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Identification of pi-a gene for resistance to blast in rice (*Oryza sativa* L.) cultivated in Mansehra

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Abstract

A molecular survey was conducted for the screening of Pi-a blast resistance gene advance lines of rice developed by Dr. Fida Muhammad Abbasi, Professor at Department of Agriculture, Hazara University Mansehra. Sequence Tag Site (STS) marker Yac72 was used in this study that amplified 905 bp for presence of Pi-a gene only in advance lines of rice respectively. These advance lines were further restricted by Hinf-1 enzyme which further differentiated the resistance and susceptible advance lines which showed degrees of resistance to the causal organism of blast (*M. grisea*) ranging from highly resistant to susceptible. After digestion of these fragments with Hinf-1, a 905-bp was cut into two fragments of ~635 bp and ~270 bp. The lines with restricted fragments of 635 bp were considered as the lines with Pi-a blast resistance gene. Among cultivated varieties Basmati-385 and Jp-5 shows the presence of pi-a gene having 635 bp and 270 bp fragments each when restricted with Hinf-1. 9 advance lines show having fragments of 635bp and 270bp indicating the presence of Pi-a blast resistance gene while 11 lines were lacking this gene. Grain length of genotypes was also measured that ranges from 4.68 to 7.98 mm. On the basis of grain length the genotypes were categorized into short, medium, long and extra-long. In this study 6 advance lines possessed extra-long, 12 long, 1 medium and only 1 have short grains.

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Introduction

The pathogen *Magnaporthe oryzae* is a type of fungus which causes blast disease in rice which is also known as one of the serious diseases of rice (*Oryza sativa*) found globally. This fungus infects both the panicles along with leaves, and stops filling of grain (Katsube and Koshimizu, 1970). A lot of rice cultivars with blast leaf resistance have been recognized (Miah *et al.,* 2013), and also introducing blast resistance traits in rice cultivars as one of the approach to overcome the occurrence of rice blast on panicles. Up till now, in addition to 80 major blast R genes has been mapped and identified through DNA markers(Bellini *et al.*, 2008); out of which 18were cloned and being used for crop safety (Roy Chowdhury *et al.,* 2012b). To prevent blast disease, different methodologies like cultural practices, fungicides and genetic resistance are interchangeably used by scientists and farmers in the world. The use of fungicides in large amount has a considerable concern for human physical condition and ecological safety. Hence, one of the most powerful strategies for controlling blast disease is utilization of several R genes with overlapped resistance spectra (Wang *et al.,* 2010; Roy Chowdhury *et al.,* 2012a).

Rice (*Oryza sativa* L.) is a staple food for more than half of the world's population (Marathi *et al.,* 2012). In rice physical grain quality plays an important role in consumer preference. Juliano and Duff (1991) concluded that improvement of physical grain quality is the second major objective of rice breeding programs after yield in many rice producing countries of the world. The physical grain quality of rice is a complex trait that is composed of many components such as appearance quality, cooking quality, eating quality and nutritional quality. Each one of these components also consists of many attributes whose values are determined not only by their physiochemical properties but also by the history and cultural traditions of the people in the human communities who consume the rice (Tan *et al.*, 1999).

Dr. Fida Muhammad Abbasi, Professor at Department of Agriculture, Hazara University Mansehra Pakistan has developed advance lines of rice. These lines are high yielding but their physical grain quality has not been properly assessed, therefore, the present study was being proposed with the aims of assessment of physical grain quality and for identification of blast resistance gene Pi-a in these advance lines.

Materials and methods

Plant Material

Advanced lines of rice developed at Department of Genetics, Hazara University, Mansehra by Professor Dr. Fida Muhammad Abbasi were evaluated for grain quality and resistance to blast**.** These advanced lines were planted at National Tea and High value Crops Research Institute (NTHRI), Shinkiari, Mansehra, Pakistan (Fig. 1).

DNA extraction and PCR analysis of advanced lines of rice for the presence of blast resistant (Pia) gene

Total genomic DNAs were extracted from selected plant material by following the method of Dellaporta *et al.,* (1983) procedure. To analyze the quantity and quality of the extracted genomic DNA, Gel electrophoresis was done on 1% agarose gel and stained with ethidium bromide. The concentration of extracted DNA was measured by spectrophotometer and was adjusted from 20 to 50ng/µL by using sterilized distilled water and stored in Eppendorf at 4°C for further use (Fig. 2).

Amplification of Pia gene was carried out by using of pair of allele specific primers yca 72 (Table 1.)Amplification reaction was carried out in 20µL reaction volumes containing 2µl genomic DNA, 0.75 µl each of primer, 1.2 µl each of dNTPs (25 mM each) , 0.4 µl of Taq DNA Polymerase (2 units , Enzynomix), 1X Taq Buffer and 2 µl $MgCl_2$(2.5mM). PCR amplification was carried out in DNA Thermal Cycler (Applied Bio System) set at: an initial denaturation of 5 min at 94°C: 35 cycles of 94°C for 45 sec: 52°C for 45 sec, and 72 °C for 2 min. one additional cycle of 7 min at 72°C was used for final extension. Amplification products were resolved by 2% agarose gel run in 1x TAE buffer.

The amplified products were observed under UV light after staining with ethidium bromide (10ug/ml). The data was scored for the presence or absence of Pia linked DNA fragments.

Determination of grain size

Physical grain quality including grain size was determined by measuring the grain length of 5 unbroken milled kernels. On the basis of average length, the grains were classified by using the scale as reported by Khush *et al.*, 1978 (Table 2).

Results and discussion

PCR analysis of advance lines of rice for the presence of Pi-a gene

A molecular survey was conducted for the molecular screening for Pi-agene for resistance to blast in advance lines of rice. A set of STS marker Yca 72 was used in this study that amplified 905bp fragment in these genotypes, respectively (Fig 3) Among the cultivated varieties, both the genotypes shows the resistance gene presence having 635 bp band when digested with Hinf-I.

Table 1. Primer sequence of Yca 72, used in the study.

Forward Primer	5' –AGGAGAAGAAGCCACCAAGG- 3'
Reverse Primer	5-'GAGCTGCCACATCTTCCTT- 3'

Table 2. Scale used for determining grain size.

Scale	Size categories	Length (mm)
1	Extra-long	More than 7.5
3	Long	6.61 to 7.5
5	Medium	5.51 to 6.6
7	Short	less than 5.5

Among advanced lines of rice, 9 genotypes showed the presence of *Pi-a* gene (635bp) and the rest were nonresistant. Out of 20 advance lines only 9 advanced lines of rice shows the presence of resistance gene when digested with Hinf-I restriction enzyme while other 11 advance lines of rice were considered as susceptible (905bp) because of lacking the pi-a gene amplified fragment.

Genotypes that possessed *Pi-a* gene fragment with 635~bpcondition includes Basmati-385, Jp-5, JP6-6-1, Line-128-2 ,Super NPT, Line-4-22-2, Line-8-2, Line 106-13, Line-12-1, Line-FH-14, Line-78-11.The rest of advance lines such as Line FHM, Line FH5-1, Line 28-1, Line 152,Line FH-6-3, Line-57L,Line-12, Line FH4-2, Line-78-4, Line-78 and Line-FH1-12 were observed to be susceptible as lacking *Pi-a* gene fragment when restricted with Hinf-I (Fig 4 and Table 3).

Table 3. Screening of cultivated varieties and advanced lines of rice for the presence of pi-a gene for resistance to blast in rice.

S. No	Genotypes	Presence/Absence of pi-a gene	S. No	Genotypes	Presence/Absence of pi-a gene
1	Basmati-385	+ ve	12	Line-FH6-3	- ve
2	Jp-5	+ ve	13	Line-12	- ve
3	Line- FHM	- ve	14	Line-FH4-2	- ve
4	Line-JP6-6-1	+ ve	15	Line-8-2	+ ve
5	Line-FH5-1	- ve	16	Line 106-13	+ ve
6	Line-128-1	- ve	17	Line-78-4	- ve
7	Line-128-2	+ ve	18	Line-78	- ve
8	Line-152	- ve	19	Line-12-1	+ ve
9	Super NPT	+ ve	20	Line-FH1-12	- ve
10	Line-57 L	- ve	21	Line-FH-14	+ ve
11	Line-4-22-2	+ ve	22	Line-78-11	+ ve

+ = Presence of Pi-a gene

- = Absence of Pi-a gene.

Table 4. Grain length of cultivated varieties and advance lines of rice (*Oryza sativa* L.).

S. No	Genotypes	Grain Length	S. No	Genotypes	Grain Length
1	Basmati-385	6.92	12	Line-FH6-3	6.80
2	Jp-5	4.65	13	Line-12	7.30
3	Line- FHM	7.87	14	Line-FH4-2	7.30
4	Line-JP6-6-1	7.45	15	Line-8-2	6.90
5	Line-FH5-1	7.98	16	Line 106-13	7.36
6	Line-128-1	7.05	17	Line-78-4	6.71
7	Line-128-2	6.96	18	Line-78	7.86
8	Super NPT	6.86	19	Line-12-1	7.41
9	Line-152	4.68	20	Line-FH1-12	7.43
10	Line-57 L	6.59	21	Line-FH-14	7.68
11	Line-4-22-2	7.61	22	Line-78-11	7.64

Mean followed by the similar letters are not significantly different from each one.

The data was scored using "+" sign for presence of gene (*Pi-a*) and "–" sign for absence of gene (Table 3 and Fig. 3)

Grain Size

Grain length of advance lines and cultivated varieties of rice used in this study was also measured that ranges from 4.6 to 7.9 mm. Maximum grain length was possessed by line-FH5-1 (7.9mm), followed by line-FHM (7.87mm). Minimum grain length was recorded for cultivated variety JP-5 (4.65 mm) followed by line-152 which was 4.68 mm. Among the two cultivated varieties genotypes *viz.*, Basmati-385, possessed long grains while only JP-5 showed short grains. Among the 20 Advance lines 6 possessed extra- long grains, 12 long, 1 medium and only one (line 152) short grains (Table 4 and Fig. 5).

Fig. 1. Advance lines of rice grown at National Tea and High value crop Research Institute (NTHRI), Mansehra, Pakistan.

The main endeavor of rice breeding program is based upon the genetic improvement of grain quality, since it builds the attractive estimation of rice assortments. The reason for this examination was to survey the variety in grain quality attributes among advance lines of rice *(Oryza sativa* L.).

Rickman et al. (2006) reported that size and shape were stable varietal properties that could be used to identify a variety. Kernel type and dimensions are considered to be important to producers, millers, processors and breeders (Slaton *et al.,* 2005; Gayin *et al.,* 2009).

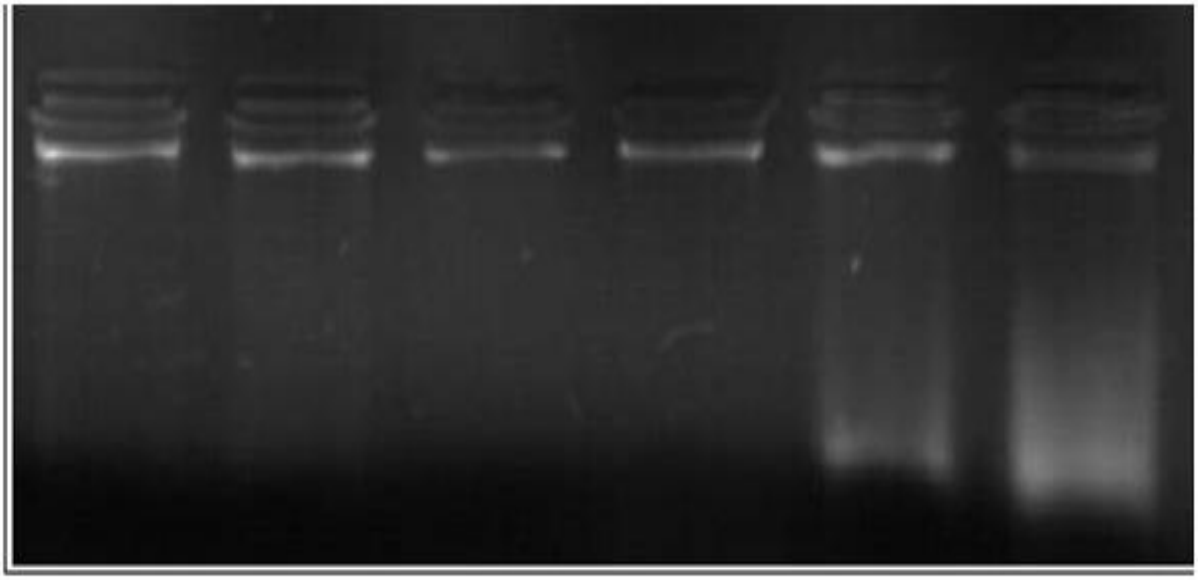

Fig. 2. Genomic DNA extracted from fresh seeds of cultivated varieties of rice using CTAB method and resolved on 2% agarose gel.

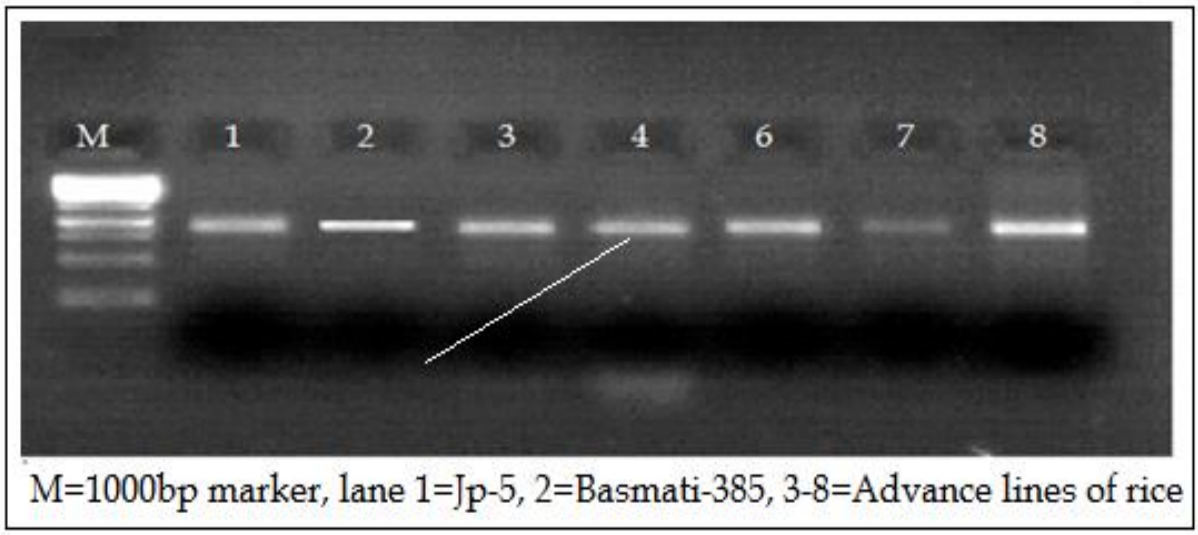

Fig. 3. PCR analysis of cultivated varieties and advance lines of rice for the presence of monomeric band. (Arrow showing 905 bp bands linked to *pi-a* gene).

Though the length and width of the seeds are varietal properties, environmental conditions during their growth could affect these qualities (Irshad, 2001).

Grain length is considered as one of the important agronomic trait for artificial selection in rice breeding. Breeders usually select plants with large seed size for high yield and appropriate grain size for milling yield and market preferences (Ali *et al.*, 2016). However, it is difficult job for breeders to improve grain size efficiently by phenotypes, since the traits are quantitatively inherited (McKenzie and Rutger, 1983). Many quantitative trait loci (QTLs) for grain size have been detected, of which four genes, grain size on chromosome 3 (GS3), grain weight on chromosome 2 (GW2), grain incomplete filling on chromosome 1 (GIF1), and seed width on chromosome 5 (qSW5/GW5), have been isolated and characterized recently (Song *et al.*, 2007; Shomura *et al.*, 2008; Wang *et al.*, 2008; Weng *et al.*, 2008). The major QTL for grain length and weight and minor QTL for grain width and thickness in rice is GS3.

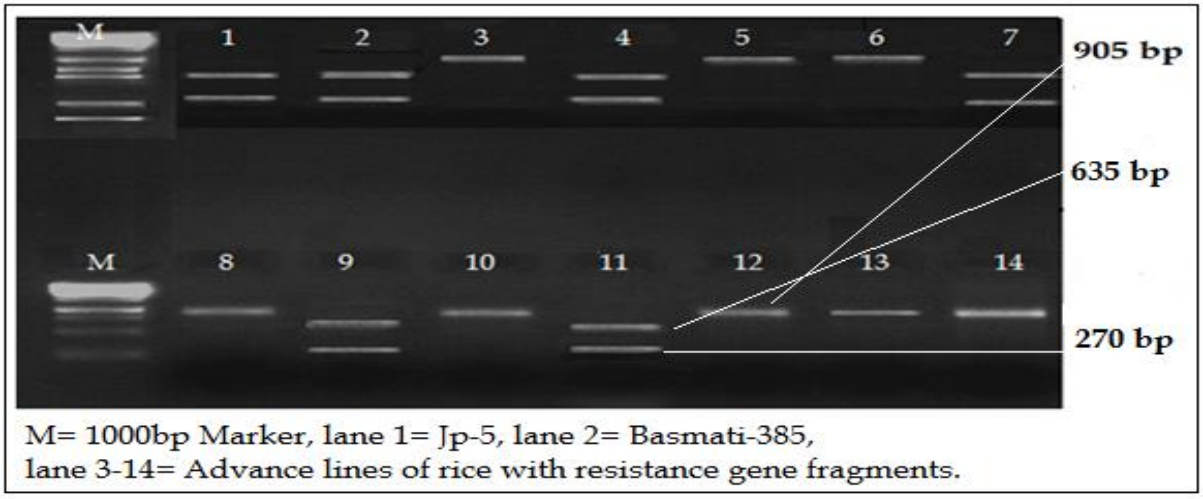

Fig. 4. Restriction fragment length polymorphism of cultivated varieties and advance lines of rice. 905 bp fragments have been digested into 635 and 270 bp fragments. (Arrow showing 635 bp bands linked to *pi-a* gene for resistant).

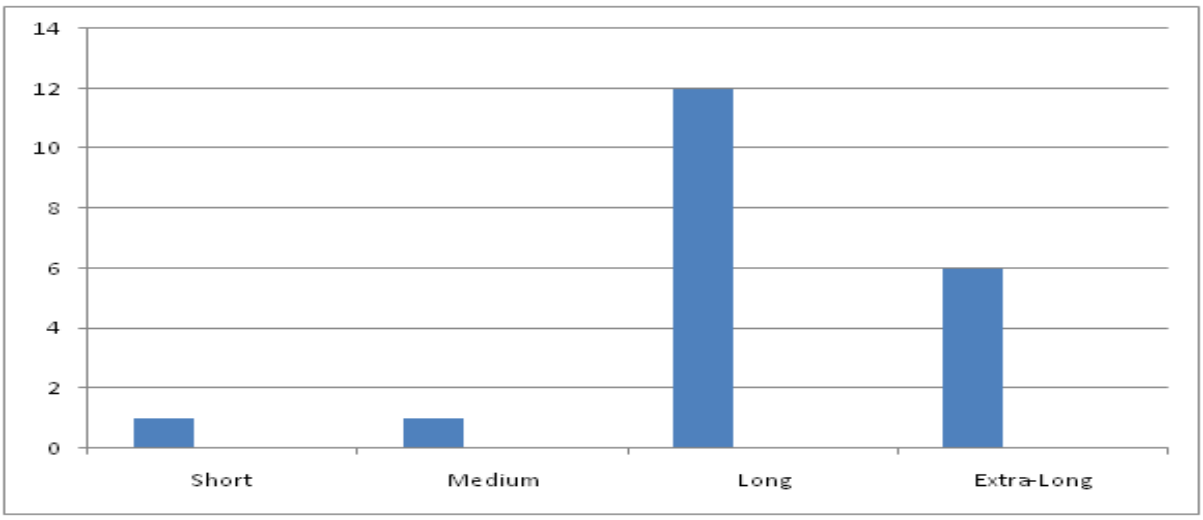

Fig. 5. Grain size of advance lines of rice used in this study. The length of each bar shows number of genotypes.

A mutation in GS3 linked with C-A is highly associated with rice grain length (Fan *et al.*, 2009).

Conclusion

Most of the advanced lines used in this study showed blast resistance gene (*pia*) having fragments size 636~bp. These advanced lines (JP6-6-1, Line-128-2, Super NPT, Line-4-22-2, Line-8-2, Line 106-13, Line-12-1, Line-FH-14, and Line-78-11) can be further analyzed for more blast resistance genes and could be used for further improvement in the breeding program. However, we recommend these advanced lines for further evaluation of other grain quality traits and replicated yield trials.

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