

# Exploring the application of molecular markers for improving resistance to bacterial blight

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Bacterial blight of rice, caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), is endemic in many parts of Asia and periodic epidemics can cause severe yield losses. Although many genes for resistance to bacterial blight are known, few have been systematically used for rice improvement. To allow the judicious use of resistance, it is important to understand the population biology of the pathogen and to understand the genetics of resistance. Molecular marker technology has contributed to both of these objectives. A series of molecular markers was developed for analysis of *Xoo* populations, including four transposable elements, a family of avirulence genes, and a set of polymerase chain reaction-based markers. One of these primers can be used for DNA fingerprinting of pathogen cells oozed directly from infected leaves. To shed light on the diversity and distribution of *Xoo*, a hierarchical analysis of variation was conducted. Pathogen populations from different Asian countries were distinct. Intensive sampling was conducted in the Philippines, revealing population differentiation between regions, sites, and even fields within a site. Certain DNA fingerprint types (haplotypes) were widely distributed (even between countries), providing evidence of pathogen movement. By subsampling the dataset, an optimized sampling strategy could be recommended for subsequent studies. Resistance genes for bacterial blight have been tagged and mapped in several laboratories. Using restriction fragment length polymorphism-based methodology, even genes with minor effects on disease (putative quantitative trait loci) have been detected. Based on inoculation data, gene combinations likely to be effective for disease reduction in the field were designed. Several rice lines carrying multiple genes have been produced by marker-assisted selection. In some cases, such as in the *Xa4* + *xa5* line, plants carrying pairs of resistance genes show

greater resistance than expected based on the performance of the individual genes. Experiments on the deployment of resistance genes are being conducted in Laguna, Philippines. These studies are making use of near-isogenic lines carrying different resistance genes, as well as mixtures and pyramid lines. Analysis of pathogen subpopulations sampled from different treatments indicated which genes are functionally distinct in this environment. Lines carrying *xa5*, expected to be resistant, showed low levels of disease due to selection of a rare pathogen lineage. Now that powerful and inexpensive methods are available for analyzing pathogen populations and for identifying resistance loci, it will be possible to deploy resistance more effectively.

Bacterial blight (BB), caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), is endemic in many parts of Asia. The disease became prominent in the 1960s, when new high-yielding varieties were first developed and introduced (Mew 1987). Varietal resistance is the main strategy for controlling the disease. The *Xa4* gene for BB resistance was introduced from TKM6 into improved varieties and has provided durable resistance to BB in China for 2 decades (Bonman et al 1992). In Southeast Asia, however, compatible races have developed. Over the 10-yr period following the release of varieties carrying the *Xa4* gene for resistance in the Philippines, pathogen races able to overcome the gene became increasingly frequent in the pathogen population (Mew et al 1992).

Perhaps because of the substantial quantitative resistance incorporated into most of the improved varieties (Koch and Parlevleit 1991a), and in part due to residual resistance conditioned by the *Xa4* gene (Koch and Parlevleit 1991b), the disease has rarely reached epidemic proportions in recent years. However, severe epidemics still occur periodically. For instance, the rice crop in the Punjab region of India was badly damaged by BB in 1995. Now, particularly as new rice types are being developed to meet the food requirements of increasing populations, there is a need to devise strategies for the deployment of our finite genetic resources for BB resistance.

Although many genes for resistance to *Xoo* are known, few of these genes have been systematically deployed in improved rice cultivars. Molecular marker technologies have been applied to the BB pathosystem, leading to greater knowledge about both pathogen and host. Many BB resistance genes have been mapped on the rice chromosomes, and several have been tagged with molecular markers, and one has recently been cloned. These advances should make it possible to deploy resistance genes more efficiently and effectively. This paper reviews recent research progress that could be of value in designing strategies for the deployment of resistance to BB.

## Progress in analysis of populations of *Xoo*

### Virulence analysis

To determine the pathotypic diversity of Philippine populations of *Xoo* and to assess differentiation of the pathogen over time and across regions, extensive surveys of the BB pathogen were conducted in the Philippines in the 1970s and 1980s (Mew et al 1992). To allow pathogen virulences to be determined, a set of differential cultivars was developed, including varieties and lines carrying *Xa4*, *Xa10*, *xa5*, *Xa7*, and *Xa18* (Mew and Vera Cruz 1979, Mew et al 1982, Vera Cruz and Mew 1989). Based on these studies, six strains of the pathogen were selected to represent the six races detected, and these have been widely used to represent the diversity of the pathogen in the Philippines, at IRRI and elsewhere. Strong spatial and temporal differentiation among pathogen collections was observed in the Philippine pathogen population (Mew et al 1992).

Although pathotypic analysis has provided a clear picture of race diversity and distribution, it does not allow the evolutionary relationships among pathotypes to be discerned. Inoculation tests require considerable time, space, and labor. In an effort to learn more about race evolution of *Xoo*, and in the hopes of developing efficient techniques for monitoring pathogen populations, molecular markers were utilized to characterize pathogen collections (reviewed by Leach et al 1995).

### Development and utilization of DNA markers for *Xoo*

Underlying initial efforts at DNA fingerprinting of *Xoo* was the hope that the groups of strains distinguished by molecular markers would correspond closely with the races distinguished by inoculation tests. The relationship between phylogeny (as inferred from DNA fingerprinting analysis) and pathotype has not turned out to be that simple. The first study of DNA fingerprint of *Xoo* was based on the repetitive element pJEL101, cloned from the genome of the pathogen itself (Leach et al 1990). Leach et al (1992) used this element to analyze a subset of the Philippine collection developed by Mew et al (1992), which included strains representing the six recognized races. Five groups of strains were distinguished at the 75% similarity level. Some groups contained strains from more than one race, and some races were found in more than one group.

To further investigate the relationship between phylogeny and virulence, Nelson et al (1994) isolated four of transposable elements from *Xoo*, one of which proved homologous to the element used in the study of Leach et al (1992). In addition to the transposable elements, an avirulence gene probe was also utilized as a hybridization probe for population analysis. This probe, cloned by Hopkins et al (1992), carried the gene *avrXa10*, a member of a family of avirulence genes in *Xoo* and other xanthomonads. Based on the results of bootstrap analysis, we found that some probes gave much more stable phenograms than others. The avirulence gene probe did not provide more useful information than the “random” fingerprinting probes. Relatively stable phenograms were derived from the restriction fragment length polymorphism (RFLP) datasets obtained using *IS1112* (the pJEL101 homolog) and *IS1113*. The phenograms derived from these probes gave relatively robust phenograms, and results from the two were fairly consistent.

From analysis using these probes, we were able to make inferences about the evolution of the Philippine races of *Xoo*. Race 1 was predominant in Central Luzon prior to the wide cultivation of varieties carrying *Xa4*, and was later replaced by race 2, which could overcome *Xa4*. Strains of race 2 were found to more closely resemble strains of race 5 than those of race 1. Race 2 was thus apparently not derived by mutation from race 1, but rather was derived from race 5 by mutation and migration. Race 3, which can overcome *Xa4* and which has become prevalent in parts of Luzon (Ardales et al 1996), is closely related to race 1.

### **Discrimination of “new” races based on DNA fingerprinting**

For three of the races, strains designated as belonging to the same race were found in distinct clusters based on DNA fingerprint analysis. Strains belonging to the same race group but representing different lineages were tested on rice varieties and near-isogenic lines carrying previously untested resistance genes. For two of the “split” races, a new pathotype could be distinguished based on reaction on the *Xa14* gene, with the “new” pathotype corresponding to one of the groupings defined by DNA fingerprinting. For race 3, however, consistent differences in reactions were not obtained by Nelson et al (1994).

To clarify the genetic and phenotypic structure of race 3, Finckh and Nelson (IRRI, unpubl. data) analyzed a larger collection of strains (120 strains of race 3, plus representatives of other races) previously classified as race 3 by Mew et al (1992). For each of the strains analyzed, DNA fingerprints were obtained using *IS1113* and *avrXa10*. Based on their DNA fingerprints, the 43 haplotypes of race 3 strains were divided into three groups, and 77 of the race 3 isolates were inoculated on expanded sets of differential cultivars. Statistically significant differences in aggressiveness on IR-BB7 carrying *Xa7* were detected between the two major groups of race 3 strains.

### **Pathogen variation in Asia**

Adhikari et al (1995) analyzed 308 strains of *Xoo* provided by scientists from seven rice-growing countries in Asia using the probes *IS1112* and *avrXa10*. They detected evidence indicating both regional differentiation (many groups of similar strains were specific to one country), and for pathogen migration between countries (some groups of the pathogen were common to more than one country). Similar observations were made by scientists in the national rice research programs of the Philippine Rice Research Institute (PhilRice) and the Central Research Institute of Food Crops (CRIFC) in Indonesia. For instance, the *IS1113* haplotype that predominates in parts of both Indonesia and the Philippines is identical (Ardales et al 1993; PhilRice, CRIFC, and IRRI, 1995, unpubl. data). This may be related to the popularity of IR64 in both countries.

### **Hierarchical analysis of pathogen diversity in the Philippines**

To determine the extent and distribution of pathogen diversity among agroecosystems, sites, and fields, we conducted a hierarchical analysis of pathogen variation along a transect on Luzon in the Philippines (Ardales et al 1996). More than 1,200 strains

were collected systematically along a 310-km transect spanning an indigenous rice-growing area in the mountainous region and the modern agroecosystem in the lowlands of Luzon. Each strain was characterized by DNA fingerprinting with *IS1113* and restriction enzyme analysis (REA) using *Pst*I, and by inoculation on a set of differential cultivars.

Nineteen haplotypes, grouping into three robust clusters, were defined using *IS1113*. *Pst*I fingerprinting revealed greater diversity (46 unique haplotypes defined), but the derived phenogram was not robust. Within a site, there was a near-perfect correspondence (>99%) between lineage or haplotype and race. Hierarchical analysis of genetic variation showed population substructuring at all levels. A high degree of genetic differentiation was seen among ecosystems and sites. On this backdrop, there was also evidence of pathogen migration: five *IS1113* haplotypes were present in more than one site and one of these haplotypes was found at 10 sites located in all six provinces sampled in the study. A subsampling exercise using the datasets obtained in this study suggested that pathogen populations could be sampled less intensively by decreasing the number of samples collected per field, without considerable loss of diversity.

### Development of PCR-based markers

To allow greater efficiency for future studies on the BB pathogen, we decided to develop polymerase chain reaction (PCR)-based markers that could be used to obtain robust DNA fingerprints from bacteria leached from lesions collected directly from the field. We experimented with various PCR-based methods, including randomly amplified polymorphic DNA (RAPD) sequence, ligation-mediated PCR, and PCR (George et al 1995; M.L.C. George, IRRI and Kansas State University, 1995, unpubl. data), and REP, ERIC and BOX, as well as PCR with various other primers (C.M. Vera Cruz, T. Adhikari, and J.E. Leach, IRRI and Kansas State University, 1995, unpubl. data). Findings in both the Kansas State laboratory and IRRI indicate that primers amplify the regions of the bacterial genome between *IS1112* elements (primers designed from the ends of the *IS1112* element). The fingerprints obtained using this method were consistent between experiments, even from bacteria oozed directly from infected leaves. The groups of strains identified based on this primer were the same as those obtained using RFLP.

### Progress in the analysis of resistance to BB

Over the last several years, substantial progress has been made in understanding the genetic basis of resistance to BB. Nineteen resistance genes have been reported (Kinoshita 1991). Near-isogenic lines (NILs) carrying individual resistance genes have been developed in three genetic backgrounds, providing superb differential sets suitable for different environments (Ogawa et al 1991, Ogawa 1993). The availability of NILs makes it possible to systematically characterize the resistance spectra of the genes in relation to pathogen strains.

Inoculation of these NILs with a range of Philippine pathogen strains revealed that the available resistance genes do not have simple complementary resistance spectra. Most of the NILs were susceptible to races 4 or 6. Among the NILs tested, only IR-BB21 gave resistance to the strains of races 4 and 6; this line, carrying *Xa21*, was resistant to all of the strains tested. Under these circumstances, it might be desirable to utilize a broad-spectrum resistance gene, such as *Xa21*, in combination with other resistance genes. Because the *Xa21* gene conditions resistance to all the isolates tested from South and Southeast Asia (Ronald et al 1996), it would not be possible to combine this gene with other resistance genes by phenotypic selection. Lines carrying multiple resistance genes could, however, be selected through the use of molecular markers closely linked to resistance genes. Through the work of researchers in several laboratories, many BB resistance genes have recently been mapped relative to molecular markers (e.g., Ronald et al 1992, Yoshimura et al 1992a,b; 1995a,b).

For example, Bordeos and Nelson (IRRI, 1994, unpubl. data) tagged a resistance gene derived from the wild rice species, *Oryza minuta*. A BB-resistant BC<sub>2</sub> progeny of the *O. sativa*/*O. minuta* cross was identified (Amante-Bordeos et al 1991). To obtain molecular markers linked to the resistance gene, we conducted bulk segregant analysis for resistant and susceptible plants selected from a BC<sub>2</sub>F<sub>3</sub> population developed from the resistant line. Pooled DNA extracts of the resistant and susceptible BC<sub>2</sub>F<sub>3</sub> lines, or DNA extracts of a resistant line and the *O. sativa* parent, were analyzed with 493 10-base primers. To determine whether a band observed in the resistant bulk or individual was associated with resistance, putative positives were reassayed using four resistant and four susceptible lines and the parents, and finally using the segregating population of 60 individuals.

Bands derived from *O. minuta* and cosegregating with resistance were amplified using primers OPAC11 (5'-CCTGGGTCAG-3') and OPL13 (5'-ACCGCCTGCT-3'). OPAC11<sub>700</sub> was located 4.7 cM from the resistance locus, while OPL13<sub>700</sub> was located 11.3 cM from the gene. Efforts to generate RFLP markers based on the RAPD markers have not been successful. Both RAPD fragments, when used as probes, hybridized with repetitive elements in the rice genome. Due to the presence of *Xa4* and perhaps other genes affecting the reaction to the BB pathogen in the recurrent *O. sativa* parent, the resistance spectrum of the gene from *O. minuta* cannot be characterized in detail at present. Crosses have been made to transfer the resistance to the IR24 genetic background.

With the availability of several resistance genes tagged with molecular markers, it has become feasible to select lines carrying a desired combination of genes. The utility of the gene pyramiding strategy would depend on the phenotype of lines carrying multiple genes, and on the ability of pathogen populations to adapt to such host genotypes. To assess the interactions between major genes, Yoshimura et al (1995b) tagged *Xa3*, *Xa4*, *xa5*, and *Xa10* and used a combination of molecular markers and inoculation assays to select lines carrying pairs of resistance genes. They compared the lesion lengths produced for diverse pathogen strains, on NILs carrying one or two genes in the IR24 genetic background. In some cases, epistatic interactions were

detected, so that lesions developed more slowly on the pyramid line than on either parental line for some isolates. For instance, lesion lengths for PXO71 (race 4) were shorter on the *Xa4* + *xa5* pyramid than on either the *Xa4* or *xa5* line at the maximum tillering stage (Yoshimura et al 1995b). Such effects were also seen for some strains of race 6.

In rice breeding programs at IRRI, and in national rice improvement programs in the Philippines, Indonesia, and India, *Xa4*, *xa5*, *Xa7*, and *Xa21* are being transferred to commercially important rice varieties (L. Sebastian, PhilRice, 1995, pers. commun.; M. Bustamam, CRIFC, 1995, pers. commun.; N. Huang, IRRI, 1995, pers. commun.). *Xa21* has recently been isolated by the University of California, Davis laboratory (Ronald et al 1996). This will make it possible to deploy the gene more easily, by direct gene transfer into varieties with various genetic backgrounds.

Genes for quantitative resistance to BB were mapped in two populations. A preliminary analysis of quantitative resistance was conducted for a recombinant inbred (RI) population derived from the cross Asominori/IR24 (A. Yoshimura, T. Mew, and R. Nelson, Kyushu University and IRRI, 1994, unpubl. data). Asominori is considered to have a high level of horizontal resistance to BB in Japan. RFLP data at 115 marker loci were obtained in Japan for the 67 lines in the population. Isolates PXO71 (race 4) and PXO99 (race 6) were inoculated to each of the RI lines at IRRI. For both isolates, a continuous distribution of lesion lengths was observed for the population. The general linear model procedure of SAS was used to examine the association between marker loci and effects on lesion length. For both of the isolates used, the known BB resistance locus *Xa1*, and markers linked to it, had a significant effect ( $P > 0.01$ ) on lesion length, though even the more resistant group had long (susceptible-type) lesions. For each of the isolates, three other loci were also identified with effects significant at the 5% level. Among these, only marker Xnpb333, on chromosome 10, showed significant effects for both of the isolates tested.

The IR64/Azucena doubled haploid (DH) mapping population was also used for mapping of quantitative trait loci (QTLs) affecting reaction to *Xoo* (M. Baraoidan, R. Nelson, S. McCouch and N. Huang, IRRI and Cornell University, 1995, unpubl. data). RFLP data at 135 loci were available for this population from previous work done in the IRRI and Cornell laboratories. Three inoculation experiments were conducted on this population. Based on the first two experiments, putative QTLs were identified. The third experiment was conducted to test hypotheses established in the preceding experiments. Lines with and without the putative QTLs, and not carrying *Xa4*, were inoculated with 15 diverse strains of the pathogen. Based on general linear modeling, a large number of significant locus by isolate interactions were detected. We were most interested, however, in those loci affecting most or all of the pathogen strains tested. When loci with significant effects (at  $P < 0.05$ ) on at least 10 of the strains were considered, 18 markers defining 7 chromosomal regions on 5 chromosomes were identified. These loci, which did not coincide with regions previously known to carry major genes for BB resistance (Causse et al 1994), were associated with reductions in lesion length of 3-7 cm. Regions of chromosomes 5, 9, and 12 were associated with

significant effects on all 15 of the strains tested. Most of the alleles associated with reductions in lesion length were from IR64, but the putative QTL on chromosome 5 was derived from Azucena.

## Experiments on the deployment of resistance to BB

The experiments described above have been conducted to explore the interactions between resistance genes and individual strains of the pathogen. Although these strains were selected in an effort to represent the known diversity of the pathogen (based on inoculation experiments and DNA fingerprinting), it is probably impossible to accurately represent the actual genetic diversity of the natural pathogen population. To determine the interactions between populations of the rice host and the pathogen in the field, we have undertaken experiments on the deployment of resistance to BB. BB-infected rice leaves were collected from each of the four replications of experiments conducted in Calauan and Mabitac, Laguna. The nine host genotypes planted were a pure stand of IR20 (*Xa4*); three pure stands of NILs—IR-BB4 (*Xa4*), IR-BB5 (*Xa5*), and IR-BB10 (*Xa10*); two pure stands of pyramids—IR-BB4/10 (*Xa4* and *Xa10*) and IR-BB4/5 (*Xa4* and *Xa5*) and three mixtures—IR-BB4+10, IR-BB4+5, and IR20 + IR-BB10. The plots were naturally infected with BB. Twenty isolates were analyzed from each plot. DNA fingerprinting was done by RFLP using *IS1113* and *avrXa10* as hybridization probes for a total of 811 isolates from the two sites. There was a strong correspondence between the *IS1113* and *avrXa10* haplotypes, and between lineages and pathotypes.

There was little difference in structure of the pathogen collections taken from IR-BB4 and IR20, carrying the *Xa4* in different genetic backgrounds. Surprisingly, the collections taken from lines carrying *Xa4* and *Xa10* were also similar, although these genes are functionally distinct for strains of races 2 and 3 in greenhouse inoculation tests. The collections taken from *xa5* were, however, quite different from those taken from *Xa4* and *Xa10*. The *xa5* gene was expected to give resistance against the pathogen population, but a low level of disease was observed. Our results indicate that this unexpected susceptibility of lines carrying *xa5* was probably due to the presence of initially rare subpopulation of lineage E, rather than to a mutation arising within the predominant lineages.

## Conclusions

The effectiveness and durability of varietal resistance depend upon the host, pathogen, and environment. The BB pathogen can change to overcome host resistance, the primary method for disease control. Therefore, it is important to manage host resistance carefully. Useful molecular markers and NILs carrying single and multiple resistance genes are available to allow efficient characterization of pathogen populations, to allow careful selection of resistance genes and design of suitable resistance genotypes and deployment strategies. Field experiments and modeling studies are now

under way to determine the potential utility of using host diversity for disease management (C. Mundt, 1993-95, IRRI, pers. commun.). Resistance based on both major genes and minor genes is available and reasonably well characterized, and both types of resistance should be utilized in resistance breeding. The identification of molecular markers closely linked to resistance genes, and the cloning of such genes, should allow the efficient exploitation of major gene resistance. The availability of cloned resistance genes should make it easier to maintain both major and minor genes in breeding programs.

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## Notes

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