Exploring the Distribution of Blast Resistance Alleles at the *Pi2/9* Locus in Major Rice-Producing Areas of China by a Novel Indel Marker

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Abstract

Rice blast disease caused by the fungus *Magnaporthe oryzae* damages cereal crops and poses a high risk to rice production around the world. Currently, planting cultivars with resistance (*R*) genes is still the most environment-friendly approach to control this disease. Effective identification of *R* genes existing in diverse rice cultivars is important for understanding the distribution of *R* genes and predicting their contribution to resistance against blast isolates in regional breeding. Here, we developed a new insertion/deletion (InDel) marker, *Pigm/2/9InDel*, that can differentiate the cloned *R* genes (*Pigm*, *Pi9*, and *Pi2/Piz-t*) at the *Pi2/9* locus. *Pigm/2/9InDel* combined with the marker *Pi2-LRR* for *Pi2* was applied to determine the distribution of these four *R* genes among 905 rice varieties, most of which were collected from the major rice-producing

regions in China. In brief, nine *Pigm*-containing varieties from Fujian and Guangdong provinces were identified. All of the 62 *Pi2*-containing varieties were collected from Guangdong, and 60 varieties containing *Piz-t* were from seven provinces. However, *Pi9* was not found in any of the Chinese varieties. The newly identified varieties carrying the *Pi2/9* alleles were further subjected to inoculation tests with regional blast isolates and field trials. Our results indicate that *Pigm* and *Pi2* alleles have been introgressed for blast resistance breeding mainly in the Fujian and Guangdong region, and *Pi9* is a valuable blast resistance resource to be introduced into China.

Keywords: blast disease, InDel marker, *Pigm*, *Pi9*, *Pi2*, *Piz-t*, rice

Rice is a staple food for more than half of the world's population, making it one of the most important cereal crops. Rice blast disease caused by the ascomycete fungus *Magnaporthe oryzae* (T. T. Hebert) M. E. Barr (syn. *Pyricularia oryzae* Sacc.) is one of the most devastating diseases threatening rice production worldwide. Breeding rice cultivars with resistance (*R*) genes has been widely recognized as the most effective and environmentally friendly approach to prevent and control blast disease (Dodds and Rathjen 2010). The genetic structure of the rice blast fungus varies in geographical populations, which leads to regionally specific virulence (Wu et al. 2005). Therefore, identification of *R* genes existing in diverse rice cultivars is important for estimating the major genetic resources resistant against *M. oryzae* races in rice-producing areas (RoyChowdhury et al. 2012).

More than 100 blast *R* genes have been identified in rice, of which at least 25 have been cloned (Deng et al. 2017; Liu et al. 2013). The *Pi2/9* locus is a cluster of tandem repeats of nucleotide binding site (NBS) leucine-rich repeat (LRR) genes on the short arm of chromosome 6, and this locus contains at least nine blast *R* genes. Four

functional alleles (*Pi2*, *Piz-t*, *Pi9*, and *Pigm*) of this locus have been cloned, all of which encode NBS-LRR proteins (Deng et al. 2017; Qu et al. 2006; Zhou et al. 2006). The resistance gene *Pi2* was originally introgressed from a resistant *indica* cultivar into cultivar Co39, and its isogenic line C101A51 displayed broad-spectrum blast resistance (Chen et al. 1996; Mackill and Bonman 1992). *Piz-t* was cloned based on the *Pi2* sequence, which is a functional allele of *Pi2* in rice cultivar Toride 1 (Zhou et al. 2006). *Piz-t* and *Pi2* alleles have high similarity of DNA sequences. *Pi9* was first introduced into cultivated rice from the wild rice species *Oryza minuta*, conferring a broader spectrum of blast resistance than that of *Pi2* in the Philippines (Liu et al. 2002). In 2017, the resistance gene *Pigm* was identified from Gumei 4, an indigenous Chinese rice variety that has been used as a blast resistance donor for rice breeding in China for >50 years (Deng et al. 2017). Because the *Pi2/9* locus contains multiple broad-spectrum *R* genes, it has been considered as one of the most important loci for resistance against blast disease worldwide (Jiang et al. 2012a; Zhu et al. 2012).

Because new *R* genes against blast disease are continuously cloned and introgressed in rice cultivars, DNA markers based on the polymorphism of genomic sequence linked to them have been widely developed and used in breeding (Fu et al. 2012; Hayashi et al. 2006; Jiang et al. 2012b; Khanna et al. 2015). The utilization of marker-assisted selection (MAS) provides an efficient method for directly identifying the introduced *R* genes controlling resistance phenotypes in population, which overcomes the limitations of conventional breeding. *Pita* and *Pib* are the two earliest cloned *R* genes against blast disease (Bryan et al. 2000; Wang et al. 1999). Development of the DNA markers linked to or from portions of them aids in selecting *Pita* and *Pib* rapidly and accurately in rice varieties, providing resistant resources for rice breeding (Jia et al. 2002, 2004; Koide et al. 2010; Liu et al. 2008; Wang et al. 2007; Zhang et al. 2013). DNA markers used for distinguishing polymorphisms belonging to functional and nonfunctional alleles at the *Pi2/9* locus have also been developed (Chen et al. 2004; Jiang et al. 2015; Scheuermann and Jia 2016; Wang et al. 2019; Yin et al. 2011). However, with *Pigm*

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cloned, it still lacks an effective molecular marker to efficiently differentiate multiple functional alleles at the *Pi2/9* locus.

In this study, we developed a novel PCR-based insertion/deletion (InDel) marker for distinguishing *Pigm*, *Pi9*, *Pi2/Piz-t*, and nonfunctional alleles named *Pigm/2/9InDel* and used it to determine the distribution of these four *R* genes in 905 rice varieties mainly collected from the major rice-producing provinces in China, such as Guangdong, Fujian, Jiangsu, and Zhejiang, and the International Rice Research Institute (IRRI) in the Philippines. Nine, 62, and 60 Chinese varieties carrying *Pigm*, *Pi2*, and *Piz-t*, respectively, were identified, and the only variety carrying *Pi9* found in this study was IR0436 from the IRRI. Furthermore, inoculation tests with the blast isolates from Guangdong and Fujian showed that *Pigm*, *Pi2*, and *Pi9* are the effective *R* genes in this area.

Materials and Methods

Plant materials and *M. oryzae* isolates. Rice blast *R* gene donors C101A51 (carrying *Pi2*) and cultivar 75-1-127 (carrying *Pi9*) were provided by Dr. Guo-Liang Wang (The Ohio State University), and Gumei 4 (carrying *Pigm*), Toride 1 (carrying *Piz-t*), and the 905 rice varieties were stored at the Fujian Provincial Key Laboratory of Genetic Engineering for Agriculture, Fujian Academy of Agricultural Sciences, Fuzhou, China. The *M. oryzae* isolates RB9, RB6, RB16, RB18, CHE86061, CHNOS60-2-3, KJ201, CHL2110, and CHL768 were provided by Dr. Guodong Lu (Fujian Agriculture and Forestry University) and Dr. Qinhua Pan (South China Agricultural University). FJ501-3 and FJ86 were isolated from naturally infected rice plants in a Fujian field.

Evaluation of disease reaction. The *M. oryzae* isolates were grown on oatmeal agar plates in the dark for approximately 5 to 7 days at 28°C, and then, they were transferred to a culture room under light for approximately 5 to 7 days at room temperature for sporulation. Rice seedlings were grown in a humidity-controlled greenhouse for about 3 weeks (three to four leaves) under natural conditions. Seedlings were spray inoculated with spores of *M. oryzae* isolates with a concentration of 5×10^5 spores per milliliter. After inoculation, seedlings were maintained in the dark for 24 h at 28°C and then, kept under high humidity for about 5 to 7 days to evaluate their symptoms.

The 0 to 5 standard scale was used to score the disease reaction as previously described (Campbell et al. 2004); briefly, “0” indicates no evidence of infection, “1” indicates brown speckling (<1 mm), “2” indicates brown specks (1 to 2 mm), “3” indicates round to elliptical lesions (2 to 4 mm) with gray center and brown margins, “4” indicates spindle-shaped lesions with necrotic centers capable of sporulation, and “5” indicates coalesced type 4 lesions that have killed the majority of the leaf blade. Plants with lesion scores from 0 to 2 were considered to be resistant. Plants with lesion scores 3, 4, and 5 were considered as moderately resistant, susceptible, and highly susceptible, respectively. For the field disease evaluation, varieties were grown at the station for testing blast resistance in Shanghang, Fujian, China, in 2017. Disease evaluation was carried out on leaf and spike of seeding and maturing stages, respectively.

DNA extraction. Leaf tissues from rice seedlings were collected for DNA extraction. About 100 mg of young leaves were collected in Eppendorf tubes and frozen in liquid nitrogen. The samples were ground into fine powder with a TissueLyser (Qiagen, Haan, Germany), and genomic DNA was extracted from ground tissues using the cetyltrimethyl ammonium bromide method (Murray and Thompson 1980).

Marker development. The genomic sequences of the *Pi2/9* locus in the rice varieties C101A51 (DQ352453), 75-1-127 (DQ285630), Toride 1 (DQ352040), Gumei4 (KU905633.2), and Nipponbare (DQ454158) were retrieved from GenBank (<https://www.ncbi.nlm.nih.gov/>), and they were aligned using Clustal (Larkin et al. 2007). The PCR was performed in 25 µl, and the mixture contained 12.5 µl of 2× Mix buffer (Mg²⁺ Plus), 1 µl of each primer (10 mM), and 0.2 µl Taqase (5 U/ml). Approximately 20 to 50 ng of genomic DNA was added in a total volume of 25 µl for PCR analysis. PCR was performed with the following thermal profile: initial

denaturation at 94°C for 5 min followed by 32 cycles of denaturation at 94°C for 30 s and 60°C for 30 s and extension at 72°C for 30 s and a final extension at 72°C for 10 min. The PCR products were separated by 3% agarose gels in 1× Tris-acetate-EDTA (TAE) electrophoresis buffer. Electrophoresis was carried out at 150 to 200 mA for 45 min, and images were taken using a FluorChem SP gel imaging system (Alpha Innotech, San Leandro, CA).

Validation of the *Pigm/2/9InDel* linkage to the *R* genes in *F*₂ populations. The cultivars GufengB (*Pigm*) (Deng et al. 2017), Hui316 (susceptible), and Minghui86 (*Piz-t*) (Tian et al. 2016) maintained at the Fujian Provincial Key Laboratory of Genetic Engineering for Agriculture, Fujian Academy of Agricultural Sciences were used to make crosses with Nipponbare (susceptible), 75-1-127 (*Pi9*), and Nipponbare, respectively, and develop *F*₂ populations. The *F*₂ populations were genotyped with the *Pigm/2/9InDel*. The segregations of the *R* genes in the *F*₂ populations indicated by the *Pigm/2/9InDel* marker are shown in Supplementary Fig. S1.

Results

Development and validation of marker *Pigm/2/9InDel*. We previously developed the InDel marker *Pi9-Pro*, which can differentiate three different genotypes corresponding to the *Pi9*, *Pi2/Piz-t*, and non-*Pi2/Piz-t/Pi9* alleles (Tian et al. 2016). However, when *Pi9-Pro* was subjected to identify the *Pigm* allele (Gumei 4), it produced an amplified fragment with similar size of the fragments from nonfunctional alleles (Nipponbare and 9311) (Fig. 1A). Therefore, *Pi9-Pro* is not effective in differentiating the *Pigm* allele. To develop an efficient DNA marker for distinguishing the functional alleles at the *Pi2/9* locus, we first analyzed genomic sequences of this locus in Gumei 4 (carrying *Pigm*), 75-1-127 (carrying *Pi9*), C101A51 (carrying *Pi2*), and Toride 1 (carrying *Piz-t*) (Fig. 1B). A primer pair, called *Pigm/2/9InDel*, was designed to target three positions at the *Pigm* locus, which produced a 164-bp amplified fragment within *Pigm-R* and *Nbs4* tandem repeat and a 132-bp fragment at the downstream of *Nbs2* (Fig. 1C). When *Pigm/2/9InDel* was used to amplify *Pi9*, *Pi2*, and *Piz-t* donors, only one 132-bp band was amplified at the downstream of *Pi9* (*Nbs2*) and *Nbs5* tandem repeat in 75-1-127; two PCR amplicons with similar sizes of 166 and 165 bp were produced within *Pi2/Piz-t* (*Nbs4*) and the *Nbs2* tandem repeat, respectively, in both C101A51 and Toride 1 (Fig. 1C and D). For the nonfunctional alleles from Nipponbare and 9311, *Pigm/2/9InDel* produced bands with similar sizes of >1,000 bp.

To validate the linkage of the *Pigm/2/9InDel* marker with *Pigm*, *Pi9*, and *Pi2/Piz-t* alleles, 168, 144, and 140 plants were randomly selected from the *F*₂ populations derived from crosses of GufengB (*Pigm*) with Nipponbare, 75-1-127 (*Pi9*) with Hui316, and Minghui86 (*Piz-t*) with Nipponbare, respectively. The genotype analysis revealed that the ratios of *Pigm*-, *Pi9*-, and *Piz-t*-containing plants to non-*Pigm/Pi9/Piz-t* alleles-containing plants were 121:47 ($\chi^2 = 0.536$), 111:33 ($\chi^2 = 0.679$), and 100:40 ($\chi^2 = 0.500$), respectively, all of which fit the 3:1 ratio (Supplementary Fig. S1). These results indicated that *Pigm/2/9InDel* is effective in differentiating the *Pigm*, *Pi9*, *Pi2/Piz-t*, and nonfunctional alleles at the *Pi2/9* locus.

Distribution of functional *Pi2/9* alleles in the varieties from major rice-producing areas of China. We have developed the cleaved amplified polymorphic sequence marker *Pi2-LRR* to differentiate the *Pi2* allele from the non-*Pi2* alleles (Tian et al. 2016). Therefore, *Pigm/2/9InDel* and *Pi2-LRR* were used in combination to investigate the distribution of the functional *Pi2/9* alleles in Chinese rice varieties. A set of 905 rice varieties included 681 collected from the major rice-producing areas in China, among which 195 were from Guangdong, 151 were from Fujian, 74 were from Zhejiang, 73 were from Jiangsu, 36 were from Hunan, 34 were from Sichuan, 22 were from Yunnan, 19 were from Guangxi, and 77 were from other provinces of China; 119 were from the IRRI; and 105 were from other or unknown areas (Supplementary Table S1). These materials were genotyped with the *Pigm/2/9InDel* and *Pi2-LRR* markers to identify the resistance alleles of the *Pi2/9* locus (partial results were shown in Fig. 2A and C). The results indicated that, among

the tested varieties, 9, 62, and 60 had the *Pigm*, *Pi2*, and *Piz-t* functional alleles, respectively (Table 1). To confirm the results by *Pigm*/2/9InDel, the *Pi9*-Pro marker was used to determine the genotypes of the above functional alleles-containing varieties. The presence of *Pi9* or *Pi2*/*Piz-t* alleles identified by *Pi9*-Pro was consistent with that identified by *Pigm*/2/9InDel (Fig. 2B). Furthermore, all of the varieties carrying *Pigm*, *Pi9*, *Pi2*, or *Piz-t* were tested with the developed molecular markers *Pigm*-4, *Pi9*SNP, *Pi2*SNP, or *Pizt*-PA (Hua et al. 2015; Zeng et al. 2018). As shown in Supplementary Figure S2, the identification results by *Pigm*/2/9InDel and *Pi2*-LRR were consistent with those by *Pigm*-4, *Pi9*SNP, *Pi2*SNP, and *Pizt*-PA, which proves the effectiveness of *Pigm*/2/9InDel in identifying the functional alleles at the *Pi2/9* locus.

Interestingly, it was found that the *Pigm* allele was carried in the varieties collected from both Fujian and Guangdong but absent in varieties from other provinces, whereas all of the 62 varieties carrying the *Pi2* allele were from Guangdong (Fig. 3). The

60 varieties carrying *Piz-t* were from various race-planting areas. *Pi9* was only identified in IR0436-1 from the IRRI, but it was not found in any of the tested Chinese varieties (Fig. 3). Therefore, our results indicated that *Pigm*/2/9InDel is an efficient marker for screening rice materials with or without functional alleles of the *Pi2/9* locus.

Determining resistance of the varieties carrying the functional *Pi2/9* alleles against diverse regional rice blast isolates. To investigate the effectiveness of *Pigm*, *Pi2*, and *Pizt* alleles identified by *Pigm*/2/9InDel, we selected five *Pi2*-carrying varieties (BL123, Huazhan, Xin 1221, Xin 1223, and Guangchao 128) collected from Guangdong province, four *Pigm*-carrying varieties (Minbeiwaxian, Fuyi A, Guangkang 13A, and Gufeng A) from Fujian province, and three *Piz-t*-carrying varieties (Chenghui 707, Hang 1, and Minghui 86) to be inoculated with isolates KJ201 and CHE86061, which are avirulent to the functional alleles of the *Pi2/9* locus (Zhou et al. 2006). The varieties with nonfunctional alleles (CO39, Nipponbare,

Fig. 1. Development of the insertion/deletion (InDel) marker *Pigm*/2/9InDel for differentiating functional alleles at the *Pi2/9* locus. **A**, Identification of *Pigm*, *Pi9*, *Pi2*, and *Piz-t* alleles in Gumei 4, 75-1-127, C101A51, and cultivar Toride-1, respectively, with the *Pi9*-Pro marker, which could not distinguish *Pigm* from the nonfunctional alleles in Nipponbare and 9311. **B**, *Pigm*/2/9InDel primers are designed for targeting the genomic variation of the *Pi2/9* locus in Gumei 4, 75-1-127, C101A51, and Toride-1. **C**, Physical location of the identified InDel region by *Pigm*/2/9InDel and sizes of its amplicons at the *Pi2/9* locus. **D**, Identification of the functional alleles with the *Pigm*/2/9InDel marker. M, DNA ladder. LTR, long terminal repeat.

and Lijiangxintuanheigu [LTH]) were included as controls. Our results indicated that all of these identified varieties showed resistance against the two isolates, whereas Co39, Nipponbare, and LTH were susceptible (Table 2).

To further determine resistance of these functional alleles-containing varieties to regional blast isolates, we inoculated them with four Fujian isolates (FJ501-3, FJ86, RB18, and RB16) and four Guangdong isolates (CHL2110, CHL768, RB9, and RB6). In addition, evaluation of blast disease reactions in the field was also carried out by growing these varieties at the station for testing blast resistance in Shanghang, Fujian, China, in 2017. The results showed that all of the four *Pigm*-carrying Fujian varieties and five *Pi2*-carrying Guangdong varieties were highly resistant to the Fujian and Guangdong isolates, respectively (Table 2). However, variety Huazhan from Guangdong was susceptible to the Fujian isolate FJ86. Varieties Gumei 4 and Gufeng A from Fujian were moderately resistant and susceptible to Guangdong isolates RB9 and CHL2110, respectively, and variety Fuyi A displayed moderate resistance and susceptibility to Guangdong isolates RB9 and RB6, respectively (Table 2). It was also found that 75-1-127 carrying *Pi9* was resistant to all of the Fujian and Guangdong isolates except CHL2110. Furthermore, the results of blast evaluation in the Shanghang field by natural infection indicated that all of the varieties carrying *Pigm*, *Pi2*, or *Pi9* displayed high resistance except Huazhan, Fuyi A, and Gufeng A, which were moderately resistant (Table 2). However, the four varieties carrying the *Piz-t* allele displayed susceptibility to most Fujian and Guangdong isolates and in the field trial (Table 2), suggesting that *Piz-t* almost lost its resistance to *M. oryzae* in these provinces. Our results suggested that different functional alleles at the *Pi2/9* locus could contribute to the specific resistance to regional *M. oryzae* isolates.

Discussion

It is widely accepted that the most effective and environmentally friendly method to control blast disease is pyramiding *R* genes into rice cultivars through breeding. However, the traditional phenotype-based rice breeding strategy is inefficient and may be inaccurate in screening the desired resistant progeny under field conditions for the reason that disease reaction and severity levels are affected by many environmental factors. Development of molecular markers linked to the desired traits can improve the efficiency and accuracy of conventional resistance breeding. With increased numbers of cloned *R* genes, gene-specific markers can be developed from portions of the gene itself for MAS, and MAS has already been proven to be very helpful in rice blast resistance breeding because the blast-resistant phenotypes are usually controlled by a single or few genes (Srivastava et al. 2017). However, to control rice blast disease, it is

also important to identify the *R* genes that are carried by diverse cultivars and determine the effective *R* genes against *M. oryzae* isolates.

The *R* genes at the *Pi2/9* locus, especially *Pi2*, *Pi9*, and *Pigm*, have provided rice cultivars with broad-spectrum resistance against blast disease in the main rice-producing areas (Chen et al. 2001; Deng et al. 2017; Du et al. 2015; Li et al. 2005; Yang et al. 2008). A series of markers has been developed by differentiating the sequence polymorphism of functional alleles at this locus. For example, Hua et al. (2015) developed *Pi9*SNP, *Pi2*SNP, and *Piz*-PA markers by utilizing the sequence differences among *Pi9*, *Pi2*, and *Piz-t*, and these

Table 1. Summary of the collected varieties carrying *Pigm*, *Pi9*, *Pi2*, and *Piz-t* alleles

Alleles	Varieties
<i>Pi2</i> (62)	Aihuasizhan, BL123, Guangchao 128, Fengerzhan, Fengruanzhan, Fengsizhan, Fengtaismiao, Fengtaizhan, Fengxianzhan, Fengxiusimiao, Fengyuezhao, Guangchao 1222, Guangjinruanzhan, Guangyinzhan, Guangyuanzhan12, Gujinzhan, Helisimiao, Huahang 52, Huahangyin, Huangguangsimiao, Huangguangxinzhao, Huangguangzhan, Huangsizhan, Huangxiusimiao, Huaxiaruanzhan, Huazhan, Jinhuaruanzhan, Lifengzhan 1, Lingnansimiao, Meiyazhan, Meiyouzhan, Moliruanzhan, Molisimiao, Qifozhan, Tianjinruanzhan, Wushanfengzhan, Wushanhuadao, Wushanlizhan, Wushanyouzhao, Wuyuezhao 2, Xin 1221, Xin 1223, Xiaolixiangzhan, Xinsimiao, Xinyuanzhan, Yinfengyouzhan, Youzhan 13, Yefengzhan, Yueguangsimiao, YueJinyouzhan, Yuekang 1221, Yuekang 1222, Yuekang 1223, Yuekang 1224, Yuenongsimiao, Yuetaiyouzhan, Yueyinzhan, Yueyouxinzhao, Yuezhenzhan, Yushansizhan, Zaonantonggu, Zhongguangsimiao 5.
<i>Piz-t</i> (60)	Haobuka, Haomake(K), Jiabala, Jiazao 935, Fengxinzhao, Hang 1hao, Guisixiangnuo, Duohui 43, Minghui 86, Zhi 07, Keyou 80, Zhi 05W, Hui 68, Shuiyuan 290, Liaodong 218, FSJ010, R1259, GIZA 176, Jiazao 1hao, Jiahua 1, Xiushui09, Xiushui 128, Xiushui 110, Chunjiang 03, Chunjiang 101, Chunjiang 104, Jia 66, Xiushui 33, Xianghu30, Zhejing 41, Yongnuo 34, Ning 88, Xiushui 134, Xiushui 321, Xiushui 05, Xiushui 03, Chunjiang 110, Tai 400, Jia 58, Xiushui 123, Jia 203, Qifanzhan, Guisixiangnuo, IR0429-1, IR0432-1, Gangannuo, IR0434, IR0435, IR0445, IR0446, IR04554, IR04555, IR04556, IR04557, IR0467, IR4774-295-2-3, IR73712-68-3-1-2, IR72164-368-6-2-2-2, IR1317, IR59552-63-2-3.
<i>Pigm</i> (9)	Anfeng A, Fuyi A, Guangkang 13A, Meibeixianxian, Fengxiangsimiao 2, Hefengsimiao, Lifengzhan, Yuefengxinzhao, Yuetazaozhan.
<i>Pi9</i> (1)	IR0436-1

Fig. 2. Identification of the functional alleles at the *Pi2/9* locus in collected varieties with the insertion/deletion (InDel) marker *Pigm/2/9InDel*, the *Pi9*-Pro marker, and the *Pi2*-LRR marker. The PCR amplification patterns with **A**, *Pigm/2/9InDel*, **B**, *Pi9*-Pro, and **C**, *Pi2*-LRR. The analyzed varieties are as follows: 1, Anfeng A; 2, Fengxiangsimiao 2; 3, Fuyi A; 4, Guangkang 13A; 5, Hefengsimiao; 6, Lifengzhan; 7, Meibeixianxian; 8, Yuefengxinzhao; 9, Yuetazaozhan; 10, IR0436-1; 11, Aihuasizhan; 12, Guangchao 128; 13, Fengerzhan; 14, Fengruanzhan; 15, Fengsizhan; 16, Haobuka; 17, Haomake (K); 18, Jiabala; 19, Jiazao 935; 20, Fengxinzhao. M, DNA ladder.

markers were used to determine the presence and absence of the *R* genes in 101 rice varieties. In 2019, Wang et al. (2019) designed the marker GMR-3 to identify the *PigmR* variant in rice varieties. However, none of these markers can individually distinguish multiple functional alleles. Although *Pi9-Pro* is able to differentiate the *Pi9*, *Pi2/Piz-t*, and non-*Pi2/Piz-t/Pi9* alleles, it is not effective in distinguishing the cloned *Pigm* from the nonfunctional alleles. *Pigm/2/9InDel* developed in this study not only differentiates *Pigm*, *Pi9*, *Pi2/Piz-t*, and the nonfunctional alleles but also, separates the target alleles in F_2 populations, making it more efficient in MAS for varieties with the resistance alleles at the *Pi2/9* locus.

Among the 905 germplasms tested in this study, all 62 varieties carrying *Pi2* were collected from Guangdong province, and 9 varieties carrying *Pigm* were collected from both Fujian and Guangdong provinces. No Chinese variety contains the *Pi9* allele. In contrast, varieties from a wide range of rice-producing areas carry *Piz-t*. Most of the tested varieties carrying *Pi2* displayed high resistance to Fujian isolates and in the field trial, whereas this allele was found in few Fujian varieties, suggesting that *Pi2* is a potential resistance resource to be utilized in Fujian province. However, nearly all of the varieties from Guangdong that carry the *Pi2* or *Pigm* alleles were developed in

recent years, implying that these two *R* genes have been intentionally used in the breeding programs in this area.

Previous studies showed that *Pi2* and *Pi9* conferred high resistance against rice blast isolates from Guangdong, Fujian, Guizhou, and Hunan (Pan et al. 2008; Yang et al. 2008; Zhu et al. 2004). However, *Pi2* has become less effective after cultivars with the gene have grown over the years in Guangdong along with the evolution of *M. oryzae* population (Ling et al. 1989; Wang et al. 2012; Yang et al. 2008; Zhu et al. 2004, 2008). Likewise, *Piz-t* was first used to develop cultivars resistant to rice blast in the 1970s, and the germplasms carrying *Piz-t* have gradually lost their resistance in many rice-producing areas (Lei et al. 2000; Yang et al. 2014). However, *Pi9* and *Pigm* have still shown broad-spectrum resistance to rice blast (Wu et al. 2015; Yang et al. 2008; Yu et al. 2013; Yuan et al. 2017), which is consistent with our results. Considering that *Pi9* is not widely present in Chinese rice varieties, it could be a valuable resistance resource for use in China. In addition, our results suggested that *Pi2* and *Pigm* are not widely distributed in the varieties grown in provinces other than Fujian and Guangdong. Therefore, these resistance alleles are still tenable for large-scale rice resistance breeding in China (Wang et al. 2017).

Fig. 3. Regional distribution of all of the varieties collected in this study. The numbers of *Pigm*-, *Pi9*-, *Pi2*-, and *Piz-t*-containing varieties are shown on the pie chart. IRRI, International Rice Research Institute.

Table 2. Disease incidence of the varieties containing *Pigm*, *Pi9*, *Pi2*, or *Piz-t* alleles inoculated with various isolates of *Magnaporthe oryzae* and in the field disease trial

Gene	Cultivar	Avirulent strains		Guangdong isolates				Fujian isolates				Shanghang (2017)
		KJ201	CHE86061	RB9	RB6	CHL2110	CHL768	RB18	RB16	FJ501-3	FJ86	
<i>Pi2</i>	C101A51	0	0	0	0	0	0	0	0	0	0	0
	BL123	0	0	0	0	0	0	0	0	0	0	0
	Huazhan	0	0	0	0	0	0	0	0	0	4	3
	Xin 1221	0	0	0	0	0	0	0	0	2	0	0
	Xin 1223	0	0	0	0	0	0	0	0	0	0	0
	Guangchao 128	0	0	0	1	0	0	0	0	0	0	0
<i>Pigm</i>	Gumei 4	0	0	3	0	0	0	0	0	0	0	0
	Minbeiwaxian	0	0	0	0	0	0	0	0	0	0	0
	Fuyi A	0	0	3	4	0	0	0	0	0	0	3
	Guangkang 13A	0	0	2	0	0	0	0	0	0	0	0
	Gufeng A	0	0	0	0	4	0	0	0	0	0	3
<i>Pi9</i>	75-1-127	0	0	0	0	4	0	0	2	0	0	0
<i>Piz-t</i>	IRBLzt-T	0	0	3	1	4	0	5	4	5	5	5
	Chenghui 707	0	0	0	2	0	0	1	2	1	3	3
	Hang 1	0	0	1	3	4	4	2	4	3	5	4
	Minghui86	2	1	2	3	0	0	1	4	4	5	5
Non-functional alleles	Co39	5	5	4	4	4	5	5	5	5	5	5
	Nipponbare	4	4	5	5	3	5	5	5	5	5	5
	LTH	5	5	5	5	3	5	5	5	5	5	5

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