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Full Length Research Paper

Production and molecular characterization of wide cross derivatives in rice

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The reduced genetic variability of modern rice varieties (*Oryza sativa* L.) is of great concern because it reduces the possibilities of genetic gain in breeding programs. Introgression lines (ILs) containing genetic fragment from wild rice can be used to obtain new improved cultivars. The objective of the present study was to develop ILs from the cross between *O. sativa* x *O. longistminata* aiming to be used in rice breeding program. In the present study, 12 ILs were produced. Among them, three ILs were highly resistant to all the isolates of bacterial blight from North West Frontier Province (NWFP) of Pakistan. A 900 bp DNA fragment linked to *Xa*21 was raised in these introgression lines and in *O. longistminata* by a pair of primers confirming the presence of *Xa*21 gene in these lines. Results indicated that *Xa*21 has broad spectrum of resistance to bacterial blight and wild species are the useful source for resistance.

Key words: Bacterial blight, molecular marker, *Oryza sativa*, rice.

INTRODUCTION

Rice is the most widely cultivated food crop all over the world. Its production is constrained by diseases of fungal, bacterial and viral origin. Bacterial blight (BB) of rice caused by *Xanthomonas oryzae* pv. *Oryzae (Xoo)* was first noticed by Japanese farmers. The disease is known to occur in epidemic proportions in many parts of the world, incurring severe crop loss of up to 50% (Gnananamickam et al., 1999). Crop loss assessment studies in Pakistan have revealed that this disease reduces grain yield to varying levels, depending on the stage of crop, degree of crop susceptibility and to a great extent, conduciveness of the environment in which it occurs (Akhtar et al., 2005).

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Abbreviations: ILs, Introgression lines; **NWFP,** North West Frontier Province.

Primarily, resistance breeding is the most economical and environmentally safe approach for achieving yield stability. Currently, more than 20 genes (*Xa*1 to *Xa*27) conferring host resistance against various strain of *Xoo* has been identified (Lin et al., 1996; Zhang et al., 1998; Khush and Angels, 1999; Chen and Wang, 2002; Yang et al., 2003). Majority of these genes are from cultivated *indica* rice with *Xa*4 being the most widely used in the Indian sub-continent (Virk et al., 2004). Two genes *Xa*21 and *Xa*23 have been identified from two related "A" genome wild species, *O. longistminata* and *Oryza rufipogon*, respectively (Khush et al., 1990; Zhang et al., 1998). The severity and significance of damages caused by infection to rice in Pakistan have necessitated the development of strategy to control and manage the disease, so as to reduce crop loss. Major genes for qualitative resistance and polygenic factors controlling quantitative resistance have contributed in the breeding for disease resistance cultivars. We report here the successful incorporation of *Xa*21 gene from *O. longistminata* into JP5 and its confirmation by molecular marker.

MATERIALS AND METHODS

Development of introgression lines

We have previously identified *O. longistminata* as showing resistance reaction to all the isolates of *Xoo* from NWFP (Shah et al., 2008). This wild species was used as donor parent and JP5 as recurrent parent in the backcrosses. The recurrent parent is a famous cold tolerant variety grown in NWFP. F1 plants obtained were advanced up to the BC_4F_1 generation by continuous backcrossing and selecting phenotypically similar plants to the recurrent parent having resistance to *Xoo*. The selected BC_4F_1 plants were selfed to produce BC_4F_2 seed. Based on phenotypic similarity to their recurrent parent, BC_4F_2 plants were selected for further studies.

Isolation and multiplication of *Xoo*

Sixty diseased samples of rice leaves were collected from different areas of the NWFP and used for the isolation of *Xoo*.

Single cell culture

Single cell was taken with the help of sterilized inoculating wire loop from slimy yellowish bacterial colony developed around the infected samples and further streaked on nutrient agar plate in zigzag manner. After streaking, plates were incubated at 25 - 26°C for 3 days.

Pathogenecity test/confirmation of pathogenic nature

All isolates were subjected to the pathogenecity test to confirm their pathogenic nature by injection infiltrations technique developed by Klemet (1963) and Klemet et al. (1964).

Inoculation of rice germplasm in glass house

Distilled water (5 ml) poured in each culture plates and bacterial colonies were suspended and the concentration of inoculums was adjusted to 10^8 cfu/ml. The suspension of all isolates was bulked in plastic bucket and shacked for uniformity. The plants were sprayed with water to create wet conditions which is favorable for disease development. Inoculation was done by cutting five leaves, approximately 5 cm from the tips of each line with scissor dipped in inoculums. On the basis of diseased data, these germplasms were categorized as resistant or susceptible using standard IRRI procedure (Table 1). After 15 days, diseased data were recorded to identify the degree of pathogenecity on 0 - 4 rating scale, Standard Evaluation System IRRI, 1996.

Sequence tagged site (STS) marker assisted confirmation of the presence of *Xa*21 gene in the ILs

Young leaves at seedlings stage were harvested for the isolation of genomic DNA. The DNA was extracted following the method of Dellaporta et al. (1983). The concentration of extracted genomic DNA was measured by fluorometer. The DNA was diluted to10 µg/µl, using sterilized distilled water and stored in microfuge tubes at 4°C for further use. Amplification of *Xa*21 linked fragment was carried out by using specific primers. Amplification reaction was carried out in 25 µl reaction volumes containing 50 ng genomic DNA, 1.0 µM each of the primer for *Xa*21, 100 µM each of dATP, dCTP, dGTP, and dTTP, 0.2 unit of Taq DNA polymerase, 1X Taq polymerase buffer and 2.5 mM $MgCl_2$. DNA amplification was performed in thermal cycler programmed as: an initial denaturation of 5 min at 94°C, 35 cycles of 94°C for one minute (denaturation), 55°C for 1 min (annealing) and 72°C for 2 min (extension). One additional cycle of 10 min at 72°C was used for final extension. Amplification product resolved by electrophoresis on 1.5% of agarose gel. The amplified products were observed under Ultra Trans Illuminator after staining with ethidium bromide (10 µl/ml) and scored for the presence and absence of *Xa*21 linked DNA fragments.

Table 1. Categorization of germplasms.

Reaction	Lesion length
Resistant	1 - 5 cm
Moderately resistant	5 - 10 cm
Moderately susceptible	10 - 15 cm
Susceptible	>15 cm

Data analysis

The amplified fragment of the introgression lines were observed and compared with *O. longistminata* and JP5 for the presence (+) and absence (-) of *Xa*21 gene.

RESULTS AND DISCUSSION

Oryza longistminata, a wild relative of rice with AA genome was provided by International Rice Research Institute (IRRI), Philippines and used in our crosses as donor parent. JP5 is high yielding, widely cultivated variety of North West Frontier Province of Pakistan (NWFP) and is highly susceptible to bacterial blight. The main goal for increasing the level of resistance to bacterial blight is to achieve durable resistance in the field. Molecular and conventional approaches were used for developing resistant lines in the background of JP5. After four backcross generations, BC_4F_1 plants were selected after screening against bacterial blight. Each of the recurrent parents was selfed to produce BC_4F_2 progenies. Of these, 70 plants showing the JP5 phenotype were inoculated with all the isolates of bacterial blight. Twelve lines were selected for their better plant type. Three ILs viz. line 10 to 12 exhibited lesion length from 3.6 to 4.3 cm (Figure 1 and Table 2) showing resistant reaction to *Xoo,* demonstrating the effectiveness of resistance gene pyramiding. The recurrent parent JP5 with lesion length 26 cm exhibited highly susceptible reaction at 15 days after inoculation and lesions covering the entire inoculated leaf leading to plant death at 25 days after inoculation. The donor parent, *O. longistminata* showed high level of resistance to all the prevailing isolates of bacterial blight from NWFP with mean lesion length of 1.5 to 3.4 cm. Three ILs viz. Line 10 to 12 have been selected for use in our breeding program.

When plants were inoculated with distinct BB isolates collected from NWFP, the consistent finding was that the lines which carry *Xa*21 showed high degree of resistance

Figure 1. Resistant reactions of introgression lines derived from the cross *O. sativa* x *O. longistminata*. The plants were inoculated with the bacterial isolates collected from NWFP. Leaves 1 to 9 represent introgression lines without *Xa*21 gene and leaves 10 to 12 represent introgression lines with *Xa*21 gene.

Table 2. Reaction of parents and BC4F2 selected plants at 15 day after inoculation with 7 isolates of bacterial blight pathogen *Xanthomonas oryzae pv oryzae*.

Rice genotypes	Lesion length (cm)
JP5	26 ± 1.0
O. longistminata	1.5 ± 0.8
L1	21.9 ± 1.0
L2	20.3 ± 0.8
L3	20.2 ± 1.0
L4	20.5 ± 1.5
L5	25.1 ± 1.0
L6	15.2 ± 1.0
L7	16.6 ± 1.4
L8	24.2 ± 1.3
L9	12.7 ± 0.9
L10	3.6 ± 1.0
L11	3.8 ± 0.6
L12	4.3 ± 0.7

L = line.

over the lines lacking this major gene. This shows that *Xa*21 gene has broad spectrum of resistance to *Xoo*. Similar results were also reported by Swamy et al. (2006) who evaluated bacterial blight resistance in transgenic lines carrying *Xa*21 gene.

DNA analysis of twelve introgression lines, *O. longistminata* and JP5 exhibited two different sizes of bands. The banding pattern were either identical with that of *O.*

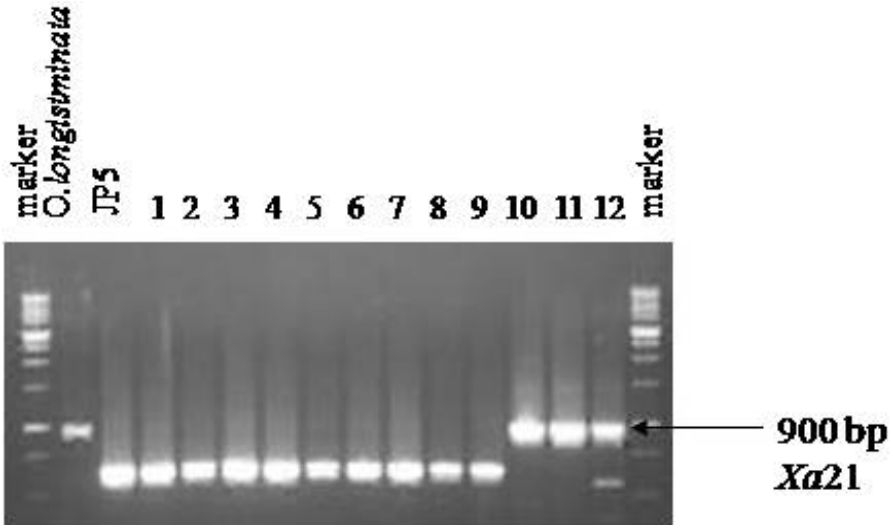

Figure 2. Marker banding pattern of selected BC_4F_2 plants and their corresponding parents from sequence tagged site marker (STS) linked to *Xa*21. Lane 1 to 9 = BC_4F_2 plants without *Xa*21, Lane 10 – 12 = BC_4F_2 plants with *Xa*21.

longistminata (having *Xa*21 gene) or with JP5 (without *Xa*21) gene. The primer pairs for *Xa*21 was previously designed from the sequence of a genomic clone RAPD 248 (Chunwongse et al., 1993) and amplified a 900 bp band from the donor. Out of 12, three ILs along with *O.longistminata* amplified 900 bp size fragment, indicating the presence of *Xa*21 gene (Figure 2). The remaining 9 lines were found to be without *Xa*21 gene. Both molecular and conventional approaches have been used by Ramalingam et al. (2001), Lee et al. (2003) and Kihupi et al. (2001) for the presence of *xa*5, *xa*13 and *Xa*21 in Chinese rice germplasm.

Marker assisted selection increases the efficiency of breeding program for selecting marker genotypes linked to target gene (Mohan et al., 1997). These ILs are the source of *Xa*21 and could be used for transferring *Xa*21 to other rice varieties.

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