

**Integrating molecular, phenotypic and *in silico* approaches
for quantitative resistance to rice blast**

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Marker-aided selection has been the hallmark of plant breeding for the past couple of years. Linkage of ideal crop traits with different types of molecular markers have been exploited for selecting advanced breeding lines with the desired plant phenotypes. In recent years, the candidate gene (CG) approach has been gaining acceptance in MAS. CGs are DNA sequences similar to known genes or conserved motifs that make it possible to infer their biological functions (Pfleiger et al., 2001). In this method, genes with known functions or are associated with a particular phenotype are used to probe mapping populations for candidate gene loci and correlated with the desired phenotype. This approach has been successfully applied to associating heterologous CG markers with disease and insect resistance in a doubled-haploid rice mapping population (Ramalingam et al., 2003). Several candidate genes for disease response mapped to previously identified disease resistance quantitative trait loci (QTLs). These probes included resistance gene analogs as well as a number of pathogenesis related proteins including chitinase, oxalate oxidase and thaumatin representing different mechanisms of resistance to pathogen. Cloned rice genes such as the rice peroxidase POX22.3 and resistance gene analogs were also mapped. This frame map has been a useful reference for selecting candidate genes involved in both pathogen recognition and general plant defense for analysis of mapping populations for improving resistance to rice blast.

While the use of resistant cultivars is the most effective and economical way to manage rice blast, durability of resistance is the primary problem, especially in upland rice and in temperate and subtropical irrigated rice (Bonman et al., 1992). Both major resistance (R) genes and quantitative trait loci (QTLs) are involved in resistance to blast. The loss of resistance to blast has been most striking and best documented when resistance is conferred by major genes (Kiyosawa, 1982). Durable resistance to blast involved major genes and QTLs (Wang et al., 1994) Recent approaches to developing cultivars with durable resistance involves advance backcross (AB) populations simultaneously analyzing for QTLs and selecting elite germplasm for commercial use. Wu et al (2004) developed an AB population from Moroberekan x Vandana to introgress genes from blast-resistant Moroberekan to high-quality variety, Vandana. Instead of using anonymous markers across the genome, they used candidate genes

involved in defense response pathways to analyze the AB population. Oxalate oxidase and 14-3-3 proteins were associated with lesion number. In another study with the same approach, Liu et al (2004) demonstrated that the candidate genes oxalate oxidase, dehydrin, PR1, chitinase and 14-3-3 accounted for 60.3% of diseased leaf area (DLA) variation. These genes also co-localized with resistance QTL identified by interval mapping.

The availability of the whole rice genome sequence from public and private sequencing efforts has provided opportunities to predict putative functions of a gene based on sequence information, thus allowing the identification of CGs. PCR-based markers can be designed based on candidate gene sequence derived from rice. SSR (simple sequence repeat) markers co-localizing with the known candidate genes can be identified and also used as markers. It has also provided insight into the genome organization of candidate genes belonging to gene families. In most cases, several copies of the gene occur in mapped loci. In other cases, while occurring in singletons, highly similar sequences can be found elsewhere in the genome. Thus, it has become imperative to use both genetic data for linkage of CGs to desired phenotypes and physical maps for precise location of the CGs in the rice genome.

The goal of the present study is to develop cultivars with durable resistance to rice blast by accumulating different mechanisms of quantitative resistance and simultaneous selection of progenies with good agronomic traits for cultivar release. To accumulate different CGs, we used phenotypic, molecular and in silico approaches. Progenies derived from the present study were screened for resistance to rice blast at blast “hotspots” in India (Almorra and Hazaribag) and the Philippines (Cavinti) and at the IRRI blast nursery. Progenies were scored for seedling blast and panicle blast reaction at appropriate stages. Progenies exhibiting a good level of resistance to rice blast and desirable morphological and agronomic traits were selected for advancement to the next generation. Advanced lines were analyzed for the presence of CGs using PCR-based markers derived from rice sequences and SSR markers co-localizing with CGs. The CGs used were identified from previous mapping populations as well as from early microarray experiments. We used in silico approaches to gain insight into the function, organization and characteristics of each candidate gene.

To accumulate different mechanisms of resistance to rice blast, 15 BC₃F₃ or BC₃F₄ parental lines derived from Vandana/Moroberekan (Wu et al., 2004) showing partial resistance and carrying positive candidate alleles were selected and crossed in all pairwise combinations. Seeds of 10 F₂ families were selected based on partial resistance of the BC₃F₃ or BC₃F₄ parental lines to seedling blast and neck blast, association of the parental lines with positive alleles, and phenotypic similarity to Vandana. Among the 10 F₂ families, 14 F₂ plants showed a good to moderate level of agronomic acceptability and high level of seedling blast resistance (0.75–3.2% DLA). Resistant lines from selected families were advanced to F₃ until F₅. At F₄, the top 10% of the lines (60 out of >600 lines) derived from the progenies of VM5/VM14, VM6/VM14, and VM82/VM14 have acceptable agronomic traits, and served as a basis for further selection and advancement to F₅. The field performance of these advanced BC lines indicated that the major QTLs have been captured in the BC lines. From the 600 lines, the top 84 lines based on reaction

to blast and morphoagronomic traits were selected for further studies. We also included in the study, 24 Vandana x Moroberekan drought tolerant lines which were not selected for blast resistance.

The intermated F₅ lines of BC3F5 Vandana x Moroberekan populations were analyzed for accumulation of CGs. The 11 defense response CGs analyzed are the following: oxalate oxidase, oxalate oxidase-like proteins, aspartyl protease, 14-3-3, PR-1, probenazole-induced protein, peroxidases, HSP90, thaumatin-like proteins, adenosylhomocysteinase, and aldose reductase. To identify informative markers for analysis, PCR primers for each CG were designed based on the 1kb upstream region, the gene-coding region and the 3' UTR. Polymorphic markers were generated for oxalate oxidases, PR-1, probenazole-induced protein, and HSP90. Thirty SSRs colocalizing with known CGs gave polymorphic fragments between Vandana and Moroberekan and were subsequently used to identify introgressed segments from Moroberekan. An additional 120 SSRs randomly dispersed in the rice genome were also used to conduct a genome scan of all progenies.

Initially, we focused on consensus DR genes for *in silico* analysis. Sequences were retrieved from the Rice Genome Program database (<http://www.tigr.org/tdb/e2k1/osa1/>). For genes occurring in gene families such as the germin-like proteins (GLP), we derived phylogenetic trees using the retrieved sequences. We also checked for conserved promoter motifs and identified *cis*-elements in the 1,000-bp upstream regions using MEME (<http://meme.sdsc.edu/>) and PLACE (Plant *Cis*-Acting Elements, <http://www.dna.affrc.go.jp/PLACE>).

Results

By combining molecular, phenotypic and *in silico* approaches, we were able to generate several lines with high of resistance to rice blast with moderate to good agronomic traits. The genome scan showed that at the CG loci, such as oxalate oxidase in chromosome 3, introgression of Moroberekan chromosomal segments in resistant lines is evident (Figure 1). It is interesting to note that for the drought-tolerant lines which were not selected for resistance to blast, introgression of Moroberekan chromosomal segments at CG loci was not evident. Single gene analysis of variance (ANOVA) correlated peroxidase with yield under blast in Almora, India ($p < 0.0001$). Both peroxidase and oxalate oxidase seem to be important for panicle blast resistance in the same location. Oxalate oxidase and thaumatin are significantly associated with seedling blast resistance at the IRRI blast nursery. In Cavinti, Philippines, thaumatin is associated with panicle blast resistance. Two-gene ANOVA ($p < 0.0001$) reveal association of oxalate oxidase*thaumatin with yield under drought conditions and with seedling blast resistance at IRRI, HSP90*thaumatin with seedling blast resistance at IRRI, and thaumatin*oxalate oxidase with panicle blast resistance in Cavinti. Molecular analyses also revealed that we were able to accumulate different CGs into several lines and that these lines have a moderate to good level of resistance to rice blast (Figure 2). We were able to identify several lines resistant to blast with several combinations of CGs good agronomic qualities

and similar yield performance with the recurrent parent, Vandana. These lines include IR78221-19-6-56, IR78221-19-6-7 and IR78221-19-6-99.

By analyzing the functional annotation, organization and characteristics of each CG, we were able to validate genetic mapping data with the rice physical map and generate additional molecular markers for analysis. For example, from published genetic maps, oxalate oxidase was mapped to chromosome 3. A look at the physical map reveal that there are four putative oxalate oxidases in tandem in the candidate gene loci. These sequences are highly identical to each other. PCR markers designed from the 3'UTR region did not get any polymorphic product. Interestingly, length polymorphism was observed in the 1kb upstream of two of these genes. There was also variation in the number of copies of cis-elements related to disease response. This is also true for the oxalate oxidase-like protein mapped to a disease QTL in chromosome 8 where we identified 12 copies of the gene in the expected loci. Phylogenetic analysis reveal the relationship of oxalate oxidase and oxalate oxidase-like protein to other germins and germin-like proteins with important roles in germination and other pathways. Chromosome 3 oxalate oxidase is also orthologous to barley oxalate oxidase which is expressed in resistant interaction to powdery mildew (Wei et al., 1998; Zhou et al., 1998).

Summary and Conclusion

We used molecular, phenotypic and in silico approaches to develop rice lines with different defense response CG combinations with the aim of producing elite breeding lines with resistance to rice blast and desirable agronomic traits. This was made possible by screening progenies derived from crosses of 15 BC₃F₃ or BC₃F₄ from Vandana/Moroberekan (Wu et al., 2004) for resistance to blast in multiple blast 'hotspots' and for good agronomic traits. Molecular markers were developed and SSRs markers colocalizing with CGs were identified. We were able to identify several promising lines that are potential candidates for commercial release in blast-prone environments. The markers used in this study are being applied to other mapping populations exhibiting resistance to rice blast.

To contribute to the practicality of this approach, we continue to investigate the genetic behavior of the QTLs in different genetic backgrounds and find consensus CGs. We also continue to develop markers derived from consensus candidate defense genes conferring partial resistance instead of just close linkage to resistance genes, and develop efficient and economical breeding procedures using molecular genotyping. Our research has therefore focused on these areas aimed at facilitating the selection of cultivars with broad-spectrum resistance against blast pathogen populations in diverse rice-growing environments.

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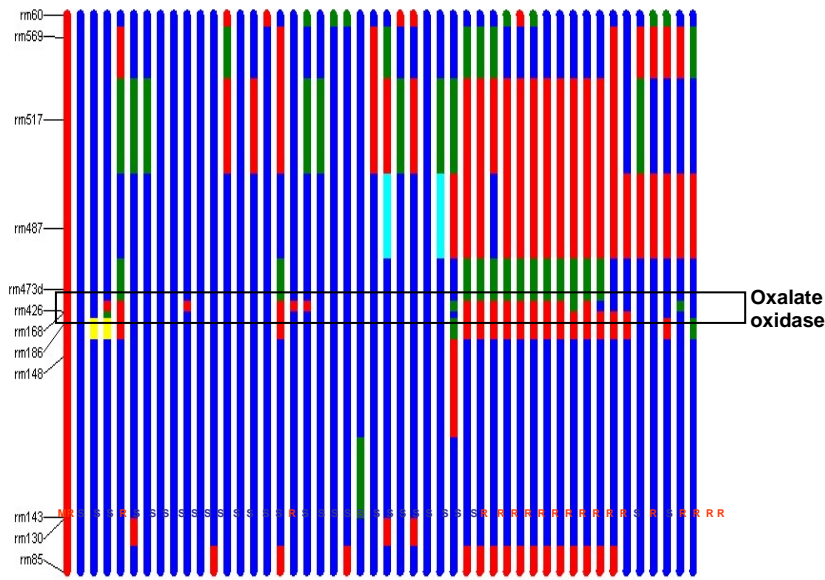


Figure 1. Genome scan of selected Vandana x Moroverekan progenies. Using 120 random SSRs and polymorphic CG-associated markers in Vandana and Moroberekan, 108 lines were targeted for genome scanning of introgressed genomic regions from Moroberekan into Vandana . These lines included 84 lines which are resistant to blast based on multilocation testing and 24 drought-tolerant V x M lines. The genome scan was generated by Graphical Genotype or GGT software (van Berloo , 1999).

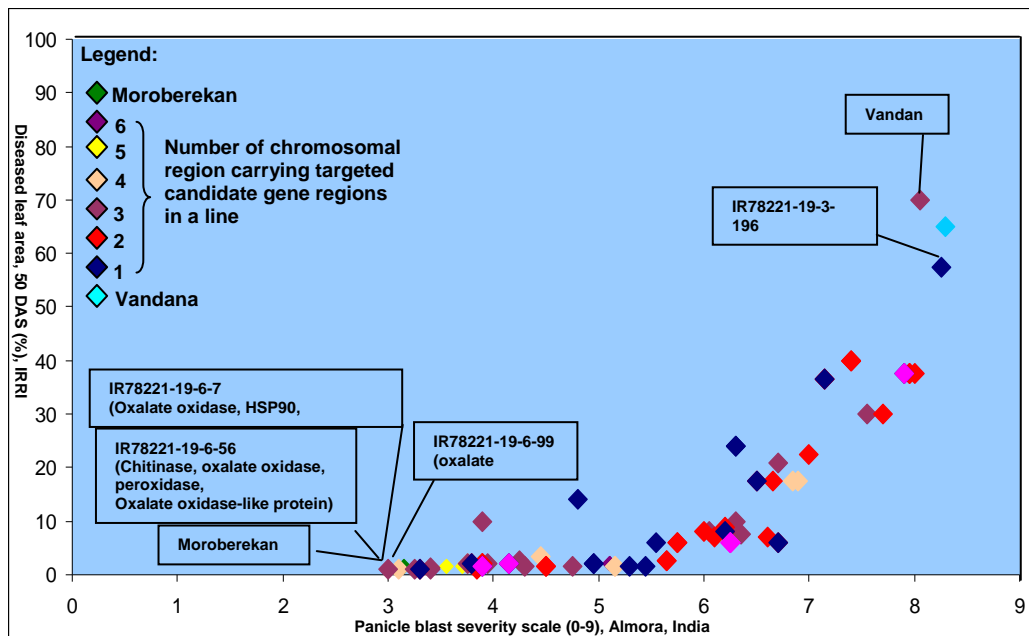


Figure 2. Lines are shown with DLA and severity scale as well as the number of candidate genes present. Highlighted lines (in boxes) with their corresponding candidate genes are being studied for expression of oxalate oxidase and peroxidase during interaction with rice blast isolate PO6-6. IR78221-19-6-56, IR78221-19-6-7 and IR78221-19-6-9 also have good yield performance.