

Inheritance of bacterial blight resistance in the rice cultivar Ajaya and high-resolution mapping of a major QTL associated with resistance

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Summary

The cultivar Ajaya (IET 8585) exhibits durable broad-spectrum resistance to bacterial blight (BB) disease of rice and is widely used as a resistance donor. The present study was carried out to decipher the genetics of BB resistance in Ajaya and map the gene(s) conferring resistance. Genetic analysis in the F₂ indicated a quantitative/additive nature of resistance governed by two loci with equal effects. Linked marker analysis and allelic tests revealed that one of the resistance genes is *xa5*. Sequence analysis of a 244 bp region of the second exon of the gene-encoding Transcription factor IIA γ (the candidate gene for *xa5*) confirmed the presence of *xa5*. Bulked-segregant analysis (BSA) revealed the putative location of the two quantitative trait loci (QTLs)/genes associated with resistance on chromosomes 5 and 8. Composite interval mapping located the first locus on Chr. 5S exactly in the genomic region spanned by *xa5* and the second locus (*qtl BBR 8.1*) on Chr. 8L. Owing to its differential disease reaction with a set of seven hyper-virulent isolates of *Xanthomonas oryzae*, a map location on Chr. 8L, which was distinct from *xa13* and data from allelism tests, the second resistance locus in Ajaya was determined to be novel and was designated as *xaAj*. A contig map spanning *xaAj* was constructed *in silico* and the genomic region was delimited to a 13.5 kb physical interval. *In silico* analysis of the genomic region spanning *xaAj* identified four putatively expressed candidate genes, one of which could be involved in imparting BB resistance in Ajaya along with *xa5*.

1. Introduction

Bacterial blight (BB) disease of rice caused by *X. oryzae* pv. *oryzae* (*Xoo*) limits rice yield in all major rice-growing regions of the world especially in irrigated lowland and rainfed conditions. The estimated yield loss due to BB under severe infection varies from 50 to 80% in tropical Asia (Khush & Ogawa, 1989). Deployment of resistant cultivars is the most economical and effective method to control the disease due to the non-availability of effective bactericidal agents. Two types of resistance, vertical and horizontal resistance, have been recognized to be in operation

in rice against *Xoo*. Vertical resistance, governed by single major genes, is race specific and can be broken down easily unless two or more genes are deployed in conjunction as gene-pyramids. Through classical genetic analysis about 34 major genes conferring resistance to various strains of *Xoo* (Chen *et al.*, 2011) have been identified and most of these have been mapped to various chromosomes (Jin *et al.*, 2007). So far, six BB resistance genes, *Xa21*, *Xa1*, *Xa26*, *Xa27*, *xa5* and *xa13* have been cloned by map-based cloning strategy (Chu *et al.*, 2006b). Horizontal resistance on the other hand is quantitative, presumably non-race specific and controlled by quantitative trait loci (QTLs). QTL mapping provides an effective approach to study complex and polygenic forms of disease resistance. Recent progress in the QTL analysis using DNA markers especially simple sequence repeats

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Table 1. Disease reaction of Ajaya along with NILs of IR24 with seven hypervirulent isolates of Xoo

Genotype	DX-066	DX-002	DX-018	DX-084	DX-109	DX-119	DX-139
Ajaya	R	MR	R	R	MR	R	R
IRBB4 (<i>Xa4</i>)	S	S	S	S	S	S	MR
IRBB5 (<i>xa5</i>)	R	MR	S	S	S	S	S
IRBB13 (<i>xa13</i>)	MR	S	MR	R	R	R	S
IRBB21 (<i>Xa21</i>)	R	MR	S	S	S	MR	S
IRBB52 (<i>xa4 + xa21</i>)	R	R	R	S	S	MR	MR
IRBB53 (<i>xa5 + xa13</i>)	MR	R	R	MR	MR	R	R
IRBB56 (<i>xa4 + xa5 + xa13</i>)	R	MR	R	R	R	R	R
IRBB59 (<i>xa5 + xa13 + xa21</i>)	R	MR	R	R	R	R	R
IRBB60 (<i>xa4 + xa5 + xa13 + xa21</i>)	R	R	R	R	R	R	R
BJ1 (<i>xa5 + xa13</i>)	R	S	MR	MR	MR	MR	R
TN1	S	S	S	S	S	S	S
BPT 5204	S	S	S	S	S	S	S

Note: DX-066 collected from Raipur (Chattisgarh); DX-002 collected from Faizabad (Uttar Pradesh); DX-018 collected from Kapurtala (Punjab); DX-084 collected from Kaul (Haryana); DX-109 collected from Kerala; DX-119 collected from Hyderabad (Andhra Pradesh); DX-139 collected from Sambalpur (Orissa).

(SSRs) allows a better understanding of the traits that are controlled by multiple genes. QTLs responsible for the BB resistance have been identified in earlier studies (Li *et al.*, 1999).

Large-scale cultivation of varieties with single resistance genes like *Xa3* and *Xa4* resulted in development of more virulent races making them ineffective (Shi *et al.*, 2001). Evolution of virulent populations of *Xoo* against the major resistance gene, *Xa21* has been reported in some parts of India and Korea (Sirisha *et al.*, 2004). Widespread and repeated use of a few resistance genes has accelerated the selection of new pathogenic races at a rate equivalent to 1.64 times the increase in specific virulence of the isolate following host-plant selection pressure after a single crop cycle (Nayak, 1986b). One of the strategies to delay breakdown of resistance is to identify new resistance genes from diverse sources especially identified broad spectrum durable resistant cultivars and to introgress them into susceptible but otherwise popular varieties.

Several varieties with resistance to BB have been released under the auspices of the All India Coordinated Rice improvement project (AICRIP) in India. The variety, Ajaya (IET 8585) was released by AICRIP in 1992 and possesses high level of resistance against most of the pathotypes in India (DRR Progress Report 1996–2009). Ajaya is commonly used as a differential cultivar and as a resistant check in AICRIP trials. However, information regarding the inheritance of BB resistance in Ajaya is not clear with one study indicating the action of two independently segregating dominant genes (Saini *et al.*, 1996), while another reported the action of a single recessive gene (Kameswara Rao *et al.*, 2003). Hence, an effort was made through the present study to clarify the

inheritance of resistance to BB in Ajaya, tag and map the gene(s) conferring BB resistance with the help of SSR markers and identify putative candidate genes responsible for BB resistance through an *in silico* approach.

2. Materials and methods

(i) Materials

The plant materials analysed in the present study consisted of the BB resistant cultivar Ajaya, one of its parents – BJ1, the BB susceptible varieties TN1, BPT 5204 (Samba Mahsuri), near isogenic lines (NILs) of IR24 possessing single BB resistance genes-*xa5* (IRBB5), *xa13* (IRBB13), *Xa21* (IRBB21), NILs possessing combinations of *Xa4*, *xa5*, *xa13* and *Xa21* and the BB differential BJ1. Crosses were made with Ajaya as donor and TN1, BPT 5204, IRBB5 and IRBB13 as recipient parents to develop F₂ and F₃ mapping populations. A population consisting of 800 F₂ individuals derived from the cross Ajaya/TN1 was used to study the inheritance of resistance to BB and for mapping the resistance gene(s) in Ajaya. An alternate population consisting of 592 F₂ individuals obtained from the cross Ajaya/BPT 5204 was used for validation of genetic distances of the linked markers. Two sets of populations, each consisting of 177 F₂ individuals derived from Ajaya/IRBB5 and 326 F₂ individuals of Ajaya/IRBB13 were used for allelism test of *xa5* and *xa13*, respectively. Seven strains of *Xoo*, collected from different hotspot locations of India were used to study the level and spectrum of BB resistance of Ajaya along with NILs possessing single or combination of BB resistance genes and susceptible parents (Table 1).

(ii) *Methods*(a) *Disease evaluation*

The parents, F₁, F₂ and F₃ populations of the above crosses were phenotyped for their reaction to BB at 55 days after sowing which coincides with the active tillering phase of the crop. The bacterial culture was grown on modified Wakimoto's medium (sucrose 20.0 g; peptone 5.0 g; Ca(NO₃)₂, 4H₂O 0.5 g; Na₂HPO₄ 0.82 g; FeSO₄, 7H₂O 0.05 g; agar 20.0 g; distilled water 1000 ml; pH 6.8–7.0) for 3 days at 28 °C. The bacterial cells were suspended in sterile distilled water such that approximately a concentration of 10⁸–10⁹ cfu/ml was maintained. The parents and the mapping populations were inoculated with the bacterial suspension by the leaf clipping method (Kauffman *et al.*, 1973). Plant reaction to the BB pathogen was recorded 14 days after inoculation. Plants were scored based on the per cent diseased leaf area (DLA) following the conditions described by Chen *et al.* (2000) and IRRI–SES system of scoring (Anonymous, 2002). The scores from 5 to 6 inoculated leaves from a single F₂ plant were averaged and categorized as highly resistant (score 1, % DLA < 5), moderately resistant (score 3, % DLA = 5–11.9), moderately susceptible (score 5, % DLA = 12–24.9), susceptible (score 7, % DLA = 25–49.9) and highly susceptible (score 9, % DLA = 50–100).

(b) *PCR analysis*

Total genomic DNA was extracted from the parents and mapping populations derived from the crosses Ajaya/TN1 and Ajaya/BPT 5204 using the procedure of Zheng *et al.* (1995). PCR was performed in a thermal cycler (Perkin–Elmer 480, USA) as per the conditions described by Chen *et al.* (1997). SSR marker resolution was done on 4% agarose gels stained with ethidium bromide and photographed under ultraviolet light. In order to test the presence of *xa5* and *xa13* in Ajaya, the *xa5*-linked CAPS marker RG556 (Huang *et al.*, 1997) and the *xa13*-linked PCR-based marker *xa13*-prom (Chu *et al.*, 2006b) were analysed in Ajaya along with IR24, IRBB5, IRBB13, TN1, BPT 5204 and the parental line BJ1. Restriction digestion for the CAPS marker RG556 was performed in a reaction mixture consisting of 3.2 µl sterile distilled water, 1.5 µl restriction buffer (10×), 0.3 µl restriction enzyme *Dra* I (3U/µl) and 20 µl PCR product by incubating at 37 °C for 1 h. The samples were then resolved in a 2% agarose gel and visualized after staining with ethidium bromide. PCR analysis of *xa13*prom was done following conditions similar to SSRs and the amplicons were resolved in 2% agarose gel.

(c) *Analysis of functional nucleotide polymorphism with respect to xa5 in Ajaya*

A primer pair was designed based on a region on chromosome 5 of Nipponbare rice sequence (Pseudo molecule5) from 415 388 to 415 630 bp, which encompasses the 2 bp functional polymorphism TC/AG in the exon II of the transcription factor IIAγ (Iyer & McCouch, 2004). The primers were used to amplify the resistant and susceptible genotypes (IRBB5, Ajaya, TN1 and BPT 5204). The primer sequences were as follows: *xa5*F: TCC CCC CAC CCC AAA AAG, *xa5*R: ACA AAC AAA ATG AGG AGT CG. PCR amplification was performed using a high-fidelity DNA polymerase–Pfu polymerase (Promega, USA) to avoid errors as per the conditions described earlier. The PCR products were resolved on a 0.8% agarose gel and eluted using Wizard[®] SV Gel and PCR clean-up system (Promega, USA). The products were cloned using Qiagen[®] PCR cloning kit (Qiagen, Germany) as per the manufacturer's instructions. Plasmids were isolated from the transformed colonies using Qiaquick plasmid isolation kit (Qiagen, Germany) and sequenced in both directions in an ABI prism 3700 automated DNA sequencer using the T7 and T13 primers of the vector. Vector sequences were removed from the sequenced fragments and the cleaned-up fragments were then aligned using Clustal W (available at the website <http://www.ebi.ac.uk/clustalw>) program of the European Bioinformatics Institute (Thompson *et al.*, 1994).

(d) *Molecular mapping using SSR markers*

Bulked segregant analysis (BSA). In order to map the BB resistance genes in Ajaya and to identify linked SSR markers, a set of 480 SSR markers spread across the rice genome were used. BSA was carried out by amplification of the resistant and susceptible parents along with the RB and SB with the polymorphic SSR markers. Markers that displayed bulk-specific amplification were analysed in the entire mapping population to study their linkage distance with respect to the resistance genes.

Fine mapping. Once the tentative chromosomal location of the resistance loci was known through BSA, all the SSR markers in the vicinity, possessing a repeat length of more than 20 bp, were selected for genotyping the F₂ population of Ajaya/TN1 for coarse mapping. Fine-mapping of the two putative resistance-associated loci was carried out using a separate set of 22 SSR markers specific to chromosome 5 (in the vicinity of *xa5*) and 45 SSR markers specific to the long arm of chromosome 8. Of the 45 markers, 39 were from the RM series (available at the website www.gramene.org) and six markers viz., RMAFM1 to RMAFM6 (see Supplementary Table S1 available at <http://journals.cambridge.org/grh>) were specifically

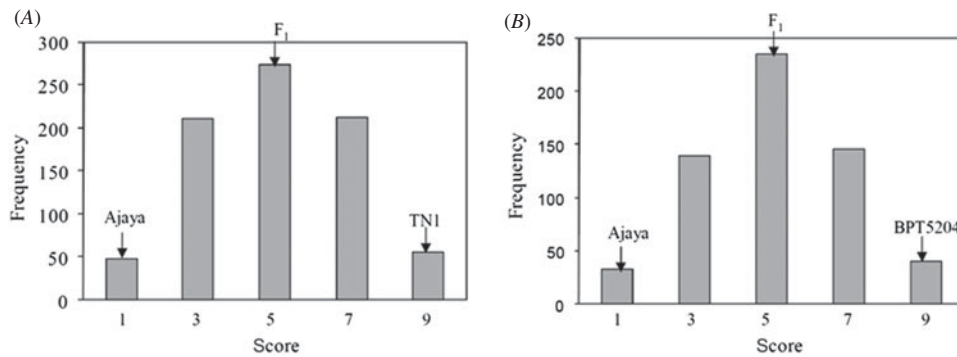


Fig. 1. Frequency distribution of the F₂ population derived from Ajaya/TN1(A) and Ajaya/BPT 5204 (B). A segregation ratio of 1 : 4 : 6 : 4 : 1 with respect to resistance, moderate resistance, moderate susceptibility, susceptibility and high susceptibility was noticed when the F₂ plants derived from the crosses Ajaya/TN1(A) and Ajaya/BPT 5204 (B) were screened for BB resistance.

designed by FASTPCR software using the flanking sequence data of the repeat motifs identified in-between the flanking markers by SSRIT tool of Gramene (available at the website <http://www.gramene.org>). Linkage analysis and map construction were performed using MAPMAKER/EXP, version 3 (Lander *et al.*, 1987). QTL analysis was done using Composite interval mapping function of Windows QTL Cartographer ver 2.5 (Zeng, 1994) using per cent DLA as trait phenotype.

(e) In silico physical mapping of BB resistance gene

The physical map of the resistance gene was constructed by bioinformatics analysis using bacterial artificial chromosome (BAC) and P1-derived artificial chromosome (PAC) clones of cv. Nipponbare released by the International Rice Genome Sequencing Project (IRGSP, 2005). The flanking markers were assigned to the respective BAC or PAC clones through BLASTN analysis. The PAC/BAC clones were aligned using the software tool Pairwise BLAST (available at the website <http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html>).

(f) Candidate gene annotation

Based on the targeted region of the resistance gene, the publicly available BAC or PAC sequences of *Oryza sativa* cv. Nipponbare were analysed by gene prediction programmes FGENESH (available at the website <http://genomic.sanger.ac.uk>), Rice GAAS (available at the website <http://ricegaas.dna.affrc.go.jp/>) and Gene Scan (available at the website <http://genes.mit.edu/GENSCAN>). The candidate genes were analysed through BLAST (available at the website <http://www.ncbi.nlm.nih.gov/blast/>) and confirmed by the TIGR Rice Genome Annotation Version 6.0 (available at the website <http://rice.plantbiology.msu.edu/cgi-bin/gbrowse/rice/>).

3. Results

(i) Genetic analysis of BB resistance in Ajaya

When screened with multiple isolates of *Xoo*, Ajaya displayed a broader spectrum of resistance as compared with the susceptible checks (TN1 and BPT 5204) and the NILs possessing the single resistance genes, *Xa4*, *xa5*, *xa13* and *Xa21* (Table 1). The resistant parent Ajaya and the susceptible parents TN1 and BPT 5204 showed clear distinction in terms of disease score with Ajaya displaying a score of 1 and the susceptible parents, TN1 and BPT 5204 displaying a score of 9. The F₁s were moderately susceptible with a disease score of 5. Out of the 800 F₂ plants screened, 48 were highly resistant, 210 were moderately resistant, 274 were moderately susceptible, 212 were susceptible and 56 were highly susceptible (Fig. 1(A), see Supplementary Table S2 available at <http://journals.cambridge.org/grh>). This fitted well in a segregation ratio of 1 : 4 : 6 : 4 : 1 ($\chi^2 = 4.27$, $P = 0.37$) with respect to high resistance, moderate resistance, moderate susceptibility, susceptibility and high susceptibility. Similar segregation pattern was observed in the F₂ population derived from the cross Ajaya/BPT 5204 (Fig. 1(B), see Supplementary Table S3 available at <http://journals.cambridge.org/grh>). The F₁s were moderately susceptible (score 5) and among the 592 F₂ plants, 32 were resistant, 139 were moderately resistant, 235 were moderately susceptible, 146 were susceptible and 40 were highly susceptible ($\chi^2 = 2.23$, $P = 0.69$). This indicated that BB resistance in Ajaya is probably controlled by two additively interacting loci with equal phenotypic effects.

(ii) Analysis with gene-linked markers

In order to test the presence of *xa5* and *xa13* in Ajaya (as predicted from its pedigree) it was analysed with linked markers RG556 (*xa5*) and *xa13*-prom (*xa13*) along with the NILs of IR24 carrying

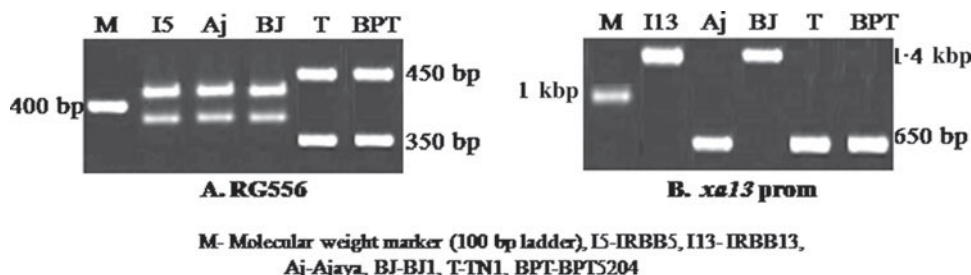


Fig. 2. Analysis of *xa5* (RG556) and *xa13* (*xa13* prom) linked markers in Ajaya. Ajaya displayed CAPS polymorphism similar to IRBB5, indicating presence of *xa5* (2A) when amplified with RG556 and restricted with *Dra*I. However, it did not show an amplification pattern specific for the resistant allele of *xa13*, when amplified with the marker *xa13* prom (2B). M – Molecular weight marker (100 bp ladder), I5 – IRBB5, I13 – IRBB13, A – Ajaya, BJ – BJ1, T – TN1 and B – BPT 5204.

TN1	GATATAGCAACTACCGTCTCATGACACATACTATTTTCATAACCACGTAATCAAATAACAG
BPT5204	GATATAGCAACTACCGTCTCATGACACATACTATTTTCATAACCACGTAATCAAATAACAG
Ajaya	GATATAGCAACTACCGAGTCATGACACATACTATTTTCATAACCACGTAATCAAATAACAG
IRBB5	GATATAGCAACTACCGAGTCATGACACATACTATTTTCATAACCACGTAATCAAATAACAG
	***** TC *****

Fig. 3. Sequence alignment of the functional polymorphic region in second exon of *xa5*. Ajaya possessed the 2 bp polymorphism (TC→AG substitution), which was similar to that of IRBB5 at the 17th and 18th nucleotide position (highlighted in box), while TN1 and BPT 5204 showed susceptible specific TC sequence.

xa5 (IRBB5), *xa13* (IRBB13), the two BB susceptible varieties TN1, BPT 5204 and the parental line BJ1. The CAPS marker RG556 amplified two fragments of sizes 1000 and 1500 bp in all the genotypes. Restriction digestion with the enzyme *Dra*I produced two fragments of sizes 390 and 410 bp in Ajaya, IRBB5 and BJ1 and two fragments of sizes 350 and 450 bp in TN1 and BPT 5204 (Fig. 2(A)). The PCR-based marker *xa13* prom, a functional marker for *xa13* amplified the resistance specific allele, a 1.4 kb fragment in IRBB13 and BJ1, whereas Ajaya amplified a 650 bp fragment as in the other susceptible genotypes TN1 and BPT 5204 (Fig. 2(B)).

(iii) Allelism tests

In order to confirm the presence/absence of *xa5* and *xa13* genes in Ajaya, it was crossed with IRBB5 and IRBB13. The F₁s and the F₂ population derived from the cross IRBB5/Ajaya was screened with *Xoo* strain DX-066, which showed resistance reaction with IRBB5 and Ajaya. The F₁ was resistant (score 1) and all the 177 F₂ plants were uniformly resistant (score 1) without any segregation for susceptibility, indicating that one of the two resistance genes in Ajaya is *xa5*. The F₁ and the F₂ population obtained from IRBB13/Ajaya were screened with *Xoo* strain DX-084, which showed resistance reaction with IRBB13 and Ajaya (score 1). The F₁ was highly susceptible (score 9) and out of 326 F₂ plants screened, 157 were resistant (score 1) and 169 were highly susceptible.

(iv) Sequence analysis for *xa5* candidate gene *TFIIAγ*

A 244 bp fragment was amplified from the second exon of the *xa5* gene candidate gene (i.e. *TFIIAγ*) encompassing the functional nucleotide polymorphism consisting of the 2 bp substitution GTC/GAG in Ajaya, IRBB5 and two susceptible genotypes IR24 and TN1 and sequenced as explained in Materials and methods. Ajaya possessed the 2 bp polymorphism (TC→AG substitution) specific for *xa5* gene, similar to that of IRBB5 at the 17th and 18th nucleotide position, while TN1 and BPT 5204 showed susceptibility-specific TC sequence at the 17th and 18th nucleotides (Fig. 3).

(v) Mapping of the second BB resistance locus in Ajaya

Genetic analysis in the F₂ plants derived from the crosses Ajaya/TN1 and Ajaya/BPT 5204 indicated that the action of two loci are involved in conferring BB resistance in Ajaya. Based on the results of analyses with the *xa5* linked marker, RG556, allelism test with IRBB 5 and sequence analysis of *xa5* candidate gene, the first locus was confirmed to be *xa5*. The second locus was found to be non-allelic to *xa13* (based on linked marker analysis), but linked to it based on allelism test with IRBB13. In order to map the second resistance locus in Ajaya, an analysis was conducted to identify SSR markers polymorphic between Ajaya and TN1. Out of 480 SSR markers tested, 112 (23.3%) were polymorphic and 368 (76.6%)

Table 2. Details of the resistance genes/QTLs in Ajaya identified by composite interval mapping

Cross	Locus	Marker interval	Chromosome	LOD	Phenotypic variance (%)	A(cM)	B(cM)	C(cM)	P(0.05)
Ajaya/TN1 F ₂	<i>xa5</i>	RM17751–RM17752	5	6.2	55	3.2	6.5	11.2	0.02
	<i>xaAj</i>	RM23499–RMAFM1	8	5.7	42.5	4.1	7.6	13.2	0.05
Ajaya/BPT 5204 F ₂	<i>xa5</i>	RM17751–RM17760	5	7	52	3.5	6.1	12	0.14
	<i>xaAj</i>	RM23499–RMAFM1	8	4.5	40	4.3	8.5	14.5	0.06

A, additive effect of Ajaya allele; B, lesion length of plants with genotype of Ajaya and C, lesion length of plants with genotype of TN1/BPT 5204.

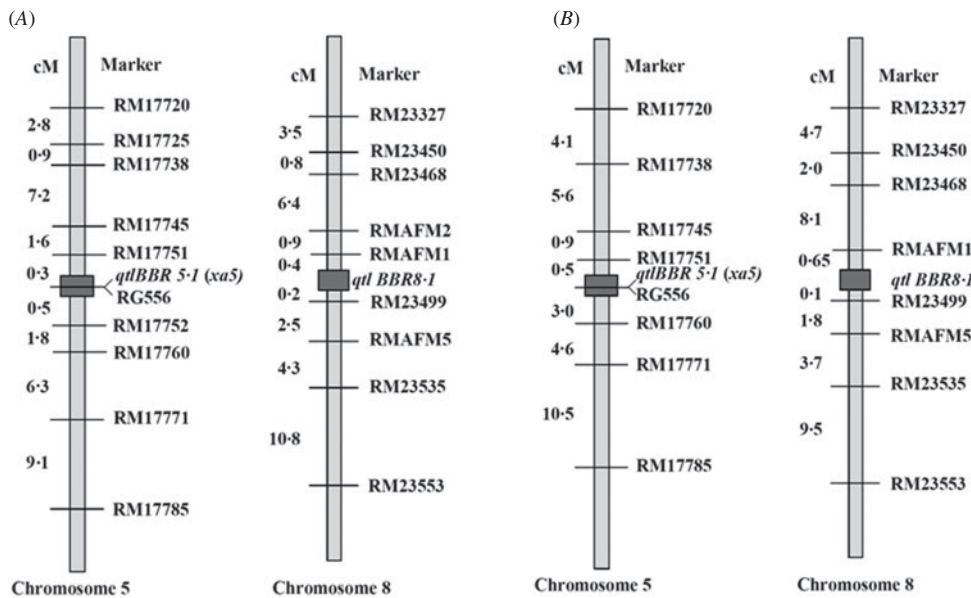


Fig. 4. Genetic linkage map of the genomic region in the vicinity of *qtl BBR 5.1 (xa5)* and *qtl BBR8.1 (xaAj)* in the F₂ mapping population derived from the cross Ajaya/TN1(A) and Ajaya/BPT 5204 (B). Two major QTLs/genes were identified to be controlling BB resistance in Ajaya. One was *xa5* located on Chr. 5 cosegregating with RG556 and the other was *xaAj* located on Chr. 8 and flanked by the SSR markers RM23499 and RMAFM1.

were monomorphic. BSA revealed linkage of the SSR marker RM17745, located on Chr. 5 and RM23535 on chromosome 8 with BB resistance. Out of 22 SSR markers tested in the vicinity of RM 17745 on the short arm of Chr. 5, 10 were polymorphic between Ajaya and TN1. Out of 45 SSR markers tested in the vicinity of RM23535 on the long arm of Chr. 8, nine were polymorphic between Ajaya and TN1. Linkage analysis was performed by genotyping the 800 F₂ plants of Ajaya/TN1 with nine SSR markers on Chr. 5, RG556 and nine SSR markers on Chr. 8. The nine SSR markers could be integrated into the linkage map of Chr. 5 and covered a span of 30.5 cM, with mean distance between the markers being 3.38 cM. The nine markers on Chr. 8 spanned a genetic distance of 29.8 cM with a mean distance of 3.31 cM. The marker order on the linkage maps of chromosomes 5 and 8 was consistent with the SSR linkage maps of Temnykh *et al.* (2000) and McCouch *et al.* (2002).

(vi) QTL analysis

Composite interval mapping (CIM) performed with a set of ten SSR markers on the short arm of chromosome 5 and nine markers on the long arm of Chr. 8 mapped the first resistance locus (tentatively named as *qtl BBR 5.1*) at a genetic distance of 0.3 cM from RM17751 and 0.5 cM from RM17752 with both the markers flanking the gene at an LOD score of 6.2. RG556 co-segregated with resistance without any recombination indicating that *qtl BBR 5.1* is *xa5*. The second resistance locus (tentatively named as *qtl BBR 8.1*) was mapped at a genetic distance of 0.2 cM from RM23499 and 0.4 cM from RMAFM1 with both of them flanking the gene (Fig. 4(A)) at an LOD score of 5.7 (Table 2). Marker-trait co-segregation analysis with the marker RM23499 revealed three recombinants in a population of 800 individuals with one recombinant from the moderately susceptible

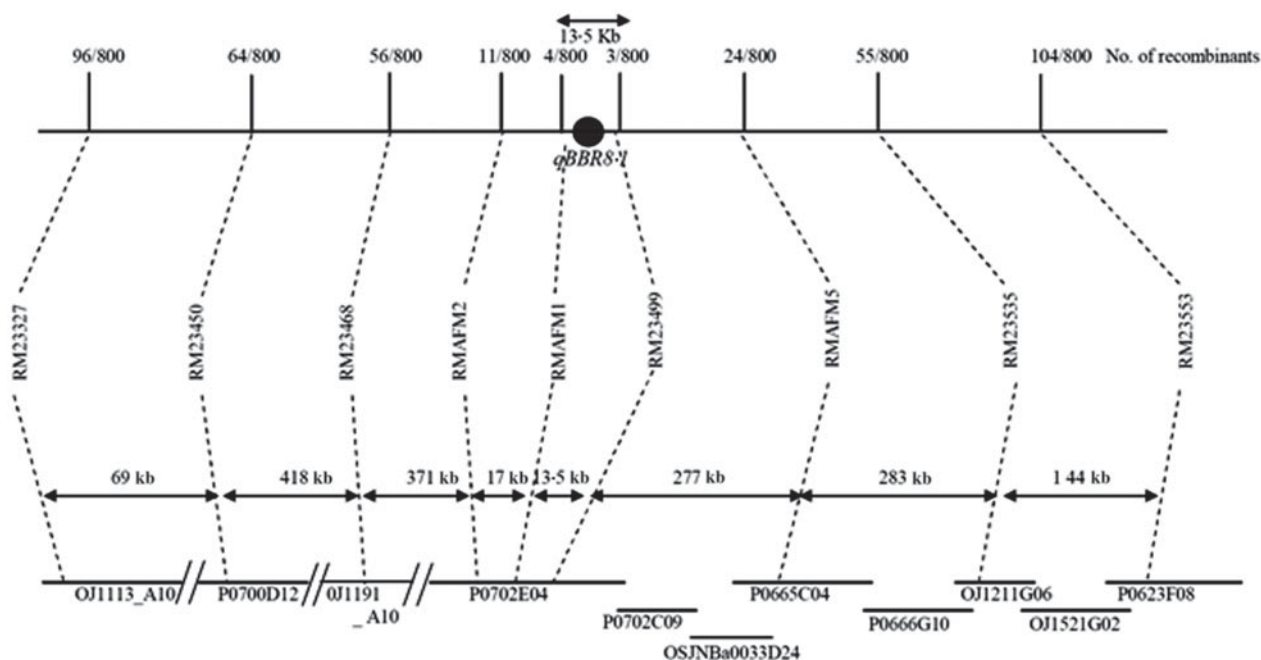


Fig. 5. Physical map of *xaAj*. Nine SSR markers were used in this study. The long horizontal lines indicate the region containing the *xaAj*. The short horizontal lines represent the BAC/PAC clones of cv. Nipponbare released by IRGSP and assembled by the corresponding markers linked to the *xaAj*. The numbers at the top above the long horizontal line are the recombination events in the mapping population. The digits between markers are physical distances in kilobase (kb). The vertical and dashed lines denote the relative positions of the corresponding markers.

class and two from the susceptible class while with respect to the marker RMAFM1, four recombinants (i.e. two from the moderately susceptible class and two from the susceptible class) were observed. All the 48 highly resistant plants showed amplification of homozygous-resistant parent-specific allele and the 56 highly susceptible plants showed the homozygous susceptible allele of RG 556 and RM23499 without any recombination. The above two loci explained 97.5% of the total variance observed. Since the second resistance locus *qBBR8.1* was observed to be non-allelic but linked to *xa13* based on allelic testing with IRBB13, it can be considered as a new gene/QTL for BB resistance.

(vii) Physical mapping

To physically map the locus *qBBR8.1*, the anchor markers RMAFM1 and RM23499 were used to land on the reference sequence of cv. Nipponbare by BLASTN analysis. The matching sequences showed that there were four PAC clones and three BAC clones, which covered the target gene i.e. OJ1113_A10, P0700D12, OJ1191_A10, P0702E04, P0665C04, OJ1211_G06 and P0623F08. The marker RM23327 was observed to be located on the BAC clone OJ1113_A10 (AP004643), RM23450 on PAC clone P0700D12 (AP004708), RM23468 on BAC clone OJ1191_A10 (AP003888), RMAFM2, RMAFM1 and RM23499 on PAC clone P0702E04

(AP005529), RMAFM5 on PAC clone P0665C04 (AP004464), RM23535 on BAC clone OJ1211_G06 (AP003948) and RM23553 on PAC clone P0623F08 (AP004632). The BAC/PAC clones identified from the IRGSP website were aligned as a contig map via pairwise BLAST analysis. The marker AFM2 aligned at position 60.574 kb on the PAC clone P0702E04, while the flanking markers RMAFM1 and RM23499 aligned at positions 77.895 and 91.330 kb, respectively. Consequently, a physical map covering the *qtBBR8.1* was generated in a 13.5 kb physical interval based on the RGP BAC/PAC contigs (Fig. 5).

(viii) Validation of the linked markers in the alternate mapping population

The utility of the linked SSR markers to predict resistance in Ajaya was validated in an alternate F_2 population derived from the cross Ajaya/BPT 5204. The *xa5* linked markers on chromosome 5 i.e. RG556, RM17720, RM17738, RM17745, RM17751, RM17760, RM17771 and RM17785 were polymorphic while RM17725 and RM17752 were monomorphic between Ajaya and BPT 5204. The markers RM23327, RM23450, RM23468, RMAFM1, RM23499, RMAFM5, RM23535 and RM23553 on chromosome 8 were polymorphic while RMAFM2 was monomorphic between the parents. The marker RG556 displayed perfect co-segregation with

Table 3. Putative candidate genes annotated in the genomic region spanned by xaAj

Accession no.	Gene no.	Location	Gene description
NP_001062339	Os08g0532300	26624938-26625234	Nuclear transport factor 2 (NTF2) domain plays an important role in the trafficking of macromolecules, ions and small molecules between the cytoplasm and nucleus
NP_001062339 BAD11651	Os08g0532400 –	26625901–26630500 26631330–26633051	Hypothetical protein with a conserved nodulin domain Unknown protein with a conserved domain of SUA7/ Transcription factor TFIIB/Brf1 subunit of TFIIB
NP_001062342	Os08g0532700	26635260–26637736	Plant peroxidase

resistance. The seven polymorphic SSR markers spanned a genetic distance of 29.2 cM on the linkage map of Chr. 5 with a mean distance of 4.17 cM between the markers, whereas the eight SSR markers on Chr. 8 spanned a genetic distance of 30.55 cM with a mean of 3.81 cM between markers. Composite interval mapping with these markers mapped the first resistance locus (*qtl BBR5.1*) in the marker interval of RM17751 and RM17760 with an LOD score of 7 and the second resistance locus (*qtl BBR8.1/xaAj*) in the marker interval of RM23499 and RMAFM1 (Fig. 4(B)) with an LOD score of 4.5 (Table 2). The gene was mapped at a distance of 0.1 cM from RM23499 with a single recombinant (in the moderately resistant class) in a population of 592 individuals while the other flanking marker RMAFM1 showed 0.65 cM distance with five recombinants (one from moderately resistant class, three from moderately susceptible and one from the susceptible class). The 32 highly resistant plants showed homozygous resistant allele and 40 highly susceptible plants showed homozygous susceptible allele with respect to RG556, RM23499 and RMAFM1. The two loci *xa5* and *xaAj* explained 92% of the total phenotypic variance observed. The marker order and the linkage distances between the markers were consistent with that observed in the F₂ population derived from Ajaya/TN1. Although there were few recombinants, no recombinants were observed in the highly resistant class with respect to the flanking markers RM23499 and RMAFM1 and so the marker combination of RG556+RM23499 and RMAFM1, could predict complete resistance in Ajaya even in the alternate background of BPT 5204.

(ix) *In silico identification of putative candidate genes for qtl BBR8.1*

In silico analysis of the intervening genomic region of 13.5 kb flanked by the closest markers RM23499 and RMAFM1 revealed the presence of four putative candidate genes (Table 3), which could be associated with *xa5* in imparting complete resistance in Ajaya.

4. Discussion

Losses due to BB are significantly higher in tropical Asia than in temperate regions because of the prevalence of virulent populations of *Xoo*. The Indian isolates of *Xoo* are reported to be more diverse and possess high level of virulence when compared with isolates from other geographic regions (Seshu, 1989). Thus, the resistance genes identified in other geographical regions may not be of significant value for resistance breeding in India. In India, where rice is grown under diverse agro-climatic conditions, a significant diversity in the *Xoo* pathogen was reported through an analysis of 67 isolates from 18 locations (Yoshitola *et al.*, 1997; Shanti *et al.*, 2001). The cultivar, Ajaya possesses high levels of resistance against most of the Indian isolates and has been observed to be durably resistant since its release in 1992 (Laha *et al.*, 2009).

The choice of durably resistant elite varieties like Ajaya as donors of BB resistance shortens the time required for developing a resistant variety through traditional/marker assisted selection, since it is an already released cultivar with some desirable agronomic features. However, in order to devise a breeding programme for transfer of BB resistance from Ajaya, knowledge about the genetics/mode of inheritance of resistance is necessary. Results from two earlier studies on inheritance of resistance to BB in Ajaya are conflicting. Hence, the present study was devised to study the genetics of BB resistance, tag and map the resistance gene(s)/QTLs in order to facilitate marker assisted introgression. In addition, the study also attempted to identify putative candidates for BB resistance in Ajaya for their possible map-based cloning in future.

Genetic analysis of the F₂ population derived from the crosses involving Ajaya with TN1 and BPT 5204 revealed quantitative inheritance of resistance, governed by two loci with equal effects. The quantitative nature of disease resistance involving 2–3 genes has been reported in many species against a variety of pathogens (Leonard, 1993). The popular IRRI bred

varieties IR28, IR26 and the differential cultivar DV85 (Wang *et al.*, 2005) were reported to possess quantitative resistance against BB. In the present study, the additive/quantitative interaction between two genes/QTLs with equal effect was discovered and one of these genes/QTLs was confirmed to be *xa5* and the same was validated in an alternate mapping population. Based on these results, the durability of resistance in Ajaya can be attributed to its quantitative nature of inheritance of resistance.

Ajaya has a complex pedigree (see Supplementary Figure S1 available at <http://journals.cambridge.org/grh>) and was originally derived from a cross between IET4141 and CR98-7216. The landrace BJ1, the predicted BB resistance donor of Ajaya has been reported to possess the BB resistance genes *xa5* and *xa13* (Ogawa *et al.*, 1987a). Since genetic analysis of F₂ populations in the present study revealed the action of two genes/QTLs involved in conferring resistance in Ajaya, its allelic status was tested with respect to the *xa5* linked marker RG556 (Blair & McCouch, 1997) and a functional marker for *xa13*, *xa13*-prom along with its parent BJ1. Iyer & McCouch (2004) reported that the functional polymorphism specific for *xa5* is a 2 bp substitution in the 17th and 18th nucleotide positions of exon-2 (i.e. TC→AG) of transcription factor IIA γ (which is the candidate gene for *xa5* located on Chr. 5) and this results in the change in a single amino acid (valine→glutamic acid) at the 39th position in the encoded protein. We observed presence of the TC→AG substitution in Ajaya, which conclusively shows that *xa5* is indeed one of the genes governing BB resistance in Ajaya.

In an earlier study, Kameswara Rao *et al.* (2003) reported that a gene non-allelic to *xa5* controls BB resistance in Ajaya and mapped it on the long arm of Chr. 5 between the markers RM39 (14.5 cM from the gene) and RM31 (17.7 cM from the gene). *xa5* has been conclusively mapped on the short arm of Chr. 5 (Blair & McCouch, 1997; Iyer & McCouch, 2004). In our study, through all the four approaches i.e. marker analysis with the closely linked CAPS marker RG556, allelism test with IRBB5, sequence analysis of the functional polymorphic region of *xa5* and linkage analysis in two populations segregating for *xa5* we have shown that *xa5* is indeed one of the candidate genes conferring resistance in Ajaya, which is in contrast to the observations of Kameswara Rao *et al.* (2003) in terms of mapping of the recessive resistance gene/QTL on Chr. 5.

The improved resistance of Ajaya to BB as compared with IRBB5, both in terms of the level and spectrum of resistance could be attributed to the additive effect of the second resistance gene/QTL identified in the present study (*xaAj*) in addition to *xa5*. Additive/dosage effects have been reported with respect to the major BB resistance genes *Xa1* and *Xa3* in

Java 14 (Kaku, 1997) and between *Xa1* and *Xa4* in IR20 (Kaku, 1999). The increased level of resistance conferred by more than one gene governing resistance to a single pathogen race has been described as quantitative complementation and has been reported in several studies involving gene-pyramiding studies for BB resistance (Yoshimura *et al.*, 1996).

Through QTL analysis in the F₂ populations derived from the crosses Ajaya/TN1 and Ajaya/BPT 5204, we fine-mapped the second resistance locus *qtlBBR 8.1* which has been designated as *xaAj*, approximately at a distance of 100 kb from another BB resistance gene *xa13*. Genetic analysis and QTL analysis in the F₂ population revealed equal contribution of resistance by the two loci in Ajaya. The BB differential DV85 was reported to carry two QTLs for BB resistance that were resolved to be *xa5* and *Xa7* (based on similar map location), with *xa5* imparting a larger effect than *Xa7* (Wang *et al.*, 2005).

When the resistance spectrum of Ajaya was studied with seven hypervirulent isolates of *Xoo*, three isolates could differentiate Ajaya and BJ1 (which possess *xa5* and *xa13*), two isolates could differentiate Ajaya and IRBB53 (which possesses *xa5* and *xa13*) and five of the seven isolates showed differential disease reaction between Ajaya and IRBB13 (which possesses *xa13*; Table 1). Based on these observations, the second resistance locus identified in the present study, i.e. *xaAj* can be considered as novel.

The gene density in the region flanking *xa Aj* is about one gene every 4.3 kb, against the published predictions of one gene every 9.9 kb (International Rice Genome Sequencing Project, 2005). In the region flanking *xa13* (Chu *et al.*, 2006a) and *Xa30t* (Cheema *et al.*, 2008), each centimorgan is roughly equivalent to 96 and 60 kb, respectively, whereas in the region flanking *xaAj*, 1 cM was calculated to be equivalent to 22.5 kb indicating a nearly 12-fold reduction in the ratio of physical/genetic sizes as compared with the average P/G ratio of 260–280 kb/cM estimated by Wu & Tanksley (1993) and Yoshimura *et al.* (1996). This reduction is apparently because of enhanced recombination in this region (hotspot for crossing over). In the *xa5* region on chromosome 5, each cM is equivalent to about 61 kb (Yang *et al.*, 1998) and 130 kb in the *Xa1* region of chromosome 4 (Yoshimura *et al.*, 1996).

Plant disease resistance genes frequently occur in the form of tightly linked clusters. Four dominant BB resistance genes *Xa3*, *Xa26t*, *Xa4* and *Xa22* have been mapped to the distal region of chromosome 11L (Yang *et al.*, 2003) and five genes, i.e. *Xa1*, *Xa2*, *Xa12*, *Xa14* and *Xa30t* in a 600 kb region on chromosome 4L (Cheema *et al.*, 2008). Recessive resistance genes generally do not cluster with other resistance genes in rice (Richter & Ronald, 2000). However, in the present study *xaAj* was mapped in a genomic region of

approximately 100 kb to *xa13*. The close linkage of *xaAj* with *xa13* based on CIM analysis was consistent with the results of genetic linkage between the two genes as revealed by allelic testing of Ajaya with IRBB13.

One of the genes annotated through *in silico* analysis in the genomic region spanned by *xaAj* encodes a nuclear transport factor which is involved in trafficking of ions and macromolecules between nucleus and cytoplasm. The second gene encodes a hypothetical protein with a conserved Nodulin domain. The candidate gene for *xa13* has been identified to be *Medicago truncatula*, nodulin gene. Nodulin-related genes are found in several species such as nematodes, insects and animals, although the biochemical functions of their proteins are unknown. Yang *et al.*, (2006) reported about 17 nodulin-related genes distributed all over the rice genome. The third gene predicted codes for a protein homologous to SUA7, which codes for the general transcription factor TFIIB or the BRF1 subunit of the transcription factor TFIIB, involved in gene transcription in association with the enzyme RNA Polymerase II. The gene *xaAj* additively interacts with *xa5* in conferring complete resistance to BB in Ajaya based on the results on genetic analysis and CIM analysis. It should be noted that *xa5* is a general transcription factor but serves as a resistance-associated protein under BB infection (Iyer & McCouch, 2004). The fourth putative candidate gene encodes a peroxidase. Peroxidases are involved in lignin biosynthesis whose accumulation was observed in the resistance mediated by *xa5*, *Xa7* and *Xa10* (Reimers & Leach, 1991). In rice, induction of peroxidases has been correlated with resistance to BB (Young *et al.*, 1995; Hilaire *et al.*, 2001). Based on these, any of the four candidate genes mentioned could be considered as a possible candidate for *xaAj* and further analysis of these genes through cloning and sequence analysis may reveal their functionality with respect to *xaAj*. Transcriptional profiling in Ajaya under BB infection revealed the differential expression of defence-related and transcription factor genes, which is in agreement with our results on *in silico* gene prediction (Kameswara Rao *et al.*, 2007).

5. Conclusion

The present study has identified two genes/QTLs governing BB resistance in Ajaya, one of which is *xa5* and the other linked to, but non-allelic to *xa13*. The second gene/QTL designated as *xaAj* was fine mapped on Chr. 8 in a physical interval of 13.5 kb close to (approximately 100 kb) another major BB resistance gene *xa13*. The gene can be deployed either singly or in combination with other major BB resistance genes *xa5*, *xa13* and *Xa21* to obtain broad-spectrum resistance against BB. The tightly linked flanking markers

identified for *xaAj*, i.e. RM23499 and RMAFM1 can be used to track the introgression of the gene into elite backgrounds. The putatively expressed genes, which could be candidates for *xaAj*, can be validated through map-based cloning. Information obtained from the present study may be valuable in understanding the molecular mechanism(s) behind quantitative resistance to *Xanthomonas* in rice.

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6. Supplementary material

The online data are available at <http://journals.cambridge.org/GRH>

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