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# Isolation and characterization of rice mutants compromised in *Xa21*-mediated resistance to *X. oryzae* pv. *oryzae*

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**Abstract** The rice gene, *Xa21,* confers resistance to diverse races of *Xanthomonas oryzae* pv. *oryzae (Xoo)* and encodes a receptor-like kinase with leucine-rich repeats in the extra-cellular domain. To identify genes essential for the function of the *Xa21* gene, 4,500 IRBB21 (*Xa21* isogenic line in IR24 background) mutants, induced by diepoxybutane and fast neutrons, were screened against Philippine race six (PR6) *Xoo* for a change from resistance to susceptibility. From two greenhouse screens, 23 mutants were identified that had changed from resistant to fully (6) or partially (17) susceptible to PR6. All fully susceptible mutants carried rearrangements at the *Xa21* locus as detected by PCR and Southern hybridization. For the partially susceptible mutants, no changes were detected at the *Xa21* locus based on Southern and PCR analyses. However, two of these mutants were confirmed via genetic analysis to have mutations at the *Xa21* locus. Partially susceptible mutants exhibited variation in level of susceptibility to different *Xoo* strains, suggesting that they may carry different mutations required for the *Xa21*-mediated resistance. The mutants identified in this study provide useful materials for dissecting the *Xa21*-mediated resistance pathway in rice.

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## Introduction

With the successful cloning and characterization of over 30 resistance (R) genes from different plant species over the last decade, we have gained significant insight into the molecular basis of disease resistance in plants. Sequence analysis of the predicted proteins reveals that R genes of diverse origin and pathogen specificity share similar structural motifs such as leucine-rich repeats (LRR), kinase domains and nucleotide binding sites (NBS) (Hulbert et al. 2001). The structures of the cloned R genes are consistent with a ligand and receptor model. However, physical interactions between an R protein with the corresponding avirulence (*avr*) gene product has been demonstrated in only three cases (Tang et al. 1996; Jia et al. 2000; Deslandes et al. 2003). The structural similarity of different R genes also suggests the existence of a common or limited number of resistance pathways in plants. Dissection of these pathways is expected to provide insight into the number of genes required to transduce a defense response, and the functions that these genes perform.

Three strategies have led to the successful identification of genes involved in transducing a resistance response: screening for mutants affecting the resistance phenotype, screening for mutants affecting specific defense responses, and yeast two-hybrid screening for proteins interacting with the cloned genes (Innes 1998). For example, a gene required for *Pto*-mediated resistance, *Prf*, was identified in a mutant screen for altered resistance to *Pseudomonas syringae* pv. *tomato* (Salmer-

on et al. 1994). *Prf* encodes an NBS/LRR-type protein, a characteristic feature of several disease R genes (Salmeron et al. 1996). Using a similar approach, *NDR1* and *EDS1* were cloned and have been shown to encode a possible membrane protein and a lipase, respectively. Subsequently, these genes were each shown to be required to transduce the signal of multiple R genes (Century et al. 1997; Innes 1998). The cloned barley gene, *Rar1,* is required for multiple R gene-mediated resistance to powdery mildew pathways (Shirasu et al. 1999).

Using a map-based cloning strategy, Song et al. (1995) cloned the *Xa21* gene, which confers resistance to *Xanthomonas oryzae* pv. *oryzae* (*Xoo),* and discovered that it belongs to a small closely linked multigene family containing eight members. The deduced amino acid sequence of *Xa21* includes a receptor-like kinase carrying LRRs in the putative extracellular domain, a single pass transmembrane domain, and a serine/threonine kinase intracellular domain. Interestingly, one of the *Xa21* gene family members, *Xa21D,* confers partial resistance to *Xoo* (Wang et al. 1998). Compared with other cloned plant R genes, the structure of *Xa21* is unique in carrying both the receptor domain LRR, presumably for recognition, and the kinase domain for subsequent signal transduction. The structure of the *Xa21* gene family member *Xa21D* is also unique as the predicted protein consists of a secreted extracellular LRR domain.

Transgenic plants expressing the cloned *Xa21* gene confer resistance to 29 out of 32 diverse *Xoo* isolates collected from eight different countries (Wang et al. 1996). Due to its broad-spectrum resistance to bacterial blight, the *Xa21* gene was introduced into multiple cultivars using transgenic and traditional breeding approaches (Tu et al. 1998; Zhang et al. 1998). The resulting resistance conferred by introduction of a single gene or locus indicates conservation of the *Xa21*-mediated defense pathway in diverse rice cultivars. However, little is known about the intermediate steps responsible for mounting the defense response that eventually leads to effective resistance. Isolation of the genes controlling the intermediate steps should shed light on the molecular basis of *Xa21*-mediated resistance. Manipulation of these genes in transgenic rice may also produce novel and broad-spectrum resistance against different pathogens. To identify such genes, we mutagenized the *Xa21*-containing line IRBB21 with diepoxybutane (DEB) and fast neutrons (FN). From 4,500 $M_2$ lines, 23 lines with fully susceptible or partially susceptible phenotypes were identified. PCR and Southern analyses revealed that all six fully susceptible mutants from four independent families carried mutations at the *Xa21* locus. In contrast, no detectable molecular changes were observed in 17 partially susceptible mutants. Genetic complementation tests showed that at least two partially susceptible mutants were altered at the *Xa21* locus. These mutants are ideal starting materials for dissecting the defense pathway in rice.

## Materials and methods

### Mutagenesis of IRBB21 with FN and DEB

About 3,000 IRBB21 (*Xa21* isogenic line) seeds were treated with FN with a dose of 20 Gy $N_f$ at the Standard Neutron Irradiation Facility, Plant Breeding Unit, International Atomic Energy Agency, Vienna, Austria in 1991. Two weeks after FN treatment, the seeds were sent to the International Rice Research Institute (IRRI) for evaluation of viability and advancement to the $M_2$ generation.

For DEB mutagenesis, a kill curve was first conducted to determine the most suitable DEB concentration for mutagenizing rice seeds. Six grams of seeds (about 276 seeds) were soaked in water overnight. Twenty seeds were each treated for 4 h with different concentrations (mM) of DEB (0, 0.3, 0.7, 1, 3, 7, 10, 13, 20, 40, 80, and 100). Treated seeds were washed thoroughly five times with water. Germination rate was calculated for each treatment. For large-scale mutagenesis, approximately 5,000 seeds were divided into two lots and treated with 1.0 and 1.5 mM DEB, respectively, at Cornell University in 1992. Treated seeds were sent to IRRI for germination test and seed propagation.

$M_1$ plants from both DEB- and FN-induced populations were allowed to self-pollinate and the resulting $M_2$ seed was harvested separately from each $M_1$ line. All 4,500 $M_2$ families (about 1,200 $M_2$ lines from FN treatment and 3,300 $M_2$ lines from DEB treatment) were planted. About 10–15 plants from each $M_2$ family were evaluated for reaction to bacterial blight in the greenhouse at IRRI.

### *Xoo* Inoculation

Philippine *Xoo* race 6 or PR6 (PXO99) was used to inoculate the two-month old $M_2$ plants. At least three fully expanded leaves from each plant were inoculated using the leaf clipping method (Kauffman et al. 1973). Plants were scored 2 weeks after inoculation by measuring the lesion length (cm). Plants with lesion lengths of more than 5 cm were transplanted into large pots to maximize the production of $M_3$ seeds. Homozygous $M_4$ and $M_5$ mutants were inoculated with an additional 11 Philippine strains: PXO61 (PR1), PXO86(PR2), PXO79(PR3), PXO71(PR4), PXO112(PR5), PXO203(PR5), PXO146(PR7), PXO207(PR7), PXO211(PR8), PXO221(PR8), and PXO87(PR9). An average of 10–12 plants per line per replicate were inoculated with each *Xoo* strain, and three replicates were used under a completely randomized design.

### Southern-blot analysis and PCR

Rice genomic DNA was isolated as described by Dellporta et al. (1984). DNA was digested with restriction enzymes and resolved on a 0.8% agarose gel and then transferred onto a Hybond-$N^+$ membrane (Amersham, UK). Probes were labeled using a megaprimer labeling kit (Amersham, UK), and rapid hybridization solution (Clonetech, USA) was used for the Southern analysis following standard procedures (Sambrook et al. 1989).

About 20 ng of genomic DNA was used in PCR containing *Xa21*- specific primers. Amplification conditions were the same as those described by Williams and Ronald (1994). Briefly, the temperature profiles for PCR amplification were as follows: 94°C for 4 min for 1 cycle, followed by 35 cycles of 94°C for 45 s, 55°–60°C (dependent of specific primer pairs) for 45 s, and 72°C for 60 s. The PCR was terminated at 72°C for 5 min. The amplicons were separated on 1.0% agarose gels. Primers used in the study were: KIN-1 (ACTGGCCATCCTCTCATCACTCTAC), KIN-2 (TCAGATCGACTTCTGCAGTGGTAT), 3′Xa21R (GATCGGTATAACAGCAAAAC), and H3FragF (ATAGCAACTGATTGCTTGG).

## Results

### Germination rate of FN- and DEB-treated seeds

Out of 3,000 FN-treated seeds, a total of 1,740 IRBB21 seeds germinated and 1,300 plants produced seeds. For DEB treatment (4 h soaking), concentrations from 0 to 100 mM were used. The germination rate (%) dropped significantly as the DEB concentration increased. No seeds germinated after treatment with a DEB concentration of more than 10 mM. To find a DEB concentration yielding 70–80% germination rate, another germination test was conducted by treating the IRBB21 seeds with 0, 1.0, 1.5, and 2.0 mM of DEB. Concentrations of 1.0 and 1.5 mM were chosen for the large-scale mutagenesis, corresponding to the germination rates of 72% and 82%, respectively. A total of 3,200 $M_1$ plants produced seeds that were used in the following mutant screen experiments.

### Screening mutants for susceptibility to *Xoo* PR6

Two screens for identification of mutants with loss of resistance to PR6 were conducted at IRRI. A total of 4,500 mutant $M_2$ families were used in both screens. The first screen was conducted in the spring of 1994. In this screen, 20 families of mutant rice plants showed partial susceptibility (lesion length between 5–8 cm) to PR6 when compared to disease symptoms in wild-type resistant IRBB21 plants (2–3 cm). Among the $M_2$ families, the phenotypes of the two mutant lines, R92 and R95, were confirmed in the $M_3$ generation and were selected for further genetic and molecular characterization (see below).

The second screen was conducted in the spring of 1997. Twenty-one plants (lesion length more than 5 cm) from 19 $M_2$ families were identified and transplanted into large pots to produce $M_3$ seeds. Disease reaction of these identified mutants to PR6 was confirmed in the $M_3$ generation both at IRRI and the Institute of Molecular Agrobiology in Singapore. Results from both locations confirmed that the mutants had a completely different disease reaction than the wild-type resistant IRBB21 plants. Based on their lesion length, the plants were separated into two groups, fully susceptible (>15 cm) and partially susceptible (5–15 cm). Typical infected leaves from partially and fully susceptible mutants are shown in Fig. 1.

To verify whether the identified mutants are truly derived from the wild-type resistant plant line, IRBB21, 11 simple sequence repeat (SSR) markers (RM101, 151, 204, 224, 264, 266, 278, 320, 333, 334, and 335, Blair et al. 2002) were used in genotyping. It was confirmed that all 21 mutants are identical to wild-type IRBB21 at the 11 SSR loci (data not shown).


**Fig. 1** Disease reaction of partially and fully susceptible mutants. Two-month old plants were inoculated with PR6 by the clipping method. Symptoms were evaluated 2 weeks after inoculation. Mutant 236 is partially susceptible and N18-238-2 is completely susceptible

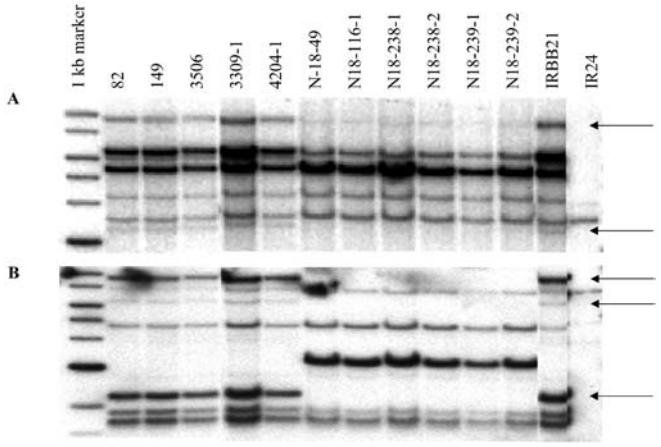


**Fig. 2A, B** Southern analysis of selected mutant samples hybridized with probes from the leucine-rich repeat (LRR) and kinase regions of *Xa21*. About 2–3 $\mu$g of *Hin*dIII-digested DNA were separated on a 0.8% agarose gel and blotted to Hybond $N^+$ membrane. The first five sample lanes contain DNA from partially susceptible diepoxybutane (DEB) mutants and the next six from fully susceptible fast neutrons (FN) mutants. $^{32}$P-labeled LRR probe (**A**) was from the *Hin*dIII-digested fragment containing the whole LRR region. The kinase probe (**B**) was from the fragment that was amplified using H3FragF and 3'Xa21R primers. *Arrows* indicate those fragments showing changes in some of the mutants

### Molecular characterization of mutants at the *Xa21* locus

Loss of resistance to *Xoo* PR6 in the identified mutants can result from either a mutation at the *Xa21* locus or changes in other genes required for the expression of *Xa21*-mediated resistance. Because *Xa21* is part of a multigene family with at least eight members (Song et al. 1995), it was expected that that DEB or FN mutagenesis of the *Xa21* locus would result in deletions or size alterations in some of the *Xa21* family members, and that these changes would be detectable by either Southern hybridization using the *Xa21*-specific sequence as a probe or by PCR analyses using *Xa21*-specific primer pairs. When DNA from the mutants was digested with the restriction enzyme *Hin*dIII and probed with either the LRR or kinase domain of the *Xa21* gene, several were observed to have altered hybridization patterns. Southern hybridization results from some selected mutants are shown in Fig. 2. Compared with the wild-type resistant IRBB21, all the partially susceptible mutants (R92, R95, 82, 149, 236, 238, 270, 284, 319, 360, 1056, 1252, 2532,

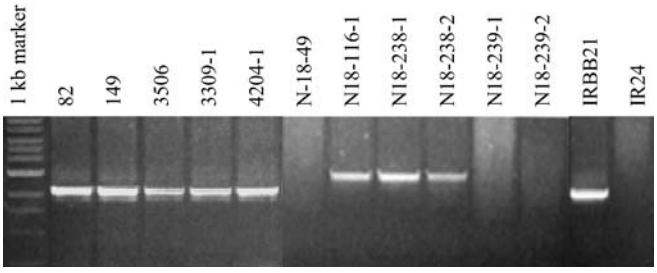


**Fig. 3** PCR analysis of mutants using *Xa21* kinase-specific primer pairs (KIN-1 and KIN-2). About 20 ng of DNA was used in PCR amplification. (Amplification conditions are described in "Materials and methods.") The PCR products were separated on 1.0% agarose gel. The mutants 82 through 4204-1 are the DEB mutants and the rest are the FN mutants

3309, 3313, 3506, and 4204-2) displayed no visible changes in the hybridization patterns when either the LRR or kinase fragments were used as probes. In contrast, all the fully susceptible mutants (N18-49, N18-116-1, N18-238-1, N18-238-2, N18-239-1, and N18-239-2) carried deletions or rearrangements in both the *Xa21* LRR and kinase domains. All of the fully susceptible mutants had similar hybridization patterns, with two LRR-hybridizing bands being absent (Fig. 2A, arrow indicated). When the kinase fragment was used as the probe in the Southern hybridization, all fully susceptible mutant lines were missing three of the original bands and had one new strong positive band (Fig. 2B, arrow indicated). It is not known why all six fully susceptible mutants, coming from four independent families, were selected only from the FN-treated population and all had large deletion patterns at the *Xa21* locus.

To further confirm the Southern blot results, PCR was used to detect mutations in these mutant lines. A specific primer pair in the kinase domain (KIN-1 and KIN-2) of the *Xa21* gene was used. All partially susceptible mutants had a 2-kb band similar to that from the wild-type resistant IRBB21 plant (Fig. 3). The six completely susceptible mutants displayed two types of amplification patterns from the kinase domain. There was no amplification in N18-49, N18-239-1, and N18-239-2, indicating deletion of at least at one of the PCR primer sites. The other three mutant lines (N18-116-1, N118-238-1, and N118-238-2) showed a larger amplification product, suggesting a rearrangement in the kinase region of these lines.

When comparing data from both the molecular analysis and phenotype evaluation, it was obvious that all of the mutants with changes at the *Xa21* locus were fully susceptible to PR6, while mutants with partial resistance had no visible deletions or re-arrangements at the *Xa21* locus, as demonstrated by both Southern blot and PCR analyses. It is possible that point mutations or small deletions could have occurred at the *Xa21* locus in some of the partially resistant mutants but that these were undetectable using the Southern or PCR methods. This was the case for two partially resistant mutants, R92 and

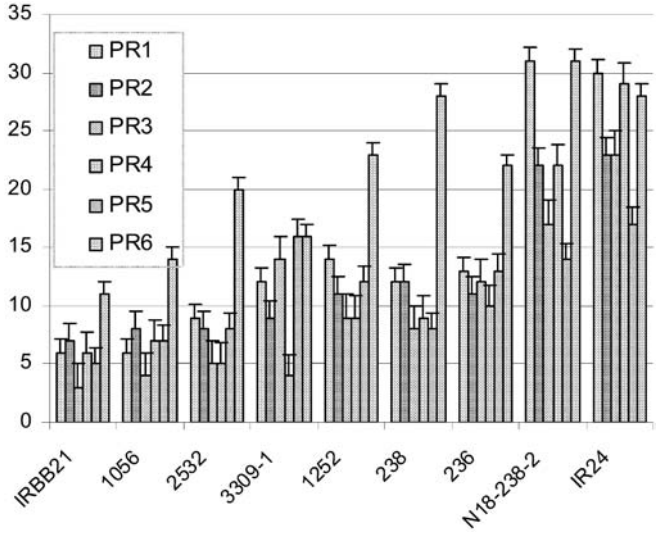


**Fig. 4** Lesion length of partially and fully susceptible mutants to six Philippine *Xoo* races. Two-month old plants were inoculated by the clipping method. The names of six races (PR1 to PR6) are described in the Materials and Methods. Lesion length (cm) was measured two weeks after inoculation. IRBB21 is the *Xa21* isogenic line and IR24 is the susceptible recurrent cultivar. 1056, 2532, 3309-1, 1252, 238 and 236 are partially susceptible mutants and N18-238-2 is a fully susceptible mutant

R95, which had a mutation at the *Xa21* locus as confirmed by genetic analysis (see below). It is also possible that the partially resistant mutants carried mutations in unlinked genes required for full expression of *Xa21*-mediated resistance. Detailed genetic analysis of all these mutants is required to verify these possibilities.

## Partially susceptible mutants show quantitative differences in their reactions to different *Xoo* races

To determine whether the partially susceptible mutants had the same spectrum of susceptibility to different *Xoo* races, they were inoculated with 12 *Xoo* strains (PR6 and an additional 11 strains representing 9 different Philippine races) in the greenhouse. To evaluate disease reaction accurately, at least three inoculations with the same strain were repeated for each mutant line. About 10–15 leaves from three to four plants were inoculated with each *Xoo* strain and average lesion length was obtained from all inoculated leaves. Compared with the susceptible recurrent cultivar IR24, most of the mutants showed a similar reaction to all *Xoo* strains tested. However, nine partially susceptible mutants exhibited a differential reaction to these *Xoo* strains. Lesion length data for six selected partially susceptible mutants (1056, 2532, 3309-1, 1252, 238, and 236) and one fully susceptible mutant (N18-238-2) are shown in Fig. 4. The most striking difference among the partially susceptible mutants was their reaction to PR6. For example, the reaction of line 238 to *Xoo* races PR1 through PR5 was similar to that of the other lines.

But the line 238 reaction to PR6 indicated a higher level of susceptibility similar to the recurrent parent IR24. Line 3309-1 was relatively susceptible to PR5 and resistant to PR4 when compared with the resistant and susceptible controls (IRBB21 and IR24, respectively). Since we have not yet mapped these mutations on the rice genome, it is not clear whether the differential reactions of the partially susceptible mutants were due to mutations in distant genes that are required for the *Xa21*-mediated resistance pathway or due to small deletions or rearrangements at the *Xa21* locus. Further characterization and mapping of these mutations should provide answers to these questions.

### Two mutants with partial resistance to *Xoo* mapped to the *Xa21* locus

Among 20 mutant lines identified in the first screen, two partially susceptible lines, R92 and R95, were selected for further genetic studies. The average lesion lengths that developed on rice leaves 14 days after inoculation with PR6 were as follows: 19.8 cm on IR24, 7.3 cm on R92, 8 cm on R95, and 1.5 cm on IRBB21 (data not shown). Bacterial growth curve analysis revealed that PR6 grew to approximately $10^9$ colony-forming units per leaf (cfu/leaf) on IR24 leaves, $10^8$ cfu/leaf on the mutant R92 and R95 leaves, and $10^7$ cfu/leaf on IRBB21 (data not shown). Therefore, the growth curves confirmed the partial resistance phenotype for R92 and R95.

To determine whether the mutations in the partially susceptible R92 and R95 lines were at the *Xa21* locus, these two lines were crossed with the susceptible line IR24 and wild-type IRBB21. The $F_1$ progeny of R92 × IRBB21 and R95 × IRBB21 were resistant to PR6 whereas the $F_1$ progeny of R92 × IR24 and R95 × IR24 were partially susceptible to PR6. These results indicate that the mutations in the R92 and R95 lines occurred at the *Xa21* locus, as they could not be complemented by IR24. Southern analysis using *Xa21* as a probe revealed no rearrangements in R92 or R95 (data not shown), indicating that if the mutations were in the *Xa21* gene, they were small or in a gene tightly linked to the *Xa21* cluster.

## Discussion

With the recent completion of the rice genome sequence (Goff et al. 2002; Yu et al. 2002), functional analysis of each gene in the rice genome is becoming the future challenge. Mutants produced by deletion or insertion mutagenesis will play an important role in assigning function to the large amount of new sequence information. The most commonly used insertional mutagenesis systems in plants are transposon and T-DNA tagging (Martienssen 1998). Although many genes have been identified using both strategies, these two systems have their limitations in gene function analysis. For example, the high frequency of secondary transposition in progeny of transposon-tagged lines could complicate gene identification from mutant populations (Izawa and Shimamoto 1998). T-DNA insertions are chemically and physically stable over multiple generations, but creation of a large number of T-DNA lines is time-consuming and expensive for most plant species. Another type of collection consisting of deletion mutants is generated either by a chemical treatment or irradiation. Compared to the insertional mutants, deletion mutants are easy to make, non-transgenic and carry multiple mutations per line. From approximately 4,500 $M_2$ lines (45,000 individual plants), we identified six mutants from four families, all induced by FN, with genomic changes at the *Xa21* locus as detected by Southern analysis or the PCR method. The mutation rate was about 0.13% at this single locus. We also identified 17 partially susceptible mutants, induced by DEB, from two screens with no detectable genomic changes at the *Xa21* locus. Genetic analysis revealed that two of them have mutations at the *Xa21* locus. Although we have not conducted a genetic test for all the mutants isolated, SSR fingerprinting provides assurance that these partially susceptible mutants are genuine mutants derived from IRBB21.

Although we cannot determine with any precision the size of genomic changes in these mutants, the results are consistent with the general prediction that FN can cause large deletions and rearrangements whereas DEB generally causes smaller deletions or point mutations. Furthermore, since the *Xa21* locus contains several members of the gene family, it is anticipated that sizable genetic lesions induced by FN will more likely lead to a complete loss of resistance, whereas small size deletions induced by DEB will result in predominantly partially susceptible mutants.

It is interesting to note that most of the mutations, in the resistance pathways identified in plant species, only partially suppress resistance mediated by individual R genes (Innes 1998). For example, the barley *Rar1* and *Rar2* genes have partial resistance phenotypes in cultivars carrying several powdery mildew R genes (Shirasu et al. 1999). The partial loss of resistance may indicate that genes required for disease resistance are redundant or part of branched pathways. Loss of any individual gene produces only small changes in resistance. Alternatively, mutations in some signal transduction components may cause lethality since these genes are essential for plant growth and development (Innes 1998). In our study, we found that all fully susceptible mutants had deletion or rearrangement at the *Xa21* locus as detected by Southern analysis or PCR. In contrast, all the partially susceptible mutants have no detectable mutations or rearrangements at the *Xa21* locus. However, it is still possible that the partially susceptible phenotype could be due to a point mutation at the *Xa21* locus that disrupts its activity. This type of mutant could maintain partial resistance, as it is known there is another active member, *Xa21D*, present at the *Xa21* locus (Wang et al. 1998). *Xa21D* confers partial resistance to *Xoo* strains and is a truncated receptor-like

kinase in which the gene was interrupted by the insertion of a retrotransposon element proximal to its kinase domain. Therefore partial resistance in the IRBB21-derived mutants would be maintained due to *Xa21D* despite loss of *Xa21* activity. It is unknown whether loss of *Xa21* function and compensation by *Xa21D* is the cause of partial susceptibility in mutants R92 and R95. However, genetic analysis confirmed that the mutation in these mutants occurred at the *Xa21* locus.

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