthomonas oryzae* pv. *oryzae*, is one of the most important bacterial diseases worldwide. It is responsible for heavy losses of global rice production, generally in the range 20% to 30% but as high as 50% in years when the disease is prevalent[1]. Chemical control is temporarily effective but often causes environmental pollution. In contrast, methods that depend on the innate defense system of plants are more acceptable and environmentally friendly. While the use of resistant cultivars remains an important strategy for biological control of BB, resistant cultivars often succumb to infection during long-term cultivation due to the emergence of new *X. oryzae* pv. *oryzae* strains. There is therefore a continuing and urgent need to identify new sources of genetic resistance for use in breeding programs to improve the BB resistance of commercial rice cultivars.

Oryza meyeriana is one of the most important wild rice resources identified in South and South-east Asia. It is adapted to survive in harsh environments and possesses many useful traits not present in cultivated rice, including high level of resistance to BB[2,3]. Although it has valuable resistance genes that could be used to broaden the existing narrow genetic base of rice and breed new resistant cultivars, it is not easy to introduce these valuable traits into cultivated rice through conventional breeding, mainly because *O. meyeriana*, with its GG genome, is only distantly related to *Oryza sativa*, which contains the AA genome[4]. The consequent sexual incompatibility between the two rice species inhibits the isolation and cloning of resistance genes in *O. meyeriana* and their utilization in breeding programs.

Somatic hybridization through the fusion of two somatic protoplasts has already been used to overcome sexual incompatibility and produce inter-species hybrids, and this technique has been improved as asymmetric somatic hybridization to reduce the co-introduction of negative traits from the donor species[5]. Using this breeding technique, we have introduced BB resistance from *O. meyeriana* into a *japonica* rice cv. Dalixiang, which has good agronomic characters but is susceptible to BB[3,6].

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Y73 is one of the progeny lines from the asymmetric somatic hybridization, and it combines the high level of BB resistance of *O. meyeriana* with the good agronomic characters of cv. Dalixiang, including high yield and good grain quality. Our long-term aim is therefore to isolate and clone the gene(s) in Y73 conferring BB resistance through a map-based cloning strategy. In this study, molecular markers linked to BB resistance genes in Y73 were screened using an F_2 population derived from the cross of Y73 with a well-studied BB susceptible *indica* cultivar IR24. This provides the basis for future work to segregate, fine map and clone the resistance genes.

2 Materials and methods

2.1 Plant materials

Y73, derived from the progeny of asymmetric somatic hybridization using *O. meyeriana* as the donor and *O. sativa* ssp. *japonica* cv. Dalixiang as the recipient, was planted in Hangzhou to survey its resistance spectrum. An F_1 population was obtained by the cross between Y73 and IR24 (*O. sativa* ssp. *indica*), and the F_2 segregating population was composed of 7125 individuals. Each individual was inoculated with *X. oryzae* pv. *oryzae* strain PXO124 as described below to evaluate resistance, and 50 extremely resistant and 50 extremely susceptible individuals were then selected to screen for linkage markers.

2.2 Pathogen inoculation

The *X. oryzae* pv. *oryzae* strains used in this study included PXO86, PXO79, PXO71, PXO112, PXO99, PXO280, PXO145, PXO87 and PXO124 from the Philippines and ZHE173 from Zhejiang Province of China. Inoculation was performed by the leaf-clipping method described by Kauffman et al.[7] and Sanchez et al.[8]. Briefly, at the booting stage (about 40 days after transplanting), the uppermost fully expanded leaves were clipped with scissors about 10 mm from the tip then dipped into the inoculum. The strains used for inoculation were grown for 48 h on potato semisynthetic agar[8] and the density was adjusted to 10^9 CFU·mL^{-1} when suspended in sterile water. Each strain was used to inoculate 4 leaves on each of 10 plants. Control plants were mock-inoculated with sterile water. Leaf length and lesion length were measured 3 weeks after inoculation and the proportion of the leaf affected was calculated.

2.3 DNA extraction

DNA was extracted from fresh young leaves of rice employing the modified CTAB method described by Wu et al.[9]. The DNA solution was diluted to 100 ng·μL^{-1} and stored at 4°C to provide the template for the polymerase chain reaction (PCR).

2.4 SSR and STS analysis

Simple sequence repeat (SSR) marker primers were synthesized by Nanjing Genscript Company. PCR amplification was performed with 20 μL volumes, containing 2 μL of PCR buffer, 1 μL of 100 ng·μL^{-1} DNA, 0.5 μL of 10 mmol·L^{-1} dNTPs, 14 μL dH_2O and 0.5 μL of 5 U·μL^{-1} Taq DNA polymerase. Amplifications were carried out on a Mastercycler Thermocycler (Eppendorf, Germany) under the following conditions: 3 min at 94°C, followed by 32 cycles of 30 s at 94°C, 45 s at 55°C and 30 s at 72°C, succeeded by 5 min at 72°C for final extension. Amplified products were separated by electrophoresis on 8% polyacrylamide gels, and the band patterns were visualized using silver staining[10,11].

Sequence-tagged site (STS) marker primers were synthesized and used in PCR as described above for SSR. The PCR conditions were: 4 min at 94°C, followed by 32 cycles of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C, succeeded by 5 min at 72°C for final extension. Amplified products were separated by electrophoresis on 3% agarose gels, and the band patterns were visualized using ethidium bromide staining.

2.5 Calculations

Linkage analysis was performed using the band-patterns of the individual plants with extremely high level of resistance or extremely high levels of susceptibility.

3 Results

O. meyeriana, one parent of the asymmetric somatic hybridization, was highly resistant to all *X. oryzae* pv. *oryzae* strains (percentage of lesion length to leaf length < 7%) while the other parent, cv. Dalixiang, was highly susceptibility to almost all of the strains. The hybrid progeny Y73 possessed a high level of the wild rice BB resistance (Fig. 1). Because Dalixiang and Y73 were *japonica*, an *indica* cultivars IR24 was selected as another parent of the segregating population to produce marker polymorphism between the parents. IR24 is a well-studied BB susceptible cultivar with a susceptible phenotype confirmed by the data in Fig. 1. The significant difference in BB resistance between Y73 and IR24 made it possible to examine segregation for resistance in the F_2 population.

As shown in Fig. 1, PXO99, PXO280 and PXO124 induced the most susceptible phenotypic response in IR24, and PXO99 produced a much longer lesion in Y73 than the other strains. Thus, PXO280 and PXO124 appeared to be most suitable for distinguishing the resistance of the

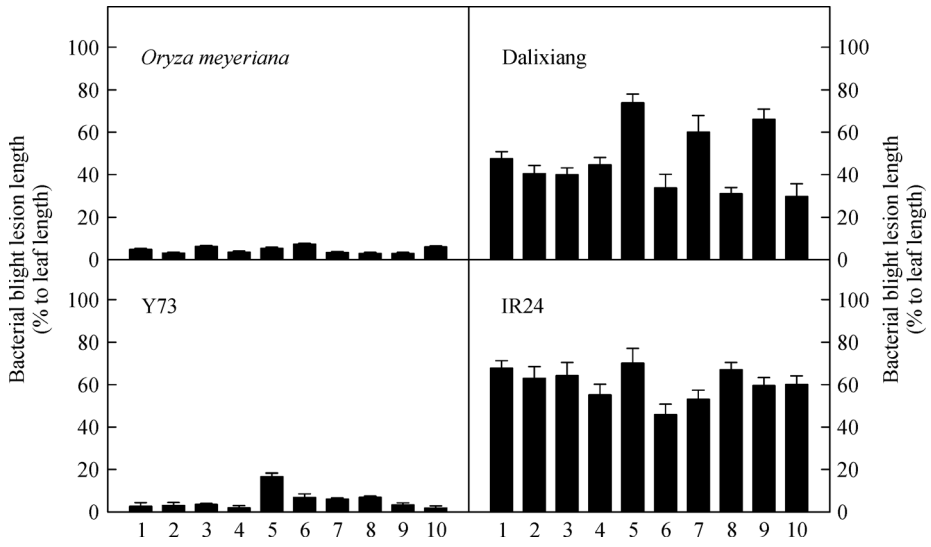

Fig. 1 Bacterial blight lesion length (% to leaf length) of *Oryza meyeriana*, Dalixiang, Y73 and IR24 at 21 days after inoculation with different *Xanthomonas oryzae* pv. *oryzae* test strains. 1 to 10 are ten different *X. oryzae* pv. *oryzae* strains of PXO86, PXO79, PXO71, PXO112, PXO99, PXO280, PXO145, PXO87, PXO124 and ZHE173, respectively. Bars show the SE of treatment means.

parents to the maximum extent. Dalixiang was highly susceptible to PXO124 but relatively resistant to PXO280, and so the loci linked to BB resistance in Y73 might come from Dalixiang not from the wild rice, if the resistance of F_2 population was evaluated by inoculating with PXO280. Based on this resistance spectrum analysis, PXO124 was finally chosen as the *X. oryzae* pv. *oryzae* strain to evaluate the resistance of the individuals in the segregating population.

Six hundred and fifteen SSR and STS markers were scanned to find those with polymorphism between the parents of Y73 and IR24. One hundred and eighty-six polymorphic markers covering 12 rice chromosomes were then used in this study. After scanning the 50 extremely resistant and 50 extremely susceptible individuals of the F_2 population, three linkage regions were identified on chromosomes 1, 3 and 5 (Fig. 2). The linkage region in chromosome 1 was between SSR markers RM5461 and RM1361 (42.5 cM interval) and the nearest marker was RM128 (Table 1a). RM128 was biased to the resistant parent Y73 in the marker type with 35 individuals resembling Y73, 13 individuals resembling IR24 and two hybrid types among the 50 resistant plants (Table 1a). The linkage region in chromosome 3 was between STS marker R03D146 and SSR RM55 (22.2 cM interval) and the nearest markers were R03D145 and R03D146 (Table 1a). The linkage region in chromosome 5 was between STS marker R05D104 and SSR RM1361 (4.5 cM interval) and the nearest markers were RM3348 and RM6972 (Table 1a). We further enlarged the resistant population to 7125 F_2 individuals expecting to narrow down the linkage region. However, the linkage ratio in the three regions considerably decreased in the enlarged population as the resistance of the individual plants became weaker than that of the 50 extremely resistant individuals (data not shown). These results indicated that the three resistance loci were polymerized in the individuals with an extremely resistant phenotype but that these loci were gradually segregated when the population was enlarged and so the resistance became weaker. Further fine mapping of these loci will require segregation of the multiple loci by constructing populations of chromosome segment substitution lines or nearly isogenic lines.

To confirm the linkage regions identified, 50 highly susceptible individuals were also scanned with the 186 polymorphic markers. The band type of the markers in the three loci was biased to the susceptible parent IR24 and the hybrid as expected (Table 1b). This result also indicated that these chromosome loci were linked to the BB resistance of Y73.

4 Discussion

Many wild species of rice have a high level of BB resistance and four resistance genes have been identified in them to date. *Xa21*, identified in *O. longistaminata*, was the first resistance gene discovered in wild rice. This gene encodes a receptor kinase-like protein with a leucine-rich repeat domain that determines race-specific recognition[12,13]. Another BB resistance gene, *Xa27,* was identified in *O. minuta* and its product is a nuclear localized type-III effector[14,15]. *Xa23* and *Xa29* were identified in *O. rufipogon* and *O. officinalis*, respectively, and both have been fine mapped[16,17]. These genes have been introduced into cultivated rice in breeding programs

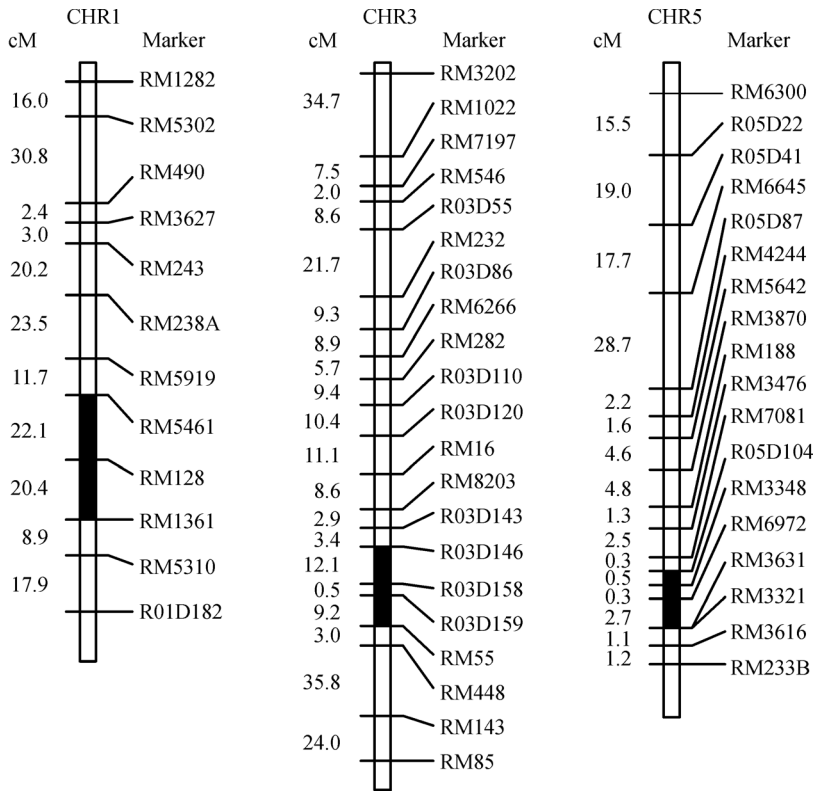

Fig. 2 Locations of the linkage regions (shown in black) on the genetic maps of rice chromosomes 1, 3 and 5. The left sides of the chromosomes show the genetic distance (cM) between adjacent markers, and the marker names are indicated on the right sides of the chromosomes.

Table 1 Analysis of linkage markers

Markers	Chromosome	Location/cM	Y73	Hybrid	IR24	χ^2
(a) Resistant						
RM5919	1	101.0	32	8	10	54.1
RM5461	1	112.7	33	6	11	60.3
RM128	1	134.8	35	2	13	74.1
RM1361	1	155.2	32	9	9	53.1
RM5310	1	164.1	32	13	5	50.9
R03D143	3	143.0	24	17	9	22.8
R03D146	3	146.4	26	17	7	28.2
R03D158	3	158.5	33	4	13	63.6
R03D159	3	159.0	33	9	8	56.9
RM55	3	168.2	25	16	9	25.8
RM7081	5	103.6	34	9	7	61.1
R05D104	5	103.9	37	8	5	75.7
RM3348	5	104.4	39	4	7	88.5
RM6972	5	104.7	39	6	5	87.1
RM3616	5	107.4	36	7	7	71.4
(b) Susceptible						
RM1361	1	155.2	9	5	36	73.3
R03D159	3	159.0	8	10	32	52.2
RM6972	5	104.7	12	5	33	61.8

Note: $\chi^2 > \chi^2_{0.05}$ (1:2:1) = 5.99.

and widely used for sustained improvement of rice productivity.

Although *O. meyeriana* has long been shown to be highly resistant to BB[2,3], the resistance mechanism is poorly understood and no resistance gene has yet been identified. The primary mapping of the resistance genes from *O. meyeriana* in this study is of great significance to the utilization of this material in breeding programs. The four BB resistance genes (*Xa21*, *Xa27*, *Xa23* and *Xa29*) identified in wild species of rice have been respectively located in chromosomes 11, 6, 11 and 1[12,14,16,17], of which *Xa29* was located within a 1.3 cM region flanked by molecular markers C904 and R596 on chromosome 1. Another four cloned genes (*Xa1*, *xa5*, *xa13* and *Xa26*) were from cultivated rice and mapped to chromosome 4, 5, 8 and 11, respectively[18,21]. *xa5* gene was mapped to a 0.5 cM interval between the markers RS7 and RM611 on the distal end of rice chromosome 5. The genome regions associated with resistance that have been identified in this study are different to the loci of the previously cloned BB resistance genes. This suggests that Y73 contains new genes conferring a high level of resistance to BB. The results are an important first step toward isolating and cloning these new genes from *O. meyeriana* and the linkage markers could also be of immediate use to assist selection and thus to improve rice resistance to a broad spectrum of BB strains.

5 Conclusions

Y73 is a progeny of asymmetric somatic hybridization between *O. sativa* cv. Dalixiang and the wild rice species *O. meyeriana*. Y73 has inherited a high level of resistance to rice BB from its wild parent. We used the F_2 population of 7125 individuals and 186 polymorphic markers to identify the linkage molecular markers to the resistance genes of *O. meyeriana*. These results suggest that the SSR marker RM128 on chromosome 1, the STS marker R03D159 on chromosome 3 and the STS marker R05D104 on chromosome 5 were linked to the rice BB resistance genes in Y73.

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Compliance with ethics guidelines Jing Wang, Chen Cheng, Yanru Zhou, Yong Yang, Qiong Mei, Junmin Li, Ye Cheng, Chengqi Yan, and Jianping Chen declare that they have no conflict of interest or financial conflicts to disclose.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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