

# Identifying favorable alleles in host defense genes for broad-spectrum resistance in rice

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Broad-spectrum resistance (BSR) is generally considered more stable than disease resistance governed by major resistance genes. We have identified rice germplasm and mutants exhibiting resistance to multiple diseases or different races of a pathogen, providing the essential materials for understanding the genetic basis of BSR. There is also an extensive list of defense genes that are considered contributors to BSR. However, because of the relatively small effects of individual defense genes, validating their function and identifying useful alleles for breeding have proved difficult. We apply forward and reverse genetics as complementary approaches to identify candidate genes and validate their functions. Expression and gene-silencing experiments suggested that genes encoding oxalate oxidase-like proteins are important factors conferring BSR. We attempted to associate sequence variation of a collection of defense genes and blast resistance in Korean germplasm, though no clear pattern of association has emerged. To reveal new candidate genes, transcriptome analysis was conducted using well-characterized resistant genotypes. We detected discrete chromosomal regions where the expression of neighboring genes is significantly correlated. Such observations lead to the hypothesis that regions of correlated expression are related to the expression of quantitative resistance. If this is true, allelic composition of multiple genes in adjacent regions could be an important selection criterion for BSR.

Keywords: durable disease resistance, mutation, transcriptome, gene silencing, candidate defense genes, natural genetic variation, haplotypes

Defense genes that contribute to disease resistance are appealing for practical breeding because their effects are in general non-race- or non-pathogen-specific. These genes, if combined and expressed appropriately, can provide broad-spectrum resistance (BSR) against multiple pathogens, and would potentially be durable. Although accumulating defense genes for BSR is a sound strategy, it is difficult to identify and validate the functions of these genes because they are often encoded by multiple members of a gene family, and the phenotypic effects of individual genes are often small. We approach this problem by gathering convergent evidence based on QTL mapping, allelic variation of individual genes, and expression analysis. In this paper, we briefly present the approach of using a set of rice germplasm and mutants that exhibit BSR to blast or bacterial blight as a means to identify genes conferring BSR. Progress is made toward validating the function of selected candidate defense genes by gene silencing and mutational analysis. Whole-genome expression analysis also provides a new pool of candidate genes for further functional validation.

## Specialized genetic stocks and candidate genes

A prerequisite to understanding the genetic basis of BSR is to identify genetic materials that exhibit the desired phenotype. Here we define broad-spectrum resistance as either resistance to multiple races of a pathogen and/or resistance to multiple pathogens. Through retrospective analysis of the agronomic performance of rice varieties in areas of high disease intensity, it is possible to identify varieties with a good “reputation” for having strong resistance in the field. One such variety is Shan-Huang-Zhan-2 (SHZ-2), an indica variety that was widely grown in the 1980s in southern China. SHZ-2 was cultivated over a large area for more than 10 years by farmers because the variety showed stable blast resistance as well as high yield. SHZ-2 was gradually replaced by new varieties with better grain quality but it remains prominent in the pedigrees of new varieties. Controlled inoculation experiments in China and in the Philippines with blast pathogen isolates also showed that SHZ-2 was resistant to >90% of the diverse isolates. Liu et al (2004) showed that disease resistance QTLs could be extracted from SHZ-2 and incorporated into new varieties.

The second source of BSR comes from systematic screening of mutants. In general, multiple-disease-resistant mutants can be grouped into two categories: those showing obvious lesion mimic phenotypes (e.g., the series of *spotted leaf* (*spl*) mutants) and those that do not have lesion mimics at least under normal growing conditions. A well-characterized lesion mimic mutant is *spl11*, which expresses enhanced resistance to multiple races of bacterial blight and blast pathogens. The wild-type gene *SPL11* encodes an E3 ubiquitin ligase, which acts as a negative regulator of host defense (Zeng et al 2004). From screening the collection of IR64 mutants (Wu et al 2005), we have identified another mutant (designated GR978) that exhibits resistance to blast and bacterial blight. But, unlike the *spl11* mutant, GR978 does not show lesion mimic under normal growing conditions, which makes this mutant particularly useful for identifying downstream genes conferring BSR.

Because of the large body of literature on disease resistance, information is abundant on the putative roles of candidate defense genes. Reverse genetics can be used for testing candidate genes in defense pathways. The completion of the rice genome (IRGSP 2005) provides the needed sequence information to extract allelic variants in natural germplasm as well as in mutants. Thus, reverse genetics is an excellent tool for validating gene function. The challenge is having the appropriate assay to detect subtle differences in resistance as well as their interactions with other genes.

## Validating the function of candidate genes and alleles

Liu et al (2004) found a strong association of disease resistance QTLs with a cluster of genes encoding oxalate oxidase-like proteins on the short arm of chromosome 8. Of the 12 members of *OsOXL* (*Oryza sativa* oxalate oxidase-like) genes located in a 2.8-Mb region, 11 members are tightly linked within 80 kb and the twelfth member more distant from the centromere. These *OsOXL* genes are labeled from 1 to 12 based on their order on the chromosome.

A key question is whether the expression patterns of the *OsOXL* genes are causally related to the level of disease resistant observed in resistant and susceptible genotypes. We examined the differential expression of these *OsOXL* genes after pathogen inoculation or wounding in four genotypes—IR64, SHZ-2, LTH, and Azucena—that

exhibit a range of resistance. SHZ-2 is the most resistant, whereas LTH is the most susceptible to blast. The time-course expression analysis suggested that six members are potential contributors to resistance, although the specific member varies with cultivar (Davidson et al, manuscript submitted). RNAi-mediated silencing of different *OsOXL* gene combinations revealed that the more genes that were suppressed, the more susceptible the plants were to blast, corroborating the results of expression analysis (Manoslava et al, manuscript in preparation). On the basis of the patterns of expression and effects of gene silencing, we concluded that *OsOXL3*, 6, 7, 8, and 9 are potential contributors of resistance.

TILLING (Targeted Induced Local Lesion IN the Genome) is applied as a reverse genetics tool to identify target mutations (Bhat et al 2007, Raghavan et al 2007). From an EMS-induced population of IR64, we recovered five missense mutations in four oxalate oxidase genes. Based on a preliminary inoculation test, only one mutant (mutation in *OsOXL7*) appeared to show reduced resistance (Fig. 1). Because individual *OsOXL* genes are hypothesized to contribute only a portion of the overall resistance, any change in resistance must be measured quantitatively. More importantly, a cumulative measurement of disease severity over growth stages and time would be relevant. Backcross lines have been produced to enable replicated tests under field conditions.

## Association analysis

If candidate defense genes play a role in resistance, one expectation would be an association between sequence variation (or unique sequence signatures) in candidate genes and quantitative disease resistance observed in germplasm. To test this hypothesis, we chose a collection of Korean rice germplasm with data on quantitative blast resistance. The Korean rice germplasm is particularly suitable for this experiment because it represents a relatively small but well-characterized set of germplasm used in blast resistance breeding. The lines have also been tested with multiple blast pathogen isolates in Korea and the Philippines.

We sequenced 11 candidate defense genes from 22 Korean and donor germplasm accessions for blast resistance (Table 1). These genes showed a modest level of single nucleotide polymorphism (SNP) in the 1-kb upstream and coding regions that defines the haplotype variation of each candidate gene locus. We observed some degree of association between the type/number of *cis*-elements and resistance in germplasm. For example, the *PR10* gene of Palgong, a resistant variety, has multiple copies of Wbox, WRKY, and ELRE in the promoter region. However, no conclusion can be drawn as any association needs to be validated using an unstructured germplasm collection or segregation analysis.

## Gene expression patterns of resistant germplasm and mutants

A limitation to using predicted defense genes is that we may miss unknown genes that are relevant to BSR. To identify additional candidate genes contributing to BSR, we analyzed the resistance transcriptomes of two genotypes that show resistance to blast. Figure 2 shows the workflow for generating expression data from GR978 and SHZ-2. Messenger RNA was isolated from leaves at 24 and 48 hours after pathogen infection and hybridized to the 22K oligoarray (Kikuchi et al 2007). In this analysis, we were particularly

interested in expression patterns in the context of chromosomal position. We applied the genome scanning technique of Spellman and Rubin (2002) to identify regions of correlated expression (RCEs) using a moving window of 2 to 20 genes.

From the resistance transcriptomes of mutant GR978 and resistant variety SHZ-2, we identified nine groups of adjacent genes that showed correlated expression, with 18 to 20 genes in each group. The average size of the RCEs is approximately 620 kb, which is about six times larger than the correlated regions (100 kb) reported by Ma et al (2005) based on their transcriptome analysis using an oligoarray containing approximately 41,000 predicted rice genes. The use of a higher density array will probably provide a more accurate estimate of sizes of the RCEs.

An interesting observation from the comparative transcriptome analysis is the detection of the same RCE independently in two resistant genotypes. Table 2 shows the genes in two overlapping RCEs on chromosome 2. The genes in this “window” showed significant correlated expression in response to pathogen infection across experiments. Some genes in the RCE are known to be involved in stress response whereas others are not obviously considered stress response genes. Such an analysis provides a new pool of candidate genes for allele mining. We are evaluating the possibility that coordinated expression of multiple genes could be contributing a single QTL, a hypothesis with practical implications for selecting genotypes for effective resistance.

## Whole-genome SNP data as a foundation for identifying favorable alleles

Through the International Rice Functional Genomics Consortium, we are generating genome-wide SNP data for diverse varieties to provide a foundation for genotype-phenotype association analysis of a large collection of germplasm (McNally et al 2006). The project involves partnership with Perlegen Sciences to apply tiling arrays to identify SNP in multiple rice genotypes relative to a reference genome. A collection of 20 diverse varieties/landraces, representing popular donor germplasm in breeding, will be investigated for genome-wide SNP. A pilot experiment has been conducted involving arrays containing 379 kb of unique sequences from a region of 684 kb on the long arm of chromosome 3. DNA obtained from 20 varieties was tested with the arrays. A total of 2,132 SNPs were detected (on average 1 SNP per 200 bp). The full SNP data set, covering approximately 100 Mb of the single-copy and low-copy sequence of the rice genome, is expected to be available in early 2007. This public database will provide the foundation for association genetics and identification of alleles in the rich rice gene pool.

## Conclusions

We have identified rice germplasm and mutants exhibiting BSR, which are essential materials for understanding the genetic basis of BSR. We have identified a set of defense genes and favorable allelic combinations based on converging evidence of QTL mapping, allelic variation of individual genes, expression analysis, and gene silencing. The use of different kinds of genetic resources and experimental evidence appears essential for validating the roles of defense genes with small but important effects. A SNP database is being developed as a platform for finding new genes and alleles. New candidate genes can be identified by transcriptome analysis. Unlike qualitative resistance where a major

gene often confers a clear-cut phenotype, quantitative resistance could be mediated by different alleles of multiple loci. The *OsOXL* gene family may provide a case to test whether allelic composition across multiple genes is important in conferring the target phenotype.

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## Notes

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Fig. 1. Preliminary evaluation of five mutants with missense mutations in oxalate oxidase (*OsOXO*) and oxalate oxidase–like protein (*OxOXL*). Mutants were inoculated with isolate PO-6-6 in the greenhouse, and disease scored over a 14-day period. Only M715, which was identified as having a mutation in *OsOXL7*, appeared to become more susceptible relative to the parent IR64. These mutants were backcrossed to IR64 to remove background mutations and to confirm the inheritance of the SNP and phenotype. Confirmation of the phenotypic effect of the mutations will be obtained using BC<sub>1</sub>F<sub>3</sub> lines.

Fig. 2. Gene expression analysis of gain-of-resistance mutant GR978 and a resistant variety (SHZ-2) provides a data set for mining candidate genes important for broad-spectrum resistance. Genes that show differential expression patterns and correlated expression patterns are identified during the early stages of infection (24 and 48 hours after infection).