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Xanthomonas oryzae pv. *oryzae* Avirulence Genes Contribute Differently and Specifically to Pathogen Aggressiveness

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Genomic copies of three *Xanthomonas oryzae* pv. *oryzae* avirulence (*avr*) genes, *avrXa7, avrXa10,* and *avrxa5,* and four homologous genes, *aB3.5, aB3.6, aB4.3,* and *aB4.5,* were mutagenized individually or in combination to study the roles of *avr* genes in one component of pathogen fitness, i.e., aggressiveness or the amount of disease *X. oryzae* pv. *oryzae* causes in susceptible rice lines. These *X. oryzae* pv. *oryzae* genes are members of the highly related *Xanthomonas avrBs3* gene family. Compared to the wild-type strain, *X. oryzae* pv. *oryzae* strains with mutations in *avrXa7, avrxa5,* and the four homologous genes caused shorter lesions on rice line IR24, which contains no resistance genes relevant to the wild-type strain. The contribution of each gene to lesion length varied, with *avrXa7* contributing the most and *avrXa10* showing no measurable effect on aggressiveness. The functional, plasmidborne copies of *avrXa7, aB4.5,* and *avrxa5* restored aggressiveness only to strains with mutations in *avrXa7, aB4.5,* and *avrxa5,* respectively. Mutations in *avrXa7* were not complemented by plasmids carrying any other *avr* gene family members. These data indicate that some, but not all, *avr* family members contribute to pathogen aggressiveness and that the contributions are quantitatively different. Furthermore, despite their sequence similarity, the aggressiveness functions of these gene family members are not interchangeable. The results suggest that selection and pyramiding resistance genes can be guided by the degree of fitness penalty that is empirically determined in *avr* gene mutations.

Pathogen avirulence (*avr*) genes were first identified on the basis of their ability to direct specific recognition leading to disease resistance in gene-for-gene plant-microbe interactions (Gabriel 1999; Leach and White 1996). Later, *avr* genes were shown to have a second function in that some also control components of pathogen fitness, including aggressiveness (the amount of disease) and symptom expression in the host-pathogen interaction (Ashfield et al. 1995; Kearney and Staskawicz 1990; Laugé et al. 1998; Lorang et al. 1994; Ritter and Dangl 1995; Swarup et al. 1991; Swords et al. 1996; Yang et al. 1994; Yang et al. 1996; Zhu et al. 1999). For example, the *Xanthomonas campestris* pv. *vesicatoria avrBs2* gene, which confers resistance in pepper containing the *Bs2* gene, also contributes to aggressiveness in pepper (Kearney and Staskawicz 1990). Genes homologous to *avrBs2* found in other xanthomonads also affect aggressiveness; mutations in the *avrBs2* homologs reduced each pathogen's ability to multiply in its respective host plant (Kearney and Staskawicz 1990).

Members of another group of avirulence genes, the *avrBs3* gene family, which is common to different species and pathovars of *Xanthomonas*, also are involved in disease expression (Swarup et al. 1991; Swarup et al. 1992; Yang et al. 1996). One *avrBs3* family member, the *X. citri pthA* gene, functions as an avirulence gene on bean and cotton when present in *X. campestris* pv. *phaseoli* and *X. campestris* pv. *malvacearum*, respectively, and controls the ability of the citrus pathogen *X. citri* to grow intercellularly and induce hyperplastic cankers on citrus (Swarup et al. 1991; Swarup et al. 1992). The *avrb6* gene of *X. campestris* pv. *malvacearum* not only functions as an avirulence gene on cotton containing the corresponding resistance gene *b6*, but also affects the extent of water soaking and the subsequent release of the pathogen to the surface of susceptible cotton leaves (Yang et al. 1994; Yang et al. 1996).

Genes in the highly conserved *avrBs3* family encode proteins that contain a central domain composed of a near-perfect repeated sequence of 34 amino acids. The number of repeats varies from 13.5 copies in Avrb6 to 25.5 copies in AvrXa7 (Leach and White 1996). The sequences of the 34 amino acid repeats are nearly identical, with a major variation occurring at amino acids 12 and 13 with respect to each repeat. The order and type of repeats in the central domain are involved in governing resistance specificity of a particular gene family member (Bonas et al. 1989; Herbers et al. 1992; Yang and Gabriel 1995a; Yang et al. 1994). The carboxy termini of the AvrBs3 family proteins, which are also highly conserved, contain features essential to avirulence function. All family members contain three nuclear localization signal (NLS) sequences (Van den Ackerveken et al. 1996; Yang and Gabriel

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1995b; Zhu et al. 1998). Inactivation of the NLS results in loss of avirulence function (Van den Ackerveken et al. 1996; Zhu et al. 1998) and, in the case of PthA from *X. citri*, reduces canker-inducing ability on citrus (Yang and Gabriel 1995b). Inactivation of the AvrBs3 NLS sequences did not impact *X. campestris* pv. *vesicatoria* virulence (Van den Ackerveken et al. 1996). An acidic transcriptional activation domain, previously found exclusively in eukaryotes, was identified in the carboxy termini of AvrBs3 family members and was shown to play a critical role in *avrXa10*-encoded avirulence function (Zhu et al. 1998; Zhu et al. 1999). Apart from the requirement for the NLS regions of PthA for symptom induction (Yang and Gabriel 1995b), the regions of the AvrBs3 family members required for aggressiveness or fitness are not known.

Some xanthomonads, such as *X. campestris* pv. *malvacearum* and *X. oryzae* pv. *oryzae*, contain multiple copies of the *avrBs3* gene family members. With the use of a mutagenesis strategy that sequentially inactivated several of the *avr* genes in *X. campestris* pv. *malvacearum*, Yang et al. (1996) showed that the *avr* genes contribute additively to the ability of the pathogen to cause water soaking on cotton. A mutant in which seven of the 10 *avr* genes had been inactivated did not cause symptoms on cotton. Interestingly, the rate of growth of the mutant bacteria in planta was identical to the wild-type strain, but 1,000-fold fewer mutant bacteria than wild-type bacteria were released to the leaf surface. Thus, the ability of *X. campestris* pv. *malvacearum* to cause disease symptom expression in cotton requires multiple members of the *avrBs3* gene family, but they are not required for in planta growth.

If the *avr* genes contribute to pathogenic aggressiveness and disease expression, determining the cost of losing these genes to pathogen fitness, which encompasses aggressiveness, disease symptom expression, and pathogen persistence, may be useful in predicting the durability of the corresponding plant-resistance gene in the field. Strains of *X. oryzae* pv. *oryzae*, the rice bacterial blight pathogen, contain more than 14 copies of *avrBs3* gene family members, including *avrxa5*, *avrXa7*, and *avrXa10*, which have been shown to have avirulence function (Hopkins et al. 1992). Here we demonstrate that some *X. oryzae* pv. *oryzae avr* gene family members play dual roles in interactions with rice in that some of these genes contribute, albeit differentially, to the aggressiveness of *X. oryzae* pv. *oryzae* to rice. We measure aggressiveness as the rate of lesion development, lesion lengths, and the final bacterial numbers. Intriguingly, the contribution of these highly related genes to aggressiveness is specific in that the loss of aggressiveness function for one gene family member cannot be complemented by another.

RESULTS

Identification of *X. oryzae* pv. *oryzae* mutants disrupted at *avrXa7*, *avrXa10*, *avrxa5* as well as other gene family members.

Marker-exchange derivative strains KL7M (*avrXa7*$^-$/*avrXa10*$^+$), KL10M (*avrXa7*$^+$/*avrXa10*$^-$), KLD1 (*avrXa7*$^-$/*avrXa10*$^-$), KLD2 (*avrXa7*$^-$/*aB3.6*$^-$), and triple mutants KLT1 to KLT4 were identified by loss of avirulence activity with the appropriate rice lines (Fig. 1) and by DNA blot analysis (Fig. 2). *X. oryzae* pv. *oryzae* strains harboring functional *avrXa7* and *avrXa10* genes elicit a hypersensitive response (HR) after infiltration of rice leaves with the corresponding *Xa7* and *Xa10* resistance genes, respectively (Fig. 1). On line IR24, which does not possess these resistance genes, strains with functional *avrXa7* and *avrXa10* genes induce water-soaked lesions. Mutants disrupted in *avrXa7* (KL7M and KLD1) lost the ability to elicit HR on IRBB7 and those disrupted in *avrXa10* (KL10M and KLD1) lost the ability to elicit HR on IRBB10 (Fig. 1). Complementation analyses with plasmids pK107 (*avrXa7*$^+$) and pK110 (*avrXa10*$^+$) restored the avirulence function on appropriate rice lines (Fig. 1).

DNA blot analyses were used to confirm and characterize the mutations. In addition, expression of appropriate-sized proteins was confirmed in complementation experiments by Western blot analyses (data not shown). In DNA blot analysis, PXO86 contains at least 14 different-sized *Bam*HI fragments that hybridize to *avrXa10* (Fig. 2). The 4.2-kb *Bam*HI fragment contains the majority of the coding region of *avrXa7* (Hopkins et al. 1992). At least three *avr* gene family members in PXO86 contain a 3.1-kb *Bam*HI fragment, whereas two of the 3.1-kb *Bam*HI fragments contain the majority of the coding regions for *avrXa10* and *avrxa5* (J. F. Bai, *unpublished*; Hopkins et al. 1992). KL7M, which contains a Tn*5* insertion in *avrXa7*, is missing the 4.2-kb *Bam*HI and 3.1-kb *Pst*I fragments that hybridize to *avrXa10* (Fig. 2). The exchange of the intact *avrXa10* with a *cat*-disrupted copy was confirmed by the reduction in intensity of the 3.1-kb *Bam*HI fragment and the absence of the 4.9-kb *Pst*I fragment in mutant KL10M (Fig. 2). Exchange mutant KLD1, which is disrupted at *avrXa7* and *avrXa10*, lacked the hybridizing 4.2-kb *Bam*HI fragment, showed reduced intensity of the 3.1-kb *Bam*HI fragment, and lacked the *Pst*I fragments at 3.1 and 4.9 kb (Fig. 2). KLD2 contained insertions in *avrXa7* and *aB3.6*, a 3.6-kb *Bam*HI fragment.

Triple mutants KLT1–KLT4 were selected to represent mutations in different *avr* gene family members on the basis of their differences in restriction fragment patterns after digestion with *Bam*HI and hybridization with *avrXa10*. In addition to mutations in *avrXa7* and *avrXa10*, KLT1–KLT4 contained insertions in *Bam*HI fragments of 3.1, 4.5 (Fig. 2), 4.3, and 3.5 kb, respectively. The mutation in KLT1 disrupts the *avrxa5* locus as demonstrated by (i) a change in the qualitative phenotype from a light HR to a water-soaked lesion in interactions with rice line IRBB5, which contains the *xa5* gene for bacterial blight resistance (data not shown); (ii) an increase in the lesion lengths on IRBB5 caused by strain KLT1 (6.25 ± 1.4 cm) relative to strain KL7M (3.06 ± 0.8 cm); and (iii) complementation of the avirulence function in the *avrxa5* mutation with the plasmid carrying the *avrxa5* gene pXO6-33 (3.1 ± 0.8 cm lesions on IRBB5).

Mutants deficient in *avrXa7* but not *avrXa10* are less aggressive to susceptible rice lines.

On rice line IR24, which does not possess relevant resistance genes, *X. oryzae* pv. *oryzae* strains with functional *avrXa7* and *avrXa10* genes induce water-soaked lesions (Fig. 1). On IR24, the water soaking of the lesions produced by the *avrXa7* mutants was much reduced compared with the wild-type strain PXO86 (Fig. 1). In comparison, mutations in the *avrXa10* locus did not cause any difference in the amount of water soaking relative to PXO86. The extensive water-soaked phenotype was restored only after complementation with *avrXa7* contained in plasmid pK107. No restoration of aggressiveness occurred after complementation with the *avrXa10* gene in pK110. These results provided a qualitative indication that *avrXa7* contributed more to pathogen aggressiveness than *avrXa10*.

Measurement of lesion lengths and final bacterial numbers after clip inoculation provided a quantitative assay (Barton-Willis et al. 1989) to compare the aggressiveness of the various *X. oryzae* pv. *oryzae* strains on the three isogenic lines, IRBB10, IRBB7, and IR24 (Table 1). In rice line IRBB10, the strain with a mutation in *avrXa10* (KL10M) produced lesions longer than 16 cm, a susceptible reaction. Strains that possessed a functional *avrXa10* produced lesions shorter than 1 cm, a resistant reaction.

On rice line IRBB7, strains deficient in *avrXa7* caused lesions that were significantly longer than those inoculated with strains containing a functional *avrXa7* gene (Table 1). Overall, the lesions on IRBB7 were much shorter than those observed on IRBB10 or IR24 because of the function of *avrXa7* as an avirulence gene (resistant reactions) or as a result of the loss of *avrXa7* as a contributor to aggressiveness.

The quantitative contribution of the *X. oryzae* pv. *oryzae* avirulence genes to aggressiveness was most obvious on line IR24, which contains no corresponding resistance genes (Table 1). On IR24, strains with a functional *avrXa7* gene, either in the genome or on a plasmid, caused long lesions that ranged from 13.2 to 17.8 cm. The lesions caused by PXO86,

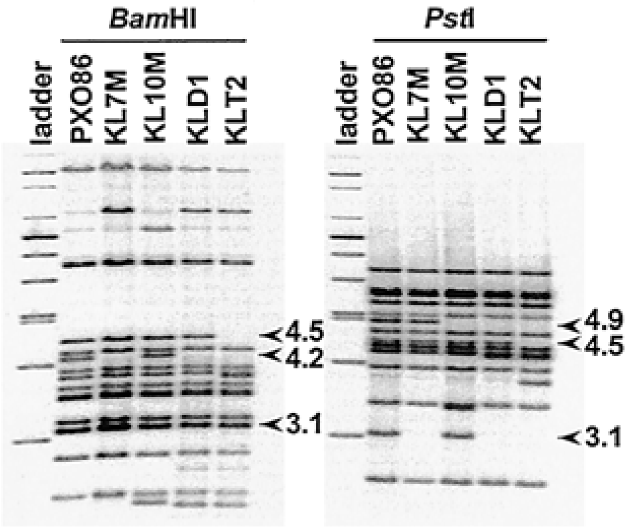
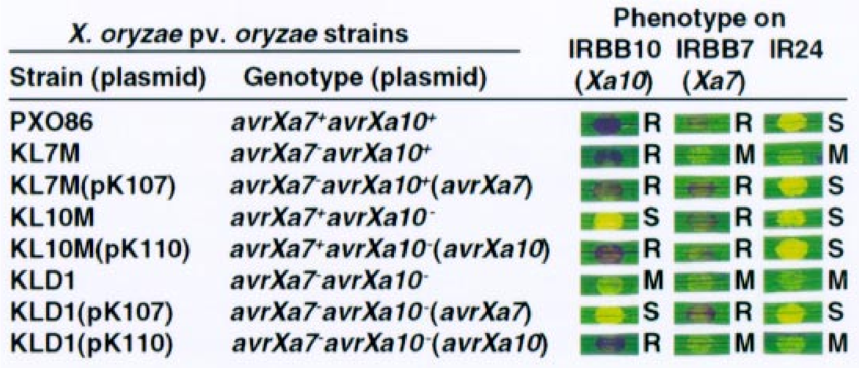


Fig. 2. DNA blot analyses of wild-type *Xanthomonas oryzae* pv. *oryzae* strain PXO86 and marker-exchange mutant strains. Blots of genomic DNA digested with *Bam*HI or *Pst*I were hybridized with pK110, which contains the *avrXa10* gene (Hopkins et al. 1992). KL7M (*avrXa7*$^-$) lacks the 4.2-kb *Bam*HI and 3.1-kb *Pst*I fragments. KL10M (*avrXa10*$^-$) shows reduced intensity of the 3.1-kb *Bam*HI fragment and lacks a 4.9-kb *Pst*I fragment. KLD1 and KLT2 lack fragments associated with *avrXa7* and *avrXa10*. In addition, KLT2 lacks 4.5-kb *Bam*HI and 4.5-kb *Pst*I fragments. The molecular marker is the 1-kb extension ladder (Gibco BRL Life Technologies, Grand Island, NY, U.S.A.).



X. oryzae pv. *oryzae* strains		Phenotype on		
Strain (plasmid)	Genotype (plasmid)	IRBB10 (*Xa10*)	IRBB7 (*Xa7*)	IR24
PXO86	*avrXa7*$^+$*avrXa10*$^+$	R	R	S
KL7M	*avrXa7*$^-$*avrXa10*$^+$	R	M	M
KL7M(pK107)	*avrXa7*$^-$*avrXa10*$^+$(*avrXa7*)	R	R	S
KL10M	*avrXa7*$^+$*avrXa10*$^-$	S	R	S
KL10M(pK110)	*avrXa7*$^+$*avrXa10*$^-$(*avrXa10*)	R	R	S
KLD1	*avrXa7*$^-$*avrXa10*$^-$	M	M	M
KLD1(pK107)	*avrXa7*$^-$*avrXa10*$^-$(*avrXa7*)	S	R	S
KLD1(pK110)	*avrXa7*$^-$*avrXa10*$^-$(*avrXa10*)	R	M	M

Fig. 1. Genotypes and host-plant interaction phenotypes of *Xanthomonas oryzae* pv. *oryzae* PXO86 (wild type), the *avrXa7* and *avrXa10* single (KL7M, *avrXa7*$^-$ and KL10M, *avrXa10*$^-$) and double (KLD1, *avrXa7*$^-$, *avrXa10*$^-$) mutants derived from PXO86, and the complemented mutant strains. Photograph was taken 4 days after infiltration of bacteria (5×10^7 CFU/ml) into 15-day-old leaves of the near-isogenic rice lines IRBB10 (*Xa10*), IRBB7 (*Xa7*), and IR24 (no relevant resistance genes). R = resistant (brown color); S = susceptible (transparent yellow is full water soaking); M = moderately susceptible (light green is moderate water soaking).

Table 1. Mutations in *avrXa7*, but not *avrXa10*, affect aggressiveness of *Xanthomonas orzyae* pv. *oryzae*; lesion lengths and final bacterial numbers after inoculation of three rice lines with *X. oryzae* pv. *oryzae* strains containing mutations in *avrXa7* and *avrXa10*

		Lesion length (cm)[a]			
***X. oryzae* pv. *oryzae* strain**	**Genotype**	**IRBB7 (*Xa7*)**	**IRBB10 (*Xa10*)**	**IR24**	**Bacterial log CFU/leaf on IR24[b]**
PXO86	*avrXa7*$^+$, *avrXa10*$^+$	0.6 b	0.5 b	17.8 a	8.6 ± 0.08 a
KL7M	*avrXa7*$^-$, *avrXa10*$^+$	3.2 a	0.6 b	3.4 c	7.9 ± 0.10 c
KL7M (pK107)	*avrXa7*$^-$, *avrXa10*$^+$ (*avrXa7*$^+$)	0.9 b	0.5 b	13.2 b	8.2 ± 0.15 b
KL10M	*avrXa7*$^+$, *avrXa10*$^-$	0.3 b	19.7 a	16.9 a	8.4 ± 0.10 a
KL10M (pK110)	*avrXa7*$^+$, *avrXa10*$^-$ (*avrXa10*$^+$)	0.3 b	0.5 b	16.7 a	8.4 ± 0.10 a

[a] *R* genes are in parentheses. Plants were inoculated by the scissors-clip method at 2 weeks after planting. Lesion length measured at 12 days after inoculation; different letters in a column indicate significant difference at P = 0.05, least significant difference.

[b] Bacterial numbers ± standard deviation at 10 days after inoculation. Different letters indicate significant difference at $P = 0.05$, least significant difference.

KL10M, and KL10M(pK110) were not significantly different in length. As a result of the loss of the plasmid from complemented strains in the plants where no antibiotic selection pressure could be maintained (data not shown), lesions induced by the complemented *avrXa7* mutant, KL7M(pK107), were significantly shorter than those caused by strains with a chromosomal copy of *avrXa7* (Table 1). However, lesions induced by these strains (>13 cm) were still significantly longer than those caused by the *avrXa7* mutant strains (<4 cm).

Lesion-length data indicated that *avrXa7* contributed more quantitatively to symptom expression than did *avrXa10.* Yang et al. (1996) demonstrated that although expression of symptoms on cotton caused by *X. campestris* pv. *malvacearum* required multiple members of the *avrBs3* gene family, in planta growth of the pathogen did not. To determine if the reduction in symptom expression in these rice–*X. oryzae* pv. *oryzae* interactions was correlated with a reduction or in planta bacterial multiplication, bacterial numbers from leaves of susceptible line IR24 were evaluated at 10 days after inoculation (Table 1). The final bacterial populations for strains that possessed a functional *avrXa7* were significantly greater than those involving strains deficient in *avrXa7* (1.7 to 3.9 × 10^8 CFU/leaf versus 7.6 × 10^7 CFU/leaf). Thus, expression of symptoms in rice and in planta growth of *X. oryzae* pv. *oryzae* requires *avrXa7* but not *avrXa10.*

avrxa5 and other *avr* family members contribute to pathogen aggressiveness.

To assess the role of other *avr* family members in aggressiveness in *X. oryzae* pv. *oryzae,* we compared the lesion lengths and rates of disease development on line IR24 by triple mutants KLT1 to KLT4, double mutants KLD1 and KLD2, and the single *avrXa7* mutant KL7M. Lesions caused by each triple mutant were shorter than those caused by mutants KL7M and KLD1, suggesting that the family members contributed to aggressiveness (Fig. 3). The lesion length differences occurred early and were detected over the entire course of lesion development. Lesions generated by the triple mutant KLT1, which carries the disrupted *avrxa5* gene, were significantly shorter than those caused by KL7M and KLD1 on IR24, demonstrating the contribution of *avrxa5* to *X. oryzae* pv. *oryzae* aggressiveness (Fig. 3). Mutant KLT2, which contains an insertion in the family member identified by a 4.5-kb *Bam*HI fragment (called *aB4.5*), caused significantly shorter lesions on IR24 rice than KL7M or KLD1 (Fig. 3). The reduction in aggressiveness caused by the mutation in *aB4.5* was restored by complementation with plasmid pK145, which contains *aB4.5* (Fig. 4). The slopes of the regression lines were calculated to measure the rate of disease development by each strain. Differences observed confirmed the reduction in aggressiveness in strains harboring mutations in all family members, except *avrXa10* (Fig. 3). These experiments demonstrate that in addition to *avrXa7*, other *avr* family members, including *avrxa5*, contribute to pathogen aggressiveness, although the contributions are not equivalent.

avrXa7 and *avrxa5* genes do not complement mutations in other *avr* gene family members for aggressiveness.

In Figure 1 and Table 1, we show that plasmidborne copies of *avrXa10* or *avrXa7* complement loss of avirulence or aggressiveness only in their identical locus, which suggests

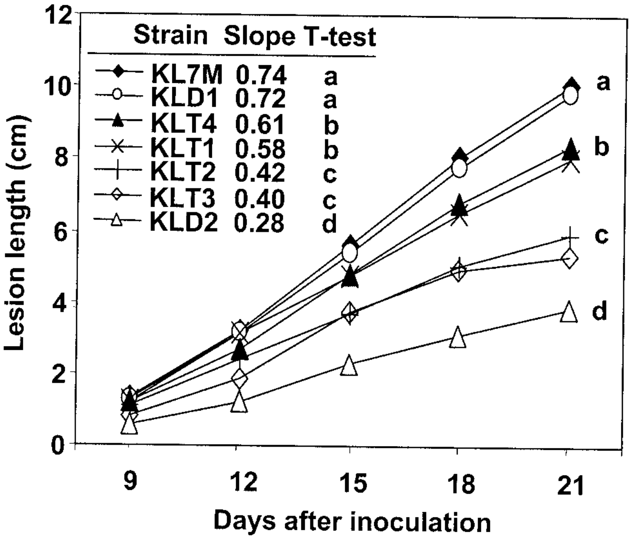
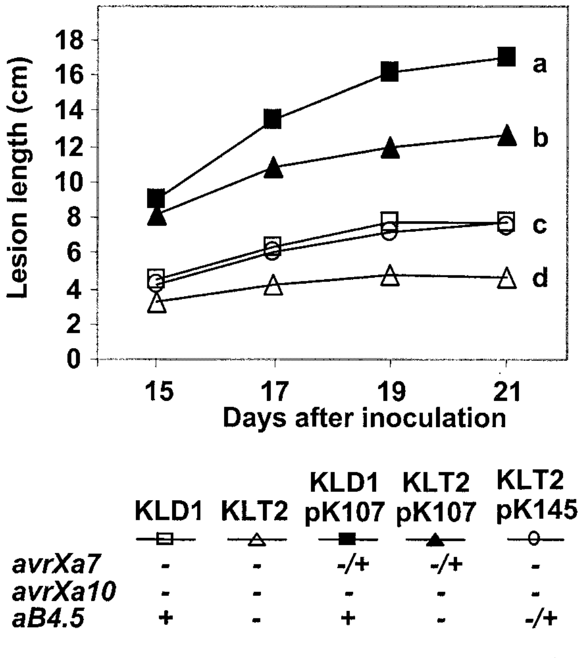


Fig. 3. Effects of mutation of *avrXa7, avrXa10,* and four additional *avr* family members (*avrxa5, aB4.5, aB4.3, aB3.5*) on lesion lengths induced by *Xanthomonas oryzae* pv. *oryzae*. Lesions caused by triple mutants KLT1 (*avrXa7*⁻, *avrXa10*⁻, *avrxa5*⁻), KLT2 (*avrXa7*⁻, *avrXa10*⁻, *aB4.5*⁻), KLT3 (*avrXa7*⁻, *avrXa10*⁻, *aB4.3*⁻), and KLT4 (*avrXa7*⁻, *avrXa10*⁻, *aB3.5*⁻) were compared with those caused by the wild-type strain PXO86 (not shown), the single mutant (KL7M; *avrXa7*⁻), and the double mutant (KLD1; *avrXa7*⁻, *avrXa10*⁻). Leaves of 4-week-old plants were inoculated by the leaf-clip method. Slopes are derived from linear regression analysis of each strain scored at different days after inoculation. Different letters indicate significant difference (P = 0.01, least significant difference) in slopes of the regression lines (inset) or lesion lengths at day 21.



	KLD1	KLT2	KLD1 pK107	KLT2 pK107	KLT2 pK145
	—□—	—△—	—■—	—▲—	—○—
avrXa7	-	-	-/+	-/+	-
avrXa10	-	-	-	-	-
aB4.5	+	-	+	-	-/+

Fig. 4. The *avrXa7* gene does not complement mutations in gene family member *aB4.5* for aggressiveness. Lesion development on susceptible rice line IR24 (no resistance gene) after clip inoculation of 4-week-old rice plants with *Xanthomonas oryzae* pv. *oryzae* strains KLD1 (*avrXa7*⁻, *avrXa10*⁻), KLT2 (*avrXa7*⁻, *avrXa10*⁻, *aB4.5*⁻), KLD1(pK107) (*avrXa7*⁻, *avrXa10*⁻(*avrXa7*⁺)), KLT2(pK107) (*avrXa7*⁻, *avrXa10*⁻, *aB4.5*⁻(*avrXa7*⁺)), and KLT2(pK145) (*avrXa7*⁻, *avrXa10*⁻, *aB4.5*⁻ (*avrXa10*⁺)). Different letters indicate significant difference (P = 0.05, least significant difference) in lesion lengths at day 21.

specificity for the aggressiveness function of these genes. Furthermore, we demonstrated that mutation of any *avr* family member (with the exception of *avrXa10*) caused a reduction in symptoms and bacterial growth, indicating that the numerous *avr* gene family members present in the genome of strain PXO86 did not complement the lesions in an individual family member. To further clarify the specificity of the aggressiveness function, we measured the ability of plasmidborne copies of the *avrXa7* and *avrxa5* genes to restore aggressiveness to triple mutants KLT1 and KLT2, which, in addition to the insertions in *avrXa7* and *avrXa10,* contain insertions in *avrxa5* and *aB4.5*. We compared lesion lengths produced on rice line IR24 by mutants KLT1, KLT2, and KLD1 and their derivatives that contained plasmid copies of *avrXa7* and/or *avrxa5.* The double-mutant strain complemented with *avrXa7*, KLD1(pK107), caused longer lesions on IR24 than did the complemented triple mutants KLT1(pK107) (not shown) and KLT2(pK107) (Fig. 4), indicating that *avrXa7* carried on pK107 could not complement the lesions in *avrxa5* and *aB4.5.* Similarly, the presence of pK145 in KLT2 only incrementally increased the lesion lengths, apparently through complementation of the *aB4.5* mutation (Fig. 4). The lesions caused by KLT2(pK145) (Fig. 4) and KLT1(pXO6-33) (not shown) were significantly shorter than those caused by KLT2(pK145) or KLT1(pK107), indicating that *aB4.5* and *avrxa5* cannot complement the *avrXa7* lesion.

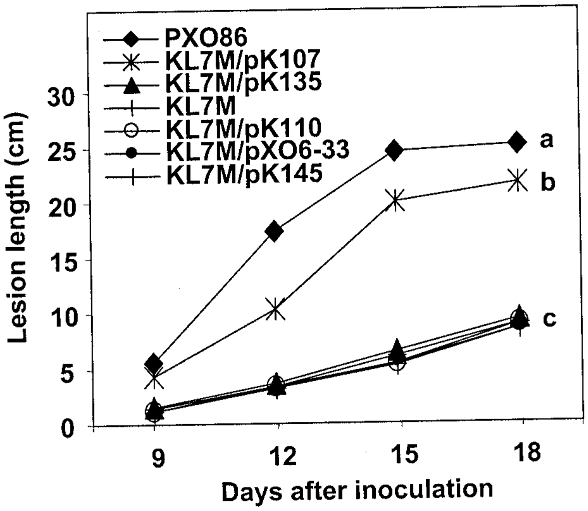


Fig. 5. The loss of aggressiveness exhibited by *avrXa7* mutants is complemented only by plasmids containing *avrXa7*. Plasmids pK107 (*avrXa7*), pK135 (*aB3.5*), pK110 (*avrXa10*), pK145 (*aB4.5*), and pK105 (*avrxa5*) were introduced into *Xanthomonas oryzae* pv. *oryzae* strain KL7M (*avrXa7*$^-$). Leaves of 4-week-old plants were inoculated by the leaf-clip method. Different letters indicate significant difference (P = 0.05, least significant difference) in lesion lengths at day 18.

To determine the loss of the *avrXa7* aggressiveness function could be complemented by other *avr* gene family members, plasmidborne versions of other genes were introduced into KL7M and the resulting transformants were inoculated to rice line IR24. The mutation in *avrXa7* was complemented only by the addition of pK107, the plasmid that carries a functional *avrXa7* (Fig. 5); i.e., the restoration of aggressiveness occurred only when KL7M was complemented with pK107. No other gene family member, including *avrxa5* or *aB4.5,* which function in aggressiveness, increased the lesion lengths of KL7M transformants. Thus, the aggressiveness functions associated with *avrXa7*, *avrxa5*, and *aB4.5* are locus specific.

DISCUSSION

Strains of *X. oryzae* pv. *oryzae* contain several copies of members of the *avrBs3* avirulence gene family. At least three of these, *avrXa7, avrXa10,* and *avrxa5*, have resistance gene-

Table 2. Bacterial strains and plasmids used in this work

Designation	Relevant characteristics and use	Source or reference
Escherichia coli		
S17-1	*294 recA*, chromosomally integrated RP4 derivative, used for biparental mating, Sm^r, Sp^r, Tp^r	Simon et al. 1983
Xanthomonas oryzae pv. *oryzae*		
PXO99A	Philippine race 6 strain, genetic lineage D. Recipient for characterization of plasmid constructs; azacytidine resistant; no *avrxa5, avrXa7*, or *avrXa10*	Hopkins et al. 1992
PXO86	Philippine race 2 strain; genetic lineage B. Parent for marker exchange, source of *avrxa5, avrXa7*, and *avrXa10*, Cp^r	Hopkins et al. 1992; Mew 1987
KL7M	PXO86 mutated at *avrXa7* locus by recombination with pK135, Cp^r, Km^r	This work
KL10M	PXO86 mutated at *avrXa10* locus by recombination with pK145, Cp^r, Km^r	This work
KLD1	Double mutant of PXO86; *avrXa7*$^-$ and *avrXa10*$^-$, Cp^r, Km^r, Cm^r	This work
KLD2	Double mutant of PXO86; *avrXa7*$^-$ *aB3.6*$^-$, Cp^r, Km^r	This work
KLT1	Triple mutant of PXO86; *avrXa7*$^-$ *avrXa10*$^-$*avrxa5*$^-$, Cp^r, Km^r, Cm^r	This work
KLT2	Triple mutant of PXO86; *avrXa7*$^-$ *avrXa10*$^-$, *aB4.5*$^-$, Cp^r, Km^r, Cm^r	This work
KLT3	Triple mutant of PXO86; *avrXa7*$^-$ *avrXa10*$^-$ *aB4.3*$^-$, Cp^r, Km^r, Cm^r	This work
KLT4	Triple mutant of PXO86; *avrXa7*$^-$ *avrXa10*$^-$ *aB3.5*$^-$, Cp^r, Km^r, Cm^r	This work
Plasmids		
pHM1	Broad host range cosmid derivative of pRI40, IncW, *parA*, Sp^r, Sm^r	R. Innes, Indiana University
pXO29-29	*avrXa7* and *aB3.5*, an *avrBs3* family member of unknown function	Hopkins et al. 1992
pXO5-15	*avrXa10* and *aB4.5*, an *avrBs3* family member of unknown function	Hopkins et al. 1992
pXO6-33	*avrxa5*, *avrXa10* and *aB4.5*	Hopkins et al. 1992
pK107	*avrXa7* gene; *aB3.5* disrupted by Tn*5*	Hopkins et al. 1992
pK110	*avrXa10* gene in pUC118, then linearized and ligated to pHM1, modified from pBavrXa10 (Young et al. 1994)	This work
pK135	pXO29-29 with *aB3.5* and a Tn*5*-disrupted *avrXa7*	Hopkins et al. 1992
pK145	pXO5-15 with *aB4.5* and a Tn*5* disrupted *avrXa10*	Hopkins et al. 1992
pK146	pXO5-15 with *cat* gene inserted into *avrXa10,* used for mutagenesis, Cm^r, Cb^r	This work

specific avirulence functions. On the basis of qualitative and quantitative assays for pathogen aggressiveness, we demonstrate that some, but not all of the *avr* gene family members contribute to pathogenic aggressiveness and that the contributions are different. Most intriguing is the finding that the contributions to aggressiveness are family-member specific and are not merely additive.

A combination of single, double, and triple mutants was used to demonstrate the contributions of different *avr* family members to aggressiveness. For the analysis of most family members, the *avrXa7*$^-$/*avrXa10*$^-$ background was selected to remove the large contribution of *avrXa7* to aggressiveness and thereby allow detection of minor contributions by other family members. The contributions of the three known *avr* genes and the four other family-member genes to aggressiveness are different and range from no detectable contribution (*avrXa10*) to a high level of contribution (*avrXa7* and *aB3.6*). Of the family members compared, only *avrXa10* does not measurably contribute to aggressiveness. On the basis of reduction in lesion lengths caused by the triple mutants, *aB3.5* (KLT4) and *avrxa5* (KLT1) contribute less to aggressiveness than *aB4.3* (KLT3) and *aB4.5* (KLT2).

As a result of the highly conserved nature of the *avr* family members, one might predict that they behave as redundant and additive effectors of aggressiveness and may complement one another (Kjemtrup et al. 2000). However, we show that although the *avr* genes may be redundant, the contributions of *avrxa5, avrXa7,* and *aB4.5* for aggressiveness are gene specific on the basis of observations that defects in *aB4.5, avrxa5,* and particularly *avrXa7* resulted in shorter lesions on IR24 that were complemented only by plasmidborne versions of *avrxa5, avrXa7* and *aB4.5,* respectively. The defects were not complemented by plasmids carrying other *avr* family members. Since *avrXa7, avrxa5* and *aB4.5,* which independently contribute to pathogen aggressiveness, cannot complement for the loss of aggressiveness function contributed by each other nor can they be complemented by other family members, we conclude that there is specificity associated with the aggressiveness function of these *avr* gene family members.

Other *avrBs3* gene family members, particularly those found in *X. campestris* pv. *malvacearum* and *X. campestris* pv. *citri,* are critical to disease-symptom expression. Yang et al. (1996) inactivated seven of 10 *avrBs3* gene family members in *X. campestris* pv. *malvacearum* and demonstrated that only five of these were needed for full water soaking on cotton leaves. The authors concluded that these five genes contributed additively. The addition of three *X. campestris* pv. *malvacearum avr* genes was sufficient to complement the asymptomatic mutant to produce fully water-soaked lesions on the susceptible cotton leaves. Although Yang et al. (1996) found that different *avr* genes contributed differently to aggressiveness, their findings differ from ours in two respects. First, the reduction in aggressiveness (in the *X. campestris* pv. *malvacearum*–cotton interaction [the release of bacteria to the plant surface]), resulting from inactivation of *X. campestris* pv. *malvacearum avr* genes, was not correlated with a reduction of in planta bacterial growth, whereas inactivation of *avrXa7* and *aB4.5* in *X. oryzae* pv. *oryzae* resulted in a reduction in aggressiveness and in final bacterial numbers. Second, gene-specific complementation for aggressiveness in the *X. campestris* pv. *malvacearum*–cotton interaction was not detected, leading to the conclusion that the effects were additive. This may be because the assay used to detect symptom differences was not quantitative. With the use of a quantitative lesion-length assay, we demonstrated that in *X. oryzae* pv. *oryzae*–rice interactions, only those genes corresponding to the mutated genes restored the aggressiveness function.

How the products of the *avrBs3* gene family function in aggressiveness is not understood. AvrBs3, the product of *avrBs3*, and PthA, the product of the *X. citri pthA* gene, cause cells of susceptible hosts to enlarge when expressed ectopically (Duan et al. 1999; Van den Ackerveken et al. 1996). While the specific mechanisms are not known, the host cell expansion and eruption likely provide a selective epidemiological advantage to the pathogen in bacterial populations. In rice interactions with *X. oryzae* pv. *oryzae*, the enhanced colonization of leaves afforded by the activity of some family members would increase the spread within the vascular system of the plant and add to the possibility of a spread to other plants (via guttation fluids), thereby increasing the epidemic potential for the pathogen. In companion field studies, we accumulated data supporting the negative epidemiological impact imposed by the loss of *avrXa7* activity, i.e., adaptation to virulence on rice line IRBB7 with the *Xa7* resistance gene as it is associated with reduced aggressiveness and lack of persistence in the field populations (Vera Cruz et al. in press).

Knowledge of the aggressiveness functions of *avr* genes in

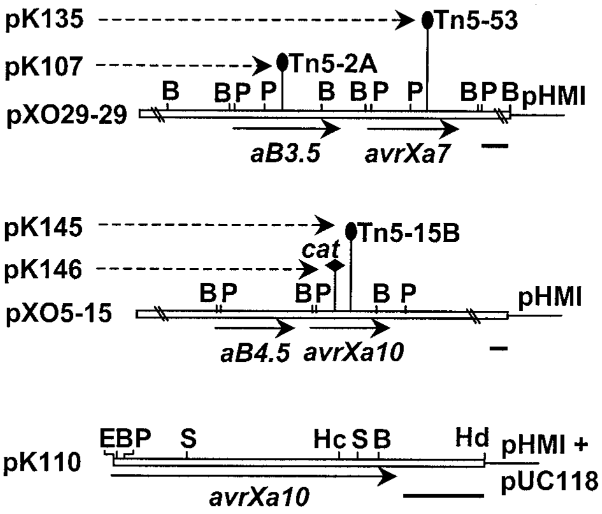


Fig. 6. Plasmid constructs used for mutagenesis and complementation studies. Open boxes = *Xanthomonas oryzae* pv. *oryzae* genomic DNA; single lines at the right of the open boxes = the broad host range cosmid cloning vector pHMI (Sm^r, Sp^r). The plasmids were derived from pXO29-29 (*avrXa7*) and pXO5-15 (*avrXa10*), which contain *X. oryzae* pv. *oryzae* genomic DNA of approximately 14.1 and 31.2 kb, respectively (Hopkins et al. 1992). *aB3.5* (3.5-kb *Bam*HI fragment adjacent to *avrXa7*) and *aB4.5* (4.5-kb *Bam*HI fragment adjacent to *avrXa10*) are members of the *avrBs3* family, with unknown function (Hopkins et al. 1992). Plasmids pK135, pK107, and pK145 were generated by insertional mutagenesis with Tn*5* (5.6 kb) (Hopkins et al. 1992). Plasmid pK146 was created by introduction of the *cat* (Cm^r) gene (1.9 kb) into the *Sph*I site located immediately preceding the repeat domain in the 5′ terminus of *avrXa10* in plasmid pXO5-15. pK110 was constructed by cloning the 4.6-kb *Eco*RI–*Hin*dIII fragment containing *avrXa10* into pUC118. The resulting plasmid was linearized with *Hin*dIII and cloned into pHMI. B = *Bam*HI; E = *Eco*RI; Hc = *Hinc*II; P = *Pst*I; S = *Sph*I; Hd = *Hin*dIII; // = additional flanking *X. oryzae* pv. *oryzae* DNA. Thick bars at the right bottom of each plasmid = 1 kb.

bacterial pathogens can be used to understand and develop durable resistance in plants. Targeted selection of a resistance gene directed against a known pervasive and important virulence factor has been used to identify plant resistance. On the basis of the aggressiveness penalty in rice observed in *avrXa7* and *avrxa5* mutations, we predict that the corresponding resistance genes *Xa7* and *xa5* would be more durable under field conditions than *Xa10* and, therefore, would be good candidates for breeding programs. In addition to the demonstration here of the importance of *avrXa7* as an aggressiveness factor in Philippine strain PXO86 (Philippine genetic lineage B) (Vera Cruz et al. 1996), we have shown that through inactivation of *avrXa7*, two other distinct genetic backgrounds of *X. oryzae* pv. *oryzae*, e.g., in strain PXO2684 (Philippine lineage C) (Vera Cruz et al. in press) and the Korean strain KXO93 (S. H. Choi, *unpublished*), also result in a substantial reduction in aggressiveness to rice. Furthermore, because complementation of mutations in the *avr* gene family members is gene specific, it is likely that deployment of genes such as *xa5* and *Xa7* will impose changes in different underlying mechanisms, thus making it more difficult and less likely for the pathogen to restore complete fitness. This point is important in the selection of resistance genes for pyramiding, i.e., the introduction of multiple resistance genes that target the same pathogen into a single rice line. Support for part of these predictions was provided by our complementary field studies, where we observed that the fitness penalty for a pathogen to adapt to virulence on *Xa7* was more severe than on *Xa10* (Vera Cruz et al. in press). Thus, with the use of microbial genetics to systematically determine the consequences of pathogen adaptation, it should be possible to proactively assess the durability of a wide array of disease-resistance genes in germ plasm.

MATERIALS AND METHODS

Rice lines and bacterial strains.

Rice lines IRBB7, IRBB10, and IRBB5 are near isogenic for bacterial blight resistance and possess the *Xa7*, *Xa10*, or *xa5* resistance genes, respectively, with IR24 as the recurrent parent (Ogawa and Khush 1989). IRBB7, IRBB10, and IRBB5 are resistant to *X. oryzae* pv. *oryzae* strain PXO86, which possesses functional *avrXa7*, *avrXa10*, and *avrxa5* genes, but are susceptible to PXO99A, which does not carry these *avr* genes (Hopkins et al. 1992) (Table 2). IR24 is susceptible to both bacterial strains. *X. oryzae* pv. *oryzae* PXO86 was used as the parental strain for marker-exchange mutagenesis experiments and PXO99A was used for screening plasmidborne constructs with Tn*5* or *cat*-insertional mutants for loss of *avr* function.

Construction of plasmids and bacterial mutants.

X. oryzae pv. *oryzae* strains KL7M and KL10M, which contain Tn*5*-disruptions in the *avrXa7* and *avrXa10* genes, respectively, were created by recombinational mutagenesis of strain PXO86 with plasmids pK135 and pK145 (Fig. 6). Plasmid pK146 (Fig. 6) was used for recombinational mutagenesis of strain KL7M (*avrXa7*::Tn*5*) to create double (KLD1 and KLD2) and triple mutants (KLT1–KLT4). Genotypes of the mutants and plasmids are shown in Table 2.

Plasmid pK110 (containing *avrXa10*) (Fig. 6), pXO6-33 (containing *avrxa5*) (Hopkins et al. 1992), and pK107 (containing *avrXa7*) (Fig. 6) were used for complementation of strains mutated at *avrXa10*, *avrxa5*, or *avrXa7*, respectively. Clone pK145, which contains the *avrBs3* family member *aB4.5*, was used to complement the corresponding mutation in KLT2. All mutations in known *avr* genes were confirmed by screening for loss of avirulence function on the appropriate rice lines and by complementation with the cloned *avr* genes. DNA blot analysis of each mutant was used to confirm changes in sizes of DNA fragments after digestion with *Bam*HI, *Pst*I, and *Sma*I and hybridization with the 3.1-kb *Bam*HI fragment from pK110, Tn*5*, or the *cat*-gene fragments. Detection of hybridized fragments in DNA blot analysis was performed with an ECL kit (Amersham, Piscataway, NJ, U.S.A.). Western blot analyses, performed as described (Young et al. 1994), were used to confirm expression of *avr* genes from plasmids during complementation experiments. AVR proteins were detected with purified (Smith and Fisher 1984) anti-AVR antibodies, which detect all AVRBs3 family members, and a chemiluminescent assay (ImmuneStar, Bio-Rad, Hercules, CA, U.S.A.).

Plant assays and in planta bacterial numbers.

To assay avirulence and aggressiveness of bacterial strains, bacterial suspensions were inoculated to rice leaves by infiltration (5×10^7 CFU/ml) (Reimers and Leach 1991) and by leaf-clipping (5×10^8 CFU/ml) (Kauffman et al. 1973). Symptom development was assessed 2 to 4 days after inoculation by infiltration. Lesion lengths after leaf-clip inoculation were measured at 2- to 3-day intervals starting at 9 or 15 days. Regression lines of the disease development were generated by PROC REG, and the slopes of the regression lines were compared through SAS PROC GLM (SAS Institute, Cary, NC, U.S.A.). Bacterial numbers were measured at 10 days after leaf-clip inoculation (Barton-Willis et al. 1989). All plant inoculation experiments contained three to four replications, with nine plants per replication, and were repeated two to four times.

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