AVRXa10 Protein is in the Cytoplasm of Xanthomonas oryzae pv. oryzae

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AVRXa10 from Xanthomonas oryzae pv. oryzae was tagged with a unique hydrophilic octapeptide (FLAG) to permit antibody-mediated identification and purification of the gene product. X. o. pv. oryzae that produced tagged AVRXa10 elicited a hypersensitive response (HR) on rice cultivars containing the resistance gene Xa-10, but not on cultivars lacking Xa-10. The tagged AVRXa10 protein purified from Escherichia coli or X. o. pv. oryzae did not elicit a hypersensitive response in rice with the Xa-10 resistance gene. Anti-FLAG monoclonal antibodies reacted with a 119-kDa protein in both E. coli and X. o. pv. oryzae cells expressing the tagged avrx10 gene. Polyclonal antibodies raised against purified AVRXa10 protein reacted with the 119-kDa protein and several additional proteins from X. o. pv. oryzae, which probably are the products of genes related to avrx10. Biochemical fractionation and immunoelectronmicroscopy analysis was used to demonstrate that AVRXa10 was located in the cytoplasm of X. o. pv. oryzae cells when grown in planta or in culture medium.

Additional keywords: gene tagging, immunocytotoxicity, electron microscopy, bacterial resistance.

Xanthomonas oryzae pv. oryzae (Ishiyama) Dye (Swings et al. 1990), the causal agent of bacterial blight of rice, interacts in a race-specific manner with rice cultivars containing different resistance genes (Mew 1987). The mechanisms by which the host and pathogen interact and result in the elicitation of disease resistance in a race-specific manner have yet to be elucidated. The products of avirulence genes cloned from both fungi and bacteria function either directly as race-specific elicitors or indirectly in governing the synthesis of race-specific elicitors. The product of the avr9 gene of Cladosporium fulvum, a 28-amino-acid peptide after posttranslational processing, directly elicits a hypersensitive response (HR), which is associated with disease resistance in tomato cultivars carrying the Cf9 locus (Schottens-Toma and de Wit 1988, van Kan et al. 1991). The avrD gene product of Pseudomonas syringae pv. tomato directs the synthesis of syringolide compounds, which elicit the HR in soybean cultivars containing the Rpg4 resistance gene (Keen et al. 1990).

A family of avirulence genes identified from X. o. pv. oryzae hybridized with avrBs3, an avirulence gene from Xanthomonas campestris pv. vesicatoria (Bonas et al. 1989, Hopkins et al. 1992). Two of these genes (avrXa7 and avrXa10) are structurally similar, each containing an internal domain with several copies of a 102-bp directly repeated sequence (Hopkins et al. 1992). The nucleotide sequence of avrXa10 consisted of a 3,306-bp open reading frame, which would code for a predicted 119-kDa protein. The number of functional avrBs3-related genes in the genome of X. o. pv. oryzae is not known. As many as 12 avrBs3 homologs are predicted in X. o. pv. oryzae strain PXO86 on the basis of restriction fragment analysis (Hopkins et al. 1992). Antiserum raised against the AvrBs3 protein was shown to cross-react with six to seven proteins in extracts of strain PXO86, indicating that several genes from the family may be expressed (Knoop et al. 1991).

Knowledge of the location of avirulence gene products in bacterial-plant interactions could provide insight regarding AVR protein function in the elicitation of resistance. Analysis of the nucleotide sequence of avrXa10 and avrBs3 revealed no apparent membrane spanning domains in the predicted protein products. Knoop and co-workers (1991) used an antiseraum directed against AvrBs3 to demonstrate that most of the AvrBs3 protein was located in the cytoplasm. However, 20–30% was associated with the insoluble protein fraction of cell extracts. In a subsequent study using immunocytotoxicism, the avrBs3 gene product was shown to be located in the cytoplasm of X. c. pv. vesicatoria in planta and in bacterial cultures (Brown et al. 1993). The cellular location of the AvrBs3 product suggests that the protein is not recognized directly by plant cells. In this study, we investigated the location of the AVRXa10 protein from X. o. pv. oryzae both in inoculated plant tissues and bacterial cultures.

Characterization of AVRXa10.

The bacterial strains, plasmids, and phage used and their sources are listed in Table 1. The avrXa10 gene was subcloned from the cosmid clone pXO5-15 (Hopkins et al. 1992) into pBluescript KS+. A portion of the downstream sequence, a 0.6-kb Smal fragment, and the promoter-distal PstI site were removed by digesting the clone with Smal and

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religating. The promoter and 5' portion of the coding region were inserted as a 1.2-kb Psrl fragment. A 1.0-kb fragment upstream of the promoter was removed by digestion with XhoI, treated with mung bean nuclease, and ligated to EcoRI linkers (Promega G1061). The resulting construct, pBavrXa10, was used for further manipulations.

The avrXa10 gene is one of approximately 12 related genes in the X. o. pv. oryzae genome. Therefore, an immunological tag (FLAG) was introduced into the C-terminal coding domain to detect the avrXa10 gene product specifically (Fig. 1A). Single-stranded DNA of pBavrXa10 was isolated (Crouse et al. 1983) from E. coli and subjected to site-directed mutagenesis (Kunkel et al. 1987) with the oligonucleotide 5'GTTGAATGCGGGAATCTTATCATCGTTATCTCAGTATTGTTTCATCCCTTGTAAATCCGCAGCCCCACG3', which anneals to avrXa10 at nucleotides 3376-3409 (Hopkins et al. 1992), to construct pBavrXa10.F1 (Fig. 1A). Seven additional codons were inserted in frame between 3390-3391 that encode the FLAG peptide DYKDDDDK (in bold). The T to A change at nucleotide 3393 created an EcoRI restriction site in pBavrXa10.F1.

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

<table>
<thead>
<tr>
<th>Designation</th>
<th>Relevant characteristics</th>
<th>Source</th>
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<tbody>
<tr>
<td><em>Xanthomonas oryzae</em> pv. <em>oryzae</em></td>
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<tr>
<td>PXO999</td>
<td>Race 6; virulent to rice with <em>Xa</em>-10 resistance gene and grows on 200 µM 5-azacytidine</td>
<td>Hopkins et al. 1992</td>
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<tr>
<td>PXO86</td>
<td>Race 2; avirulent to rice with <em>Xa</em>-10 resistance gene</td>
<td>T. Mew, IRRI, Los Baños, Philippines</td>
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<td><em>Escherichia coli</em></td>
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<td>DH5α MCR</td>
<td><em>F−</em>, mcrA, recA, 80dlacz, M15</td>
<td>Gibco BRL, Grand Island, NY</td>
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<td>BL21(DE3)</td>
<td><em>F−</em>, lacUV5, ompT*, m*, Rif*</td>
<td>Novagen, Inc., Madison, WI</td>
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<td>CT236</td>
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<td>Kunkel et al. 1987</td>
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<td>Plasmids</td>
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<tr>
<td>pUFRO27</td>
<td>Km*, lacZ*, Mob*, mob(P), Par*</td>
<td>De Feyter et al. 1990</td>
</tr>
<tr>
<td>pBluescript KS+</td>
<td>Ap*, M13; lacZ*</td>
<td>Stratagene Inc., La Jolla, CA</td>
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<td>pXO5-15</td>
<td>pHMI clone from <em>X. o. pv. oryzae</em> that contains avrXa10</td>
<td>Hopkins et al. 1992</td>
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<tr>
<td>pBavrXa10</td>
<td>pBluescript clone with a 4.6-kb <em>EcoRI</em>-Sma1 fragment that contains avrXa10</td>
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<td>pBavrXa10 clone cut with XhoI and cloned into pUFRO27</td>
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<tr>
<td>pBUavrXa10-2</td>
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![Fig. 1](image-url)

**Fig. 1.** Subclones containing avrXa10. The hatched boxes represent the avrXa10 open reading frame. Arrows indicate promoters and the orientation of transcription. In pBavrXa10.F1, avrXa10 is under the influence of its native promoter (P2) and the pBluescript lacZ promoter (P1). pBavrXa10.F1 carries a 21-nucleotide insertion that, with three nucleotides from the original clone, encodes for the FLAG peptide (DYKDDDDK). In addition, pBavrXa10.F1 carries an engineered *EcoRI* site indicated by the "*". The pET-avrXa10.F1 clone is under the influence of the pET-22b+ **TTlac** promoter (P3) and lacks the avrXa10 promoter. Restriction enzyme cleavage sites are indicated: B, *BamHI*; E, *EcoRI*; K, *KpnI*; P, *PstI*; S, *SmaI*; X, *XhoI*.**
spanning nucleotides 3391 to 3396. Mutant clones of pBavxl0.F1 were identified by cleaving with EcoRI. To test for avirulence activity, both pBavxl0 and pBavxl0.F1 were introduced into the broad-host-range plasmid pUFRO27. The plasmids were cleaved with either XbaI or KpnI, to orient the avrXa10 and avrXa10.F1 gene with respect to the pBluescript lacZ promoter, and ligated to pUFRO27 to generate pBuvavxl0.F1, pBuvavxl0.F2, pBuvavxl0, and pBuvavxl0-2. The endogenous avrXa10 promoter by itself (pBuvavxl0.F2 and pBuvavxl0-2) did not allow for levels of expression of the AVRxa10 protein that were sufficient for detection by immunocytochemistry in E. coli (data not presented).

Both clones pBavvl0.F1 and pBavvl0.F1 were introduced to the X. o. pv. oryzae strain PXO99 A by electroporation as described (Choi and Leach 1994). The transformants were inoculated to rice cultivars IR24 and IR-BB10 as previously described (Reimers and Leach 1991) to test for the ability to elicit an HR. Rice cultivar IR-BB10 is near-isogenic to IR24 and contains the Xa-10 gene for bacterial blight resistance (Ogawa and Khush 1989; Ogawa and Yamamoto 1987). IR24 lacks the Xa-10 gene and is susceptible to all strains of X. o. pv. oryzae used in this study. In interactions involving avrXa10 with and without the immunological tag, and the resistance gene Xa-10 (IR-BB10), a dark brown color was observed throughout the infiltrated site within 24-48 hr, whereas in the interactions without Xa-10 (IR24) the infiltration site remained water soaked. The immunological tag (FLAG) was introduced into three other locations in the N-terminal, repeat, and C-terminal domains; however, the avirulence activity was lost in these mutants (data not presented).

To detect AVRxa10.F1, total protein extracts from X. o. pv. oryzae strain PXO99 A containing pBuvavxl0.F1 were analyzed in Western blots. Xanthomonas strains were grown in Terrific Broth (TB; Tartof and Hobbis 1987) supplemented with carbenicillin (100 μg/ml) and kanamycin (100 μg/ml), on a rotary shaker (250 rpm) at 28°C. Proteins were extracted and separated on sodium dodecyl-sulfate (SDS)-polyacrylamide gels (10% PAGE) as described (Laemmli 1970) and either stained with Coomassie Blue R-250 or transferred electrophoretically to nitrocellulose membranes (Schleicher & Schuell, Keene, NH) using Towbin buffer (Towbin et al. 1979). Anti-FLAG IgG M2 monoclonal antibody (IBI/Kodak, New Haven, CT), which detects the FLAG peptide, specifically recognized a protein of approximately 119 kDa from cell extracts of X. o. pv. oryzae strain PXO99 A harboring the tagged avrXa10 gene (Fig. 2A, lane 3). A PXO99 A transformant harboring the untagged avrXa10 gene did not contain protein that reacted with the anti-FLAG antibody (Fig. 2A, lane 2).

To prepare AVRxa10 polyclonal antiserum, plasmid pBavvl0.F1 was digested partially with BamHI to isolate a 4.6-kb fragment. The 5' terminus of the fragment was at the BamHI site in the coding region for the N-terminus of the peptide and included the portions coding for the FLAG sequence and carboxy-terminus. The 4.6-kb BamHI fragment was inserted into the expression vector pET-22b (+) (Novagen, Inc. Madison, WI) to generate pET-avrXa10.F1 (Fig. 1B). For the production of protein, E. coli BL21(DE3) containing pET-avrXa10.F1 was induced with 1 mM isopropyl-β-thiogalactopyranoside (IPTG). The protein was extracted by sonification and purified by chromatography on anti-FLAG M2 affinity gel, as described by the manufacturer (IBI/Kodak). For immunization of female white New Zealand rabbits, the protein (250 μg) was excised from SDS-polyacrylamide gels and emulsified with Freund's incomplete adjuvant (1:1). Antibodies for Western analysis were purified from the serum by protein A column chromatography (Sigma, St. Louis, MO) and multiple cross adsorption to BL21(DE3) acetone powder as described (Harlow and Lane 1988). The antibodies were used at a dilution of up to 1:5,000 relative to the original antiserum.

Polyclonal antibodies to AVRxa10 reacted only with the 119-kDa AVRxa10 protein in E. coli harboring the avrXa10 gene (Fig. 2B; lane 2). However, the antibodies also reacted with several protein bands in addition to the 119-kDa band in extracts from both PXO99 A and PXO86 (Fig. 2B; lanes 4–7). Antiserum raised to AvrB3 cross-reacted with several proteins in extracts from X. o. pv. oryzae strain PXO86 and also
in other pathovars of X. campestris (Knoop et al. 1991). Because several avirulence genes (avrB6, avrBs3, avrBs3-2, avrBsp, and avrXa10) from the avrBs3-related family of Xanthomonas show a high degree of sequence identity (> 90%, Bonas et al. 1989; Canteros et al. 1991; Bonas et al. 1993; De Feyter et al. 1993; Hopkins et al. 1992), cross-reactivity with AVRXa10 antiserum is likely due to similarity of epitopes in the protein products of the avrBs3-family. Preimmune serum did not react with any of these proteins (data not shown).

E. coli with pET-avrXa10.F1 that expressed the AVRXa10.F1 protein did not elicit the HR on IR-BB10 (data not presented). Affinity purified AVRXa10.F1 from E. coli also did not elicit an HR on IR-BB10. Similarly, purified AVRXa10.F1 protein from X. o. pv. oryzae did not elicit the HR when infiltrated into rice cultivars IR-BB10 or IR24 (at a maximum concentration of 1 mg/ml). These data indicate that the AVRXa10 protein is not directly involved in the elicitation of resistance. However, we cannot rule out the possibility that AVRXa10 may be modified to an active state before being exported from the bacterium, as a result of interacting with the plant.

Localization of AVRXa10 protein.

The location of AVRXa10 in cells of X. o. pv. oryzae expressing tagged AVRXa10 was determined by biochemical fractionation and immunolectronmicroscopy. For biochemical fractionation, X. o. pv. oryzae cells were grown in TB overnight and harvested by centrifugation. Cells were washed and resuspended to an OD450 of 0.7 in cold (4°C) fractionation buffer (10 mM Tris HCl [pH 8.0], 200 mM NaCl, 0.5 mM EDTA, 2 mM 2-mercaptoethanol) containing RNase A (10 μg/ml), DNase I (10 μg/ml), phenylmethylsulfonyl fluoride (PMSF) (100 μg/ml), Leupeptin (1 μg/ml), and Pepstatin A (1 μg/ml). Bacterial cells were compressed and passed through a prechilled French pressure cell (16,000 lbs/in²) three times and centrifuged at 235,000 × g for 4 hr at 4°C to separate soluble and insoluble fractions. To obtain total membranes, the insoluble fraction was resuspended in fractionation buffer, layered on a 20–60% sucrose density gradient, centrifuged at 55,000 × g for 1.5 hr at 4°C, and collected from the sucrose gradient.

For extraction of extracellular proteins, X. o. pv. oryzae cells were grown in 100 ml each of TB and a medium re-

![Fig. 3. Immunogold localization of tagged AVRXa10 protein. A, B, Cells of X. o. pv. oryzae PXO99^a (pBUavrXa10) and X. o. pv. oryzae PXO99^a (pBUavrXa10.F1), respectively, grown in liquid cultures. C, D, Cells of X. o. pv. oryzae PXO99^a (pBUavrXa10) and X. o. pv. oryzae PXO99^a (pBUavrXa10.F1), respectively, in the vessels of leaves of IR-BB10 at 24 hr after inoculation. Sections were labelled with anti-FLAG antibody. Bar = 0.5 μm.](image-url)
ported to induce hrp genes in X. c. pv. vesicatoria, MM1 (Schulte and Bonas 1992), overnight. Cultures were treated either in the presence or absence of 500 μM PMSF, 5 μM Pepstatin A, and 5 μM Leupeptin for 1 hr before cells were pelleted. In addition, leaf tissues from IR24 and IR-BB10 were macerated and extracted in 10 mM Tris buffer (pH 8.0), and 10-ml aliquots were added to the MM1 medium. Extracellular proteins were precipitated by addition of ammonium sulfate to a concentration of 95% (w/v). The precipitate was collected by centrifugation, dissolved in TBS buffer (50 mM Tris-HCL [pH 7.4], 150 mM NaCl) with 0.1% bovine serum albumin, and dialyzed overnight at 4°C in TBS. All fractions were compared densitometrically after analysis in Western and dot blots with both AVRxA10 polyclonal antibodies and anti-FLAG M2 antibody.

AVRxA10 was detected primarily in the cytoplasmic (soluble) fraction, with less than 12% present in the membrane (insoluble) fraction. Similar results were reported for the related AvrB53 protein from X. c. pv. vesicatoria (Knoop et al. 1991). However, no AVRxA10 protein was detected in concentrated culture supernatants of X. o. pv. oryzae cells or from intercellular fluids of rice leaves from IR-BB10 and IR24 after infiltration with X. o. pv. oryzae PX099A (pBUavrXa10.F1). These data indicate that the AVRxA10 protein is not located outside the bacterial cells, although the amount or forms of AVRxA10 present may have limited detection of the protein outside the bacterium.

The location of AVRxA10 was determined in inoculated leaves and in bacteria grown overnight in TB by using both AVRxA10 antibody and anti-FLAG antibody in immunoelectronmicroscopy. One site per leaf of 10-day-old seedlings was inoculated with X. o. pv. oryzae PX099A (pBUavrXa10) and PX099A (pBUavrXa10.F1) as previously described (Reimers and Leach 1991). For each treatment, two leaves were combined from each of four different pots, and the inoculated sites were sampled at 3, 6, 12, and 24 hr after inoculation. Leaf tissues and bacterial cell pellets were treated with 2.5% glutaraldehyde in CAB at 4°C overnight and 30 min, respectively. Fixed leaf tissues and bacterial cells were dehydrated and infiltrated with LR White as described by the manufacturer (Data Sheet #305A, Polyscience, Inc.).

Thin sections, placed onto parlodion-covered nickel grids, were immunogold-labeled and stained as described (Lutkenhaus and Bi 1991). The sections were examined with an electron microscope (Hitachi H-300, Hitachi Instruments Inc., San Jose, CA) at magnifications of 30,000х to 40,000х. The gold particles in and around the bacteria were counted in 40 fields per treatment, nonspecific labeling was subtracted, and mean numbers ± standard error of the mean (SEM) of gold particles were determined. Each treatment consisted of two to four replicates.

Gold particles were distributed randomly within the cytoplasm of X. o. pv. oryzae PX099A (pBUavrXa10.F1) cells both from cultures and in rice cultivars IR-BB10 (Fig. 3B and D). A few gold particles were found in the rice tissue (cell walls, vacuoles, cytoplasm, and intercellular spaces), but the number of particles bound to host tissue was similar to the nonspecific labeling observed on sections that were not treated with the anti-FLAG antibody. Specific gold-labeling when the anti-FLAG antibodies was used was absent in PX099A cells containing an untagged avrXa10 gene (Fig. 3A and C). The number and distribution of gold particles at 3, 6, and 24 hr after inoculation were the same in cells of X. o. pv. oryzae PX099A (pBUavrXa10.F1) grown either in IR24 or IR-BB10 rice leaves (Fig. 4). The mean number of gold particles associated with cells of X. o. pv. oryzae PX099A (pBUavrXa10.F1) grown in culture was 4.6 ± 0.46. The majority of the gold particles were in the cytoplasm of the cultured bacteria, with less than 5% of the particles associated with the membrane. In addition, the results suggest that avrXa10 is expressed constitutively and is not induced to higher levels in planta. Previously, avrB53, pha4, and avrb6 were shown to be expressed constitutively (Knoop et al. 1991; Swarup et al. 1992; De Feyer et al. 1993). Similar experiments were performed using the anti-AVRxA10 antibody; the mean numbers of gold particles observed on X. o. pv. oryzae PX099A (pBUavrXa10.F1) grown in IR24 and IR-BB10 for 24 hr after infiltration were 14.5 ± 1.2 and 14.1 ±

![Fig. 4. Quantitative analysis of the distribution of immunogold particles within cells of X. o. pv. oryzae PX099A(pBUavrXa10.F1) 3, 6, and 24 hr after inoculation into rice leaves of IR24 (hatched bars) and IR-BB10 (solid bars). Location of gold particles indicated underneath bars (cytoplasm, inner membrane, outer membrane, and extracellular). Sections were labeled with anti-FLAG antibody. Mean numbers of gold particles recorded at each site in 200 separate bacteria are given (bars = SEM).](image-url)
0.87, respectively. The three- to four-fold increase in the number of bound gold particles with the polyclonal antiserum probably was due both to the increase in available antigenic sites and to the presence of products from genes related to avrXa10.

The detection of AVRXa10 protein in the cytoplasm by immunocytochemistry is consistent with the results obtained for AvrBs3 protein in X. c. pv. vesicatoria (Brown et al. 1993). By using antiserum that reacts specifically to a known extracellular plant peroxidase in immunogold labeling experiments, we detected the peroxidase in the intercellular spaces and vessels of the same tissues used for detection of AVRXa10 (Young and Leach, unpublished results). Thus, extracellular proteins were not washed from these spaces during fixation processes. We cannot rule out the possibilities that, if AVRXa10 was exported, the amounts outside the cell may have been too low to detect immunologically or that the protein may have been modified such that it was elicitor-active but not recognized by the antiserum.

Since AVRXa10 and AvrBs3 (Brown et al. 1993) are located in the cytoplasm and were not detected outside the bacterial cells, it is unlikely that these avr gene products interact directly with plant cells. Thus, the mechanism by which disease resistance is elicited in a race-specific manner in these plant-bacterial interactions remains unclear. Studies on the function of AVRXa10 and related proteins in the bacterium will offer valuable insight into the role of this family of proteins in plant-pathogen interactions.

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LITERATURE CITED


