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Marker-assisted breeding of Chinese elite rice cultivar 9311 for disease resistance to rice blast and bacterial blight and tolerance to submergence

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Abstract Rice (*Oryza sativa* L.) is the staple food crop for more than half of the world's population. The development of hybrid rice is a practical approach to increase rice production. However, rice production was frequently affected by biotic and abiotic stresses. Rice blast and bacterial blight are two major diseases in rice growing regions. Rice plantation is also frequently affected by short-term submergence or seasonal floods in wet seasons and drought in dry seasons. The utilization of natural disease resistance (*R*) genes and stress tolerance genes in rice breeding is the most economic and efficient way to combat or adapt to these biotic and abiotic stresses. Rice cultivar 9311 is widely planted rice variety, either as inbred rice or the paternal line of two-line hybrid rice. Here, we report the pyramiding of rice blast *R* gene *Pi9*, bacterial blight *R* genes *Xa21* and *Xa27*, and submergence tolerance gene *Sub1A* in 9311 genetic background through backcrossing and marker-assisted selection. The improved rice line, designated as 49311, theoretically possesses 99.2% genetic background of 9311. 49311 and its hybrid rice, GZ63S/49311, conferred disease resistance to rice blast and bacterial blight

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and showed tolerance to submergence for over 18 days without significant loss of viability. 49311 and its hybrids had similar agronomic traits and grain quality to 9311 and the control hybrid rice, respectively. The development of 49311 provides an improved paternal line for two-line hybrid rice production with disease resistance to rice blast and bacterial blight and tolerance to submergence.

Keywords Rice · Marker-assisted selection · Gene pyramiding · Rice blast · Rice bacterial blight · Submergence tolerance

Introduction

Rice (*Oryza sativa* L.) is a staple food crop for more than half of the world's population. Rice yield has doubled or even tripled in most rice-producing countries in the world over the past three decades (Zhang 2007). This is due to two types of genetic improvements during the first "Green Revolution" starting from 1960s. The first genetic improvement is the utilization of semi-dwarf gene (*sd-1*), which reduces plant height and thereby increases the harvest index (Spielmeyer et al. 2002). The second genetic improvement involves the development of hybrid rice (Yuan et al. 1994). However, the rice yield and production are still frequently affected by biotic stress, including various diseases caused by pathogens, pests and weeds, and abiotic stress, such as flood and drought due to global warming and climate change.

Bacterial blight of rice, caused by the bacterium *Xanthomonas oryzae* pv. *oryzae*, is one of the most destructive bacterial diseases that is prevalent throughout the major rice-growing regions, especially in Asia (Gnanamanickam et al. 1999). Rice plants infected with *X. oryzae* pv. *oryzae* strains at the maximum tillering stage could result in a 20% yield loss, and, for severe cases, the yield loss could be more than 50% (Mew et al. 1993). On host side, rice has co-evolved disease resistance (*R*) genes to counteract bacterial infection. The utilization of *R* genes in rice breeding is still the most effective and economic way to control the bacterial disease. So far, nearly 40 bacterial blight *R* genes have been identified and they are designated in a series from *Xa1* to *Xa40* (Busungu et al. 2016; Kim et al. 2015). Among which, *Xa21* and *Xa27* are two dominant *R* genes that provide broad-spectrum disease resistance to *X. oryzae* pv. *oryzae* (Gu et al. 2004; Ikeda et al. 1990). *Xa21* and *Xa27* share most of their resistance spectrum and differ in resistance specificity to a few *X. oryzae* pv. *oryzae* strains (Gu et al. 2004). For instance, *Xa21* confers specific resistance or moderate resistance to *X. oryzae* pv. *oryzae* strains K202 and ZHE173, while *Xa27* is specifically resistant or moderately resistant to *X. oryzae* pv. *oryzae* strains A3842, NXO260, and JW89011 (Gu et al. 2004). *Xa21* encodes a pattern recognition receptor kinase with an extracellular leucine-rich repeat domain (Song et al. 1995). XA21 recognizes a tyrosine-sulfated protein RaxX from *X. oryzae* pv. *oryzae* and triggers hallmarks of the plant immune response (Pruitt et al. 2015). *Xa27* is a transcription activator-like (TAL) effector-dependent *R* gene, whose expression is induced by TAL effector AvrXa27 from pathogens (Gu et al. 2005). XA27 is localized to the apoplast of rice cells and activates disease resistance to *X. oryzae* pv. *oryzae* (Wu et al. 2008). The differences in resistance specificity and signaling provide opportunity for us to pyramid *Xa21* and *Xa27* in different rice varieties for enhanced, broad-spectrum, and probably durable disease resistance to *X. oryzae* pv. *oryzae* (Luo and Yin 2013; Luo et al. 2012, 2016).

Rice blast, caused by the fungus *Magnaporthe oryzae*, is one of the most important fungal diseases in rice. The fungal disease has caused nearly 19 million tons of rice yield loss per year (Moffat 1994). Like the host resistance to *X. oryzae* pv. *oryzae*, rice has co-evolved *R* genes for resistance to *M. oryzae*. So far, more than 70 rice blast *R* genes have been identified (Zheng et al. 2016; Liu et al. 2010). Among which, the *Pi9* gene in rice line 75-1-127 was introgressed from the wild species *Oryza minuta* (Liu et al. 2002). *Pi9* provides high and broad-spectrum resistance to 43 *M. oryzae* isolates collected from 13 countries (Liu et al. 2002). *Pi9* has been deployed in rice breeding program (Koide et al. 2011; Luo and Yin 2013; Khanna et al. 2015; Ni et al. 2015; Luo et al. 2016).

Submergence and drought are two major constraints to rice production in rain-fed farmlands, both of which can occur sequentially during a single crop cycle. Rice variety FR13A was identified to be tolerant to submergence (Xu and Mackill 1996). The submergence tolerance is controlled by the *Sub1A* gene, which was mapped on rice chromosome 9 (Xu and Mackill 1996). *Sub1A* encodes an ethylene-response-factor (ERF)-like transcription factor that dampens ethylene production and gibberellic acid (GA) responsiveness,

in avoidance of carbohydrate starvation, allowing plants to endure submergence and causing quiescence in growth that correlates with the capacity for regrowth upon desubmergence (Xu et al. 2006; Fukao et al. 2006). Interestingly, *Sub1A* also increases the recovery of plants from drought at the vegetative stage through the reduction of leaf water loss, lipid peroxidation, and increase in expression of genes associated with acclimation to dehydration (Fukao et al. 2011). The *Sub1A* gene has been introduced into several rice cultivars through marker-assisted selection, and the improved rice lines with the *Sub1A* gene are tolerant to submergence (Luo et al. 2016; Luo and Yin 2013; Neeraja et al. 2007).

Rice cultivar 9311 is widely planted indica variety, either as inbred rice or the paternal line of two-line hybrid rice. Liang-You-Pei-Jiu (LYP9), a two-line hybrid rice variety derived from the cross between Pei-Ai 64 and 9311, has 20–30% more yield than the other rice varieties in large area of cultivation (Yuan 1997). Hybrid rice derived from 9311 covered 10.73 million hectare in China, accounting for 51.2% of total two-line hybrid rice during the period of 1993–2005 (Si et al. 2011). However, 9311 is susceptible to some *X. oryzae* pv. *oryzae* strains or *M. oryzae* isolates (Luo et al. 2012; Li et al. 2015). In addition, floods and drought often affect rice production in Yangzi River valley in China during typhoon and summer seasons, where two-line hybrid rice is widely cultivated. Therefore, it is desirable to develop new parental lines for two-line hybrid rice with disease resistance to rice blast and bacterial blight and tolerance to submergence. The objectives of this study were to introduce *Pi9*, *Sub1A*, *Xa21*, and *Xa27* genes into 9311 genetic background through backcrossing and pyramid the four genes in a single line through marker-assisted selection. Besides for disease resistance and submergence tolerance, the improved rice line and its derived hybrid rice should maintain similar agronomic traits and grain quality to 9311 and the control hybrid rice, respectively.

Materials and methods

Rice cultivars

Cultivar 9311, an elite indica variety, was used as the recipient line for breeding in this study. Wan Hui 21 (WH21) is an *Xa21* introgressive line in Mianhui 725 genetic background (Luo et al. 2012). IR64 (*Sub1ASub1A*) is a near-isogenic line (NIL) of the *Sub1A* gene in IR64 genetic background. 75-1-127 is a *Pi9* introgressive line in IR31917 genetic background (Liu et al. 2002). 1892S and Guangzhan 63S (GZ63S) are two thermosensitive-genic male sterility (TGMS) lines and were used as the maternal lines in two-line hybrid rice production.

PCR-based molecular markers

The molecular marker for the *Pi9* gene was the co-dominant sequence-tagged site (STS) marker NBS2-1 derived from the *Pi9* gene (Luo et al. 2016). The molecular marker for the *Sub1A* gene was the co-dominant cleaved amplified polymorphic sequence (CAPS) marker Sub1A4, which was developed from the *Sub1A* gene. The PCR products of Sub1A4 were digested with BstXI to generate polymorphism between IR64 (*Sub1ASub1A*) and 9311 alleles. The molecular marker for the *Xa21* gene was the co-dominant STS marker 21 derived from the *Xa21* gene (Chen et al. 2000). The molecular marker for the *Xa27* gene was the co-dominant simple sequence repeat (SSR) marker RMXa27, which is located at 35 kb to the upstream of the *Xa27* gene (Luo and Yin 2013). Oligo primers for PCR amplification of the molecular markers are listed in Table S1.

PCR amplification of molecular markers was performed on a PTC-100 programmable thermal controller (MJ Research, USA). The PCR reaction mixture of 20 μl consisted of 10 ng of rice genomic DNA, 0.15 mM of each dNTPs, 0.15 mM of each primer, 2 μl of 10× PCR buffer, and 1 unit of Taq polymerase (QIAGEN, Germany). For PCR amplification of marker RMXa27, 4 μl of 5 × Q-solution (QIAGEN) was added to the PCR reaction mixture. Template DNA was initially denatured at 94 °C for 2 min followed by 35 cycles of PCR amplification with the following parameters: 30 s of denaturation at 94 °C, 40 s of primer annealing at 55 °C for marker 21, 60 °C for markers RMXa27, NBS2-1, and Sub1A4, and 1 to 1.5 min of primer extension at 72 °C according to the marker fragment length. Finally, the reaction mixture was maintained at 72 °C for 5 min before completion. The PCR product of the marker Sub1A4 was digested by BstXI for 3–4 h. The amplified products were resolved on a 1.5% agarose gel for NBS2-1 and Sub1A4, a 2.0% agarose gel for marker 21, and a 3.0% agarose gel for marker RMXa27 in 1× TAE buffer.

Blast inoculation

M. oryzae isolate ZB13, an isolate collected from Anhui, China, was used for inoculation experiments. ZB13 was grown on prune agar medium (40 ml prune juice, 5 g/l lactose, 5 g/l sucrose, 1 g/l yeast extract, 20 g/l agar, pH 6.5) at 28 °C in darkness for 3–5 days and then at 26 °C under constant illumination for 5–6 days for conidia formation. Three-week-old seedlings were inoculated with 20–25 ml of conidia suspension at the density of 1×10^6 spores/ml and containing 0.25% gelatin. The inoculated plants were placed in darkness in a dew chamber for 24 h at 25 °C and 90% of humidity and then moved to a growth chamber and grew under a 12/12 h (light/dark) photoperiod at 25–28 °C and 90% of humidity for 6 days. The disease phenotype was scored at 7 days after inoculation.

Bacterial blight inoculation

X. oryzae pv. *oryzae* strains were grown on PSA medium (10 g/l peptone, 10 g/l sucrose, 1 g/l glutamic acid, 16 g/l bacto-agar, and pH 7.0) at 28 °C for 2 days. The bacterial cells were suspended in sterile water at an optical density of 0.5 (OD600). Bacterial blight inoculation was carried out according to the leaf-clipping method (Kauffman et al. 1973). Disease scoring was measured at 14 days after inoculation according to the method described previously (Gu et al. 2004).

Evaluation for submergence tolerance

Tests of rice lines for submergence tolerance were performed in open water tanks and repeated for two times. For each experiment, 60 17-day-old seedlings planted evenly in three pots were completely submerged in water for 18 days. The plants were photographed before and after submergence treatment. The submergence-treated plants were allowed to recover for 7 days and then were photographed and scored for viability. The plant survival was indicated by having minimum growth of a new green leaf. The statistical analysis was carried out with Duncan's multiple range tests (Duncan 1955).

Field trails and collection of important agronomic traits

To evaluate the performance of rice lines in field condition, four field trials were conducted for 49311 and 9311 in Hefei, China, in the summer seasons of 2014 and 2015 and in Lingshui, China, in the winter seasons of 2013/2014 and 2014/2015. Meanwhile, two field trials were conducted for hybrids in Hefei in the summer seasons of 2014 and 2015. In each field trial, paired parental lines or hybrids were planted near to each other in the plots at the size of 12 m^2. Three repeat plots were planted for each parental line or hybrid rice variety. The major agronomic traits, including growth duration, plant height, productive panicle per square meter, panicle length, total grains per panicle, seed setting rate, 1000-grain weight and yield, were collected from 10 plants grown in each plot and a total of 30 plants were scored for each variety (Luo et al. 2016). Statistical analysis was performed on the function of Microsoft Office Excel 2007 using a two-tailed *t* test for paired samples.

Evaluation of grain quality

The evaluation of rice grain quality was conducted with the rice grains harvested from the four field trials for 49311 and 9311 and the two field trials for hybrid rice. Rice grain quality characteristics, including grain length, ratio of length/width, degree of chalkiness, amylose content, gel consistency, and alkali spreading value, were scored according to the method described previously (Cruz and Khush 2000).

Results

Marker-assisted breeding of 49311

To pyramid the *Pi9*, *Sub1A*, *Xa21*, and *Xa27* genes into 9311 genetic background, we firstly generated backcross lines containing *Pi9*, *Sub1A*, *Xa21*, or *Xa27* in 9311 genetic background by marker-assisted selection using 9311 as the recurrent female parent and 75-1-127 (*Pi9Pi9*), IR64 (*Sub1ASub1A*), WH21 (*Xa21Xa21*), IRBB27 (*Xa27Xa27*) as the donor lines (Fig. S1). In each generation, the genotypes at the *Pi9*, *Sub1A*, *Xa21*, and *Xa27* loci in cross or backcross progeny were determined with molecular markers NBS2-1, Sub1A4, 21, or RMXa27 (data not shown). Theoretically, after 6 rounds of backcrossing, the BC6F1 plants would have possessed 99.2% of the genetic material of the recurrent female parent 9311. In addition, the B6F1 (*Pi9pi9*), B6F1 (*Sub1Asub1A*), B6F1 (*Xa21xa21*), and B6F1 (*Xa27xa27*) plants displayed similar morphological

phenotypes to 9311 (data not shown). To combine *Sub1A* and *Xa27* in a single line, B6F1 (*Sub1Asub1A*) was crossed with B6F1 (*Xa27xa27*) to produce F1-127 (*Sub1Asub1A*, *Xa27xa27*) (Fig. S1). Similarly, to combine *Xa21* and *Pi9* in a single line, B6F1 (*Xa21xa21*) was crossed with B6F1 (*Pi9pi9*) to produce F1-921 (*Pi9pi9*, *Xa21xa21*) (Fig. S1). F1-127 (*Sub1Asub1A*, *Xa27xa27*) was then crossed with F1-921 (*Pi9pi9*, *Xa21xa21*) to pyramid the four genes in a single line (Fig. S1). Eighty-one F1 plants were obtained from the cross and five of them carried heterozygous alleles at the *Pi9*, *Sub1A*, *Xa21*, and *Xa27* loci (Fig. S1). The 5 F1 plants were self-crossed to generate F2 populations containing 786 individuals (Fig. S1). The genotypes of the 786 F2 plants at the *Pi9*, *Sub1A*, *Xa21*, and *Xa27* loci were determined with co-dominant molecular markers NBS2-1, Sub1A4, 21, and RMXa27, respectively (data not shown). Three F2 plants, F2-39 and F2-201 and F2-753, were identified to contain homozygous alleles at the four loci for selection (Fig. S2). They also showed similar morphological phenotype in growth and development to 9311. The F2 plant F2-39 was designated as 49311 (genotype: *Pi9Pi9*, *Sub1ASub1A*, *Xa21Xa21*, *Xa27Xa27*) and selected for further study. In addition to carrying homozygous *Pi9*, *Sub1A*, *Xa21*, and *Xa27* genes, 49311 theoretically still possesses 99.2% genetic background of 9311 after genetic crosses for gene pyramiding.

Evaluation of 49311 and its derived hybrid rice for disease resistance

The *Pi9* donor line 75-1-127 conferred high disease resistance to all 43 isolates from 13 countries (Liu et al. 2002). To evaluate 49311 and its hybrids for disease resistance to rice blast, the seedlings of 75-1-127, 9311, 49311, GZ63S, GZ63S/9311, and GZ63S/49311 were challenged with the *M. oryzae* isolate ZB13. 75-1-127 was highly resistant to ZB13, while 9311, GZ63S, and their hybrid GZ63S/9311 were completely susceptible to the *M. oryzae* isolate (Fig. 1a). No blast lesion was observed on 49311 or its hybrid GZ63S/49311, demonstrating that the *Pi9* gene in 49311 and its hybrid GZ63S/49311 conferred disease resistance to ZB13 (Fig. 1a). Furthermore, compared to 9311 that was susceptible in field condition, 49311 conferred high resistance to rice blast in the field trial in Lingshui, Hainan, China, in the winter season of 2015/2016 (Fig. 1b).

Rice lines 49311, 9311, GZ63S, GZ63S/9311, GZ63S/49311, IRBB27, and WH21 were evaluated for disease resistance to bacterial blight using 27 *X. oryzae* pv. *oryzae* strains collected from 10 countries (Table 1). 9311 was susceptible to 9 *X. oryzae* pv. *oryzae* strains and moderately susceptible to 3 *X. oryzae* pv. *oryzae* strains (Table 1). WH21, carrying *Xa21* in Mianghui 725 genetic background, conferred resistance

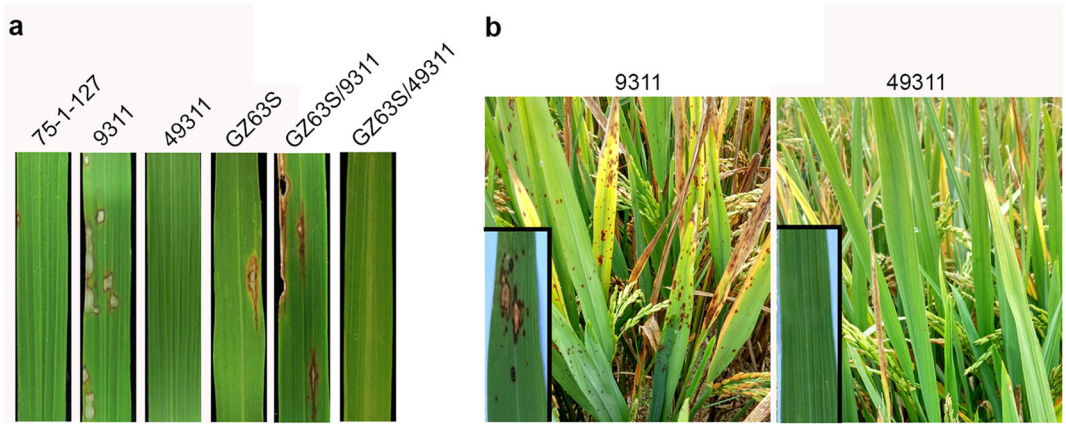

Fig. 1 Disease evaluation for rice blast resistance. **a** Phenotypes of rice blast on rice lines grown in greenhouse. Images were taken at 1 week after inoculation with *M. oryzae* isolate ZB13. 75-1-127, *Pi9* donor line; 9311, recurrent parent; 49311, improved 9311; GZ63S, TGMS line; GZ63S/9311, F1 plants derived from the cross between GZ63S and 9311; GZ63S/49311, F1 plants derived from the cross between GZ63S and 49311. **b** Phenotypes of rice blast on 9311 and 49311 plants grown in fields in Lingshui, Hainan, China, in the winter season of 2015/2016. A typical rice leaf from 9311 with rice blast infection and a healthy leaf from 49311 with no pathogen infection are shown as *insets*, respectively

Table 1 Disease evaluation of rice lines to rice bacterial blight

X. oryzae pv. *oryzae* strain	Origin	WH21	IRBB27	9311	49,311	GZ63S	GZ63S/9311	GZ63S/49311
Aust-2031	Australia	20.1 ± 1.4 (S)	0.1 ± 0.1 (R)	2 ± 0.6 (R)	0.1 ± 0.0 (R)	1 ± 1.2 (R)	0.3 ± 0 (R)	0.3 ± 0.1 (R)
Aust-R3	Australia	15.7 ± 0.7 (S)	0.1 ± 0.0 (R)	2.1 ± 0.9 (R)	0.1 ± 0.0 (R)	0.1 ± 0.1 (R)	0.1 ± 0.0 (R)	0.1 ± 0.0 (R)
GD1358	China	1.9 ± 0.7 (R)	0.1 ± 0.0 (R)	8.4 ± 3.5 (MS)	0.4 ± 0.3 (R)	14 ± 3.9 (S)	17.6 ± 1.7 (S)	0.2 ± 0.1 (R)
HB17	China	4.4 ± 0.9 (MR)	3.5 ± 1.9 (MR)	4.2 ± 1.4 (MR)	0.3 ± 0.2 (R)	2.7 ± 1.7 (R)	4.9 ± 1.6 (MR)	0.2 ± 0.1 (R)
HB21	China	3.0 ± 1.6 (R)	0.1 ± 0.0 (R)	12.8 ± 4.9 (S)	0.2 ± 0.1 (R)	12.3 ± 1.8 (S)	1.2 ± 0.2 (R)	0.1 ± 0.1 (R)
HLJ72	China	3 ± 1.1 (R)	0.2 ± 0.1 (R)	7.4 ± 2 (MS)	0.1 ± 0.0 (R)	1.3 ± 0.3 (R)	0.4 ± 0.4 (R)	0.1 ± 0 (R)
JS49-6	China	4.4 ± 1.8 (MR)	0.1 ± 0.0 (R)	5.6 ± 2.5 (MR)	0.1 ± 0.0 (R)	4.7 ± 2.3 (MR)	3.5 ± 1.8 (MR)	0.2 ± 0.1 (R)
LN57	China	2.2 ± 0.2 (R)	0.1 ± 0.0 (R)	2.9 ± 1.5 (R)	0.1 ± 0.1 (R)	1.6 ± 0.5 (R)	0.1 ± 0.0 (R)	0.1 ± 0.0 (R)
NX42	China	4.4 ± 0.9 (MR)	0.1 ± 0.0 (R)	3.4 ± 0.7 (MR)	0.1 ± 0 (R)	7.8 ± 2.8 (MS)	1.3 ± 0.5 (R)	0.2 ± 0.0 (R)
ZHE173	China	4.5 ± 1.8 (MR)	25.5 ± 5.4 (S)	1.8 ± 1 (R)	0.2 ± 0.1 (R)	2.2 ± 0.7 (R)	3.6 ± 1.2 (MR)	0.3 ± 0.0 (R)
CIAT1185	Columbia	1.6 ± 1.0 (R)	0.1 ± 0 (R)	9.2 ± 3.3 (S)	0.1 ± 0.1 (R)	7.5 ± 2.1 (MS)	12.7 ± 0.9 (S)	0.1 ± 0.1 (R)
A3842	India	19.3 ± 4.0 (S)	0.3 ± 0.2 (R)	11.9 ± 5.3 (S)	0.1 ± 0.1 (R)	21 ± 5.7 (S)	14.1 ± 3.3 (S)	0.2 ± 0.1 (R)
A3857	India	13.5 ± 4.9 (S)	0.1 ± 0.0 (R)	20.4 ± 4.9 (S)	0.2 ± 0.1 (R)	18.4 ± 5.4 (S)	41.5 ± 4.6 (S)	0.1 ± 0.1 (R)
IXO56	Indonesia	4.3 ± 1.2 (MR)	0.3 ± 0.1 (R)	17 ± 2.2 (S)	1.5 ± 0.4 (R)	22.2 ± 3.4 (S)	21.8 ± 2.5 (S)	2.4 ± 1.1 (R)
H75373	Japan	14.6 ± 4.7 (S)	0.1 ± 0.0 (R)	3.2 ± 1.1 (MR)	0.1 ± 0.0 (R)	1.7 ± 0.7 (R)	2.6 ± 1.2 (R)	0.1 ± 0.1 (R)
T7174	Japan	5.9 ± 2.2 (MR)	0.1 ± 0.0 (R)	1.8 ± 1 (R)	0.1 ± 0.0 (R)	0.5 ± 0.3 (R)	0.2 ± 0.0 (R)	0.1 ± 0.0 (R)`
JW89011	Korea	19.1 ± 6.5 (S)	0.1 ± 0.0 (R)	2.1 ± 0.7 (R)	0.1 ± 0.0 (R)	0.2 ± 0.0 (R)	0.1 ± 0.0 (R)	0.1 ± 0.0 (R)
K202	Korea	4.5 ± 1.2 (MR)	28 ± 3.2 (S)	0.7 ± 0.5 (R)	0.5 ± 0.5 (R)	1 ± 0.9 (R)	0.8 ± 0.6 (R)	0.8 ± 0.2 (R)
NXO260	Nepal	11.6 ± 2.0 (S)	0.1 ± 0.1 (R)	19.1 ± 4.1 (S)	0.2 ± 0.1 (R)	25.5 ± 6.2 (S)	27.5 ± 3.4 (S)	2.6 ± 3.1 (R)
PXO86 (R2)	Philippines	2.1 ± 0.7 (R)	0.1 ± 0.1 (R)	12.2 ± 2.4 (S)	0.2 ± 0.1 (R)	15.9 ± 3.7 (S)	18.2 ± 2.9 (S)	1.5 ± 0.4 (R)
PXO79 (R3)	Philippines	1.3 ± 0.4 (R)	0.1 ± 0.1 (R)	10.9 ± 1.6 (S)	0.1 ± 0.1 (R)	10.4 ± 4.3 (S)	13.5 ± 2.4 (S)	1.4 ± 0.2 (R)
PXO71 (R4)	Philippines	2.5 ± 1.4 (R)	5.8 ± 4.3 (MR)	5.3 ± 1.6 (MR)	0.1 ± 0.1 (R)	1.6 ± 0.8 (R)	1.6 ± 1.2 (R)	0.2 ± 0.0 (R)
PXO113 (R4)	Philippines	2.2 ± 1.0 (R)	2.8 ± 0.7 (R)	6 ± 0.6 (MR)	0.2 ± 0.1 (R)	2.6 ± 1.1 (R)	1.2 ± 0.8 (R)	0.2 ± 0.1 (R)
PXO112 (R5)	Philippines	2.2 ± 0.8 (R)	0.4 ± 0.3 (R)	3.6 ± 1.2 (MR)	0.1 ± 0.0 (R)	1 ± 0.7 (R)	0.2 ± 0.1 (R)	0.2 ± 0.1 (R)
PXO99 (R6)	Philippines	9.2 ± 0.8 (S)	0.1 ± 0.1 (R)	23.6 ± 2.1 (S)	0.2 ± 0.1 (R)	15 ± 2.2 (S)	21.4 ± 5.7 (S)	0.2 ± 0.1 (R)
R-7	Thailand	8.1 ± 0.3 (MS)	2.3 ± 2.3 (R)	0.4 ± 0.3 (R)	0.2 ± 0.1 (R)	0.6 ± 0.4 (R)	2.6 ± 0.6 (R)	0.2 ± 0.1(R)
2	Thailand	12.3 ± 4.4 (S)	13.4 ± 8.2 (S)	7.5 ± 1.1 (MS)	0.4 ± 0.1 (R)	4.2 ± 2.6 (MR)	2.6 ± 1.5 (R)	0.3 ± 0.2 (R)

Mean ± SD, *R* resistant, lesion length (LL) ≤ 3.0 cm; *MR* moderately resistant, 3.0 cm < LL ≤ 6.0 cm; *MS* moderately susceptible, 6.0 cm < LL ≤ 9.0 cm; *S* susceptible, LL > 9.0 cm. WH21, *Xa21* donor line; IRBB27, *Xa27* donor line; 9311, recurrent parent; 49311, improved rice line in 9311 genetic background; GZ63S, thermosensitive-genic male sterility line; GZ63S/9311, F1 hybrid rice derived from the cross between GZ63S and 9311; GZ63S/49311, F1 hybrid rice derived from the cross between GZ63S and 49311

to 10 *X. oryzae* pv. *oryzae* strains and moderate resistance to 7 *X. oryzae* pv. *oryzae* strains (Table 1). IRBB27, carrying *Xa27* in IR24 genetic background, was resistant to 22 *X. oryzae* pv. *oryzae* strains and moderately resistant to 2 *X. oryzae* pv. *oryzae* strains (Table 1). 49311, carrying both *Xa21* and *Xa27* in 9311 genetic background, provided high resistance to all 27 *X. oryzae* pv. *oryzae* strains tested (Table 1). The TGMS line GZ63S was susceptible to 9 *X. oryzae* pv. *oryzae* strains and moderately susceptible to 2 *X. oryzae* pv. *oryzae* strains (Table 1). The control hybrid rice GZ63S/9311 was susceptible to 9 *X. oryzae* pv. *oryzae* strains (Table 1). Like 49311, the two-line hybrid rice GZ63S/49311 was highly resistant to all 27 *X. oryzae* pv. *oryzae* strains tested (Table 1). The results demonstrated that 49311 and its two-line hybrid rice provided broad spectrum disease resistance to *X. oryzae* pv. *oryzae* strains.

Test of 49311 and its derived hybrid rice for submergence tolerance

Seventeen-day-old seedlings of IR64 (*Sub1ASub1A*), 9311, 49311, GZ63S, GZ63S/9311, and GZ63S/49311 were evaluated for submergence tolerance (Fig. 2a). After 18 days of submergence treatment, 9311, GZ63S, and GZ63S/9311 turned yellow, while IR64 (*Sub1ASub1A*), 49311, GZ63S/49311 remained in green or light green color (Fig. 2b). Plant viability was scored according to the emergence and growth of new leaves after 7 days of recovery from 18 days of submergence treatment. Most of the 49311 (viability = 85.9 ± 9.2%), GZ63S/49311 (viability = 85.6 ± 7.7%), and IR64 (*Sub1ASub1A*) (viability = 95.3 ± 8.7%) plants survived after 18 days of submergence, while most of the 9311 (viability = 3.1 ± 4.0%), GZ63S (viability = 2.6 ± 4.6%), and their hybrid GZ63S/9311 (viability = 6.1 ± 6.0%) plants died, with a few plants survived but in poor health condition (Fig. 2c, d). The results demonstrated that the *Sub1A* gene in 49311 and its two-line hybrid rice GZ63S/49311 conferred submergence tolerance.

Major agronomic traits of 49311 and its derived hybrid rice

We conducted 4 field trials for 49311 and 9311 and 2 field trials for the two-line hybrid rice lines (1892S/49311, 1892S/9311, GZ63S/49311, and GZ63S/9311). Major agronomic traits were scored from the plants in field trials (Table 2). Both 49311 and 9311 had similar growth duration (Table 2). Similar growth duration was also recorded for hybrid rice lines between 1892S/49311 and 1892S/9311 or between GZ63S/49311 and GZ63S/9311 (Table 2). There was no much difference in plant height between 49311 and 9311, between 1892S/49311 and 1892S/9311, or between GZ63S/49311 and GZ63S/9311 (Table 2). 49311 and 9311 produced similar number of effective panicles per square meter and the length of their panicles was comparable to each other (Table 2). Similarly, there was no significant difference in the number of effective panicles per square meter and in the panicle length between 1892S/49311 and 1892S/9311 or between GZ63S/49311 and GZ63S/9311 (Table 2). 49311 and 9311 had comparable scores in total grains per panicle, filled grains per panicle, seed setting rate, and 1000-grain weight (Table 2). The scores for the four agronomic traits were comparable between 1892S/49311 and 1892S/9311 or between GZ63S/49311 and GZ63S/9311 (Table 2). Finally, similar yields were obtained in field trials between 49311 and 9311, between 1892S/49311 and 1892S/9311, or between GZ63S/49311 and GZ63S/9311 (Table 2). In summary, 49311 and its derived hybrid rice lines had similar major agronomic traits to 9311 and the control hybrid rice, respectively. The results indicated that the introgression and pyramiding of the *Pi9*, *Sub1A*, *Xa21*, and *Xa27* gene in 49311 or its derived hybrid rice lines had no significant influence on their major agronomic traits.

Grain quality of 49311 and its derived hybrid rice

Grain quality of 49311 and its derived hybrid rice was measured with the grains harvested from field trials. Both 49311 and 9311 were characterized as long grain rice based on ratio of length to width, whereas the grains of the four hybrid rice lines (1892S/49311, 1892S/9311, GZ63S/49311, and GZ63S/9311) were classified as medium grain rice (Table 3). The degree of chalkiness was scored as "5" for 49311, 9311, 1892S/49311, and 1892S/9311, whereas it was measured as "1" for GZ63S/49311 and GZ63S/9311, indicating that the latter two rice lines had better grain quality than the former four rice lines in terms of degree of chalkiness (Table 3). Grains from all rice lines had low amylose content and soft gel consistency (Table 3). However, 49311, 9311, GZ63S/49311, and GZ63S/9311 had high alkali spreading value and low gelatinization temperature, whereas

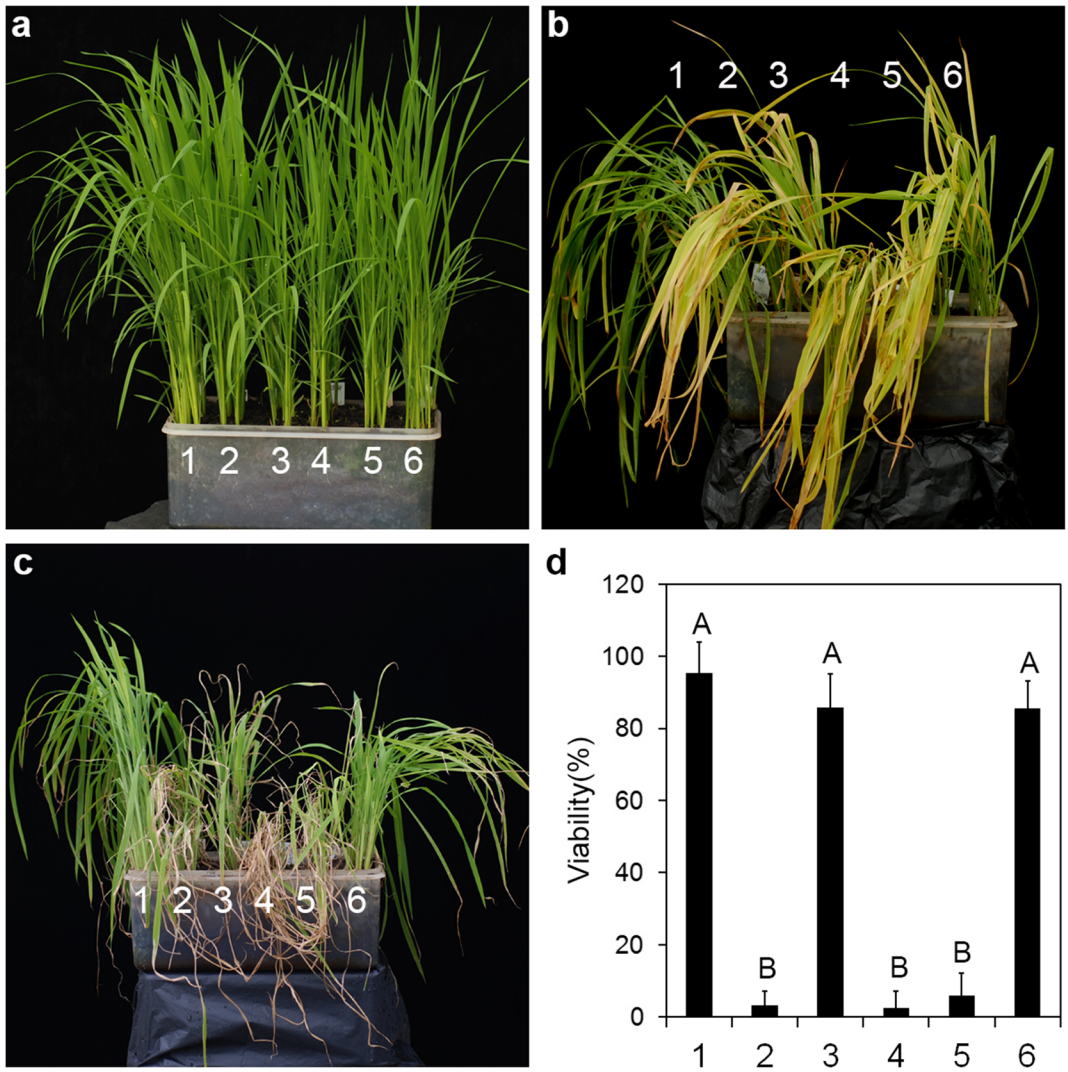

Fig. 2 Test of rice lines for submergence tolerance. **a** Plants at 17 days after germination and before submergence treatment. **b** Plants after 18 days of submergence treatment. **c** Plants after 7 days of recovery from 18 days of submergence treatment. **d** Viability of plants after 7 days of recovery from 18 days of submergence treatment. The data represents mean ± SD (n = 6, 15–22 plants for each variety in each treatment). The different letters (A or B) indicate significant difference between varieties at $P = 0.01$ levels of probability according to Duncan's multiple range tests. *Rice lines*: *1*, IR64 (*Sub1ASub1A*); *2*, 9311; *3*, 49311; *4*, GZ63S; *5*, GZ63S/9311; *6*, GZ63S/49311

1892S/49311 and 1892S/9311 had intermediate alkali spreading values and intermediate gelatinization temperature (Table 3). In all experiments, there were no significant difference in grain quality between 49311 and 9311, between 1892S/49311 and 1892S/9311, or between GZ63S/49311 and GZ63S/9311. The results demonstrated that the introgression and pyramiding of the *Pi9*, *Sub1A*, *Xa21*, and *Xa27* gene in 49311 or its derived two-line hybrid rice lines had no significant influence on their grain quality.

Discussion

Marker-assisted selection is an indirect selection in plant or animal breeding, which is based on a molecular marker co-segregated or linked to the gene that controls the trait of interest. It is an efficient and precise selection system that allows for selection of recessive allele, selection at seedling stage before visible phenotype developed and pyramiding several useful traits in a single line without conducting traditional phenotypic evaluation

Table 2 Major agronomic traits of rice lines

	Inbred rice[a]			Hybrid rice[b]					
				I			II		
Traits	9311	49311	*P* value[c]	1892S/9311	1892S/49311	*P* value	GZ63S/9311	GZ63S/49311	*P* value
Growth duration (days)	141.5 ± 6.4	141.5 ± 4.9	1.00	132.0 ± 1.4	132.5 ± 0.7	0.50	136.5 ± 0.7	137 ± 1.4	0.50
Plant height (cm)	109.3 ± 19.3	115.3 ± 16.9	0.45	125.9 ± 3.1	125.3 ± 2.2	0.76	133.3 ± 5.3	132.9 ± 4.9	0.78
Effective panicles/m^2	182.3 ± 30.0	188.0 ± 32.1	0.63	183.3 ± 13.9	176.6 ± 14.0	0.48	190.3 ± 15.9	192.8 ± 13.4	0.70
Panicle length (cm)	22.0 ± 1.5	22.7 ± 1.6	0.21	24.9 ± 0.7	24.3 ± 0.5	0.08	23.9 ± 1.6	24.8 ± 1.1	0.29
Filled grains/panicle	153.2 ± 19.5	154.9 ± 37.8	0.90	194.3 ± 16.1	199.6 ± 13.6	0.23	177.6 ± 15.9	189.2 ± 14.4	0.33
Total grains/panicle	183.9 ± 33.9	176.8 ± 46.6	0.67	229.0 ± 20.7	234.3 ± 21.3	0.48	235.5 ± 13.4	230.2 ± 15.9	0.45
Seed setting rate (%)	84.6 ± 9.9	88.3 ± 5.8	0.14	84.9 ± 2.1	85.4 ± 4.0	0.78	75.6 ± 7.8	82.2 ± 3.4	0.17
1000-grain weight (g)	29.3 ± 1.1	30.0 ± 1.4	0.22	27.5 ± 1.2	26.6 ± 1.2	0.13	26.2 ± 1.8	27.0 ± 0.7	0.21
Grain yield (t/ha)	8.2 ± 1.1	8.5 ± 1.8	0.59	9.7 ± 0.7	9.4 ± 1.1	0.41	8.8 ± 1.3	9.8 ± 0.6	0.18

Mean ± SD was obtained from 6 or 12 samples with each sample containing 10 plants. Statistical analysis for *P* value was performed on the function in Microsoft Office Excel 2007 using a two-tailed *t* test for paired samples

[a] Twelve samples (n1 = n2 = 12) were collected from 2 field trials in Lingshui, China, in the winter seasons of 2013/2014 and 2014/2015 and 2 field trials in Hefei, China, in the summer seasons of 2015 and 2016)

[b] Six samples (n1 = n2 = 6) were collected from 2 field trials in Hefei, China, in the summer seasons of 2015 and 2016

for each trait. In this study, resistance to rice blast and tolerance to submergence were evaluated with young seedlings. The *Pi9* or *Sub1A* plants could survive after treatment with fungal pathogens or submergence; however, they are weak as compared to the non-treated control plants or need time to recover upon desubmergence. This makes it inefficient and inconvenient to select the two genes in each generation by phenotypic evaluation and selection. Furthermore, the plants cannot be challenged simultaneously with the two kinds of stress. Both *Xa21* and *Xa27* are broad-spectrum *R* genes for bacterial blight resistance. The two *R* genes have different resistance spectrum with overlapping resistance specificity (Gu et al. 2004). Although disease evaluation and selection for either *Xa21* or *Xa27* can be performed by using specific incompatible *X. oryzae* pv. *oryzae* strains for the corresponding *R* genes, it would be inconvenient to do it concurrently for the two *R* genes on individual plants in a large population. In addition, since *Xa21* and *Xa27* differ in perception of pathogens and signalling in activation of downstream disease resistance pathways (Pruitt et al. 2015; Song et al. 1995; Gu et al. 2005), the pyramiding of *Xa21* and *Xa27* in 49311 would not only broaden the resistance spectrum to *X. oryzae* pv. *oryzae* and but also provide durable resistance to the bacterial pathogens. Breeding of rice varieties with multi-resistance to biotic and abiotic stresses have become a major tool as part of integrated management programs for diseases, pests, fertilizers, and water. It leads to a reduction in the usage of pesticides, fungicides, bactericides, chemical fertilizers, and water, and at the same time, the environment and human health are being safeguarded (Zhang 2007).

Rice genetic background may affect the performance of *R* genes for disease resistance to *X. oryzae* pv. *oryzae* (Cao et al. 2007; Luo et al. 2012). For instance, the *Xa21* gene in IRBB21 of IR24 genetic background was resistant to *X. oryzae* pv. *oryzae* strains Aust-2031, Aust-R03, A3857, H75373, PXO99, R-7, and moderately resistant to NXO260 (Gu et al. 2004). However, the *Xa21* gene in WH21 of Mianhui 725 genetic background was susceptible to Aust-2031, Aust-R03, A3857, H75373, PXO99, NXO260, and moderately susceptible to R-7 in this study (Table 1). The resistance spectrum of 49311 or GZ63S/49311 to *X. oryzae* pv. *oryzae* covered both *Xa21* and *Xa27* resistance specificities (Table 1). The result indicates that the performance of *Xa21* and *Xa27* in 49311 or GZ63S/49311 was not affected by the genetic backgrounds of 9311 and GZ63S. The additional resistance specificity of 49311 or GZ63S/49311 to Thailand 2 might be derived from

Table 3 Grain quality of rice lines

	Inbred rice[a]		Hybrid rice[b]			
			I		II	
Trait	9311	59,311	1892S/9311	1892S/49311	GZ63S/9311	GZ63S/49311
GL[c] (mm)	6.7 ± 0.2 (Long)	6.7 ± 0.2 (Long)	6.4 ± 0.1 (Medium)	6.4 ± 0.2 (Medium)	6.5 ± 0.2 (Medium)	6.5 ± 0.1 (Medium)
L/W ratio[d]	2.8 ± 0.5 (Medium)	2.9 ± 0.1 (Medium)	2.9 ± 0.1 (Medium)	2.9 ± 0.1 (Medium)	3.0 ± 0.2 (Medium)	3.0 ± 0.2 (Medium)
DC[e] (%)	17.1 ± 12.2 (5)	18.7 ± 12.7 (5)	15.0 ± 14.7 (5)	15.1 ± 15.8 (5)	7.8 ± 3.3 (1)	7.6 ± 6.0 (1)
AC[f] (%)	16.7 ± 1.8 (Low)	17.4 ± 2.0 (Low)	15.9 ± 0.3 (Low)	15.8 ± 0.5 (Low)	17.3 ± 1.0 (Low)	16.5 ± 0.7 (Low)
GC[g] (mm)	63.8 ± 34.1 (Soft)	67.5 ± 4.0 (Soft)	79.0 ± 1.4 (Soft)	84.0 ± 1.4 (Soft)	83.5 ± 10.6 (Soft)	84.5 ± 20.5 (Soft)
ASV and GT[h]	6.1 ± 0.1 (Low)	6.6 ± 0.5 (Low)	4.5 ± 0.7 (Intermediate)	4.7 ± 0.9 (Intermediate)	6.2 ± 0.3 (Low)	6.2 ± 0.3 (Low)

[a] The grain quality of inbred rice was examined for 4 times with the rice seeds harvested from 2 field trials in Lingshui, China, in the winter seasons of 2013/2014 and 2014/2015 and 2 field trials in Hefei, China, in the summer seasons 2015 and 2016

[b] The grain quality of hybrid rice was examined for 2 times with the rice seeds harvested from 2 field trials in Hefei, China, in the summer seasons of 2015 and 2016

[c] *GL* grain length. Category of grain length: short, grain length ≤ 5.5 mm; medium, 5.5 mm < grain length ≤ 6.6 mm; long, 6.6 mm < grain length ≤ 7.5 mm); very long, grain length > 7.5 mm

[d] *L/W ratio* ratio of length to width (L/W). Grain shape based on L/W ratio: bold, L/W ratio ≤ 2.0, medium, 2.0 < L/W ratio ≤ 3.0; slender, L/W ratio > 3.0

[e] *DC* degree of chalkiness. Scale for DC: 9, DC > 20%; 5, 10% < DC ≤ 20%; 1, 0 < DC ≤ 10%; 0, DC = 0

[f] *AC* amylose content. Classification of AC: high, AC > 25%; intermediate, 20% < AC ≤ 25%; low, 10% < AC ≤ 20%; very low, 2% < AC ≤ 10%; waxy, AC ≤ 2%

[g] *GC* gel consistency. Classification of GC: hard, GC ≤ 40 mm; medium, 40 mm < GC ≤ 60 mm; soft, GC > 60 mm

[h] *ASV* alkali spreading value, *GT* gelatinization temperature, grade of GT estimated by ASV: low (GT < 70 °C), 5.5 ≤ ASV ≤ 7; intermediate (70 °C ≤ GT < 74 °C), 3.5 ≤ ASV < 5.5; intermediate high (74 °C ≤ GT < 74.5 °C), 2.5 ≤ ASV < 3.5; high (74.5 °C ≤ GT < 80 °C), 1 ≤ ASV < 2.5

9311 or GZ63Z as they showed either moderate resistance or moderate susceptibility to Thailand 2 (Table 1).

It is important that crop genotypes and the genes to be employed are compatible for a successful pyramiding strategy. Both 49,311 and 9311 had similar major agronomic traits in field trials, so did their derived hybrid rice lines. The results indicate that the multi-gene incorporation in 49311 did not cause deleterious effect. In fact, earlier reports suggested that rice is compliant to be improved with pyramiding strategy for effective, stable, and inheritable disease resistance without any penalty for yield or grain quality (Chen et al. 2000; Luo and Yin 2013).

In summary, we have introduced and pyramided the *Pi9*, *Sub1A*, *Xa21*, and *Xa27* genes in rice cultivar 9311, which has high yield and good grain quality. The improved rice line 49311 provided disease resistance to rice blast and rice bacterial blight and tolerance to submergence. Furthermore, the introgression and pyramiding of the *Pi9*, *Sub1A*, *Xa21*, and *Xa27* genes in 49311 or its derived two-line hybrid rice had no significant influence on their yield and grain quality. The development of 49311 provides an improved paternal line for two-line hybrid rice production.

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