

RESEARCH ARTICLE

Promoter variants of *Xa23* alleles affect bacterial blight resistance and evolutionary pattern

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Abstract

Bacterial blight, caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), is the most important bacterial disease in rice (*Oryza sativa* L.). Our previous studies have revealed that the bacterial blight resistance gene *Xa23* from wild rice *O. rufipogon* Griff. confers the broadest-spectrum resistance against all the naturally occurring *Xoo* races. As a novel executor *R* gene, *Xa23* is transcriptionally activated by the bacterial avirulence (Avr) protein AvrXa23 via binding to a 28-bp DNA element (*EBE_{AvrXa23}*) in the promoter region. So far, the evolutionary mechanism of *Xa23* remains to be illustrated. Here, a rice germplasm collection of 97 accessions, including 29 rice cultivars (*indica* and *japonica*) and 68 wild relatives, was used to analyze the evolution, phylogeographic relationship and association of *Xa23* alleles with bacterial blight resistance. All the ~ 473 bp DNA fragments consisting of promoter and coding regions of *Xa23* alleles in the germplasm accessions were PCR-amplified and sequenced, and nine single nucleotide polymorphisms (SNPs) were detected in the promoter regions (~131 bp sequence upstream from the start codon ATG) of *Xa23/xa23* alleles while only two SNPs were found in the coding regions. The SNPs in the promoter regions formed 5 haplotypes (*Pro-A, B, C, D, E*) which showed no significant difference in geographic distribution among these 97 rice accessions. However, haplotype association analysis indicated that *Pro-A* is the most favored haplotype for bacterial blight resistance. Moreover, SNP changes among the 5 haplotypes mostly located in the *EBE/ebe* regions (*EBE_{AvrXa23}* and corresponding *ebes* located in promoters of *xa23* alleles), confirming that the *EBE* region is the key factor to confer bacterial blight resistance by altering gene expression. Polymorphism analysis and neutral test implied that *Xa23* had undergone a bottleneck effect, and selection process of *Xa23* was not detected in cultivated rice. In addition, the *Xa23* coding region was found highly conserved in the *Oryza* genus but absent in other plant species by searching the plant database, suggesting that *Xa23* originated along with the diversification of the *Oryza* genus from the grass family during evolution. This research offers a potential for flexible use of novel *Xa23* alleles in rice breeding programs and provide a model for evolution analysis of other executor *R* genes.

Introduction

Plants could co-evolve in response to changes of pathogens [1]. Resistance of a plant to a pathogen with Avr protein effectors, which can be secreted and internalized into the plant cells through type-III secretion pathway, is due to the recognition by plant surveillance system [2]. Accordingly, plants have evolved resistance (*R*) genes to interact with their cognate *avr* genes and activate host immune responses [3, 4]. However, intensive diversifying selection allowed the pathogen to diversify its effector genes and escape recognition by the plant resistance gene, resulting in loss of the *R* gene-mediated resistance [1]. The constant interactions between hosts and pathogens are thought to play an important role in the evolution of *R* genes in plants and *avr* genes in pathogens. Thus, an in-depth study of the molecular evolution of *R* genes will be of significance for identifying novel or “hidden” resistant alleles and unravelling the role of pathogen-imposed selection of *R* genes [5–8].

The polymorphism and molecular evolution of plant *R* genes have been extensively studied [9–13] and found three kinds of distinctly regular patterns [8, 14–16]. The first is conserved type with little variation in the population or species, which identifies conservative *avr* genes and accounts for 63% of the *R* genes of rice genome, such as *Pi-ta* [17]. The second is the opposite type with abundant variation in population or species, which shares the mutations among different alleles by recombination and identifies conservative and non-conservative *avr* genes, such as *Rpp13* and *Rpp8* [18, 19]. The third is present and absent *R* genes, such as *Rpm1* and *Rpm5* [20]. Although, in contrast to plants, pathogens have always been the dominant force in such arm-races, it may be a feasible way to identify the disease resistance genes against the pathogen by following the variation pattern. However, previous studies have mostly focused on nucleotide-binding site leucine-rich repeat (*NBS-LRR*) type *R* genes and there is no in-depth investigation on genetic diversity analysis of the plant executor *R* genes which is triggered by activator-like effector (TALE) to activate defense response in plants [21–25].

Bacterial blight (BB) caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) is one of the most harmful and overwhelming diseases in rice [25]. This disease is rampant in humid tropic and temperate regions and reduces rice production up to 50%–90% [26]. To date, three executor *R* genes have been identified and cloned in rice [25]. *Xa10* and *Xa27* are two executor *R* genes, encoding 126- and 113-aa proteins respectively, with no conserved domains of any known *R* gene product [21, 24]. *Xa27* is the first cloned executor *R* gene in plants that provides disease resistance to *Xoo* strains obtained from different Asian countries [21, 27] and the *XA27* protein depends on its N-terminal signal-anchor-like sequence to localize to the apoplast [28]. The resistant allele *Xa27* in the rice line IRBB27 and the susceptible allele *xa27* in IR24 contain the identical coding sequences but only the resistant allele expresses *Xa27* upon infection by *Xoo* strains expressing AvrXa27, a natural TALE protein [21]. Expressing of *Xa27* depends on the interaction between its promoter and AvrXa27. Similarly, *Xa10* gene in rice confers race-specific but narrow-spectrum resistance to *Xoo* strains containing transcription activator-like effector (TALE) gene *avrXa10* [29, 30]. Unlike *Xa27*, the tested susceptible cultivars (Nipponbare, IR24, IRBB5 and 93–11) do not have an identical open reading frame (ORF) of *Xa10* [24].

The *Xa23*, a recently cloned BB resistance executor *R* gene identified from wild rice (*O. rufipogon*) is transcriptionally activated by AvrXa23, a transcription activator-like effector (TALE) encoded in *Xoo* strains [31]. Because the *avrXa23* is widely present in all tested naturally occurring *Xoo* strains, the rice with *Xa23* exhibit extremely broad-spectrum resistance against bacterial blight [32]. *Xa23* is an intronless gene encoding a 113-amino acid protein with three predicted trans-membrane domains [25]. CBB23, a near isogenic line obtained through transfer of *Xa23* from the wild rice into cultivated *indica* rice (*O. sativa* ssp. *indica*) variety JG30,

confers the broadest resistance to *Xoo* strains [32]. Comparatively, the recessive allele *xa23* in JG30 which is susceptible to *Xoo* strains contains the identical coding region of *Xa23*. The main nucleotide polymorphism between these two alleles (*Xa23* in CBB23 and *xa23* in JG30) is located in their respective promoter regions. The 28-bp $EBE_{AvrXa23}$ (*AvrXa23* binding element) is present in the promoter of *Xa23* allele and can interact with *AvrXa23*. Through the analysis of polymorphisms in coding and promoter regions of *Xa23/xa23* alleles in cultivated rice and their wild relatives, we would investigate whether the promoter mutation of *Xa23* plays an important role in resistant varieties and study the origin of this allele and trace its ancestry among the genetically divergent subpopulations of rice.

In this work, we analyzed nucleotide diversity in the promoter regions (-131 bp upstream sequence from the start codon ATG) and coding regions of *Xa23/xa23* alleles from 97 different rice varieties/accessions including 29 cultivated rice (consist of 20 *indica* and 9 *japonica*) and 68 wild relatives (consist of 41 *O. rufipogon*, 10 *O. nivara*, 14 *O. officinalis wall*, 1 *O. latifolia desy*, 1 *O. glumaepatula* and 1 *O. alta swallen*). The major objectives of this study were: (1) to find out homologs of *Xa23* across different plant species; (2) to analyze the nucleotide diversity of *Xa23/xa23* alleles; (3) to detect the association between *Xa23/xa23* haplotypes and BB resistance as well as the haplotypes distribution in rice; and (4) take the evolutionary model of *Xa23* as a direction or route map for other plant executor *R* genes to develop more BB resistant resources and produce valuable materials for rice breeding.

Materials and methods

Plant materials and growth conditions

A total of 97 rice varieties/accessions (20 *indica*, 9 *japonica* and 68 wild relatives) (S1 Table) were used for sequencing and nucleotide diversity analysis of *Xa23/xa23* alleles. The 97 rice materials are in forms of DNA, rice leaves and rice seed samples. Among them, seeds of 51 rice materials were planted in a paddy field in Beijing (39°54'N, 116°23'E), China.

Database search and identification of *Xa23* homologs

We blasted the $EBE_{AvrXa23}$ (28-bp) and *ORF* (342-bp) sequences of *Xa23* allele from CBB23 in NCBI (National Center for Biotechnology Information, <http://www.Ncbi.nlm.nih.gov>), Gramene (A comparative resource for plants, <http://www.gramene.org/>) and RGAP (Rice Genome Annotation Project, <http://rice.plantbiology.msu.edu/index.shtml>). We also searched the $EBE_{AvrXa23}$ sequence in Rice SNP-Seek Database (<http://www.oryzasnp.org/iric-portal>) which consist of Oracle database having a total number of rows with SNP genotypes near to 60 billion (20 M SNPs \times 3 K rice lines) and web interface for convenient querying [33, 34] and Phytozome (<http://phytozome.jgido.gov/pz/portal.html>). Altogether 39 plant species were selected to retrieve the $EBE_{AvrXa23}$ and *Xa23* coding sequences. The *Xa23* in CBB23 and *xa23* in JG30 were used as reference sequences [25].

DNA extraction, PCR and sequencing

Genomic DNA was extracted from ~ 100 mg of rice leaves by using CTAB method [35]. Based on the known sequence of *Xa23* in CBB23, considering that the flanking sequences of *Xa23* gene are highly complex (one transposon and four repeat sequences) may affect the amplification results, we used the Primer Premier 5.0 (Premier Biosoft, Palo Alto, CA) to design several pairs of primers (Fig 1, S1 Fig, S2 Table) to make the amplification results more accurate. Depending on the positions of the forward and reverse primers, they can be matched with each other for amplification of the corresponding *Xa23* alleles in different *Oryza* species.

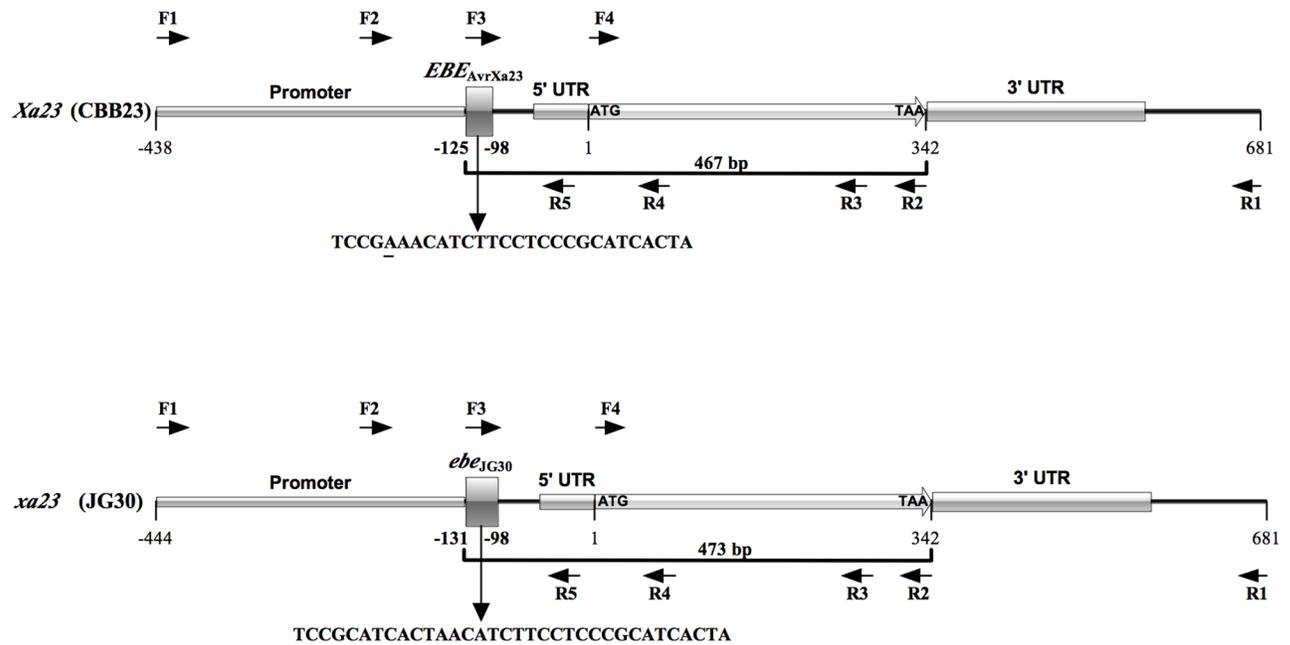


Fig 1. A schematic presentation of gene structure of *Xa23/xa23* alleles and primer positions. The resistant *Xa23* allele in CBB23 and susceptible *xa23* allele in JG30 were used to show the gene structure and primer locations. Overlapping primers were designed to amplify DNA fragments covering the *EBE_{AvrXa23}* in CBB23, the corresponding *ebe* region in JG30 and their coding regions; arrows represent the locations and the orientations of the primers. The numbers indicate the positions and lengths of different regions in the two alleles. The alignment regions are the sequences ranging from the first nucleotide of the *EBE/ebe* to the last nucleotide of the stop codon. Because of the *ebe_{JG30}* has 7-bp polymorphic nucleotides (6-bp insertion and 1-bp substitution, underlined) compared to the *EBE_{AvrXa23}*, the corresponding alignment regions in length are 467-bp in *Xa23* of CBB23 and 473-bp in *xa23* of JG30, respectively.

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Polymerase Chain Reaction (PCR) was performed in a Veriti 96-Well Thermal Cycler using highly-efficient KOD polymerase in a total volume of 20 μ l reaction mixture. Briefly, reaction mixture contained 100 ng of genomic DNA, 0.5 μ M of each primer, 0.2 mM of each dNTP, 2 \times PCR buffer (10 mM Tris-HCl, pH 8.8, 1.5 mM MgCl₂) 10 μ L, and 0.2 unit (1U/ μ L) of KOD. The PCR profile consists of 3 min initial denaturation at 94°C, 35 cycles of amplification with 30 s DNA denaturation at 94°C, 30 s annealing at 60°C and a final elongation at 72°C with 30 s-60 s depending on the length of different fragment. Subsequently, all amplified products were visualized on 1% agarose gels and sequenced.

Statistical analysis

The genomic sequences of *Xa23* alleles obtained from 97 rice accessions were aligned and assembled by *Clustal X* version 2.0 [36] and *BioEdit* [37, 38]. The aligned file was used as an input format for analysis into *DnaSP* version 5.0 [39]. The number of polymorphic sites, including single nucleotide polymorphisms (SNPs), insertions and deletions (InDels) in promoter and coding regions were determined according to *DnaSP* version 5.0 [39]. Nucleotide diversity was also analyzed by estimating average number of nucleotide diversity per pair (π) [40] and number of segregating sites (θ_w) [41]. Different neutral test such as Tajima's *D* [42] and Fu and Li's *D* [43] were calculated separately for *indica*, *japonica* and wild rice by using *DnaSP* to determine whether the locus is departed from neutrality. Phylogenetic network analysis of the entire *Xa23/xa23* alleles was performed through *Network* version 4.6 [44].

Assessment of rice resistance to *Xoo* strain PXO99^A

The *Xoo* strain PXO99^A was cultured in PSA medium at 28°C for 48 hours. Bacterial suspensions ($OD_{600} = 1.0$) were used for inoculation on fully expanded rice leaves at the seedling stage. The pathogenicity assay on the PXO99^A was performed using the leaf-clipping method [45]. Disease symptoms were recorded two weeks after inoculation and measured by lesion length [46].

Results

Homologs search of *Xa23* allele in different species

Our previous work indicated that *Xa23* is a single copy gene in the rice variety CBB23 and the 28-bp $EBE_{AvrXa23}$ localized in the *Xa23* promoter is the core element for interaction with the pathogen effector AvrXa23. To find out the homologs of *Xa23* gene across different species, we performed a blast with CDS_{Xa23} (342-bp) and $EBE_{AvrXa23}$ (28-bp) sequences separately in Gramene (<http://www.gramene.org/>), NCBI (National Center for Biotechnology Information, <https://blast.ncbi.nlm.nih.gov/Blast.cgi>) and RGAP (Rice Genome Annotation Project, <http://rice.plantbiology.msu.edu/index.shtml>). As a result, we found *Xa23* homologous only in the *Oryza* genus; no significant homologous sequences with an *E-value* < 1 has been found in other plant species. In *Oryza*, the coding regions of *Xa23* alleles ranged from 327 bp to 492 bp in length and the nucleotide sequence identity compared with *Xa23* in CBB23 ranged from 88% to 100% (Table 1, S2 Fig), indicating a highly conserved coding region of *Xa23* alleles. Moreover, we didn't find any $EBE_{AvrXa23}$ sequence present in cultivated rice by Rice SNP-Seek Database retrieval, suggesting that the $EBE_{AvrXa23}$ is absent in the cultivated rice surveyed.

Polymorphism and haplotypes of *Xa23* alleles

We collected 97 representative rice varieties/accessions (S1 Table) to amplify the *Xa23* alleles by using overlapping gene-specific primers (Fig 1, S2 Table). The *Xa23/xa23* alleles from the 97 rice accessions were sequenced and aligned for their nucleotide diversity analysis. Including sites with alignment gaps, the length of total alignment (sequence starting from the *EBE/ebe* to the stop codon TAA, Fig 1) is 473 bp. Comparative analysis in *DnaSP*, excluding sites with gaps/missing data (in total 461 available sites), 11 SNPs and 3 insertions and deletions (InDels) events were detected in the aligned 473 bp (Fig 2A). Varied DNA polymorphisms were observed in promoter and coding regions of the *Xa23* alleles (Table 2).

Based on the detected 11 SNPs and 3 InDels events, the 97 rice varieties/accessions were divided into 10 haplotypes (H1-H10 in Fig 2, S1 Table). CBB23 was defined as haplotype H2. The wild rice accessions showed more various haplotypes, in which haplotypes H1, H6 and H9-H10 containing completely different SNPs were all represented by 68 wild relatives, while *indica* haplogroup contained only three haplotypes (H3, H5 and H8) which were defined by two SNPs at positions -126 bp and -88 bp upstream from ATG and one InDel, and haplotype H7 was only identified in *japonica* varieties. Notably, four haplotypes (H3, 4, 5 and 8) were shared in both wild and cultivated rice analyzed in this study and their distribution ratio was not significantly different (Fig 2A).

Compared with the coding region, more variations occurred in the promoter regions (Fig 2). Five haplotypes were formed by 9 SNPs and 2 InDels events within the promoter regions of *Xa23* alleles; these haplotypes were designated *Pro-A*, *Pro-B*, *Pro-C*, *Pro-D*, *Pro-E* (Fig 2B). Furthermore, haplotypes H1 consist of 15 (15.46%) wild rice and H2 (CBB23) belonged to *Pro-A*, haplotype H3 consist of 13 (13.40%) *indica*, 3 (3.10%) *japonica* and 13 (13.40%) wild rice belonged to *Pro-B*, and H4-H6 together account 5 (5.15%) *indica*, 4 (4.12%) *japonica* and 19

Table 1. Summary of sequence comparison among the coding regions of *Xa23* alleles in *Oryza* species with reference to CBB23.

Species & Accession	Coding region (bp)		Protein (a.a.)	
	Total	Identity (%)	Total	Identity (%)
<i>Oryza sativa indica</i> ASM465v1	342	99	113	100
<i>Oryza sativa japonica</i> IRGSP-1.0	342	100	113	100
<i>Oryza rufipogon</i> OR_W1943	333	100	110	100
<i>Oryza glumaepatula</i> ALNU02000000	342	99	113	99
<i>Oryza meridionalis</i> _v1.3	342	99	113	99
<i>Oryza longistaminata</i> _v1.0	339	88	112	77
ABB94457	492	100	163	100
<i>Oryza barthii</i> _v1	336	88	110	80
<i>Oryza glaberrima</i> AGI1.1	330	88	109	81
<i>Oryza punctata</i> AVCL00000000	327	88	108	83

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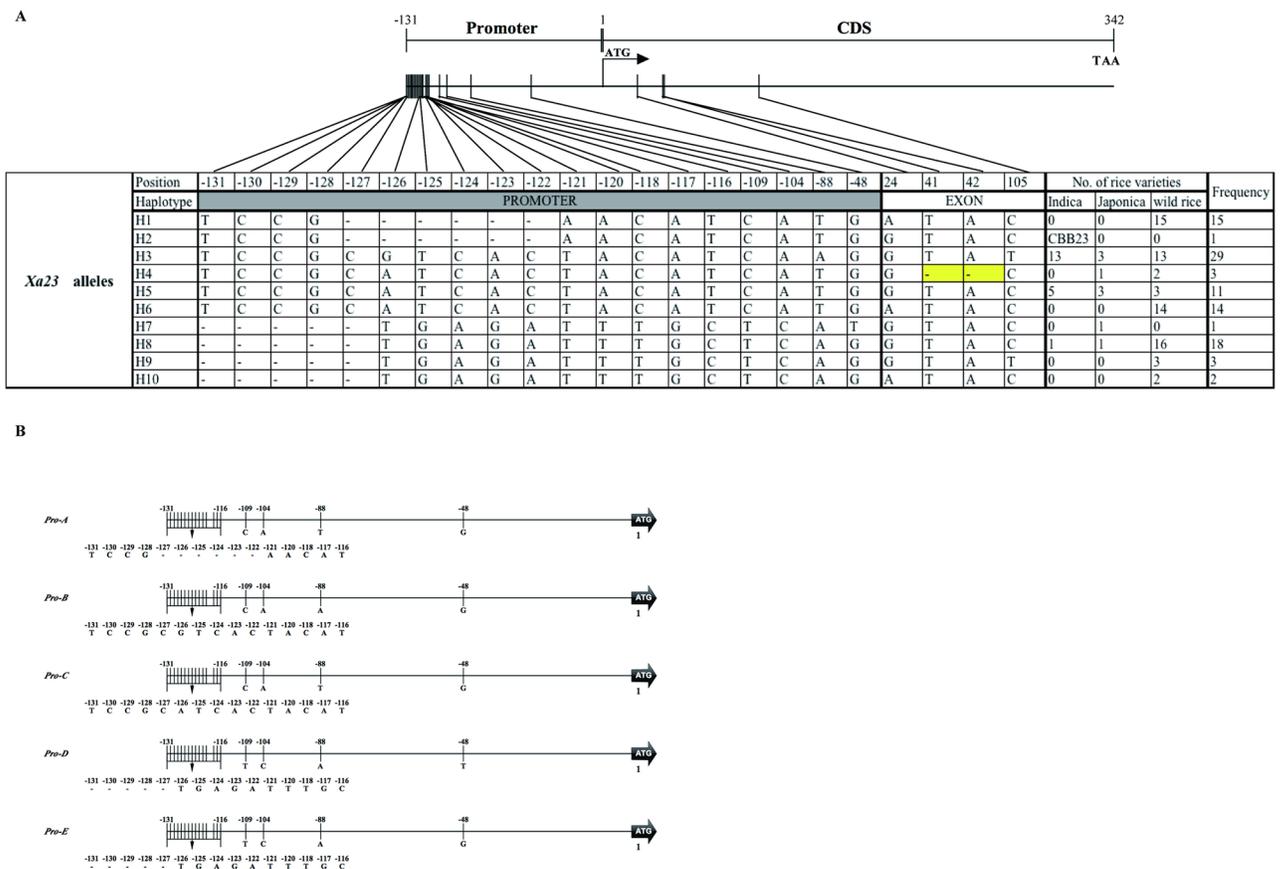


Fig 2. Haplotype analysis of the *Xa23* gene region in the 97 rice varieties/accessions. (A) The *Xa23/xa23* alleles contain the sequence covering promoter region (-131 bp upstream sequences from ATG start codon) and the coding region. The entire aligned length of the 473-bp genome sequence is shown in graphic on the top. The numbers on the top row shows the positions (cf. ATG start codon) of nucleotide polymorphisms in the region starting from the *EBE/ebe* to the stop codon TAA. The “-” indicate deletions, yellow represents missense mutations in the coding region. Ten haplotypes (H1-H10) were detected in the 97 representative rice varieties/accessions, which consist of 22 *indica*, 7 *japonica* and 68 wild rice. The total number of every haplotype and the number of every haplotype in different rice species are shown in the right columns. (B) Haplotypes in the promoter regions of *Xa23* alleles. Five haplotypes were formed by 9 SNPs and 2 InDels in the *Xa23/xa23* promoter regions.

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Table 2. Polymorphism and neutral test in different regions of *Xa23/xa23* alleles.

Species	<i>Xa23</i> alleles	Total sites (excluding sites with gaps)	Number of haplotype	S	InDel	π	θ_w	Fu and Li's <i>D</i>	Tajima' <i>D</i>	<i>K</i>	Ka/Ks(Jukes & Cantor)
Whole population	entire gene	461	9	11	3	0.00855	0.00464	0.76414	2.21473*	3.944	0.00864
	coding region	340	3	2	1	0.00261	0.00114	0.68782	1.98002	0.886	0.00261
	promoter region	121	5	9	2	0.02527	0.01445	0.57301	1.87651	3.058	0.02616
Indica	entire gene	463	4	9	0	0.0035	0.00548	-2.23543	-1.24143	1.621	0.00352
	coding region	342	2	1	0	0.0014	0.00082	0.64952	1.26176	0.47895	0.0014
	promoter region	121	4	8	0	0.00944	0.01864	-2.61649*	-1.66331	1.14211	0.00966
Japonica	entire gene	466	4	14	2	0.01264	0.01184	1.28707	0.32335	5.88889	0.01284
	coding region	340	2	1	1	0.00147	0.00108	0.8404	0.98627	0.5	0.00147
	promoter region	126	4	13	1	0.04277	0.04088	1.26193	0.22156	5.38889	0.04544
Wild rice	entire gene	461	7	10	3	0.00938	0.00453	1.38919	2.90562**	4.32485	0.00948
	coding region	340	3	2	1	0.00256	0.00123	0.71782	1.79613	0.86874	0.00256
	promoter region	121	4	8	2	0.02856	0.0138	1.28344	2.75975**	3.4561	0.02964

π , nucleotide diversity (average of nucleotide differences per site between two sequences); θ , Watterson estimator (no. of segregating sites); *K*, average nucleotide difference; Tajima's *D*, Fu and Li's *D*, test for neutral selection.

*Significant at $P < 0.05$;

**Significant at $P < 0.01$.

entire region: start from EBE/ebe to the TAA stop codon (-131 bp upstream sequences from ATG start codon to the TAA stop codon); promoter region: start from EBE/ebe to the ATG start codon (-131 bp upstream sequences from ATG start codon).

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(19.59%) wild rice belonged to *Pro-C*, H7 containing only 1 (1.03%) *japonica* belonged to *Pro-D* and H8-H10 consist of 1 (1.03%) *indica*, 1 (1.03%) *japonica* and 21 (21.65%) wild rice belonged to *Pro-E* (Fig 3). These results suggest that, among the five haplotypes in promoter regions, the haplotype *Pro-A* presented only in CBB23 and wild rice, whereas other four haplotypes (*Pro-B, C, D, E*) were distributed in both cultivated and their wild relatives; moreover, haplotype *Pro-B* seems like the major haplotype at *xa23* locus in the rice accessions used in this work.

The association between variations in coding regions of *Xa23* alleles and BB resistance phenotypes

In order to discover the association between the nucleotide diversity and BB resistant function, we first used a representative panel of 22 rice accessions (8 *indica*, 3 *japonica* and 11 wild relatives, S3 Table) to study the relationship between coding region variations and BB resistance. The coding regions of *Xa23* alleles were highly conserved across the 22 rice accessions, and two synonymous SNPs in total were identified at 24 (S1: G/A) and 105 (S2: T/C) positions in the coding regions. The two SNPs (S1 and S2) were found to be grouped into three genotypes A1 (S1G/S2C), A2 (S1G/S2T) and A3 (S1A/S2C) (S3 Table).

Focused on the 22 rice accessions, we found that 3 *indica*, 2 *japonica* and 4 wild rice containing A1, 4 *indica* and 1 *japonica* containing A2 and 2 wild rice containing A3 were

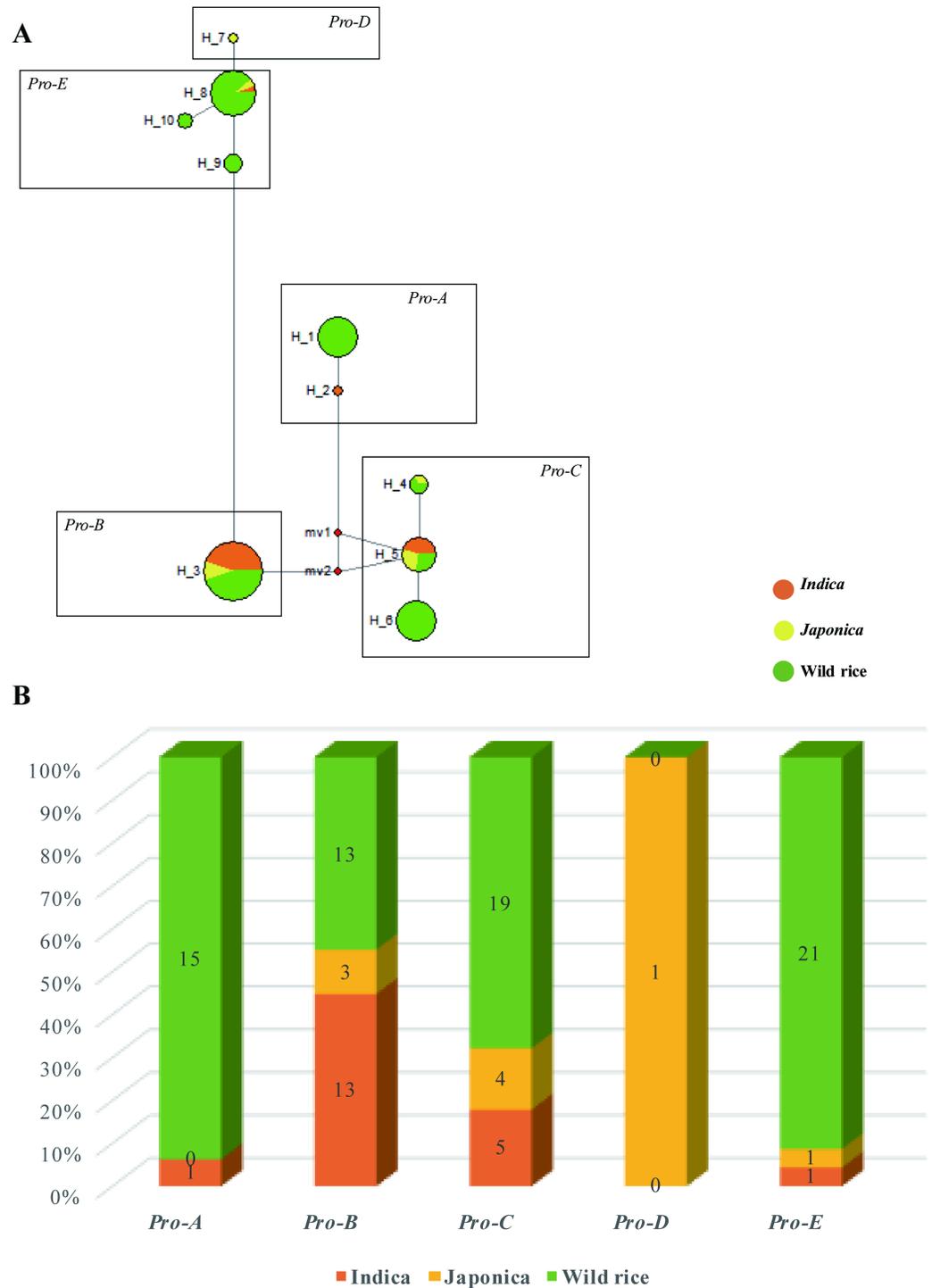


Fig 3. Haplotypes distribution of *Xa23* alleles. (A) Haplotype network of the *Xa23/xa23* alleles in 97 rice accessions. Haplotype frequencies are proportional to the area of the circles. The proportion of wild rice and two cultivated subgroups (*indica* and *japonica*) in each haplotype is represented by different colors. The five haplotypes (*Pro-A*, *B*, *C*, *D* and *E*) were formed based on their corresponding promoter regions. (B) The specific numbers of the five haplotypes (*Pro-A*, *B*, *C*, *D* and *E*) in 97 rice varieties/accessions.

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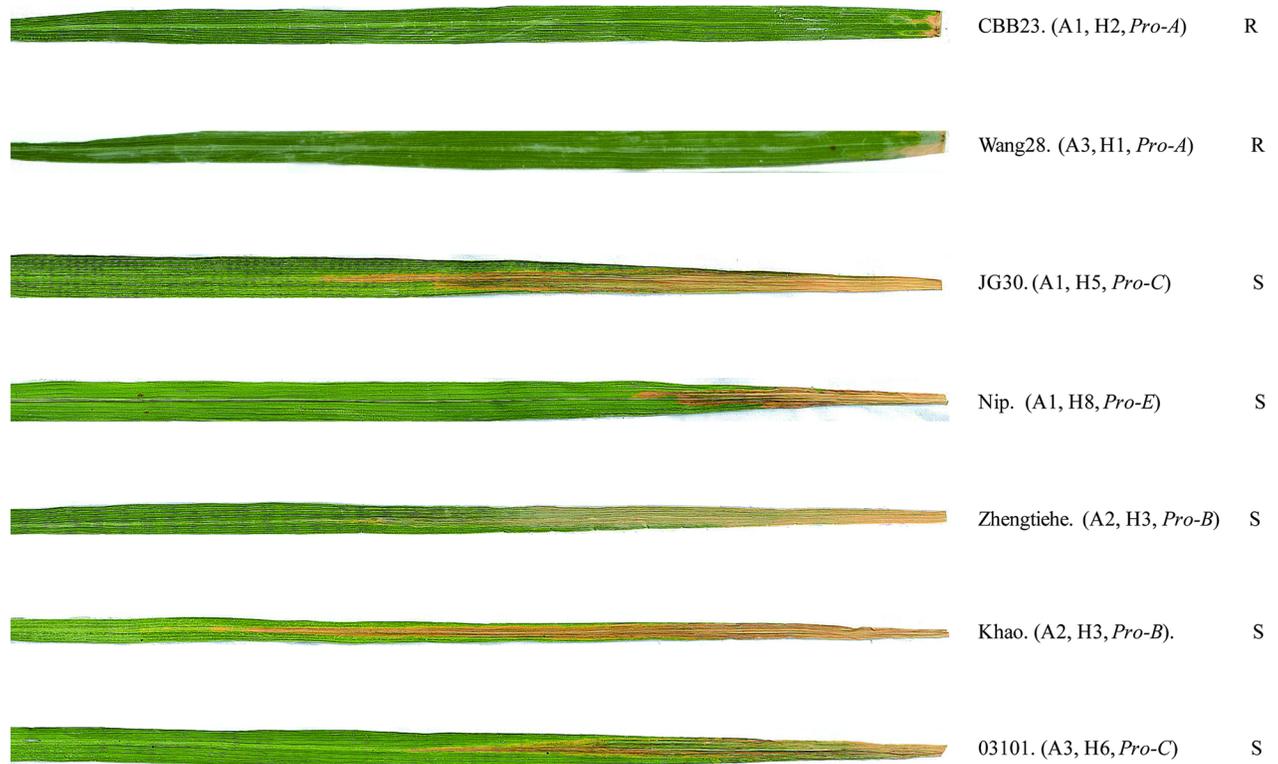


Fig 4. Disease reactions of representative rice accessions with different genotypes to PXO99^A. Representative leaves of rice accessions CBB23 (H2, *Pro-A*, *indica*), Wang28 (H1, *Pro-A*, *rufipogon*), JG30 (H5, *Pro-C*, *indica*), Nipponbare (Nip. H8, *Pro-E*, *japonica*), Zhengtiehe (H3, *Pro-B*, *indica*), Khao Dawk Mali 105 (Khao. H3, *Pro-B*, *indica*) and 03101 (H6, *Pro-C*, *rufipogon*) show the resistant (R) and susceptible (S) lesions caused by *Xoo* strain PXO99^A. Photographs were taken 14 days after inoculation. These six rice accessions contained 3 different types of coding regions, among which CBB23, JG30 and Nipponbare represent A1 (S1G/S2C), Zhengtiehe and Khao Dawk Mali 105 represent A2 (S1G/S2T), Wang28 and 03101 represent A3 (S1A/S2C).

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susceptible to *Xoo* strain PXO99^A, whereas 1 *indica* containing A1 and 5 wild rice containing A3 all exhibited resistance (Fig 4, S3 Table). Consequently, our association study indicated that S1 and S2 have no correlation ($r^2 = 0.137$, $p = 0.13477$) with the resistance/susceptibility response to PXO99^A. Therefore, the variations in the coding regions of *Xa23* alleles did not significantly affect rice resistance to *Xoo* strain PXO99^A.

The association between promoter haplotypes of *Xa23* alleles and BB resistance phenotypes

Because the coding region polymorphism of *Xa23* alleles did not significantly influence the BB resistance phenotypes, we selected 51 rice varieties/accessions (13 *indica*, 5 *japonica* and 33 wild relatives, Table 3) to further study the association between the promoter region haplotypes (*Pro-A*, *B*, *C*, *D* and *E*) and BB resistance phenotypes. As a result, we found *Pro-A* is the key haplotype in conferring resistance to *Xoo* strain PXO99^A (Table 3, S3 Table, S3 Fig). It was also noteworthy that the major difference between susceptible haplotypes (*Pro-B*, *C*, *D* and *E*) and haplotype *Pro-A* is due to mutations in their corresponding *EBE/ebe* regions (Figs 1 and 2), which directly affect the interaction between *Xa23* and *AvrXa23*. Therefore, *Xa23* (containing haplotype *Pro-A*) confers the resistance to bacterial blight disease, and the different *ebe* regions contained by different *xa23* alleles may be the major reason for BB resistance elimination in rice breeding.

Table 3. *Xa23* haplotypes based on promoter regions and corresponding BB resistance phenotypes.

Variety/Accession	Species	Haplotype	Phenotype
CBB23	<i>indica</i>	<i>Pro-A</i>	R
93-11	<i>indica</i>	<i>Pro-B</i>	S
CO 39	<i>indica</i>	<i>Pro-B</i>	S
fr 13 a	<i>indica</i>	<i>Pro-B</i>	S
MADHABSAR	<i>indica</i>	<i>Pro-B</i>	S
Zhengtiehe	<i>indica</i>	<i>Pro-B</i>	S
Khao Dawk Mali 105	<i>indica</i>	<i>Pro-B</i>	S
da 16	<i>indica</i>	<i>Pro-B</i>	S
t 1	<i>indica</i>	<i>Pro-B</i>	S
JG30	<i>indica</i>	<i>Pro-C</i>	S
LANI KHAMA	<i>indica</i>	<i>Pro-C</i>	S
5024S	<i>indica</i>	<i>Pro-C</i>	S
Basmati 1	<i>indica</i>	<i>Pro-C</i>	S
Hongguo	<i>japonica</i>	<i>Pro-B</i>	S
66756	<i>japonica</i>	<i>Pro-C</i>	S
n 22	<i>japonica</i>	<i>Pro-C</i>	S
IRGC 5441	<i>japonica</i>	<i>Pro-D</i>	S
Nipponbare	<i>japonica</i>	<i>Pro-E</i>	S
03-8	<i>O. rufipogon</i>	<i>Pro-A</i>	R
03-9	<i>O. rufipogon</i>	<i>Pro-A</i>	R
03-19	<i>O. rufipogon</i>	<i>Pro-A</i>	R
04-110	<i>O. rufipogon</i>	<i>Pro-A</i>	R
04-102	<i>O. rufipogon</i>	<i>Pro-A</i>	R
03-66	<i>O. rufipogon</i>	<i>Pro-A</i>	R
Wang13	<i>O. rufipogon</i>	<i>Pro-A</i>	R
2511-1	<i>O. rufipogon</i>	<i>Pro-A</i>	R
Wang28	<i>O. rufipogon</i>	<i>Pro-A</i>	R
03-15	<i>O. rufipogon</i>	<i>Pro-A</i>	R
03-20	<i>O. rufipogon</i>	<i>Pro-A</i>	R
03-27	<i>O. rufipogon</i>	<i>Pro-A</i>	R
03-28	<i>O. rufipogon</i>	<i>Pro-A</i>	R
03-104	<i>O. rufipogon</i>	<i>Pro-A</i>	R
04-72	<i>O. rufipogon</i>	<i>Pro-A</i>	R
KHM08-37	<i>O. rufipogon</i>	<i>Pro-B</i>	S
KHM08-51	<i>O. rufipogon</i>	<i>Pro-B</i>	S
NEP09-79	<i>O. rufipogon</i>	<i>Pro-B</i>	S
2511-2	<i>O. rufipogon</i>	<i>Pro-C</i>	S
03-14	<i>O. rufipogon</i>	<i>Pro-E</i>	S
03-16	<i>O. rufipogon</i>	<i>Pro-E</i>	S
03-26	<i>O. rufipogon</i>	<i>Pro-E</i>	S
04-103	<i>O. rufipogon</i>	<i>Pro-E</i>	S
04-108S	<i>O. rufipogon</i>	<i>Pro-E</i>	S
HL_8	<i>O. rufipogon</i>	<i>Pro-E</i>	S
BC2701	<i>O. rufipogon</i>	<i>Pro-B</i>	S
VN04-9	<i>O. nivara</i>	<i>Pro-B</i>	S
VN04-10	<i>O. nivara</i>	<i>Pro-B</i>	S
VN07-00	<i>O. nivara</i>	<i>Pro-B</i>	S

(Continued)

Table 3. (Continued)

Variety/Accession	Species	Haplotype	Phenotype
VN07-10	<i>O. nivara</i>	Pro-B	S
03-101	<i>O. latifolia desy</i>	Pro-C	S
94	<i>O. alta swallen</i>	Pro-C	S
84	<i>O. glumaepatula</i>	Pro-C	S

R, resistant; S, susceptible.

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Genetic diversity of *Xa23* alleles in cultivated and wild rice

In the whole germplasm population, the average number of nucleotide difference, ‘*K*’ of the entire gene region (from the *EBE/ebe* to the stop codon TAA) was estimated to be 3.944. The genetic diversity, ‘ π ’ of *Xa23* alleles was 0.00855 and ‘ θ_w ’ equal to 0.00464 in the promoter region (-131 bp upstream sequence from the ATG start codon) were 9- to 12- fold higher than that in coding region. The test of neutrality give a significant positive Tajima’s *D* value ($P < 0.05$) in the entire *Xa23* genomic region. Considering the population stratification, we also tested these parameters within the three populations (*indica*, *japonica* and wild rice). The values of the π and θ_w in the promoter region were 6- to 22- fold, 29- to 37- fold and 11- to 11- fold higher than in coding region in *indica*, *japonica* and wild rice, separately. Moreover, compared with wild rice (average proportion of pairwise differences per base pair, $\pi = 0.00938$), a 62.69% reduction of sequence diversity was found in *O. sativa* ssp. *indica* varieties ($\pi = 0.0035$), while *japonica* has higher nucleotide diversity ($\pi = 0.01264$) than the *indica*. In addition, the positive Tajima’s *D* value reached a significant level in wild rice ($P < 0.05$) in the promoter region and the entire gene region of wild rice. However, the Fu and Li’s *D* value showed a negative value and reached a significant level in the promoter region of *indica* varieties (-2.61649, $P < 0.05$) (Table 2). These results suggest that *Xa23* was most likely undergone the bottleneck founder effect in rice during domestication and breeding.

Discussion

Functional variations in the alleles of *R* genes which lead to different phenotypes due to polymorphisms such as SNPs and InDels of core DNA fragments. As a natural phenomenon, *R* genes usually maintain different allelic forms in the population to protect plants from evolving pathogens [47, 48]. The high-end next generation sequencing technology in combination with a wide range of genetic resources in rice, provides us with the opportunity to discover potential resistance genes or alleles and trace their evolutionary pattern as well as to reveal the key elements regulating resistance. In the present study, we have analyzed the sequence polymorphisms, phylogeographic relationship and association of *Xa23* alleles with bacterial blight resistance. This research may help to uncover genetic *Xa23* variants in cultivated and wild rice species.

Moreover, at the species level, the nucleotide diversities of *Xa23* alleles (0.0035 for *indica*, 0.01264 for *japonica* and 0.00938 for wild rice) was found to be comparatively much higher as compared to genome-wide average level of the two subspecies ($\pi_{\text{silent}} = 0.0021$ for *indica* and 0.0011 for *japonica* [49]). This may be resulted from a wider geographical distribution of the rice germplasm used in this work, as it comprised rice varieties worldwide. We also observed a much higher diversity in wild rice (*O. rufipogon* and *O. nivara*) than the cultivated *O. sativa*, as well as a decrease in diversity among *O. sativa*, which may be due to the bottleneck effect [49–51]. In addition, the non-coding regions always evolve more rapidly and show higher sequence

polymorphism than coding regions under natural conditions [52], which is often discovered in most of the genes observed; and high polymorphism is expected at the locus involved in pathogen recognition [53]. In this study, among the 97 rice accessions, the nucleotide diversity in promoter of *Xa23* alleles ($\pi = 0.02527$) was about ten times than that of the coding region. The similar results appeared in *indica*, *japonica* subpopulations and wild relatives, indicating the presence of higher diversity in the promoter region of *Xa23* alleles. In details, most polymorphisms were found in the $EBE_{AvrXa23}$ and the corresponding *ebe* regions which can affect the recognition between *AvrXa23* encoded by *Xoo* strains and *Xa23* promoter. In addition to the $EBE_{AvrXa23}$ contained only by haplotype *Pro-A* identified in the resistant *Xa23* promoter, three types of *ebe* region were found in the other four haplotypes (*Pro-B*, *C*, *D*, *E*) which accounted for a large proportion of *Xa23* haplotypes (Fig 3), and coding regions of *Xa23/xa23* alleles are highly conservative. So we speculate that the additional *ebe* might play a critical role when $EBE_{AvrXa23}$ loses its recognition function for the *Xoo* effector *AvrXa23*. In this circumstance, the new *Avr* effector evolved in the pathogen *Xoo* may be recognized by the three types of *ebe* regions and thus stimulate the BB resistance function. Therefore, the high diversification of the promoter region provided the flexibility for *R* gene to adapt to different environments or to meet a variety of developmental requirements.

On the other hand, it will be useful to study the different alleles where amino acid changes had occurred, after fusing these newly found coding sequences with a functional $EBE_{AvrXa23}$ by promoter engineering [54, 55]. Since CBB23 has been widely adopted in rice breeding programs [56, 57], and several *Xa23*-containing hybrid rice varieties have been released to farmers in recent years. Thus, the method that combine the known $EBE_{AvrXa27}$, $EBE_{AvrXa10}$ and $EBE_{AvrXa23}$ to build up a functional *EBE* element which capable of interacting with various *Avr* effectors is feasible and will lead to more extensive resistance and longer time application of single *R* gene.

In summary, we have analyzed the genetic polymorphism of *Xa23* in rice. This analysis is the first of its kind for executor *R* genes and may be helpful in molecular evolutionary studies and mining useful alleles for rice improvement. The main contributions and potential effects of these variants on BB resistance managements have not been analyzed. In natural population, maintenance of allelic diversity in resistance genes seems like a result of co-evolution between host and pathogen [47]. Previous researches also suggested that pathogen may be an important selective agent during the process of *R* gene evolution [58]. Therefore, following the phenotypic screening of pathogens with specific *Avr* genes, the inclusion of more individuals in the analysis will help determine the co-evolutionary relationship between pathogens and host resistance.

Supporting information

S1 Fig. Alignment of nucleotide sequences between the resistant *Xa23* allele in CBB23 and susceptible *xa23* allele in JG30. The 1720-bp sequence of *Xa23* allele in CBB23 was used as the reference. The numbers at right side indicate nucleotide positions of the CBB23 and JG30 sequences. The 28-bp $EBE_{AvrXa23}$ and 34-bp ebe_{JG30} are highlighted in green. The 7-bp polymorphic nucleotides (6-bp insertion and 1-bp substitution) in JG30 are highlighted in purple. The coding regions of *Xa23/xa23* alleles in CBB23 and JG30 are both highlighted in blue. The primers are used for amplifying the alignment regions covering entire *EBE/ebe* and coding regions. The arrows indicate the primers and their amplification directions. (PDF)

S2 Fig. Comparison of coding regions of *Xa23/xa23* alleles in *Oryza* species. The multiple sequence alignment was constructed using CLC Sequence Viewer 7 program. The *Xa23* in

CBB23 is used as a reference. The different residues are shown in red. The consensus sequence of all the *Xa23/xa23* alleles along with percentage conservation of residue is also shown. The numbers at right side indicate the length of each sequence.

(PDF)

S3 Fig. Disease responses of five haplotypes of *Xa23/xa23* alleles to PXO99^A. These 18 representative rice accessions (8 *indica*, 3 *japonica* and 7 wild relatives) were inoculated with *X. oryzae* pv. *oryzae* strain PXO99^A using leaf-clipping method and bacterial blight lesions were measured 14 days after artificial inoculation. Y-axis is showing the lesion length. The *Pro-A, B, C, D* and *E* at the top indicate the five haplotypes of *Xa23/xa23* alleles. W, wild rice; I, *indica*; J, *japonica*; R, resistant; S, susceptible.

(PDF)

S1 Table. Summary of rice used for polymorphism and haplotype analysis.

(PDF)

S2 Table. Primers used for amplification and sequencing in this research.

(PDF)

S3 Table. The SNPs in coding regions of *Xa23* alleles and corresponding bacterial blight resistance phenotypes.

(PDF)

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