



# Superior *japonica* rice variety YJ144 with improved rice blast resistance, yield, and quality achieved using molecular design and multiple breeding strategies

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**Abstract** Yanfeng 47 (YF47) is an elite *japonica* rice variety cultivated in China on nearly 2 million hectares over the past 20 years. However, YF47 is highly susceptible to rice blast (*Magnaporthe oryzae*), one of the most destructive rice diseases. In this study, we developed novel TPAP (tetra-primer ARMS-PCR) functional markers for the genes *Pita*,

*Pib*, and *Pid2*, all of which afford broad-spectrum resistance to blast. A collection of 91 *japonica* rice germplasms with similar ecological characteristics to YF47 were screened, and Wuyunjing 27 (WYJ27) with *Pita* and *Pib* alleles and P135 with the *Pid2* allele were identified. Furthermore, the corresponding positive *Pita*, *Pib*, and *Pid2* alleles were transferred into YF47 using single, mutual, and backcrosses, together with molecular marker-assisted selection (MAS) and anther culture technology. These genetic materials, carrying one, two, or three functional alleles, were generated within 3 years, and compared to YF47, they all showed improved resistance to naturally inoculated rice blast. Further improved lines (IL) 1 to 5 (all containing *Pita*, *Pib*, and *Pid2* alleles) were evaluated for yield performance, and when no fungicide was applied, all lines except IL-4 showed increased traits compared with those of YF47. IL-5, renamed Yanjing 144 (YJ144), showed yield increases in the Liaoning province regional variety comparison test and superior appearance quality compared to YF47. Our work provides a molecular design strategy for pyramiding multiple beneficial genes to rapidly improve rice blast resistance, yield, and quality using multiple breeding strategies.

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**Keywords** Rice blast resistance · Molecular design breeding · TPAP functional marker · Multiple genes pyramiding · Anther culture technology

## Introduction

Food security has become one of the world's most important problems (Seck et al. 2012), and crop production faces unprecedented challenges, including climate change and the outbreak of COVID-19. Rice is the primary food crop for over half the world's population (Pennisi, 2010), with close to 1.5 billion tons produced each year. However, extreme environmental conditions create biotic and abiotic stress that may severely affect production. Rice blast, caused by *Magnaporthe oryzae*, is one of the most destructive diseases affecting rice (Dean et al. 2012). Numerous studies have shown that it affects both yield and quality and yield loss estimates could reach 30–50% under temperature and humidity conditions favorable to outbreaks of *M. oryzae*. This is equivalent to destroying enough rice to feed more than 60 million people each year (Skamnioti and Gurr, 2009). From 2013 to 2017, the average annual area affected by rice blast in China was 75 million ha (<https://www.natesc.org.cn>, accessed 17 July 2020). The disease is broadly distributed and survives in a wide range of environmental conditions. Its predominant groups are complex and dynamic, which greatly increases the difficulty of prevention (Wang et al. 2017).

Over long-term co-evolution, rice has developed multiple mechanisms to resist pathogens, including pathogen-associated molecular pattern (PAMP)-induced immune responses (PAMP-triggered immunity, PTI) and pathogenic effector proteins (effector-induced immune response mechanism immunity, ETI) (Akerley et al. 1995; Nakahara and Masuta, 2014). The widespread incorporation in modern rice varieties of multiple resistance genes with durable resistance to rice blast is recognized as the most effective and environment-friendly approach (Miah et al. 2013). Multiple rice blast resistance genes have been cloned successfully: *Pish*, *Pit*, *Pi37*, *Pib*, *Pi21*, *Pigm*, *Pi9*, *Piz-t*, *Pi2*, *Pid2*, *Pid3*, *Pi25*, *Pi36*, *Pi5/Pi3/Pi-i*, *Pi56*, *Pia*, *PiCO39*, *Pi1*, *Pik*, *Ptr*, *Pik-h/Pi54*, *Pik-p*, *Pik-m*, *pb1*, and *Pita* (Li et al. 2015; Zhao et al. 2018; Zhou et al. 2018). Based on their encoded protein structures and molecular functions, these cloned genes can be divided into four categories: (1) nucleotide binding site–leucine-rich repeat (NBS-LRR) proteins, including the above genes with the exception of *Pid2*, *Pi21*, and *Ptr*; (2) receptor-like

protein kinases, such as *Pid2*; (3) proline-rich domain proteins, including *Pi21*; (4) armadillo-rich repeat proteins, such as *Ptr*. Furthermore, most rice blast resistance genes have been identified and cloned in a dominant inheritance pattern, whereas only a small number function in a pattern of recessive inheritance, such as *Pi21* (Chen et al. 2006; Fukuoka et al. 2009). The identification, cloning, and deep understanding of the mechanisms of these blast resistance alleles have already provided an enriched gene bank that has been widely used in traditional and hybrid rice breeding.

Marker-assisted selection (MAS) is a rapid, accurate, and effective tool for identifying target genes (Feng et al. 2019). In addition, it is little affected by environmental conditions (Ye et al. 2001; Chen et al. 2013). Currently, MAS technology is widely used to improve rice yield, optimize quality and enhance resistance and permit multiple breakthrough achievements (Wang et al. 2005; Chen et al. 2018). To date, MAS has also been widely used to improve rice blast resistance. Single resistance genes, though, cannot effectively deal with multiple pathotype infections simultaneously; thus, multiple studies used polymerized rice blast genes to improve the blast resistance of susceptible varieties (Jiang et al. 2012; Zhao et al. 2018), including enhanced Jin 23B and Kongyu 131 through *Pi1* and *Pi2* pyramiding, pyramiding blast resistance genes *Piz5* and *Pi54* into the elite Basmati rice restorer line PRR 78, and breeding the high-yielding and durable resistance rice variety Longliangyou 3189 by using *Pigm* (Koide et al. 2010; Singh et al. 2013; Deng et al. 2017; Yang et al. 2019; Yin et al. 2021). However, it is challenging to pyramid multiple favorable genes because of the laborious molecular detection techniques and long breeding periods involved (Yang et al. 2019). Anther culture technology produces haploid plants from pollen microspores that can become diploid either naturally or via treatment with active mitotic substances. This technique could homogenize breeding materials within two generations of rice breeding (Lee et al. 2003; Zhao et al. 2015). It was estimated that more than 40 rice varieties in China between 1980 and 2020 were successfully generated using this approach. However, few studies have reported where the combination of MAS with anther culture has generated superior rice varieties.

Accurate and convenient molecular markers are the basis of MAS technology (Jeppe and Thomas, 2003). At present, alleles of the multiple resistance genes in rice are demonstrated by the diversity of single-nucleotide polymorphism (SNP); therefore, cost-effective identification of functional molecular markers for SNP diversity is crucial (Abdiel et al. 2021). For this, cleaved amplification polymorphism sequence-tagged sites (CAPS), sequence-related amplified polymorphism (SPAP), and kompetitive allele-specific PCR (KASP) have been developed (Abdiel et al. 2021; Zhang et al. 2021). Among these, KASP is new, low-cost, and convenient, but it needs more expensive instrument support (Zhang et al. 2021). The others depend on DNA sequencing or restriction enzyme digestion, which is time-consuming (Jeppe and Thomas, 2003; Zhang et al. 2021). Tetra-primer ARMS-PCR (TPAP) is a rapid genotyping method for SNP identification and has been used in SNP analyses from plant science to medical science (Ye et al. 2001; Chen et al. 2013). In its design, two outer primers are designed to amplify the positive control DNA fragment, and two inner primers each containing a mismatched nucleotide are developed corresponding to the SNP diversity (Ye et al. 2001); ultimately, only one PCR amplification could identify the SNP diversity.

YF47, an elite *japonica* rice variety approved for the Chinese provinces of Liaoning and Shandong in 2001 and 2009, respectively, has high yield and wide adaptability characteristics (<https://www.ricedata.cn/variety/varis/600002.htm>, accessed 21 July 2020). This variety also received national certification in 2006 and is cultivated on nearly 2 million hectares in the Chinese provinces of Liaoning, Hebei, Shandong, and Jiangsu (Lu et al. 2017). However, because YF47 is highly susceptible to rice blast disease (Liu et al. 2014; Li et al. 2017), improving resistance is important for ensuring food security in northern China. Pyramiding multiple blast resistance genes is an effective way to improve resistance (Jiang et al. 2012; Zhao et al. 2018). It was reported that *Pi2/Pi9/Pigm* allele clusters mainly occur in *indica* rice with increased rice blast resistance (Deng et al. 2017; Xie et al. 2019), however, in the northeast, *Pita* presents a great opportunity to improve rice blast resistance due to the corresponding avirulence gene *Avr-Pita*, which is widespread in the area (Wang et al. 2014,

2016; Xie et al. 2019). Furthermore, owing to the prevalence of blast strain ZB15 in the area in recent years (Liu et al. 2014; Li et al. 2017), its corresponding high resistance gene *Pid2* should be considered a good application prospect. *Pib* is resistant to Chinese strains ZB13 and ZC15, and regarding the progress of rice blast resistance breeding in Jiangsu province (the *japonica* rice area in eastern China) (Wang et al. 2012), pyramiding *Pita* and *Pib* achieved a good application value. Therefore, based on a comprehensive consideration of the defense mechanisms of resistance genes to improve the rice blast resistance of YF47, *Pita*, *Pib*, and *Pid2* were selected as candidate genes. In this study, TPAP functional markers for *Pita*, *Pib*, and *Pid2* alleles were developed and used to evaluate the alleles in a collection of 91 *japonica* rice germplasms. Two donor parents, WYJ27 with *Pita* and *Pib* and P135 with *Pid2*, were crossed with the receptor parent YF47, following a mutual intercross and backcross to YF47 to produce highly blast-resistant lines. A range of breeding strategies were employed, including multiple-gene pyramiding and anther culture. The derived materials with one, two, or three of them introduced with the *Pita*, *Pib*, and *Pid2* genes showed improved resistance to rice blast on natural inoculation. One line IL-5 (also named YJ144) carrying three resistance genes was further selected and shown to exhibit improved resistance, yield, and quality. Taken together, this work provides a novel and rapid strategy in modern rice breeding to generate superior varieties with comprehensively improved traits.

## Materials and methods

### Plant materials

Plant materials comprised two groups. In group A were control rice varieties that were used to develop functional molecular markers: LJHG—susceptibility check, K013—resistance check for *Pita*, K014—resistance check for *Pib*, and Digu—resistance check for *Pid2* (Wang et al. 2012, 2015). Group B varieties were used to improve the rice blast resistance of YF47: WYJ27 with *Pita* and *Pib*, P135 with *Pid2* and the recurrent parent YF47. P135 is a *japonica* accession carrying *Pid2* and produced by crossing YF47

with Digu. Detailed information on these rice varieties is shown in Table 1.

#### Design strategy for *Pita*, *Pib*, and *Pid2* markers

Following the procedure in Ye et al. (2001), the online primer design software Primer 1 was used to design four primers for each gene: one pair of outer primers to amplify a short stretch of genomic DNA as a positive control; one forward inner primer and one reverse inner primer designed according to the functional SNP. One deliberate mismatched base at the -3 site of the 3'-end of the extension was introduced into each inner primer (Table 2).

#### Crossing and selection

In 2013, WYJ27 and P135 were separately crossed with YF47 in Panjin (41.07° N, 122.03° E), and the seeds of  $F_{1-1}$  and  $F_{1-2}$  were planted in winter in Hainan (18.15° N, 109.30° E), followed by  $F_{1-1} \times F_{1-2}$  to get the seeds of  $F_{1-3}$ . In 2014, 25 strains of  $F_{1-3}$  plants carrying *Pitalpita*, *Pibl/pib*, and *Pid2/pid2* were obtained through molecular detection in Panjin; furthermore, YF47 was hybridized with  $F_{1-3}$ , and  $BC_1F_1$  lines ( $n=102$ ) were planted in Hainan. Based on molecular detection,  $BC_1F_{1-7}$  (*Pitalpita*, *Pibl/pib*, *Pid2/pid2*,  $n=13$ ) plants were identified and anther culture was carried out at the late uninucleate stage when 7000 anthers were collected. From these, about 630 embryoids were induced; callus induction was 9%. Finally, 151 green seedlings were successfully generated, giving a plant differentiation rate of 24%. In 2015, 115  $H_1$  plants were obtained to carry out genotypic identification in Panjin. Ultimately, improved lines of no resistance gene ( $n=6$ ), *Pita* ( $n=26$ ), *Pib* ( $n=15$ ), *Pid2* ( $n=22$ ), *Pita + Pib*

( $n=22$ ), *Pita + Pid2* ( $n=15$ ), *Pib + Pid2* ( $n=10$ ), and *Pita + Pib + Pid2* ( $n=5$ ) were obtained.

#### Anther culture operation scheme

The culture medium composition followed the methods described by Lee et al. (2003): At the late uninucleate stage, the spike neck wrapped with young spikes were dropped out and pretreated at 10 °C for 5 h, N6 was used as basal media for the first step of callus induction, adding 2,4-D (2 mg/L), NAA (1 mg/L), KT (1 mg/L), LH (0.5 g/L), sucrose (50 g/L), and agar (4.2 g/L). Further, MS was used as the callus differentiation medium, adding 6-BA (2 mg/L), NAA (0.5 mg/L), IAA (0.5 mg/L), IBA (0.1 mg/L), inositol (0.1 g/L), MET (2 mg/L), LH (0.5 g/L), sucrose (30 g/L), and agar (4.2 g/L). The pH value of culture medium was 5.8.

#### Identification of rice blast resistance

Rice blast resistance was monitored in 2019 and 2020 in the city of Dandong (40.07° N, 124.23° E). This is a mountainous area with high humidity and heavy fog, with the most serious susceptibility to rice blast disease in Liaoning province. According to previous studies, 44 races of 6 groups (ZA, ZB, ZC, ZD, ZE, and ZF) of the fungus were prevalent in this area in recent years (Liu et al. 2014; Li et al. 2017). A total of 124 rice accessions (including 121 filial generation accessions, YF47, WYJ27, and P135) were tested in a randomized complete block design with three replications. Resistance identification was conducted by natural inoculation according to Jiang et al. (2012), with slight changes: every replication was planted at a high density (50 plants/m<sup>2</sup>) to create a hot and humid environment. Menggudao (susceptible variety) was planted at both sides of each row and around the

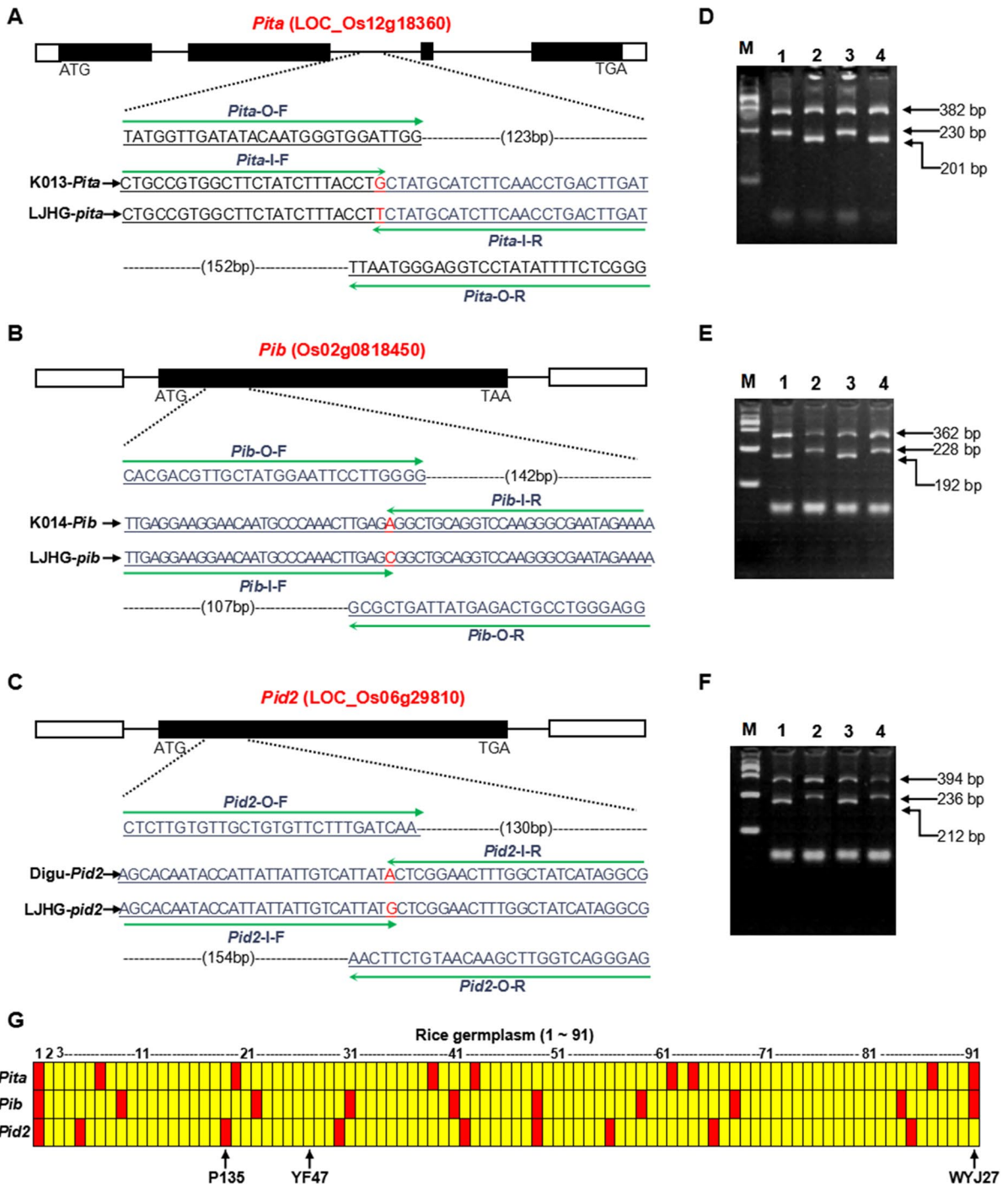
**Table 1** Plant materials used in this study

Variety (or line) name	Subspecies	Origin	Genotype
LJHG	<i>japonica</i>	Yunnan, China	<i>pita</i> , <i>pib</i> , <i>pid2</i>
K013	<i>indica</i>	Jiangsu, China	<i>Pita</i>
K014	<i>indica</i>	Jiangsu, China	<i>Pib</i>
Digu	<i>indica</i>	Sichuan, China	<i>Pid2</i>
Yanfeng 47 (YF47)	<i>japonica</i>	Liaoning, China	<i>pita</i> , <i>pib</i> , <i>pid2</i>
Wuyunjing 27 (WYJ27)	<i>japonica</i>	Jiangsu, China	<i>Pita</i> , <i>Pib</i>
P135	<i>japonica</i>	Liaoning, China	<i>Pid2</i>

**Table 2** Molecular markers used in this study

Gene	Marker Name	Primer Sequence (5'-3')	Annealing temperature	Extension time	Cycles	Produce Size (bp)	
<i>Pita</i>	TPAP- <i>Pita</i>	<i>Pita</i> -I-F: CTCTGCCGTGGCTTCTATCTTTACTITG <i>Pita</i> -I-R: ATCAAGTCAGGTTGAAGATGCATGGA <i>Pita</i> -O-F: ATGGTTGATATACAATGGGTGGATTGG <i>Pita</i> -O-R: CCCGAGAAAATATAGGACCTCCCAATTA	55 °C	30 s	32	230 bp (G allele) 201 bp (T allele) 382 bp (Positive control)	
	TPAP- <i>Pib</i>	<i>Pib</i> -I-F: TTGAGGAAAGGAACAATGCCCAAACTTGGC <i>Pib</i> -I-R: TTTTCTATTCGCCCTTGGACCTGCAGTCT <i>Pib</i> -O-F: CACGACGTTGCTATGGAAATTCCTTGGGG <i>Pib</i> -O-R: TCCTCCCAGGCAGTCTCATATCAGCGC	56 °C	30 s	32	228 bp (A allele) 192 bp (C allele) 362 bp (Positive control)	
		TPAP- <i>Pid2</i>	<i>Pid2</i> -I-F: AGCACAATACCATTATTATTGTCATTCTG <i>Pid2</i> -I-R: CGCCTATGATAGCCAAAAGTCCCGCGT <i>Pid2</i> -O-F: CTCTTGTTGCTGTGTCTTTTGATCAA <i>Pid2</i> -O-R: CTCCTGACCAAGCTTGTACAGAAAGTT	58 °C	30 s	32	212 bp (A allele) 236 bp (G allele) 394 bp (Positive control)

The deliberate introduction of a mismatched base is shown in red



entire population. Diseased straw collected from the previous year was used around each accession and also around Menggudao. The leaf blast disease index at the tillering and heading stage was recorded using the 0–5 scale rating system, and neck blast severity

was recorded as a percentage of infection in the neck of rice panicle at the maturity stage (Bonman et al. 1986; Jiang et al. 2012).



◀ **Fig. 1** TPAP primer design and detection of *Pita*, *Pib*, and *Pid2* alleles. Design strategy for TPAP-*Pita* (A), TPAP-*Pib* (B), and TPAP-*Pid2* (C) markers. Functional SNPs are shown in red; underlines show primer binding sites. PCR assay of *Pita* (D) between resistant (K103, lanes 1 and 3) and susceptible (LJHG, lanes 2 and 4) genotypes in two repeats; *Pib* (E) between resistant (K104, lanes 2 and 4) and susceptible (LJHG, lanes 1 and 3) genotypes in two repeats; *Pid2* (F) between resistant (Digu, lanes 1 and 3) and susceptible (LJHG, lanes 2 and 4) genotypes in two repeats. M, marker 2000. G Genotype analysis of *Pita*, *Pib*, and *Pid2* on 91 rice germplasm using TPAP-*Pita*, TPAP-*Pib*, and TPAP-*Pid2*. Nos. 1–2 are the resistance check and susceptibility check of *Pita*, *Pib*, and *Pid2*, corresponding to K013 and LJHG, K014 and LJHG, and Digu and LJHG, respectively; Nos. 3–48, 49–63, 64–67, 68–82, 83–87, and 88–91 are *japonica* germplasms collected from Liaoning, Ningxia, Tianjin, Xinjiang, Jilin, and Jiangsu provinces, respectively. The yellow regions represent susceptible genotypes, and the red regions represent the resistance genotypes YF47, Yanfeng47, and WYJ27, Wuyunjing27

#### Investigation of yield-related characters and rice quality

Yield-related characters were investigated in Panjin during 2019 and 2020. In 2019, the five improved lines carrying the three blast resistance genes were investigated with and without the application of fungicides, and with YF47 as the control. In 2020, yield-related characters of YJ144 and YF47 were investigated with and without the application of fungicides. Test materials were grown under irrigated conditions in a randomized complete block design with three replications, in six-row plots, with each row 10 m long, and a plant spacing of 30×13.3 cm. Ten plants were harvested to investigate yield traits: number of effective panicles, number of filled grains per panicle, and 1000 grain weight. Rice grain from a 1-m<sup>2</sup> area was harvested and used to calculate yield. In 2020, YJ144 and YF47 were harvested and stored at room temperature for 3 months, according to Ren et al. (2016). A 600-g sample of grain was processed to analyze the milling quality, and 30 plump grains were selected to measure grain length and width. After processing into polished rice, the chalkiness degree was assessed using an SC-E rice appearance quality analyzer (Wanshen, China). The amylose content, alkali spread value, and gel consistency were determined as described previously (Butardo et al. 2011), and the protein content was calculated by using the N-protein conversion coefficient 5.95 described by Hu et al.

(2014). The eating quality score was determined by sensory tasting, followed the methods described by Lestari et al. (2009).

The regional variety comparison test in Liaoning province was conducted both in 2019 and in 2020 with three replications in eight-row plots. Each row was 10 m long, with a plant spacing of 30×13.3 cm. This was replicated in six cities: Tieling (TL), Shenyang (SY), Liaozhong (LZ), Linghai (LH), Dandong (DD), and Panjin (PJ).

#### DNA isolation, PCR amplification, and electrophoresis detection

A plant genomic isolation kit (Tiangen, Wuhan, China) was used to extract rice genomic DNA. PCR was prepared in a total volume of 20 μL containing 10 μL of 2×*Tap* PCR Master Mix (Tiangen, Wuhan, China), 0.3 μL of each outer primer (4 pmol/μL), 0.2 μL of each inner primer (4 pmol/μL), and 1 ng of genomic DNA diluted with ddH<sub>2</sub>O to 20 μL. The PCR procedure was as follows: 95 °C for 5 min; 32 cycles of 95 °C for 30 s, 55–58 °C for 30 s, 72 °C for 50 s; and a final extension at 72 °C for 10 min. Agarose gels (5%) were used to separate PCR products, which were then stained with ethidium bromide, and photographed with a GEL DOC 1000 system (Bio-Rad, Hercules, CA, USA).

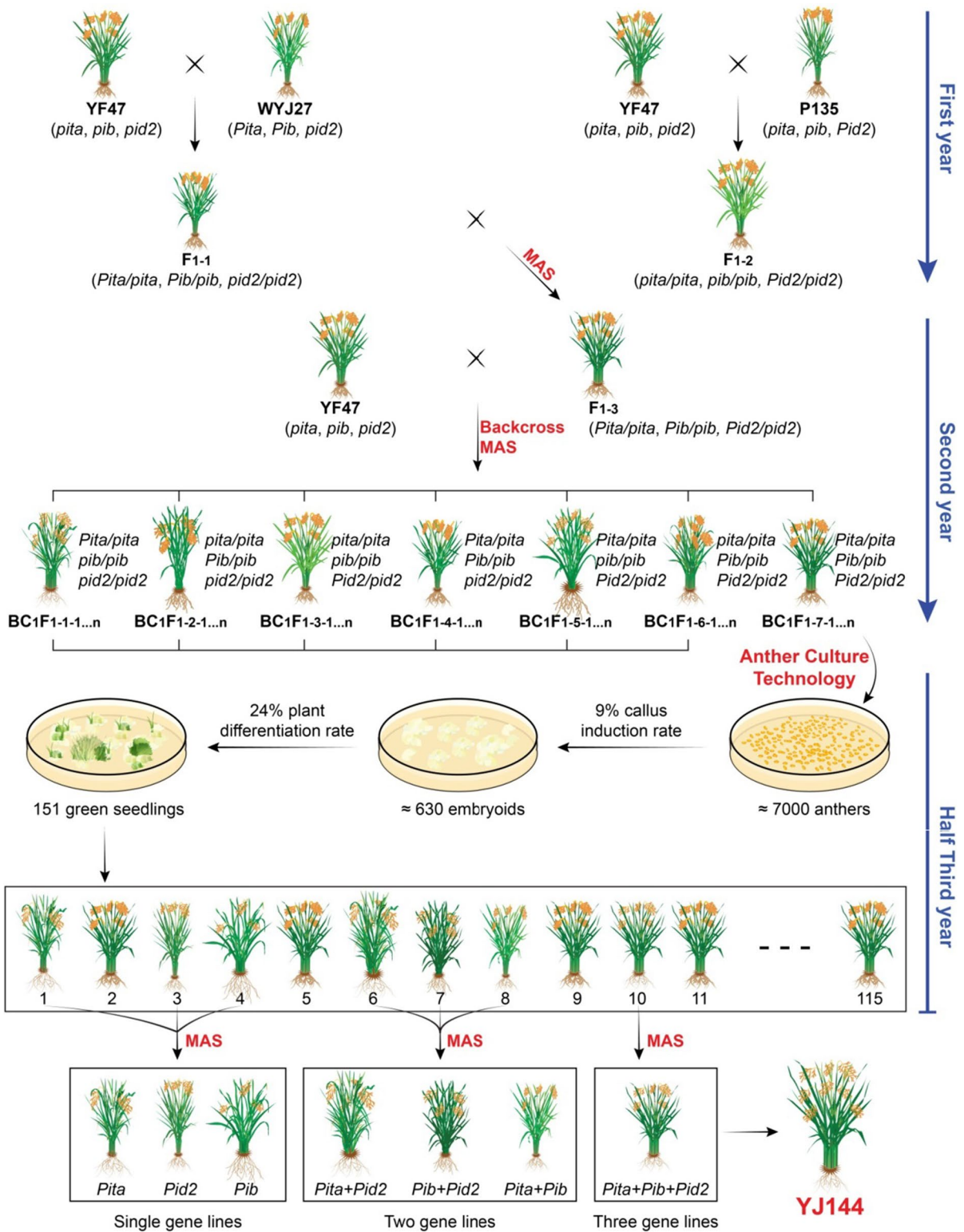
#### Statistical analyses

The data and chart analyses were performed using Graph Pad Prism v. 5.0 and Microsoft Office PowerPoint 2007.

## Results

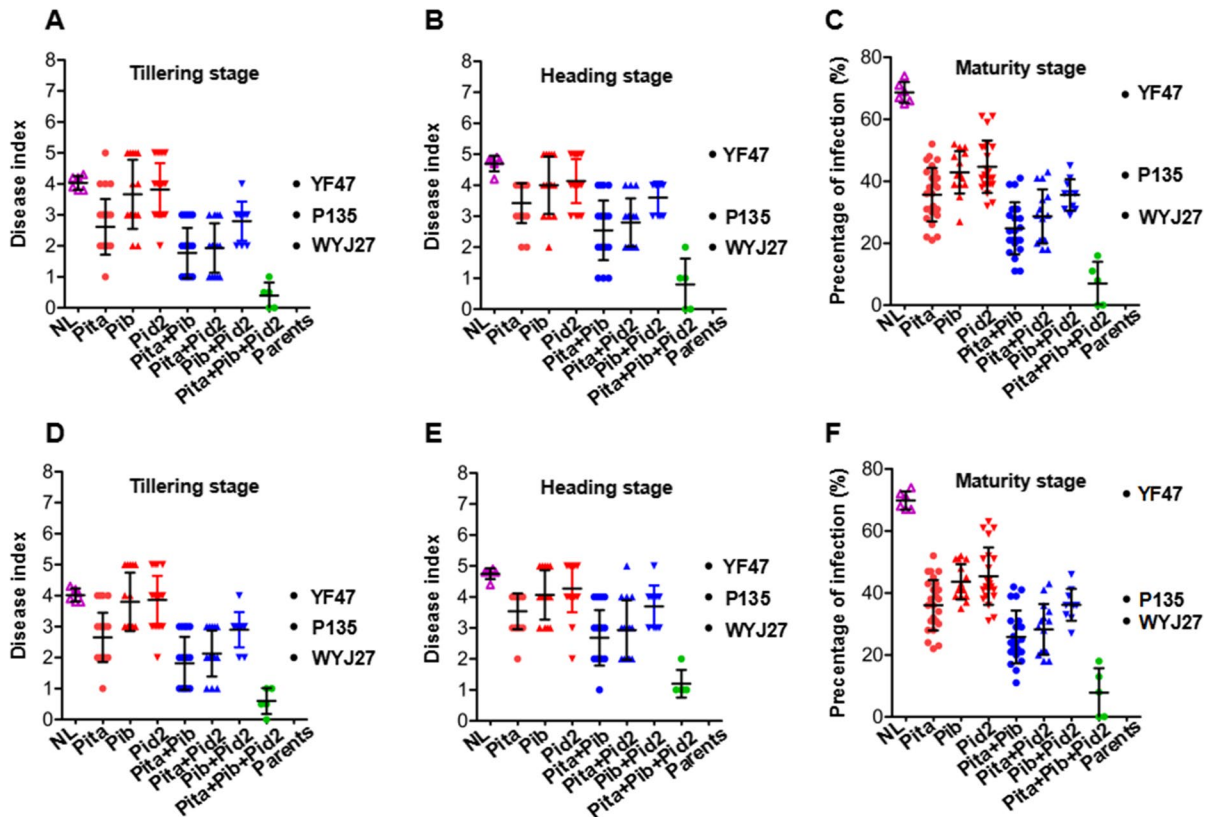
#### Developing novel TPAP molecular markers for *Pita*, *Pib*, and *Pid2*

*Pita* encodes a cytoplasmic membrane receptor protein containing an NBS domain and a leucine-rich domain (LRD), and its disease-resisting mechanism is due to its detection of the expression product of avirulent gene *Avr-Pita* in rice blast, thus triggering rapid local defenses (Bryan et al. 2000). Two other useful alleles function in rice blast resistance. One is *Pib*, which belongs to the NBS-LRR disease-resistant gene group and encodes a protein product comprising one



**Fig. 2** Construction of genetic materials carrying various rice blast resistance genes





**Fig. 3** Determination of rice leaf blast resistance and neck blast resistance. Rice leaf disease index at the tillering (A) and heading stage (B) in 2019. C Rice neck blast scores at the maturity stage in 2019. Rice leaf disease index at the tillering

(D) and heading (E) stages in 2020. F Rice neck blast scores at the maturity stage in 2020. The horizontal/vertical lines show mean  $\pm$  SD. NL, no resistance gene lines

NBS and 17 LRR. The other is *Pid2*, which encodes a transmembrane receptor protein kinase (RLK) and confers high resistance to the Chinese rice blast strain ZB15 (Wang et al. 1999, 2015). One major task of rice breeders is to use these genes effectively to generate novel highly resistant varieties. Functional molecular markers have been developed for *Pita* and *Pib* detection, with two pairs of primers and double PCR amplification needed (Fjellstrom et al. 2004; Wang et al. 2012), while *Pid2* allele detection still requires sequencing (Wang et al. 2015). However, the detection primers for *Pita*, *Pib*, and *Pid2* are inefficient for pyramiding multiple genes because the process is time-consuming (Fjellstrom et al. 2004; Kim et al. 2016). Therefore, rapid, simple, and low-cost functional molecular markers for *Pita*, *Pib*, and *Pid2* detection are urgently needed.

First, based on the functional nucleotide polymorphism sites of *Pitalpita*, *Piblpib*, and *Pid2lpid2* (*LOC\_Os12g18360* and *LOC\_Os06g29810* collected from the MSU database, and *Os02g0818450* from RAP-DB), molecular markers were designed for TPAP-*Pita*, TPAP-*Pib*, and TPAP-*Pid2* (Fig. 1A–C) (Wang et al. 1999, 2015; Bryan et al. 2000). Referring to the previous studies, K013, K014 and Digu were chosen as the resistance checks for *Pita*, *Pib*, and *Pid2*, respectively, and LJHG was used as the susceptibility check (Wang et al. 2012, 2015). For *Pita*, the marker TPAP-*Pita* amplified 230-bp and 201-bp DNA fragments corresponding to the resistance and susceptibility alleles, and a 382-bp fragment was the positive control (Fig. 1D). For *Pib*, the marker TPAP-*Pib* amplified 228-bp and 192-bp DNA fragments corresponding to the resistance and susceptibility alleles, and a 362-bp DNA fragment

was the positive control (Fig. 1E). For *Pid2*, the marker TPAP-*Pid2* amplified 212-bp and 236-bp DNA fragments corresponding to the resistance and susceptibility alleles, and a 394-bp DNA fragment was the positive control (Fig. 1F). To confirm the precise application of molecular markers, we sequenced the PCR products using outer primers of TPAP-*Pita*, TPAP-*Pib*, and TPAP-*Pid2*, and confirmed the accuracy of the above constructed molecular markers (Fig. S1–S3).

Generation and resistance analysis of blast resistance materials using the crosses of YF47 as a receptor parent, and WYJ27 and P135 as donor parents

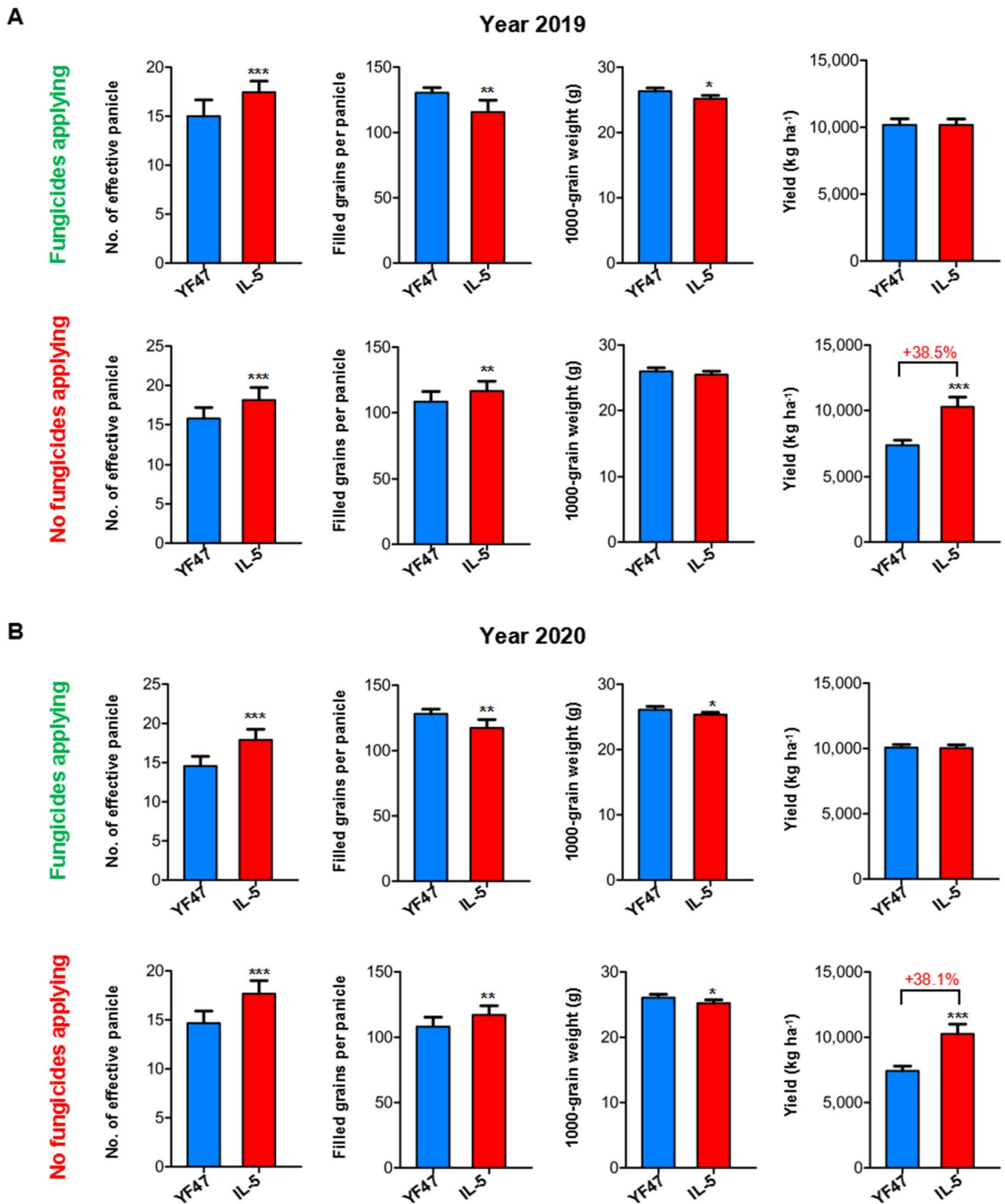
To investigate the benefit that blast-resistant alleles confer, we studied 91 *japonica* varieties (or lines) from different provinces: Liaoning, Ningxia, Tianjin, Xinjiang, Jilin, and Jiangsu (Table S1). Most of them show similar ecological characteristics and were further used for genotype analysis of *Pita*, *Pib*, and *Pid2*. It was found that some cultivars contained one or two functional alleles; however, no one rice variety contained all three (Fig. 1G). Next, considering genetic relationship, plant architecture, rice yield, and quality, WYJ27 (with *Pita* and *Pib* alleles) and P135 (with the *Pid2* allele) were selected as donor parents to improve rice blast resistance of YF47. As a result, a series of rice lines carrying one, two, or three resistant genes were identified using multiple strategies of rice breeding, including single, mutual, and backcrosses together with MAS (Fig. 2). To accelerate the breeding process further, the anther culture technique was used to generate these genetic materials. A total of 7000 anthers were collected from BC<sub>1</sub>F<sub>1-7</sub> plants at the late uninucleate stage. From these, about 630 embryoids were induced; callus induction was 9%. Finally, 151 green seedlings were successfully generated, giving a plant differentiation rate of 24% (Fig. 2).

To further evaluate the resistance levels of those generated materials, we recorded a leaf and neck blast disease index in both 2019 and 2020 using natural inoculation (Fig. 3). In 2019, at the tillering stage, the leaf blast disease index for no resistance gene lines (NL,  $n=6$ ), *Pita* ( $n=26$ ), *Pib* ( $n=15$ ), *Pid2* ( $n=22$ ), *Pita+Pib* ( $n=22$ ), *Pita+Pid2* ( $n=15$ ), *Pib+Pid2* ( $n=10$ ), and *Pita+Pib+Pid2* ( $n=5$ ) was recorded as means of 4.1, 2.6, 3.7, 3.8,

1.8, 1.9, 2.8, and 0.4, respectively (Fig. 3A). At the heading stage, the corresponding means were 4.8, 3.4, 4.1, 4.1, 2.5, 2.8, 3.6, and 0.8, respectively (Fig. 3B). The receptor parent YF47 had a leaf blast disease index of 4 and 5 at the tillering and heading stages, respectively. The neck blast severity was recorded as infection percentages of 68.6, 35.7, 42.9, 44.7, 24.8, 28.7, 35.6, and 7.0%, respectively, whereas 68.0% was observed for YF47 (Fig. 3C). In 2020, the disease index increased slightly but showed a similar pattern that of 2019 (Fig. 3D–F). The other two donor parents, WYJ27 and P135, were also used to investigate the disease index. They both showed improved leaf blast disease index and reduced percentage of infection over the control parent YF47 (Fig. 3). Taken together, *Pita* showed the best improvement against rice blast, while *Pib* exhibited slightly stronger resistance than *Pid2*. It is noteworthy that blast resistance increased with the number of functional alleles in the line.

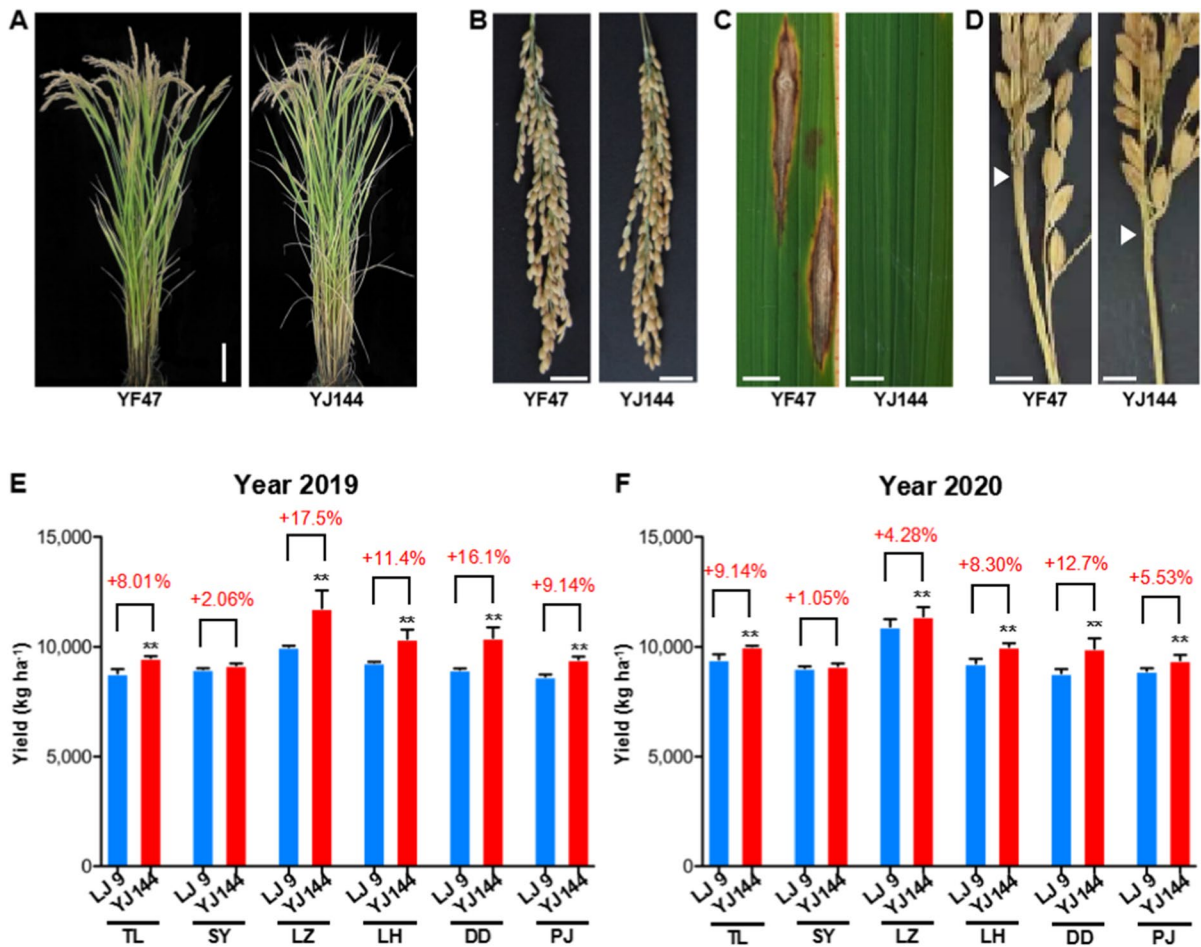
Agronomic traits of YF47 and the improved lines carrying three resistance genes with and without application of fungicides

The field performances in Liaoning province were tested for five improved lines IL-1 to IL-5, all with introduced *Pita*, *Pib*, and *Pid2* alleles with YF47 as a control. Their yield-related traits, including the number of effective panicles, filled grains per panicle, 1000 grain weight, and total yield per hectare, were investigated with and without the application of fungicides. In 2019, under the application of fungicides, IL-5 showed an increased number of effective panicles, whereas IL-2 and 4 exhibited the opposite outcome. IL-3, 4, and 5 all showed reduced numbers of filled grains per panicle, while the five lines all showed decreased 1000 grain weight. As a result, each of the three lines (IL-1, 2, and 4) exhibited a reduction in yield compared to YF47. The other two lines, IL-3 and 5, had yields equal to that of YF47 (Fig. 4A and Fig. S4A). In 2019, without application of fungicides, IL-5 showed an increased number of effective panicles, whereas IL-1, 2, and 4 exhibited the opposite effect. IL-1, 2, 3, and 5 all had an increased number of filled grains per panicle. Also, lines IL-1 to 4 all showed decreased 1000 grain weight. As a result, after rice blast infection, all lines except IL-4 exhibited better yield



**Fig. 4** Yield-related trait comparison of YF47 and IL-5. Yield-related trait comparison of YF47 and IL-5 in 2019 (A) and 2020 (B), with and without application of fungicides.

IL-5, improved line 5. The data shows mean  $\pm$  SD; \* $p < 0.05$ , \*\* $p < 0.01$ , and \*\*\* $p < 0.001$



**Fig. 5** Plant architecture, rice blast resistance, and yield performance of YJ144. **A** Plant architecture comparison of YJ144 and YF47. Scale bar, 10 cm. **B** Panicle architecture comparison of YJ144 and YF47. Scale bar, 2 cm. **C** Rice leaf (**C**) and neck (**D**) blast disease index of YJ144 and YF47. Yield comparison of LJ9 and YJ144 in the regional variety comparison test in

six cities of Liaoning province in 2019 (**E**) and 2020 (**F**). LJ9 (Liaojing9) was a control in the regional variety comparison test. TL, SY, LZ, LH, DD, and PJ represent the Liaoning cities of Tieling, Shenyang, Liaozhong, Linghai, Dandong, and Panjin, respectively. The data shows mean  $\pm$  SD; \*\* $p < 0.01$

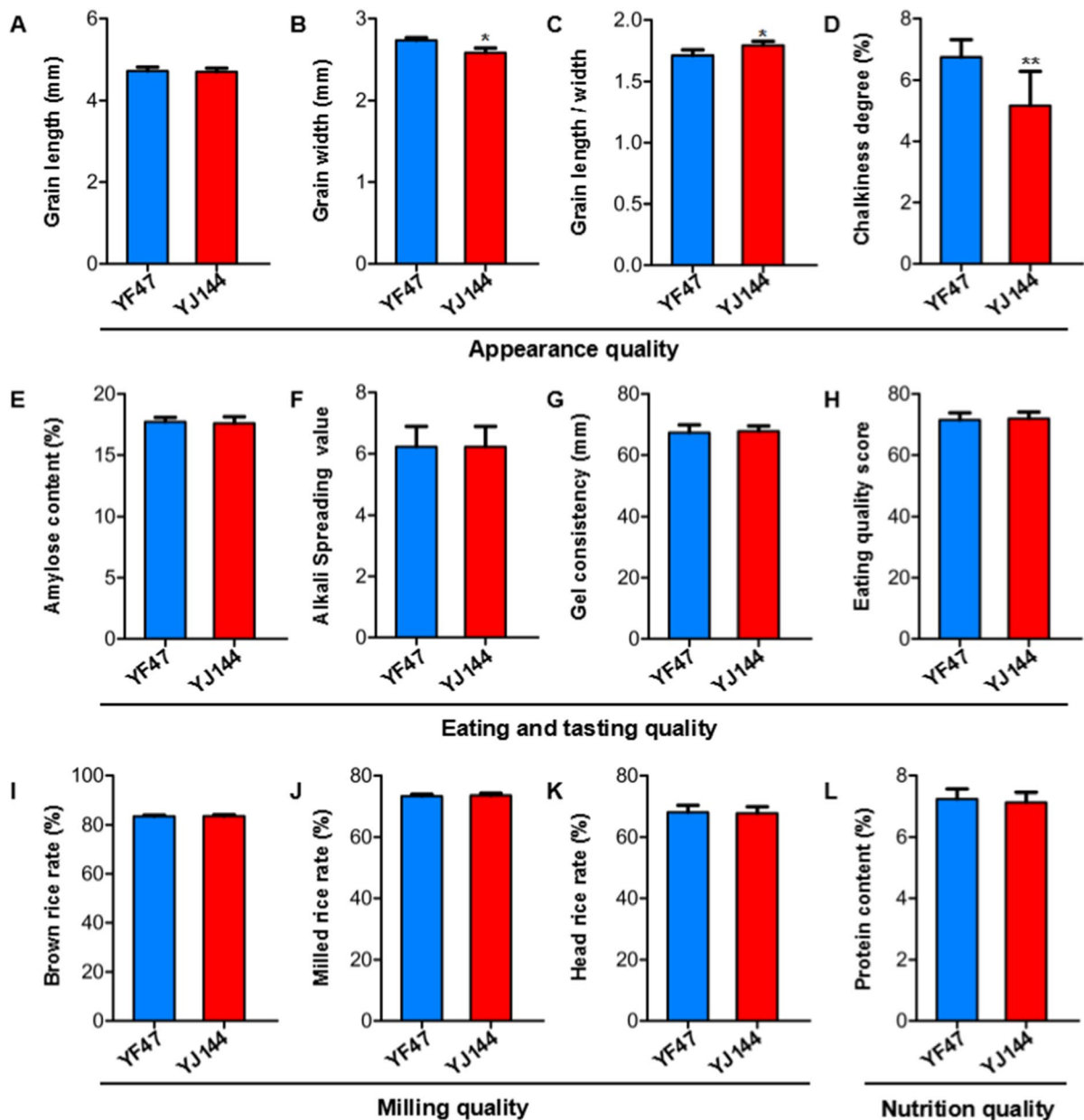
performance compared to that of YF47 (Fig. 4A and Fig. S4B). Thus, IL-3 and 5 are two candidate lines with huge for rice breeding.

To evaluate further the performance of IL-5, with and without application of fungicides, similar experiments were conducted in 2020. We compared the yield-related parameters of YF47 and IL-5. As shown in Fig. 4B, under the application of fungicides, the effective panicle number of IL-5 was significantly higher than that of YF47 ( $p < 0.01$ ), while the filled grains per panicle and 1000 grain weight were significantly lower than those of YF47 ( $p < 0.05$ ), resulting in nearly the same yield.

However, where fungicides were not applied, the effective panicle number and the filled grains per panicle of IL-5 were both significantly higher than those of YF47 ( $p < 0.01$ ), while its 1000 grain weight was significantly lower ( $p < 0.05$ ), resulting in higher yield for IL-5 ( $p < 0.001$ ).

YJ144, a high blast resistance rice line, exhibited increased yield traits in the Liaoning province regional variety comparison tests

Of five improved lines, each containing three rice blast resistance genes, IL-5 had the minimum



**Fig. 6** Comparison of rice quality between YF47 and YJ144. Appearance quality comparison of YF47 and YJ144, including grain length (A), grain width (B), the ratio of grain length to width (C), and degree of chalkiness (D). Comparison of milling quality of YF47 and YJ144, including the brown rice (E), milled rice (F), and head rice rates (G). H Nutrition qual-

ity comparison of YF47 and YJ144. Eating and tasting quality comparison of YF47 and YJ144, including amylose content (I), alkali spread value (J), gel consistency (K), and eating quality score (L). The data show mean  $\pm$  SD; \* $p < 0.05$  and \*\* $p < 0.01$

disease index for leaf blast and neck blast, and showed increased yield both in 2019 and 2020 (Figs. 3 and 4 and Fig. S4). We named this line YJ144. Then, we conducted phenotypic and morphological analyses

of YF47 and YJ144. As shown in Fig. 5A–B, YJ144 showed similar plant architecture and panicle morphological architecture compared to YF47. Moreover, YJ144 had higher rice blast resistance (Fig. 5C–D),



consistent with their relative leaf and neck blast disease index, as indicated in Fig. 3. To confirm that the three functional alleles (*Pita*, *Pib*, and *Pid2*) had been successfully introduced, we sequenced the appropriate functional sites of these genes within YF47 and YJ144. The results confirmed that YJ144 contained these novel functional sites, as shown in Fig. S1–S3.

To examine the performance of YJ144 in field trials, in 2019 and 2020, it was entered in the regional variety comparison tests in Liaoning province. Liaojing 9 (LJ9) is an officially designated control variety with medium yield used in regional comparison tests in Liaoning province. Its planting has covered nearly 0.6 million hectares over the past 30 years (<https://www.ricedata.cn/variety/varis/600590.htm>, accessed 25 July 2020). This trial encompassed tests in six Liaoning cities: Tieling (TL), Shenyang (SY), Liaozhong (LZ), Linghai (LH), Dandong (DD), and Panjin (PJ). In both 2019 and 2020, YJ144 exhibited significant yield increases of 4.3 to 17.5% at all six sites apart from SY. Yields at SY also increased in 2019 and 2020 but not significantly (Fig. 5E–F). These findings suggest that YJ144 exhibited superior yield capacity and could be used as an important *japonica* germplasm with both high blast resistance and yield.

Appearance quality of YJ144 is improved compared to that of YF47

To ascertain whether the introduction of blast-resistant genes could affect rice quality, we determined some rice quality indicators: appearance, milling, nutrition, eating, and tasting. YJ144 and the control YF47, both harvested in 2020, were tested. As shown in Fig. 6A–C, the grain lengths of YJ47 and YJ144 were very similar, while the grain width of YF47 was greater than that of YJ144, which resulted in a significantly higher length-to-width ratio for YJ144. Additionally, the chalkiness of YJ144 was significantly lower than that of YF47 (Fig. 6D), probably resulting from its greater length-to-width ratio. However, grain shape and chalk differences between YF47 and YJ144 did not give rise to significant changes in milling quality, including the brown, milled, and head rice rates (Fig. 6E–H). To assess nutritional, eating and cooking quality, we compared protein and amylose contents, the alkali spreading value, and gel consistency, which are recognized as the most critical

quality factors for eating and cooking. Ultimately, none of the characteristics showed any significant change between YF47 and YJ144 (Fig. 6H and I–L). Taken together, our findings demonstrated that YJ144 showed improved appearance quality compared to YF47.

## Discussion

Accurate and rapid detection of rice blast resistance is fundamental for resistance breeding. In previous studies, plants were artificially inoculated with pathogen races (Deng et al. 2006; Wang et al. 2014), but the type of pathogen inoculated was limited and the operation was complicated and easily affected by artificial factors. One problem was the nature of the fungus found in Liaoning province, which consists of at least 44 races in 6 groups (ZA, ZB, ZC, ZD, ZE, and ZF), the predominant ones of which are complex and dynamic (Liu et al. 2014; Li et al. 2017). In this study, we used natural infection through dense planting, application of diseased straw, and cultivation of a susceptible variety in the surrounding plot. Under these natural conditions, plants were infected by numerous *M. oryzae* pathotypes simultaneously; therefore, this is likely to reflect environmental factors and test the effects of resistance genes more accurately. Under these conditions, YF47 is readily susceptible to *M. oryzae*, whereas WYJ27 with *Pita* and *Pib* alleles, and P135 with the *Pid2* allele, exhibited increased resistance (Fig. 3). In addition, resistance to rice blast improved with an increased number of resistance genes, including relevant *Pita*, *Pib*, and *Pid2* alleles (Fig. 3). Natural infection showed good and stable results in rice blast resistance detection, similar to the conclusions reported by Jiang et al. (2012).

Numerous studies showed that the aggregation of multiple blast genes is prone to durable disease resistance, caused by the adaptation to different physiological races. The additive effect among genes could also lead to a high pyramiding effect (Xiao et al. 2020; Xu et al. 2021). In this study, improved lines with single a resistance gene represented susceptible or moderate resistance, but two or three pyramiding genes presented improved or superior resistance to rice blast, respectively. According to the previous study (Wang et al. 1999, 2015; Bryan et al. 2000), *Pita* and *Pib* belong to the NBS-LRR type, and *Pid2* belongs

to receptor-like protein kinases, resulting in different molecular functions. The encoded product of the *Pita* gene can interact with the *AVR-Pita* expression product of *Magnaporthe grisea* to induce disease resistance; the *Pib* is resistant to the Chinese strain ZB15, and *Pid2* is resistant to Chinese strains ZB13 and ZC15. More resistance genes' pyramiding could respond to broader physiological race changes, and, according to Wang et al. (2012), *Pita* has the better improved application value on rice blast resistance than *Pib* in Jiangshu province in China; this is similar to our findings; also, *Pita+Pib* pyramiding has a superior joint effect (Wang et al. 2012); thus, we speculated that the above factors are the main reasons for the high pyramiding effect as shown in Figs. 3, 4, and 5.

In this study, using TPAP technology, we developed a set of functional markers to detect *Pita*, *Pib*, and *Pid2*, where only one time PCR amplification was required for accurate identification at each locus. Compared with the previous study, *Pib* and *Pid2* detection needed two pairs of primer amplification or sequencing (Fjellstrom et al. 2004; Wang et al. 2015), respectively. For *Pita*, Ramkumar et al. and Hayashi et al. developed allele-specific and linkage molecular markers (Hayashi et al. 2006; Ramkumar et al. 2015). Our markers had a mismatch deliberately inserted, which enhanced amplification accuracy by providing a lower annealing temperature, an advantage of the better amplification conditions. Subsequently, a total of 91 *japonica* rice germplasms were collected from northern districts that presented similar ecological environments, and were screened within the loci of *Pita*, *Pib*, and *Pid2*. WYJ27 was found to contain *Pita* and *Pib* alleles, while P135 had *Pid2* alleles. Both were selected as donor parents to improve the rice blast resistance of YF47. Overall, the above work could provide both cost-effective TPAP molecular markers and *japonica* rice germplasms with *Pita*, *Pib*, and *Pid2* alleles, which could be used as the basic elements for MAS in rice breeding.

Compared with traditional breeding strategies, MAS can significantly improve selection efficiency; however, pyramiding multiple genes still requires many generations (Jiang et al. 2012; Baliyan et al. 2018). In this study, using multiple breeding strategies (single cross, mutual intercross, and backcross) combined with MAS, we successfully obtained BC<sub>1</sub>F<sub>1-7</sub> lines carrying *Pitalpita*, *Piblpib*, and

*Pid2/pid2*. Then, through anther culture, which is more commonly used in *japonica* rice than in *indica* rice due to its high rates of induction and differentiation (Lee et al. 2003; Wang et al. 2020), a series of derivative lines with different numbers of resistance genes were also created by using MAS (Fig. 2). As a result, the generation of stable materials was achieved within 3 years and showed much greater efficiency than conventional or marker-assisted breeding. In contrast to conventional breeding, CRISPR/Cas technology is widely used to modify gene components, in both the animal and plant kingdoms, by deleting negative genetic elements responsible for undesired traits or introducing gain-of-function mutations through precise genome editing. For example, the application of CRISPR/Cas has to date improved several rice crop characteristics, including yield, quality, and disease resistance (Wang et al. 2013; Zhou et al. 2015). However, it is difficult to edit three genes in their functional sites simultaneously. It also takes more than 1 year to eliminate the hygromycin B resistance gene and the noise background through one or two consecutive crosses between the transgenic line and the receptor parent. Further, the generated lines could be used only as intermediates in rice breeding. Our research provides a strategy for rapidly breeding rice cultivars with durable resistance to rice blast.

To date, large numbers of genes controlling vital agronomic traits have been characterized ([www.ricedata.cn](http://www.ricedata.cn), accessed 29 July 2020). High yield, multiple resistance, and superior quality are primary objectives of genetic improvement; however, these goals are difficult to coordinate (Yu et al. 2018), such as *Pi21* being separable from a closely linked gene conferring poor flavor (Fukuoka et al. 2009), and *Pigm R* reducing 1000 grain weight and rice yield. Recently, a rice genome navigation system, RiceNavi, was developed for quantitative trait nucleotides (QTN) pyramiding and breeding route optimization, and it is easy to employ multiple useful alleles effectively to generate novel rice varieties for rice breeders (Wei et al. 2021). In this study, we first developed a genetic improvement strategy by combining primer construction, applying various breeding methods, and conducting a comprehensive investigation of resistance, yield, and quality. Subsequently, for better coordinate yield, quality, and blast resistance, we identified *Pita*-, *Pib*-, and *Pid2*-resistant genes in 91 rice germplasm; next, a high-yield parent carrying *Pita* and *Pib*

(WYJ27) and a superior quality parent possessing *Pid2* (P135, with low chalkiness) were selected for genetic improvement, with a widely adaptable variety YF47 as the recurrent parent. Moreover, BC<sub>1</sub>F<sub>1</sub> plants with *Pita*, *Pib*, and *Pid2* were used for anther culture. Further, the blast resistance, yield components and rice quality of improved lines were investigated in depth. Ultimately, an ideal line, YJ144, was generated. Our molecular design strategy for pyramiding multiple beneficial genes generated superior varieties with comprehensively improved traits.

**Author contribution** T.M. and G.S. designed and performed the experiments, and analyzed data; M.Z., S.A., Z.S., S.H., G.J., L.X., and S.T. participated in improved lines' construction and data analysis; S.A., G.Y., X.W., G.S., and T.M. wrote the manuscript and prepared the illustrations. G.S. and P.H. conceived the idea and supervised the project. All authors have read and agreed to the published version of the manuscript.

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## Declarations

**Data and materials availability** All data are given in the manuscript.

Not applicable.

**Consent for publication** The authors give consent for the publication.

**Conflict of interest** The authors declare no competing interests.

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