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Nucleotide diversity of *Pita*, a major blast resistance gene and identification of its minimal promoter

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ABSTRACT

Improvement of host plant resistance is one of the best methods to protect the yield from biotic stresses. Incorporation of major resistance genes or their variants into elite rice varieties will enhance the host plant resistance and its durability. Allele mining is a preferred choice to discover the novel allelic variants of major genes from wide range of germplasm. 'True' allele mining includes coding and noncoding regions, which are known to affect the plant phenotype, eventually. In this study, major blast resistance gene, *Pita* was analyzed by allele and promoter mining strategy and its different allelic variants were discovered from landraces and wild *Oryza* species. Polymorphisms at allelic sequences as well as transcription factor binding motif (TFBM) level were examined. At motif level, MYB1AT is present in $Pita^{Tadukan}$ and other resistance alleles, but was absent in the susceptible allele. Core promoter was demarked with 449 bp, employing serial promoter deletion strategy. Promoter with 1592 bp upstream region could express the *gfp* two fold higher than the core promoter. The identified *Pita* resistance allele ($Pita^{Konibora}$) can be directly used in rice blast resistance breeding programs. Moreover, characterization of *Pita* core promoter led to deeper understanding of resistance gene's regulation and the identified core promoter can be utilized to express similar genes in rice.

1. Introduction

Rice is one of the most important food crops and is a primary source of energy for more than half of the world population. The population of rice consumers is steadily increasing due to enhancement in living standards of the people in developing countries and hence the demand for rice is increasing continuously (Khush and Jena, 2009). The actual yield of irrigated lands is nearly half of its actual potential (10 tons/ha), mainly due to biotic and abiotic stresses (Siddiq, 2010). Among the biotic stresses, rice blast is the most devastating fungal disease which is caused by the filamentous ascomycete fungus *Magnaporthe oryzae* (anamorph *Pyricularia oryzae*). Rice blast disease causes considerable yield loss (Khush and Jena, 2009) which is as high as 100% during an epidemic. The loss due to rice blast was estimated to be 157 million tons and led to multiple social crises (Lin et al., 2007).

Increasing yield potential and productivity through genetic and cultural practices, and minimizing the yield losses due to biotic and abiotic factors without any harmful effects on the environment are considered as two important strategies to sustain rice production. As the chemical fungicides are hazardous to environment, strengthening the host plant resistance is the best strategy to face this biotic stress problem. To date, nearly 100 blast resistance genes were reported and the number is increasing steeply; among them, 19 genes have been cloned and characterized (Das et al., 2012). *Pita*, a NBS-LRR domain containing blast resistance gene, located at rice chromosome 12 (Bryan et al., 2000), provides wide spectrum resistance against different isolates of *M. oryzae* in India. Discovery of novel alleles of this gene will help in devising effective gene deployment strategies for durable resistance. It has been well proven that alleles of many agronomically important genes were lost during evolution and domestication of the crop (Costanzo and Jia, 2010; McCouch et al., 2007). Identification and exploitation of such untapped beneficial alleles favor crop improvement. Allele mining — dissection of naturally occurring variations at candidate genes, is the most commonly used strategy to identify novel alleles of gene of interest from a wide range of germplasm. The availability of diversified rice germplasm, genome sequences and many in silico bioinformatics tools made allele mining simpler and more efficient. So far, the allele mining and nucleotide diversity were studied at coding regions of the gene (Huang et al., 2008; Lee et al., 2011), since mutation at coding regions of a gene is known to affect protein structure, function and thus phenotype of the plant. However, many studies reported that differences in the noncoding region also influence the phenotype (Fiume et al., 2004; Fu et al.,

Abbreviations: TFBM, transcription factor binding motif(s); *gfp*, green fluorescent protein; Del, deletion; ED, evolutionary distance.

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2005). A well known example is a mutation in the upstream region of *Xa13* (dominant allele) led to recessive *xa13* which conferred resistance to bacterial blight in rice (Chu et al., 2006). Hence, a 'true' allele mining should consider both coding and non-coding regions i.e. upstream sequence and introns also. The upstream sequence is known to have various transcription factor binding motifs (TFBMs), to which transcription factors (TFs) bind and influence the gene expression. The arrangement and presence/absence of the TFBMs affect the TFs interaction that leads to differential gene expression (Lengar and Joshi, 2009). Influence of these regulatory motifs on gene expression can be analyzed by promoter deletion analysis (Guo et al., 2010; Wang and Oard, 2003). Upstream sequences of many rice blast resistance genes, including *Pita*, have not been characterized, till today. Core promoter is a minimal upstream region, possessing essential TFBMs, required to express the gene. The identification and analysis of core promoter lead to better understanding of gene expression and decipher the role of essential TFBMs in gene regulation. To the best of our knowledge, this is the first report on core promoter of a rice blast resistance gene, which provided better knowledge on regulation of resistance genes.

Based on the present status of the *Pita* gene, the present study was designed i) to identify novel alleles of *Pita*, major blast resistance gene; ii) to analyze the diversity of the novel *Pita* alleles from landraces and wild *Oryza* species; iii) to analyze motif level diversity at promoter region; and also iv) to demark the *Pita* gene's core promoter region.

2. Materials and methods

2.1. Plant materials

Plant materials of this study include twenty four land races collected from North Eastern region of India and 110 accessions of different *Oryza* wild species and cultivars (which were collected from different parts of the world) were obtained from International Rice Research Institute (IRRI), Philippines and Directorate of Rice Research, India (Supplementary Table 1). Fifty seeds of each cultivar were germinated in petri-plates and the seedlings were transplanted to earthen pots and maintained at glass house at 25 ± 3 °C.

2.2. Screening of plant materials for blast resistance

All the 24 landraces and 110 different accessions of *Oryza* wild species were screened for rice blast resistance for twice with three replicates in the consecutive seasons. TN1 and HP2216 were included for screening as susceptible controls, while Tadukan (harboring *Pita*) was included as resistant control, along with other test varieties. Screening was performed based on Peng and Shishiyama (1988) method with little modifications. Briefly, spore suspension (approximately 1×10^5 spores per ml mixed with 0.2% of Tween-20) of ALM-2 isolate (collected from rice blast hot spot — Almora, India; highly virulent and not compatible with *Pita*) was sprayed onto 15 day old plants using glass automizer. Sprinklers were used to maintain artificial humidity of 80 to 90%. In the case of the wild rice species, in which multiplication of seeds was difficult, the leaves were collected and screened by punch inoculation method (with ~1×10^5 spores per ml mixed with 0.2% of Tween-20) (Dillon et al., 1997). Twelve days after inoculation, the seedlings were scored based on disease severity with 0–9 scale (Supplementary Table 2). The dominant molecular markers, viz., YL153, YL154, YL155, and YL87 (Jia et al., 2002) were used to screen the plant materials to confirm the presence of *Pita*/*pita* alleles.

2.3. Primer designing for allele and promoter mining

The *Pita* gene sequence was downloaded from NCBI (acc. no. AF207842) along with its flanking sequences (500 bp), which was used to design the primers. Using the online tool, Primer 3 (Rozen and Skaletsky, 2000), and fast PCR (Kalendar, 2009), primers were designed to amplify the candidate gene. Primers have been designed in such a way that the forward primer binds before the transcription start site (TSS) and the reverse primer binds after the terminator region of that gene (Fig. 1). The targeted region covers a part of upstream region, introns and part of 3′ UTR region. In the case of promoter mining, 2 Kb upstream region of *Pita* was downloaded from RAP-DB website, which was used to design the primers (Supplementary Table 3). The allelic and promoter regions were amplified in 20 μl PCR mix containing 1× buffer (20 mM Tris–HCl (pH 8.8 at 25 °C), 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 0.1 mg/mL BSA, 0.1% (v/v) Triton X-100, 2 mM $MgSO_4$), 0.25 mM of each dNTPs, 5 pM of each primers and 1 U of *Pfu* DNA polymerase (Fermentas, USA). Template quantity: 300 to 500 ng of genomic DNA was used to amplify *Pita* allelic region (as the amplicons are ~5 Kb in size), while 100 ng DNA was used to amplify 2 Kb promoter region. Thermal profile followed was: initial denaturation at 94 °C for 5 min followed by 35 cycles of denaturation at 94 °C for 1 min; primer annealing at 56 to 58 °C for 1 min (Supplementary Table 3), and extension at 72 °C for 1 min/Kb, followed by final extension at 72 °C for 10 min.

2.3.1. Allelic sequence analysis

The raw sequences of *Pita* alleles of selected genotypes were compared with $Pita^{Tadukan}$ sequence using NCBI alignment tool (http://blast.ncbi.nlm.nih.gov/). Pair wise alignment of the alleles was also done using MEGA version 4.1 — ClustalW (Tamura et al., 2007). Evolutionary distances (θ_π) of the alleles, in comparison with the reference allele, were calculated by MEGA tool with parameters of gap/missing data: complete deletion; model: maximum calculate likelihood; with other default settings. Standard error was calculated by bootstrap value with 1000 replicates. Phylogenic tree was constructed by comparing all the sequences by MEGA — Neighbor-Joining (NJ) method with the parameters of phylogeny test: bootstrap (1000 replicates); gaps/missing data: complete deletion; with other default settings. Gene structure (length of open reading frame (ORF), number of exons/introns, etc.) and protein sequences were predicted using online tool softberry — FGENESH (http://linux1.softberry.com). Number of SNPs, InDels, and non-synonymous/synonymous value of the allelic sequences were calculated using, DnaSP (version 5.10.01) (Librado and Rozas 2009). Motifs were analyzed with TFBM database, PLACE (Higo et al., 1999) and Weeder (Pavesi et al., 2004). Weeder tool was installed locally and run in large mode.

2.4. Promoter serial deletion analysis

Targeted, different lengths of *Pita* upstream regions were amplified by specific primers. The longest length of promoter region was amplified with forward primer, located at −1592 bp and the reverse primer, located at −1 bp of the *Pita* upstream sequences and the resulting amplicon was referred as Del I. In order to eliminate the motifs, present at last ~500 bp of 5′ end of Del I, second forward primer was designed at −935 bp and resulting amplicon was referred as Del II. To eliminate the motifs at last ~500 bp of 5′ end of Del II, third forward primer was designed at −449 bp and the resulting amplicon was named as Del III. The primer, designed at −1 bp, was used as common reverse primer for amplifying Del I, II and III (Fig. 2; Supplementary Table 4). The individual PCR amplicons (Del I, II and III) were inserted before the promoter less *gfp* gene of binary vector, pCXGFP, (accession number: FJ905225) as demonstrated by Chen et al. (2009). Briefly, adenine overhangs of the amplicons were ligated with thymine overhangs of the vector using Ligase enzyme (Promega, USA).

Orientation of the inserted upstream sequence in the vector pCXGFP was checked by PCR with orientation specific primers (Supplementary Table 4). The primers were designed in such a way that if the promoter region was in right direction, the primers will amplify 546 bp fragment and otherwise it will not (Supplementary Fig. 1). These confirmed constructs were mobilized into *Agrobacterium tumefaciens* EHA 105 by