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# Identification of the novel recessive gene *pi55*(t) conferring resistance to *Magnaporthe oryzae*

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The elite rice cultivar Yuejingsimiao 2 (YJ2) is characterized by a high level of grain quality and yield, and resistance against *Magnaporthe oryzae*. YJ2 showed 100% resistance to four fungal populations collected from Guangdong, Sichuan, Liaoning, and Heilongjiang Provinces, which is a higher frequency than that shown by the well-known resistance (*R*) gene donor cultivars such as Sanhuangzhan 2 and 28zhan. Segregation analysis for resistance with $F_2$ and $F_4$ populations indicated the resistance of YJ2 was controlled by multiple genes that are dominant or recessive. The putative *R* genes of YJ2 were roughly tagged by SSR markers, located on chromosomes 2, 6, 8, and 12, in a bulked-segregant analysis using genome-wide selected SSR markers with $F_4$ lines that segregated into 3 resistant (R):1 susceptible (S) or 1R:3S. The recessive *R* gene on chromosome 8 was further mapped to an interval ≈1.9 cM/152 kb in length by linkage analysis with genomic position-ready markers in the mapping population derived from an $F_4$ line that segregated into 1R:3S. Given that no major *R* gene was mapped to this interval, the novel *R* gene was designated as *pi55*(t). Out of 26 candidate genes predicted in the region based on the reference genomic sequence of the cultivar Nipponbare, two genes that encode a leucine-rich repeat-containing protein and heavy-metal-associated domain-containing protein, respectively, were suggested as the most likely candidates for *pi55*(t).

**rice blast, recessive resistance gene, *pi55*, resistance inheritance, gene mapping**

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Blast, caused by the ascomycete fungus *Magnaporthe oryzae*, is one of the most devastating diseases of rice worldwide [1]. Generally, it is responsible for large losses in yield in epidemically favorable areas and seasons [2]. Since the 1990s, the disease has occurred in all rice-growing regions in China with more than 3800000 $hm^2$ infected each year, which caused the loss of hundreds of millions of kilograms of rice [3]. Guangdong Province (GD) is the main rice cropping area in South China and the double cropping system in combination with the unique ecosystem characterized by high temperature and humidity is highly beneficial to infection and spread of this fungus. In recent years, huge outbreaks occurred more frequently in GD [4]. Deployment of resistant cultivars has been considered as the most cost-effective and environmentally friendly means by which to minimize crop losses because of the disease. Such cultivars, however, often remain effective only for a few years because of the high pathogenic variability and complexity of the fungus from which resistance-breaking isolates rapidly emerge [5]. There is, therefore, an urgent need to develop

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cultivars that carry durable resistance (*R*) genes against the fungus.

Genetic characteristics of blast resistance are complicated. Resistance is controlled by one or more dominant *R* genes, and by an incompletely dominant or recessive *R* gene in some scenarios. All of these different *R* genes are either mutually independent or related [6]. The study of genetic resistance to rice blast was initiated in the 1920s in Japan. Sasaki identified one dominant *R* gene in cultivar Tsurugi by genetic segregation analysis and artificial inoculation [7]. Subsequently, extensive genetic research on the resistance to rice blast was undertaken in the 1960s in Japan [8]. In China, such research was initiated in the 1980s, when *M. oryzae* races were investigated nationwide [9]. During the last two decades, substantial research progress in blast resistance has been made with the availability of various molecular markers and the whole genome sequences of the rice subspecies cv. Nipponbare (*japonica*) and cv. 93-11 (*indica*). Currently, over 85 dominant *R* genes and 350 quantitative trait loci (QTLs) have been identified in a variety of rice resources [10], which have been mapped on all of the 12 rice chromosomes, including six genes (*Pi36*, *Pi42*, *Pi33*, *Pi11*, *Pi29* and *Pi-GD-1*) on chromosome 8 [11,12]. Thirteen dominant *R* genes and two QTLs have been cloned by a map-based cloning approach [13,14]. The mapping and cloning of such *R* genes is the basis for a marker-aided selection (MAS) strategy applied in rice blast resistance breeding programs.

Although many types of recessive *R* genes are documented in plants, the resistance mechanism, which is generally distinct from that of dominant *R* genes, has remained largely obscure. Identification of additional recessive *R* genes is needed [15,16]. In rice, most recessive *R* genes identified are responsible for resistance to bacterial blight and few to blast. Ten of 33 bacterial blight *R* genes are recessive [17], and only one (*pi21*) confers resistance to blast [18]. Fukuoka and colleagues identified four blast QTLs in Japanese upland rice cv. Owarihatamochi used a $F_4$ population, and further identified the major effective QTL on chromosome 4 as *pi21* used a backcross population, which was ultimately isolated via a map-based cloning approach [19,20]. In addition, Liang *et al.* [21] reported that the Yunnan native rice cv. SB70L carries a recessive *R* gene corresponding to *M. oryzae* isolate 95-8-3C, identified by genetic segregation analysis using $F_2$, $F_3$ and $BC_1$ populations. Increasing evidence showed that functional recessive *R* genes are derived from susceptible dominant alleles via natural and artificial mutation, such as *xa5* and *xal3*, which might impart more durable resistance against the respective pathogens [22].

The elite *indica* cv. Yuejingsimiao 2 (YJ2), which is characterized by a high level of grain quality and yield, and resistance to *M. oryzae*, has been authorized as the leading cultivar since 2006 and as a control cultivar in regional tests of new cultivars in 2011 [23]. YJ2 has been used extensively as one of the most important parental cultivars in current rice breeding programs. To understand the genetic basis of the superior and stable blast resistance in YJ2, a series of genetic segregation and mapping analyses were performed that resulted in identification of a novel recessive *R* gene on chromosome 8 that is designated *pi55*(t).

## 1 Materials and methods

### 1.1 Isolates

A total of 297 isolates selected from four *M. oryzae* populations, which were collected in Guangdong (60 isolates), Sichuan (66 isolates), Liaoning (108 isolates), and Heilongjiang (63 isolates) Provinces in China, were subjected to resistance spectrum analysis. These isolates included all of the seven types (A to G) of Chinese races (data not shown). Four representative isolates (CHL688, CHL1743, CHL440, and CHL381) were chosen for genetic analysis of YJ2 resistance. Inoculations and disease evaluations were conducted in a greenhouse as previously described by Pan *et al.* [24].

### 1.2 Comparative analysis of resistance spectra

Five key parental cultivars used in blast resistance breeding programs in GD (YJ2, Qingliuai, Sanhuangzhan2, 28zhan and Zhenzhuai), as well as two reference cultivars (Nipponbare and 93-11), were selected for comparative analysis of resistance spectra to the four *M. oryzae* populations in 2007 and 2008. Each resistance spectrum was represented by the resistance frequency: the number of avirulent isolates compared with the total number of isolates tested for a rice cultivar in every population. The resistance frequencies were determined after statistical analysis using Microsoft Excel software (Figure 1).

### 1.3 Genetic crosses and progeny

YJ2 was crossed with cv. Tsuyuake, which carries *Pik-m*, in the early season of 2007. The $F_2$ population was derived from 12 $F_1$ individuals in the late season of 2007, and 1700 $F_3$ lines were developed from $F_2$ plants by the single seed transfer method in 2008. Then, 210 $F_3$ lines were randomly selected and used to construct a $F_4$ population (assigned from D64-1 to D64-210; Appendix Table S1 in the electronic version) for subsequent resistance segregation and gene mapping analyses.

### 1.4 Segregation analysis in $F_2$ and $F_4$ populations

The $F_2$ population was inoculated with a stable and highly virulent isolate, CHL688, which is avirulent to YJ2 and virulent to Tsuyuake. The $F_4$ population was inoculated with four isolates, two (CHL688 and CHL440) that were

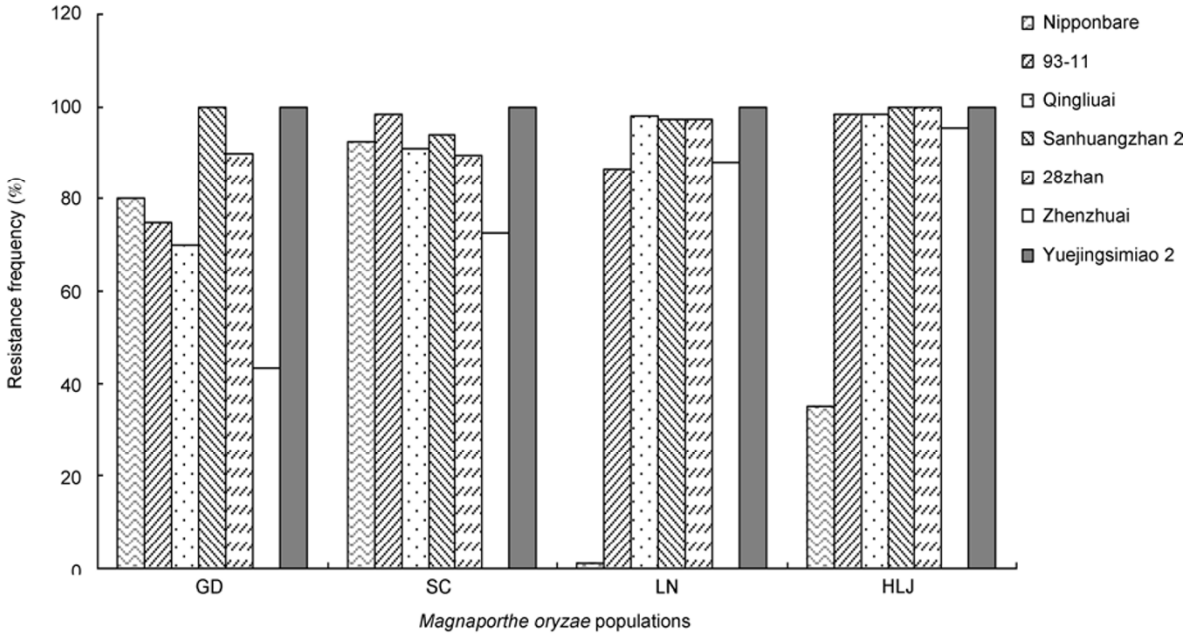


**Figure 1** Comparison of resistance frequencies of rice cv. Yuejingsimiao 2 and six additional cultivars against four *Magnaporthe oryzae* populations.

virulent to Tsuyuake, and two (CHL1743 and CHL381) that were avirulent to Tsuyuake (Table S1). With regard to the latter population, 15–16 plants for each $F_4$ line were planted in a row (some plants died after inoculation). Twenty and six $F_4$ lines were randomly selected and expanded as mapping populations for *de novo* inoculation with CHL688. Among these lines, four $F_4$ lines that segregated into 3R:1S or 1R:3S were randomly selected for subsequent bulked-segregant analysis (BSA). Seedling cultivation, inoculation, and leaf sampling were performed as described previously [24].

### 1.5 DNA extraction and gene pool construction

Genomic DNA was extracted from frozen leaves using the CTAB method. Two contrast pools were made by mixing equimolar amounts of DNA from either 10 resistant or 10 susceptible $F_4$ individuals. On the basis of the linkage maps established by Temnykh and colleagues [25,26], a set of 180 simple sequence repeat (SSR) markers, which were selected equally from all of the 12 rice chromosomes, were used for BSA analysis (Appendix Table S2 in the electronic version).

### 1.6 Linkage analysis

On the basis of the BSA analysis, only a recessive *R* gene on chromosome 8 was the focus of the present research. Gene mapping was achieved by three rounds of linkage analysis. The first round was carried out with candidate markers obtained by BSA to screen recombinants on both sides of the target locus. The second round was carried out with additional SSR markers adopted from the GRAMENE database (http://www.gramene.org) in the target region defined by the flanking markers obtained in the first round of linkage analysis. The third round was carried out with new SSR markers as well as sequence-tagged site (STS) markers developed in the present study. Candidate marker search and primer designation were carried out as described previously [27]. The new markers, which showed clear polymorphism between the parents, were subjected to the third round of linkage analysis with the recombinant progeny. Genetic distance between marker loci was estimated by the ratio $r=N_r/2N_T$, when $N_r$ is the actual number of the recombinants that occurred in the interval, and $N_T$ is the total number of individuals in the mapping population, therefore $2N_T$ is the number of gametes.

### 1.7 Construction of genetic and physical map *in silico*

A genetic map of the *R* gene locus was constructed using the genomic position-ready markers, which comprised the SSR and STS markers identified by the three rounds of linkage analysis. To construct the physical map of the locus, all of the markers used for linkage analysis were located on the respective bacterial artificial chromosome (BAC) clones of cv. Nipponbare released by IRGSP by BLASTN analysis (http://www.ncbi.nlm.nih.gov/BLAST). A contig map that covered the *R* locus was constructed with those clones by Pairwise BLAST analysis (http://www.ncbi.nlm.nih.gov/blast/bl2seq/bl2.html). Collectively, the physical map spanning the *R* gene locus was constructed *in silico* based on the Nipponbare sequences.

### 1.8 Candidate gene prediction

Candidate genes for the target *R* gene were annotated on the

basis of publicly available BAC or PAC sequences of the reference cv. Nipponbare in the targeted region, using the gene prediction programs FGENESH (http://genomic. sanger.ac.uk) and RiceGAAS (http://ricegaas.dna.affrc.go.jp/).

## 2 Results

### 2.1 Resistance spectrum

Resistance profiles of the seven cultivars tested against the four fungal populations are shown in Figure 1. YJ2 expressed 100% resistance to the four populations, which indicated that YJ2 is resistant to all of the seven types of Chinese blast races. YJ2 and Sanhuangzhan2 exhibited the same resistance frequency against the GD and Heilongjiang populations, and YJ2 showed higher resistance than Sanhuangzhan2 to the Sichuan and Liaoning populations. YJ2 and 28zhan had the same resistance frequency against the Heilongjiang population, but YJ2 showed higher resistance to the remainder of the populations. In terms of their resistance the cultivars were ranked as YJ2>Sanhuangzhan2>28zhan>93-11>Qingliuai>Zhenzhuai>Nipponbare. Therefore, YJ2 is indeed an excellent cultivar with broad-spectrum resistance to *M. oryzae*.

### 2.2 Resistance pedigree

YJ2 was derived from a cross between a soft aromatic rice line 'Yuexiangzhan/Zhongerranzhan' and a high yielding and resistant line 'Wufengzhan/Jinchaosimiao' (Appendix Figure S1 in the electronic version). Genealogy analysis revealed three clades of resistance donors that contributed to the blast resistance of YJ2. One clade of resistance donors included IR36, Qingliuai, Teqing, and IR37704-131-2-1; a second clade comprised Jingxian21, Fengaizhan, and 28zhan; and the third clade consisted of Yuexiangzhan, Zhongerruanzhan, and Wufengzhan. Hence, the genetic basis of blast resistance in YJ2 is divergent and complex.

### 2.3 Resistance inheritance

The $F_2$ population segregated into 504 resistant (R) and eight susceptible (S) individuals when inoculated with the isolate CHL688, which fits a 63R:1S ratio ($\chi^2$=1.56; $P$>0.20) and indicated that YJ2 carried three or more *R* genes for resistance to the isolate. To understand the *R* gene constitution of YJ2, 210 $F_4$ lines were inoculated with the four isolates CHL688, CHL440, CHL1743, and CHL381. About 50% of these lines expressed full resistance without segregation against the four isolates, which indicated that multiple *R* genes were involved, of which one is a broad-spectrum *R* gene involved in the blast resistance of YJ2 (Appendix Table S1 in the electronic version). To rapidly identify the potential new *R* gene(s) in YJ2, 26 $F_4$ lines were expanded and subjected to segregation analysis after *de novo* inoculation with CHL688. Four lines (D64-107, -110, -134, and -195) retained full resistance without segregation, three lines (D64-129, -171, and -197) segregated into 63R:1S, five lines (D64-62, -85, -89, -156, and -157) segregated into 15R:1S, six lines (D64-35, -59, -86, -98, -133, and -165) segregated into 3R:1S, and two lines (D64-61 and -208) segregated into 1R:3S. The remaining six lines (D64-2, -109, -128, -147, -153, and -193) showed a segregation ratio of between 15R:1S and 3R:1S, which might be because of the involvement of a combination of dominant and recessive *R* genes (Appendix Table S1 in the electronic version). Taken together, the results indicate blast resistance of YJ2 was controlled by multiple *R* genes, including dominant and recessive genes.

### 2.4 *R* constitution of Yuejingsimiao 2

To further understand the *R* constitution of YJ2, four of the 26 $F_4$ lines (D64-35, -61, -133, and -193) were selected for BSA analysis. A set of 180 SSR markers, selected from the whole genome of rice with an average genetic interval of 10 cM, were used to determine the chromosomal position of the target *R* genes (Appendix Table S2 in the electronic version). Candidate markers linked with *R* genes were located on chromosomes 2 (D64-133), 6 (D64-35), 8 (D64-61), and 12 (D64-193) (Table 1; Appendix Figure S2 in the electronic version). Interestingly, the candidate markers on chromosomes 2, 6, and 12 were linked with the known *R* gene loci *Pib*, *Pi2*/*Pi9*, and *Pita*/*Pita*-2, respectively, but those on chromosome 8 were not associated with any

**Table 1** Segregation of blast resistance in the four $F_4$ populations derived from a cross between the rice cultivars Yuejingsimiao 2 and Tsuyuake, and their candidate linkage markers in the respective populations obtained by bulked-segregant analysis

| $F_4$ line[a)] | Reaction pattern[b)] | | Total | $\chi^2$ (3R:1S or 1R:3S)[c)] | Candidate markers (chromosome)[d)] |
|---|---|---|---|---|---|
| | Resistant (R) | Susceptible (S) | | | |
| D64-35 | 135 | 50 | 185 | 0.30 | RM162 (6) |
| D64-61 | 27 | 98 | 125 | 0.60 | RM502 (8) |
| D64-133 | 75 | 17 | 92 | 1.75 | RM498 (2) |
| D64-193 | 88 | 44 | 132 | 4.45* | RM7102 (12) |

a) Four representative $F_4$ lines selected based on the bulked-segregant analysis. b) Reaction patterns of each $F_4$ line were scored after inoculation using isolates that are avirulent to Yuejingsimiao 2 and highly virulent to Tsuyuake. c) $\chi^2$ test was adopted for two segregation ratios. *, $P$=0.05. d) The representative markers on the four chromosomes obtained by bulked-segregant analysis.

known *R* gene locus. Therefore, it was inferred that the *R* genes in YJ2 were composed of three known dominant *R* genes plus an unknown recessive gene.

### 2.5 Mapping of novel *R* gene

To tag the new recessive *R* gene locus, 26 highly resistant and 84 extremely susceptible progenies derived from D64-61 were used as a mapping population. Chromosome walking to the *R* gene locus, *in silico*, was achieved by three rounds of linkage analysis. The first round was carried out with the candidate markers RM223 and RM502 obtained by BSA analysis (Table 2). Twenty-eight and nine distinct recombinants were identified at the RM223 and RM502 loci, respectively, which suggested that the *R* gene locus was flanked with these two markers with genetic distances of about 14.0 and 4.1 cM, respectively (Figure 2A).

The second round of linkage analysis was carried out with eight additional significantly polymorphic SSR markers (Table 2, Figure 2) in the interested region adopted from the GRAMENE database. Twenty-five, 22, 12, seven, and seven recombinants derived from RM223 were identified at the marker loci RM284, RM556, RM80, RM3845, and RM3452, respectively, towards the centromere; seven, seven, and six recombinants derived from RM502 were identified at the marker loci RM23478, RM5493, and RM1345, respectively, towards the telomere. The locus was defined further by the markers RM3452 and RM1345 with genetic distances of 3.2 and 2.7 cM, respectively (Figure 2A). Because no major *R* gene had been identified previously in this region, the novel *R* gene in YJ2 was tentatively designated as *pi*55(t).

To further narrow down the location of the gene locus, the third round of linkage analysis was carried out with two SSR and 12 STS markers, which were developed in the smaller region defined with the second-round markers (Table 2). The recombinants derived from RM3452 on the centromere side were detected at the H8, H25, H56, H64, H33, H34, H35 and H2 loci; and those derived from RM1345 on the telomere side were detected at the H66, H49, H41, H42, H44, and H17 loci. Thus, the *pi55*(t) locus was closely flanked by H2 and H66 with genetic distances of 0.5 and 1.4 cM, respectively (Figure 2A).

### 2.6 The physical position and candidates of *pi55*(t)

To physically map the *pi55*(t) locus, all anchor markers

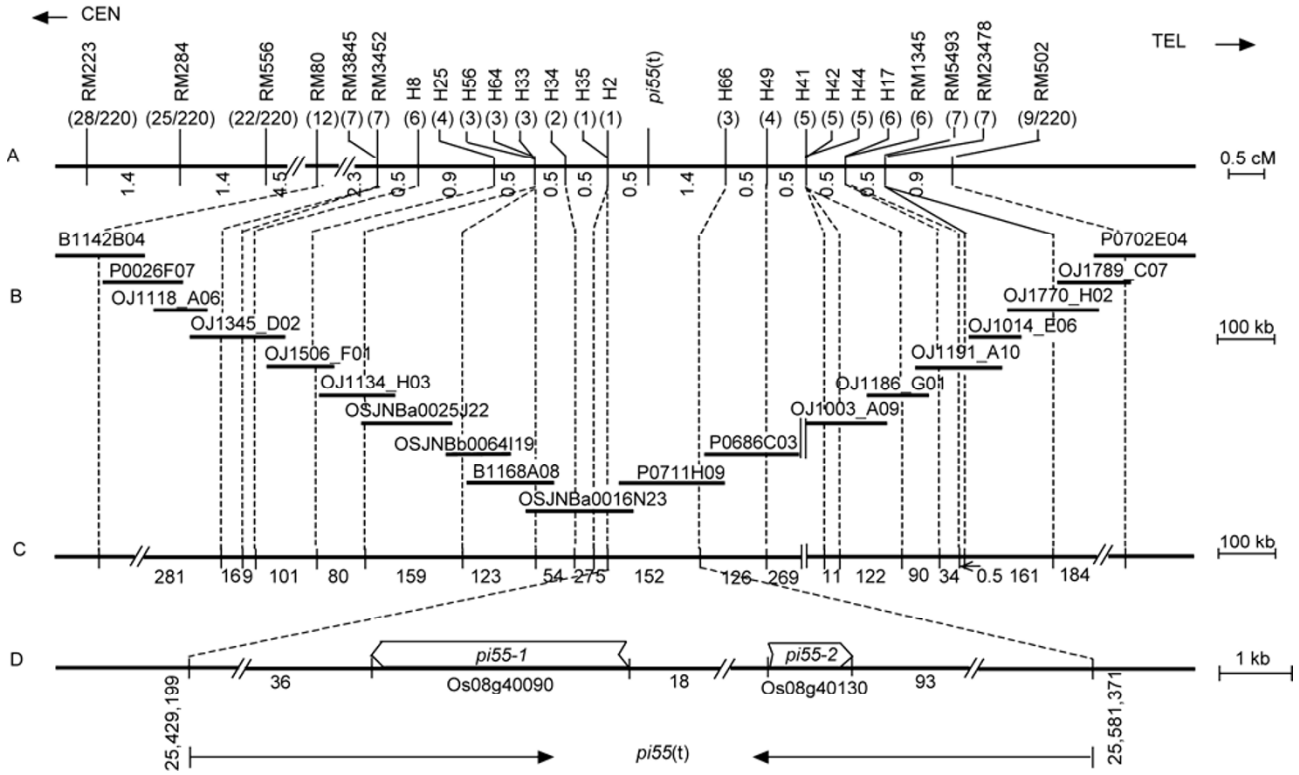


**Figure 2** Genetic and physical maps of the *pi55*(t) locus. A, Genetic map. The numbers below the map are relative genetic distances in cM and the corresponding markers are listed above the map. The fraction numbers in parentheses are the numbers of recombinants/gametes, and constant numbers in parentheses are the numbers of recombinants, which were detected in the interval between the adjacent marker loci including *pi55*(t). B, Contig map. The short horizontal lines represent BAC/PAC clones of the reference cv. Nipponbare, which were released by the IRGSP and anchored by the corresponding markers linked to the *pi*55(t) locus. The dashed lines designate the relative positions of the corresponding markers. C, Physical map. The numbers below the map are relative physical distances (in kb) estimated based on the reference sequence of cv. Nipponbare. D, The most promising candidate genes for *pi*55(t), which were annotated based on the reference sequence of cv. Nipponbare in the target region by the RiceGAAS system (http://ricegass.dna.affrc.go.jp). CEN, centromere; TEL, telomere.

**Table 2** PCR-based markers related to the *pi55*(t) locus

| Marker[a)] | Type | Primer sequence (5′→3′)[b)] | Genome location (bp)[c)] | Annealing temp (°C)[d)] | Expected size (bp) |
|---|---|---|---|---|---|
| First round of linkage analysis (BSA) | | | | | |
| RM223 | SSR | F: GAGTGAGCTTGGGCTGAAAC | 20740818–20740837 | 58 | 185 |
| | | R: GAAGGCAAGTCTTGGCACTG | 20740965– 20740946 | | |
| RM502 | SSR | F: GCGATCGATGGCTACGAC | 26582836–26582853 | 58 | 265 |
| | | R: ACAACCCAACAAGAAGGACG | 26583100– 26583081 | | |
| Second round of linkage analysis | | | | | |
| RM284 | SSR | F: ATCTCTGATACTCCATCCATCC | 21233214–21233193 | 58 | 148 |
| | | R: CCTGTACGTTGATCCGAAGC | 21233067–21233086 | | |
| RM556 | SSR | F: ACTCCAAACCTCACTGCACC | 22430725–22430706 | 58 | 93 |
| | | R: TAGCACACTGAACAGCTGGC | 22430633–22430652 | | |
| RM80 | SSR | F: TTGAAGGCGCTGAAGGAG | 24569414–24569431 | 55 | 120 |
| | | R: CATCAACCTCGTCTTCACCG | 24569533–24569514 | | |
| RM3845 | SSR | F: AGCTCGATCTCCTCTCTAGACC | 24850879–24850858 | 56 | 211 |
| | | R: GCTTCAGCCTTCAGGTCAAC | 24850669–24850688 | | |
| RM3452 | SSR | F: GGCAGCCCATCAACTAGATC | 24867502– 24867521 | 58 | 190 |
| | | R: TTGCAAACCCTAGTCCAAGC | 24867691–24867672 | | |
| RM1345 | SSR | F: ACCACCACGCCATTAGAGAC | 26235635–26235654 | 55 | 124 |
| | | R: TGAGCATCCCGTGCTGTC | 26235758–26235741 | | |
| RM5493 | SSR | F: GACAAAACACAAAGCAGGAC | 26236354–26236335 | 58 | 201 |
| | | R: TAACAAACCAACCAACCAAG | 26236154–26236173 | | |
| RM23478 | SSR | F: CGACGCAGGGTTTAGATAGAGTGC | 26398076– 26398099 | 55 | 178 |
| | | R: GTTCTCGTTCCGATGGCTAGACG | 26398253–26398231 | | |
| Third round of linkage analysis | | | | | |
| H8 | STS | F: CAAGCACGCGGATATGGAT | 24877302–24877320 | 62 | 95 |
| | | R: GGGACGCTACTACCACTGACAT | 24877396–24877375 | | |
| H25 | STS | F: TGCCATTCATATAGGTTT | 24978580–24978597 | 55 | 197 |
| | | R: CTTCCCATGTTTGCTCCAGT | 24978776–24978757 | | |
| H56 | SSR | F: GTTCAGCACACACAACCATA | 25058979–25058998 | 55 | 290 |
| | | R: CCCATACATACACATCTCCA | 25059268–25059249 | | |
| H64 | STS | F: GCCCCACCATTTTTAGAT | 25218324–25218341 | 55 | 126 |
| | | R: GCGCTTACGTGGCAACTA | 25218449–25218432 | | |
| H33 | STS | F: GCAGGGAGGAAGCAAATCA | 25341460–25341478 | 60 | 180 |
| | | R: AAAACCATCGGCGTCAAAC | 25341639–25341621 | | |
| H34 | STS | F: TGCGAGCGTGATTTTAGGG | 25396171–25396189 | 55 | 160 |
| | | R: ATAGCACCCATAGTATTTAG | 25396330– 25396311 | | |
| H35 | STS | F: TGTCTCCCTAACCTTCTTGC | 25423634–25423653 | 55 | 176 |
| | | R: CTACCTCAATGTTTGCTACC | 25423809–25423790 | | |
| H2 | SSR | F: ATGACGACGACGGCGAGGAG | 25429199–25429218 | 60 | 89 |
| | | R: CTCCCGCTTCGCCCTGCTCT | 25429287–25429268 | | |
| H66 | STS | F: TTCTCTTCCCTTTGTATGC | 25581242–25581260 | 58 | 130 |
| | | R: TATCTTGCCTGGGCGACC | 25581371–25581354 | | |
| H49 | STS | F: CCCAATCTCTAACTGGTGCC | 25707876– 25707895 | 55 | 82 |
| | | R: CAGCGACATCTCTAAGTTGGC | 25707957–25707937 | | |
| H41 | STS | F: CTAAAGAATACCGTGTGCG | 25977546–25977564 | 56 | 128 |
| | | R: CATTTGGCACATCTCACG | 25977673–25977656 | | |
| H42 | STS | F: GAACAAAACACAGCATTAGC | 25989522–25989541 | 56 | 138 |
| | | R: AGCCTGACCCAGTTGTTGA | 25989659–25989641 | | |
| H44 | STS | F: GCGAGATGGGGAGATAGAT | 26111624–26111642 | 55 | 111 |
| | | R: CCGTATAGGAAAGTGAATCG | 26111734–26111715 | | |
| H17 | STS | F: GTTCTTCTTCCCTGCTGATG | 26201644–26201663 | 58 | 109 |
| | | R: CATCTCAGGATTCCACCAA | 26201752–26201734 | | |

a) Molecular markers were summarized based on three rounds of linkage analysis. Markers with prefix RM were adopted from the GRAMENE website (http://www.gramene.org), and those with H were developed in this study. b) F, forward; R, reverse. c) Locations of genetic markers in the reference genomic sequence of cv. Nipponbare were obtained from BLAST search (http://www.ncbi.nlm.mih.gov.blast). d) All PCR runs began with one cycle at 94°C for 3 min, followed by 35 cycles at 94°C for 30 s, 55–62°C for 45 s, and 72°C for 1–1.5 min; with a final extension at 72°C for 7 min. Amplicons were separated by electrophoresis on 6% and 8% polyacrylamide gels, respectively.

used in the chromosome walking to the locus were located on the reference genomic sequence of cv. Nipponbare by BLASTN analysis. The markers anchored with BAC or Pi artificial chromosome (PAC) clones of Nipponbare were assembled as a contig map by Pairwise BLAST analysis. There were 19 clones in the contig map that covered the *pi55*(t) locus (Figure 2B). Subsequently, a physical map spanning the *pi55*(t) locus was constructed, *in silico*, based on the contig map (Figure 2C). The locus flanked by H2 and H66 was located on the two overlapping clones, OSJNBa0016N23 and P0711H09, with a physical distance of about 152 kb (25,429,199–25,581,371 bp; Figure 2C). The reference sequence of this physical interval was subjected to gene annotation. A total of 26 potential genes were predicted in this region based on the reference genomic sequence of cv. Nipponbare (Table 3). Among these genes, Os08g40090, which contains a conserved leucine-rich repeat domain, and Os08g40130, which encodes a heavy-metal-binding domain, were considered as the most promising candidate genes for *pi55*, and were designated as *pi55-1* and *pi55-2* (Figure 2D), although other genes, such as Os08g40170, which encodes cyclin dependent kinase B2-1, and Os08g40200, which encodes a serine/threonine phosphatase family protein, could not be ruled out as potential candidates.

## 3 Discussion

*R* genes that convey broad-spectrum and durable resistance are essential for modern breeding programs. The results from the present research showed that the elite rice cv. Yuejingsimiao 2 possessed high resistance to the *M. oryzae* populations collected from GD, SC, LN, and HLJ, which was better than the resistance shown by two well-known *R* gene donor cultivars, Sanhuangzhan2 and 28zhan. YJ2 is, therefore, an outstanding blast resistance *R* gene donor that is used in breeding programs in the above regions. Given that YJ2 has been authorized as an elite cultivar and is characterized by a high level of grain quality and yield, it could be amenable to breeding programs without the deteriorative genetic linkage effect derived from the native *R* gene donors such as Moroberekan, Tetep, Sanhuangzhan2, and 28zhan.

Numerous cultivars that express broad-spectrum and durable resistance against *M. oryzae* have been identified, such

**Table 3** Candidate genes for *pi55*(t) that were annotated in the target region (H2–H66)

| Number | Annotated gene[a)] | Predicted protein function | Amino acid | Most promising candidates |
|---|---|---|---|---|
| 1 | Os08g40010 | expressed protein | 398 | |
| 2 | Os08g40020 | selenium-binding protein | 513 | |
| 3 | Os08g40030 | cup-shaped cotyledon3 | 341 | |
| 4 | Os08g40040 | hypothetical protein | 269 | |
| 5 | Os08g40050 | retrotransposon protein, | 814 | |
| 6 | Os08g40060 | expressed protein | 174 | |
| 7 | Os08g40070 | hypothetical protein | 113 | |
| 8 | Os08g40080 | expressed protein | 120 | |
| 9 | Os08g40090 | Leucine-rich repeat-containing protein | 577 | *pi55-1* |
| 10 | Os08g40100 | expressed protein | 118 | |
| 11 | Os08g40110 | peptidase | 458 | |
| 12 | Os08g40120 | conserved hypothetical protein | 256 | |
| 13 | Os08g40130 | heavy-metal-associated domain-containing protein | 92 | *pi55-2* |
| 14 | Os08g40140 | geranylgeranyl transferase type-2 subunit beta | 320 | |
| 15 | Os08g40150 | AT hook motif domain-containing protein | 355 | |
| 16 | Os08g40160 | thylakoid lumen protein, chloroplast precursor | 243 | |
| 17 | Os08g40170 | cyclin-dependent kinase B2-1 | 327 | |
| 18 | Os08g40180 | 3-hydroxy-3-methylglutaryl-coenzyme A reductase | 562 | |
| 19 | Os08g40190 | expressed protein | 101 | |
| 20 | Os08g40200 | Ser/Thr protein phosphatase family protein | 429 | |
| 21 | Os08g40210 | expressed protein | 110 | |
| 22 | Os08g40220 | hypothetical protein | 80 | |
| 23 | Os08g40230 | expressed protein | 390 | |
| 24 | Os08g40240 | calvin cycle protein CP12 | 142 | |
| 25 | Os08g40250 | expressed protein | 1441 | |
| 26 | Os08g40260 | OsSPL15-SBP-box gene family member | 1141 | |

a) Candidate genes were annotated based on the reference genomic sequence of cv. Nipponbare by the Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/) and RiceGAAS (http://ricegass.dna.affrc.go.jp).

as Moroberekan, Tetep, IR64, Maowangu, Sanhuangzhan2, Gumei2, Xiangzi3150, and Zhe733, all of which contain more than two *R* genes. Wang *et al.* [28] identified two major genes, *Pi5* and *Pi*7, in the African cv. Moroberekan, but its durable resistance was believed to be controlled by both major genes in combination with 10 QTLs. Liu *et al*. [29] found that three major *R* genes (*Pi-GD-1*, *Pi-GD-2*, and *Pi-GD-3*) and five QTLs were responsible for the broad-spectrum blast resistance in cv. Sanhuangzhan2. In addition, Lee *et al.* [12] identified that two major *R* genes, *Pi42*(t) and *Pi43*(t), were involved in the durable blast resistance of cv. Zhe733. In the present study, three well-known dominant *R* genes and one novel recessive gene, *pi55*, were identified in YJ2. It is noteworthy to understand whether a combination of dominant and recessive *R* genes would be a superior means of achieving high resistance to a dynamic pathogen population.

Various populations, such as near isogenic lines (NIL), recombinant inbred lines (RIL), doubled haploid lines and $F_2$ populations, are used for gene analysis and mapping. The $F_2$ population is commonly used for blast *R* gene mapping, as half of the 85 major *R* genes have been identified in $F_2$ populations. For example, Yang *et al*. [30] identified the major *R* gene *Pi41* in cv 93-11 using a $F_2$ population. Similarly, Kumar *et al.* [31] mapped the major *R* gene *Pi42* in cv. DHR9. For cultivars that carry more than two *R* genes, it is preferable to use advanced populations such as NIL and RIL populations, as well as monogenic $F_3$ and $F_4$ lines. For example, Yu *et al.* [32] constructed a NIL population with cv. CO39 and tagged the blast *R* genes *Pi2* and *Pi4* on chromosomes 6 and 12, respectively. Huang *et al*. [33] identified two major *R* genes, *Pi47* and *Pi48*, in cv. Xiangzi 3150 using an RIL population. Pan *et al.* [34] localized *Pi13* and *Pi14* on chromosomes 6 and 2 using four monogenic $F_3$ lines in cv. Maowangu. Fukuoka *et al*. [19,20] identified four blast QTLs in cv. Owarihatamochi using a $F_4$ population, and then isolated the most significant locus as *pi21* using a backcross population. In the present research, four major *R* genes were identified rapidly in YJ2 by BSA analysis with four $F_4$ lines derived from a cross between YJ2 and Tsuyuake. The novel recessive gene *pi55*(t) was finely mapped on chromosome 8 by three rounds of linkage analysis. Collectively, these results demonstrated that the complex *R* gene constitution of a cultivar could be dissected when these genes were separately segregated into each $F_3$ or $F_4$ population.

On the basis of the structural characteristics of encoded proteins, over 70 *R* genes have been cloned in plants, which can be divided roughly into four categories: nucleotide binding site—leucine-rich repeat, receptor-like kinase, leucine-rich repeat—transmembrane domain, and transmembrane domain—coil coiled proteins [35]. With regard to three recessive genes, *pi21*, *xa5*, and *xa13*, isolated in rice, each encodes novel types of proteins. The bacterial blight *R* genes *xa5* and *xal3* encode a transcriptional factor TFIIAγ [36], and a protein that is homologous to the nodules gene *MiN3* [37], respectively. In addition, the blast *R* 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, and has lost 18 and 42 bp segments in the proline-rich region, which might lead to the slow-blast development in the resistant cultivar [20]. It was considered to represent a novel mechanism underlying blast resistance in rice. In the present study, a total of 26 candidate genes were predicted in the target region, of which two are suggested as the most promising candidates for *pi55*(t). One is Os08g40090, which encodes a leucine-rich repeat-containing protein, and the other is Os08s40130, which encodes a protein similar to *pi21*. Notably, *pi21* imparts partial resistance, and *pi*55(t) conveys full resistance, against *M. oryzae*. Further research is focused on isolation of the *pi*55(t) gene, which will help to elucidate the functional difference between *pi55*(t) and *pi21*.

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