Stable quantitative trait locus (QTL) for sheath blight resistance from rice cultivar CR 1014

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Abstract Sheath blight disease of rice causes substantial crop losses and resistance sources are rare. A moderately resistant genotype CR 1014 was identified and hybridized with highly susceptible genotype Swarna-Sub1. In the F_2 and $F_{2:3}$ generations, three QTLs (*qShB-1.1, qShB-1.2* and *qShB-1.3*) were mapped in chromosome-1. In F_5 generation of the same cross and F_4 generation of an alternative mapping population (Tapaswini/CR 1014), only the major QTL *qShB-1.1* was recorded consistently with high LOD score (> 5.0). This stable QTL was co-localized with *qShB1* reported earlier from *Oryza nivara*. A typical leucine rich repeat (LRR) motif containing gene (*LOC_Os01g65650*) and a chitin-inducible gibberellin-responsive protein coding non-LRR gene (*LOC_Os01g65900*) located within *qShB-1.1* with high expression levels in leaf and shoot were predicted as putative candidate genes among others. Nearly 27.8% reduction in relative lesion height was recorded among several near isogenic lines of Swarna-Sub1 carrying the QTL region from CR 1014.

Keywords Disease resistance · Horizontal resistance · Near isogenic lines · Novel source · *Rhizoctonia solani*

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Introduction

Nearly half of the people living in different continents of the world consume rice as their major staple food. The two most populous countries (India and China) of the world are inhabited by people who depend mainly on rice for meeting the calorie requirements. As the crop is grown in highly diverse ecosystems ranging from below mean sea levels to mountains and semi-arid to humid climates, the challenges for growing rice are also diverse. To ensure food security, it is of utmost need to develop rice varieties that not only possess higher yield potential, but also tolerate the dynamic and ever evolving biotic stresses. Due to climate change and other factors, many pests which were hitherto considered as minor have evolved as major threat for the crop. Sheath blight (ShB) of rice caused by the fungus *Rhizoctonia solani* Kühn (Teleomorph:

Thanatephorus cucumeris (Frank) Donk) has evolved as a major disease of rice especially in the areas that follow intensive cultivation practices (Molla et al. 2020). In case of severe disease incidences, the yield loss may reach up to 50% (Singh et al. 2004). Semi-dwarf cultivars which generally require high crop densities with higher doses of fertilizers (especially nitrogen) provide favorable microclimate for rapid disease development and spread (Savary et al. 1995). Use of chemical fungicides is the only available option which leads to increased cost of cultivation and shows negative impact on environment. Till date, researchers have not achieved much success in deploying effective and heritable resistance against this pathogen in improved breeding lines of rice. Only some partially resistant lines were identified from the landraces and other germplasm (Srinivasachary and Savary 2011; Molla et al. 2020).

Rice researchers from different countries have identified nearly 200 Quantitative Trait Loci (QTL) with limited effects (Zarbafi and Ham 2019; Molla et al. 2020). Li et al. (1995) identified six QTLs from 'Teqing' which could collectively explain approximately 47% of the phenotypic variation in the F_4 populations of Lemont/Teqing. Pinson et al. (2005) also mapped total 12 QTLs from the same cross explaining 5–18% of PVE individually. Pan et al. (1999) mapped three major QTLs (*Rh-2*, *Rh-3* and *Rh-7*) located on chromosomes 2, 3 and 7 respectively from genotype Jasmine 85 and the QTLs could explain 14.4%, 26.1% and 22.2% of the phenotypic variation in the population of Lemont/Jasmine 85. Zou et al. (2000) also identified six QTLs from the same cross out of which two were from the susceptible parent Lemont. Beside these, some other important QTLs were mapped from different moderately resistant genotypes like Jingxi-17 (Kunihiro et al. 2002), Minghui 63 (Han et al. 2002), 4011 (Che et al. 2003), WSS2 (Sato et al. 2004), Pecos (Sharma et al. 2009), Tetep (Channamallikarjuna et al. 2010), HJX74 (Zhu et al. 2014), RP2068-18-3-5 (Nymagoud 2015), ARC10531 (Yadav et al. 2015) etc. When compared with number of genes/QTLs reported for other biotic stresses of rice like bacterial blight, blast, brown plant hopper or gall midge; the number of genes or QTLs identified in case of ShB is negligible (Molla et al. 2019). In fact, effective transfer of ShB disease resistance in susceptible cultivars through molecular marker assisted selection leading to development of commercial rice cultivars is rare. However, imparting heritable resistance has become necessary considering the significance of the disease and its impact on rice cultivation. With recent advances in phenotyping and genotyping, mapping of genes/QTLs have become easier and routine in genetic research. However, mapping of genes/QTLs for sheath blight especially from novel sources other than those already reported holds great significance for rice breeders. Even the moderately resistant genotypes available to map the QTLs are limited in number and several research groups even used the same parent for mapping the QTLs or rediscovered the similar QTLs (Zarbafi and Ham 2019). Due to unavailability of any major QTL/gene(s) governing the resistance, there is need for accumulating multiple QTLs from different backgrounds which shows positive QTL × QTL interaction (Kar et al. 2018; Molla et al. 2020).

The present study reports identification of a new source of ShB resistance from CR 1014, a high yielding indica cultivar developed by National Rice Research Institute (formerly Central Rice Research Institute), Cuttack in 1988 for medium deep water-logged ecosystem. This genotype showed similar disease response compared to the widely reported resistant genotype 'Tetep' and performed much better when compared with some other reported sources like Jasmine-85 and Teqing with respect to a highly virulent strain of *Rhizoctonia solani* (CRRI-RS-8/MTCC-12232). QTL was mapped from the $F_{2:3}$ mapping population derived from crossing of CR 1014 with susceptible cultivar Swarna-Sub1 and further validated in the mapping population of Tapaswini/CR 1014 and different other generations of Swarna-Sub1/CR 1014 as well as its backcross populations. Moreover, backcross derived Near Isogenic Lines (NILs) of Swarna-Sub1 carrying the identified ShB resistance QTL from CR 1014 in Swarna-Sub1 background were developed and phenotyped for ShB resistance. The QTL transfer led to improvement of disease resistance in multiple introgression lines. Putative candidate genes from the QTL region were also predicted.

Materials and methods

Plant materials

Seventy rice genotypes, including released varieties and landraces were evaluated for identification of new source for sheath blight disease resistance. The details of the genotypes are listed in Supplementary Table 1. The genotypes Tetep, Jasmine-85 and Teqing were used as resistant checks whereas the genotypes Annapurna and Lemont were used as susceptible checks based on the available literature. All the genotypes reported here are available in the gene bank of ICAR-National Rice Research Institute, Cuttack, India.

Evaluation of rice genotypes for sheath blight resistance

A total of 70 genotypes were screened during *Kharif* (rainy) season of the years 2013 and 2014. The highly humid and warm weather of Cuttack during the rainy seasons is highly favorable for the development of sheath blight disease. The detailed weather data of the crop growing periods (July to November) at the trial location from the year 2013 to 2018 is provided in Supplementary Table 2. The 70 genotypes were screened in pots with three replications per genotype in the net house through direct sowing from seeds in three different blocks after randomization. Statistical analysis for ANOVA was performed using the R-software (R Core Team 2013).

Pathogen multiplication and artificial inoculation

For artificial inoculation, a highly virulent strain (CRRI-RS-8) of the pathogen *Rhizoctonia solani* was utilized. The strain has been registered and deposited as Type Culture in the National Microbial Type Culture Collection (Type Culture Number: MTCC12232, CSIR-IMTECH, Chandigarh, India) repository. At maximum tillering stage, the rice genotypes were inoculated artificially with the strain CRRI-RS-8 following the standard methodology of Yoshimura and Nishizawa (1954). After 15 days of inoculation, data on total plant height and lesion height were recorded for calculation of relative lesion height (RLH). The relative lesion height (RLH) was calculated by using the following formula.

$$RLH(\%) = [\text{Lesion length (cm)}/\text{Total plant height (cm)}] \times 100$$

The score was recorded based on standard evaluation system (SES) for sheath blight disease developed by International Rice Research Institute, Philippines (2002). Under this scoring system, there are six categories (0, 1, 3, 5, 7 and 9) of rating, where '0' is considered as immune and '9' is considered as highly susceptible. The scores of 1, 3, 5 and 7 are considered as resistant, moderately resistant, moderately susceptible and susceptible, respectively. The range of RLH for those six categories are 0, 1–20, 21–30, 31–45, 46–65 and > 65%.

Development of mapping populations

Among the genotypes screened, only one genotype CR 1014 was found to be moderately resistant besides the check Tetep. This genotype was crossed (as male parent) with two highly susceptible genotypes Swarna-Sub1 and Tapaswini. Both these genotypes are high yielding semi-dwarf varieties cultivated widely in eastern India for shallow lowlands and irrigated ecologies, respectively. Total 654 F_2 progenies were generated from the Swarna-Sub1/CR 1014 and the individual F_2 plants were subsequently advanced till F_5 generation using single panicle descent method. The final population size in F_5 was 630. A randomly selected subset of 216 F_2 plants was used for QTL mapping in F_2 and $F_{2:3}$. In case of Tapaswini/CR 1014, 192 plants were advanced up to F_3 generation through single seed descent and further generations were advanced through single panicle descent method. The QTL mapping was carried out initially in F_2 and then in the $F_{2:3}$ populations of Swarna-Sub1/CR 1014. Subsequently, QTLs were also validated in F_4 population of Tapaswini/CR 1014 and a larger F_5 population of Swarna-Sub1/CR 1014.

Phenotypic evaluation of mapping populations

Different mapping populations and advanced progenies from susceptible × resistant crosses were phenotyped under field conditions during the *Kharif* seasons of the years 2015–2018 in augmented design (Federer 1956). Twenty-five days old seedlings of respective genotypes were transplanted in the

experimental field of ICAR-National Rice Research Institute, Cuttack. Compared to standard agronomic practice of N:P:K application (80:40:40 kg per ha), we applied additional doses of nitrogenous fertilizer (100:40:40 kg per ha) to promote disease development. Minimum water level of 10 cm was maintained in the field till 50% plants reached flowering stage. After the genotypes reached to maximum tillering stage, artificial inoculation with pathogen was done. Disease scoring was carried out on 15th day after inoculation. For the populations from F_3 to F_5 generations, 10 plants of each line of respective generations were evaluated and average value of RLH was calculated for the corresponding lines. The genotypes Tetep, Teqing, Jasmine 85, CR 1014, Lemont, Annapurna, Swarna, Swarna-Sub1 and Tapaswini were used as replicated checks during the evaluation of all the mapping populations in augmented design. For disease scoring, the height from base to longest leaf tip was recorded. For the plant height of a genotype reported in this study, the height was recorded from base to tip of panicle at 25 days after flowering. Statistical analysis for ANOVA was performed using the R-software (R Core Team 2013).

DNA isolation, PCR, parental polymorphism survey and genotyping of mapping populations

The total genomic DNA of plants was isolated by the CTAB (cetyl trimethyl ammonium bromide) miniprep method described by Dellaporta et al. (1983). Isolated DNA were re-suspended in TE (10 mM Tris-Cl, pH 8.0. 1 mM EDTA.) and stored at $-$ 20 °C. The quality of DNA was measured by 0.8–1.0% agarose gel electrophoresis containing ethidium bromide (0.5 μg/ml) by using $0.5 \times$ TBE buffer at constant voltage of 100 V for 30 min and visualized under UV light. The DNA was quantified using Nano-Drop spectrophotometer (Thermo Scientific, USA). Polymerase chain reaction (PCR) was performed by using reaction mixture which contain 25 ng template DNA, 5 pM of each of the reverse and forward primers, 2.5 mM dNTPs, 01 μl of $10 \times$ PCR buffer (150 mM Tris-HCl, pH 8.75; 500 mM KCl, 20 mM $MgCl_2$, and 1% TritonX-100) and 0.2 unit of Taq DNA polymerase in a total reaction volume of 10 μl. The PCR reaction was carried out through touchdown method where first 10 cycles out of the 30 followed gradual reduction of annealing temperature from 62 to 55 °C. From 11th

cycle onwards constant annealing temperature of 55 °C was followed. The amplified products were resolved by using 3.5% Metaphor Agarose gel containing ethidium bromide and were visualized under gel imaging system (Typhoon FLA, General Electric, USA). The amplicon sizes were detected using 100 bp ladders as marker.

Total 2086 evenly distributed rice sequence tagged microsatellite sites (STMS) markers covering the 12 chromosomes of rice (details available from http://www.gramene.org) were utilized for studying the parental polymorphism between the ShB susceptible genotypes Swarna-Sub1 and ShB tolerant genotype CR 1014. Polymorphic marker combinations were identified and used for genotyping of 216 F_2 plants of Swarna-Sub1/CR 1014 for construction of linkage map and QTL analysis. In case of Tapaswini/CR 1014, 192 F_4 plants were genotyped for validating the QTLs. In case of F_5 RILs of Swarna-Sub1/CR 1014, 630 plants were genotyped for validating the QTLs.

Construction of linkage map and QTL analysis

Linkage map construction and QTL analysis were carried out by using integrated QTL software, IcMapping, Version4.0, available from http://www.isbreeding.net (Meng et al. 2015). Based on the 216 F_2 genotype data, the linkage groups and order of markers were generated by the software which was further used as input files for QTL analysis. Transformation of recombination frequencies into genetic distances (cM) were calculated based on Kosambi mapping function (Kosambi 1944). For all the mapping populations, Kolmogorov–Smirnov test for goodness of fit were performed on phenotypic data to test normality of distribution at $p \leq 0.05$ (Massey 1951) and then were further included for analyzing QTLs for ShB resistance by the inclusive composite interval additive mapping (ICIM-ADD) program. Single marker analysis (SMA) and composite interval mapping (CIM) were performed by the software to identify QTLs for ShB resistance. The QTLs identified from F_2 and $F_{2:3}$ populations of Swarna-Sub1/CR 1014 were validated in 192 F_4 lines of Tapaswini/CR 1014 and 630 F_5 Recombinant Inbred Lines (RILs) of Swarna-Sub1/CR 1014 with the same software. To cross check the results, we also analyzed the same datasets with another software QTL-Cartographer version 2.5 (Wang et al. 2012).

The QTLs detected in present study were co-localized with the QTLs reported in literature. The physical position of markers in present study and previous reports were elucidated from the reference genome of Nipponbare (IRGSP 1.0).

Development of backcross population and QTL validation in near isogenic lines (NIL)

Besides advancement of the F_1 plants of Swarna-Sub1/CR 1014 through selfing cycles, they were also backcrossed with the recurrent parent Swarna-Sub1 to develop 423 BC_1F_1 progenies. Subsequently one more backcrossing was done, and 396 BC_2F_1 progeny lines were obtained. This population was advanced from single sampled seed up to BC_2F_3 with a final population size of 315. In BC_2F_2 and BC_2F_3 generations, 10 plants of corresponding lines were artificially inoculated for disease scoring in the field. A selected sub-set of 45 lines showing disease score of 5 or better in BC_2F_2 were additionally screened in pots in BC_2F_3 besides the screening in the field conditions. All the lines with disease score of '3' among the 45 lines were at-least 30 cm taller than Swarna-Sub1. However, we phenotypically selected the lines with plant heights lesser, similar, or up to 15 cm higher than Swarna-Sub1. Total 11 such lines showed the score of '5' in both seasons of field screening as well as pot screening for one season. Single representative plant of those 11 genotypes of '5' score and 11 highly susceptible lines of '9' score from BC_2F_3 were initially pooled to produce two contrasting bulks and then tested with the markers linked with the QTLs. Further, all the individual plants were also profiled with the QTL linked markers. Besides, the markers identified as polymorphic between the two parents were used for profiling of all the homozygous genotypes (of '5' disease score and plant height up to 15 cm higher than Swarna-Sub1) in order to check the recovery of background genome. Any genotype carrying the genomic region of QTL for ShB resistance from CR 1014 in homozygous condition and having background genotype > 90% like Swarna-Sub1 were considered as QTL-NILs.

In silico analysis of the QTL region

For predicting the putative candidate genes, we followed a strategy suggested by Chattopahyay et al.

(2019). Physical positions of the identified QTLs (*qShB-1.1, qShB-1.2* and *qShB-1.3*) were marked in the *Oryza sativa* var. japonica (Nipponbare) genome using the flanking markers from Garamene database (http://www.gramene.org) (McCouch 2002). MSU identifiers of the genes present within the QTLs were downloaded from Rice Expression Database (http://expression.ic4r.org) (Xia et al. 2017). Protein sequences of these genes were downloaded from the Rice Genome Annotation Project database (http://rice.plantbiology.msu.edu) (Kawahara et al. 2013). Leucine Reach Repeat (LRR) motif containing proteins were identified and number of LRR motif present in each LRR containing protein were analyzed using Protein Pattern Recognition tool available at Sequence Manipulation Suit 2 (https://www.bioinformatics.org/sms2) (Stothard 2000). LxxLxxLxLxxC/Nxx was considered as typical- (McHale et al. 2006) and LxxLxLxxC/N as minimal- (Padmanabhan et al. 2009) LRR motifs. The extracellular LRR (eLRR) proteins were screened by the presence of LT/SGxIP motif (Padmanabhan et al. 2009) following the LRR motif. Other non-LRR genes present in the QTL regions but having probable role in defense response were manually identified by critically analyzing their annotation and literature search. An *in-silico* expression profile of leaf and shoot tissues for the selected genes located within the QTL regions was obtained from Rice Expression Database which contain RNA-Seq data of nine different tissues from 24 projects consisting of a total of 284 experiments (Xia et al. 2017). An expression heat map was generated to compare the gene expression profile of the selected LRR-protein and non-LRR-protein encoding genes using Microsoft Excel package.

Results

Identification of source for sheath blight resistance

Among the genotypes screened for response to sheath blight inoculation, only two genotypes (Tetep and CR 1014) consistently showed disease score of '3' (moderately resistant category) against the highly virulent strain CRRI-RS-8 over 2 years in all replications. Even the two checks Jasmine-85 and Teqing showed disease score of '7' over 2 years. Among the 70 genotypes screened over 2 years, none of the

genotypes showed even stable score of '5' (moderately susceptible category) over 2 years. The genotypes Tapaswini, Swarna, Swarna-Sub1, Annapurna, Lemont, Ratna, Saket 4, Heera, Sattari, Khitish, Panidhan, CR Dhan 601and TN1 consistently showed score of '9' (highly susceptible category) over 2 years. Rest of genotypes showed disease score of '7' (susceptible category) in at least one season of their evaluation and '5' or '7' or '9' in another season. No genotypes with disease score of '0' (immune category) or '1' (resistant category) were identified. Disease response of the genotypes during the 2 years of evaluation has been represented Supplementary Table 1 and their analysis of variance (ANOVA) have been presented Supplementary Table 3. The disease response of Swarna-Sub1 and CR 1014 at 14 days after artificial inoculation is represented in Fig. 1.

Phenotype distribution of mapping populations

The frequency distribution for disease response of the different populations used in the study has been represented in Fig. 2. Except F_2 generation, all the other values have been derived from mean disease response of 10 sampled plants of different generations for the respective lines. The frequency distributions in all the generations were continuous in nature and fitted to normal distribution which typically progressed towards symmetric bell shape with successive generations. The BC_2F_2 and BC_2F_3 populations also displayed near symmetric bell shape distribution. F_2 population was more skewed compared with others. Maximum numbers of genotypes were distributed near the mean values of their respective populations. However, when compared for the two extremes, all the populations showed significant bias towards susceptibility. The random subsets of the populations used to map the QTLs in F_2 and $F_{2:3}$ of Swarna-Sub1/CR 1014 also displayed normal distribution. The results of ANOVA of different generations in augmented design have been presented in Supplementary Table 4.
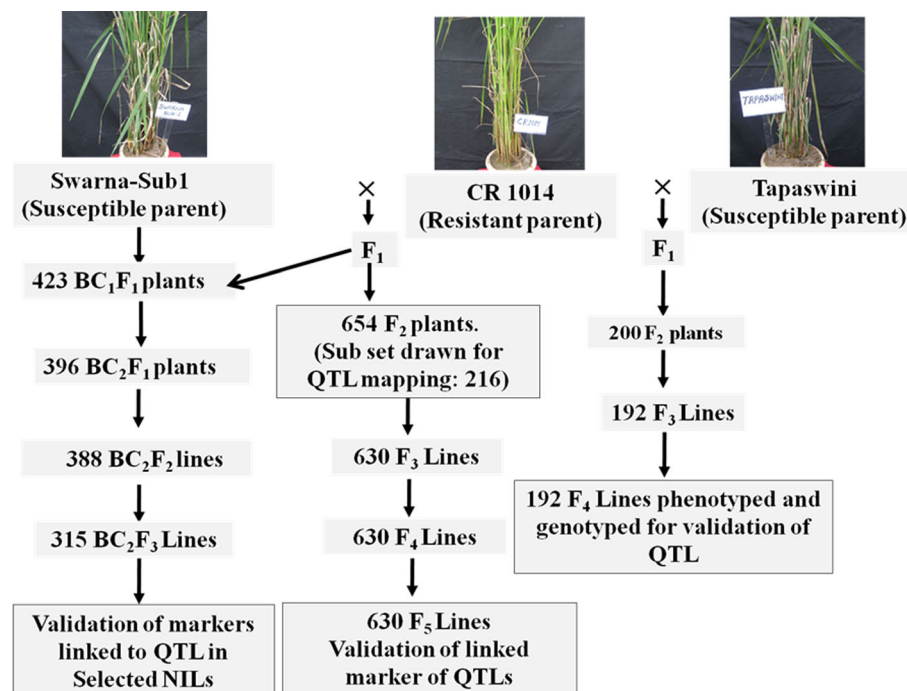
Fig. 1 Flow chart representing the methodologies followed for development of different mapping populations, identification and validation of QTLs for ShB disease resistance. As common resistance source, CR 1014 was crossed with two different susceptible parents Swarna-Sub1 and Tapaswini. In case of Swarna-Sub1/CR 1014, populations were advanced through both selfing series for development of recombinant inbred lines (RILs) and recurrent backcrossing for development of near isogenic lines (NILs)
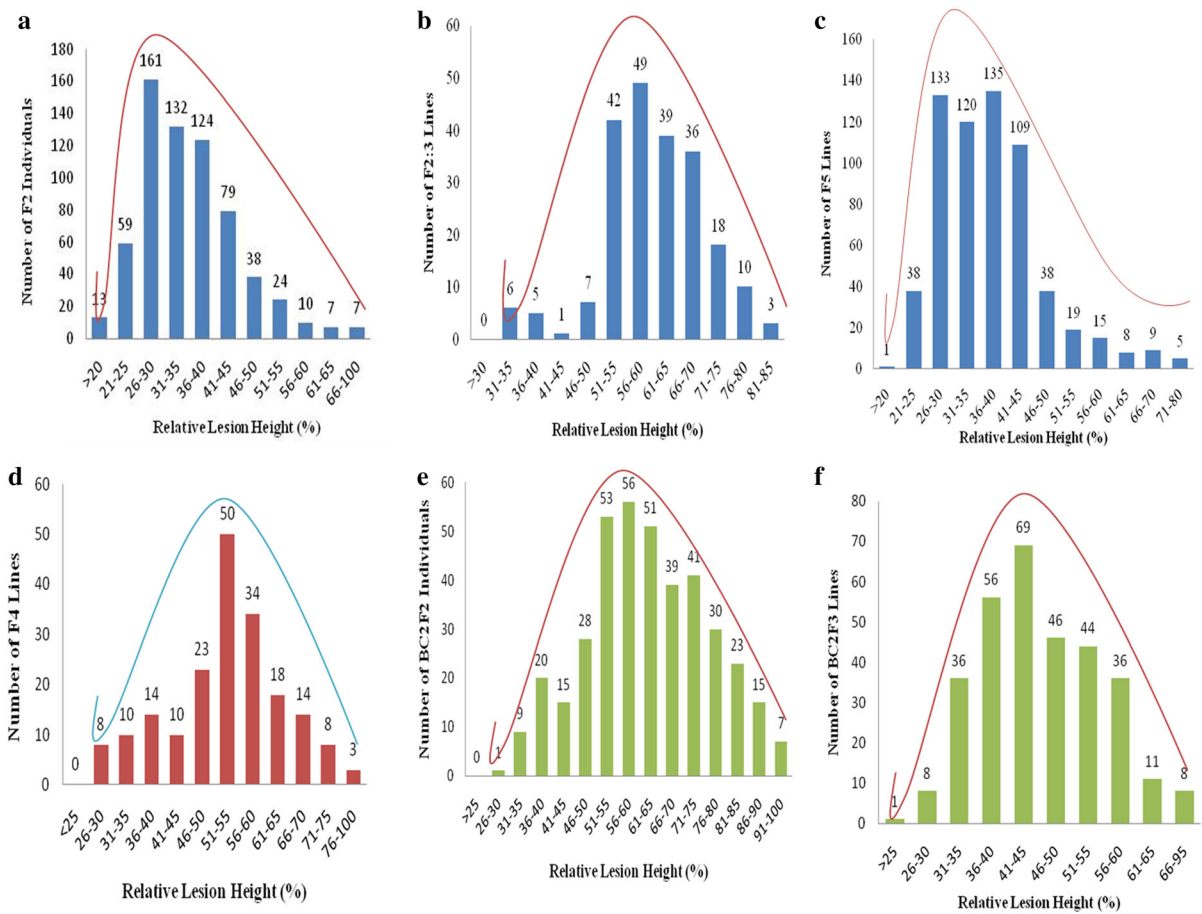
Fig. 2 Frequency distribution curve of ShB disease response in terms of relative lesion heights (RLH) of different populations developed from contrasting parents for sheath blight resistance. **a** 654 F_2 individuals of Swarna-Sub1/ CR 1014; **b** 216 $F_{2:3}$ lines of Swarna-Sub1/ CR 1014; **c** 630 F_5 lines of Swarna-Sub1/CR 1014; **d** 192 F_4 lines of Tapaswini/ CR 1014; **e** 388 BC_2F_2 lines of (Swarna-Sub1/ CR 1014) × Swarna-Sub1; **f** 315 BC_2F_3 lines of (Swarna-Sub1/ CR 1014) × Swarna-Sub1

Linkage map construction

A total of 2086 SSR markers spread over all the 12 rice chromosomes were used for parental polymorphism survey between the susceptible (Swarna-Sub1) and tolerant (CR 1014) parents (Supplementary Datasheet 1). Total 120 SSR markers were found polymorphic between the two parents (Supplementary Datasheet 2). The randomly selected sub-set of 216 F_2 plants of the cross Swarna-Sub1/CR 1014 were genotyped using those 120 polymorphic markers. Nearly 4.34% missing data points was recorded in the entire population. Nearly 21.95% marker data points were homozygous for Swarna-Sub1 and 25.77% for CR 1014. The heterozygote data points in F_2 were 47.92%.

The LOD score threshold was kept as more than three to avoid inclusion of any false linkage between two markers during map construction. Out of 120 markers, 11 markers could not be placed in any linkage groups. The remaining 109 markers were grouped into 18 different linkage groups belonging to the 12 chromosomes of rice covering approximately 2007.94 cM map distance. The details of different linkage groups have been represented in Supplementary Table 5 and Fig. 3.

Identification and validation of QTLs

All the 654 F_2 plants derived from the contrasting cross of Swarna-sub1/CR 1014 were inoculated and a
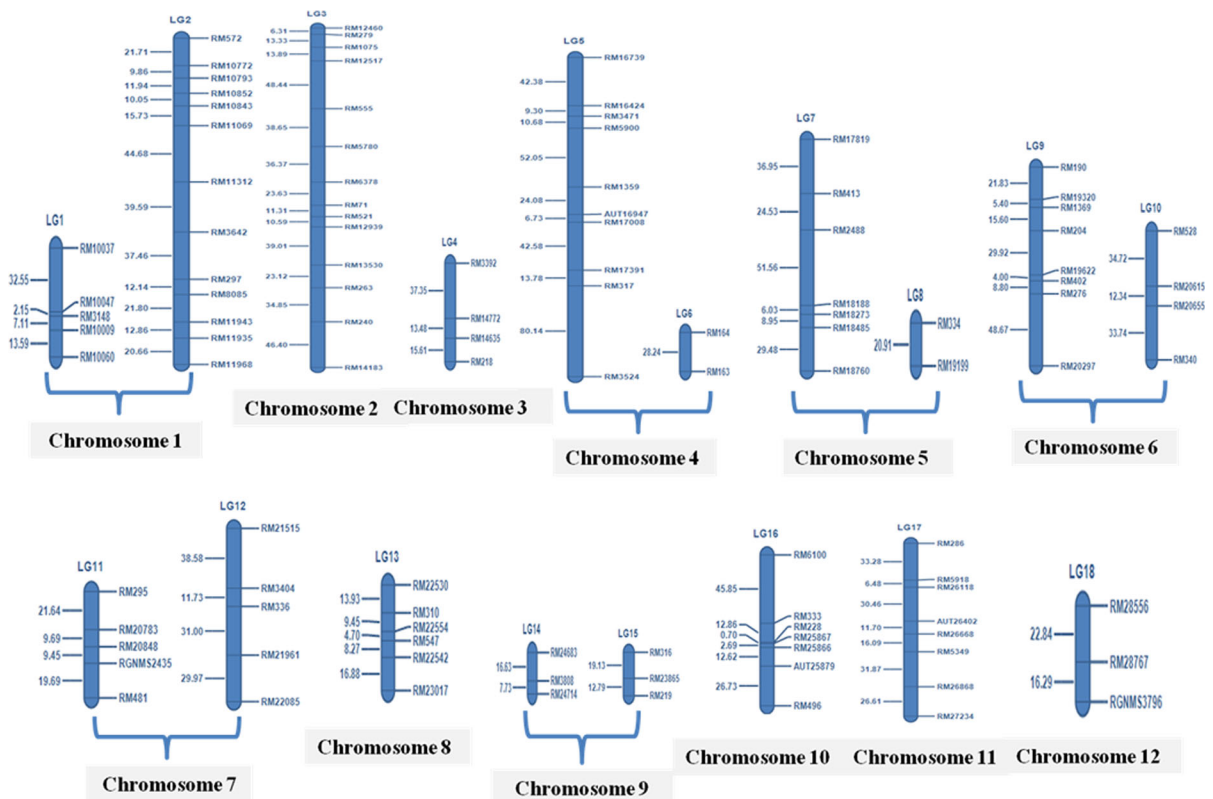
Fig. 3 Linkage map of 109 polymorphic sequence tagged microsatellite sites (STMS) markers distributed over 12 rice chromosomes in F_2 population of Swarna-Sub1/ CR 1014. The random subset of 216 plants were sampled to carry out preliminary mapping of QTL based on relative lesion height (RLH) of single plant in F_2. The same population was further advanced and 216 $F_{2:3}$ genotypes were phenotyped.

polymorphic markers of chromosomes 1, 4, 5, 6, 7 and 9 were distributed over two linkage groups (LG) each. Total 18 linkage groups were identified

In case of F_2 genotyping and phenotyping, two QTLs were mapped in Linkage Group-2 (LG-2) (Table 1; Fig. 4a). One QTL *qShB-1.1* was mapped in the marker interval of RM11935-RM11943 with a LOD score of 10.22. The QTL explained 25.23% of PVE. Another QTL *qShB-1.2* showed LOD score of only 2.5. During mapping of $F_{2:3}$, the QTL *qShB-1.2* was not detected. However, the *qShB-1.1* was detected again at nearly similar position of LG-2 (at marker interval of RM11935-RM11968) with a LOD score of 5.11(Table 1; Fig. 4b). The PVE explained by the QTL was 13.95%. A new QTL detected in Linkage Group-1 (*qShB-1.3*) was having lesser LOD score (2.71).

During subsequent validations (Fig. 1), out of those three QTLs, only the QTL *qShB-1.1* was detected consistently. In the F_4 population of Tapaswini/CR 1014, the QTL was detected with LOD score of 13.15 and contributing 26.90% of PVE. In the F_5 generation of Swarna-sub1/CR 1014, the same QTL was validated in a larger population of 630 genotypes where the PVE (%) was 31.55 at LOD score of 20.56 (Table 1). It may also be noted that with advancement of selfing generations, reduction dominance effect and subsequent increase of component of the additive effect of the QTLs was also observed.

Total 388 BC_2F_2 lines were phenotyped for sheath blight disease score and their response showed normal distribution. Total 45 lines showed '5' or better disease score. In BC_2F_3 generation, 315 lines could be phenotyped which again showed normal distribution (Fig. 2). The sub-set of 45 lines showing disease score of 5 or better in BC_2F_2 were additionally screened in pots under net house condition in BC_2F_3 besides the

Table 1 Identification and validation of sheath blight resistance QTLs in different generations of Swarna-Sub1/CR 1014 and F$_4$ lines of Tapaswin/iCR 1014

| Population	Generation	Population size	QTLs identified or valiadted	Chromosome number (linkage group)	Marker interval	Phenotype (PVE %)	LOD score	Additive effect	Dominance effect
Swarna-Sub1/CR 1014	F$_2$	216	qShB-1.1	1(LG2)	RM11935-RM11943	25.23	10.23	− 9.3182	− 1.992
			qShB-1.2	1(LG2)	RM11069-RM11312	12.59	2.51	− 5.0911	− 4.8491
	F$_{2:3}$	216	qShB-1.3	1(LG1)	RM10037-RM10047	9.31	2.72	0.9732	− 5.7764
			qShB-1.1	1(LG2)	RM11935-RM11968	13.95	5.11	− 5.7706	− 3.3865
	F$_5$	630	qShB-1.1	1	RM11935-RM11968	31.54	20.56	− 5.6764	–
Tapaswini /CR 1014	F$_4$	192	qShB-1.1	1	RM11935-RM11968	26.90	13.15	− 6.8968	− 2.098
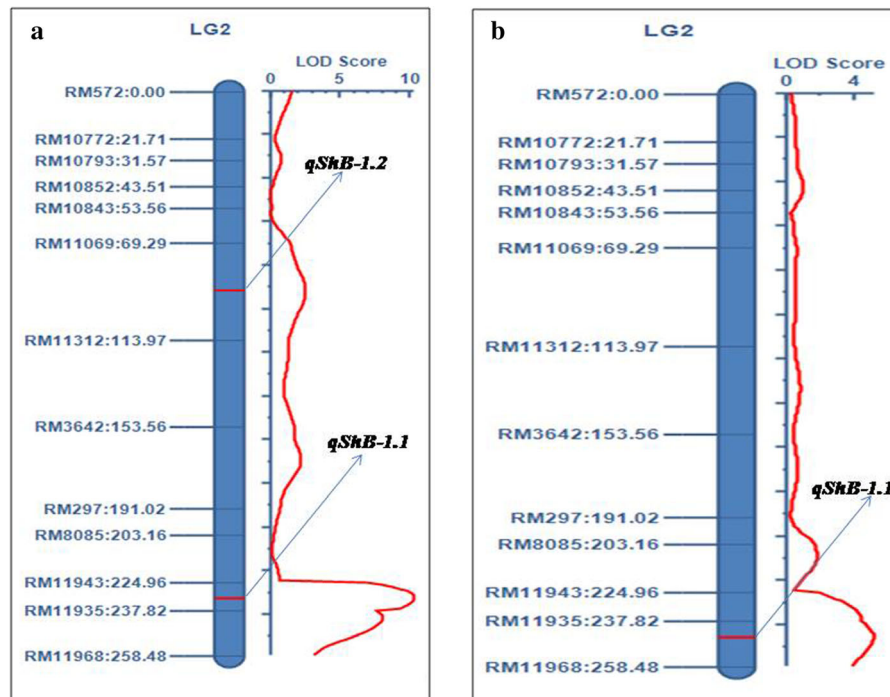
Fig. 4 Graphical representation of quantitative trait loci (QTLs) for sheath blight (ShB) resistance mapped in linkage group 2 (LG2) of chromosome 1 from the contrasting cross of Swarna-Sub1/ CR 1014. **a** QTLs *qShB-1.1* and *qShB-1.2* mapped in F_2 generation; **b** the QTL *qShB-1.1* was again mapped in $F_{2:3}$ generation

screening in the field conditions. All the lines with disease score of '3' among the 45 lines were at-least 30 cm taller than Swarna-Sub1 and were carrying the QTL *qShB-1.1* from CR 1014. However, we also phenotypically selected the lines with plant heights lesser, similar or up to 15 cm higher than Swarna-Sub1 among those 45 lines. Total 11 such lines showed the score of '5' in both seasons of field screening as well as pot screening for one season (in BC_2F_3). DNA from all the 11 lines with stable score of '5' was pooled and tested with the markers which were associated with the QTLs *qShB-1.1*, *qShB-1.2* and *qShB-1.3*. The pool was compared with the bulked DNA of 11 random genotypes showing score of '9' in field evaluation. The pool with score of '5' showed heterozygosity whereas the susceptible pool showed homozygosity for the markers associated with *qShB-1.1* (Fig. 5a, b). For markers of rest of the two QTLs, the susceptible pool also showed heterozygosity. The individual 22 genotypes were then profiled with RM11935, RM11943 and RM11968. Among the 11 genotypes consistently showing disease score of '5', seven were homozygous for the alleles of CR 1014;

three were heterozygote and only one genotype showed alleles of Swarna-Sub1 for all the three linked markers. All the 11 highly susceptible genotypes showed homozygous alleles similar to Swarna-Sub1 (Fig. 5c). Chi square test for goodness of fit among the 22 genotypes also revealed highly significant deviation (at $P = 0.001$) from expected segregation ratio of marker alleles (9.37: 6.25: 84.38 for CR 1014-Homozygote: Heterozygote: Swarna-Sub1-Homozygote) in BC_2F_3. However, no such allelic relation to phenotypic features was recorded with respect to the four markers associated with *qShB-1.2* and *qShB-1.3* in those genotypes. Six plants out of the seven showing homozygous alleles of CR 1014 from the QTL region were further profiled with the rest 102 polymorphic markers belonging to 18 linkage groups. The extent of background recovery in homozygous condition varied from 82.10 to 91.50% among those six BC_2F_3 progenies. The progeny line S/C-BC_2F_3-PLN-72 showed maximum background recovery. This line was identified as *qShB-1.1* NIL (near isogenic line) of Swarna-Sub1.
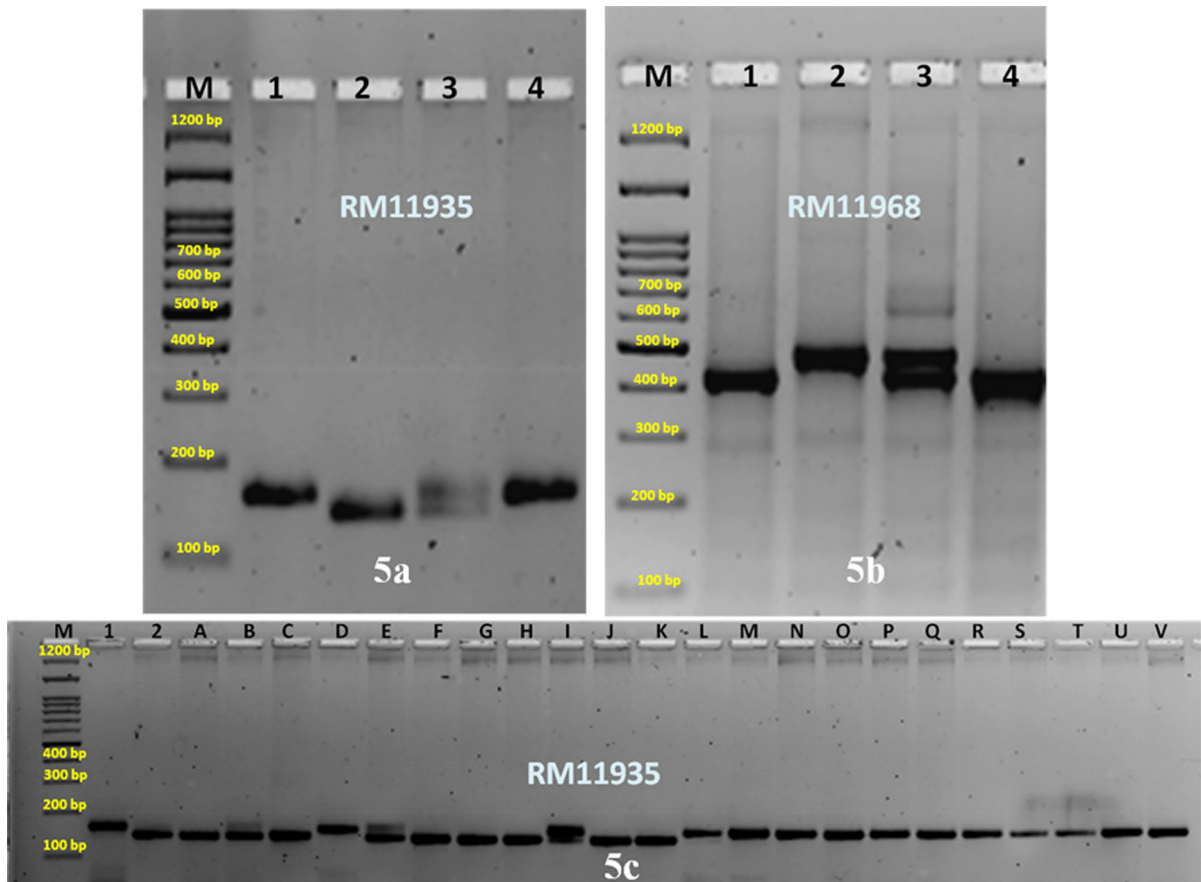
Fig. 5 Bulk segregant analysis (BSA) of contrasting pools of genotypes from BC₂F₃ generation of (Swarna-Sub1/CR 1014) × Swarna-Sub1. Based on response to sheath blight disease inoculation, DNA from eleven genotypes showing consistent disease score of '5' over 2 years in both pot and field screening were pooled together to constitute the resistant bulk. Another bulk was constituted with 11 highly susceptible genotypes showing consistent disease score of '9' over 2 years in field screening. 5a: profiling of parents and contrasting bulks through *qShB-1.1* linked marker RM11935. 5b: profiling of parents and contrasting bulks through *qShB-1.1* linked marker RM11968; 5c: Profiling of individual 22 genotypes from the two contrasting bulks and their parental lines with RM11935. M: 100 bp ladder, 1: Swarna-Sub1, 2: CR 1014, 3: Resistant bulk (include genotypes A to K of Fig. 5c), 4: Susceptible bulk (include genotypes L to V of Fig. 5c)

Putative candidate genes from QTL region

All three QTLs detected by us belong to chromosome 1 of rice. The stable QTL *qShB-1.1* was flanked by RM11935 (homologus position: 37.8 Mb of Nipponbare chromosome 1) and RM11968 (homologus position: 38.3 Mb of Nipponbare chromosome 1) covering ∼ 0.51 Mb region and containing 81 genes. The other QTL *qShB1.2* was flanked by RM11069 (homologus position: 19.3 Mb of Nipponbare chromosome 1) and RM11312 (homologus position: 24.0 Mb of Nipponbare chromosome 1) covering ∼ 4.84 Mb region and containing 673 genes. The QTL *qShB1.3* were flanked by RM10037 and RM10047 (homologus position: 0.7 Mb of Nipponbare chromosome 1) covering ∼ 0.18 Mb region and containing 26 genes (Supplementary Datasheet 2). While analyzing the protein sequences, we found that *qShB-1.1*, *qShB-1*.2 and *qShB-1*.3 contain 2, 25 and 3 LRR-motif containing genes, respectively. Among these 1, 6 and 1 genes, respectively in *qShB-1.1*, *qShB-1*.2 and *qShB-1*.3, encoded eLRR-motif contain protein. Our heat map analysis revealed the LRR encoding genes (*LOC_Os01g65650* and *LOC_Os01g65920*) of *qShB-1.1* were expressed highly in leaf and shoot tissues (Fig. 6). *LOC_Os01g65650* (identical to *OsCEPR2* or

C-Terminally Encoded Peptide Receptor2) encode a typical eLRR-containing protein with receptor-like Tyrosine Kinase activity. We generated a high quality 3-D protein model (QMEAN = − 3.03) of *LOC_Os01g65650* encoded protein using SERK2 (PDB ID: 5GQR). Apart from LRR-protein encoding genes we also identified nine genes (two in *qShB-1.1* and seven in *qShB1.2*) which are highly expressed in leaf and/or shoot and might be relevant in controlling disease resistance (Fig. 7). Interestingly, *LOC_Os01g65900*, a gene located in the major and consistent QTL *qShB-1.1*, encodes a chitin-inducible gibberellin-responsive (CIGR) protein and shows high expression both in leaf and shoot tissues.
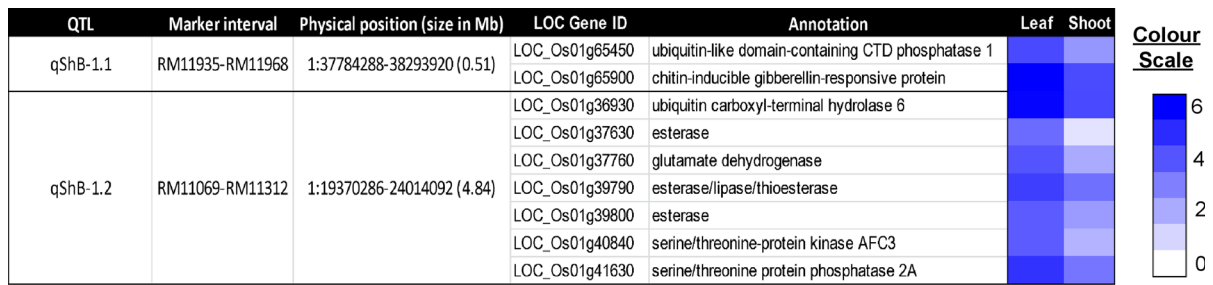
Discussion

Sheath blight is considered as one of the most devastating disease of rice after rice blast in tropical as well temperate rice growing areas of the world, especially Asia. However, when compared with other major biotic stresses of rice, the successful deployment of resistance genes with respect to this disease is largely unsuccessful (Teng et al. 1990; Molla et al. 2020). This is particularly due to lack of availability of resistance sources among the germplasm and quantitative nature of inheritance of the disease response. Vertical resistance showing clearly distinguishing results in terms of resistance and susceptibility is yet to be discovered for sheath blight. Due to this reason, gene for gene hypothesis based pathotyping of the different physiological races or strains of the causal organism is also not possible. Moreover, the resistance
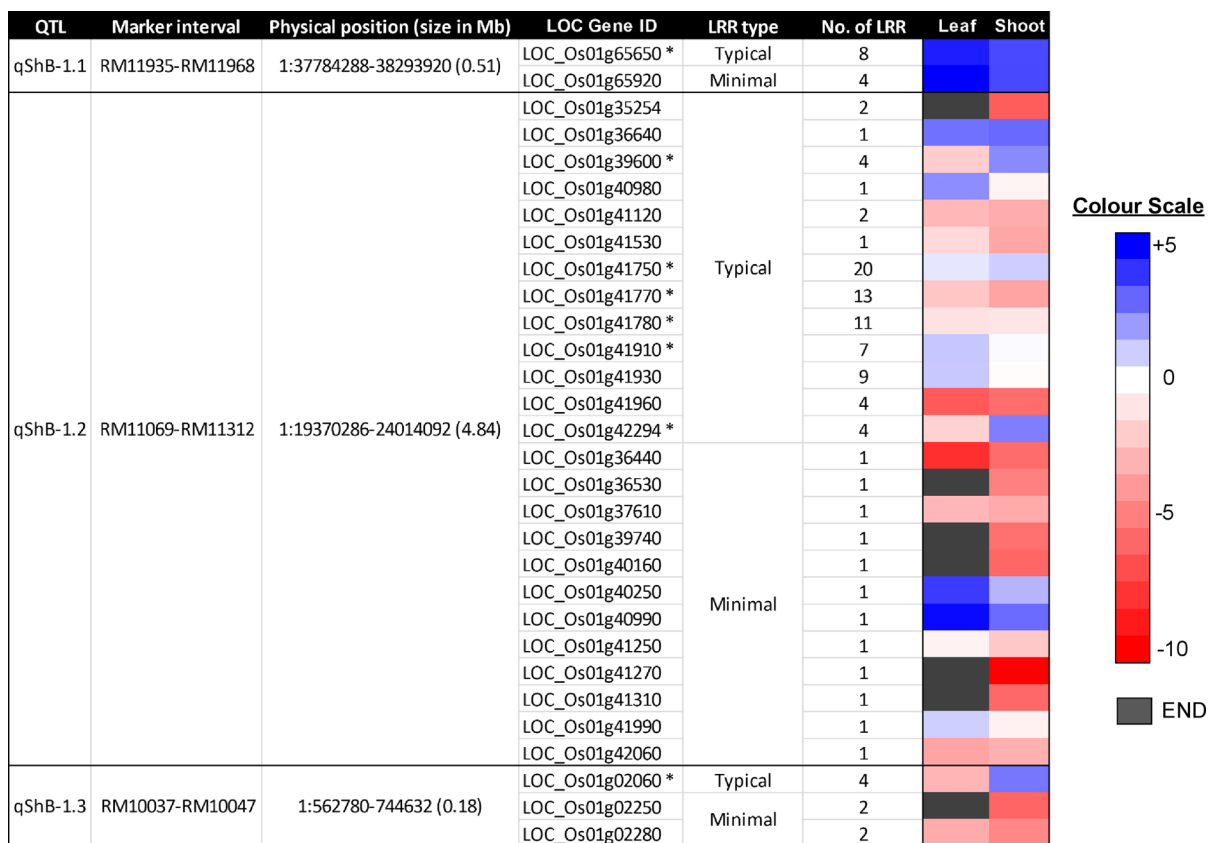
Fig. 6 Expression heat map of leucine rich repeat (LRR) containing genes in leaf and/or shoot tissues of rice from the QTL regions mapped from Swarna-Sub1/CR 1014. Physical positions of flanking markers based on Gramene database (http://www.gramene.org) (McCouch 2002). MSU identifiers of the genes (LOC Gene ID) and expression profile of leaf and shoot tissues as per Rice Expression Database (Xia et al. 2017). Color scale + 5 to − 10 used for heat map generation

QTL	Marker interval	Physical position (size in Mb)	LOC Gene ID	Annotation	Leaf	Shoot	Colour Scale
qShB-1.1	RM11935-RM11968	1:37784288-38293920 (0.51)	LOC_Os01g65450	ubiquitin-like domain-containing CTD phosphatase 1			
			LOC_Os01g65900	chitin-inducible gibberellin-responsive protein			
qShB-1.2	RM11069-RM11312	1:19370286-24014092 (4.84)	LOC_Os01g36930	ubiquitin carboxyl-terminal hydrolase 6			
			LOC_Os01g37630	esterase			
			LOC_Os01g37760	glutamate dehydrogenase			
			LOC_Os01g39790	esterase/lipase/thioesterase			
			LOC_Os01g39800	esterase			
			LOC_Os01g40840	serine/threonine-protein kinase AFC3			
			LOC_Os01g41630	serine/threonine protein phosphatase 2A			

Fig. 7 Expression heat map of genes lacking leucine rich repeat (LRR) in leaf and/or shoot tissues of rice from the QTL regions mapped from Swarna-Sub1/CR 1014. Physical positions of flanking markers based on Gramene database (http://www. gramene.org) (McCouch 2002). MSU identifiers of the genes (LOC Gene ID) and expression profile of leaf and shoot tissues as per Rice Expression Database (Xia et al. 2017). Color scale 0–6 was used for heat map generation

in germplasm is found only in genotypes which are taller and late maturing (Sharma et al. 2009; Liu et al. 2014). Even with taller and late maturing genotypes, only moderate resistance with consistent disease score of '3' is the best achieved response till now (Yadav et al. 2014; Dey et al. 2016). This fact lead to the perception among various rice researchers that the disease can't be tackled in modern high yielding varieties which are mostly semidwarf in nature and transgenic route is one of the attractive solution in this regard (Molla et al. 2013, 2016; Karmakar et al. 2016). Still, it was possible to identify few QTLs from a limited number of moderately resistant genotypes (Zarbafi and Ham 2019; Molla et al. 2020). Whenever any new and stable source of resistance is detected among germplasm, it needs to be mapped immediately to look for the presence of any novel and effective QTLs. As vertical resistance for this disease is not achievable, attaining horizontal resistance through deployment of genomic regions from multiple resistance sources with smaller but cumulative effect is looked upon as a solution to this menace (Han et al. 2002). This disease rate limiting approach is rather practical when compared with initial inoculums limiting approaches. There is also scope of obtaining race non-specific durable resistance by adopting such approaches. Any QTL/gene which reduces the rate of disease development in highly susceptible genotypes even in a limited but stable manner is quite likely to play crucial role to achieve that objective (Zarbafi and Ham 2019).

The moderately resistant genotype CR 1014 identified in the present study consistently showed the disease response at par with Tetep, which is one of the most widely reported resistance source till date

(Sharma et al. 2009; Channamallikarjuna et al. 2010; Yadav et al. 2015, Dey et al. 2016). Although earlier studies reported Jasmine85 and Teqing as moderately resistant genotypes (Li et al. 1995; Pan et al. 1999; Zou et al. 2000; Pinson et al. 2005), both genotypes showed susceptible reaction to the virulent strain CRRI-RS-8 used in the study. Hence this new genotype (CR 1014) identified in our study holds significance for mapping QTLs and for studying the effect of the identified QTLs in the susceptible background. To map the QTLs for sheath blight, earlier studies used quantitative value or percentage of relative Lesion Height (RLH) (Prasad and Eizenga 2008; Fu et al. 2011; Liu et al. 2014; Yadav et al. 2015; Wen et al. 2015; Zeng et al. 2015) while few researchers have also used disease rating scores (Li et al. 1995; Che et al. 2003). However, the widely followed SES rating system of International Rice Research Institute (IRRI 2002), categorizes the genotypes in different class intervals which make such scoring-based data unsuitable for QTL mapping. The phenotypic variation of RLH in all the mapping populations followed normal distribution although certain skewness towards the susceptibility was also recorded. In mapping populations involving other parental combinations, similar skewness towards susceptibility along with overall normal distribution for RLH were reported earlier (Sharma et al. 2009; Channamallikarjuna et al. 2010; Yadav et al. 2015).

Distinct non-overlapping polymorphic markers distributed all over the genome are another prerequisite for mapping of QTLs. For the two contrasting parents Swarna-Sub1 and CR 1014, only 5.86% of the tested markers were found to be polymorphic. The low level of polymorphism may have arisen due to narrow

genetic variation owing to their common gene pool. The 120 polymorphic markers between the two contrasting genotypes are physically distributed over the 12 chromosomes. However, only 109 markers could be assigned to different linkage groups. These 11 markers were not possible to assign to any linkage group either due to their segregation distortion or due to unavailability of polymorphic markers close to them. Due to those gaps in the genome coverage, the derived linkage map was distributed over 18 linkage groups. Although we screened 231 and 126 markers for chromosomes-3 and 12 respectively, only five and four ploymorphic SSR markers were detected. Out of those, only four and three markers were assigned on their corresponding linkage maps. Due to such large gap of marker intervals in those chromosomes, some QTLs may remain undetected. Chances of failing to detect the minor QTLs and their interaction effects with other QTLs increases substantially under such circumstances. To overcome the problem, the fixed RIL populations available with us may be genotyped with genome wide distributed SNP markers in future.

We started mapping of QTL in F_2 generation itself where the same plant was genotyped and phenotyped. However, there is always chance of error in phenotyping as the value of respective genotype will come from only single plant. Still, we utilized it to carry out preliminary mapping of gene/QTL. To increase the precision level, the F_2 derived F_3 (designated as $F_{2:3}$) population were phenotyped where multiple plants of a particular line (full sib family) can be evaluated and average value may be considered for mapping the QTL(s). In both F_2 and $F_{2:3}$ populations, we could map two QTLs. However, one QTL (qShB-1.1) was common in both the populations. During further validation of the detected QTLs in alternative populations (192 F_4 lines of Tapaswini/CR 1014 and 630 F_5 Recombinant Inbred Lines of Swarna-Sub1/CR 1014) using the linked markers, the qShB-1.1 was only found as consistent. The other two QTLs (qShB-1.2 and qShB-1.3) were not recorded further. However, this does not rule out the possibility of presence of other QTLs in the populations as QTL detection largely depends on type of population, density of genome coverage and precision of phenotyping. The status of QTLs reported here is specifically related to the population size, phenotyping value and genome coverage used by us. However consistent presence of the QTL qShB-1.1 across populations phenotyped at different years (over 4 years) with high LOD score and almost similar phenotypic variation ($F_{2:3}$ onwards) and gene action indicate this as stable QTL. Based on the physical position of markers associated with the QTLs detected by us, we compared the earlier reported QTLs for sheath blight for co-localization. The QTL qShB1.2 was identified at 19.3–24.0 Mb homologus position of Nipponbare chromosome 1. Jia et al. (2012) reported a QTL qSBR-1 linked with marker RM11229 at 22.3 Mb Nipponbare chromosome-1 homologous position in a core collection of 217 genotypes. No reported QTL was found near the qShB1.3 identified in our study. The stable QTL qShB-1.1 was flanked by RM11935 (homologus position: 37.8 Mb of Nipponbare chromosome 1) and RM11968 (homologus position: 38.3 Mb of Nipponbare chromosome 1). Xu et al. (2011) reported a QTL, qShB1 at approximate physical position of 38.80–39.02 Mb in a doubled haploid (DH) population of Baiyeqiu/Maybelle where this particular QTL was only consistently detected over 2 years (2007 and 2008) with LOD scores of 5.18 and 8.03. In another study, qShB1 was recorded at approximate Nipponbare reference physical position of 38.8–39.07 Mb with LOD score of 4.7 in the BC_2F_2 mapping population of *O. nivara/O. sativa* c.v. Bengal. The QTL from *O. nivara* also revealed the influence of plant height and heading date on sheath blight resistance (Eizenga et al. 2013). Unlike many other studies reported earlier, we could not detect multiple QTLs from the single population. Shiobara et al. (2013) initially detected eight QTLs for sheath blight resistance in a biparental population where finally only one stable QTL was detected over 3 years. As QTL regions represent a set of genes present as a block in a region of the genome, their effect will vary with the parental combinations due to difference of QTL alleles. Some minor QTLs in a biparental population may behave as major QTL in another population and vice-versa. One QTL qshb7.3 (located in chromosome 7) was identified from two different sources, Tetep and ARC10531. However, their contributions to total phenotypic variations were 10.02% and 21.76%, respectively (Channamalikarjuna et al. 2010; Yadav et al. 2015).

A QTL for sheath blight resistance will be considered as effective and of any practical applications only when it improves the disease response of a highly susceptible genotype. We simultaneously developed

large backcross population of Swarna-Sub1/CR 1014 using Swarna-Sub1 as recurrent parent. Before genotyping the backcross derived lines, the QTLs were validated in large F_5 population of this cross and F_4 population of Tapaswini/CR 1014. The sub-set of backcross derivatives with lesser plant height than the donor parent CR 1014 and showing consistent score of '5' over years were genotyped with the markers associated with the three QTLs. Except a single genotype, all the other genotypes whose full sib lines showed '5' score for the disease in field screening of BC_2F_2 and BC_2F_3 as well pot screening of BC_2F_3 were carrying qShB-1.1. Out of ten genotypes, seven were carrying homozygous flanking marker alleles of CR 1014 and three were heterozygotes. Heterozygotes are also expected as all the genotypes in BC_2F_1 will be heterozygote for loci where CR 1014 alleles will be detected, and their proportion is theoretically 25% of the population. In BC_2F_2 and BC_2F_3, the proportion of CR 1014-Homozygote:Heterozygote:Swarna-Sub1-Homozygote are expected to be 6.25:12.5:81.25 and 9.37:6.25:84.38, respectively. It is again interesting to record that the random sample of all the genotypes which were consistently categorized as highly susceptible showed complete absence of marker alleles of CR 1014 associated with qShB-1.1. For the two other QTLs, there was no such pattern and the genotypes of the two different sets mostly showed homozygous alleles of Swarna-Sub1, besides few heterozygotes. As we have not applied any selection based on genotyping during the development of backcross population, this high degree of bias of majority of the genotypes with stable and improved score for disease response (from '9' to '5' score) towards the consistently detected QTL region from donor parent CR 1014 indicate the effectiveness of the QTL for improving the resistance. However, despite of the presence of the QTL qShB-1.1 among those 10 backcross progenies, none of them have shown the consistent disease score of '3' like the donor parent CR 1014. Only four lines with plant heights ranging from 130-140cm showed score of '3' and all of them also carried the QTL qShB-1.1. The average plant height of Swarna-Sub1 ranges from 95–100 cm whereas in case of CR 1014 it is 145–150 cm. After deploying the stable QTL qShB-1.1 from moderately resistant donor CR 1014 in the background of highly susceptible recurrent parent Swarna-Sub1 through repeated backcrossing; we could improve the highly susceptible genotype (score

'9') to moderately susceptible (score '5') while applying strong selection pressure for reduced plant height. In case of qShB1 mapped from O. nivara in the background of O. sativa at almost the same physical position, the influence of plant height and heading date on sheath blight resistance from the QTL was reported (Eizenga et al. 2013). It should also be noted that unlike most of the QTL or gene mapping experiments, we could not develop the biparental population from two extreme types (Immune or resistant vs. highly susceptible) of genotypes. Till date only moderately resistant (score '3') sources are available against this pathogen, that also in very few cases. Among the BC_2F_3 lines showing disease score of '5' and homozygosity for qShB-1.1 loci, the genotype S/C-BC2F3-PLN-72 was identified as QTL-NIL due to its highest background recovery of the recurrent parent. This near isogenic line holds importance as breeding material due to the additional benefit of submergence resistance and improved sheath blight resistance when compared with the mega variety Swarna. In any earlier report, direct transfer of any QTLs leading to improvement of disease response to better than '5' score was not achieved. Shiobara et al. (2013) identified backcross derived inbred lines (BILs) of popular Japanese variety Koshihikari carrying the stable QTL qSBR-9 from resistant genotype Jarjan with better resistance to sheath blight but the disease score was limited to '5'. One QTL (qshb7.3, located in chromosome 7) identified from two different sources Tetep (Channamalikarjuna et al. 2010) and ARC10531 (Yadav et al. 2015) was found to be highly effective in backcross derivatives of BPT5204. Coincidentally, BPT5204 has one parent (Mahsuri) common with Swarna in its pedigree. As the two effective QTLs (qShB-1.1 from CR 1014 and qshb7.3 from Tetep as well as ARC10531) are located in different chromosomes, they may be pyramided in the same background for study of QTL × QTL interaction as well as to find out the scope of improving the resistance to further higher level. The genotype S/C-BC2F3-PLN-72 may be used as recurrent parent for this purpose. Several QTLs reported from the other sources may also be tested to check the disease reactions in combination with QTL of CR 1014. Even if some QTLs lead to only one scale increase (say '7' from '9' or '5' from '7') in disease response, that should also be given due importance considering the nature of the disease.

The QTL *qShB-1.1* identified in our study is expected to play an important role to combat sheath blight disease. Additionally, the gene action recorded for the QTL in different populations are mainly additive in nature which provide the scope of using the QTL in fixed lines. The mapping populations used by us are now fixed and immortal mapping populations like F_6-RIL population of Swarna-Sub1/CR 1014 (population size 630) and Tapaswini/CR 1014 (population size 192) as well as BC_2F_4 population of Swarna-Sub1/CR 1014 (population size 315) are available with us. These populations will serve as suitable resources for fine mapping and multilocation testing of the identified QTL. Besides, there is always chance that we may have missed several minor QTLs while mapping the QTLs from F_2 and $F_{2:3}$ populations due to limited population size and genome coverage. By employing high throughput genotyping platforms and multi-location testing, more comprehensive studies are now possible with the available populations. However, before using the BC_2F_4 population for mapping purpose, segregation distortion must be checked as we have lost a sizeable portion (73 out of 388) of the population during advancement from BC_2F_2 to BC_2F_3 due to very severe disease reaction and other natural factors in some of those lines.

Among the candidate genes predicted in the QTL region identified by us, *LOC_Os01g65900* located in QTL *qShB-1.1*, encodes a chitin-inducible gibberellin-responsive (CIGR) protein. Chitin is present in the cell wall of the pathogen *Rhizoctonia solani* and absent in plants. Day et al. (2004) have reported that the *CIGR* proteins have important role in both plant defense and development. Kovi et al. (2011) have reported that the gene *LOC_Os01g65900* play important role in governing plant height and have very high expression levels in leaf sheath of tall plants compared to short plants. Except these two reports, much information about this gene in rice is not available. In case of wheat and barley it has been found that some specific plant height controlling genes also play important role in conferring disease resistance (Gosman et al. 2009; Saville et al. 2012). Sheath blight resistance in rice is often found to be related with increased plant height and delayed heading date. A series of events could be hypothesized here. Due to *R. solani* infection, the rice plant possibly senses the fungal chitin, and as a result, *CIGR* gene is activated and influences the growth of the plant. Such changes in growth of plants may make the plant more tolerant of sheath blight disease. Hence the role of this *LOC_Os01g65900* in rice needs to be studied with further details. Another factor needs to be considered here; since the SES scoring system depends on relative lesion height, there is a high likelihood of bias toward increased plant height among the resistant lines of a population (Molla et al. 2020). The other important gene identified in the QTL region was *LOC_Os01g65650* (identical to *OsCEPR2* or C-Terminally Encoded Peptide Receptor2) which encode a typical eLRR-containing protein with receptor-like Tyrosine kinase activity and have high expression in leaf and shoot. The role of typical leucine rich repeat motif containing genes in plant defense is well known. Although the function of the gene is yet to be studied in rice, the role of C-Terminally Encoded Peptide Receptor proteins in stress response and plant development has been characterized recently in greater detail in case of *Arabidopsis thaliana* (Yu et al. 2019). The presence of these candidate genes within the QTL region also indicate the possible dual role of *qShB-1.1* in plant growth (especially plant height) and defense response to sheath blight which is also recorded in our study as well as other co-localized QTL from same region (Eizenga et al. 2013). On proper validation of the two putative candidate genes for the identified QTL *qShb-1.1*, they could be utilized in breeding program for enhancing sheath blight resistance.

Conclusion and future perspectives

Addressing the problem of sheath blight disease through genetic approach is one of the major challenges for rice researchers. As the conventional gene for gene hypothesis is not applicable, the study of inheritance remains limited to only quantitative approaches. Lack of availability of parents with high level of resistance and pleiotropic gene action of sheath blight resistance with plant height and heading date further complicates the situation in many cases. Despite of these limitations, germplasm screening is carried out and whenever a new source is identified even with moderate resistance, it needs to be utilized for identification of QTLs. The present study identified CR 1014 as new source of resistance at par with the best checks and a stable QTL *qShB-1.1* was identified and validated through experiments conducted over a

period of 6 years. Near isogenic line for the QTL *qShB-1.1* improved the disease response of the susceptible parent Swarna-Sub1 to two scales of higher score which demonstrated the effectiveness of the QTL. Still, the transfer of this single QTL only converted a highly susceptible genotype to a moderately susceptible genotype. This indicates that only single QTL from one source will not be effective to turn a genotype to resistant one. There is need for selective stacking of multiple QTLs from other genotypes in a single background through marker assisted recurrent selection to further increase the level of resistance while carefully avoiding the pleiotropic effect of QTLs for plant height and heading date. Selection for moderate alleles, rather than strong alleles may also serve the purpose better in such cases.

Compliance with ethical standards

The authors declare that the experiments conducted for this publication comply with the current laws of India.

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

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