6 SUMMARY
Rice is one of the most important food crops in the world, and its production is severely affected by devastating fungal diseases, such as rice blast. Though a range of fungicides are available to control this disease, they are not very effective and their use causes environmental pollution and adds costs to the farmers. Hence, exploitation of host resistance is being considered as the best option to combat this disease. Though more than 80 rice blast resistance genes are reported, only few genes like $P_{ih} (Pi54)$, $P_{ita}$ and $P_{ib}$ are reported to provide broad spectrum resistance to the wide range of isolates of $M. oryza$ in India and elsewhere. Incorporation of such major rice blast genes in elite rice varieties may improve the resistance significantly and save the crop from damage by the disease. For successful pyramiding of these genes, identification and selection of novel/superior alleles of the selected gene(s) is very important, which can be achieved by allele mining approach.

Allele mining refers to identification and exploitation of the natural variation of the candidate gene/loci. Allele mining is performed in coding as well as non-coding region as many recent reports have shown that the polymorphism at the non-coding region (5’ UTR and intron) affects the gene expression and hence the phenotype of the trait. Transcription factor binding motifs (TFBMs) or simply motifs play major role in gene expression.
Arrangement and presence/absence of the motifs may affect the expression pattern of the candidate gene. For proper expression of the gene, necessary motif element should be present in the upstream region. The minimum upstream region, which contains necessary motifs to express the candidate gene, is referred as core promoter. Identification of core promoter can be done with deletion analysis. Based on the above mentioned points, the research work was carried out to identify the novel/superior alleles and TFBMs of major blast resistance genes – *Pi54, Pita* and *Pib* and also to identify the core promoter of the *Pita* gene by deletion analysis.

Selected plant materials (24 diverse landraces and 110 accessions belonging to eight different wild *Oryza* species) were screened with differential isolates for the selected genes. Among the materials analyzed, landraces, Ammana Bavo and Boha thulasi joha showed complete resistance with *Pi54* specific differential isolate. Konibora and Punsimutt with *Pita* specific differential isolate and Sercher and Krengosa with *Pib* specific differential isolate showed resistant phenotypic pattern. While, Podumoni Ahu and Bizor II, landraces showed extreme susceptibility for all the differential isolates. These highly resistant and complete susceptible genotypes were selected for the allele mining study. In case of wild *Oryza* species also, three extreme resistant and three complete susceptible accessions were selected for this study. The presence of the selected genes was confirmed by molecular markers also. In
order to analyze the \textit{Pi54} allelic diversity, six different genotypes, having distant genomes (AA to EE) were also included for the allele mining study.

For the first time, sixteen novel alleles of \textit{Pi54} were identified, which can be used in the blast breeding programs. High nucleotide diversity was found in alleles derived from wild \textit{Oryza} species than in the landraces. The distant wild species, \textit{O. alta} and \textit{O. australiensis}, which belong to CCDD and EE genome respectively, had the highest nucleotide variations among all the analyzed \textit{Pi54} alleles. Phylogeny and evolutionary distance analysis also confirmed the above results. The presence of \textit{Pi54} in wide range of \textit{Oryza} species indicated that this gene might have originated long time ago and it should be functionally significant and provides resistance against wide range of isolates.

Among the many nucleotide polymorphisms found, 45 SNPs and four InDels differentiated the resistance and susceptible alleles. Among those differentiating polymorphism, one InDel, which was 144 bp and located in the exonic region of the allele, was targeted to develop the functional marker, which was validated in diverse cultivars and in BC\textsubscript{1}F\textsubscript{2} mapping population. The results showed that the marker is polymorphic in most of the resistant–susceptible genotype combinations and this marker is more accurate than the earlier reported markers for \textit{Pi54}. This is the first report for the development of
functional marker for *Pi54* to differentiate the resistance and susceptible alleles, which will be very useful in marker assisted breeding program of *Pi54*.

Different *Pi54* promoter alleles were also sequenced and the nucleotide diversity revealed that promoter region also showed polymorphism among the resistance and susceptible *Pi54* alleles. The distribution of many defense related motifs were also found all over the promoter region in all the resistance promoter alleles. In *Pi54* promoter alleles derived from the landraces, ten different motifs (whose function is reported from other systems) and a novel motif i.e ACCCCAGG differentiated the resistance and susceptible promoter alleles.

Two landraces *viz.* Ammana Bavo and Boha thulasi joha showed better phenotypic reaction (score of 0) than Tetep (*Pi54* donor, with score of 1), The higher resistance in these two landraces might be because of the acquired and accumulated mutations in the allelic and promoter regions of *Pi54*.

Five novel alleles were identified for *Pita*. Among the *Pita* alleles, the nucleotide diversity was less and alleles showed 99% similarity with the reference sequence. This may be due to fewer mutations and selection pressure that operates in these alleles in the nature. *Pita* promoter alleles also conserved like allelic sequences. However, motif level polymorphisms were observed between the promoter alleles and novel motifs could be predicted by reliable motif predicting tools.
Three *Pib* novel alleles were identified and all the alleles showed higher nucleotide diversity and structural differences by the presence of (3-5) ORFs in comparison with the reference sequence. The Ka/Ks ratio also indicated that these alleles are under the selection pressure as the values were very high. At the promoter level, 1.5 Kb InDel was observed between the resistance and susceptible promoter alleles. Due to the accumulated polymorphisms between the alleles, motif level differences were also recorded.

The *Pita* promoter allele of resistant landrace, Konibora was selected for core promoter analysis. Three constructs were prepared with three different lengths of promoter alleles (-1 to -449 bp, -1 to 935 bp and -1 to 1592 bp) and these constructs were transferred to Taipei 309 through *Agrobacterium* mediated transformation. The presence and orientation of the promoter constructs in the plants were confirmed by PCR analysis. Fluorescence of the GFP and cDNA analysis confirmed that all the three constructs could express the GFP and the core promoter was demarkated with -449 bp. Quantification of cDNA revealed that -1592 bp lengthy promoter had expressed GFP two fold higher than other constructs. The possible motifs, which may be responsible for this higher GFP expression, were identified.

Thus 16, 5 and 3 novel alleles of *Pi54, Pita* and *Pib* respectively were isolated from different landraces and wild *Oryza* species. In case of *Pi54*, superior alleles were identified from the landraces, Ammano Bavo and Boha
Tulasi Joha, which showed better resistance than Tetep. Functional marker for \textit{Pi54} also was developed to differentiate the \textit{Pi54} resistant and susceptible genotypes. Different sources of resistance also were identified for \textit{Pita} (Konibora and Punsimutt) and \textit{Pib} (Sercher and Krengosa). Analysis of allelic sequences confirmed that the alleles derived from wild species had more divergence than the alleles derived from landraces. Distribution and polymorphic TFBMs analysis revealed the significance of the motifs. Core promoter analysis led to the demarcation of length of \textit{Pita} core promoter and motifs were identified, which were probable response for the higher expression of Del III \textit{Pita} promoter.

In continuation of this study, the identified superior alleles (\textit{Pi54}^{AB} and \textit{Pi54}^{BTJ}) can be validated for their efficacy by transferring these alleles to the susceptible genotype. After confirmation of their strong resistance, these alleles can be used in molecular breeding with/ without the \textit{Pi54} allele derived from Tetep for developing broad and durable resistant varieties. The identified novel resistance alleles of \textit{Pib} and \textit{Pita} also can be good resource for blast resistance breeding programs. Upon the nucleotide sequence analysis, many polymorphisms were identified, which differentiated resistant and susceptible \textit{Pi54} alleles. These observed nucleotide changes (SNPs and InDels) in the coding and non-coding regions can be used for knowing the different haplotypes distribution and their haplotype structure among population. The TFBMs, which differentiated resistant and susceptible promoter alleles of \textit{Pi54}, \textit{Pita} and \textit{Pib} by their presence/ absence, can be validated for their role in
resistance reaction by motif deletion analysis. For finding the rice based constitutive promoters, the expression of constructs with different *Pita* promoter length can be compared with expression of CMV 35s promoter, so as to replace the 35s promoter with rice specific plant promoter in trans-gene expression studies.