# Identification of a Family of Avirulence Genes from *Xanthomonas oryzae* pv. *oryzae*

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Races of *Xanthomonas oryzae* pv. *oryzae*, the causal agent of bacterial blight of rice, interact with cultivars of rice in a gene-for-gene specific manner. Multiple DNA fragments of various sizes from all strains of *X. o.* pv. *oryzae* hybridized with *avrBs3*, an avirulence gene from *Xanthomonas campestris* pv. *vesicatoria*, in Southern blots; this suggests the presence of several homologs and possibly a gene family. A genomic library of a race 2 strain of *X. o.* pv. *oryzae*, which is avirulent on rice cultivars carrying resistance genes *xa-5*, *Xa-7*, and *Xa-10*, was constructed. Six library clones, which hybridized to *avrBs3*, altered the interaction phenotype with rice cultivars carrying either *xa-5*, *Xa-7*, or *Xa-10* when present in a virulent race 6 strain. Two avirulence genes, *avrXa7* and *avrXa10*, which correspond to resistance genes *Xa-7* and *Xa-10*, respectively, were identified and partially characterized from the hybridizing clones. On the basis of transposon insertion mutagenesis, sequence homology, restriction mapping, and the presence of a repeated sequence, both genes are homologs of avirulence genes from dicot xanthomonad pathogens. Two *Bam*HI fragments that are homologous to *avrBs3* and correspond to *avrXa7* and *avrXa10* contain a different number of copies of a 102-bp direct repeat. The DNA sequence of *avrXa10* is nearly identical to *avrBs3*. We suggest that *avrXa7* and *avrXa10* are members of an avirulence gene family from xanthomonads that control the elicitation of resistance in mono- and dicotyledonous plants.

*Additional keyword*: gene-for-gene hypothesis.

Bacterial blight of rice, caused by *Xanthomonas oryzae* pv. *oryzae* (Ishiyama) Dye (Swings *et al.* 1990), is a major disease that is prevalent throughout Asia (Ou 1985). Races of *X. o.* pv. *oryzae* have been defined by use of rice cultivars containing different resistance genes (Mew 1987). Race-specific interactions such as those observed between *X. o.* pv. *oryzae* and rice are thought to follow the gene-for-gene model (Ellingboe 1976; Flor 1955), which predicts that incompatible interactions are the consequence of positive functions encoded by avirulence genes in the pathogen and corresponding resistance genes in the host. By analogy, races of *X. o.* pv. *oryzae* should contain different complements of avirulence genes that correspond to individual rice resistance genes.

Avirulence genes have been cloned from several different species and pathovars of *Xanthomonas* (Bonas *et al.* 1989; Canteros *et al.* 1991; De Feyter and Gabriel 1991; Gabriel *et al.* 1986; Minsavage *et al.* 1990; Ronald and Staskawicz 1988; Swanson *et al.* 1988; Swarup *et al.* 1992; Whalen *et al.* 1988). An avirulence gene corresponding to *Xa-10* was previously reported on a 2.5-kb fragment from *X. o.* pv. *oryzae* (Kelemu and Leach 1990). However, subsequent analysis of this clone showed the presence of a phosphate-binding protein gene that does not have avirulence activity (C. M. Hopkins, F. F. White, and J. E. Leach, unpublished).

Whether this gene plays a role in the plant-pathogen interaction is unclear.

The avirulence genes *avrBs3* and *avrBsP*, both from *X. campestris* pv. *vesicatoria* (Doidge) Dye, are particularly intriguing from two perspectives. First, *avrBs3* and *avrBsP* contain a 102-bp sequence in the coding region; this sequence is repeated 17.5 and six times, respectively (Bonas *et al.* 1989; Canteros *et al.* 1991). The close similarity between the two genes, with the exception of rearrangements in the repeat structure, as well as the requirement of the repetitive region for avirulence activity of *avrBsP* has led to the suggestion that the repeat domain controls, in part, the specificity of avirulence activity (Canteros *et al.* 1991). Second, DNA sequences related to *avrBs3* from *X. c.* pv. *vesicatoria* were detected in seven additional pathovars of *X. campestris* that cause disease on diverse dicotyledonous plants (Bonas *et al.* 1989). Recently, homologs of *avrBs3* from *X. c.* pv. *malvacearum* (Smith) Dye and *X. c.* pv. *citri* (Hasse) Dye were shown to have avirulence activity (Swarup *et al.* 1992). Although avirulence activity has yet to be demonstrated for many of the *Xanthomonas* pathovars with sequences homologous to *avrBs3*, the presence of *avrBs3* homologs with avirulence function in several pathovars implies a common resistance mechanism may be operating in diverse dicot hosts. We were interested if there were *avrBs3* homologs that function as avirulence genes in *X. o.* pv. *oryzae* and if common resistance mechanisms may be operating in dicot and monocot hosts. Evidence for such common resistance mechanisms was presented by Whalen *et al.* (1988), who showed that a non-host avirulence gene from *X. c.* pv. *vesicatoria* controlled the induction of resistance in monocot and dicot hosts. We report here that avirulence genes from a monocot pathogen, *X. o.* pv. *oryzae*, are members of a multiple gene family with sequence similarity to *avrBs3*.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, bacteriophage, and media.**
Bacterial strains, plasmids, and phage used in this study are listed in Table 1. Strains of *E. coli* were cultured in Luria-Bertani medium (Miller 1972) at 37° C. Strains of *X. o.* pv. *oryzae* were grown in either nutrient (Difco Laboratories, Detroit, MI) or peptone-sucrose medium (Tsuchiya *et al.* 1982) at 28° C. For culture on solid medium, 1.5% agar was added. Casein peptone glucose agar (CPGA; Kelman 1954) was used for enumeration of bacteria from plants. The concentrations of antibiotics incorporated into media were carbenicillin (Cb), 100 μg/ml; cephalexin (Cp), 20 μg/ml; kanamycin (Km), 50 μg/ml; nalidixic acid (Nal), 50 μg/ml; spectinomycin (Sp), 50 μg/ml; and streptomycin (Sm), 50 μg/ml.

**Plant material and plant inoculations.** Cultivar Cas 209 contains the *Xa-10* gene for bacterial blight resistance (Yoshimura *et al.* 1983). IR24 is susceptible to all strains of *X. o.* pv. *oryzae* used in this research. Cultivars IR-BB5, IR-BB7, and IR-BB10 are near-isogenic to IR24 and contain *xa-5*, *Xa-7*, and *Xa-10*, respectively (Ogawa and Khush 1989; Ogawa and Yamamoto 1987).

Seeds were planted and incubated in growth chambers as previously described (Reimers and Leach 1991). Leaves of seedlings (10–11 days after planting) were inoculated with bacterial suspensions ($5 \times 10^9$ cfu/ml in sterile distilled water) by infiltrating bacteria into the intercellular spaces with a needleless syringe (Reimers and Leach 1991). One site per leaf was inoculated, except during the initial screening of transposon mutants when three individual mutants were infiltrated into three different sites on the same leaf. After infiltration, plants were returned to the growth chamber and incubated under continuous light at 30° C and 75% relative humidity (RH) for 24 hr. At the end of the light period, the normal light regime (12-hr light/12-hr dark) was resumed. Plants were scored at 2, 4, and 5 days after infiltration because incompatible responses involving *xa-5* and *Xa-7* take longer to develop than those involving *Xa-10* (4–5 days for *xa-5*, 3–4 days for *Xa-7*, and 1–2 days for *Xa-10*).

**Recombinant DNA techniques.** Genomic DNA isolation from bacteria and Southern blot hybridization conditions were as described (Leach *et al.* 1990). Colony blots, performed as described by Maas (1983), were washed after hybridization under high stringency conditions (Leach *et al.* 1990). Otherwise, standard molecular biological techniques were used (Ausubel *et al.* 1991). Restriction enzymes, calf intestinal alkaline phosphatase, T4 DNA ligase, and nick translation kits were purchased from Bethesda Research Laboratories (BRL, Gaithersburg, MD) or Promega Biotech (Madison, WI). Reactions were performed as described by the manufacturers.

**Construction of a genomic DNA library.** Genomic DNA from a race 2 strain of *X. o.* pv. *oryzae* (PXO86) was partially digested with *Sau*3AI and fractionated in a 0.5% agarose gel. Fragments larger than 25 kb were extracted from the agarose by electroelution. The cosmid vector pHMI was digested to completion with *Bam*HI and treated with calf intestinal alkaline phosphatase. The *X. o.* pv. *oryzae* genomic and vector DNA fragments were ligated,

packaged into lambda phage *in vitro* by use of Gigapack Plus packaging kits (Stratagene, La Jolla, CA), and transduced into *E. coli* HB101 (resistant to nalidixic acid, Nal$^r$). Colonies were selected for resistance to Sp and Sm (50

**Table 1.** Bacterial strains, plasmids, and phage used in this study

| Designation | Relevant characteristics | Source or reference |
|---|---|---|
| *Xanthomonas oryzae* pv. *oryzae* | | |
| PXO86 | Race 2; avirulent to rice with *xa-5*, *Xa-7*, *Xa-8*, and *Xa-10* resistance genes | T. Mew, International Rice Research Institute, Los Baños, Philippines |
| PXO99$^A$ | Race 6; virulent to rice with *xa-5*, *Xa-7*, *Xa-8*, and *Xa-10* resistance genes; grows on 200 μM 5-azacytidine | S. H. Choi and J. E. Leach |
| PXO99$^A$2A | Marker-exchange mutant with functional *avrXa7* gene in the chromosome of PXO99$^A$ | This study |
| PXO99$^A$15A | Marker-exchange mutant with functional *avrXa10* gene in the chromosome of PXO99$^A$ | This study |
| *Escherichia coli* | | |
| DH5α F′ | F′ *recA*, φ80d*lacZ*, ΔM15 | Bethesda Research Laboratories |
| S17-1 | 294 *recA*, chromosomally integrated RP4 derivative; Tp$^r$, Sm$^r$ | Simon *et al.* 1983 |
| HB101 | F$^-$, *recA*, Nal$^r$, Sm$^r$ | Boyer and Roulland-Dussoix 1969 |
| TB1 | JM83, *lacZ*, *hsdR* | Baldwin *et al.* 1984 |
| Plasmids | | |
| pHMI | Sp$^r$, Sm$^r$, *cos*, *parA*, IncW, derivative of pRI40 (Innes *et al.* 1988) | R. Innes, Indiana University |
| pBluescript | Ap$^r$, M13 | Stratagene Inc., La Jolla, CA |
| pRK2013 | Km$^r$, Tra$^+$, Mob$^+$, ColE1 replicon | Friedman *et al.* 1982 |
| pEC83 | pLAFR3 clone with *avrBs3* | Minsavage *et al.* 1990 |
| pXO5-15 | pHMI clone from *X. o.* pv. *oryzae* that contains *avrXa10* | This study |
| pXO29-29 | pHMI clone from *X. o.* pv. *oryzae* that contains *avrXa7* | This study |
| pXO6-33 | pHMI clone from *X. o.* pv. *oryzae* that contains *avrxa5* and *avrXa10* | This study |
| pBSavrXa10 | pBluescript clone with a 3.1-kb *Bam*HI fragment from pXO5-15 | This study |
| pBSavrXa7 | pBluescript clone containing a 4.1-kb *Bam*HI fragment from pXO29-29 | This study |
| pBSavrBs3 | pBluescript clone containing 3.3-kb *Bam*HI fragment internal to *avrBs3* | This study |
| pBSA10L | pBluescript clone containing 4.5-kb *Bam*HI fragment from pXO5-15 | This study |
| pBSA7L | pBluescript clone with 3.7-kb *Bam*HI fragment from pXO29-29 | This study |
| Phage | | |
| Tn5-B20 | *lac* fusion, Km$^r$ | Simon *et al.* 1989 |

μg/ml of each). Restriction enzyme analysis of 17 randomly selected clones revealed unique DNA fragmentation patterns for each and an average DNA insert size of 34 kb. On the basis of an insert size of 34 kb, 1,010 clones are needed to represent each gene at least once with a probability of 99% if the *X. o.* pv. *oryzae* genome size is assumed to approximate that of *Pseudomonas fluorescens* (Trevisan) Migula (7.4 × $10^3$ kb) (Bak *et al.* 1970; Clarke and Carbon 1976). A $^{32}$P-labeled 2.5-kb fragment of *X. o.* pv. *oryzae* DNA containing a single-copy sequence (Kelemu and Leach 1990) hybridized with three colonies of the approximately 1,400 that were screened.

**Bacterial conjugation.** Donor and recipient strains for triparental matings (Ditta *et al.* 1980) were prepared as described (Kelemu and Leach 1990). The bacterial mixtures (*X. o.* pv. *oryzae* PXO99[A], *E. coli* HB101 with the library clones, and *E. coli* HB101[pRK2013]) were spotted onto sterile 1.5-cm² pieces of nylon membrane (GeneScreenPlus, Du Pont, Wilmington, DE) on nutrient agar and incubated at 28° C. After 24 hr, the membrane was transferred to peptone-sucrose agar (PSA) + Sp + Sm + Cp (to inhibit growth of *E. coli*) and incubated at 28° C for an additional 48 hr. The bacteria were rinsed from the membrane by vortexing in 1 ml of sterile water, and the suspension was plated on PSA + Sp + Sm + Cp and incubated 3–5 days at 28° C. Biparental matings with S17-1 (Simon *et al.* 1983) as the donor strain were performed on membranes as described above.

**Enumeration of bacterial multiplication.** Bacterial suspensions (5 × $10^5$ cfu/ml) of strains PXO86, PXO99[A], and transconjugants containing *avr* genes (PXO99[A][pXO5-15] or PXO99[A][pXO29-29]) or mutated *avr* genes (PXO99[A][pTn5-35] or PXO99[A][pTn5-53]) were infiltrated into one site per rice leaf (Reimers and Leach 1991). Three leaves from each treatment were harvested at 0.5, 24, 48, 72, and 96 hr post-infiltration and ground separately. Each homogenate was diluted serially in sterile distilled water and plated in duplicate onto CPGA that was supplemented with cycloheximide (75 μg/ml) for reducing fungal contamination. Colonies were counted 3–4 days after plating; values from duplicate plates were averaged to obtain one replication. Treatments were replicated three times within each experiment, and the experiment was performed three times.

**Transposon mutagenesis and marker-exchange mutagenesis.** Clones of *X. o.* pv. *oryzae* DNA in pHMI were mutagenized in *E. coli* strain TB1 by using λB20::Tn5 (Simon *et al.* 1989). Plasmid DNA was isolated from Km[r], Sp[r], and Sm[r] transductants and transformed into *E. coli* S17-1. The transposon-containing plasmids were analyzed by restriction endonuclease cleavage and transferred into *X. o.* pv. *oryzae* PXO99[A] by biparental conjugation. Mutants were tested for pathogenicity and induction of resistance in appropriate rice cultivars.

Active avirulence gene fragments were introduced into the chromosome of PXO99[A] by marker gene replacement. Mutated clones containing Tn5 insertions outside of *avrXa7* (Tn5-2A; Fig. 1) and *avrXa10* (Tn5-15A; insertion to right of *avrXa10* as shown in Fig. 1, but outside the mapped region) were selected. Transconjugants containing these mutations (pXO29-29[Tn5-2A] and pXO5-15[Tn5-15A], respectively) were cycled four times in nutrient broth containing Km, then plated on PSA + Km. Single colonies that were resistant to Km but susceptible to Sm and Sp were selected. Marker-exchange mutants were analyzed by inoculation to plants and by Southern blot hybridization experiments with the cosmid vector (pHMI) and the internal *Hpa*I fragment from Tn5 as probes.

**Estimation of repeat number.** *Bam*HI fragments from pXO29-29 and pXO5-15 that hybridized to *avrBs3* (Fig. 1) were cloned into pBluescript II (Table 1). The clones were partially digested with restriction enzyme *Bal*I (IBI, New Haven, CT) as described (Ausubel *et al.* 1991), and the fragments were separated on a 1% agarose gel. The clone pBSavrBs3 containing the 3.3-kb *Bam*HI fragment from *avrBs3* was used as a control. Southern blot analysis was performed with $^{32}$P-labeled pBSavrBs3 as a probe.

**DNA sequencing.** Nested deletions of pBSavrXa10 were generated in pBluescript II clones as described by Ausubel *et al.* (1991). Sequence was also obtained from pBluescript clones containing the 4.5-kb *Bam*HI, 4.5-kb *Pst*I, and 2.0-kb *Bam*HI-*Pst*I fragments adjacent to the 3.1-kb *Bam*HI insert of pBSavrXa10. Single-stranded template was prepared as described (Viera and Messing 1987) and primed with either T3, T7, or synthetic primers. The dideoxy sequencing method of Sanger *et al.* (1977) was performed with a Sequenase Version 2.0 kit used according to the manufacturer's recommendations (U.S. Biochemical, Cleveland, OH). Microgenie software (Beckman, Palo Alto, CA) and the FASTP program (Pearson and Lipman 1988) were used for sequence analysis.

**Peroxidase assays.** Apoplastic fluids were extracted from rice leaves at 24 and 48 hr after infiltration with bacterial suspensions as described (Reimers *et al.* 1992). We monitored the appearance of a cationic peroxidase (pI 8.6) in the apoplastic fluids by using nondenaturing, cathodic polyacrylamide gel electrophoresis (Thomas and Hodes 1981). Peroxidase activity was detected by incubating the
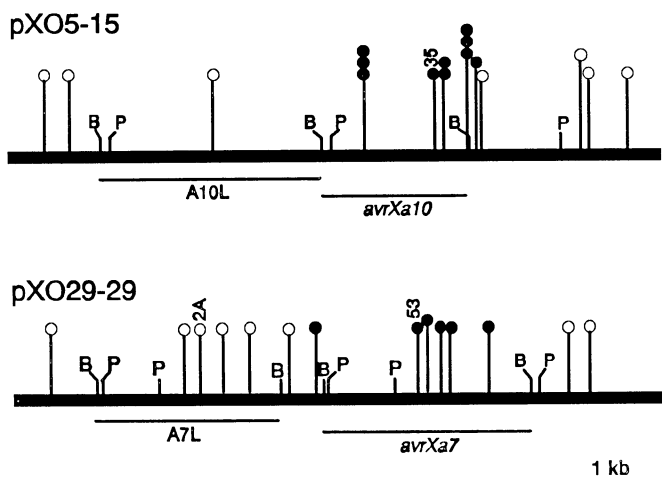


**pXO5-15**

A10L

avrXa10

**pXO29-29**

A7L

avrXa7

1 kb

**Fig. 1.** Partial restriction map of clones with *avrXa7* and *avrXa10* activity. Positions of transposon Tn5-B20 insertions that do (●) and do not (○) inactivate the avirulence phenotypes are shown. Transposon insertions used for marker exchange and physiological studies are indicated. B, *Bam*HI; P, *Pst*I. *Bam*HI fragments that hybridized with the 3.3-kb *Bam*HI fragment of *avrBs3* are indicated below the restriction maps with horizontal lines.

gels in 100 mM sodium phosphate containing 18 mM guaiacol, 0.01% 3-amino-9-ethylcarbazole, and 0.03% $H_2O_2$ at 30° C for 15 min (Reimers et al. 1992). The experiment was repeated twice.

## RESULTS

**Identification of avirulence genes from *X. o.* pv. *oryzae*.** Forty-three clones from a genomic library of strain PXO86 hybridized with the 3.3-kb *avrBs3* BamHI fragment. Six of the clones, when mobilized into the virulent recipient strain PXO99$^A$, conferred an avirulent phenotype on transconjugants with the inoculation of the appropriate cultivar (Table 2). The phenotypes of the reactions varied with the resistance gene involved in the interaction. Interactions involving *Xa-10* resulted in a dark brown color throughout the infiltrated site within 24–48 hr, whereas with *xa-5* and *Xa-7* a dark ring formed around the perimeter of the watersoaked site at 48 hr, and the tissue within the site became tan to brown after 72 hr. Compatible interactions in both combinations remained watersoaked in the infiltration site through 5 days, after which time the watersoaked lesion had spread, and the leaf had wilted. The resistance phenotype that was observed after inoculation of 10-day-old seedlings with transconjugants PXO99$^A$ (pXO6-33) containing *avrxa5* and *avrXa10*, PXO99$^A$ (pXO29-29) containing *avrXa7*, and PXO99$^A$(pXO5-15) containing *avrXa10* also was observed after inoculation of 4-wk-old rice plants (Barton-Willis et al. 1989; data not shown). The interactions between transconjugants and traditional (non-isogenic) cultivars Cas 209 (*Xa-10*), IR1545-339 (*xa-5*), and DV85 (*xa-5, avrXa7*) were consistent with the reactions observed on near-isogenic cultivars (data not shown).

The clones containing *avrXa7* (pXO29-29) and *avrXa10* (pXO5-15) were subjected to further analysis by transposon insertional mutagenesis, and the positions of the insertions were determined by restriction digestion mapping. Insertions that resulted in inactivation of *avrXa7* and *avrXa10* were located either within the 4.1-kb BamHI fragment of

pXO29-29 and the 3.1-kb BamHI fragment of pXO5-15, respectively, or immediately adjacent to these regions (Fig. 1). Mutations in the 3.7-kb BamHI fragment of pXO29-29 (A7L) and the 4.5-kb BamHI fragment of pXO5-15 (A10L), which are adjacent to the respective *avr* genes, had no effect on avirulence activity (Fig. 1).

The phenotypes of incompatible interactions involving cosmid-borne copies of the avirulence genes were characterized by more intense lesions (darker brown) than those observed with PXO86. The copy number of the cosmid vector pHMI, which has a pSa replicon, in *X. o.* pv. *oryzae* is unknown; however, the copy number of pSa replicons in *E. coli* was estimated to be two to three per cell (Tait et al. 1982). To determine if the differences in phenotype resulted from increased copy number of the *avr* genes on the cosmid, we generated marker-exchange mutants containing active copies of *avrXa7* (PXO99$^A$2A) and *avrXa10* (PXO99$^A$15A) in the PXO99$^A$ genome by using clones with Tn5 insertions outside of the *avr* genes (pTn5-2A and pTn5-15A, respectively) (Table 2). Our assumption was that exchange would occur into one of the numerous *avrBs3*-related sequences in the PXO99$^A$ genome or into adjacent homologous regions. Absence of cosmid DNA and presence of the transposon in the genomic DNA were confirmed by Southern blot analysis (data not shown). The insertion sites of the marker-exchange mutants were not characterized. Resistant host reactions to the marker-exchange mutants were less intense than those observed when the clone was present on a cosmid, but were equivalent to the responses observed after infiltration with PXO86. Susceptible interactions were similar in all combinations.

**Table 2.** Interactions of *Xanthomonas oryzae* pv. *oryzae* strains and transconjugants with near-isogenic rice cultivars

| | Rice cultivar[a] | | | |
|---|---|---|---|---|
| Strain or transconjugant | IR-BB5 | IR-BB7 | IR-BB10 | IR24 |
| *X. o.* pv. *oryzae* | | | | |
| PXO86 (race 2) | R[b] | R | R | S |
| PXO99$^A$ (race 6) | S[c] | S | S | S |
| PXO99$^A$(pHMI) | S | S | S | S |
| PXO99$^A$(pXO29-29) | S | R | S | S |
| PXO99$^A$(pXO7-29) | S | R | S | S |
| PXO99$^A$(pXO23-38) | S | R | S | S |
| PXO99$^A$(pXO5-15) | S | S | R | S |
| PXO99$^A$(pXO7-2) | S | S | R | S |
| PXO99$^A$(pXO6-33) | R | S | R | S |
| PXO99$^A$2A | S | R | S | S |
| PXO99$^A$15A | S | S | R | S |

[a] IR-BB5, IR-BB7, and IR-BB10 contain the *xa-5*, *Xa-7*, and *Xa-10* genes, respectively, and are near-isogenic to the backcross parent IR24.

[b] R indicates the infiltration site turns brown, characteristic of an incompatible interaction.

[c] S indicates the infiltration site is watersoaked, characteristic of a compatible interaction.
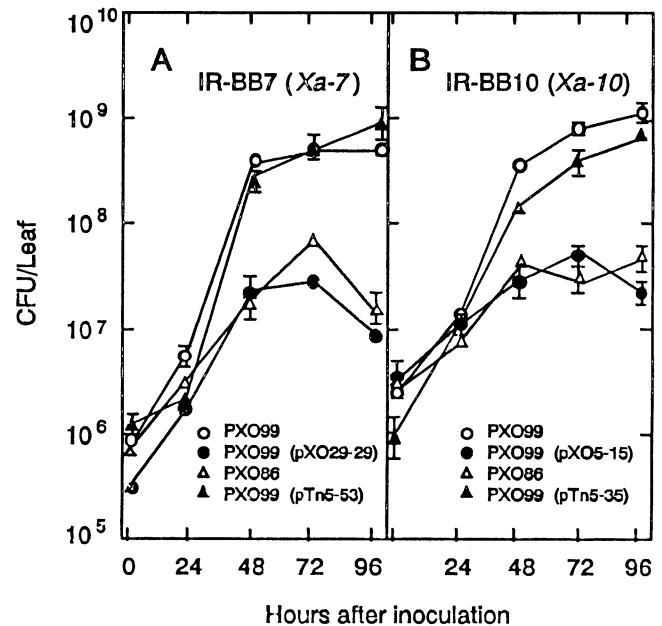


**Fig. 2.** Time course of bacterial growth in leaves of rice cultivars **A**, IR-BB7 and **B**, IR-BB10. Leaves were infiltrated with bacterial suspensions and were sampled for 96 hr after infiltration. A, IR-BB7 infiltrated with *Xanthomonas oryzae* pv. *oryzae* PXO99$^A$ (O); PXO86 (△); PXO99$^A$(pXO29-29) (●); PXO99$^A$(pTn5-53) (▲). B, IR-BB10 infiltrated with *X. o.* pv. *oryzae* PXO99$^A$ (O); PXO86 (△); PXO99$^A$(pXO5-15) (●); PXO99$^A$(pTn5-35) (▲). Values are means from three repetitions, and vertical bars represent ±1 SEM. Similar results were obtained in three independent experiments.

**In planta** growth curves and induction of peroxidase activity. The authenticity of the resistance reactions that were induced by *avrXa10* and *avrXa7* were further corroborated by analysis of *in planta* bacterial growth and the induction of peroxidase activity. In the compatible interactions, bacterial numbers increased steadily until 48 hr after inoculation to approximately $1 \times 10^8$ colony-forming units per leaf, after which the rate of multiplication decreased, and bacterial numbers did not substantially increase (Fig. 2). In incompatible interactions, the multiplication rate decreased between 24 and 48 (*Xa-10*) or 48 and 72 hr (*Xa-7*) (approximately $5 \times 10^7$ colony-forming units per leaf) (Fig. 2). Multiplication rates of the transconjugants carrying the *avr* genes on pXO5-15 and pXO29-29 were similar to those observed for strain PXO86 in rice cultivars with the corresponding resistance genes (Fig. 2). If the cosmid-borne avirulence gene was inactivated by insertion of Tn5, the multiplication rate of transconjugants was similar to that of the virulent parental strain PXO99$^A$ (Fig. 2). Growth curves were similar for all strains in IR24, which carries no resistance genes (data not shown).

An increase in the activity of a cationic peroxidase has previously been shown to be correlated with incompatible reactions (Reimers *et al.* 1992). To determine if this activity was also associated with interactions involving the cloned avirulence genes, we measured peroxidase activity of plants inoculated with transconjugants carrying the active and transposon-inactivated *avrXa7* and *avrXa10*. The activity of the cationic peroxidase increased by 24 hr in the incompatible interactions (PXO86 and PXO99$^A$ [pXO5-15]) with IR-BB10 (Fig. 3). The activity of the peroxidase had not increased in incompatible interactions with *Xa-7* (PXO86 and PXO99$^A$[pXO29-29]) at 24 hr, but had increased by 48 hr. In the compatible combinations (PXO99$^A$, PXO99$^A$[pTn5-35], and PXO99$^A$[pTn5-53] with *Xa-10* and *Xa-7* [Fig. 3], and all strains with IR24 [not shown]), the peroxidase activity increased by 48 hr after inoculation, but the final levels were less than the activity observed in the incompatible combination.
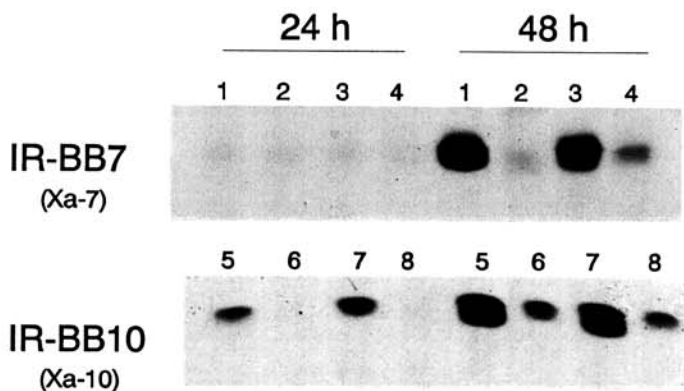
**Hybridization of *X. o.* pv. *oryzae* genomic DNA with *avrBs3*.** The internal 3.3-kb *Bam*HI fragment from *avrBs3* (Bonas *et al.* 1989) hybridized with multiple *Bam*HI fragments in DNA from strains of *X. o.* pv. *oryzae* under high stringency conditions ($0.5\times$ SSC [$1\times = 0.15$ M sodium chloride, 0.015 M sodium citrate, pH 7.0], 65° C) (Fig. 4). Approximately 12 DNA fragments from race 2 strains of *X. o.* pv. *oryzae* hybridized with pBSavrBs3 ranging in size from 2.8 to greater than 12 kb. Differences in banding patterns were apparent among 18 strains representing six races. However, restriction fragment patterns were similar within race groups. For example, race 6 strains were distinguishable from the other five races by the presence of a band of relatively high molecular weight (Fig. 4). Race 2 strains contained two high molecular weight bands that distinguished them from strains of other races, with the exception of one race 3 strain, PXO143 (Fig. 4). Genomic DNA from more than 100 different *X. o.* pv. *oryzae* strains from various geographic areas (including parts of Asia, Australia, South America, and North America) were tested for hybridization with *avrBs3*. DNA from all strains contained multiple fragments that hybridized with the *avrBs3* probe, with the exception of the strains from the United States (Jones *et al.* 1989), which showed no hybridization signal under high stringency conditions (data not shown).

**Restriction analysis of repeat domains.** The 3.3-kb *Bam*HI fragment of *avrBs3* is internal to the gene and contains the entire repeat domain. The *Bam*HI fragments internal to *avrXa7* and *avrXa10* as well as the adjacent *avrBs3*-hybridizing, *Bam*HI fragments with unknown activity (A7L and A10L; Fig. 1) were analyzed for the presence of a domain with a directly repeated 102-bp sequence analogous to that found in *avrBs3*. The 0.9-kb
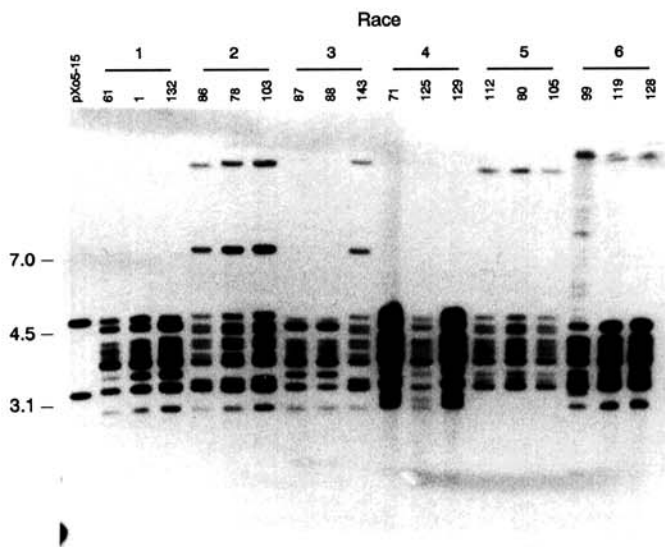


**Fig. 3.** Peroxidase activity in extracellular fluids from rice leaves extracted at 24 and 48 hr after infiltration with bacterial suspensions. Rice cultivars IR-BB7 and IR-BB10 were infiltrated with suspensions of *Xanthomonas oryzae* pv. *oryzae* PXO86 (1 and 5); PXO99$^A$ (2 and 6); PXO99$^A$(pXO29-29) (3); PXO99$^A$(pTn5-53) (4); PXO99$^A$(pXO5-15) (7); PXO99$^A$(pTn5-35) (8). Extracellular proteins (0.2 μg/lane) were separated in a nondenaturing, cathodic gel (Thomas and Hodes 1981). Peroxidase activity was detected with a mixture of the substrates guaiacol and 3-amino-9-ethylcarbazole.



**Fig. 4.** Southern blot analysis of genomic DNA from strains of *Xanthomonas oryzae* pv. *oryzae* races 1–6. DNA (3 μg) was digested with *Bam*HI, and the blot was probed with a $^{32}$P-labeled pBSavrBs3, which contains the 3.3-kb *Bam*HI internal to *avrBs3* (Bonas *et al.* 1989). Strains of *X. o.* pv. *oryzae* (and race) are PXO61 (1), PXO1 (1), PXO132 (1), PXO86 (2), PXO78 (2), PXO103 (2), PXO87 (3), PXO88 (3), PXO143 (3), PXO71 (4), PXO125 (4), PXO112 (5), PXO80 (5), PXO105 (5), PXO99 (6), PXO119 (6), PXO128 (6). Plasmid pXO5-15, which contains *avrXa10*, was digested with *Bam*HI. Size markers are in kilobases.

BamHI fragment of pXO29-29 between A7L and *avrXa7* (Fig. 1) did not hybridize to pBSavrBs3 and, therefore, was not analyzed for repeats. The BamHI fragments of *avrXa7*, *avrXa10*, A7L, and A10L were inserted into pBluescript to create pBSavrXa7, pBSavrXa10, pBSA7L, and pBSA10L, respectively. The presence and number of directly repeated sequences in each plasmid were demonstrated by partial digestion with BalI and Southern blot analysis with pBSavrBs3 as a probe. Each direct repeat from *avrBs3* contains a unique BalI site; thus, a partial digestion with BalI created a ladder of 102-bp units. We estimated that *avrXa7* and *avrXa10* contained at least 25 and 15 bands, respectively, corresponding to the number of repeats (Fig. 5). The adjacent *avrBs3*-homologous sequences A7L and A10L had 17 and 19 bands, respectively. On the basis of comigration with *avrBs3*, the tested *avrBs3*-hybridizing fragments from X. o. pv. oryzae contain repeats of approximately 102 bp in size.

**Nucleotide sequence of *avrXa10*.** The nucleotide sequence of *avrXa10* (Fig. 6) was determined from deletions of pBSavrXa10 and cloned adjacent fragments. A 3,306-bp open reading frame, which would code for a 1,102-amino acid, 116-kDa protein, was identified. The region 118 bp upstream of the translational start site of *avrXa10* is 96.6% identical to the corresponding region of *avrBs3* and contains putative Shine-Delgarno, −10 and −35 sequences similar to *avrBs3* (Fig. 6). The 5′ region of *avrXa10* between the putative translational start site and the beginning of the 102-bp repeats (which would correspond to the "N-terminal domain" of the protein) is 93.2% identical at the nucleotide level (92.0% at amino acid level) to the corresponding region of *avrBs3*. Similarly, the 3′ or C-terminal region, between
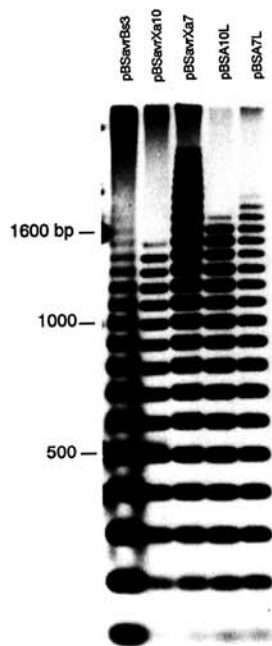


**Fig. 5.** Southern blot analysis of fragments from plasmids pBSavrBs3, pBSavrXa10, pBSavrXa7, pBSA10L, and pBSA7L generated by partial digestion with BalI for estimation of repeat number in cloned avirulence genes from *Xanthomonas oryzae* pv. *oryzae*. The blots were hybridized with ^{32}P-labeled *avrBs3*.
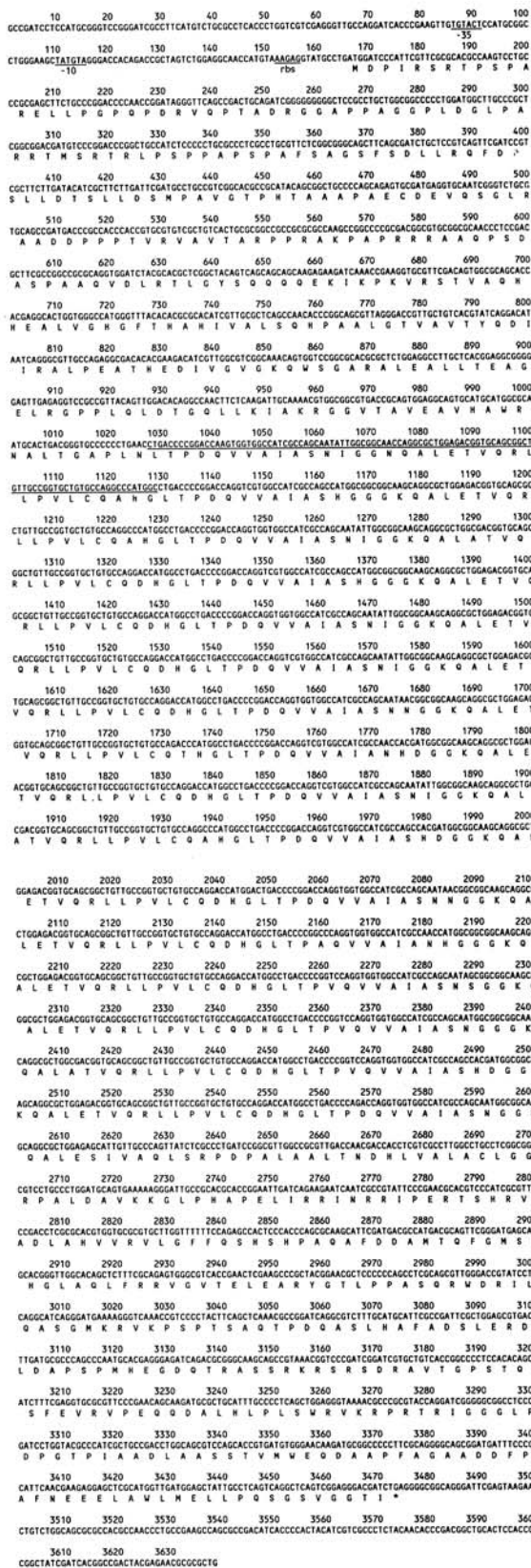


**Fig. 6.** Nucleotide and putative amino acid sequence of *avrXa10*. Possible ribosomal binding site, −10, and −35 sequences, and the first of the 102-bp repeats are underlined.

the *Bal*I site of the last repeat and the putative translational stop codon, is 95.1 and 90.5% identical to *avrBs3* at the nucleotide and deduced amino acid levels, respectively. The presence of a "C" residue at position 3,449 in *avrXa10* in place of a "G" in *avrBs3* forms a serine codon rather than a stop codon. As a result, *avrXa10* has eight more codons than *avrBs3*, and the predicted protein of *avrXa10* extends eight amino acids longer than *avrBs3* at the C terminus.

The repeat of *avrXa10* is 102 bp in length, which is the same length as that of *avrBs3* (Fig. 7), and is present in 15.5 directly repeated copies. The sequence of each copy in *avrXa10* is almost identical to the other copies with the exception of a 6-bp region (starting at nucleotide number 34), which we hereafter refer to as the variable region. The variable region alternates between six possible nucleotide sequences with no discernible order. The 102-bp repeat represents a repeat domain of 34 amino acids in the predicted protein sequence (Fig. 8). Most of the nucleotide variations do not result in amino acid substitutions with the exception of the variable region, which represents codons 12 and 13 in the protein repeat domain. Codon 12 encodes either asparagine or histidine, whereas codon 13 encodes isoleucine, glycine, asparagine, aspartate, or serine. No apparent order is discernible from the amino acid sequence in the repeat domain, except histidine at codon 12 is only present with glycine or aspartate at codon 13.

The *avrXa10* gene has two fewer copies of the repeat than *avrBs3*. The fourth codon of the *avrBs3* repeat encodes either glutamate or glutamine compared to aspartate, alanine, or valine for the *avrXa10* repeat, and the 32nd codon in *avrBs3* is for alanine, compared to aspartate, alanine, or threonine in *avrXa10*. In addition to the amino acid combinations present in the variable region of *avrBs3*, *avrXa10* contains the combinations histidine-glycine (repeats 2, 4, and 12) and asparagine-asparagine (repeats 7 and 11).

## DISCUSSION

We have identified clones from *X. o.* pv. *oryzae* that contain three avirulence genes (*avrxa5, avrXa7, avrXa10*), which control bacterial elicitation of resistance in rice cultivars carrying the *xa-5*, *Xa-7*, and *Xa-10* resistance genes,
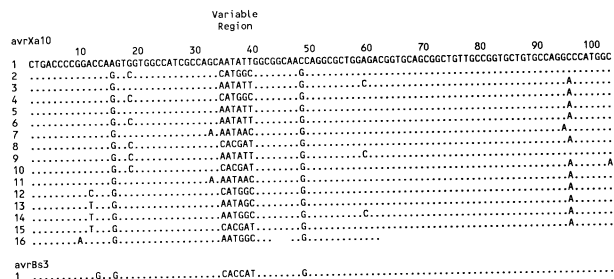
respectively. All of these genes were found on cosmid clones containing sequences homologous to *avrBs3*, an avirulence gene from the pepper pathogen *X. c.* pv. *vesicatoria* (Bonas *et al.* 1989). Two of the genes, *avrXa7* and *avrXa10*, were localized by transposon mutagenesis to regions of the clone that hybridized to *avrBs3*. The identification of a 102-bp repeated sequence within the active regions of *avrXa7* and *avrXa10* and other closely linked homologous sequences along with the DNA sequence of *avrXa10* indicates that these genes are most likely homologs of *avrBs3*. The third gene (*avrxa5*) is contained within a clone that hybridized with *avrBs3* and is likely to be an *avrBs3* homolog. The *avrBs3* homologs in *X. o.* pv. *oryzae*, therefore, represent a family of genes of which some are involved in the induction of gene-specific resistance in rice. In contrast, *avrBs3* and the *avrBs3*-like gene *avrBsP* are the only related avirulence genes found in *X. c.* pv. *vesicatoria* (Canteros *et al.* 1991).

Recently, a similar family of genes has been identified in *X. c.* pv. *malvacearum* (Swarup *et al.* 1992). We also detected multiple copies of *avrBs3*-related sequences in *X. o.* pv. *oryzicola* (Fang, Ren, Chen, Chu, Faan, and Ulu) Dye (Swings *et al.* 1990), *X. c.* pv. *vasculorum* (Cobb) Dye, *X. c.* pv. *translucans* (Jones, Johnson and Reddy) Dye, *X. c.* pv. *secalis* (Reddy, Godkin and Johnson) Dye, and *X. c.* pv. *undulosa* (Smith, Jones and Reddy) Dye (data not shown). Relatively few of the *avrBs3* homologs detected in *Xanthomonas* spp. have been established as avirulence genes, and some may have no avirulence activity. The widespread occurrence of the genes may reflect their involvement
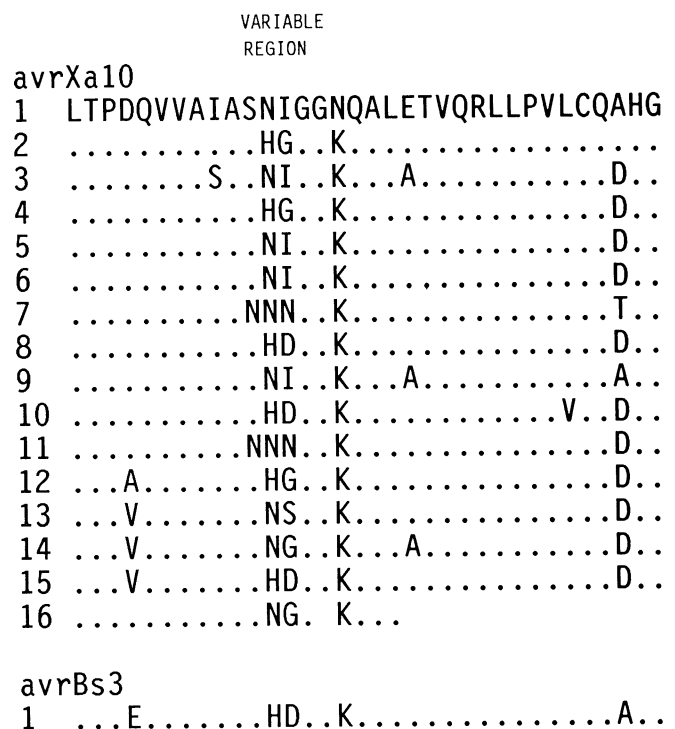


**Fig. 7.** Nucleotide sequence of the avrXa10 repeat domain. Dots represent identical nucleotide compared to repeat 1 of *avrXa10*. The repeat number is indicated to the left of each repeat. The first repeat of *avrBs3* is shown for comparison (Bonas *et al.* 1989). The 6-bp variable region is indicated above the repeat region.



**Fig. 8.** Amino acid sequence of the *avrXa10* repeat domain. The repeat number is indicated on the left, and the 2-aa (amino acid) variable region is indicated above. The first repeat of *avrBs3* (Bonas *et al.* 1989) is below for comparison.

in pathogenicity (Swarup *et al.* 1992) or, alternatively, in functions unrelated to pathogen-plant interactions.

The total number of functional *avrBs3*-related genes in the genome of *X. o.* pv. *oryzae* is not known. However, the number of *avrBs3* homologs can be estimated from the number of *Bam*HI fragments that were observed in Southern analysis. Without considering doublets or multiple copies on larger fragments, we found that total genomic DNA from race 2 strains, for example, contained approximately 12 hybridizing *Bam*HI fragments. From the work described here, at least two of the fragments are contained within active avirulence genes. An antiserum that was raised against the AvrBs3 protein was previously shown to cross-react with six to seven proteins in an extract of strain PXO86, indicating that several genes may be expressed and encode antigenically similar proteins to *avrBs3* (Knoop *et al.* 1991). Although the number of *avrBs3*-related genes that are transcribed and translated cannot be precisely determined from the data, we have shown that multiple avirulence genes are expressed in *X. o.* pv. *oryzae*. On the basis of this information, models for avirulence gene action in *X. o.* pv. *oryzae* will have to account for expression of multiple genes whose products may be closely related, but have different specificities.

Incompatible interactions between *X. o.* pv. *oryzae* and rice are characterized by an increase in the activity of an extracellular cationic peroxidase and a decrease in the rate of bacterial multiplication (Barton-Willis *et al.* 1989; Reimers *et al.* 1992; Reimers and Leach 1991). The timing of both these events corresponds with the phenotypic reaction observed on the plant leaf. However, the timing of incompatible interactions between different avirulence gene-cultivar combinations varies. Incompatible interactions involving cultivars carrying *Xa-10* were characterized by an increase in the extracellular cationic peroxidase within 16–24 hr, which coincided with a decrease in the rate of bacterial multiplication (beginning at 24 hr) (Reimers *et al.* 1992). In incompatible interactions with *avrXa7*, the increase in cationic peroxidase activity and the decrease in the rate of bacterial multiplication were not observed until 48 hr. The timing and intensity of the browning response characteristic of resistance also were delayed in incompatible interactions with *avrXa7* as compared with *avrXa10*. Previously, differences in interactions between PXO86 (a wild-type race 2 strain carrying both *avrXa7* and *avrXa10*) and plants with *Xa-7* or *Xa-10* resistance genes could have been explained as the effect of other genes in PXO86. However, we have demonstrated that the timing of the different interactions when *avrXa7* and *avrXa10* are in the same genetic background (PXO99[A]) is the same as that observed in the wild-type strain (PXO86) (Figs. 2,3). We, therefore, conclude that the timing of the physiological and phenotypical reactions is dependent on the specific avirulence-resistance gene combination. In addition, differences observed in the phenotypes of interactions after inoculation with PXO99[A] containing the chromosomal or plasmid-borne avirulence genes indicate that avirulence genes may have a dosage effect in specific interactions. However, more extensive characterization (e.g., growth curves and insertion mapping) of the marker-exchanged mutants (PXO99[A]2A and PXO99[A]15A) is necessary to confirm a dosage effect.

The cosmid clones with the functional avirulence genes contain additional copies of the *avrBs3* homologous sequence with unknown function in tandem (Fig. 1). In contrast, *avrBs3* and *avrBsP*, the two avirulence genes from *X. c.* pv. *vesicatoria*, are not adjacent to one another, and tandem, nonfunctional sequences are not present (Bonas *et al.* 1989; Canteros *et al.* 1991). Clone pXO6-33, which encodes both *avrxa5* and *avrXa10* activities, contains two *Bam*HI fragments similar in size to those present in pXO5-15, which encodes only *avrXa10* activity. We are investigating whether pXO5-15 and pXO6-33 are overlapping clones and, if so, whether *avrxa5* activity is encoded by the homolog A10L and is affected during cloning. Alternatively, the *avrXa10* gene may be represented in more than one copy on the genome or the same gene may encode *avrxa5* and *avrXa10* activities in pXO6-33.

The *X. o.* pv. *oryzae* avirulence genes, *avrXa7* and *avrXa10*, are very similar to *avrBs3*. We can account for the differences in the sizes of the *Bam*HI fragments contained within the genes (*avrBs3*, 3.3 kb; *avrXa7*, 4.1 kb; *avrXa10*, 3.1 kb) by the differences in estimated copy numbers of the 102-bp repeat structure (17.5, 25, and 15.5, respectively). Bonas *et al.* (1989) reported that the sequence of the *avrBs3* repeat motif is highly conserved, that is, few differences in the nucleotide sequences were observed among the repeats. Comparison of repeat units from *avrXa10* with those of *avrBs3* suggests that the repeat motif of *avrXa10* also is highly conserved even among different *Xanthomonas* species, with the exception of a 6-bp variable region (position 34 of the repeat). Herbers *et al.* (1992) have shown that the specificity of the cloned *avrBs3* gene can be changed by deleting specific repeats within *avrBs3*. We propose that the variation in sequence in the variable region of different genes suggests that the region has some relevance to the specificity of each protein product. The arrangement of specific repeats within the gene, the sequence of a specific repeat unit, or a combination of arrangement and sequence may provide the distinguishing features of each gene with regard to avirulence specificity. The high degree of similarity between the sequence of the N-terminal and C-terminal domains of *avrXa10* and *avrBs3* also supports the notion that the specificity of the different avirulence genes lies within the repeat domain.

Our work adds to the growing list of related genes that function to confer avirulence and extends the types of plants with which they interact from dicots (pepper, Bonas *et al.* 1989; tomato, Canteros *et al.* 1991; and cotton, Swarup *et al.* 1992) to a monocot (rice). Although nothing is known about how the avirulence genes function to confer resistance, it is tempting to speculate that the sequence relatedness of the *avr* genes implies similarity in avirulence gene function and in the mechanism of host recognition.

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