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A novel bacterial blight resistance gene from *Oryza nivara* mapped to 38 kb region on chromosome 4L and transferred to *Oryza sativa* L.

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Summary

Bacterial blight (BB) of rice caused by *Xanthomonas oryzae* pv *oryzae* (*Xoo*) is one of the major constraints to productivity in South-East Asia. The strategy of using major genes, singly or in combination, continues to be the most effective approach for BB management. Currently, more than two dozen genes have been designated but not all the known genes are effective against all the prevalent pathotypes. The challenge, therefore, is to continue to expand the gene pool of effective and potentially durable resistance genes. Wild species constitute an important reservoir of the resistance genes including BB. An accession of *Oryza nivara* (IRGC 81825) was found to be resistant to all the seven *Xoo* pathotypes prevalent in northern states of India. Inheritance and mapping of resistance in *O. nivara* was studied by using F_2, BC_2F_2, BC_3F_1 and BC_3F_2 progenies of the cross involving *Oryza sativa* cv PR114 and the *O. nivara* acc. 81825 using the most virulent *Xoo* pathotype. Genetic analysis of the segregating progenies revealed that the BB resistance in *O. nivara* was conditioned by a single dominant gene. Bulked segregant analysis (BSA) of F_2 population using 191 polymorphic SSR markers identified a ~35 centiMorgans (cM) chromosomal region on 4L, bracketed by RM317 and RM562, to be associated with BB resistance. Screening of BC_3F_1 and BC_2F_2 progenies and their genotyping with more than 30 polymorphic SSR markers in the region, covering Bacterial artificial chromosome (BAC) clone OSJNBb0085C12, led to mapping of the resistance gene between the STS markers based on annotated genes LOC_Os04g53060 and LOC_Os04g53120, which is ~38·4 kb. Since none of the known *Xa* genes, which are mapped on chromosome 4L, are effective against the *Xoo* pathotypes tested, the BB resistance gene identified and transferred from *O. nivara* is novel and is tentatively designated as *Xa30*(*t*). Homozygous resistant BC_3F_3 progenies with smallest introgression region have been identified.

1. Introduction

Bacterial blight (BB) of rice, caused by *Xanthomonas oryzae* pv *oryzae* (*Xoo*) (Ishiyama, Dye), is found worldwide and is particularly endemic to much of Asia (Mew *et al*., 1993; Nino-Liu *et al*., 2006). At seedling stage (Kresek), the disease may cause complete wilting or death of effective tillers, whereas at maximum tillering stage, it results in blighting of leaves. Losses due to its outbreak can reach up to 50% depending on stage, weather, location and variety (Reddy *et al*., 1979; Mew *et al*., 1993). The *Xoo* pathogen is highly variable and over 30 races of it have been reported worldwide (Nino-Liu *et al*., 2006; Gonzalez *et al*., 2007). Because of variability in virulence, breeding of resistant cultivars always confronts difficulties in terms of durability of resistance. Genome structure of *Xoo* revealed the presence of a large number of effector (*avr*) genes and insertion sequences (Ochiai *et al*., 2005; Lee *et al*., 2005), which may be playing a major role in generating high degree of genetic diversity and race differentiation (Ochiai

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Table 1. *Avirulence/virulence (effective/ineffective) of Xoo pathotypes prevalent in Punjab (India) against known BB resistance genes*

Pathotype	Avirulence	Virulence
I	*Xa4*, *xa5*, *xa8*, *xa13*, *Xa21*	*Xa1*, *Xa2*, *Xa3*, *Xa7*, *Xa10*, *Xa11*, *Xa12*, *Xa14*, *Xa16*
II	*Xa4*, *xa5*, *Xa7*, *xa8*, *xa13*, *Xa21*	*Xa1*, *Xa3*, *Xa10*, *Xa11*, *Xa14*
III	*Xa4*, *xa5*, *Xa7*, *xa8*, *xa13*, *Xa21*	*Xa1*, *Xa2*, *Xa3*, *Xa10*, *Xa11*, *Xa14*, *Xa16*
IV	*Xa4*, *xa5*, *xa8*, *xa13*	*Xa1*, *Xa2*, *Xa3*, *Xa7*, *Xa10*, *Xa11*, *Xa12*, *Xa14*, *Xa21*, *Xa16*
V	*Xa3*, *xa8*, *xa13*, *Xa21*,	*Xa1*, *Xa2*, *Xa4*, *xa5*, *Xa7*, *Xa10*, *Xa11*, *Xa12*, *Xa14*, *Xa16*
VI	*xa8*, *Xa4*, *xa5*, *xa13*, *Xa21*	*Xa1*, *Xa2*, *Xa3*, *Xa7*, *Xa10*, *Xa11*, *Xa12*, *xa13*, *Xa14*, *Xa16*
VII	*xa13*, *Xa21*	*Xa1*, *Xa2*, *Xa3*, *Xa4*, *xa5*, *Xa7*, *xa8*, *Xa10*, *Xa11*, *Xa12*, *Xa14*, *Xa16*

et al., 2005). In Punjab State of India, for example, two pathotypes were reported in the 1980s, but this number has now grown to seven (Lore & Raina, 2005; J. S. Lore, personal communication), despite the fact that BB-susceptible cultivars occupied a large area under cultivation during this period. Chemical control of BB is not possible because the effective and economical chemical control measures are not available (Gnanamanickam *et al.*, 1999); therefore, the preferred strategy for BB management has been through development and deployment of resistant cultivars (Khush, 2001).

Currently, more than two dozen genes conferring resistance on BB have been identified and these are designated *Xa1* to *Xa29*(*t*) (Nino-Liu *et al.*, 2006). A majority of these are from cultivated rice, except *Xa21*, *Xa23*, *Xa27* and *Xa29*, which have been transferred from *Oryza longistaminata, Oryza rufipogon, Oryza minuta and Oryza officinalis*, respectively (Khush *et al.*, 1990; Zhang *et al.*, 1998; Gu *et al.*, 2003; Tan *et al.*, 2004). Not all the known genes are effective, but deployment of a few genes has been highly successful. *Xa4*, for example, has been used most widely in the tropics and in many parts of China (Sun *et al.*, 2003; Mew *et al.*, 2004; Vikal *et al.*, 2004), whereas *Xa1* and *Xa3* are widely used in Japan and Korea (Mew *et al.*, 2004). In the North Western Plains of India, except for *xa13*, none of the known *Xa* genes are effective singly against all the prevalent *Xoo* pathotypes. Also appearance of new pathogen virulences warrants expanding the gene pool of effective and potentially durable genes (Mew *et al.*, 2004). Most of the known BB resistance genes have been tagged with DNA markers (He *et al.*, 2006) and six, viz. *Xa1*, *xa5*, *xa13*, *Xa21*, *Xa26* and *Xa27*, have been cloned (Dai *et al.*, 2007). Keeping in view the evolutionary potential of *Xoo*, identification of new genes or pyramiding of two or more genes is required for ensuring control of the disease. Here, we report identification and mapping of a new dominant BB resistance gene from *Oryza nivara* and its transfer to cultivated rice, *Oryza sativa*.

2. Materials and methods

(i) *Plant material and population development*

O. nivara acc. IRGC 81825 (hereafter referred to as acc. 81825) procured from IRRI (International Rice Research Institute; Los Baños, Laguna, Philippines) showed a high level of resistance to all the seven *Xoo* pathotypes tested (Vikal *et al.*, 2007). This accession was crossed as male to *O. sativa* cv PR114. The F_1 plants were self-pollinated and backcrossed to PR114 to generate F_2 and BC_1F_1 progeny. The resistant BC_1F_1 plants were again backcrossed to PR114 to generate BC_2F_1 that were further self-pollinated to generate BC_2F_2 generation. BB-resistant BC_2F_1 plants were again crossed to the recurrent parent PR114 to generate BC_3F_1 progeny. Individual BC_2F_2 plants were again selfed to generate BC_2F_3 progeny. The F_2, BC_3F_1, BC_2F_2 and BC_2F_3 progenies generated were used for studying inheritance, mapping and stability of the resistance gene introgressed from *O. nivara*.

(ii) *Screening with Xoo*

The *O. nivara* acc. 81825 was resistant to all the seven *Xoo* pathotypes prevalent in Punjab (Vikal *et al.*, 2007) and PR114 was resistant to five but susceptible to two. Pathotype VII, which was virulent on PR114 but avirulent on *O. nivara* acc. 81825, was used for screening the segregating progenies. The *Xoo* pathotype VII was virulent on *Xa1*, *Xa2*, *Xa3*, *Xa4*, *xa5*, *Xa7*, *xa8*, *Xa10*, *Xa11*, *Xa12*, *Xa14*, *Xa16* and DV85 (*xa5*+*Xa7*) but avirulent on *xa*13 and *Xa21* (Table 1). The cultures, originally isolated from a single colony, were streaked on Walkimoto medium (10 g sucrose, 5 g peptone and 20 g agar; total volume made 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 concentration of bacterium was standardized to a density of 10^8–10^9 cells per ml and used

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for inoculations. The BC_3F_1 and BC_2F_2 progenies were screened during 2006 and BC_2F_3 during 2007. Individual plants were inoculated at maximum tillering stage following clip inoculation (Kauffman *et al.*, 1973). The disease severity was recorded 14–16 days after the inoculation. In this, lesion length in each plant was measured on five leaves and averages worked out. Plants having lesion length up to 5·0 cm were classified as resistant and the ones with more than 5·0 cm were classified as susceptible. This was based on the alley formed while classifying plants as resistant or susceptible. Chi-square test, after Yates correction (χ^2_C), was used to test goodness of fit for ascertaining the number of genes governing the BB resistance. In addition to inoculations at maximum tillering stage, both *O. nivara* acc 81825 and PR114 were inoculated with pathotype VII at seedling stage (1-month-old seedlings) as well using the same procedure as described above.

(iii) *Marker analysis*

DNA from parents and the segregating generations was isolated from field-grown plants of F_2, BC_3F_1 and BC_2F_2 progenies using the CTAB (cetyl trimethyl ammonium bromide) method. *In vitro* DNA amplification through PCR was performed in a reaction volume of 25 μl, containing 50–75 ng template DNA, 100 μM of each dNTP, 1 × PCR reaction buffer (10 mM Tris/HCl + 50 mM KCl + 0·01 %, w/v, gelatin, pH 8·3), 1·5 mM $MgCl_2$, 0·25 μM of each forward and reverse primers and 1·0 unit of Taq polymerase. The PCR amplification involved the following thermal profile: initial denaturation at 94 °C for 4 min followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 50–60 °C (depending on the primer) for 1 min and extension at 72 °C for 2 min followed by final extension at 72 °C for 7 min. A negative control, without template DNA, was included in each plate during every amplification reaction. The amplification products were resolved on 2·5 % high-resolution agarose (Amresco) prepared in 0·5 × TBE buffer. After electrophoresis, gels were stained with ethidium bromide for 20 min, rinsed in deionized water and photographed under ultraviolet light using the gel documentation system (Fotodyne). Parental polymorphism and bulked segregant analysis (BSA) (Michelmore *et al.*, 1991) were taken up simultaneously. In this, equal quantity of DNA from ten highly resistant and ten highly susceptible plants from F_2 population of the cross PR114/*O. nivara* acc. 81825 was bulked to generate resistant bulks (RB) and susceptible bulks (SB), respectively. *In vitro* DNA amplification of parents and the RB and SB was done using SSR markers selected at an interval of 5–10 centiMorgans (cM), from each of the 12 linkage groups presented by Temnykh *et al.* (2001). Once the chromosomal region harbouring the BB resistance gene was identified, based on BSA, the exact position of the gene was determined by analysing individual plants in segregating BC_3F_1 and BC_2F_2 progenies with polymorphic SSR markers selected from Temnykh *et al.* (2001) and IRGSP (2005 – Supplementary Table 18).

(iv) *Data analysis and linkage mapping*

Goodness of fit for all the loci to an expected 1 : 1 (in BC_3F_1) or 1 : 2 : 1 (in BC_2F_2) was tested using χ^2 analysis. Linkage analysis of polymorphic markers was performed with the software package Mapmaker (Lander *et al.*, 1987) for f_2 backcross and f_2 intercross for BC_3F_1 and BC_2F_2 progenies, respectively. Recombination frequencies were converted to cM using Kosambi's mapping function (Kosambi, 1944). Multipoint analysis was performed using an initial LOD (logarithm of the odds) threshold of 5·0 and maximum recombination fraction of 0·30. The polymorphic markers were ordered using the 'Compare' command. Additional markers were added afterwards on this frame using the 'Try' command. The final order was verified with the 'Ripple' command with a window size of 5 and LOD threshold of 10·0. The linkage map was finally drawn using the software MapChart, version 2.1 (Voorrips, 2002), and graphical genotypes of individual plants were generated using the software GGT32 (Berloo, 1999; www.dpw.wageningen-ur.nl/pv/pub/ggt/).

(v) *Quantitative trait locus (QTL) analysis*

As presented above, the BB reaction was recorded as lesion length in cm, and plants in the segregating populations were classified as resistant or susceptible depending on whether lesion length was up to 5·0 cm or more. Although an alley was formed, both in BC_3F_1 and BC_2F_2 populations around the lesion length of 5·0 cm, still the variation was continuous. It was therefore analysed for QTL as well. The QTLs were detected and localized by single marker regression (SMA) and interval mapping (IM) using MapManager QTXb20 software (Manly *et al.*, 2001). In this analysis, the lesion length data of the individual plants were entered along with their genotypic data. The 'marker regression' function ($P = 0·01$) was used to detect possible single marker loci associated with the QTL. Thereafter, the IM was used for localization and estimation of effects of the QTL. Significant ($P < 0·05$) and highly significant ($P < 0·01$) threshold levels were determined by the permutation test function of MapManager, which is based on the statistical methods developed by Churchill & Doerge (1994). A likelihood ratio statistic (LRS) value of 4·6 is equivalent to 1 LOD. Phenotypic variance (R^2) explained by the

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Table 2. *Frequency of resistant and susceptible plants in the BC_3F_1, BC_3F_2, BC_2F_2 and BC_2F_3 populations*

Population	Number of plants: Resistant (<5 cm)	Segregating	Susceptible (>5 cm)	Total	χ^2_C	*P* value
BC_3F_1	36	–	38	74	0·014 (1:1)[a]	0·906
BC_3F_2	806	–	289	1095	1·059 (3:1)	0·303
BC_2F_2	290	–	106	396	0·569 (3:1)	0·451
BC_2F_3	115	175	106	396	5·750 (1:2:1)	0·056

[a] Ratios in parentheses are the ones expected, based on one-gene segregation. The average lesion length in PR114 and *O. nivara* acc. 81825 against pathotype VII was 16·4 and 0·5 cm, respectively.

QTL was estimated as difference between the total variance and the residual variance expressed as the percentage of the total variance.

3. Results

(i) *Inheritance of BB resistance in* O. nivara *acc. 81825*

The *O. nivara* acc. 81825 showed resistance to all the seven *Xoo* pathotypes tested at maximum tillering stage. It was resistant to pathotype VII at seedling stage as well. Thus, the resistance gene in *O. nivara* acc. 81825 is effective at all the growth stages. Inheritance of BB resistance in *O. nivara* acc. 81825, based on an F_2 progeny of the cross PR114/*O. nivara* acc. 81825, was reported earlier (Kaur *et al.*, 2005). Since our objective was simultaneous mapping and transfer of the gene to cultivated rice and preliminary evidence of segregation distortion for several SSR loci in the F_2 progeny of the cross PR114/*O. nivara* acc. 81825, we generated BC_2F_2, BC_2F_3, BC_3F_1 and BC_3F_2 progenies for confirming the inheritance and mapping of the gene. The average lesion length in BC_3F_1 ranged from <0·5 to 20·4 cm. Of the 74 BC_3F_1 plants, 36 were resistant and 38 susceptible (Table 2), which fitted well to the expected 1:1 segregation ($\chi^2_C=0{\cdot}014$, Table 2). In BC_2F_2, the average lesion length ranged from 0·5 to 24·2 cm (Fig. 1). Of the 396 BC_2F_2 plants, 290 showed resistant reaction, whereas 106 showed susceptible reaction (Table 2), which again fitted well to one-gene segregation ($\chi^2_C=0{\cdot}569$). Of the 396 BC_2F_3 progenies, 115 were homozygous resistant, 175 were segregating and 106 were homozygous susceptible, which fitted well to the expected 1:2:1 segregation (Table 2). Similarly, out of 1095 BC_3F_2 plants, 809 were resistant and 289 susceptible, which again fitted well to 3:1 (Table 2) expected of a single gene segregation. Thus, the BB resistance gene in *O. nivara* acc. 81825 behaves as a single dominant gene even when transferred to cultivated rice *O. sativa*.

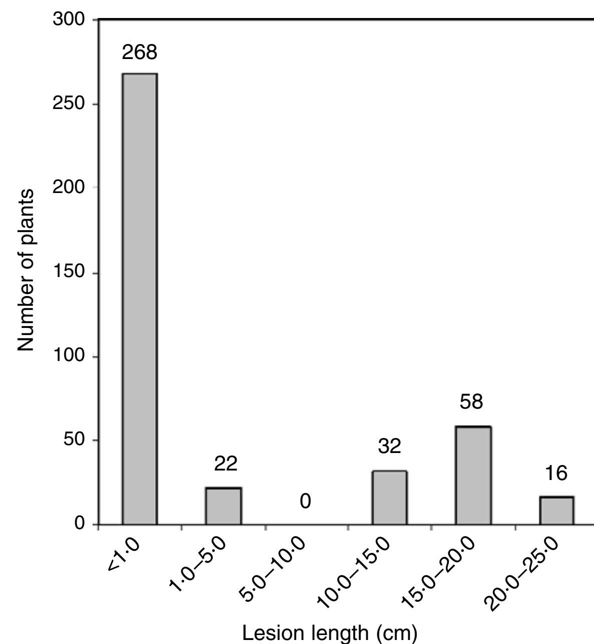

Fig. 1. The distribution of average lesion length in BC_2F_2 progeny of the cross PR114/*O. nivara* acc. 81825/2*PR114. The average lesion lengths of *O. nivara* acc. 81825 and PR114 were 0·5 and 16·4 cm, respectively.

(ii) *Parental polymorphism and BSA*

Parental polymorphism and BSA (Michelmore *et al.*, 1991) were conducted simultaneously. SSR markers from the map of Temnykh *et al.* (2001) were selected at an interval of 5–10 cM, from all the 12 linkage groups. A total of 402 SSR markers were tested for parental polymorphism and BSA. Out of the total 402 SSR markers tested, 211 (52·5%) were monomorphic and 191 (47·5%) were polymorphic when resolved in 3·0% high-resolution agarose gels. Parental polymorphism ranged from as low as 21·7% for chromosome 12 to 61·3% for chromosome 7. All the 402 markers were simultaneously tested for BSA using the DNA of RB and SB prepared from the F_2 population of the cross PR114/*O. nivara* acc. 81825. Any marker that assorts independent of resistance gene will show

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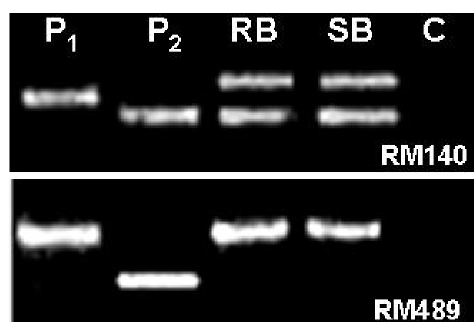

Fig. 2. Banding pattern of parents, RB and SB for RM140 and RM489. RM140 shows both parent bands in RB as well SB, whereas RM489 shows only P1 (*O. nivara*) band, both in RB and SB. P2 is *O. sativa* cv PR114 and C is control (no DNA).

both parent bands in RB as well as SB, whereas a marker linked to a dominant resistance gene in coupling phase will show only susceptible parent band in the SB, but in RB both resistant and susceptible parent bands will appear, though with varied intensity. Of the 191 polymorphic markers, as many as 48 markers showed distorted segregation based on BSA, i.e. both the RB and SB had either PR114 allele or *O. nivara* allele only (Fig. 2). Thirteen of the distorted markers map on chromosome 5. Markers RM567, RM127, RM317 and RM252 located on distal end of chromosome 4L showed the banding pattern expected of a marker linked to the target gene (Fig. 3). RM317 showed only susceptible parent band in SB and resistant parent band in RB, although RB was expected to have bands from both the parents. Markers RM252, RM537 and RM551 that map distal to RM317, towards the centromere (Temnykh *et al.*, 2001), also showed distorted segregation. The region bracketed by RM317 and RM567 was about 35 cM (Fig. 4*a*, Temnykh *et al.*, 2001).

(iii) *Mapping of BB resistance gene in* O. nivara *acc. 81825*

Although the BSA was based on F_2 progeny of the cross PR114/*O. nivara* acc. 81825, mapping was done using BC_3F_1 and BC_2F_2 progenies due to distorted segregation for several SSR loci as described above. For chromosome 4, 32 SSR markers were tested for polymorphism from the map of Temnykh *et al.* (2001) and 45 from IRGSP (2005) (Table 3). Out of the total 77 SSR markers tested, 49 (63·6%) were polymorphic (Table 3). Of 49 polymorphic markers, 34 were analysed on 74 BC_3F_1 plants. Twenty-one of these showed expected 1 : 1 segregation for homozygotes (for PR114 allele) and heterozygotes and 13 showed significant deviation from the expected 1 : 1, with χ^2 values ranging from 4·4 to 46·4. All the markers that showed segregation distortion had PR114 allele in higher frequency and map towards the centromere, whereas the ones that did not show the distortion map towards telomere.

Analysis of 74 BC_3F_1 plants for BB reaction and genotypic data for 34 SSR markers, using the software package Mapmaker, mapped BB resistance gene from *O. nivara* between RM17499 and RM17502, which are 4·0 cM apart (Fig. 4*b*). Only 26 of the 34 markers could be mapped using this population. Markers RM252, RM537 and RM551 that showed segregation distortion in favour of PR114 allele did not show linkage with other markers but formed a separate linkage group (data not shown). The linkage map thus generated with 26 SSR markers spans 27·3 cM region (Fig. 4*b*), which is almost similar to map distance between RM317 and RM567 (Fig. 4*a*) as reported by Temnykh *et al.* (2001). Since the BB resistance gene showed co-segregation with RM17499, an additional 336 BC_2F_2 plants were used for fine mapping of the gene. Six SSR markers, RM17480, RM17483, RM17488, RM17496, RM17499 and RM17502 (IRGSP, 2005; Supplementary Table 18), were analysed in 336 BC_2F_2 plants. All these markers showed expected 1 : 2 : 1 frequency of individuals homozygous for PR114 alleles, heterozygous, and homozygous for *O. nivara* allele, respectively. Mapmaker analysis showed the BB resistance gene to map between RM17499 and RM17502, which are about 176 kb apart and flank the BAC clone OSJNBb0085C12. To further narrow down the region, 14 SSRs were identified from the BAC clone OSJNBb0085C12 and their primers designed. However, none of the 14 SSRs showed

Table 3. *Parental polymorphism for SSR markers between PR114 and* O. nivara *acc. 81825 in chromosome 4*

Marker series	Markers tested	Monomorphic markers	Polymorphic markers	% Polymorphism
RM1 to RM600 (Temnykh *et al.*, 2001)	32	13	19	59·4
RM17396 to RM17663 (IRGSP, 2005)	45	15	30	66·7
Total	77	28	49	63·6

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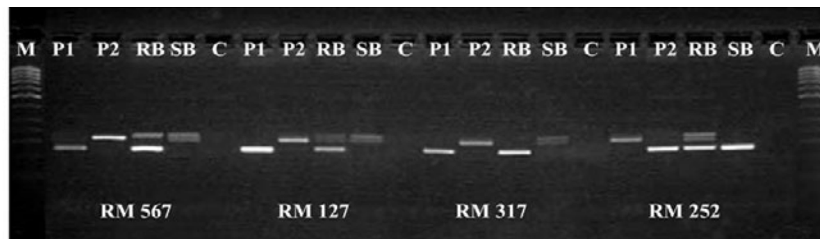

Fig. 3. Bulk segregant analysis showing association of RM567, RM127, RM317 and RM352 with BB resistance gene in *O. nivara*. P1 is *O. nivara*, P2 is PR114 and C is control (no DNA). SB shows the presence of PR114 band only, whereas the RB shows the presence of bands from both the parents as expected of a dominant gene, except for RM317 wherein RB had only *O. nivara* band.

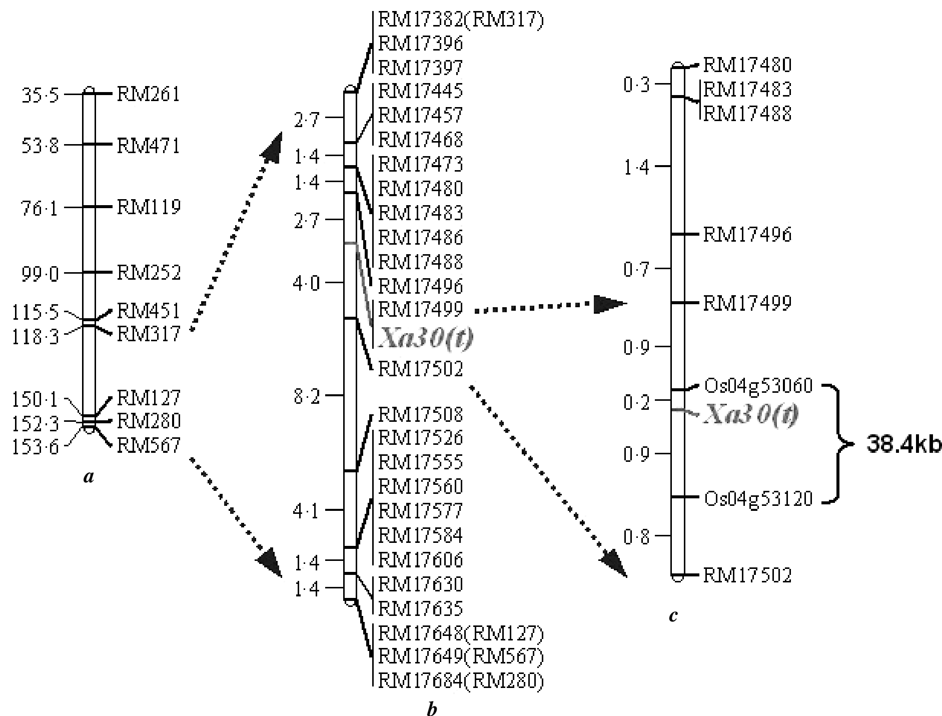

Fig. 4. Linkage map of chromosome 4L showing map position of *Xa30*(*t*). (*a*) Linkage map of 4L based on Temnykh *et al.* (2001); (*b*) linkage map of 4L based on 74 BC_3F_1 plants and (*c*) based on 336 BC_2F_2 plants. BSA identified markers RM317, RM127 and RM567 to be associated with the BB resistance gene.

polymorphism between the parental lines. Subsequently, 17 gene-based primers were designed from 12 loci, including five nucleotide-binding site–leucine-rich repeat (NBS–LRR) loci, annotated from BAC clone OSJNBb0085C12 (http://rice.plantbiology.msu.edu/cgi-bin/gbrowse/rice/). Primers designed from two loci, LOC_Os04g53060 (5′-TGGAAACAAGGAAGGTTTCG-3′F and 5′-TGGACTGAGATGAGGTGCTG-3′R) and LOC_Os04g53120 (5′-ATGAATGGATTCGCCAGAT-3′F and 5′-CGTCTCTTCAGCCTCAAACC-3′R), were polymorphic, whereas the other 15 markers were monomorphic even after restriction with several restriction enzymes for which restriction sites were present in the Nipponbare genome. The locus LOC_Os04g53060 showed amplification in cultivated parent line PR114 but no amplification in *O. nivara*, whereas the locus LOC_Os04g53120 showed amplification in both the parents. Analysis of 336 BC_2F_2 plants with six SSR and two gene-based markers showed that the gene maps between LOC_Os04g53060 and LOC_Os04g53120 at a distance of 0·2 cM from LOC_Os04g53060 and 0·9 cM from LOC_Os04g53120 (Fig. 4 *c*). Thus the BB resistance gene from *O. nivara* maps on chromosome 4L in 1·1 cM region bracketed by annotated loci LOC_Os04g53060 and LOC_Os04g53120 (http://rice.plantbiology.msu.edu/cgi-bin/gbrowse/rice/).

(iv) *Mapping BB resistance as QTL*

The BB resistance gene could be mapped as a major gene after classifying the plants as resistant or susceptible, based on lesion length, as presented in the preceding section. The BC_3F_1 population was also

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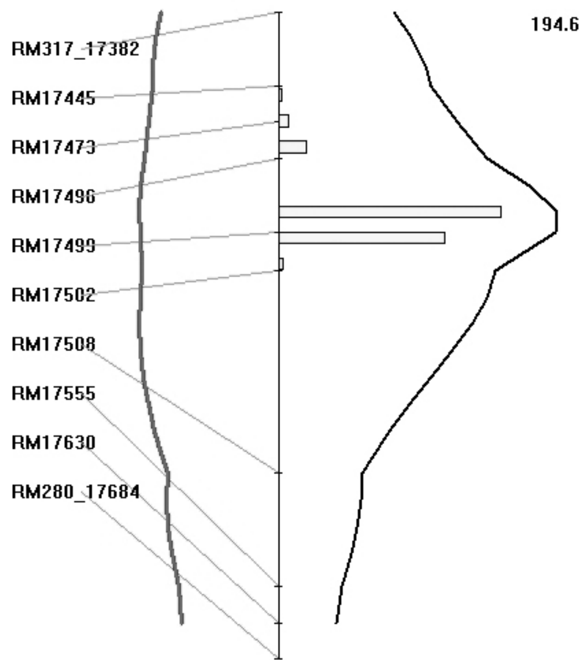

Fig. 5. Map location of BB resistance QTL transferred from *O. nivara*. A partial linkage map of chromosome 4L is shown along. Curves in black represent the LRS values for the markers and curves in grey represent the regression coefficients (additive effects). The numerical value on top of the figure is the highest LRS values for the QTLs detected on chromosome 4L. Histogram represents estimates of confidence interval by bootstrap resampling.

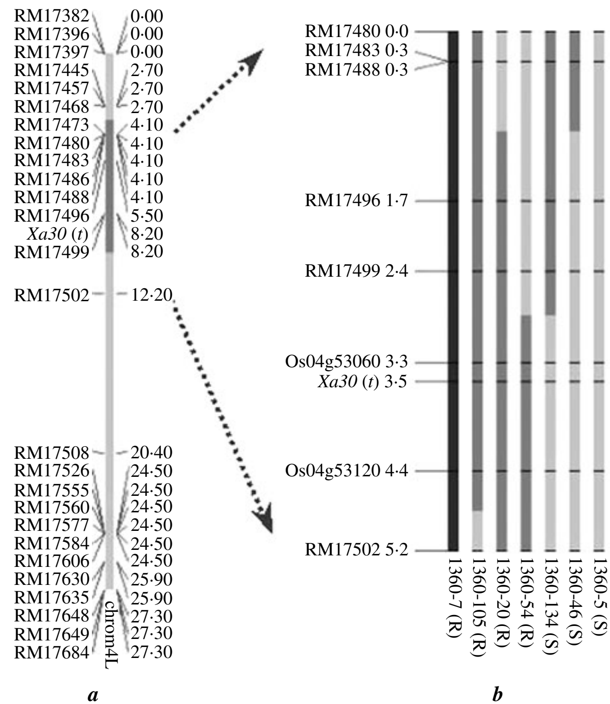

Fig. 6. Graphical genotype for chromosome 4L of a BB-resistant BC_3F_1 plant (no. 1358-15) (*a*) and seven BC_2F_2 plants (*b*). Numbers below bars in (*b*) are the BC_2F_2 plant numbers; 1360-7, 1360-105, 1360-20 and 1360-54 were resistant to BB, whereas 1360-134, 1360-46 and 1360-5 were susceptible. Black region indicates introgression from *O. nivara* chromatin homozygous condition; dark grey as heterozygous regions and light grey indicates PR114 chromatin. Numerical values on the right in (*a*) and the values adjoining the markers in (*b*) are the map distances in cM.

analysed for QTL, based on lesion length. Both SMA and IM were used for identifying the regions associated with BB resistance. Markers that showed co-segregation were removed as redundant loci by the software for analysis. With IM, the region between markers RM17499 and RM17502 had the highest LRS value of 194·6 (which equates to a LOD value of 42·0) and it explained 93% of the total phenotypic variation (Fig. 5). The threshold LRS value ($P<0{\cdot}001$), based on 1000 permutations, was 18·1. Thus the QTL for BB resistance mapped on chromosome 4L is highly significant. Similarly, IM of the BC_2F_2 population of 336 plants identified an interval between markers LOC_Os04g53060 and LOC_Os04g53120 as having the highest LRS value, being 460 (which equates to a LOD score of 100·0), and it again explained 93% of the total phenotypic variation.

(v) *Graphical genotypes of the BC_3F_1 and BC_2F_2 plants*

Graphical genotypes were generated for all the 74 BC_3F_1 plants for 27·3 cM region using 26 SSR markers and for 336 BC_2F_2 plants using six SSR markers. Among the 36 resistant BC_3F_1 plants, the smallest introgression, around the BB resistance gene, was observed in plant no. 1358-15 for the chromosomal region spanning SSR markers RM17473 and RM17499 (Fig. 6*a*), which is ~476 kb. Similarly out of 336 BC_2F_2 plants, graphical genotype of four resistant and three susceptible plants are presented in Figure 6*b*. All the resistant plants had introgression in the region spanning the loci LOC_Os04g53060 and LOC_Os04g53120. Plant no. 1360-105 was resistant but did not show any introgression for RM17502 thus ruling out the possibility of this marker associated with BB resistance. Plant no. 1360-54 was resistant and had introgression for LOC_Os04g53060, LOC_Os04g53120 and RM17502 but did not show introgression for RM17499, thereby indicating that RM17499 is also not associated with BB resistance. Graphical genotypes of seven BC_2F_2 plants presented in Figure 6*b* thus confirm the mapping data, which showed BB resistance gene from *O. nivara* being located between

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Fig. 7. BB reaction of PR114 (2), *O. nivara* acc. 81825 (3) and a BC_3F_2 plant (4) when inoculated at maximum tillering stage with *Xoo* pathotype VII. Sample 1 is uninoculated leaf of PR114.

LOC_Os04g53060 and LOC_Os04g53120, which is about 38·4 kb.

(vi) *Transfer of BB resistance gene from* O. nivara *into* O. sativa *cv PR114*

Out of 74 BC_3F_1 plants, 36 were resistant and 38 susceptible. Lesion length in resistant plants ranged from <1·0 to 3 cm with 31 plants having less than 1 cm lesion length. Since graphical genotypes were generated for all the BC_3F_1 plants, plant no. 1358-15 was identified as having the smallest introgression around the region harbouring BB resistance. This plant was self-pollinated and, based on flanking markers linked to the BB resistance gene, homozygous BC_3F_2 plants were identified from its progeny. These plants were resistant with lesion length less than 1 cm (Fig. 7). The BC_3F_3 progenies that were homozygous for BB resistance gene and phenotypically like PR114 have been identified and are available for use in breeding programmes.

4. Discussions

(i) *Polymorphism and segregation distortion*

Overall, 47·5% polymorphism was observed between *O. sativa* cv PR114 and *O. nivara* acc. 81825 for all the 12 chromosomes. McCouch *et al.* (2002) observed 86% polymorphism between a japonica cultivar, Nipponbare, and an indica cultivar, Kasalath, when they resolved a set of 545 SSR markers in 3·0% agarose gel, whereas He *et al.* (2006) observed only 17% polymorphism between two indica lines, Zhenzhuai and IR24. The polymorphism level observed between the parental lines PR114 and *O. nivara* acc. 81825, although not as high as in the indica–japonica crosses, seems sufficiently high for map-based cloning of the BB resistance gene from *O. nivara*. Out of 191 polymorphic SSR markers tested in parents and the RB and SB, 48 markers showed segregation distortion wherein both the RB and SB had only either PR114 allele or *O. nivara* allele. The phenomenon of segregation distortion has been frequently reported in wide crosses in many crop species and is well documented in intraspecific and interspecific crosses in rice (Xu *et al.*, 1997; Lorieux *et al.*, 2000; Heuer & Miézan, 2003). The preferential transmission or segregation distortion might be due to gamete elimination, pollen killer genes and the phenomena of restriction in recombination as well. The work of Nakagahra *et al.* (1972) led to the suggestion that the gametophytic loci were responsible for the partial or total elimination of gametes carrying one of the parental alleles. Although 48 loci showed segregation distortion in this cross, the region harbouring the BB resistance gene in *O. nivara* showed normal segregation. However, markers RM252, RM551 and RM537 that map on 4L towards the centromere (Temnykh *et al.*, 2001) showed distortion in favour of *O. sativa* allele. Sterility gene *S28*(*t*) from *Oryza glumaepatula* has been mapped on chromosome 4 (Sobrizal *et al.*, 2002) in the same region where the above mentioned SSR markers map. In crosses between *O. sativa* and *O. glumaepatula*, the sterility gene *S28*(*t*) causes elimination of *O. sativa* allele (Sobrizal *et al.*, 2002), but in the present cross, *O. sativa* alleles were favoured.

(ii) *BB resistance gene in* O. nivara *acc. 81825 is novel*

The BB resistance gene under study was mapped to ~176 kb region on chromosome 4L using BC_3F_1 population. Four other BB resistance genes, *Xa1*, *Xa2*, *Xa12* and *Xa14*, are also reported on 4L (Ogawa *et al.*, 1978; Taura *et al.*, 1987; Yoshimura *et al.*, 1998; He *et al.*, 2006), but none of these are effective against any of the seven pathotypes prevalent in Punjab (Table 1), and hence allelic test is not possible. The *Xa1* was closely linked to *Xa12* and *Xa2* with *Xa1*

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mapping towards the centromere and *Xa2* towards the telomere with a recombination distance of 4·0 cM between them (Kinoshita & Takashi, 1991). Physical mapping and gene sequencing, however, placed *Xa2* towards the centromere and *Xa1* towards telomeric region (Yoshimura *et al*., 1998; He *et al*., 2006). The *Xa2* has been mapped to a 190 kb region on overlapping BAC clones OSJNBa0008M17, OSJNBa0093O08 and OSJNBa0058K23 (He *et al*., 2006). The *Xa1* gene (Os04g0622600) annotated in RAP-DB (http://rapdb.dna.affrc.go.jp/cgi-bin/gbrowse/IRGSP40/) corresponds to gene LOC-Os04g53160, whose co-ordinates span the genome sequences 31 437 628 bp to 31 443 630 bp (http://rice.plantbiology.msu.edu/cgi-bin/gbrowse/rice/). Both major gene and QTL mapping data and the graphical genotypes of BC_3F_1 and BC_2F_2 plants show that the gene described in the present study maps between markers LOC_Os04g53060 (start position 31 386 340 bp) and LOC_Os04g53120 (end position 31 425 732 bp), which are about 38·4 kb apart and cover the BAC clone OSJNBb0085C12. The BAC clone OSJNBb0085C12 harbours five NBS-LRR disease resistance gene motifs, viz. LOC_Os04g52970 (31334008–31339348), LOC_Os04g53000 (31346084–31347211), LOC_Os04g53050 (31381060–31385140), LOC_Os04g53060 (31386340–31388283) and LOC_Os04g53160 (31437628–31443630). In addition, LOC_Os04g53120 (31419036–31425732), a putative NB-ARC-domain-containing protein, is also present on this BAC clone. The *Xa1* locus corresponds to LOC_Os04g53160 (31437628–31443630), whereas *Xa2* may correspond to the locus LOC_Os04g52780 (31201695–31206034), which maps on another BAC clone OSJNBa0058K23 and is an LRR-receptor protein kinase as postulated by He *et al*. (2006). None of the four BB resistance genes, *Xa1*, *Xa2*, *Xa12* and *Xa14*, on 4L are effective against any of the seven pathotypes prevalent in Punjab, but the gene identified in the present study is resistant against all the seven pathotypes. All the evidence suggests that the gene is novel. High-resolution mapping around this locus is in progress, and final characterization will be possible after cloning and sequencing of the gene. This gene, therefore, is tentatively designated *Xa30*(*t*). The homozygous BB-resistant BC_3F_3 progenies in the background of PR114 will serve as a source of this gene. The closely linked SSR marker RM17499 and LOC_Os04g53120 could be used as diagnostic markers for marker-assisted breeding and gene pyramiding as the locus LOC_Os04g53160 is not amplified in *O. nivara*. Since no known dominant BB resistance gene is effective against prevalent pathotypes in North Western India, the new gene could be useful for development of BB-resistant F_1 hybrids and varieties. Locus LOC_Os04g53120 is a putative NB-ARC-domain-containing protein and is a part of the locus *Xa1* along LOC_Os04g53160 (http://rapdb.dna.affrc.go.jp/cgi-bin/gbrowse/IRGSP40/) that has functions similar to *Xa1*. Therefore, locus LOC_Os04g53060 (=Os04g621900 – RAP-DB), which is an NBS-LRR and is a 1944 bp long gene, is a candidate gene for *Xa30*(*t*).

Plant disease resistance genes frequently occur in the form of a tandem repeat multigene family. Four BB resistance genes, *Xa3*, *Xa26*(*t*), *Xa4* and *Xa22*, have been mapped to the terminal region of the long arm of chromosome 11(Lin *et al*., 1996; Yoshimura *et al*., 1996; Sun *et al*., 2003; Yang *et al*., 2003). All these genes are tightly linked and may belong to a multigene family (Yang *et al*., 2003). BB resistance genes *Xa1*, *Xa2*, *Xa12*, *Xa14* and *Xa30*(*t*), which map in ~600 kb region on chromosome 4L, may also belong to a multigene family. The chromosomal region between RM17499 and RM17502 where *Xa30*(*t*) maps is around 176 kb and shows a recombination rate of ~60 kb/cM, which is high to moderately high as reported in certain other high recombination regions in rice (Wu *et al*., 2003). The high frequency of recombination in resistance-gene-rich regions may be the cause for the generation of new resistance gene specificities.

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