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The broad bacterial blight resistance of rice line CBB23 is triggered by a novel transcription activator-like (TAL) effector of *Xanthomonas oryzae* pv. *oryzae*

CHUN-LIAN WANG[1,2,†], TENG-FEI QIN[1,†], HONG-MAN YU[2], XIAO-PING ZHANG[1], JIN-YING CHE[1], YING GAO[1], CHONG-KE ZHENG[1], BING YANG[2,*] AND KAI-JUN ZHAO[1,*]

[1]*National Key Facility for Crop Gene Resources and Genetic Improvement (NFCRI), Institute of Crop Science, Chinese Academy of Agricultural Sciences (CAAS), Beijing 100081, China*

[2]*Department of Genetics, Development and Cell Biology, Iowa State University, Ames, IA 50011, USA*

SUMMARY

Bacterial blight (BB), caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), is not only a disease devastating rice production worldwide, but also an ideal model system for the study of the interaction between plants and their bacterial pathogens. The rice near-isogenic line (NIL) CBB23, derived from a cross between a wild rice *Oryza rufipogon* accession (RBB16) and a susceptible *indica* rice variety (Jingang 30), is highly resistant to all field *Xoo* strains tested so far. Although the BB resistance of CBB23 has been widely used in rice breeding programmes, the mechanism of its extremely broad-spectrum resistance remains unknown. Here, we report the molecular cloning of an avirulence gene, designated as *avrXa23*, from *Xoo* strain PXO99[A]. We validate that AvrXa23, a novel transcription activator-like effector, specifically triggers the broad-spectrum BB resistance in CBB23. The prevalence of *avrXa23* in all 38 *Xoo* strains surveyed may explain the broad-spectrum feature of BB resistance in CBB23. The results will significantly facilitate the molecular cloning of the corresponding resistance (*R*) gene in the host, and provide new insights into our understanding of the molecular mechanism for broad-spectrum disease resistance in plants.

Keywords: *avrXa23*, broad-spectrum resistance, rice, TAL effector, *Xa23*, *Xoo*.

INTRODUCTION

Rice bacterial blight (BB), caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), is one of the most devastating rice diseases worldwide, and host genetic resistance is regarded as the most effective approach to the management of this disease (Mew, 1987). Over the last two decades, scientists have exerted tremendous efforts to identify and isolate BB-resistance genes. Apart from cultivated rice, wild rice has proven to be another important and rich genetic resource for BB resistance (Khush *et al*., 1989) and, indeed, some elite BB-resistance genes, such as *Xa21* (Ikeda *et al*., 1990), *Xa23* (Zhang *et al*., 2001) and *Xa27* (Amante-Bordeos *et al*., 1992; Gu *et al*., 2004), have been identified from wild rice *Oryza longistaminata*, *O. rufipogon* and *O. minuta*, respectively. *Xa21* and *Xa27* have been molecularly cloned and studied extensively (Chen *et al*., 2010; Gu *et al*., 2005, 2009; Song *et al*., 1995).

The rice near-isogenic line (NIL) CBB23 was derived from a cross between an *O. rufipogon* accession (RBB16) and a susceptible *indica* rice variety, Jingang 30 (JG30; Zhang *et al*., 2001). Our previous studies have revealed that a single resistance (*R*) gene, designated as *Xa23*, in CBB23 confers completely dominant and high resistance to all the naturally occurring *Xoo* races tested (Wang *et al*., 2009; Zhang *et al*., 2001), and have delimited the *Xa23* locus on the long arm of chromosome 11 between the molecular markers 69B and CP02662, a region with numerous repetitive sequences (Fan *et al*., 2006; Wang *et al*., 2006). Although the BB resistance of CBB23 has been widely used in rice breeding programmes in China, the mechanism for its extremely broad-spectrum resistance has yet to be elucidated.

Transcription activator-like (TAL) effectors are a class of proteins identified in many plant-pathogenic *Xanthomonas* spp., including *Xoo* (Boch and Bonas, 2010; Bonas *et al*., 1989; Hopkins *et al*., 1992). The pathogens inject their TAL effectors through a type III secretion system (TTSS) into plant cells, primarily to activate the expression of host genes that contribute to disease (Yang *et al*., 2006). However, plants have evolved promoter elements that trap TAL effectors to trigger plant defence (Gu *et al*., 2005; Romer *et al*., 2007; Strauß *et al*., 2012). All TAL effectors have a conserved architecture: an N-terminus required for type III secretion, a central region consisting of a varying number of near-perfect 34-amino-acid repeats, and a C-terminus containing nuclear localization signals (NLSs) and an acidic transcription activation domain (AD) (Boch and Bonas, 2010). TAL effectors activate host gene expression by binding to promoter sequences of target genes. The number and order of the 34-amino-acid repeats determine the specificity between a TAL effector and its target DNA sequence (Boch and Bonas, 2010). Amino acid polymorphism

*Correspondence: Email: zhaokaijun@caas.cn; byang@iastate.edu

†These authors contributed equally to this work.

among the central repeats exists and is concentrated at residues 12 and 13 of each repeat, referred to as the repeat-variable di-residue (RVD) (Moscou and Bogdanove, 2009), which mediates specific DNA recognition. The code of DNA recognition between RVDs of the TAL effector and the nucleotides of its target site has been deciphered (Boch *et al*., 2009; Moscou and Bogdanove, 2009).

To investigate the molecular interaction between CBB23 and *Xoo*, we generated and identified some Tn5-tagged PXO99^{A} mutants that were virulent on CBB23 (Wang *et al*., 2009). Here, we report an extensive survey of the resistance spectrum of CBB23, the molecular cloning of the avirulence (*avr*) gene *avrXa23* in *Xoo* and the molecular mechanism of the broad-spectrum BB resistance of CBB23.

RESULTS

CBB23 displays a broad spectrum of BB resistance

Plants of CBB23, JG30, IRBB21 and ZW1, a *japonica* rice line with the *Xa23* locus transferred from CBB23, were inoculated by the leaf-cutting method with 39 naturally occurring *Xoo* strains and three Tn5-insertion mutants of PXO99^{A} at the booting stage. CBB23 showed high resistance to all 39 *Xoo* strains, including PXO99^{A} and nine other *Xoo* strains from the Philippines for which the reaction patterns have been documented previously (Zhang *et al*., 1998, 2001), but was susceptible to the three PXO99^{A} mutants, P99M2, P99M4 and P99M5 (Table 1, Fig. 1). By contrast, IRBB21, a rice variety containing the broad-spectrum resistance gene *Xa21* (Ikeda *et al*., 1990; Khush *et al*., 1991), was susceptible to nine of the 39 *Xoo* strains (Table 1). As a control, JG30, the recurrent parent of CBB23, was highly susceptible to all the naturally occurring *Xoo* strains and the PXO99^{A} Tn5-insertion mutants (Table 1, Fig. 1). Notably, ZW1 showed the same spectrum of *Xoo* resistance as that of CBB23, indicating that *Xa23* functions well in both *indica* and *japonica* genetic backgrounds. According to Gu *et al*. (2004), IRBB27 (*Xa27*) is susceptible to *Xoo* strains ZHE173, PXO71 and AXO1947 (Table 1). As *Xa21* and *Xa27* are widely recognized as broad-spectrum BB-resistance genes, it appears that *Xa23* confers a broader spectrum of BB resistance than these two important genes.

Screening of cosmid clones containing TAL effector gene(s) in PXO99^{A}

As no naturally occurring *Xoo* strain virulent to CBB23 has been identified, we generated and identified several Tn5-tagged PXO99^{A} mutants that were virulent in CBB23, and found that the Tn5 insertions were located in gene(s) related to TAL effector(s) in some of the virulent mutants, including P99M5 (Wang *et al*., 2009). Based on these findings and the fact that there is a large repertoire of TAL effector genes in PXO99^{A} (Salzberg *et al*., 2008), we speculated that the *avr* gene corresponding to *Xa23* could be a member of TAL effector-coding genes. Thus, we performed the selection of cosmid clones containing TAL effector gene(s) from the genomic library of PXO99^{A} by Southern blotting with the central repeat fragment of *avrXa7* as a probe. A total of 34 TAL effector-containing cosmids was selected and individually transformed into P99M5 through electroporation (Table 2).

The cosmid clone C15 harbours the *avr* gene corresponding to *Xa23*

To determine whether the screened PXO99^{A} genomic cosmids harbour the *avr* gene corresponding to *Xa23*, we inoculated CBB23 leaves with the 34 individual cosmid-derived transformants and measured the disease responses accordingly (Table 2). Inoculation assay showed that, except for P99M5-C15, all the transformants were virulent on both CBB23 and JG30 plants; the lesion length was in the range 9–13 cm, comparable with that of the control strain P99M5 (Table 2). However, the lesion length caused by transformant P99M5-C15 was still as long as 12 cm on JG30, but only 1.5 cm on CBB23 (Table 2). These results clearly indicated that the cosmid pHM1/99lib-15 (C15) completely complemented the avirulence defect of P99M5 (Table 2, Fig. 2a). In other words, the cosmid C15 harbours the cognate *avr* gene of *Xa23*.

avrXa23 encodes a novel TAL effector

Subcloning and transposon-tagging sequencing of the cosmid C15 were performed to reveal the identities of the TAL effectors. Sequencing analysis revealed that C15 had an insertion of a DNA fragment containing full-length talC9a and talC9b, and truncated talC9c, of the TAL effector cluster 9 in the genome of PXO99^{A} (Salzberg *et al*., 2008). As talC9c is *avrXa27* (Gu *et al*., 2005), we reasoned that the *avrXa23* gene could be either talC9a or talC9b. Thus, we digested plasmid DNA of C15 with *Sph*I, and isolated the *Sph*I fragments L and Mi (Fig. 2b), containing the central repeats of talC9b and talC9a, respectively. The *Sph*I fragments L and Mi were then individually cloned into plasmid pSK/avrXa7ΔSphF (*avrXa7* with its repeat region deleted), resulting in plasmids pSK-L and pSK-Mi, respectively. Next, pSK-L and pSK-Mi were separately linearized by *Hind*III digestion and ligated with *Hind*III-digested pHM1 vector, resulting in plasmids pHM-L and pHM-Mi, respectively. Finally, pHM-L and pHM-Mi were separately introduced into competent cells of the Tn5-insertion mutant P99M5, resulting in strains P99M5-L and P99M5-Mi, respectively. pHM-L was also separately introduced into competent cells of the Tn5-insertion mutants P99M2 and P99M4, resulting in strains P99M2-L and P99M4-L, respectively. Inoculation assay showed that CBB23 is still susceptible to P99M5-Mi, but highly resistant to P99M5-L,

Table 1 CBB23 is highly resistant to all 39 naturally occurring *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) strains tested.

			Reaction pattern of rice*			
Xoo strain	Origin of *Xoo*	CBB23†	JG30†	ZW1†	IRBB21†	IRBB27‡
1 HLJ-72	China	R	S	R	R	R
2 HB17	China	R	S	R	R	R
3 NX42	China	R	S	R	R	R
4 ZHE173	China	R	S	R	R	**S**
5 GD1358	China	R	S	R	R	R
6 LN57	China	R	S	R	R	R
7 JS49-6	China	R	S	R	R	R
8 HuB05-6	China	R	S	R	S	
9 HAN06-1	China	R	S	R	S	
10 HAN08-2	China	R	S	R	S	
11 Yun96-11	China	R	S	R	R	
12 Yun98-1	China	R	S	R	R	
13 Yun98-5	China	R	S	R	R	
14 LN44	China	R	S	R	R	
15 JL691	China	R	S	R	R	
16 PXO61	Philippines	R§	S	R	R	
17 PXO86	Philippines	R§	S	R	R	R
18 PXO79	Philippines	R§	S	R	R	R
19 PXO71	Philippines	R§	S	R	R	MS
20 PXO112	Philippines	R§	S	R	R	R
21 PXO99	Philippines	R§	S	R	R	R
22 PXO145	Philippines	R§	S	R	R	
23 PXO280	Philippines	R§	S	R	R	
24 PXO339	Philippines	R§	S	R	R	
25 PXO341	Philippines	R§	S	R	S	
26 T7174	Japan	R	S	R	R	R
27 T7147	Japan	R	S	R	R	
28 T7133	Japan	R	S	R	R	
29 KXO19	Korea	R	S	R	R	
30 KXO85	Korea	R	S	R	S	
31 KXO112	Korea	R	S	R	R	
32 KXO559	Korea	R	S	R	MS	
33 KXO576	Korea	R	S	R	S	
34 KXO657	Korea	R	S	R	MS	
35 KXO660	Korea	R	S	R	R	
36 PP8511	Bangladesh	R	S	R	R	
37 CN9417	Bangladesh	R	S	R	R	
38 TL9924	Bangladesh	R	S	R	R	
39 AXO1947	Africa	R	S	R	S	S
40 P99M2	Mutant of PXO99	S	S	S	R	
41 P99M4	Mutant of PXO99	S	S	S	R	
42 P99M5	Mutant of PXO99	S	S	S	R	

*MS, moderate susceptible; R, resistant; S, susceptible.

†The inoculation assays were mainly performed during 2008–2010 in Beijing. For *Xoo* strain AXO1947, the assays were carried out in 2009 at Iowa State University.

‡Data of reaction patterns of IRBB27 are from Gu *et al*. (2004).

§Consistent reaction patterns have been reported previously (Zhang *et al*., 1998, 2001).

P99M2-L and P99M4-L (Fig. 2c), indicating that the avirulence activity of P99M5 was specifically restored by plasmid pHM-L. As the L fragment was released from talC9b, we thought that talC9b might be the *avrXa23* gene.

To further confirm the function of talC9b, we transferred pHM-L and pHM-Mi plasmids separately into JZ-8, a strain of *Xanthomonas oryzae* pv. *oryzicola* (*Xoc*) which is highly virulent in causing bacterial leaf streak disease in CBB23 and JG30. Virulence assay clearly showed that JZ-8-L (JZ-8 harbouring the pHM-L plasmid) became avirulent on CBB23 plants, but was still virulent on JG30, whereas JZ-8-Mi (JZ-8 harbouring pHM-Mi) remained as virulent as wild-type JZ-8 on CBB23 plants (Fig. 2d). On the basis of these observations, we concluded that talC9b is the *avrXa23* gene. Bioinformatic analysis revealed that *avrXa23* (the nucleotide sequence of *avrXa23* has been registered in GenBank with the accession number GU732172.1) encodes a TAL effector of 1238 amino acids, containing three C-terminal NLSs, a transcription AD and a novel central region of 26.5 direct repeats of mostly 34 amino acids (Fig. 2e). The most likely *avrXa23* in the Japanese *Xoo* strain MAFF 311018 is XOO1136, which also encodes 26.5 repeats

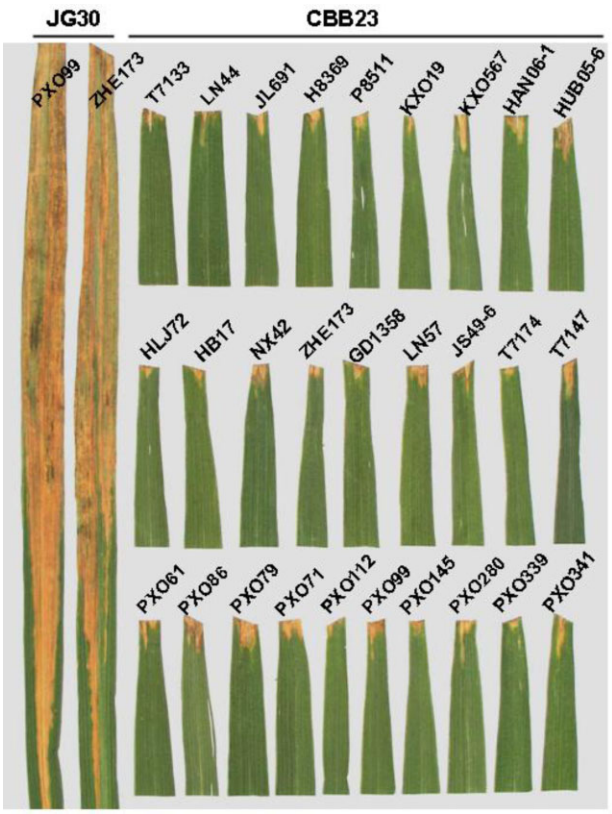

Fig. 1 Disease responses of CBB23 and JG30 to representative *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) strains. Bacterial suspension was inoculated into rice leaves using the leaf clipping method. Photographs were taken at 14 days post-inoculation.

with 99% identity at both the nucleotide and predicted amino acid levels to the repeat region of *avrXa23* (Fig. 2e; Ochiai *et al*., 2005).

Mutation analysis of talC9b in *Xoo* mutants further confirms its *avrXa23* activity

In our previous study, we could not determine, based on the flanking sequences, which member of the TAL effector gene family was disrupted by Tn5 insertion in mutants P99M2, P99M4 and P99M5, all derived from PXO99^{A} (Wang *et al*., 2009). In light of the aforementioned evidence for talC9b being *avrXa23*, we performed a more detailed molecular analysis on the mutants. We designed polymerase chain reaction (PCR) primer P1 within the promoter region of talC9b, and performed PCRs using different primer combinations between P1 and each of the four primers locating in the Tn5-DNA (Fig. 3a). PCR analysis and sequencing of the PCR products revealed that Tn5-DNA indeed presented in talC9b in mutants P99M2, P99M4 and P99M5. The Tn5-DNA inserted at nucleotide positions 282 and 2018 in P99M4 and P99M5, respectively, in sense orientation, and at 671 in P99M2, in antisense orientation (Fig. 3a). Therefore, the nested PCRs with primer combinations P1/P2 and P1/P3 amplified 1143- and 1979-bp fragments, respectively, from mutant P99M2 (Fig. 3b). Similarly, nested PCRs with primer combinations P1/P4 and P1/P5 amplified 760- and 1229-bp fragments from P99M4, and 2496- and 2965-bp fragments from P99M5, respectively (Fig. 3b). Southern blotting of the genomic DNA digested with *Sph*I and probed with Tn5-DNA as probe also showed the expected 1346-, 1883- and 2377-bp hybridization bands in P99M2, P99M4 and P99M5, respectively (Fig. 3c).

In addition, we created a PXO99^{A} mutant with cluster 9 of the TAL effector genes deleted (referred to as P99ΔC9). Like the Tn5-insertion mutants, P99ΔC9 became compatible with CBB23, albeit with a shorter lesion length (*c*. 8 cm) than in JG30 (*c*. 14 cm), and reintroduction of pHM-L completely restored the avirulence to P99ΔC9 in CBB23 (Fig. 3d). All of these observations further confirmed that talC9b is *avrXa23*.

Table 2 Virulence assays of P99M5 transformants harbouring cosmids containing transcription activator-like (TAL) effector gene(s) from PXO99^{A}.

Cosmid	Transformant*	Caused BB lesion length (cm)† on CBB23	Caused BB lesion length (cm)† on JG30
C1: pHM1/99lib-1	P99M5-C1	10.0 ± 0.98 Aa	11.0 ± 0.53 Aa
C2: pHM1/99lib-2	P99M5-C2	11.5 ± 1.01 Aa	11.0 ± 1.01 Aa
C4: pHM1/99lib-4	P99M5-C4	10.0 ± 1.45 Aa	11.0 ± 1.95 Aa
C5: pHM1/99lib-5	P99M5-C5	13.0 ± 0.52 Aa	13.0 ± 1.03 Aa
C6: pHM1/99lib-6	P99M5-C6	13.0 ± 1.03 Aa	14.0 ± 0.50 Aa
C7: pHM1/99lib-7	P99M5-C7	9.0 ± 2.04 Aa	9.0 ± 1.54 Ab
C8: pHM1/99lib-8	P99M5-C8	10.0 ± 0.46 Aa	9.0 ± 1.03 Ab
C9: pHM1/99lib-9	P99M5-C9	11.0 ± 1.50 Aa	10.0 ± 1.50 Aa
C11: pHM1/99lib-11	P99M5-C11	12.0 ± 1.51 Aa	10.0 ± 1.95 Aa
C12: pHM1/99lib-12	P99M5-C12	11.0 ± 1.47 Aa	13.0 ± 0.51 Aa
C13: pHM1/99lib-13	P99M5-C13	10.0 ± 0.99 Aa	12.0 ± 1.04 Aa
C14: pHM1/99lib-14	P99M5-C14	12.0 ± 0.48 Aa	11.0 ± 1.53 Aa
C15: pHM1/99lib-15	P99M5-C15	1.50 ± 0.51 Cc	12.0 ± 1.02 Aa
C16: pHM1/99lib-16	P99M5-C16	14.0 ± 0.51 Aa	14.0 ± 0.47 Aa
C19: pHM1/99lib-19	P99M5-C19	11.0 ± 0.53 Aa	12.0 ± 0.96 Aa
C20: pHM1/99lib-20	P99M5-C20	10.0 ± 0.97 Aa	10.0 ± 1.00 Aa
C21: pHM1/99lib-21	P99M5-C21	13.0 ± 0.46 Aa	12.5 ± 1.00 Aa
C22: pHM1/99lib-22	P99M5-C22	12.0 ± 1.00 Aa	10.0 ± 0.54 Aa
C23: pHM1/99lib-23	P99M5-C23	13.0 ± 0.53 Aa	12.0 ± 0.56 Aa
C25: pHM1/99lib-25	P99M5-C25	9.0 ± 1.04 Ab	11.0 ± 0.49 Aa
C26: pHM1/99lib-26	P99M5-C26	12.0 ± 0.52 Aa	13.0 ± 0.51 Aa
C27: pHM1/99lib-27	P99M5-C27	11.0 ± 0.53 Aa	12.0 ± 0.49 Aa
C28: pHM1/99lib-28	P99M5-C28	10.5 ± 0.50 Aa	12.0 ± 0.96 Aa
C29: pHM1/99lib-29	P99M5-C29	11.0 ± 1.04 Aa	12.0 ± 0.50 Aa
C31: pHM1/99lib-31	P99M5-C31	9.0 ± 0.96 Ab	10.0 ± 0.50 Aa
C32: pHM1/99lib-32	P99M5-C32	13.0 ± 0.97 Aa	12.0 ± 0.50 Aa
C33: pHM1/99lib-33	P99M5-C33	11.0 ± 0.98 Aa	12.0 ± 0.95 Aa
C34: pHM1/99lib-34	P99M5-C34	10.0 ± 0.99 Aa	10.0 ± 0.48 Aa
CK1	PXO99^{A}	1.20 ± 0.41 Cc	12.0 ± 1.04 Aa
CK2	P99M5	13.0 ± 1.51 Aa	13.5 ± 0.98 Aa

BB, bacterial blight.

*Each of the cosmids (C1–C34) was introduced into virulent mutant P99M5 and two transformants were used for leaf clipping inoculation in leaves of CBB23 and JG30. PXO99^{A} and P99M5 were used for inoculation as controls.

†Lesion length was the mean ± SD of 10 leaves, measured at 14 days post-inoculation. The values followed by a common letter are not significantly different as determined by least-significant difference (LSD) test at $P < 0.05$ (LSD0.05 = 4.49, small letter 'a') or $P < 0.01$ (LSD0.01 = 6.45, capital letter 'A'), respectively.

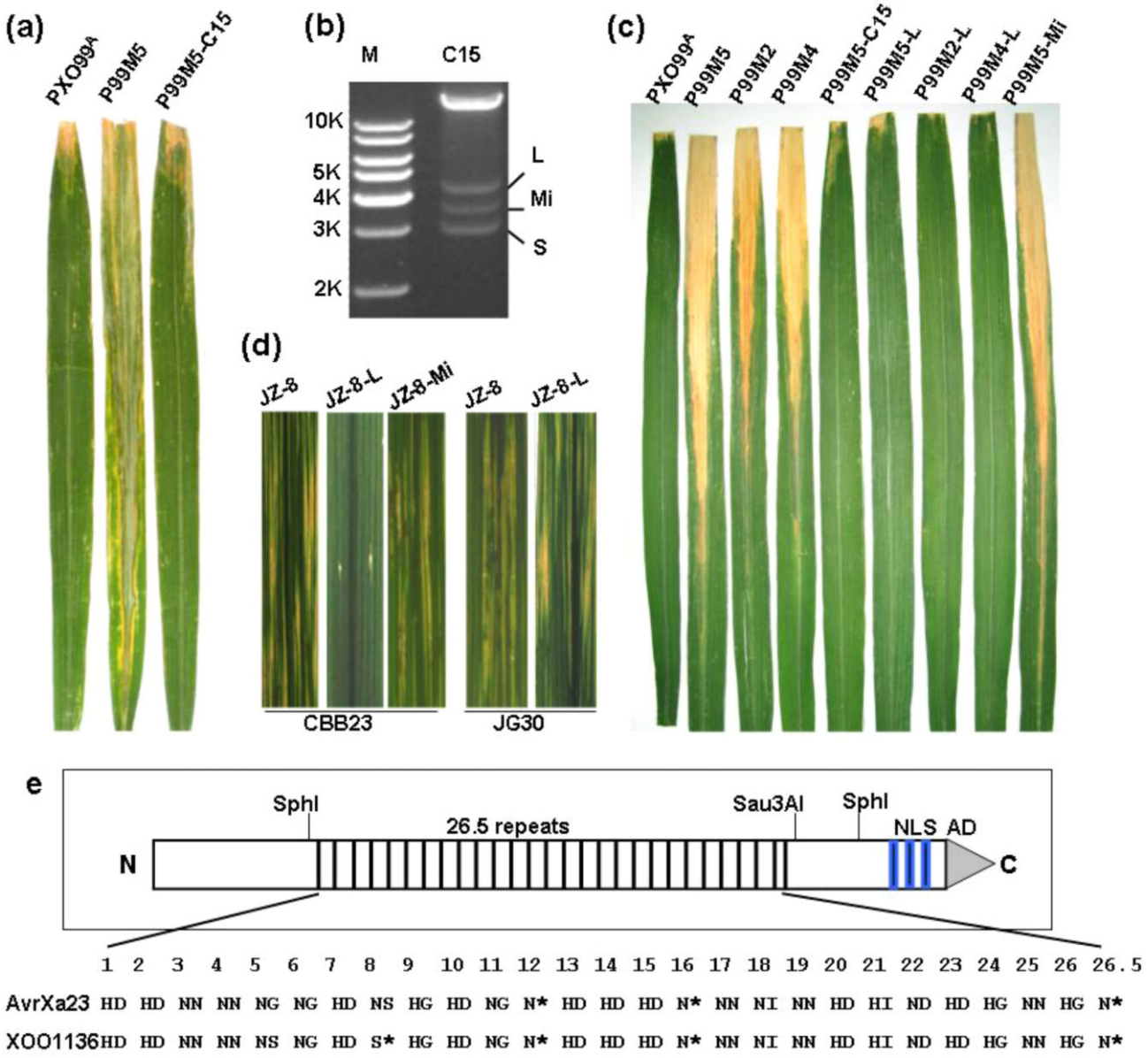

Fig. 2 Molecular cloning of *avrXa23*. (a) Virulence assays of the wild-type *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) strain PXO99^{A}, mutant P99M5 and the complementary strain P99M5-C15 (P99M5 harbouring the cosmid C15) in CBB23 by artificial leaf clipping inoculation. Representative leaves are presented to show the lesions caused by the strains. (b) Cosmid C15, digested by *Sph*I, released three *Sph*I fragments, L (large), Mi (middle) and S (small), containing the central repeats of talC9b, talC9a and talC9c, respectively. M, molecular ladder. (c) Virulence assays of virulent mutants P99M2, P99M4, P99M5 and their transformants in CBB23 by artificial leaf clipping inoculation. The transformants harbour the cosmids pHM1-L or pHM1-Mi, which contain the N- and C-terminal coding regions of *avrXa7*, but with the central repeat region being replaced with the *Sph*I fragments of L and Mi from cosmid C15, respectively. Representative leaves are presented to show the lesions caused by the different *Xoo* strains. PXO99^{A} was used as a control. (d) Virulence assays of virulent *Xanthomonas oryzae* pv. *oryzicola* (*Xoc*) strain JZ-8 and its transformants JZ-8-L (JZ-8 harbouring plasmid pHM1-L) and JZ-8-Mi (JZ-8 harbouring plasmid pHM1-Mi) in CBB23 and JG30 by artificial leaf piercing inoculation. Representative leaves are presented to show the lesions caused by the *Xoc* strains. Photographs were taken at 4 days post-inoculation. (e) Schematic representation of AvrXa23. Thin boxes represent the 26.5 repeats which are also represented by the di-amino acids at the positions 12 and 13 of each repeat of AvrXa23 from PXO99^{A} and its relative (XOO1136) from the Japanese *Xoo* MAFF 311018. Three blue bars indicate the nuclear localization signals (NLSs). The activation domain (AD) is represented by a grey triangle. The N- and C-termini are indicated by 'N' and 'C', respectively. The *Sph*I restriction sites and the *Sau3*AI site flanking the central repeat region of *avrXa23* are also indicated.

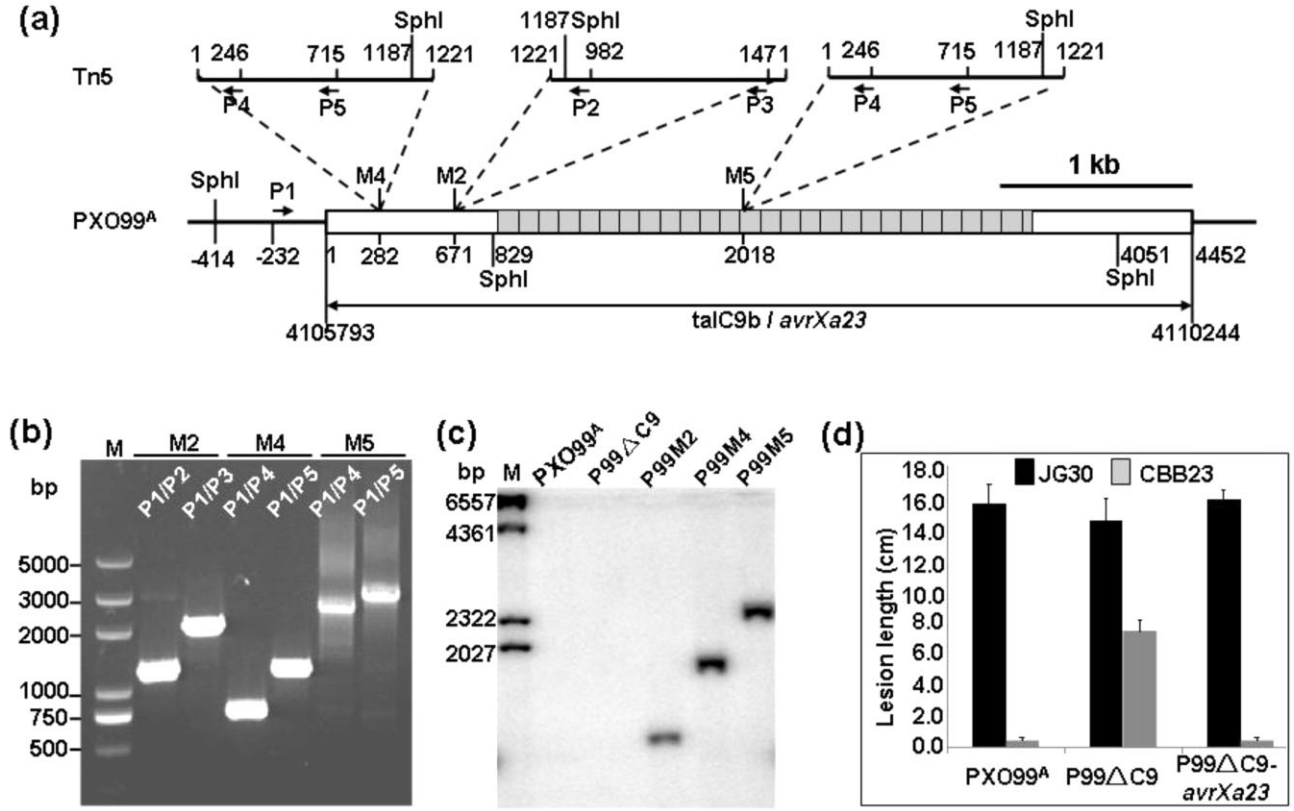

Fig. 3 Characterization of *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) mutants. (a) Schematic representation of insertion sites and orientations of Tn5-DNA in mutants P99M2, P99M4 and P99M5. Black bars represent the genome of PXO99^{A} or the Tn5-DNA. Boxes represent talC9b located in genome region 4105793–4110244 of PXO99^{A} (GenBank: NC_010717.1). Grey boxes represent the 26.5 repeats of talC9b. Numbers under the boxes and a nearby bar indicate the relative nucleotide positions from the first 'A' in the open reading frame (ORF) of talC9b. Numbers above the bars indicate the nucleotide positions of the 1221-bp Tn5-DNA. Broken lines indicate the Tn5 insertion sites in P99M2 (M2), P99M4 (M4) and P99M5 (M5). The nucleotide positions of the polymerase chain reaction (PCR) primers (P1–P5) and *Sph*I restriction sites are also indicated. (b) Nested PCR analysis of mutants P99M2 (M2), P99M4 (M4) and P99M5 (M5). Primer combinations and the size (bp) of the molecular ladder (M) bands are shown. (c) Southern blotting of *Xoo* strains. Genomic DNA of *Xoo* strains were digested with *Sph*I, separated in 1.2% (w/v) agarose gel and transferred to a nylon membrane. The 569-bp PCR fragment amplified from Tn5-DNA using primers P3 and P5 (a) was labelled with ^{32}P-dCTP and used as the DNA probe. PXO99^{A} and P99ΔC9 were used as negative controls. M, molecular ladder. (d) Virulence assays of the talC9b knockout mutant P99ΔC9 and its complementary strain P99ΔC9-*avrXa23* in CBB23 by artificial leaf clipping inoculation. Wild type *Xoo* PXO99^{A} and susceptible rice JG30 were used as controls. The lesion length was the mean ± SD of five leaves measured at 14 days post-inoculation.

DISCUSSION

Our studies in the past decade have demonstrated that CBB23 is highly resistant to all the representative *Xoo* strains collected from China, the Philippines, Japan, Korea and Bangladesh, including some strains that overcame the resistance mediated by *Xa21* and *Xa27*, the well-known *R* genes with broad-spectrum BB resistance (Table 1). Indeed, we have not identified any naturally occurring *Xoo* isolate that can overcome the *Xa23*-dependent resistance (Wang *et al*., 2009). Thus, the *Xa23* locus in CBB23, originally from wild rice *O. rufipogon* (Zhang *et al*., 2001), confers the broadest spectrum resistance to BB of rice.

Strong and dominant resistance to BB fits a classic gene-for-gene interaction between rice and *Xoo*. We reasoned that there would be an *avr* gene in *Xoo* corresponding to the *R* gene *Xa23* in rice (Wang *et al*., 2009). In the present study, we cloned the *avr* gene *avrXa23* from *Xoo*, which is talC9b that encodes a novel TAL effector with 26.5 central repeats. By introducing talC9b/*avrXa23* into both CBB23-compatible *Xoo* mutants and *Xoc* strain JZ-8, we demonstrated that the novel TAL effector AvrXa23 specifically triggers *Xa23*-dependent BB and bacterial leaf streak disease resistance (Figs 2 and 3c). The latter resistance, once *Xa23* is cloned, may be engineered against *Xoc* if the expression of *Xa23* can be activated appropriately, as demonstrated for *Xa27* (Hummel *et al*., 2012).

As CBB23 was highly resistant to all the naturally occurring *Xoo* strains tested so far, and the broad-spectrum resistance was specifically triggered by TAL effector AvrXa23, we speculated that

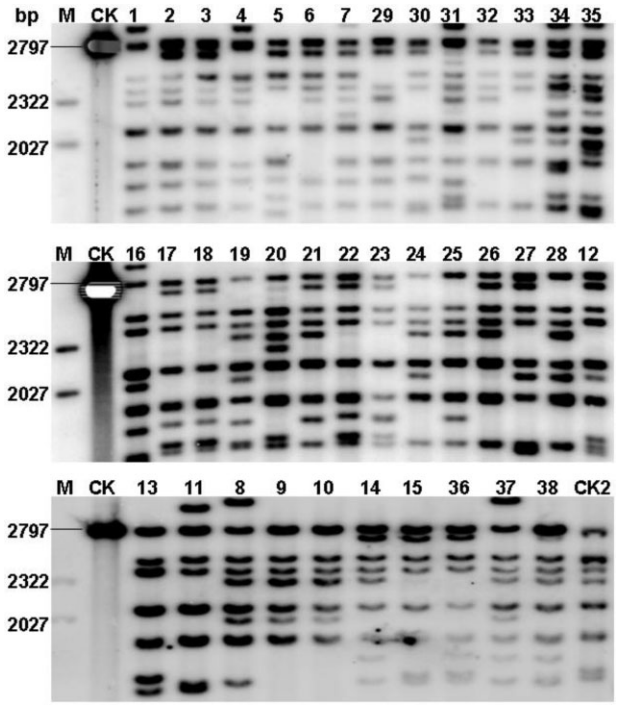

Fig. 4 Southern blotting of 38 *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) strains. Genomic DNA of *Xoo* strains was digested with *Sph*I and *Sau3*AI, separated in 1.2% (w/v) agarose gel and transferred to a nylon membrane. The *Sph*I-*Sau3*AI fragment containing the central repeat region of *avrXa23* was labelled with ^{32}P-dCTP and used as a DNA probe. M, molecular ladder; CK, the 2797-bp *Sph*I-*Sau3*AI fragment of *avrXa23*; 1–38, numbers of the 38 *Xoo* strains in Table 1; CK2, P99ΔC9.

avrXa23, or its functional equivalent, resides in all *Xoo* strains tested. For example, the sequenced Japanese *Xoo* strain (MAFF 311018) contains a TAL effector that is 99% identical to PXO99^{A} *avrXa23*. The two effectors have 26.5 repeats that differ only in two repeats (Fig. 2e). Southern blotting showed that the expected 2797-bp hybridization band (the *Sph*I-*Sau3*AI fragment containing the 26.5 central repeats of *avrXa23*) presented in all the 38 *Xoo* strains (Fig. 4), indicating that *avrXa23* may be ubiquitous among *Xoo* strains. This might explain the extremely broad spectrum of CBB23 for BB resistance, although experimental evidence for such prevalence is needed.

Molecular cloning of *avr* genes in pathogens and their cognate host *R* genes is necessary to fully understand the interaction between the pathogen and the host at the molecular level. So far, several *avr* genes from *Xoo* have been cloned, such as *avrxa5* (Bai *et al*., 2000; Hopkins *et al*., 1992), *avrXa7* (Hopkins *et al*., 1992; Vera Cruz *et al*., 2000), *avrXa10* (Hopkins *et al*., 1992; Zhu *et al*., 1998) and *avrXa27* (Gu *et al*., 2005). Primarily, *Xoo* strains inject their TAL effectors into rice cells to activate host susceptibility genes that contribute to disease susceptibility (Yang *et al*., 2006); however, rice has evolved promoter elements that trap the TAL effectors to trigger plant defence (Gu *et al*., 2005). In the case of *avrXa27*, the rice *R* gene *Xa27* contains a TAL effector binding site in its promoter that directs transcriptional activation by the corresponding TAL effector AvrXa27, resulting in the hypersensitive response (HR), a local programmed cell death that inhibits pathogen growth within the infected site (Gu *et al*., 2005). The same defence mechanism has also been identified in pepper, demonstrated by the interactions between the host *R* gene *Bs3* and the pathogen TAL effector AvrBs3 (Romer *et al*., 2007), and between *Bs4c* and AvrBs4 (Strauß *et al*., 2012). It seems that transcriptional activation of so-called executor-type *R* genes may be a common mechanism for plants encountering bacterial infection by exploiting the recognition of pathogen TAL effectors (Strauß *et al*., 2012). In this regard, it is conceivable to hypothesize that *Xa23* in CBB23 is also an executor-type *R* gene, as the *Xa23*-dependent BB resistance is specifically triggered by the TAL effector AvrXa23. Consequently, the isolation of *avrXa23* will certainly facilitate the molecular cloning of *Xa23*, which would further promote the investigation of the molecular interaction between *Xoo* and CBB23.

Because *avrXa23* seems to be conserved in all *Xoo* isolates, we thought that AvrXa23 might contribute to the virulence of *Xoo* for infection or growth in host plants. However, we did not observe a significant difference between the wild-type PXO99^{A} and its *avrXa23*-disrupted mutant (P99M5) in lesion length in the highly susceptible JG30 (Table 2). Whether AvrXa23 contributes virulence to *Xoo* needs to be clarified by studies using more rice varieties in strictly controlled conditions. Based on the virulence of the mutants P99M2, P99M4 and P99M5 in CBB23 (Table 2), it is possible that *Xa23*-containing rice varieties might lose BB resistance if *Xoo* could mutate *avrXa23* under the high selection pressure generated by widespread and long-term adoption of *Xa23*-containing rice. Considering the fact that the *avrXa23*-disrupted mutants were still avirulent on IRBB21 (Table 2), we speculate that diversified usage of *Xa23*, *Xa21* or other *Xa* genes could make *Xa23*-dependent BB resistance durable.

EXPERIMENTAL PROCEDURES

Plant varieties and bacterial strains

The susceptible variety JG30 and near-isogenic resistant lines CBB23 (harbouring *Xa23*) and IRBB21 (harbouring *Xa21*, originally from the International Rice Research Institute, Los Baños, Philippines) are *indica* varieties of rice. ZW1 is a BC_5F_4 line derived by transferring the *Xa23* locus from CBB23 into the *japonica* rice variety Wuyujing 3 through genetic crossing and backcrossings. The rice lines are stocks in Kai-Jun Zhao's laboratory at the Chinese Academy of Agricultural Sciences. Rice plants were planted in the field or glasshouse at 28–32 °C. *Xoo* strain AXO1947 is a stock in Bing Yang's laboratory at Iowa State University; other *Xoo* strains used in this study are stocks in Kai-Jun Zhao's laboratory (Table 1). *Xoc* strain JZ-8 was kindly provided by Xiaoxiang Li, Rice Research Institute, Hunan Academy of Agricultural Sciences, Hunan, China.

Screening of cosmid clones containing TAL effector gene(s) of PXO99^{A}

Genomic DNA of PXO99^{A} was partially digested with *Sau3A*I and separated through agarose gel electrophoresis for DNA fragments larger than 20 kb. The DNA was ligated into *BamHI*-digested cosmid vector pHM1 and packaged into lambda phages, and the phages were transduced into *Escherichia coli* XL1-Blue MRF' cells, resulting in a cosmid library. The library was screened for TAL effector-containing plasmids with a ^{32}P-labelled repetitive DNA of *avrXa7* as a probe. A collection of 34 representative cosmid clones carrying varying numbers of TAL effector genes was used in this study (data not shown).

Creation of talC9b knockout mutant of PXO99^{A} (P99ΔC9)

Unique fragments upstream and downstream of TAL effector cluster 9 were PCR amplified with primers Tal9-F1 (5'-ATCAAGGCAATGGCCTT CAACGCAC-3') and Tal9-R1 (5'-AATATCCGGGTAGGCGCAATCACTTCG GCTTGCCATCGAAGTGGCAG-3'), and Tal9-F2 (5'-AGCCTACACAATCGCT CAAGACGTCATCCCCGAAACAAAAACAGC-3') and Tal9-R2 (5'-AGTGGT ACGCCAAGGTCAACTAC-3'), respectively. The two fragments and a fragment of the kanamycin resistance gene cassette were overlap PCR amplified, and the replicon was cloned into the A/T cloning vector pGEM-T, as described by Song and Yang (2009). The resulting plasmid was electroporated into PXO99^{A} competent cells, and kanamycin-resistant and ampicillin-sensitive transformants were selected for further molecular and genetic confirmation, as described by Song and Yang (2009).

Transformation of *X. oryzae* cells with plasmid DNA

Competent cells of the *Xoo* mutants P99M2, P99M4, P99M5 and *Xoc* strain JZ-8 were prepared as described previously (Wang *et al*., 2009). PPS medium (cloth-filtered juice from cooked potato, 300 g; peptone, 5 g; sucrose, 15 g; $Na_2HSO_4{\cdot}12H_2O$, 2 g; $Ca(NO_3)_2{\cdot}4H_2O$, 0.5 g, per litre) was used to grow *X. oryzae* cells. An aliquot of 50 μL of bacterial competent cells was mixed with 10 ng (0.5 μL) of plasmid DNA for electroporation using a Bio-Rad electroporation instrument (Bio-Rad, Hercules, CA, USA). An electric field of 15 kV/cm, 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 (20 g tryptone, 5 g yeast extract, 4.8 g MgSO4, 3.6 g dextrose, 0.5 g NaCl and 0.2 g KCl per liter) 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 onto PPS medium containing appropriate antibiotics, and incubated at 28 °C for 3 days. Non-electroporated competent cells were used as a control. Positive clones were picked for further analysis.

Pathogenicity assessment of *Xanthomonas* strains or their transformants

The pathogenicity of *Xoo* strains or their transformants was evaluated using the leaf tip clipping method (Wang *et al*., 2009). *Xoo* strains were cultured in liquid PPS medium, with shaking at 250 rpm for 42 h at 28 °C. Bacterial inoculum was adjusted to ~10^5 colony-forming units (cfu)/mL in water and inoculated onto fully expanded rice leaves at the booting stage. Each strain was inoculated on 5–10 rice plants and three to five fully expanded leaves per plant. Disease symptoms were scored by lesion ratio against the whole leaf or lesion length (cm). Disease symptoms were scored 2 weeks after inoculation by visual assessment of the percentage of lesion to whole leaf. Leaves with a lesion area of less than 15%, 15–20% and greater than 20% were classified as resistant (R), moderate susceptible (MS) and susceptible (S), respectively, as described previously (Zhang *et al*., 1996).

For pathogenicity assay for *Xoc* strain JZ-8 or its transformants, bacterial inoculum was prepared similarly as for *Xoo*. Adult rice plants were inoculated by piercing the leaves with a needle when they were submerged in the bacterial suspension in a Petri dish, as described previously (Li *et al*., 2011). Disease symptoms were recorded and photographs were taken at 4 days post-inoculation.

All virulence assays were repeated at least three times.

PCR and Southern blot analysis of *Xoo* mutants

Genomic DNA of *Xoo* strains was isolated as described previously (Wang *et al*., 2009). PCRs were performed to check the Tn5-insertion locations in *Xoo* mutants P99M2, P99M4 and P99M5, using primers P1 (5'-AAGTGGGTTCACTCGCTGTCAGCACAG-3'), P2 (5'-GGCAGAGCATTACGCT GACT-3'), P3 (5'-ATTCAACGGGAAACGTCTTG-3'), P4 (5'-CTGATTGCCC GACATTATCG-3') and P5 (5'-ACTGAATCCGGTGAGAATGG-3'). The reaction (20 μL) contained 50 ng of template DNA, 1 × PCR buffer, 0.6 mmol/L deoxynucleoside triphosphates (dNTPs), 0.15 mmol/L of each primer and 0.75 U KOD *Taq* polymerase (TOYOBO, Osaka, Japan). PCR was initiated at 95 °C for 3 min, followed by 35 cycles of amplification at 94 °C for 40 s, 60 °C for 40 s, 72 °C for 1 min, and a final extension at 72 °C for 10 min.

For Southern blot analysis, 1 μg of bacterial genomic DNA was digested with *Sph*I alone or together with *Sau3A*I, separated on a 1.2% (w/v) agarose gel and transferred to a nylon membrane (Hybond-N+, Amersham Pharmacia Bio-tech, Amersham, UK). A 569-bp PCR fragment amplified from Tn5-DNA using primers P3 and P5 (Fig. 3a), or the *Sph*I-*Sau3A*I fragment containing the central repeat region of *avrXa23* (Fig. 2e), was used as DNA probe, and labelled with ^{32}P-dCTP by the random priming method (Sambrook *et al*., 1989). Hybridization was performed in a solution containing 5 × saline-sodium citrate (SSC), 5 × Denhardt's solution, 0.6% (w/v) sodium dodecylsulphate (SDS), 10% (w/v) dextran sulphate and 20 mg/L denatured salmon sperm DNA at 65 °C for 14 h. Filters were washed twice in 2 × SSC, 0.1% SDS at 65 °C for 10 min, once in 1 × SSC, 0.1% SDS at 65 °C for 10 min, and once in 0.5 × 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).

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