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ORIGINAL ARTICLE

High-resolution genetic mapping of a novel bacterial blight resistance gene *xa-45(t)* identified from *Oryza glaberrima* and transferred to *Oryza sativa*

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Abstract

Key message **A novel recessive bacterial blight resistance locus designated as a *xa-45(t)* was identified from *Oryza glaberrima* accession IRGC 102600B, transferred to *O. sativa* and mapped to the long arm of chromosome 8 using ddRAD sequencing approach. The identified QTL spans 80 kb region on Nipponbare reference genome IRGSP-1.0 and contains 9 candidate genes. An STS marker developed from the locus LOC_Os08g42410 was found co-segregating with the trait and will be useful for marker-assisted transfer of this recessive resistance gene in breeding programs.**

Abstract Bacterial blight, caused by *Xanthomonas oryzae* pv. *oryzae*, is one of the major constraints of rice productivity in Southeast Asia. In spite of having 44 bacterial blight resistance genes from cultivated rice and wild species, the durability of resistance is always at stake due to the continually evolving nature of the pathogen and lack of suitable chemical control. Here, we report high-resolution genetic mapping of a novel bacterial blight resistance gene tentatively designated as a *xa-45(t)* from an introgression line derived from *Oryza glaberrima* accession IRGC 102600B. This introgression line was crossed with the susceptible rice *indica* cultivar cv. Pusa 44 to generate F_2 and $F_{2:3}$ populations for inheritance and mapping studies. The inheritance studies revealed the presence of single recessive locus controlling resistance to the *Xanthomonas* pathotype seven. A high-density linkage map was constructed using double-digest restriction-associated DNA sequencing of 96 F_2 populations along with the parents. The QTL mapping identified a major locus on the long arm of rice chromosome 8 with a LOD score of 33.22 between the SNP markers C8.26737175 and C8.26818765. The peak marker, C8.26810477, explains 49.8% of the total phenotypic variance and was positioned at 202.90 cM on the linkage map. This major locus spans 80 kb region on Nipponbare reference genome IRGSP-1.0 and contains 9 candidate genes. A co-segregating STS marker was developed from the LOC_Os08g42410 for efficient transfer of this novel gene to elite cultivars.

Introduction

Bacterial blight (BB), caused by *Xanthomonas oryzae* pv. *Oryzae* (*Xoo*), is an important disease of rice (*Oryza sativa* L.) causing significant yield losses annually in irrigated and rain-fed lowland ecologies throughout Asia (Mew 1987; Chen et al. 2011). In general, the yield losses due to bacterial blight disease range from 20 to 30%, but in epidemic years, it may cause up to 50% reduction in yield (Ou 1985; Noh et al. 2007; Gu 2008). Breeding for bacterial blight resistance with deployment of R genes in high-yielding rice cultivars is the key economical and environment-friendly approach for the management of this disease. Globally, 44 different R genes (*Xa/xa*) conferring resistance against *Xoo* have been identified (Nino-Liu et al. 2006; Cheema et al. 2008; Wang et al. 2009;

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Kim et al. 2015; Zhang et al. 2015; Kim 2018; Kim and Reinke 2019). Out of these, 17 genes, namely *xa5*, *xa8*, *xa13*, *xa15*, *xa19*, *xa20*, *xa24(t)*, *xa25*, *xa26(t)*, *xa28(t)*, *xa31(t)*, *xa32(t)*, *xa33(t)*, *xa34*, *xa41(t)*, *xa42* and *a 44 (t)*, are recessive and the rest is dominant (Chen et al. 2011; Liu et al. 2011; Busungu et al. 2016; Kim and Reinke 2019). A total of nine genes have been cloned, of which six (*Xa1*, *Xa3/Xa26*, *Xa10*, *Xa21*, *Xa23* and *Xa27*) are dominant and three are (*xa5*, *xa13* and *xa25*) recessive (Iyer and McCouch 2004; Kim et al. 2015). High-resolution mapping and candidate gene analysis approach plays a crucial role in map-based cloning projects in rice (Ashikari et al. 1999; Blair et al. 2003). The bacterial blight resistance genes are mostly nucleotide-binding site–leucine-rich repeat (NBS-LRR) genes and the cell surface pattern recognition receptors (Song et al. 1995; Yoshimura et al. 1998; Sun et al. 2004). Out of three cloned recessive genes, the *xa5* encodes a mutated subunit of the basal transcription factor IIA on chromosome 5 (TFIIAg5) (Iyer and McCouch 2004; Jiang et al. 2006), whereas *xa13* and *xa25* belong to the SWEET multiple gene family (Chu et al. 2006; Liu et al. 2011). Of the dominant genes, *Xa1* encodes an NB-LRR-type protein (Yoshimura et al. 1998), whereas *Xa3/Xa26* and *Xa21* encodes an LRR receptor kinase-like protein (Song et al. 1995; Sun et al. 2004; Xiang et al. 2006). The other three dominant genes, viz. *Xa 10*, *Xa23* and *Xa27*, encode for executor R protein (Gu et al. 2005; Tian et al. 2014).

The genus *Oryza* comprises two distinct cultivated, viz. *O. sativa* L. (Asian rice) and *O. glaberrima* Steud. (African rice) and 22 wild species (Brar and Singh 2011). The *O. glaberrima* is the native cultivated rice of economic importance in West Africa and has many useful traits such as tolerance to drought, soil acidity, iron toxicity, phosphorus deficiency, as well as to biotic stresses such as resistance to African gall midge, nematodes, bacterial blight, blast, etc. (Sanchez et al. 2013; Tanaka et al. 2014; Sikirou et al. 2018), but are very poor yielder. The major obstacle in the transfer of useful variability from *O. glaberrima* to *O. sativa* is the presence of reproductive barriers which cause interspecific hybrid sterility (Sano 1986; Heuer and Miézan 2003; Li et al. 2018). The phenomenon of segregation distortion (SD) was well documented in wide crosses of many crop species (Zhang 2007; Li et al. 2012; Yang et al. 2012; Wang et al. 2013; Xu et al. 2004). In rice, SD is frequently observed in inter-subspecies crosses (Maekawa et al. 1981; Lin et al. 1992; Xu et al. 1997; Zhao et al. 2006; Reflinur et al. 2014) and inter-species crosses (Causse et al. 1994; McCouch et al. 1988; Lorieux et al. 2000; Koide et al. 2012). However, with the advancement in genomics and phenomics facility, it became possible to utilize these species in rice improvement programs. One such example is the development of NERICA (New Rice for Africa) varieties with increased rice productivity in Africa by combining the high yielding quality of Asian rice with locally adapted African rice (Sie 2008; Somado et al. 2008).

Though many bacterial blight resistance genes have been deployed in resistance breeding programs, breakdown of resistance in many rice cultivars and in near isogenic lines containing bacterial blight resistance genes either singly or in combination was observed. This could be either due to a narrow resistance spectrum of deployed gene or rapid evolution in the virulence behavior of *Xoo* pathotypes prevalent in a particular region. Mega rice cultivars of Punjab like PR114, PR116, PR120, PAU 201 and many others released variety became susceptible to one or the other virulent strain of *Xoo*. The probable explanation could be the presence of a high degree of pathogenic variation in *Xanthomonas*, often leading to the breakdown of resistance (Vera Cruz et al. 2000; Suh et al. 2013). In the Punjab State of India alone, 10 pathotypes have evolved over a very short period (Lore et al. 2011; Neelam et al. 2016). None of the previously reported genes currently exhibit resistance to all the prevalent pathotypes in India. Thus, expanding genetic diversity by identifying novel resistance genes from both cultivated rice gene pool as well as wild relatives of rice and subsequently their deployment in breeding programs can help in developing rice cultivars with durable BB resistance to *Xoo* (Suh et al. 2009; Kumar et al. 2012).

Punjab Agricultural University (PAU), India, is maintaining an active collection of around 1600 wild species germplasm received from the International Rice Research Institute, Los Baños, Philippines, and ICAR-National Rice Research Institute (ICAR-NRRI), Cuttack, India (formerly CRRI, Central Rice Research Institute). These accessions were screened against *Xoo* pathotypes prevalent in Punjab State of India, over several years, starting from 2001 (Vikal et al. 2007; Neelam et al. 2016). Among these, thirteen accessions of *O. glaberrima* were found resistant to seven *Xoo* pathotypes (Vikal et al. 2007), prevalent in Punjab State of India (Lore et al. 2011), and one accession was used for the development of mapping population. Here, we report high-resolution mapping of bacterial blight resistance gene designated as *xa-45(t)* and development of markers for marker-assisted transfer of this recessive resistance gene into other backgrounds.

Materials and methods

Plant material and population development

The *O. glaberrima* accession IRGC102600B (single plant selection from the original accession IRGC102600) from Liberia procured from IRRI, Philippines, showed a high level of resistance to all the seven *Xoo* pathotypes prevalent

in Punjab (Vikal et al. 2007). The accession was crossed as male to *O. sativa* cv. Pusa44, a widely adapted genotype in Punjab, which is high yielding, lodging tolerant, but susceptible to bacterial blight (Fig. 1). The F_1 was made by crossing Pusa44 with *O. glaberrima*. The F_1's were completely male sterile; therefore, the BC_1F_1 seeds were generated by mass pollination of F_1 plants with Pusa44 pollen, in isolation, without clipping of florets. This was done because clipping of the florets of the F_1 plants leads to 100% shattering of florets within 24 h of pollination. Pollination in each F_1 plant was continued for 4–5 days to ensure sufficient seed set. All the BC_1F_1 showed partial to complete pollen sterility. All the BC_1F_1 plant was bagged to generate BC_1F_2 seeds. Out of more than 100 BC_1F_1 plants, only eight BC_1F_1 plants showed partial fertility and set seeds upon selfing. The BC_1F_2 populations of the eight plants were inoculated with *Xoo* pathotype PbXo7 and two progenies showed segregation for BB resistance and all others were susceptible. Selfed seeds from each BC_1F_2 plant were collected from one population and again screened against *Xoo* pathotype PbXo7 to confirm the reaction of the BC_1F_2 plants and ascertaining their genotype. Since pollen sterility was still a concern, the progenies homozygous for resistance to BB were further backcrossed two times to Pusa 44 and selection continued to generate a stable and agronomically desirable BC_3F_8 introgression line (Pusa 44/*O. glaberrima* IRGC102600B//3* Pusa 44), designated as IL 274 (Vikas 2010). The IL 274 was again crossed to Pusa 44 to generate F_2 and $F_{2:3}$ population for mapping and inheritance studies.

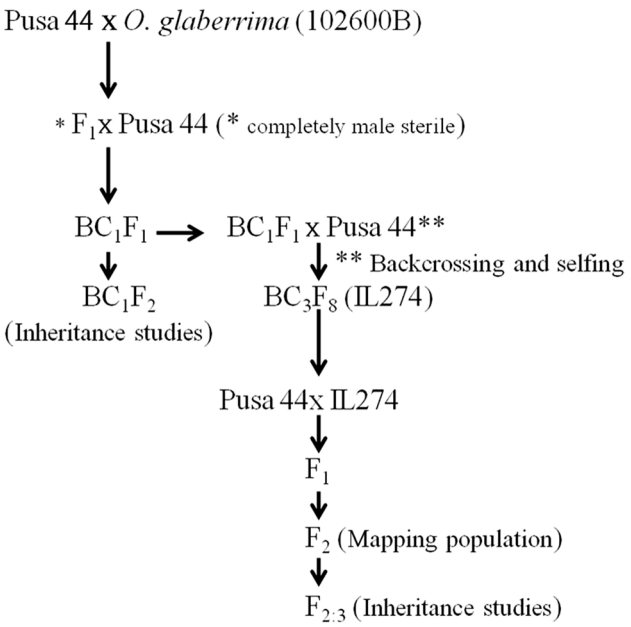

Fig. 1 Population development strategies for mapping and inheritance studies of bacterial blight resistance gene in a cross of Pusa44 and O. *glaberrima* 102600B

Screening with *X. oryzae* pv. *oryzae* pathotype PbXo-7

Bacterial culture of *Xanthomonas* pathotype PbXo7 (Lore et al. 2011), originally isolated from a single colony, was streaked on Walkimoto medium (10 g sucrose, 5 g peptone and 20 g agar, total volume made up to 1000 ml using distilled water) slants in test tubes under aseptic conditions. After streaking, the cultures were incubated for 72 h at 28 °C. The bacterial growth was scrapped and homogenized by vigorous shaking. The bacterial suspension was standardized to the inoculum density of 10^9 cells/ml. This virulent culture was used for inoculation of the parents (IL 274 and Pusa 44), F_1 plants, F_2 and $F_{2:3}$ progenies at maximum tillering stage following 'clip inoculation technique' (Kauffman et al. 1973) during the *kharif* crop season 2014 to 2016.

Disease assessment

The disease data were recorded 14 days after inoculation (DAI). The disease reaction on the basis of lesion length (cm) was classified according to the scale given by Cottyn and Mew (2004). The lesion length up to 5.0 cm (disease score 1–3) was classified as resistant, between 5 and 10 cm (disease score 3–5) as moderately resistant, 11–15 cm (disease score 5–7) as moderately susceptible and greater than 15 cm (disease score 7–9) as susceptible. For the inheritance study, at least five leaves from each of 312 F_2 individual plants were inoculated and the disease score was recorded using the standard scoring system. Seeds were harvested from individual F_2 plants and planted as F_3 progeny with 15 plants per progeny. Five leaves of each plant were inoculated with pathotype PbXo-7 and lesion length recorded after 14 days of inoculation. The average of the lesion length of five leaves was used for analysis. The Chi-square test was used to test the goodness of fit for ascertaining the number of genes governing the BB resistance.

DNA extraction and SNP genotyping

Large-scale DNA extraction protocol was followed to isolate high-molecular-weight DNA from 25- to 30-day-old field-grown F_2 plants using CTAB (cetyl trimethyl ammonium bromide) method. DNA was quantified using a spectrophotometer (Eppendorf Biophotometer). The quality and integrity of genomic DNA were also checked on 0.8% agarose gel and normalized by adding Tris EDTA buffer. 1000 ng DNA from each F_2 plant and the parents was used for double-digest restriction site-associated DNA sequencing (ddRAD-seq). The ddRAD sequencing was outsourced to SciGenom (www.scigenom.com). Briefly, the Illumina HiSeq 2000/2500 sequencing technology was used for fetching out SNPs. The enzymes used for double

digestion of the samples are SphI and MlucI. Approximately, 100–120 MB data per sample was obtained for further analysis.

Linkage map construction

High-quality SNP markers with less than 5% missing data were retained for the construction of a genetic map. The Chi-square test was performed to exclude markers with segregation distortion or $\chi^2 > 10$. Linkage groups (LGs) were generated with RECORD (REcombination Counting and ORDering) software (Van Os et al. 2005), using the Kosambi mapping function. Linkage groups (LGs) were drawn using MapChart V2.2 (Voorrips 2002). The markers with similar segregation patterns (based on a similarity of ≥ 0.95) were placed in the same genetic bin and only one of the markers was retained.

The composite interval mapping (CIM) function of winQTL cartographer software (Wang et al. 2012) was used for mapping QTL(s) for BB resistance with a window size of 10 cM. The stepwise threshold logarithm of the odds (LOD) scores for the QTL was calculated based on 1000 permutations at $P \leq 0.05$ (Churchill and Doerge 1994). Markers used as cofactors in the CIM model were selected by forward and backward regression analyses. The proportion of observed phenotypic variance attributable to the QTL was estimated by the coefficient of determination (R^2) using maximum likelihood for composite interval mapping.

Markers development

To enrich the QTL region, we have used available SSR markers within the defined region (International Rice Genome sequencing Project 2005). Further, we have designed new primers harboring SSRs in the whole 80 kb region on chromosome 8 for getting more number of polymorphic markers. In addition to this, gene specific primers were also designed from the nine putative candidate genes within the QTL region. In total, 39 primer pairs were designed for saturating the identified region. The markers were amplified in a set of 10 µl PCR reactions containing 60 ng of genomic DNA, 10 pmol of each primer, 2.5 mM, each of the four dNTPs, 1 units of Taq DNA polymerase, 1X PCR buffer and 1.5 mM $MgCl_2$. Reactions were performed in a thermal cycler (Eppendorf), programmed at 94 °C for 5 min, followed by 35 cycles of 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C with a final extension of 7 min at 72 °C. PCR amplified products were resolved in 2.5% agarose gel, and individual alleles were scored by comparing to parental alleles.

Sequencing of xa13 gene (SWEET11) in IRBB13, IL274 and Pusa44

The *Oryza sativa* Japonica sequence (LOC4346153) was used for designing the overlapping primers from position 26,725,954–26,728,807 bp covering a total length of 2853 bp on reference sequence. The amplicons were purified using Wizard® SV 96 PCR clean-up/Gel extraction kit from Promega, USA, as per manufacturer's protocol. Sequencing reaction was performed using ABI Big-dye Terminator v3.1 chemistry and sequenced using ABI Sequencer 3730XL. Hi-fidelity long-read DNA polymerase (*Phusion Taq*) from Promega, USA, was employed to obtain the required amplicon size. A minimum of three replications was carried out for the confirmation of single nucleotide polymorphism (SNPs).

Results

Inheritance of bacterial blight resistance using BC_1F_2 population

The disease reaction of Pusa 44, *O. glaberrima* 102600B and F_1 of the cross Pusa44/*O. glaberrima* 102600B is shown in Fig. 2. The susceptible reaction of the F_1 plants indicated the recessive nature of BB resistance gene in *O. glaberrima* 102600B. Further, the BC_1F_1 progeny was inoculated with *Xoo* pathotype PbXo7. All the BC_1F_1 plants were susceptible, thereby confirming the recessive nature of BB resistance. Only two of the BC_1F_2 progeny

Fig. 2 Differential bacterial blight disease reactions after 14 days of inoculation with *Xanthomonas* pathotype PbXo-7. **a** Pusa 44, **b** *O. glaberrima* 102600B, **c** F_1 hybrid

designated as 532 and 535 with normal segregation for BB was used for inheritance studies. In population 532, out of 129 plants, only 27 were resistant and 102 susceptible, whereas in population 535, out of 191 plants, 51 were resistant and 140 susceptible (Table 1). Assuming single gene segregation, the Chi-square values were non-significant for both the populations with P values < 0.29 and < 0.59 for population 532 and 535, respectively. The population of 535 was tall and leafy and had a high level of sterility and shattering; hence, BC_1F_3 progenies of this population could not be tested, but the majority of the plants in a population 532 set seed. A total of 129 BC_1F_3 progenies of this population were screened against *Xoo* pathotype PbXo7 during 2006. In each progeny, 29 plants were inoculated and data recorded as homozygous resistant (HR), homozygous susceptible (HS) or segregating (Seg). Of the 129 progenies tested, 27 were homozygous resistant, 35 were homozygous susceptible and 67 showed segregation. The Chi-square value fitted to the expected for 1:2:1 HR/Seg/HS (Table 1) with P value < 0.55. This confirmed that the bacterial blight resistance in *O. glaberrima* 102600B was governed by a single recessive gene.

Mapping of bacterial blight resistance using BC_1F_2 population

Population of 535 was initially used for parental polymorphism and bulked segregant analysis. From the rice linkage map (Temnykh et al. 2001), parental polymorphism and BSA were carried out using 159 SSR markers spanning all the twelve linkage groups. Out of 159 primers tested, 86 showed polymorphism between the parents. The level of polymorphism between Pusa44 and *O. glaberrima* 102600B varied from 20.0% in chromosome 10–71.4% in chromosome 5, with an overall polymorphism of 54.0% (Table S1). Segregation distortion was observed for several markers throughout the genome where only one parent band was observed in both resistant and susceptible bulks (Figure S1); hence, this population was discontinued for gene mapping studies. The introgression of *O. glaberrima* allele was observed on chromosome 5 and 6 but was unable to map the gene, primarily due to segregation distortion and a smaller number of markers (Figure S2). We continued further selfing and backcrossing to generate several populations, including one BC_1F_6:BC_2F_{10} lines.

Inheritance of bacterial blight resistance using IL 274

In order to map the BB resistance gene, we attempted a new cross between *O. sativa* cv. Pusa 44 with the BC_3F_8 introgression line IL 274 (Pusa 44/*O. glaberrima* IRGC102600B//3* Pusa 44). The F_1 plant of the above-said cross was susceptible when inoculated with *Xanthomonas* pathotype PbXo7. Out of 312 F_2 individuals, 221 plants were found susceptible and 91 plants resistant (Table 2). The segregation ratio of susceptible to resistant plants agreed with 3: 1 segregation ratio of the single recessive gene for BB resistance in the introgression line. Out of 312 $F_{2:3}$ progeny, 74 were homozygous resistant, 158 segregating and 80 homozygous susceptible, which fitted well to the expected 1:2:1 segregation ($\chi^2_c = 0.271, \chi^2_{0.75,2} = 0.575$). Thus, it could be deduced that the gene governing BB resistance in the introgression line IL274 is governed by a single recessive gene. The phenotypic data on bacterial blight were also recorded on F_4 and F_5 progenies in consecutive years 2017 and 2018 (Fig. 3).

Table 1 Frequency of resistant and susceptible plants/progenies in BC_1F_2 and BC_1F_3 populations of the cross Pusa44/*O. glaberrima*/Pusa44 during 2005

Population	No. of plants/progenies			Total	χ^2
	Resistant (< 5 cm)	Segregating	Susceptible (> 5 cm)		
BC_1F_2 (#532)	27	–	102	129	1.14 (1:3)
BC_1F_2 (#535)	51	–	140	191	0.29 (1:3)
BC_1F_3 (#532)	27	67	35	129	1.18 (1:2:1)

Lesion length in *O. glaberrima* 102600B, Pusa44 and their F_1 was 0.5 cm, 38.5 cm and 32.0 cm, respectively

(–) Indicates that in BC_1F_2 population, the segregating plants for disease reaction were not observed

Table 2 Frequency of resistant and susceptible plants in F_2 and $F_{2:3}$ populations and Chi-square analysis

Population	Number of plants			Total	χ^2_c	χ^2_t
	Resistant	Segregating	Susceptible			
F_2	91	–	221	312	2.57	$\chi^2_{(0.05,\,1)} = 3.84$
$F_{2:3}$	74	158	80	312	0.27	$\chi^2_{(0.75,\,2)} = 0.57$

(–) Indicates that in F_2 population no segregating plants for disease reaction were observed

Fig. 3 Differential bacterial blight disease reactions after 14 days of inoculation with *Xanthomonas* pathotype PbXo-7. **a** Pusa 44, **b** IL 274, **c**–**h** F_5 progenies with resistance and **i**–**n** susceptible reactions

Construction of linkage map and segregation distortion

In total, 19,458 SNP markers were obtained with ddRAD-sequencing (Table 3). The SNP markers with indel or heterozygote SNP in either of the parents were removed, and only SNPs in the homozygous state (AA, CC, TT and GG) were used for scoring the polymorphism between *O. sativa* cv. Pusa 44 and the introgression line IL274. With this approach, we were left with only 1754 polymorphic SNPs between the parents for further linkage analysis. The SNPs markers with distorted segregation were removed using Chi-square test. A total of 652 markers were thus used for construction of the linkage map using RECORD.

Table 3 Distribution of SNP markers on all the 12 rice chromosomes and the length of genetic map

Linkage group	Total number of SNPs	Total no. of polymorphic markers	Polymorphic markers placed in LGs	Length of genetic map (cM)
Chr01	2270	36	2	23.67
Chr02	2351	292	131	325.48
Chr03	2184	36	2	6.19
Chr04	1453	170	72	308.67
Chr05	1367	49	15	29.68
Chr06	1631	239	98	481.31
Chr07	1513	15	–	–
Chr08	1343	200	87	247.92
Chr09	1197	80	25	155.39
Chr10	1110	26	–	–
Chr11	1955	592	220	847.86
Chr12	1084	19	–	–

(–) Indicates that no SNP markers were placed on rice chromosome 7, 10 and 12

The genetic map spanned a total of 2426.17 cM on 12 chromosomes of rice except for 7, 10 and 12, where all the identified polymorphic markers showed a complete deviation from the expected Mendelian frequencies. Only two markers mapped on rice chromosome 1 and 3 after the Chi-square test. In most of the cases, the markers were skewed toward the introgression line allele (Table 4). The genetic length of the smallest LG was for chromosome 3 (6.14 cM), and the largest was on chromosome 11 (847.86 cM).

QTL analysis and predicted genes

Composite interval mapping with 652 SNP markers revealed a sharp peak at the SNP marker interval C8.26737175 and C8.26818765 at LOD score 33.22 indicating major effect QTL (Figs. 4, 5) on chromosome 8. The peak marker, C8.26810477, was positioned at 202.90 cM on linkage map and explains 49.8% of the total phenotypic variance. This marker interval spans a physical distance of 80 kb on the Nipponbare reference genome IRGSP v1 (http://rice.plantbiology.msu.edu/cgi-bin/gbrowse/rice/). The QTL region flanked by the two SNPs markers C8.26737175 and C8.26818765 contains nine predicted genes (Table 5) including seven genes with assisted functions, one hypothetical protein and one expressed protein.

Markers development

Out of 39 primer pairs designed within the identified QTL region, only one of the primer pair designed from

Table 4 Segregation distortion of the SNP markers on rice chromosomes

Chromosome	No. of distorted markers	No. of markers skewed toward *O. sativa* cv. Pusa44 homozygote	No. of markers skewed toward Introgression line IL274 homozygote	Number of markers skewed toward heterozygote
1	12	4	8	–
2	98	36	61	1
3	19	0	19	–
4	54	25	29	–
5	15	10	5	–
6	87	32	52	3
7	9	1	8	–
8	62	37	21	4
9	25	18	6	2
10	8	0	8	0
11	260	160	72	28
12	8	3	4	1

(–) Indicates that no markers were skewed toward the heterozygotes

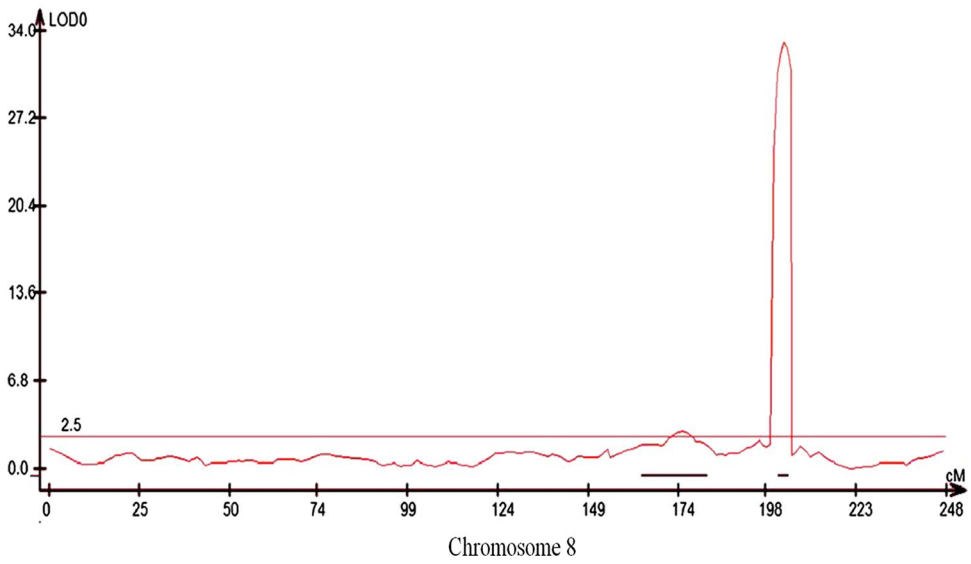

Fig. 4 Schematic view of the QTL identified for bacterial blight resistance on the long arm of chromosome 8 of rice with a LOD score of 33.22. The horizontal line represents the threshold line for QTL identification

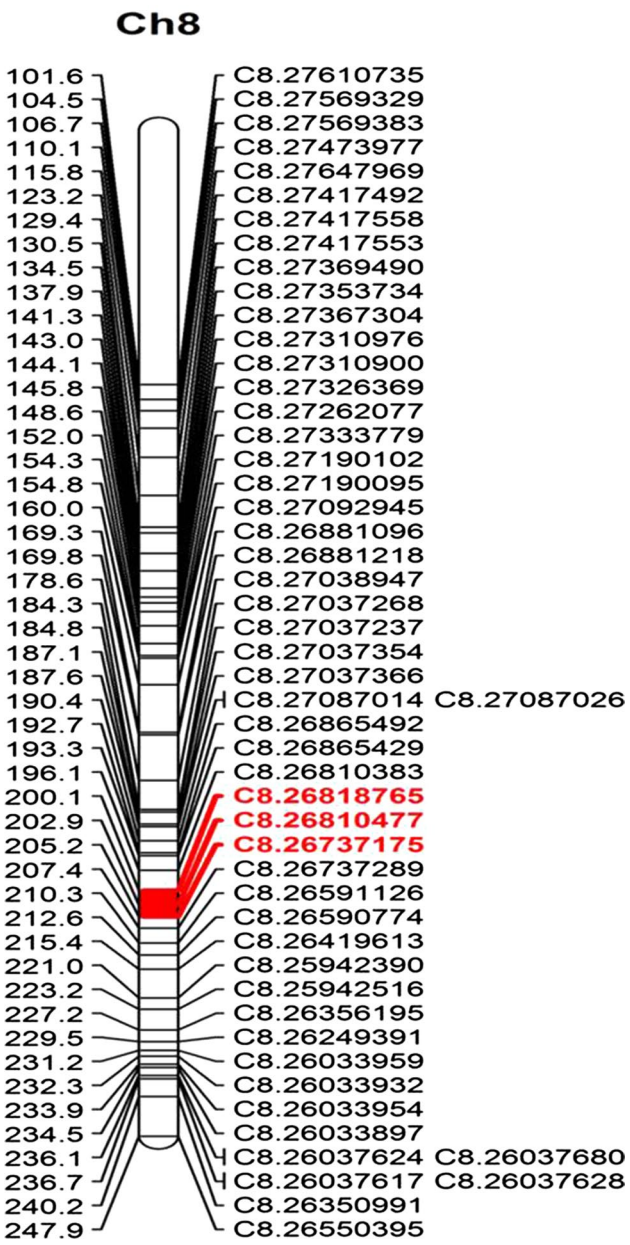

Fig. 5 Linkage map of rice chromosome 8 showing position of a *xa-45(t)* between linked SNP markers (red color) C8.26818765 and C8.26737175. The numbers shown on the left side of the map represent the distance between markers in cM (color figure online)

the LOC_Os08g42410 showed polymorphism between the parents. This marker was analyzed on F_5 population (273 plants). This marker is co-segregating with the phenotype (Fig. 6, Table 6). Five recombinant plants (3581-40-3-3, 3581-72-3-3, 3581-93-3-3 3581-193-3-3 and 3581-272-3-3) were observed with this marker. Our observation indicated that the location of the *xa-45(t)* (QTL location, chr8: 26737175…26818765) is fairly close to the position of *xa13* (LOC_Os084346153, chr8:26725954…26728807), another cloned recessive bacterial blight resistance gene on chromosome 8. Therefore, we also applied the STS marker for *xa13* (F-GGCCATGGCTCAGTGTTTAT, R-GAGCTC CAGCTCTCCAAATG) to the same F_5 population. This marker is also found as co-segregating with the trait (Figure S3). However, 11 recombination points were observed between the *xa-45(t)* marker and the *xa13* marker on the whole population. For the individuals 3581-13-3-3, 3581-93-3-3, 3581-193-3-3, 3581-269-3-3, 3581-272-3-3 and 3581-273-3-3 both of the markers showed opposite results, i.e., with one marker they are showing resistant genotype scores and with another it is the susceptible genotype score (Table S2). The individuals 3581-129-3-3, 3581-235-3-3, 3581-250-3-3, 3581-267-3-3 and 3581-268-3-3 had a resistant genotype score (*B*) converted to heterozygote (*H*) with either of the markers. This also provides an indication that both of the genes are different.

Comparative sequence analysis of *xa13* gene with other diploid wild species of rice

We performed a comparative sequence analysis of the *xa13* gene from nine diploid wild *Oryza* species and three of the cultivated *Oryza* species (Fig. 7). The genomic sequence of the cloned *xa13* gene, i.e., LOC4346153 (SWEET11), was used as reference sequence for the analysis. A number of polymorphic sites were found at the nucleotide positions 232, 358, 359, 442, 694 and 728 between the reference sequence and *O. glaberrima* assembly. Phylogenetic analysis demonstrated high gene collinearity across the AA genome *Oryza* species. The *O. rufipogon*, *O. barthii* and *O. nivara* share a common clade with *O. sativa japonica* group, whereas *O. glumaepatula* and *O. glaberrima* fall closer to *O. sativa*. Two of the AA genome species, *O. longistaminata* and *O. meridionalis*, showed less similarity with the reference sequence as well as among themselves. The *O. punctata* (BB) genome and *O. brachyantha* (FF) genome did not grouped, indicating non-collinearity of *xa13* gene across other diploid genome species. On contrary, the comparative analysis of 80 kb region containing a *xa-45(t)* candidate genes in Nipponbare reference sequence aligned with the Chr8 of *O. glaberrima* genome (nucleotide position 26737175–26818765) showed a higher degree of genomic rearrangement. This was evident by several polymorphic sites, including several INDELS of 3-5 bp (Figure S4).

Comparison of *xa13* gene sequence in IRBB13, IL274 and Pusa 44

We analyzed the *xa13* gene sequence (from two of the resistant lines, viz. a NIL containing *xa13* (IRBB13), introgression line (IL274) and the susceptible check, Pusa44. To our surprise, this gene is having similar sequences

Table 5 Details of the nine candidate genes in the BB resistance QTL interval introgressed from *O. glaberrima* acc. IRGC 102600B in the background of Pusa44

Sr. no.	Gene	Putative functions
1	LOC_Os08g42360	Hypothetical protein
2	LOC_Os08g42370	Zinc finger DHHC domain-containing protein, putative, expressed
3	LOC_Os08g42380	Mitochondrial import inner membrane translocase subunit Tim, putative, expressed
4	LOC_Os08g42390	Glycerophosphoryl diester phosphodiesterase family protein, putative, expressed
5	LOC_Os08g42400	No apical meristem protein, putative, expressed
6	LOC_Os08g42410	Transketolase, putative, expressed
7	LOC_Os08g42420	Expressed protein
8	LOC_Os08g42430	Membrane-associated DUF588 domain-containing protein, putative, expressed
9	LOC_Os08g42440	CCT/B-box zinc finger protein, putative, expressed, involved in flowering

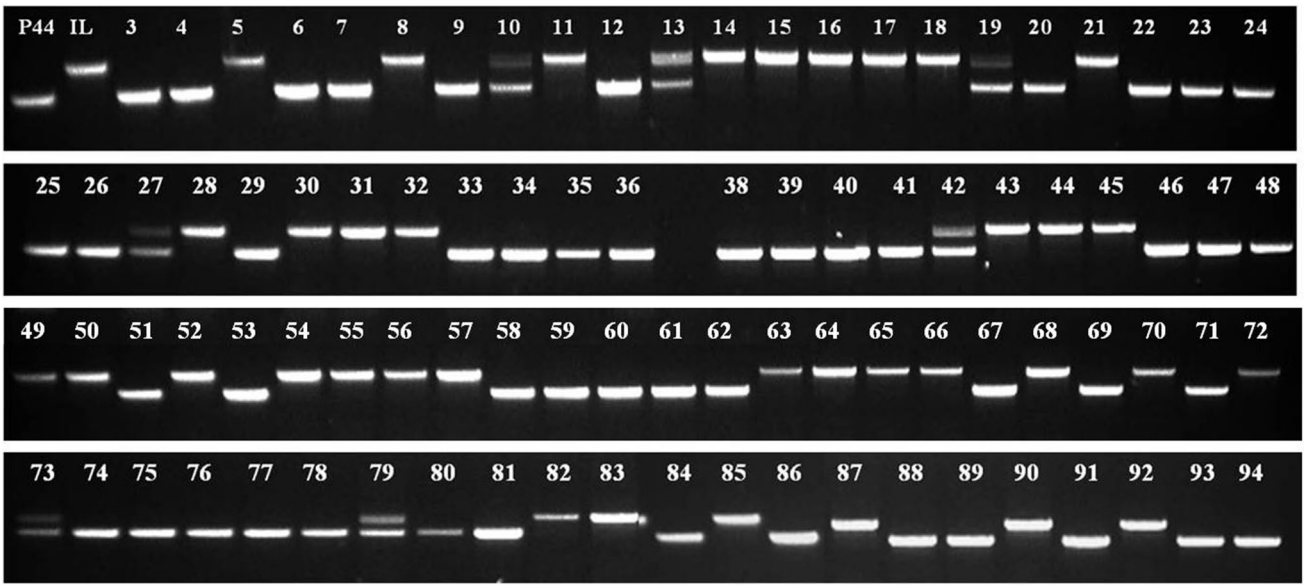

Fig. 6 Genotyping of 94 F_5 progenies along with susceptible and resistant parents using gene-based marker developed from the LOC_Os08g42410 on agarose gel (1.5%)

in all the three lines except at six nucleotide positions when compared to the reference sequence (Figure S4). At the nucleotide positions 1148 and 1586, the reference sequence and Pusa44 had the nucleotide C as compared to G in IL274 and IRBB13, whereas at the nucleotide position 2223, G/A transition was seen between these pairs. The IRBB13 and IL274 differ at the nucleotide position 2238(A/C), whereas singletons in Pusa44 were observed at nucleotide positions 1964 (G/C) and 2001(T/C). However, having similar genomic sequence does not always correlate well with the expression of the gene. There might be a difference in the expression level or it may be induced after inoculation with the *Xanthomonas* in resistant genotypes. Another possible explanation could be differences in the promoter region of susceptible allele as compared to the resistant allele. Similar findings were also reported in the case of the bacterial blight resistance gene *Xa23* and *Xa27*. In the case of *Xa23*, the susceptible allele has an identical open reading frame as that of resistant allele but it lacks the TALE binding elements in the promoter region for Avrxa23, whereas the *Xa27*-resistant allele (IRBB27) and the susceptible allele *xa27* in IR24 contain the identical coding sequences but the resistant allele expression induced only after inoculation with *Xanthomonas* (Wang et al. 2015). Further, investigation is needed to decipher the mechanism governing resistance in the *xa-45(t)* to conclude that if it's only an allele of the *xa13* or a new gene.

Phenotypic evaluation of *xa45(t)* and *xa13* against 10 key pathotypes of *X. oryzae* pv. *oryzae*

To date, 10 pathotypes of *XOO oryzae* pv. *oryzae* have been identified in Northern India (Lore et al. 2011, 2013). Since *xa13* locus is co-located with *xa-45(t)* on chromosome 8,

Table 6 The phenotypic and genotypic data of F_5 population (IL274/Pusa44) with the markers LOC_ Os08g42410 and *xa*13

ID	Pedigree	BB	Os08g42410	*xa13*	ID	Pedigree	BB	Os08g42410	*xa13*
1	Pusa 44	23.48	A	A	48	3581-52-3-3	15.4	A	A
2	IL274	2.4	B	B	49	3581-53-3-3	3.3	B	B
3	3581-1-3-3	12.3	A	A	50	3581-54-3-3	1.9	B	B
4	3581-2-3-3	13.1	A	A	51	3581-55-3-3	9.4	A	A
5	3581-3-3-3	2.1	B	B	52	3581-56-3-3	2.6	B	B
6	3581-7-3-3	9.8	A	A	53	3581-57-3-3	15.3	A	A
7	3581-8-3-3	12.4	A	A	54	3581-58-3-3	3.1	B	B
8	3581-9-3-3	2.9	B	B	55	3581-59-3-3	2.5	B	B
9	3581-10-3-3	18.6	A	A	56	3581-60-3-3	2.5	B	B
10	3581-11-3-3	8.9	H	H	57	3581-61-3-3	3.0	B	B
11	3581-13-3-3	3.2	**B**	**A**	58	3581-62-3-3	13.0	A	A
12	3581-14-3-3	15.2	A	A	59	3581-63-3-3	14.8	A	A
13	3581-15-3-3	8.8	H	H	60	3581-64-3-3	15.9	A	A
14	3581-16-3-3	3.4	B	B	61	3581-65-3-3	16.2	A	A
15	3581-17-3-3	2.8	B	B	62	3581-66-3-3	19.5	A	A
16	3581-18-3-3	3.6	B	B	63	3581-67-3-3	2.8	B	B
17	3581-19-3-3	2.4	B	B	64	3581-68-3-3	2.7	B	B
18	3581-20-3-3	1.9	B	B	65	3581-69-3-3	2.5	B	B
19	3581-21-3-3	8.8	H	H	66	3581-70-3-3	3.0	B	B
20	3581-22-3-3	12.7	A	A	67	3581-71-3-3	15.1	A	A
21	3581-23-3-3	3.6	B	B	68	3581-72-3-3	1.6	B	B
22	3581-24-3-3	20.5	A	A	69	3581-74-3-3	16.3	A	A
23	3581-25-3-3	13.5	A	A	70	3581-75-3-3	2.2	B	B
24	3581-27-3-3	20.8	A	A	71	3581-76-3-3	16.2	A	A
25	3581-28-3-3	11.8	A	A	72	3581-**77-**3-3	2.8	B	B
26	3581-29-3-3	15.0	A	A	73	3581-78-3-3	6.6	H	H
27	3581-30-3-3	16.7	**H**	**A**	74	3581-79-3-3	14.2	A	A
28	3581-31-3-3	3.1	B	B	75	3581-80-3-3	19.1	A	A
29	3581-32-3-3	10.9	A	A	76	3581-81-3-3	16.3	A	A
30	3581-33-3-3	2.9	B	B	77	3581-82-3-3	12.7	A	A
31	3581-34-3-3	1.9	B	B	78	3581-83-3-3	14.2	A	A
32	3581-35-3-3	1.8	B	B	79	3581-84-3-3	8.9	H	H
33	3581-36-3-3	6.03	A	A	80	3581-85-3-3	16.1	A	A
34	3581-37-3-3	10.5	A	A	81	3581-86-3-3	12.6	A	A
35	3581-38-3-3	9.84	A	A	82	3581-87-3-3	3.02	B	B
36	3581-39-3-3	19.8	A	A	83	3581-88-3-3	2.2	B	B
37	3581-40-3-3	1.96	B	B	84	3581-89-3-3	2.5	B	B
38	3581-41-3-3	15.34	A	A	85	3581-90-3-3	16.2	A	A
39	3581-42-3-3	13.1	A	A	86	3581-91-3-3	14.4	A	A
40	3581-43-3-3	14.3	A	A	87	3581-92-3-3	2.6	B	B
41	3581-44-3-3	8.6	A	A	88	3581-93-3-3	1.8	**A**	**B**
42	3581-45-3-3	15.7	**H**	**A**	89	3581-94-3-3	17.0	A	A
43	3581-47-3-3	1.9	B	B	90	3581-95-3-3	2.1	B	B
44	3581-48-3-3	2.5	B	B	91	3581-96-3-3	16.0	A	A
45	3581-49-3-3	2.5	B	B	92	3581-97-3-3	2.0	B	B
46	3581-50-3-3	14.4	A	A	93	3581-98-3-3	14.3	A	A
47	3581-51-3-3	11.3	A	A	94	3581-99-3-3	15.1	A	A

The bold letter signifies recombination point between the STS marker Os0842410 and xa13 in the individuals 3581-13-3-3, 3581-30-3-3, 3581-45-3-3 and 3581-93-3-3

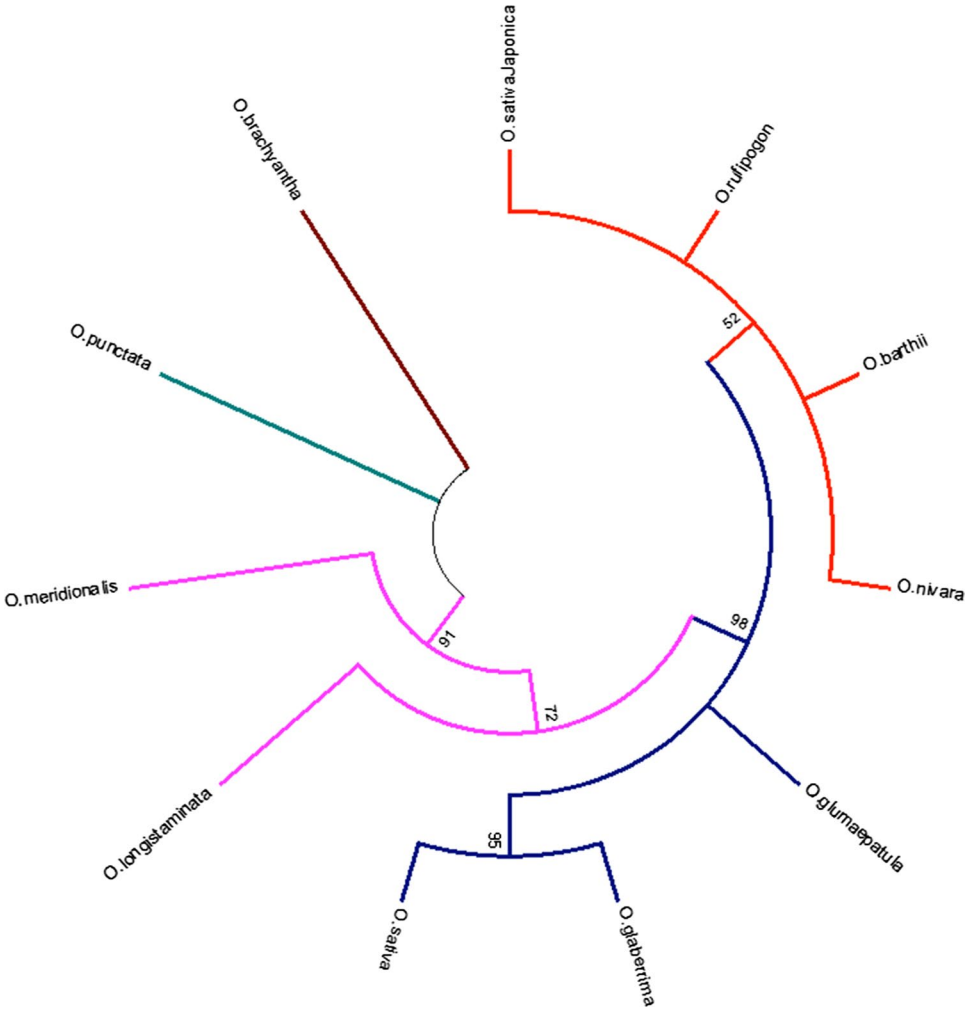

Fig. 7 Comparative sequence analysis of *xa13* gene with diploid species of rice. The molecular phylogeny was inferred using the Maximum Likelihood method with 1000 bootstrap using MEGA software. The different node colors indicate the presence of different mutations as compared to the reference

we investigated the disease reaction of the introgression line (IL274) carrying bacterial blight resistance gene *xa45(t)*, IRBB13 carrying *xa13* gene, resistant donor *O. glaberrima* acc. 102600B, susceptible recipient parent Pusa 44 and susceptible check IR 24 against 10 key pathotypes of *Xoo* (Table 7, Fig. 8). The IL 274 and the donor *O. glaberrima* acc. 102600B showed resistant reaction to all the 10 pathotypes of *Xoo*; however, IRBB 13 (*xa13*) showed susceptible reaction to the pathotype PbXo-8. The cultivar Pusa 44 and IR24 (*Xa18*) were susceptible to all the tested pathotypes, thus confirming the novelty of the new gene.

Discussion

The durability of resistance depends upon the prevalence of the pathogen population in a particular area. Pathogen *Xanthomonas* is highly variable and showed diverse reaction to rice cultivars possessing genetic resistance to bacterial blight under field conditions. The pathogen population has been divided into many groups in different countries based on local rice cultivars/near isogenic lines (Adhikari et al. 1999; Noda et al. 2001; Lore et al. 2011). The pathogen population from Northern India are comparatively more aggressive and became virulent to most of the single BB resistance genes such as *Xa1, Xa3, Xa4, xa5, Xa7, xa8, Xa10, Xa11, xa13, Xa21, Xa23, Xa38* and even some of the *Xanthomonas* strains showing virulence on pyramided genes such as *Xa4* + *xa5*, *Xa4* + *Xa21*, *xa5* + *Xa21* and *xa13* + *Xa21* in India (Lore et al. 2011). In the present study, we identified and mapped a new bacterial blight resistance gene from *O. glaberrima* on the long arm of chromosome 8. None of the bacterial blight resistance gene is reported from *O. glaberrima* previously. It is important to note that the most widely used resistance gene *xa13* shows susceptibility to *Xoo* pathotype PbXo-8, whereas *xa-45(t)* showed resistance against all the 10 pathotypes present in North India. This infers that the addition of this new gene or allele to the bacterial blight resistance gene pool will definitely broader the genetic base and hence will be helpful in the creation of durable resistance.

Table 7 Bacterial blight reactions of the resistant and susceptible checks along with IRBB13 against ten *Xanthomonas* pathotypes

Genotype	Gene	Reaction to pathotypes									
		PbXo-1	PbXo-2	PbXo-3	PbXo-4	PbXo-5	PbXo-6	PbXo-7	PbXo-8	PbXo-9	PbXo-10
IL 274	*xa-45(t)*	R	R	R	R	R	R	R	R	R	R
IRBB13	*xa13*	R	R	R	R	R	R	R	**S**	R	R
O. glabrrima acc. 102600B	Resistant donor	R	R	R	R	R	R	R	R	R	R
Pusa44	Susceptible recipient	S	S	S	S	S	S	S	S	S	S
IR24	*Xa18* (susceptible check)	S	S	S	S	S	S	S	S	S	S

Fig. 8 Differential disease reaction of **a** Pusa 44, **b** IL274, **c** IRBB 8 after inoculation with *Xanthomonas* pathotype PbXo-8. Pusa 44 showed bacterial blight score of 9, IL274 showed immune reaction with the score 1, whereas IRBB 13 showed susceptible reaction with disease score of 7

Segregation distortion

In our analysis, we observed that the markers were distorted either toward one of the alleles from *O. sativa*/*O. glaberrima*. The presence of sterility barriers between *O. sativa* and *O. glaberrima* in early hybrid generations is evident in many earlier studies (Sano et al. 1979; Sano 1986; Ikehashi and Araki 1986; Pham and Bougerol 1993; Doi et al. 1998; Lorieux et al. 2000; Li et al. 2004; Reflinur et al. 2014). Till date, approximately ten hybrid sterility loci as gamete eliminator or pollen killer between *O. sativa* and *O. glaberrima* were identified (Li et al. 2018). The introgression line alleles were over represented on chromosome 2, 3, 6 and 10. This may be attributed to the presence of gametophytic and sterility genes on these chromosomes (Lin et al. 1992; Xu et al. 1997) that might lead to the partial female gamete abortion. Approximately, 60% of the markers were skewed toward introgression line allele on chromosome 2. Maekawa et al. (1981) reported the presence of the gametophyte gene (*ga-6*) on chromosome 2 causing significant deviation of the markers from the normal Mendelian ratio. Another hybrid sterility gene *S29 (t)* on chromosome 2 was also observed to cause female gamete abortion in remote cross of rice (Zhu et al. 2005). Liu et al. (1997) mapped two of the minor genes on chromosome 2 and 12 which jointly cause reduction in the female fertility even in the presence of wide- compatibility genes. Segregation distortion of the marker locus due to the presence of gametophyte genes on chromosomes 6 (*ga-1*) was reported way back by Iwata et al. (1964) and on chromosome 3 (*ga*-2 and *ga*-3) by Nakagahra et al. (1972, 1986). It is noteworthy that the region around *waxy* locus on chromosome 6 was reported as distortion hotspot showing one-locus sporo-gametophytic distortion (Sano 1990; Lorieux et al. 2000). According to this model, the Sa gametes (*O. sativa* genotype) will abort in heterozygous F_1 plants and only S gametes (*O. glaberrima* genotype) will be represented in F_2 population (Sano et al. 1979; Heuer and Miézan 2003). Another pollen killer gene *S1* is also located on chromosome 6. This gene could behave as gamete eliminator or pollen killer depending on the activity of modifiers and genetic

background (Sano 1990). They observed higher female sterility in plants carrying distal segments of chromosome 6 of *O. glaberrima* due to preferential abortion of female gametes (*O. sativa* alleles). Aluko et al. (2004) also observed skewness of the markers toward *O. glaberrima* allele specifically on chromosomes 3 and 6 while studying grain quality traits in the interspecific cross between *O. sativa* and *O. glaberrima*. Similarly, transgressive segregation has been reported in crosses of *O. sativa* with *O. rufipogon* as well, due to a gene block of sterility genes on chromosome 6 (Matsubara et al. 2003). Lin et al. (1992) also observed strong distortion of the markers on chromosomes 3, 7, 8, 11 and 12 in a hybrid between *indica* and *japonica* crosses. However, as per our observation, the *O. sativa* alleles were in higher frequency of chromosome 11. This could owe to the fact that the sterility/gametophytic gene's behavior modifies due to the existence of multiple alleles or interaction among them including the presence of neutral alleles.

Putative candidate genes

Within the 80 kb region, 9 putative genes were predicted (Table 5) using rice genome annotation project database (http://rice.plantbiology.msu.edu). The probable function of these genes is discussed here. The Zinc finger, DHHC domain-containing protein has been reported in brachypodium, maize, sorghum to be involved in palmitotransferase function. This gene family has been reported to play a role in reversible posttranslational lipid modification of long chain fatty acid, usually the 16-carbon palmitate, covalently attaches to a cysteine residue(s) throughout the protein via a thioester bond. They regulate various biological processes during growth and development and reproduction (Gleason et al. 2006; Xiang et al. 2010; Yuan et al. 2013; Li and Qi 2017). The genome-wide transcriptome analysis revealed enhanced expression of this gene against bacterial blight disease (Jung et al. 2014). Mitochondrial import inner membrane translocase subunit Tim is intermembrane chaperone that participates in the import and insertion of some multipass transmembrane proteins into the mitochondrial inner membrane in arabidopsis, brachypodium, maize, sorghum and rice (Werhahn et al. 2001; Lister et al. 2004).

The glycerophosphoryl diester phosphodiesterase family protein is basically responsible for maintaining cellular phosphate homeostasis under phosphate starvation and organization of primary cell wall (Rest et al. 2004; Cheng et al. 2011). The no apical meristem protein is a NAC4 domain-containing protein in *O. sativa japonica* group, whereas it is putatively un characterized protein in *O. sativa* indica group. The NAC family transcription factors play an important role in the regulation of the various plant physiological process and response to stress. Their involvement in activating defense responses in rice, innate immune responses and host recognition of pathogens and further recognition triggered early signaling pathways is well characterized (Skamnioti and Gurr 2009; Valent and Khang 2010). The transketolase is involved in biological processes such as acetyl-CoA biosynthetic process from pyruvate, protein phosphorylation and also act as precursors for producing thymine in C3 cycle. The thymine biosynthesis is activated during plant response to abiotic and biotic stresses, including inoculation with *Xanthomonas* (Wang et al. 2006). Its molecular function includes ATP binding, protein serine/threonine kinase activity and pyruvate dehydrogenase activity. The protein serine/threonine kinases are well known to be involved in signal transduction and defense mechanism either directly as resistance genes or downstream components of defense pathways (Martin et al. 1993; Meskiene and Hirt 2000; Frye et al. 2001; Afzal et al. 2008). The membrane-associated DUF588 domain-containing protein may have catalytic activity, and these proteins are predicted to contain 3 or 4 transmembrane helices. In rice, only three BBX genes (CCT/B-box Zinc finger) have been identified and known to be involved in the regulation of flowering time (Huang et al. 2012). Based on the above-described functions of putative candidate genes in the QTL region, LOC_Os08g42370, LOC_Os08g42400 and LOC_Os08g42410 are worth further investigation in their role in response to bacterial blight infection.

PCR-based markers for marker-assisted selection

A PCR-based marker was developed from the LOC_Os08g42410 for marker-aided transfer of this recessive R gene. This can be of immediate use in rice bacterial blight breeding programs as being the recessive gene; desirable phenotypes are not expressed in heterozygotes. Being closely located to *xa13,* an STS marker for *xa13* could also be used for MAS of a *xa-45(t)*. However, the bacterial blight resistance provided by the *xa-45(t)* has a wider spectrum than the *xa13* gene which indicates presence of superior alleles from *O. glaberrima*. This new gene was incorporated in mega rice variety Pusa 44 and it was released as new variety PR127 in Northern India during the year 2018 (POP 2018).

Conclusion

In summary, we identified a novel gene or an allele from *O. glaberrima* of the existing *xa13* gene from *O. sativa*, conferring broad spectrum resistance to all the ten *Xoo* pathotypes of Northern states of India. Though, further confirmation is needed with the allelic test. A cross between NIL

carrying *xa13* (IRBB 13) and IL274 will be carried out in the next *kharif* season to validate the concept. In addition, we also developed co-dominant marker for the transfer of this recessive gene to the elite cultivar for the deployment of bacterial blight resistance in breeding programs.

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Author contribution statement KS and KN designed the experiment and performed the phenotyping and genetic mapping using F_2 population. JSL provided *Xanthomonas* cultures and assisted in phenotyping of mapping population. KS, RM, VG, DB, BKG and RK contributed in the phenotyping and genotyping of backcrossed derivatives. KN and KS analyzed the data and wrote the manuscript. KS and GSM involved in the field trials and the release of the rice variety PR127 with *xa-45(t)*.

Compliance with the ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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