

Extensive Variation in Alleles of *Pi54* Makes This Gene Show Broad-Spectrum Resistance to Blast

T. R. Sharma

National Research Centre on Plant Biotechnology, IARI, Pusa Campus New Delhi -110012. Email: trsharma1965@gmail.com;trsharma@nrcpb.org

Abstract

Rice blast resistance is one of the extensively studied traits at phenotypic, genetic and molecular levels. In spite of all the advances in biological research, the mechanism of rice-*Magnaporthe* interaction is still puzzling. Host resistance is often governed by single resistance (R) genes, the products (receptor) of which directly or indirectly interact with specific elicitors produced by avirulence (*avr*) genes. Considerable progress has been made in our understanding of gene-for-gene resistance (R-*avr* interactions) in many host pathogen systems. In our endeavour to understand the molecular basis of R: *Avr* interaction in blast resistance, we identified a new gene *Pi54* (*Pik^h*) from Tetep, cloned it and functionally validated. Induction of resistance in rice by transferring single blast resistance gene *Pi54* has shown that it activates complex resistance mechanisms in rice during infection thus provides resistance to plant phenotype against different and divers strains of *M. oryzae*. The mining of alleles and orthologues of *Pi54* gene from local land races and wild species of rice has shown some very interesting features which can be exploited in future for blast resistance breeding programmes.

Keywords: Rice, blast, *Magnaporthe*, resistance gene, alleles

Introduction

Rice blast, caused by *Magnaporthe oryzae* considered as one of the most important constraints in production. This pathogen can infect rice crop at all the stages of plant growth and yield loss may go as high as 80% during favourable environmental conditions. Genetic and genomics information have been generated in rice-*M. oryzae* system which plays an important role in cloning and characterization of resistance genes and alleles from germplasm lines. It has been reported that rice blast resistance is mainly controlled by single dominant gene; more than 84 genes have already been identified in different rice lines. Besides, 347 QTLs responsible for resistance to blast fungus have also been identified in rice. Before the decoding of rice genome sequence in 2005, only two genes for blast resistance i.e. *Pib* and *Pita* were cloned in Japanese and US laboratories, respectively. Then by using positional cloning approach in conjunction with the draft sequence of rice genome third blast resistance gene *Pi-k^h* (*Pi54*) was cloned and functionally validated from Tetep (Sharma et al 2005b). This gene is now being transferred in commercial varieties of rice using marker assisted selection (Singh et al 2011). Till date, 19 blast resistance genes have been cloned and characterized in different countries.

Identification of *Pik^h/Pi54* blast resistance gene

Rice line Tetep, an *indica* type line possesses durable resistance to blast pathogen across India.

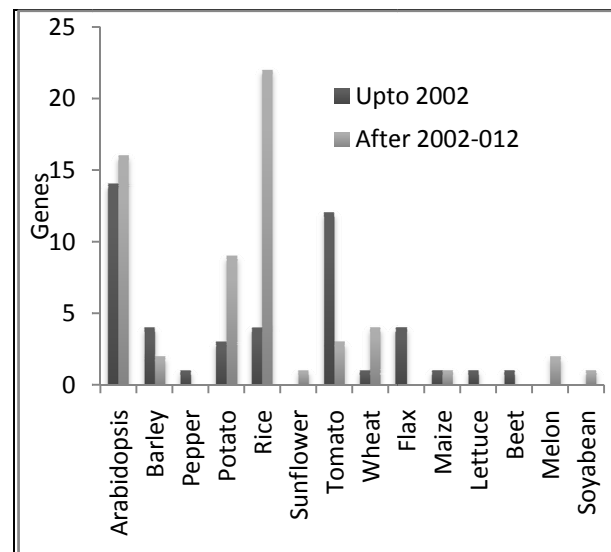


Figure 1. Status of R-genes cloned in different species before and after the high throughput sequencing efforts in different crops

Kiyosawa (1981) studied the genetics of blast resistance in rice line Tetep and showed that this line contains a blast resistance gene which is allelic to *Pi-k^h* identified from rice line HR22. We obtained very high variability in pathogenicity of isolates of *M. oryzae* prevalent in North-Western Himalayan region of India (Sharma et al 2002). Besides, it was also found that *indica* rice Tetep containing *Pi-k^h* (in addition to other genes) was highly effective against the pathogen population of North-Western Himalayan region of India (Sharma et al 2002).

Molecular mapping and cloning of *Pi-k^h* (*PI54*)

For the mapping of *Pi-k^h* gene with DNA markers an F₂ mapping population consisting of 205 plants was generated by crossing Tetep with a susceptible rice line HP2216. Inoculation with specific isolate (PLP-1) of *M. oryzae* at seedling stage showed that the *Pi-k^h* gene was inherited as a single dominant gene in this population (Sharma et al 2005a). Later a high resolution map was constructed and gene was mapped between two SSR markers, TRS26 and TRS33 at 0.7 cM and 0.5 cM, respectively and the *Pi-k^h* gene was cloned and characterized (Sharma et al 2005b). High titre genomic library was also prepared for the identification of a genomic clone containing *Pi-k^h* gene with its complete upstream and downstream sequences from the rice blast resistant line Tetep (Madhav et al 2008). With the advances in molecular genetics and genomics of rice, the *Pik* locus has now been mapped more precisely. Since there are two reports on the mapping of *Pi-k^h* gene from different rice lines (Sharma et al 2005b; Xu et al 2008), there is some confusion in the naming of this gene. The name of *Pi-k^h* gene cloned from the rice line Tetep has thus been designated as per the standard guidelines of Committee on Gene Symbolization, Nomenclature and Linkage (CGSNL) and its physical location on rice chromosome 11, which is ~2.5Mb away from the *Pik* locus mapped recently. Hence, *Pi-k^h* gene cloned from Tetep was designated as *Pi54* (Sharma et al 2010). Functional validation of the *Pi-k^h* (*Pi54*) gene using complementation assay has been reported (Rai et al 2011). The blast resistance candidate gene *Pi-k^h* (*Pi54*) was cloned into a plant transformation vector and the construct was used to transform a *japonica* cultivar of rice Taipei 309, which is susceptible to *M. oryzae*. Transgenic lines containing single insertions of *Pi-k^h* (*Pi54*) were found to confer a high degree of resistance to diverse isolates of *M. oryzae*. Successful complementation of *Pi-k^h* (*Pi54*) gene confirmed that the gene is responsible for resistance to *M. oryzae* in transgenic lines developed during this study.

Structural organization of *Pi-k^h* (*PI54*) locus

To understand the structural organization of *Pi-k^h* (*Pi54*) locus, an analysis of 50 kb upstream and downstream sequences flanking to this locus (100 kb region) was

performed (Kumar et al 2007). In this region, 15 genes in the sequence of *japonica* and 16 genes in *indica* lines were predicted and annotated. Average percent GC content of *japonica* and *indica* genes in this region was 49.3 and 53.15%, respectively. Both *japonica* and *indica* sequences were polymorphic for microsatellites having mono-, di-, tri-, tetra-, and penta-nucleotide repeats in the microsatellite regions. Sequence analysis of specific blast resistant *Pi-k^h* (*Pi54*) allele of Tetep and susceptible allele of Nipponbare showed differences in number and distributions of motifs involved in phosphorylation, resulting resistance phenotype in rice line Tetep (Kumar et al 2007).

Resistance gene dependent accumulation of expressed sequence tags (ESTs) was studied in a blast resistant, *indica* rice cultivar Tetep after challenge inoculation with an incompatible race of *M. oryzae* (Dikshit et al 2009). From the rice cDNA library constructed from the RNA isolated after challenge inoculation of the host, sequence of 287 randomly selected cDNA clones from the rice cDNA library constructed from the RNA isolated after challenge inoculation of the host was obtained and submitted in NCBI Genbank (Acc Nos. DN475717 - DN475431). Of these 63% ESTs were highly representative of the rice transcriptomes studies in this experiment. The unique transcripts (178) were also identified after assembly of the ESTs and categorized into 17 functional groups (Dikshit et al 2009). Physical positions of the unigenes on the rice pseudomolecules i.e. chromosomes was also identified. This information can be effectively used in understanding molecular basis of disease resistance in *M. oryzae*-rice system.

Expression and comparative analysis of *Pi54* mediated resistance

To understand the genome wide co-expression of genes in the transgenic rice Taipei 309 (TP) containing *Pi54* gene, microarray analysis was performed at 72 h post inoculation of *M. oryzae* strain PLP-1 (Gupta et al 2011a). A total of 1154 differentially expressing genes were identified in TP-*Pi54*, 587 were up-regulated and 567 genes were found to be down-regulated. In this line, 107 genes were exclusively up-regulated and 58 genes down-regulated. Various defense response genes like callose, laccase, phenylalanine ammonia lyase and peroxidase, and genes related to transcription factors like NAC6, Dof zinc finger, MAD box, bZIP, WRKY were found to be up-regulated in the transgenic line. The enzymatic activities of six plant defense response enzyme namely peroxidase, polyphenol oxidase, phenylalanine ammonia lyase, β -glucosidase, β -1, 3 glucanase and chitinase were found to be significantly high at different stages of inoculation by *M. oryzae*.

This study suggests activation of defense response and transcription factor related genes and higher expression of key enzymes in rice line TP-*Pi54* thus leading to incompatible interaction (Gupta et al 2011a). In order to understand the presence of zinc finger domains in resistance (R) proteins 70 R-genes sequences of various crops were analyzed and found that 26 genes have typical zinc finger domain (Gupta et al 2011b). The size of individual zinc finger domain within the R genes varied from 11 to 67 amino acids in length, whereas the proteins containing these domains varied from 263 to 1305 amino acids. The pair-wise identity analysis showed that the LIM, zinc finger domain of rice blast resistance protein Pi21 was found to have 12.3% similarity with the NFX type zinc finger of Pi54 protein. Fifteen zinc finger resistance proteins were highly divergent compared to the zinc finger domain of Pi54 protein of rice. We concluded that Pi54 protein has a small zinc finger domain of NFX type integrated with LRR at -C terminal end (Gupta et al 2011a). This domain is different from all other cloned resistance genes and might play an important role in blast resistance phenotypes.

Allele mining for blast resistance genes

Allele mining is the process of identifying alleles of the gene responsible for a given trait and their variants in other genotypes. Different approaches used for allele mining and alleles mined for different genes in rice have recently been reviewed (Sharma et al 2012). Thakur et al (2012) and Sharma et al (2012) have reported molecular dynamics of blast resistance gene *Pi-ta* in Indian land races of rice. They selected a total of 48 out of 529 land races of rice based on resistant phenotype at three different geographical locations of India. A total of 64 and 34 haplotypes have been identified from the nucleotide polymorphism of 268 rice accession and 48 land races, respectively. A total of 22 new *Pi-ta* protein variants were identified, of which 5 novel *Pi-ta* variants (PV 2, PV 5, PV 6, PV 7 and PV 10) have been identified from Indian land races of southern (PV 2, PV 5, PV 7) and northern regions (PV 6 and PV 10). This study helps to understand the variability present in the land races of the Indian sub-continent, which can be employed for selection of better alleles for blast resistant breeding programmes. Similarly alleles for blast resistance genes have also been mined from 48 land races of rice. Large insertions and SNP variations were obtained (Fig. 2). These variations have been used for designing allele specific markers which can be used for MAS by the rice breeders. In an effort to identify more effective forms of this gene, an orthologue of *Pi54* named as *Pi54rh* was cloned from the blast resistant wild species of rice, *Oryza rhizomatis*, using allele mining approach (Das et al 2012). The *Pi54rh* belongs to CC-NBS-LRR family of disease resistance

genes with a unique zinc finger (C₃H type) domain. The 1447 bp *Pi54rh* transcript comprises of 101 bp 5'-UTR, 1083 bp coding region and 263 bp 3'-UTR, driven by pathogen inducible promoter. We showed the extracellular localization of Pi54rh protein and the presence of glycosylation, myristoylation and phosphorylation sites that implicated its role in signal transduction process. This is in contrast to other blast resistance genes that are predicted to be intra-cellular NBS-LRR type resistance proteins. Functional validation of cloned *Pi54rh* gene using complementation test showed high degree of resistance to eight isolates of *M. oryzae* collected from different geographical locations of India. This allele provides broad spectrum resistance to *M. oryzae* hence can be used in resistance breeding programme in rice.

Co-evolution of *Pita* and *Avr-Pita* genes under Indian conditions

Blast resistance gene *Pita* has been cloned in 2002 in USA (Bryan et al 2000). We mined its alleles from local land races of rice and sequence analysis was performed. There were very little variations in terms of InDels and single nucleotide polymorphism (SNPs) in this allele across the land races. There appears to be little pathogen pressure on this allele under natural conditions in India. We also mined pathogen avirulence gene *Avr-Pita* from 70 isolates of *M. oryzae* collected from different parts of the country. Sequence analysis showed that the *Avr-Pi54* allele in the pathogen is highly variable. Hence pathogen is trying to mutate and may overcome the resistance gene *Pita* (Sharma et al, unpublished)

Recent advances in rice blast resistance

The two innate immune systems used by plants in response to the pathogen infection, are known as pathogen-associated molecular pattern (PAMP)-triggered innate immunity and effector-triggered immunity (Jones and Dangl 2006). The early response to pathogen infection is usually induced by PAMP-triggered innate immunity by recognition of PAMPs by trans-membrane receptors. Whereas the effector-triggered immunity involves interaction between host receptors and pathogen effectors, either directly or indirectly, and usually induces hypersensitive reaction leading to cell death. This phenomenon has not been studied in case of *Pi54* mediate resistance. However, there are some examples which clearly show the significance of this study. A small GTPase, Rac1, plays a key role in rice (*Oryza sativa*) innate immunity as part of a complex of regulatory proteins. The rice RACK1 (for receptor for activated c-kinase 1) has been identified as an interactor with Rac1 (Nakshima et al 2008). It has been reported that rice contains two RACK1 genes, RACK1A and RACK1B, and the RACK1A protein interacts with the GTP form of Rac1.

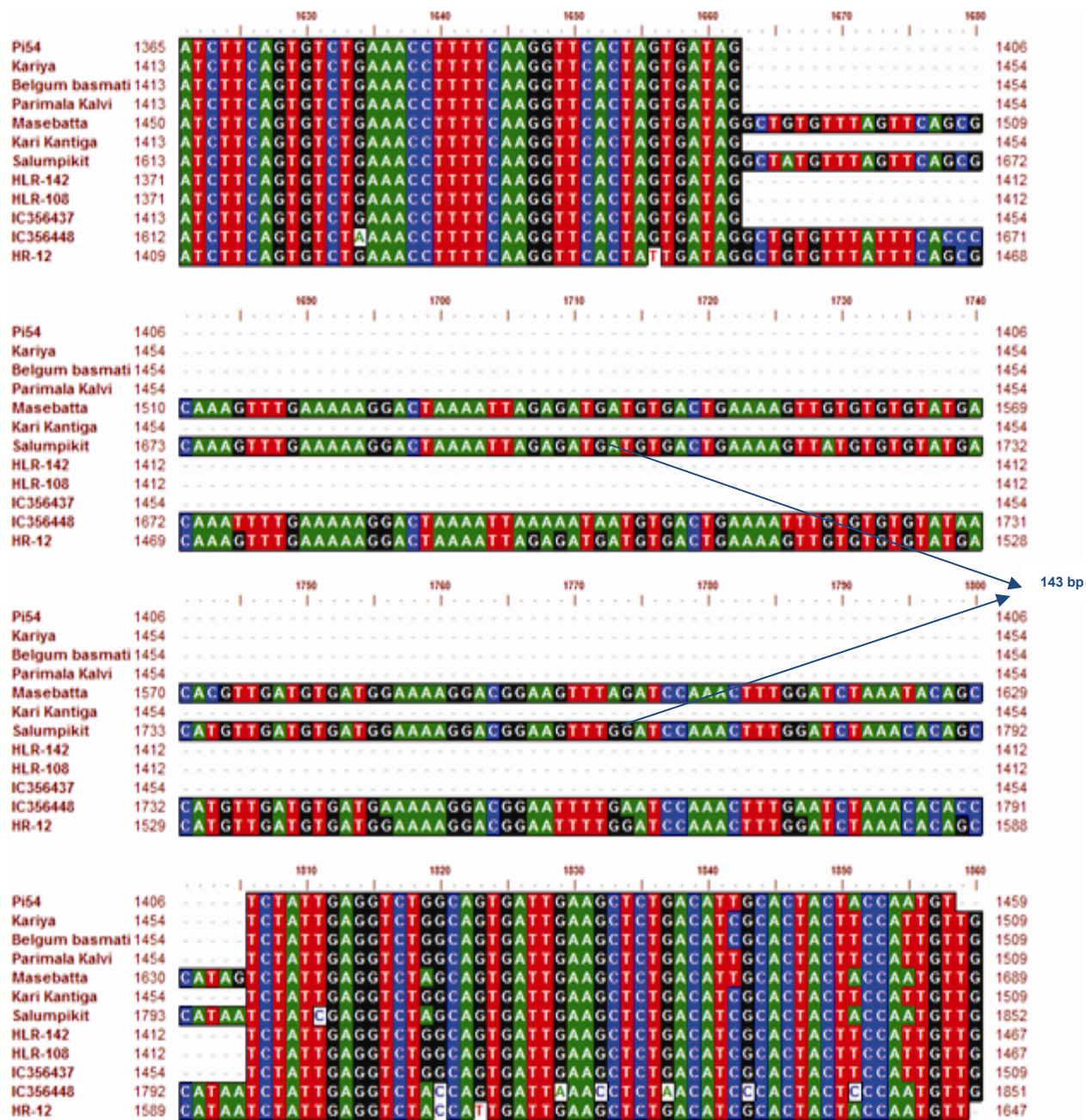


Figure 2. Large insertions of 143 bp in Pi54 alleles cloned from different land races of rice

Rac1 positively regulates RACK1A at both the transcriptional and posttranscriptional levels. Similarly, emerging evidence suggests that E3 ligases play critical roles in diverse biological processes, including innate immune responses in plants. However, the mechanism of the E3 ligase involvement in plant innate immunity is unclear. Li et al (2011) reported that a rice gene, *OsBB1*, encoding a RING finger protein with E3 ligase activity, mediates broad-spectrum disease resistance. The expression of *OsBB1* was induced by *M. oryzae*, as

well as chemical inducers, benzothiadiazole and salicylic acid. Biochemical analysis revealed that *OsBB1* protein possesses E3 ubiquitin ligase activity in vitro. The overexpression of *OsBB1* in transgenic plants conferred enhanced resistance to multiple races of *M. oryzae*. This indicates that *OsBB1* modulates broad-spectrum resistance against the blast fungus. Protection of plants by means of non host resistance is a rule rather than exception. In spite of the fact that most of the plants are resistant to many potential pathogens,

the molecular basis of non-host resistance are poorly understood. Zellerhoff et al (2010) reported the transcriptional responses of one particular genotype of barley (*Hordeum vulgare* sub sp. *vulgare* cv. Ingrid) to three different pairs of adapted (host) and non-adapted (non host) isolates of fungal pathogens namely, *Blumeria* (powdery mildew), *Puccinia* (rust), and *Magnaporthe* (blast). Non host resistance against each of these pathogens was associated with changes in transcript abundance of distinct sets of non host-specific genes, although considerable overlap was obtained in general (not non host-associated) transcriptional responses to the different pathogens. The powdery mildew- and blast-induced differences in transcript abundance between host and non host interactions were significantly correlated with differences between a near-isogenic pair of barley lines that carry either the *Mlo* wild-type allele or the mutated *mlo5* allele, which mediates basal resistance to powdery mildew. Moreover, during the interactions of barley with the different host or non host pathogens, similar patterns of overrepresented and underrepresented functional categories of genes were found. The results suggest that non host resistance and basal host defense of barley are functionally related and that non host resistance to different fungal pathogens is associated with more robust regulation of complex but largely non-overlapping sets of pathogen-responsive genes involved in similar metabolic or signalling pathways.

The penetration resistance to *M. oryzae* in multiple mutants, including pen2 NahG pmr5 agb1 and pen2 NahG pmr5 mlo2 plants, was severely compromised and that fungal growth was permitted in penetrated epidermal cells (Nakao et al 2011). Furthermore, rice *Pi21* enhanced movement of infection hyphae from penetrated *Arabidopsis* epidermal cells to adjacent mesophyll cells. These results indicate that PEN2, PMR5, AGB1, and MLO2 function in both penetration and post-penetration resistance to *M. oryzae* in *Arabidopsis*, and suggest that the absence of rice *Pi21* contributed to *Arabidopsis* NHR to *M. oryzae*. In the plant pathogenic fungus *M. oryzae*, there is also an association between chromosome ends and genes that control interactions with the host. The ability of *Magnaporthe* to infect a given host species - or, in some cases, a specific cultivar of a species is controlled by avirulence genes which code for secreted proteins that are translocated in the host cytoplasm. To date, approximately half of the 20-plus avirulence genes that have been identified in *M. oryzae* map very close to telomeres (Farman 2007). Starnes et al (2012). has recently revealed two related non-LTR retrotransposons (*M. oryzae* telomeric retrotransposons - MoTeRs) inserted in the telomere repeats in perennial rye grass. This contrasts with rice pathogen telomeres which are

uninterrupted by other sequences. Genetic evidence indicates that the MoTeR elements are responsible for the observed instability. Hence, understanding molecular basis of rice blast resistance though has been studied very extensively in some labs of the world, its application for the development of long lasting resistance to rice blast yet to be realized.

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