Inhibition of Resistance Gene–Mediated Defense in Rice by *Xanthomonas oryzae* pv. *oryzicola*

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Xanthomonas oryzae pv. *oryzae* and the closely related *X. oryzae* pv. *oryzicola* cause bacterial blight and bacterial leaf streak of rice, respectively. Although many rice resistance (*R*) genes and some corresponding avirulence (*avr*) genes have been characterized for bacterial blight, no endogenous *avr/R* gene interactions have been identified for leaf streak. Genes *avrXa7* and *avrXa10* from *X. oryzae* pv. *oryzae* failed to elicit the plant defense-associated hypersensitive reaction (HR) and failed to prevent development of leaf streak in rice cultivars with the corresponding *R* genes after introduction into *X. oryzae* pv. *oryzicola* despite the ability of this pathovar to deliver an *AvrXa10*:Cya fusion protein into rice cells. Furthermore, coinoculation of *X. oryzae* pv. *oryzicola* inhibited the HR of rice cultivar IRBB10 to *X. oryzae* pv. *oryzae* carrying *avrXa10*. Inhibition was quantitative and dependent on the type III secretion system of *X. oryzae* pv. *oryzicola*. The results suggest that one or more *X. oryzae* pv. *oryzicola* type III effectors interfere with *avr/R* gene–mediated recognition or signaling and subsequent defense response in the host. Inhibition of *R* gene–mediated defense by *X. oryzae* pv. *oryzicola* may explain, in part, the apparent lack of major gene resistance to leaf streak.

Additional keywords: phytopathogenic bacteria.

The ability of plants to perceive and defend against pathogen attack is partially governed by the interaction between plant resistance (*R*) genes and pathogen avirulence (*avr*) genes. These gene-for-gene interactions underlie cultivar and race specificity in the resistance of plants to pathogenic viruses, bacteria, and fungi (Flor 1971; Keen 1990). *R* gene products directly or indirectly recognize corresponding *avr* gene products and trigger downstream signaling to activate defense responses (Martin et al. 2003). In the absence of either the *R* gene or the *avr* gene, plants fail to mount sufficient defense responses and succumb to disease. The hypersensitive reaction (HR) is an early, *R* gene–mediated response to avirulence signals. HR is localized host-cell death associated with limitation of pathogen spread in host-plant tissues (Hammond-Kosack and Jones 1996; Klement 1982; Wright and Beattie 2004).

In phytopathogenic bacteria, most known Avr signals are proteins delivered to plant cells via a specialized secretion pathway called the Hrp or type III secretion system (TTSS) (Bogdanove et al. 1996; Buttner and Bonas 2002; Cornelis and Van Gijsegem 2000). The TTSS is broadly conserved among gram-negative animal and plant pathogens as a specific path-

way for delivery of virulence factors into host cells (Hueck 1998). Cya, an adenylate cyclase of *Bordetella pertussis*, activity of which depends on calmodulin produced in the host cell, has been used in translational fusions as a quantitative reporter for type III effector delivery in both animals and plants (Casper-Lindley et al. 2002; Sory and Cornelis 1994).

Many Avr proteins, in the absence of a corresponding *R* gene, contribute demonstrably to virulence (Bai et al. 2000; Swarup et al. 1991). The general term effector is used for all substrates of the TTSS delivered to host cells. The dual roles of some type III effectors is probably a consequence of the evolution in plants of the ability to recognize those effectors and mount resistance as mediated by *R* genes (Alfano and Collmer 2004). Recently, the functions of some type III effectors have been elucidated and some have been shown to suppress plant defense responses, including both *R* gene–mediated and nonhost HR as well as cell-wall fortification and other resistance mechanisms (Abramovitch and Martin 2004; Chang et al. 2004).

Bacterial blight, caused by *Xanthomonas oryzae* pv. *oryzae*, is one of the most important diseases of rice (Mew et al. 1993). The disease is characterized by a high degree of race and cultivar specificity, with over 30 races of *X. oryzae* pv. *oryzae* and more than 25 bacterial blight *R* genes identified (Lee et al. 2003; Noda et al. 1996; Yang et al. 2003). Effectors found in a major class of *X. oryzae* pv. *oryzae* are members of the *avrBs3/pthA* family (Bai et al. 2000; Bonas et al. 1989; Hopkins et al. 1992; Yang and White 2004; Yang et al. 2000, 2005; Zhu et al. 1998). Of these, *avrXa7* and *avrXa10* were cloned from the *X. oryzae* pv. *oryzae* PXO86 (race 2; Hopkins et al. 1992) and correspond to the *R* genes *Xa7* and *Xa10*, respectively. In PXO86 and KXO85, *avrXa7* contributes markedly to disease (Bai et al. 2000; Yang and White 2004). In strains lacking *avrXa7*, other members of the gene family act as major virulence factors (Yang and White 2004). The protein structures of this family share a highly conserved N-terminus, a central region of 34 aa, direct, near-perfect repeats, which vary in number, and a C-terminal region containing functional nuclear localization signals (NLS) and an acidic transcription activation domain (AAD) (Buttner and Bonas 2002; Szurek et al. 2001; Zhu et al. 1998). Type III secretion and delivery of *AvrBs3* is directed by sequences in the N-terminus (Szurek et al. 2002), which is nearly identical to and interchangeable with the N-termini of *AvrXa7* and *AvrXa10* (Hopkins et al. 1992; Yang and White 2004; Yang et al. 2000, 2005). The NLS are required for avirulence activity of some but not all members of the *AvrBs3* family, indicating that different members are recognized differently (Ballvora et al. 2001). The AAD at the C-terminus suggests an interaction with plant transcriptional machinery during infection and determines *R*-protein specificity for some mem-

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bers (Yang and White 2004, 2005; Zhu et al. 1998, 1999). The repeat domains of AvrBs3 and AvrXa7 also contribute to their *R*-gene specificity (Herbers et al. 1992; Yang et al. 2005).

X. oryzae pv. *oryzicola* is closely related to *X. oryzae* pv. *oryzae*, showing more than 90% similarity by DNA-DNA hybridization (Swings et al. 1990). Unlike *X. oryzae* pv. *oryzae*, which invades the xylem to infect systemically, *X. oryzae* pv. *oryzicola* is a nonvascular pathogen (Ou 1985). Leaf streak can also cause severe yield losses, though it occurs more sporadically than blight (Mew 1993). Like *X. oryzae* pv. *oryzae*, *X. oryzae* pv. *oryzicola* harbors multiple members of the *avrBs3/pthA* family (Yang and White 2004), but no function has yet been ascribed to them. Notably, despite some effort (Das 1977; Rao et al. 1972), no endogenous *avr* and *R*-gene combination has been identified for *X. oryzae* pv. *oryzicola* and rice. We therefore investigated whether *X. oryzae* pv. *oryzicola* has the capacity to express and deliver members of the AvrBs3/PthA family using *avrXa7* and *avrXa10* and rice cultivars containing *Xa7* or *Xa10*.

RESULTS

Expression of *X. oryzae* pv. *oryzae* *avr* genes in *X. oryzae* pv. *oryzicola* does not cause avirulence to rice cultivars with corresponding *R* genes.

To determine whether *avr* genes of *X. oryzae* pv. *oryzae* function in *X. oryzae* pv. *oryzicola*, the plasmids pHMavrXa10 and pHMavrXa7 (Table 1), which carry *avrXa10* and *avrXa7*, respectively, fused to the FLAG epitope tag (Brizzard et al. 1994), were transformed into *X. oryzae* pv. *oryzicola* strains BLS303 and BLS256 (Table 1). None of the resulting strains induced HR when infiltrated into leaves of corresponding rice

cultivars IRBB10 and IRBB7, which carry resistance genes *Xa10* and *Xa7*, respectively. Instead, transformants caused lesions that were water-soaked in appearance and typical of the parental strains (Fig. 1). Both plasmids conferred the ability to elicit HR in the respective cultivars when introduced into *X. oryzae* pv. *oryzae* PXO99^A (Fig. 1). Population growth of BLS303(pHMavrXa10) in IRBB10 was not significantly different from that of BLS303 (Fig. 1). Western blot analysis using anti-FLAG antibody confirmed expression of AvrXa10 in BLS303(pHMavrXa10) (data not shown). Thus, *avrXa10*, and most likely *avrXa7*, was expressed in *X. oryzae* pv. *oryzicola* but failed to render the pathogen avirulent.

X. oryzae pv. *oryzicola* harboring *avrBs3* elicits the HR of pepper in a *Bs3*-dependent manner.

To determine if the apparent lack of avirulence function was specific for rice or due more generally to a failure in type III secretion, the ability of *X. oryzae* pv. *oryzicola* to deliver AvrBs3 and elicit a *Bs3*-dependent response in pepper was examined. pHMavrBs3, identical to pHMavrXa10 except for the coding region of the *avr* gene (Table 1), was transformed into *X. oryzae* pv. *oryzicola* strains BLS303 and BLS256 as well as *X. oryzae* pv. *oryzae* PXO99^A, and the resulting strains were infiltrated into leaves of six-week old plants of pepper varieties ECW30R, which carries *Bs3*, and the near-isogenic line ECW, which lacks the resistance gene. The parental *X. oryzae* pv. *oryzicola* and *X. oryzae* pv. *oryzae* strains did not elicit an HR in ECW30R (Fig. 2, top panels). However, the transformants carrying pHMavrBs3 did elicit an HR (Fig. 2, bottom panels). None of the strains elicited an HR on ECW (data not shown). Thus, *X. oryzae* pv. *oryzicola* effectively expresses and translocates AvrBs3 into pepper, indicating that the inability of AvrXa7

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics ^z	Source or reference
<i>Escherichia coli</i>		
TOP10	F ⁻ <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>) φ80 <i>lacZAM15</i> Δ <i>lacX74</i> <i>deoR</i> <i>recA1</i> <i>ara</i> Δ139 Δ(<i>ara-leu</i>)7697 <i>galU</i> <i>galK</i> <i>rpsL</i> (Str ^r) <i>endA1</i> <i>nupG</i>	Invitrogen, Carlsbad, CA, U.S.A.
<i>Xanthomonas oryzae</i> pv. <i>oryzae</i>		
PXO99 ^A	Azacytidine resistant derivative of Philippine race 6 strain PXO99, virulent to rice cultivars IR24, IRBB7, and IRBB10	Choi and Leach 1994
PXO99 ^A ME7	Previously unnamed marker exchange mutant of PXO99 ^A with Tn5- <i>gusA1</i> insertion number 7, in <i>hrcU</i> (in the <i>hrpC</i> operon; Zhu et al. 2000, Fig. 1), type III secretion system-deficient, Km ^r	Zhu et al. 2000
JXO1	Japanese race 1 strain. Avirulent to IRBB2	Yang and White 2004
<i>X. oryzae</i> pv. <i>oryzicola</i>		
BLS256	Philippines isolate	J. Leach
BLS303	Philippines isolate	J. Leach
BLS303hrcC ⁻	BLS303 with insertion of pTOPOhrcC174-781 in <i>hrcC</i> , TTSS-deficient, Km ^r	This study
Plasmids		
pBluescript II KS(+)	Cloning vector, Ap ^r	Stratagene, La Jolla, CA, U.S.A.
pCH43	pUC118 carrying 4.79-kb fragment of <i>X. oryzae</i> pv. <i>oryzae</i> PXO86 genome containing <i>avrXa10</i> and flanking DNA, Ap ^r	Hopkins et al. 1992
pCRBluntII	TOPO cloning vector, Km ^r	Invitrogen
pDD62	Derivative of pVSP1 (Van Der Bij et al. 1996), broad host range, high copy, Km ^r	Mudgett et al. 2000, B. Staskawicz
pHM1	Derivative of pRI40 (Innes et al. 1988) containing multiple cloning site of pUC19, broad host range, low copy, Sp ^r	R. Innes
pHMavrBs3	pHM1 carrying <i>avrBs3</i> with FLAG epitope tag near 3' end, fused with <i>lacZ</i> promoter, Sp ^r	Zhu et al. 1998
pHMavrXa10	pHM1 carrying <i>avrXa10</i> with FLAG epitope tag near 3' end, fused with <i>lacZ</i> promoter, Sp ^r	Zhu et al. 1998
pHMavrXa7	pHM1 carrying <i>avrXa7</i> with FLAG epitope tag near 3' end, fused with <i>lacZ</i> promoter, Sp ^r	Yang et al. 2000
pKEB26	pDD62 carrying <i>avrXa10</i> with native promoter, downstream of and oriented with <i>lacZ</i> promoter of pDD62, Km ^r	This study
pKEB27	pDD62 containing the 30-bp <i>Bam</i> HI to <i>Xho</i> I fragment of pBluescript II KS(+), Km ^r	This study
pKEB45	Similar to pHMavrXa10, but with a 1,232-bp fragment encoding residues 2 to 406 of Cya inserted in-frame in the <i>Sal</i> I site at bp 2,945 of <i>avrXa10</i>	This study
pMJH20	Source of DNA fragment encoding residues 2 to 406 of Cya, Ap ^r	Miao et al. 1999
pTOPOhrcC174-781	pCRBluntII containing bp 174 to 781 (608 bp) of the <i>hrcC</i> open reading frame of <i>X. oryzae</i> pv. <i>oryzicola</i> BLS256, Km ^r	This study
pZWavrXa10	<i>avrXa10</i> cloned into pBluescript II KS+, downstream of and oriented with the <i>lacZ</i> promoter.	Zhu et al. 1998

^z Ap = ampicillin, Km = kanamycin, and Sp = spectinomycin.

and AvrXa10 to render *X. oryzae* pv. *oryzicola* avirulent in rice is not likely due to a failure in delivery.

X. oryzae pv. *oryzicola* and *X. oryzae* pv. *oryzae* deliver equivalent amounts

of an AvrXa10:Cya fusion protein into rice cells.

To determine whether a quantitative difference in the abilities of *X. oryzae* pv. *oryzicola* and *X. oryzae* pv. *oryzae* to deliver AvrXa10 into rice cells could account for the lack of HR to *X. oryzae* pv. *oryzicola* expressing *avrXa10*, Cya was used as a reporter. A construct similar to pHMavrXa10 but encoding a fusion of Cya to the C-terminus of AvrXa10, pKEB45 (Table 1), was made and introduced into *X. oryzae* pv. *oryzicola* BLS303 and *X. oryzae* pv. *oryzae* PXO99^A. As controls, pKEB45 was also introduced into corresponding TTSS-defi-

cient mutant strains BLS303hrcC⁻ and PXO99^AME7 (Table 1), and the empty vector pHM1 was introduced into BLS303 and PXO99^A. The resulting transformants were inoculated to leaves of rice cv. IR24, which lacks *Xa10*, and after 8 h, Cya activity in the inoculated tissue was assessed, using a quantitative immunoassay to measure cyclic AMP levels (discussed below). Inoculation of BLS303(pKEB45) or PXO99^A(pKEB45) resulted in equivalent, high levels of cyclic AMP relative to those resulting from inoculation of the TTSS-deficient strains or the strains carrying pHM1, indicating that both *X. oryzae* pv. *oryzicola* and *X. oryzae* pv. *oryzae* deliver AvrXa10 into rice cells in similar amounts (Fig. 3).

Coinoculated *X. oryzae* pv. *oryzicola* inhibits the rice HR to avirulent *X. oryzae* pv. *oryzae* in a TTSS-dependent fashion.

The above results suggested that *X. oryzae* pv. *oryzicola* might specifically interfere with resistance responses to Avr proteins in rice. To test this idea, IRBB10 rice plants were coinoculated with wild-type *X. oryzae* pv. *oryzicola* BLS303 and *X. oryzae* pv. *oryzae* PXO99^A(pHMavrXa10) at a 1:1 ratio. As with strains of *X. oryzae* pv. *oryzicola* inoculated alone, HR did not occur (Fig. 4A). The same result was observed upon coinoculating plants of cv. IRBB7 with BLS303 and PXO99^A(pHMavrXa7) or cv. IRBB2, containing *Xa2*, with BLS303 and *X. oryzae* pv. *oryzae* JXO1 (Table 1), which is avirulent to plants containing *Xa2* (data not shown). At the same time, *X. oryzae* pv. *oryzae* PXO99^A(pHMavrXa10) mixed at the same ratio with the virulent parental strain PXO99^A and coinoculated in the same manner to IRBB10 plants did elicit an HR (Fig. 4A) that was indistinguishable in extent and timing from that elicited by PXO99^A(pHMavrXa10) alone (Fig. 1A). Symptoms over time were compared among IRBB10 leaves inoculated with *X. oryzae* pv. *oryzae* PXO99^A(pHMavrXa10), *X. oryzae* pv. *oryzicola* BLS303, and a 1:1 mixture of the two (Fig. 4B). One day after inoculation, incomplete but discernible HR of infiltrated tissue was apparent for *X. oryzae* pv. *oryzae* PXO99^A(pHMavrXa10) only; the coinoculated tissue and tissue inoculated with *X. oryzae* pv. *oryzicola* BLS303 alone showed no symptoms. Water-soaking became visible in the tissue infiltrated with the coinoculum and with *X. oryzae* pv. *oryzicola* BLS303 after two days and to a similar extent. The earlier appearance of HR compared with water-soaking and the lack of HR by day 1 following coinoculation indicates that water-soaking is not masking the HR in this experiment. This observation provides evidence that *X. oryzae* pv. *oryzicola* actively interferes

Fig. 1. Neither *avrXa10* nor *avrXa7* in plasmid pHM1 renders *Xanthomonas oryzae* pv. *oryzicola* avirulent to rice plants containing a corresponding *R* gene. **A**, Symptoms at two days following syringe infiltration of *X. oryzae* pv. *oryzicola* (Xoc) BLS303 or *X. oryzae* pv. *oryzae* PXO99^A carrying either pHM1 or pHMavrXa10 into leaves of rice cv. IRBB10, which carries the *Xa10* resistance gene, or of the same strains carrying pHMavrXa7 into leaves of IRBB7, which carries *Xa7*. Virulent strains cause dark lesions water-soaked in appearance. Avirulent strains induce the hypersensitive reaction, visible as lighter brown collapsed tissue. **B**, Plots of bacterial populations in leaves over eight days for BLS303 and BLS303(pHMavrXa10), compared with PXO99^A and PXO99^A(pHMavrXa10), inoculated to rice cv. IRBB10 by syringe infiltration. Capped, vertical bars represent standard deviation of values (CFU per square centimeter of leaf) from three samples. Experiments were repeated three times with similar results.

Fig. 2. *Xanthomonas oryzae* pv. *oryzicola* (Xoc) strains BLS256 and BLS303 and *X. oryzae* pv. *oryzae* (Xoo) PXO99^A transformed with *avrBs3* induce the hypersensitive reaction (HR) of pepper plants carrying the *Bs3* gene. Leaves of pepper cv. ECW30R were infiltrated using a syringe with the indicated strains carrying no plasmid (–) or pHMavrBs3, encoding AvrBs3. Photographs were taken two days after inoculation. HR is apparent as light brown collapsed tissue.

with rice resistance responses to the selected Avr proteins and that the interference takes place following secretion of these proteins from the bacterial cell.

We next determined whether the inhibition of rice HR to avirulent *X. oryzae* pv. *oryzae* depends on the TTSS of *X. oryzae* pv. *oryzicola* by using BLS303hrcC⁻. This strain was non-pathogenic and failed to prevent an HR when coinoculated with PXO99^A(pHMavrXa10) (Fig. 4A). Together, the results support a model in which *X. oryzae* pv. *oryzicola* specifically prevents or suppresses rice defense responses to avirulence

Fig. 3. *Xanthomonas oryzae* pv. *oryzicola* (Xoc) and *X. oryzae* pv. *oryzae* (Xoo) deliver equivalent amounts of an AvrXa10:Cya fusion protein into rice cells. Shown are mean cyclic AMP concentrations for three technical replicates of two replicate samples each of leaves of rice cv. IR24, which lacks *Xa10*, 8 h following syringe infiltration of wild-type (WT) or Hrp-deficient (*hrp*⁻) strains carrying either pKEB45 or the empty vector pHM1. pKEB45 is similar to pHMavrXa10 but contains an in-frame, 3' fusion to *avrXa10* of a fragment encoding the calmodulin-dependent adenylate cyclase of *Bordetella pertussis*. Capped vertical bars represent standard deviation. This experiment was repeated three times with similar results.

signals via the plant intracellular activity of one or more endogenous type III effector proteins.

X. oryzae pv. *oryzicola* defense inhibition is quantitative.

Coinoculations of IRBB10 plants were carried out using a series of different ratios of *X. oryzae* pv. *oryzae* PXO99^A(pHMavrXa10) to *X. oryzae* pv. *oryzicola* BLS303. The HR occurred at the highest ratios, indicating that HR inhibition by *X. oryzae* pv. *oryzicola* in this context is quantitative (Fig. 5A, panel 1). The same series of coinoculations of PXO99^A(pHMavrXa10) was also carried out with the virulent *X. oryzae* pv. *oryzae* parental strain PXO99^A. At relatively low ratios of avirulent to virulent bacteria, *X. oryzae* pv. *oryzae* also inhibited the HR (Fig. 5A, panel 3). At the lowest ratio tested, symptoms over time were similar to those resulting from the inoculation of a 1:1 mixture of avirulent *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* shown in Figure 4B (data not shown). Thus *X. oryzae* pv. *oryzae* also quantitatively inhibits rice defense but, apparently, less effectively than *X. oryzae* pv. *oryzicola*. To assess whether the transition from watersoaking to HR as ratios of avirulent to virulent strain increased correlated with an *Xa10*-dependent suppression of bacterial population growth, populations of PXO99^A(pHMavrXa10) were measured (by plating with selection for the plasmid) over time following coinoculation with BLS303 or PXO99^A at selected ratios both in cv. IRBB10 and in cv. IR24 (Fig. 5B). For PXO99^A(pHMavrXa10) coinoculated with PXO99^A, reduced growth in IRBB10 relative to IR24 was evident at low ratios, but the relative reduction in growth became greater with increasing proportions of the avirulent strain, consistent with a weak defense-inhibitory effect of PXO99^A. In coinoculations with BLS303, populations of PXO99^A(pHMavrXa10) followed similar patterns of growth in IRBB10 and IR24 at all but the highest ratio tested, at which significantly reduced population growth in IRBB10 relative to IR24 was observed (data not shown). The overall growth patterns in IR24 of PXO99^A(pHMavrXa10) coinoculated with BLS303, however,

Fig. 4. *Xanthomonas oryzae* pv. *oryzicola* (Xoc) inhibits hypersensitive reaction (HR) of rice cv. IRBB10 to *X. oryzae* pv. *oryzae* (Xoo) carrying *avrXa10* in a type III secretion-dependent manner. **A**, Leaves 2 days following inoculation with the indicated strains suspended individually to an optical density at 600 nm (OD₆₀₀) = 1.0 and infiltrated together at a 1:1 ratio or (for BLS303hrcC⁻) suspended to an OD₆₀₀ = 0.5 and infiltrated alone. **B**, Leaves 1, 2, and 3 days postinoculation (DPI) with the indicated strains or strain mixture, prepared and infiltrated as in A. Dark lesions appearing water-soaked are typical for virulent *X. oryzae* pv. *oryzicola* and *X. oryzae* pv. *oryzae* strains. Light-brown collapsed tissue indicates the HR. A lighter, tan-colored ring visible in some panels is the result of wounding with the syringe.

Fig. 5. *Xanthomonas oryzae* pv. *oryzicola* (Xoc) inhibits hypersensitive reaction (HR) of rice cv. IRBB10 to *X. oryzae* pv. *oryzae* (Xoo) carrying *avrXa10* in a quantitative manner unaffected by copy number of the *avrXa10* construct, and inhibition activity is weakly conserved in *X. oryzae* pv. *oryzae*. **A**, Leaves 3 days after inoculation with mixtures of *X. oryzae* pv. *oryzicola* BLS303 or *X. oryzae* pv. *oryzae* PXO99^A with PXO99^A carrying either pHMavrXa10 (panels 1 and 3) or pKEB26 (panels 2 and 4). Strains were suspended individually to an optical density at 600 nm = 0.5 and were mixed at the indicated ratios before infiltration. An asterisk indicates that the corresponding strain suspension was replaced with an equivalent volume of water. Note that, at the minimum concentration tested in coinoculations, the avirulent *X. oryzae* pv. *oryzae* strain by itself elicited a robust HR. **B**, Plots of populations of PXO99^A(pHMavrXa10) in rice cv. IRBB10 (solid lines) or cv. IR24 (dashed lines) over 8 days following coinoculation in the indicated ratios with BLS303 or PXO99^A.

were similar to growth patterns in IRBB10 of PXO99^A (pHMavrXa10) coinoculated with PXO99^A, suggesting that BLS303, which multiplies faster than PXO99^A in susceptible rice (Fig. 1B), may have directly or indirectly suppressed population growth of PXO99^A(pHMavrXa10) to a degree similar to, and masking the potential effect of, the *R* gene. In neither experiment did a divergence in growth pattern correlate strictly with the appearance of the HR, though the divergence followed the same overall trend as the HR, increasing with increasing ratios of avirulent to virulent strains.

Expression of *avrXa10* from a higher-copy plasmid in *X. oryzae* pv. *oryzicola* leads to avirulence.

Plasmid pKEB26 consists of the *avrXa10* gene with its native promoter in tandem with the *lacZ* promoter (as in pHMavrXa10) in the vector pDD62 (Table 1), which is a higher copy-number plasmid than pHMavrXa10. We reasoned that pKEB26 would permit higher levels of *avrXa10* expression and possibly alter the balance between the secretion of AvrXa10 and one or more presumed inhibitors. In fact, inoculation of transformants of either *X. oryzae* pv. *oryzicola* BLS303 or BLS256 carrying pKEB26 induced HR on IRBB10 plants, just as the control strain PXO99^A(pHMavrXa10) or PXO99^A(pKEB26), which was also tested (Fig. 6 and data not shown). Although this result contradicts the result with pHMavrXa10, the induction of an HR by *X. oryzae* pv. *oryzicola* strains carrying pKEB26 confirms that this pathovar can

deliver AvrXa10 into rice cells and provides evidence for a stoichiometric relationship between AvrXa10 and one or more presumed inhibitors in determining the plant response. AvrXa10 expression from pKEB26 might result in a simple increase in the proportion of AvrXa10 in the plant cell, or AvrXa10 may competitively impede secretion of other type III effectors of *X. oryzae* pv. *oryzicola*, including one or more presumed inhibitors. While these possibilities could not be distinguished phenotypically in *X. oryzae* pv. *oryzicola* due to the lack of an HR in rice, an experiment was performed to determine if AvrXa10 expression from pKEB26 blocks *avrXa7*-mediated HR elicitation by *X. oryzae* pv. *oryzae*. pHMavrXa7 was introduced into *X. oryzae* pv. *oryzae* PXO99^A containing pKEB26, and the resulting strain PXO99^A(pKEB26, pHMavrXa7) and appropriate control strains were inoculated to IRBB7 plants and IRBB10 plants. PXO99^A(pKEB26, pHMavrXa7) elicited an HR on both IRBB10 and IRBB7 (Fig. 7). Thus, expression of *avrXa10* from pKEB26 does not block type III secretion to an extent that prevents signaling by *avrXa7*. Notably also, pKEB26 did not interfere with the abilities of either BLS303 or PXO99^A to cause water-soaking in cv. IR24 (Fig. 6), indicating that it does not impede traffic necessary for virulence. Coinoculation experiments at different ratios were also conducted using PXO99^A carrying pKEB26 as the avirulent strain, and no discernible difference was observed in the ability of *X. oryzae* pv. *oryzicola* BLS303 to inhibit HR elicitation by this strain and to inhibit HR elicitation by PXO99^A(pHMavrXa10) (Fig. 5A, panels 1 and 2). Also, as for *X. oryzae* pv. *oryzicola*, no difference was observed in the ability of *X. oryzae* pv. *oryzae* PXO99^A to inhibit the HR elicited by PXO99^A(pKEB26) or PXO99^A(pHMavrXa10) (Fig. 5A, panels 3 and 4). Thus, inhibition is overcome when pKEB26 is in *X. oryzae* pv. *oryzicola* but not when it is in a coinoculated *X. oryzae* pv. *oryzae* strain.

DISCUSSION

X. oryzae pv. *oryzae* and *X. oryzae* pv. *oryzicola* each carry multiple members of the *avrBs3/PthA* gene family (Yang et al. 2000). Defining the functions of these genes will advance our understanding of the molecular basis of bacterial blight and of bacterial leaf streak of rice and ultimately contribute to our ability to more effectively control these and other diseases caused by xanthomonads. In *X. oryzae* pv. *oryzae*, different members of the family function as major virulence determinants in different strains (Yang and White 2004). The only isolated avirulence determinants from *X. oryzae* pv. *oryzae* are members of the family, *avrXa7* and *avrXa10*, and the recently

Fig. 6. Expression of *avrXa10* from a high-copy number plasmid renders *Xanthomonas oryzae* pv. *oryzicola* avirulent to rice plants containing *Xa10*. Leaves of rice cv. IRBB10 were inoculated by syringe infiltration with *X. oryzae* pv. *oryzicola* (Xoc) BLS303 or *X. oryzae* pv. *oryzae* (Xoo) PXO99^A carrying pHMavrXa10 or the high-copy number equivalent pKEB26, as indicated. As a control, cv. IR24, lacking *Xa10*, was inoculated with the strains carrying pKEB26, as shown. Leaves were photographed 2 days following inoculation. Virulent strains cause dark lesions water-soaked in appearance. Avirulent strains induce the lighter colored hypersensitive reaction.

Fig. 7. Expression of *avrXa10* from the high-copy number plasmid pKEB26 does not block signaling mediated by *avrXa7*. Leaves of rice cv. IRBB7 and cv. IRBB10 as a control were inoculated by syringe-infiltration with *Xanthomonas oryzae* pv. *oryzae* PXO99^A containing pKEB26 and pHM1, pKEB26 and pHMavrXa7, or pAvrXa7 alone, as indicated. Leaves were photographed 4 days following inoculation. Note that the hypersensitive reaction (HR) of IRBB7 triggered by the presence of pHMavrXa7 is less pronounced than the HR of IRBB10 triggered by pKEB26 but is not blocked by the presence of pKEB26.

cloned *avrXa27* (Gu et al. 2005). One might expect similar functions for the *avrBs3*-like genes in *X. oryzae* pv. *oryzicola*. Evidence suggests, however, that the genes in the two pathogens may function differently. First, an *avrBs3*-like gene that complements mutations in the homologous major virulence determinant genes of *X. oryzae* pv. *oryzae* could not be recovered from *X. oryzae* pv. *oryzicola* (Yang and White 2004). Second, host genes induced by *X. oryzae* pv. *oryzae* in a manner dependent on *avrBs3*-like genes in the pathogen are not induced by *X. oryzae* pv. *oryzicola* (A. Sugio and F. White, unpublished results). Also, curiously, no endogenous *avr/R* gene combination has been described for *X. oryzae* pv. *oryzicola* and rice. Experiments were, therefore, designed to first determine if *avr* genes of the *avrBs3/pthA* family from *X. oryzae* pv. *oryzae* would operate in *X. oryzae* pv. *oryzicola*. Specifically, *avrXa7* and *avrXa10* were introduced into *X. oryzae* pv. *oryzicola*, and the resulting strains were tested for avirulence in rice plants with corresponding *R* genes. Neither gene conferred avirulence in either of two strains of *X. oryzae* pv. *oryzicola*. Further investigation led to the unexpected discovery that *avrXa7* and *avrXa10* failed to function because *X. oryzae* pv. *oryzicola* inhibits *R* gene-mediated defense in rice, putatively via the action of one or more endogenous type III effectors.

Several lines of evidence support this conclusion. First, the lack of an effective resistance response in rice was not likely due to a failure of *X. oryzae* pv. *oryzicola* to express and deliver AvrXa7 and AvrXa10, since the same strains transformed with an equivalent *avrBs3* construct could induce a *Bs3*-dependent HR in pepper and because *X. oryzae* pv. *oryzicola* could deliver an AvrXa10:Cya fusion protein to rice cells in an amount equivalent to that delivered by *X. oryzae* pv. *oryzae*. Second, coinoculated *X. oryzae* pv. *oryzicola* blocked HR to avirulent *X. oryzae* pv. *oryzae* and this ability depended on a functional *X. oryzae* pv. *oryzicola* TTSS, indicating that one or more *X. oryzae* pv. *oryzicola* type III effectors likely interfere with resistance from within the plant cell. Third, inhibition of rice HR to *X. oryzae* pv. *oryzae* expressing AvrXa10 by coinoculated *X. oryzae* pv. *oryzicola* could be overcome by increasing the proportion of the avirulent *X. oryzae* pv. *oryzae* strain, consistent with the notion that inhibition depends on the activity of a type III-secreted protein or proteins that can be overwhelmed by greater amounts of the elicitor. Also, expression of *avrXa10* from a high-copy number plasmid in *X. oryzae* pv. *oryzicola* resulted in HR, confirming the ability of *X. oryzae* pv. *oryzicola* to deliver this protein in rice and further suggesting a stoichiometric effect in determining the rice response.

A number of observations bear on whether inhibition involves blocking recognition of Avr signals, dampening of downstream signaling, or suppression of responses. In this study, *X. oryzae* pv. *oryzicola* prevented rice defense triggered by distinct Avr proteins, suggesting that inhibition might be targeting processes downstream of recognition. However, these effectors are structurally similar; an inhibitor might interfere similarly with recognition of either protein. In coinoculations, *X. oryzae* pv. *oryzicola* also blocked Xa2-mediated HR to *X. oryzae* pv. *oryzae*, but it is not known whether the corresponding avirulence signal is like AvrXa7 and AvrXa10. Failure of *X. oryzae* pv. *oryzicola* to inhibit the AvrBs3-dependent response in pepper suggests that an inhibitor does not target the Avr proteins directly. Inhibition could be directed at shared structures in the *R* proteins or occur via modification of a common target of the Avr proteins that is involved in recognition of the Avr proteins (Kim et al. 2005). If inhibition targets signal transduction, failure to prevent *Bs3*-mediated response in pepper might be due to a difference in the *Bs3* vs. the *Xa7* and *Xa10* pathways or divergence of shared signaling components between pepper and rice. If inhibition targets a component of resistance responses

common to most or all *R*-gene pathways, it might be expected to inhibit defense in a plant closely related to rice, such as maize. This is not the case however, as *X. oryzae* pv. *oryzicola* induces HR in maize varieties carrying the *Rxo1* gene. This induction is due to *avrRxo1*, a gene distinct from the *avrBs3/pthA* family that is conserved in all *X. oryzae* pv. *oryzicola* isolates examined (Zhao et al. 2004). Recently, transgenic rice carrying *Rxo1* was shown to be resistant to *X. oryzae* pv. *oryzicola* (Zhao et al. 2005), favoring the idea that *X. oryzae* pv. *oryzicola* inhibits only certain pathways. On the other hand, *Rxo1* may “trump” (Abramovitch and Martin 2005) *X. oryzae* pv. *oryzicola* by suppressing defense inhibition activity in its recognition of AvrRxo1 (could AvrRxo1 be the inhibitor?). To better define the process targeted for inhibition by *X. oryzae* pv. *oryzicola*, diverse *avr/R* pairs need to be tested. Cloning of one or more putative inhibitors and expression in avirulent *X. oryzae* pv. *oryzae* strains will best enable assays of the effect on different *R* gene-mediated responses. An intriguing possibility is that members of the *avrBs3/pthA* family themselves function in defense inhibition.

A difference in the effect on defense inhibition of expressing *avrXa10* from a high- vs. a low-copy plasmid in *X. oryzae* pv. *oryzicola* and expressing it in a high- vs. a low-copy plasmid in *X. oryzae* pv. *oryzae* coinoculated with *X. oryzae* pv. *oryzicola* suggested that there is no significant difference in the amount of secretion of *avrXa10* due to the different plasmids, but that expression of *avrXa10* from the high-copy plasmid overcame inhibition in *X. oryzae* pv. *oryzicola* due to an effect on delivery of one or more putative inhibitors from the bacterium. The hypothesis that type III secretion is limiting and that overexpression of an effector can compromise delivery of other effectors remains to be tested conclusively, but it is consistent with observations in *X. oryzae* pv. *oryzae* that complementation of a mutation in a virulence determinant of the *avrBs3/pthA* family using a plasmid-borne clone often fails to fully restore virulence in quantitative assays (Bai et al. 2000; Yang and White 2004; Yang et al. 2000); this failure could be due to a reduction in secretion of other virulence factors. pKEB26 did not disrupt watersoaking on the susceptible rice cv. IR24 or prevent HR mediated by pHMavrXa7 in *X. oryzae* pv. *oryzae*, but the former observation was not quantitative and the latter might have been affected by the fact that *avrXa7* itself was on a multi-, albeit low, copy plasmid. The HpaB protein, required for secretion of AvrBs3 and several other effectors (Buttner et al. 2004), provides an example of a possible rate-limiting factor for type III secretion.

Recent evidence suggests that diverse type III effectors contribute to virulence by inhibiting plant-defense responses (Abramovitch and Martin 2004; DebRoy et al. 2004; Metz et al. 2005). Our demonstration that *X. oryzae* pv. *oryzicola* inhibits *R* gene-mediated defense in its host plant in a TTSS-dependent manner supports the notion that defense inhibition is a general mechanism in bacterial pathogenesis of plants and an important function of the TTSS. Further, our results demonstrate that one pathogen can inhibit host defense against another, otherwise-avirulent pathogen in the same plant.

The quantitative nature of defense inhibition by *X. oryzae* pv. *oryzicola* suggests an intriguing question; aside from disabling basal immunity and crippling host resistance responses mediated by *R* genes, might there be a role for defense inhibitors in modulation of cell death during disease development? HR is a rapid form of programmed cell death (PCD) associated with successful defense against pathogens. Yet some evidence suggests that necrogenic plant pathogens may rely on controlled elicitation of plant PCD to colonize plant tissues or to generate lesions to aid in pathogen dispersal (Brunings and Gabriel 2003; Greenberg and Yao 2004). If manipulation of

PCD is involved in disease development, a role for some bacterial effectors might be to dampen PCD so that it occurs only to an extent that promotes pathogen exploitation of host tissues and survival. Whether this is indeed the case for *X. oryzae* pv. *oryzicola*, *X. oryzae* pv. *oryzae*, and other necrogenic pathogens remains to be explored.

Outbreaks of bacterial leaf streak can have devastating impact, but they are sporadic (Mew 1993). Quantitative resistance to *X. oryzae* pv. *oryzicola* exists in some rice varieties (Tang et al. 2000), and there may be yet-unknown native *R* genes that prevent the disease from becoming more prevalent. Nevertheless, the ability of *X. oryzae* pv. *oryzicola* to inhibit host defense provides a plausible explanation for the lack of known rice *R* genes for bacterial leaf streak.

Our results provide the first demonstration that *X. oryzae* pv. *oryzicola* depends on the TTSS for pathogenesis and the first formal demonstration that AvrXa10 is delivered to rice cells, both by *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola*. These findings suggest that the many *avrBs3/pthA* family members present in *X. oryzae* pv. *oryzicola* are, in fact, expressed and their products secreted. The roles of these genes in pathogen-host interaction remain an important target for research. In addition to virulence functions, one might predict that avirulence functions might be revealed by heterologous expression of the genes in *X. oryzae* pv. *oryzae* inoculated to differential rice cultivars.

Also to be considered, the ability of *X. oryzae* pv. *oryzicola* to block elicitation of the HR by coinoculated avirulent *X. oryzae* pv. *oryzae* implies that *R* genes to bacterial blight and perhaps other diseases may not be effective when *X. oryzae* pv. *oryzicola* is infecting the plant. Further elucidation of rice defense inhibition by *X. oryzae* pv. *oryzicola* is therefore a priority as well. Such elucidation should lead to a better understanding of the role of plant defense inhibition in pathogenesis generally and could contribute to the development of new strategies for disease control that target the defense inhibition mechanism and, in the case of bacterial leaf streak, uncover useful gene-for-gene interactions.

MATERIALS AND METHODS

DNA manipulation.

Standard methods of DNA manipulation were carried out as described (Sambrook et al. 1989). Plasmids were isolated using the Wizard miniprep (Promega, Madison, WI, U.S.A.) and the HiSpeed midiprep (Qiagen, Valencia, CA, U.S.A.) kits. *X. oryzae* pv. *oryzicola* genomic DNA for polymerase chain reaction (PCR) was extracted using the DNeasy kit (Qiagen).

Bacterial strains, plasmids, and culture conditions.

The bacterial strains and plasmids used in this study are listed in Table 1. pHMavrXa10, pHMavrXa7, and pHMavrBs3 have been described in detail previously (Yang et al. 2000; Zhu et al. 1998). Briefly, pHMavrXa10 is *avrXa10* cloned in pBluescript KS (+), such that it is downstream of and oriented with the *lacZ* promoter (making pZWavrXa10; Table 1), and inserted into pHM1 (opposite the *lacZ* promoter of that plasmid). The *lacZ* promoter is expressed constitutively in *Xanthomonas* spp. (Soby and Daniels 1996). A 24-bp sequence encoding the FLAG epitope (Brizzard et al. 1994) is inserted at an engineered *SalI* site near the 3' end of the open reading frame of *avrXa10*. Replication in *Xanthomonas* spp. is directed by the origin of pHM1. pHMavrXa7 and pHMavrBs3 are identical to pHMavrXa10 except for the gene-specific sequence between the conserved *SphI* sites in the *avr* genes. pKEB26 was constructed as follows. The *BamHI* to *XhoI* fragment of the mul-

tip cloning site (MCS) of pBluescript II KS(+) (Table 1) was inserted between these sites in pDD62 (Table 1), creating pKEB27 with unique sites *BamHI*, *EcoRI*, *EcoRV*, *HindIII*, and *XhoI* in the MCS. The 4.8-kb *EcoRI* to *HindIII* fragment of pCH43 (Table 1), containing the *avrXa10* open reading frame and 252 bp upstream, was then inserted between these sites in pKEB27, resulting in pKEB26. In pKEB26, *avrXa10* is downstream of and oriented with the *lacZ* promoter of pKEB27. To construct pTOPOhrcC174-781, a 608-bp internal fragment (base pairs 174 to 781) of the *hrcC* open reading frame was amplified from *X. oryzae* pv. *oryzicola* BLS256 genomic DNA by PCR, using primers SM20 (forward primer, 5'-CACCTTGACCTGTGCGGCATT-3') and SM21 (reverse primer, 5'-TTTGTCCGCCAGTCCATC-3'), designed based on the *hrcC* sequence of *X. oryzae* pv. *oryzae* (GenBank accession number AB115081). The PCR product was cloned in pCRBluntII by the TOPO cloning reaction (Invitrogen, Carlsbad, CA, U.S.A.). pKEB45 was constructed similarly to pHMavrXa10. First, a 1,232-bp fragment encoding residues 2 to 406 of Cya was amplified from plasmid pMJH20 (Table 1) with primers that add a *SalI* site on each end. This fragment was then inserted in the *SalI* site at base pair 2,945 of *avrXa10* in pZWavrXa10 (Table 1). Finally, the resulting plasmid was linearized using *HindIII* and was inserted into the *HindIII* site of pHM1. *Escherichia coli* was cultured in Luria-Bertani medium (Sambrook et al. 1989) at 37°C. *X. oryzae* strains were grown in glucose yeast extract (GYE; 20 g of glucose and 10 g of yeast extract per liter) medium or nutrient broth yeast extract (NBY; Vidaver 1967) medium at 28°C. Ampicillin (100 µg/ml), kanamycin (25 µg/ml), and spectinomycin (100 µg/ml) were added to growth media as appropriate for plasmid selection.

Plasmid transformation.

Plasmids were introduced by electroporation into *E. coli* cells supplied with pCRBluntII according to the manufacturer's protocol (Invitrogen) and into *Xanthomonas* cells as described previously (Tsuge et al. 2001). Transformation of *Xanthomonas* strains was confirmed by reisolation and transfer of plasmids to *E. coli*.

Plant materials and inoculations.

Rice plants of the Indica variety IR24 and its near-isogenic lines, IRBB2 containing *Xa2*, IRBB7 containing *Xa7*, and IRBB10 containing *Xa10*, were grown in LC-1 soil mixture (SunGRO, Bellevue, WA, U.S.A.) in a growth chamber under cycles of 12 h of light at 28°C and 12 h of dark at 25°C. Fertilizer (Peters Professional, St. Louis) and iron chelate micronutrient (Becker Underwood, Ames, IA, U.S.A.) were applied with watering every 2 days at rates of 0.25 and 4.5 g/liter, respectively. Plants were inoculated at four weeks after sowing by infiltration of leaves with bacterial suspension using a needleless syringe (Schaad et al. 1996). Pepper plants of cv. ECW30R carrying the *Bs3* resistance gene were grown in a standard potting mix in a greenhouse and were inoculated by syringe infiltration at six weeks. For inoculations, overnight bacterial cultures were washed twice and were resuspended with sterile H₂O. Unless otherwise indicated, strains inoculated individually were suspended to an optical density at 600 nm (OD₆₀₀) = 0.5, and strains inoculated together were suspended to an OD₆₀₀ = 1.0 before mixing. Following inoculation, plants were incubated at room temperature under constant fluorescent light in the laboratory.

Bacterial population growth measurement.

Infiltrated areas of rice leaves were removed with a number 1 cork borer and were ground in liquid nitrogen in 1.5-ml mi-

crocentrifuge tubes by using a plastic pestle (Kontes pellet pestle; Kontes, Vineland, NJ, U.S.A.). Samples were mixed with 0.5 ml of sterile water, and then, serial dilutions were made and spotted (10 μ l per spot) in triplicate on GYE agar plates with appropriate antibiotics. Plates were incubated at 28°C until single colonies could be counted. The number of CFU per square centimeter of leaf area was then estimated, and standard deviation was calculated using colony counts from the three triplicate spots from each of three samples per timepoint per inoculum. Experiments were repeated at least three times.

Construction of BLS303*hrcC*⁻.

pTOPO*hrcC*174-781, which is not replicated in *Xanthomonas* spp., was introduced into *X. oryzae* pv. *oryzicola* BLS303. Recombinants were selected on GYE plates containing kanamycin. Disruption of *hrcC* by single homologous recombination was assayed by PCR, using the M13forward(-20) primer corresponding to the vector sequence upstream of the insert in pTOPO*hrcC*174-781 and primer SM25 (5'-ATGGCTCCTGC CTGTACCA-3'), which corresponds to the 3' end of *hrcC*. Each of five isolates showing disruption of *hrcC* was inoculated to rice and confirmed to be nonpathogenic. One was chosen arbitrarily for coinoculation experiments and was designated as BLS303*hrcC*⁻.

Immunodetection of AvrXa10.

X. oryzae pv. *oryzicola* BLS303(pHMavrXa10), alongside *X. oryzae* pv. *oryzae* PXO99^A (pHMavrXa10) and *X. oryzae* pv. *oryzicola* BLS303 as positive and negative controls, respectively, were grown in NBY to OD₆₀₀ = 0.7. As described above, AvrXa10 encoded by pHMavrXa10 contains the FLAG epitope (Brizzard et al. 1994). For each culture, cells (2 ml) were resuspended in 75 μ l of 1 $\times$ Laemmli buffer (Laemmli 1970), and cultures were heated to 100°C for 3 min to obtain total cellular proteins. Polyacrylamide (10%) gel electrophoresis and Western blotting of samples was performed using a standard protocol (Bowen et al. 1980). Mouse monoclonal anti-FLAG antibody (Sigma, St. Louis) followed by rabbit anti-mouse antibody conjugated to peroxidase (Pierce, Rockford, IL, U.S.A.) and ECL Plus Western blotting detection system (Amersham, Piscataway, NJ, U.S.A.) with Blue sensitive autoradiography film (Midwest Scientific, St. Louis) were used according to manufacturer protocols to detect AvrXa10.

Cya reporter assay.

Rice leaves were infiltrated with bacterial suspensions at OD₆₀₀ = 0.5 by needleless syringe. After inoculation (8 h), two replicate samples of 5-cm lengths containing four inoculated spots each were collected, frozen in liquid nitrogen, and were ground with a plastic pestle to a powder. The ground leaf tissues were resuspended in 250 μ l of 0.1 M HCl and were centrifuged briefly. The supernatants were assayed with the Correlate-EIA cAMP immunoassay kit (Assay Designs, Ann Arbor, MI, U.S.A.) according to the manufacturer's directions, with three technical repeats for each sample. The amount of total proteins in each sample for normalization was determined by the BCA protein assay kit (Pierce).

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