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ORIGINAL PAPER

A putative candidate for the recessive gall midge resistance gene *gm3* in rice identified and validated

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Abstract

Key message **We report here tagging and fine-mapping of *gm3* gene, development of a functional marker for it and its use in marker-assisted selection.**

Abstract The recessive rice gall midge resistance gene, *gm3* identified in the rice breeding line RP2068-18-3-5 confers resistance against five of the seven Indian biotypes of the Asian rice gall midge *Orseolia oryzae*. We report here tagging and fine-mapping of *gm3* gene, development of a functional marker for it and demonstrated its use in marker-assisted selection (MAS). A mapping population consisting of 302 F_{10} recombinant inbred lines derived from the cross TN1 (susceptible)/RP2068-18-3-5, was screened against gall midge biotype 4 (GMB4) and analyzed with a set of 89 polymorphic SSR markers distributed uniformly across the rice genome. Two SSR markers, RM17480 and gm3SSR4, located on chromosome 4L displayed high degree of co-segregation with the trait phenotype and flanked the gene. *In silico* analysis of the genomic region spanning these two markers contained 62 putatively expressed genes, including a gene encoding an NB-ARC (NBS-LRR) domain containing protein. A fragment of this gene was amplified with the designed marker, NBcloning 0.9 Kb from the two susceptible TN1, Improved Samba Mahsuri (B95-1) and two resistant cultivars, RP 2068-18-3-5 and Phalguna (with *Gm2* gene). The amplicons were observed to be polymorphic between the susceptible and resistant genotypes and hence were cloned and sequenced. A new primer, gm3del3, which was designed based on sequence polymorphism, amplified fragments with distinct size polymorphism among RP2068-18-3-5, Phalguna and TN1 and B95-1 and displayed no recombination in the entire mapping population. Expression of the candidate NB-ARC gene in the susceptible TN1 and the resistant RP2068-18-3-5 plants following infestation with GMB4 was analyzed, through real-time reverse transcription PCR. Results showed twofold enhanced expression in RP2068-18-3-5 plants, but not in TN1 plants, 120 h after infestation. Amino acid sequence and structure analysis of the proteins coded by different alleles of *gm3* gene showed deletion of eight amino acids due to an early stop codon in RP2068-18-3-5 resulting in a change in the functional domain of the protein. The gm3del3 was used as a functional marker for introgression of *gm3* gene into the genetic background of the elite bacterial blight resistant cultivar Improved Samba Mahsuri (B95-1) through MAS.

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Introduction

The Asian rice gall midge, *Orseolia oryzae* (Wood-Mason) is a serious pest of rice in India and other Asian

countries causing significant yield losses (Widowsky and O'Toole 1996). Since the larvae of the insect feed inside the plant and remain enclosed within the galls, chemical control is not very effective. Cultivation of resistant varieties is the most effective and desirable way to manage the pest. Over 75 gall midge resistant rice varieties have been developed and released for commercial cultivation in India. However, extensive cultivation of single resistance gene containing varieties over a large area has resulted in breakdown of resistance due to evolution of virulent gall midge biotypes (Bentur et al. 2003). One of the options suggested for delaying emergence of virulent biotypes is to pyramid two or more resistance (R) genes that have not been deployed earlier and are mechanistically diverse (Cohen et al. 2004).

Of the 11 gall midge resistance genes in rice reported so far, eight genes viz., *Gm1*, *Gm2*, *Gm4*, *Gm5*, *Gm6*, *Gm7*, *Gm8* and *Gm11* have been tagged and mapped (Yasala et al. 2012). A cluster of four genes on chromosome 4 and of two genes on chromosome 8 was reported. These regions are rich in plant defense related genes. Out of these, only two genes, *Gm1* and *Gm8* confer resistance without the expression of hypersensitive reaction (HR-type, Bentur and Kalode 1996). All the other genes confer resistance associated with HR (Bentur et al. 2003). Resistance conferred by *gm3* gene is also HR+ type. Katiyar et al. (2000) suggested RAPD marker OPQ12 located on chromosome 4 to be linked to *gm3* gene but did not provide experimental data. This gene was reported from the breeding line RP2068-18-3-5 (Kumar et al. 1998), which is a derivative of the cross Swarnadhan/Velluthacheera. The latter is a land race from southern India which was reported to posses multiple pest resistance (Kalode et al. 1977). Extensive multi-location evaluation of this line has shown its wide spectrum of resistance against five of the seven biotypes prevalent in the country (Bentur et al. 2011). Hence, *gm3* offers a good candidate for gene pyramiding. However, development of closely linked markers is a prerequisite for development of gene pyramids through marker-assisted selection (MAS).

In the present study we report tagging and fine-mapping of *gm3* and development of a functional marker (gm3del3) suitable for use in MAS. This functional marker is also able to distinguish the sequence polymorphism between *Gm2* and *gm3* genes. We validated the candidate gene encoding for NB-ARC domain containing protein by real- time PCR analysis indicating its role in *gm3* mediated defense response. Further, we have used gm3del3 marker for introgression of *gm3* gene in the genetic background of Improved Samba Mahsuri (B95-1) possessing bacterial blight (BB) resistance.

Materials and methods

Plant material and mapping populations

The gall midge resistant rice breeding line RP2068-18-3-5 was crossed with the gall midge susceptible rice variety TN1.Two mapping populations were developed from this cross; first one consisting of 112 plants in F_2 generation was used to reconfirm the genetics of gall midge resistance, while the second mapping population consisting of 302 recombinant inbred lines (RILs) in F_{10} generation was used for gene tagging and mapping studies. Three other gall midge resistant rice varieties Phalguna (possessing *Gm2*), Dukong#1 (*Gm6*) and RP2333-156-8 (*Gm7*) were also included in the analysis since the map position of these resistance genes has been reported to be very proximal to each other on Chr. 4. For gene introgression, we used a recently released rice variety, Improved Samba Mahsuri (B95-1) having three BB resistance genes *xa5*, *xa13* and *Xa21* (Sundaram et al. 2008). BC_2F_2 population from the cross Improved Samba Mahsuri x RP2068-18-3-5 was used to validate the marker and introgress *gm3* into this cultivar.

Screening for gall midge resistance

Screening for gall midge resistance was carried out using gall midge biotype 4 (GMB4), in the greenhouse as described by Himabindu et al. (2010). F_2 plants were scored as susceptible or resistant based on the presence or absence of gall and confirmation of resistance through dissection of plants. Presence of tissue necrotic spot at the feeding site (indicating hyper sensitive reaction—HR) was taken as confirmation of resistance. In case of F_{10} RILs, if all the plants ($n \geq 10$) exhibited galls, the line was scored as homozygous susceptible and as homozygous resistant if all the plants registered no damage and displayed HR reaction when dissected. On the other hand, if a line showed mixture of R and S plants, it was rated as heterozygous. Of the 302 RILs 24 recorded heterozygous reaction. Reaction of BC_2F_3 plants against the gall midge biotypes GBM1, GMB4M and GMB5 was tested using a single tiller egg infestation method and reaction was rated based on survival or mortality of the maggots after 10 days of infestation. For real-time reverse transcription PCR studies, test plants (RP2068-18-3-5 and TN1) were raised in plastic trays as described by Himabindu et al. (2010) and exposed to 50 females and 25 males of gall midge biotype 4 when the plants were 15 day old. Egg hatching was monitored by examining plants periodically and both test plants were cut at the base for extracting RNA at specified time as described in detail by Rawat et al. (2012).

Molecular genotyping and linkage analysis

Total genomic DNA was isolated from the leaves of the parents, F_2, F_{10} RIL populations and BC_2F_2 plants, using the method of Dellaporta et al. (1983). A set of 423 rice microsatellite (SSR) markers uniformly spaced across the 12 chromosomes was used to detect polymorphism between the selected parents through PCR amplification (Chen et al. 1997). Some more markers were designed based on sequence information of the desired region of the genome (Table 1). The PCR products were resolved on 3 % agarose (US Biochemicals, USA) and in 0.5× TBE buffer (Sigma, USA), stained with Ethidium bromide (0.5 pg/ml) and documented under UV light (Sambrook and Russell 2001). The size of the amplified fragments was calculated using Alphaease software (Alpha Inotech, USA) with a 100-bp ladder (MBI Fermentas, Lithuania) as size reference standard. In all, 89 polymorphic SSR markers were used to test possible linkage with the trait through bulked-segregant analysis (Michelmore et al. 1991) in R and S bulk consisting of 10 F_{10} RILs each. Linkage analysis and map construction were performed using MAPMAKER/EXP, version 3 (Lander et al. 1987) as described by Biradar et al. (2004) using the RIL mapping population.

Identification of putative candidate genes and designing functional markers

Physical position of the markers on the *japonica* genome was identified through NCBI-BLAST search (http://www.ncbi.nlm.nih.gov), while it was also identified on the draft *indica* genome sequence (http://rice.genomics.org.cn/rice/index2.jsp) by keeping the forward and reverse primer sequences as the reference points, opening the specific rice chromosome sequence through the software Bioedit (available at http://www.mbio.ncsu.edu/BioEdit/bioedit/html) and using the 'find' command of the software. Using these markers as reference points, the genomic sequence between the markers was downloaded from *indica* genome from http://rise.genomics.org.cn/rice/index2Jsp. The downloaded sequences were then analyzed using the software package FGENESH (http://www.softberry.com) for open reading frames (ORFs) and annotated using BLAST-P utility (http://www.ncbi.nlm.nih.gov) to identify the putative function of each gene present in the marker delimited region.

Table 1 Details of designed primers used in fine-mapping of *gm3* and validation of the candidate NB-ARC gene

S. no	Name	Primer sequences (5′–3′)	Tm (°C)
1	gm3SSR4	F: AAGCCTTACCTCC CTCA	55
		R: TCAYGAAGCCAC CAGGGC	
2	NBcloning 0.9 Kb	F: AAGCCTTACCTCC CTCAAGTGG	55
		R: TCATGAAGCCACC AGGCACTGTA	
3	gm3del3	F: CTGCCAGAGATGG GCCTTCCA	55
		R: CGTACAAATTCCTG TACCACTC	
4	NB-ARC qRT primer	F: TCTGGCCTGCAC-GAAGC	60
		R: GGCAAACGCCTACC-CAGGA	

Cloning and sequencing of different alleles of candidate NB-ARC gene

Three separate primer pairs were designed to amplify three different regions of the putative candidate gene (NB-ARC) for *gm3*. Genomic DNA from RP2068-18-3-5, Phalguna and TN 1 were amplified using these primers through PCR and the amplified polymorphic fragments with each of the primer pairs were cloned using the PROMEGA PCR Cloning Kit (Qiagen, USA) as per the manufacturer's instructions. The cloned DNA samples were sequenced by using automated sequencing facility available commercially at Bioserve Biotechnologies (Hyderabad, India). A DNA sequence similarity search was carried out among the sequenced fragments using the BLAST utility available online (Altschul et al. 1997). Based on the alignments, single nucleotide polymorphisms (SNPs), additions and deletions, if any, were identified among TN1, RP2068-18-3-5 and Phalguna fragments. Three more primer pairs were designed targeting the deletion region from the sequence alignment data for the fragment of the gene NB-ARC at 32.05 MB (Table 1). All the three primer pairs were used to amplify the genomic DNA from the susceptible and the resistant parents. The primer pair giving robust amplification and displaying clear polymorphism was used for further analysis.

Quantitative real-time PCR analysis

Real-time PCR was performed using Applied Biosystems 7500 real-time PCR System with the SYBR green chemistry (Applied Biosystems, USA) according to the manufacturer's instructions. Gene-specific primers for real-time PCR were designed (Table 1) from the unique region of NB-ARC gene using the Primer Express software Version 3.0 (Applied Biosystems). Rice ubiquitin gene, *OsUBC* (accession no. AK059694) was used as the endogenous control. PCR reactions were carried out in 10 µl reaction containing 2 µl of first strand cDNA, 1× PCR buffer,

125 μM dNTPs, 1.5 mM $MgCl_2$, 0.2 μM primers and 1U Taq polymerase. The thermal profile used was: 94 °C for 2 min; 35 cycles of 94 °C for 20 s, annealing at 60 °C for 20 s, 72 °C for 30 s and final extension of 72 °C for 5 min. Melt curve analysis was also performed after completion of PCR cycles to check specificity of the PCR amplification. To calculate mean relative expression levels, cDNAs from two independent biological samples in three technical replications each were used. Relative transcription levels are presented graphically, and mean values were tested for difference using *t* test.

Protein analysis

The amplified nucleotide sequence (representing 30 % of *gm3* gene) of TN1 was analyzed for six frame translation using Bioedit six (http://www.mbio.ncsu.edu/bioedit/bioedit.html). The largest open reading frame (ORF) was selected and submitted for BLAST-P analysis to NCBI (http://www.ncbi.nlm.nih.gov/). Translation of nucleotide sequences to protein sequences for the four genotypes (TN1, B95-1, Phalguna and RP2068) was done using Bioedit software. Alignment of protein sequences of the NB-ARC domain containing protein, expressed [Oryza sativa Japonica Group] (gb|ABA93781.1) with those of four selected genotypes was done using Bioedit. The initial alignment was refined manually taking into account the domain predictions of the protein and to match the sequence conservation. As a result a multiple sequence alignment was generated.

Introgression of *gm3* gene into elite rice cultivar B95-1

RP2068-18-3-5, with *gm3* gene, was crossed with the elite, fine-grained, BB resistant rice variety Improved Samba Mahsuri (B95-1). F_1 plants were backcrossed to B95-1 to generate a BC_1F_1 population consisting of eight individual plants. These plants were tested for heterozygosity for the target BB genes (*Xa21*, *xa13* and *xa5*) using the gene-linked markers (Sundaram et al. 2008) and for *gm3* with gm3del3. A single BC_1F_1 plant with grain-type similar to Improved Samba Mahsuri and with all four genes in heterozygous state was then backcrossed to generate BC_2F_1 population. The BC_2F_1 plants were then screened using gene-linked markers for heterozygosity with respect to *Xa21*, *xa13*, *xa5* and *gm3*. A single 'positive' backcross plant, nearly identical to the recurrent parent morphologically, was selfed to generate BC_2F_2 plants. Sixteen of such plants were genotyped for presence of *Xa21, xa13, xa5* and *gm3* genes. One of these plants, designated as BC_2F_2-5, had three of the genes (*Xa21*, *xa13* and *gm3*) in homozygous condition and it was advanced to the next generation. BC_2F_3 plants were phenotyped for resistance against *Xanthomonas oryzae oryzae* (*Xoo*) strain DX-020 and GMB4 as per the standard protocol. These F_3 plants were further tested against GMB1, GMB4M and GMB5 biotypes of the pest, for grain quality, background genome analysis with 57 polymorphic markers on chromosomes other than on chromosome 4 and foreground genotyping with 15 polymorphic markers on chromosome 4 encompassing *gm3*.

Results

Inheritance of gall midge resistance in the rice genotype RP2068-18-3-5

The inheritance pattern of gall midge resistance in RP2068-18-3-5 was studied in F_2 and F_{10} RILs populations derived from cross TN1/RP2068-18-3-5 against GMB4 in the greenhouse. Of the 112 F_2 plants tested, 26 plants were observed to be resistant and 86 susceptible. The segregation data fitted well ($\chi^2 = 0.18$; $P = 0.671$) with the ratio of 1R: 3S, expected of a single recessive gene controlled resistance. Of the 302 F_{10} RILs tested, 125 lines were resistant with nil damage and 153 lines were susceptible with 100 % damage. The remaining 24 lines with >10 % and <90 % damage were scored as heterozygous. Thus F_{10} families segregated in an approximate ratio of 1R: 1S ratio ($\chi^2 = 2.8$; $P = 0.09$) further supporting our earlier observation of involvement of a single gene governing gall midge resistance (i.e. *gm3*) in RP2068-18-3-5.

Fine-mapping of *gm3* gene

Parental polymorphism survey between TN1 and RP2068-18-3-5 using 423 Simple Sequence Repeat (SSR) markers, revealed 89 markers (20.3 %) to be polymorphic. Among these, 53 markers were with dinucleotide repeat motif, 27 and nine with tri and tetra nucleotide repeat motifs, respectively. Highest number of polymorphic markers were on chromosomes 4 (18 markers) and 1 (14 markers). The least number (1) of polymorphic markers was on chromosome 3. When checked with 89 polymorphic markers in R and S bulks consisting of 10 RILs each, no pattern of linkage with the trait was detected with the markers located on the rice chromosomes other than chromosome 4. Three SSR markers RM17468, RM17473, RMl7480 and gm3SSR4 located on chromosome 4 displayed distinct association with the trait phenotype in BSA. All the four putatively linked SSR markers showed close linkage with the trait phenotype (Table 2) in co-segregation analysis of all 125 resistant and 153 susceptible F_{10} lines with RM17468, RM17473 and RMl7480 to be located on one side of the gene at a genetic distance of 2.8, 2.3 and 2.1 cM, respectively, while gm3SSR4 was located on the other side of the gene at a genetic distance of 1.0 cM. Thus, markers

Table 2 Marker-trait co-segregation pattern with four polymorphic SSR markers and the gene-specific gm3del3 marker in F_{10} RILs of the cross TN1 X RP2068-18-3-5

Marker	Phenotype R/S lines[a]	Total plants	No. of lines showing the marker allele			χ^2 (1R:1S)	*P*	Recombination (%)
			R[b]	H[a]	S[b]			
RM17468	R lines	125	120	Nil	5			
	S lines	153	3	Nil	150			
	Total lines tested	278	123	Nil	155	0.258	0.611	2.8
RM17473	R lines	125	119	1	5			
	S Lines	153	1	Nil	152			2.3
	Total lines tested	278	120	1	157	0.294	0.587	
RM17480	R lines	125	120	Nil	5			
	S lines	153	1	1	151			2.1
	Total lines tested	278	121	1	157	0.208	0.644	
gm3SSR4	R lines	125	122	Nil	3			
	S lines	153	Nil	Nil	153			1.0
	Total lines tested	278	122	Nil	156	0.032	0.858	
gm3del3	R lines	125	125	0	0			
	H lines	24	0	24	0			
	S lines	153	0	0	153		1.000	0.0
	Total lines tested	302	125	24	153			

[a] Heterozygous lines were not tested with SSR marker

[b] R-RP2068 allele; H-allele of both parents; S-TN1 allele

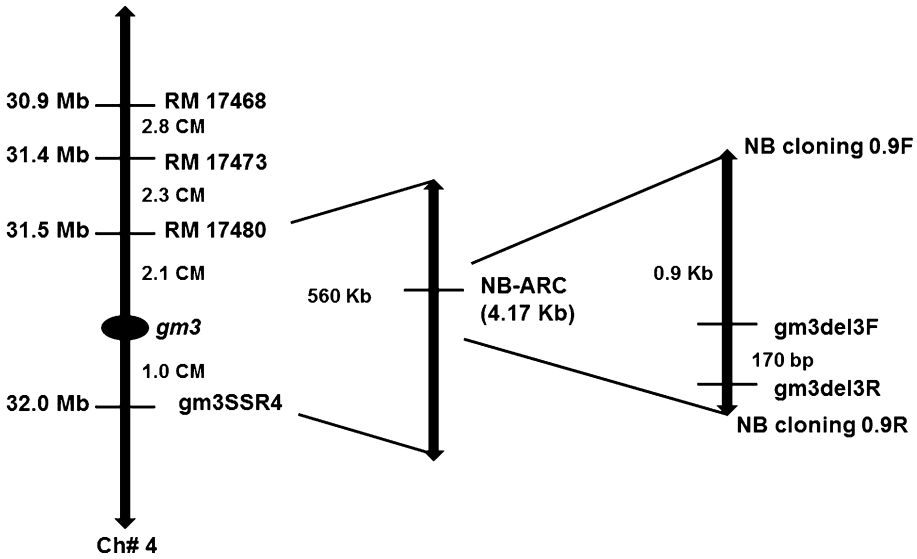

Fig. 1 Fine-mapping of *gm3* candidate gene NB-ARC with designed (gm3SSR4) and known SSR markers (RM17468, RM17473 and RM17480) on rice chromosome 4. Figures on *left* indicate physical position in mega base pair (Mb) and on *right* indicate genetic distance in cM

RM17480 and gm3SSR4 were the closest and flanking *gm3* in RP2068-18-3-5.

Identification of putative candidate gene for *gm3*

In silico analysis of *japonica* rice genome revealed 62 genes in the region spanning ~560 kb spanning the flanking RM17480 and gm3SSR4 markers (Fig. 1). Of these, a gene encoding putative NB-ARC protein (LOC_Os04g52970.1) at 31.553 MB region was suspected to be a candidate gene in view of the earlier reports of role of this class of genes in conferring resistance to insects and pathogens. Three primers designed based on the sequence information of this gene amplified fragments from the genomic DNA of the parents. However, one of the primer pairs designated as NBcloning 0.9 Kb amplified a fragment of size 1.2 kb in TN1 and 0.9 kb in RP2068-18-3-5. The polymorphism was also distinct between the resistant and susceptible bulks, and also among these and Phalguna rice variety (possessing *Gm2*). Sequence analysis of the amplified fragment revealed a large deletion of 320 bp at the starting point of the sequence in the resistant

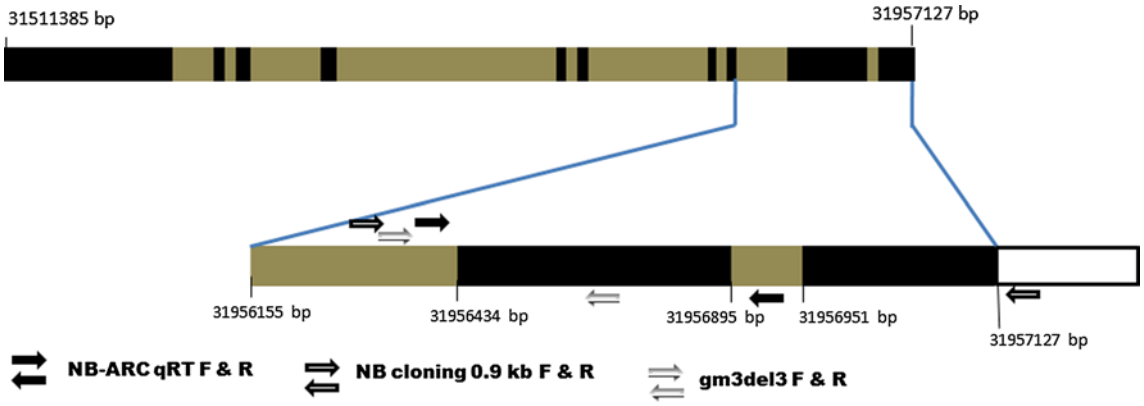

Fig. 2 Schematic illustration of the putative candidate gene for *gm3,* NB-ARC. The forward primer of the candidate gene-specific marker NBcloning 0.9 kb has been designed by targeting the eighth exon of the gene and the reverse primer targets a region in the 3′ UTR of the gene and the marker amplifies a produce of size 0.9 kb. Another candidate gene-specific marker gm3del3F & R amplifies a smaller fragment of size 213 bp and targets the eighth exon and the adjacent intron. In addition to these markers, another primer pair targeting the eighth and ninth exons of the gene has been designed for qRT-PCR experiments

RP2068-18-3-5 fragment in comparison with TN1 fragment (Fig. 2). Further deletion of 92 bp was observed in RP2068-18-3-5 as compared to the Phalguna fragment. Based on the sequence polymorphism a new primer pair, gm3del3 was designed for genotyping the entire set of 302 F_{10} RIL mapping population (including 125 resistant, 153 susceptible and 24 heterozygous RILs), the parents, Phalguna and R and S pools (Fig. 3; Table 2). The marker gm3del3 amplified RP2068-18-3-5 specific fragment in all the 125 resistant RILs, and TN1 allele in all the 153 susceptible RILs and amplified both RP2068-18-3-5 and TN1 alleles in all the 24 heterozygous lines (Table 2) indicating complete co-segregation along with the trait phenotype with no recombinants. Thus this marker can be considered as the gene-specific/functional marker for detection and introgression of *gm3*, and the gene encoding an NB-ARC protein as the candidate gene for *gm3*.

Validation of the candidate gene encoding a NB-ARC protein through real-time reverse transcription PCR

Expression of the NB-ARC gene was analyzed in RP2068-18-3-5 and TN1 plants infested with GMB4 at two time points: 24 and 120 h after infestation (Fig. 4). At 24 h time point, no change in the transcript level was observed in both the genotypes when compared with their respective uninfested controls. But at 120 h, a twofold increase in the transcript level was noticed in the resistant RP2068-18-3-5 with respect to the control. However, in susceptible rice TN1 there was no significant increase in the transcripts at 120 h of GMB4 infestation.

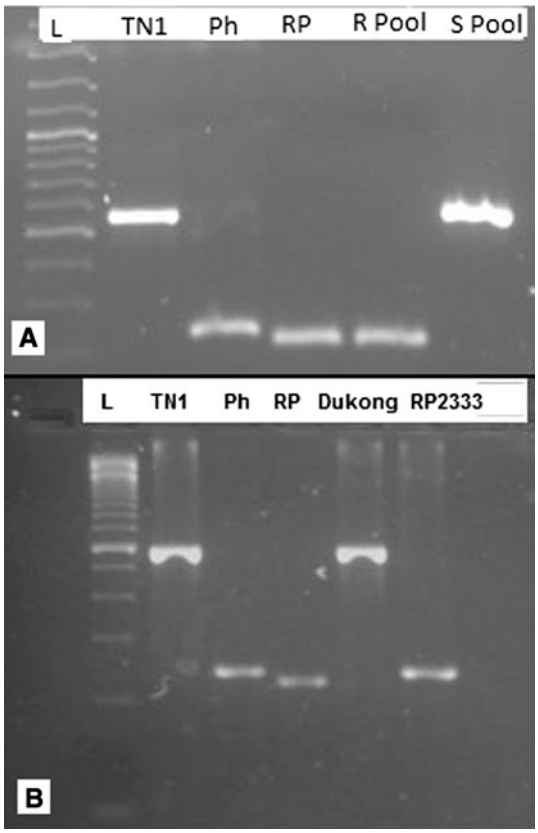

Fig. 3 **a** Amplification pattern of genomic DNA from *TN1*, Phalguna (*Ph*), RP2068-18-3-5 (*RP*), resistant pool (*R pool*) and susceptible pool (*S pool*) of F_{10} RILs of the cross TN1/RP2068-18-3-5 with gm3del3 marker. **b** Amplification pattern of genomic DNA from (1) TN1, (2) Phalguna, (3) RP2068-18-3-5, (4) Dukong #1 and (5) RP 2333-156-8 with gm3del3 marker

Protein analysis

Six frame translation of the TN1 allele sequence of the gene resulted in 33 possible ORFs within the sequence.

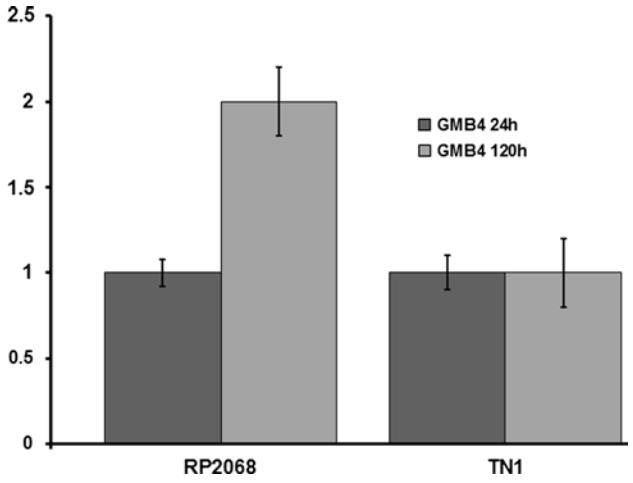

Fig. 4 Relative expression profiles of NB-ARC (LOC_Os04g 52970.1) in RP2068-18-3-5 and TN1 after gall midge biotype 4 infestation as revealed by real-time reverse transcription PCR. *Error bars* represent Mean ± S.D. *Darker color bars* represent 24 h time point and *lighter color bars* represent 120 h time point after gall midge infestation. Sequence details of the primer used in the study, NB-ARC qRT, is given in Table 1

Further, two out of 33 ORFs were the longest. These two ORFs were subjected to BLAST-P analysis. One ORF that aligned with the NB-ARC domain containing protein, expressed [Oryza sativa Japonica Group] (gb|ABA93781.1) in the database was picked up for further analysis. All four sequences and the NB-ARC protein were aligned together to check for sequence similarity. Differences based on reference NB-ARC sequence were noted at the following amino acid positions between the susceptible (B95-1 and TN1) and resistant (Phalguna and RP 2068) lines: D1364Y, R1366S, D1367G. In addition M1361T and M1361R were noted between the susceptible lines and Phalguna or RP 2068, respectively. Differences in amino acid at positions, R1341H and P1358L were also noted between the susceptible lines and RP 2068. Interestingly, eight amino acid deletions, resulting from an earlier stop codon, were observed for RP 2068 when compared with the two susceptible lines (Fig. 5). This could be reasoned with the length of polymorphism between the susceptible and the resistant lines.

Introgression of gall midge resistance gene *gm3* in B95-1 (Improved Samba Mahsuri)

From the cross B95-1/RP2068-18-3-5//B95-1*2, 16 BC_2F_2 plants were genotyped for presence of *Xa21*, *xa13* and *gm3* genes and five plants had *gm3* genes in homozygous condition (Fig. 6) and also had *Xa21*. Among these five plants,

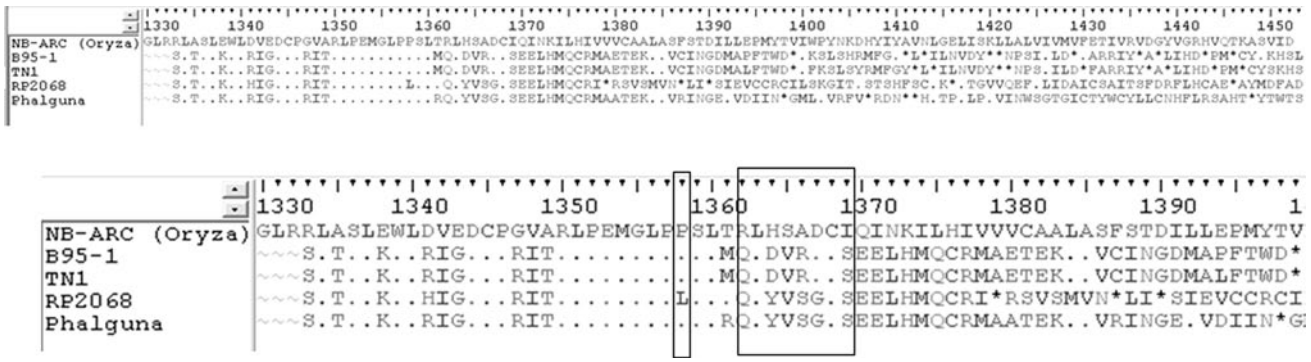

Fig. 5 Protein sequence alignment of the selected genotypes, B95-1, TN1, Phalguna and RP 2068 with the NB-ARC protein (gb|ABA93781.1)

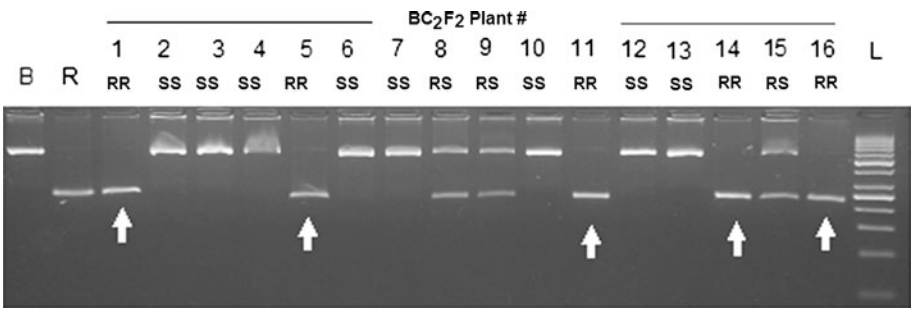

Fig. 6 Genotyping of 16 BC_2F_2 plants from the cross of B95-1/RP2068-18-3-5//B95-1*2 with gm3del3 marker

only one plant BC_2F_2-5 had *xa13* gene also in homozygous condition (supplementary Fig. 1). Therefore, BC_2F_2-5 was subjected to background phenotyping to know the recovery of recurrent parent (B95-1) genome with a set of 60 polymorphic SSR and other markers located on chromosomes other than chromosome 4 (Supplementary Table 1). Results suggested BC_2F_2-5 line to have 86.7 % genome of Improved Samba Mahsuri (B95-1). Further, a genomic region of the size ~6 Mb (17 % of chromosome 4 region) from the donor line (RP 2068-18-3-5) has been introgressed along with *gm3* gene, based on analysis with 15 SSR markers in the vicinity of the gene (Supplementary Table 2). The grain features of the line BC_2F_2-5 resembled the donor parent with medium slender grains (supplementary Fig. 2; Supplementary Table 3). Phenotyping of BC_2F_3 plants of this line confirmed high level of resistance against both BB and gall midge (1, 4, 4 M biotypes) (Table 3, supplementary Table 4). Thus *gm3* was successfully introgressed into the elite variety Improved Samba Mahsuri through MAS.

Molecular allelism test

To identify whether the gene-specific marker gm3del3 could distinguish *Gm2*, *gm3*, *Gm6* and *Gm7* present very closely on chromosome 4, we used the marker for amplification in TN1 (no R gene), Phalguna (with *Gm2*), RP2068-18-3-5 (with *gm3*), Dukong #1 (with *Gm6*) and RP 2333-156-8 (with *Gm7*). Results showed polymorphism in amplification pattern as gm3del3 amplified TN1 specific allele in Dukong #1 while it amplified *Gm2* specific allele in RP 2333-156-8 (Fig. 3). Thus, the test established that the NB-ARC alleles from different gall midge resistant genotypes showed substantial differences in amplicon size, consistent with the hypothesis that NB-ARC alleles encode *Gm2*, *gm3*, *Gm6* and *Gm7*.

Discussion

The rice–gall midge interaction has been well characterized genetically. Three of the R genes, *Gm2* (Phalguna), *Gm6* (Dukong#1) and *Gm7* (RP2333-156-8) have been mapped onto the rice chromosome 4 (Tan et al. 1993; Mohan et al. 1994; Sardesai et al. 2002). *Gm2* and *Gm6* have been tagged to the same RFLP marker RG214 (Mohan et al. 1994; Katiyar et al. 2001). Likewise, *Gm7* and *Gm2* were found to be tightly linked and tagged by the AFLP marker SA598 and RAPD marker F8 (Mohan et al. 1994; Sardesai et al. 2002). Though *Gm2* and *Gm7* were claimed to be non-allelic (Kumar et al. 1999), mapping data of Sardesai et al. (2002) suggested these to be tightly linked and possibly allelic. Likewise, *Gm6* was claimed to be a new gene

Table 3 Phenotyping of BC_2F_3 lines and genotyping of corresponding BC_2F_2 plants of the cross B95-1 (improved Samba Mahsuri)/RP 2068-18-3-5 against gall midge biotype GMB4 and Bacterial Blight pathogen (BB) isolate DX-020 in the greenhouse

BC_2F_2 plant no.	Reaction against GMB4		Reaction against BB Isolate DX-020		Genotype with gm3del3	Genotype with pTA248
	Plant damage (%)	Rating	Lesion (Cms)	Rating		
1	0	R	2.3	R	RR	RR
2	100	S	11	S	SS	SS
3	PD	–	2.1	R	SS	RS
4	100	S	10.9	S	SS	RR
5	0	R	2.2	R	RR	RR
6	100	S	2	R	SS	RR
7	80	S	3.9	MR	SS	RS
8	38	S	2.5	R	RS	RS
9	60	S	11.1	S	RS	SS
10	100	S	11.5	S	SS	SS
11	0	R	2.4	R	RR	RR
12	100	S	6	MS	SS	RS
13	100	S	9	S	SS	SS
14	0	R	2	R	RR	RR
15	58	S	9.2	S	RS	SS
16	0	R	2	R	RR	RR
B95-1	100	S	1.3	R	SS	RR
RP2068-18-3-5	0	R	12.5	S	RR	SS

Genotyping was done with markers for *gm3* (gm3del3) and *Xa21* (pTA248)

PD plants dead, *RR* allele specific to resistant parent, *SS* allele specific to susceptible parent, *RS* alleles of both the parents

only on the basis that it conferred resistance against the Chinese biotype of the rice gall midge (Katiyar et al. 2001). No allelism tests were conducted to establish this. Further, the claim that *Gm6* does not confer resistance to the Indian gall midge biotypes was not substantiated (Tiwari et al. 2005). Kumar et al. (1998) reported the recessive gene *gm3* from the breeding line RP2068-18-3-5 but this study did not involve a cross between this line and Phalguna to establish that these two are independent genes. Probably this was not felt necessary since *Gm2* in Phalguna was found to be dominant while *gm3* was recessive. Significantly, *gm3* gene conferred resistance against gall midge biotypes GMB4 and GMB4M, whereas *Gm2* gene did not provide resistance against these biotypes (Tiwari et al. 2005; Vijayalakshmi et al. 2006).

Resistance genes have often been reported to be located in clusters (Dickinson et al. 1993; Century et al. 1995; Salmeron et al. 1996; Rossi et al. 1998). Such clustered genes can induce resistance to diverse pathogens as exemplified by *Rx* and *Gpa2* genes in potato, which are tightly linked, specifying resistance against the potato X virus and the potato cyst nematode, respectively (van der Vossen et al. 2000). Similar cluster of R genes such as the *cf2* and *cf5* for the fungus, *Cladosporium falvum* and *Mi* for the root-knot nematode, *Meloidogyne* spp. on chromosome 6 of the tomato genome was reported (Dickinson et al. 1993). However, Rossi et al. (1998) showed that the tomato resistance genes, *Meu-1* to the aphid, *Macrosiphum euphorbiae* and *Mi* to nematode which were earlier reported as tightly linked was the same gene. At least eight blast resistance genes were reported in a cluster on chromosome 11 along with a number of BB resistance genes in rice (Wang et al. 2009). Likewise, four clusters of brown planthopper resistance genes were reported (Jena and Kim 2010; Fujita et al. 2013). Two clusters are on chromosome 3 while one each is on chromosomes 4 and 12, respectively. Two other gall midge resistance genes *Gm4* and *Gm8* were localized on chromosome 8 (Yasala et al. 2012). It is possible that the genes *Gm2*, *gm3*, *Gm6* and *Gm7* in the cluster on chromosome 4 arranged in tandem may be functioning in consonance to confer varying spectrum of resistance to different biotypes of the gall midge. Thus R genes in a cluster may show different specificities to the same organism, or confer resistance to different organisms or a single R gene may confer resistance to the different organisms. Alternatively, identity of *Gm2*, *gm3*, *Gm6* and *Gm7* might not have been well established in the earlier reports, as discussed above. The present study suggested that *Gm2* and *Gm7* could be identical and allelic to *gm3*, since the functional marker gem3del3 amplified similar fragments from the rice genotypes carrying these genes while *Gm6* appears to be an independent gene. Xiang et al. (2006) demonstrated that four of the BB resistance genes *Xa3*, *Xa6*, *Xa9* and *Xa26* mapped on to chromosome 11 were indeed one gene. Thus it is also likely that some of these gall midge resistance genes may be alleles. More studies as suggested by Zhou et al. (2010) in case of maize resistance gene Rxo1 are needed to resolve this.

Although most plant R genes are dominant, recessive genes have also been reported in many plant–insect, plant-pathogen relationships. Recessive resistance genes are well studied in rice-BB system. Nine out of the 38 BB resistance genes are recessive. Two of the 12 blast resistance genes (*pi21* and *pi55*) are recessive (He et al. 2012). In contrast, scanty information is available regarding the phenomenon of recessive genes in plant–insect interaction (Iyr-Pascuzzi and McCouch 2007). Rice-brown planthopper (BPH) system provides an excellent opportunity to examine such recessive genes. Out of 27 BPH resistance genes, 10 genes are recessive (Jena and Kim 2010; Fujita et al. 2013). It has also been suggested that recessive genes may more aptly be viewed as mutations in the dominant susceptibility alleles and may also function in a gene-for-gene manner (Iyr-Pascuzzi and McCouch 2007). Iyer and McCouch (2004) showed that *xa5* conferring broad spectrum BB resistance encoded the small subunit of the transcription factor TFIIA, while *xa13*, another recessive gene, encoded a protein related to *MtN3* (White and Yang 2009). The blast resistance gene *pi21* encodes a protein with a heavy metal transfer/detoxify domain in the N-terminal and a proline-rich domain in the C-terminal. However, the candidate gene for *pi55* is suggested to encode leucine-rich repeat (LRR)-containing protein (He et al. 2012). This is probably the only recessive gene in rice reported to code NBS-LRR class of resistance protein. In the present study, we suggest that the recessive *gm3* gene in RP2068-18-3-5 encodes a protein from NB-ARC class, which is identical to NB-LRR class of resistance proteins. Thus resistance by the recessive genes may also be a part of induced defense and may be equally prone to be overcome through selection pressure. Virulence against *gm3* resistance is reported in GMB5 and GMB6 populations (Bentur et al. 2003). However, such virulence is not widespread and *gm3* may still be deployed in elite rice varieties in India.

Many of R genes in plants against pests/pathogens belong to an ancient family encoding proteins with nucleotide-binding site (NBS) and LRR domains (Bai et al. 2002). NBS-LRR genes are abundant in plant genomes, with approximately 150 genes described in the *Arabidopsis* genome (Meyers et al. 2003) and over 500 genes in the rice genome (Bai et al. 2002). The NBS motif belongs to a larger domain family, while the NB-ARC domain is shared by the proteins involved in regulation of cell death in animals and resistance in plants. These genes encode cytoplasmic receptor-like proteins that recognize elicitor proteins expressed by the pathogen and further trigger the

downstream signals leading to disease resistance (DeYoung and Innes 2006). Numerous resistance genes of this class have been cloned from different plant species. Of the 12 blast *R* genes studied by map-based cloning, 11 encode NBS-LRR resistance proteins (Takahashi et al. 2010; Lee et al. 2009). Among the eight BB resistance genes cloned, *Xa1* and *Xa26* are also member of the NBS-LRR class of plant disease resistance genes (Sun et al. 2004). Of the 27 BPH-resistance genes, only *Bph14* has been characterized at the molecular level. It encoded a coiled-coil, nucleotide binding, and LRR (CC-NB-LRR) protein (Du et al. 2009). An NBS-LRR gene is suspected to be the candidate gene at *Gm4* locus on chromosome 8 as revealed by linkage analysis of the F_{10} mapping population derived from the cross TN1/Abhaya (Himabindu 2009). This gene needs functional validation. *In silico* analysis of different regions of the rice genome containing gall midge resistance genes by Yasala et al. (2012) has shown seven NBS-LRR genes in *Gm2-gm3-Gm6-Gm7* region and another two genes in *Gm8* region and one gene in *Gm11* region. Of the seven NBS-LRR genes, two genes with TIGR locus IDs viz., LOC_Os04g52970.1 and LOC_Os04g53120.1 have been suggested to be candidate genes for *gm3* & *Gm2* (present study) and *Xa1* (Yoshimura et al. 1998), respectively.

Plant NBS-LRR proteins act through a network of signaling pathways and induce a series of plant defense response, such as activation of an oxidative burst, calcium and ion fluxes, mitogen-associated protein kinase cascade, induction of pathogenesis-related genes and the HR-hypersensitive response (Hammond-Kosack and Parker 2003). The *gm3* gene is also associated with gall midge resistance involving HR. Our transcriptomics studies also revealed enrichment of five ESTs of NBS-LRR and two ESTs of NB-ARC genes in SSH library of Suraksha (HR+ type, carrying *Gm11* gene) against gall midge (Rawat et al. 2013). Of these ESTs, expression of one EST encoding NBS-LRR gene (GenBank ID: HO088625) was validated by real-time PCR. But such an involvement of NBS-LRR/NB-ARC mediated defense was not noticed in HR-type of gall midge resistance conferred by *Gm1* gene in Kavya (Rawat et al. 2012).

Complete co-segregation of the gene-based marker with the phenotype in the mapping population and real-time PCR results strongly suggested NB-ARC gene (LOC_Os04g52970.1) to be the candidate for *gm3* and gm3del3 to be a functional marker. The marker developed in the present study, gm3del3, targeting the NB-ARC gene, distinguished the susceptible TN1 and B95-1, from the three resistant genotypes (i.e. RP2068-18-3-5, Phalguna and RP2333-156-8, possessing *gm3, Gm2 and Gm7*, respectively) suggesting allelic nature of these resistance genes as NB-ARC is a strong candidate for resistance. It is evident from the sequence analysis of the PCR product with gm3del3 marker that deletion in the susceptible alleles has resulted in gain of function i.e. resistance. Similar observations were made by Ramkumar et al. (2011) with respect to the functional allele of the major blast resistance gene *Pi54*. Others have also reported that the alleles of NBS-LRR/NB-ARC class of R genes that confer resistance have a truncated size compared to the non-effective alleles (Katiyar et al. 2001; Sardesai et al. 2002; Jain et al. 2004; Himabindu 2009). Though we did not sequence the entire NB-ARC gene, the segment of the gene amplified by NBcloning 0.9 Kb marker had truncated version in Phalguna and RP2333-156-8 compared to TNl and a further smaller version in RP2068-18-3-5. We believe that such a truncation with deletion of a complete exon resulted in resistance function of the allele in Phalguna. Further truncation involving a part of another exon in RP2068-18-3-5 might have resulted in acquisition of broader range of resistance and altered the dominance status of the *Gm2* gene.

The NB-ARC protein identified from BLAST analysis using query sequence (TN1) contained functional domain at the position 225…501. However, the query sequence had partial alignment with C-terminal end of the NB-ARC containing protein (gb|ABA93781.1). It is noteworthy that amino acid variations were observed between susceptible and resistant lines for these partially aligned sequences. Furthermore, early stop codon in the resistant line RP 2068 could have resulted in sequence deletion at the C-terminal end. Our results support earlier findings by Rairdan and Moffett (2006) that a deletion at the CC-NB-ARC region disrupts the protein function. Similarly we suggest that the deletion observed here could also affect the functionality of the protein. Additional studies involving cloning these genes/alleles and transformation of a susceptible rice genotype with the cloned genes and phenotyping the transformants with different gall midge biotypes may validate this hypothesis.

We used the functional marker gm3del3 for targeted introgression of *gm3* in the elite background of the rice variety, Improved Samba Mahsuri, through MAS breeding. Functional markers were used to track the introgression of the target BB and gall midge resistance gene(s) in each backcross generation and also among BC_2F_2 plants. This is the second report wherein resistance genes conferring BB resistance have been pyramided/stacked with gene conferring resistance against gall midge in rice (Sama et al. 2012). Since we did not follow a more rigorous marker-assisted backcross breeding (MABB) involving a stringent background selection, our best selected BC_2F_2-5 plant had a moderate level of 87 % genome recovery of the recurrent parent. Foreground selection also ensured minimal introgression (17 % segment of chromosome 4) of the donor parent genome linked to *gm3* gene. Nevertheless, as a proof of concept, we have introgressed two BB resistance genes *xa13*, *Xa21* and one

gall midge resistance gene *gm3* in the elite background of Improved Samba Mahsuri with similar grain features and resistance to the target pests. Efforts are on at DRR to pyramid the recessive gene *gm3* with *Gm8* and *Gm4* in the genetic background of elite varieties like Improved Samba Mahsuri to confer multiple and durable resistance.

Conclusions

We have tagged and fine-mapped the recessive gall midge resistance gene, *gm3* in the rice breeding line RP2068-18-3-5. Further a functional marker gm3del3 was developed for the putative candidate gene NB-ARC for *gm3*. We also validated expression profile of this gene through real-time reverse- transcription PCR, which strongly supports the candidacy of the gene. Since *gm3* is relatively less deployed and conferred HR+ type of resistance against five of the seven gall midge biotypes, it was pyramided by using the functional markers with BB resistance genes *Xa21*and *xa13* in elite rice cultivar Improved Samba Mahsuri.

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Conflict of interest We declare that none of the authors has any conflict of interest with reference to the publication of results contained in the manuscript.

Ethical standards We declare that we have followed the highest standards of ethical practices in reporting the results of our research contained in this paper.

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