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Characterization of Akhanaphou, an unique landrace from North-East India and its RIL population for rice leaf and neck blast resistance

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Abstract

Rice blast is continues to be the major constraint in sustainable rice production throughout the world. Although many genetic resources harboring single R-genes are available for blast resistance, wide genetic variations exist in the blast fungus lead to breakdown of these resistant varieties soon after its release. To control such a deadly disease, there is a need to identify QTLs which offer durable partial resistance. In this endeavor, we identified Akhanaphou, a unique rice landraces of Manipur showing a high level of resistance to leaf and neck blast across various locations. Upon genetic characterization, we found resistance in Akhanaphou governed by QTLs and two major genes *i.e Pi38* and *Pitp*. Three best stabilized recombinant inbred lines (RILs) showing resistance for leaf and neck blast and having significant homozygosity at various loci were identified based on gene profiling, phenotyping, and agronomic evaluation studies. The shortlisted RILs are valuable genetic resources for the development of blast resistance in rice improvement programs.

Keywords: Akhanaphou, Neck blast, Gene profiling, *Pi38* and *Pitp*

Introduction

In rice, since from the identification of *Pia,* the first blast-R gene (1), more than 100 blast resistance genes and more than 350 QTLs for blast resistance have been identified in various germplasm (2). The wide genetic variation available in *Magnoporthe* may be the driving force in the evolution of rice blast R-genes. Use of resistant cultivar is the primary and most economical approach to control blast disease (3). But increments of novel pathotypes have been reported to cause a breakdown of resistant rice varieties soon after its release (1, 4). Identification of novel R-genes/QTLs or mining the new alleles of blast R-genes with broad spectrum resistance and pyramiding different R-genes/QTLs with different resistance spectra into the elite cultivars are the alternative ways to achieve durable and broad-spectrum blast resistance (6 - 10). Therefore the continuous search for new resistance sources of the blast is important for sustainable rice production.

Ramkumar and coworkers (7) demonstrated that Amano Bavo and Boha Tulasi Joha (two landraces) have novel alleles of *Pi54* and offer higher level of resistance. Wang and coworkers (8) reported the high level of panicle and leaf blast resistance in *japonica* landrace Jiangnanwan. Upon characterizing, RIL population developed from Jiangnanwan identified and fine mapped Pi-*jnw1*gene responsible for panicle blast resistance. It is well established from earlier studies that rice landraces are a good source of identification and mining of novel blast R-genes/QTLs.

India is known for having rich rice diversity and particularly the North Eastern part of India is one of the hotspots for rice biodiversity in the world. Such landraces are worthy candidates for a detailed examination of the blast resistant genes (11). Present study carried out with motivation to identify and characterize novel source of leaf and neck blast resistance. For this work, we selected Akhanaphou (7) which is one of the unique landraces of North-East India, showing high-level of resistance to both leaf and neck blast. Genetic characterization of Akhanaphou and its population for inheritance of leaf as well as neck blast resistance at two different locations revealed the presence of multiple genes. Gene profiling was employed for determining the known blast R-genes. Based on mulitiparametric tests like phenotyping and agronomic evaluation and gene profiling, best RIL lines were selected which can be used for introgression of blast resistance during varietal development program.

Material and Methods

Plant material : Akhanaphou (*indica)* is a popular landrace of Manipur possessing a high level of resistance to leaf and neck blast. One hundred and three RILs (Recombinant Inbreed Lines) (Akhanaphou X Leimaphou) were developed by SSD (Single Seed Descent) method at NEH-RC (North East Hilly-region Research Center), Manipur up to F_8 generation and seeds were received through personal communication. Leimaphou (*indica*) is a commercially important cultivar of rice from Manipur which is popular for its cooking quality and high yield but showing high susceptibility for leaf and neck blast. RIL population was maintained and forwarded at IIRR (Indian Institute of Rice Research) for the next two successive generations by using SSD method. An F_2 population was also developed using same donor and recurrent parent of RILs *i.e.* Akhanaphou and Leimaphou respectively.

Phenotyping of Akhanaphou, RILs and F_2s for blast resistancc : Akhanaphou was extensively screened for leaf blast as well as neck blast resistance as done by Rama Devi and coworkers (12). Screening for leaf blast resistance was done at two locations, IIRR, Rajendranagar, Hyderabad and at NEH-RC, Manipur. However, screening for neck blast was done only at one location *i.e.* NEH-RC, Manipur. Screening for the leaf blast resistance was carried out on UBN (Uniform Blast Nursery) at IIRR, Rajendranagar for the three seasons (Nov-2013, Nov-2014, and Sept-2015) with two replications. At NEH-RC, Manipur, Akhanaphou was screened under field condition for both leaf and neck blast resistance in two successive seasons (Aug-2014 and Aug-2015) with two replications.

At IIRR, Rajendranagar, the entire nursery bed was surrounded from all sides by two rows of HR12 to function as a spreader source for the pathogen. About 30-40 ml of the spore suspension (NLR-1 isolate) of the blast pathogen (approximately 10^5 spores per ml mixed with Tween-20 @ 0.2%) was sprayed on 15-day old seedlings using a glass atomizer. The inoculum was also provided by placing pieces of infected leaves over the test material. High humidity (95%) was maintained using sprinklers. The observation on disease reaction was recorded when the susceptible check was severely infected by the blast. For F_2s, individual plants in each line were scored based on 0–9 scale (13). For RILs, ten plants from each line were scored and the average score was used for further QTL analysis.

Screening for leaf and neck blast was done in the field conditions, under natural disease pressure and also augmented with artificial inoculums. For leaf blast screening, modified UBN conditions were maintained and pathogen inoculums was augmented with a spray of leaf blast infected leaves and a daily spray of water for maintaining high humidity. For neck blast screening, individual plants were syringe inoculated with a spore suspension (a mixture of pathogen's pure cultures). Phenotyping for leaf blast was done based on 0-9 scale (13).

Field resistance of Akhanaphou for leaf and neck blast was also checked across different parts of the country in AICRP (All India Coordinated Research Project) on rice during Kharif-2012. In the AICRP on rice trials, Akhanaphou was checked for its reaction to the leaf blast at 23 centers whereas; the neck blast resistance was checked at six centers.

The RIL population and F_2 populations were also screened for the leaf blast resistance at IIRR, Rajendranagar during Feb-2012 and Sep-2015 respectively whereas, for leaf and neck blast resistance at NEH-RC, Manipur during Kharif-2014, Kharif-2015 with two replications.

Study of inheritance of blast resistance : Chi-square test for goodness of fit was applied to study the inheritance of blast resistance in Akhanaphou. Obtained Resistance:Susceptible ratio in F_2 population was used to test fitness for Mendelian 3:1 monogenic ratio and also other ratios such as dihybrid ratio, trihybrid ratio, and ratios of epistatic interactions.

Genomic DNA isolation and Gene profiling study : Genomic DNA of individual RILs, Akhanaphou and Leimaphou were isolated using modified CTAB (Cetyltrimethylammonium bromide) method (14). The quality of isolated DNA was checked using 0.8% agarose gel electrophoretically and quantified using the Nanodrop (Thermo Fisher, USA). Gene profiling study was conducted for Akhanaphou and Leimaphou as done by Rama Devi and coworkers (12). The SSR (Simple Sequence Repeats) markers linked to nine important blast resistant genes *viz*., *Pi54, Pi9, Pib, Pi20, Pita-2, Pitp, Pik-s, Pi33* and *Pi38* were used to know whether the Akhanaphou and Leimaphou contain any of these genes by comparing the marker allele data with positive and negative controls (Supplementary Table 1). Further, their segregation was also checked across RIL population.

Agro-morphological evaluation of the RILs : During Kharif-2014, RIL population was grown along with donor and recurrent parents. Observations were recorded for the following agro-morphological parameters (i) days to 50 % flowering, (ii) plant type, (iii) average plant height, (iv) number of tillers per plant, (v) number of productive tillers per plant, (vi) panicle exertion and (vii) seed type

Identification of the best RILs : Based on phenotyping for the blast resistance, evaluation of agronomic traits, gene profiling analysis and presence of QTL (15); RIL population was evaluated for identification of good RIL lines for the blast resistance.

Results

Characterization of Akhanaphou for the blast resistance : Akhanaphou showed a high level of resistance to leaf blast (score-2) at IIRR, Rajendranagar, and NEH-RC, Manipur, whereas for neck blast, it showed resistance reaction (score-1). Leimaphou showed a highly leaf blast susceptible reaction (8-9 score) at both locations whereas; for neck blast it recorded '8' score. During 2012-AICRP on rice screening, Akhanaphou showed an average score of '3.1' for leaf blast and '2.7' for neck blast (Table 1).

Genetics of blast resistance : Out of 196 F_2 plants screened for leaf blast resistance at Rajendranagar, 171 plants showed resistance reaction while the remaining 25 plants showed susceptible reaction. While screening for the leaf blast resistance at Manipur, out of 80 F_2 plants, 25 plants found to be resistant and 55 plants found to be susceptible. In case of neck blast resistance out of 80 plants screened, only 27 plants recorded resistance and 53 plants recorded susceptible reaction.

Obtained Resistance:Susceptible ratios of F_2 population, for leaf and neck blast resistance screened at Rajendranagar and Manipur did not fit into any Mendelian ratios (Table 2). So, it is concluded that the blast resistance of Akhanaphou is governed by polygenes rather than a single gene. The graph plotted for the frequency distribution of disease severity of the leaf blast and neck blast at two locations showed normal distribution which support proposed hypothesis.

Gene profiling analysis : Gene profiling analysis revealed the presence of *Pi38* and *Pitp* genes in Akhanaphou. Since, it showed similar marker alleles as positive control (Tadukan) in the case of *Pi38* with two linked flanking markers *i.e.* RM206 and RM21. Similarly, Akhanaphou also showed similar marker allele as a positive control (Tetep) with RM246 indicating the presence of *Pitp.* In contrary, Leimaphou showed the presence of allele similar to the negative control in case of *Pi38* and *Pitp* genes (Supplementary Table 1 and Fig. 1).

Segregation of *Pi38* and *Pitp* were observed among RILs, there was a good segregation among RILs for these two markers. Out of 103 RILs, 15 RILs were positive for *Pi38* genes, 10 RILs were positive for *Pitp* gene; whereas, 3 RILs were positive for both the genes *Pi38* and *Pitp*. The total of 81 RILs did not show the presence of any of these genes. Details of the analysis are given in Table 3.

Phenotyping reaction of RILs : At IIRR, Rajendranagar, 60 RILs showed resistance reaction with a 0-4 score and 41 showed a susceptible reaction with a 5-9 score. At Manipur 34 RILs recorded a resistance reaction with a 0-4 score and 69 RILs recorded a susceptible reaction with a 5-9 score for leaf blast disease. In case of neck blast screening 34 RILs showed a resistance reaction with 0-3 score whereas, 69 RILs recorded a susceptible reaction with a 0-3 score.

Identification of best RILs : Out of 103 RILs, only 22 RILs found to contains one or two blast R-genes. Among 22 RILs, 15 and 10 RILs found to contain *Pi38* and *Pitp* gene respectively. Only three RILs found to contain both the blast R-genes (Table 3). Disease reaction pattern of RILs were decided based on phenotyping at three different environments namely 1) screening for leaf blast at IIRR, Rajendranagar, Hyderabad, 2) screening for leaf blast at NEH-RC, Manipur and 3) screening for neck blast at NEH-RC, Manipur. RILs showing leaf blast resistance at any one environment were

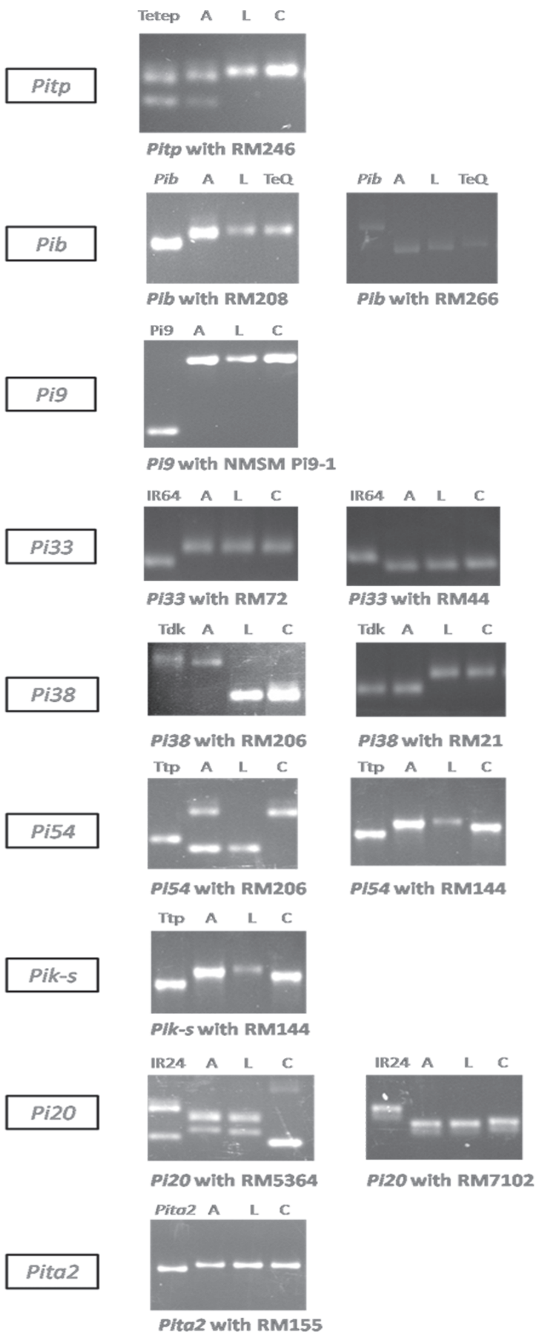

Fig. 1. Gene profiling of Akhanaphou with linked SSR markers.
A-Akhanaphou, L-Leimaphou, C-Co-39, Tdk-Tadukan, Ttp-Tetep, TeQ- Te-Qing, *Pib*- IRBLb-IT13[CO], *Pita*2- IRBLta2-Pi, *Pi*9- IRBL9-W[LT].

Table 1-1. Nine important blast resistant genes and related information such as chromosomal location, linked markers, their distance (cM) from respective R-gene, positive control and negative control.

Sr. No.	Blast	Chromosome resistant gene	Linked location	Positive markers	Negative control	Akhanaphou control	Leimaphou
1	***Pitp***	1	RM246	Tetep	Co-39*	+	-
2	***Pib***	2	RM208RM266	IRBLb-IT13[CO]	Te-Qing	-	-
3	***Pi9***	6	NMSMPi9-1	IRBL9-W[LT]	Co-39*	-	-
4	***Pi33***	8	RM72RM44	IR64	Azucena	-	-
5	***Pi38***	11	RM206RM21	Tadukan	Co-39	++	-
6	***Pi54***	11	RM206RM144	Tetep	Co-39*	-	-
7	***Pik-s***	11	RM144	Tetep	Co-39*	-	-
8	***Pi20***	12	RM1337				
			RM5364				
			RM7102	IRBL20-IR24	Co-39*	-	-
9	***Pita-2***	12	RM55	IRBLta2-Pi	Co-39*	-	-

*As standard negative control given for blast R-genes were not available with us, Co-39 is considered as negative control for these blast R-genes in the gene profiling study. The rice blast resistance gene scored as the presence (+) and absence (-) of amplicon

Table 1. Characterization of Akhanaphou for the blast resistance

Sr. No.	Phenotyping for the blast	Recorded score for Akhanaphou	Recorded score for Leimaphou	Recorded score for C101LAC	Recorded score for C101A51	Recorded score for HR12
	I. Phenotyping for the leaf blast resistance at Rajendranagar					
1	Feb-2012	1	9	5	5	9
2	Nov-2014	2	8	2	2	9
3	Sept-2015	3	8	3	2	9
	II. Phenotyping for the leaf blast resistance at NEH-RC, Manipur					
1	Aug-2014	3	9	4	3	9
2	Aug-2015	2	8	3	4	9
	III. Phenotyping for the neck blast resistance at NEH-RC, Manipur					
1	Aug-2014	1	7	3	3	9
2	Aug-2015	1	7	3	4	9
	IV. Phenotyping for the leaf blast resistance in the ACRIP trials					
1	2012	3.1	-	6.1	7.2	8.0
	V. Phenotyping for the neck blast resistance in the ACRIP trials					
1	2012	2.7	-	7.1	8.2	8.2

- data not available
C101LAC & C101A51-positive controls
HR12-negative control

Table 2. Chi-square table for F_2 (Akhanaphou X Leimaphou) populations screened for the leaf blast resistance at IIRR, Rajendranagar, Hyderabad and screened for leaf and neck blast at NEH-ICAR, Research Center, Lamphelpat, Manipur

Blast type and location	Phenotyping for leaf blast at Rajendranagar	Phenotyping for leaf blast at Manipur	Phenotyping for neck blast at Manipur
Resistant	171	25	27
Susceptible	25	55	53
Total	196	80	80
Chi-square value for monohybrid ratio	15.67	81.66	72.60
Chi-square value for dihybrid ratio	256.00	177.82	172.40

Table value for monohybrid ratio: 3.84
Table value for dihybrid ratio: 7.81

Table 3. Segregation of *Pi38* and *Pitp* gene among RILs (Akhanaphou X Leimaphou)

RILs positive for	Number of RILs	RILs
Pi38	15	RIL: 2, 3, 12, 18, 24, 27, 50, 58, 62, 68, 71, 91, 96, 100, 102
Pitp	10	RIL: 13, 20, 34, 44, 46, 50, 59, 69, 71, 91
Pi38 + Pitp	3	RIL: 50, 71, 91

considered as resistant to leaf blast disease. Among the 15 RILs which contain the *Pi38*, five RILs *viz*., RIL: 2, 3, 27, 91 and 96 showed leaf blast resistance whereas, out of 10 RILs which contain the *Pitp*, three RILs showed leaf blast resistance namely, RIL: 13, 34 and 91. Interestingly, nine RILs *viz*., RIL: 13, 18, 20, 27, 34, 44, 91, 100 and 102 containing either *Pi38* or *Pitp* showed neck blast resistance. Based on all these parameters along with another study results (15), 3 RILs namely RIL: 13, 34 and 91 were considered as best for blast resistance (Table 4).

Discussion

Blast disease is a devastating disease of rice, threatening the global rice production particularly from tropical and subtropical rice growing regions of the world. Though, many methods and practices are available for control of blast disease in the field, resistance breeding is the most economic, effective and feasible method for control of blast disease. Deployment of resistant genes and QTL is one of the important approaches of resistance breeding since a lot of genetic variation for the resistance exists among the rice germplasm. In the changing climatic scenario, the neck blast has become a serious threat and endemic in many rice growing areas. Hence, the identification of genes or QTL for both leaf and neck blast resistance is a continuing a challenging part in rice improvement program. Among the 100 blast R-genes identified so far, only one gene *i.e. Pb1*, identified from Modan (Indonesian) rice variety, confers resistance specifically for neck blast (16).

Landraces are one of the important components of germplasm of any crop. Landraces

Table 4. Comparison of Akhanaphou, Leimaphou and Three good donor RILs for blast resistance along with their details

RIL No.	Akhanaphou	Leimaphou	13	34	91
Screening of RILs for presence of Pi genes					
Pi38	+	-	-	-	+
PitP	+	-	+	+	+
Distribution of QTLs among RILs					
qLNBL-5	+	-	+	+	+
qLNBL-7	+	-	-	+	-
Evaluation of RILs for agronomic traits					
Flowering days	104	92	84	96	103
Plant type	Erect	Spreading	Erect	Erect	Erect
Dwarf/Tall/Medium	Tall	Dwarf	Dwarf	Medium	Tall
Panicle emergence	Well exerted	Partly exerted	Partly exerted	Partly exerted	Well exerted
Grain type	Medium bold	Long bold	Medium bold	Medium bold	Long bold
Average productive tillers	5	7	4	4	4
Average plant height	133	78	52	91	134
Phenotyping of RILs for leaf blast at Rajendranagar					
Disease reaction	Resistant	Susceptible	Resistant	Resistant	Resistant
Phenotyping of RILs for leaf blast at NEH-RC, Manipur					
Disease reaction	Resistant	Susceptible	Resistant	Resistant	Resistant
Phenotyping of RILs for neck blast at NEH-RC, Manipur					
Disease reaction	Resistant	Susceptible	Resistant	Resistant	Resistant

are “balanced populations in equilibrium with both the environment and pathogens, and are genetically dynamic” (17). The North Eastern part of India is known for rich biodiversity including rice and also one of the hot spots of rice blast disease particularly for neck blast with existence of high genetic diversity of *M. oryzae* (18, 19). In North Eastern India, wide genetic variation and high pathogen pressure of blast pathogen contributes to the evolution of many novel gene(s). Among several landraces, Akhanaphou is one of the unique landraces from Manipur having a high level of resistance to leaf and neck blast, whose genetic base for resistance is not known.

In the extensive and rigorous phenotyping, Akhanaphou proved resistant against leaf and neck blast. Interestingly it showed a high resistant reaction to neck blast with 1 score during both of the seasons. There was no change in disease reaction showed by Akhanaphou during UBN and field evaluation for leaf blast; though the pathogen pressure at Manipur was very high. After confirming high level of blast resistance of Akhanaphou we had gone for inheritance study in F_2 population. In inheritance study, based on chi-square test and from graphs showing normal frequency distribution of disease severity, it was confirmed that the trait of leaf and neck blast resistance of Akhanaphou is under polygenic control. To date approximately 350 QTL resistances to leaf blast have been mapped for blast resistance in rice which provide evidence for polygenic control of blast resistance in many rice germplasm (2, 20 - 23).

After confirming polygenic inheritance of blast resistance, gene profiling study was done to know the presence of major blast R-genes in Akhanaphou. For this study, nine blast R-genes which are being used more often in blast resistance breeding programs in India were selected (24). Though gene specific and functional markers are available for some of the blast R-gene, we did not use them in gene profiling because they are allele specific. Different alleles of the same gene can be present in a population, which cannot be identified with gene specific and functional markers. Gene profiling is a rapid method to know the presence of known blast R-genes using tightly linked markers. Moreover, it is difficult and time-consuming process to conduct allelic tests for all the known blast R-genes. Similar approach was followed earlier for identification of alleles or better performing alleles from the diverse germplasm through PCR-based approach (12, 18, 25, 26, 27, 28, 29). Gene profiling of ten major rice blast resistance genes has been determined in 192 rice germplasm accessions using SSR markers by Singh and coworkers (27). Rama Devi and coworkers checked the allelic status of seven important blast R-genes in the 326 introgression lines derived from six different wild species using gene-based markers (12). Gene profiling revealed that Akhanaphou contain *Pi38* and *Pitp* blast R-genes. As both of these genes were identified for leaf blast and genetics study revealed polygenic nature of blast resistance, we hypothesized that, there may be some novel genes or QTLs which contribute to true blast resistance in Akhanaphou.

We developed RIL population by crossing Akhanaphou with Leimaphou, an elite variety with good agronomic traits such as good yield and famous for cooking quality. RIL population is stabilized population and achieves significant homozygosity, for almost all of the loci after 6-7 generations. Akhanaphou is a landrace and many undesirable traits are associated with it such as low yield, tallness, grain shattering nature *etc*. Direct use of Akhanaphou in rice breeding program as donor will cause more linkage drag which is highly undesirable. Use of RILs showing blast resistance rather than Akhanaphou will reduce linkage drag in crossing program. RHLs (Recombinant Heterogeneous Lines) developed by the crossing of two donor RILs will also act as valuable and useful breeding material for blast resistance breeding. Identification of good RILs showing blast resistance along with good agronomic traits will act as good candidates for direct varietal release. In order to shortlist good donors, two type of analysis was done. In the first analysis, RILs containing either *Pi38* or *Pitp* or both genes were checked for their disease reaction. Interestingly nine RILs *viz.,* RIL: 13, 18, 20, 27, 34, 44, 91, 100 and 102 which contain either *Pi38* or *Pitp* showed neck blast resistance. These results help to infer that *Pi38* and *Pitp* may also contribute to neck blast resistance of Akhanaphou. Surprisingly, RILs which do not contain either *Pi38* or *Pitp* but still showed blast resistance were also identified which includes 16 RILs showing leaf blast resistance and 42 RILs showing neck blast resistance whereas, 8 RILs showing resistance to both leaf and neck blast. These results are in supports of our hypothesis that, leaf and neck blast resistance in Akhanaphou is under the polygenic control. RILs which do not contain either *Pi38* or *Pitp* but still showing leaf or/and neck blast resistance will be more informative and helpful in novel QTL mapping approach. Moreover, use of RIL population rather than F_2s will also advantageous to identify minor effect QTLs. The results obtained in our recent studies are in support of polygenic nature of blast resistance of Akhanaphou. The mapping studies conducted in Akhanaphou has leads to identification of two novel QTLs *i.e. qLNBL*-5 and *qLNBL-7*; for leaf and neck blast resistance on chromosomes 5 and 7 respectively (15).

In conclusion, Akhanaphou found to be a reliable resource for leaf and neck blast resistance. Extensive phenotypic analysis (at two different locations with artificial and field screening as well as screening at the diverse location through AICRP on rice) revealed the broad spectrum nature of resistance in Akhanaphou. Akhanaphou contains two major blast R-genes *i.e. Pi38* and *Pitp*. The identified stable breeding lines (RIL: 13, 34 & 91) which contains either or both of the genes and having good agronomic traits can be explored in rice improvement program. They can even used for direct varietal release after multi-location trials. The breeding lines which do not have major blast R-genes but yet showing resistance can be explored for identification of novel QTLs. Thus the identified unique landrace Akhanaphou and its population can be further exploited for deciphering the complete blast resistance mechanism.

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