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Molecular characterization of the *avrXa7* locus from *Xanthomonas oryzae* pv. *oryzae* field isolates

Grisel Ponciano[a], Kimberly Webb[a,b], Jianfa Bai[a], Casiana Vera Cruz[b], Jan E. Leach[a,*]

[a]*Department of Plant Pathology, 4024 Throckmorton Plant Sciences Center, Kansas State University, Manhattan, KS 66508-5502, USA*
[b]*Division of Entomology and Plant Pathology, International Rice Research Institute, Manila, Philippines*

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Abstract

The effector gene *avrXa7* from *Xanthomonas oryzae* pv. *oryzae* has avirulence function in rice with the *Xa7* resistance gene and confers pathogenic fitness (aggressiveness). Field strains of *X. oryzae* pv. *oryzae* displayed a diversity of phenotypes on rice ranging from complete to partial loss of these functions. To understand the molecular basis for variation in *avrXa7* function, we sequenced the alleles from seven field strains. The *avrXa7* gene is an *avrBs3/pthA* family member and contains nuclear localization signal sequences and an acidic activation domain (AAD) in the 3′ end and a central region containing repeated sequences, all of which are important for avirulence and aggressiveness. Sequence analysis revealed changes in the *avrXa7* alleles ranging from a point mutation to multiple mutations spread throughout the alleles. Some strains with identical mutant alleles exhibited different levels of aggressiveness to rice, suggesting the presence of second mutations. A point mutation found at the beginning of the AAD of one *avrXa7* allele was reconstructed in the wild type gene; this mutant exhibited partial loss of avirulence and aggressiveness. Our data suggest that adaptation of *X. oryzae* pv. *oryzae* to *Xa7* rice fields involves not only alterations in *avrXa7*, but may include changes in other gene family members resulting from recombination between family members.

Keywords: *Xanthomonas oryzae* pv. *oryzae*; *Oryza sativa*; Avirulence gene; Effector gene; Hypersensitive response

1. Introduction

Avirulence (*avr*) genes from phytopathogenic bacteria encode effectors that, depending on the host plant genotype (i.e. the presence or absence of a resistance gene), either trigger a resistance response or cause disease [5,20,25]. Many of these bacterial effectors contribute to various aspects of virulence or pathogenic fitness such as intercellular multiplication, aggressiveness, and ability of the pathogen to exit to the leaf surface (for review, see Refs. [8,16]). Loss of *avr* effector gene function has great implications in disease control practices that are based on single resistance (*R*) genes. That is, mutations in the *avr* effector gene could lead to a loss of recognition by the corresponding *R* gene product and result in a breakdown of resistance. Indeed, several naturally occurring mutations in bacterial effectors have been described; analysis of the sequences of these genes revealed that the mutations resulted from insertions, deletions, rearrangements, or loss of plasmids carrying the effector genes [6,9,13,14,21,24]. The relevance of these changes in field populations has been analyzed for only a few pathogen avirulence genes [14,24]. Based on these studies, it has been proposed that if the *avr* effector gene has a high fitness value to the pathogen, then strains harboring mutations in those genes would be less likely to establish in field populations, and the corresponding *R* genes would be more durable (for review, see Ref. [16]). That is, if the mutations in *avr* effector genes negatively impact pathogen fitness, then the ability of the mutant to cause disease or persist in the population would be reduced.

The *avrXa7* gene from *Xanthomonas oryzae* pv. *oryzae* encodes an avirulence effector that is recognized by the *Xa7*

Abbreviations: AAD, acidic activation domain; AUDPC, area under disease progress curve; HR, hypersensitive response; NLS, nuclear localization signal; LZ, leucine zipper; pv., pathovar; TTS, type III secretion; *X. oryzae* pv. *oryzae*, *Xanthomonas oryzae* pv. *oryzae*.

* Corresponding author. Tel.: +1 785 532 1367; fax: +1 785 532 5692.
E-mail address: jeleach@ksu.edu (J.E. Leach).

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rice resistance gene product and is also important for pathogen aggressiveness [1]. This gene is a member of the *avrBs3/pthA* gene family of effectors that is common in *Xanthomonas* species (for review, see Refs. [7,15,17]). AvrXa7 effector activity is dependent of an intact type III secretion (TTS) system [26] and secretion of the protein, like other AvrBs3/PthA effectors, is proposed to be mediated by a signal sequence in the amino terminus [22]. Molecular characterization of several members of the family reveals that three key structures of the gene products, the central repeat region, the nuclear localization signal (NLS), and an acidic activation domain (AAD), are essential for virulence and for host R protein recognition [23,29,31]. The central repeat region, which is composed of several nearly identical repeated sequences of 34 amino acid residues, is the structure that governs host recognition [10,26,27]. An imperfect leucine zipper (LZ) immediately downstream the repeat region has been identified but its function in avirulence or virulence has not been studied [7].

In a field study, Vera Cruz et al. [24] identified strains of *X. oryzae* pv. *oryzae* that had gained virulence to rice with the *Xa7* gene for resistance. Based on the lesion lengths they caused on *Xa7* rice, these strains were divided into two groups, those that had completely lost *avrXa7* avirulence function (races 9a and c) and those that had partially lost *avrXa7* avirulence function (race 9b). Molecular analysis of the first group indicated that an undetermined genetic rearrangement causing a polymorphism at the *avrXa7* locus in race 9a strains, and mutations in the 3′ end of *avrXa7* in a race 9c strain are the causes of loss of avirulence to *Xa7* rice [24]. The majority of the field strains isolated from rice with *Xa7*, however, exhibited a wide range of both avirulence and virulence on rice. This population of *X. oryzae* pv. *oryzae* strains, called collectively race 9b, continues to persist at a high frequency in field plots planted to rice containing *Xa7* over a 10 year period, although they have not yet caused epidemics on rice with *Xa7* (K. Webb, J. Leach and C. Vera Cruz, unpublished work).

Our goal was to determine the molecular changes in the *avrXa7* effector gene that were associated with phenotypic changes in *X. oryzae* pv. *oryzae*-rice interactions.In this report, we describe the interaction phenotypes of a subset of race 9b strains that represent the broad range in virulence to *Xa7*-containing rice. The sequences of seven *avrXa7* alleles from these field strains were compared to determine the range of molecular changes that could account for adaptation of the strains to *Xa7* rice. We demonstrate that the changes in phenotype do not always correlate with *avrXa7* change, suggesting that second site mutations may be involved. Using genetic complementation, one mutation in an *avrXa7* variant was demonstrated to account for the intermediate avirulence and aggressiveness phenotypes. Our work documents that while in some cases natural variation at the *avrXa7* allele can regulate pathogen aggressiveness in nature, additional changes at other sites may moderate the impact of the variation at *avrXa7*.

2. Materials and methods

2.1. Bacterial strains and plasmids

A subset of 52 field strains of *X. oryzae* pv. *oryzae* that were isolated from IRBB7 rice were selected from the study of Vera Cruz et al. [24] for greenhouse inoculations to evaluate interaction phenotypes with rice. A second subset of 14 strains from this study and six additional strains isolated from IR24 were selected for more comprehensive evaluation in greenhouse experiments (Table 1). PXO557 was used for sequence analysis and was collected from IR24 in 1998 from the same field in Calauan sampled by Vera Cruz et al. [24].

X. oryzae pv. *oryzae* strain KL7M, which contains a Tn*5* insertion that inactivates *avrXa7* [1], was used in complementation tests for aggressiveness phenotypes. Strain PXO99A does not contain *avrXa7* and was selected for growth on 5-azacytidine to reduce impact of restriction modification systems on plasmid stability [4,11]. This strain is an excellent recipient for recombinant plasmids, and was therefore used for analysis of avirulence phenotypes. All *X. oryzae* pv. *oryzae* strains were maintained as described previously [11]. Immunoblot assays confirming expression of variant alleles were performed as described [30] using antibodies generated to recognize the sequence PASQRWDRILQ immediately upstream of NLSa (a gift from

Table 1
Description of *X. oryzae* pv. *oryzae* field isolates used for greenhouse inoculations

Strain	Collection date	Collection site	Cultivar source	Original race designation
PXO0314	1993	Calauan	IRBB7	9b
PXO346	1994	Mabitac	IRBB7	9c
PXO348	1993	Calauan	IRBB7	9b
PXO350	1993	Calauan	IRBB7	9b
PXO351	1993	Mabitac	IRBB7	9b
PXO352	1993	Mabitac	IRBB7	9b
PXO354	1994	Calauan	IRBB7	9b
PXO356	1994	Calauan	IRBB7	9b
PXO357	1995	Calauan	IRBB7	9b
PXO360	1995	Calauan	IRBB7	9b
PXO368	1994	Calauan	IRBB7	3
PXO419	1993	Calauan	IR24	9b
PXO429	1993	Calauan	IR24	9b
PXO433	1993	Calauan	IR24	9b
PXO441	1993	Mabitac	IR24	9b
PXO448	1993	Mabitac	IRBB7	9b
PXO449	1993	Mabitac	IRBB7	9b
PXO459	1994	Mabitac	IR24	9b
PXO557	1998	Calauan	IR24	9b
PXO1865	1994	Calauan	IRBB7	3[a]
PXO2684	1994	Mabitac	IRBB7	9c

[a] In the first study, this isolate grouped as a clear race 3 (lesion lengths of 5.6 cm on IRBB7 and 24.9 on IR24). However, in the second study, this isolate had lost aggressiveness, presumably due to changes resulting from laboratory culturing.

S. Tsuyumu), and a chemiluminescent assay (ImmuneStar, BioRad, Hercules, CA, USA).

2.2. Plant assays

Rice cultivar IRBB7 is near-isogenic for the bacterial blight resistance gene *Xa7*; IR24, which contains no relevant R genes, is the recurrent parent for IRBB7 [18]. To assay avirulence and virulence, bacterial suspensions were inoculated to rice leaves by infiltration (5×10^7 and 5×10^8 CFU/ml) [19] or by leaf-clipping (5×10^8 CFU/ml) [12].

For greenhouse studies to evaluate the virulence and avirulence of the 52 strains from IRBB7, three seedlings per replication of each of the rice cultivars IR24 and IRBB7 were leaf-clip inoculated [12]. Lesion lengths (cm) from two replications of each cultivar were scored at 14 days. Plant inoculation experiments to characterize the subset of 20 strains using area under the disease progress *c*urve (AUDPC) [3] were also conducted in the greenhouse using the leaf-clipping method of rice cultivars IR24 and IRBB7 in a split plot design. Each bacterial strain was inoculated in three replications (six plants per replication) with repeated measurements taken over the six plants per replication, and each experiment was repeated three times (54 plants total per bacterial strain inoculation). Lesion lengths (cm) were measured at 6, 9, 12, 15 and 18 days after inoculation. AUDPC was calculated using SAS (SAS Institute, Cary, NC) and ANOVA derived with the proc mixed program. Groupings were determined using pair-wise comparisons and *P*-values less than 0.05.

To assess virulence and avirulence of transformants containing the *avrXa7* variant allele, symptom development was assessed 3 days after inoculation by infiltration and at 9, 13, 17 and 21 days after inoculation by leaf-clipping. Disease development data were analyzed using the general linear model of SAS. For each treatment, disease development was regressed against time (days) and linear regression models were fit to the data. To determine differences among the treatments, the slopes of the models were compared. When slopes of two lines were not significantly different, the estimates of the *Y* intercepts were compared to determine if one line was significantly different from another. Bacterial populations were measured at 0, 4 and 10 days after leaf-clip inoculation as previously described [2]. All plant inoculation experiments contained 4–8 replications, with six plants per replication, and were performed two times.

2.3. Sequence analysis of avrXa7 variants from field strains

The sequences of *avrXa7* alleles from the field isolates of *X. oryzae* pv. *oryzae* were determined from cloned fragments and PCR products. Because each strain of *X. oryzae* pv. *oryzae* contains over 12 copies of the *avrBs3/pthA* gene family, the fragments specifically containing *avrXa7* alleles were obtained by digesting genomic DNA of each strain separately with the following restriction enzymes: *Bam*HI (fragment containing the majority of *avrXa7* ORF and only lacking the last 174 bp of the 3′ end), and *Eco*RI and *Pst*I (fragments containing the missing 174 bp of the 3′ end from the *Bam*HI digest). Digested DNA fragments were separated in 1% (for the *Bam*HI and *Pst*I digests) and 1.5% (for the *Eco*RI digest) agarose gels (20 cm W × 18 cm L) at 50 V for 25–36 h at 4 °C. The 4.2 kb *Bam*HI, 1.3 kb *Eco*RI and 3.1 kb *Pst*I fragments were excised from the gels. The DNA fragments were extracted and purified from the gel (Qiagen, Valencia, CA 91355). The 4.2 kb *Bam*HI fragment was cloned into pBluescript II KS (Stratagene) for sequence analysis of the 5′ and 3′ ends using *avrXa7* specific primers 5′AACCCTCCGACGCTTC3′ (avr7N primer), 5′CAGGGGGGCACCCGTCAGTGC3′ (p7NR primer), 5′GCCCAGTTATCTCGCCC3′ (avr7hinc for primer), 5′CGATTGATTCTTCTGAT3′ (avr7hinc primer) and 5′GATCCTGGTTACGCGG3′ (avr7BamHI primer) in combination with vector specific primers 5′AATTAACCCTCACTAAAGGG3′ (T3 primer), 5′GTAATACGACTCACTATAGGGC3′ (T7) and 5′GTAAAACGACGGCCAGT3′ (M13–20 primer). Sequence of the 170 bp 3′ terminus of each *avrXa7* allele, which was absent from the 4.2 kb *Bam*HI fragment, was obtained from products that were amplified from the *Eco*RI or *Pst*I fragments, where appropriate, using the *avrXa7* specific forward primers 5′GGGCCAAACCGTCCCCTACT3′ (Cterm1 primer, for the *Eco*RI template DNA) or 5′GCCCAGTTATCTCGCCC3′ (avr7hinc for primer, for the *Pst*I template DNA) in combination with the *avrXa7* specific reverse primer 5′TCGGCAGGGATTCGTGTAA3′ (9bRev primer). Sequence of the repeat region of each allele was determined from independent insertion clones derived by insertion of the EZ::TN<KAN-2> transposon into the 4.2 kb *Bam*HI cloned fragments (Epicentre, Madison, WI 53713). Primers 5′ACCTACAACAAAGCTCTCATCAACC3′ (KAN-2 FP-1 primer) and 5′GCAATGTAACATCAGAGATTTTGAG3′ (KAN-2 RP-1 primer) were used for sequencing reactions. All sequencing was performed by the Kansas State University Sequencing and Genotyping facility. Sequences were analyzed using ClustalW multiple sequence alignment program version 1.8 from the Baylor College of Medicine Human Genome Sequencing Center.

The *avrXa7* 4.2 Kb *Bam*HI fragment is difficult to separate from the 4.3 kb *Bam*HI fragment of another *avrBs3/pthA* homolog (*aB4.3*) found in these strains of *X. oryzae* pv. *oryzae*. To be certain that we were characterizing *avrXa7* alleles from the field strains, the partial sequence (i.e. the amino and carboxy termini only without the repeats) of the *aB4.3* homolog from two field strains (PXO348 and PXO441) was obtained and used for comparisons.

2.4. Construction of pH0314

Plasmid pH0314 containing the *avrXa7* allele from the field strain PXO0314 was constructed by ligating the *Bam*HI

digestion product obtained from the 1.3 kb *Eco*RI fragment of the PXO0314 *avrXa7* allele with pUC81 that had been partially digested with *Bam*HI. Plasmid pUC81 contains the wild type *avrXa7* gene from PXO86 [1]. The resulting plasmid pH 0314 was linearized with *Hin*dIII and cloned into the broad host range vector pHM1 (R. Innes). Plasmids were introduced into *X. oryzae* pv. *oryzae* strains PXO99A and KL7M by electroporation [4] and virulence and avirulence gene activities of strains carrying the plasmids (genes in trans) were assessed by infiltration and leaf-clip inoculation assays as described above.

3. Results

3.1. A X. oryzae pv. oryzae field population exhibits a wide spectrum of virulence to Xa7 rice

The changes in *avrXa7* avirulence and aggressiveness (a measurable component of virulence in interactions of *X. oryzae* pv. *oryzae* and rice) functions of a subset of *X. oryzae* pv. *oryzae* strains collected by Vera Cruz et al. [24] were reassessed by inoculating the strains to rice cultivars IRBB7 (*Xa7* resistance gene) and IR24 (no *Xa7*) under greenhouse conditions. The 52 strains, collected between 1993 and 1995 from two field sites in the Philippines, have genomic profiles that are almost identical [24], suggesting they are closely related descendents. Most of the strains (47) were designated as race 9b; the remaining five were designated race 9a and c. Our greenhouse inoculations show that collectively these strains exhibit a wide range of virulence to IRBB7 rice from low virulence (4.9 cm lesion length) to highly virulent (23.9 cm lesion length) (Fig. 1). Thus, although some strains had apparently lost most or all avirulence function of *avrXa7* (lesions >19 cm), a large group of strains exhibited some level of avirulence gene function (lesions ranging from 5 to 19 cm).

On IR24 rice, the strains caused a wide spread in lesion lengths, ranging from 19.3 to 29.6 cm (Fig. 1). Strains that showed the longest lesions on IRBB7 (19–23.9 cm) were frequently reduced in aggressiveness on IR24 (23.1–25.0 vs 29.6 cm), suggesting that reduction or loss of *avrXa7* function reduced aggressiveness as previously described [24]. Because the lesions on IRBB7 were in general shorter than those observed on IR24, it is possible that none of the strains had completely lost *avrXa7* avirulence function. Alternatively, this difference in aggressiveness could be due to a genetic difference that remains in the two near-isogenic rice cultivars.

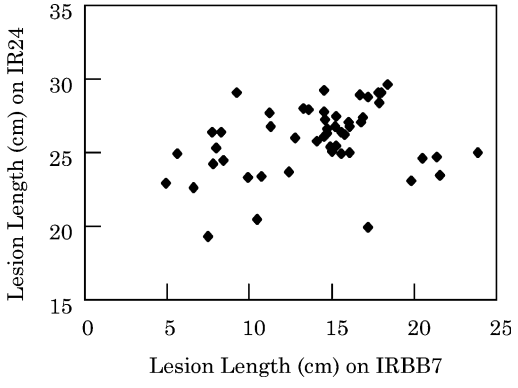

Fig. 1. Relationship between lesion lengths induced by 52 field strains of *X. oryzae* pv. *oryzae* on rice lines IRBB7 (containing the *Xa7* resistance gene) and IR24 (no *Xa7*). Plants were inoculated in the greenhouse.

The demonstration of a wide range in lesion lengths on IRBB7 and IR24 suggests that mutations affecting the avirulence function of *avrXa7* may result in partial loss of gene function and intermediate phenotypes. To more carefully characterize the range in virulence and aggressiveness, the interaction phenotypes of 20 strains (Table 1) were evaluated on rice with and without *Xa7* under greenhouse conditions. The degree of virulence of these strains was estimated as the AUDPC from the inoculated rice leaves. Based on the AUDPC, the population of strains exhibited a continuum in their interaction phenotype on rice with *Xa7*, ranging from avirulent (e.g. PXO1865 and PXO351) to virulent (e.g. PXO346, PXO441 and PXO419) (Fig. 2A). The diversity of phenotypes was also apparent on the susceptible cultivar IR24. That is, strains ranged from low aggressiveness (e.g. PXO1865, PXO2684) to highly aggressive (PXO419, PXO357) (Fig. 2B). Some strains that exhibited virulence to rice with *Xa7* (apparent loss of avirulence function) did not exhibit a reduction in aggressiveness function to IR24 rice (e.g. strains PXO441 and PXO419). Thus, loss of avirulence function was not always associated with a reduction in aggressiveness in *X. oryzae* pv. *oryzae*/rice interactions.

3.2. Molecular changes in avrXa7 locus of X. oryzae pv. oryzae field strains and their relationship to interaction phenotypes in rice

To determine if the molecular basis for the observed diverse reactions of the race 9b strains on rice was associated with changes in the *avrXa7* gene, we sequenced the *avrXa7* alleles from a subset of six strains (PXO0314, PXO348, PXO356, PXO357, PXO441, and PXO448) that exhibited different degrees of avirulence and aggressiveness. GenBank accession numbers for the alleles from each strain are AY626404 (PXO0314), AY626405 (PXO348), AY626406 (PXO356), AY626410 (PXO357), AY626408 (PXO441), and AY626409 (PXO448). In addition, we sequenced the allele from strain PXO557 (AY626407), which was isolated from IRBB7 rice in a later experiment (1998). This strain was of interest because separate experiments (not shown) demonstrated that PXO557 maintains much of its avirulence and is slightly reduced in virulence function (average lesion lengths on IRBB7 are 6.1 cm and on IR24 are 24.0 cm).

Since strains of *X. oryzae* pv. *oryzae* harbor more than 12 homologs of *avrXa7* [11], we confirmed that our analyses

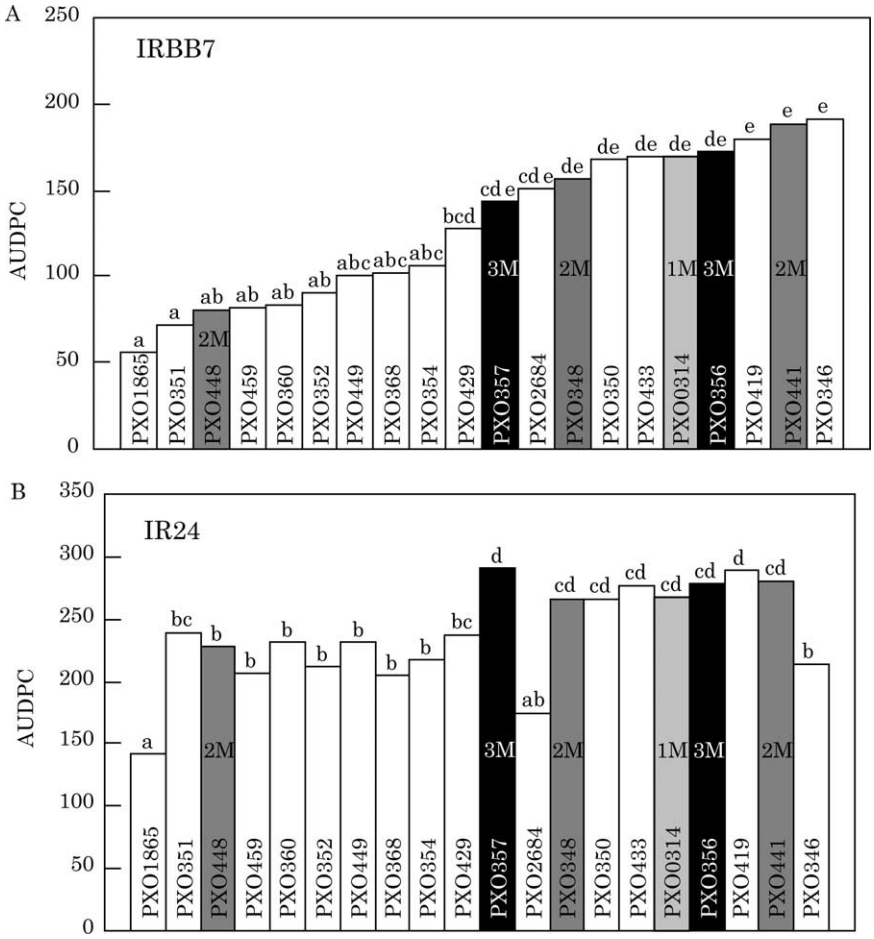

Fig. 2. Area under the disease progress curve (AUDPC) for bacterial blight on (A) resistant IRBB7 (*Xa7*) and on (B) susceptible IR24 (no *Xa7*) rice cultivars. Plants were inoculated in the greenhouse with 20 field strains of *X. oryzae* pv. *oryzae*. Lower case letters indicate significant differences at $P=0.05$.

involved the appropriate *avrXa7* allele by comparing the 5′ and 3′ ends flanking the repeat region of each allele with those of wild type *avrXa7* as well as those of a homolog of similar size (*aB4.3* [1], Fig. 3A). The 5′ and 3′ ends of the seven alleles were more similar to wild type *avrXa7* compared to the same termini of the *aB*4.3 homolog. Immunoblot analysis confirmed expression of all *avrXa7* alleles sequenced (data not shown).

DNA sequence analysis of the seven alleles revealed four different types of *avrXa7* mutants (*1–4M*) with changes in the 5′ and 3′ ends as well as in some repeats (Fig. 3). The *2M* mutant alleles from strains PXO348, PXO441 and PXO448 were identical to each other, but differ from the wild type allele because they contain three fewer repeat units (repeats 13, 14 and 15 are missing) as well as changes in the N-terminus (Fig. 3). The finding that the alleles from these three isolates were identical was unexpected because the three strains vary significantly in their interactions with IRBB7 and IR24 rice in the greenhouse (Fig. 2). Strain PXO448 shows significantly less disease on IRBB7 than PXO348 and PXO441, suggesting PXO448 maintains more avirulence function than the other isolates (Fig. 2A). Additionally, PXO348 and PXO441 are more aggressive to rice with no *R* gene (IR24) than PXO448 (Fig. 2B).

The *3M* mutant alleles from strains PXO356 and PXO357 are identical to each other, but harbor several point mutations that alter the repeats, amino and carboxy termini, and also, contain an additional 120 bp repeat unit relative to the wild type allele (Fig. 3). Strains PXO356 and PXO357 both exhibit reduced avirulence function (more disease on IRBB7) and maintain aggressiveness to IR24 (Fig. 2).

Sequences flanking the central repeats of mutant allele *4M* from strain PXO557 contain the same mutations as PXO356 and PXO357 (Fig. 3A), however, the entire repeat region of *4M* is identical to wild type *avrXa7* (Fig. 3B). Analysis of virulence and avirulence in separate experiments (not shown) suggested that PXO557 maintains much of its avirulence and is slightly reduced in virulence function (average lesion lengths on IRBB7 are 6.1 cm and on IR24 are 24.0 cm).

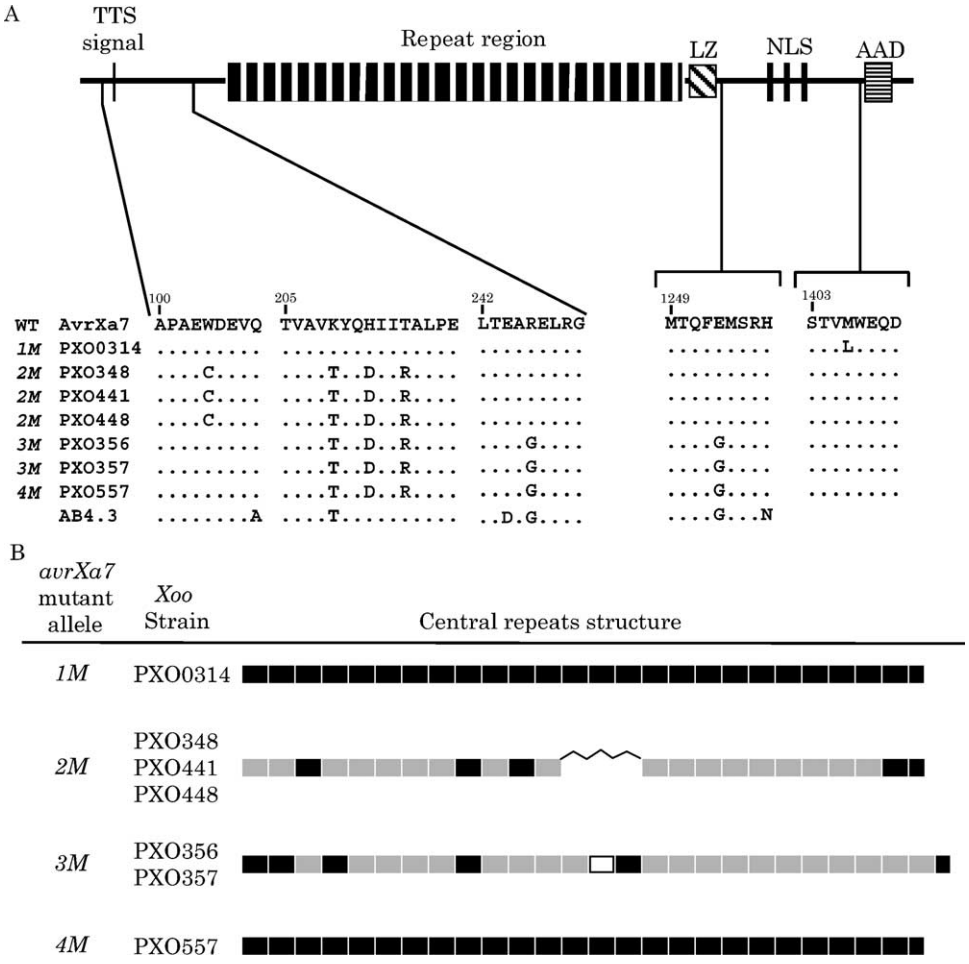

Fig. 3. Schematic of the *avrXa7* effector gene from *X. oryzae* pv. *oryzae* summarizing location of mutations in the mutant alleles from field strains. A, structure of *avrXa7* gene and location of N- and C-termini mutations in alleles from seven *X. oryzae* pv. *oryzae* field strains. Deduced amino acid sequence from the *aB4.3* homolog, another gene family member slightly larger than *avrXa7*, is also shown. TTS, type three secretion; LZ, leucine zipper; NLS, nuclear localization signal; AAD, acidic activation domain; *1–4M*, mutant *avrXa7* alleles 1–4; WT, wild type; Position of the amino acid residues shown are indicated by the numbers above the wild type sequence. B, arrangement of the 102 bp repeats from wild type *avrXa7* (black blocks) and mutant (gray blocks) alleles from the seven *X. oryzae* pv. *oryzae* field strains. Empty block represents a novel 120 bp repeat unit.

3.3. A point mutation in the acidic activation domain of avrXa7 impairs X. oryzae pv. oryzae avirulence and virulence functions in rice

Sequence analysis of mutant allele *1M* from PXO0314 revealed a single change relative to the wild type *avrXa7* allele (Fig. 3A). The change was a point mutation that conferred a single amino acid change in the C-terminus (M1406L). This change is located at the beginning of the AAD, a protein domain that is essential for both avirulence and virulence functions of *avrXa7* [26,31]. Interestingly, in our greenhouse assays, PXO0314 exhibited a loss of *avrXa7* avirulence function (Fig. 2A), but the reduction in aggressiveness function was not significant (Fig. 2B).

The AAD domain of *avrXa7* was demonstrated to be important to both avirulence and aggressiveness functions [26], yet, in our greenhouse assays the strain PXO0314 harboring the mutation at the beginning of the AAD domain was significantly reduced only in avirulence function and not aggressiveness functions. This raised the possibilities that (1) the point mutation indeed only impacted the avirulence function or (2) that the point mutation in *avrXa7* could reduce both functions, but that the strain might harbor a second site mutation that allows it to maintain aggressiveness. To further characterize the effects of the point mutation in the PXO0314 *avrXa7-1M* allele on gene functions, the nucleotide change was reconstructed in the wild type *avrXa7* gene to create the plasmid pH 0314. The modified *avrXa7* gene was introduced into two different strains of *X. oryzae* pv. *oryzae*, strain PXO99A, which has no *avrXa7* avirulence gene [11], and strain KL7M, which contains a Tn*5* insertion that inactivates *avrXa7* avirulence and aggressiveness functions [1]. Transformant PXO99A(pH 0314) conferred an intermediate HR and intermediate multiplication in IRBB7 rice relative to the transformants harboring the empty vector, PXO99A

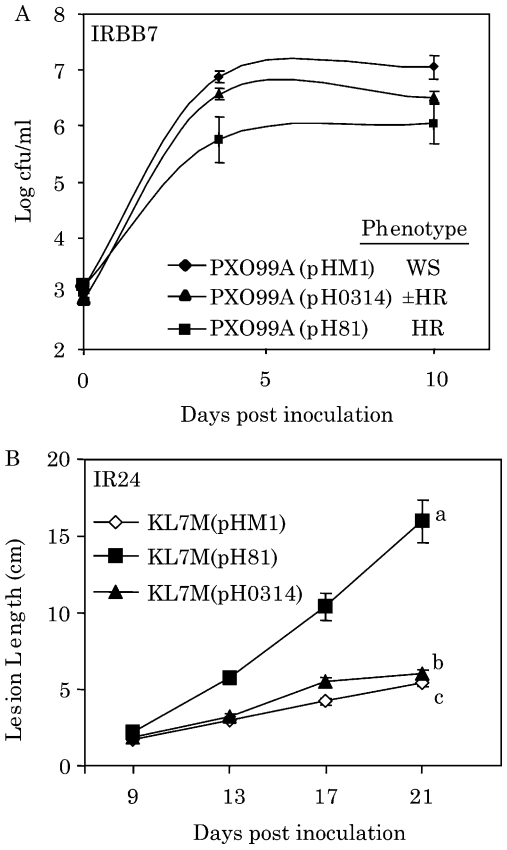

Fig. 4. Expression of *avrXa7* allele harboring a point mutation at the beginning of the AAD domain reduces bacterial numbers and confers an intermediate hypersensitive response to *X. oryzae* pv. *oryzae* strains PXO99A (A) and does not restore aggressiveness function to KL7M (B). A, multiplication of PXO99A(pH 81), which harbors plasmid-borne wild type *avrXa7* allele, PXO99A(pH 0314), which harbors the point mutation in *avrXa7* identical to the field strain PXO0314 allele, and PXO99A(pHM1), which contains the empty vector. B, lesion length induction of a *X. oryzae* pv. *oryzae* strain KL7M harboring wild type *avrXa7* (pH 81), the point mutation from field strain PXO0314 in *avrXa7* (pH 0314), and the empty vector (pHM1). Different letters indicate significant differences at $P=0.005$.

(pHM1), or the wild type *avrXa7* allele PXO99A(pH 81) (Fig. 4A). Bai et al. [1] demonstrated that complementation to aggressiveness for *avrXa7* is gene specific, that is, that mutation in the *avrXa7* gene cannot be complemented by other homologs and that *avrXa7* could not complement for mutations causing reduction of aggressiveness in other homologs. Thus, to measure the effects of the mutation on aggressiveness function, we used transformants in the strain KL7M that is mutated in *avrXa7*. Complementation with the mutant *avrXa7* allele from PXO0314 did not restore aggressiveness to KL7M on susceptible IR24 rice (Fig. 4B). Lesions caused by KL7M(pH 0314) were significantly shorter than those caused by KL7M(pH 81), which is complemented with wild type *avrXa7*. These observations demonstrate that the point mutation in *avrXa7* compromised the aggressiveness function conferred by the *avrXa7* allele.

4. Discussion

In this study, we demonstrate that (1) field strains of *X. oryzae* pv. *oryzae* exhibit a range of virulence on rice with *Xa7* and (2) the *avrXa7* effector gene alleles from these variants contain a variety of mutations. To understand the evolution of the *avrXa7* alleles in the field population, we attempted to correlate the interaction phenotypes of field strains with the sequence information from their *avrXa7* alleles. Although a correspondence between the interaction phenotypes of some strains and the sequences of their *avrXa7* alleles was observed (i.e. PXO356 and PXO357 completely lost avirulence function), other strains with identical *avrXa7* mutant alleles did not exhibit the same interaction phenotypes (i.e. PXO448 and PXO441 show moderate avirulence and complete loss of avirulence functions, respectively). Although not specifically tested, this suggests that some field strains may contain second site mutations or recombination events that complement the effects of the *avrXa7* mutations. For example, field strain PXO0314 exhibits partial loss of *avrXa7* avirulence function, but insignificant reductions in aggressiveness functions (Fig. 2A and B). However, when the *avrXa7* mutant allele, which contains a point mutation at the beginning of the AAD domain, was used for complementation tests, it partially restored avirulence function, but did not enhance aggressiveness (Fig. 4B). This suggests that strain PXO0314 may harbor an additional mutation that complements the loss of aggressiveness function of *avrXa7*, or that aggressiveness of PXO0314 is maintained by recombination with a second homolog. The reduction in aggressiveness function caused by the mutation of the *avrXa7* allele was only measurable by isolation and expression of the allele in an *avrXa7* deficient strain (Fig. 4B).

The *X. oryzae* pv. *oryzae* field strains we analyzed exhibited a wide range of virulence to *Xa7* containing rice. However, in comparing the same strains between the two different greenhouse experiments (the first set of experiments with the 52 isolates and the second set with the 20 isolates) the relative ranking of some isolates changed. For example, in the first experiments, PXO448 and PXO348 induced similar lesion lengths (16.9 and 17.2 cm on IRBB7, and 27.4 and 28.8 cm on IR24, respectively), whereas in the second set of experiments, these isolates produced significantly different lengths of lesions on both hosts (10.9 cm (AUDPC value of 81) and 19.3 cm (AUDPC value of 157) on IRBB7, and 25.6 cm (AUDPC value of 228) and 31.4 cm (AUDPC value of 266) on IR24, respectively. At this time, it is not clear if these differences in relative ranking are due to experimental variation, or due to the differences in experimental conditions (temperature and light). We have observed that in the cool, wet season in the Philippines, lesions caused by some race 9b isolates are significantly longer on IRBB7 than in hot, dry seasons (Vera Cruz et al., unpublished work).

Yang and Gabriel [28] proposed that the *avrBs3/pthA* family members are hot spots for recombination in the genome of *Xanthomonas* species. Indeed, most of the mutant *avrXa7* alleles characterized in this study likely resulted from recombination events with other *avrBs3/pthA* family members in the *X. oryzae* pv. *oryzae* genome. For example, the mutant allele *avrXa7-2M*, which was found in three distinct strains, contains a few changes in the 5′ region, and has a very different repeat region (three fewer repeats, and amino acid changes in all but five repeats relative to wild type *avrXa7*). The last repeats and the 3′ terminus are identical to the *avrXa7* wild type allele, suggesting recombination occurred upstream of those regions. Depending on where recombination occurs, the intergenic recombination events may not only result in the alteration of the repeat region leading to loss of *avrXa7* avirulence function, but could also generate new host specificities by creating novel repeat regions in the recombinant gene. We did not evaluate the possible creation of new host specificities; this would require inoculation to a wide variety of rice lines with different resistance genes. It is also possible that the homolog recombinant gene could inherit partial or complete fitness functions from *avrXa7*. Such events could explain the wide range in aggressiveness observed in interactions on IR24 and strains PXO348, PXO441 and PXO448, all of which contain the *avrXa7-2M* allele. This genetic plasticity could be the key for successful adaptation of *X. oryzae* pv. *oryzae* in the field population.

Apart from the essential role of the repeat region in interactions with the host, other regions of the *avrBs3 /pthA* genes are also important for gene function (i.e. the NLS and AAD; for review see Refs. [15,25]). Our sequence analysis detected mutations within the amino and carboxy termini that have not previously been linked to avirulence or virulence functions. Because, based on sequence homology, these regions do not appear to encode for protein structure(s) with known function, their potential roles can only be speculated. For example, these regions may be important for maintenance of the overall protein conformation. This could also be the effect of the point mutation at the beginning of the AAD domain of *avrXa7* that we identified in the allele of strain PXO0314. The function of AADs, in general, is to allow interaction with the transcriptional machinery and activate transcription. While the ability of AvrXa7 to promote transcription has not been proven, the protein has been shown to bind to double stranded DNA [26]. If AvrXa7 indeed binds DNA and activates transcription, the point mutation in the vicinity of the AAD may result in disruption of the domain conformation resulting in a weaker interaction with other proteins from the transcription machinery. A reduction in transcription capacity could explain the partial avirulence phenotype of PXO0314 observed on IRBB7.

Previous cloning and characterization of an allele from a field isolate of *X. oryzae* pv. *oryzae* that had adapted to rice with *Xa7* had shown an association between the mutations in the *avrXa7* gene and the host interaction phenotypes [24]. Furthermore, separation of avirulence and virulence functions had been previously described in another *avrBs3/pthA* family member, *pthA* from *X. axanopodis* pv. *citri* [29]. These findings and the wide range in avirulence and aggressiveness phenotypes exhibited by the field strains lead us to initially hypothesize that the molecular basis for these phenotypes was based on mutations within the *avrXa7* alleles. However, because of the likelihood that recombination events generated second site mutations, we cannot conclude from our experiments that mutations within each *avrXa7* allele accounted for the observed change in host interaction phenotypes. This would require that each allele be cloned and used in complementation studies as was done for *avrXa7-1M* in this study and for the mutant allele from PXO2684 in another study [24].

We have proposed that loss of *avrXa7* function at the bacterial population level is detrimental because *avrXa7* is a major contributor of pathogenic fitness [24]. However, it is now clear that two types of mutations may occur that negate this effect. First, if mutations at the *avrXa7* allele can occur that cause the loss of avirulence function but do not affect fitness functions, then the importance of *avrXa7* to the pathogen is reduced. Second, if recombination events occur that result in loss of avirulence function but transfer *avrXa7* fitness function to other homologs in the genome, then the importance of *avrXa7* will be masked. Although both of these possibilities are feasible, the fact remains that rice with the *Xa7* resistance gene still does not show bacterial blight epidemic in our field sites after 10 years of planting, even though cultivars surrounding the *Xa7* rice show epidemic (K. Webb and C. Vera Cruz, unpublished work). Experiments are now underway to determine the genetic structure of the current population to find out if a mutant type is building up that is fit, aggressive, and virulent to IRBB7.

We have demonstrated here and elsewhere [24] that changes in virulence to *Xa7* can be attributed to specific changes in the *avrXa7* mutant allele, and that these mutations also affect pathogenic fitness. However, mutations at some *avrXa7* alleles may not account for the phenotypes exhibited. Although the identity of some of the characterized alleles suggests that they arose from the same recombination event, it is possible that subsequent mutations or recombination events in the recipient allele restored or destroyed *avrXa7* functions. Future efforts to determine the molecular structure of recombinant homologs will be important to understand the evolution of these genes in the field and to understand why *Xa7* remains effective.

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