

## Note

# Breeding for three-line japonica hybrid rice combinations with high resistant starch content using molecular marker-assisted selection

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Resistant starch (RS) is beneficial for human health, and especially for diabetics. Considering the high cost and low productivity of the Jiangtangdao 1 rice variety with high RS content, breeding high RS rice varieties exhibiting high productivity is essential. A molecular marker-assisted selection strategy was applied to increase RS content in a three-line hybrid rice variety. The functional rice variety Jiangtangdao 1, which contains *sbe3-rs* (on chr2) that controls the RS content, was used as the high RS content donor parent. Subsequently, male sterile maintainer and restorer lines containing homozygous *sbe3-rs* were bred using molecular marker-assisted selection combined with traditional breeding methods. The male sterile line was crossed with the restorer lines to identify the optimal hybrid combination with a high RS content. We obtained four combinations for which the yields were >50% higher than those of the control Jiangtangdao 1. In addition, there was no significant difference in the RS content between the combinations and Jiangtangdao 1. The hybrid rice plants with high RS content exhibited favorable agronomic traits and therefore have broad prospects for commercial application.

**Key Words:** hybrid rice, Jiangtangdao 1, molecular marker-assisted selection, resistant starch, *sbe3-rs*.

## Introduction

With increasing awareness of healthy and nutritious food, consumers have begun to pay attention to foods with health benefits. In recent years, researchers have focused on the properties of bioactive compounds in functional foods to control various aspects of diabetes (Rudkowska 2009). The presence of resistant starch (RS) has received extensive attention. RS is defined as the sum of starch and its degradation products that are not absorbed in the small intestine of healthy individuals and can be completely or partially fermented into short chain fatty acids by microorganisms in the colon (Ma *et al.* 2018, Topping and Clifton 2001). It has a series of physiological effects; for example, it leads to slower glucose release, lower glycemic response, and better insulin sensitivity. In addition, it can facilitate body weight management, prevent heart diseases, cancer, diabetes, and cardiovascular diseases, and enhance intestinal calcium and iron absorption (Kwak *et al.* 2012, Maki *et al.* 2012,

Sajilata *et al.* 2006, Shu *et al.* 2009).

Type-II diabetes mellitus (DM) is a metabolic disorder characterized by hyperglycemia, insulin resistance,  $\beta$ -cell dysfunction, and impaired insulin secretion (Zhang and Zhang 2012). It affects approximately 321 million people globally, of which cardiovascular complications are expected to be the cause of death for half of them (Weidman-Evans *et al.* 2014). It is estimated that 552 million people will have DM by 2030 worldwide (Zeng *et al.* 2016). In China, approximately 114.4 million people either have been diagnosed with DM or are considered prediabetic. China has the highest number of patients with diabetes globally, and consequently, diabetes exerts considerable pressure on the healthcare system due to the associated medical costs (Jiang *et al.* 2019).

Rice is an important food crop globally. It is a major source of nutrients and carbohydrates in Asia, and starch is the main component of rice (Bao *et al.* 2017). Enzymes in the digestive tract hydrolyze rice starch and convert it into glucose. After meeting the energy requirements of the body, the extra calories from starch are stored as glycogen or fat for later use. A high consumption rate of rice could lead to health problems such as obesity, DM, and colon diseases (Hu *et al.* 2012). Conversely, consuming rice with high RS content could ameliorate diabetes in Asia,

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particularly in China. Therefore, breeding novel rice varieties with high RS content has attracted the attention of breeders. Rice varieties or many mutants with high RS content have been identified, such as Goami No. 2, Goami No. 3 (Zeng *et al.* 2016), RS111 (Yang *et al.* 2006), and Jiangtangdao 1 (Yang *et al.* 2012). Jiangtangdao 1, which was bred by the Shanghai Academy of Agricultural Sciences (SAAS), is rich in RS (approximately 13%). We have previously shown that the postprandial glucose concentration of type II diabetic patients consuming Jiangtangdao 1 (Youtian) was significantly lower than that of those consuming ordinary rice (Shi *et al.* 2014).

A putative gene, *sbe3-rs* (GenBank: JQ937272.1), which controls the RS content in Jiangtangdao 1, has been reported (Yang *et al.* 2012), and the mutated high RS allele is recessive to the regular low RS locus. Yang *et al.* (2012) developed a cleaved amplified polymorphic sequence (CAPS) marker that could be used in marker-assisted selection (MAS) for breeding high RS rice cultivars. MAS is used extensively to transfer favorable alleles from a donor to an elite variety (Chen *et al.* 2011, Yang *et al.* 2015, Zhai *et al.* 2002), and it has been touted as a highly efficient breeding method since it can facilitate the rapid and precise selection of a target gene (Bai *et al.* 2018). Considering the high cost and low productivity of Jiangtangdao 1, breeding high RS hybrid rice varieties with high productivity through MAS techniques is essential. The development of hybrid rice based on heterosis or hybrid vigor is a practical approach to increase rice production with yields approximately 20–30% higher than those of inbred rice varieties (Bai *et al.* 2018). In China, more than half of the total rice-growing area is cultivated using hybrid rice varieties (Bai *et al.* 2018). Hybrid rice breeding has enhanced rice quality and yield, in addition to improving several other agronomic traits (Jiang *et al.* 2015, Zhai *et al.* 2002). This study aimed to breed a male sterile line (MS) and a restorer line of rice with high RS content using *sbe3-rs* based on the MAS system. Subsequently, we identified an optimal three-line hybrid combination with high RS content, high yield, and good quality based on MAS and pedigree selection.

## Materials and Methods

### Plant materials

The three-line hybrid system comprised a MS line (A line), maintainer (B line), and restorer (R line). To breed three-line japonica hybrid rice combinations with high RS content, Jiangtangdao 1 carrying *sbe3-rs* was used as the donor parent of the high RS content gene. 49A (a Boro II type (BT) MS line) and 49B (the maintainer line of 49A) with good agronomic traits, which are preserved in our lab, were used as the receptor parent lines, while whereas R1 japonica restorer lines of high quality and with good agronomic traits were used as the recipient parents to improve the RS content.

### DNA extraction and MAS of *sbe3-rs*

Total DNA was extracted from fresh leaves using the cetyltrimethylammonium bromide method as reported previously (Murray and Thompson 1980). A CAPS marker developed for high RS was used to screen parents and populations of introgression lines (Yang *et al.* 2012). A 571-bp fragment was amplified by polymerase chain reaction (PCR) using genomic DNA as a template. The primer sequences for PCR-*SpeI* were as follows: F: 5'-ATGTGATGTGCTGGATTTGG-3' and R: 5'-TGTGGTTTTTCATACCGTTCTTA-3'. The PCR products were digested with *SpeI* (TaKaRa, Dalian, China). The reaction mixture with a total volume of 20  $\mu$ L contained 17.5  $\mu$ L of PCR product, 2  $\mu$ L of 10 M buffer, and 0.5  $\mu$ L of *SpeI*. It was incubated at 37°C for 2 h. The digested products were separated on a 1% agarose gel for the genotyping of individuals. A point mutation led to the loss of the restriction enzyme site *SpeI*; the *sbe3-rs* mutant gene was not digested with *SpeI*, whereas the wild-type was digested.

### Evaluation of pollen fertility

During backcross, the MS lines were examined for pollen sterility and self-seed setting in bagged panicles. The spikelets were collected at one or two days prior to anthesis and fixed in Carnoy's fluid. The pollen grains were collected from the top, middle, and bottom of 10 panicles, stained with 1% (w/v) iodine and potassium iodide (I<sub>2</sub>-KI) solution, and then observed under a light microscope to determine pollen sterility. All irregularly-shaped, yellowish, or unstained pollen grains were scored as sterile, whereas round and dark-brown pollen grains were scored as fertile. Pollen fertility was estimated as the percentage of fertile pollen grains among all the pollen grains examined.

### Evaluation of restoring ability, productivity, and quality of hybrid rice combinations

The MS line and the newly developed restorer lines were crossed. The F<sub>1</sub> plants were grown in an experimental farm of the SAAS to evaluate spikelet fertility and productivity. In all cases, using Jiangtangdao 1 as a control, the following agronomic traits were assessed: plant height, panicle length, number of grains per panicle, spikelet fertility, and 1000-grain weight. We fully harvested four hybrid combinations to estimate the theoretical yield per hectare. The data were analyzed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). The means and standard deviations are listed in **Table 1**.

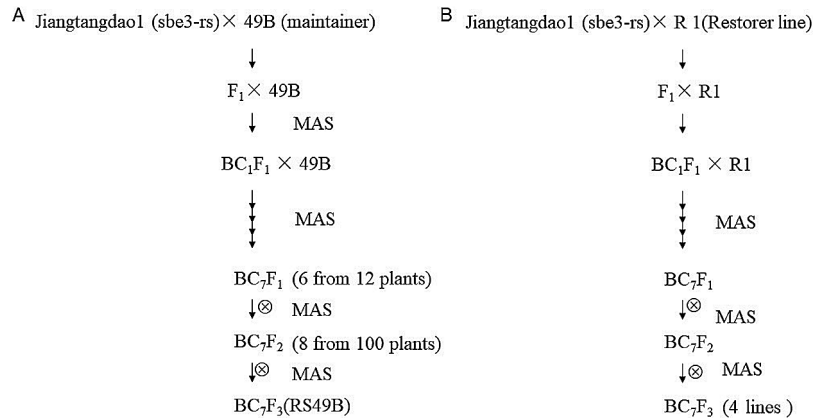
### Measurement of resistant starch content

RS was measured using the Megazyme RS Assay kit (Megazyme, Co., Wicklow, Ireland), which is widely used for RS determination in crops, according to the manufacturer's instructions. The grain samples were treated with 10 mg/mL pancreatic  $\alpha$ -amylase and 3 U/mL amyloglucosidase (AMG) enzyme to hydrolyze and solubilize the non-RS. After terminating the enzymatic reaction by adding

**Table 1.** Agronomic traits and RS content of the F1 hybrids in different hybrid combinations

Hybrid combination	Plant height/cm	Panicle length/cm	grains/panicle	Spikelet fertility %	1000-grain weight/g	Theoretical yield kg/hectare	Yield increase over check variety (Jiangtangdao 1)
Jiangtangdao 1	98.6 ± 4.3	20.6 ± 0.8	99.4 ± 20.8	95.5 ± 3.5	19.7 ± 0.2	5230.74	–
RS49Ax RS307	111 ± 4.8	20.3 ± 0.7	138.6 ± 22.3	90.9 ± 3.1	22.6 ± 0.3	8076.76**	54.4
RS49Ax RS308	96.5 ± 3.4	22.9 ± 0.8	156.3 ± 25.8	92.1 ± 3.2	21.1 ± 0.2	9000.01**	72.1
RS49Ax RS309	101 ± 2.9	24.0 ± 0.6	168.3 ± 26.4	90.7 ± 2.9	22.2 ± 0.3	7922.94**	51.5
RS49AxRSR286	107.5 ± 3.2	21.7 ± 0.7	149.6 ± 23.1	90.2 ± 4.1	22.3 ± 0.4	8307.65**	58.8

\*\* symbols adjacent to means indicate significant differences at the 5% or 1% significance level based on the *t*-test.



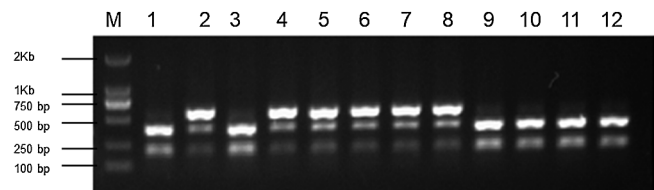
**Fig. 1.** Process of selecting maintainer and restorer lines with high RS content via the introgression of *sbe3-rs* (donor parent: Jiangtangdao 1) into maintainer line 49B and restorer line R1 using MAS approaches. MAS: marker assistance selection; RS: resistant starch.

99% ethanol solution, RS was recovered as a pellet by centrifugation at 1,500 g for 10 min and the supernatant was decanted. RS in the pellet was dissolved in 2 mol/L KOH before the pellet was washed twice with 50% ethanol solution. Subsequently, starch in the solution was hydrolyzed quantitatively into glucose with AMG. D-Glucose was measured with glucose oxidase/peroxidase reagent at 510 nm against the reagent blank. The percentage of RS was calculated using the formula provided by the manufacturer. All analyses were performed in triplicate.

## Results

### Development of maintainer and restorer lines carrying *sbe3-rs* via MAS

The F<sub>1</sub> plants carrying the target resistance gene *sbe3-rs* obtained from the cross of MS maintainer line 49B (with good agronomic traits) with Jiangtangdao 1 (carrying *sbe3-rs* as donor parent) were selected using a CAPS marker to identify plants with heterozygous *sbe3-rs* for backcrossing with the MS maintainer line 49B (Fig. 1A). The 49B line was used as the recurrent parent for continuous backcrossing for six generations, and the BC<sub>n</sub>F<sub>1</sub> plants carrying heterozygous *sbe3-rs* were selected by MAS. Six of 12 BC<sub>7</sub>F<sub>1</sub> individual plants were identified as containing *sbe3-rs* (Fig. 2). Homozygous plants in the BC<sub>7</sub>F<sub>2</sub> population were obtained in the self-crossing generation by MAS (8 from 100 plants). Finally, we developed a MS maintainer line

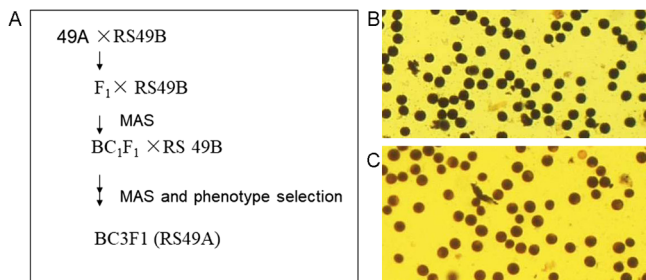


**Fig. 2.** Marker selection for *sbe3-rs* in the BC<sub>7</sub>F<sub>1</sub> maintainer population using CAPS marker. Because the point mutation resulted in the loss of the restriction enzyme site of *SpeI*, the BC<sub>7</sub>F<sub>1</sub> plants carrying *sbe3-rs* had three bands. M: marker DL2000, 1–12: 12 plants of the BC<sub>7</sub>F<sub>1</sub> populations. 2, 4–8 are heterozygote genotypes. CAPS: cleaved amplified polymorphic sequence.

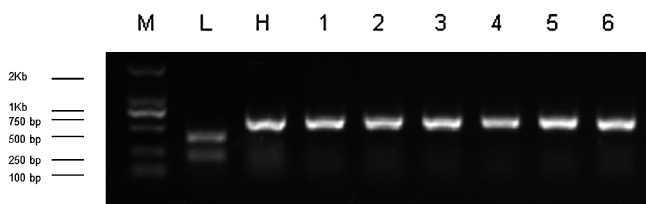
with RS starch content, RS49B (BC<sub>7</sub>F<sub>3</sub>), via the introgression of *sbe3-rs* (donor parent: Jiangtangdao 1) into 49B through MAS (Fig. 1A). The breeding of a restorer line with high RS was performed similar to that of RS49B. In the BC<sub>7</sub>F<sub>2</sub> population, we developed eight restorer lines that harbored homozygous *sbe3-rs*. RS49A was crossed with the eight restorer lines. The F<sub>1</sub> hybrids were grown to evaluate spikelet fertility and productivity. Finally, we developed four stable restorer lines in BC<sub>7</sub>F<sub>3</sub> with restoring ability, and named them RSR307, RSR308, RSR309, and RSR286.

### Selection of male sterile line RS49A via MAS and conventional phenotypic selection

Male sterile line 49A was crossed with RS49B, and then



**Fig. 3.** Scheme for selecting MS line carrying homozygous *sbe3-rs* (A) and pollen fertility analysis by potassium iodide staining (B: full pollen fertility control; C: RS49A). MS: male sterile. The full pollens of RS49A exhibiting sterility with starch grain filling and light color aborted.



**Fig. 4.** Verification of the lines developed with homozygous *sbe3-rs* using CAPS marker. M: marker DL2000; L: low RS plant control; H: *sbe3-rs* homozygous genotype; 1: RS49A; 2: RS49B; 3–6: RSR307, RSR308, RSR309, and RSR286.

the F<sub>1</sub> plants were backcrossed with RS49B as the recurrent parent to produce BC<sub>1</sub>F<sub>1</sub>. MAS for *sbe3-rs* was performed in BC<sub>1</sub>F<sub>1</sub> to identify a homozygous *sbe3-rs* plant, which was simultaneously tested for pollen fertility. Finally, we obtained an MS line with a high RS homozygous genotype and designated it as RS49A (Fig. 3A). RS49A was sterile when it flowered in August in Shanghai. The light-colored pollens of RS49A with starch grain filling were aborted in the three-nucleus stage. RS49A belonged to the Boro II type (BT) MS line (Fig. 3C). The seed setting in bagged panicles was very low and typical abortion or round abortion was observed in RS49A.

### Evaluation of restoring ability and agronomic traits of hybrid rice

The development of a three-line MS line (RS49A), a maintainer line (RS49B), and four restorer lines (RSR307, RSR308, RSR309, and RSR286) with a high RS homozygous gene *sbe3-rs* was verified using the CAPS marker. Fig. 4 shows that *sbe3-rs* in all MS lines, maintainer lines, and four restorer lines could not be digested by *SpeI* (Fig. 4). To identify the most favorable hybrid combination with high RS, RS49A was crossed with the RS307, RSR308, RSR309, and RSR286 restorer lines. The agronomic traits of the F<sub>1</sub> hybrids were evaluated. As expected, the spikelet fertility rates were greater than 90%. The 1000-grain weight of the combinations increased when compared to that of the control Jiangtangdao 1. The theoretical yields of all of these combinations were close to or

**Table 2.** RS content in different varieties

Hybrid combination	RS content (%)
Jiangtangdao 1	13.60 ± 1.23
49B	0.46 ± 0.12
R1	0.52 ± 0.13
RSR1	12.89 ± 1.15
RS49A× RS307	13.51 ± 1.23
RS49A× RS308	13.56 ± 1.25
RS49A× RS309	14.2 ± 1.64
RS49A× RS286	13.1 ± 1.32

>8000 kg/hectare, which is approximately 50% higher than that of Jiangtangdao 1. Particularly, the yield of the RS49A/RSR308 combination (9,000.01 kg/hectare) was 72.1% higher than that of the control (Table 1). The RS content in the RS49A/RSR307, RS49A/RSR308, RS49A/RSR309, and RS49A/RSR286 combinations was more than 13%, which was 10-fold higher than that in 49B or R1 (Table 2). There were no significant differences in the RS content between the hybrid combinations and Jiangtangdao 1.

### Discussion

Improving rice quality including post-harvest processing quality, cooking and eating quality, and nutritional value is one of the key objectives of rice breeding (Butardo *et al.* 2019). Numerous genes and quantitative trait loci associated with rice quality have been cloned, and some of them are applied in breeding practices. For example, *Waxy*, which encodes a granule-bound starch synthase, influences starch quality by affecting the rice amylose content (Larkin and Park 2003, Wang *et al.* 1995). RS has received considerable attention for both its potential health benefits and functional properties (Bao *et al.* 2017, Maki *et al.* 2012, Sajilata *et al.* 2006).

In our previous study, we mapped *sbe3-rs*, controlling high RS, in Jiangtangdao 1 (Yang *et al.* 2012). It is a novel recessive allele of *OsSBEIIb* responsible for the biosynthesis of RS (Yang *et al.* 2016). RS content in steamed rice with the *OsSBEIIb* mutant is high (Tsuiki *et al.* 2016). Further, Tanaka *et al.* (2018) reported that starch synthase IIIa and *SBEIIb*-deficient mutant rice could ameliorate pancreatic insulin secretion in rats. These results are consistent with our previous studies of Jiangtangdao 1 with *sbe3-rs*. However, due to low productivity, the high-RS rice varieties with *sbe3-rs* are not applied widely. The three-line hybrid rice system has been extensively used in China to increase rice grain yield. According to the statistics of the Ministry of Agriculture and Rural Affairs of the People's Republic of China, the planting area of three-line hybrid rice has reached 4.9 million hectares in China in 2015, which is approximately 55% of the total hybrid rice production in China (Bai *et al.* 2018). China's hybrid japonica rice development space is huge, which will be an important future grain yield growth point. However, a three-line

hybrid japonica rice variety with high RS content has not been reported. In the present study, our objective was to develop high-RS varieties to breed three-line hybrid japonica rice varieties using MAS for the first time.

Hybrid rice developed based on heterosis can significantly increase rice yield, improve quality, and enhance resistance to diseases and pests compared to those for inbred rice. Numerous studies on the improvement of hybrid rice resistance to bacterial blight, rice blast, and brown planthopper have been conducted using MAS (Jiang *et al.* 2015, Luo *et al.* 2012, Wang *et al.* 2016). In the present study, for the first time, we attempted to transfer *sbe3-rs* into a high-yield hybrid rice variety with high RS content. RS content determination is a time-consuming and laborious process. We used MAS combined with a traditional breeding method to breed the hybrid combinations, which decreased the cost and improved breeding efficiency considerably. We selected a combination with good combining ability to improve its RS content. A high-RS hybrid combination breeding strategy was established based on crossing, backcrossing, selfing, and MAS. Considering that *sbe3-rs* is a recessive gene, to obtain hybrid rice with a high RS content, all the male-sterile lines, maintainer lines, and restorer lines should contain homozygous *sbe3-rs*. The RS content in the hybrids was relatively low when only the MS line or restorer line had *sbe3-rs* (data not shown). Based on genetic engineering and MAS, we obtained four hybrid combinations for which the yield was >50% higher than that of conventional high-RS rice, Jiangtangdao 1, and their comprehensive agronomic traits were also superior to those of the control (Table 1). The RS contents of the four hybrid combinations were more than 13%, which was not significantly different from that of Jiangtangdao 1.

Although we obtained three-line hybrid combinations with high RS content for the first time, the high cost and low productivity of hybrid rice seeds remain the major limiting factors for the large-scale adoption of hybrid rice. There are different types of cytoplasmic male sterility in rice, such as wild abortive type (WA), Honglian type (HL), BT, and Dian type 1 (Bai *et al.* 2018). RS49A is a BT-type male sterility and a gametophytic sterility, which is not as stable as the WA or HL type. It can be self-crossbred easily at high temperature, and it produces hybrid seeds that are not pure. To promote the development of a high-RS hybrid rice, in the future, we plan to breed more types of japonica rice with cytoplasmic sterility such as the HL type. In addition, there are several advantages of the two-line hybrid breeding system. For example, no maintainer line is required and nearly all the normal cultivars can restore male fertility of the photoperiod-sensitive genic MS line (Bai *et al.* 2018, Si *et al.* 2011). Therefore, we will also attempt to breed two-line hybrids with high RS content.

### Author Contribution Statement

RY, JB, and ZP conceived and designed the experiments.

JB and RY performed the experiments and prepared the manuscript. RY wrote the paper. CW, GL, and XR participated in data analysis and assisted in drafting the manuscript. All authors have read and approved the final draft.

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