Avoidance of Host Recognition by Alterations in the Repetitive and C-Terminal Regions of AvrXa7, a Type III Effector of *Xanthomonas oryzae* pv. *oryzae*

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avrXa7 is a member of the *avrBs3/pthA* gene family. The gene is a critical type III effector in several strains of *Xanthomonas oryzae* pv. *oryzae* (virulence activity), and in the presence of the *Xa7* host gene for resistance, controls the elicitation of resistance in rice (avirulence activity). The ability of strains containing *avrXa7* to adapt to the presence of *Xa7* in the host population is dependent, in part, on the genetic plasticity of *avrXa7*. The potential for the conversion of *avrXa7* to a virulence effector without *Xa7*-dependent elicitor activity was examined. Internal reorganization of *avrXa7* by artificially deleting a portion of the central repetitive region resulted in gene *pthXo4*, which retained virulence activity and lost *Xa7*-dependent avirulence activity. Similarly, spontaneous rearrangements between repetitive regions of *avrXa7* during bacterial culture gave rise to gene *pthXo5*, which also had virulence activity without *Xa7*-dependent avirulence activity. *pthXo5* appeared to be the result of recombination between *avrXa7* and a related gene in the genome. Loss of avirulence activity and retention of virulence activity also resulted from replacement of a portion of the C-terminal coding region of *avrXa7* with the corresponding sequence from *avrBs3*. The results demonstrated the potential for a critical virulence effector to lose avirulence activity while retaining effector function. The results also demonstrated that features of both repetitive and nonrepetitive C-terminal regions of AvrXa7 are involved in avirulence specificity.

Additional keyword: type III secretion.

Avirulence genes of plant pathogens encode elicitors that are often recognized by elicitor-specific resistance (*R*) genes. The recognition process is advantageous for the host plant in preventing or reducing the growth of potentially damaging pathogens. At the same time, *R* genes create selective pressure on the pathogen population for the emergence of strains with reduced elicitor activity. If the *R* genes correspond to elicitors that are also critical effectors for virulence, the genes create selective pressure for both the avoidance of re-

sistance and a possible reduction in virulence. The imposition of a fitness cost to the pathogen by the host may lengthen the time required for the emergence of a highly virulent pathogen population, since adaptation may require more genetic changes than simple loss of the avirulence gene. In addition to representing an important evolutionary strategy for host plants, deployment of critical elicitor-specific *R* genes has been proposed as a practical strategy for durable *R* genes in crop plants (Lauge et al. 1998; Tai et al. 1999). However, elicitor-specific resistance in the host may be countered by the adaptive mechanisms of the pathogen, and the effectiveness of *R* gene deployment will depend, in part, on the prevalence (and transfer rate) of alternative virulence effectors and the plasticity of specific effectors.

Many avirulence genes of plant pathogenic bacteria encode effector proteins of the type III secretion pathway, and a number of genes have phenotypic virulence activity. The avirulence activity results from the entry of effectors into the host cells via the type III system and recognition by an elicitor-specific *R* gene-mediated pathway. The virulence activity is the mechanism by which the effector facilitates pathogenicity. AvrBs2 was the first type III effector with dual activities (Kearney and Staskawicz 1990; Swords et al. 1996). In the presence of the *Bs2* gene for resistance, AvrBs2 elicits resistance in pepper plants. Loss of *avrBs2* in *X. campestris* pv. *vesicatoria* results in reduced bacterial growth (Kearney and Staskawicz 1990; Swords et al. 1996). In the absence of *Bs2*, bacteria with *avrBs2* have a competitive advantage over bacteria lacking the avirulence gene (Wichmann and Bergelson 2004), and *avrBs2* alleles are appearing in field populations with reduced elicitor activity (Gassmann et al. 2000). AvrBs2 is particularly interesting in that the gene is widespread in a variety of *Xanthomonas* pathogens.

Another dual active avirulence gene is *avrXa7* from *X. oryzae* pv. *oryzae*, the causal organism of bacterial blight of rice (Hopkins et al. 1992). Bacteria containing the *avrXa7* gene elicit a resistance response in the presence of the rice gene *Xa7* (Ogawa et al. 1991; Sidhu et al. 1978). In susceptible rice varieties, *avrXa7* is required for virulence in some strains of *X. oryzae* pv. *oryzae* (Bai et al. 2000; Yang et al. 2000). The mode of action of AvrXa7 as a virulence or avirulence effector is unknown. However, the protein is a member of an effector family and the product of the *avrBs3/pthA* gene family, whose members are found in many strains of *Xanthomonas* (Bonas et al. 1989; De Feyter and Gabriel 1991; Hopkins et al. 1992; Swarup et al. 1991) and at least one strain of *Ralstonia solanacearum* (Salanoubat et al. 2002). The various proteins of the *avrBs3/pthA* family differ primarily in the number and apparent nature of near-identical, 34 direct repeats in the central

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Nucleotide sequence data for fragments of *pthXo4*, *avrXa7-A38*, *pthXo5*, and *avrXa7-sacB50* are available in the GenBank database under accession numbers AY497527, AY497528, AY497529 and AY497530, respectively.

*The e-Xtra logo stands for “electronic extra” and indicates that Figures 1, 3, and 6 appear in color online.

portion, or repetitive region, of the protein (White et al. 2000). The repetitive regions contribute to the function and specificity of the protein products of the *avrBs3/pthA* gene family during the elicitation of resistance and virulence on the respective host species (Herbers et al. 1992; Yang et al. 1994, 2000). The effector function is also controlled by features of the C-terminal region. All of the members encode C-terminal nuclear localization motifs, which in *avrBs3* and *avrXa7*, have been demonstrated to be required for virulence and avirulence activities (Szurek et al. 2001; Yang et al. 2000). A functional eukaryotic transcriptional activation domain is also present at the extreme C terminus (Zhu et al. 1998, 1999) and is required for the virulence and avirulence activities of *avrXa7* and *avrBs3* (Szurek et al. 2001; Yang et al. 2000). Aspects of the C-terminal coding region also can influence the specificity of the avirulence activity of a given gene. For example, *AvrBs3* does not function as an elicitor in *X. oryzae* pv. *oryzae* on rice containing *Xa7*. However, replacement of the C-terminal region of *AvrBs3* with the C-terminal domain of *AvrXa7* results in a protein with *Xa7*-dependent elicitor activity (Yang and White 2004). Mutations in the C-terminal coding sequence have been identified in field studies that inactivate both activities of *avrXa7* (Vera Cruz et al. 2000).

Evidence indicates that the members of the *avrBs3/pthA* gene family are critical effectors in a variety of diseases, including bacterial blight of rice. *avrXa7* is one of four major effector genes of the *avrBs3/pthA* gene family that have been identified in *X. oryzae* pv. *oryzae* (Yang and White 2004). Three additional genes, named *pthXo1*, *pthXo2*, and *pthXo3*, are present in field isolates and have the equivalent virulence activity of *avrXa7* without the *Xa7*-dependent avirulence activity (Yang and White 2004). Theoretically, *avrXa7* loss could be compensated by horizontal gene transfer of a gene without *Xa7*-dependent avirulence activity or the population could be supplanted by a strain harboring effector genes without avirulence activity. Alternatively, new versions of *avrXa7* may arise spontaneously within a strain by internal rearrangement or intergenic recombination between related genes within a strain. Individual strains of *X. oryzae* pv. *oryzae* have an estimated 12 to 14 genes of the *avrBs3/pthA* gene family (Hopkins et al. 1992), and the copies may represent a reservoir of genes for the generation of new alleles as suggested for *X. campestris* pv. *malvacearum* (Yang and Gabriel 1995). To assess the po-

tential for changes involving *avrXa7* itself as a mechanism for adaptation to the presence of *Xa7* in the host population, the effects of sequence rearrangements in the repetitive and C-terminal coding regions of *avrXa7* on virulence and avirulence activities were investigated.

RESULTS

Generation of a new *pth* gene by internal deletions of *avrXa7*.

To determine if internal rearrangements of *avrXa7* alone could result in loss of *Xa7*-dependent avirulence activity without loss of virulence activity, deletions of *avrXa7* were constructed by partially digesting pZWavrXa7 with *MscI*, which cleaves each repeat once, and rejoining the fragments under conditions favoring intramolecular ligation. A total of 47 clones were recovered from the process and were tested for virulence and *Xa7*-mediated avirulence activities after transfer to PXO99^AME2. PXO99^AME2 is a low-virulence strain, due to the mutation of the major virulence effector *pthXo1* and without *avrXa7* avirulence activity (Table 1). Clones containing *avrXa7* restore the strain to full virulence and, in the case of wild-type *avrXa7*, competence for eliciting *Xa7*-dependent avirulence activity. Both virulence and avirulence activities were lost by the deletion process in the majority of clones, which were not analyzed further. Two clones, *avrXa7*-Δ18 and *avrXa7*-Δ38, were identified in which single activities were affected. *avrXa7*-Δ18 had lost avirulence activity, which in this system, consists of a hypersensitive reaction (HR) and darkening of the tissue at the inoculated site in 48 to 72 h, in the presence of *Xa7* in rice cultivar IRBB7, while retaining virulence activity on IR24, a near-isogenic rice cultivar without *Xa7*. *avrXa7*-Δ38 retained avirulence activity on IRBB7 while having lost virulence activity on IR24. The central *SphI* fragment of *avrXa7* was replaced by either the *SphI* fragment of *avrXa7*-Δ18 or *avrXa7*-Δ38, to test whether mutations affecting each activity were, in fact, due to changes within the central *SphI* fragment. The new construct involving *avrXa7*-Δ18 was renamed *pthXo4*, while the construct involving *avrXa7*-Δ38 was given the same name as the original clone (Table 1). In PXO99^AME2, *pthXo4* conferred the same phenotypes as *avrXa7*-Δ18 on resistant and susceptible rice leaves (data not shown). The strain with *pthXo4* did not elicit a HR on plants

Table 1. Bacterial plasmids and strains used in this study

Designation	Genotypes or relevant characteristics	Source or reference
Plasmids^a		
pUC19	Resistance to carbenicillin (Cb')	Yanisch-Perron et al. 1985
pHM1	Broad host range resistance to spectinomycin (Sp')	Hopkins et al. 1992
pZWavrXa7	<i>avrXa7</i> in pBluescript II KS+ unique <i>HindIII</i> site after <i>avrXa7</i> stop codon	Yang et al. 2000
pHMavrXa7	pZWavrXa7 in pHM1 (Cb', Sp')	This study
pZWavrXa7-Δ18	<i>avrXa7</i> -Δ18, <i>MscI</i> partial deletion derivative of pZWavrXa7	This study
pZWavrXa7-Δ38	<i>avrXa7</i> -Δ38, <i>MscI</i> partial deletion derivative of pZWavrXa7	This study
pZWpthXo4	<i>pthXo4</i> , <i>SphI</i> fragment from pZWavrXa7-Δ18 in pZWavrXa7	This study
pZWavrXa7 ^{SK}	<i>nptI-sacB</i> cassette inserted in <i>PstI</i> site of seventh repeat in <i>avrXa7</i> , resistance to kanamycin (Km')	This study
pHMavrXa7 ^{SK}	pZWavrXa7 ^{SK} in pHM1	This study
pZWavrXa7-sacB50	<i>avrXa7-sacB50</i> , deletion of pZWavrXa7 ^{SK}	This study
pZWavrXa7-sacB29	<i>avrXa7-sacB29</i> , deletion of pZWavrXa7 ^{SK}	This study
pZWpthXo5	<i>pthXo5</i> , <i>SphI</i> fragment from pZWavrXa7-sac29 in pZWavrXa7	This study
pZWavrBs3	<i>avrBs3</i> in pBluescript II KS+	Zhu et al. 1998
pZWavrXa7-3HS	<i>avrXa7-3HS</i> , <i>HincII-SalI</i> fragment of <i>avrBs3</i> replacement of <i>HincII-SalI</i> of <i>avrXa7</i> in pZWavrXa7	This study
<i>Escherichia coli</i> strains		
XL1-Blue MRF'	<i>F'proAB lacI^qZAM15 Tn10</i> (Tet')	Stratagene
C2110	Nal ^r Rif ^r (<i>polA1</i> , <i>rha</i> , <i>his</i>)	Leong et al. 1982
<i>Xanthomonas oryzae</i> pv. <i>oryzae</i>		
PXO99 ^A	Philippine race 6; azacytidine resistant clone of PXO99	Hopkins et al. 1992
PXO99 ^A ME2	PXO99 ^A with EZ::TN<KAN2> inserted into <i>pthXo1</i>	Yang et al. 2004

^a All modified versions of pZWavrXa7 in pHM1 have pHM prefix.

with *Xa7* (Fig. 1A, IRBB7, leaf 2) compared with a hypersensitive reaction on IRBB7 caused by the PXO99^AME2 with the original *avrXa7* (Fig. 1A, IRBB7, leaf 3). Both strains had water-soaked symptoms that begin to streak up and down the leaf from the initial site of inoculation by day 3 on the susceptible rice (Fig. 1A, IR24, leaves 2 and 3, respectively). By comparison, the vector control strain PXO99^AME2 (pHM1) has water-soaked lesions limited to the inoculation site (Fig. 1A, IR24, leaf 1). Strains containing *avrXa7*-Δ38 elicited an HR on IRBB7, albeit with less tissue darkening, suggestive of a weaker reaction (Fig. 1A, IRBB7, leaf 4) in comparison with the reaction conferred by *avrXa7* (Fig. 1A, IRBB7, leaf 3). PXO99^AME2 with *avrXa7*-Δ38 had the same lesion phenotype as the vector control on the susceptible line (Fig. 1A, IR24, leaves 4 and 1, respectively).

The effect on avirulence and virulence of the genes was quantified using lesion length measurements after leaf-clip inoculation of IRBB7 and IR24. Avirulence activity was measured using the fully virulent strain PXO99^A containing each gene,

Fig. 1. Avirulence and virulence activities of *pthXo4* and *avrXa7*-Δ38. **A**, Inoculation phenotypes on resistant and susceptible cultivars. *pthXo4* and *avrXa7*-Δ38 were tested for avirulence activity in strain PXO99^AME2 on rice cultivar IRBB7 (left upper panel) and virulence activity on IR24 (right upper panel). Leaves were photographed three days after inoculation for avirulence and virulence. Leaf 1, PXO99^AME2 (pHM1); 2, PXO99^AME2 (*pthXo4*); 3, PXO99^AME2 (*avrXa7*); and 4, PXO99^AME2 (*avrXa7*-Δ38). Avirulence activity is characterized by brown discoloration at the inoculation site, which was shown on leaves 3 and 4 of IRBB7. Virulence activity is characterized by water-soaked lesions extending above and below the inoculated site. **B**, Measurements of lesion lengths on IRBB7. *pthXo4* and *avrXa7*-Δ38 were transferred to PXO99^A and tested for avirulence on rice cultivar IRBB7 by leaf-clip inoculation. Column 1, PXO99^A (pHM1); 2, PXO99^A (*pthXo4*); 3, PXO99^A (*avrXa7*); and 4, PXO99^A (*avrXa7*-Δ38). **C**, Measurement of lesion lengths on IR24. *pthXo4* and *avrXa7*-Δ38 in PXO99^AME2 (columns 2 and 4, respectively) were compared with inoculations with PXO99^AME2 with pHM1 or *avrXa7* (columns 1 and 3, respectively). Lesions in both experiments were measured in centimeters 10 days after inoculation. Letters above columns in B and C indicate significant differences at the $P < 0.05$ level.

in order to distinguish the effects of resistance from loss of virulence. *pthXo4* had no effect on the length of lesions when PXO99^A with and without the gene was inoculated on IRBB7 (Fig. 1B, columns 2 and 1, respectively), while *avrXa7* in PXO99^A reduced the length of lesions by 73% (Fig. 1B, column 3). *avrXa7*-Δ38 caused a reduction of only 40% (Fig. 1B, column 4), again indicating a resistance reaction that is weaker than the wild-type *avrXa7*. Measurements of lesion length due to the genes in PXO99^AME2 on the susceptible cultivar indicated that *pthXo4* had approximately 80% the activity of *avrXa7* (Fig. 1C, columns 2 and 3, respectively). *avrXa7*-Δ38 had no measurable virulence activity in PXO99^AME2 (Fig. 1C, column 4). The central *SphI* fragments of both genes were sequenced (discussed below).

A new effector gene without avirulence activity can arise spontaneously from *avrXa7*.

The *pthXo4* gene was derived from in vitro manipulation of *avrXa7*. The potential for variants of *avrXa7* to arise spontaneously was examined, using a negative selection scheme that allowed the identification of strains in which de novo rearrangements of *avrXa7* had occurred. Negative selection was accomplished using *sacB*. A gene cassette containing *sacB*, which encodes levansucrase and confers sensitivity to *Xanthomonas* and other species at sucrose concentrations above 5%, and a gene for resistance to kanamycin (*nptII*) was inserted at the *PstI* site in the seventh repeat of *avrXa7*, creating pZWavrXa7^{SK} (Fig. 2). pZWavrXa7^{SK} was, in turn, cloned in the wide host range vector pHM1 (pHMavrXa7^{SK}) and was introduced into PXO99^AME2. Due to the *sacB*/*nptII* cassette, pHMavrXa7^{SK} does not confer virulence or avirulence activity. PXO99^AME2 (pHMavrXa7^{SK}) was plated on 5% sucrose and spectinomycin (to select for strains that retained pHMavrXa7^{SK}). Sucrose-resistant colonies were then tested for an increase in virulence or *Xa7*-mediated avirulence activity. Sucrose-resistant colonies arose at a frequency of 1.1×10^{-3} . A total of 78 sucrose-resistant colonies were visually screened for avirulence and virulence activity by inoculation of IRBB7 and IR24, respectively (data not shown). Five strains had only *Xa7*-mediated avirulence activity, three strains had only virulence activity, and the remainder had neither activity. No strains were recovered with both activities. Plasmids were retrieved from the strains that had gained either activity, were transferred anew to PXO99^AME2, and were retested for the virulence and avirulence properties, and the central *SphI* fragments containing the repetitive regions were partially sequenced (data not shown). After sequencing, the five clones with avirulence activity were determined to have identical repetitive regions, and three clones with virulence activity had identical sequences (data not shown). The gene in pZWavrXa7-sacB50 was named *avrXa7*-

Fig. 2. Map of *avrXa7*^{SK}. The gene is 4,341 bp in length, not including the *sacB*/*nptII* element in the seventh repeat. Functional regions indicated are the three nuclear localization signal motifs (NLS1, 2, 3) and an acidic activation domain (AD) in the 3' end. Restriction enzyme cleavage sites: B = *Bam*HI; P = *Pst*I; and S = *Sph*I.

sacB50. The gene in pZWavrXa7-*sacB29*, as a pathogenicity gene without avirulence activity, was renamed *pthXo5*.

The central *SphI* fragment of *avrXa7*, as represented in pZWavrXa7, was replaced with the central *SphI* fragments of *avrXa7-sacB50* or *pthXo5* individually, to ensure that the associated phenotypes were attributable to regions within the *SphI* fragment. The clones, in turn, were cloned into the broad host range vector pHM1 and were introduced into PXO99^ΔME2. PXO99^ΔME2 with the parental gene *avrXa7* elicited a HR on IRBB7 (Fig. 3A, IRBB7, strain 2), while the same strain with *pthXo5* did not elicit a HR (Fig. 3A, IRBB7, strain 3). PXO99^ΔME2 with either gene produced a virulent response on the susceptible cultivar IR24 (Fig. 3A, IR24, strains 2 and 3, respectively). PXO99^ΔME2 with *avrXa7-sacB50*, on the other hand, elicited a HR on IRBB7 (Fig. 3A, IRBB7, strain 4), as expected. However, the strain also elicited a HR on IR24 (Fig. 3A, IR24, strain 4). An additional cultivar was tested to assess whether the reaction was specific to IR24 and IRBB7 or was a general reaction of all rice lines. PXO99^ΔME2 with *avrXa7-sacB50* gave neither a HR nor a virulent phenotype on rice cultivar Nipponbare (Fig. 3A, NIP, strain 4). The virulence contribution of *pthXo5* in PXO99^ΔME2 was the same whether IR24 or IRBB7 was inoculated (Fig. 3B and C, columns 2, respectively). However, the potency based on lesion length was approximately 80% of the effect due to *avrXa7* on the susceptible cultivar IR24 (Fig. 3C, compare columns 2 and 3). The effects of *avrXa7-sacB50* on lesion length were not measured. IRBB7, which is near-isogenic to IR24, may also contain the newly recognized *R* gene or the *R* gene in IR24 may be an allele of *Xa7*.

The central *SphI* fragments of the genes that were generated by enzymatic or selection techniques were sequenced and compared with the parental and previously characterized genes of the *avrBs3/pthA* family (Fig. 4). *pthXo4* suffered a deletion of 12 repeat units from *avrXa7*, starting at repeat 7 and extending through repeat 19, while *avrXa7-Δ38* was missing repeats 17 to 23 of the original *avrXa7* (Fig. 4). No sequence polymorphisms were observed in the nonrepetitive regions of the *SphI* fragments of either gene in comparison with *avrXa7*, indicating that the phenotypes due to the genes were due to the repetitive domain rearrangements (data not shown). Sequence analysis indicated that *avrXa7-sacB50* contained 10 repeats and was the result of a 16-repeat deletion of *avrXa7*^{SK} (Fig. 4). *pthXo5* contained 19 repeats. Alignment analysis indicated that *pthXo5* differed from *avrXa7*^{SK} by the absence of a 17-repeat section of the original gene and the presence of a new 10-repeat section starting at repeat 6 and ending at repeat 23 of the original gene (Fig. 4). Presumably, *pthXo5* was the result of recombination with another gene with the same last three repeats and, possibly, some of the same 5' proximal repeats or, alternatively, deletion of seventeen repeats of the original gene and subsequent insertion of ten new repeats from another endogenous gene in PXO99^ΔME2.

Replacement of the C-terminal region of AvrXa7 resulted in loss of Xa7-dependent elicitor activity.

The above approaches demonstrated that changes within the repetitive region altered the activity of *avrXa7*. At the same time, changes in the C-terminal nonrepetitive region can also alter avirulence specificity. A previous study indicated that *avrBs3* in *X. oryzae* pv. *oryzae* could function with *Xa7*-dependent avirulence manner in the rice system if the C-terminal coding region was replaced with the C-terminal coding sequences from *avrXa7* (Yang and White 2004). The results indicated that the C-terminal coding region could have a profound effect on the avirulence activity of AvrBs3/PthA effectors. Therefore, the effect of partial replacement of the C-terminal

coding region of *avrXa7* on elicitor activity was examined by replacing the sequences with the equivalent region of *avrBs3*. The *HincII-SalI* fragment of *avrXa7*, which is 448 bp in length and encodes 149 amino acids of the C-terminal portion (Fig. 5), was replaced with the *HincII-SalI* fragment of *avrBs3* (Fig. 5). The region encodes 12 differences in amino acid residues between AvrXa7 and AvrBs3 (Fig. 5). The hybrid gene *avrXa7-3HS* was introduced into PXO99^ΔME2 and tested on IRBB7 and IR24. The gene lost the ability to elicit a *Xa7*-dependent HR on IRBB7, which was observed with *avrXa7* (Fig. 6A, IRBB7, leaf 2), and conferred a virulent phenotype on PXO99^ΔME2 when the strain was inoculated on IRBB7 (Fig. 6A, IRBB7, leaf 3) or IR24 (Fig. 6A, IR24, leaf 3).

Fig. 3. Avirulence and virulence activities of *pthXo5* and *avrXa7-sacB50*. **A**, Inoculation phenotypes on three rice varieties. *pthXo5* and control genes were tested for avirulence and virulence activities in strain PXO99^ΔME2 on rice cultivars IRBB7 (left upper panel) and IR24 (middle upper panel), respectively. *avrXa7-sacB50* in strain PXO99^ΔME2 was tested on IRBB7, IR24, and Nipponbare (right upper panel). Pictures were taken three days after inoculation. Strains are indicated below each leaf. Strain designations: 1, PXO99^ΔME2 (pHM1); 2, PXO99^ΔME2 (*avrXa7*); 3, PXO99^ΔME2 (*pthXo5*); and 4, PXO99^ΔME2 (*avrXa7-Δ50*). NIP, Nipponbare. **B**, Effect of *pthXo5* on lesion lengths in rice cultivar IRBB7. Column 1, PXO99^ΔME2 (pHM1); 2, PXO99^ΔME2 (*pthXo5*), and 3, PXO99^ΔME2 (*avrXa7*). Lesions were measured 10 days after inoculation. Letters above columns indicate significant differences at the *P* < 0.05 level. **C**, Effect of *pthXo5* on lesion lengths in rice cultivar IR24. Same designations and conditions as for B.

Measurements of lesion lengths after inoculation with PXO99^ΔME2 with *avrXa7* or *avrXa7-3HS* were of equivalent lengths indicating no loss of virulence activity (Fig. 6B). Thus, changes in the C-terminal region can cause the loss of avirulence activity without loss of effector function.

DISCUSSION

The effectiveness of crop protection strategies involving genetic resistance depends, in part, on the ability of individual members of the pathogen population to adapt to the deployment of elicitor-specific *R* genes in the predominant host plant population. Here, an analysis of *avrXa7* was performed to assess the potential for variants in corresponding gene *avrXa7* to arise that circumvent the concomitant avirulence activity of the wild-type gene while retaining the virulence function. In the first approach, simple partial deletion of repeats of *avrXa7* was shown to give rise to a functional effector capable of evading the *Xa7*-mediated recognition process. Although performed artificially, deletions of 12 repeats could presumably occur within any recombination-competent strain of *X. oryzae* pv. *oryzae* by an intragenic recombination event or intermolecular exchange between two copies of *avrXa7*. Spontaneous, or in vivo, generation of a new pathogenicity gene was observed by the second approach. *pthXo5* gained virulence activity without the associated avirulence activity of *avrXa7* by a recombination event occurring within a bacterium. The recombination event occurred in strain PXO99^ΔME2, which is derived from a highly virulent strain, PXO99, from The Philippines and has a disruption of the major pathogenicity gene *pthXo1* (Hopkins et al. 1992; Yang and White 2004). However, the sequence of *pthXo5* indicates that the gene was not the result of a simple recombination between *avrXa7*^{SK} and *pthXo1*, the major effector gene of PXO99^Δ, but rather, recombination between *avrXa7*^{SK} and another gene in this strain (Yang and White 2004). That *pthXo4*, which theoretically could be derived from *avrXa7*^{SK}, was not observed by spontaneous rearrangement may be due to a greater frequency of intergenic recombination compared with intragenic recombination or simply due to chance.

In any event, the repetitive regions of *pthXo4* and *pthXo5* are not identical to any previously characterized *avrBs3/pthA* family member and are the fourth and fifth pathogenicity genes to be identified from *X. oryzae* pv. *oryzae*. Three naturally occurring genes, *pthXo1* to *pthXo3*, were identified in diverse strains of *X. oryzae* pv. *oryzae*, in a recent study (Yang and White 2004). The genes were named using the precedent of *pthA* (pathogenicity) from *X. axonopodis* pv. *citri* (Yang et al. 1994), and none of the *pth* genes have known corresponding resistance genes in their respective hosts. The appearance of new effectors without *Xa7*-mediated recognition and the presence of alternate effectors in extant strains indicate that a diverse group of functionally equivalent or nearly equivalent virulence effectors of the *AvrBs3/PthA*

family exist in natural populations or can be generated by recombination within an individual bacterium.

Concurrently, it was discovered that exchanging the C-terminal coding region of *avrXa7* with the similar (but not identical) region of *avrBs3* resulted in the loss of *Xa7*-mediated avirulence activity and retention of effector function. This analysis was prompted by the recent observation that *avrBs3* conferred *Xa7*-mediated avirulence activity to *X. oryzae* pv. *oryzae* if a portion of the C-terminal coding region of *avrXa7* is used in place of the *avrBs3* terminal region (Yang and White 2004). Therefore, critical structural features for avirulence specificity also reside in the C-terminal region. Mutations were also identified in the C-terminal coding region of *avrXa7* from field strains occurring on plants harboring *Xa7* (Vera Cruz et al. 2000). However, the genes from the field strains, to the extent they were characterized, appear to have lost both virulence and avirulence activities (Vera Cruz et al. 2000).

The process leading to *pthXo5* was biased, due to the *sacB* insertion in the seventh repeat. Gene recovery required, minimally, the removal of repeat 7. Nevertheless, both *pthXo5* and *pthXo4* suffered rearrangements starting at the sixth repeat, which may indicate the presence of critical repeats involved in *Xa7* recognition at or near this position. Furthermore, the lack of genes with single and relatively short repeat deletions may reflect intimate or close linkage of structural domains required for avirulence and domains required for effector virulence function. In other words, most deletions caused the loss of both activities. However, all of the derived genes were not sequenced, and therefore, it is unknown whether all possible deletions were generated and tested. The virulence activities of *pthXo5* also may have been generated de novo from the new arrangements of repeats and did not necessarily involve deletion of repeats from the original *avrXa7*. However, more detailed analyses and understanding of *avrXa7* function are required before the role of specific repeats in virulence and avirulence function are known. It is also interesting to note that the nonfunctional *avrXa7* gene product in strain PXO2684 had two amino acid replacements (LEARG) as compared with *AvrXa7* (FEARY), which are also variant in *AvrBs3* (LEARS) (Vera Cruz et al. 2000). These amino acid residues may have important roles in the function and specificity of *avrXa7* gene product. The studies of *AvrXa7* variants may, therefore, provide insight as to important regions of the protein for elicitor and effector activity.

The results have implications for practical considerations in the deployment of *Xa7* resistance in areas where *avrXa7* is a predominant avirulence gene and, possibly, a predominant effector. The evidence here supports the prediction that virulent strains can emerge, provided the processes employed here readily occur in the field. At the same time, the actual timing of the emergence of highly virulent strains on rice plants with the *Xa7* gene in field or natural situations is difficult to predict precisely, due to many other factors that might affect the

Effector																																
AvrXa7-Δ38		NI	HG	NI	NI	NS	HD	NN	HD	HD	HD	NS	N*	N*R	HD	HD	NS	-----										N*	NS	N*		
PthXo4		NI	HG	NI	NI	NS	HD	NN	-----										NI	NG	NN	NI	N*	NS	N*							
AvrXa7-sacB50		NI	HG	NI	NI	NS	HD	-----										NI	N*	NS	N*											
PthXo5		NI	HG	NI	NI	NS	HD	-----										NI	HD	HG	HD	NI	N*	NS	NI	NI	HG	--		N*	NS	N*
AvrXa7		NI	HG	NI	NI	NS	HD	NN	HD	HD	HD	NS	N*	N**	HD	HD	NS	NS	NN	NN	NI	NG	NN	NI	N*	NS	N*					
PthXo1		NN	HD	NI	HG	HD	NG	N*	HD	HD	NI	NG	NG	NI	HD	NG	NN	NG	NI	NI	NI	NI	N*	ND	N*							
Repeat #		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26					

Fig. 4. Alignment of variable regions from repetitive region of *AvrXa7* and its derivatives. Each repeat is represented schematically by the twelfth and thirteenth variable residues. Single amino acid code was used for each amino residue. An asterisk (*) represents missing thirteenth residue in some repeats. R indicates the additional six amino acid residue repeat within repeat 13 of the original *AvrXa7*. The repeats are aligned to possible similar repeat segments in the other proteins. Dash lines represent gaps in alignment. The underlined residues of *PthXo5* designate the new repeats derived from other *avrBs3/pthA* genes. Also shown for comparison is the repetitive region of *PthXo1*.

growth of the bacteria. Field studies in The Philippines have provided evidence that *avrXa7* is a predominant avirulence gene within regional bacterial populations. However, breakdown of the *Xa7* resistance has not been observed for three years covering six growing seasons (Vera Cruz et al. 2000). The results are not necessarily incongruent. Three years may be insufficient time for the detection of new effector genes arising in the population, or conditions were not optimal for selection of more virulent individuals. Alternatively, IRBB7, the line used in the field studies, may have additional resistance genes, which are not found in the near-isogenic line IR24 and must also be overcome by the pathogen population, that are delaying emergence of more highly virulent strains. Though near-isogenic, marker studies with IR24 and IRBB7 indicate that the lines have considerable amounts of genetic material from both parents, particularly in the region of *Xa7* (Porter et al. 2003; Yoshimura et al. 1996).

Field studies with plant pathogenic bacteria with mutations in type III effectors have demonstrated that the effectors can have important contributions to environmental fitness in terms of increased pathogen population and transmission to uninfected plants (Wichmann and Bergelson 2004). The type III system is critical for pathogenicity of *X. oryzae* pv. *oryzae* under glasshouse conditions and, undoubtedly, can be expected to be critical for natural field infections (Zhu et al. 2000). The presence of multiple copies of *avrBs3/pthA* genes may, as proposed by Yang and Gabriel (1995), represent the evolution of a system for the rapid generation of new *pth* genes in the event of host recognition or avoidance of the extant *pth* genes. It should also be noted that a repetitive region is also present in AvrBs2, which is another effector in which changes in elicitor activity without loss of virulence function may be advantageous (Swords et al. 1996). Rice is an ancient crop intensively managed under conditions favorable for bacterial disease, and the extensive amount of genetic material makes the rice/*X. oryzae* pv. *oryzae* pathogen system an attractive subject for studies of host-pathogen interactions. A better understanding of the diversity and plasticity of AvrBs3/PthA effectors of *X. oryzae* pv. *oryzae* should provide insight into the design and deployment of resistance genes in rice.

Fig. 5. Replacement of *avrXa7* C-terminal coding region with the corresponding fragment from *avrBs3*. The *HincII*/*SalI* fragment is shown as unfilled box. The repetitive region is represented by the gray box. The amino acid sequences of the corresponding regions are shown below with polymorphic amino acid residues in bold and underlined.

MATERIALS AND METHODS

Bacterial strains, plasmids, and DNA manipulations.

Bacterial strains and plasmids used in this study are listed in Table 1. Standard culture and DNA techniques were used for *Escherichia coli* and recombinant DNA manipulations (Ausubel et al. 1988). *X. oryzae* pv. *oryzae* was grown in either nutrient broth (Difco Laboratories, Detroit) or tryptone sucrose agar (TSA) medium (in grams per liter: tryptone, 10; sucrose, 10; glutamic acid, 1; Difco Bacto agar, 15) at 28°C. Plasmids were introduced into *E. coli* and *X. oryzae* pv. *oryzae* by electroporation. Antibiotics used in this study were (in micrograms per milliliter) carbenicillin, 100; cephalixin, 10; kanamycin, 50; and spectinomycin, 100.

Plant materials and virulence assays.

Rice lines IRBB7, which contains the resistance gene *Xa7*, and IR24 were used for avirulence and virulence tests, respectively (Ogawa et al. 1991). Virulence assays were performed on 14- and 20-day-old rice plants by needleless syringe infiltration (Reimers and Leach 1991) and leaf-clip inoculation, respectively, in growth chambers set at 28°C with 85% humidity for a 12-h day and night cycle (Kauffman et al. 1973).

Fig. 6. Avirulence and virulence activities of *avrXa7-3HS*. **A**, Effect of *avrXa7-3HS* on inoculation phenotype of resistant and susceptible rice. PXO99^ΔME2 with *avrXa7-3HS* was inoculated on IRBB7 (left panel) and IR24 (right panel). Leaf 1, PXO99^ΔME2 (pHM1); 2, PXO99^ΔME2 (*avrXa7*); and 3, PXO99^ΔME2 (*avrXa7-3HS*). **B**, Effect of *avrXa7-3HS* on lesion lengths. Strains were inoculated by leaf-clip inoculation on IRBB7 (left panel) and IR24 (right panel). Column 1, PXO99^ΔME2 (pHM1); 2, PXO99^ΔME2 (*avrXa7*); and 3, PXO99^ΔME2 (*avrXa7-3HS*). Different letters above each column indicate significant differences below the *P* < 0.05 level.

Symptoms were scored by measuring lesion lengths for clipping inoculation and observation of water-soaking after syringe infiltration at the intervals indicated in the text. One-way analysis of variance (ANOVA) statistical analyses were performed on all measurements. The Tukey's studentized range HSD (honest significant difference) test was used for post-ANOVA pair-wise tests for significance, which was set at 5% ($P < 0.05$).

Deletion construction.

For in vivo deletion selection, the 3.8-kb *nptI-sacB* cartridge (Ried and Collmer 1987) was inserted into the *PstI* site in the seventh repeat of *avrXa7*, as presented in the clone pZWavrXa7, to create *avrXa7^{SK}*. *avrXa7^{SK}* was then cloned into the broad host range vector pHM1, and the plasmid was named pHMavrXa7^{SK} and was transferred to PXO99^AME2. Since PXO99^AME2 is resistant to kanamycin, PXO99^A was used to test the frequency of the loss of both resistance to kanamycin and sensitivity to elevated sucrose levels (5%) due to *nptI* and *sacB*, respectively, and 98% of the clones that lost *sacB* also had lost resistance to kanamycin. PXO99^AME2 with pHMavrXa7^{SK} was plated on TSA with 5% sucrose and spectinomycin. A total of 78 colonies were assayed for virulence and avirulence on IR24 and IRBB7, respectively. The pHM1 clones were recovered from selected strains and introduced back into PXO99^A and PXO99^AME2. The strains were assayed for virulence and Xa7-dependent avirulence activities to determine if the activities associated with the original strain were due to the gene on pHM1 from each strain. In vitro deletions of *avrXa7* were generated by partially digesting pZWavrXa7 with *MscI*, which cleaves once in each repeat, were gel size-fractionated, were self-ligated, and were transformed into *E. coli* XL1-blue. Plasmids with deletion of one to ten repeats (42 clones total) were divided into three pools, were digested with *HindIII*, and were ligated into pHM1. Clones were transformed into PXO99^AME2 by electroporation. Cells were plated on the TSA with spectinomycin, and 47 individual transformants were tested for virulence and avirulence on IR24 and IRBB7.

Gene sequencing and characterization of *avrXa7* derivatives.

The *SphI* fragments with the repetitive regions were sequenced in their entirety. The fragments were cloned into pBluescript SK (Stratagene, La Jolla, CA, U.S.A.) and were sequenced from both ends, using the T3 and T7 primers. The internal sequence was derived by transposition tagging with EZ::TN<KAN-2> (Epicentre Technologies, Inc, Madison, WI, U.S.A.) and sequencing using outwardly directed primers derived from the right and left ends of the transposon. The *SphI* fragments of candidate clones were exchanged with the *SphI* fragment of *avrXa7* in pZWavrXa7 (Table 1), were linearized with *HindIII*, and were ligated into the broad host range vector pHM1. The ligated DNA was electroporated into *E. coli* C2110, and the transformed cells were plated on Luria-Bertani agar plates containing spectinomycin and carbenicillin. Plasmid configuration was verified by restriction enzyme analysis, and plasmids were electroporated into the respective strains. The DNA sequencing was performed at the sequencing facilities of Kansas State University. The sequences of the *SphI* fragments of *pthXo4*, *avrXa7-Δ38*, *pthXo5*, and *avrXa7-sacB50* were deposited as GenBank accession AY497527, AY497528, AY497529 and AY497530, respectively.

Construction of *avrXa7-3HS*.

The plasmid pZWavrBs3 was digested with *HincII* and *SalI* and the fragment was gel-purified. The *SalI* restriction site was previously introduced immediately downstream of the second

SphI site in the 3' end of *avrXa7* and *avrBs3* upon addition of a FLAG epitope (Zhu et al. 1998). The *HincII/SalI* fragment of *avrBs3* was exchanged with the corresponding fragment of *avrXa7* to create *avrXa7-3HS* from pZWavrXa7, which contains coding sequences of all N-terminal, the repetitive region, and 44 additional amino acids at the proximal C-terminus of *AvrXa7*, and the approximately 400-bp *HincII/SalI* fragment of the C-terminal coding region of *avrBs3*. The resulting plasmid was cloned in pHM1 and was introduced into PXO99^AME2 by electroporation

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