The virulence factor AvrXa7 of *Xanthomonas oryzae* pv. *oryzae* is a type III secretion pathway-dependent nuclear-localized double-stranded DNA-binding protein

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***AvrXa7* is a member of the *avrBs3* avirulence gene family, which encodes proteins targeted to plant cells by a type III secretion apparatus. AvrXa7, the product of *avrXa7*, is also a virulence factor in strain PXO86 of *Xanthomonas oryzae* pv. *oryzae*. Avirulence and virulence specificities are associated with the central repeat domain, which, in *avrXa7*, consists of 25.5 direct repeat units. Mutations in three C-terminal nuclear localization signal motifs eliminated avirulence and virulence activities in rice and severely reduced nuclear localization in a yeast assay system. Both pathogenicity functions and nuclear localization were restored on the addition of the sequence for the nuclear localization signal motif from SV40 T-antigen. The loss of avirulence activity because of mutations in the acidic transcriptional activation domain was restored by addition of the activation domain from the herpes simplex viral protein VP16. The activation domain was also required for virulence activity. However, the VP16 domain could not substitute for the endogenous domain in virulence assays. In gel shift assays, AvrXa7 bound double-stranded DNA with a preference for dA/dT rich sequences. The results indicate that products of the *avrBs3*-related genes are virulence factors targeted to host cell nuclei and have the potential to interact with the host DNA and transcriptional machinery as part of their mode of action. The results also suggest that the host defensive recognition mechanisms are targeted to the virulence factor site of action.**

avirulence | rice | disease

Two avirulence genes have been cloned from *Xanthomonas oryzae* pv. *oryzae*, the causal agent of bacterial blight of rice. Bacteria containing *avrXa7* or *avrXa10* elicit resistance on rice cultivars containing the resistance genes *Xa7* and *Xa10*, respectively (1). Both genes are members of the *avrBs3* gene family, which are found in a variety of species of *Xanthomonas*. The members of the family are nearly identical but differ in the number and apparent nature of a series of near-identical 102-bp direct repeats in the central portion of the coding sequence (reviewed ref. 2). The activities of *avrBs3*-related genes depend on a type III secretory pathway known as the hypersensitive reaction and pathogenicity or Hrp pathway (3, 4). Like a variety of type III-dependent proteins, the products of the *avrBs3* family are secreted from the bacteria and translocated into the host cells (reviewed in ref. 5). Avirulence genes were named for the elicitation of disease resistance by the bacteria harboring the genes on inoculation to the appropriate cultivar of an otherwise susceptible plant host. However, the function of many avirulence genes from the perspective of the bacterium is their role in the virulence. In fact, many bacterial avirulence genes are dual-acting proteins (reviewed in ref. 6). These proteins elicit resistance in one context and are involved in the virulence of the bacterium on susceptible host plants. A variety of genes in the *avrBs3* gene family are dual acting, including *pthA* from *Xanthomonas citri* (7), *avrb6* and *pthN* from *X. campestris* pv. *malvacearum* (8–10), and *avrXa7* (11, 12).

The biochemical functions of protein products of the *avrBs3* family are unknown. Critical structural features involved in specificity for avirulence and virulence lie within the repeat domains (13–16). For example, replacement of the repeat coding region in *avrXa10* with the corresponding region of *avrXa7* converts *avrXa10* to a gene with avirulence specificity for *Xa7* and loses activity toward *Xa10* (14). Exchange of the repeat domains of *avrb6* and *pthA* demonstrated that specificity for virulence on the respective hosts was also controlled by the repeat domain (16). All members of the *avrBs3* family also encode functional nuclear localization signal (NLS) motifs and acidic transcriptional activation domains (ADs) in the C-terminal coding regions (14, 17). Experimental evidence for *avrBs3* and *avrXa10* has indicated that the NLS and AD motifs are required for avirulence (14, 18, 19). The C-terminal features suggest that the proteins, in their capacity as virulence factors, may be targeted to the cell nucleus. However, no evidence has been reported indicating whether the NLS or AD was critical for the role of these proteins as virulence factors. We therefore examined the role of the various structural features as represented in *avrXa7* on the virulence of *X. oryzae* pv. *oryzae*. Furthermore, the similarities of gene products to eukaryotic transcription factors also led us to examine the potential of AvrXa7 protein to bind DNA.

Materials and Methods

Strains, Plasmids, and DNA Manipulations. Standard cultural methods and recombinant DNA techniques were used for *Escherichia coli* (20). *X. oryzae* pv. *oryzae* strain PXO99^{A}, which lacks an endogenous copy of *avrXa7* or *avrXa10*, was used for avirulence activity testing (1). Strain PXO86mx53, which was obtained by marker exchange of transposon mutant p29–29::Tn5B20–53 (1), was used for virulence testing. The plasmid pZW*avrXa10* contains the *avrXa10* coding sequence fused to the *lacZ* promoter of pBluescriptKS+ (Stratagene) (14). The 3.2-kb *Sph*I fragment of *avrXa7* was derived from cosmid p29–29, which is a genomic clone from strain PXO86 (1). AD mutations in *avrXa7* were prepared by replacing the *Sph*I fragment of previously con-

Abbreviations: NLS, nuclear localization signal; AD, transcriptional activation domain.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. AF275317).

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structed mutants of *avrXa10* with the *Sph*I fragment of *avrXa7* (14, 19). Codon numbering refers to corresponding codons in *avrXa10*. Methods for yeast manipulations were as described (21). Sequencing was performed at the Iowa State University and Kansas State University Sequencing Facilities.

Pathogenicity Tests. Rice line IR24 is susceptible to PXO99 and PXO86 and was used for virulence assays. Rice lines IRBB10 and IRBB7 contain resistance genes *Xa10* and *Xa7* and were used for assaying avirulence activities of *avrXa10* and *avrXa7*, respectively. Virulence assays were performed on 2-week-old rice plants by needleless syringe infiltration (14) and leaf-clip inoculation in growth chambers (22). Symptoms were scored by measuring lesion lengths after 11 days for the leaf-clip inoculations and noting the appearance of water soaking after 3 days for the syringe infiltrations. Lesion length measurements are averages of 25 leaves.

Nuclear Localization Assays. The primers 5′-ACCTCTGCAGCAGAGTGGGCGTCACCGAATTCGAGC-3′ and 5′-TCAGAAGCTTCACTGAGGTTTATCATCGTCATCCTTGTAATCCAATAGCTCCATCAACCATGC-3′ were used to amplify the C-terminal coding region from pZW22 (*avrXa10*$^{nls123-}$) (14). The second primer contains a FLAG epitope encoded immediately upstream of the stop codon. The PCR DNA was partially digested with *Eco*RI, completely with *Hin*dIII, and then ligated into pZW*avrXa7* and pKSXa7(PH), resulting in pZW*avrXa7*M123 and pKS*avrXa7*M123, respectively. The plasmid pKSXa7(PH) was generated by ligating a partially digested *Pst*I-*Hin*dIII fragment from pZW*avrXa7* into pBluescriptKS+. Both pKS*avrXa7* and pKS*avrXa7*M123 were cut with *Xho*I, partially digested with *Bam*HI, and then ligated into vector pNIA (23) to generate pNIAXa7 and pNIAXa7 M123, respectively. Primers NLS1SV408 5′-CAGTAGATCTCCGACCAAAGAAGAAGCGCAAGGTCTCAGCTCAAACGCCGGATCAGGCGTC-3′ and NLS 5′-CAGTAGATCTCTCATGGCGCGTTAAGCGCCCACGCACTTCAGCTCAAACGCCGGATCAGG-3′, which contain NLS sequences of SV40 (RPKKKRKV) and native NLS3 (RVKRPR), respectively, were used with 3′ end primer wt2rev 5′-TCCTAAGCTTGACTGAGCCTCACTGAGGCAATAGCTCC-3′, which contains a stop codon and a *Hin*dIII site. Template DNA was pZW*avrXa7*M123. The *Bgl*II-*Hin*dIII fragment of the PCR product was ligated into pZW*avrXa7*M123 to generate plasmids pZW*avrXa7*SV40 and pZW*avrXa7*MN3, respectively. pZW*avr*Xa7SV40 was used to generate pNIAXa7SV40. The plasmids pZW*avrXa7*, pZW*avrXa7*M123, pZW*avrXa7*SV40 and pZW*avrXa7*MN3 were cut with *Hin*dIII and ligated into pHM1, then conjugated into PXO99A and PXO86mX53, respectively, by mating with the helper strain HB101 containing pRK2013. Respective pNIA fusion plasmids (pNIA, pNIAXa7, pNIAXa7 M123, and pNIAXa7SV40) were transformed into yeast strain L40, which contained reporter genes *His3* and *LacZ*. The transformed yeast cells were used to measure β-galactosidase activity for *lacZ* activation as described previously (19). Activation of *His3* was assessed by growth on histidine-deficient media.

DNA-Binding Assays. The *avrXa7* coding region from pZW*avrXa7* was cut by *Hin*dIII, partially with *Pst*I, and then ligated into expression vector pPROEX (GIBCO/BRL). Primer 5′-GGGGTACCAGGAGATCTGCCATGGACCCCATTCGTTCG-3′ was used to add a *Bgl*II site upstream of the start codon of *avrXa7*, and the DNA was then cut with *Bgl*II and *Hin*dIII and ligated into pGEX vector (Amersham Pharmacia Biotech). The respective fusion proteins of AvrXa7 with 6His- or GST-tag were expressed and purified by affinity chromatography according to the supplier (Amersham Pharmacia Biotech). The DNA was labeled with ^{32}P by end labeling for PolydA, T, C, G, A/T, and G/C and by PCR for random oligonucleotides, which are 5′-CAGGGCATGTGGATCCCN$_{25}$GGGCCTGCAGGAATTCGA-3′, where N$_{25}$ indicates 25 randomly synthesized bases. Purified protein (approximately 30 ng) was incubated in 12 mM Tris (pH 7.9)/12% glycerol/35 mM KCl/0.07 mM EDTA/8.5 mM $MgCl_2$ with 1–2 ng of labeled DNA at room temperature for 20 min. The reaction products were separated on a 6% native PAGE gel with a running buffer of 25 mM Tris (pH 8.3)/190 mM glycine/1 mM EDTA.

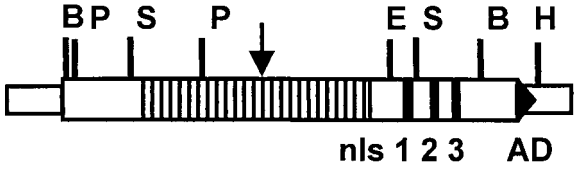

Fig. 1. Map of *avrXa7*. The repeat domain is shown as a series of boxes in the middle of the gene. nls 1 2 3, NLS sequences 1, 2, and 3. AD, acidic transcriptional activation domain. E, *Eco*RI; P, *Pst*I; S, *Sph*I; H, *Hin*dIII. Arrow indicates position of Tn5-B20::53 insertion used to generate PXO86mx53 (see *Results*).

Results

***AvrXa7* Contains a Unique and Unusual Repeat Region That Determines Avirulence Specificity for *Xa7* and Virulence on Rice.** The features of *avrXa7* are shown in Fig. 1. The sequence of the 3.2-kb *Sph*I revealed that *avrXa7* had 25.5 repeats, and the order of the repeats, as defined by the twelfth and thirteenth codons of each repeat, were unique in comparison to previously characterized members of the family (Fig. 2*A*). The repeat coding region of *avrXa7* is unusual compared with most other members of the *avrBs3* family because of the presence of four repeat units 99 bp in length rather than 102 bp (Fig. 2*A*), having an 18-bp duplication within repeat 13 (Fig. 2*B*). The shorter repeat units appear to be missing the thirteenth codon of the prototypical repeat unit. Replacement of the repeat region of pZW*avrXa10* with the repeat region of *avrXa7* by using the central *Sph*I fragment changes the specificity from *Xa10* to *Xa7* when the avirulence gene was present in strain PXO99 (Table 1). The composite gene in pZW*avrXa7* was used here because further experiments required the introduction of mutations that were originally constructed in *avrXa10*. Because the C-terminal coding regions are interchangeable, C-terminal mutations could be readily transferred to *avrXa7* simply by replacing the C-terminal coding region of pZW*avrXa7* with the C-terminal coding region of *avrXa10*. To determine whether the composite gene had the virulence properties of the original *avrXa7*, pZW*avrXa7* was introduced into PXO86mx53, and the resulting strain was tested for virulence (Table 1). Strain PXO86mx53 has a mutation in the endogenous copy of *avrXa7* and retains an active copy of *avrXa10*. PXO86mx53 has no avirulence activity on IRBB7 rice containing *Xa7* and impaired virulence on the susceptible rice line IR24 (Table 1). The decrease in virulence of PXO86mx53 is evidenced by reduced water soaking, a phenomenon caused by release of water into the intercellular spaces from cells in the infected area (Fig. 3*A*), reduced leaf lesion lengths (Fig. 3*B*), and lower bacterial populations in the leaves (data not shown). The gene in pZW*avrXa7* was capable of restoring full virulence as well as avirulence specificity for *Xa7* (Table 1; Fig. 3). Therefore, the repeat region of *avrXa7* was sufficient to convert *avrXa10* into a gene with the virulence and avirulence properties of *avrXa7*. We refer to the composite gene hereafter simply as *avrXa7*.

The AD Is Required for Virulence and Avirulence Activities. Deletion of the C-terminal coding region by the introduction of a stop

A

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  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
GENE
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*
AvrXa10  NI HG NI HG NI NI NN HD NI HD NN HG NS NG HD NG
AvrBs3   HD NG NS NG NI NI NI HD HD NG NS NS HD HD HD NG HD NG
AvrBs3-2 NI NG NI NI NG NG NI NS NG NI NS NG HD HD NS HD NG NG
Avrb6    HD NI NG HD HD NI HD NI NS HD HD HD NN NG
PthA     NI NG NI NI NG HD NG HD NG NG NG NG NS HD HD NG NG
PthN     NI HD HD NI HD NI NG NI NN HD NI NG N* NN
PthB     NI NI NN NI HD NS NS NN NG HD NN NI NG
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B

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REPEAT 13
1  CTGACCCCGGACCAGGTCGTGGCCATCGCCAGCAAT***GGCGGCAAGCAGGCGCTGGAGACGGTGCAGCGG
1   L  T  P  D  Q  V  V  A  I  A  S  N  *  G  G  K  Q  A  L  E  T  V  Q  R

CTGTTGCCGGTGCAGCGGCTGTTGCCGGTGCTGTGCCAGGACCATGGC 102
 L  L  P  V  Q  R  L  L  P  V  L  C  Q  D  H  G    39
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Fig. 2. Sequence analysis of *avrXa7*. (*A*) Schematic representation of the repeat domain of the *avrXa7* gene product by using the single amino acid code for the twelfth and thirteenth codons of the repeat units. GenBank accession nos. for proteins are as follows: AvrXa7, AF275317; AvrXa10, AAA92974; AvrBs3, CAA34257; AvrBs3–2, S34809; Avrb6, AAB00675; PthA, AAC43587; PthN, AAB69865; PthB, AAD01494. (*B*) Unusual repeat thirteen of *avrXa7*. Duplicated region is underlined. Asterisks indicate positions of missing nucleotides or amino acid residues of the prototypic repeat unit.

codon (TGA1064) or replacement of the C-terminal codons 1080, 1085, and 1088 (FLL) in the AD with alanine codons was previously shown to result in the loss of transcriptional activation and avirulence activity because of *avrXa10* (19). The mutations did not result in changes in the apparent stability of the protein in *Xanthomonas*, and the effects of the mutations could not be abrogated by expression of the genes within the host cells (14, 19). The 3.2-kb *Sph*I fragment from *avrXa7* was used to replace the corresponding fragment from *avrXa10*^{TGA1064} and *avrXa10*^{FLL} to create *avrXa7* versions of the same mutations (*avrXa7*^{TGA} and *avrXa7*^{FLL}). Both mutations caused the loss of avirulence and virulence because of *avrXa7* (Table 2; Fig. 4). Similarly to AvrXa10, no differences were detected in the amount of protein synthesized in the bacterium (data not shown). Transient expression of *avrXa7* in the host cells was not tested. Loss of avirulence activity could be restored to *avrXa7*^{TGA} by the introduction of the AD of VP16 (Table 2; Fig. 4). However, the AD of VP16 could not restore virulence activity to *avrXa7*^{TGA} (Table 2, Fig. 4).

Table 1. Dual activity of *avrXa7*

Strain	BB7 (*Xa7*)	BB10 (*Xa10*)	IR24
PXO99 (pHM1)*	S	S	S
PXO99 (p29-29-2A)†	R	S	S
PXO99 (pZW*avrXa10*)‡	S	R	S
PXO99 (pZW*avrXa7*)‡	R	S	S
PXO86 (pHM1)	R	R§	S
PXO86mx53 (pHM1)	I	R¶	I
PXO86mx53 (p29-29-2A)	R	S	S
PXO86mx53 (pZW*avrXa7*)	R	S	S

S, susceptible reaction; R, resistant; I, impaired.
*Vector alone.
†Cosmid clone with *avrXa7* (1).
‡*Sph*I fragment of *avrXa7* exchanged with *Sph*I fragment of *avrXa10* (14).
§PXO86 contains a chromosomal copy of *avrXa10* (1).
¶Although growth is poor because of the impaired growth of the bacteria, BB10 plants display a resistant phenotype.

NLS Motifs Are Required for Avirulence and Virulence Activities of AvrXa7. The three consensus NLS motifs in the C-terminal coding portion of *avrXa7* were altered to nonconsensus sequences, creating *avrXa7 M123* (pZW*avrXa7*M123; Fig. 5*A*). To directly measure the effect of the NLS mutations on nuclear localization, the ability of AvrXa7 to localize to nuclei was assayed in a yeast nuclear localization reporter system (Fig. 5*B*). In this system, the coding sequences of the gene are fused to the coding sequence for the LexA DNA-binding protein that lacks a NLS motif. The yeast strain contains reporter genes (*lacZ* and *His3*) that are regulated by a minimal yeast core promoter and tandem copies of the LexA consensus binding site. Expression of the *lacZ* and *His3* genes depends on the presence of a NLS in the LexA fusion protein. When full-length wild-type *avrXa7* was fused in frame to the coding region for the DNA-binding domain of LexA, the yeast strain was capable of growth without the addition of exogenous histidine and had high levels of β-galac-

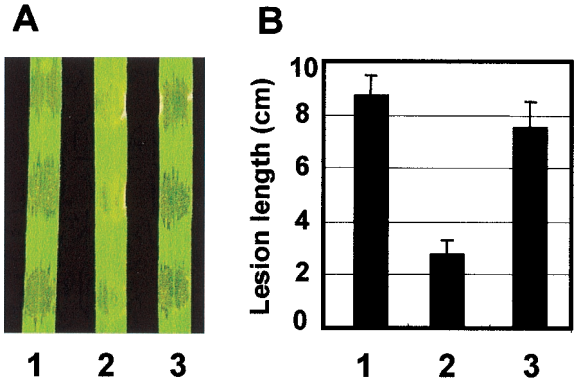

Fig. 3. Repeat domain of *avrXa7* controls virulence specificity. (*A*) Leaves were inoculated with wild-type PXO86 (leaf 1); PXO86mx53 (pHM1) with the mutated copy of *avrXa7* and the cloning vector alone (leaf 2); and PXO86mx53 (pZW*avrXa7*) (leaf 3). Leaves were photographed 3 days after inoculation. Dark areas represent water-soaked tissue. (*B*) Lesion length measurements (cm) 11 days after leaf-clip inoculation.

Table 2. Requirement of the AD for *avrXa7* activity

Strain	IRBB7 (*Xa7*)	IR24
PXO99 (pHM1)	S	S
PXO99 (pZW*avrXa7*)	R	S
PXO99 (pZW*avrXa7*)^{TGA}*	S	S
PXO99 (pZW*avrXa7*)^{FLL}†	S	S
PXO86 (pHM1)	R	S
PXO86mx53 (pHM1)	I	I
PXO86mx53 (pZW*avrXa7*)	R‡	S
PXO86mx53 (pZW*avrXa7*^{TGA})	I	I
PXO86mx53 (pZW*avrXa7*^{FLL})	I	I
PXO99 (pZW*avrXa7*^{VP16})	R	S
PXO86mx53 (pZW*avrXa7*^{VP16})	R‡	I

S, susceptible reaction; R, resistant; I, impaired.
*TGA indicates TGA stop codon at position 1064 of AvrXa10.
†FLL indicates replacement of phenylalanine, leucine, and leucine with alanines at positions 1080, 1085, and 1088, respectively, in reference to AvrXa10.
‡Resistance response is weak but evident.

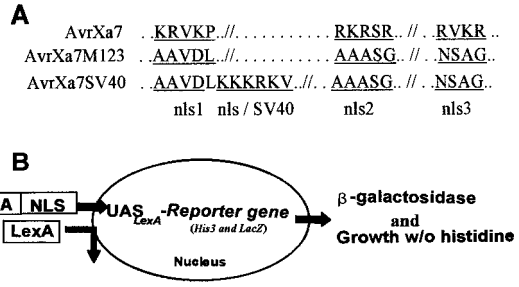

Fig. 5. NLS mutations and yeast nuclear localization assay. (*A*) Amino acid replacements at NLS 1, 2, and 3 are underlined. Only regions of NLS motifs are shown. KKKRK was introduced into AvrXa7 M123 to create AvrXa7SV40. (*B*) Each gene was fused to the LexA coding DNA-binding domain in pNIA vector and expressed in yeast. Strain L40 contains LexA-binding sites (UAS_{LexA}) immediately upstream of *lacZ* and *His 3* genes. Induction of *lacZ* and *His3* depends on the nuclear localization of the LexA fusion product. Induction leads to increased β-galactosidase activity and the ability of the yeast strain to grow in histidine-deficient media.

tosidase activity (Table 3). Fusion of AvrXa7 M123 to LexA resulted in weak growth on histidine-deficient media and a 76% reduction in the level of β-galactosidase (Table 3). Addition of the NLS from SV40 to *avrXa7 M123* (pZW*avrXa7*SV40) resulted in the restoration of histidine-free growth and 77% of the β-galactosidase activity because of the wild-type protein (Table 3). *AvrXa7 M123* and *avrXa7SV40* were then introduced into *X. oryzae* pv. *oryzae* and tested for avirulence and virulence activities. When introduced into PXO99^{A} and tested on IRBB7, *avrXa7 M123* had no avirulence activity (Fig. 6). In PXO86mx53, a*vrXa7 M123* also had no virulence activity (Fig. 6). The addition of the SV40 T-antigen NLS (AvrXa7SV40, Fig. 5*A*) or NLS 3 of AvrXa7 (data not shown) restored both activities (Fig. 6). Protein levels in yeast or bacteria were unchanged by the NLS mutations (data not shown).

AvrXa7 Binds DNA. The requirement for nuclear localization and transcriptional activation prompted us to consider whether the function of AvrXa7 might involve interaction with host DNA. DNA binding was assayed by gel shift assays by using AvrXa7 protein and ^{32}P-labeled DNA oligonucleotides. In this assay, evidence for DNA binding is indicated by the slower migration of the labeled oligonucleotides that are bound to protein. AvrXa7 was synthesized in *E. coli* as a GST fusion. GST alone had no DNA-binding activity (Fig. 7*A*). None of the four single-stranded homomeric oligonucleotides [single-stranded (ss)DNA] were retarded by GST/AvrXa7 (Fig. 7*A*). The migration of both PolydA/dT and PolydC/dG was retarded by the GST/AvrXa7 fusion. The amount of label in the retarded bands was greater for dA/dT than dC/dG. A second assay was performed by using polyhistidine-tagged AvrXa7 and ^{32}P-labeled random double-stranded DNA oligonucleotides. In this assay, ssDNA oligonucleotides of each base, PolydA/dT or PolydC/dG were added in 100-fold excess over the labeled random oligonucleotides. As with the GST fusion, AvrXa7 caused the gel retardation of labeled random oligonucleotides, and binding to the oligonucleotides was completely inhibited by the addition of dA/dT (Fig. 7*B*). Slight inhibition was observed with both PolydG and PolydC/dG. Thus, AvrXa7 will bind PolydA/dT and, to a lesser extent, PolydC/dG. Similarly, PolydC/dG is not a good competitor against a pool of random oligonucleotides (Fig. 7*B*).

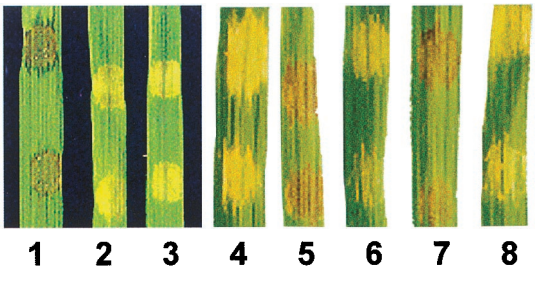

Fig. 4. Virulence activity of AvrXa7 requires C-terminal AD. Rice leaves were syringe inoculated with PXO86mx53 (leaves 1–3) or PXO99 (leaves 4–8) containing the following genes: 1, avrXa7; 2, *avrXa7*^{TGA}; 3, *avrXa7*^{VP16}; 4, vector control; 5, *avrXa7*; 6, *avrXa7*^{TGA}; 7, *avrXa7*^{VP16}; 8, *avrXa7*^{VP16}. Leaves 1–3 and 8 are from IR24. Leaves 4–7 are from IRBB7 (*Xa7*).

Discussion

We have demonstrated that the specificity for avirulence activity and virulence effects of *avrXa7*, a dual-acting avirulence gene of the *avrBs3* family, depended on the unique structural features of the products of this gene family. Specificity for enhancing virulence and *Xa7*-dependent avirulence activity on rice depended on the repeat domain, and both activities required functional NLS and AD motifs. The virulence properties of the *avrBs3* family were first demonstrated by the identification of *pthA* and *avrb6* (7, 16). The latter two genes have repeat domains of 13.5 and 18.5 units, respectively, and a unique repeat domain as defined by the twelfth and thirteenth codons of each repeat unit. Exchange of the domains of *avrb6* and *pthA*, which have effects on cotton and citrus species, respectively, also resulted in a switch in the virulence specificities of the genes (16). Replacement of the repeat region of *avrXa10*, which has no apparent contribution to virulence, with the repeat region of *avrXa7* also

Table 3. Effect of nls123 mutations on nuclear localization in yeast assay

Gene	Histidine deficiency*	% β-galactosidase activity†
pNIA	−	3.1 ± 1.1
pNIAXa7	+	100
pNIAXa7M123	±	20.0 ± 3.8
pNIAXa7SV40	+	76.3 ± 2.4

*Histidine deficiency was measured as the ability to grow on minimal media with 20 mM 3-AT
†Activity is expressed as percent of activity of *o*-nitrophenyl β-D-galactopyranoside (ONPG) produced per hour by pNIAXa7.

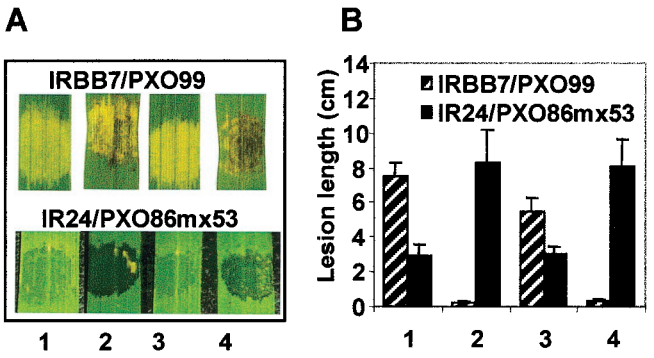

Fig. 6. Effects of NLS mutation on the avirulence and virulence activities of *avrXa7*. (*A*) Leaves of IRBB7 rice containing the resistance gene *Xa7* (*Upper*) and the susceptible variety IR24 (*Lower*) were syringe inoculated with PXO99 or PXO86mx53, respectively, containing the following genes: (1) pHM1, vector alone; (2) pZW*avrXa7*; (3) pZW*avrXa7*M123; (4) pZW*avrXa7*SV40. (*B*) Lesion length measurements 11 days after leaf-clip inoculations of IRBB7 with PXO99 (hatched bars) or IR24 with PXO86mx53 (solid bars). Numbering is as for *A*.

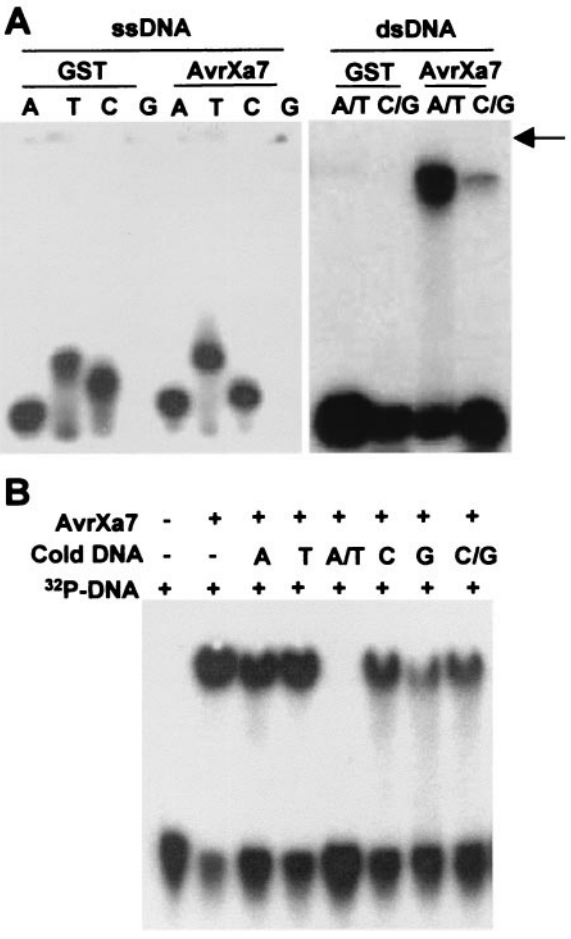

Fig. 7. AvrXa7 is a DNA-binding protein. (*A*) Single-stranded and double-stranded oligonucleotides were end labeled with ^{32}P, mixed with AvrXa7 or GST (glutathione *S*-transferase), and subjected to polyacrylamide electrophoresis. Arrow indicates position of well. (*B*) DNA-binding competition to AvrXa7. Random oligonucleotides were labeled with ^{32}P and mixed with AvrXa7 and the indicated unlabeled DNA in 100-fold excess. A, T, C, G, indicate unlabeled single-stranded homopolymers; A/T and C/G indicate unlabeled double-stranded polymers. +/− indicate presence or absence of component.

created a gene with all of the properties of *avrXa7*. The dual-acting genes are presumably under selective pressure to maintain virulence function while avoiding recognition by the host defense system. The adaptive process is presumably reflected in the variety of repeat domains in the genes from different strains and within strains. The length of a given pathogenicity gene may reflect the particular configuration of variable regions necessary for virulence that was arrived at by recombination and mutation. Strains of *X. oryzae* pv. *oryzae* contain an estimated 12 to 14 copies of the genes, which may provide ample substrate for the generation of new repeat combinations (1). How the particular repeat domains mediate specificity for virulence or resistance remains a mystery.

The requirement for NLS motifs had previously been demonstrated only for the avirulence activity of *avrBs3* and *avrXa10* (14, 18). These findings demonstrated that removal of the NLS motifs also resulted in the loss of virulence activity. As with AvrBs3 and AvrXa10 or any other avirulence protein, AvrXa7 has not been directly observed in the host cytoplasm or nuclei after transfer from the bacterium. Corroborative evidence, however, is abundant. Studies with truncated AvrBs3 and PthA demonstrated that the NLS motifs in the C-terminal portion of the protein could direct a β-glucuronidase fusion protein to onion cell nuclei (17, 18). Full-length fusions of AvrXa10 with the Gal4 DNA-binding domain were directed to *Arabidopsis* cell nuclei on the basis of transcriptional activation activity (14). The NLS signals of AvrXa7 fall into the class that function in a broad range of eukaryotic cells (24). Therefore, we used a yeast one-hybrid system in this work in an attempt to assess the behavior of full-length AvrXa7. The results indicated that full-length AvrXa7 was efficiently localized to yeast nuclei provided the endogenous NLS or heterologous SV40 motifs were intact. The formal possibility remains that the NLS sequences of AvrXa7 and related proteins are required, in general, for secretion through the type III apparatus and/or stability in the plant cells. However, a role for the NLS motifs in secretion is unlikely because of several considerations. Some secreted proteins from *Xanthomonas* do not have the NLS (or AD) motifs, as noted below for AvrBsP, yet remain functional (25, 26), and secretion signals for the type III systems appear, in part, to be encoded in the N-terminal mRNA structure (27). In regards to stability in the plant cells, mutations of the NLS motifs had no effect on the stability of the C terminus when fused to β-glucuronidase (18). Therefore, these proteins can localize to the nuclei of their respective host cells, and, indeed, we conclude that the proteins must localize to the nuclei to perform their function.

A domain with the features of an acidic-type AD is found at the C terminus of proteins encoded by the *avrBs3* gene family (19). The AD is required for the virulence and avirulence activities of AvrXa7. Avirulence activity could be restored by introduction of the heterologous domain. However, the VP16 domain could not substitute for the endogenous domain in virulence assays. Therefore, regardless of the actual function of the domain, virulence and avirulence activities have different requirements. If the activation domain is indeed functional, the interaction of AvrXa7 with the transcriptional machinery and recognition as mediated by *Xa7* (or AvrXa10 by *Xa10* or AvrBs3 by *Bs3*) may be a relatively general phenomenon, whereas virulence may require a specific interaction with a host factor, possibly a transcription cofactor. Whether the domain acts as an activator *in vivo* has yet to be demonstrated. Activation activity has been demonstrated only after fusion of the protein to a DNA-binding domain from a well-characterized transcription factor. The ability of AvrXa7 by itself to promote transcription of a gene either in yeast or a plant has not been demonstrated. However, we were able to demonstrate that the protein is a

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general double-stranded DNA-binding protein. Further work will be required to determine which features of AvrXa7 are responsible for DNA binding and whether high-affinity sites can be identified.

The requirement of the AD and NLS for both virulence and avirulence activities of AvrXa7 is curious. The finding suggests that either the host defense factors are targeted to the site of action of the virulence factor or that R-gene products may be components of a complex that is targeted by the virulence factor. In tomato, the AvrPto protein, which also has virulence properties (28, 29), requires a myristylation motif for activity, suggesting that the protein is targeted to an inner membrane surface (personal communication). In *Arabidopsis*, RPM1 has been localized to the inner cytoplasmic membrane (30). Interestingly, the corresponding avirulence protein AvrRpm1, which functions in virulence for strain PsmM2 of *Pseudomonas syringae* pv. *maculicola*, also has a myristylation motif (31) and recently has been found associated with the cytoplasmic membrane on expression within the host cell (32). Loss of the myristylation site in AvrRpm1 results in loss of avirulence and virulence activities (32). Therefore, localization of the resistance gene product to the site of effector protein function may be a more general phenomenon and not specific to AvrXa7 and related proteins. Localization to the site of action may ensure that the virulence protein is effectively intercepted and the defense response initiated rather than usurped during pathogen challenge. Specific R-gene products may be targeted to and function as "guardians" of specific defense signaling complexes (33). At the same time, not all members of the *avrBs3* family appear to depend on the NLS sequences for avirulence activity and therefore are not necessarily intercepted in the nucleus. *AvrBs3–2*, which is also referred to as *avrBsP* and represents another member of family with avirulence activity on tomato, was initially cloned with a truncated C-terminal coding region and therefore lacks the NLS coding regions (25, 34). One presumes that the AvrBs3–2 protein is localized to the nucleus yet intercepted in the cytoplasm.

Little is known about the function of the type III dependent virulence factors of plant pathogenic bacteria. The similarity of AvrXa7 and related proteins, regardless of the precise mechanism, suggests that all members of the family are likely to have a requirement to localize to the nuclei of their respective host plants and have the potential to interact with the host transcriptional machinery. *AvrXa7* is the only *avrBs3*-related gene of *X. oryzae* pv. *oryzae* to be demonstrated to have virulence properties. The fact that PXO99 and other strains do not have *avrXa7* activity yet remain fully virulent suggests either not all strains of *X. oryzae* pv. *oryzae* require an *avrBs3* member for full virulence or that genes without *Xa7* recognition can also function in virulence. Further characterization of *avrXa7* and related genes in *X. oryzae* pv. *oryzae* will hopefully allow identification of the critical repeat configurations for avirulence and virulence activities.

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