

# Sustainable disease resistance in rice: current and future strategies

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## Abstract

Through the Asian Rice Biotechnology Network, marker-aided analyses of pathogens and host plant resistance have been practiced by several national breeding programs resulting in the production of elite or commercial lines with multiple disease resistance genes. A unique feature of ARBN is the continuing effort to capture new findings from host-pathogen interaction research and apply them in breeding. This network approach is essential for sharing resources and providing sustained training in the adoption of tools and genetic knowledge in individual breeding programs. Several studies have demonstrated the successful application of candidate genes selection for enhancing blast and bacterial blight resistance in breeding lines and varieties. Advances in genomic research will provide new approaches to determine the relationship between genetic variation, disease resistance phenotypes and performance in the field. Prospect for achieving sustainable disease control in Asia is good provided that breeding programs are enabled to access and apply tools to address local problems.

## Media Summary

Since 1993, several rice breeding programs in Asia have collaborated under the Asian Rice Biotechnology Network to develop disease resistance varieties through the application of DNA marker technology. Such collaborative effort has led to the production of elite or commercial lines with multiple disease resistance genes. This network approach is essential for sharing resources and providing sustained training in the adoption of tools and genetic knowledge to individual breeding programs. Advances in genomics research will provide new tools to determine gene function and how they can be used to enhance the resilience of the rice crop. Prospect for achieving sustainable disease control in Asia is good provided that breeding programs are enabled to access and apply tools to address local problems.

## Key Words

Candidate defense genes, collaborative network, functional genomics, marker-aided selection

## Introduction

The humid tropical environments in Asia are highly conducive to rice disease epidemics. The leading disease problems are blast (caused by *Magnaporthe grisea*), bacterial blight (caused by *Xanthomonas oryzae* pv *oryzae*), sheath blight (caused by *Rhizoctonia solani*), tungro viruses (caused by RTSV and RTBV), brown spot (caused by *Bipolaris oryzae*) and a number of grain discoloration diseases (Mew et al. 2004). The degree of severity of these diseases differs according to the production systems but together they account for 1 to 6% of yield loss depending on various estimates (Savary et al. 2000). This relatively low level of loss is largely attributed to the past and on-going efforts of disease management. With a few exceptions, control of rice diseases has been through host plant resistance. However, because of the dynamic nature of host pathogen interactions, rice diseases are constant threats to rice productivity. For example, the control of bacterial blight has been successful through the use of major genes such as *Xa4* in southeast and south Asia, *Xa1* and *Xa3* in Japan and Korea for a considerable time until recently when these genes became less effective due to the emergence of new pathogen races (Leung et al. 2002).

Control of blast through major R genes has been only partially successful because of the unusually high degree of variation exhibited by the blast fungus. For the control of tungro viruses, the history of using virus resistance is relatively short, such that the effectiveness and durability of using major resistance genes or quantitative resistance remain to be seen.

Plant pathologists and breeders in Asia face two main problems in managing rice diseases through host resistance. First, for diseases with rapidly evolving pathogen populations (such as blast and bacterial blight), the main issue is to achieve effective and durable resistance. This involves the identification and deployment of both qualitative and quantitative resistance at the genotypic and cropping system levels. Second, there is a need to discover genes conferring resistance to diseases such as sheath blight where no host resistance is known. This will involve gene discovery that could be aided by advances in genomic research. This report summarizes several successful cases of applying marker-aided selection (MAS) in disease resistance breeding in Asia and highlight new avenues to find new genes for resistance to multiple diseases through the applications of genomic tools.

#### *Capitalizing on knowledge of host-pathogen interaction*

Advances in understanding of host-pathogen interactions in plants have provided the basis for continuing improvement in disease control strategy. We have benefited from an expanding knowledge of the structure and function of major genes for disease resistance. It is now known that most major resistance (R) genes (recognition function) share structural similarity that bear signature motifs of nucleotide binding sites (NBS), leucine rich repeats (LRR), and kinases. *Xa21* of rice is among the first major R genes cloned and it belongs to one of the classes of R genes that carry the NBS-LRR and a transmembrane kinase domain (Song et al. 1995). Such sequence conservation of major resistance genes has been exploited for the identification of resistance genes in different species. Survey of the rice genome sequence showed that there are about 600 NBS-LRR type sequences (Bai et al. 2002). Although only a handful of major R genes in rice have been cloned and functionally confirmed by the complementation experiments, resistance gene analogs (RGA) (considered candidate resistance genes) and markers linked to major genes have been widely used as MAS tools (Liu et al. 2002; Ramalingam et al. 2003; Jeon et al. 2003).

The second advance in understanding disease resistance in rice comes from the extensive analysis of natural populations of *M. grisea* and *X. oryzae* pv *oryzae* (*Xoo*) (Mew et al. 2004). The population analysis was aided by the development of a large series of near isogenic lines (NILs) useful for diagnostics of pathogen virulence. An understanding of pathogen virulence and how it evolves in relation to host resistance genes has helped identify resistance genes that have a high probability of being durable (Vera Cruz et al. 2000; Leach et al. 2001). The NILs also serve as donors of resistance genes for breeding. Most recently, the genome of *M. grisea* has been sequenced (<http://www-genome.wi.mit.edu/annotation/fungi/magnaporthe/>), providing even greater opportunities to monitor genomic changes and evolution of specific virulence factors in the pathogen.

For most breeding programs, the primary interest is to translate these advances to practical use at the farm level. This need was recognized when Rockefeller Foundation's International Rice Biotechnology Program began investing in rice biotechnology and development of human resources in developing countries (see review by O'Toole et al. 2001). In the early 1990's, we envisioned that molecular marker-aided studies of pathogens and resistance breeding were mature enough that could be adopted by the National Agricultural Research System (NARS) but a collaborative network was necessary to team up plant pathologists and breeders to share resources and technical experiences. This strategy was implemented by the formation of the Asian Rice Biotechnology Network (ARBN) with support from the Asian Development Bank. Since then, the Network has enabled collaboration and partnerships that prove productive until this day.

#### *Network approach: Asian Rice Biotechnology Network*

The objectives of ARBN are twofold: (a) to build and support the capability of Asian national rice improvement programs to apply biotechnology tools to solve problems affecting rice production, and (b) to conduct collaborative research that would result in rice varieties with improved resistance to diseases and insects. We organized research teams in six countries (People's Republic of China, India, Indonesia, Philippines, Thailand, and Vietnam) to apply DNA marker technology to first understand the genetic

variability and structure of pathogen and insect populations as a basis to identify useful and durable resistance genes. Resistance genes are then evaluated and incorporated into high-yielding local varieties. Finally, varieties with new genes are deployed strategically to sustain the use of the resistant germplasm.

#### *Adding value to varieties to match local needs*

Between 1993-97, research teams used molecular markers and virulence tests to determine the pathogen population structure of *Xoo* and *M. grisea* in Indonesia, the Philippines, and eastern India. This provides a regional picture of the prevalent pathogen populations (George et al 1997, Shanti et al. 2001) and hence leading to the identification of appropriate resistance genes for particular locations.

As of 2002, ARBN has achieved impact through addressing the bacterial blight problem prevalent in Indonesia, the Philippines, northern India, and irrigated areas in China (**Figure 1**). Bacterial blight is particularly serious in hybrid rice, and because of the yield potential in hybrid rice, solving the bacterial blight problem is particularly important in the high productivity area. In early 2002, Indonesia released two varieties--Angke and Conde-- both containing multiple resistance genes against bacterial blight resistance. In China, hybrid rice You218 carrying bacterial blight resistance was released in 2001. Significant yield gain provided by these varieties has been demonstrated in farmer's field. The yield advantage of these MAS products over the standard popular varieties ranged from 11.4-31.4% (**Table 1**). Because the new varieties are derived from existing popular varieties, the MAS-improved varieties or derivatives from them are well accepted by farmers and consumers, and they are expected to cover conservatively 0.8 million hectare in four countries. Assuming that bacterial blight epidemic occurs in 10% of the time over the production area, the yield gain is estimated to be 0.8 million metric tons of paddy rice per cropping season in India, Indonesia, the Philippines and China. This translates into a gain of about US\$20.5 million per cropping season.

**Table 1. Marker aided selection (MAS)-improved varieties and their corresponding increase in yield developed by research teams from the Philippines, Indonesia, India and China.**

Country	Background commercial /Yield standard	Released (R) / Near-release (NR)	Yield (t/ha)	Gain over yield standard (%)
Philippines <sup>a</sup>	IR64	AR32-19-3-2 (NR)	5.1	0
	IR64	AR32-19-3-3 (NR)	6.7	31.4
	IR64	AR32-19-3-4 (NR)	6.1	19.6
	BPI Ri10	AR32-4-3-1 (NR)	6.0	17.6
	BPI Ri10	AR32-4-58-2 (NR)	6.5	27.5
	PSB Rc28	Yield standard	5.1	-
Indonesia <sup>b</sup>	IR64	Angke (Bio-1) (R)	5.4	20.0
	IR64	Conde (Bio-2) (R)	5.4	20.0
	IR64	Yield standard	4.5	-
India <sup>c</sup>	PR106	IET17948 (PR106-P2) (NR)	8.2	22.4
	PR106	IET17949 (PR106-P9) (NR)	7.9	17.9
	PR106	Yield standard	6.7	-
China <sup>d</sup>	Zhong 9A/Zhonghui 218	Hybrid Guofeng No. 2 (NR, R)	7.8	11.4
	II-3A/Zhonghui 218	Hybrid II You 218 (NR, R)	8.3	18.6
	Shanyou 46	Yield standard	7.0	-

<sup>a</sup>In the Philippines, representative yield data are averages of tests in farmers' fields in 2002 WS. Tests were conducted in Nueva Ecija, Southern Leyte, Eastern Samar, and Mindoro, Philippines.

<sup>b</sup>In Indonesia, representative yield data were based on test at Batang, Indonesia in 2001 WS.

<sup>c</sup>Representative yield data were based on 2002 cropping season at farmers' fields in 4 districts of Punjab State.

<sup>d</sup>In Southern China, representative yield were based on adaptability and yield potential trial of hybrid rice containing bacterial blight resistance.



Figure 1. Two bacterial blight resistant varieties released by Department of Agriculture, Indonesia in 2002, (A) Angke and (B) Conde. Angke and Conde carry *Xa4 + xa5* and *Xa4 + Xa7*, respectively. They were selected by a combination of phenotypic and marker-aided selection. Marker-aided selection is necessary because no pathogen strains in Indonesia are available to detect the presence of *xa5* and *Xa7*. (C) Disease resistance and yield performance of mixtures (e.g. AR32-19-3-3: IR64, 1:1) and pure stands of near-released varieties (Bio2 = Conde) with different bacterial blight resistance genes evaluated by the Philippine Rice Research Institute in the Philippines. The mixtures perform well under disease stress and have the desirable effect of prolonging the effectiveness of the resistance genes used in the varieties.

Advanced elite lines with resistance to other diseases (blast and tungro) and insect (gall midge, Katiyar et al. 2001; Huang et al. 2003) are near the final stage of field testing in several breeding programs under the ARBN. Approximately 20 elite lines or varieties are expected to be released for cultivation within 2-3 years. Because of the wide spread nature of disease and insect problems, the economic impact of these disease and pest resistant varieties will be equivalent to, if no more than, that estimated for bacterial blight.

#### *Deployment of resistant varieties*

In addition to developing varieties, an important objective of ARBN is to use the improved varieties in a manner that will preserve the effectiveness of the valuable genetic resources. The MAS-improved lines developed by ARBN teams make it possible to test different deployment strategies to prolong the usefulness of the elite lines and sustain productivity in farmers' field. Near-isogenic MAS products (nearly identical but with different resistance genes) are used as mixtures to prolong the effectiveness of individual resistance genes. Yield performance and disease resistance were evaluated in Angke (IR64+*xa5*) and Conde (IR64+*Xa7*), and IR64 for several seasons in Indonesia and the Philippines.

In terms of yield, the 1:1 mixture of Angke and Conde (5.9 tons/ha) was similar to Conde (5.9 tons/ha) and IR64 (5.6 tons/ha) when tested in the Philippines. Yield from Angke was lower at 5.4 tons/ha. The yield of AR32-19-3-3 was highest (6.2 tons/ha) among the varieties and lines evaluated. An average of 5.3% yield gain over IR64 was observed in Conde and in the mixture. A yield gain of 10.7% was observed in AR32-19-3-3. No significant yield differences were observed between Conde and their 1:1 mixtures. The mixture strategy using lines with individual genes was also tested in Indonesia to determine the effectiveness of Angke and Conde and their sister lines at farmers' fields. Lower disease incidence and severity were observed in Angke and Conde and sister lines relative to IR64 in West and Central Java. In the wet season 2002 trial, the pure lines and 1:1 mixture of IR64 and MAS products showed less disease than IR64. However, the IR64:Conde mixture showed significantly lower disease than the IR64:Angke mixture, suggesting that *Xa7* might confer a higher level of resistance than *xa5*. AR32-19-3-3 (*Xa4+xa5+Xa21*) produced by PhilRice showed the least amount of disease among the MAS products.

These results suggest that by producing elite lines with different resistance genes, we broaden our option of deploying them spatially or temporally. The use of mixture does not necessarily lead to yield gain but may help "soften" the selection of virulence that would overcome R genes pyramided in a single variety.

### *Candidate gene approach to accumulate qualitative and quantitative resistance*

So far the selection work has been primarily based on markers linked to resistance genes with the exception for *Xa21* where a PCR marker developed from the kinase domain of the gene was used. There are two disadvantages of using linked markers. First, unless a marker is tightly linked to the target gene, it could be uncoupled from the target in breeding cycles after a large number of meioses. Second, polymorphism in linked markers does not lend itself to a functional understanding of the gene and its allelic variants responsible for the phenotype. In other words, it is not possible to interpret the relationship between molecular polymorphism and function with linked markers. In contrast, if molecular variation, such as single nucleotide polymorphism (SNP), is described within a gene, it is then possible to determine the relationship between nucleotide variation and function. Such information will enable us to interpret molecular variation (functional SNP) in the rice gene pool and possibly in orthologs in the genomes of allied species.

Under ARBN, we have advocated the use of candidate genes involved in plant defense as a source of new markers for MAS. We have assembled over 1,000 cloned disease-response genes or EST from rice, maize, barley and oats that are reported to be associated with plant defense based on DNA sequence information, gene tagging, and mapping. We experimentally tested the utility of this candidate gene approach in two breeding populations with the objective of accumulating quantitative resistance to blast.

To identify candidate genes contributing to blast resistance, we generated and analyzed an advanced backcross population derived from Vandana/Moroberekan for blast resistance (Wu et al., 2004). An advanced backcross population consisting of 80 BC<sub>3</sub>F<sub>3</sub> lines derived from Vandana/Moroberekan was analysed for blast resistance and genotyped with candidate genes and SSR markers. Six candidate defense response genes (thaumatin, three NBS-LRR sequences from maize, and two resistance gene analog (RGA) markers) and one SSR marker (RM21) were significantly associated with partial blast resistance in the population ( $P=0.01$ ). These markers accounted for phenotypic variation ranging from 9.6 to 29.4%, and contributed to 76% of the total variation of percent diseased leaf area (DLA) observed under natural infection. Four candidate genes (oxalate oxidase, 14-3-3 protein, and two RGA markers) and four SSR markers (RM21, RM168, RM215 and RM250) were significantly associated with resistance to a single pathogen isolate PO6-6. These markers accounted for 9.1 to 28.7% of the phenotypic variation. A moderate correlation ( $r = 0.48$ ,  $P < 0.01$ ) was found between levels of partial resistance measured in the greenhouse and under natural conditions. Cluster analysis of DNA profiles showed that the BC<sub>3</sub> population was genetically similar (>85%) to the recurrent parent Vandana. Field performance of the advanced BC lines indicated that the major QTLs have been captured in the BC lines. In this study, only a small proportion of alleles were from Moroberekan. One possible explanation is that the loci contributing to disease resistance may not be polymorphic, thus precluding their use as informative markers.

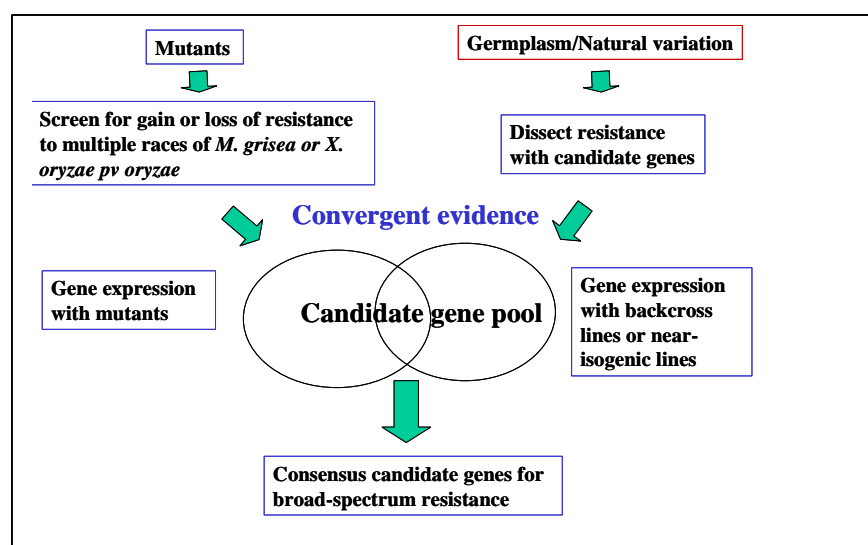
To accumulate different mechanisms of blast resistance, we inter-crossed 15 BC<sub>3</sub>F<sub>5</sub> lines and selected progeny for blast resistance under field conditions. We identified five blast resistant lines carrying a Moroberekan segment (chromosome 3) which contains a cluster of four putative oxalate oxidase genes. We further identified DNA polymorphisms in the upstream non-coding regions in two of these genes. Whether there is a causal relationship between these allelic variants and blast resistance remains to be tested.

In a collaboration between Guangdong Academy of Agricultural Sciences and IRRI, we applied a collection of candidate defense genes to identify genes conferring blast resistance in a durable blast-resistant variety Sanhuanzhan 2 (SHZ-2) and to introgress durable blast resistance from SHZ-2 into Texianzhan 13 (TXZ-13), a popular indica cultivar in South China with high yield and good quality but poor blast resistance (Liu et al. 2004). Five defense response (DR) genes, encoding putative oxalate oxidase, dehydrin, PR-1, chitinase, and 14-3-3 protein, were found to be significantly associated with quantitative resistance to blast in SHZ-2, and together accounting for 60.3% of the DLA variation. These five DR genes were used to select lines derived from SHZ-2 x TXZ 13 backcross populations. Two selected advanced BC lines with the five effective DR genes showed much stronger leaf and panicle blast resistance than their recurrent parent TXZ-13, and had the same high yield and good quality as TXZ-13 in multi-location tests in Guangdong, China in 2002 and 2003.

Although the application of candidate gene-aided selection has proven successful in accumulating quantitative disease resistance in rice, a limitation of this approach has been the relatively low level of variation of candidate genes based on restriction site polymorphism. To associate DNA sequence with function, abundant molecular variation at each of the candidate gene loci is needed. Our current work is to increase our capacity to detect polymorphism through SNP detection in the selected advanced lines. TILLING (Targeting Induced Local Lesions IN Genome) is a reverse genetics strategy for detecting point mutations in targeted genetic loci in mutants (Henikoff and Comai 2003; Till et al. 2003). An extension of this technique called EcoTILLING detects polymorphism of selected loci in natural populations using a reference DNA mixed with a queried DNA (Comai et al. 2004). We are applying TILLING as a fingerprinting tool to increase our ability to detect haplotypic variation in defense response genes and to associate haplotypic variation or SNP with performance in advanced backcross lines. Primers are designed for ~20 candidate genes with evidence for contributing to disease resistance based on microarray expression analysis and QTL analysis. These genes are used as targets to survey allelic variation in the germplasm pool and for associating the variation with disease resistance in advance breeding lines.

#### *Discovering genes conferring broad-spectrum resistance*

The candidate defense genes used so far are based on sequence information and functional evidence from other plant species. They most likely represent only a small fraction of the defense genes in the rice genome. To expand the arsenal of defense genes for manipulation and to discover novel genes for broad-spectrum resistance, we need to take a genome-wide and unbiased approach to identify defense-related genes. Our approach is to first identify genetic materials that exhibit broad-spectrum resistance followed by genome-wide analysis of gene expression patterns that co-segregate with resistance phenotypes (Figure 2).



**Figure 2. A framework to apply germplasm and mutant resources to associate genetic variation at candidate genes with phenotypes responsible for broad-spectrum resistance.**

#### *Mutants and advanced lines exhibiting BSR*

We define broad-spectrum resistance (BSR) to diseases as genetic mechanisms conferring resistance to multiple races of a pathogen and/or multiple diseases caused by different pathogens. Mutational analysis indicates that many single gene mutations can confer a BSR phenotype but a majority of them have pleiotropic effects on development. Nonetheless, these mutants provide a suitable platform to understand the genetic pathways involved in resistance to multiple pathogens.

We have identified a number of lesion mimic mutants exhibiting BSR to blast and bacterial blight (Yin et al. 2000). One of these lesion mimics mutations, *spl11*, has been recently isolated by map-based cloning. The *Spl11* locus encodes a U-Box E3 ubiquitin ligase that presumably controls ubiquitination of target proteins involved in programmed cell death and defense response (Zeng et al. 2002, Zeng et al. 2004). Identification of a pivotal point in defense response is particularly useful; we can now place a number of candidate defense genes in relation to the position of *Spl11* in the defense pathways. We have also

identified two IR64 mutants (designated GR978 and GR282) with gain of resistance to both blast and bacterial blight (Sugiyama et al. unpublished). These mutants show quantitative resistance to blast and bacterial blight without any apparent lesion mimic phenotypes. Expression analysis of these mutants show distinct patterns, therefore suggesting different mechanisms involved for the observed resistance phenotypes.

#### *Whole-genome approach to establish causal relationship between candidate genes and phenotypes*

With the available rice genome sequence (Goff et al. 2002; Yu et al. 2002; Feng et al. 2002; Sasaki et al. 2002; Kikuchi et al. 2003), it is possible to produce genome-wide gene arrays to examine gene expression simultaneously. Jansen and Nap (2000) proposed a whole-genome segregation analysis to establish the causal and interactive relationships between gene expression levels and QTL. This approach has been illustrated in the genetic analysis of genetic expression in human, mice and maize (Schadt et al. 2003). We see promise of applying this approach to find additional genes contributing to durable resistance. The main ingredients of this analysis is an immortalized segregating population that exhibit a full range of resistance phenotypes and a cost-effective means of gene expression assay. We are pursuing co-segregation analysis of quantitative resistance with whole-genome expression profiles using the 62,000-oligo chip developed by Beijing Genomics Institute in China. Our initial experiments involve comparing differential gene expression at different time points in the resistant SHZ-2 and susceptible LTH parents under pathogen challenge. Then an appropriate time for assay will be applied to about 100 RI lines derived from SHZ-2 x LTH that show a full range of resistance phenotypes. We expect this segregation genomics approach to reveal the causal relationship between gene expression and phenotypic performance, and hence providing a rich source of candidate genes for selection breeding.

#### *Functional verification*

Through expression analyses of mutants and near-isogenic lines with resistance against bacterial, fungal, and viral pathogens, we have begun to accumulate information on a set of genes whose expression is consistently correlated with resistant phenotypes. The next challenge is to establish a direct relationship between expression polymorphism and molecular variation, and finally predict performance of the trait. Currently, two approaches are used to validate the phenotypic contribution of candidate genes. As described above in the selection of advanced backcross lines, we use candidate genes as predictors of performance to produce gene pyramids and validate their performance in the field (Liu et al. 2004). Through this method, we have demonstrated that breeding lines with multiple candidate genes show a high level of quantitative resistance against blast in multiple locations in China. To generalize the utility of these alleles, they have been introduced into different popular cultivars in Vietnam (Quang et al. Agricultural Genetics Institute, Vietnam, unpublished data) and in Korea (Han et al. Rural Development Administration, Korea, unpublished data). Second, we apply association genetics analysis to test for significant association between molecular variation at a single or multiple loci with phenotypes. Using TILLING as a haplotyping tool, we have begun surveying intragenic variation in disease-response genes in a germplasm collection of 1,400 lines and to associate haplotypic variation with functional performance. Using these multiple approaches, we hope not only to identify genes involved in BSR but also to validate their utility in practical breeding.

#### **Conclusions**

The variable nature of plant pathogens requires a sustained investment to keep diseases in check. Many rice farmers in Asia prefer stable secured yield of durably resistant varieties to sporadic high yield of varieties that may succumb to disease pressure. Thus, our strategy has been to add stable resistance to popular varieties that are renowned for their wide adaptation and production stability across environments. Pyramiding of disease resistance genes is often complicated by the masking effect of major R genes. The use of markers in disease resistance breeding is not only for improving efficiency but a necessity for accumulating different defense mechanisms against different races of pathogen or multiple pathogens. It appears that many breeding institutions in Asia have the capacity to implement marker-aided breeding; however, we are limited by the knowledge of what gene or gene combinations are effective and durable. Whole-genome analyses of well-characterized genetic materials can provide a powerful approach to identify these genes. The collaborative mechanisms in research and training fostered by ARBN have proven to be effective to enable access and applications of the new technologies. We expect this model will continue to strive in functional genomics research. We see bright prospect of

sustainable disease control provided that breeding programs are empowered to choose and apply the appropriate technologies to address local problems.

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