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ORIGINAL ARTICLE

Genetic mapping and molecular marker development for *Pi65(t)*, a novel broad-spectrum resistance gene to rice blast using next-generation sequencing

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Abstract
Key message **A novel *R* gene was mapped to a locus on chromosome 11 from 30.42 to 30.85 Mb, which was proven to be efficient in the improvement of rice blast resistance.**
Abstract Rice blast is a devastating fungal disease worldwide. The use of blast resistance (*R*) genes is the most important approach to control the disease in rice breeding. In the present study, we finely mapped a novel resistance gene *Pi65(t),* conferring a broad-spectrum resistance to the fungus *Magnaporthe oryzae*, using bulked segregant analysis in combination with next-generation sequencing technology. Segregation in a doubled haploid (DH) population and a BC_1F_2 population suggested that resistance to blast in Gangyu129 was likely conferred by a single dominant gene, designated *Pi65(t)*; it was located on chromosome 11 from 30.20 to 31.20 Mb using next-generation sequencing. After screening recombinants with newly developed molecular markers, the region was narrowed down to 0.43 Mb, flanked by SNP-2 and SNP-8 at the physical location from 30.42 to 30.85 Mb based on the Nipponbare reference database in build 5. Using the software QTL IciMapping, *Pi65(t)* was further mapped to a locus between InDel-1 and SNP-4 with genetic distances of 0.11 and 0.98 cM, respectively. Within this region, 4 predicted *R* genes were found with nucleotide binding site and leucine-rich repeat (NBS-LRR) domains. We developed molecular markers to genotype 305 DH lines and found that InDel-1 was closely linked with *Pi65(t).* Using InDel-1, a new rice variety Chuangxin1 containing *Pi65(t)* was developed, and it is highly resistant to rice blast and produces a high yield in Liaoning province of China. This indicated that *Pi65(t)* could play a key role in the improvement of rice blast resistance.

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W. Zheng and Y. Wang contributed equally to this work.

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Introduction

Rice blast, caused by *Magnaporthe oryzae* (*M. oryzae*), is the most important fungal disease in rice (*Oryza sativa*) worldwide (Ou 1985). An outbreak of blast can devastate rice yields, completely destroying crops in the most extreme cases. The use of host resistance has been proven to be the most effective and economical method to control rice blast (Fukuoka et al. 2009; Jeung et al. 2007).

To date, 84 rice blast *R* loci have been identified (Huang et al. 2011; Liu et al. 2010; Ma et al. 2014; Xu et al. 2014). Among them, 24 have been cloned (*Pb1*, *Pia*, *Pib*, *Pid2*, *Pid3*, *Pik*, *Pikh, Pik-m*, *Pik-p*, *Pish*, *Pit*, *Pita*, *Piz-t*, *Pi1*, *Pi2*, *Pi5*, *Pi9*, *Pi21*, *Pi25*, *Pi36*, *Pi37*, *Pi54*, *Pi56* and *PiCO39*) (Chauhan et al. 2002; Liu et al. 2013a, b). The

majority of rice blast *R* genes are associated with a hypersensitive response (HR), according to the gene-for-gene concept, and race specificity is the key feature of this *R* gene-mediated disease resistance (Fukuoka et al. 2009; Jia et al. 2000). Due to highly frequent variation in the *M. oryzae* population (Dean et al. 2005), the resistance of new rice varieties simply with a single *R* gene can be lost quickly, especially when such a variety is grown in large areas (Fukuoka et al. 2009; Hulbert et al. 2001; Qu et al. 2006). The way to solve this problem and control rice blast is pyramiding multiple *R* genes into a single rice variety, and deployment of rice varieties with broad-spectrum resistance is another practical means to control blast disease (Chen et al. 1995; Hittalmani et al. 2000). Therefore, it is more and more important to screen rice germplasm with broad-spectrum resistance and identify broad-spectrum *R* genes.

Molecular mapping has been extensively used for the identification of *R* genes; it can provide the starting point for gene cloning and marker-assisted selection in rice breeding (Hittalmani et al. 2000; Jeung et al. 2007). This is usually conducted by genotyping segregating populations derived from bi-parental crosses; however, it is time-consuming and laborious (Michelmore et al. 1991; Salvi and Tuberosa 2005). The bulked segregant analysis (BSA) provides a simple approach to identify genes by genotyping a pair of bulked DNA samples from two sets of individuals with contrasting extreme phenotypes (Michelmore et al. 1991). Over the past decades, BSA technologies have been used frequently to map important traits in many crops (Cheng et al. 2015; Jeon et al. 2003; Liu et al. 2005). The rapid development of next-generation sequencing (NGS) technologies gives a further push to BSA strategies for gene mapping. The technique for gene mapping using NGS in combination with BSA has been developed (Das et al. 2015; Fekih et al. 2013; Sun et al. 2013; Takagi et al. 2015; Takagi et al. 2013; Takagi 2013) and demonstrated in the identification of genes in maize (*Zea mays* L.) (Xia et al. 2014), cucumber (*Cucumis sativus* L.) (Lu et al. 2014), cotton (*Gossypium* spp.) (Chen et al. 2015), wheat (*Triticum aestivum* L.) (Trick et al. 2012), rice (*Oryza sativa* L.) (Abe et al. 2012; Yang et al. 2013), and sunflower (*Helianthus annuus* L.) (Livaja et al. 2013).

The *japonica* rice variety Guangyu 129 is resistant to many Chinese *M. oryzae* races/isolates and was predicted to carry novel blast *R* gene (Wang et al. 2015). The objectives of the current study were to finely map a rice blast *R* gene using a BSA-NGS approach in the DH and BC_1F_2 populations derived from the crosses of Gangyu129/Liaoxing1 and DH181/Liaoxing1, respectively. Results will certainly benefit the improvement of rice blast resistance in breeding program.

Materials and methods

Plant materials

A mapping population of 305 DH lines was developed from the Gangyu129/Liaoxing1 cross. Gangyu129 and its parent Danjing4 have been highly resistant to rice blast for many years in China. Gangyu129 was resistant to 8 isolates (ZA1, ZA9, ZB1, ZB13, ZC1, ZE1, ZF1 and ZG1) (Fig. S1a), whereas Liaoxing1 was susceptible to these isolates (Fig. S1b). The line DH181 of the DH population is highly resistant to rice blast, whereas its agronomical traits are similar to Liaoxing1. A BC_1F_2 population of 850 individuals was developed from the DH181/Liaoxing1 cross.

Inoculation and disease evaluation

Field and greenhouse inoculations were used to evaluate blast reactions of the DH and BC_1F_2 populations. Natural inoculation was conducted in the field, in Dagushan town of Dandong, Liaoning province, China, where rice blast is very serious every year. Field trials were conducted in randomized complete blocks with three replicates. Plots of each line consisted of two 2 m rows of 20 plants with 30 cm spacing between rows. Two rows of control variety (Lijiangxintuanheigu) were planted every 10 rows. No fungicide was used during the entire growth period. The seeds were sown in mid-April, and seedlings were transplanted by the end of May. Incidents of rice blast infection were scored at seedling and tillering stages using a disease assessment scale of 0–5 (Pan et al. 1996). Artificial inoculation was conducted in greenhouse. At 56 days after sowing (5−6 leaves stage), seedlings were spray-inoculated with a mixture of 8 isolates (ZA1, ZA9, ZB1, ZB13, ZC1, ZE1, ZF1 and ZG1) that were isolated from the field in 2012 (Wang et al. 2013), and these are prevalent isolates of *M. oryzae* in China. After being sprayed with a suspension of the mixture, the inoculated plants were placed in darkness in dew chambers at 100 % relative humidity and 26 °C for 24 h, and then transferred to a greenhouse and maintained at 26 °C. The inoculated seedlings were scored 7 day after inoculation following Pan et al. (1996).

Genetic analysis

Genetic analysis of resistance to rice blast was performed on the DH and BC_1F_2 populations. A Chi-square test (χ^2) was used to evaluate the goodness of fit of observed and expected segregation ratios in the DH and BC_1F_2 populations.

Specific-locus amplified fragment (SLAF) library construction and high-throughput sequencing

First, we carried out a SLAF pre-design experiment (Sun et al. 2013). The enzymes and sizes of restriction fragments were evaluated using training data. To maintain the sequence depth uniformity of different fragments, a tight length range was selected (about 30–50 bp); a pilot PCR amplification was performed to check the reduced representation library features within this target range, which would ordinarily include fragments with similar amplification features on the gel. When non-specifically amplified bands appeared on the gel, we then repeated the pre-design step to produce a new scheme. We constructed the SLAF library in accordance using the pre-designed scheme. Then, two DNA pools were constructed, one R-pool and one S-pool, by mixing an equal amount of DNA from 50 resistant and 50 susceptible DH lines, respectively. Finally, pair-end sequencing was performed according to the selected SLAFs using an Illumina high-throughput sequencing platform, followed by SNP genotyping and evaluation.

Sequence clustered and association analysis

All SLAF pair-end reads with clear index information were clustered based on sequence similarity. To reduce computing requirements, identical reads were merged, and sequence similarity was detected using one-to-one alignment by BLAST (Kent 2002). SLAFs with two, three, or four tags were considered to be polymorphic markers.

The parameters SNP-index and Δ(SNP-index) (Abe et al. 2012; Takagi 2013) were calculated to identify candidate regions for rice blast disease resistance. An SNP-index is the proportion of reads harboring the SNPs that are different from the reference sequence. We calculated Δ(SNP-index) by subtracting the SNP-index of R-pool from that of S-pool. Thus, SNP-index is equal to 0 if all short reads contain genomic fragments from Liaoxing1, while SNP-index is equal to 1 if all short reads were from Gangyu129. The SNP-index graphs for R-pool and S-pool, and the corresponding Δ(SNP-index), were plotted.

The Δ(SNP-index) value should not be significantly different from 0 in a genomic region that has no target genes (Takagi 2013). We calculated statistical confidence intervals of Δ(SNP-index) for all the SNP positions with given read depths under the null hypothesis of no *R* genes, and plotted them along with Δ(SNP-index). If the Δ(SNP-index) value of a marker was bigger than the threshold at the 99 % level of significance, the marker was called diff_marker, and regions with three or more consecutive diff_markers were identified as trait-related candidate regions.

Marker development and recombinants screening

To screen recombinants from the BC_1F_2 population, polymorphic markers between the two parental lines were developed in the predicted region of rice chromosome 11. All SSR markers within the mapping region were used for polymorphism identification. To develop more polymorphic markers between Gangyu129 and Liaoxing1, we downloaded the sequence of predicted genes (http://rapdblegacy.dna.affrc.go.jp/) in the mapped region and designed SNP markers based on the sequence of those genes (Table S1). After PCR and sequencing, we observed much difference in sequences between the two varieties—based on that difference, we developed one InDel and nine SNP markers to screen the recombinants to finely map the locus for rice blast resistance in Gangyu129. Primers for the InDel and SNP markers were designed using Primer 5. (http://www.PromerBiosoft.com).

The fine mapping strategy

We investigated 850 BC_1F_2 plants for their genotypes using polymorphic markers. Based on the difference between the genotypes as assessed using polymorphic markers, six recombinants were screened, and comparison of the donor region to the phenotypes among multiple recombinants enabled us to narrow down the location of the *Pi65(t)* region. This mapping strategy, based on recombinant-derived progeny, effectively minimizes experimental errors that may result from genetic background noise and environmental variation (Zuo et al. 2014).

Linkage analysis and marker-assisted selection

Besides the polymorphic markers in the predicted region, we screened some SSR markers in other region of rice chromosome 11. The linkage analysis was performed using the software QTL IciMapping (Li et al. 2008) and recombination values were converted to centiMorgens using the Kosambi mapping function (Kosambi 1943). Using the tightly linked marker InDel-1, we selected individuals that carried *R* gene from the DH or BC_1F_2 population, and then backcrossed with the susceptible parent Liaoxing1, an adapted variety in Liaoning province. Based on phenotypic and genotypic identification, the homozygous resistant plants were selected, with agronomical traits similar to the recurrent parent from the backcross population.

Table 1 Genetic analysis of resistance to rice blast in the DH population derived from the Gangyu129/Liaoxing1 cross and the BC_1F_2 population derived from DH181/Liaoxing1 cross

Population	Phenotypic identification	Total number of plants observed	Resistant plant	Susceptible plant	χ^2	$\chi^2_{0.05}$
DH population	Natural inoculation	305	160	145	0.738	3.841
	Artificial inoculation	305	158	147	0.397	3.841
BC_1F_2 population	Natural inoculation	850	655	195	1.922	3.841
	Artificial inoculation	850	647	203	0.566	3.841

χ^2 value of the DH population is for $\chi^2_{1:1}$ and the χ^2 value of the BC_1F_2 population is for $\chi^2_{3:1;}$ all the values are smaller than 3.841, indicating that the resistance to *M. oryzae* in Gangyu129 was conferred by a single dominant gene

Table 2 Summary of the sequencing results data

Sample	Sample ID	Read length (bp)	Total reads	Total nucleotides (bp)	Q20 percentage	GC percentage
Resistant pool	R-pool	80	26,100,402	2,218,534,170	85.13	47
Susceptible pool	S-pool	80	33,249,048	2,826,169,080	85.15	47
Gangyu129 (R)	M	80	15,571,945	1,323,615,325	81.88	47
Liaoxing1 (S)	P	80	13,731,020	1,167,136,700	80.41	48

Resistant and susceptible pools were formed by mixing an equal amount of DNA from 50 resistant and 50 susceptible DH lines, respectively

Results

Phenotypic and genetic analysis

To investigate the inheritance of the resistance to *M. oryzae* in Gangyu129, reciprocal crosses between Gangyu129 and Liaoxing1 were performed. Both F_1 plants were resistant to rice blast; the DH population showed a 1:1 segregation in both the natural and artificial inoculation tests, with $\chi^2_{1:1} = 0.738$ and 0.397, respectively (Table 1); the BC_1F_2 population showed a 3:1 segregation in both the natural and artificial inoculations, with $\chi^2_{3:1} = 1.822$ and 0.566, respectively (Table 1). These indicated that the resistance to *M. oryzae* in Gangyu129 was likely conferred by a single dominant gene, temporarily designated *Pi65(t)*.

Sequencing and SNP identification

After SLAF library construction and high-throughput sequencing, a total of 7.53 Gb of data, including 88,652,415 valid single-end reads, was procured with an averaged read length of 80 bp (Table 2). The total number of SLAF tags was 172,419 with an average coverage of 339.78 folds. The average sequence depths of SNP markers were tenfold in parents and 50-fold in pools. The Q20 ratio ranged from 80.41 to 85.13 %. Based on the results of SLAF positioning on the genome, the SLAF tag numbers on each chromosome were calculated (Table 3), and a distribution diagram of SLAF tags on each chromosome is shown in Fig. 1a. The SLAF tags were distributed equally on each chromosome and the rice genome has been successfully simplified. A total of 172,419 high-quality SLAF tags were developed, 9092 of which were polymorphic according to analysis of allele numbers and the differences between gene sequences. The polymorphic SLAF tags were referred to as markers with a polymorphism rate of 5.27 %. Summary statistics for marker numbers are shown in Table S2, and a distribution diagram of markers on each chromosome is shown in Fig. 1b.

Table 3 The abundance of SLAF tags, polymorphic markers and Diff_markers on each chromosome

Chr ID	SLAF tag number	Polymorphic marker number	Diff_marker number
Chr1	20,253	1103	0
Chr2	15,159	550	0
Chr3	17,205	534	0
Chr4	17,536	711	0
Chr5	14,152	1354	0
Chr6	14,222	663	0
Chr7	14,179	464	0
Chr8	13,121	773	0
Chr9	11,181	376	0
Chr10	10,298	1232	1
Chr11	12,644	486	36
Chr12	12,469	846	0
Total	172,419	9092	37

Marker the polymorphic SLAF tag; *Diff_marker* the Ä (SNP-index) value of the marker is bigger than the threshold at 99 % significance level

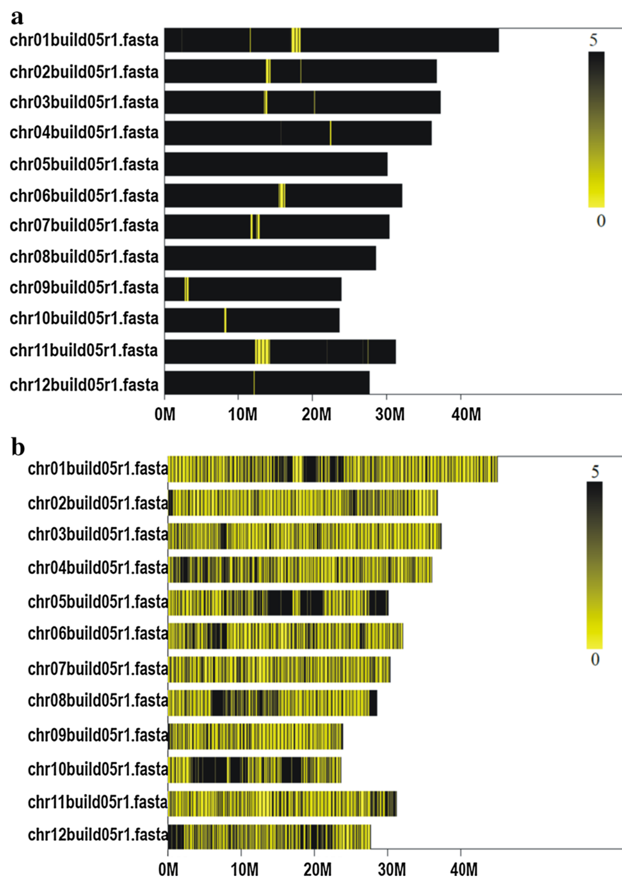

Fig. 1 **a** SLAF tags (*black lines*) distributed on 12 rice chromosomes. **b** Polymorphic markers (*black lines*) distributed on 12 rice chromosomes

Association analysis

The SNP-index was calculated for each SNP identified. SNP-index graphs were generated for the R-pool (Fig. 2a) and S-pool (Fig. 2b) by plotting the average SNP-index against the position of each sliding window in the Nipponbare genome assembly. By combining the information of SNP-index in the R-pool and S-pool, Δ(SNP-index) was calculated and plotted against the genome positions (Fig. 2c). It was expected that the SNP-index graphs of the R-pool and S-pool would be identical for the genomic regions that are irrelevant to the phenotypic difference, whereas the genomic region(s) harboring *R* genes would exhibit unequal contributions from R and S parental genomes. In addition, the SNP-index of these regions for R- and S-pools would appear as mirror images (Takagi 2013).

The Δ(SNP-index) value should be significantly different from 0 if a genomic region harbors a major QTL of the target genes. In this study, the threshold of Δ(SNP-index) value was 0.8122 at the 99 % significance level. Further, the Δ(SNP-index) values of 37 markers (diff_marker) were bigger than 0.8122, and they were found on Chr10 and Chr11 (Fig. 2c; Table 2). Among them, only 1 diff_marker was mapped on Chr10, whereas the other 36 were all located on Chr11, from 30.20 to 31.20 Mb based on the Nipponbare reference database in build 5 (http://rapdblegacy.dna.affrc.go.jp/). These demonstrated that a major QTL/gene conferring rice blast disease resistance was present at the 30.20–31.20 Mb region on chromosome 11 in the rice variety Gangyu129.

Developing SSR, Indel and SNP markers to narrow down the region of *Pi65(t)*

Based on the annotation of Nipponbare reference database in build 5 (http://rapdblegacy.dna.affrc.go.jp/), about 101 predicted genes were located within the region on chromosome 11 from 30.20 to 31.20 Mb. To screen the recombinants and refine the *Pi65(t)* gene, 42 SSR markers were selected on chromosome 11 to identify the polymorphism between Gangyu129 and Liaoxing1. The results showed that RM286, RM332, RM27181 and RM27364 were polymorphic between the two varieties. Based on the next-generation sequencing data and the sequence of mapped genes in Nipponbare from 30.20 to 31.20 Mb, we designed 9 SNP markers to amplify DNA from Gangyu129 and Liaoxing1 (the sequence and location of the SNP markers are shown in Table S1). Using SNP-3, we obtained two PCR products for 928 and 947 bp from Liaoxing1 and one PCR product for 947 bp from Gangyu129. The sequence alignment indicated an InDel for 19 bp between Gangyu129 and Liaoxing1 (Fig. S2a). We developed an InDel marker (InDel-1) based on the sequence difference, with the result of polyacrylamide gel electrophoresis using InDel-1 (Fig. S2b). Three primers (SNP-1, InDel-1 and RM27364) were used for a recombination test by polyacrylamide gel electrophoresis in the BC_1F_2 populations derived from DH181/Liaoxing1. Six recombinants were recovered within the region on chromosome 11 from 30.20 to 31.20 Mb. To find the position where the recombinant events occurred, SNP-1 to SNP-9 were used to amplify DNA from the recombinants and two parents. Comparing the sequence, graphic genotypes and disease reaction, we found six recombinants, and they were products of single-crossovers. No recombinants were detected using SNP-3, SNP-4, SNP-5, SNP-6 and SNP-7. Together, the mapping data indicated that the *Pi65(t)* locus falls within an interval of 0.43 Mb on chromosome 11 flanked by SNP-2 (30.42 Mb) and SNP-8 (30.85 Mb) (Fig. 3). Based on the Nipponbare reference database in build 5, 20 predicted genes were located within the interval of 0.43 Mb; among them, 19 were related to disease resistance (Table 4).

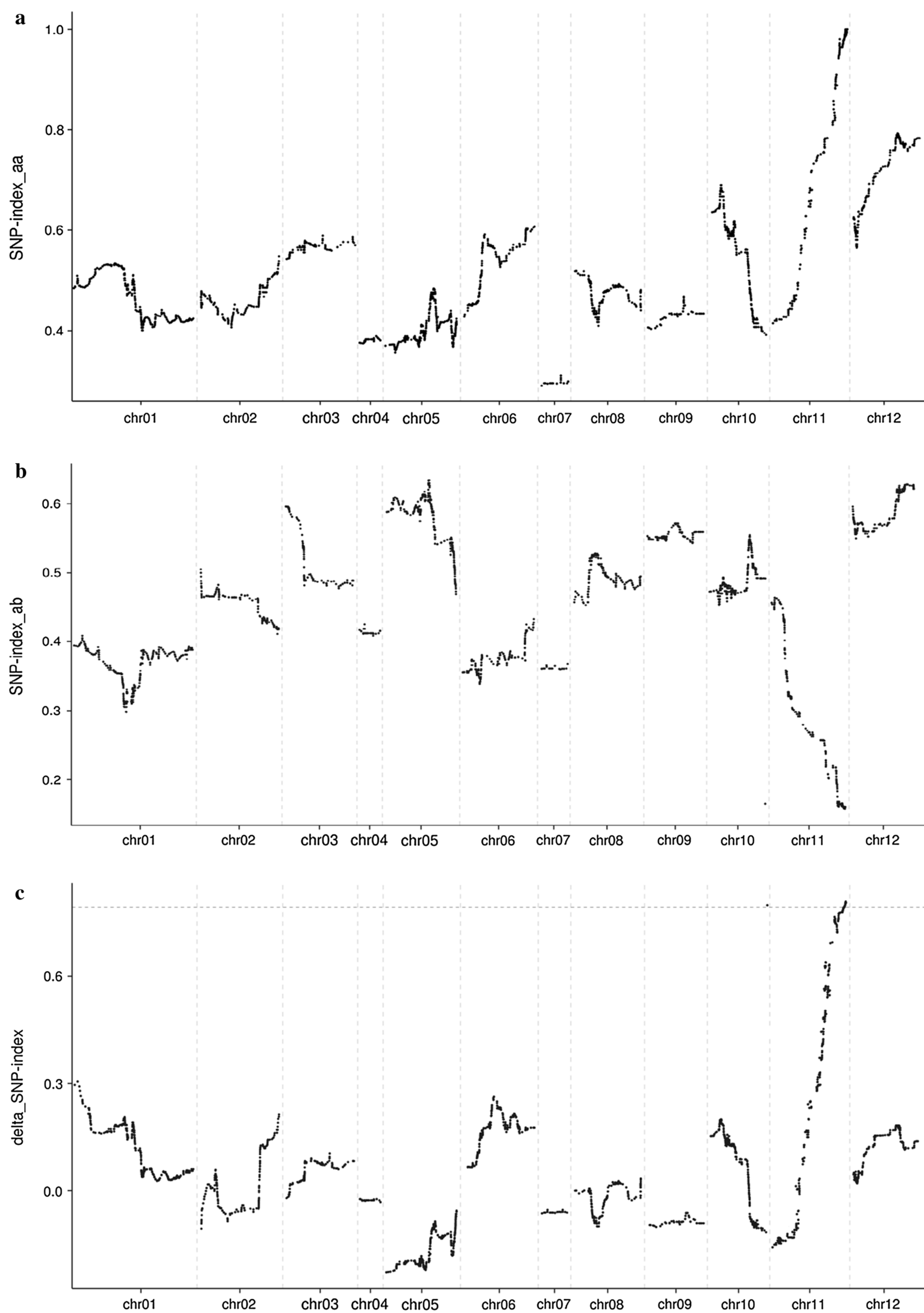

Fig. 2 SNP-index graphs of R-pool (**a**), S-pool (**b**) and Δ(SNP-index) graph (**c**) from SLAF-seq analysis. *X*-axis represents the position of 12 rice chromosomes and *Y*-axis represents the SNP-index. A candidate gene (*Pi65(t)*) location was identified in rice chromosome 11 (30.2–31.2 Mb interval) with the criteria that **a** the SNP-index in R-pool was near 1, **b** SNP-index in L-pool was near 0, and **c** the Δ(SNP-index) was over the confidence value ($P < 0.01$)

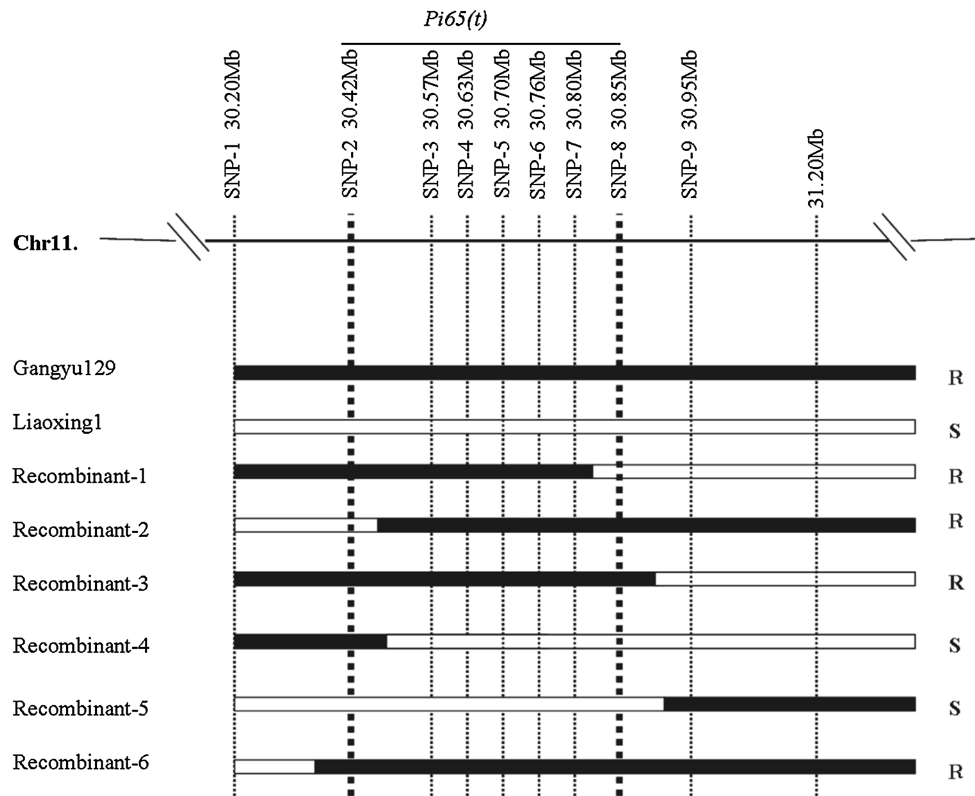

Fig. 3 Graphical genotypes of the recombinants spanning the *Pi65(t)* region using SNP-1 to SNP-9. The *black bar* is the introgression region from Gangyu129 (R). The *white bar* is the Liaoxing1 genome (S). Nine SNP markers are localized on the physical map. Numbers below the markers are the physical distance from the top on chromosome 11 based on the Nipponbare reference database in build 5. Six recombinants were detected in the region defined by the nine polymorphic markers. Recombinant-1 was from a crossover between SNP-7 and SNP-8 with a resistant phenotype. Recombinant-2 and recombinant-4 were from crossovers between SNP-2 and SNP-3, with resistant and susceptible phenotypes, respectively. Recombinant-3 and recombinant-5 were from crossovers between SNP-8 and SNP-9, with resistant and susceptible phenotypes, respectively. Recombinant-6 was from a crossover between SNP-1 and SNP-2 with a resistant phenotype. Together, these recombinants indicated that the *Pi65(t)* locus is within an interval of 0.43 Mb on chromosome 11 flanked by SNP-2 (30.42 Mb) and SNP-8 (30.85 Mb)

Linkage analysis and marker-assisted selection

One InDel, nine SNP and four SSR markers on chromosome 11 showed polymorphisms between the resistant and susceptible parents. The 14 polymorphic markers were then used to analyze the genetic distance between *Pi65(t)* and the markers. Using the software QTL IciMapping, the *R* gene *Pi65(t)* was mapped to a locus between InDel-1 and SNP-4 with genetic distances of 0.11 and 0.98 cM, respectively (Fig. S3; Table S3), and its physical position is at 30.57–30.63 Mb. Within this region, 4 predicted *R* genes were found with nucleotide binding site and leucine-rich repeat (NBS-LRR) domains (Table 4). Using InDel-1, DH170 carrying *Pi65(t)* was screened and then backcrossed with Liaoxing1. Via phenotypic and genotypic identification, we got 14 homozygous resistant plants with similar agronomical traits to Liaoxing1 from the backcross population and they carried the rice blast *R* gene *Pi65(t)*. Combined with yield and quality tests, we have successfully bred a new rice variety "Chuangxin1" that is highly resistant to rice blast in Liaoning province of China.

Discussion

Pi65(t) is a novel broad-spectrum *R* gene to rice blast

In the present study, we mapped a gene, *Pi65(t)*, with broad-spectrum resistance to rice blast, into an interval of 0.43 Mb on chromosome 11. Firstly, *Pi65(t)* was located within the region on chromosome 11 from 30.20 to 31.20 Mb. Then, the markers SNP-1, RM27364 and InDel-1 were chosen to select recombinants and delimited the introgression region of *Pi65(t)*. Secondly, nine SNP markers were developed to complete the chromosome walk to the target region. The *Pi65(t)* locus is located in a region of chromosome 11 with known blast *R* genes, including *Pi54*, *Pi66(t)*, *Pi-k*, *Pik-m* and *Pik-p*.

Table 4 Predicted genes within the region on chromosome 11 from 30.42 to 30.85 Mb on chromosome 11

Gene ID	Physical position (Mb)[a]	Swissprot_annotation
Os11g0691700	30.423	LRR receptor-like serine/threonine-protein kinase EFR
Os11g0691800	30.424	LRR receptor-like serine/threonine-protein kinase FLS2
Os11g0691900	30.428	LRR receptor-like serine/threonine-protein kinase FLS2
Os11g0692000	30.434	LRR receptor-like serine/threonine-protein kinase FLS2
Os11g0692100	30.435	LRR receptor-like serine/threonine-protein kinase FLS2
Os11g0692300	30.448	Probable LRR receptor-like serine/threonine-protein kinase
Os11g0692500	30.455	LRR receptor-like serine/threonine-protein kinase FLS2
Os11g0694100	30.556	Wall-associated receptor kinase 3
Os11g0694150	30.564	Wall-associated receptor kinase 2
Os11g0694200	30.565	Wall-associated receptor kinase-like 5
Os11g0694400	30.570	LRR receptor-like serine/threonine-protein kinase EFR
Os11g0694500	30.574	LRR receptor-like serine/threonine-protein kinase EFR
Os11g0694600	30.575	Probable LRR receptor-like serine/threonine-protein kinase
Os11g0694850	30.599	Probable LRR receptor-like serine/threonine-protein kinase
Os11g0695000	30.607	Probable LRR receptor-like serine/threonine-protein kinase
Os11g0695600	30.635	Probable LRR receptor-like serine/threonine-protein kinase
Os11g0695700	30.643	LRR receptor-like serine/threonine-protein kinase FLS2
Os11g0695800	30.650	LRR receptor-like serine/threonine-protein kinase FLS2
Os11g0699532	30.804	–
Os11g0700100	30.853	Putative disease resistance RPP13-like protein 3

[a] Physical position of the predicted genes based on the Nipponbare reference database in build 5

Pi54 was mapped to a 2-cM interval between RM224 and Y6855RA in the same region. *Pi-k*, *Pik-m* and *Pik-p* are located between the SSR markers RM1233 and RM224 on the long arm of chromosome 11, with respective distances of 1.6 and 0.2 cM to the linked markers (Ashikawa et al. 2008). *Pi66(t)* was mapped to an interval on Chr11 with the physical position from 27.7 to 27.84 Mb based on the Nipponbare reference database in MSU 7.0 (Liu et al. 2015). To differentiate these resistance genes, we constructed an integrated physical map via analysis of the positions of *Pi54, Pi66(t), Pi-k*, *Pik-m* and *Pik-p* (Fig. S4). In this physical map, the *Pi65(t)* locus was defined by SNP-2 and SNP-8, which were located from 30.42 to 30.85 Mb on chromosome 11; *Pi54* is located at 27.46 Mb (Sharma et al. 2010); *Pi66(t)* is located at 29.9–30.0 Mb based on the Nipponbare reference database in build 5 and the *Pi-k*, *Pik-m* and *Pik-p* loci are located at the genomic positions 30.17 Mb. Further, in our previous study, the allele at *Pik* locus in Gangyu129 was proved to have no resistance function (Wang et al. 2015). Thus, the resistance in Gangyu129 is conferred by a novel broad-spectrum *R* gene because no other blast *R* genes were mapped to the same region on chromosome 11. However, it is possible that the broad-spectrum resistance was attributed to the synergy of multiple *R* genes that were linked very closely in the mapping interval. For this, we are performing gene transformation to confirm the function of the candidate genes.

Development and utilization of molecular markers for *Pi65(t)*

The traditional phenotypic evaluation of resistance to *M. oryzae* in rice breeding is labor-intensive, time-consuming, and easily influenced by environmental factors. With high-resolution genetic and physical mapping strategies, molecular markers associated with disease resistance can be effectively deployed in crop breeding (Boyd et al. 2013). A number of PCR-based markers have also been developed for several blast *R* genes, such as *Pita* (Jia et al. 2002), *Piz*, *Piz-t*, *Pit*, *Pik*, *Pik-m*, *Pik-p*, *Pita-2* (Hayashi et al. 2006). The DNA markers offer an efficient and rapid means to select blast *R* genes. However, because of sequence similarity and clustering of *Pi* genes, an effective marker must be highly specific. In this study, we developed an InDel marker (InDel-1) located at 30.57 Mb on Chr11 and it is tightly linked with *Pi65(t)* resistance genotype. Because the sequences of genes at 30.42 and 30.57 Mb on Chr11 are very similar, two fragments of 120 bp and 139 bp were amplified with Indel-1 from Liaoxing1, and one fragment of 139 bp from Gangyu129 (Fig. S2b). We were able to screen the homozygous resistant individuals by selecting those plants with only a 139 bp using Indel-1, and the individuals that had two bands were all heterozygotes or homozygous susceptible plants at the *Pi65(t)* locus.

Though *Pi65(t)* has not been cloned, we could improve the resistance of Liaoxing1 using Indel-1. To improve the

resistance of Liaoxing1 to rice blast, we screened a resistant line (DH170) from the DH population derived from Gangyu129 (R)/Liaoxing1 (S). DH170 was identified to carry *Pi65(t)* using InDel-1, and we then backcrossed it with Liaoxing1. In the F_6 population derived from DH170/Liaoxing1, via phenotypic and genotypic identification we screened 14 homozygous resistant plants with similar agronomical traits to Liaoxing1 and they carried the rice blast *R* gene *Pi65(t)*. Combined with tests of yield and quality, we have thus bred a new rice variety "Chuangxin1" that is highly resistant to rice blast in Liaoning province of China, indicating that *Pi65(t)* would play a key role in the improvement of rice blast resistance in northern China.

Reliability and advantages of the gene mapping strategy using SLAF-seq and BSA

In the current study, a rice blast *R* gene *Pi65(t)* was finely mapped using SLAF-seq in combination with BSA; this has many advantages. Firstly, the SNP markers of the genetic map are much denser compared with SSR or other markers. Using SLAF-seq, we detected 9092 SNP markers in total and more than 700 markers were mapped to every chromosome, which is conducive to map a gene to a small region. Secondly, it saves the labors and shortens the process of the research. In view of the recent rapid development in sequencing technology, we foresee that the method will dramatically accelerate gene mapping and crop improvement in a cost-effective and time-saving manner.

Author contribution statement W. J. Zheng and Y. Wang performed the experiments and wrote the paper. L. L. Wang, Z. B Ma, J. M. Zhao, P. W, L. X. Zhang and Z. H. Liu performed the experiments. X. C. Lu designed the experiments.

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Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest in regard to this manuscript.

Ethical standards We declare that these experiments comply with the ethical standards in China, where they were performed.

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