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AvrXa10 Contains an Acidic Transcriptional Activation Domain in the Functionally Conserved C Terminus

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The *avrXa10* gene of *Xanthomonas oryzae* pv. *oryzae,* the causal agent of bacterial blight of rice, is a member of the *avrBs3* avirulence gene family and directs the elicitation of resistance in a gene-for-gene manner on rice lines carrying the resistance gene *Xa10*. The carboxyl (C) terminus of AvrXa10 has a previously undescribed domain that is structurally similar to the acidic activation domain of many eukaryotic transcription factors in addition to three nuclear localization signal (NLS) sequences. Removal of the C-terminal 38 codons containing the putative activation domain, but retaining the NLS sequences, was concomitant with the loss of avirulence activity. The C-terminal coding regions of *avrBs3* and *avrXa7* can be replaced by the corresponding region of *avrXa10,* and the genes retained specificity for the resistance genes *Bs3* in pepper and *Xa7* in rice, respectively. The *avrBs3* and *avrXa7* avirulence activities of the hybrid genes were also lost upon removal of the terminal 38 codons. When fused to the coding sequence of the Gal4 DNA binding domain, AvrXa10 activated transcription in yeast and *Arabidopsis thaliana*. Removal of the carboxyl region severely reduced transcriptional activation. AvrXa10 would have to be localized to the host cell nucleus to function autonomously in transcriptional activation. Consistent with this requirement, mutations in all three NLS sequences of *avrXa10* caused a loss in avirulence activity. The findings demonstrate the requirement of the C terminus for AvrXa10 function and the potential for the members of this family of avirulence gene products to enter the host nucleus and alter host transcription.

Avirulence (*avr*) genes control the ability of a pathogen to elicit a resistance response, generally involving a rapid cell death or hypersensitive reaction (HR), on plant cultivars that contain specific corresponding genes for resistance (R-genes). The products of some *avr* genes and the corresponding R-genes presumably interact and result in the elicitation of a resistance reaction (reviewed in Alfano and Collmer 1996; Leach and White 1996; Yang et al. 1997). *avr* genes can also control aspects of pathogenicity in the pathogen/host interaction. Presumably, therefore, some *avr* gene products are secreted into the host cell, where the proteins, in the case of susceptible interactions, promote, or previously promoted under some circumstances, the virulence of the bacteria. The *avrXa10* gene of *Xanthomonas oryzae* pv. *oryzae* is a member of the bacterial *avrBs3* avirulence gene family (Hopkins et al. 1992). Members of the family have been identified in many species and pathovars of *Xanthomonas* (Bonas et al. 1989, 1993; Canteros et al. 1991; De Feyter and Gabriel 1991; De Feyter et al. 1993; Hopkins et al. 1992; Swarup et al. 1992). *avrBs3,* the type member of the gene family, is dependent on the *hrp* secretory pathway (Knoop et al. 1991) and will elicit resistance when delivered to the plant via gene transfer by *Agrobacterium tumefaciens,* indicating that the protein products of this family are delivered to plant cells during bacterial infection (Van den Ackerveken et al. 1996). Several members of the gene family are also involved in pathogenicity. The *pthA* (*pth*ogenicity*A*) gene, for example, controls the ability of *X. citri* to grow intercellularly and induce hyperplastic cankers on citrus (Swarup et al. 1991, 1992), while the *avrb6* gene determines the extent of watersoaking, a symptom of pathogenicity, and subsequent release of *X. campestris* pv. *malvacearum* to the leaf surface of cotton plants (Yang et al. 1994, 1996).

The predicted protein product of *avrXa10* and other members in the *avrBs3* family have remarkable features in their structure. The middle third of the protein consists of a near-perfect repeat sequence of 34 amino acids varying in number from 13.5 copies for Avrb6 to 25.5 copies in AvrXa7 (Bonas et al. 1989, 1993; De Feyter et al. 1993; Hopkins et al. 1992; Yang et al. 1994). The sequences of the repeats are nearly identical, with most of the variation occurring in the codons at the twelfth and thirteenth positions with respect to each repeat. The order or type of repeats in the central domain are suspected to be involved in the resistance specificity of the particular family member (Bonas et al. 1989; Herbers et al. 1992; Yang et al. 1994; Yang and Gabriel 1995a). Another notable feature of the protein is the presence of three nuclear localization signal (NLS) sequences (termed nlsA–C) in the carboxyl (C) coding region (Yang and Gabriel 1995b). NLS sequences are present in all members of the family including *avrXa10* (Yang and Gabriel 1995b), and loss of AvrBs3 activity was observed after their removal (Van den Ackerveken et al. 1996). The requirement for NLS sequences suggests that some members of the AvrBs3 family enter the host nucleus and interact with some component of the host nuclear machinery.

The C terminus of the *avrXa10* protein product is similar to those of the other members of the gene family. The requirement of the C terminus, in general, or the NLS sequences,

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specifically, for the activity of *avrXa10* was unknown prior to this work. We report the identification of an additional domain with the features and activity of an acidic transcriptional activation domain near the extreme terminus of the protein. We have constructed mutations and performed domain swapping experiments to determine if the C-terminal coding region of *avrXa10* is functionally conserved and required for avirulence and transcription activation activity. The results demonstrate the potential for the gene product of *avrXa10* and related proteins to interact with the host transcriptional machinery in the elicitation of both resistance and disease.

RESULTS

The C-terminal region of *avrXa10* is functionally conserved.

The *avrXa10* gene is typical of the *avrBs3* family and consists of three regions; the amino-terminal coding region; the central repeat region; and the C-terminal region (Fig. 1). The different members of the family are highly similar in structure with the exception that each has a varying number of 102-bp repeats in the central repeat domain. The specific encoded amino acid sequence of each repeat is also variable, particularly at the twelfth and thirteenth codons. The number of repeats in *avrXa10* is 15.5, while *avrBs3* and *avrXa7* have 17.5 and 25.5 copies, respectively (Fig. 1). The C-terminal region has three NLS sequences (Fig. 1, nlsA–C), as previously described (Yang and Gabriel 1995a). The region also has a domain with similarity to eukaryotic acidic transcription activation domains in the last 38 codons (Fig. 1, AD). The general feature of acidic activation domains is the interspersion of acidic residues (Fig. 2, E and D) with bulky and hydrophobic residues (Fig. 2, bold letters; for reviews see Goodrich et al. 1996 and Ptashne and Gann 1997). The region in AvrXa10 occurs roughly between the tryptophan residue (W) at position 1063 and the proline residue (P) at 1093 (Fig. 2). This region of AvrXa10 differs from the corresponding regions of AvrBs3, Avrb6, and PthA, which are the predicted gene products of other members of the *avrBs3* family, only in that AvrXa10 extends for another eight codons and has three amino acid residue differences (Fig. 2).

To determine if the C-terminal coding domain of *avrXa10* was functionally conserved, portions of *avrXa10* were interchanged with *avrBs3* and *avrXa7*. First, the central region of *avrXa10* in pFWX10-F2, which also encodes a FLAG epitope in the C-terminal region (Fig. 1, FL), was replaced with the corresponding region of *avrBs3* with the conserved *Sph*I sites on either side of the repeat region used to create pFWB3S-F2 (Fig. 1). The avirulence activity of the chimeric gene, when transferred to *X. campestris* pv. *vesicatoria*, had *avrBs3* activity and, when transferred to *X. oryzae* pv. *oryzae*, had lost *avrXa10* avirulence activity (Table 1). Since the 2.3-kb *Sph*I fragment of *avrBs3* still had 401 bp of the C-terminal region of *avrBs3* including nlsA, the region in pFWB3S-F2 was then replaced with the *Hinc*II/*Xba*I fragment of pFWX10-F2 to create pFWB3SH-F2. This gene now has the nearly complete *avrXa10* coding sequence with the exception of the *avrBs3*

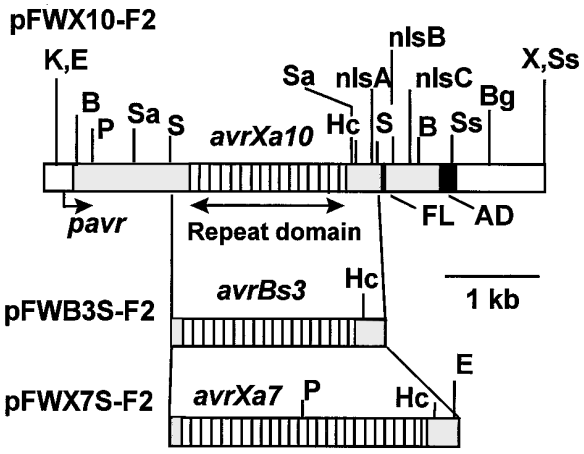

Fig. 1. Map of *avrXa10* in pFWX10-F2. Gray filled bars represent amino-terminal and C-terminal coding regions. Open region with vertical hatches represents central repeats. Solid bars indicate sites of FLAG epitope (FL) and putative transcription activation domain (AD) in C-terminal coding region. B, *Bam*HI; Bg, *Bgl*II; E. *Eco*RI; H, *Hind*III; Hc, *Hinc*II; K, *Kpn*I; P, *Pst*I; S, *Sph*I; Sa, *Sau*3AI; Ss, *Sst*I; X, *Xba*I; nls, nuclear localization signal. Arrows indicate endogenous promoter of *avrXa10*. Two bars below *avrXa10* represent fragments from *avrBs3* and *avrXa7* used to replace *Sph*I fragment of *avrXa10* in indicated plasmids.

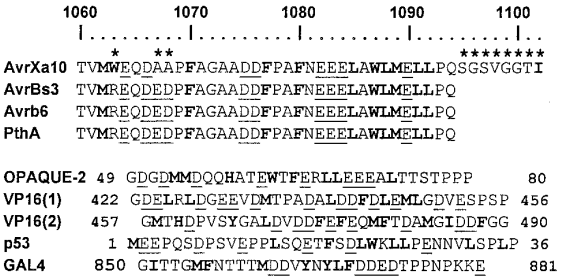

Fig. 2. Putative acidic transcription activation domains of AvrXa10. Acidic residues are underlined. Bulky or hydrophobic residues are in bold. Asterisks indicate differences in amino acid sequence of AvrXa10 compared with AvrBs3. Also shown are previously characterized activation domains of following eukaryotic transcription factors: OPAQUE-2, maize seed protein regulatory protein (Lohmer et al. 1991); VP16, herpes viral protein domain 1 (Cress and Triezenberg 1991); VP16, domain 2 (Cress and Triezenberg 1991); p53, tumor suppressor protein (Matlashewski et al. 1984); GAL4, yeast galactose regulatory protein (Laughon and Gesteland 1984).

Table 1. Requirement of the C terminus for avirulence activity

	Resistance gene and reaction[a]		
Plasmid (avirulence gene)	***Xa10***	***Xa7***	***Bs3***
pFWX10-F2 (*avrXa10*)	R	S	S
pFWB3S-F2 (*avrBs3*)	S	NT	R
pFWB3SH-F2 (*avrBs3*)	S	NT	R
pFWX7S-F2 (*avrXa7*)	S	R	NT
pZW17 ($avrXa10^{TGA1064}$)	S	NT	NT
pZW18 ($avrXa7^{TGA1064}$)	NT	S	NT
pZW19 ($avrBs3^{TGA1064}$)	NT	NT	S

[a] The *avrXa7* and *avrXa10* activities were tested with *Xanthomonas oryzae* pv. *oryzae* strain PXO99A on IRBB7 and IRBB10 rice containing *Xa7* and *Xa10*, respectively. The *avrBs3* activity was tested in *X. campestris* pv. *vesicatoria* 83-15 on pepper ECW-30R containing *Bs3*. Each plasmid was first cloned in pHM1 and introduced into the respective strain. Each strain gave a susceptible reaction on the near-isogenic line lacking the resistance gene. R, resistant; S, susceptible; NT, not tested.

repeat region and retained *avrBs3* avirulence activity (Table 1). The 2.1-kb *Sph*I fragment of *avrXa10* was also replaced with the 3.1-kb *Sph*I fragment of *avrXa7* to create pFWX7S-F2 (Fig. 1). The gene in pFWX7S-F2 had no *avrXa10* avirulence activity and gained *avrXa7* activity (Table 1). All of the genes were tested on the near-isogenic lines (ECW for *avrBs3* and IR24 for *avrXa7* and *avrXa10*) that lack the respective R-genes, and none were found to elicit an HR (data not shown). Therefore, the carboxyl coding region of *avrXa10* could function for *avrBs3* and *avrXa7* avirulence activities.

The C-terminal region is required for avirulence activity.

The requirement for the C-terminal activation domain for avirulence was tested by introducing a stop codon followed by a *Pst*I site into *avrXa10* in pFWX10-F2 at nucleotide position 3437 (amino acid residue 1064) to create $avrXa10^{TGA1064}$ in pZW17. The truncated gene was introduced into *X. oryzae* pv. *oryzae* strain $PXO99^{A}$, which does not harbor an endogenous copy of *avrXa10* or *avrXa7*, and the strain was then inoculated on rice plants containing the resistance gene *Xa10*. The strain containing $avrXa10^{TGA1064}$ failed to elicit an HR on the resistant plants (Fig. 3). The requirement for the C-terminal coding sequences for *avrXa7* and *avrBs3* activities was also tested by replacing the central *Sph*I fragment of $avrXa10^{TGA1064}$ with the respective *Sph*I fragments of *avrXa7* and *avrBs3*. In each case, the truncated gene did not confer avirulence activity toward the respective resistant cultivar, indicating that the avirulence activities of *avrXa7* and *avrBs3* also require the C-terminal sequence (Table 1).

Fig. 3. The TGA1064 mutation results in the loss of avirulence activity due to *avrXa10*. Avirulence activity of pFWX10-F2 and pZW17 was tested on IRBB10 containing resistance gene *Xa10* after cloning into pHM1, introduction of each construct to *Xanthomonas oryzae* pv. *oryzae* PXO99A, and infiltration of strains containing plasmids into rice leaves. Lane 1, pFWX10-F2 (*avrXa10*); lane 2, pZW17 (*avrXa10TGA1064*); lane 3, pBluescript II KS^{+} (vector without avirulence [*avr*] gene). Host reaction is indicated at top of leaf. R, hypersensitive response (HR) or resistant; S, susceptible. HR occurred by 24 h. Photos were taken 3 days after inoculation to allow for expression of susceptible symptomology.

Transcription activation by *avrXa10*.

The transcriptional activation ability of *avrXa10* and the involvement of the C-terminal residues were tested first in a yeast one-hybrid system by creating in-frame fusions of the gene for the DNA binding domain of Gal4 (Gal4DB), as represented in the plasmid pAS2-1, and portions of *avrXa10*. The fusions are expressed under the control of the yeast ADH1 promoter, and the plasmid replicates as an autonomous plasmid in yeast. If *avrXa10* encodes a transcriptional activation domain, the yeast strain containing the fusion gene will express the structural genes for *HIS3* and *lacZ*, which are under the control of Gal4-activated promoters in the strain Hf7c, and the strain will grow in histidine-deficient media and synthesize β-galactosidase.

Three restriction endonuclease fragments from *avrXa10* were fortuitously in-frame with the Gal4DB domain coding sequence including a genomic 5.0-kb *Pst*I fragment, containing the entire *avrXa10* coding region with the exception of the first 78 bp and extending approximately 1.8 kb beyond the stop codon, a 2.0-kb *Sau*3A fragment containing the *avrXa10* repeat region, and a 0.8-kb *Eco*RI/*Bgl*II fragment containing 527 bp of the carboxyl coding region. The fragments were representative of the near-complete *avr* gene, the repeat coding region, and the C-terminal region, respectively. The *Eco*RI/*Bgl*II fragment was cloned from pFWX7S-F2. Both the 5.0-kb *Pst*I and 0.8-kb *Eco*RI/*Bgl*II fragments conferred on HF7c the ability to grow without addition of histidine and synthesize β-galactosidase (Table 2; pAS2-1/*avrXa10P* and pAS2-1/*avrXa10EBg*). The *Sau*3A clone of the repeat domain of *avrXa10* or pAS2-1 alone had no activation activity (Table 2; pAS2-1/*avrXa10Sau*).

The requirement of the C-terminal acidic domain was tested for transcriptional activation in yeast with $avrXa10^{TGA1064}$ from pZW17. The 3.3-kb *Pst*I fragment, which was created in the construction of $avrXa10^{TGA1064}$, was inserted into pAS2-1 to create pAS2-1/$avrXa10^{TGA1064}$. The plasmid was tested for activation activity in yeast and found to be severely impaired in the activation activity compared with the wild-type gene as measured by β-galactosidase activity, yet still conferred on Hf7c the ability to grow on histidine-free media (Table 2). Growth of Hf7c with pAS2-1/$avrXa10^{TGA1064}$ could be retarded by the inclusion of the HIS3 inhibitor 3-AT in the histidine-free medium, whereas Hf7c with pAS2-1/*avrXa10P* or pAS2-1/*avrXa10EBg* was not inhibited (Fig. 4). Thus, the near-complete AvrXa10 or the C-terminal region alone were

Table 2. Transcription activation by *GAL4DB::avrXa10* gene fusions in yeast

Plasmid	β-gal activity[a]	Growth on his^{-} plates[b]
pAS2-1	0.0 ± 0.1	–
pAS2-1/*avrXa10P*	10.2 ± 0.1	+
pAS2-1/*avrXa10Sau*	0.0 ± 0.1	–
pAS2-1/*avrXa10EBg*	27.9 ± 0.5	+
pAS2-1/$avrXA10^{TGA1064}$	0.1 ± 0.1	+[c]
pGAL4	248.0 ± 4.4	+

[a] Activity of β-galactosidase (β-gal) with oNPG as substrate was determined as described in Materials and Methods and expressed as Miller units (Miller 1972). Measurements are averages (with standard deviation) of three independent assays.

[b] Growth of yeast strains containing the indicated plasmid on minimal synthetic media without histidine.

[c] Growth on histidine-deficient plates was inhibited by 5 mM 3-AT.

capable of activating transcription in yeast, and the activity was severely reduced by deletion of the C-terminal domain.

Transcription activation in plants.

The activation activity of *avrXa10* was also assayed in *Arabidopsis thaliana* plants containing a Gal4-promoted reporter gene. The reporter gene consisted of 10 tandem UAS_G located immediately upstream from a minimal 35S promoter, which was linked to the coding sequences of the bacterial *uidA* gene for β-glucuronidase (GUS). Genes encoding Gal4DB::AvrXa10 fusions from pAS2-1 were cloned behind the cauliflower mosaic virus (CaMV) 35S promoter and introduced into the leaf cells via *Agrobacterium tumefaciens*-mediated transfer (see Materials and Methods). Each strain was inoculated into eight leaves. β-glucuronidase activity was detected in four of eight leaves upon introduction of pYB1 (*Gal4DB::avrXa10*) or pYB3, which contains a hybrid gene (*Gal4DB::VP16AD*) encoding Gal4DB and the VP16 activation domain under the control of the CaMV 35S promoter (Fig. 5; leaves 1 and 3, respectively). On the other hand, no leaves were scored positive with *GAL4DB::avrXa10*^{TGA1064} (pYB2) or *Gal4DB* (pYB4) alone (Fig. 5; leaves 2 and 4, respectively). Although the efficiency of GUS expression remains unexplained, an intron-containing *uidA* gene that was expressed by the complete 35S promoter also gave four of eight leaves positive for GUS activity (data not shown).

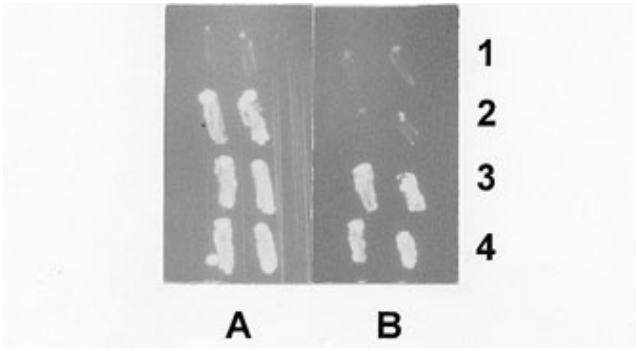

Fig. 4. AvrXa10 activates transcription in yeast. Portions of *avrXa10* were cloned in the Gal4 DNA binding protein fusion vector pAS2-1. Plasmids were introduced into yeast strain Hf7c and streaked on histidine-deficient media. **A,** Without histidine. **B,** Without histidine and supplemented with 5 mM 3-AT. Lane 1, pAS2-1; lane 2, pAS2-1/*avrXa10*^{TGA1064}; lane 3, pAS2-1/*avrXa10EBg*; lane 4, pAS2-1/*avrXa10P*.

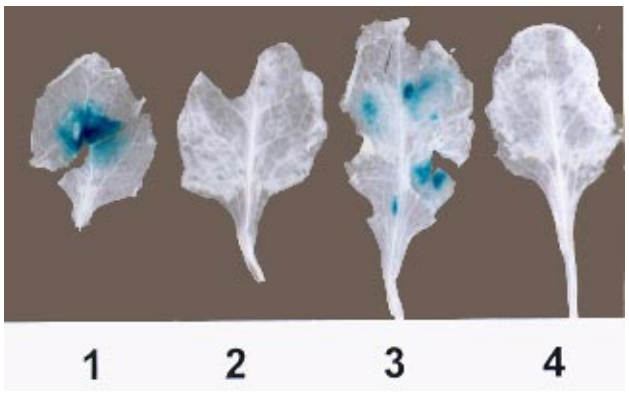

Fig. 5. *Gal4BD::AvrXa10* activates transcription of *UASG::GUS* in *Arabidopsis thaliana*. *Gal4 BD::avrXa10* and its truncated derivative were cloned in plant expression vector pMON410 under the cauliflower mosaic virus (CaMV) 35S promoter. *A. thaliana* plants containing *UASG::GUS* were infected by *Agrobacterium tumefaciens* EHA101 with (lane 1) pYB1 (*Gal4BD::avrXa10P);* (lane 2) pYB2 (*Gal4BD*::*avrXa10*^{TGA1064}); (lane 3) pYB3 (*Gal4DB::VP16AD)*; and (lane 4) pYB4 (*Gal4DB* alone). Leaves were stained with 5-bromo-4-chloro-3-indolyl-β-glucuronidase (X-Gluc) 3 days after inoculation. Blue color indicates areas of β-glucuronidase (GUS) activity.

The requirement for the NLS sequences of *avrXa10*.

If the C-terminal region of *avrXa10* does indeed function in transcriptional activation, the protein must be localized to the plant cell nucleus. The C-terminal region of *avrXa10* complemented the corresponding region of *avrBs3*, indicating that the NLSs of *avrXa10* were functional. The requirement of the NLS sequences for *avrXa10* activity was tested by replacing codons in the sequences with nonconsensus sequences (see Materials and Methods). The constructs pZW20 (*avrXa10*$^{nlsB-}$), pZW21 (*avrXa10*$^{nlsBC-}$), and pZW22 (*avrXa10*$^{nlsABC-}$) harbored mutations in one (nlsB), two (nlsB and nlsC), and three (nlsA, nlsB, and nlsC) NLS sequences, respectively. The loss of avirulence on *Xa10*-containing rice was observed only after replacement of all three sequences (Fig. 6, lanes 4 and 5). Strains containing an *avrXa10* derivative with nlsA and nlsC together or nlsA alone retained avirulence activity, although the inoculations with pZW20 and pZW21 consistently gave slightly weaker reactions in terms of the browning (Fig. 6, lanes 2 and 3, respectively). The reaction symptoms were within the range observed with the wild-type gene. Genes with single copies of nlsB or nlsC were not tested. Therefore, the C-terminal coding region of *avrXa10* could functionally

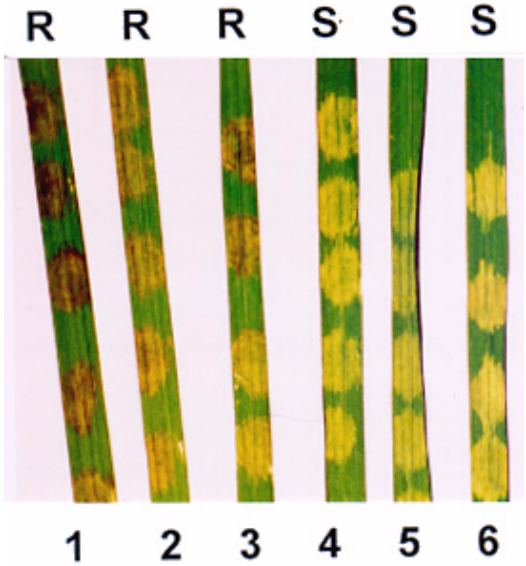

Fig. 6. Effect of mutations in nuclear localization signal (NLS) sequences of *avrXa10* on avirulence activity. Mutations were introduced into *avrXa10* and transferred into PXO99A. Strains were inoculated into leaves of IRBB10 containing *Xa10*. Leaves were inoculated with the strain containing the following genes: lane 1, *avrXa10*; lane 2, *avrXa10*$^{nlsB-}$; lane 3, *avrXa10*$^{nlsBC-}$; lanes 4 and 5, *avrXa10*$^{nlsABC-}$; lane 6, pHMBS (vector alone). Host reaction is indicated at top of leaf. R, Hypersensitive response or resistant; S, susceptible. Resistant reactions occurred by 24 h. Photographs were taken 3 days after inoculation.

meet the NLS requirements of *avrBs*, and at least one NLS sequence was required for *avrXa10* activity.

Proteins were extracted from *X. oryzae* pv. *oryzae* strains that contained pFWX10-F2 (*avrXa10*), pZW17 (*avrXa10*^{TGA1064}), pZW22 (*avrXa10*$^{nlsABC-}$), and pHMBS (vector control), and analyzed by Western blot (immunoblot) analysis with anti-FLAG monoclonal antibody to determine if the mutations affected the relative level of protein, compared with the wild-type gene. The analysis revealed no differences in the levels of protein from the wild-type, truncated, and NLS-deficient avirulence genes, while no band corresponding to AvrXa10 was detected with the vector control (Fig. 7).

DISCUSSION

The results presented here demonstrate that the C-terminal region of *avrXa10* encodes a functional, eukaryotic-like, acidic transcription activation domain, and that the same region is required for avirulence activity. The activation domain in the C terminus, although not as active in yeast as the endogenous Gal4 transcription factor, was active in both yeast and *A. thaliana*. The region is highly conserved among members of the *avrBs3* family and required by at least three members of the family, including systems with both monocot (*avrXa10*/*Xa10* and *avrXa7*/*Xa7* in rice) and dicot (*avrBs3*/*Bs3* in pepper) hosts. By inference from the close sequence relatedness among the family members, some of the related proteins in other pathovars and species of *Xanthomonas* are also likely to have the same requirement. Based on structural similarities and the results of the activation activity of the region contained in the *Eco*RI/*Bgl*II fragment, the extreme C-terminal domain appeared to contain the domain that was primarily responsible for transcription activity. This suspicion was borne out by the results of the truncation of *avrXa10* at residue 1064. Truncation of *avrXa10* at residue 1064, which removes 38 amino acid residues and leaves the repeat region and NLS residues intact, severely reduced transcriptional activation and eliminated avirulence activity.

The results also indicated that *avrXa10* encodes one or more additional domains with transcriptional activation activity in yeast, since the truncated protein was not completely devoid of activity, as evidenced by the growth of the yeast cells on histidine-deficient media. The ability to grow in the absence of HIS3 expression is an extremely sensitive assay, and very low levels of expression can lead to prototrophic growth on histidine-deficient media. The level of transcriptional activation can be titrated by addition of increasing levels of 3-AT (Vidal et al. 1996). Growth by the yeast in histidine-deficient media due to pAS2-1/*avrXa10*^{TGA1064} could be inhibited by low levels of 3-AT (5 mM), whereas the C terminus domain permitted growth in the presence of relatively high levels of the inhibitor (>50 mM; W. Zhu and F. F. White, *unpublished results*). The levels of β-galactosidase activity due to pAS2-1/ *avrXa10*^{TGA1064} were not significantly different from the control strain without an activation domain under the conditions of the assay. Therefore, both phenotypes indicate that the activity of the other domain (or domains) is weaker than the activity of the C-terminal domain. The weak domains have yet to be identified, and their biological significance, if any, remains unknown. The acidic nature of the FLAG peptide may contribute to activation. However, the FLAG is not the sole cause of the weak activation since similar experiments have been performed without the FLAG, and fusion without the C-terminal domain permitted growth of the strain on histidine-deficient media. The 2.0-kb *Sau*3A1 of the repeat domain in pAS2-1 would not support growth on histidine-deficient plates, indicating that the repeat region is not an activator in the yeast system.

The results presented here do not demonstrate that avirulence activity requires transcription activation. The residues for activation and avirulence may simply be juxtaposed or even coincidental. A range of peptides have been identified, including sequences from bacterial genomes, that are capable of activating transcription in the Gal4 one-hybrid system (Estruch et al. 1994; Ma and Ptashne 1987; Ma et al. 1988), and none, in the case of bacterially derived activators, were shown to be bona fide transcription factors. Presumably any sequence that conforms approximately to the proper structural features can promote transcription to varying degrees when linked to a specific DNA binding domain. On the other hand, a number of circumstances exist in the case of the *avrBs3* family that add to the plausibility of the Avr protein functioning as a transcription activator. Although secretion and localization of the proteins through the *hrp* secretion apparatus and into the plant cells have not been directly documented, expression in the plant leads to a *Bs3*-specific HR (Van den Ackerveken et al. 1996). Avirulence activities of *avrBs3*, *avrXa10*, and *avrXa7* are dependent on a functional *hrp* system (Knoop et al. 1991; W. Zhu and F. F. White, *unpublished results*). The apparent requirement for at least one NLS in AvrBs3 and AvrXa10, and, again, the requirement of NLS for activity of AvrBs3 whether delivered by *Xanthomonas* spp. via the *hrp* system or *Agrobacterium tumefaciens*-mediated transfer indicates that the proteins are potentially targeted to the host nucleus (Van den Ackerveken et al. 1996). The intact *avrBs3* gene product after delivery by either *Xanthomonas* spp. or *A. tumefaciens* has yet to be detected in the host nucleus. However, the results with the Gal4DB domain, which contains an endogenous NLS, demonstrate that the nearly full-length AvrXa10 protein was capable of nuclear localization. We have evidence that further supports a transcription activation model

Fig. 7. Mutations at 1064 or nuclear localization signal (NLS) do not affect levels of AvrXa10 in *Xanthomonas oryzae* pv. *oryzae*. Proteins were extracted from PXO99A containing the following plasmids: Lane 1, pFWX10-F2 (*avrXa10*); lane 2, pBluescript II KS^{+} (vector alone); lane 3, pZW17 (*avrXa10*^{TGA1064}); lane 4 pZW22 (*avrXa10*$^{nlsABC-}$); lane 5, molecular mass standards in kilodaltons. Proteins were separated by 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), blotted to nitrocellulose, and probed with anti-FLAG M2 monoclonal antibody. Predicted sizes: for AvrXa10-F2 and AvrXa10$^{nlsABC-}$, 116 kDa; for AvrXa10^{TGA1064}-F2, 112 kDa. The 4-kDa difference was not resolved in the gel conditions used.

from experiments whereby mutations in specific residues of the domain have been found to affect both transcriptional activation and avirulence, and, by substitution of the endogenous domain of AvrXa10 with the domain from the herpes virus VP16 transcription factor, a hybrid protein with avirulence activity can be constructed (W. Zhu, B. Yang, L. B. Johnson, and F. F. White, *in preparation*).

An alternative explanation of the results is that the truncation at residue 1064 and the changes in the NLS sequences altered the instability of the protein either in the bacterium or plant and yeast cells, or the changes interfered with the delivery of the protein through the *hrp* secretory apparatus. No differences in protein levels of AvrXa10 in the bacterium were detected for the alterations presented here, and similar results were reported for changes in the NLS sequences of *avrBs3* (Van den Ackerveken et al. 1996). The protein products of the *avrBs3* family have not been detected in the host nucleus or cytoplasm after delivery by *Xanthomonas* spp. or *A. tumefaciens* and, therefore, changes in protein stability in the plant cell remain a possibility for the NLS or C-terminal mutations reported here or by others (Van den Ackerveken et al. 1996). Interference with protein secretion remains a possible although, in our opinion, less likely reason for the effect of the mutations. Signals for protein transfer in type III systems such as the *hrp* apparatus appear to be exclusively located in the amino-terminal coding region of the protein (Michiels and Cornelis 1991; Schesser et al. 1996) or possibly, as reported in one case, in the amino-terminal mRNA structure (Anderson and Schneewind 1997), although the secretory signals of the *avrBs3* family of proteins, specifically, have not been characterized. The elicitation activity of *avrBs3-2,* another family member, was observed whether the gene was expressed as the full-length copy or was truncated in the central repeat region (Bonas et al. 1993; Canteros et al. 1991). The mechanism of action of this member of the family presumably also requires secretion into the host cell via the *hrp* system yet functions in the absence of the NLS and activation domain sequences.

R-genes that correspond to *avrBs3* and related genes have not yet been characterized. However, if indeed nuclear localization and transcription activation are required for avirulence activity, models for recognition will have to accommodate some interesting twists in the story for at least some members of the *avrBs3* family. The most compelling model for the function of the protein products of the *avrBs3* family and a variety of other *avr* gene products postulates the secretion of the proteins from the bacteria into the plant cell. Once in the cell, the proteins presumably function in mediating the host/pathogen interaction (Gopalan et al. 1996; Leister et al. 1996; Scofield et al. 1996; Tang et al. 1996; Van den Ackerveken et al. 1996). The AvrPto protein, which is not a member of the AvrBs3 family, has been shown to interact with the protein product of *Pto,* the product of the R-gene corresponding to *avrPto* (Scofield et al. 1996; Tang et al. 1996). In the case of *Pto* resistance, the interaction of the Avr protein and the R-gene product is likely to occur in the cytoplasm and triggers a resistance reaction through an as yet poorly understood signal transduction cascade (Salmeron et al. 1996; Zhou et al. 1995, 1997). If the AvrXa10 interacts with an R-gene product, the interaction presumably occurs in the nucleus, or the complex, once formed, is translocated to the nucleus as proposed for the interaction of AvrBs3 and the product of the R-gene *Bs3* (Van den Ackerveken et al. 1996). However, equally consistent with the data is the model whereby some members of the AvrBs3 family may elicit resistance by induction of an R-gene and specificity may, in fact, be a DNA binding site. At the present time, both protein/protein and protein/DNA interaction models are plausible. The recent observation that *Xa1,* the gene for resistance to bacterial blight in rice, was induced upon inoculation with the incompatible bacterial strain is intriguing, although it is unknown if *avrXa1* is a member of the *avrBs3* family (Yoshimura et al. 1998).

Not all avirulence activities of the *avrBs3* family members are likely to be dependent on nuclear localization and/or the conserved C-terminal region containing the activation domain. The *avrBs3-2* gene (also referred to as *avrBsP*) is a member of the *avrBs3* family and has avirulence activity on tomato. Again, this gene was initially cloned without the carboxyl coding region and, therefore, would not appear to require these regions (Bonas et al. 1993; Canteros et al. 1991). In this case, then, the AvrPto/Pto model may be more apropos, and the *avrBs3-2* gene product may be intercepted in the cytoplasm.

Despite their avirulence activity, the function of the AvrXa10-like proteins is presumably for the enhancement of virulence. In parallel to resistance models, members of the *avrBs3* family may function as transcription factors (or cofactors) in susceptible interactions. The *avrXa10* gene has not been shown to have a role in pathogenicity. However, the gene occurs as but one of an estimated 12 to 14 members of the *avrBs3* family in the strain from which the gene was isolated (Hopkins et al. 1992). Thus, while the avirulence activity may be distinct, the role in pathogenicity may not be apparent due to the activity of related members without *Xa10* specificity. Other members of the *avrBs3* family have been shown to have effects on aspects of pathogenicity (Chakrabarty et al. 1997; Swarup et al. 1991, 1992), and the activation domain is highly conserved at the amino acid sequence level in all known gene members (Bonas et al. 1989; Hopkins et al. 1992; Yang et al. 1994). Therefore, the characterization of the transcription activation domain in members of the *avrBs3* family may add further insight into the mechanisms of bacterial pathogenesis. The identification of host genes, whose transcription may be affected by the protein products of the *avrBs3* family in resistance and disease, will provide the basis for studies regarding the possible function of these proteins as transcription factors.

MATERIALS AND METHODS

Strains, plasmids, and general methods.

Standard cultural methods were used for *Escherichia coli* (Ausubel et al. 1988). Where appropriate, carbenicillin was added at 50 µg per ml and spectinomycin at 100 µg per ml. Unless otherwise noted, the *E. coli* strain DH5αMCR was used (Life Technologies, Bethesda, MD). Sequencing was performed at the Iowa State University Sequencing Facility. All strains of *X. oryzae* pv. *oryzae* were derived from PXO99^{A}, which was maintained as described previously (Hopkins et al. 1992). Liquid cultures of *Xanthomonas* spp. were grown in Terrific Broth (TB; Ausubel et al. 1988). The *avrXa10* gene used in this study was derived from pBavrXa10 as previously described (Young et al. 1994). The 5.0-kb *Pst*I genomic frag-

ment was cloned from cosmid pXO5-15 (Hopkins et al. 1992). The FLAG epitope (DYKDDDDK) was added immediately downstream of the second *Sph*I site in the C-terminal region with the oligonucleotide 5′-GCGTCTTTGCATGCAGATTA CAAGGACGACGACGACAAGAAGGGTCGACCCAGCC CAATG-3′ to create pFWX10-F2 by the mutagenesis procedure of Kunkel et al. (1987). The oligonucleotide contains a unique *Sal*I fragment, which is not shown in Figure 1. Further details of *avrXa10* cloning and testing for avirulence activity have been described (Hopkins et al. 1992; Young et al. 1994). The *avrXa10* gene was introduced into *X. oryzae* pv. *oryzae* at the *Kpn*I site of the wide host range plasmid pHM1 as previously described (Young et al. 1994). The pHM1 vector was kindly provided by Brian Staskawicz and contains the pUC19 polylinker and a gene for resistance to spectinomycin.

The yeast strain Hf7c (*Saccharomyces cerevisae*), which contains both the *HIS3* and *lacZ* genes under the control of Gal4-regulated promoters, was used for testing transcriptional activation activity. The shuttle vector pAS2-1 containing the Gal4 DNA binding domain sequence under the control of the *ADH1* promoter and a polylinker at the C terminus was used for generating fusions (Harper et al. 1993). The plasmid was maintained in yeast by selection for tryptophan auxotrophy and in *E. coli* by selection for resistance to carbenicillin. Hf7c and pAS2-1 were obtained from Clontech Laboratories (Palo Alto, CA). The plasmid pGAL4 was used as a positive control (Fields and Song 1989). Methods for yeast manipulations were as described by the supplier. The inhibitor of the HIS3 activity, 3-amino-1,2,4-triazole (3-AT) was purchased from Sigma Chemical (St. Louis, MO).

Mutagenesis.

Changes in the DNA sequence of *avrXa10* (GenBank accession number J03710; Hopkins et al. 1992) were made by the procedure of Kunkel et al. (1987). The plasmid pFWX10-F1 was introduced into *E. coli* CJ236, which was subsequently infected with the helper phage M13K07, and single-stranded DNA was prepared as described (Kunkel et al. 1987). The TGA1064 mutation was introduced with the oligonucleotide 5′ - AAGGGGGCCGCCTGCAGTCACCAGATCTCGGTGC TGGACGC-3′, which, in addition to adding a stop codon, altered the terminal tryptophan residue at 1063 to isoleucine. TGA1064 also incorporated a new *Pst*I site into the DNA sequence after the stop codon. NlsA was altered with 5′-GAGCTGAAGTAGGGGAAAGATCTACTGCTGCCATCC CTGATGCCTGGAG-3′, which changed the translated sequence from KRVKP to AAVDL. The nlsB site was altered with oligonucleotide 5′-TGACAGCACGATCGGATCCTGA TGCTGCTGCGCTGCTTGCCCGCGTCTG-3′, which converted the sequence RKRSR to AAASG (starting at position 996 in AvrXa10) and introduced a *Bam*HI site. The nlsC site was altered with 5′-CCCGATCCTGGTAGCCGGTGCTG CTGAATTCCAGCTGAGGGGCAAATG-3′, which converted the sequence RVKRPRTR to NSAAPATR (starting at position 1032) and introduced an *Eco*RI site. The FLAG epitope was added to pZW22 as described for pFWX10-F2 above. All constructs with changes were sequenced at the region of interest and, in cases in which avirulence activity was lost, the repeat domain was swapped to a wild-type carboxyl coding sequence and tested for the ability to elicit an HR. The latter was done to ensure that mutations did not occur in the repeat domain in the mutagenesis process.

Avirulence activity testing.

Rice and tomato plants were grown in a growth chamber that was maintained at 25°C and 70% humidity. Plants were inoculated with bacteria that were prepared as described with a 1 cc plastic needleless syringe (Hopkins et al. 1992). HRs were visually scored. Rice lines IRBB7 and IRBB10 containing the resistance genes *Xa7* and *Xa10*, respectively, were used for avirulence tests of *avrXa7* and *avrXa10*. The *avrBs3* activity was tested in *X. campestris* pv. *vesicatoria* on near-isogenic pepper lines ECW-30R (*Bs3*) and ECW.

Enzyme activity assays.

Yeast cells were streaked on selective media and grown at 28°C for 2 days. Assays for β-galactosidase activity in HF7c were performed as described by the supplier (Clontech Laboratories) in triplicate. Histological staining for β-glucuronidase was performed according to Martin et al. (1992). Eight to 12 leaves were inoculated with each strain and stained with 5-bromo-4-chloro-3-indolyl-β-glucuronidase (X-Gluc) for β-glucuronidase activity.

Plant transcriptional activation assay.

Agrobacterium tumefaciens EHA101 was used for transient assays of transcriptional activation in plant leaves (Hood et al. 1986). Gal4 DNA binding protein (Gal4DB) fusions with AvrXa10 and derivatives were cloned from pAS2-1 as *Hin*dIII fragments and inserted into pBluescript II KS^+ (Stratagene, La Jolla, CA). The fragments were then inserted into pMON410 after cleavage with *Kpn*I and *Eco*RI and removal of the gene for hygromycin resistance (Rogers et al. 1987). The gene for the Gal4DB domain without a transcription activator domain, as represented in pAS2-1, was used as a negative control. The gene 35S-TMV-Gal4-VP16-3'NOS, which encoded the Gal4DB domain fused to the activation domain of VP16 (Ulmasov et al. 1995; kindly provided by Tim Ulmasov), was inserted into pMON410 as a positive control. *Agrobacterium* strains were grown in LB media (Ausubel et al. 1988) containing 100 μg per ml of spectinomycin, resuspended in AB media (Chilton et al. 1974) containing 100 μM acetosyringone and cultured for 5 h at 28°C prior to inoculation. Bacteria were inoculated into the leaves of *Arabidopsis thaliana* (ecotype Nossen) containing pAT73, which has the bacterial *uidA* gene (GUS) under the control of the minimal CaMV 35S promoter and 10 UAS_G binding sites (kindly provided by Juan Estruch, Novartis Seeds). Eight leaves (two per plant) were inoculated with each strain. Leaves that stained positive at any inoculation site were scored as positive.

Immunodetection assays.

Protein was extracted from $PXO99^A$ containing the individual constructs after overnight growth on TB. The bacteria were pelleted by centrifugation and resuspended in 20 mM phosphate buffer, pH 7.2. The suspension was sonicated in four 15-s pulses for 1 min. Protein was concentrated by centrifugation in Centricon 100 concentrators (100 kDa molecular mass cutoff; Amicon, Beverly, MA), fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-

PAGE), blotted, and treated with M2 anti-FLAG monoclonal antibody as previously described (Young et al. 1994).

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