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Xa21D Encodes a Receptor-like Molecule with a Leucine-Rich Repeat Domain That Determines Race-Specific Recognition and Is Subject to Adaptive Evolution

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The rice *Xa21* gene confers resistance to *Xanthomonas oryzae* pv *oryzae* in a race-specific manner. Analysis of the inheritance patterns and resistance spectra of transgenic plants carrying six *Xa21* gene family members indicated that one member, designated *Xa21D*, displayed a resistance spectrum identical to that observed for *Xa21* but conferred only partial resistance. *Xa21D* encodes a receptor-like protein carrying leucine-rich repeat (LRR) motifs in the presumed extracellular domain. The *Xa21D* transcript terminates shortly after the stop codon introduced by the retrotransposon *Retrofit*. Comparison of nucleotide substitutions in the LRR coding regions of *Xa21* and *Xa21D* provided evidence of adaptive selection. Both functional and evolutionary evidence indicates that the *Xa21D* LRR domain controls race-specific pathogen recognition.

INTRODUCTION

Receptor kinases (RKs) play a key role in important cellular processes in plants and animals (Fantl et al., 1993; Song et al., 1995; Becraft et al., 1996; Heldin and Ostman, 1996; Stein et al., 1996; Ten Dijke et al., 1996; Torii et al., 1996; Li and Chory, 1997). Three functional domains are commonly associated with RK proteins: an extracellular domain, a transmembrane domain, and an intracellular catalytic domain. Studies of animal RKs have revealed a common mechanism for RK-mediated cellular signaling (Hunter, 1995; Pawson, 1995; Heldin and Ostman, 1996). In this model, ligand binding to the extracellular receptor domain induces receptor dimerization and subsequent activation of the intracellular kinase domain. The specificity of the interaction with the ligand is controlled by amino acid residues in the extracellular domain (Heldin and Ostman, 1996).

Plant RKs can be divided into six subclasses based on the protein motif in the presumed extracellular domains (Walker, 1994; Becraft et al., 1996). The largest subclass of plant RKs is the leucine-rich repeat (LRR) group, which encodes proteins with an extracellular domain containing 20 to 25 imperfect repeats of a 24–amino acid leucine-rich motif. The LRR subclass of plant RKs includes proteins that govern pollen development, plant elongation, regulation of meristem and flower development, disease resistance, and brassinosteroid signal transduction, as well as other functions that remain to be determined (Chang et al., 1992; Valon et al., 1993; Song et al., 1995; Torii et al., 1996; Clark et al., 1997; Li and Chory, 1997). Plant LRRs have also been found in secreted proteins (polygalacturonase inhibitor proteins or PGIPs) (De Lorenzo et al., 1994) and in membrane-bound resistance gene products (Dixon et al., 1996). LRR domains are present in a variety of proteins involved in peptide ligand recognition, cell adhesion, and various other functions and are thought to mediate protein–protein interactions (Braun et al., 1991; Kobe and Deisenhofer, 1994).

The cloning and characterization of the rice *Xa21* gene demonstrated that LRR-containing RKs function in plant disease resistance. *Xa21* confers race-specific resistance to *Xanthomonas oryzae* pv *oryzae* (Mew, 1987; Nelson et al., 1994; Song et al., 1995; Wang et al., 1996; S.H. Choi, personal

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communication) in transgenic plants. The predicted protein product of *Xa21* carries LRRs in the presumed extracellular domain and a serine/threonine kinase in the presumed cytoplasmic domain (Song et al., 1995; W.-Y. Song, L.-Y. Pi, D.-L. Ruan, D. Braun, J.C. Walker, and P.C. Ronald, unpublished data). *Xa21* is a member of a multigene family located on rice chromosome 11 (Ronald et al., 1992; Song et al., 1995). Seven *Xa21* gene family members, designated A1, A2, B, C, D, E, and F, were cloned and grouped into two classes based on sequence similarity (Song et al., 1997). The Xa21 class contains *Xa21* as well as members D and F. The A2 class contains members A1, A2, C, and E. Within each class, family members share striking nucleotide sequence identity (98.0% average identity for the members of the Xa21 class; 95.2% average identity for the members of the A2 class). In contrast, there is low sequence identity between members of the two classes (63.5% identity between *Xa21* and A2) (Song et al., 1997). A GC-rich region located immediately downstream of the start codon is highly conserved among all of the sequenced family members.

Based on models of mammalian RKs, we have proposed that the *Xa21* LRR domain interacts with a presumed ligand to determine the race-specific resistance response (Ronald, 1997). In support of this idea, recent work with the *M* locus of flax demonstrated that alterations in the LRR domain play a significant role in the evolution of rust resistance genes and production of new recognitional specificities (Anderson et al., 1997). Similarly, the amino acid differences in the LRRs of the *Xa21* gene family members suggest that members may have evolved to recognize different races and/or may confer altered resistance phenotypes. We tested this hypothesis in two ways. First, we further characterized the resistance phenotype and inheritance patterns of transgenic rice plants carrying family members A1, A2, C, D, E, and F to eight *X. o. oryzae* races. We found that members A1, A2, C, E, and F conferred no observable resistance phenotype in transgenic plants, whereas Xa21 class member D, designated *Xa21D*, conferred the same resistance spectrum as did *Xa21.* However, the resistance level in the *Xa21D* transgenic plants was intermediate to that observed for *Xa21.* The presumed open reading frame (ORF) of *Xa21D* encodes a receptor-like molecule lacking the transmembrane and kinase domains.

Second, we characterized nucleotide substitution patterns in members of the *Xa21* gene family to gain insight into the function and evolution of particular coding domains. For the investigation of function, it is important to discriminate between nucleotide substitutions that lead to amino acid replacements (nonsynonymous substitutions) and nucleotide substitutions that do not alter amino acids (synonymous substitutions). The ratio of these two types of substitutions is particularly informative. In most protein-coding genes, the ratio of nonsynonymous to synonymous substitutions is <1; this observation is consistent with functional constraints against amino acid replacements (Kimura, 1983). Conversely, a ratio significantly >1 indicates that adaptive selection events have fueled divergence between genes (Hughes and Nei, 1988; Messier and Stewart, 1997).

Evidence of adaptive selection is rare but appears to be most common in gene regions that function in host and pathogen recognition (Endo et al., 1996). A comparison of nucleotide substitutions in the LRR coding regions of *Xa21* and *Xa21D* revealed that although *Xa21* and *Xa21D* share 99.1% sequence identity, nonsynonymous substitutions occur significantly more frequently than do synonymous substitutions in the LRR; this result is consistent with the LRR's putative role in ligand binding. These two approaches unambiguously demonstrate that the *Xa21* locus carries two functional resistance genes (*Xa21* and *Xa21D*), that the LRR domain is subject to adaptive evolution, and that this region governs race-specific pathogen recognition.

RESULTS

Expression of *Xa21* Gene Family Members

To assess expression of the *Xa21* gene family members, a cDNA library was constructed using mRNA isolated from leaves infected with *X. o. oryzae* race 6 at various time intervals (Song et al., 1995). The genomic clone RG103, which hybridizes with the LRR coding sequence, and an amplified DNA fragment encoding the XA21 kinase domain (McCouch et al., 1988; Ronald et al., 1992; Wang et al., 1996) were used to screen the library. Fifteen cDNA clones were identified and partially sequenced. Eight of the clones hybridized with only the LRR probe RG103, which corresponds to *Xa21* (RC2, 1.0 kb; RC9, 1.4 kb; and RC17, 1.6 kb) and *Xa21D* (RC1, 1.4 kb; RC3, 0.5 kb; RC4, 1.0 kb; RC10, 1.0 kb; and RC12, 1.0 kb). The five cDNAs carrying the *Xa21D* LRR coding region were distinguished from the highly similar *Xa21* sequence by the presence of 17 bp that are unique to *Xa21D* (Song et al., 1997). Five clones hybridizing only with the *Xa21* kinase probe corresponded to *Xa21* (RC8, 0.5 kb; RC13, 1.2 kb; and RC15, 0.8 kb) and C (RC6, 1.7 kb; RC7, 1.7 kb). No cDNA corresponding to the expected *Xa21D* kinase sequence was found. Only RC5 (3.2 kb, member C) and RC16 (1.5 kb, *Xa21*) hybridized with both the LRR and kinase probes.

Sequence analysis (800 bp in the 5′ region and 900 bp in the 3′ region) of RC5 indicates that this cDNA carries the ORF of member C beginning 10 bp before the ATG codon and ending 53 bp after the stop codon. The intron of member C is spliced, as predicted by genomic sequence analysis (Song et al., 1997). In summary, seven, three, and five cDNAs were found for *Xa21*, member C, and *Xa21D*, respectively. No cDNAs for A1, A2, E, and F were identified after screening >3 million plaques, suggesting that these members are not expressed. Alternatively, they are expressed at very low levels or under different conditions than those assayed.

Transgenic Rice Plants Carrying Family Members A1, A2, C, E, and F Do Not Confer Resistance to *X. o. oryzae*

In a previous study, 16 clones hybridizing with RG103 were isolated from bacterial artificial chromosome and cosmid libraries (Song et al., 1995). Two of these clones carried the *Xa21* coding sequence (pB821 and pC822) and the other 14 clones carried the coding sequences of members A1, A2, C, *Xa21D*, E, and F. These clones were introduced by particle bombardment into the *X. o. oryzae*–susceptible rice cultivar TP309 (Song et al., 1995). We previously demonstrated that only transgenic plants carrying *Xa21* conferred high levels of resistance to *X. o. oryzae* race 6.

In this study, we further characterized the transgenic plants carrying the other gene family members. Of the 14 original genomic clones carrying members A1, A2, C, *Xa21D*, E, and F, only five carried the entire coding sequence for an RK ORF and were designated pB833 (A1), pB843 (A2), pB853 (C), pB812 (*Xa21D*), and pB806 (F) (Figure 1). The RK ORF on pB37 (E) was truncated by the insertion of a transposable-like element called *Truncator* (Song et al., 1997). Eighty-seven independently transformed lines (T_0) carrying these six constructs were generated. All T_0 lines were self-pollinated to produce T_1 progeny. Two to three T_0 families per construct were chosen for detailed inoculation experiments with six *X. o. oryzae* isolates representing six Philippine races (Table 1). The selected T_0 families were assayed by DNA gel blot analysis to confirm that they carried the corresponding transgenes (data not shown). No resistant plants were identified in 468 T_1 individuals from the 13 T_0 families carrying members A1 (104-6, 104-8, and 104-29), A2 (109-8, 109-11, and 109-15), C (110-4, 110-7, and 110-21), E (103-9 and 103-27), and F (5-11 and 5-16) (Table 1), suggesting that these five *Xa21* family members conferred no resistance to the six *X. o. oryzae* races tested.

Xa21 Gene Family Member *Xa21D* Confers Partial Resistance to *X. o. oryzae*

In contrast to the susceptibility to *X. o. oryzae* race 6 observed in transgenic plants carrying the five *Xa21* gene

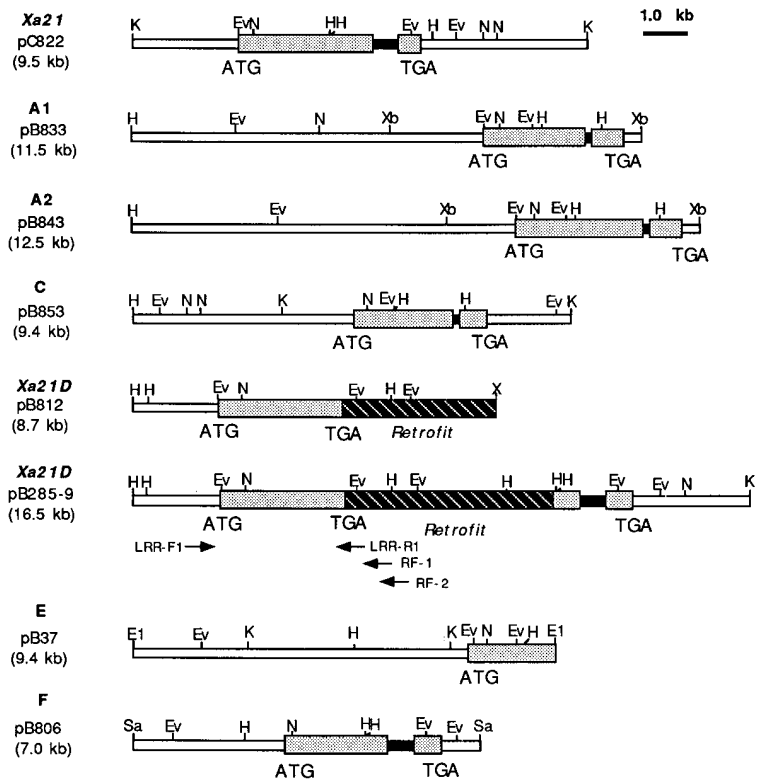

Figure 1. Restriction Maps of Eight Constructs Carrying the *Xa21* Gene Family Members Used to Generate Transgenic Lines.

The name of the family member, the construct, and the insert size are shown at left. The shaded bars indicate coding sequences for the RK-like proteins. Start and stop codons are indicated. The black bars represent introns. The *Retrofit* transposon-like sequence is indicated by hatched bars (Song et al., 1997). Primers used in the PCR and RT-PCR experiments are indicated below pB285-9. Selected restriction sites EcoRI (E1), EcoRV (Ev), HindIII (H), KpnI (K), NotI (N), SalI (Sa), and XbaI (X, Xb) are shown.

Table 1. Disease Reaction of Transgenic Plants Transformed with the *Xa21* Gene Family Members[a]

			X. o. oryzae Races								
Member	Construct	T_0 Line	R1[b]	R2[c]	R3[d]	R4[e]	R5[f]	R6[g]	K1[h]	K2a[i]	K2b[j]
A1	pB833	104-29	9.5 ± 1.3[k]	10.5 ± 1.2	9.1 ± 1.4	11.3 ± 0.9	10.4 ± 1.1	11.5 ± 1.3	ND[l]	ND	ND
A2	pB843	109-11	11.5 ± 1.1	10.2 ± 1.3	10.4 ± 0.8	13.5 ± 1.6	8.5 ± 1.2	12.7 ± 1.2	ND	ND	ND
C	pB853	110-4	10.3 ± 0.8	9.5 ± 1.2	11.7 ± 0.8	13.4 ± 1.1	9.6 ± 1.3	13.9 ± 1.0	ND	ND	ND
Xa21D	pB812	105-15	6.0 ± 0.8	4.8 ± 1.0	5.4 ± 0.9	6.2 ± 1.1	4.2 ± 1.2	5.3 ± 0.8	14.5 ± 1.2	11.6 ± 1.1	13.4 ± 1.2
Xa21D	pB812	105-22	5.5 ± 1.2	5.8 ± 1.2	5.6 ± 1.1	6.5 ± 1.0	4.8 ± 1.1	6.3 ± 1.2	16.4 ± 1.3	12.2 ± 1.2	12.5 ± 1.4
Xa21D	pB812	105-47	5.3 ± 0.9	5.4 ± 1.0	6.0 ± 1.3	6.7 ± 1.3	5.0 ± 1.4	5.8 ± 0.9	15.5 ± 1.0	12.9 ± 1.4	11.6 ± 1.0
Xa21D	pB285-9	14	6.4 ± 0.7	5.6 ± 0.9	5.4 ± 1.3	5.8 ± 0.7	5.0 ± 0.9	6.6 ± 0.7	15.0 ± 1.4	11.3 ± 1.2	13.3 ± 1.0
Xa21D	pB285-9	64	6.2 ± 0.9	5.8 ± 1.1	5.9 ± 1.1	6.5 ± 1.2	4.9 ± 1.0	6.3 ± 0.9	14.5 ± 1.2	12.6 ± 1.3	14.4 ± 1.5
Xa21D	pB285-9	65	6.4 ± 0.9	5.7 ± 1.2	5.8 ± 1.2	6.7 ± 1.3	4.7 ± 1.4	6.1 ± 0.9	16.5 ± 1.3	13.6 ± 1.2	13.8 ± 1.4
E	pB37	103-27	12.0 ± 1.2	11.5 ± 1.2	9.7 ± 1.3	12.0 ± 1.2	9.8 ± 0.9	13.2 ± 1.1	ND	ND	ND
F	pB806	5-16	10.3 ± 0.8	11.0 ± 0.7	10.8 ± 1.1	14.5 ± 0.9	11.5 ± 1.2	13.9 ± 1.0	ND	ND	ND
Control											
Xa21	pC822	106-17	1.0 ± 0.3	0.5 ± 0.3	1.1 ± 0.3	1.9 ± 0.1	0.75 ± 0.3	1.5 ± 0.1	14.5 ± 0.9	13.8 ± 0.8	14.7 ± 0.7
TP309	None		12.0 ± 0.7	9.5 ± 0.8	11.0 ± 0.7	13.7 ± 1.0	9.4 ± 0.6	12.5 ± 0.6	16.4 ± 1.2	14.0 ± 0.9	15.3 ± 0.8

[a] The name of family member, construct (diagrammed in Figure 1), and the T_0 parental line are indicated. Two-month-old progeny were inoculated with *X. o. oryzae* races 1 to 6 and three *X. o. oryzae* Korean isolates representing two races. T_1 progeny from two or three independently transformed T_0 lines were evaluated for pB833 (*Xa21* family member A1), pB843 (*Xa21* family member A2), pB853 (*Xa21* family member C), pB37 (*Xa21* family member E), and pB806 (*Xa21* family member F). For simplicity, data from only one independently transformed line are shown because the other two or three lines for these five constructs gave similar results. T_1 progeny from the independently transformed T_0 lines pB812 and pB285-9 (*Xa21* family member *Xa21D*) are listed separately. T_2 progeny from lines 106-17 were used to evaluate *Xa21* (Wang et al., 1996). For all segregating lines, the presence of the corresponding transgene was determined by DNA gel blot hybridization with RG103. TP309 is the recipient used in the transformation studies. The lesion length (in centimeters) is the average of at least 18 leaves, and the standard error is shown.

[b] R1, Philippine isolate PXO61 (race 1).

[c] R2, Philippine isolate PXO86 (race 2).

[d] R3, Philippine isolate PXO79 (race 3).

[e] R4, Philippine isolate PXO113 (race 4).

[f] R5, Philippine isolate PXO112 (race 5).

[g] R6, Philippine isolate PXO99A (race 6).

[h] K1, Korean isolate DY89031 (race K1).

[i] K2a, Korean isolate CK89021 (race K2).

[j] K2b, Korean isolate JW89011 (race K2).

[k] Average lesion length of T_1 or T_2 progeny carrying the transgene (cm) ±SE.

[l] ND, no data.

family members A1, A2, C, E, and F, a significant reduction in lesion length was observed among six out of the 17 T_0 lines generated with the construct pB812 carrying *Xa21D.* These six lines (105-15, 105-20, 105-22, 105-31, 105-35, and 105-47) showed >50% lesion length reduction (5.3- to 6.6-cm lesion length) compared with the susceptible recipient line TP309 (12.5-cm lesion length) when inoculated with *X. o. oryzae* race 6 (Figure 2A and Table 1). DNA gel blot analysis confirmed the presence of the *Xa21D* transgene in these six T_0 plants (data not shown). DNA gel blot analysis of the other 11 T_0 lines indicated either lack of *Xa21D*-hybridizing bands or bands indicative of DNA rearrangements (data not shown).

Three of these partially resistant T_0 lines (105-15, 105-22, and 105-47) were self-pollinated to produce T_1 progeny and analyzed for resistance to six *X. o. oryzae* races (Table 1). All T_1 progeny from two of the T_0 parents (105-15 and 105-47) (10 plants per T_0 line tested) were partially resistant to *X. o. oryzae* race 6. Hybridization of DNA extracted from 105-15 and 105-47 with the probe RG103 revealed the presence of several RG103-hybridizing bands suggestive of multiple insertions (data not shown). T_1 progeny from a third T_0 plant, 105-22, carrying clone pB812, segregated for resistance to *X. o. oryzae* race 6 in a 3:1 ratio (30R:9S; $\chi^2 = 0.08$; $P = 0.78$). The partial resistance phenotype cosegregated with the presence of the transgene as revealed by polymerase chain reaction (PCR) and DNA gel blot analysis (Figures 3A and 3B). All plants carrying pB812 were partially resistant to Philippine *X. o. oryzae* races 1 to 6 and susceptible to three Korean *X. o. oryzae* isolates representing two races (Table 1). Plants lacking pB812 were fully susceptible to all eight *X. o. oryzae* races (data not shown). These results indicate

that the presence of the transgene is required for partial resistance to the six *X. o. oryzae* races and that transgenic lines carrying construct pB812 displayed the same resistance spectrum as did transgenic lines carrying *Xa21* (Wang et al., 1996). The only observable phenotypic difference was that the level of resistance in the pB812 transgenic plants was intermediate to that observed for the *Xa21* lines (Table 1). Similarly, growth of an avirulent race of *X. o. oryzae* in transgenic plants carrying construct pB812 was intermediate to that of the resistant *Xa21* donor line (IRBB21) and the susceptible line TP309 6 to 10 days after infection (Figure 4), whereas the virulent race K1 grew to the same levels in all three lines (data not shown).

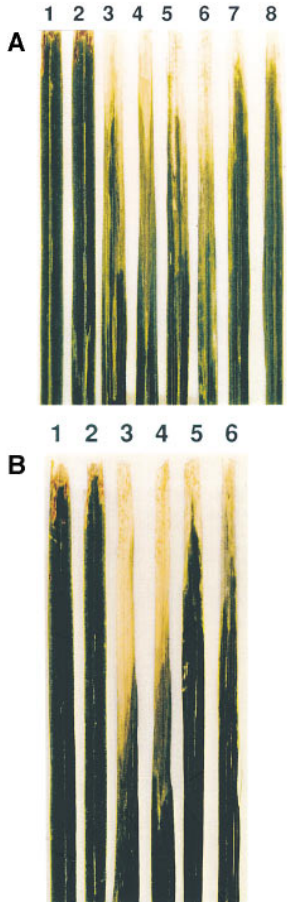

Figure 2. Resistance Phenotype of *Xa21D* Transgenic Plants.

Resistance of T_0 transgenic plants transformed with construct pB812 **(A)** and pB285-9 **(B)** to *X. o. oryzae* race 6. Plants were photographed 12 days after inoculation at the leaf tip. Lengths of lesions are indicated in the text.

(A) Leaves 1 and 2 are from IRBB21, leaves 3 and 4 are from IR24, leaves 5 and 6 are from TP309, and leaves 7 and 8 are from a transgenic line containing pB812.

(B) Leaves 1 and 2 are from IRBB21, leaves 3 and 4 are from TP309, and leaves 5 and 6 are from a transgenic line containing pB285-9.

The *Xa21D* ORF Encodes a Receptor Kinase–like Protein Lacking the Transmembrane and Kinase Domains

Restriction mapping, sequence analysis, and comparison of the 8.7-kb clone pB812 with the *Xa21* genomic sequence revealed that the RK ORF encoded in pB812 lacked the transmembrane and kinase domains. The RK ORF was prematurely truncated because of the presence of a stop codon in the last LRR preceding the transmembrane domain (Song et al., 1997). The truncated ORF encodes a 612–amino acid protein, including the signal peptide, and GC-rich and LRR domains (Figure 5; Song et al., 1997). Up to *Retrofit* insertion, *Xa21D* is 99.1% identical to *Xa21* in nucleotide sequence (Song et al., 1997).

To determine whether full resistance could be restored in transgenic plants carrying the entire RK ORF, including the 4.8-kb *Retrofit* coding sequence, a second, larger clone (pB285-9; 16.5 kb) was made from cosmid 285 (Figure 1; Song et al., 1995). Cosmid clone 285 carries, in addition to the 8.7-kb region present on pB812, the entire *Retrofit* coding region as well as a flanking region carrying transmembrane and kinase domains downstream of the *Retrofit* stop codon (Song et al., 1997). The predicted amino acid sequence of the transmembrane and kinase domains of XA21D is 99.8% identical to XA21, with only two amino acid differences in the kinase domain. Twenty-nine independently transformed lines were generated from the 16.5-kb pB285-9 construct using particle bombardment. From each independently transformed line, six clonal plants were derived. Inoculation tests indicated that 120 plants (representing 20 independently transformed lines) were susceptible and 54 plants (representing nine independently transformed lines) were partially resistant to *X. o. oryzae* race 6 (Figure 2B). DNA gel blot analysis of DNA extracted from three susceptible and seven partially resistant T_0 plants indicated that only the resistant plants carried the expected 5.0-kb HindIII DNA fragment hybridizing with RG103 (Figure 3C; Song et al., 1997). The 5.0-kb band corresponds to the LRR domain and the 5′ end of *Retrofit* (Figure 1). Three independently transformed T_0-resistant lines (designated 14, 64, and 65) were selected for further analysis. These T_0 plants were self-pollinated, and the resulting T_1 progeny were inoculated with *X. o. oryzae* race 6. All progeny (22 plants from the three families) displayed partial resistance to *X. o. oryzae* race 6.

DNA gel blot analysis with the LRR probe RG103 and the kinase probe (Wang et al., 1996) indicated that all 22 T_1 plants contained multiple hybridizing bands, suggestive of multiple insertions. DNA gel blot hybridization of NotI-digested DNA of 22 T_1 individuals from these three families with the LRR and kinase probe revealed the presence of the expected 10-kb fragment carrying the entire *Xa21D* coding

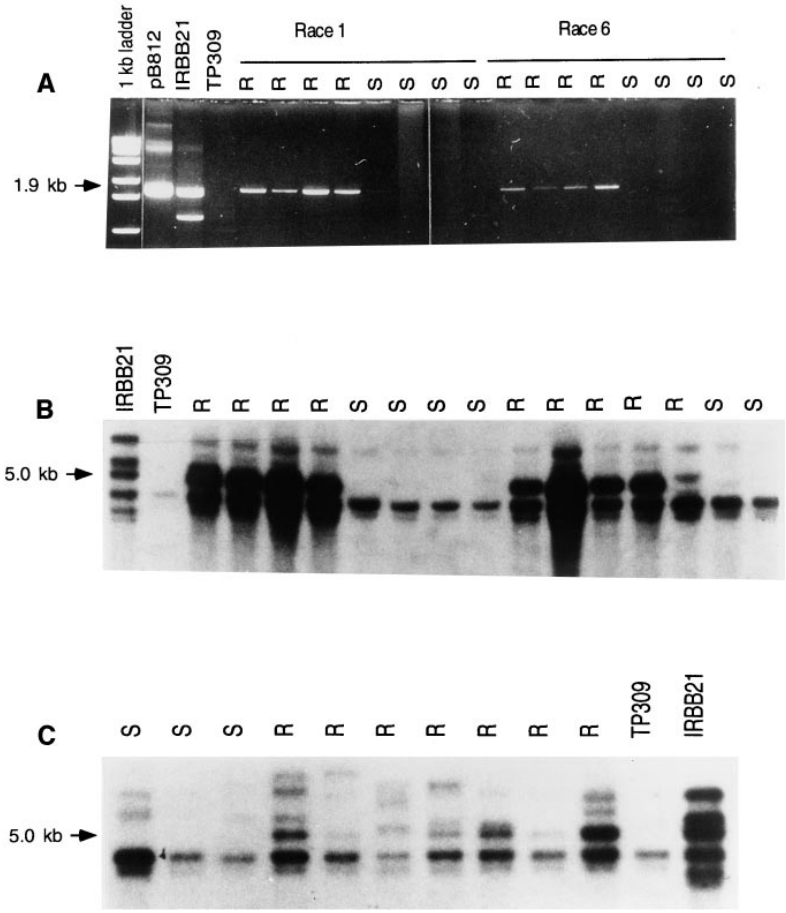

Figure 3. PCR and DNA Gel Blot Analyses of Transgenic Plants.

(A) PCR analysis of T_1 transgenic plants transformed with construct pB812. PCR amplification of *Xa21D*-specific DNA fragments was performed using primer pairs LRR-F1 and RF-1 (see Figure 1). IRBB21 and TP309 represent the donor line of *Xa21D* and the recipient cultivar used in transformation experiments, respectively. The PCR products were separated in a 1.5% agarose gel. The 1.9-kb PCR product is specific to transgenic plants carrying pB812. Partially resistant and susceptible phenotypes that were inoculated with *X. o. oryzae* race 1 or race 6 are designated as R and S, respectively.

(B) and **(C)** DNA gel blot analysis of T_1 transgenic plants transformed with construct pB812 and DNA gel blot analysis of T_0 transgenic plants transformed with pB285-9, respectively. In both **(B)** and **(C)**, DNA extracted from the transgenic plants was digested with HindIII and hybridized with the restriction fragment length polymorphism marker RG103, which hybridizes with the LRR region of the *Xa21* gene family members. The 5.0-kb hybridizing band is specific to *Xa21D.* IRBB21 and TP309 represent the donor line of *Xa21D* and the recipient cultivar used in the transformation experiments, respectively. Partially resistant and susceptible phenotypes that were inoculated with *X. o. oryzae* race 6 are designated as R and S, respectively.

region, the *Retrofit* insertion, and the 3′ kinase domain (data not shown; Figure 1). The lesion length and in-the-plant bacterial growth displayed by these T_1 individuals inoculated with *X. o. oryzae* race 6 were intermediate to that of *Xa21*-containing and TP309 lines (Table 1 and Figure 4). These results demonstrate that construct pB285-9 containing the LRR, *Retrofit*, and kinase domains conferred the same level of resistance as did plants carrying the 8.7-kb construct pB812.

Family Member *Xa21D* Is Expressed in Transgenic Plants Carrying pB812 and pB285-9

To determine whether the *Xa21D* transcript was found in transgenic plants carrying pB285-9 and pB812, RNA was isolated from plants generated from both pB812 (T_1 plants) and pB285-9 (T_0 plants). Reverse transcriptase–PCR (RT-PCR) was conducted using a 5′ primer located precisely before the *Xa21D* ATG (LRR-F1) with three different 3′ primers.

LRR-R1 anneals 21 to 39 bp upstream of the stop codon introduced by *Retrofit*, RF-1 anneals 147 to 167 bp downstream of the stop codon introduced by *Retrofit*, and RF-2 anneals 244 to 264 bp downstream of the stop codon introduced by *Retrofit* (Figure 1). RT-PCR products with the expected lengths were amplified using the two primer pairs, LRR-F1 and LRR-R1 (1.8 kb) and LRR-F1 and RF-1 (1.9 kb) (Figure 6). However, primers LRR-F1 and RF-2 did not amplify the expected 2.1-kb product, suggesting that the transcript terminates shortly after the *Xa21D* stop codon introduced by *Retrofit* in both pB812- and pB285-9–transformed plants. The same primer pair (LRR-F1 and RF-2) amplified a 2.1-kb DNA fragment from plasmid and genomic DNA, indicating that the failure to amplify the 2.1-kb product from the cDNA was not due to failure of the primers to anneal to target sequences (Figure 6; data not shown).

To test whether sequences downstream of *Retrofit* carrying the *Xa21D* kinase coding region are present in the *Xa21D* transcript, two kinase-specific primers (KIN1-F and KIN2) were used to amplify RT-PCR products from pB285-9 transgenic plants. Although these two primers successfully amplified the *Xa21* kinase domain from *Xa21*-containing plants (data not shown), no amplification was observed using the same cDNA preparations from which the previously described 1.8- and 1.9-kb LRR RT-PCR products were successfully amplified. This result indicates that the *Xa21D* transcript carries at least 167 bp of *Retrofit* sequences but does not contain sequences 264 bp downstream of the stop codon introduced by *Retrofit.*

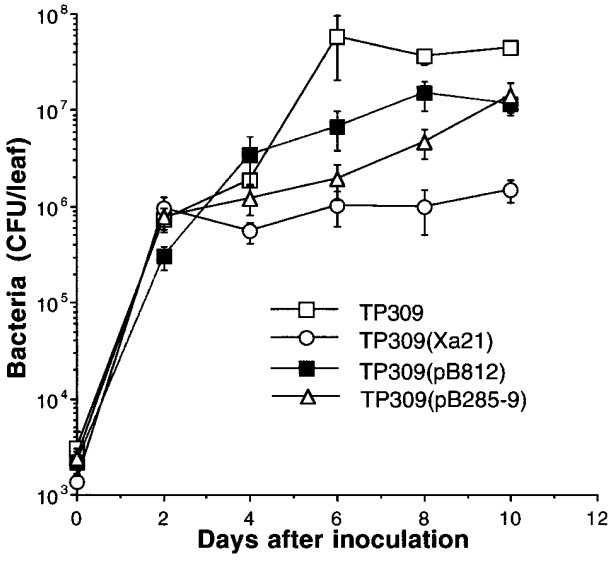

Figure 4. Growth Curve Analysis of *X. o. oryzae* Race 6 in Transgenic and Control Lines.

Open square, TP309; closed square, TP309 carrying pB812 (transgenic line 105-22); triangle, TP309 carrying pB285-9 (transgenic line 64); circle, TP309 carrying Xa21. Bars show standard error. CFU, colony-forming units.

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A  MISLPLLLFVLLFSALLLCPSSS                          23

B  DDDGDAAGDELALLSFKSSLLYQGGQSLASWN                55
   TSGHGQHCTWVGVVCGRRRRRHPHR                       80

C        XX LXLXX
         VVK LLLRSSN LSGIISPS                      98
   LGNLSFLRE LDLGDNY HSGEIPPE                      122
   LCRLSRLQL LELSDNS IQGSIPAA                      146
   IGACTKLTS LDLSHNQ LRGMIPREI                     171
   GASLKHLSN LYLHKNG LSGEIPSA                      195
   LGNLTSLQE FDLSFNR LSGAIPSS                      219
   LGQLSSLLN MNLGQNN LSGMIPNS                      243
   IWNLSSLRA FCVSENK LGGMIPTNA                     268
   FKTLHLLEV IYMGTNR FHGKIPAS                      292
   VANASHLTR LQIDGNL FSGIITSG                      316
   FGRLRNLTE LYLWRNL FQTREQEDWGFISD                346
   LTNCSKLQT LNLGENN LGGVLPNSF                     371
   SNLSTSLSF LALHLNK ITGSIPKD                      395
   IGNLIGLQH LYLCNNN FRGSLPSS                      419
   LGRLKNLGI LLAYENN LSGSIPLA                      443
   IGNLTELNI LLLGTNK FSGWIPYT                      467
   LSNLTNLLS LGLSTNN LSGPIPSE                      491
   LFNIQTLSIMINVSKNN LEGSIPQE                      516
   IGHLKNLVE FHAESNR LSGKIPNT                      540
   LGDCQLLRH LYLQNNL LSGSIPSA                      564
   LGQLKGLET LDLSSNN LSGQIPTS                      588
   LADITMLHS LNLSFNS FVGEVPT                       611
   M                                               612
```

Figure 5. Predicted Amino Acid Sequence of *Xa21D.*

The 14 amino acids that differ between the *Xa21D* and *Xa21* LRR regions are boldface and underlined; DNA sequence data indicate that residues 196 and 384 have experienced two nucleotide substitutions leading to two separate amino acid replacements.

(A) Deduced potential signal sequence.

(B) Unknown function.

(C) LRR. The coding regions for domains A and B comprise the GC-rich region. The *Xa21* LRR consensus is defined as LXXLXXLXXLXLXXNXLSGXIPXX (Song et al., 1995); the predicted solvent-exposed region of the β-strand/β-turn structure is indicated as XXLXLXX (Jones and Jones, 1997; Parniske et al., 1997).

The RT-PCR products (1.8 and 1.9 kb, respectively) from pB812 transgenic plants (105-22) amplified with the two primer pairs (LRR-F1 and LRR-R1, LRR-F1 and RF-1) were cloned into pGEM-T vector (Promega, Madison, WI) and sequenced. Sequence analysis showed that the two clones representing the RT-PCR products have a DNA sequence identical to the corresponding regions in the *Xa21D* genomic clone and *Xa21D* cDNAs isolated from the IRBB21 cDNA library. These results indicate that transgenic lines carrying two different *Xa21D* constructs expressed the same *Xa21D* transcript as did the wild-type line IRBB21.

Nucleotide Substitution in the LRR Domain of the *Xa21* Gene Family

Functional assays indicate that the *Xa21D* gene, which encodes a truncated RK-like protein consisting of signal peptide and GC-rich and LRR domains, confers partial resistance to six *X. o. oryzae* races. These studies identify the LRR as a region of interest for detailed characterization of patterns of nucleotide substitution. We estimated the ratio of nonsynonymous to synonymous nucleotide substitutions between the LRR region of all seven *Xa21* gene family members. Comparisons between all members of the *Xa21* gene family except *Xa21* and *Xa21D* produced a ratio much less than 1 (range 0.0301 to 0.456). However, the ratio of nonsynonymous to synonymous substitutions between the LRR domains of *Xa21* and *Xa21D* is significantly greater than 1 (ratio $= 5.14$; $Z = 2.45$; $P < 0.01$). The LRR regions of *Xa21* and *Xa21D* contain 16 nonsynonymous nucleotide substitutions and only one synonymous nucleotide substitution. (These totals do not include the premature stop codon within *Retrofit* sequences that interrupts the end of the LRR domain of *Xa21D*, nor do they take into account that residues 296 and 384 shown in Figure 5 have two amino acid replacements between *Xa21* and *Xa21D*.) A G test with these numbers also documents a significant bias toward nonsynonymous substitutions ($P = 0.042$). These results suggest that adaptive selection events have fueled divergence between *Xa21* and *Xa21D.*

If amino acid differences between the LRR domains of *Xa21* and *Xa21D* have been fueled by natural selection, then the location of amino acid replacements can provide insight into regions of functional importance. For example, Parniske et al. (1997) found elevated rates of nonsynonymous substitution in amino acid residues of LRR domains that are predicted to be solvent exposed, suggesting that these residues act in ligand binding. Twelve of the 16 nonsynonymous nucleotide substitutions between *Xa21* and *Xa21D* occurred in LRR residues that are predicted to be solvent exposed as part of the β-strand/β-turn structure (Figure 5; Jones and Jones, 1997). Under the null hypothesis that amino acid substitutions accrue randomly in the LRR domain, the clustering of 12 substitutions within these solvent-exposed residues is unexpected ($P < 0.001$). Hence, amino acid replacements between *Xa21* and *Xa21D* have occurred preferentially in the β-strand/β-turn structure. Ten of these 12 substitutions are in nonconsensus residues. In addition, individual LRR repeats contain different numbers of nonsynonymous nucleotide substitutions. Individual LRR repeats contain different numbers of nonsynonymous nucleotide substitutions. For example, the 10th LRR repeat contains four nonsynonymous nucleotide substitutions (two of which are within residue 301), repeats 8 and 13 contain two nonsynonymous nucleotide substitutions (residue 384 has changed twice), and the remaining 20 repeats contain either one or zero nonsynonymous substitutions (Figure 5). Under the null hypothesis that nonsynonymous nucleotide substitutions accrue randomly along the LRR, the clustering of four substitutions within a single LRR repeat is expected to occur infrequently ($P = 0.008$). This result suggests that the 10th LRR repeat is subject to particularly strong selection.

DISCUSSION

Role of the LRR Domain in Ligand Recognition and Signal Transduction

In the past several years, disease resistance genes that encode resistance to diverse pathogens have been isolated

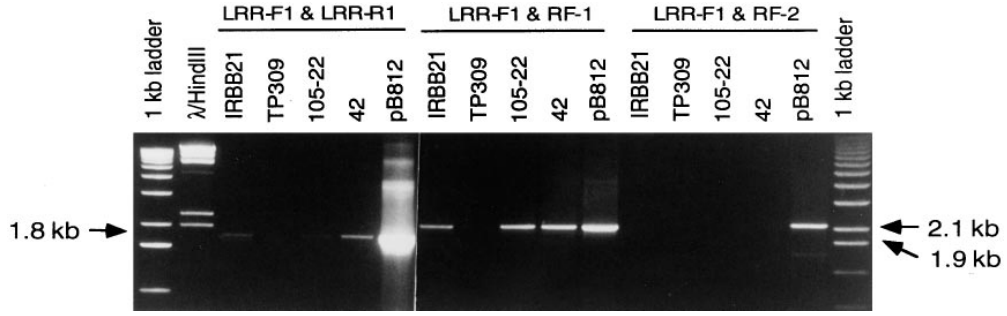

Figure 6. RT-PCR Products from Transgenic Plants Transformed with pB812 and pB285-9.

Primers LRR-F1, LRR-R1, RF-1, and RF-2 correspond to the sequences before the *Xa21D* ATG initiation codon 21 to 39 bp 5′ to the stop codon introduced by *Retrofit*, 147 to 167 bp 3′ to the stop codon introduced by *Retrofit*, and 244 to 264 bp 3′ to the stop codon introduced by *Retrofit*, respectively. Line 105-22 is a partially resistant line transformed with pB812. Line 42 is a partially resistant line transformed with pB285-9. The 1.8-, 1.9-, and 2.1-kb PCR products were separated on a 1.0% agarose gel, and the lengths are indicated. IRBB21 and TP309 represent the donor line of *Xa21D* and the recipient cultivar used in the transformation experiments, respectively.

from several species. Interestingly, resistance genes from tomato, Arabidopsis, tobacco, flax, and rice encode similar protein motifs, suggesting that these genes play a role in ligand recognition and signal transduction. For instance, the presumed extracellular domain of the tomato *Cf* and *Xa21* gene products consists of LRRs similar to the extracellular domain of human gonadotrophin receptors. In the case of gonadotrophin receptors, researchers have demonstrated that the extracellular domains can be exchanged between receptors without loss of receptor function. In these hybrid receptors (luteinizing hormone, chorionic gonadotrophin, and follicle-stimulating hormone receptors), the hormone specificity is determined by the hormone binding site present in the LRR (Braun et al., 1991). Based on the results of domain swaps between LRR-type receptors in animal systems, it is likely that plant LRR domains are also responsible for ligand recognition. In support of this hypothesis, domain swap experiments with resistance genes at the L locus of flax indicate that important determinants of specificity reside in the LRR domain (J. Ellis, personal communication). Similarly, differences in the LRR domains of the *Cf* gene products are thought to be responsible for ligand binding specificity (Dixon et al., 1996).

Based on the deduced amino acid sequence of *Xa21*, we have proposed that the XA21 LRR domain is extracellular and that its function is to bind a polypeptide produced by the pathogen (or plant cell). Ligand binding to the LRR would cause receptor dimerization, activation of the intracellular kinase domain, and subsequent signaling events leading to disease resistance (Ronald, 1997). In support of this hypothesis, we have shown that the XA21 catalytic domain is capable of autophosphorylation in *Escherichia coli* (W.-Y. Song, L.-Y. Pi, D.-L. Ruan, D. Braun, J.C. Walker, and P.C. Ronald, unpublished data). In this study, we investigated the function and evolution of the LRR domain in race-specific recognition.

Xa21D Encodes a Receptor-like Protein and Confers Race-Specific Resistance in Transgenic Plants

Previous work has demonstrated that the seven cloned gene family members (A1, A2, *Xa21*, C, D, E, and F) fall into two distinct classes designated the Xa21 class (family members *Xa21*, D, and F) and the A2 class (family members A1, A2, C, and E). The LRR domain of XA21 and A1 share a low level of identity (59.5%) and differ in the number of LRRs (23 versus 22, respectively) (Song et al., 1997). The amino acid differences in the LRRs of the *Xa21* gene family members suggest that members may have evolved to recognize different races and/or may confer altered resistance phenotypes. To test this idea, we assayed the race-specific resistance conferred by the seven cloned gene family members in transgenic plants. We found that *Xa21* family members A1, A2, C, E, and F do not confer resistance to the *X. o. oryzae* isolates tested. In contrast, two members (*Xa21* and *Xa21D*) do confer resistance. Interestingly, transgenic plants carrying *Xa21D* or *Xa21* exhibit identical resistance spectra, namely, resistance to six Philippine races of the pathogen and susceptibility to three Korean isolates representing two races.

The presumed secreted extracellular LRR encoded by *Xa21D* is unique among the cloned disease resistance genes. The predicted protein product of *Xa21D* encodes a 612–amino acid protein carrying the signal peptide and GC-rich and LRR domains (Song et al., 1997). It lacks the transmembrane domain characteristic of CF9 and the kinase domain characteristic of XA21. The predicted structure of the *Xa21D* gene product is highly similar to that of SLG (for *S* locus glycoprotein) and PGIP, which are secreted into the plant extracellular matrix (De Lorenzo et al., 1994; Nasrallah et al., 1994), suggesting that *Xa21D* may also be secreted and function extracellularly. The ability of *Xa21D*, encoding an LRR but lacking the transmembrane and kinase domains, to transduce a partial resistance response in a race-specific manner supports the hypothesis that the LRR plays the key role in *X. o. oryzae* recognition. Our results contrast with the *Pto*/*avrPto* system, in which evidence suggests that the PTO kinase interacts physically and highly specifically with the *avr* gene product AvrPTO, intracellularly (Scofield et al., 1996; Tang et al., 1996).

Adaptive Selection Events Have Played a Role in the Divergence of *Xa21* and *Xa21D*

To further investigate the function and evolution of the LRR domain, we compared DNA sequences among members of the *Xa21* gene family and estimated the ratio of the nonsynonymous to synonymous nucleotide substitutions. A similar analysis of alleles at the class I major histocompatibility complex (MHC) loci in human and mouse revealed that this ratio was >1 (Hughes and Nei, 1988). This observation was important both because it established a model for the evolutionary pressures acting on genes that function in pathogen recognition and because it documented the role of adaptive selection events in maintaining diversity at the antigen recognition site. The evolutionary advantage to diversity at the antigen recognition site of the MHC is clear: the greater the diversity, the better the ability to recognize, bind, and defend against a broad array of pathogens (Hughes and Nei, 1988).

The ratio of nonsynonymous to synonymous substitutions in the LRR region of *Xa21* and *Xa21D* is also >1, which implies that adaptive selection events have played a role in the divergence of these two genes. Given the demonstrated role of *Xa21* and *Xa21D* in pathogen defense, this observation suggests that the function of the LRR region could be analogous to that of the antigen recognition site of the MHC. It should also be noted that very few homologous genes exhibit a ratio of nonsynonymous to synonymous substitution >1. For example, a survey of GenBank showed that only 17 (0.5%) of 3595 groups of homologous sequences have a

ratio >1, and nine of these 17 genes express surface antigens of parasites or viruses (Endo et al., 1996). Thus, this phenomenon appears to be most prevalent in genes that encode proteins involved in host and pathogen recognition systems. Altogether, the pattern of sequence diversity between the LRR regions of *Xa21* and *Xa21D* is consistent with an important role for the LRR in pathogen recognition. These results corroborate functional studies, establishing that the *Xa21D* gene, which lacks transmembrane and kinase coding domains, is sufficient to confer race-specific resistance.

If the LRR domain of *Xa21* and *Xa21D* has a role in ligand binding, it is likely that some LRR repeats function primarily in a structural role and/or play a role in dimerization, whereas other repeats play an active role in ligand binding. It is expected that regions that bind ligand will be subject to stronger adaptive selection than regions that play a structural role. For example, the antigen recognition site of class I MHC proteins is subject to strong adaptive selection events, but structural regions of the protein are not (Hughes and Nei, 1988). Similarly, the predicted solvent-exposed residues of the β-strand/β-turn region of the LRR domain of tomato *Hcr9s* genes exhibit increased ratios of nonsynonymous to synonymous nucleotide substitution relative to other residues in the LRR domain, and this has been cited as evidence that solvent-exposed residues play a role in ligand binding (Parniske et al., 1997). Divergence between *Xa21* and *Xa21D* has also occurred predominantly at nonconsensus amino acid residues that are predicted to be solvent exposed. This result supports the idea that the consensus residues of the repeats play a structural role, whereas specificity of interactions with other proteins is due to the specific composition of nonconsensus residues in the solvent-exposed β-strand/β-turn structure (Kobe and Deisenhofer, 1994; Parniske et al., 1997).

We have also shown that the 10th LRR repeat has an excess of nonsynonymous substitutions relative to other LRR repeats. This observation suggests that the 10th repeat of the LRR of *Xa21* and *Xa21D* is subject to particularly strong selection, and it identifies the 10th repeat as an additional candidate for a region of the LRR domain that is active in ligand binding. We should note that our experimental results indicate that plants carrying *Xa21* and *Xa21D* have the same specificity for the tested *X. o. oryzae* races; therefore, there is no evidence for different ligand binding properties between the LRR of *Xa21* and *Xa21D.* However, the partial resistance (versus full resistance) conferred by *Xa21D* may be due to altered ligand binding encoded by the 10th LRR repeat. Similarly, it is possible that *Xa21* and *Xa21D* confer resistance to distinct *X. o. oryzae* races or pathogens not yet assayed.

Comparisons of the LRR domain between other members of the *Xa21* gene family did not produce ratio estimates >1. This result does not preclude a defense function for other members of the gene family, because examination of the ratio of nonsynonymous to synonymous substitutions tests for only one facet of adaptive selection. However, the results are consistent with functional assays. Only the *Xa21* and *Xa21D* genes have thus far been shown to function in disease resistance, and only the *Xa21* and *Xa21D* genes exhibit a nonsynonymous to synonymous ratio >1. The A2 gene class is highly diverged from the Xa21 class; based on sequence comparison, it appears that these two gene classes diverged before the split of rice and maize ~50 million years ago (Wolfe et al., 1989). It is possible the A2 class has also diverged functionally from the *Xa21* class and does not function in defense.

Insertion of the Retrotransposon-like Element *Retrofit* in *Xa21D* Alters the Predicted Protein Product

Based on sequence comparisons with other *Xa21* gene family members, it is likely that *Xa21D* arose by duplication of a progenitor *Xa21* gene with subsequent integration of the retrotransposon *Retrofit* (Song et al., 1997). In support of this idea, the *Xa21* and *Xa21D* coding regions are 99.1% identical up to the point of *Retrofit* insertion and 99.8% identical downstream of the *Retrofit* insertion. Insertion of *Retrofit* introduced a stop codon truncating the presumed RK ORF. It is plausible that *Xa21D* displayed full resistance to *X. o. oryzae* before *Retrofit* insertion, because the predicted amino acid sequences in the LRR, transmembrane, and kinase domains are nearly identical. Alternatively, the progenitor XA21D RK-like protein may have also conferred partial resistance due to reduced levels of the protein, minor alterations in the LRR domain that affect ligand binding affinity, or impaired downstream signaling compared with *Xa21.*

We therefore investigated the possibility that pB285-9 transgenic plants carrying *Xa21D* express an entire RK-like protein resulting from the splicing or suppression of *Retrofit* and thereby confer full resistance. The demonstration that transposable element insertions can either alter intron structure or simply function as novel introns has been reported previously. For instance, mutant alleles of *bronze* (*bz-m13*) and *anthocyaninless2* (*a2-m1*) carrying defective transposable element (*defective Suppressor-mutator* [*dSpm*]) insertions in the coding regions maintain structural gene expression in the absence of the transposable element *Spm* due to splicing of the *dSpm* sequence from the pre-mRNA as either part of a novel intron (*bz-m13*; Kim et al., 1987; Raboy et al., 1989) or simply as an intron (*a2-m1*; Menssen et al., 1990). As noted by Bunkers et al. (1993), the splicing event removes nearly all of the *dSpm* sequence, maintains the ORF, and produces an mRNA encoding an altered but functional protein. Similar phenomena have been observed with the maize *Dissociation* transposable elements (reviewed in Wessler, 1989) and with a Drosophila retrotransposon (Fridell et al., 1990).

In the case of *Xa21D*, we have no evidence that the retrotransposon-like element *Retrofit* is spliced out of the transcript, permitting expression of an entire RK-like protein.

Transgenic plants containing the shorter clone pB812, carrying coding sequences terminating in the *Xa21D* LRR, displayed the same resistance phenotype as did transgenic plants containing the larger clone pB285-9, carrying the additional coding sequences for *Retrofit*, and the transmembrane and kinase domains. In addition, no cDNA clones encoding the *Xa21D* kinase domain were identified, and no RT-PCR products amplified from the transgenic plants using primers in the *Xa21D* kinase region were found. Finally, characterization of RT-PCR clones from transgenic plants carrying the two different *Xa21D* constructs revealed that at least 167 nucleotides from *Retrofit* are present in the *Xa21D* transcripts, supporting the idea that *Retrofit* is not spliced out of *Xa21D.*

Our data indicate that *Retrofit* insertion has created a novel protein, representing a new class of plant disease resistance genes, through truncation of a duplicated progenitor *Xa21* gene. The predicted product of *Xa21D* does not carry the transmembrane-spanning or kinase domains that are found in XA21. In plants, there is evidence that transposon-induced truncations can affect protein expression and localization. For instance, the maize *R-sc* gene is a member of the *R* gene family of transcriptional activators that regulate anthocyanin biosynthesis. The *r-m9* mutant allele, a derivative of *R-sc*, had a reduced but significant amount of aleurone pigmentation due to the presence of a 2.1-kb *Dissociation* insertion near the 3′ end of the coding region (Alleman and Kermicle, 1993). The reduced activity of *r-m9* results in part from inefficient nuclear localization of the truncated R protein (Liu et al., 1996). Similarly, it is likely that the *Xa21D* gene product is extracellular and does not span the plasma membrane, as is predicted for XA21. The reduced activity of *Xa21D* compared with *Xa21* may therefore be due to aberrant localization of the protein, altered ligand binding affinity, and/or deletion of the entire serine/threonine kinase domain.

Model for the Mode of Action of *Xa21D*-Encoded Protein

How can a presumed secreted receptor-like protein lacking a transmembrane domain and kinase domain transduce a cellular defense response? In mammals, receptors can transduce signals to downstream protein products by formation of heterodimers between related family members. For example, the type II receptor of transforming growth factor β (TGFβ) binds its ligand TGFβ and subsequently interacts with type I receptors that lack ligand recognition capability. The receptors form an oligomeric complex competent for signal transduction (Massague, 1996). Similarly, the bacterial chemotaxis receptor Tar forms heterodimers containing one full-length transmembrane Tar receptor and one truncated Tar molecule lacking the cytoplasmic domain (Gardina and Manson, 1996; Tatsuno et al., 1996). Ligand binding to the Tar extracellular domain results in heterodimer formation. Dimerization permits the transmission of conformational change from the extracellular domain to the cytoplasmic domain and subsequent signal transduction (Ullrich and Schlessinger, 1990).

The Tar and TGFβ receptors provide working models for receptor-mediated signaling pathways in plants. In Brassica, SRK (for *S* locus receptor kinase) and SLG appear to be necessary for the self-incompatibility phenotype. SRK spans the plasma membrane, whereas SLG accumulates extracellularly in the papillar cell wall (Nasrallah et al., 1994; Stein et al., 1996). It has been hypothesized that heterodimerization of the S receptor domains of SRK and SLG initiates the signaling cascade via the kinase domain of SRK after binding of a pollen-derived ligand (Hiscock et al., 1996; Stein et al., 1996).

Similar models have been proposed for *Cf-2*– and *Cf-9*–mediated disease resistance in tomato and CLV1-mediated meristem development in Arabidopsis (Dixon et al., 1996; Clark et al., 1997). The *Cf* genes encode transmembrane receptor-like proteins that lack a cytoplasmic domain. The extracellular domains of CF-2 and CF-9 consist almost entirely of LRRs. Comparisons among these and other CF family members' LRR domains revealed that there is a highly conserved subdomain and a variable subdomain. The highly conserved LRR subdomains are thought to be involved in homodimerization or heterodimerization, whereas the variable subdomains are responsible for specific binding to pathogen ligands (Dixon et al., 1996). Upon ligand binding, the *Cf* gene product may interact with a transmembrane protein kinase that also carries an intracellular domain. In this model, heterodimerization activates the intracellular kinase enzyme, which triggers downstream events resulting in resistance (Dixon et al., 1996). Finally, the *clv1-6* mutation in Arabidopsis, which results in a truncated protein with most of the kinase domain of CLV1 deleted, has a weak phenotype, indicating that the kinase domain may be partially redundant with another protein that interacts to control shoot and floral meristem size (Clark et al., 1997).

Similar to models proposed for TGFβ in mammals, Tar in bacteria, and CF, CLV1, and SLG in plants, we hypothesize that the LRR domain encoded by *Xa21D* is necessary for ligand binding. Upon ligand binding, XA21D forms a heterodimer with the LRR domain of an endogenous RK present in the recipient (normally susceptible) cultivar TP309 (Figure 7). After heterodimerization, the intracellular kinase domain is activated, leading to a phosphorylation cascade that ultimately restricts pathogen growth. In support of this model, DNA gel blot and RT-PCR analyses showed that the susceptible line TP309 used as a recipient in the *Xa21D* transformation experiments expresses a full-length *Xa21* family RK-like gene (L.-Y. Pi, W.-Y. Song, and P.C. Ronald, unpublished results).

An alternative model to the heterodimer hypothesis is that *Xa21D* ligand binding takes place intracellularly. For example, an *X. o. oryzae*–produced molecule may be secreted into the plant cell via type III secretion apparatus, as has been shown for the *X. campestris* pv *vesicatoria avr* gene

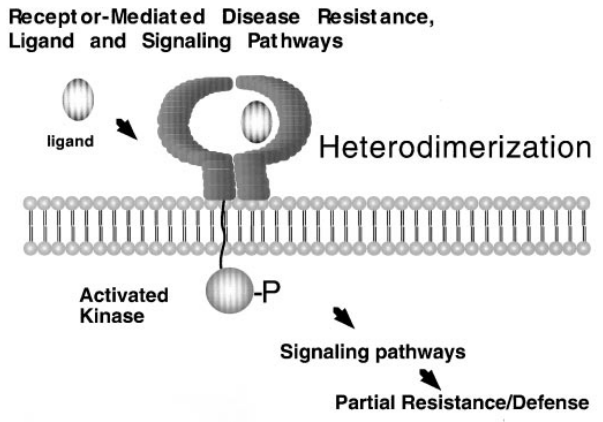

Figure 7. Model for Copy *Xa21D*-Mediated Partial Disease Resistance (Adapted from Staskawicz et al., 1995).

The secreted truncated LRR (shown at right) is extracellular and forms a heterodimer with the endogenous TP309 RK (which lacks recognitional specificity; shown at left). Upon ligand binding to XA21D, heterodimerization and activation of the intracellular kinase occur, activating expression of the partial resistance phenotype.

product *avrBs3* (Van den Ackerveken et al., 1996), and interact with an intracellularly localized *Xa21D* and other intracellularly localized proteins capable of transmitting the defense response. This model seems less plausible because of the presence of the XA21D signal peptide domain and the overall structural similarity to SLG and PGIP, which are known to be secreted proteins. Cellular localization of the *Xa21D*-produced protein in the cells of partially resistant transgenic plants during bacterial infection is needed to distinguish these possibilities.

METHODS

Construction of *Xa21D* Clone pB285-9

To construct the 16.5-kb clone pB285-9, cosmid clone pB285 (Song et al., 1995) containing member *Xa21D* was digested with KpnI and self-ligated. The ligation mix was transformed into *Escherichia coli* DH5 α-competent cells, and the transformants were selected on Luria-Bertani plates containing ampicillin. DNA from these colonies was hybridized with the leucine-rich repeat (LRR) probe (RG103; Song et al., 1995) and the kinase probe (Wang et al., 1996). The resulting subclone pB285-9 contained the signal peptide, GC-rich, LRR, *Retrofit*, transmembrane, and kinase domains (Figure 1).

Plant Transformation

Generation of transgenic rice plants carrying constructs pB812, pB833, pB843, pB853, pB37, and pB806 was reported in a previous study (Song et al., 1995). In this study, transformation of pB285-9 was performed according to Zhang et al. (1996). Twenty-nine independently transformed lines were grown in a growth chamber under the following conditions: 24°C and 90% humidity for 14 hr without light; and 28°C and 85% humidity for 10 hr with light (metal halide and incandescent bulbs; Sylvania, Manchester, NH). At least six plants for each line were kept for inoculation. T_0 plants were self-pollinated, and T_1 progeny were grown in a greenhouse for 5 weeks and moved to the growth chamber 1 week before inoculation.

Inoculation and Resistance Scoring

To assess resistance phenotypes, six Philippine isolates representing *Xanthomonas oryzae* pv *oryzae* races 1 to 6 (PXO61, PXO86, PXO79, PXO113, PXO112, and PXO99A, respectively) and three isolates from South Korea representing two new races that are virulent on *Xa21*-containing lines (DY89031 race K1, CK89021 race K2, and JW89011 race K2; Wang et al., 1996; S.H. Choi, personal communication) were used in the inoculation tests (isolates kindly provided by J. Leach [Kansas State University, Manhattan, KS] and S.H. Choi [National Agricultural Experiment Station, Suwon, South Korea]). Groups of isolates that share a common pattern of virulence to a set of host cultivars are called races. The isolates were grown for 72 hr at 30°C on peptone sucrose agar (Tsuchiya et al., 1982). Six-week-old plants were cut $\sim$4 cm from the tip of fully expanded leaves with scissors dipped in a bacterial suspension at 10^9 cells per mL (Kauffman et al., 1973). After inoculation, plants were maintained in a growth chamber with the same conditions as given above. For each experiment, lesion length was measured on at least 18 infected leaves from six independent plants 12 days after inoculation.

To verify the presence of transgenes, genomic DNA was extracted from young leaves, as described by Dellaporta et al. (1984), and digested with appropriate informative enzymes (XbaI for A1 and A2; KpnI for C; HindIII for Xa21D and E; and SalI for F). The digested DNAs were separated by gel electrophoresis and blotted to a Hybond N^+ membrane (Amersham), according to the manufacturer's instructions. The restriction fragment length polymorphism marker RG103 (McCouch et al., 1988; Ronald et al., 1992; Song et al., 1995) that hybridizes with the LRR region of *Xa21* and a 230-bp kinase fragment amplified from the *Xa21* gene (Wang et al., 1996) were used in the hybridization studies. The expected size of RG103-hybridizing bands based on restriction mapping of each construct (Figure 1) was observed in these plants (data not shown).

Growth Curve Analysis

Growth of *X. o. oryzae* race 6 PXO99Az in transgenic and control lines was performed as described by Song et al. (1995) (Figure 4). T_4 progeny from line 105-22, T_2 progeny from line 64, and T_4 progeny from line 106-17 were used for pB812, pB285-9, and *Xa21* growth curve analysis, respectively (Table 1 and Figure 4). Because lines 64 and 105-22 were segregating for the transgene before growth curve analysis, we performed polymerase chain reaction (PCR) with *Xa21D*-specific primers (LRR-F1 and LRR-R1) to assay the presence of the transgene in individual plants. Primer LRR-F1 (5′-GTCTTG-CCTTGCACTTCTGCACGA-3′) corresponds to the sequence precisely before the *Xa21D* ATG initiation codon. Primer LRR-R1 (5′-GCTGTTGAAAGAAAGGTT-3′) corresponds to the sequence 21 bp before the insertion site of *Retrofit.* DNA was extracted from

leaves by using a previously described rapid DNA isolation method (Williams and Ronald, 1994), and the 1.8-kb target sequences were amplified by PCR. PCR amplification conditions were the same as those described by Williams and Ronald (1994). Three independent experiments gave similar results.

Analysis of Transgenic Plants

For analysis of transgenic plants generated from pB812 (*Xa21D*), we designed primer pairs (LRR-F1, see above, and RF-1) based on the sequence in the *Xa21D* and *Retrofit* regions. Primer RF-1 (5′-CCTCTACCGTGGCTTACAGT-3′) corresponds to the sequence 147 to 167 bp downstream of the stop codon introduced by *Retrofit* (Figures 1 and 3A). DNA extraction and PCR amplification conditions were the same as those described above.

RNA Isolation and Reverse Transcriptase–PCR Amplifications

An RNeasy minikit (Qiagen, Chatsworth, CA) was used to isolate total RNA from 150 to 200 mg of rice leaf tissue. Poly(A)$^+$ RNA, fractionated from total RNA using a Qiagen Oligotex spin column, was used as a template in a reverse transcriptase (RT)–mediated PCR. RT-PCR (StrataScript RT-PCR kit) was conducted following protocols provided by the manufacturer (Stratagene, La Jolla, CA).

Three primer pairs were used to amplify DNA fragments from the synthesized cDNAs (LRR-F1 with LRR-R1, RF-1, and RF-2; Figures 1 and 6). The sequences of primer LRR-F1, LRR-R1, and RF-1 are shown above. Primer RF-2 (5′-GTGGAAAAAGGCTCTGATAC-3′) corresponds to the sequence 244 to 264 bp after the stop codon introduced by *Retrofit* (Figure 1). Two primers (KIN1F, 5′-AGCAGACCAGAGGGACTTGAAT-3′; and KIN2, 5′-TCAGATCGACTTCTGCAGTGGTAT-3′) corresponding to the kinase domains of *Xa21D* and *Xa21* were used to amplify the cDNAs isolated from transgenic plants transformed with the pB285-9 construct.

DNA Sequencing and Sequence Analysis

RT-PCR fragments were cloned into pGEM-T vector, according to the manufacturer's (Promega) protocols. The clones were sequenced using the ABI PRISM 377 DNA sequencer (Perkin-Elmer, Foster City, CA). The sequence was analyzed with Lasergene (DNAstar, Madison, WI) and Sequencer (Gene Codes Corp., Ann Arbor, MI) 3.0 software.

Amino acid sequences of all *Xa21* gene family members were aligned with the Clustal program (Thompson et al., 1994). The amino acid alignments were adjusted manually and used to guide nucleotide sequence alignments. For all analyses, functional domains and LRR repeats were defined as given in Figure 3 of Song et al. (1995).

The number of nucleotide substitutions per synonymous site (d_s) and the number of nucleotide substitutions per nonsynonymous nucleotide site (d_n) were estimated by the method of Nei and Gojobori (1986). Differences between d_n and d_s within the LRR domain were examined with a Z-test, with the variance of $d_n - d_s$ estimated by 10,000 bootstrap resamplings over codons. We also applied a 2×2 contingency table G test to test for differences in nonsynonymous and synonymous substitution rates (e.g., Zhang et al., 1997). The probability of clusters of nonsynonymous substitutions within the solvent-exposed residues of a single LRR repeat was calculated with the binomial distribution, as given by Leicht et al. (1995).

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