

The Cloned Gene, *Xa21*, Confers Resistance to Multiple *Xanthomonas oryzae* pv. *oryzae* Isolates in Transgenic Plants

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The cloned rice gene, *Xa21*, confers resistance to multiple pathogen isolates of *Xanthomonas oryzae* pv. *oryzae* in transgenic plants. The resistance phenotype was stably transmitted to T1 progeny and inherited as a single locus. The T1 progeny were tested for resistance to 32 *X. oryzae* pv. *oryzae* isolates from eight countries. Both the engineered line and the donor line showed resistance to 29 isolates and susceptibility to three isolates. The identical resistance spectrum of both lines indicates that the presence of a single member of a multigene family, *Xa21*, is sufficient to confer multi-isolate resistance. The results presented here have important implications for engineering disease resistance in crop plants.

Additional keywords: bacterial blight; *Oryza sativa*.

Most plant species carry genes governing highly effective resistance to microbial attack. Often, resistance genes are clustered in specific regions of the plant genome (Shepherd and Mayo 1972; Farrara et al. 1987; Richter et al. 1995) leading to the hypothesis that resistance genes are members of large multigene families that have diverged, generating different pathogen recognition specificities (Pryor 1987; Pryor and Ellis 1993; Dangl 1995; Michelmore 1995). In support of this hypothesis, a large cluster of alleles or tightly linked genes at the *Mla* locus in barley has been characterized, and at least 23 have been differentiated by their specific reaction to unique isolates of *Erysiphe graminis* (DeScenzo et al. 1994). Furthermore, seven rust resistance specificities map to the *M* locus in flax, which appears to be an array of closely linked genes spanning over 0.5 cM (Ellis et al. 1995). Finally, of the recently cloned resistance genes, most are members of multigene families (Staskawicz et al. 1995; Song et al. 1995). Thus, it appears that many resistance loci are made up of an array of linked genes related in sequence and governing diverse resistance specificities.

Demonstration of the resistance specificity of a single gene in such complex loci requires detailed molecular and phenotypic analysis. For instance, the *Pto* locus, carries at least three genes controlling resistance to *Pseudomonas syringae* pv. *tomato* (*Pto* and *Prf*) and sensitivity to the insecticide fenthion (*Prf* and *Fen*) (Staskawicz et al. 1995). The evidence

that *Pto* and *Fen* are distinct genes was revealed only by mutational and sequence analysis (Martin et al. 1994; Salmeron et al. 1994; Salmeron et al. 1996). The presence of two nearly identical functional *Cf-2* genes in the *Cf-2* locus is another example indicating that molecular and phenotypic characterization of individual genes at complex resistance loci is necessary to distinguish the resistance specificities of clustered genes (Dixon et al. 1996).

In rice, the genetics of resistance to several plant pathogens has been well characterized (Ronald et al. 1992; Yu et al. 1991; Causse et al. 1994; Wang et al. 1994). A locus for resistance to bacterial blight was transferred from the wild species *Oryza longistaminata* to the cultivated rice line IR24 generating the introgression line IRBB21 (Khush et al. 1990). This locus, *Xa21*, was found to confer resistance to all known *Xanthomonas oryzae* pv. *oryzae* races in India and the Philippines (Khush et al. 1990, Ikeda et al. 1990). We have recently cloned a gene at the *Xa21* locus, called *Xa21*. *Xa21* is a member of a small multigene family with at least seven members. Most of these family members are linked, suggesting that *Xa21* is part of a complex locus (Ronald et al. 1992; Song et al. 1995). We have previously demonstrated that transgenic lines expressing the *Xa21* gene confer resistance to a single isolate of *X. oryzae* pv. *oryzae* race 6 (Song et al. 1995). Compared with other cloned plant resistance genes, the structure of *Xa21* represents a previously uncharacterized class. The deduced amino acid sequence of *Xa21* encodes a receptor kinase-like protein carrying leucine rich repeats (LRRs) in the putative extracellular domain, a single pass transmembrane domain, and a serine threonine kinase intracellular domain.

This study was undertaken to determine if the multi-isolate resistance observed for line IRBB21 was due to a single gene or multiple genes at the *Xa21* locus. For example, the locus may encode a single gene product, *Xa21*, that specifies resistance to multiple pathogen isolates, or the locus may be composed of a cluster of tightly linked genes, each of which recognizes a unique isolate-specific determinant.

In this study, we report that transgenic plants expressing the cloned *Xa21* gene confer multi-isolate resistance to 29 diverse isolates from eight countries. Three *X. oryzae* pv. *oryzae* isolates were identified that can overcome the resistance of *Xa21* in both transgenic plants and the donor cultivar IRBB21. The resistance spectrum of the engineered line is identical to that of the donor line, indicating that the single cloned gene is sufficient to confer multi-isolate resistance.

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RESULTS

Xa21 is inherited as single gene in transgenic progeny.

To investigate the inheritance of the transgene, 281 T1 plants from transgenic line 106-17 containing the cloned resistance gene *Xa21* (Song et al. 1995) were tested for resistance to six isolates of *X. oryzae* pv. *oryzae* representing six Philippine races. About 40 to 50 plants were inoculated for each race. Resistance to each race segregated in a 3:1 ratio, which is consistent with a single locus insertion in the rice genome of the parental line 106-17 (Table 1).

To test for cosegregation of the resistance phenotype with the transgene, primer pairs (U1 and I1, Fig. 1) were developed that specifically amplified a 1.4-kb DNA fragment of *Xa21* that was polymorphic to fragments amplified from other family members. The availability of these primer pairs allowed us to quickly detect the presence of the *Xa21* transgene in the T1 progeny. A 1.4-kb fragment was amplified from the resistant donor IRBB21 and the plasmid used for transformation (pC822) (Fig. 2). All of the resistant T1 progeny contained the expected 1.4-kb band corresponding to the *Xa21* intron/kinase

DNA fragment (Fig. 1). The 1.4-kb PCR product was absent in all susceptible T1 progeny. A 1.3-kb fragment was amplified from the recipient cultivar TP309 and the susceptible control IR24 corresponding to a polymorphic family member present in these two lines. These results indicated that the transgene cosegregates with resistance to the six Philippines races.

Southern analysis was used to verify the PCR results. Since the *EcoRV* restriction sites are present in the beginning and end of the *Xa21* gene (Fig. 1), cleavage with this enzyme generates a 3.8-kb DNA fragment carrying most of the *Xa21* genomic region (Fig. 1). Control DNA as well as DNA from nine resistant and seven susceptible T1 progeny was digested with this enzyme, electrophoresed on an agarose gel, and hybridized with the 230-bp kinase specific probe. A 3.8-kb hybridizing band was detected in clone pC822, the donor cultivar IRBB21, and the resistant T1 progeny (Fig. 3). A similar result was obtained when the probe RG103 corresponding to the LRR region of *Xa21* was used (data not shown; Song et al. 1995). We also tested three additional enzymes *EcoRI*, *HindIII* and *PstI* with the LRR probe RG103 and the kinase probe. All probe/enzyme combinations detected the expected fragment of the transgene in the resistance progeny (data are not shown). Thus, the Southern results confirmed that the resistance to the six *X. oryzae* pv. *oryzae* races cosegregated with the *Xa21* transgene.

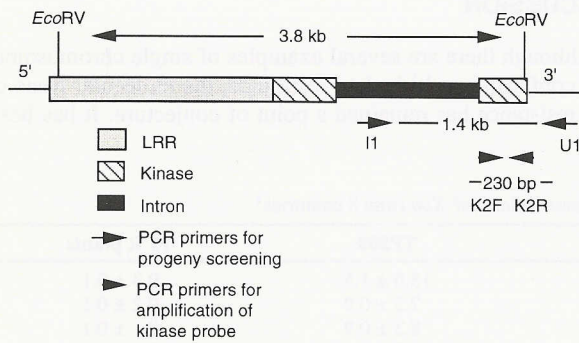


Fig. 1. Restriction map and probe/primer combinations used in analysis of transgenic plants expressing the *Xa21* gene. The primer pairs U1 and I1 amplify a 1.4-kb DNA fragment from the *Xa21* gene that was used in genotypic analysis of the T1 progeny (see Fig. 2). The primer pairs K2F and K2R amplify a 230-bp fragment which was used as a probe in Southern analysis of transgenic plants (see Fig. 3). Restriction enzyme sites, the open reading frame and intron are indicated.

Table 1. Segregation of resistance to six Philippine *Xanthomonas oryzae* pv. *oryzae* races in transgenic rice plants expressing the *Xa21* gene^a

Isolate	R plants	S plants	Total	χ^2	P
PXO61(race 1)	40	12	52	0.10	0.97-0.90
PXO86(race 2)	38	10	48	0.44	0.90-0.50
PXO79(race 3)	31	10	41	0.01	0.97-0.90
PXO113(race 4)	40	12	52	0.10	0.97-0.90
PXO112(race 5)	31	8	39	0.42	0.90-0.50
PXO99A(race 6)	38	11	49	0.17	0.90-0.50

^a Six-week-old T1 progeny plants were inoculated with *X. oryzae* pv. *oryzae*. For each isolate, at least nine leaves from three individual plants were inoculated. The lesion length (cm), the average of nine infected leaves, was measured 12 days after inoculation. The goodness of fit to a 3:1 ratio is indicated.



Fig. 2. PCR analysis of T1 transgenic plants. PCR amplification of *Xa21* specific DNA fragments was carried out using primer pairs U1 and I1 (see Fig. 1). IRBB21, TP309, and IR24 represent the donor line of *Xa21*, the recipient cultivar used in transformation experiments and a susceptible indica cultivar, respectively. pC822 carries the *Xa21* gene used to generate the transgenic line 106-17 (Song et al. 1995). The PCR products were separated in a 1.5% agarose gel. The 1.4-kb PCR product specific to all samples carrying the *Xa21* gene is indicated. Resistant and susceptible phenotypes are designated. One-kilobase ladder (NEB) and λ HindIII DNA fragments are used as markers in lanes 1 and 2, respectively.

Transgenic progeny carrying *Xa21* are highly resistant to 29 isolates from eight different countries.

To further test if the resistance spectrum of the *Xa21* transgenic progeny was similar to that of the donor line IRBB21, we inoculated both rice lines with *X. oryzae* pv. *oryzae* isolates from India, Indonesia, Colombia, China, Philippines, Thailand, Nepal and Korea (Table 2). The *X. oryzae* pv. *oryzae* isolates from these countries are quite divergent based on pathotype and lineage analysis (Leach et al. 1992; Adhikari et al. 1995; Nelson et al. 1995). It was found that transgenic progeny carrying *Xa21* are highly resistant to 29 isolates from these countries. The lesion length of IRBB21 and the T1 resistant transgenic progeny inoculated with *X. oryzae* pv. *oryzae* were less than 1.0 cm. The recipient cultivar TP309 was susceptible to these isolates displaying lesion length ranging from 4.5 to 17.0 cm.

Lesion length analysis revealed that the transgenic *Xa21* plants showed greater resistance to at least 16 isolates as compared to that of the donor cultivar IRBB21 (Song et al. 1995; Table 2). This increased resistance may be due to the high copy number of the *Xa21* gene in the transgenic plants. About a 5 to 10 times stronger *Xa21*-specific hybridization signal was observed in the transgenic *Xa21* plants as compared to the donor line IRBB21, indicating multiple insertions in a single locus in the transformed lines (Fig. 3). These results are consistent with reports that multiple copies of transgenes are often inherited as a single locus in transgenic lines generated by

particle bombardment (Christou et al. 1989; Cooley et al. 1995).

Three Korean isolates are virulent on the *Xa21* transgenic plants and the *Xa21* donor IRBB21.

Three *X. oryzae* pv. *oryzae* isolates (DY89031, CK89021, and JW89011) were previously found to be virulent on the *Xa21* donor cultivar at maximum tillering stage under field conditions (S. H. Choi, personal communication). We inoculated the transgenic progeny with these three isolates to test if the resistance spectrum of the donor line was identical to that of the transgenic line. We found that both the *Xa21* transgenic plants and the donor line showed susceptibility to these three isolates. The lesion length in the *Xa21* transgenic plants ranged from 12 to 16 cm, which is similar to the lesion length of the nontransgenic control (TP309) and the donor cultivar IRBB21 (Fig. 4). PCR analysis confirmed the presence of the gene in the transgenic lines tested. Thus these experiments indicate that transgenic lines carrying the single gene *Xa21* show an identical resistance spectrum to that of the donor cultivar IRBB21.

DISCUSSION

Although there are several examples of single chromosomal loci conferring multi-isolate resistance, the molecular basis of this resistance has remained a point of conjecture. It has been

Table 2. Transgenic plants carrying the cloned gene *Xa21* confer resistance to 29 diverse isolates of *Xoo* from 8 countries^a

Strain	Origin	IRBB-21	TP309	T1 R plants
PXO61 (Race 1)	Philippines	0.7 ^b ± 0.1 ^c	13.0 ± 1.5	0.3 ± 0.1
PXO86 (Race 2)	Philippines	0.5 ± 0.1	7.3 ± 0.9	0.2 ± 0.1
PXO79 (Race 3)	Philippines	0.9 ± 0.2	8.3 ± 0.9	0.1 ± 0.1
PXO113 (Race 4)	Philippines	2.1 ± 0.2	16.5 ± 1.5	0.4 ± 0.1
PXO112 (Race 5)	Philippines	1.5 ± 0.2	7.3 ± 0.6	0.3 ± 0.2
PXO99A (Race 6)	Philippines	2.3 ± 0.3	16.6 ± 1.1	1.0 ± 0.2
POX150 (Race 7)	Philippines	1.0 ± 0.1	13.5 ± 0.2	0.2 ± 0.1
K212	Korea	0.6 ± 0.2	8.0 ± 1.2	0.4 ± 0.1
K202	Korea	0.5 ± 0.4	4.5 ± 0.8	0.2 ± 0.1
JH89031	Korea	0.8 ± 0.1	13.2 ± 1.5	0.3 ^d
SN89023	Korea	1.3 ± 0.2	3.0 ± 0.5	0.3 ± 0.1
NX261	Nepal	0.8 ± 0.2	6.0 ± 1.5	0.2 ± 0.1
NXO228	Nepal	0.5 ± 0.1	9.0 ± 1.2	0.3 ± 0.1
NXO212	Nepal	0.8 ^d	5.0 ± 1.1	0.3 ^d
NXO256	Nepal	0.7 ^d	9.5 ± 1.4	0.4 ^d
A3846	India	0.6 ± 0.2	9.5 ± 1.2	0.5 ± 0.2
A3842	India	0.6 ^d	7.0 ± 0.8	0.2 ± 0.1
A3857	India	0.5 ^d	6.5 ± 0.9	0.4 ± 0.1
IXO100	India	0.5 ^d	10.0 ± 1.1	0.3 ^d
IXO56	Indonesia	0.4 ± 0.1	11.5 ± 1.3	0.4 ± 0.2
IXO57	Indonesia	0.6 ± 0.1	10.5 ± 0.9	0.5 ± 0.1
IXO58	Indonesia	0.2 ^d	7.5 ± 0.1	0.4 ^d
1	Thailand	0.4 ± 0.1	8.3 ± 0.9	0.4 ± 0.1
2	Thailand	0.5 ^d	9.0 ± 1.3	0.4 ± 0.1
3	Thailand	0.2 ^d	5.5 ± 0.6	0.2 ^d
R-7	Thailand	0.5 ± 0.1	11.0 ± 1.1	0.4 ± 0.1
CIAT1185	Colombia	0.8 ^d	10.0 ± 1.2	0.3 ^d
HB21	China	0.4 ^d	9.3 ± 0.1	0.1 ^d
NX42	China	0.4 ± 0.1	8.5 ± 1.0	0.3 ^d
ZHE173	China	0.5 ± 0.1	6.5 ± 0.8	0.4 ^d

^a Six-week-old T1 progeny plants were inoculated with *X.oryzae* pv. *oryzae*. For each isolate, at least nine leaves from three individual plants were inoculated. The lesion length is the average of 9 infected leaves. The standard error of the mean is indicated.

^b Lesion length (cm).

^c Standard error.

^d Standard error < 0.04.

proposed that these loci are composed of a cluster of tightly linked genes, each of which recognizes unique pathogen determinants (produced by pathogen avirulence genes) (Ronald et al. 1992). Alternatively, it has been hypothesized that these loci carry single resistance genes capable of recognizing conserved or diverse pathogen determinants (Ronald et al. 1992; Kearney and Staskawicz 1990). The availability of cloned genes isolated from such complex loci now allows experimental investigation of these hypotheses.

The donor line IRBB21 containing the *Xa21* locus was shown to be highly resistant to all tested *X. oryzae* pv. *oryzae* races in India and the Philippines (Khush et al. 1990). Whether the multi-isolate resistance observed in IRBB21 was controlled by a single gene or a cluster of tightly linked genes was not known. We have previously isolated the gene *Xa21* from IRBB21 and demonstrated that it is a member of a small multigene family located at the *Xa21* locus (Ronald et al. 1992; Song et al. 1995). Transgenic plants expressing *Xa21* were resistant to a single isolate of *X. oryzae* pv. *oryzae* race 6. In this paper, we demonstrate that the multi-isolate resistance observed for the introgression line IRBB21 is due to the presence of a single member of the *Xa21* gene family. This result was obtained by cloning one member of the gene family, transforming it into a susceptible line, and inoculating the resulting transgenic progeny with diverse *X. oryzae* pv. *oryzae* isolates from various locations around the world. Transgenic lines expressing *Xa21* conferred resistance to 29 isolates from eight countries and showed susceptibility to three Korean isolates. The resistance spectrum of the transgenic line precisely reflected the resistance spectrum of the donor cultivar IRBB21.

Plant disease resistance genes have been successfully utilized in classical breeding programs for crop protection for nearly a century. The recent availability of cloned resistance genes now provides additional tools for genetic engineering of improved plant cultivars by transformation. Bacterial blight disease caused by *X. oryzae* pv. *oryzae* is one of the most important diseases in Asia and Africa. Introduction of resistant cultivars is the most effective means of controlling the disease (Ogawa 1993). We have found that the *Xa21* transgenic plants (japonica background) have the same resistance spectrum as

the wild species donor (*O. longistaminata*) and the introgression line IRBB21 (indica background), indicating that the gene functions in diverse rice genotypes. Transformation of the cloned gene into existing rice cultivars would have direct applications in rice breeding programs. Resistant cultivars can be generated using transformation techniques within 2 years, compared with 7 to 10 years using a classical breeding approach.

Several advantages can be envisioned for using transformation technology as a tool to generate new cultivars. First, lines expressing low to high levels of resistance can be selected. For example, some of the *Xa21* transgenic lines show increased resistance to *X. oryzae* pv. *oryzae* as compared with

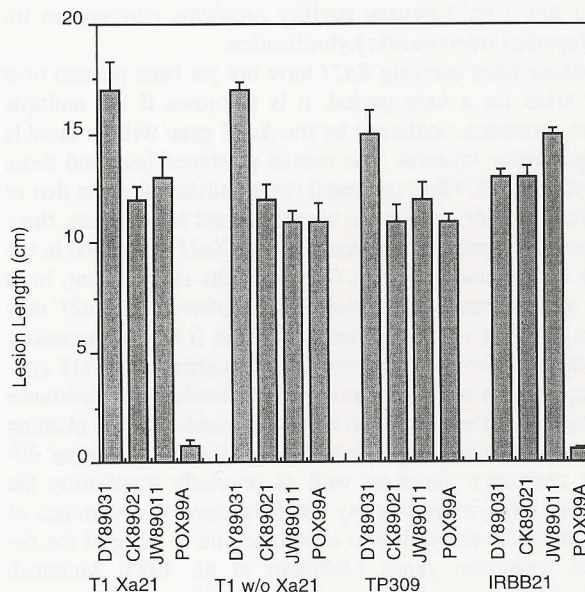


Fig. 4. Transgenic rice plants expressing *Xa21* and the donor cultivar IRBB21 are susceptible to three Korean isolates. The lesion length (cm) is the average of nine inoculated leaves. Six-week-old IRBB21 and eight-week-old T1 transgenic and TP309 plants were inoculated. Susceptible segregants lacking the *Xa21* gene as reflected by PCR analysis are indicated as T1 w/o *Xa21*. The standard error of the mean is indicated.

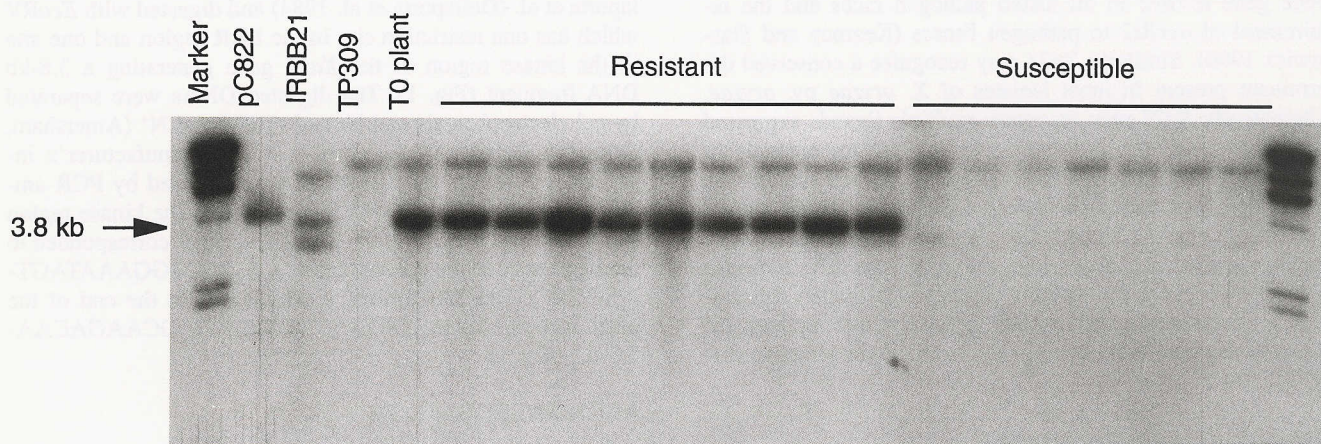


Fig. 3. Southern analysis of T1 transgenic plants. DNA extracted from the transgenic plants was digested with *EcoRV* and hybridized with the amplified 230-bp kinase fragment (see Fig. 1). The 3.8-kb hybridizing band specific to the *Xa21* expressing plants and the clone pC822 is indicated. Resistant and susceptible phenotypes are designated. The marker shown in the first and last lane is λ HindIII digest.

the donor line (Song et al. 1995). This increased resistance may be due to the high copy number of the transgene in these plants (Fig. 2). Other studies are consistent with this hypothesis. For instance, a correlation between copy number and expression level of an introduced gene was observed in transgenic potato plants (Stochhaus et al. 1987). Similarly, smaller bacterial populations observed in *Pto* transgenic plants as compared to wild type may have been the result of higher abundance of the *Pto* protein in transgenic tomato (Martin et al. 1993). Secondly, transformation techniques can be used to improve valuable cultivars currently containing some quantitative or qualitative resistance to disease, leading to increased durability (Wang et al. 1994). Finally, transformation of japonica type cultivars with a resistance gene derived from an indica line would bypass sterility problems common to indica/japonica interspecific hybridization.

Because lines carrying *Xa21* have not yet been planted over large areas for a long period, it is unknown if the multiple isolate resistance conferred by the *Xa21* gene will be durable in a particular location. The results presented here and those reported by S.H. Choi (personal communication) show that of the six *X. oryzae* pv. *oryzae* isolates tested from Korea, three isolates are capable of overcoming the *Xa21* resistance in the donor and engineered lines. These results suggest that, in at least some locations, the resistance conferred by *Xa21* may not be effective against all isolates. Thus, it will be necessary to determine the best strategy for deployment of *Xa21* containing lines in order to maximize the durability of resistance in a particular location. Such a strategy would include planting a mixture of genetically diverse rice cultivars containing different resistance genes as well as regularly monitoring the pathogen population to assay the frequency of occurrence of races that have the ability to overcome one or more of the deployed resistance genes (Adhikari et al. 1995; McIntosh 1992). Such strategies have been effective for controlling wheat stem rust in Australia and wheat stripe rust in Oregon (McIntosh 1992; Mundt 1994).

Determining the functional importance of the pathogen molecule that interacts with the *Xa21* gene product may help predict the durability of the resistance gene *Xa21*. For example, the pepper resistance gene *Bs2* is predicted to be durable due to the extensive conservation of the corresponding avirulence gene *avrBs2* in all tested pathogen races and the requirement of *avrBs2* to pathogen fitness (Kearney and Staskawicz 1990). Similarly *Xa21* may recognize a conserved determinant present in most isolates of *X. oryzae* pv. *oryzae*. Alternatively *Xa21* may recognize multiple ligands expressed from diverse *X. oryzae* pv. *oryzae* isolates. An example of such broad specificity was illustrated by the characterization of the recently cloned *Arabidopsis RPM1* gene which confers resistance to bacterial pathogens expressing two seemingly unrelated avirulence genes (Grant et al. 1995). The presence of resistance genes recognizing conserved pathogen determinants or multiple ligands would be an efficient strategy for reducing the total number of genes required for effective disease control.

MATERIALS AND METHODS

T1 transgenic plants.

Nine independent transgenic lines (T0) containing the *Xa21*

gene that displayed resistance to *X. oryzae* pv. *oryzae* race 6 (Song et al. 1995) were self-pollinated. The progeny (T1) of the resistant line 106-17 were used for the studies in this report.

Bacterial isolates, inoculation, and resistance scoring.

Thirty-two isolates from eight countries were used in the inoculation tests (kindly provided by J. Leach, Kansas State, and S. H. Choi, Korea). Only the Philippine isolates were classified into races. The isolates from other countries have not been classified into races since an international race identification system is not available for *X. oryzae* pv. *oryzae*. The isolates were grown for 72 h at 30°C on peptone sucrose agar (PSA) (Tsuchiya et al. 1982). Six-week-old, greenhouse-grown plants were cut about 4 cm from the tip of fully expanded leaves with scissors dipped in a bacterial suspension (Kauffman et al. 1973). After inoculation, plants were kept in a growth chamber with the following conditions: 24°C and 90% humidity for 14 h without light, 28°C and 85% humidity for 10 h with light (MH and MPS bulbs). For each experiment, lesion length was measured on nine leaves from three independent plants 12 days after inoculation.

PCR analysis.

DNA was extracted from 3-week-old seedlings using a previously described rapid DNA isolation method (Williams and Ronald 1994). PCR (polymerase chain reaction) amplification conditions were the same as described by Williams and Ronald (Williams and Ronald 1994). For analysis of segregation of the transgene in the T1 progeny, we designed primer pairs based on the *Xa21* intron/kinase region (Fig. 1). The U1 (untranslated) primer corresponded to the 3' untranslated region of *Xa21* (5'-CGATCGGTATAACAGCAAAAC-3'). The I1 (intron) primer corresponded to the center of the intron (5'-ATAGCAACTGATTGCTTGG-3'). These primers amplified a 1.4-kb DNA fragment that was specific to the *Xa21* gene and not to other family members and was not present in the cultivar TP309 (recipient for the *Xa21* transgene in transformation studies, 11) (Figs. 1 and 2).

Southern analysis.

DNA was extracted from each plant as described by Dellaporta et al. (Dellaporta et al. 1984) and digested with *EcoRV* which has one restriction site in the LRR region and one site in the kinase region of the *Xa21* gene generating a 3.8-kb DNA fragment (Fig. 1). The digested DNAs were separated by gel electrophoresis and blotted to Hybond N⁺ (Amersham, Arlington Heights, IL) according to the manufacturer's instruction. A kinase-specific probe was prepared by PCR amplification of a 230-bp DNA fragment from the kinase region using K2F and K2R primers. The K2F primer corresponded to the beginning of the second exon (5'-GTGCTGGAAATAGTAAACC-3'). The K2R primer corresponded to the end of the open reading frame (ORF) (5'-CCTGAGAGCAAGACAAAC-3') (Fig. 1).

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