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Generation and characterisation of Tn5-tagged *Xanthomonas oryzae* pv. *oryzae* mutants that overcome *Xa23*-mediated resistance to bacterial blight of rice

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Abstract *Xanthomonas oryzae* pv. *oryzae* causes bacterial blight of rice. *Xa23*, a bacterial blight resistance gene identified originally in wild rice, *Oryza rufipogon*, is dominant and resistant to all *X. oryzae* pv. *oryzae* field isolates tested. The corresponding avirulence gene *avrXa23* is unknown. Here we report the generation of a random insertion mutant library of *X. oryzae* pv. *oryzae* strain PXO99 using a Tn5-derived transposon tagging system, and identification of mutant strains that are virulent on CBB23, a near-isogenic rice line containing *Xa23*. A total of 24,192 Tn5 inserted clones was screened on CBB23 by leaf-cutting inoculation and at least eight of them caused lesions on CBB23 comparable to those on JG30, the susceptible recurrent parent of CBB23. Polymerase chain reaction and Southern blot analysis showed that all the eight mutants, designated as P99M1, P99M2, P99M3, P99M4, P99M5, P99M6, P99M7 and P99M8, have a single Tn5-insertion in their genomes. The flanking DNA sequences of the Tn5-insertion sites were isolated by PCR-walking and sequenced. Bioinformatic analysis of the flanking sequences, by aligning them with the whole genome sequences of *X. oryzae* pv. *oryzae* strains PXO99, KACC10331 and MAFF311018 through NCBI, revealed that the Tn5-insertions disrupted genes that encode TAL effector AvrBs3/PthA, ISXo1 transposase, Type II secretion system protein-like protein or outer membrane protein, glycogen synthase, cytochrome C5 and conserved hypothetical protein. Further identification of

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these mutants will facilitate the molecular cloning of avirulence gene *avrXa23*.

Keywords *avrXa23* · Flanking sequence · PXO99 · Transposon · Virulent mutant · *Xoo*

Abbreviations

BB bacterial blight
NIL near-isogenic line
Xoo *Xanthomonas oryzae* pv. *oryzae*

Introduction

The rice bacterial blight (BB), caused by *Xanthmonas oryzae* pv. *oryzae* (*Xoo*), was first reported in Japan in 1884. It has been the most devastating bacterial disease of rice since the 1960s worldwide (Mew 1987). In severely infested fields, the disease can cause yield losses as high as 50% (Ezuka and Kaku 2000).

Xoo is a rod-shaped, obligately aerobic and Gram-negative bacterium. *Xoo* infects rice by entering plant tissues through wounds, hydathodes, stomata or contaminated seeds, causing disease symptoms of water soaking to yellowish stripes on leaf blades, and eventually resulting in death of the infected leaves (Niño-Liu et al. 2006). *Xoo* has been classified into different races based on their host range to rice cultivars with various differential resistance genes (Swings et al. 1990). Rice bacterial blight is the classic gene-for-gene interaction between rice and *Xoo* (Flor 1971), and *Xoo* has been used as an important model to study pathogenic mechanism of host–pathogen interactions.

In our previous studies, a novel bacterial blight resistance gene *Xa23* has been identified from *Oryza rufipogon* (Zhang et al. 1998) and it has been transferred into an indica rice variety JG30 by cross and back crosses, resulting in the development of the near-isogenic line (NIL) CBB23. A series of experiments on CBB23 have shown that *Xa23* is a complete dominant, host all-growth-stage effective BB-resistance gene. CBB23 was resistant to all known races of *Xoo* tested, including the PXO99 (Philippine race 6) that has the broadest host range (Zhang et al. 2001). In order to investigate the interaction between CBB23 and *Xoo*, we attempted to find a *Xoo* strain virulent to CBB23. However, no natural or laboratory *Xoo* stain that can infect CBB23 has been identified.

Transposons are valuable mutagenic tools for genetic and molecular analysis in bacteria (Voelker and Dybvig 1998). Recently, a Tn5-based transposome system has been successfully applied for mutagenesis of various bacteria including *Xoo* (Davies et al. 2000; Guilhabert et al. 2001; Hoffman et al. 2000; Sun et al. 2003). Here, we report the construction and screening of a Tn5-tagged mutant library of *Xoo* strain PXO99 and the identification of eight mutants virulent to CBB23.

Materials and methods

Plant and bacterial strain

Rice near-isogenic line CBB23 harbouring BB-resistance gene *Xa23* and its susceptible recurrent parent JG30 were planted in the field or greenhouse at 28–32°C in daylight. *Xoo* strain PXO99 was originally provided by Dr. Vera Cruz, International Rice Research Institute (IRRI).

Preparation of *Xoo* competent cells

PXO99 competent cells were prepared basically following the method described by Sun et al. (2003), except the PPS medium (ferv-filtering juice of 300 g potato, 5 g peptone, 15 g sucrose, 2 g $Na_2HPO_4{\cdot}12H_2O$ and 0.5 g $Ca(NO_3)_2{\cdot}4H_2O$ was used to culture the *Xoo* cells in this study.

Electroporation of *Xoo* with transposon DNA

Insertional mutagenesis of PXO99 was performed by adapting a previously described method (Lee et al. 2004; Sun et al. 2003; Park et al. 2007) using the EZ-Tn5™ <KAN-2>Tnp Transposome™ Kit (Epicentre Technologies, Madison, WI). Aliquots of 50 μl PXO99 competent cells were mixed with 10 ng (0.5 μl) transposome of the EZ-Tn5 Kit and electroporated using a Bio-Rad electroporation instrument. An electric field of 15 kV cm^{-1} (with a resistance of 200 Ω and a capacitance of 25 μF) was applied. After pulse delivery, cells were immediately transferred into 1 ml of SOC medium (Sambrook et al. 1989) in a 2 ml round-bottomed polypropylene tube. After incubation at 28°C with constant shaking for 1.5 h, the electroporated cells were plated on PPS medium containing

kanamycin (25 μg ml^{-1}), and then incubated at 28°C for 2 days. Non-electroporated PXO99 competent cells were used as a control. The numbers of PXO99 mutants were counted for each electroporation. For the mutant library, 24,192 clones were picked at random and stored in sixty-three 384-well plates in 15% glycerol at −86°C.

Pathogenicity assessment of *Xoo* mutants

The pathogenicity of the PXO99 and its mutants was evaluated using the leaf-cutting method described by Kauffman et al. (1973). In the first round screening of the library, each of the *Xoo* mutant clones was cultured in the liquid PPS medium containing 30 μg ml^{-1} kanamycin, shaking at 250 rpm for 42 h at 28°C. Bacterial suspension was adjusted to a concentration about 1×10^9 cfu ml^{-1} with sterile distilled water (SDW) and inoculated on fully expanded leaves of CBB23 at booting or flowering stage. Each mutant clone was inoculated on three to five fully expanded leaves of a single CBB23 plant. Two weeks after inoculation, disease symptoms were surveyed and the leaves with lesion percentage (of the whole leaf) equal to or >15% were collected for isolation of virulent mutant candidates. To isolate a virulent mutant candidate, the diseased leaf was clipped into several pieces of 2×1 cm^2, soaked in 70% ethanol for 30 s and washed with SDW for 10 s, and soaked in 1 ml SDW for about 20 min. The solution was spread on solid PPS medium containing 30 μg ml^{-1} kanamycin for a single colony.

In the second round screening, each of the virulent mutant candidates was inoculated on 10 CBB23 plants. The inoculation method was the same as that described above. A third round assessment of pathogenicity was performed for the eight virulent mutants confirmed in the second round screening. In this experiment, each virulent mutant was inoculated on 10 CBB23 plants and 10 JG30 plants at the tillering stage. For each plant, three fully expanded leaves were inoculated. Two weeks after inoculation, the length (cm) of both the lesion and the inoculated leaf were measured by a ruler. Disease symptoms were scored as both the lesion length and the ratio against the whole leaf.

Extraction of *Xoo* genomic DNA and PCR analysis

Total genomic DNA of PXO99 and its mutants was isolated as described by Leach et al. (1990). PCR was performed to check the inserted Tn5-DNA fragment using the forward primer Tn5F (5′-ATTCAACGG GAAACGTCTTG-3′) and the reverse primer Tn5R (5′-ACTGAATCCGGTGAGAATGG-3′). The expected PCR product is 569 bp in length (Fig. 3a). The PCR reaction (25 μl) contained 100 ng of template DNA, 1x PCR buffer, 0.5 mM dNTPs, 0.2 μM each primer, and 1.0 U Taq polymerase (TaKaRa, Japan). The reactions were heated to 95°C for 5 min followed by 35 cycles of amplification at 94°C for 30 s, 59°C for 30 s, 72°C for 1 min and a final extension at 72°C for 7 min.

Southern blot analysis

One microgram of genomic DNA of PXO99 or its virulent mutant was digested with *Sph*I, separated on 0.8% (*w*/*v*) agarose gel and then transferred to a nylon membrane (Hybond-N+, Amersham Pharmacia Biotech). A 569 bp Tn5-DNA fragment amplified from the EZ-Tn5™ <KAN-2>Tnp Transposome™ DNA template by PCR using primers Tn5F and Tn5R was used as the DNA probe (Fig. 3a), labelled with ^{32}P-dCTP by the random priming method (Sambrook et al. 1989). Hybridisation was performed in a solution containing 5x SSC, 5x Denhardt's solution, 0.6% (*w*/*v*) SDS, 10% (*w*/*v*) dextran sulphate, and 20 mg l^{-1} denatured salmon sperm DNA at 65°C for 14 h. Filters were washed twice in 2x SSC, 0.1% SDS at 65°C for 10 min, once in 1x SSC, 0.1% SDS at 65°C for 10 min, and once in 0.5x SSC, 0.1% SDS at 65°C for 10 min. Blots were exposed on a PhosphorImager plate and signals were detected by the Molecular imager® FX (Bio-Rad).

Isolation and analysis of the flanking sequences at Tn5-insertion sites

The flanking sequences of the Tn5-insertion sites were isolated by PCR-walking (Cottage et al. 2001). The adaptor (AP) consists of two oligonucleotides LAP (5′-CTAATACGACTCACTATAGGGCTC GAGCGGCCGCCCGGGGAGGT-3′) and NAP (5′-ACCTCCCC-H_2N-3′). The 3′ end of NAP was labelled with -NH_2 to guarante the blunt-end ligation between the adaptor and *Xoo* DNA fragments. The adaptor specific primers AP1 (5′-GGATCCTAATAC GACTCACTATAGGGC-3′) and AP2 (5′-CTA TAGGGCTCGAGCGGC-3′) were designed based

on the sequence of LAP. The Tn5 specific primers or walking primers TnFP1 (5′-GGCAGAGCAT TACGCTGACT-3′) and TnFP2 (5′-ACCTACAA CAAAGCTCTCATCAACC-3′) were designed for isolation of the forward flanking sequences. Similarly, the Tn5 specific primers TnRP1 (5′-CTGATTGCCC GACATTATCG-3′) and TnRP2 (5′-GCAATGTAA CATCAGAGATTTTGAG-3′) were designed for isolation of the reverse flanking sequences (Fig. 3a).

DNA digestion and ligation were performed simultaneously. The reaction mixture (10 μl) contained 0.2 μg genomic DNA of *Xoo*, 1 μl annealed adaptor (25 μM), 0.4 μl blunt-cutting enzyme *Dra*I (15 U μl^{-1}, TaKaRa, Japan), 1 μl T4 DNA ligase buffer (10x) and 1 μl T4 DNA ligase (3 U μl^{-1}, TaKaRa, Japan). The restriction–ligation mixture was incubated at 25°C for 16 h and then heat inactivated at 65°C for 10 min. Forty microliters SDW was added to the restriction–ligation mixture.

For the primary amplification in the PCR-walking, the PCR reaction mixture (20 μl) consisted of 5 μl of the restriction–ligation mixture, 2 μl 10x polymerase buffer, 0.6 μl Taq DNA polymerase (2.5 U μl^{-1}, Ausable Biotechnology Co., Ltd), 1.6 μl dNTPs (2.5 mM each), 2 μl AP1 primer (2 μM), and 2 μl walking primer TnRP1 (2 μM) or TnFP1 (2 μM) and 6.8 μl H_2O. The reactions were heated to 94°C for 5 min followed by 35 cycles of amplification at 94°C for 50 s, 58°C for 50 s, 72°C for 2 min 30 s and a final extension at 72°C for 10 min. The second round of amplification was performed using AP2 and TnRP2 or TnFP2 as nested primers, and the 50x or 100x diluted solution of the primary amplification as the template. The PCR programme was the same as the primary amplification except the annealing temperature was 60°C or 62°C. The PCR products were retrieved and purified for sequencing. The Tn5-insertion site was determined by aligning both the forward and reverse flanking sequences with the whole genome sequences of *Xoo* strains PXO99, KACC10331 and MAFF311018 through NCBI.

Results

Construction of the PXO99 mutant library

To achieve a high efficiency of transformation, we firstly tested different media for PXO99 cell culture, various competent cell concentrations and electric fields for electroporation. Using the optimised competent cells and electroporation conditions described in the Materials and methods, about 2.6×10^4 kanamycin-resistant clones were obtained by transforming 20 ng (1 μl) of transposome mixture into PXO99 competent cells. Controls without adding the transposon complex failed to yield any colony on the selective PPS plates supplemented with kanamycin, indicating that formation of kanamycin-resistant colonies did not occur spontaneously.

In total, 24,192 *Xoo* transformants were collected and stored at −86°C in sixty-three 384-well plates with PPS liquid medium containing 15% sterile glycerol. To test the stability of the Tn5-transposon DNA in the mutants, 14 mutants picked randomly were subjected to 15 successive subcultures, and the presence of the Tn5-transposon in the transformants was confirmed by PCR with Tn5-specific primers Tn5F and Tn5R (data not shown).

Screening of the library for virulent PXO99 mutants

The mutants virulent on *Xa23*-containing rice were identified for their ability to cause disease symptoms on the otherwise resistant rice plants. The first round of screening revealed 121 putative virulent strains out of 24,192 kanamycin-resistant clones. The virulence assay on the 121 candidate mutants in the second round of screening confirmed that at least eight mutants, designated as P99M1, P99M2, P99M3, P99M4, P99M5, P99M6, P99M7 and P99M8, caused longer lesions on CBB23 plants than the wild-type PXO99. To further test the virulence of the eight selected mutants, a third round assessment of pathogenicity was performed on both CBB23 plants and JG30 plants. As shown in Fig. 1, the wild-type PXO99 was avirulent on CBB23 plants (Fig. 1a) but strongly pathogenic on JG30 plants (Fig. 1b). However, the eight PXO99 mutants were strongly pathogenic on CBB23 plants even if the virulence of P99M6 was weaker compared with the other seven mutants (Fig. 1a). The mean lesion length caused by P99M6 on CBB23 leaves was about 30% of the whole leaf length, and that caused by other mutants reached 70–90% (Fig. 1a,c), indicating that the eight mutants lost their elicitor activities of avirulence on *Xa23*-containing rice plants. Notably, the virulence of all the eight mutants on JG30 plants was not changed compared with that of the wild-type PXO99 (Fig. 1b,c).

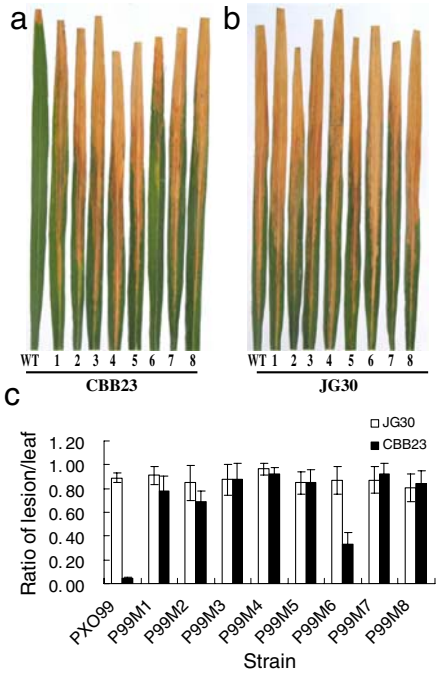

Fig. 1 Virulence assays of the eight mutational PXO99 strains performed on rice NILs CBB23 (with *Xa23*) and JG30 (without *Xa23*) at the tillering stage by artificial leaf-cutting inoculation. Each strain was inoculated on 10 CBB23 plants and 10 JG30 plants. Three fully expanded leaves were inoculated for each rice plant. *WT* wild-type of PXO99. *Numbers 1–8* the eight mutants P99M1, P99M2, P99M3, P99M4, P99M5, P99M6, P99M7 and P99M8. Representative leaves are shown with lesions caused by the strains on CBB23 (**a**) and JG30 (**b**). The virulence of the mutants is also shown by the statistical ratio of lesion length against the whole leaf (**c**)

Molecular characterisation of the PXO99 mutants

The eight pathogenic mutants that were clearly virulent on CBB23 plants, were initially analysed by PCR using Tn5F and Tn5R primers. A 569 bp expected DNA fragment was amplified from the genomic DNA of the eight mutants (Fig. 2a), indicating the presence of the Tn5-insertion in the genomes of the eight pathogenic *Xoo* mutants. The presence of the transposon insertions in the eight mutants was further confirmed by Southern hybridisation analysis. A single hybridisation band was detected by a Tn5-derived probe (Fig. 3a) for each of the eight mutants, indicating that there was a single Tn5-insertion in the genomes of the mutants (Fig. 2b). The differences in size of the hybridisation bands among the eight mutants indicated that the transposon inserted at different sites in the genome of PXO99 (Fig. 2b).

Analysis on the Tn5-insertion sites in the mutational PXO99 strains

The flanking sequences of the Tn5-insertion sites were isolated by PCR-walking (Cottage et al. 2001). The relationships among the Tn5-DNA, the flanking DNA of *Xoo*, the adaptor and all the primers used in PCR-walking are shown in Fig. 3a. Both the forward and the reverse flanking sequences were isolated and analysed to confirm the Tn5-insertion sites from two orientations (Table 1). The results showed that the Tn5-transposon inserted at different sites in the genomes of P99M2, P99M4 and P99M5, but the disrupted genes encode the same class proteins belonging to TAL effector AvrBs3/PthA (Table 1). Since the TAL effector AvrBs3/PthA proteins were encoded by a big gene family and the genes with various copies of repeat sequences, the flanking sequences of the Tn5-

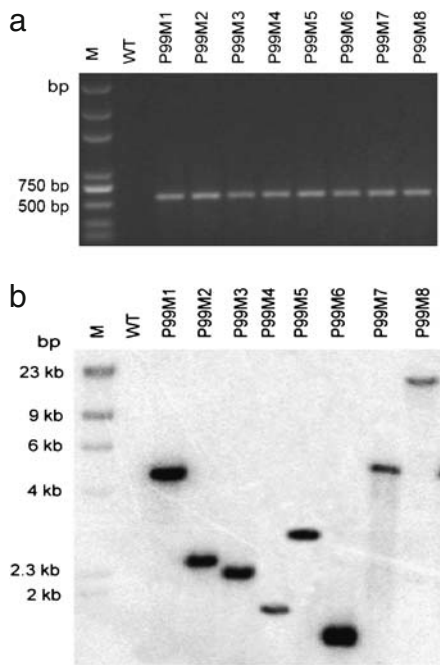

Fig. 2 Molecular characterisation of the eight mutational PXO99 strains. **a** Identification of the Tn5-DNA inserted in the genome of the mutants by PCR using primers Tn5F and Tn5R; the expected PCR product is 569 bp in size. **b** Southern blot analysis for the copy number of Tn5-insertion in the mutants. Single-copy of Tn5-insertion was detected in all the eight pathogenic mutants

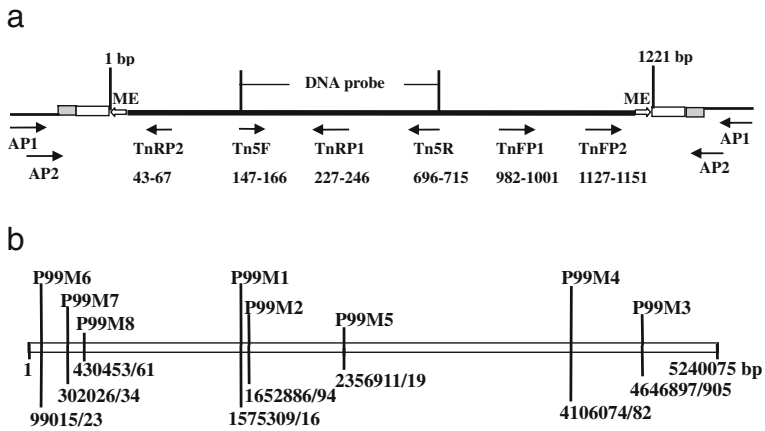

Fig. 3 Analysis of the Tn5-DNA insertion sites in the mutational PXO99 strains. **a** Schematic representation of a Tn5-DNA insertion site in the genome of *Xoo*. The 1,221 bp Tn5-DNA is shown by the *black bar* with *white-box arrows* at the ends; the *white-box arrows* indicate the mosaic ends (*ME*). *White bars* represent the flanking sequences of *Xoo*. The adaptor used to isolate the flanking sequences is marked by "▭". *Black arrows under the bars* represent the primers used in this study. *Numbers under primer names* correspond to the nucleotide positions of the primers. The position of the probe used for Southern blotting is indicated. **b** Schematic representation of the distribution of the eight Tn5-DNA insertion sites in the genome of PXO99. *White bar* represents the 5,240,075 bp whole genome sequence of PXO99. The Tn5-DNA insertion sites of the eight mutants are shown by both the mutant names (*above the bar*) and the nucleotide positions (*under the bar*)

insertion sites of P99M2, P99M4 and P99M5 aligned to multiple sites in a single genome of *Xoo* strains. A similar situation occurred to P99M1, in which the Tn5-dirupted gene encodes ISXo1 transposase, a protein encoded by a gene cluster (Rajeshwari and Sonti 2000). The exact Tn5-insertion site was determined in the other mutants P99M3, P99M6, P99M7 and P99M8, since the flanking sequences of each mutant aligned to only one site in the genome of the *Xoo* strains. The Tn5-dirupted genes in P99M3, P99M6, P99M7 and P99M8 encode a Type II secretion system protein-like protein or an outer membrane protein, a glycogen synthase, a conserved hypothetical protein and a cytochrome C5 protein, respectively (Table 1). The Tn5-DNA insertion sites of the eight mutants in the genome of PXO99 are shown in Fig. 3b.

Discussion

Bacterial blight is a classic gene-for-gene interaction between rice and *Xoo*. The cloning of avirulence (*avr*) genes in the pathogen and their cognate host resistance (R) genes is necessary to fully understand the interaction between the pathogen and the host at the molecular level. To date, several *avr* genes from *Xoo* have been cloned, such as *avrXa3* (JXO III) (Li et al. 2004), *avrxa5* (PXO86) (Hopkins et al. 1992; Bai et al. 2000), *avrXa7* (PXO86) (Hopkins et al. 1992; Vera Cruz et al. 2000), *avrXa10* (PXO86) (Hopkins et al. 1992; Zhu et al. 1998) and *avrXa27* (PXO99) (Gu et al. 2005). It seems more difficult to clone a pair of R gene and the corresponding *avr* gene, and this may be the reason that only the pair of *avrXa27*/*Xa27* genes has been cloned so far. In our study, the molecular cloning of *Xa23* gene will be accomplished (Wang et al. 2006). To investigate the interaction between *Xoo* and CBB23 at the molecular level, we are attempting to clone the *avrXa23*.

In the present study, we constructed a large Tn5-tagged PXO99 mutant library to guarantee the tagging on the *avrXa23* gene. According to the formula $P=1-(1-X/G)n$ (Sun et al. 2003), the probability of finding one transposon insert for a certain gene in our library is 99.7%. The mutant library would have six times the coverage of the PXO99 genome.

In the first round of library screening, our major objective was to obtain the virulent mutants that could overcome *Xa23*-mediated resistance of CBB23. Therefore, all the possible diseased leaves, with a lesion percentage about 15%, were collected to isolate the candidates of virulent mutants. This should be the major reason that the pathogenicity of most of the virulent mutant candidates was not repeatable in

the second round screening. In addition, since the second round of screening was carried out during middle-August to middle-September in the field in Beijing, the lesions might not be fully expanded because of the lower temperature. Furthermore, the eight virulent mutants were chosen for further identification because of their apparent stronger pathogenicity shown in the second round of screening. In other words, more virulent mutants, with various levels of virulences, could to be identified from the 121 virulent mutant candidates in the future.

On the other hand, we assume that Tn5-insertion mutations from virulent to avirulent (loss-of-function) could be obtained more easily compared with those from avirulent to virulent (gain-of-function), because the Tn5-insertion could not only disrupt the genes encoding virulence factors but also those genes that are important to the normal physiological functions of *Xoo*, since interruptions to such genes will reduce their ability of setting and amplifying *Xoo* on the host plants. On the contrary, a gain-of-function mutant that has an enhanced ability to grow on the host could be generated only when the avirulence gene and its regulatory genes were interrupted. Since the avirulence genes of *Xoo* are actually a disadvantage to its infection potential on rice and may be infrequent, the mutation rate of such genes is low. This might, to some extend, explain the relative lower frequency of identification of strong virulent mutants from the large PXO99 mutant library.

We determined the Tn5-insertion sites by isolating both the forward and the reverse flanking sequences and aligning the flanking sequences to whole genome sequences of three *Xoo* strains. This strategy should be good enough when the Tn5-insertion site locates in a single-copy sequence (genes) such as those in P99M3, P99M6, P99M7 and P99M8. However, with mutants P99M1, P99M2, P99M4 and P99M5, since

Table 1 Location of Tn5-insertions of the eight mutational PXO99 strains

Mutant strain	Flanking sequence	Aligned Tn5-insertion sites in genomes of *Xoo* strains[a]			Proteins encoded by the disrupted gene (genome of *Xoo* strain)
		PXO99A (A)	KACC10331 (B)	MAFF311018 (C)	
P99M1	Forward	1575316/7th, —	2853628/7th, —	31836/5th, —	ISXo1 transposase (A, C); Putative (ISXo8) transposase (B)
	Reverse	1575309/8th, —	2853635/6th, —	31843/4th, —	
P99M2	Forward	1652886/5th, —	3225347/8th, —	1240958/7th, —	TAL effector AvrBs3/PthA (A); Avirulence protein (B); Avirulence protein AvrBs3/pth family (C)
	Reverse	1652894/5th, —	3225339/8th, —	1240966/7th, —	
P99M3	Forward	4646905/6th	788949/8th	757309/8th	Type II secretion system protein-like protein or outer membrane protein (A, B, C)
	Reverse	4646897/6th	788957/8th	757317/8th	
P99M4	Forward	4106074/3rd, —	3231109/8th, —	1235910/1st, —	TAL effector AvrBs3/PthA (A); Avirulence protein (B); Avirulence protein AvrBs3/pth family (C)
	Reverse	4106082/3rd, —	3231117/8th, —	1235902/1st, —	
P99M5	Forward	2356919/0th, —	2409648/7th, —	1239533/4th, —	TAL effector AvrBs3/PthA (A); Avirulence protein (B); Avirulence protein AvrBs3/pth family (C)
	Reverse	2356911/0th, —	2409656/7th, —	1239525/4th, —	
P99M6	Forward	99015/4th	105202/1st	71758/9th	Glycogen synthase (A, B, C) or Putative signal protein with GGDEF domain (A)
	Reverse	99023/4th	105210/1st	71750/9th	
P99M7	Forward	302034/5th	4789816/5th	4784905/4th	Conserved hypothetical protein (A, B, C)
	Reverse	302026/5th	4789824/5th	4784913/4th	
P99M8	Forward	430453/2nd	4660056/7th	4656090/1st	Cytochrome C5 (A, B, C)
	Reverse	430461/2nd	4660048/7th	4656082/1st	

[a] Multiple aligned sites in a single genome are indicated with "—"

the flanking sequences aligned to multiple sites of repeated sequences in the *Xoo* genomes, the exact Tn5-insertion sites are still to be determined in association with other approaches such as nested PCRs.

Both the Southern blotting and the flanking sequence analysis demonstrated that the Tn5-transposon inserted at different sites in genomes of the eight virulent mutants. Since the disrupted genes in three out of eight mutants encode the same protein, TAL effector AvrBs3/PthA, we speculate that the avrXa23 is most likely a member of TAL effectors. However, based on the Tn5-insertion site of P99M3, the avrXa23 could also be an outer membrane protein involved in the secretion system of *Xoo*. Tn5-insertion site information from P99M6 and P99M8 suggests that glycogen synthase and cytochrome C5 protein might also involve the pathway of *Xa23*-mediated resistance in rice. Up to now, we have no idea about the gain of virulence in P99M1 and P99M7 through disruptions of the genes encoding ISXo1 transposase and a hypothetical protein. Since the disrupted genes in P99M6, P99M7 and P99M8 have no homology with all the *avr* genes cloned so far, the pathogenic mechanism of *Xoo* seems so complicated that much of it needs to be completely characterised. To obtain more information, we will look into the neighbouring genes around the Tn5-insertion sites, as well as the disrupted genes themselves. But most importantly, only the isolation of the disrupted genes or their neighbouring genes, and functional complementary experiments will reveal the *avrXa23* gene.

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