The *avrRxo1* Gene from the Rice Pathogen *Xanthomonas oryzae* pv. *oryzicola* Confers a Nonhost Defense Reaction on Maize with Resistance Gene *Rxo1*

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Submitted 23 September 2003. Accepted 11 February 2004.

Maize lines that contain the single dominant gene *Rxo1* exhibit a rapid hypersensitive response (HR) after infiltration with the rice bacterial streak pathogen *Xanthomonas oryzae* pv. *oryzicola*, but not with the rice bacterial blight pathogen *X. oryzae* pv. *oryzae*. The avirulence effector gene that corresponds to *Rxo1*, designated *avrRxo1*, was identified in an *X. oryzae* pv. *oryzicola* genomic library. When introduced into *X. oryzae* pv. *oryzae*, clones containing *avrRxo1* induced an HR on maize with *Rxo1*, but not on maize without *Rxo1*. The *avrRxo1* gene is 1,266 bp long and shows no significant homology to any database sequences. When expressed in an *X. oryzae* pv. *oryzae* *hrpC* mutant that is deficient in the type III secretion system, *avrRxo1* did not elicit the HR, indicating that the *avrRxo1*–*Rxo1* interaction is dependent on type III secretion. Transient expression of *avrRxo1* in onion cells after biolistic delivery revealed that the protein product was associated with the plasma membrane. Transient expression in maize lines carrying *Rxo1* resulted in cell death, suggesting that *AvrRxo1* functions from inside maize cells to elicit *Rxo1*-dependent pathogen recognition.

Additional keywords: bacterial leaf streak, nonhost resistance.

Xanthomonas oryzae pathovars cause two serious diseases of rice. *X. oryzae* pv. *oryzicola* causes the bacterial streak disease and *X. oryzae* pv. *oryzae* causes bacterial blight. More than 20 rice genes that confer resistance to bacterial blight are available and are widely used in breeding programs to control the disease (Leung et al. 2002). In contrast, no single-gene resistance to bacterial streak has been identified in rice. This is particularly alarming because bacterial streak is increasing in importance throughout Asia, and especially in China, where many of the high-yielding hybrids are very susceptible (Xie et al. 1990).

Single-gene resistance to *X. oryzae* pv. *oryzicola* has not been found in rice; therefore, alternative strategies are needed to control this disease. One possibility is to identify a nonhost resistance (*R*) gene, (i.e., a gene that recognizes and signals a defense response in a plant that is not a host for *X. oryzae* pv. *oryzicola*) and to introduce that *R* gene into rice. Recognition of bacterial pathogens and induction of defense

responses by nonhost plants have been reported previously (Leach and White 1996). For example, the effector genes *avrA* and *avrD* from *Pseudomonas syringae* pv. *tomato* (a tomato pathogen) are recognized by two soybean resistance genes, *Rpg2* and *Rpg4*, respectively (Ashfield et al. 1995; Keen et al. 1991). An effector gene, *pthA*, from *X. citri* that is essential for pathogenicity on citrus also controls the elicitation of hypersensitive responses (HR) on the two nonhosts bean and cotton (Swarup et al. 1992). In a few cases, both the host resistance gene and the pathogen effector (*avr*) genes have been genetically characterized (e.g., *avrPphB* from *P. syringae* pv. *phaseoli* [Jenner et al. 1991] and the *R* gene from *Rps5 Arabidopsis* [Simonich and Innes 1995; Warren et al. 1999]). Although it is unlikely that the elicitation of the HR in these situations is solely responsible for the inability of the pathogen to colonize the nonhost plants, it is intriguing that the nonhost plants contain genes whose products recognize specifically with effectors from bacteria that are not typically pathogens of those plants and that these interactions result in execution of a signal cascade that is related to defense responses.

Recently, we demonstrated that a single dominant gene, called *Rxo1*, in maize controls elicitation of the hypersensitive response to *X. oryzae* pv. *oryzicola* (Zhao et al., in press). In addition to the possible utility for controlling bacterial leaf streak in rice, the identification of the maize and *X. oryzae* pv. *oryzicola* genes that activate defense responses provides a useful model system for studying nonhost resistance in cereals. Here, we describe the identification and characterization of the effector gene from *X. oryzae* pv. *oryzicola* that corresponds to *Rxo1*. Because no rice resistance genes are available for bacterial leaf streak, no effector genes that directly function in avirulence had been identified from this pathogen. *X. oryzae* pv. *oryzicola*, like its close relative *X. oryzae* pv. *oryzae*, contains several copies of the *avrBs3-pthA*-family of effector genes common to the genus *Xanthomonas* (Ryba-White et al. 1995). In addition, the pathogen contains genes similar to type III secretion system (TTSS) genes from *X. oryzae* pv. *oryzae* (Raymundo and Leach 1994); the TTSS functions to deliver bacterial pathogen effector genes to plant cells in many host–pathogen interactions (Buttner and Bonas 2002). Here, we report that the *avrRxo1* gene encodes a novel effector not related to the *avrBs3-pthA* family, and provide indirect evidence that the pathogen effector gene product (*AvrRxo1*), which localizes to the plant plasma membrane, interacts with *Rxo1* inside maize cells to elicit cell death.

RESULTS

X. oryzae pv. *oryzicola* strains cause an HR on maize with *Rxo1*.

Forty *X. oryzae* pv. *oryzicola* strains collected from different geographical regions in the Philippines and China were tested for their interaction phenotypes in maize lines B73, which carries *Rxo1*, and Mo17, which does not carry *Rxo1*. All 40 strains induced a strong HR when infiltrated into seedling leaves of line B73, but not when infiltrated into Mo17 (Fig. 1). In a separate study, an F₂ population and a population of recombinant inbred (RI) lines derived from a cross of B73 × Mo17 also were screened by infiltration with *X. oryzae* pv.

oryzicola. In the F₂ population, the HR phenotype segregated at a 3:1 ratio; whereas, in the RI population, the HR phenotype segregated at a 1:1 ratio (Zhao et al., in press). Together, these experiments suggest a single gene, designated *Rxo1*, from maize that controls recognition of *X. oryzae* pv. *oryzicola* and determines the HR phenotype (Zhao et al., in press). Two *X. oryzae* pv. *oryzicola* strains, PXO86 and PXO99A, did not induce the HR in B73 or Mo17.

Identification of the bacterial effector gene *avrRxo1*.

All 40 *X. oryzae* pv. *oryzicola* strains induced an HR on maize lines with *Rxo1* but not on lines without *Rxo1*; therefore, we speculated that the genome of pathovar *X. oryzae* pv. *oryzicola* carried a conserved effector gene whose product was recognized by *Rxo1*. To identify the effector gene, a genomic library constructed from strain BLS256 was introduced into *X. oryzae* pv. *oryzicola* PXO99A by triparental mating (Hopkins et al. 1992). Kanamycin-resistant transconjugants were tested for the ability to induce HR on B73. A transconjugant carrying cosmid VB1C, with an insert size of approximately 40 kb, incited a strong HR on B73 (*Rxo1*) but not Mo17 (*rxo1*) (Table 1), suggesting that VB1C carries the effector gene *avrRxo1* that corresponds to *Rxo1*.

Subcloning of VB1C revealed that the avirulence function was contained within a 1.797-kb *SphI* fragment. The 1.797-kb *SphI* fragment contained two putative open reading frames (ORFs) (Figs. 2 and 3). The 1,266-bp ORF encodes a predicted protein of 421 amino acids (aa) (GenBank accession number AY395713), whereas the smaller 294-bp ORF codes for a putative protein of 98 aa (GenBank accession number AY395712). The promoter region of ORF1 contains an imperfect plant-inducible promoter (PIP-box) sequence motif (Fenselau and Bonas 1995) at 89 to 110 bp upstream of the putative translational start site (Fig. 2). There is no obvious promoter adjacent to ORF2. A sequence homology search of ORF1 and ORF2 and their predicted proteins did not yield significant similarity to any known gene or protein. The predicted amino acid sequence of the ORFs were examined using InterProScan and GCG (Madison, WI, U.S.A.) software to identify conserved motifs that might provide clues as to their function. Several possible functional domains were revealed in ORF1, including a eukaryotic thiol (cysteine) protease active site (69-LPHSVPIAGAS-79), an ATP/GTP binding site motif A (P-loop) (160-GNTATGAS-167), nine putative N-myristoylation sites, and a putative nuclear localization sequence (NLS) (122-PERRKLH-128) (Fig. 2). Analysis of the predicted protein product of ORF2 suggests a pI of 5.1, which, along with its

Fig. 1. A, Phenotype of interactions between *Xanthomonas oryzae* pv. *oryzicola* strain BLS256 and maize inbred lines B73 (*Rxo1/Rxo1*) and Mo17 (*rxo1/rxo1*). Infiltration of B73 with *X. oryzae* pv. *oryzicola* strain BLS256 results in a hypersensitive response that appears within 2 to 3 days. Infiltration of Mo17 shows no reaction. **B**, Transient expression of the *avrRxo1* in maize cells expressing the endogenous *Rxo1* gene causes cell death. Many fluorescent foci were observed after cobombardment of B73 (1) or Mo17 (2) with HBT-SGFPTYG-NOS (encoding the green fluorescent protein) and pUC19 (empty plasmid) indicating no cell death was induced. When Mo17 (*rxo1/rxo1*) was cobombarded with HBT-SGFPTYG-NOS and the construct containing *avrRxo1*, HBT-AVRRXO1-NOS, there are many fluorescent foci (4) indicating no cell death. However, when B73 (*Rxo1/Rxo1*) was cobombarded with HBT-SGFPTYG-NOS and HBT-AVRRXO1-NOS, no green foci were observed (3). This indicates that AvrRxo1 functions inside maize cells to elicit *Rxo1*-dependent pathogen recognition and cell death. **C**, Green fluorescent protein (GFP)-labeled AvrRxo1 locates to the plant cell plasma membrane. Transient expression of HBT-GFP-GFP-NOS (1) in onion cells showed that the GFP protein was located to both the cytosol and nucleus. Expressing HBT-AVRRXO1-GFP-GFP-NOS (2) in onion cells showed that the AvrRxo1-GFP fusion proteins are located predominantly on the plasma membrane.

small size and putative α -helix at its carboxyl terminus, is consistent with a chaperone-like protein (Page and Parsot 2002). The G+C content of the two ORFs was 51.1%, which is below the normal range of *Xanthomonas* genes (approximately 63 to 71%) (Starr 1981). These adjacent ORFs were flanked by nearly identical sequences that show similarity to transposase IS1478 from *X. campestris* pv. *campestris* (gi:1927196) (Chen et al. 1999) (Fig. 3).

Southern blot analysis of genomic DNA from seven different *X. oryzae* pv. *oryzicola* strains indicated that there is only one copy each of ORF1 (Fig. 4) and ORF2 (not shown) in the genome. All *X. oryzae* pv. *oryzicola* genomes tested contained fragments that hybridized to both ORFs (BLS101, BLS175, BLS256, BLS288, BLS290, BLS295, and BLS298) (Fig. 4). In addition, neither ORF1 nor ORF2 hybridize with genomic DNA from *X. oryzae* pv. *oryzae* (strains PXO61, PXO71, PXO79, PXO86, PXO99, and PXO112). The *Rxo1* gene in maize cosegregates with a gene conferring resistance to *Burkholderia andropogonis* (Zhao et al. in press), which causes bacterial leaf stripe of maize, sugarcane, and sorghum. Thus, we asked if strains of *B. andropogonis* contained homologues of *avrRxo1*. Under hybridization conditions of low or moderate stringency, the *B. andropogonis* strains tested (Pa3459, Pa3549, and Pa3319) did not hybridize to *avrRxo1* (data not shown).

To determine which ORF was responsible for induction of the HR on B73 maize with *Rxo1*, the second ORF was removed by digestion of plasmid pavrRxo1 (which contains both ORFs) with *Hind*III and religation to form plasmid p9-1H3, which only carries the 1.43-kb *Sph*I-*Hind*III fragment (Fig. 3). *X. oryzae* pv. *oryzae* PXO99A transformants containing plasmid p9-1H3, with a functional ORF1 and a truncated ORF2, elicited an HR on B73, demonstrating that ORF1 is sufficient for elicitation of the HR (Table 1). This result also was confirmed by inoculation onto the RI lines derived from a B73 $\times$ Mo17 cross (data not shown).

AvrRxo1 function is dependent on the TTSS.

Secretion of the products of most characterized *Xanthomonas* *avr* genes requires a functional TTSS. To determine if AvrRxo1 function in plants is dependent on type III secretion, clone pavrRxo1 was expressed in PXO99^AMX, an *hrpC* mutant of *X. oryzae* pv. *oryzae* PXO99^A. The disruption in *hrpC* mutates the HrcC protein, and results in a nonfunctional TTSS (Zhu et al. 2000). Although PXO99^A(pavrRxo1) incites the HR on maize line B73 (*Rxo1*), PXO99^A9MX (pavrRxo1) does not incite the HR in maize lines B73 or Mo17 (*rxo1*) (Table 1). These results suggest that AvrRxo1 requires the *Xanthomonas* TTSS to elicit the HR in maize.

avrRxo1 induces cell death when expressed inside the plant cell.

A transient expression assay system (Bryan et al. 2000; Gopalan et al. 1996; Leister et al. 1996; Mindrinos et al. 1994;

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1  GATCGCAGCATGCGACGATTTTATAGCTTCGCTTCGCTGCTCATATAGTT
52  CGTAGAGTGTGCGAGTTGCGATTGCAATCGCTCTTAATGTTTGCAGGGTCA
103  GGCACACCTGATTGCATTGGACCTTTTACATGAAAAACA
                                     M K N K
154  AGACAGACATTGCTGGCATAGCCGAGCTTTCTACGTCCTCATGTTTCCCTTG
      T D I A G I A G L S T S H V S F D
205  ATGACGCAAGATCCAAAGCGTCTAGATGGATCAACAGCTCTACGCCGG
      D A E S K S V L D G S T Q S T P E
256  AGTACAAATCCAGACCCCGCTTGATCAGTTGCGCGCTAGGCCCGCTGGTC
      Y K F Q T P L D Q L P A R P A G P
307  CGAAGAGCAGTAACACAACATGATGCTTACATTCATGAATCACTGCCGCATT
      K S S N T T D A Y I H E S L P H S
358  CGGTTCCAATCGCAGGTGCATCGACCTCCGCGGTGACACATCAACAGGCTA
      V P I A G A S T S A V T H Q Q A T
409  CTCAGCGTAAAGCCACCGATGAGATCTAGTGTGGGCGAGCTCCCATCTG
      Q R K G T D E I Y G L G S L P S A
460  CTGGTCCAGGCGGATGGGAGTATCTGCGAATCCGGGAACTGGCATCCCG
      G P G R W E Y L A N P G N W H P E
511  AGCGCGTAAAGTACACGAAAGCTATTAGATCAAGCGCATCTTCTGCTT
      R R K L H E K L L D Q A R S S A L
562  TGACCCCTGGCAGAAATCCCTTGAGAGTATGGCTGCCAACCGACATGTTTG
      T L A E S L E S D G C Q P T L F A
613  CGCTCGCGGAAATACCGCAACGGTAAACACCGATTGCGACAAAAAGA
      L R G N T A T G K T R I A T K K I
664  TCCCGGTGCTCGCAGCAGCTCTGAAGAACAGACAGTGGGAAAGGTTGCGTGA
      P V L A A A L K K T A G K G C V N
715  ATCCAGATGATTCAGAGGTTTCGCTTGGCAATCCGAGACAGGCCCAAGA
      P D V F K S S L A K S E T G A K I
766  TATTAGTTTGGCACAAGTGCATAGCGAGTCATCTTTCTTGGCAGCCGGT
      F S S A Q V H S E S S F L A D R F
817  TTGAAGCGGACTTCGCTCTCAAGACCGGCGAGGTGCAATCGCTAGCA
      E G G L R S Q K T G S G A I A S I
868  TTGTGTGCAAGCGCTTGTCAGAGAGTACGAATGATAGCTACATAC
      V V D K R L S R E Y E I D S Y I Q
919  AACTAGCGAAAGAAACCGGTAGAAAAGTGAATGTGCGATATCGACGCGC
      L A K E T G R K V E L C D I D A P
970  CCCTAGAGAAATCTTTGGTGGGAGTTTTCGACGCAAAACCGGAAGTGAAG
      L E N S L V G V L Q R K P E G E D
1021  ATCCTAGGCTTCGCTACCTCTGATCTCGAGTGGATTTGTAGCCGTACGGA
      P R P P Y P V V S S G F V A V R S
1072  GCAATAGAATGTACGTGATAGACAGGTTCATGTCGATCCGAGCCTGGGAA
      N R M Y V I D R F I A D P S L G N
1123  ACTACCGACTATTCGGAACAGCTGAAGATGTTGAGAGGTCATGGTTGCTA
      Y R L F G T A E D G E K V M V A S
1174  GCGTGATGGCGGTGAGTTTAGTGTAGAGAATGCTGAGTTGTATGAAAAA
      V I G G E F S V E N A E L Y E K I
1225  TCAGTCCCAACAGCTCTCGGTACCGGCGGATTTGGCTGATAAGGTAATCG
      T S P Q L S V T G D L A D K V I D
1276  ACAAGGAATTGATTGATCGTCTGAAATAATATAGCTGACCCCGAGCGGG
      K E L I D R L E N N I A D P E R A
1327  CGGCCAAGACGCGAGCCGCTTGAAATAATACAGCGGTAAATCATGTCGG
      A K T R A A L E K Y S G K S W S A
1378  CCGCGCTAGCTGCTACAGCGAGCTAATTTGATTGAAAGGGGAAAGCAAT
      A L A A H S E L I * M
1429  GAAAACTTTGACAGGCGCAGCAGCTCAATTTTCATAAGAAGCTTAAGGA
      K T L T G A D A L E F H K K L K E
1480  ACGCAATAAGCCTTACATGCTTCCGACCTCGAGTTAGCACTGGTGCACGC
      R N K A L H A S D L E L A L V H A
1531  CGATGCTGTCGGAAGAAGCGGTTCGATCTCGAGGAGCTGAGAAAAATTTG
      D A V G K E R F D L E L E K I C
1582  CGATACCGAGATGCGGGGCGCTTACTGATGCGAAAGAGCGAAACGACAT
      D T S D A G R L T D A K E R N D I
1633  ATATGAACGGATGACTACGTTGAGTATCCCAACGTTATGACGTTAAAGGA
      Y E R M Y Y V E Y P N V M T L K E
1684  ATTCGCTCATATCGTCGAGACATTTTCTCGTGGTCATGA
      F A H I V E T L F S W S *

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Fig. 2. Deduced amino acid sequence of AvrRxo1. Open reading frame (ORF)1 (nucleotides 144 to 1,408) is essential for inducing the hypersensitive response on B73 (*Rxo1/Rxo1*). ORF1 and ORF2 (nucleotides 1,427 to 1,723) are flanked by IS-like sequences. Putative elements are designated as: PIP-box, bold and underlined; start and stop codons, italic; nine N-myristylation sites, italic and underlined; nuclear localization sequence, underlined; eukaryotic thiol (cysteine) protease, bold and italic; and ATP/GTP binding site motif A (P-loop), bold.

Table 1. Interaction phenotypes with maize and *Xanthomonas oryzae* pv. *oryzicola* and *X. oryzae* pv. *oryzae* strains expressing *avrRxo1*^A

Bacterial strain (plasmid)	Bacterial genotype	Interaction phenotype on maize inbred lines	
		B73	Mo17
<i>X. oryzae</i> pv. <i>oryzicola</i> BLS256	<i>avrRxo1</i> ⁺	HR	NR
<i>X. oryzae</i> pv. <i>oryzae</i> PXO99 ^A (pHM1)	<i>avrRxo1</i> ⁻	NR	NR
<i>X. oryzae</i> pv. <i>oryzae</i> PXO99 ^A (pVB1C)	<i>avrRxo1</i> ⁺ , ORF2 ⁺	HR	NR
<i>X. oryzae</i> pv. <i>oryzae</i> PXO99 ^A (pavrRxo1)	<i>avrRxo1</i> ⁺ , ORF2 ⁺	HR	NR
<i>X. oryzae</i> pv. <i>oryzae</i> PXO99 ^A (p9-1H3)	<i>avrRxo1</i> ⁺ , ORF2 ⁻	HR	NR
<i>X. oryzae</i> pv. <i>oryzae</i> PXO99 ^A 9MX (pavrRxo1)	<i>avrRxo1</i> ⁺ , <i>hrpC</i> ⁻	NR	NR

^a Maize leaves were infiltrated with bacterial suspensions and scored for phenotype at 2 to 3 days post infiltration. HR, hypersensitive response; NR, no visible response; ORF, open reading frame.

Tang et al. 1996) was adopted to determine if the *avrRxo1* gene functions to induce cell death when expressed inside of plant cells. Green fluorescent protein (GFP) activity was used to monitor the physiological state of transformed cells. Co-bombardment of maize seedling leaves with HBT-SGFPTYG-NOS, which carries the GFP gene, and the plasmid pUC19 resulted in numerous brightly-fluorescing foci in both *Rxo1/Rxo1* (B73) and *rxo1/rxo1* (Mo17 and H95) genotypes (Fig. 1B; Table 2). In contrast, when leaves were cobombarded with particles coated with HBT-SGFPTYG-NOS and the plasmid carrying *avrRxo1*, HBT-AVRRXO1-NOS, no fluorescing foci were observed in the *Rxo1/Rxo1* genotype, whereas many fluorescing foci were observed in the *rxo1/rxo1* genotypes (Table 2). These data suggest that AvrRxo1 functions to elicit cell death inside the plant cell.

AvrRxo1 is localized to the plasma membrane.

Several Avr effector proteins are localized at the plant cell membrane (*avrRpm1*, *avrPto*, and *avrRpt2*) (Mackey et al. 2002; Shan et al. 2000a) or in the nucleus (*avrBs3* and *avrXa10*) (Van den Ackerveken et al. 1996; Zhu et al. 1998). The deduced AvrRxo1 protein contains nine putative myristoylation sites and one putative NLS, raising the possibilities that AvrRxo1 might be membrane associated or localized to the nucleus (Fig. 2). To determine the subcellular location of AvrRxo1 in plant cells, a construct containing a double GFP fusion (HBT-AVRRXO1-GFP-GFP-NOS) was expressed in onion cells (Fig. 1C) and maize cells (data not shown). Control cells

were bombarded with the fusion lacking the *avrRxo1* gene (HBT-GFP-GFP-NOS). GFP fluorescence was observed in all parts of the control cells. However, in those cells expressing the AvrRxo1-GFP fusions, fluorescence was located predominantly in the plasma membranes. This suggests that AvrRxo1 is membrane associated and functions in the cytoplasm. To determine whether the AvrRxo1-GFP fusion expressed avirulence function, the construct containing the AvrRxo1-GFP fusion also was cobombarded with HBT-SGFPTYG-NOS onto maize lines B73 (*Rxo1/Rxo1*) and Mo17 (*rxo1/rxo1*). Although fluorescent cells were observed in the Mo17 line, very few fluorescent cells were observed in B73 (data not shown), which suggested that the AvrRxo1-GFP fusion construct still carries the *avrRxo1* effector gene function.

The *avrRxo1/Rxo1* interaction results in reduced bacterial numbers in maize.

The appearance of an HR following interactions of *avr* and *R* genes typically is associated with a defensive response. However, because maize is not a host for *X. oryzae* pv. *oryzicola*, we tested the effect of the interaction between *avrRxo1* and *Rxo1* on bacterial multiplication in maize with (B73) and without (Mo17) *Rxo1*. Maize plants were infiltrated with *X. oryzae* pv. *oryzicola* BLS256 and *X. oryzae* pv. *oryzae* strains PXO99^A(pHM1), which harbors the empty vector, and PXO99^A(pavrRxo1), which contains both the *avrRxo1* gene and ORF2 driven by the native promoter. In Mo17, numbers of bacteria with or without the *avrRxo1* gene increased over time by roughly 0.5 to 1.0 log CFU/ml (Fig. 5). Similarly, in B73, which harbors *Rxo1*, numbers of *X. oryzae* pv. *oryzae* PXO99^A(pHM1) without *avrRxo1* increased over 4 days. In interactions involving both the *Rxo1* gene (B73) and *avrRxo1* gene (BLS256 with endogenous *avrRxo1* or PXO99^A harboring a plasmid-borne copy of *avrRxo1*), bacterial numbers did not increase but, instead, declined significantly by 4 days after infiltration. These results confirm that *avrRxo1-Rxo1* interactions induce a defense response in maize.

DISCUSSION

In this study, we identified an effector gene, *avrRxo1*, from the rice bacterial streak pathogen, *X. oryzae* pv. *oryzicola*, that confers an HR in maize lines carrying the *Rxo1* resistance gene. The interaction between *avrRxo1* and *Rxo1* is considered a nonhost interaction because i) *X. oryzae* pv. *oryzicola* is not a reported pathogen of maize and, in our assays, does not cause disease symptoms or multiply to high levels in maize, and, ii) in interactions where both the *avrRxo1* and *Rxo1* gene are present, populations of *X. oryzae* pv. *oryzicola* decline rapidly and substantially (Fig. 5). The interaction is a gene-for-gene type of interaction because the bacterial effector gene *avrRxo1* is sufficient to induce an HR in maize with *Rxo1* either after delivery from the bacteria (Fig. 1A) or after transient

Fig. 3. Schematic map of the 1.7-kb fragment containing the *avrRxo1* (open reading frame [ORF]1, 1,266 bp, spotted box). ORF2 (297 bp, open box) shares characteristics with molecular chaperones. Putative promoter region is shown as a hatched box. ORF1 and ORF2 are flanked by IS-like sequences (block arrow).

Table 2. Transient expression of AvrRxo1 in cells of maize line B73 (*Rxo1/Rxo1*) but not Mo17 (*rxo1/rxo1*) results in cell death

Plasmids introduced by bombardment	Number of fluorescing foci/cm ² of leaf surface maize line ^a	
	B73 (<i>Rxo1/Rxo1</i>)	Mo17 (<i>rxo1/rxo1</i>)
HBT-SGFPTYG-NOS + pUC19	12.4 ± 4.1	15.0 ± 0.3
HBT-AVRRXO1-NOS + HBT-SGFPTYG-NOS	0	10.8 ± 5.4

^a Plasmids were introduced into maize cells by bombardment. The number of fluorescent foci resulting from expression of the green fluorescent protein (GFP) per square centimeter of bombarded leaf was counted using a fluorescent microscope. Counts of fluorescent foci from four independent experiments were averaged and the number of fluorescent foci per square centimeter was calculated. Numbers of fluorescing foci per square centimeter within a column were compared by TTEST using the SAS program. Cells of maize line B73 (*Rxo1/Rxo1*) bombarded with both constructs containing the *avrRxo1* and GFP genes exhibited significantly reduced numbers of fluorescent foci ($P = 0.0045$) relative to those bombarded with the GFP construct alone. Mo17 (*rxo1/rxo1*) cells did not exhibit significant differences between treatments ($P = 0.3965$). These results indicate that transient expression of AvrRxo1 in maize line B73 (*Rxo1/Rxo1*) results in cell death.

expression in maize cells with *Rxo1* (Fig. 1B). The interaction, therefore, is a nonhost, gene-for-gene type of interaction.

Although most of the more than 40 avirulence effector genes cloned from plant-pathogenic bacteria encode very diverse proteins, the cultivar-specific avirulence genes cloned from *Xanthomonas* spp. generally are the exception, in that most identified to date are members of the *avrBs3-pthA* family of highly related genes (Leach and White 1996). Avirulence genes that are not related to the *avrBs3-pthA* family have been identified from *Xanthomonas* spp., including the *avrBs1* (Ronald and Staskawicz 1988), *avrBs2* (Swords et al. 1996), and *avrRxv* (Whalen et al. 1993) genes from *X. campestris* pv. *vesicatoria*. The nonhost *avrRxo1* gene is not a member of the *avrBs3-pthA* family, and is not related to any other type of *Xanthomonas* avirulence gene or to any other protein in the public databases.

Most effector genes reported so far from *Xanthomonas* spp. and other plant-pathogenic bacteria are dependent on secretion through the TTSS for avirulence function. The *avrRxo1* has an imperfect PIP-box upstream of the start codon, which is a common hallmark of other pathogenicity operons whose protein products are secreted by the TTSS (Astua-Monge et al. 2000). The dependency of *avrRxo1* on the TTSS for avirulence function was confirmed by expression of the gene in a TTSS-deficient mutant (*hrpC* mutant) of *X. oryzae* pv. *oryzae*. Although expression of *avrRxo1* in wild-type *X. oryzae* pv. *oryzae* allowed induction of the HR on maize plants with *Rxo1*, the *hrpC*-deficient strain expressing *avrRxo1* was unable to induce the HR. These data also indicate that the TTSS apparatus is functionally conserved in *X. oryzae* pv. *oryzicola* and *X. oryzae* pv. *oryzae*.

Several TTSS effector proteins of animal-pathogenic bacteria require a specific chaperone to assist in the secretion process or to stabilize the protein prior to secretion (Hueck 1998; Page and Parsot 2002; Parsot et al. 2003). At least three avirulence effectors from plant-pathogenic bacteria require specific chaperones (Gaudriault et al. 2002; van Dijk et al. 1999). Chaperones normally are encoded by a small ORF adjacent to the effector proteins. Although they lack conserved domains, most share some specific characteristics. The *X. oryzae* pv.

oryzicola ORF2, which is immediately adjacent to the 3' end of *avrRxo1*, encodes a protein that has characteristics of a chaperone (i.e., it is a small, acidic peptide with a putative C-terminal amphiphilic α -helix). However, an intact ORF2 was not required for the HR-inducing function of *avrRxo1*. Although ORF2 apparently is not essential for *avrRxo1* function, we cannot conclude at this point whether it interacts with AvrRxo1 and plays a role in stabilization or secretion or whether it is relevant to pathogenesis or avirulence.

After delivery into plant cells, several bacterial effector proteins have been shown to localize to the plasma membrane (Nimchuk et al. 2000). For example, AvrPto contains a myristoylation site at its extreme N-terminus, and this site was demonstrated by mutagenesis to be important for plasma membrane localization and function (Shan et al. 2000b). Sequence analysis revealed at least nine putative myristoylation sites in AvrRxo1; however, none of the putative myristoylation sites were near the N terminus. Transient expression experiments, on the other hand, demonstrated that AvrRxo1 is localized to the plasma membrane of plant cells. It is possible that AvrRxo1 contains an N-terminal signal peptide that is processed to expose an internal myristoylation site for anchoring to the membrane. Alternatively, there may be other unknown signals that drive the AvrRxo1 protein to the plasma membrane. Nevertheless, the membrane localization of AvrRxo1 provides insights into where the corresponding RXO1 protein is located. If the two gene products interact directly, or interact through another common protein or proteins, the RXO1 protein also may co-localize at the membrane. For example, both *R* gene products RPS2 and RPM1 and their corresponding bacterial effector proteins (AvrRps2 and AvrRpm1, respectively) are localized to the plant plasma membrane (Axtell and Staskawicz 2003; Boyes et al. 1998).

Although database homology searches did not provide a clear picture of the function of the AvrRxo1 protein, searches for conserved motifs provided clues. Putative functional domains identified included a eukaryotic thiol (cysteine) protease active site (69-LPHSVPIAGAS-79) and an ATP/GTP binding site motif A (P-loop; 122-GNTATGAS-128). Other effector proteins, such as AvrPphB and AvrRpt2 from *P. syringae*, are

Fig. 4. DNA blot analysis revealed only one copy of *avrRxo1* in the *Xanthomonas oryzae* pv. *oryzicola* genome (lanes 8 to 14). No homologues could be detected in the *X. oryzae* pv. *oryzae* genome (lanes 2 to 7). Bacterial genomic DNA was digested by *SphI* and probed with the 1.2-kb open reading frame 1 of the *avrRxo1* gene. Lane 1, lambda DNA/*HindIII*; lanes 2 to 7, strains of *X. oryzae* pv. *oryzae*, including PXO61 (lane 2), PXO71 (lane 3), PXO79 (lane 4), PXO86 (lane 5), PXO99 (lane 6), and PXO112 (lane 7); lanes 8 to 14, strains of *X. oryzae* pv. *oryzicola*, including BLS101 (lane 8), BLS175 (lane 9), BLS256 (lane 10), BLS288 (lane 11), BLS290 (lane 12), BLS295 (lane 13), and BLS298 (lane 14).

Fig. 5. Multiplication of *Xanthomonas oryzae* pv. *oryzicola* (BLS256), *X. oryzae* pv. *oryzae* (PXO99^Δ), and *X. oryzae* pv. *oryzae* PXO99^Δ(*pavrRxo1*) in maize lines Mo17 and B73. Bacteria were infiltrated at 10⁵ CFU/ml.

cysteine proteases whose proteolytic activity is essential for elicitation of the HR in *Arabidopsis* spp. (Axtell and Staskawicz 2003; Shao et al. 2002). Although we cannot conclude that the motifs found in *AvrRxo1* are important to function, their identification enables the design of mutagenesis and other experimental studies to understand the function of *AvrRxo1* in the plant cell.

The low G+C content of *avrRxo1* (51.1%) relative to other *Xanthomonas* genes (63 to 71%) is suggestive that the gene may not have originated in *X. oryzae* pv. *oryzicola*. In other cases where bacterial avirulence effector genes also have a lower G+C content than the normal range for genes within their species, the effector genes also are frequently flanked by highly conserved sequences, often with similarity to insertion sequences, transposases, or bacteriophage sequences, that may have a role in horizontal transfer between different bacterial species or genera (Alfano et al. 2000; Arnold et al. 2001; Jackson et al. 1999; Kim et al. 1998; Kjemtrup et al. 2000; Staskawicz et al. 1987). We demonstrated that *avrRxo1* is flanked by two sequences similar to the insertion element IS1478 from *X. campestris* pv. *campestris* (gi:1927196). Interestingly, Kim and associates (1998) demonstrated that three of four avirulence genes from *P. syringae* with known virulence functions were flanked by transposons or other mobile sequences. Based on this finding, they speculated that avirulence effector genes that are important to virulence or fitness are more frequently flanked by mobile sequences, and suggested that this could account for the maintenance and transfer of these genes in bacterial populations. All *X. oryzae* pv. *oryzicola* strains tested contain the *avrRxo1* gene, suggesting that *avrRxo1* plays an important role in pathogen

virulence or fitness. Currently, we have been unable to test this hypothesis because, even after repeated attempts with different strategies, we were unable to obtain knock-out mutants of the *avrRxo1* gene in *X. oryzae* pv. *oryzicola*.

Bacterial leaf streak is a disease of increasing importance in rice with the expanding use of highly susceptible hybrid rice in Asia. Although thousands of rice accessions were screened for resistance to the disease, no simply inherited resistance genes that could offer control of bacterial leaf streak disease were found (Adhikari et al. 1999; Khush 1977). The identification of a gene-for-gene type of resistance interaction in maize that results in a resistance-like phenotype to *X. oryzae* pv. *oryzicola* provides the opportunity to explore interspecific transfer of resistance genes (i.e., from maize to rice) to control disease. This will require the cloning of the *Rxo1* gene that corresponds to *avrRxo1*. The cloning of the *avrRxo1* gene may facilitate the cloning of the corresponding *Rxo1* gene from maize. Testing candidate resistance genes by stable transformation requires over 6 months; the use of a biolistic delivery or transient assay system with *avrRxo1* can be applied to rapidly test the functions of candidate *Rxo1* genes in maize leaves. Candidate *Rxo1* genes can be cobombarded with the *avrRxo1* genes into leaves of maize lines without endogenous *Rxo1*, and, by monitoring of the expression of reporter genes (either GUS or GFP), the induction of cell death by interactions involving *avrRxo1/Rxo1* can be determined in cells of detached leaves. Cloning of the corresponding *Rxo1* gene will allow us to study how the two gene products interact to initiate resistance and will provide an excellent model system for studying signal transduction in monocots.

Table 3. Bacterial strains and plasmids used in this work

Designation	Relevant characteristics and use ^a	Source or reference
<i>Xanthomonas oryzae</i> pv. <i>oryzicola</i> BLS256	Induces HR on maize line B73 (with <i>Rxo1</i>); used for construction of cosmid library	Raymundo and Leach 1994
<i>X. oryzae</i> pv. <i>oryzae</i> PXO99A	Grows on 200:μM 5-Azacytidine; no reaction when infiltrated into maize lines B73 or Mo17 and does not hybridize to <i>avrRxo1</i>	Hopkins et al. 1992
PXO86	No reaction when infiltrated into maize lines B73 or Mo17 and does not hybridize to <i>avrRxo1</i>	T. Mew, International Rice Research Institute
<i>Escherichia coli</i> DH5αMCR	...	Bethesda Research Laboratories
DH5α	...	Bethesda Research Laboratories
HB101	F ⁻ , rec A, Nal ^r , Sm ^r	Boyer and Roulland-Dussoix 1969
Plasmids pUFR034	Km ^r , Nm ^r , IncW, pSA oriR, parA locus from <i>Agrobacterium</i> , lacZ Cb ^r ; used in subcloning and as control in transient assay	De Feyter et al. 1990
pUC19	Km ^r , Tra ⁺ , Mob ⁺ , ColE1 replicon	Friedman et al. 1982
pRK2013	Broad-spectrum cosmid vector that replicates in <i>X. oryzae</i> pv. <i>oryzicola</i> , <i>X. oryzae</i> pv. <i>oryzae</i> , and <i>E. coli</i> ; Sp ^r , Sm ^r , cos, parA, IncW, derivative of pR140	R. Innes, Indiana University
pVB1C	Cosmid pURF034 clone from BLS256 library that confers HR to B73 maize. Insert size ≈40 kb.	This study
pavrRxo1	The 1.797-kb <i>SphI</i> fragment subcloned into pHM1 from VB1C that contains the <i>avrRxo1</i> gene (ORF1) and the adjacent ORF2; confers HR on B73 maize	This study
p9-1H3	Subclone in pHM1 that contains only ORF1 (<i>avrRxo1</i>) created by removal of ORF2 by digestion with <i>HindIII</i> ; confers HR on B73 maize	This study
HBT-SGFPTYG-NOS	Plasmid carrying synthetic (codon optimized) GFP driven by a strong, constitutive, chimeric promoter made from the CaMV 35S enhancer fused to the basal promoter of the maize C4PPDK gene	Sheen et al. 1995
HBT-AVRRXO1-NOS	The SGFP gene in pHBT-SGFP-NOS was replaced by the <i>avrRxo1</i> gene to study in planta expression of <i>AvrRxo1</i>	This study
HBT-GFP-GFP-NOS	Two GFP ORFs are tandemly arrayed in frame	This study
HBT-AVRRXO1-GFP-GFP-NOS	The <i>avrRxo1</i> gene was fused in frame between the promoter and tandem GFP sequences of HBT-GFP-GFP-NOS	This study

^a HR, hypersensitive response; Nal, nalidixic acid; Sm, streptomycin; Km, kanamycin; Nm, neomycin; Cb, carbenicillin; ORF, open reading frame, SGFP, synthetic green fluorescent protein.

MATERIALS AND METHODS

Bacterial strains and plasmids.

Bacterial strains used in this study are listed in Table 3. An additional 39 strains of *X. oryzae* pv. *oryzicola* from The Philippines and China were used to determine the presence or absence of *avrRxol*; a list of these strains is available from the authors upon request. Strains of *Escherichia coli* were cultured in Luria-Bertani (Miller 1972) or nutrient medium (Difco Laboratories, Detroit) at 37°C. *X. oryzae* pv. *oryzicola* and *X. oryzae* pv. *oryzae* strains were grown in either peptone-sucrose agar (PSA) (Tsuchiya et al. 1982) or nutrient medium at 28 to 30°C. For culture on solid medium, 1.5% agar was added. Unless otherwise mentioned, antibiotics were incorporated into media where appropriate at the following concentrations: kanamycin (Km) at 50 µg/ml, cephalixin (Cp) at 20 µg/ml, spectinomycin (Sp) at 50 µg/ml, and carbenicillin (Cb) at 100 µg/ml. *X. oryzae* pv. *oryzae* PXO99^A (Hopkins et al. 1991) was used as a recipient to test *avrRxol*-containing constructs and strain PXO99^A86mx9 (Km^r) (Zhu et al. 2000), which harbors an insertion in the *hrpC* gene of the TTSS, was used to determine whether *avrRxol* function required transfer through the TTSS.

The plasmid HBT-SGFPTYG-NOS (pUC18) was obtained from J. Sheen (Harvard Medical School, Boston). The *gfp* gene in these constructions encodes a synthetic GFP (SGFP-TYG) that was synthesized based on optimal codon usage for humans, maize, and *Arabidopsis* spp. (Chiu et al. 1996; J. Sheen, *personal communication*). The chromophore of the native protein (SYG) was mutated to TYG, to yield a protein with a single excitation peak by blue light. The promoter region of the construction (HBT) consists of the CaMV 35 S enhancer fused to the basal promoter and 5' untranslated region of the maize C4PPDK gene (Chiu et al. 1996; Sheen et al. 1995) and the 3' region consists of *NOS* terminator.

Plasmid HBT-AVRRXO1-NOS was constructed by digestion HBT-SGFPTYG-NOS with *Bam*HI/*Pst*I to replace the GFP coding region with the *avrRxol* ORF1 (*avrRxol*). During amplification of ORF1 by polymerase chain reaction (PCR), *Bam*HI and *Pst*I sites were appended to the 5' and 3' ends, respectively. The primers used to amplify the *avrRxol* ORF were Bamforward: 5'-CGGGATCCCGATGAAAAACAAGACAGACAT-3' and Pstreverse: 5'-CTGCAGGCGAATTA GCTCGCTGTGAG-3'. Plasmid HBT-GFP-GFP-NOS was constructed by insertion of an in-frame second GFP coding region in front of the original GFP gene in the plasmid HBT-SGFPTYG-NOS. The second GFP coding region was PCR amplified as a *Bam*HI-*Bgl*II fragment with primers GFPBamforward: 5'-CCGTGGATCCATGGTGAGCAAGGGCGA-3' and GFPBglIIReverse: 5'-GAAGATCTCTTGACAGCTCGTCC-3'. Plasmid HBT-AVRRXO1-GFP-GFP-NOS was constructed by introduction of a *Bam*HI fragment containing the ORF1 of *avrRxol* into plasmid HBT-GFP-GFP-NOS. The primers used to amplify the ORF1 of *avrRxol* were Bamforward: 5'-CGGGATCCCGATGAAAAACAAGACAGACAT-3' and Bamreverse: 5'-CGCGGATCCGCGAATTAGCTCGCTGTGAG-3'.

Recombinant DNA techniques and DNA gel blot analysis.

Total DNA isolation from *X. oryzae* pv. *oryzicola*, *X. oryzae* pv. *oryzae*, and *B. andropogonis* were as described (Leach et al. 1990). Gel electrophoresis and DNA blot transfer were by standard techniques (Sambrook et al. 1989). Plasmid DNA was prepared following the alkaline lysis method or the Qiagen (Valencia, CA, U.S.A.) plasmid midiprep protocol. Labeling of DNA probes, DNA blot hybridization, and signal detection were either as specified in the ECL direct nucleic acid labeling and detection handbook (Amersham Life Science,

Buckinghamshire, England) or as described by Feinberg and Vogelstein (1983). For moderate-stringency washes of DNA blots hybridized with ³²P-labeled probes, blots were washed with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) one time, then two times in 0.5× SSC.

Restriction enzymes, calf intestinal alkaline phosphatase (CIAP), and T4 DNA ligase were purchased from Promega Biotech (Madison, WI, U.S.A.) or Bethesda Research Laboratories (BRL, Gaithersburg, MD, U.S.A.); shrimp alkaline phosphatase from Amersham Life Sciences, Inc. (Cleveland OH, U.S.A.); *E. coli* DH5α and DH5αMCR competent cells from Life Technologies (Gibco BRL, Gaithersburg, MD, U.S.A.); and Fast-Link ligation and screening kit from Epicenter Technologies (Madison, WI, U.S.A.).

Genomic DNA library construction.

A genomic library of *X. oryzae* pv. *oryzicola* BLS256, which confers an HR on maize line B73 but not on Mo17, was constructed in the broad-host-range cloning vector pUFR034 (Km^r) (De Feyter et al. 1990). CsCl-purified bacterial genomic DNA was digested partially with *Sau*3AI and treated with calf intestinal alkaline phosphatase (Sambrook et al. 1989). Vector pUFR034 was digested to completion with *Bam*HI. The genomic and vector DNA fragments were ligated, and packaged into lambda phage in vitro by use of Gigapack II packaging extract (Stratagene, La Jolla, CA, U.S.A.). After transduction into *E. coli* DH5αMCR, white colonies were selected on Luria-Bertani agar plates containing X-gal, IPTG, and kanamycin. Approximately 97% of the colonies contained recombinant plasmids. For the library, 1,500 colonies were picked randomly and stored in microtiter plates in 15% glycerol at -80°C (Sambrook et al. 1989). Plasmid DNA from 30 randomly selected clones was isolated, digested with *Eco*RI and *Hind*III, and separated in agarose gels. The size of the cloned genomic fragments ranged from approximately 30 to 40 kb and the fragmentation patterns were all different.

Bacterial transformations and conjugations.

Two methods were used to transfer plasmid DNA from the library clones into PXO99A. The first method was a triparental mating (Kelemu and Leach 1990). Briefly, the library clones in *E. coli* DH5αMCR (donor) were grown overnight at 37°C on Luria-Bertani agar (LA) with kanamycin and then transferred to the wells of microtiter plates each containing 80 µl of nutrient broth using a flame-sterilized multipronged inoculator. The microtiter plates were incubated at 37°C with shaking for 4 to 6 h. Eighty microliters of a 4- to 6-h nutrient broth culture of the strain *E. coli* HB101(pRK 2013) (helper) was added to each of the wells containing the library clones and the bacteria were incubated at 37°C for 1 h. Nutrient agar plates containing kanamycin and cephalixin (used to inhibit *E. coli*) were seeded with 0.1 ml of a 16-h nutrient broth culture of *X. oryzae* pv. *oryzae* PXO99A (60 to 100 Klett units) and air dried. The mated bacteria were replica plated onto the lawn of PXO99A, and the plates were air dried and incubated at 28°C until transconjugants appeared. The transconjugants were picked and purified by streaking on nutrient agar containing kanamycin and cephalixin.

Electrotransformation was used to transfer plasmid DNA into PXO99A and was performed as described (Choi and Leach 1994). Plasmid DNA was introduced into competent PXO99A cells by electroporation (600 V amplitude, 5-millisecond pulse length) using a T-100 electroporator system (Bio-technical and Experimental Research Inc., BTX, San Diego, CA, U.S.A.) and an electroporation chamber with a 0.56-mm gap. After electroporation, the cells were recovered from the electroporation chamber by pipetting with 600 µl of peptone

sucrose broth. The cell suspension was incubated at 30°C with shaking at 200 rpm for 4 h, diluted, and spread on peptone sucrose agar containing kanamycin.

Plant assays.

Maize lines B73 and Mo17 were used to test the ability of the bacterial strains, transconjugants, and electrotransformants to elicit the HR. Line B73 (harbors the *Rxo1* resistance gene) gives an extensive HR (complete necrosis of the area infiltrated with the bacteria) to inoculation with *X. oryzae* pv. *oryzicola* BLS256, whereas Mo17 (does not harbor *Rxo1*) gives no reaction. The second and third leaves of 7- to 12-day-old maize seedlings were infiltrated with cell suspensions of bacteria (approximately 1×10^8 CFU/ml) prepared from 48- to 72-h-old PSA plate cultures as described (Reimers and Leach 1991). *X. oryzae* pv. *oryzicola* BLS256 and *X. oryzae* pv. *oryzae* PXO99^A were used for comparison. Bacterial suspensions were infiltrated into the intercellular spaces with a needleless syringe (Reimers and Leach 1991). Plants were examined for the induction of the HR 2 and 3 days after infiltration.

For bacterial population studies, B73 and Mo17 maize leaves were infiltrated with bacterial suspensions adjusted to 10^5 CFU/ml. Leaves were sampled at 0, 1, 2, and 3 days after infiltration by removing leaf disks from the infiltration sites with a 10-mm cork borer (six disks pooled per sample) with three repetitions. The experiment was repeated three times and data from one representative experiment is shown. To assess bacterial numbers, leaf disk samples were ground in 1 ml of sterile ddH₂O and the homogenate was serially diluted and plated on NB with appropriate antibiotics (Hopkins et al. 1992).

Subcloning the *avrRxo1* gene.

To subclone *avrRxo1*, VB1C cosmid DNA was partially digested with *Sau3A*, and the DNA fragments were separated by electrophoresis in 0.7% (wt/vol) low melting agarose in 1× Tris acetate buffer. Fragments ranging in size from 4 to 15 kb were extracted from the gel and purified by using a GeneClean kit (Vista, CA, U.S.A.). The purified fragments were ligated into vector pHM1, which had been digested with *Bam*HI and treated with calf intestinal alkaline phosphatase. The ligation mixture was transformed into *E. coli* DH5α and spectinomycin-resistant colonies were selected.

Plasmid DNA was extracted from the clones and transferred to PXO99^A by electrotransformation (Choi and Leach 1994). The electrotransformants were inoculated on B73 to test their ability to elicit the HR. Two subclones, VS87 and VS96, were identified that conferred an HR on B73, but not on Mo17 (Fig. 3). After digestion with either *Sac*II or *Sph*I, one common fragment was observed. The 1.7-kb *Sph*I fragment was subcloned to pUC19, linearized with *Bam*HI, and then cloned to pHM1. Both orientations exhibited the HR, suggesting that the endogenous promoter functioned. The entire 1.7-kb fragment was sequenced at the Kansas State University DNA Sequencing Facility by primer walking.

To determine which of the two ORFs functioned as *avrRxo1*, the second ORF was removed by digestion with *Hind*III (Fig. 3) and re-ligated to construct plasmid p9-1H3.

Transient expression of *AvrRxo1* in maize cells.

Three-week-old maize seedling leaves were detached, placed on 0.15% water-agar plates, and cobombarded using the particle inflow gun as described by Finer and associates (1992). Plasmids to be cobombarded (either HBT-SGFPTYG-NOS/pUC19 or HBT-SGFPTYG-NOS/pHBT-*avrRxo1*-NOS) were mixed 1:1 equimolar with M-10 tungsten particles (Atlantic Equipment Engineers, Bergenfield, NJ, U.S.A.) prior to bombardment. After bombardment, plates were maintained at 25°C with

16-h light, 8-h dark photoperiod. After bombardment (24 h), green fluorescent foci were observed using a Leica FLZIII fluorescent dissecting microscope with a GFP3 filter set. Transient assays using onion cells (derived from locally purchased white onion bulbs) followed the same procedure.

ACKNOWLEDGMENTS

This work was supported by USDA-NRI #2001-35319-10014 and the Kansas Agricultural Experiment Station (04-110-J). E. Ardales was supported by a Rockefeller postdoctoral fellowship, and A. Raymundo was supported by a Rockefeller Biotechnology Career Development award.

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