Molecular tools for characterization of rice blast pathogen (*Magnaporthe grisea*) population and molecular marker-assisted breeding for disease resistance

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Rice blast caused by the fungal pathogen, *Magnaporthe grisea* (anamorph: *Pyricularia grisea*) limits rice yield in all major rice-growing regions of the world, especially in irrigated lands and dry upland environments where predisposition factors favour disease development to epidemic proportions. Deployment of host resistance is by far the most effective means of control. The dynamic evolution of the blast fungus in response to different rice genotypes complicates breeding for blast resistance. In order to prolong the useful life of resistance genes, a knowledge of population genetics and evolutionary biology of the pathogen is required. The population structure and virulence composition of the blast fungus have been analysed in terms of genetic diversity, fertility and virulence characteristics. A global atlas of *M. grisea* and a rice blast database have been constructed based on the information. This report discusses the molecular tools that have been used for characterizing *M. grisea* populations in epidemic areas and describes how the molecular data generated through these methods are linked to breeding for durable blast resistance. Molecular breeding approach has been deployed in several countries across the world including India for the improvement of blast resistance in high-yielding commercial rice cultivars.

**Blast** is considered the principal disease of rice because of its wide distribution and high incidence under favourable conditions (Figure 1). It is a potentially damaging disease in upland environment where drought and soil stress predispose the rice crop to severe attacks by the pathogen. Yield loss due to blast can be as high as 50% when the disease occurs in epidemic proportions.

*Magnaporthe grisea* (Hebert) Barr (syn: *Pyricularia grisea* Sacc.), a filamentous heterothallic ascomycetous fungus is the causal organism of blast. The genus *Magnaporthe* collectively parasitizes more than 50 hosts, individual isolates have limited host range and cross-infectivity is relatively rare. The ability of this fungus to quickly overcome resistance within a short time after the release of a new cultivar has made breeding for resistance a constant challenge. An understanding of the structure and dynamics of pathogen population is essential for prudent implementation of strategies for management of the disease. Recent work suggests that diverse individual isolates can be grouped on the basis of DNA sequence patterns into a limited number of lineages, each of which has a characteristic host range. In this review, we highlight a set

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Figure 1. Symptoms of rice blast. *a*, leaf blast; and *b*, neck blast.

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of molecular tools that are currently being used to study the population dynamics of the rice blast fungus, and explore a promising new concept which utilizes such molecular data to breed for durable resistance to rice blast.

**Characterization of genetic variation in the pathogen**

The extent of genetic variation and instability in *M. grisea* has been a topic of long-standing debate among blast researchers. Theories centred around mitotic recombination, parasexual recombination, hyphal fusion, etc. were advanced to explain the high levels of variation encountered in the blast pathogen. Considerable effort has indeed gone into designing new strategies to understand and document genetic variation in *M. grisea*.

**Pathogenicity tests**

Differences in pathogenicity between individual isolates have been used for a long time to assess variation in natural pathogen populations. Such assessments are primarily based on Flor’s (ref. 28) ‘gene-for-gene’ concept for which avirulence genes provide an important source of markers. Races of *M. grisea* have been distinguished among pathogen isolates depending on the rice cultivars they successfully infect. Strains with different virulence on standard sets of cultivars are considered to represent different pathotypes. However, pathogen assays have often led to highly exaggerated estimates of variability since these are dependent on several parameters such as climate, inoculum concentration and nitrogen status of the soil. The set of differentials used in pathotype assays is also an important parameter that influences estimates of variability. Standard sets of differential cultivars have been developed by blast researchers in order to classify local isolates into pathotypes. Yet, the absence of a universally applicable set of differential cultivars has handicapped the comparison of pathotypic structure of populations across countries. Also, the fact that traditional varieties used as differentials may harbour more than one gene for blast resistance complicates analysis of host-pathogen interactions.

The use of lines carrying single known genes for resistance to define pathotypic diversity of isolates could conceptually overcome this difficulty. Several near-isogenic lines (NILs) carrying blast resistance genes in different backgrounds have been established at the International Rice Research Institute (IRRI). These include, IR49830-7-1-2-2 NILs (elite rainfed lowland rice), LTH NILs (japonica background) and CO39 NILs (indica background). These elite genetic stocks can be used for evaluating the performance of individual resistance genes, and for characterizing pathogen populations as well as for map-based cloning and construction of gene pyramids.

The development of NILs involves identification of resistance genes from wild/cultivated varieties and transferring them via hybridization into a susceptible background. Backcrossing is generally the method of choice for generating NILs. For instance, the set of CO39 NILs has been developed through backcrossing. The cultivar CO39, developed in Coimbatore, is highly susceptible to most if not all *M. grisea* isolates. To date, no resistance gene has been characterized from this line. It is an early duration (95 days to maturity) crop and was therefore chosen as an excellent recurrent parent. The major genes that have been introduced into this background are derived from four donor varieties each carrying a major blast resistance gene. The donors are (i) ‘LAC23’ from Liberia from which *Pi-1* has been derived; (ii) ‘C101A51’ or ‘5173’ from Colombia in South America from which *Pi-2* has been derived; (iii) ‘Pai-Kan-Tao’ from China from which *Pi-3* and *Pi-4* have been derived, and (iv) ‘Tetep’ from Vietnam from which *Pi-4b* has been derived. The use of NILs for pathotype characterization has not only simplified the inference of resistant/susceptible interactions but has also made it more meaningful.

**Molecular markers**

The use of molecular markers in population genetic studies has unravelled epidemiologic information to levels of precision not previously possible. Unlike traditional markers, molecular markers are direct manifestations of genetic content and can therefore serve as reliable indices of genetic or pathotypic variation. They are not influenced by environmental factors and hence are highly reproducible. Besides, these are cost-effective and less cumbersome.

The utility of proteins, isozymes and nucleic acids (DNA, RNA) as potential markers to define variation in the blast pathogen has been explored by many researchers. Hunst *et al.* sought to establish a possible relationship between the presence of mycoviruses (ds RNA) and physiologic races in the pathogen. The electrophoretic profile of enzymes in *M. grisea* has been studied in order to gain information on the genetic structure of the fungus. Such studies were inconclusive in defining the nature and extent of genetic variation in this organism.

Restriction fragment-length polymorphism (RFLP) analysis is a valuable tool to characterize genetic variation among populations. RFLPs associated with rDNA cistron, mitochondrial genome and single copy regions of the nuclear genome such as CUT1, MFG1, and II.V1 have been used to study variation among populations of *M. grisea*. Avirulence gene probes, *Avr Pw-1*, *Avr Pw-2*, *Avr2-Yama*, AVR1-CO39 (refs 23, 43–45) and repetitive elements in the *M. grisea* genome such as MGR (*Magnaporthe grisea* repeat
element)\(^4\) and grasshopper\(^4\) have also been used to characterize population variation among strains of different host origin and to show genetic isolation among them. Pathogenicity tests have confirmed that the rice and nonrice (finger millet) strains of \textit{M. grisea} are distinctly different and do not cross-infect. Another recent report by Kumar \textit{et al.}\(^4\) also supports this finding.

\textbf{MGR--DNA RFLP analysis}

Of the nine classes of repetitive elements that have been identified in the \textit{M. grisea} genome\(^4\), a family of dispersed middle repetitive DNA sequences called MGR has been most widely used to fingerprint the pathogen. This sequence is diagnostically conserved in \textit{M. grisea} genomes; strains from rice typically show between 50 and 60 bands when digested with \textit{Eco}RI and probed with MGR while strains from nonrice hosts show fewer than 5 bands\(^4\).\(^6\). Cluster analysis of the banding patterns delineates strains into discrete groups which can be inferred to represent genetically related 'lineages'. In spite of being tedious to use, MGR--RFLP analysis is still the most robust among molecular techniques available to study the population structure of the blast pathogen. It has been widely used by researchers to characterize blast fungus populations in China, India (Figure 2), Thailand, USA, Europe, South America, and West Africa (Table 1).

\textbf{Pot2-based rep-PCR analysis}

A dispersed repetitive element, \textit{Pot2} has been isolated and subsequently characterized from the \textit{M. grisea} genome\(^6\). This element shares structural features with MGR586 (ref. 46) and represents one of the major repetitive DNAs shared by isolates of \textit{M. grisea} that infect rice and those that infect nonrice hosts. It is present in approximately 100 copies per haploid genome.

Primers specific for blast pathogen have been used\(^5\) in a PCR at the IRRI to amplify DNA sequences in the \textit{M. grisea} genome that lie between copies of the repetitive element \textit{Pot2}. The fingerprint profiles consisted of 30 or fewer resolved fragments. Cluster analysis of the fingerprint patterns placed the isolates into discrete groups that closely correspond with the MGR--RFLP lineage groupings. This technique thus combines the simplicity of the PCR with the polymorphism detected by RFLP and can be used to characterize local pathogen populations. The fact that the \textit{Pot2} element is present in both rice and non-rice infecting isolates of \textit{M. grisea} in equal copy numbers broadens its utility relative to other host-specific repetitive elements. Several rice researchers have used this technique to characterize \textit{M. grisea} populations\(^5\)\(^1\)-\(^5\) (Figure 3).

\textbf{Other markers}

Random Amplified Polymorphic DNA (RAPD)\(^5\)\(^8\)-\(^6\)\(^1\), Amplified Fragment Length Polymorphisms (AFLP)\(^5\)\(^2\) and Sequence Characterized Amplified Regions (SCAR)\(^2\) analyses have also been used to fingerprint \textit{M. grisea} strains from different regions.

\textbf{Management of blast}

In developing countries, poor farmers cannot afford to control blast disease by the application of chemicals and pesticides. Chemical control of plant pathogens is most effective and yet the use of chemicals is not generally desired due to the serious environmental threat it poses. Besides, their continuous use leads to the resurgence of resistant races of the pathogen under selection pressure. Although biocontrol agents for blast have been successfully deployed to combat the disease in the laboratory, greenhouse and field tests\(^6\)\(^3\)-\(^6\)\(^5\), the feasibility of such strategies on a commercial scale still remains to be tested. Use of resistant cultivars is the best alternative to overcome yield losses. The viability of the pathogen and the history of resistance breakdown have led to the develop-
ment of a number of different plant breeding approaches to achieve durable blast resistance.

**Resistance genes**

Resistance to blast may be conditioned by major genes or by quantitative trait loci (QTLs)\(^{66}\). Major genes are those that prevent completion of the life cycle of incompatible races of *M. grisea*. QTLs are those that reduce the sporulation of the pathogen within a compatible interaction. The deployment of major gene resistance will minimize selection pressure and thereby prevent evolution of resistance in the pathogen population\(^{67}\).

More than 30 blast resistance genes (Table 2) and QTLs have been identified in rice by conventional genetic studies based on linkage analyses and recombination frequencies\(^{58}-^{70}\). Some major genes for blast resistance have been identified in recombinant inbred lines (RILs)\(^{70}\). The resistant genes are clustered in several regions of the rice genome. Nine loci have been reported on chromosome 11 and five blast resistance genes have been reported on chromosome 6. More recently, a number of resistance genes have also been tagged to molecular markers, facilitating their identification in segregating populations after hybridization.

**Partial resistance**

Parlevliet\(^{71}\) describes partial resistance as an incomplete quantitative resistance based on minor genes. It is characterized by compatibility between the pathogen and the plant with reduced development of disease compared to plants with no partial resistance\(^{22,72}\). This form of resistance is suitable for low to moderately blast-conducive environments\(^{72-73}\). Genetic studies indicate that partial resistance is under oligo or polygenic control and can be affected by the environment\(^{71-74}\). Several researchers have suggested that there are minor genes that play an important role in maintaining an acceptable level of disease under field conditions\(^{52,75-78}\). Such genes would be difficult to identify and characterize in the presence of major genes as these have epistatic interactions among themselves. Their presence could also affect the accuracy of classification of lines for complete resistance to blast\(^{70}\).

**Gene pyramiding**

Johnson\(^{79}\) describes durable resistance as that which remains effective while a cultivar possessing it is widely cultivated. Gene pyramiding is one of the strategies recommended to increase durability of resistance\(^{80-83}\). This term refers to the combining of two or more major genes for resistance in a single plant genotype\(^{84}\). While the use of single major genes limits the useful life span of resistant cultivars to few years, gene pyramiding could delay resistance breakdown by conferring 'horizontal resistance' effective against all prevalent

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**Table 1. Lineage composition** of *M. grisea* in rice-growing countries of the world

<table>
<thead>
<tr>
<th>Country</th>
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<th>Reference</th>
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<td>S. India</td>
<td>29</td>
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<td>N. India</td>
<td>18</td>
<td>Dayakar(^{22})</td>
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<td>Kumar et al.(^{84}), Zeigler(^{126})</td>
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<td>56</td>
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<td>Japan</td>
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<td>Sone and Zomik(^{123})</td>
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<td>16</td>
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<td>Philippines</td>
<td>11</td>
<td>Chen(^{125})</td>
</tr>
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<td>Thailand</td>
<td>51</td>
<td>Mekwatanakarm et al.(^{115})</td>
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<td>Vietnam</td>
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<td>Vien and Trung(^{116})</td>
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<td>Latin America</td>
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<td></td>
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<td>Colombia</td>
<td>6</td>
<td>Correa-Victoria et al.(^{126})</td>
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<tr>
<td>Brazil</td>
<td>18</td>
<td>Filipi(^{e}) et al.(^{127})</td>
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<td>USA</td>
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<td>West Africa</td>
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<td>Chipili et al.(^{128})</td>
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<tr>
<td>Europe</td>
<td>5</td>
<td>Roumen et al.(^{129})</td>
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</table>

*The lineage analyses were made by MGR-RFLP fingerprinting.

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**Figure 3.** Pot 2-based rep-PCR profiles for *M. grisea* isolates collected from Kerala. These isolates have known MGR lineage assignments (in parentheses).
pathotypes of a pathogen. Combinations of resistance genes are thought to provide broader spectra of resistance through both ordinary gene action and quantitative complementation that results in durable resistance (Figure 4).

**Lineage-exclusion hypothesis**

The organization of the blast fungus population into well-defined lineages and their distribution in specific geographic locations have led researchers to employ resistance genes targeted against pathogen populations prevalent in that region. This concept was proposed by Zeigler et al.\(^{85}\), and has been called the 'lineage-exclusion' hypothesis. In many regions, it might be useful to combine or pyramid two or more genes in a cultivar since resistance genes effective against members of a lineage might not be so against members of another lineage. On the other hand, the combination of resistance genes can confer resistance to the entire population by effective complementation. This strategy thus allows judicious use of host resistance, which is essential for resistance to be durable.

The identification of useful genes has been greatly facilitated by the development of NILs as they carry single major genes in a susceptible background. These lines are screened for resistance against diverse members of different pathogen lineages and the genes that perform best are identified for subsequent use in breeding programmes.

In southern India, the major blast resistance genes *Pi*-1 and *Pi*-2 excluded all the 29 *M. grisea* lineages in detailed exclusion assays\(^{66-68}\) (Table 3). The same combination of genes is also considered useful to confer resistance in China (Shen, personal communication, USA, and Latin America (Levy, unpublished results) to the pathogen lineages prevalent in those countries.

Sivaraj et al.\(^{89}\) proposed a model to support gene pyramiding based on lineage-exclusion. They consider traditional plant breeding as a strategy of pathotype-exclusion which leads to frequent resistance breakdown when appropriate pathotypes appear within one or two years after such resistance is deployed in large areas. In lineage

<table>
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<td><em>Pi-b</em> (Pi-s)</td>
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<td>Shinoda et al.(^{130}), Kiyosawa(^{10})</td>
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<td>Shinoda et al.(^{130}), Kiyosawa(^{10})</td>
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<td>Shinoda et al.(^{130}), Kiyosawa(^{10})</td>
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<td>9 or 12</td>
<td>Shinoda et al.(^{130}), Kiyosawa(^{10})</td>
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<td><em>Mi-Pi-c</em></td>
<td>11</td>
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<td><em>Pi-z</em></td>
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<td>Kiyosawa(^{153}), Shinoda et al.(^{130})</td>
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<tr>
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<td><em>Pi-sh</em></td>
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<td>Imbe and Matsumoto(^{136})</td>
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(1) - tentative designation, pending allelism tests with previously identified genes.

Figure 4. Blast-susceptible rice cultivar CO39 introgressed with *Pi*-1 + *Pi*-2 genes for blast-resistance shows resistance to blast in pathogenicity tests. (Greenhouse experiment)
Table 3. Virulence spectrum of M. grisea lineages from southern India on near-isogenic rice cultivars bearing single resistance genes. (The number of susceptible interactions is indicated; dark boxes indicate that no member of a lineage is compatible)

<table>
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"The near-isogenic lines are CI01LAC (P1-1(t)), CI01A51 (P1-2(t)), CI04PKT (P1-3(t)), CI01PKT (P1-4a(t)), CI04PKT.4123 (P1-4b(t)), and CI03KTP (P1-1b), (Mackill and Bonman)" 29

exclusion, the conventional strategy is modified as a phylogenetic pathotype-exclusion. Lineage-exclusion presumes that lineage-specific avirulences represent an evolutionary genetic barrier to pathotype diversification within the lineage. The pyramided resistance (for instance, with P1-1 and P1-2 blast resistance genes) will be durable in places where compatibility to the component resistance genes is distributed among the prevalent lineages (Figure 5).

Breeding for resistance and marker-assisted selection

A breeding programme with blast resistance as its principal objective should be structured such that major genes are combined to exclude the known lineages in a target region, and supported by a high level of general resistance conferred by QTLs.

In breeding for disease and pest resistance at present, the segregating populations derived from crosses between the resistant sources and otherwise desirable and productive genotypes are selected either under natural disease or pest hotspots or under artificially created disease and pest nurseries or by infesting individual plants under controlled environments. These procedures are time-consuming and expensive and are prone to be ambiguous. Besides, there are always susceptible plants that escape attack. Screening of plants with different pathogens and their pathotypes simultaneously or even sequentially is difficult if not impossible.

Molecular markers offer great scope for improving the efficiency of conventional plant breeding by carrying out

Blast Resistance Breeding Strategies (P1-1+P1-2 Pyramid)

Blast Population

Pathotype (race) Exclusion Frequent Breakdown

Lineage (MGR) Exclusion

Durable Resistance

Source of Breakdown

Figure 5. Blast resistance breeding strategies using a two-resistance gene P1-1 + P1-2 pyramid against a rice blast population with isolates that are differentially compatible with each resistance gene. Conventional pathotype (race) exclusion directed against pathotypes not yet detected, leads to frequent breakdown. Lineage-exclusion directed against the distribution of virulence and avirulence among genetic lineages predicts durable resistance when each lineage is avirulent with at least one component resistance.
selection not directly on the trait of interest but on molecular markers linked to that trait. Molecular markers are especially advantageous for agronomic traits that are otherwise difficult to tag such as resistance to pathogens. Durability of resistance has been increased in several crops by incorporating genetically diverse major resistance genes. Marker-assisted selection (MAS) is of enormous use in gene pyramiding where the presence of more than one gene has to be confirmed99-92.

With the use of molecular techniques, it would now be possible to hasten the transfer of desirable genes among varieties. Techniques which are particularly promising in assisting selection for desirable characters involve the use of molecular markers such as RAPD93, RFLP, microsatellites94, AFLP95, and PCR-based DNA markers such as SCAR96,97, Sequence Tagged Sites (STS), Cleaved Amplion Polymorphisms (CAPs)98,99, etc. Detailed reviews on the application of these techniques in plant improvement are available100-102.

Availability of tightly linked genetic markers for resistance genes will help in identifying plants carrying these genes simultaneously without subjecting them to pathogen or insect attack in early generations. Thus with MAS it is now possible for the breeder to conduct many rounds of selection in a year without depending on the natural occurrence of the pest or pathogen and theoretically without the pest or pathogen as well.

The essential requirements for MAS in a plant-breeding programme are:

(a) Markers should cosegregate or be closely linked with the desired trait.
(b) An efficient means of screening large populations (for the molecular markers) should be available.
(c) The screening technique should have high reproducibility across laboratories, be economical to use and user-friendly.

The potential benefits of MAS strategy have been widely discussed103-105 but actual examples of the application of this approach are few at present.

Molecular breeding for blast resistance

The lineage-exclusion breeding approach is being followed in several laboratories around the world to develop rice varieties durably resistant to blast. Work was started at IRRI in the Philippines and the Center for International Agricultural Research (CIAT) in Colombia and is now being carried out by research groups in India106-110, Thailand115 and Vietnam116. It is very likely that many other nations will include this work in their regular rice breeding programmes.

In India, careful pathogen analysis for the rice blast fungus population has been carried out both in southern India117 and in the Central Himalayan region117-119 (Table 1). To a limited extent, analyses have also been performed in other regions120. The wealth of information available on the genetic diversity of M. grisea has been incorporated into pyramiding of blast resistance genes in elite high-yielding indica rice varieties.

A PCR-based marker (RG64) for the blast resistance gene Pi-2 has been developed at IRRI108. The marker was used to identify blast-resistant germplasm. Three genes for rice blast resistance Pi-1, Pi-2 and Pi-4 have been incorporated into 10 agronomically superior rice varieties including IR36, IR50, IR64 and IR72. The selection of these genes was made possible via their close linkage to RFLP markers114. The improved lines have also been tested in hotspots and are reported to be resistant to blast. A gene pyramiding approach to introduce ‘lineage-excluding’ genes (Pi-2, Pi-9) into elite commercial cultivars has been pursued at the Central Rice Research Institute, Cuttack110.

Interest in mapping the entire rice genome has resulted in generation of molecular markers covering all the 12 chromosomes of rice. These include several RFLP markers and different classes of PCR-based markers. Of special importance is the class known as ‘microsatellites’ which are hyper-variable, one to four base pair repeats dispersed throughout the rice genome. Microsatellites have proved to be particularly valuable in such crosses where other markers (viz. RFLPs, RAPDs, etc.) fail to distinguish the two parental genotypes. This is true when
two closely related species (such as 2 indica varieties) are involved in a cross. Work is currently in progress to intro-
gress the Pti-1 and Pti-2 genes for blast resistance (identi-
fied on the basis of lineage-exclusion assays) from a CO39 pyramid line into elite indica rice varieties such as
IR50 and Jyothi111-113. Microsatellite markers have been
used to identify blast-resistant plants in BC1 and F2
progenies (Babujee and Mc Couch, unpublished results)
(Figure 6).

Concluding remarks

The urgent need to increase global production of rice can-
not be over emphasized, in the light of the ever-increasing
population. Management of available land and protection
of crops against devastating pests can go a long way
in increasing rice production. The lineage-exclusion
approach can be an effective strategy to manage rice blast,
the success of which hinges on the extent of knowledge
about the structure and dynamics of pathogen population.
The reliability of molecular techniques in defining the
variation present in pathogen populations has hastened the
otherwise laborious and time-consuming process.

In order to facilitate greater understanding of the genetics
of pathogenesis and host–plant resistance and to serve as a
guide to breeders for resistance gene deployment, a rice
blast database has been created20. The rice blast database
focuses on the blast pathogen M. grisea and has three
major components: (i) genome (maps, markers and genes),
(ii) population (strains, isolates, lineage relationships and
passport data), and (iii) pathotype (host–pathogen inter-
actions). This publicly accessible database located at Cornell
University, NY, USA can be browsed on the www site:

The simplicity of molecular techniques and their
cost-effectiveness should urge rice breeders to integrate
these techniques to conventional breeding. In future,
molecular breeding will help in the introduction of dura-
ibly blast-resistant rice cultivars thereby sustaining rice
yields.

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