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ORIGINAL PAPER

Genetic analysis and molecular mapping of a novel recessive gene *xa34*(t) for resistance against *Xanthomonas oryzae* pv. *oryzae*

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Abstract A new bacterial blight recessive resistance gene *xa34*(t) was identified from the descendant of somatic hybridization between an *aus* rice cultivar (cv.) BG1222 and susceptible cv. IR24 against Chinese race V (isolate 5226). The isolate was used to test the resistance or susceptibility of F_1 progenies and reciprocal crosses of the parents. The results showed that F_1 progenies appeared susceptibility there were 128R (resistant):378S (susceptible) and 119R:375S plants in F_2 populations derived from two crosses of BG1222/IR24 and IR24/BG1222, respectively, which both calculates into a 1R:3S ratio. 320 pairs of stochastically selected SSR primers were used for genes' initial mapping. The screened results showed that two SSR markers, RM493 and RM446, found on rice chromosome 1 linked to *xa34*(t). Linkage analysis showed that these two markers were on both sides of *xa34*(t) with the genetic distances 4.29 and 3.05 cM, respectively. The other 50 SSR markers in this region were used for genes' fine mapping. The further results indicated that *xa34*(t) was mapped to a 1.42 cM genetic region between RM10927 and RM10591. In order to further narrow down the genomic region of *xa34*(t), 43 of insertion/deletion (Indel) markers (BGID1-43) were designed according to the sequences comparison between *japonica* and *indica* rice. Parents' polymorphic detection and linkage assay showed that the Indel marker BGID25 came closer to the target gene with a 0.4 cM genetic distance. A contig map corresponding to the locus was constructed based on the reference sequences aligned by the *xa34*(t) linked markers. Consequently, the locus of *xa34*(t) was defined to a 204 kb interval flanked by markers RM10929 and BGID25.

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Introduction

Bacterial blight (BB) of rice, caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is one of the oldest known diseases and was first noticed by the farmers of Japan in 1884 (Tagami and Mizukami 1962). Subsequently, its incidence has been reported from different parts of Asia, northern Australia, Africa, and the USA and has become one of the three serious rice diseases. Enhancing genetic resistance has proven to be the most effective method to control the BB disease. The resistant mechanism of *Oryza sativa* (*Os*)–*Xoo* pathosystem attracted many researchers. Many articles have reported that plants develop defense mechanisms to recognize pathogens and protect themselves from assault when they are attacked by various pathogens during their life (Baker et al. 1997). These defense reactions are triggered by recognition of the pathogen's avirulence (*Avr*) genes and host disease resistance (*R*) genes. The *R*-*Avr* gene recognition can trigger hypersensitive response (HR), a form of programmed cell death at the infected site.

Recessive inheritance is an obvious character in the rice-*Xoo* pathogenic system (Iyer-Pascuzzi and McCouch 2007). The *Os–Xoo* pathosystem provides an excellent opportunity to examine recessive resistance in plant–bacterial interactions. Until now, at least 33 BB resistance genes have been identified and designated in a series from *Xa1* to *Xa33* (t), etc. (Khush and Angeles 1999; Tan et al. 2004; Xiang et al. 2006; Chen et al. 2008; Natarajkumar et al. 2009; Wang et al. 2009). Of all the BB resistance genes, ten recessive genes have been identified: *xa5* (Petpisit et al. 1977), *xa8* (Sidhu et al. 1978; Singh et al. 2002), *xa13* (Ogawa et al. 1987), *xa15* (Nakai et al. 1988, 1990; Ogawa 1996), *xa19* (Taura et al. 1991), *xa20* (Taura et al. 1992), *xa24* (Khush and Angeles 1999), *xa26b* (Lee et al. 2003), *xa28* (Lee et al. 2003) and *xa32*(t) (Ruan et al. 2008). Of these, only *xa5* and *xa13* have been cloned (Chu et al. 2006; Iyer and McCouch 2004). Though the identification of *xa5* and *xa13* has stimulated research in molecular mechanisms of plant recessive gene-for-gene resistance, more need to be identified and characterized for clarifying the recessive resistance system.

In South China, *Xoo* race V is the prevailing race attacking the conventional and hybrid paddy rice. The *indica* rice cultivar BG1222 derived from Sri Lanka, is high resistant to the Chinese *Xoo* race V in South China (Zeng et al. 2002). In the present study, a new recessive gene, which confers a high resistance to Chinese *Xoo* race V, was identified in BG1222. Here, we are reporting the results of fine genetic and physical mapping of the target resistance gene in BG1222 against race V, in an effort to replicate it using the map-based cloning approach. The objective of the study is to identify a number of molecular markers tightly linked to target gene in BG1222 for marker-assisted selection of this gene in breeding programs and to construct a physical map containing the locus with overlapping BAC/PAC clones in order to provide candidates for isolation and characterization of this gene.

Materials and methods

Plant materials

BG1222, an *indica* rice cultivar from Sri Lanka, has demonstrated a good resistance to BB disease (Zeng et al. 2002). The F_1 progenies and F_2 population, developed as a cross of BG1222 (*O. sativa* ssp. *indica*) carrying the resistance gene and highly susceptible cultivar IR24 (*O. sativa* ssp. *indica*), were used to analyze the dominance or recessiveness of the BB resistance gene and map it in BG1222. F_1 progenies and F_2 population of the reciprocal crosses (BG1222/IR24 and IR24/BG1222) were inoculated with Chinese race V to determine the *Xoo* resistance gene numbers and construct the F_2 mapping population used for genetic and physical mapping of the target gene. F_1 progenies (IR24/BG1222) and their backcross BF_1 (IR24/BG1222/BG1222) were detected for determining the *Xoo* resistance gene numbers of BG1222 by inoculating race V. To distinguish the gene in BG1222 and known BB recessive resistance gene *xa5* which are both resistant to Chinese race V, allelic test was carried out according to the resistance response of F_1 progenies and resistance/susceptibility ratio of F_2 population derived from the cross between BG1222 and IRBB5(*xa5*).

Disease evaluation

Chinese typical *Xoo* race V (strain 5226, incompatible with BG1222 and compatible with IR24) was selected to evaluate the resistance of F_1 progenies and the F_2 individuals derived from the cross between BG1222 and IR24 by the leaf-clipping method at the booting stage (Kauffman et al. 1973). BB resistance to strain 5226 was evaluated by scissors-clipping three of the youngest leaves of each plant approximately 2 cm below the leaf tips with a bacterial suspension having an $OD_{600} = 0.6$. The inoculum was prepared from bacteria revived from glycerol stocks and grown for 72 h in nutrient yeast sucrose broth at 30°C. After inoculation, the plants were maintained in a growth chamber relative humidity above 80%, night temperatures of 28°C and day temperatures of 32°C. The plants were considered as resistant or susceptible as determined by the average lesion length which was measured for three leaves after 20 days of inoculation (Kauffman et al. 1973). Leaf tissue of these plants was stored at −70°C for DNA extraction from a portion of the uninoculated tissue harvested at the time of bacterial blight inoculation. The controls used during this process were both parents of the F_2 population.

SSR markers detection and linkage analysis

Plant DNA was prepared from frozen leaves of rice plants using the CTAB method (Murray and Thompson 1980). The F_2 population was genotyped with simple sequence repeat (SSR) markers adopted from the ''Gramene'' database (http://www.gramene.org; International Rice Genome Sequencing Project 2005). The detection procedures were followed as defined by Blair and McCouch (1997). The recessive class analysis (RCA; Zhang et al. 1994) was used to identify polymorphic molecular markers linked to the resistance gene. Linkage analysis was conducted with Mapmaker/Exp (version 3.0) with a threshold LOD score of 3.0 (Lincoln et al. 1992). The recombination frequencies were transformed into centimorgans according to the Kosambi function (Kosambi 1944).

Indel markers designed and linkage analysis

For fine mapping the target gene, a series of Indel markers were designed. First, we ascertained the exact region of the target gene based on the results that SSR detected, then the reference sequences of *japonica* and *indica* between the target regions were downloaded from the rice database (http://rgp.dna.affrc.go.jp/IRGSP/) and these sequences were compared by the BLAST tool at the NCBI website (http://www.ncbi.nlm.nih.gov). Finally, the Indel markers with more than 10-bp consecutive insertions or deletions were designed with the software FastPCR 5.4 (http://www.biocenter.helsinki.Fi/bi/programs/fastpcr.htm).

Genetic and physical mapping of BB resistance gene

The genetic map of the resistance gene was constructed according to the genetic distances of the SSR markers which were linked to the targeted 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). The PAC/BAC clones were aligned using the software tool Pairwise BLAST (http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html) derived from the gene-anchored markers of the target gene.

Candidate gene annotation

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

Results

Resistance inheritance of BG1222

Chinese typical *Xoo* races were divided into 9 pathotypes (I–IX), of which a new race (designed as IX) was identified in Guangdong (Zeng et al. 2005), South China. BG1222 is resistant to all of the races I, II, III, IV, V, VIII and IX (Zeng et al., 2005). Since *Xoo* race V is the prevailing race in South China, it was used to analyze the resistance inheritance of the donor BG1222. An isolate 5226 of race V was selected to inoculate BG1222 and IR24, F_1 progenies and F_2 individuals and both of the parents at the booting stage. All the individuals were measured for lesion length after 20 days of inoculation. The evaluated results showed that BG1222 is highly resistant to the isolate 5226 with an average lesion length of 0.45 ± 0.15 cm, and IR24 was highly susceptible to the tested pathogen with an average lesion length of 25.4 ± 2.55 cm (Fig. 1). F_1 descendants showed a clearly susceptible reaction to strain 5226 with an average lesion length of 20.2 ± 2.36 cm (Fig. 1). Reciprocal crosses were carried out between BG1222 and IR24. Using a lesion length of 5 cm as the dividing point, the numbers of resistant (R, lesion length < 5 cm) and susceptible (S, lesion length $\geqq 5$ cm) F_2 individuals in two crosses were 128R:378S and 119R:375S, respectively, which both fitted the expected 1R:3S segregation ratio against the isolate 5226 (Table 1), indicating that the resistance of BG1222 was controlled by a single recessive gene against Chinese race V. The same results of reciprocal crosses also suggested that the resistance of BG1222 is controlled by nuclear genes, not by cytoplast. To further determine the number of *Xoo*

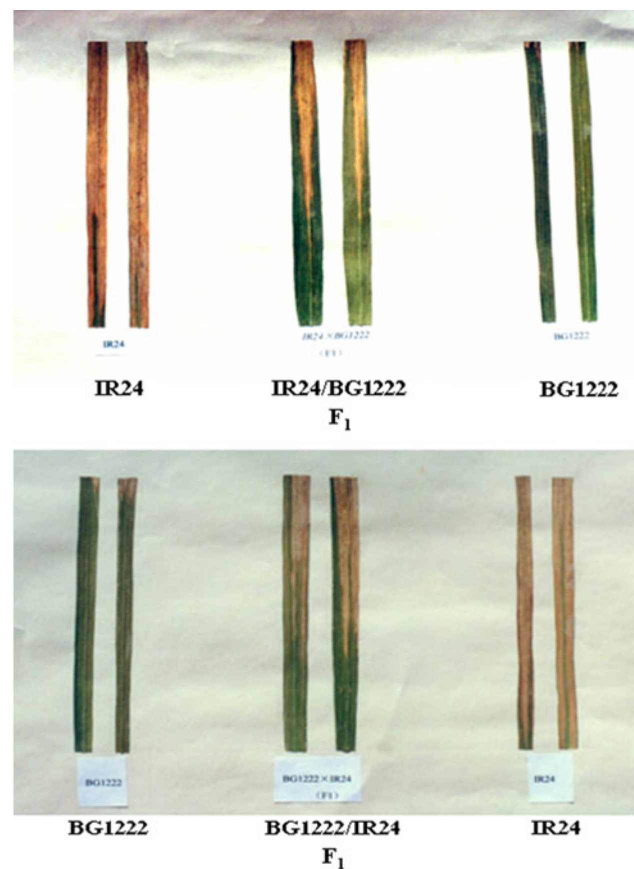

Fig. 1 Reactions of BG1222, IR24 and the reciprocal crosses (BG1222/IR24 and IR24/BG1222) F_1 progenies to Chinese typical *Xanthomonas oryzae* pv.*oryzae* race V (strain 5226, incompatible with BG1222 and compatible with IR24)

Table 1 Resistance response of F_1 and F_2 of reciprocal crosses between BG1222 and IR24 against Chinese race V

Cross (P1/P2)	Resistance response of parents		F_1 resistance response	F_2 resistance response					
	P1	P2		Numbers of R	Numbers of S	Total numbers	Segregation ratio	χ^2	*P*
IR24/BG1222	S	R	S	128	378	506	1 : 3	0.0237	0.90–0.75
BG1222/IR24	R	S	S	119	375	494	1 : 3	0.2186	0.75–0.50

S susceptible, *R* resistant

Table 2 Resistance response of F_1 and B_1F_1 of backcross between BG1222 and IR24 against Chinese race V

Cross (P1/P2)	F_1 resistance response	B_1F_1 of backcross	Numbers of R	Numbers of S	Segregation ratio	χ^2	*P*
IR24/BG1222	S	IR24/BG1222/BG1222	38	42	1 : 1	0.20	0.75 ~ 0.50

S susceptible

Table 3 Allelism test between BG1222 and IRBB5 and resistance response of F_1 and F_2 of the cross against Chinese race V

Cross (P1/P2)	Resistance response of parents		F_1 resistance response	F_2 resistance response					
	P1	P2		Total numbers	Numbers of R	Numbers of S	Segregation ratio	χ^2	*P*
BG1222/IRBB5	R	R	S	314	139	175	7 : 9	0.0342	0.90 ~ 0.75

R resistant, *S* susceptible

resistance genes in BG1222, F_1 progenies and its B_1F_1 individuals were inoculated with race V. Resistance response results showed that all F_1 progenies were susceptible, and segregation ratio of B_1F_1 individuals was 1R:1S (Table 2), indicating that the resistance of BG1222 is exactly controlled by a single gene against race V.

Allelic test

Until now, only one recessive *Xoo* resistance gene *xa5* identified is resistant to *Xoo* Chinese race V in South China (Zeng et al. 2002). To determine whether the *xa5* gene is an allele of the recessive gene in BG1222 whose *Xoo* resistance gene also shows high resistance to Chinese race V, the F_1 progenies from the cross between BG1222 and IRBB5 were inoculated with isolate 5226 of race V. The segregation of resistant and susceptible plants was observed in this cross with the ratio of 7R:9S (Table 3), indicating that the recessive resistance gene in BG1222 is not allelic to *xa5*.

Construction of *R* genes' mapping population

For mapping the recessive resistance gene in BG1222 against race V, all the resistant individuals of the reciprocal crosses between BG1222 and IR24 inoculated with the isolate 5226 at the booting stage were combined as a big well-mixed F_2 mapping population. The distribution of the lesion length in the total 1,000 plants was bimodal with an apparent valley at approximate 5 cm (Fig. 2). Integrating the two reciprocal crosses F_2 population, the number of resistant (R, lesion length < 5 cm) F_2 individuals was 247. So 247 R individuals were used as the population to map the target gene because of the high efficiency of their recessive class (Zhang et al. 1994).

Genetic mapping of the target gene

To ascertain the chromosomal position of the target gene, a total of 320 known SSR markers were selected from 12 rice chromosomes with intervals of about 8 cM to survey R (resistance)/S (susceptible) parents and pools through the bulked-segregant analysis (BSA) approach. The results indicated that two SSR markers, RM493 and RM446 on chromosome 1, showed obvious polymorphisms for the R/S parents and pools. The linkage analysis of the two markers showed that 21 and 15 distinct recombinants in the mapping population were identified at RM493 and RM446 loci with the genetic distances of 4.29 and 3.05 cM. Since the 21 and 15 recombinants of RM493 and RM446 were

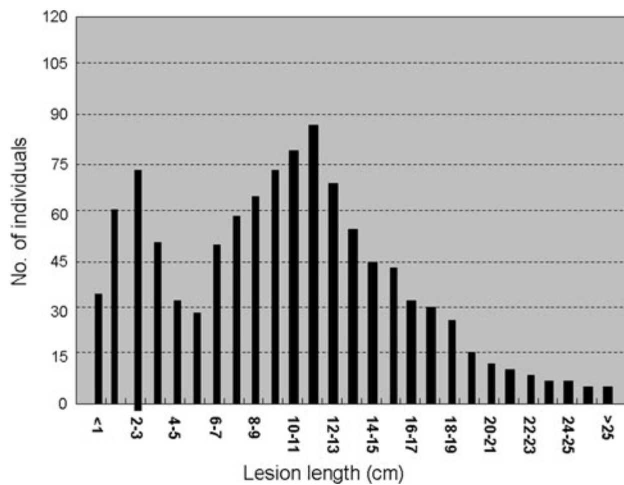

Fig. 2 Distribution of lesion length after inoculation with Chinese *Xanthomonas oryzae* pv. *oryzae* race V (strain 5226) in a sample containing 1000 individuals from two F_2 populations derived from a reciprocal crosses between BG1222 and IR24

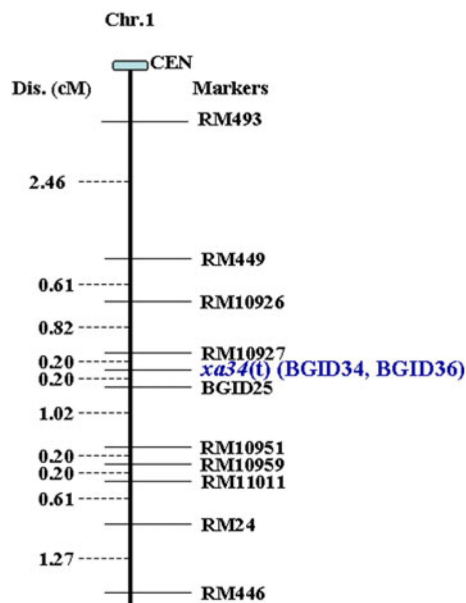

Fig. 3 High resolution genetic map of the *xa34*(t) region on the short arm of chromosome 1. The chromosome is represented as a filled bar. SSR and Indel markers used in this phase of the study are indicated to the right of the bar; the distances between markers are indicated to the left

different, the target gene was mapped to a 7.34 cM genetic region between RM493 and RM446 (Fig. 3). Therefore, the two markers were identified as the initial point in mapping the target gene.

For fine mapping the target gene, an additional 50 SSR markers located in the target region were adopted from the "Gramene" database. The 50 SSR markers were first screened between the two parents and results showed that 23 SSR markers were polymorphic in this case. Only seven distinctly polymorphic markers were selected to carry out the linkage assay according to the 36 recombinants around the target gene. The results depicted three polymorphic markers on the RM493 side (RM449, RM10926 and RM10927) were linked to the gene at genetic distances of 1.22, 0.40 and 0.20 cM farthest to nearest; and four additional polymorphic markers on the RM446 side (RM24, RM11011, RM10959 and RM10951) were linked to the locus at genetic distances of 2.23, 1.62, 1.42 and 1.22 cM, respectively (Fig. 3). The fine mapping results revealed that the target gene was further defined in a 1.82 cM region between RM10926 and RM10951 where two and six recombinants from those identified at RM493 and RM446 site were detected (Fig. 3).

To narrow down the region surrounding the target gene, additional 43 Indel markers in the target region were designed to analyze the polymorphism between R/S parents. The surveyed results showed that 15 Indel markers exhibited polymorphism between the two parents. Only three markers BGID25, BGID34 and BGID36 (Table 4) were located in the first mapping region between RM10927 and RM10951 (Fig. 3). BGID25 detected two recombinants on the side of RM10951 whereas BGID34 and BGID36 were co-segregated with the gene (Fig. 4), all the F_2 homozygous resistance individuals showed the same band patterns to IR24. Thus, the target gene has been defined to a 0.80 cM region between RM10927 and BGID25. Since only one dominant *Xoo* resistance gene *Xa29*(t) was identified on the long arm of chromosome 1 (Tan et al. 2004), no other BB *R* genes were reported in the region, the target gene is denominated as *xa34*(t).

Physical map construction of *xa34*(t)

To physically map the *xa34*(t) gene, all the anchor markers were used to land on the reference sequences of cv. Nipponbare by BLASTN analysis. The sequences matching results showed that there were four PAC/BAC clones which covered the target gene region: B1111C09, P0416G11, B1150E06 and B1096D03. Of these, the marker RM10926 was landed on the BAC clone B1111C09, RM10930 on PAC clone P0416G11, RM10936 and RM10940 on BAC clone B1150E06, RM10951 on BAC clone B1096D03 (Fig. 5). The BAC/PAC clones identified from the IRGSP website were aligned as a contig map covering the *xa34*(t) gene via Pairwise BLAST analysis. Consequently, a physical map covering the *xa34*(t) gene was generated in combination with the genetic map (Fig. 5). Finally, the target gene was mapped to a region between markers RM10929 and RM10951 in a 0.42 cM genetic and 204 kb physical intervals based on the RGP BAC/PAC contigs (Fig. 5).

Table 4 Primer sequences of polymorphic insertion/deletion (Indel) markers used in gene mapping

Marker name	Forward (5′–3′)	Reverse (5′–3′)	PCR condition	Gel	Products size (bp)
BGID25	cccaattatgtagtaggcaactcc	tcgtgagcacatacatgttgca	A	I	148
BGID34	actaagctcattggcaaagctg	agccatattttgagaccgagggag	A	I	106
BGID36	cgagcacatcctcctcattgcg	gtaggtggctacggcgat	A	I	180

A After preheating 4 min at 94°C, 35 cycles (30 s at 94°C, 45 s at 58°C, 1 min at 72°C), followed by 5 min at 72°C

I 8% acrylamide

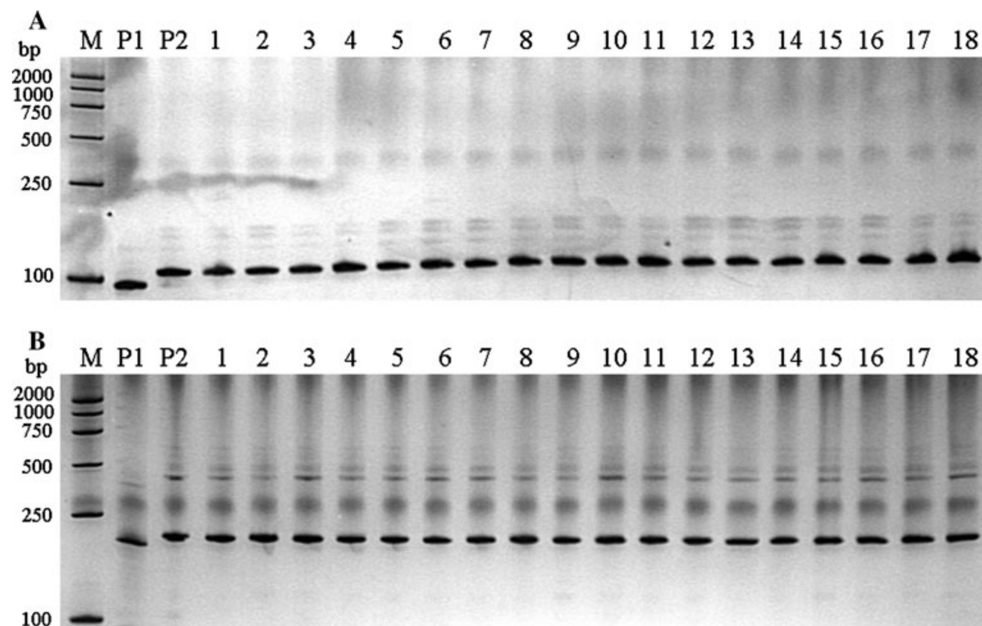

Fig. 4 Development of two PAGE-based Indel markers for the *Xanthomonas oryzae* pv. *oryzae* resistance genes *xa34*(t). **a** PCR product pattern of co-segregated marker BGID34 for the *xa34*(t) gene. **b** PCR product pattern of co-segregated marker BGID36 for the *xa34*(t) gene. M indicates DNA marker DL2000. P1 indicates resistant parent BG1222. P2 indicates susceptible parent IR24. 1–18 indicate the F_2 homozygous resistance individuals

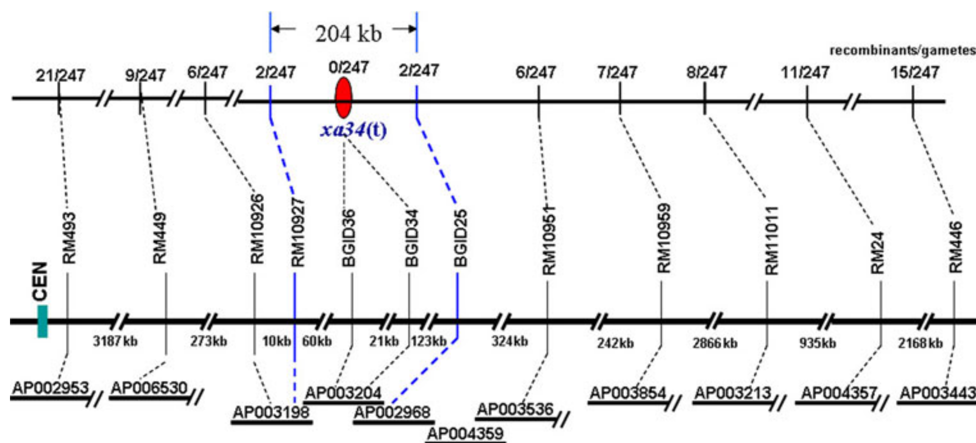

Fig. 5 Physical map of the *xa34*(t) gene. Twelve SSR markers were used in this study. The *long horizontal lines* indicate the region containing the *xa34*(t) gene. The *short horizontal lines* represent the BAC/PAC clones of cv. Nipponbare released by IRGSP and assembled by the corresponding markers linked to the *xa34*(t) gene. The numbers on upside of the above *long horizontal lines* are the recombination events in the mapping populations (recombinants/gametes are indicated). The digits between markers are physical distances in kilobase (kb). The *vertical and dashed lines* denote the relative positions of the corresponding markers

Candidate genes prediction of *xa34*(t)

The *xa34*(t) gene was determined to be within a 204 kb region as calculated by softwares FGENESH, RiceGAAS, and GeneScan. These programs only predicted 21 ORFs. Among the target ORFs, seven encode hypothetical protein unclassified, three encode expressed protein without gene ontology annotation found, five encode putative retrotransposon protein unclassified, the other seven encode glutathione S-transferase, putative TNP-like transposable element, laccase precursor, E3 ubiquitin-protein ligase MGRN1, heat shock protein DnaJ N-terminal domain-containing and 3-dehydroquinate dehydratase/shikimate 5-dehydrogenase protein, respectively. No knowable encoded proteins of BB resistance genes cloned are present in the objective region.

Discussion

Utilization of recessive *Xoo* resistance genes might be an effective strategy to control BB disease. Recessive resistance function is derived from the mutation of dominant susceptibility ('S') alleles, such as *Xa5* and *Xa13* (Chu et al. 2006; Iyer and McCouch 2004). *S* genes are significant for bacterial virulence whereas mutations in matching effector genes may bear a fitness cost (Leach et al. 2001), suggesting that recessive resistance genes may be more durable (Iyer-Pascuzzi and McCouch 2007). Many resistance genes 'break down' when they have been widely used for numerous years in a large population over an extended area which is conducive for disease development. Exploitation of durable resistance genes will help to solve this problem. Though *xa5* and *xa13* are not broad spectrum resistance genes, they provide stronger and more diversified levels of resistance used with *Xa4* and *Xa21* than when used alone (Huang et al. 1997; Shanti et al. 2001). The present study indicates that *xa34* is a novel recessive *Xoo* resistance gene conferring an extensive resistance to bacterial blight. BB resistance genes *Xa3*, *Xa4* and *Xa21* have been widely used for Chinese rice breeding programs in the past (Chen et al. 2001; Zhai et al. 2002), however, the resistances of these genes have weakened, many races in South China could infect the cultivars carrying *Xa3*, *Xa4* and *Xa21* (Zhang et al. 2002). In South China, only a few known BB resistance genes such as *xa5*, *Xa7*, *Xa23*(t) and *xa34*(t) (the present) show a broad spectrum resistance to the local races of *Xoo* (unpublished data). Thus, identification of *xa34*(t) is promising for rice breeding programs based on the recessive resistance genes strategy. The fine mapping of *xa34*(t) using PCR-based molecular markers provides a convenient tool for marker-assisted selection of *xa34*(t).

The *xa34*(t) gene has been defined to a 204 kb region near centromere of rice chromosome 1. Base on TIGR Rice Genome Annotation Version 6.0, the 204 kb region harboring the *xa34*(t) gene had been analyzed, which indicated that this region contained 21 genes. But the structures of these candidate genes were different from all the other *Xoo* resistance genes which have been cloned, suggesting that *xa34*(t) might be a new type of BB resistance gene.

Among the identified *Xoo* recessive resistance genes, only two, *xa5* and *xa13,* have been characterized (Chu et al. 2006; Iyer and McCouch 2004). The *xa5* allele provides race specific resistance to *Xoo* and encoded the small subunit of transcription factor IIA (TFIIAγ). The *xa5* sequence assay showed that a 2-nucleotide substitution at position 39 in the second exon of the gene resulted in an amino acid change from valine (V39) in susceptible cultivars to glutamic acid in resistant (Iyer and McCouch 2004). The second cloned *Xoo* recessive resistance gene *xa13*, encodes unique 307-amino acid plasma membrane-localized proteins that show 68% sequence similarity to the MtN3 protein induced by *Rhizobium* spp. in legume nodule development. The gene expression result showed that promoter mutations in *Xa13* cause down-regulation of expression during host-pathogen interaction, resulting in the fully recessive *xa13* that confers race-specific resistance (Chu et al. 2006). Some reporters such as Kottapalli propose that the recessive gene mediated resistance mechanism is more complex and might not be governed by a single gene through extensive in silico analyses of the genomic environment around *xa5* and *xa13* regions (Kottapalli et al. 2007). Although *xa5* and *xa13* represent two kinds of resistant mechanisms, and characterization of both genes has stimulated research in the molecular mechanisms of plant recessive gene-for-gene resistance to bacterial pathogens. Moreover, it is not clear how the *Xoo* recessive resistance genes mediate resistance to BB. It is necessary to further make clear why so many recessive resistance genes exist in the rice-*Xoo* pathogen system, much more empirical data is needed. The *xa34*(t) gene might be a novel recessive resistance gene that confers a new type of plant disease resistance based on our research. A complete and accurate description of *xa34*(t) will help to reveal the recessive resistant mechanisms in the rice-*Xoo* pathogen system.

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