

Research Paper

# Marker-Assisted Breeding of Thermo-Sensitive Genic Male Sterile Line 1892S for Disease Resistance and Submergence Tolerance



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**Abstract:** Rice line 1892S is an elite thermo-sensitive genic male sterile (TGMS) line for two-line hybrid rice production. However, 1892S is susceptible to rice blast, bacterial blight and submergence. Here we reported the introduction of blast resistance (*R*) gene *Pi9*, bacterial blight *R* gene *Xa21* and submergence tolerance gene *Sub1A* into 1892S genetic background through backcrossing and marker-assisted selection. The improved TGMS line 31892S and its hybrids conferred disease resistance to rice blast and bacterial blight, and showed submergence tolerance for over 14 d without significant loss of viability. The sterility-fertility conversion of 31892S was similar to that of 1892S. 31892S and its derived hybrid rice had similar agronomic traits and grain quality with 1892S and the control hybrid rice, respectively. The newly developed 31892S provided an improved TGMS line for two-line hybrid rice production with disease resistance to rice blast and bacterial blight, and submergence tolerance with no yield penalty or change in grain quality.

**Key words:** thermo-sensitive genic male sterility; bacterial blight; rice blast; submergence tolerance; disease resistance; marker-assisted selection

Rice (*Oryza sativa* L.) is a major staple food for approximately one-half of the world's population. However, rice plantation is frequently affected by biotic and abiotic stresses, which cause severe yield loss and have an adverse impact on food security. Rice blast, caused by the fungus *Magnaporthe oryzae*, is the most devastating fungal disease in rice plantation in the world. The fatal leaf and panicle blast can even cause yield loss between 10% and 30% with high incidence of infected crops (Sakthivel et al, 2009). To counteract *M. oryzae* infection, rice and its wild species have evolved disease resistance (*R*) genes. The rice blast *R* gene *Pi9* in line 75-1-127 was introgressed

from the wild species *Oryza minuta* (Liu et al, 2002). *Pi9* shows high and broad-spectrum resistance to 43 *M. oryzae* isolates collected from 13 countries (Liu et al, 2002), and 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, 2017). Bacterial blight of rice, caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), is one of the important bacterial diseases that is prevalent throughout the major rice-growing countries, especially in Asia (Gnanamanickam et al, 1999). Plants infected at the maximum tillering stage could have 50% yield reduction (Mew et al, 1993). Rice and its wild relatives have evolved dominant or recessive *R*

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genes that confer disease resistance to bacterial blight. *Xa21* is a dominant *R* gene that provides broad-spectrum resistance to *Xoo* strains (Ikeda et al, 1990), which has been widely deployed in rice breeding for bacterial blight resistance since late 1990s (Huang et al, 1997; Zhang et al, 2006; Perez et al, 2008; Luo et al, 2012, 2014, 2016).

Flood and drought are two major abiotic stresses that adversely affect rice production in Asian countries. Rice variety FR13A was identified to confer submergence tolerance for up to two weeks at the seedling stage (Xu and Mackill, 1996). A major locus *Sub1A* for submergence tolerance in FR13A has been cloned (Xu et al, 2006). The mechanism of *Sub1A* for submergence tolerance is to dampen ethylene production and gibberellic acid responsiveness during submergence, so as to economize carbohydrate reserves and to prolong endurance of rice plants (Xu et al, 2006). Interestingly, *Sub1A* also enhances 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* locus from FR13A has been introduced into popular rice varieties, including Swarna, KDML105, Mianhui 725, IR64 and 9311 through marker-assisted selection (Neeraja et al, 2007; Septiningsih et al, 2009; Luo and Yin, 2013; Luo et al, 2016, 2017). The improved rice lines with the *Sub1A* locus are tolerant to submergence and maintain high yield, good grain quality and other elite agronomic properties of the recurrent parents.

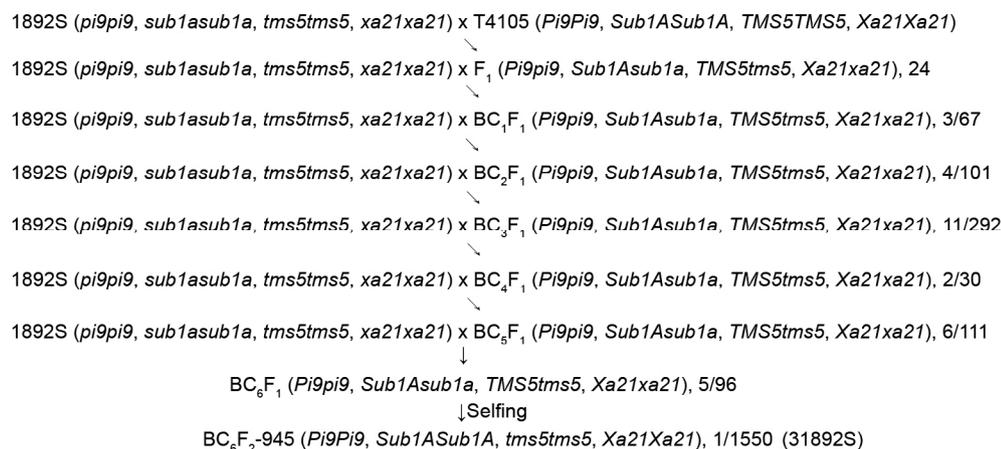
The development of hybrid rice, known as the second ‘green revolution’ in China, has contributed significantly to rice production and food security in China for over 40 years (Yuan, 2014). So far, two systems on hybrid rice seed production have been developed and successfully commercialized. The three-line hybrid rice system includes a cytoplasmic male sterile (CMS) line, a maintainer line and a restorer line for a commercial production of rice hybrids. The CMS line is used as a maternal line to cross with the restorer line as a pollen donor for hybrid seed production. The maintainer line maintains the sterility of the CMS line and is used to produce the seeds of the CMS line. The two-line hybrid rice system has only two parental lines, a photoperiod-sensitive genic male sterile (PGMS) line or a thermo-sensitive genic male sterile (TGMS) line as the maternal line and the pollen donor as the paternal line. Unlike a CMS line in three-line hybrid rice system that requires

a maintainer line for its propagation, a PGMS or TGMS line can be propagated by self-pollination under permissive conditions of photo and/or temperature combinations. In addition, as the male sterile trait of a PGMS or TGMS line is controlled by a single recessive nuclear gene, any conventional rice varieties can be used as pollen donors to produce F<sub>1</sub> hybrids. Therefore, the two-line hybrid rice technology simplifies the system for hybrid rice seed production, increases the probability of identifying heterotic hybrid combination, especially in yield and grain quality, and saves the cost on hybrid seed production. Annong S-1 is an *indica* TGMS rice line that carries male sterile gene *tms5* (Deng et al, 1999). The *tms5* gene was cloned in 2014 (Zhou et al, 2014). The elite TGMS line 1892S also carries the *tms5* gene (Zhou et al, 2014). 1892S has been used as the maternal line for more than 25 two-line hybrid rice varieties released in China, which had total plantation area of more than 1.696 million hectares during 2006–2014 (Yang et al, 2016). However, 1892S is susceptible to most of the isolates or strains of rice blast or bacterial blight pathogens (Song et al, 2016). Our unpublished preliminary study also indicated that, like most of rice varieties, 1892S is susceptible to submergence. Here we reported the development of an improved TGMS line by pyramiding *Pi9*, *Sub1A* and *Xa21* into 1892S genetic background for disease resistance to rice blast and bacterial blight and submergence tolerance through backcrossing breeding and marker-assisted selection.

## RESULTS

### Marker-assisted breeding of 31892S

To introduce and pyramid the *Pi9*, *Sub1A* and *Xa21* genes in 1892S genetic background, 1892S was used as the female to cross with donor line T4105, carrying *Pi9*, *Sub1A* and *Xa21* genes in KDML105 genetic background, followed by backcrossing using 1892S as the recurrent female (Fig. 1). Marker-assisted selection was conducted to select positive F<sub>1</sub> and backcross plants at each generation (Fig. 1 and Table S1). In summary, 24 F<sub>1</sub>, 3 BC<sub>1</sub>F<sub>1</sub>, 4 BC<sub>2</sub>F<sub>1</sub>, 11 BC<sub>3</sub>F<sub>1</sub>, 2 BC<sub>4</sub>F<sub>1</sub>, 6 BC<sub>5</sub>F<sub>1</sub> and 5 BC<sub>6</sub>F<sub>1</sub> plants were identified to carry heterozygous alleles at the *Pi9*, *Sub1A* and *Xa21* loci (Fig. 1). The 5 BC<sub>6</sub>F<sub>1</sub> plants were self-pollinated and produced a BC<sub>6</sub>F<sub>2</sub> population with 1 550 individuals. Genotyping analysis demonstrated that one BC<sub>6</sub>F<sub>2</sub> plant (BC<sub>6</sub>F<sub>2</sub>-945) carried homozygous alleles at the *Pi9*, *Sub1A*, *tms5* and *Xa21* loci (Fig. 1). Subsequently, the BC<sub>6</sub>F<sub>2</sub>-945 plant was designated as 31892S. The



**Fig. 1. Marker-assisted breeding of 31892S.**

The donor line T4105 is a BC<sub>4</sub>F<sub>2</sub> plant containing homozygous *Pi9*, *Sub1A* and *Xa21* genes in KDML105 genetic background. The number of total plants screened and the number of positive plants identified are indicated in each generation. Only one plant, BC<sub>6</sub>F<sub>2</sub>-945, was identified from 1 550 BC<sub>6</sub>F<sub>2</sub> individuals to contain homozygous alleles at the *Pi9*, *Sub1A*, *tms5* and *Xa21* loci. BC<sub>6</sub>F<sub>2</sub>-945 was designated as 31892S.

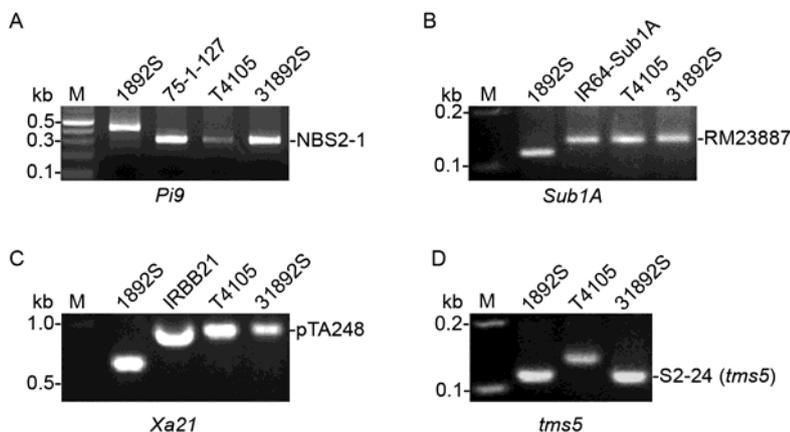
patterns of molecular markers for the *Pi9*, *Sub1A* and *Xa21* loci in 3189S were similar to those in T4105 and the corresponding original donor lines, 75-1-127, IR64-Sub1A and IRBB21 (Fig. 2-A to -C). 31892S also carried homozygous *tms5* genes from 1892S (Fig. 2-D) and showed sterile phenotype at restrictive conditions. 31892S and 1892S had similar morphological phenotype and growth duration. 31892S was allowed to set seeds at permissive conditions in the autumn season in Hefei, Anhui Province or in the winter season in Lingshui Hainan Province, China for further study.

**Disease resistance of 31892S and its F<sub>1</sub> hybrids against rice blast and bacterial blight**

To evaluate 31892S and its F<sub>1</sub> hybrids for rice blast resistance, the seedlings of rice lines 75-1-127, 1892S, 31892S, RH003, and F<sub>1</sub> hybrids 1892S/RH003 and 31892S/RH003 were challenged with two field *M. oryzae* isolates, 11-17-1-2-2 and 11-3-1-1-2. 75-1-127 and 31892S were resistant to the two isolates, whereas 1892S was susceptible to both *M. oryzae* isolates (Fig. 3-A and -B). As for the F<sub>1</sub> hybrids, the control 1892S/RH003 was susceptible to 11-17-1-2-2, whereas 31892S/

RH003 was resistant to the *M. oryzae* isolate (Fig. 3-A). As RH003, the paternal line of the F<sub>1</sub> hybrids, was resistant to 11-3-1-1-2, and both 1892S/RH003 and 31892S/RH003 F<sub>1</sub> hybrids conferred disease resistance to the *M. oryzae* isolate (Fig. 3-B). 31892S also showed disease resistance to rice blast at the maturity stage in field, while neck blast infection was observed in 1892S plants (Fig. 3-C and -D). The results collectively demonstrated that the *Pi9* gene in 31892S and its F<sub>1</sub> hybrid 31892S/RH003 conferred disease resistance to rice blast.

31892S and its F<sub>1</sub> hybrids were tested for bacterial blight resistance with 24 *Xoo* strains collected from 9 countries. The original *Xa21* donor line IRBB21 conferred resistance or moderate resistance to 21 strains. 1892S was susceptible or moderately susceptible to 18 strains (Table 1). RH003 was susceptible or moderately susceptible to 20 strains. The control F<sub>1</sub> hybrid 1892S/RH003 was susceptible or moderately susceptible to 20 strains. Collectively, among the 24 *Xoo* strains tested, 18 strains could cause susceptible or moderately susceptible phenotypes on 1892S, RH003 and 1892S/RH003. The improved line 31892S was resistant or moderately



**Fig. 2. Genotyping of molecular markers at the selected loci in 31892S.**

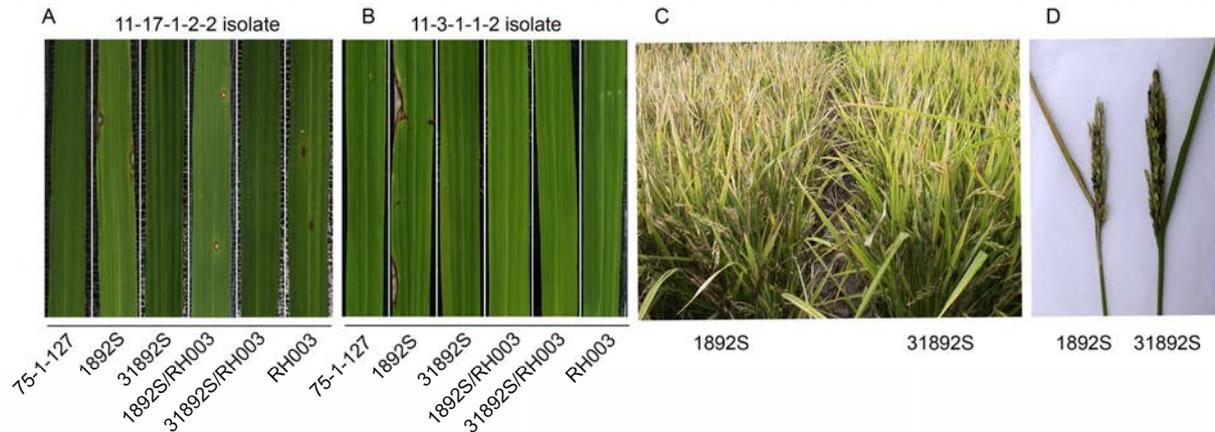
A, Genotyping of sequence-tagged site (STS) marker NBS2-1 at the *Pi9* gene. 75-1-127 is the original donor line of the *Pi9* gene.

B, Genotyping of simple sequence repeat (SSR) marker RM23887 closely linked to the *Sub1A* gene. IR64-Sub1A is the original donor line of the *Sub1A* gene.

C, Genotyping of STS marker pTA248 co-segregated with the *Xa21* gene. IRBB21 is the original donor line of the *Sub1A* gene.

D, Genotyping of SSR marker S2-24 closely linked to the *tms5* gene in 1892S.

M, Marker.



**Fig. 3. Disease evaluation of rice lines for resistance to rice blast.**

**A and B**, Disease evaluation of rice lines to rice blast. Two-week-old rice seedlings were inoculated with *M. oryzae* isolates 11-17-1-2-2 (**A**) and 11-3-1-1-2 (**B**). The images were taken at 7 d after blast inoculation.

**C**, Phenotypes of 1892S and 31892S in field. Image was taken at 30 d after heading in the field trial in Hefei, Anhui Province, China, in the summer season of 2017.

**D**, A typical flag leaf and panicle of 1892S with neck blast infection and a healthy flag leaf and panicle of 31892S with no blast infection.

75-1-127, *Pi9* introgressive line; 1892S, Thermo-sensitive genic male sterile (TGMS) line; 31892S, Improved TGMS line in 1892S genetic background; RH003, Paternal line used for hybrid  $F_1$  production; 1892S/RH003,  $F_1$  hybrid derived from the cross between 1892S and RH003; 31892S/RH003,  $F_1$  hybrid derived from the cross between 31892S and RH003.

resistant to 17 of the 18 1892S-compatible *Xoo* strains, and the improved  $F_1$  hybrid 31892S/RH003 conferred resistance or moderate resistance to 23 of the 24 *Xoo* strains tested, including the 18 compatible *Xoo* strains on 1892S, RH003 and 1892S/RH003 (Table 1). The results demonstrated that the *Xa21* gene in 31892S and its  $F_1$  hybrid 31892S/RH003 provided broad spectrum resistance to *Xoo* strains.

### Submergence tolerance of 31892S and its $F_1$ hybrids

Fourteen-day-old seedlings of IR64-Sub1A, 1892S, 31892S, RH003, 1892S/RH003 and 31892S/RH003 were tested for submergence tolerance (Fig. 4-A). After 14 d of complete submergence, 1892S, RH003 and 1892S/RH003 plants turned yellow, while IR64-Sub1A, 31892S and 31892S/RH003 plants maintained green or light green (Fig. 4-B). Plant viability was evaluated based on the emergence and growth of new leaves over 7 d of recovery from the 14 d of complete submergence. Most of the IR64-Sub1A ( $85.6\% \pm 5.1\%$ ), 31892S ( $85.6\% \pm 13.5\%$ ) and 31892S/RH003 ( $78.9\% \pm 12.6\%$ ) plants survived from the 14 d of submergence, while most of the 1892S ( $4.4\% \pm 1.9\%$ ), RH003 ( $8.9\% \pm 1.9\%$ ) and 1892S/RH003 ( $6.7\% \pm 0.0\%$ ) plants died (Fig. 4-C and -D). The results indicated that the *Sub1A* gene in 31892S and its  $F_1$  hybrids conferred tolerance to submergence.

### Sterility-fertility alteration of 31892S

Sterility-fertility alteration of spikelets on panicles of

31892S and 1892S plants was examined with bagged panicles during the summer season of 2017 in Hefei, China. The spikelets on panicles of 31892S were completely sterile when the panicles were bagged on 27 July 2017 to 7 September 2017 (Fig. 5-A). Spikelet fertility was partially resumed when the panicles were bagged on 10–19 September 2017 (Fig. 5-A). Therefore, the stable period of complete male sterility of 31892S was at least 43 d, which was similar to the bagged panicles of 1892S (Fig. 5-B). After sterility-fertility alteration, the spikelet fertility of the bagged panicles of 31892S ranged from  $11.1\% \pm 7.2\%$  to  $24.7\% \pm 11.1\%$ , which was similar to that of 1892S (from  $11.2\% \pm 6.5\%$  to  $21.8\% \pm 7.1\%$ ) (Fig. 5-A and -B). No pollen grain was observed in the spikelets of 31892S or 1892S before sterility-fertility alteration, and no filled seed was set on the panicles when they were grown under restrictive conditions (Fig. 5-C to -E). Fertile pollen grains were observed in the panicles of 31892S and 1892S after sterility-fertility alteration as the temperature dropped to permissive levels (Fig. 5-F and -G). Both 31892S and 1892S had normal spikelet fertility and similar seed-setting rate when grown in the winter season in Lingshui, China (Fig. 5-H). In summary, the fertility-sterility alteration of 31892S was similar to that of 1892S.

### Agronomic traits and grain quality of 31892S and its $F_1$ hybrids

Three field trials were conducted for 1892S and 31892S

**Table 1. Disease evaluation of IRBB21, 1892S, 31892S and F<sub>1</sub> hybrids to rice bacterial blight.**

Strain	Origin	IRBB21	1892S	31892S	RH003	1892S/RH003	31892S/RH003
Aust-2031	Australia	2.4 ± 0.6 (R)	6.0 ± 1.3 (MR)	5.1 ± 1.4 (MR)	3.8 ± 1.5 (MR)	5.0 ± 2.3 (MR)	4.3 ± 1.0 (MR)
Aust-R3	Australia	2.7 ± 0.4 (R)	1.0 ± 0.3 (R)	0.8 ± 0.3 (R)	0.7 ± 0.3 (R)	0.8 ± 0.2 (R)	1.4 ± 0.3 (R)
GD1358	China	2.6 ± 0.7 (R)	11.2 ± 2.5 (S)	4.6 ± 0.7 (MR)	7.7 ± 2.8 (MS)	9.4 ± 2.9 (S)	5.2 ± 1.4 (MR)
HB17	China	2.3 ± 0.5 (R)	17.8 ± 3.7 (S)	2.5 ± 0.9 (R)	23.1 ± 4.0 (S)	24.0 ± 4.4 (S)	3.1 ± 2.1 (MR)
HB21	China	2.2 ± 0.3 (R)	20.9 ± 4.5 (S)	4.4 ± 1.0 (MR)	20.4 ± 4.1 (S)	24.7 ± 1.9 (S)	3.8 ± 1.0 (MR)
HLJ72	China	2.0 ± 0.5 (R)	3.2 ± 0.7 (MR)	2.6 ± 0.4 (R)	9.1 ± 2.5 (S)	11.5 ± 3.2 (S)	3.4 ± 0.8 (MR)
JS49-6	China	1.7 ± 0.4 (R)	16.6 ± 2.7 (S)	1.5 ± 0.6 (R)	14.3 ± 3.0 (S)	19.2 ± 3.3 (S)	3.5 ± 1.6 (MR)
LN57	China	3.3 ± 0.6 (MR)	13.1 ± 2.7 (S)	1.4 ± 0.5 (R)	15.4 ± 3.6 (S)	18.4 ± 2.1 (S)	3.1 ± 1.1 (MR)
NX42	China	2.0 ± 0.7 (R)	17.5 ± 2.9 (S)	1.6 ± 0.8 (R)	19.2 ± 3.7 (S)	21.6 ± 4.9 (S)	3.8 ± 0.9 (MR)
ZHE173	China	1.6 ± 0.6 (R)	14.2 ± 2.7 (S)	1.1 ± 0.4 (R)	14.9 ± 2.3 (S)	13.4 ± 2.8 (S)	2.7 ± 0.6 (MR)
CIAT1185	Columbia	2.1 ± 1.0 (R)	16.5 ± 2.0 (S)	5.6 ± 1.3 (MR)	17.3 ± 6.1 (S)	15.6 ± 3.2 (S)	5.9 ± 1.5 (MR)
IXO56	Indonesia	4.2 ± 0.9 (MR)	15.1 ± 1.6 (S)	4.8 ± 2.3 (MR)	17.6 ± 3.7 (S)	16.3 ± 2.0 (S)	5.6 ± 1.3 (MR)
H75373	Japan	2.0 ± 0.4 (R)	10.6 ± 1.8 (S)	1.1 ± 0.2 (R)	20.9 ± 3.7 (S)	18.9 ± 3.8 (S)	1.5 ± 0.5 (R)
T7174	Japan	1.9 ± 0.7 (R)	0.7 ± 0.4 (R)	1.2 ± 0.4 (R)	18.8 ± 4.3 (S)	17.9 ± 1.9 (S)	2.2 ± 0.4 (R)
JW89011	Korea	21.9 ± 1.9 (S)	0.8 ± 0.4 (R)	1.3 ± 0.4 (R)	1.1 ± 0.4 (R)	1.5 ± 0.8 (R)	1.1 ± 0.3 (R)
K202	Korea	3.0 ± 1.1 (R)	12.2 ± 2.4 (S)	0.6 ± 0.2 (R)	16.4 ± 5.1 (S)	18.8 ± 2.7 (S)	1.8 ± 0.9 (R)
PXO86 (R2)	the Philippines	1.2 ± 0.4 (R)	16.8 ± 1.9 (S)	4.6 ± 1.7 (MR)	17.1 ± 2.3 (S)	21.9 ± 0.8 (S)	5.5 ± 1.9 (MR)
PXO79 (R3)	the Philippines	1.7 ± 0.4 (R)	16.9 ± 2.1 (S)	4.8 ± 0.8 (MR)	11.9 ± 2.5 (S)	14.0 ± 3.2 (S)	4.2 ± 0.9 (MR)
PXO71 (R4)	the Philippines	2.5 ± 0.6 (R)	15.7 ± 2.9 (S)	1.4 ± 0.6 (R)	21.1 ± 3.0 (S)	21.7 ± 2.4 (S)	2.4 ± 0.7 (R)
PXO113 (R4)	the Philippines	2.5 ± 0.7 (R)	17.6 ± 2.9 (S)	2.0 ± 0.6 (R)	18.8 ± 1.6 (S)	21.1 ± 1.7 (S)	2.2 ± 1.0 (R)
PXO112 (R5)	the Philippines	3.3 ± 0.6 (MR)	4.0 ± 0.7 (MR)	0.7 ± 0.4 (R)	5.2 ± 1.4 (MR)	4.3 ± 2.1 (MR)	1.2 ± 0.3 (R)
2	Thailand	12.6 ± 2.6 (S)	12.1 ± 2.4 (S)	4.4 ± 1.0 (MR)	16.1 ± 2.6 (S)	8.9 ± 2.6 (MS)	6.0 ± 1.2 (MR)
R-7	Thailand	2.4 ± 0.5 (R)	20.4 ± 4.1 (S)	4.7 ± 1.0 (MR)	30.1 ± 5.1 (S)	25.4 ± 4.2 (S)	4.0 ± 1.8 (MR)
1947	Cameroon	18.2 ± 3.7 (S)	6.8 ± 2.1 (MS)	8.6 ± 2.5 (MS)	9.0 ± 3.2 (MS)	11.3 ± 4.1 (S)	19.8 ± 5.2 (S)

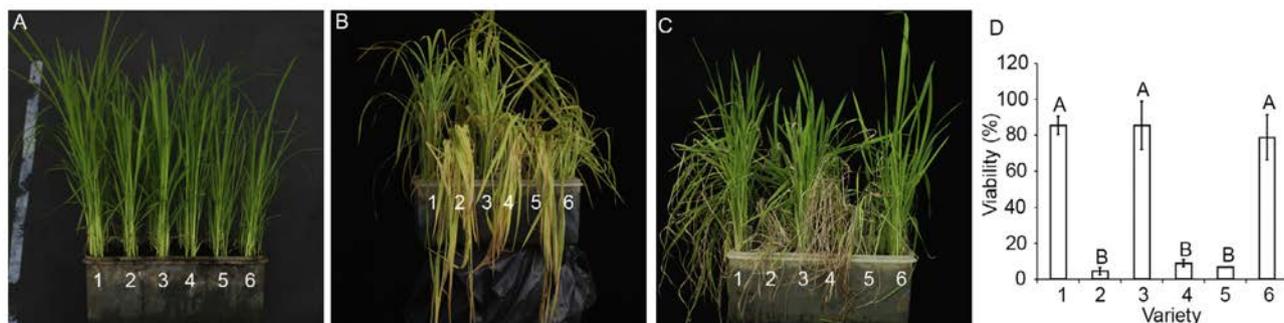
R, Resistance (lesion length ≤ 3.0 cm); MR, Moderate resistance (3.0 cm < lesion length ≤ 6.0 cm); MS, Moderately susceptible (6.0 cm < lesion length ≤ 9.0 cm); S, Susceptible (lesion length > 9.0 cm).

The lesion length of bacterial blight is shown in Mean ± SD (n = 15).

with one in the summer season of 2017 in Hefei, China, and two in the winter seasons of 2015/2016 and 2016/2017 in Lingshui, China. Meanwhile, two field trials were conducted for the F<sub>1</sub> hybrids, 1892S/RH003 and 31892S/RH003, in the summer seasons of 2016 and 2017 in Hefei, China. Important agronomic traits were collected from the plants in the field trials, and grain quality was measured. 31892S and 1892S had similar growth duration, plant height, productive panicle number

per plant, panicle length and total number of spikelets per panicle in all field trials under either restrictive or permissive conditions (Table 2). There was no significant difference in spikelet fertility, 1000-grain weight and yield when they were grown under permissive conditions (Table 2). The results indicated that 31892S and 31892S/RH003 had similar important agronomic traits to 1892S and 1892S/RH003, respectively (Table 2).

The grain quality of TGMS lines and hybrid rice (F<sub>2</sub>



**Fig. 4. Evaluation of rice lines for submergence tolerance.**

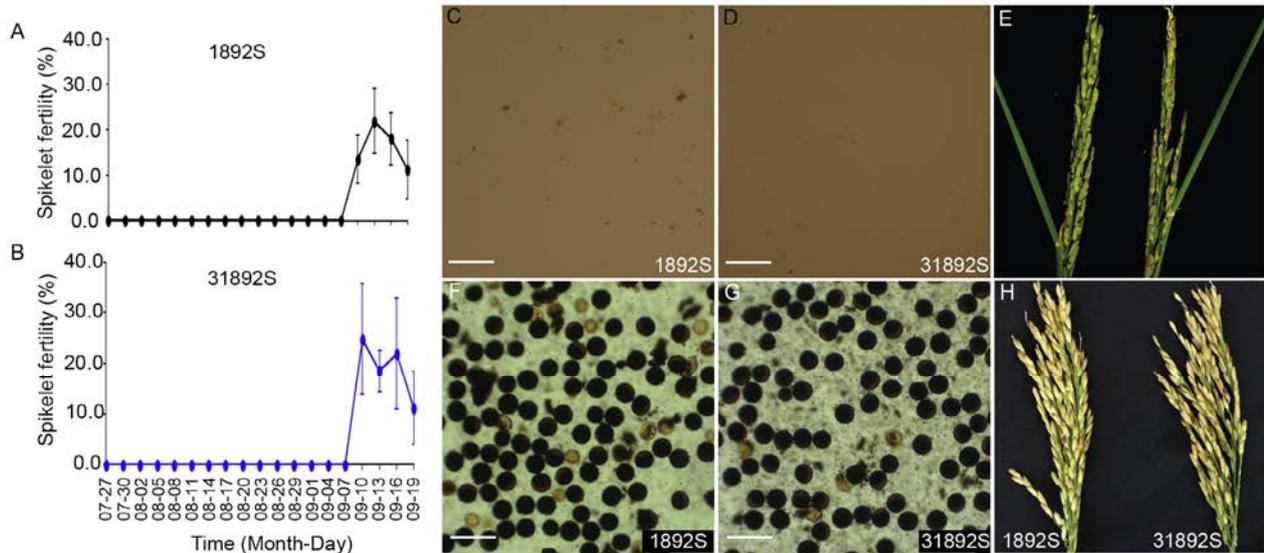
A, Phenotypes of plants at 14 d after germination and before submergence treatment.

B, Phenotypes of plants after 14 d of submergence treatment.

C, Phenotypes of plants after 7 d of recovery from submergence treatment.

D, Viability of plants after 7 d of recovery from submergence treatment. The data represent Mean ± SD (n = 3, about 30 plants for each line in each treatment). The different capital letters indicate significant difference among rice lines at the 0.01 level according to the Duncan’s multiple range tests.

1, *Sub1A* donor line IR64-Sub1A (genotype: *Sub1ASub1A*); 2, 1892S; 3, 31892S; 4, RH003; 5, 1892S/RH003; 6, 31892S/RH003.



**Fig. 5. Sterility-fertility conversion of 1892S and 31892S.**

**A and B**, Spikelet fertility of bagged panicles on 1892S (**A**) and 31892S (**B**) plants at different bagged time in 2017.

**C and D**, Pollen grains of 1892S (**C**) and 31892S (**D**) plants grown under restrictive conditions with panicles flowering on 14 August, 2017.

**E**, Panicles of 1892S and 31892S plants grown under restrictive conditions in Hefei, China with panicles flowering on 14 August 2017.

**F and G**, Pollen grains of 1892S (**C**) and 31892S (**D**) after sterility-fertility alteration with panicles flowering on 18 September 2017 in Hefei, China.

**H**, Panicles of 1892S and 31892S plants grown under permissive conditions in the winter season of 2016/2017 in Lingshui, China.

In **C**, **D**, **F** and **G**, Pollen grains were stained with 1% I<sub>2</sub>-KI solution. Scale Bars, 100 μm.

grains) were measured. Both 31892S and 1892S were characterized as medium grain rice based on ratio of length to width (Table 3). They had very low degree of chalkiness (DC) and low amylose content (AC) (Table 3). They were also classified as soft rice with low gel consistency (GC) and intermediate alkali spreading value (ASV) (Table 3). The results indicated that 31892S retains similar grain quality to 1892S. In fact, the quality of the F<sub>2</sub> grains harvested from the F<sub>1</sub> hybrids is more important than that of the parental lines as they are consumed by people rather than being used as the seeds for reproduction. F<sub>2</sub> grains of both 31892S/

RH003 and 1892S/RH003 were characterized as medium grain rice (Table 3). They had similar DC and intermediate AC, and were also classified as soft rice with similar GC and ASV (Table 3). The results demonstrated that both 31892S and its derived hybrid rice had similar grain quality to 1892S and the control hybrid rice, respectively.

## DISCUSSION

Hybrid rice has been successfully used for commercial rice production in China for over 40 years. However, the hybrid F<sub>1</sub> production and plantation are still

**Table 2. Agronomic traits of 1892S, 31892S and their F<sub>1</sub> hybrids.**

Trait	TGMS line under restrictive condition <sup>a</sup>		TGMS line under permissive condition <sup>b</sup>		F <sub>1</sub> hybrid <sup>c</sup>	
	1892S	31892S	1892S	31892S	1892S/RH003	31892S/RH003
Growth duration (d)	123	123	136.3 ± 4.0	136.0 ± 4.6	138.0 ± 1.1	138.0 ± 1.1
Plant height (cm)	76.3 ± 0.5	80.2 ± 4.8	67.5 ± 7.1	67.9 ± 9.0	121.0 ± 5.9	126.7 ± 1.4
Productive panicles per plant	NA	NA	6.4 ± 1.1	6.6 ± 1.0	8.7 ± 1.1	8.7 ± 1.5
Panicle length (cm)	19.3 ± 0.1	19.7 ± 0.3	15.7 ± 1.3	16.7 ± 1.4	23.5 ± 0.6	23.7 ± 0.6
Total spikelet number per panicle	181.8 ± 7.5	175.6 ± 14.5	146.5 ± 5.3	138.1 ± 26.4	251.1 ± 34.6	232.4 ± 32.3
Spikelet fertility (%)	NA	NA	82.2 ± 8.2	72.3 ± 12.0	87.2 ± 4.6	82.8 ± 5.8
1000-grain weight (g)	NA	NA	23.3 ± 0.5	23.7 ± 0.5	22.4 ± 0.2	23.4 ± 1.1
Yield (t/hm <sup>2</sup> )	NA	NA	5.8 ± 0.9	5.1 ± 0.9	10.5 ± 1.6	9.6 ± 1.8

<sup>a</sup> Field trial for thermo-sensitive genic male sterile (TGMS) lines was conducted in Hefei, China, in the summer season of 2017 (*n* = 3).

<sup>b</sup> Two field trials for TGMS lines were conducted in Lingshui, China, in the winter seasons of 2015/2016 and 2016/2017 (*n* = 6).

<sup>c</sup> Two field trials for hybrid rice plantation were conducted in Hefei, China, in the summer seasons of 2016 and 2017 (*n* = 6).

Data represent Mean ± SD. Statistical analysis for *P*-value was performed in Microsoft Office Excel 2007 using a two-tailed *t* test for paired samples, and there were no significant difference in all.

NA, Not available.

**Table 3. Grain quality of 1892S, 31892S and F<sub>2</sub> grains of hybrid rice.**

Characteristic	TGMS line		F <sub>2</sub> grains of hybrid rice	
	1892S	31892S	1892S/RH003	31892S/RH003
Grain length (mm)	6.3 ± 0.1	6.4 ± 0.1	6.2 ± 0.3	6.3 ± 0.4
Ratio of length to width	2.9 ± 0.1	2.9 ± 0.1	2.9 ± 0.0	3.0 ± 0.1
Degree of chalkiness (%)	8.3 ± 0.6	9.0 ± 0.9	16.8 ± 3.4	17.7 ± 4.0
Amylose content (%)	14.8 ± 0.2	15.4 ± 0.8	22.7 ± 0.7	22.9 ± 0.4
Gel consistency (mm)	87.0 ± 8.2	91.0 ± 7.2	68.5 ± 37.5	74.0 ± 35.4
Alkali spreading value	4.2 ± 0.3	4.7 ± 0.6	5.3 ± 0.7	6.1 ± 0.1

The evaluation of grain rice quality of 1892S and 31892S was done with rice seeds from the field trials in the winter seasons of 2015/2016 and 2016/2017 in Lingshui, China. The evaluation of grain quality of hybrid rice was done with rice seeds collected from the field trials in the summer seasons of 2016 and 2017 in Hefei, China.

TGMS, Thermo-sensitive genic male sterile.

affected by biotic and abiotic stresses. The utilization of natural resistance in parental lines is still an efficient, economical and sustainable way to combat against the biotic and abiotic stresses. Previously, we have introduced and pyramided the *Pi9*, *Xa21* and *Sub1A* genes in WH725 and 9311 genetic backgrounds and developed two elite paternal lines for two- and/or three-line hybrid rice production (Luo et al, 2016, 2017). In this study, we successfully introduced and pyramided the *Pi9*, *Xa21* and *Sub1A* genes in 1892S genetic background and developed an improved TGMS line 31892S for two-line hybrid rice. 31892S and its F<sub>1</sub> hybrids conferred disease resistance to rice blast and bacterial blight and provided submergence tolerance. 1892S, an elite TGMS line, has been used as female to produce more than 25 commercial F<sub>1</sub> hybrids, which have been widely grown in midstream and downstream Yangze Valley (Yang et al, 2016). The development of 31892S will provide enhanced disease resistance to rice blast and bacterial blight and submergence tolerance in two-line hybrid rice production.

Backcrossing is a traditional but still efficient way for the introgression of gene(s) from a donor line into a recurrent female line. However, if several genes from different donor lines need to be introduced and pyramided into a single recurrent female line, co-current backcrossing has to be conducted in each generation before gene pyramiding at BC<sub>4</sub>F<sub>1</sub> or higher backcrossing generations (Luo and Yin, 2013; Luo et al, 2016, 2017). Marker-assisted selection can provide precise selection without doing phenotypic selection. However, co-current backcrossing and subsequent gene pyramiding are still labour-intensive and time-consuming. In this study, T4105, which carries the *Pi9*, *Xa21* and *Sub1A* genes in KDML105 genetic background, was selected as the single donor line for the three genes. Marker-assisted selection of the *Pi9*, *Xa21* and *Sub1A* loci was conducted in a single medium-size backcrossing population at

each generation from BC<sub>1</sub>F<sub>1</sub> to BC<sub>6</sub>F<sub>1</sub>. A large population was only generated at the BC<sub>6</sub>F<sub>2</sub> generation in order to screen for individual plants that contained homozygous allele at the *Pi9*, *Xa21* and *Sub1A* loci. The breeding strategy saved at least one generation of time at the gene pyramiding stage. Most importantly, 31892S and its F<sub>1</sub> hybrids showed resistance to rice blast and bacterial blight and provided submergence tolerance.

Undesirable genes, linked with a selected gene or locus, which is also known as linkage drag (Brinkman and Frey, 1977), is always a concern in marker-assisted breeding. Backcrossing and marker-assisted selection can reduce the size of introgression window more rapidly and more efficiently. The original *Pi9* donor line 75-1-127 is a BC<sub>3</sub> offspring, derived from the wild cross between *O. sativa* cv. IR31917-45-3-2 ( $2n = 24$ , AA genome) and *Oryza minuta* Acc. 101141 ( $2n = 48$ , BBCC genome), followed by backcrossing using the cultivar as the recurrent female (Amante-Bordeos et al, 1992). IRBB21 is a near-isogenic line of the *Xa21* gene in IR24 genetic background, which was generated through intensive backcrossing after the *Xa21* locus was introgressed from the African wild rice *O. longistaminata* ( $2n = 24$ , AA genome) to cultivated rice (Khush et al, 1990). The *Sub1A* locus was originally identified in the Indian landrace FR13A (Mackill et al, 1993). IR64-Sub1A is a BC<sub>3</sub>F<sub>2</sub> line of the *Sub1A* gene in IR64 genetic background (Septiningsih et al, 2009). T4105, the common donor line for the *Pi9*, *Xa21* and *Sub1A* genes, is a BC<sub>4</sub>F<sub>2</sub> plant with the three genes pyramided in KDML105 genetic background (Luo and Yin, 2013). Taken together, for all the three genes in this study, at least 15 generations of crossing and/or backcrossing have been conducted with *indica* varieties as the female or recurrent female after they were introgressed from the wild species or the native landrace. 31892S and its F<sub>1</sub> hybrid 31892S/RH003 had similar agronomic traits

and grain quality to 1892S and the control F<sub>1</sub> hybrid 1892S/RH003, respectively. No pleiotropic phenotype, yield penalty or change in grain quality was observed. The results indicated that linkage drag did not occur during the introducing and pyramiding of the *Pi9*, *Xa21* and *Sub1A* genes in 1892S genetic background. Similar results were also observed in our previous work on introducing and pyramiding the three genes in WH725 and 9311 genetic backgrounds (Luo et al, 2016, 2017). It is estimated that 31892S may harbour narrow introgression windows at the *Pi9*, *Xa21* and *Sub1A* loci derived from *O. minuta*, *O. longistaminata* and FR13A, respectively.

## METHODS

### Rice materials

1892S is an elite TGMS line carrying *tms5* gene. T4105 was a sister line of T5105 in KDML105 genetic background with the *Pi9* gene from 75-1-127, the *Sub1A* gene from IR64-Sub1A and the *Xa21* gene originally from IRBB21 (Luo and Yin, 2013). RH003 is a paternal line for F<sub>1</sub> hybrid production.

### PCR-based molecular markers

The molecular markers were NBS2-1 for the *Pi9* gene (Luo et al, 2016), RM23887 for the *Sub1A* locus (Neeraja et al, 2007), S2-24 for the *tms5* locus (Xu et al, 2011; Zhou et al, 2014) and pTA248 for the *Xa21* locus (Chunwongse et al, 1993). The nature of the molecular markers and their DNA primers are summarized in Table S1. The molecular markers were amplified by PCR on a BIO-RAD T100 programmable thermal cycler (Life Science Research, USA). The PCR reaction mixture of 20 µL consisted of < 200 ng of rice genomic DNA, 0.15 mmol/L of each primer, 10 µL of 2× PCR GoTaq® Green Master Mix (Promega, USA). Template DNA was denatured at 94 °C for 3 min, followed by 35 cycles of PCR amplification with the following parameters: 30 s of denaturation at 94 °C; 30 s of primer annealing at 55 °C for markers pTA248, RM23887 and S2-24, and 60 °C for marker NBS2-1; 30 s of primer extension for RM23887, S2-24 and NBS2-1, and 90 s of primer extension for pTA248 at 72 °C. Finally, the reaction mixture was maintained at 72 °C for 5 min before completion. The amplified products were electrophoretically resolved on 1.5% agarose gel for pTA248 and NBS2-1, and 3.0% agarose gel for RM23887 and S2-24 in 1× Tris-acetate-EDTA buffer.

### *M. oryzae* isolates and blast inoculation

Field *M. oryzae* isolates 11-17-1-2-2 and 11-3-1-1-2 were collected from rice fields in Anhui Province, China. The isolates were grown on prune agar medium with 40 mL prune juice, 5 g/L lactose, 5 g/L sucrose, 1 g/L yeast extract, 20 g/L agar and pH 6.5 at 28 °C in darkness for 7 d and at 26 °C under light for 4 d. For blast inoculation, two-week-old rice plants

were inoculated with a spore suspension at the density of  $1 \times 10^5$  spores/mL. The inoculated plants were placed in darkness in a dew chamber for 24 h at 25 °C and 90% of humidity. The inoculated plants were then transferred to a growth chamber at 25 °C–28 °C and grown under a 12 h/12 h (light/dark) photoperiod and 90% humidity. The disease reaction was scored at 7 d after inoculation.

### *Xoo* strains and bacterial blight inoculation

Twenty-four *Xoo* strains collected from nine countries were used for bacterial blight inoculation. *Xoo* strains were grown on peptone-sucrose agar (PSA) medium (10 g/L peptone, 10 g/L sucrose, 1 g/L glutamic acid, 16 g/L agar and pH 7.0) at 28 °C for 2 d. The bacterial cells were collected and suspended in sterile water at an optical density of 0.5 ( $OD_{600}$ ). Fully expanded leaves of 6-week-old rice plants were inoculated with *Xoo* strains by the leaf clipping method (Kauffman et al, 1973). The lesion length was measured at 14 d after inoculation.

### Evaluation of rice for submergence tolerance

Tests of rice plants for submergence tolerance were conducted in an open water tank and repeated for three times. In each experiment, 20 plants were tested for each rice line. Fourteen-day-old plants grown in pot soil were completely submerged in water. The submergence-treated plants were allowed to recover for 7 d before scored for viability. The plant survival was indicated by having minimum growth of a new green leaf (Luo et al, 2016). The statistical analysis was carried out by the method of new multiple range test (Duncan, 1955).

### Dynamic observation of fertility expression in the field

Tests on fertility-sterility alteration of TGMS lines were conducted in 2017 in Hefei, China. One hundred seeds for each TGMS line were sown at 10-day intervals from 21 April to 11 July 2017. Thirty uniform and healthy rice seedlings at the five-leaf stage were transplanted in fields at a raw spacing of 20.0 cm × 20.0 cm. Ten panicles from each TGMS line were bagged for self-pollination at 3-day interval during the heading period from 27 July to 19 September 2017. Spikelet fertility of the bagged panicles was scored at 25 d after heading. Pollen grains from top five florets of primary panicles were stained with 1% I<sub>2</sub>-KI solution and examined under a ZEISS Axioplan Upright microscope.

### Design of field trials and collection of important agronomic traits

Field trials for the TGMS lines were conducted in Lingshui, China, in the winter seasons of 2015/2016 and 2016/2017 and in Hefei, China in the summer season of 2017. Field trials for the F<sub>1</sub> hybrids were conducted in Hefei, China, in the summer seasons of 2016 and 2017. For each rice line, three randomly selected plots were planted with each at 12 m<sup>2</sup>. Major agronomic traits were collected as described previously (Luo et al, 2016). *P*-value of statistical analysis for each trait was

calculated according to a two-tailed *t*-test for paired samples.

### Evaluation of grain quality

The characteristics of rice grain quality, including grain length, ratio of length/width, degree of chalkiness, amylose content, gel consistency and alkali spreading value (ASV), were measured according to the method described previously (Cruz and Khush, 2000).

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### SUPPLEMENTAL DATA

The following material is available in the online version of this article at <http://www.sciencedirect.com/science/journal/16726308>; <http://www.ricescience.org>.

Table S1. Molecular markers for genes used in this study.

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