Eur J Plant Pathol (2009) 123:235–240
DOI 10.1007/s10658-008-9356-4

Identification and fine mapping of the new bacterial blight resistance gene, *Xa31(t)*, in rice

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Received: 26 October 2007 / Accepted: 10 July 2008 / Published online: 12 August 2008

Abstract Zhachanglong (ZCL), a regional rice variety from Yunnan province in southwest China, has a high level of resistance to a broad spectrum of *Xanthomones oryzae* pv. *oryzae* (*Xoo*) isolates. In a previous study, a bacterial blight (BB) resistance (*R*) gene, *Xa22(t)*, with resistance against *Xoo* strain *Px061* on chromosome 11 was identified in ZCL. Here, we report another BB *R*-gene, tentatively named *Xa31(t)*, with resistance against *Xoo* strain OS105 and susceptible to *Px061* identified in ZCL. To determine the location of *Xa31(t)*, 102 polymorphic RFLP markers on 12 rice chromosomes were selected for bulked segregation analysis (BSA). Twelve RFLP markers on chromosome 4 detected DNA polymorphisms between ZCL and Zhengzhu Ai (ZZA), as well as in the OS105-resistant and -susceptible bulks from F_2 populations derived from ZCL×ZZA. Genetic linkage analysis and fine mapping localised *Xa31(t)* within a genetic distance of 0.2 cM between two RFLP markers, G235 and C600, on the end of the long arm of chromosome 4, using two F_2 populations from the cross ZCL×ZZA and two F_3 populations, consisting of 3,311 plants with 301 F_3 random families and 3,333 plants with 303 F_3 *Px*o61-susceptible families, derived from the same F_2 populations from the cross ZCL×ZZA. Using two flanking markers, G235 and C600, to screen the MH63 BAC library, the *Xa31(t)* locus was limited to one BAC clone with a length of about 100 kb.

Keywords Rice · Bacterial blight · Resistance gene · Genetic/physical map

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Bacterial blight (BB) of rice (*Oryza sativa*), caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), is one of most serious diseases in rice, decreasing rice yields by 5–20% (Mew 1987). The genetic basis of host resistance to bacterial blight has been studied in depth, and about 30 BB-resistance (*R*) genes conferring resistance against various strains of *Xoo*, identified from cultivated rice and wild rice species, have been mapped on six of 12 rice chromosomes, respectively (Nishimura 1961; Sun et al. 2003; Tan et al. 1998; Chu et al. 2006; Jin et al. 2007). So far, six of 30 BB *R*-genes, *Xa21*, *Xa1*, *Xa26*, *xa5*, *Xa27* and *xa13*, have been cloned successfully by map-based cloning strategy, and characterized as encoding five types of proteins, suggesting multiple mechanisms of *R*-gene-mediated *Xoo* resistance (Song et al. 1995; Yoshimura et al. 1998; Iyer and McCouch 2004; Sun et al. 2004; Gu et al. 2005; Chu et al. 2006). Some of these *R*-

genes have been widely used in rice breeding for BB resistance and many useful cultivars have been released in Asian countries.

Zhachanglong (ZCL), a land rice from the Yunnan province in southwest China, a centre of diversity for Asian cultivated rice (*Oryzae sativa*), shows a high level of resistance to 16 of the 17 BB strains tested (Lin et al. 1996); one BB *R*-gene, *Xa22(t)*, has been identified and fine mapped on the long arm of chromosome 11 (Wang et al. 2003). Here, we present evidence that another BB *R*-gene with a special resistance spectrum to *Xoo* strains in ZCL, designated as *Xa31(t)*, is identified. Genetic linkage analysis has localised this gene on the nearby end of the long arm of the chromosome 4, and a physical map of *Xa31(t)* locus has been constructed, delimiting *Xa31(t)* to about 100-kb segment of genomic DNA.

Two F_3 family populations, including 3,311 plants with 301 F_3 random families and 3,333 plants with 303 F_3 *P*xo61-susceptible families, respectively derived from 301 randomly selected F_2 plants and 303 extremely *P*xo61-susceptible F_2 plants in the same F_2 population from a cross between ZCL (resistant parent carrying BB *R*-genes, *O. sativa* subsp. *japonica*) and Zhengzhu Ai (ZZA; susceptible to all *X. oryzae* pv. *oryzae* isolates, *O. sativa* subsp.*indica*, *O. sativa* subsp. *indica*), were constructed. Two *Xoo* pathogen strains, *P*xo61 (the Philippine *X. oryzae* pv. *oryzae* race 1, provided by T. W. Mew) and OS105 (the Chinese *X. oryzae* pv. *oryzae* race, provided by L. H. Zhu in Nanjing Agricultural University) were prepared and used to assay resistance in the two family populations by methods described previously (Wang et al. 2003).

In the F_3 random families derived from the 301 random F_2 individuals, the segregation ratios for resistance against *Xoo* pathogen strains, *Pxo*61 and OS105, of homozygous resistant families, heterozygous resistant families, and homozygous susceptible families were 1:2:1 (data not shown), and 7:8:1 (χ^2= 0.678, *P*=0.8–0.7) (Table 1), respectively. In the F_3 *P*xo61-susceptible families derived from the 303 extremely *P*xo61-susceptible F_2 individuals, the segregation ratio of homozygous OS105-resistant families (*n*=79), heterozygous OS105-resistant families (*n*=155) and homozygous OS105-susceptible families (*n*=69) was 1:2:1 (χ^2=0.8463, *P*=0.70–0.50) (Table 1), and no *P*xo61-resistance family was detected. The results suggest that there are two independently-inherited BB *R*-gene loci in ZCL, one acting on the same resistance as *Xa22(t)* against both *Xoo* pathogen strains, *P*xo61 and OS105 (Lin et al. 1996; Wang et al. 2003), and another for resistance against *Xoo* pathogen strain OS105 and susceptible to *P*xo61 (*P*xo61S–OS105R).

To confirm that the *P*xo61-susceptible and OS105-resistant (*P*xo61S–OS105R) *R*-gene locus differs from the *Xa22(t)* locus on chromosome 11 in ZCL, 102 polymorphic RFLP markers on 12 rice chromosomes were selected for bulked segregation analysis (BSA). Twelve RFLP markers on chromosome 4 detected DNA polymorphisms between ZCL and ZZA, as well as in the OS105-resistant and -susceptible bulks from F_2 populations derived from ZCL×ZZA (Fig. 1). These polymorphic markers were first used for small-scale linkage mapping of the *R*-gene locus in a small population of 78 OS105-susceptible F_2 individuals. Linkage analysis revealed that the *P*xo61S–OS105R locus was located on the end of the long arm of chromosome 4 and co-segregated with markers C600 and G235, flanked by G264 and RG214 with a genetic distance of 1.28 cM (Fig. 2).

Table 1 Segregation of resistance to OS105 in the F_3 populations

Populations	No. of homo-R families	No. of hetero-R families	No. of homo-S families	No. of families	χ^2	*P*
Extreme F_3 population	79 (1)	155 (2)	69(1)	303	0.8463	0.70–0.50
Random F_3 population	136 (7)	144 (8)	21 (1)	301	0.678	0.80–0.70

Xoo isolates were grown on potato semisynthetic agar medium (Wakimoto 1954) for 3 days at 30°C. Cells were collected by washing and suspending in sterilised water to a cell density of ~6×10^8 ml^{-1}. Every F_3 family consisted of 11 plants in one row. The longest two lesions of undamaged leaves per plant were measured 20 days after inoculation by the leaf clipping method. If all lesion lengths of the 11 plants in one family were the same as or < the resistant parents, this family is a homozygous resistant family. If all lesion lengths of the 11 plants in one family were the same as or > the susceptible parent, it is a homozygous susceptible family. If the lesion lengths of the 11 plants in one family were very different, it is a heterozygous resistant family

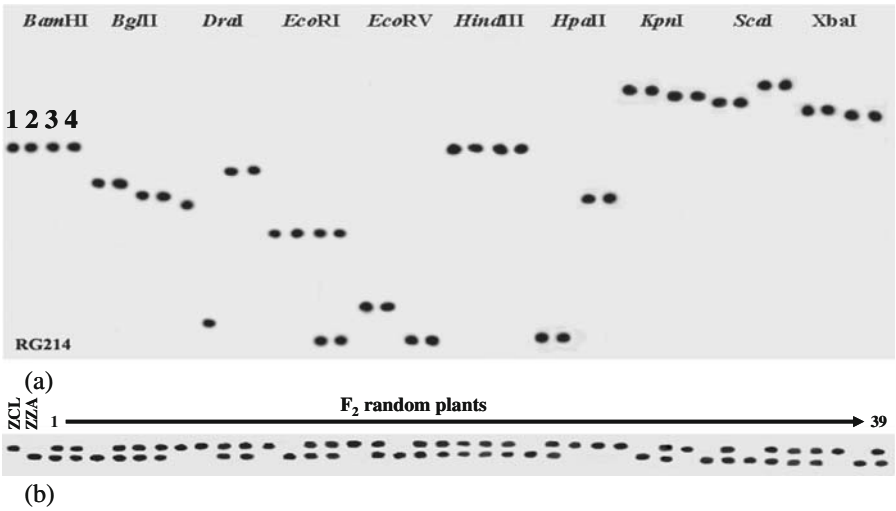

Fig. 1 Southern hybridisation for genetic linkage analysis of *Xa31(t)* with RFLP marker RG214. Genomic DNA was isolated from the fresh leaves of field-grown plants using the CTAB method of Zhang et al. (1992). Genomic DNA from 20 OS105-resistant without carrying *Xa22(t)* and 20 OS105-susceptible individuals was pooled to make up the resistant and susceptible DNA bulks, respectively. The standard protocol was used for blotting and hybridisation (Sambrook et al. 1989). (**a**) The polymorphism of RFLP marker RG214 between genomic DNAs of two parents, ZCL and ZZA, and two bulks, which were digested by different restriction enzymes; *1* and *4* resistant bulk and susceptible bulk, *2* and *3* ZCL and ZZA. (**b**) Genetic linkage analysis in the F_2 population with RG214

To determine the location of the *P*xo61S–OS105R locus in the G264 and RG214 region on chromosome 4, four RFLP markers spanning this region, including C600, G235, R78 and RG329, was then used on the large OS105-susceptible F_3 population derived originally from ZCL×ZZA for fine-scale mapping of the Pxo61S–OS105R *R*-gene locus. Genetic linkage analysis localised both C600 and G235 in the region within 0.1 cM on either side of the Pxo61S–OS105R locus (Fig. 2) and thus demonstrated that the Pxo61S–OS105R locus is a different BB *R*-gene from *Xa22(t)* in ZCL.

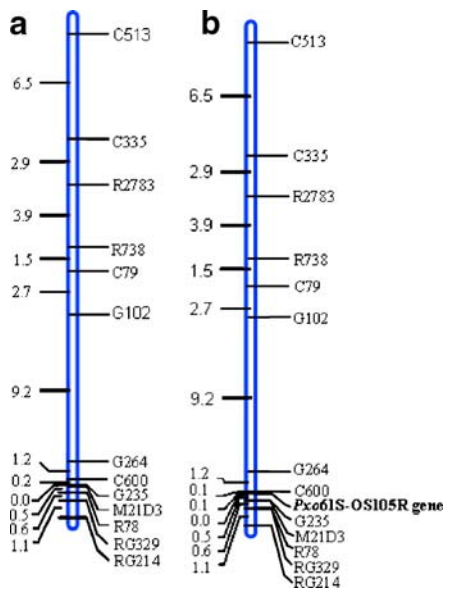

Fig. 2 Fine genetic map of gene *P*xo61S–OS105R (*Xa31(t)*) on chromosome 4. (**a**) Linkage map with 13 RFLP markers on rice chromosome 4; (**b**) *P*xo61S–OS105R gene linkage map in the OS105-susceptible F_2 population of 210 individuals derived from ZCL×ZZA

There are four BB *R*-gene loci (*Xa1*, *Xa2*, *Xa12* and *Xa14*) on chromosome 4 identified (Yoshimura 1993; Kinoshita 1998; Taura et al. 1992), of which *Xa1* was localised in the same C600 and G235 region (Yoshimura et al. 1996, 1998) as *P*xo61S–OS105R locus on the end of the long arm of chromosome 4. To confirm that the *P*xo61S–OS105R locus differs from the four known BB *R*-gene loci on rice chromosome 4, four discriminating varieties of IRBB1, IRBB2, Java14 and TN1 carrying *Xa1*, *Xa2*, *Xa12* and *Xa14*, respectively, two F_7 families of L011 and L246 carrying *Xa22(t)* without *Xa31(t)*, and two F_8 families of B104 and B038 carrying *Xa31(t)* without *Xa22(t)*, originally derived from the cross ZCL×ZZA, were used for analysing their resistant spectra against 10 selected *Xoo* strains (Table 2). The variety IRBB1 with *Xa1*, similar to the control of discriminating variety, ZZA, was susceptible to all the *Xoo* strains tested. The variety, Java14, with *Xa12* was resistant

Table 2 Comparison of resistant spectra to 10 different *Xoo* strains between *Xa31(t)* and the other BB resistance genes located on chromosome 4

Strain	ZCL	ZZA	Xa1[a]	Xa2[b]	Xa12[c]	Xa14[d]	Xa22(t)[e]	Xa22(t)[f]	Xa31(t)[g]	Xa31(t)[h]
OS105	R	S	S	S	S	S	R	R	R	R
LN44	R	S	S	S	S	S	R	R	S	S
HB17	R	S	S	R	R	R	R	R	R	R
T2	R	S	S	R	S	R	R	R	S	S
Pxo61	R	S	S	S	S	S	R	R	S	S
Px071	R	S	S	S	S	S	R	R	S	S
Pxo79	R	S	S	S	S	S	R	R	S	S
Pxo86	R	S	S	S	S	S	R	R	S	S
Pxo99	S	S	S	S	S	S	S	S	S	S
Pxo112	R	S	S	R	S	R	R	R	R	R

There were 11 plants in every row for one variety, and four repeats for every variety. The longest two lesions of undamaged leaves per plant were measured on the 20th day post-inoculation by the leaf clipping method (Kauffman et al. 1973)

R resistant reaction, *S* susceptible reaction

[a] IRBB1

[b] IRBB2

[c] Java14

[d] TN1

[e,f] F_7 families L011 and L246 with *Xa22(t)* and without *Xa31(t)* from the cross ZCL×ZZA

[g,h] F_8 families B104 and B038 without *Xa22(t)* and with *Xa31(t)*

only to the *Xoo* strain HB17; the two varieties IRBB2 and TN1, respectively carrying *Xa2* and *Xa14*, were resistant to three *Xoo* strains HB17, T2 and *Pxo*112. Two F_7 families of L011 and L246 carrying *Xa22(t)* without *Xa31(t)*, similar to the control of variety ZCL, were resistant against all the tested *Xoo* strains, but *P*xo99, and two F_8 families of B104 and B038 carrying *Xa31(t)* without *Xa22(t)*, originally derived from the cross ZCL×ZZA, were resistant against *Xoo* strains OS105, HB17 and *P*xo112. The results demonstrate that the resistant spectrum of the newly mapped *P*xo61S–OS105R gene on chromosome 4 in ZCL was evidently different from that of *Xa1*, *Xa2*, *Xa12* and *Xa14*.

For checking if *P*xo61S–OS105R gene was the allele of *Xa1*, three PCR primer pairs, A, B and C, designed according to flanking DNA sequences of the second intron, NBS and LRR domains of *Xa1*, respectively, were used for amplifying genomic DNAs of IRBB1, Nipponbare, ZCL and two F_3 families carrying the *P*xo61S–OS105R gene without *Xa22(t)* (Fig. 3). Each of three primer pairs amplified a single expected-band in genomic DNA of IRBB1

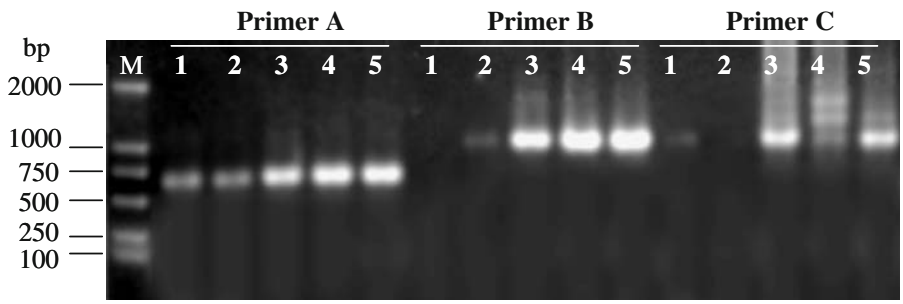

Fig. 3 The results of amplification with the special primers for *Xa1*. *M* DL 2000 marker, *primer pairs A*, *B* and *C* were designed for amplifying three DNA sequences of the second intron, NBS and LRR domains in *Xa1*, respectively; *1* and *2* the genomic DNAs were isolated from seedling leaves of two F_3 families carrying the *P*xo61S–OS105R gene without *Xa22(t)*; *3* to *5* the genomic DNAs isolated from seedling leaves of varieties ZCL carrying *Xa22(t)* and *Xa31(t)*, *Nipponbare* without carrying *Xa1* and IRBB1carrying *Xa1*, respectively

carrying *Xa1*, as well as in genomic DNA of ZCL. Two of three primer pairs, A and B, amplified a single expected-band in genomic DNA of *Nipponbare*. In genomic DNAs of two F_3 families carrying the *P*xo61S–OS105R gene, however, only one of three primer pairs, primer A for amplifying the DNA sequence covering the second intron in *Xa1*, detected a single band. The results further demonstrate that the novel BB-resistant gene in ZCL, tentatively designated as *Xa31(t)*, is distinctly different from *Xa1*.

To construct a physical map covering the target region, two flanking RFLP markers, G235 and C600, on each side of the *Xa31(t)* locus were used to screen the BAC library from Minghui63 (*O. sativa* subsp. *indica*) consisting of over 26,000 clones, or approximately eightfold genome equivalents (Peng et al. 1998). One positive BAC clone M21D3, by screening with probe C600, and two positive BAC clones, M21D3 and M5E9, by screening with probe G235, were selected from the library. The results of both G235 and C600 hybridising with the same BAC clone, M21D3, indicated that *Xa31(t)* was localised to the BAC clone (Fig. 4a). The insert size of BAC clone M21D3, estimated by PFGE electrophoresis after digestion with *Not* I, was about 100 kb. The R end of BAC clone M21D3, isolated by thermal asymmetric interlaced-PCR (TAIL-PCR) (Liu and Whittier 1995) and Vector-Hexamer PCR (Herring et al. 1998), was then used for recombination event assay in the mapping population with 210 F_2 plants derived from the cross ZCL and ZZA; linkage analysis confirmed that the R end of M21D3 cosegregated with the flanking marker G235 of *Xa31(t)*. Therefore, *Xa31(t)* present in ZCL lies within a segment of about 100 kb between the markers G235 and C600 (Fig. 4).

A BB *R*-gene *Xa22(t)*, which provides resistance against a wide spectrum of *Xoo* strains in a variety of ZCL, has been identified and fine-mapped on rice chromosome 11, using two F_2 populations from the cross ZCL×ZZA (Wang et al. 2003). The same F_2 populations and two F_3 populations, consisting of 3,311 plants with 301 F_3 random families, and 3,333 plants with 303 F_3 *P*xo61-susceptible families derived from the same F_2 populations from the cross ZCL×ZZA, were further used for genetic analysis of resistance against different *Xoo* strains. A novel BB *R*-gene *Xa31(t)*, which is resistant against three tested *Xoo* strains OS105, HB17 and *P*xo112, but susceptible to seven tested *Xoo* strains *P*xo61, *P*x071, *P*xo79, *P*xo86, *P*xo99, LN44 and T2, was identified in ZCL. Genetic linkage analysis and fine-mapping localised *Xa31(t)* within a genetic distance of 0.2 cM between two RFLP markers, G235 and C600, on the end of the long arm of chromosome 4.

Up to now, five BB *R*-genes, *Xa1*, *Xa2*, *Xa12*, *Xa14* and *Xa31(t)* are mapped on rice chromosome 4, of which *Xa1*, *Xa2* and *Xa31(t)* have been localised within the same region between two RFLP markers, G235 and C600, on the end of the long arm of chromosome 4, characterising, with respective specificity, resistance to *Xoo* strains (Table 2). Interestingly, a gall midge-resistance gene was also mapped in the same region on the end of the long arm of chromosome 4 (Rajyashri et al. 1998). It is suggested

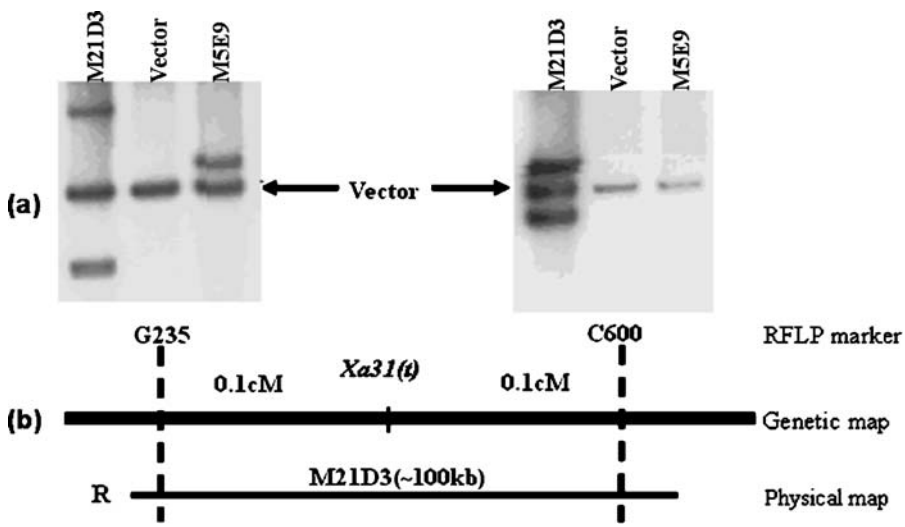

Fig. 4 Genetic and physical maps encompassing the *Xa31(t)* locus present in ZCL on chromosome 4. (**a**) Southern hybridisation of the two selected positive BAC clones of M21D3 and M5E9, digested by *Not I*, with two *Xa31(t)*-flanking RFLP markers, G235 (*left*) and C600 (*right*); (**b**) Genetic (*upper*) and physical (*underside*) maps covering the *Xa31(t)* locus

that the G235–C600 region, rich in resistance genes, on the end of the long arm of chromosome 4, must have played an important role in response to different *Xoo* strains; characterising *Xa31(t)* by transformation testing of the candidate fragment and comparing its sequence with other four BB R-genes in this region will reveal characteristics of their evolution.

Acknowledgements This work was supported, in part, by a grant from the National Special Programme for Research and Industrialisation of Transgenic Plants of China and the National High Technology Research Programme of China. We thank S. D. Tanksley's group at Cornell University, T. Sasaki of the Japanese Rice Genome Research Programme for providing the RFLP probes, and Q. Zhang for help in many aspects of this study.

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