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Breeding Rice lines for physio-functional food through *indica* 'Zhaxima' × *japonica* 'Nanjing 46' haploid technique

Q. Q. Tian[1,2], X. Li[1,2*], C. M. Lu[3] and X. W. Fang[1]

Abstract

Resistant starch (RS) encompasses those forms of starch which are not accessible to human digestive enzymes and are fermented in the colons producing short chain fatty acids. The plant materials containing RS are few in the world. In this contribution, the culture ability of callus from anthers of F1 plants from, landraces, 'Zhaxima'(*Oryza sativa* var. *indica*, high-RS rice line with 7.705 ± 0.142, g/100 g) × 'Nanjing 46' (*Oryza sativa* var. *japonica*, rice variety with RS content (g/100 g) of 0.200 ± 0.001 crosses were studied for obtaining high RS rice plants. The results showed that when M8 basic induction medium was added with 1.5 mg /L 2,4-D、2 mg /LNAA and 0.3 mg /L KT, the inductivity of callus was high as 32.14% for 21 d after pretreatment at 4 °C for 3 d; When MS differentiation basic medium was added with 2 mg /LKT and 3 mg /L ABA, the frequency of regeneration for callus was 50.3% with only a regeneration frequency of 4.55% grown into green seedlings. The RS content in the seeds was between those of the two parents and was partially normally distributed, the highest RS contents of the regenerated plants was as high as 7.66 ± 1.197%. This produced an efficient technology for regenerating stable rice lines with high RS and good eating quality using anthers culture.

Keywords: Rice (*Oryza sativa* L.)/anther culture/regeneration/resistant Starch

Introduction

Increases in the incidence of type-2 diabetes are being observed throughout the world (Zhou et al. 2016). Starch is a major dietary source of carbohydrate. It is composed of two types of molecules, amylose (Am) and amylopectin (Ap) (Jobling 2004). Based on its enzymatic digestion characteristics, starch can be classified into rapidly digestible starch (RDS), slowly digestible starch (SDS), and resistant starch (RS) (Sajilata et al. 2006). RS is a small fraction of starch that is resistant to hydrolysis by exhaustive α-amylase and pullulanase treatment in vitro (Haralampu 2000). RS encompasses those forms of starch, which are not accessible to human digestive enzymes and are fermented in the colon to produce short chain fatty acids (Ashwar et al. 2015). Consumption of foods high in resistant starch (RS) can help to control type-2 diabetes, because its slow digestion and absorption by the small intestine decrease postprandial glucose and insulin responses (Raigond et al. 2015). In addition, RS has similar physiological effects to dietary fiber, affecting body weight and energy balance and increasing lipid excretion to reduce calorie intake and decrease the serum lipid levels (Jacobasch et al. 1999). These unique physical functions of RS have received increasing attention from plant researchers in recent years (Yang et al. 2012; Zhou et al. 2016). Thus, improvement of the amounts and properties of RS in foods is an important goal (Zhou et al. 2016).

As the primary dietary source of carbohydrates in the world, rice (*Oryza sativa* L.) plays an important role among cereal crops in meeting energy requirements and nutrient intake. However, the RS content in ordinary rice variety is low, generally about 1%, which is not enough to confer the associated health benefits (Frei et al. 2003). In addition, rice with high content of RS also has inferior eating quality, resulting in lower market prices (Raigond et al. 2015).

* Correspondence: jspplx@jaas.ac.cn; 1779119573@qq.com
[1]Institute of Food Crops, Jiangsu Academy of Agricultural Sciences, Jiangsu High Quality Rice R&D Center, Nanjing Branch, China National Center for Rice Improvement, Nanjing 210014, China
[2]Jiangsu Collaborative Innovation Center for Modern Crop Production, Yangzhou 225009, China
Full list of author information is available at the end of the article

Therefore, many studies have focused on elevating the RS content in rice cultivars via mutation breeding and bioengineering (Hu et al. 2004; Shen et al. 2006; Kubo et al. 2008; Wani et al. 2012). Higher amylose content in starch is generally suggestive of increased RS content, thus producing increased levels of amylose through breeding is a possible method to increase endogenous RS content. Two methods have been used with success: inbreeding of mutants containing genes for high amylose production and the inhibition of starch-branching enzyme (SBE) (Deupuis et al. 2014). Many rice mutants with elevated RS content have been identified, including RS111 (Yang et al. 2006) and 'Jiangtangdao 1' (Zhu et al. 2010). 'Teqing Resistant Starch' (TRS) is another high-amylose and high RS transgenic line developed by modifying antisense RNA inhibition for SBE in rice (Wei et al. 2010). A putative gene, *sbe3-rs* of RS was also identified and the codominant cleaved amplified polymorphic sequence (CAPS) marker could be used in marker-assisted breeding to develop rice cultivars with elevated RS, which is otherwise difficult to accurately assess in crop (Yang et al. 2012). Although much progress has been made in obtaining the rice cultivars high in RS, three problems remain to be solved. Firstly, only few rice germplasms high in RS have been reported. Secondly, many rice varieties high in RS belong to the *indica* subspecies, with low yield, and are very difficult to cross rapidly with *japonica* rice plants by conventional breeding as this process of breeding needs quite a long time of 5–10 years. Thirdly, the safety of transgenic rice varieties high in RS need to be verified in longer time. Currently, safe, rapid and efficient techniques for breeding rice varieties with both good eating quality and high RS content are lacking.

Doubled haploids (DHs) in plants have complete homozygosity and can be achieved in one generation from hybrid plants. DH production includes two major steps: haploid induction and chromosome doubling. Chromosome doubling of haploid plants has been routinely and successfully performed using colchicine. However, the success and efficiency of haploid induction varies among crop species (Niu et al. 2014). In rice, many theoretical and applied problems remain to be solved, such as the low fertility of *indica–japonica* F1 hybrids, the lower rate of plant regeneration and the lower seed setting rate of regenerated plants. The factors limit the application of the rice breeding technology by anther culture (Zhao et al. 2012).

In this study, a safe and highly efficient technique for generating new rice lines from the anthers of F1 hybrids of 'Zhaxima' × 'Nanjing 46' was introduced to obtain rice lines both high in RS and excellent in eating quality. Some genetic characteristics of the regenerated rice plants were also studied to elucidate these traits in the parent *indica* and *japonica* plants for tissue culture ability. The genetic characteristics of the partial-*japonica* type hybrid were helpful to obtain more regeneration of green fertile plants, and the RS content in the seeds of these regeneration plants was all higher than in the male parent with lower content of RS.

Materials and methods

Materials

The *indica* variety 'Zhaxima' from Yunnan was the female parent (stamens removed), and it has high RS content in the seeds and is a landraces (Lin et al. 2013). The *japonica* variety 'Nanjing 46' was male parent, and is known for its good eating quality (Wang et al. 2009). The F1 cross 'Zhaxima' × 'Nanjing 46' was designed to combine the RS quality of the former with the eating quality of the latter and was produced in the summer of 2012 in Nanjing (118°46′E, 32°24′N 30 m above sea level), China.

Anther culture of the F1 population was carried out as follows. 'Zhaxima', 'Nanjing 46', and the F1 hybrids were planted in the experimental field of Jiangsu Academy of Agricultural Sciences, Hainan, China, in the spring of 2013. The anthers of the F1 population were collected for anther culturing in the laboratory. Then, anther-culture regenerants were planted in the experimental field of Jiangsu Academy of Agricultural Sciences, Nanjing, China in the summer of 2013.

Regeneration plant callus induction from anthers of F1 hybrids

The media used in the different stages of callus induction were summarized in Table 1. Anthers were cold pretreated in the dark at 4 °C for 1–10 d on induction medium (IM) based on M8 basal medium (Mei et al. 1988). Seven differentiation media (DM) supplemented with different hormone compositions were used in this study (Table 1). Individual dishes were labelled with the anther type and location in the inflorescence (top, middle, base), sealed with Parafilm, and incubated in the dark at 26–28 °C for callus induction. Four different supplemented MS media (Murashige & Skoog 1962) were used for differentiation (Table 1). Calli, at least 2 mm in size from anthers, were transferred to MS basal semisolid medium supplemented with different plant hormone compositions and incubated at 28 °C under a 16-h light photoperiod supplied by cool white fluorescent lamps (66 μmol $m^{-2} s^{-1}$) for plant regeneration. Tissues producing green buds were transferred to rooting medium (RM) and cultured at 26 ± 2 °C under light for rooting. The plantlets were cultured at 26–28 °C with a 16-h photoperiod at a photosynthetic photon flux density of 200 μmol $m^{-2} s^{-1}$. Before being transferred to the experimental field, the plantlets were hardened in their tubes by adding sterile water to drown their roots and cultured for 3 d in a culture chamber. When the

Table 1 Plant hormone components in the callus induction, differentiation, and rooting media[a]

Medium	Medium number	Basic medium	ρ(2,4-D) mg•L^{-1}	ρ (NAA) mg•L^{-1}	ρ (KT) mg•L^{-1}	ρ (ABA) mg•L^{-1}
Callus induction medium	IM-1	M8[b]	1.0	3.0		
	IM-2		1.5	2.0		
	IM-3		1.5	2.0	0.3	
	IM-4		2.0	1.0		
	IM-5		2.0	1.0	0.3	
	IM-6		3.0	1.0		
	IM-7		3.0	0.5	0.3	
Differentiation medium	DM-1	MS			2.0	2.0
	DM-2				2.0	3.0
	DM-3		0.2		2.5	2.0
	DM-4		0.5		2.0	2.0
Rooting medium	RM	MS				
Seedling medium	SM	1/2MS				

[a] Abbreviations: *2,4-D* 2,4-dichlorophenoxyacetic acid, *NAA* 1-naphthylacetic acid, *KT* Kinetin, *ABA* Abscisic acid, *IM* Induction medium, *DM* Differentiation medium, *MS* Murashige and Skoog medium (Chu et al. 1975), *RM* Rooting medium, *SM* Seedling medium, M8[b]: the basic composition as (Mei et al.1998)

height of the plants reached 5–6 cm, they were transferred to clay pots (pot with 0.1 m diameter and 0.6 m high) filled with soil, fertilized with slow-release fertilizer, and kept in a growth chamber at 28–30 °C with a 16/8 h (day/night) photoperiod, and finally planted in the field.

Measurements of stomatal guard cell size of the leaf epidermis and plant height of regeneration plants

To detect the ploidy level of regeneration plants, the size of the guard cell hypodermis on the flag leaf was measured at the rice jointing stage (Liang 1979). Clear nail polish was painted on the leaf epidermis, allowed to dry, then pulled off. Subsequently, the stomatal guard cells of epidermis were observed under a microscope. The perimeters of 100 randomly selected guard cells on each leaf blade were measured. Ten blades were observed for each regeneration plant. Plant height was directly measured at the heading stage using a ruler.

Molecular analysis of the regeneration plant subspecies type by insertion–deletion (InDel) markers

'Nipponbare' was the *japonica* control, and 'Nanjing 16' was the *indica* control. Ten seeds of each regeneration plant were randomly selected and germinated at 37 °C. Then, seedlings were grown in an incubator at 28 °C until the leaves reached the heart embryo, one of embryo developmental stages on shape of callus, when 0.1 g of fresh leaves were sampled. The regeneration materials were sampled at the tillering stage. DNA was extracted from all samples by the CTAB method (Murray & Thompson 1980), and the polymorphisms were used to identify the *japonica* and *indica* types based on modified protocols of Shen et al. (2003) and Lu et al. (2009). Insertion–deletion (InDel) markers are codominant. Using PCR and electrophoresis of the DNA of *indica*, 'Nanjing 16', and *japonica*, 'Nipponbare' (as the standard), the genotype of each tested cultivated and wild rice sample was determined based on the InDels (Additional file 1: Table S1). Samples consistent with the 'Nanjing 16' bands were recorded as the homozygous *indica* genotype (II), samples matching the banding pattern of 'Nipponbare' samples were identified as the homozygous *japonica* genotype (JJ), and those with both 'Nanjing 16' and 'Nipponbare' bands were considered to be *indica* and *japonica* heterozygous genotype (IJ). The mean gene frequency (*F*) of all InDel sites was calculated using the following formulas (Shen et al. 2003; Lu et al. 2009),

$$\textit{japonica}\ \text{gene frequency}\ Fi = \frac{2\sum_1^N Xii + \sum_1^N Xij}{2N}$$

$$\textit{indica}\ \text{gene frequency}\ Fj = \frac{2\sum_1^N Xii + \sum_1^N Xij}{2N}$$

where X_{ii} is a specific indel locus whose position is the same as a 'Nanjing 16' band and denotes an *indica* homozygous genotype (ii); X_{jj} is a specific indel site whose position is the same as a 'Nipponbare' band and denotes a *japonica* homozygous genotype (jj); X_{ij} is a specific indel site whose location matches that of both 'Nanjing 16' and 'Nipponbare' and indicates a heterozygous *indica* and *japonica* genotype (ij) and N is the number of indel primer pairs (sites) included (Additional file 2 Table S2).

Wide compatibility variety (WCV) gene sequences have been studied and their functional motifs are known (Ikehashi & Araki 1984; Ji et al. 2005; Ikehashi & Araki 1986; Morinaga & Kuriyama 1958; Qiu et al.

2005;Yanagihara et al. 1995). The S5 WCV gene has been sequenced in *indica* (accession number EU889295; S5-i), *japonica* (EU889294; S5-j), and *japonica* 02428 (EU889293; S5-n). These sequences differ in a 69-bp deletion upstream and a 67-bp deletion downstream of the ATG at the translation initiation point; these deletions lead to loss of function and prevent S5-i and S5-j interaction. According to the deletion sequence of both sides of the gene (Yang et al. 2009), the primers S5136-Forward (5′-ATCAACCCATTTCCTTTCCT-3′) and S5136-Reverse (5′-ATACGCTCGATCGGATTAAC-3′) were designed.

Measurements of RS content in the regeneration plants

RS was measured using the Megazyme RS assay kit (Megazyme, Co. Wicklow, Ireland), which has been widely employed for RS determination in crops (McCleary et al. 2002). The grain sample was treated with 10 mg/mL pancreatic α-amylase and 3 U/mL amyloglucosidase (AMG) enzymes for hydrolysis and solubilization of non-resistant starch. After the enzymatic reaction was terminated by adding 99% ethanol, RS was recovered as a pellet by centrifugation (approx. 3000 *g*, 10 min). RS in the pellet was dissolved in 2 mol L^{-1} KOH before being added into the reaction solution and was repeatedly washed and decanted. Then, starch in the solution was quantitatively hydrolyzed to glucose with AMG. D-glucose was measured with glucose oxidase/peroxidase (GOPOD) at 510 nm wavelength against the reagent blank. All analyses were repeated three times for error control.

Data analysis

Data parameters were calculated as follows:

Callus induction rate = (number of calluses) / (number of pollen grains inoculated) × 100%.
Differentiation rate = (number of plantlets obtained from callus) / (number of calluses inoculated) × 100%.
Plant regeneration rate = (number of plantlets) / (number of inoculated calluses) × 100%.
Resistant Starch (g/100 g sample) = ΔE × F × 100/0.1 × 1/1000 × 100/W × 162/180 = ΔE × F/W × 9.27,
Where ΔE = absorbance (reaction) read against the reagent.

blank; F = conversion from absorbance to micrograms = 100 (mg glucose)/absorbance of 100 mg glucose; 100/0.1 = volume correction (0.1 mL taken from 100 mL); 1/1000 = conversion from micrograms to milligrams; W = dry weight of sample analyzed [= "as is" weight ′ (100-moisture content)/100]; 100/W = factor to present starch as a percentage of sample weight; 162/180 = factor to convert from free glucose, as determined, to anhydroglucose as occurs in starch; 10.3/0.1 = volume correction (0.1 mL taken from 10.3 mL) for samples containing 0–10% RS where the incubation solution is not diluted and the final volume is 10.3 mL (McCleary et al. 2002). One-way analysis of variance of all data was performed using SPSS 17.0 (IBM, Chicago, IL, USA).

Results

Establishment of anther regeneration technology from the hybrid cross "Zhaxima" and "Nanjing 46"

After pretreatment at 4 °C for 0–10 d, the anthers were cultured on M8 induction medium with different hormones. The anthers were induced to form callus during 21 d in the dark. The frequency of callus induction was as high as 32.14% on the IM-3 medium after pretreatment at 4 °C for 3 d (Table 2). The results also showed that anthers from both the basal and middle parts of the panicle had higher callus induction frequency than those from top part (Table 2). The callus was then transferred to DM medium, where it began to form green tissues under light treatment over 14 *D. callus* with green tissue was cultured for about 30 d on DM-2, with a greening rate of about 4.55% (Fig. 1). The green plantlets could take root on RM for about 20 d. The regeneration plants were placed in the experimental field during the clover heart stage. Notably, the regeneration plants also had a very high rate of albinism. We obtained the seeds of rice plants from anthor culture of F1 from, landraces, 'Zhaxima'(*Oryza sativa* var. *indica*,) × 'Nanjing 46' (*Oryza sativa* var. *japonica*) crosses that year. In this contribution, the plant regeneration rate from anther culture was as high as 4.55%, but the albino rate of regeneration plants was as high as 47.5%. We further analyzed the morphological and molecular characteristics of the regeneration plants got in this work, in order to provide the basis for improvement of anther culture in rice.

Analysis of indica and japonica types of the high regenerated plants

According to 35 InDel sites (Fig. 2), the *indica* and *japonica* types of samples (ii, jj and ij) were calculated. Table 3 shows the standard characteristics of *indica* and *japonica* types based on the frequency calculations. The *japonica* genotype frequencies of 'Zhaxima' and 'Nanjing 46' were 0 and 1, respectively, demonstrated that these parents were typical *indica* and *japonica* types, respectively. Type 1 plants had frequencies of 0.37 *indica* and 0.63 *japonica*, and were a partial-*japonica* type. In contrast, type 2 plants had frequencies of 0.63 *indica* and 0.37 *japonica*, indicated a partial-*indica* type. The frequencies of type 3 were 0.41 *indica* and 0.59 *japonica*, an intermediate type. We also analyzed the yellow and albino regeneration plants in this study. The *indica* frequency of yellow plants was 0.44 and their *japonica* frequency was 0.56, while the frequencies of the albino ones were 0.53 and 0.47, respectively; Both lines were of the intermediate type. These results indicated that *japonica* plants were better suited to anther culture than *indica*

Table 2 Effects of different hormone components and treatment times on callus induction

Days after cold pretreatment(d)	Sampling area of spikes	Callusing induction rate of plated anthers (%) anthers (%)						
		IM-1	IM-2	IM-3	IM-4	IM-5	IM-6	IM-7
0	Top	6.21 ± 0.21b	1.91 ± 0.05e	7.82 ± 0.21a	2.53 ± 0.08d	2.61 ± 0.07d	1.31 ± 0.03f	3.61 ± 0.10c
	miiddle	6.51 ± 0.21b	2.05 ± 0.08e	8.03 ± 0.24a	2.84 ± 0.08d	2.81 ± 0.07d	1.41 ± 0.04f	3.81 ± 0.11c
	Bassal	8.03 ± 0.29a	2.92 ± 0.11d	8.12 ± 0.22a	3.13 ± 0.08c	3.01 ± 0.07d	1.51 ± 0.03e	4.11 ± 0.12b
1	Top	8.21 ± 0.29b	6.05 ± 0.19c	12.03 ± 0.33a	4.03 ± 0.12e	4.41 ± 0.13d	4.02 ± 0.12e	4.52 ± 0.13d
	miiddle	8.52 ± 0.31b	6.71 ± 0.23c	13.14 ± 0.33a	4.52 ± 0.17e	4.82 ± 0.11d	4.12 ± 0.12f	4.82 ± 0.11d
	Bassal	9.32 ± 0.32b	7.65 ± 0.20c	14.03 ± 0.32a	4.91 ± 0.14d	5.02 ± 0.11d	4.22 ± 0.11e	5.04 ± 0.15d
2	Top	9.33 ± 0.31c	9.11 ± 0.26c	12.51 ± 0.25b	7.05 ± 0.19e	15.05 ± 0.35a	8.03 ± 0.24d	8.06 ± 0.14d
	miiddle	9.51 ± 0.33e	11.02 ± 0.34c	19.05 ± 0.34a	7.52 ± 0.22 g	15.51 ± 0.36b	10.03 ± 0.17d	9.03 ± 0.27f
	Bassal	10.05 ± 0.35e	12.06 ± 0.38d	13.73 ± 0.31b	7.63 ± 0.22f	16.04 ± 0.38a	12.03 ± 0.26d	12.81 ± 0.51c
3	Top	10.54 ± 0.39f	14.02 ± 0.46d	23.45 ± 0.43b	10.03 ± 0.30 g	28.04 ± 0.74a	18.04 ± 0.44c	12.03 ± 0.36e
	miiddle	11.04 ± 0.41 g	15.04 ± 0.40d	32.14 ± 0.26a	12.10 ± 0.26f	25.04 ± 0.77b	20.03 ± 0.41c	13.02 ± 0.39e
	Bassal	11.51 ± 0.36d	16.41 ± 0.45b	22.83 ± 0.41a	14.03 ± 0.32c	22.14 ± 0.86a	22.04 ± 0.46a	14.03 ± 0.42c
4	Top	13.20 ± 0.41d	17.03 ± 0.48b	12.11 ± 0.33e	13.06 ± 0.39d	18.02 ± 0.54a	12.03 ± 0.26e	14.04 ± 0.42c
	miiddle	14.03 ± 0.45e	17.51 ± 0.30c	18.52 ± 0.37b	14.06 ± 0.32e	19.03 ± 0.47a	14.02 ± 0.42e	15.04 ± 0.45d
	Bassal	16.04 ± 0.54c	18.71 ± 0.44b	12.93 ± 0.28e	15.03 ± 0.35d	20.03 ± 0.51a	15.03 ± 0.45d	16.03 ± 0.48c
5	Top	18.12 ± 0.54a	18.02 ± 0.52a	11.92 ± 0.35b	8.22 ± 0.24c	12.03 ± 0.36b	4.21 ± 0.11d	2.62 ± 0.07e
	miiddle	18.54 ± 0.54a	18.52 ± 0.44a	12.51 ± 0.31b	8.41 ± 0.15d	11.02 ± 0.33c	4.43 ± 0.13e	2.71 ± 0.08f
	Bassal	19.91 ± 0.69a	18.93 ± 0.45b	12.72 ± 0.29c	8.61 ± 0.25e	10.52 ± 0.21d	4.81 ± 0.14f	3.02 ± 0.09 g
6	Top	15.33 ± 0.46b	16.03 ± 0.44a	10.07 ± 0.30c	6.01 ± 0.18d	6.04 ± 0.24d	3.03 ± 0.08e	2.41 ± 0.06f
	miiddle	16.51 ± 0.56	16.51 ± 0.46	16.04 ± 0.26	6.54 ± 0.19	6.21 ± 0.17	3.21 ± 0.08	2.51 ± 0.07
	Bassal	18.03 ± 0.62a	17.03 ± 0.51b	14.09 ± 0.32c	7.01 ± 0.20c	6.71 ± 0.13e	3.41 ± 0.10f	2.81 ± 0.08 g
7	Top	5.51 ± 0.19b	10.03 ± 0.35a	4.52 ± 0.11e	5.02 ± 0.15d	5.81 ± 0.17c	2.02 ± 0.05f	2.02 ± 0.06f
	miiddle	6.03 ± 0.22b	12.03 ± 0.38a	4.61 ± 0.13d	5.51 ± 0.16c	6.05 ± 0.17b	2.23 ± 0.06e	2.21 ± 0.06e
	Bassal	6.52 ± 0.21b	13.11 ± 0.42a	4.81 ± 0.14d	6.02 ± 0.18c	6.23 ± 0.17c	2.41 ± 0.07e	2.42 ± 0.07e
8	Top	5.02 ± 0.20b	8.03 ± 0.22a	4.04 ± 0.12c	3.02 ± 0.09d	5.06 ± 0.15b	0.81 ± 0.01f	1.81 ± 0.04e
	miiddle	5.11 ± 0.19c	8.51 ± 0.24a	4.22 ± 0.12d	3.24 ± 0.08e	5.51 ± 0.11b	1.02 ± 0.02 g	2.03 ± 0.06f
	Bassal	5.52 ± 0.19c	9.04 ± 0.26a	4.42 ± 0.13d	3.43 ± 0.09e	6.05 ± 0.11b	1.21 ± 0.04 g	2.31 ± 0.06f
9	Top	4.41 ± 0.17b	6.51 ± 0.16a	3.81 ± 0.09c	1.42 ± 0.04e	4.51 ± 0.13b	0	1.71 ± 0.04d
	miiddle	4.51 ± 0.17c	7.01 ± 0.18a	4.03 ± 0.11d	1.51 ± 0.04f	5.04 ± 0.14b	0	1.91 ± 0.04e
	Bassal	4.91 ± 0.18c	7.52 ± 0.22a	4.21 ± 0.12d	1.61 ± 0.03f	5.22 ± 0.14b	0	2.12 ± 0.04e
10	Top	0	0	2.01 ± 0.08b	1.21 ± 0.03d	2.81 ± 0.08a	0	1.32 ± 0.03c
	miiddle	0	0	2.31 ± 0.02b	1.32 ± 0.02c	3.02 ± 0.09a	0	1.21 ± 0.03d
	Bassal	0	0	2.24 ± 0.03b	1.42 ± 0.04c	3.11 ± 0.09a	0	1.41 ± 0.03c

Note: The media consisted of M8 basal medium (Mei et al. 1988) supplemented with 60 g•L^{-1} sucrose and 8 g•L^{-1} agar and different hormone complements as follows: IM-1: 1 mg•L^{-1} 2,4-D and 3 mg•L^{-1} NAA; IM-2: 1.5 mg•L^{-1} 2,4-D and 2 mg•L^{-1} NAA; IM-3: 1.5 mg•L^{-1} 2,4-D, 2 mg•L^{-1} NAA, and 0.3 mg•L^{-1} KT; IM-4: 2 mg•L^{-1} 2,4-D and 1 mg•L^{-1} NAA; IM-5: 2 mg•L^{-1} 2,4-D, 1 mg•L^{-1} NAA, and 0.3 mg•L^{-1} KT; IM-6: 3 mg•L^{-1} 2,4-D and 1 mg•L^{-1} NAA, IM-7: 3 mg•L^{-1} 2,4-D and 0.5 mg•L^{-1} NAA. 2,4-D, 2,4-dichlorophenoxyacetic acid; NAA, 1-naphthylacetic acid; KT, kinetin; ABA, abscisic acid, IM, induction medium. Different letters above the bars indicate significant differences ($P < 0.05$).

ones. WCVs with S5-n are able to produce highly fertile hybrids when crossed with both *indica* and *japonica* varieties. Using the S_{5-136} primers detected, the two parents together with their regenerated plants did not contain the wide compatibility gene, S_5. We also observed that the seed-setting rate of the F1 generation was very low, which indicated that the genetic obstacles between the *indica* and *japonica* without WCVs might be one of the reasons for the low fertility. As the explant, anther from the hybrid with *indica japonica* incompatibility was one of the main

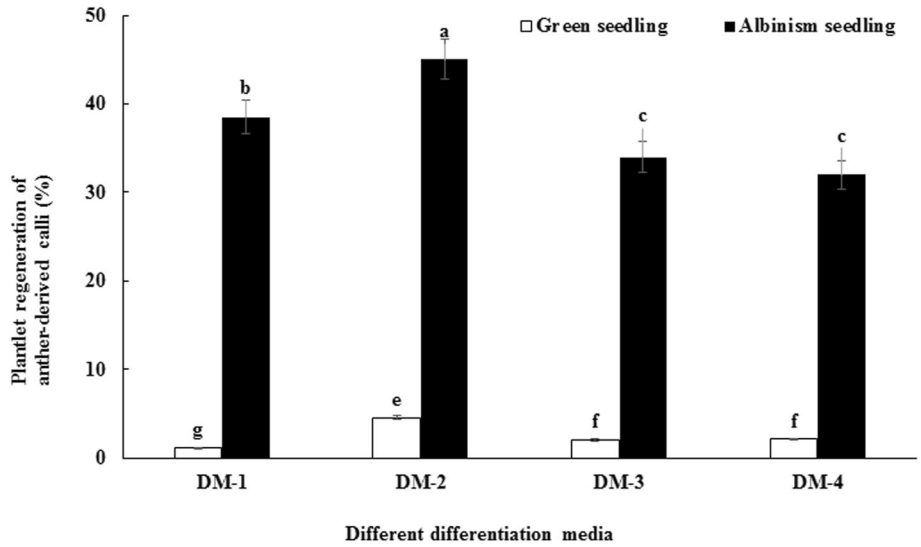

Fig. 1 Effect of phytohormone components on callus differentiation rate. MS was the basic medium (Murashige & Skoog 1962): sucrose 30 g•L^{-1} and agar 8 g•L^{-1}. DM-1: 2 mg•L^{-1} KT and 2 mg•L^{-1} ABA; DM-2: 2 mg•L^{-1} KT and 3 mg•L^{-1} ABA; DM-3: 0.2 mg•L^{-1} 2,4-D, 2.5 mg•L^{-1} KT, and 2 mg•L^{-1} ABA; DM-4: 0.5 mg•L^{-1} 2,4-D, 2 mg•L^{-1} KT, and 2 mg•L^{-1} ABA. 2,4-D, 2,4-dichlorophenoxyacetic acid; NAA, 1-naphthylacetic acid; KT, kinetin; ABA, abscisic acid; DM: differentiation medium. Different letters above the bars indicate significant differences ($P < 0.05$)

reasons for low regeneration rate. A noteworthy phenomenon was that the higher culture ability of hybrid rice cross *indica* and *japonic*a seems partial male such as *japonica*, Nanjing 46 while the equal distribution of *indic*a and *japonica* characteristics was not suitable for regenerating green plants and setting seeds.

Plant height and seed-set of the regenerants of the green plants

Based on plant height, the 40 regeneration lines could be divided into three types (Table 4, Fig. 3). Type 1 plants grew to 72.1 ± 4.5 cm in plant height, similar to the male parent 'Nanjing 46'. The stomatal perimeter on the lower epidermis of type 1 plants (59.58 ± 2.20 μm) was larger than those of both parents ('Nanjing46': 55.97 ± 1.24 μm; 'Zhaxima': 45.98 ± 0.54 μm), and all of those plants set seed. Type 2 plants were 128.8 ± 2.7 cm in plant height and did not set seeds with abnormally hooked anthers and also had a larger stomatal perimeter on the lower epidermis (63.47 ± 3.40 μm). Type 3 plants were the shortest at 58.9 ± 1.4 cm in plant height and also had the smallest lower-epidermal stomatal

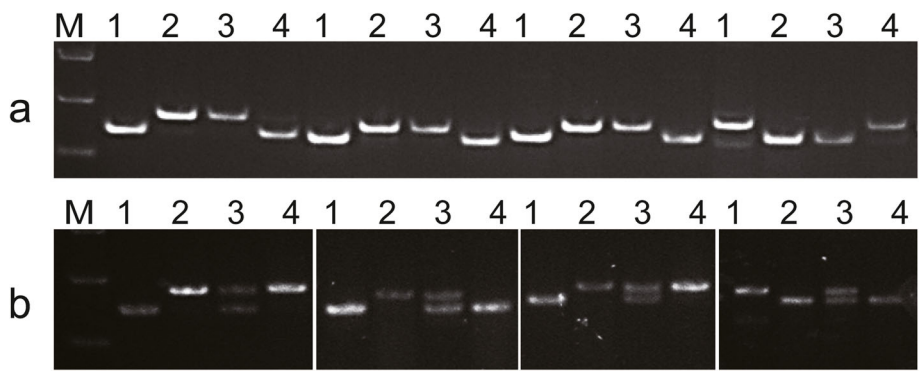

Fig. 2 Amplification results of 'Nanjing 46', 'Zhaxima', F1 hybrids, and regeneration plants using indel primer (R3M23, R4M17, R4M43, and R5M30). In row "a", lane 1 is Nipponbare, lane 2 is Nanjing 16, lane 3 is Zhaxima, and lane 4 is Nanjing 46. In row "b", lane 1 is Nipponbare, lane 2 is Nanjing 16, lane 3 is the F1 hybrid, and lane 4 is the regenerated plants

Table 3 *Indica* (F_i) and *japonica* (F_j) gene frequencies in rice samples[a]

Material	*Indica* gene frequency (F_i)	*Japonica* gene frequency (F_j)	Rice Types
Nipponbare	0	1	Typical *japonica*
Nanjing 16	1	0	Typical *indica*
Nanjing 46 (male parent)	0	1	Typical *japonica*
Zhaxima (female parent)	1	0	Typical *indica*
F1 hybrids	0.49	0.51	Intermediate
Regenerating green plants (type I)	0.37	0.63	Partial-*japonica*
Regenerating green plants (Type 2)	0.63	0.37	Partial-*indica*
Regenerating green plants (type 3)	0.41	0.59	Intermediate
Regenerating yellow plants	0.44	0.56	Intermediate
Regenerating albino plants	0.53	0.47	Intermediate

[a]: For the numerical range, please refer to Additional file 2: Table S2

perimeter (51.9 ± 2.4 μm) of the three type plants. Furthermore,the rice lines in type 3 had significantly smaller spikelet than those of the other two regenerated plants and also did not produce seeds. According to Liang (1979) and Choe et al. (2012), the shortest rice types may be haploid, which are significantly less tall than the high and the intermediate types. Low rate of natural doubling in the regeneration plants was also the main reason for low regeneration rate in this work.

Resistant starch (RS) content of regenerated rice lines

We obtained seeds from regeneration plants of 12 rice lines and determined their RS contents in their seeds (Table 5). 'Zhaxima' and 'Nanjing 46' contained 7.705 ± 0.142% and 0.200 ± 0.000% RS, respectively. The RS contents of the regenerated plants lay between those of the two parents, exhibiting the partial normal distribution. The RS contents of the regeneration plants were all higher than that of male parent, Nanjing 46. The rice plants with RS content between 0 and 3% accounted for 83.37% of the regenerated plants, while 16.67% had RS of 3–8%. Furthermore, the amylose starch contents in these regenerated plants were also reduced. Although the regeneration rate was low, the trait of the fertile regeneration plant with higher content of resistant starch has the obvious super mother advantage with good eating quality, which is easier to improve.

Discussion

Resistant starch (RS) has the potential to protect against diabetes and reduce the incidence of diarrhea, inflammatory bowel disease, colon cancer, and chronic renal and hepatic diseases (Zhou et al. 2016). The cultivation of rice varieties with unique medical value is not only a focal point in breeding but also has particularly received widespread attention among nutritionists. Resistant starch has novel functions similar to dietary fiber that can regulate metabolism. However, breeding rice varieties high in RS by time-consuming conventional methods is inefficient and difficult (Hu et al. 2004), because the RS-rich materials more belong to landraces and their yields are lower. The current focus of breeders is mainly how to obtain the crop high in RS. Some high RS rice varieties, such as 'RS111', 'Zhefu201', 'AMF18', Goami No. 2, Gongmi No. 3, Jiangtangdao 1 and 'Yitang1', have been bred using chemical mutagens or aerospace radiation mutagenesis or conventional hybridization (Shen et al. 2006; Kubo et al. 2008; Bai et al. 2012; Lee et al. 2006; Matsumoto et al. 2012). Although these methods have proven successful in crop breeding, it is highly random, not very efficient and also time-consuming (Rahman et al. 2007). With the rapid development of molecular biology, scientist have also tried to breed high RS rice by transgenic techniques (Deupuis et al. 2014) by inhibiting the activity of the SBEs. SBEs are one of the four major enzyme classes involved in starch biosynthesis in plants, and their activities play a crucial role in determining the structure and physical properties of starch granules (Tetlow & Emes 2014). Although inhibiting the activity of the SBE can get higher RS rice lines, modification of SBEs in planta also influences the degradation of starch reserves in developing seeds, thus impacting seedling vigor, this also in turn deteriorates the good eating quality of the rice grain (Nakamura et al. 2010; Sawada et al. 2009; Xia et al. 2011; Nakamura et al. 2012). Zhou's discovery provides an opportunity to increase RS content of cooked rice, especially in the *indica* varieties, which predominates in southern Asia (Zhou et al. 2016). However, because of security concerns about transgenic plants and, they are difficult to grow on large tracts of land. In this study, we produced an efficient technology for

Table 4 External peripheral long of the stoma, stem length and the seed rate in regeneration plants from anthers

Plant number	stomatal perimeter in the lower epidermis (μm)	Plant height (cm)	Seed setting	Average of the stomatal perimeter in the lower epidermis (μm)	Average of plant height (cm)	Spikle feature	Types
2 (1)	63.01 ± 1.12	73.5 ± 3.1	Seeding	59.58 ± 2.2	72.1 ± 4.5	Normal	I
3 (1)	60.73 ± 3.16	75.4 ± 2.1	Seeding				
1 (2)	60.27 ± 4.23	71.8 ± 2.1	Seeding				
7 (3)	61.06 ± 2.72	70.3 ± 2.4	Seeding				
8 (3)	63.24 ± 2.51	70.7 ± 1.4	Seeding				
10 (3)	54.92 ± 4.13	69.1 ± 1.3	seeding				
11 (3)	62.02 ± 2.30	76.5 ± 2.5	seeding				
12 (3)	59.45 ± 4.40	72.2 ± 1.6	seeding				
4 (4)	55.29 ± 2.18	80.8 ± 2.1	Seeding				
5 (4)	57.19 ± 1.72	67.1 ± 1.6	Seeding				
6 (4)	57.14 ± 2.72	68.4 ± 2.6	Seeding				
13 (6)	60.68 ± 2.42	70.7 ± 3.9	Seeding				
34 (1)	62.27 ± 2.15	133.2 ± 2.6	No seeding	63.47 ± 3.4	128.8 ± 2.7	spikes size is normal, but the anthers top is hooked	II
25 (2)	57.44 ± 4.09	123.3 ± 5.1	No seeding				
26 (2)	64.38 ± 2.64	124.3 ± 4.7	No seeding				
38 (2)	73.63 ± 3.04	135.8 ± 3.2	No seeding				
23 (3)	57.41 ± 2.33	119.5 ± 2.7	No seeding				
24 (3)	62.10 ± 4.29	121.5 ± 3.3	No seeding				
27 (3)	65.38 ± 6.31	126.3 ± 2.9	No seeding				
28 (3)	61.99 ± 3.04	130.1 ± 2.8	No seeding				
30 (3)	68.19 ± 5.07	130.5 ± 3.1	No seeding				
37 (4)	61.92 ± 1.50	135.5 ± 2.7	No seeding				
39 (4)	65.75 ± 2.74	137.2 ± 3.1	No seeding				
29 (7)	63.27 ± 4.13	130.4 ± 2.7	No seeding				
31 (8)	66.64 ± 5.47	131.9 ± 2.4	No seeding				
32 (6)	59.49 ± 1.34	132.1 ± 1.9	No seeding				
33 (6)	64.84 ± 3.27	133.1 ± 3.7	No seeding				
35 (6)	67.58 ± 3.16	133.3 ± 4.1	No seeding				
36 (6)	52.74 ± 1.37	133.6 ± 2.1	No seeding				
40 (6)	66.44 ± 2.71	140.3 ± 2.1	No seeding				
22 (1)	64.54 ± 1.27	95.5 ± 5.1	No seeding				
18 (4)	50.59 ± 3.02	58.4 ± 1.2	No seeding	51.9 ± 2.4	58.9 ± 1.4	spikes shape abnormalities, smaller and whiter	Ш
9 (4)	59.69 ± 3.34	71.3 ± 1.5	No seeding				
15 (6)	45.78 ± 1.23	54.4 ± 1.1	No seeding				
17 (6)	49.84 ± 1.43	58.1 ± 1.3	No seeding				
20 (6)	48.46 ± 2.63	59.0 ± 1.2	No seeding				
14 (8)	54.05 ± 1.12	53.8 ± 1.3	No seeding				
16 (8)	51.90 ± 3.02	58.1 ± 1.4	No seeding				
19 (8)	54.95 ± 1.32	58.5 ± 1.1	No seeding				
21 (9)	54.92 ± 1.67	60.9 ± 1.6	No seeding				
Zhaxima (female parent)	45.98 ± 0.54	150.2 ± 2.4					
Nanjing 46(male parent)	55.97 ± 1.24	70.5 ± 3.1					
F1	65.61 ± 2.12	130.0 ± 2.8					

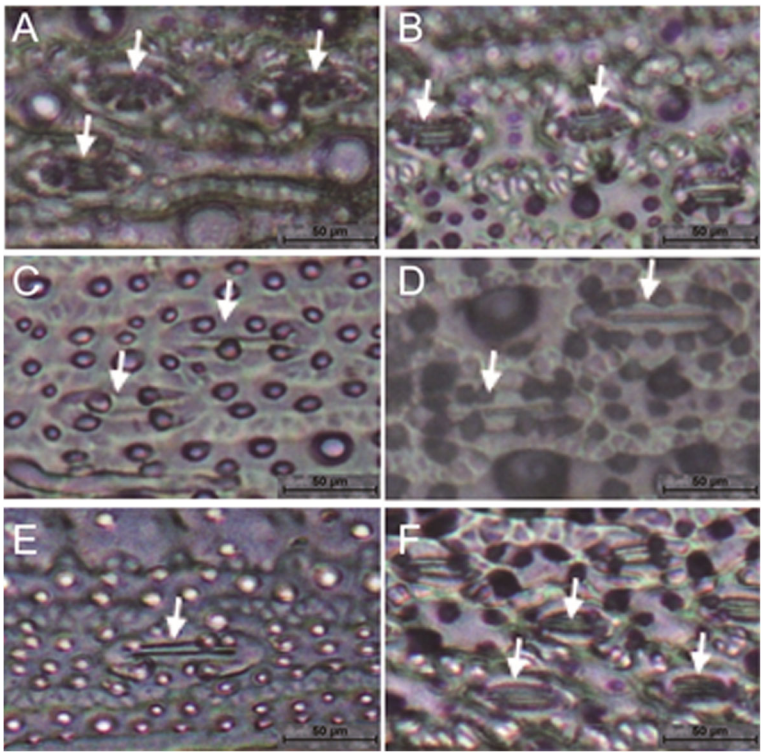

Fig. 3 Guard cells of 'Zhaxima', 'Nanjing 46', F1 hybrids, and regenerated plants. **a** female parent; **b** male parent; **c** F1 hybrid; **d** type 1 plants (height: 72.1 ± 4.5 cm); **e** type 2 plants (height: 128.8 ± 2.7 cm); **f** type 3 plants (height: 58.9 ± 1.4 cm)

Table 5 Amylose starch content and resistant starch (RS) content of regenerated rice plants [a]

Plant number	Apparent amylose content (%)	RS content (g/100 g)
2 (1)	12.953 ± 0.167	0.662 ± 0.140
3 (1)	15.987 ± 0.168	2.559 ± 0.412
1 (2)	17.986 ± 0.215	1.925 ± 0.141
7 (3)	16.397 ± 0.398	1.777 ± 0.211
8 (3)	15.496 ± 1.398	2.276 ± 0.412
10 (3)	14.289 ± 0.986	2.05 ± 0.069
11 (3)	16.987 ± 2.197	5.282 ± 0.074
12 (3)	17.149 ± 2.341	2.195 ± 0.069
4 (4)	18.563 ± 1.698	2.195 ± 0.054
5 (4)	15.064 ± 2.691	0.5869 ± 0.021
6 (4)	14.023 ± 3.219	1.451 ± 0.266
13 (6)	23.782 ± 2.876	7.659 ± 1.197
Zhaxima (female parent)	27.910 ± 1.698	7.705 ± 0.142
Nanjing 46 (male parent)	11.051 ± 0.324	0.200 ± 0.001

[a] Values are mean ± standard deviation of duplicate analyses
Note: Plant number m(n) indicates the serial number (m) and the number of regenerating plants from the same callus (n)

regenerating stable rice lines with high RS and good eating quality using anthers culture.

Androgenesis (anther or microspore culture) is one of two basic approaches for developing haploids in higher plants. In vitro process, microspore cells with haploid genomes develop into embryo-like structures on culture medium. The embryo-like structures further develop into haploid plantlets (Jauhar et al. 2009). With the rapid development of modern biotechnology, research on androgenesis and haploid breeding in *Gramineae* is gradually deepening, and some progress has been made in obtaining haploid grasses via anther technology. Although haploids have been successfully developed via in vitro culture of unfertilized ovules and ovaries in more than 20 angiosperm species since 1976 (Wu et al. 2004), anther or microspore culture in crops has experienced obstacles in haploid production, such as high rates of albinism, low response rates of some genotypes, and long periods for the inducing and regenerating processes, which could cause detrimental gametoclonal variation and mixed-ploidy plants (Niu et al. 2014). Cold pretreatment and using anther collected from the top or middle spike position can improve the ability of anther culture (Table 2). There may have been a synergistic effect between 2,4-D concentration and the cold pretreatment, because cold pretreatment is linked to the embryogenic capacity of plant tissues to acquire a specific hormonal status. Tian et al. (2015) reported that

pretreatments of low temperature on rice (*Oryza sativa* L.) anthers changed polysaccharide and protein composition of the anther walls and increased pollen fertility and then callus induction. Furthermore, the molecular characteristics of the two parents are the most important determinants of anther regeneration efficiency. In this study, we selected the female parent, *indica* 'Zhaxima' with high RS content and the male parent, *japonica* Nanjing 46′ with good eating quality of grain for anther cultural. Our results showed that the higher culture ability of hybrid rice cross *indica* and *japonica* seemed partial male such as *japonica*, Nanjing 46, while the equal distribution of *indica* and *japonica* characteristics was not suitable for regenerating green plants and setting seeds.

The ploidy level of microspore-derived regenerant varies among cereals. Microspores when cultured in vitro can spontaneously double the gametophytic chromosome number, but, for unknown reasons, this does not occur in all cases (Jähne & Lörz 1995). In wheat anther culture of a range of genotypes, 20–50% of green regenerants were doubled (Henry & Buyser 1999), while in barley, up to 87% of plants were spontaneous dihaploids (Hoekstra et al. 1993), and in rice up to 72% were reported (Cho & Zapata 1988). Ploidy identification in plant by pressing young root to count chromosome number in cell was a classical method. For this conventional method of ploidy identification of plants using root have certain limitations, because the rice plants from the rice regeneration must be conducted by the grain for the identification of resistant starch. Getting the green plants with seeds is necessary for rice breeding with high content of resistant starch. If the roots of plants were destroyed, the growth of the plants might be affected, conferring the lower seed-setting of rice varieties. We adopted the stomatal perimeter method for identifying ploidy. In this work, based on the observation of stomatal perimeter of the rice plants, combined with other characteristics such as plant height and seed setting rate, we also clearly distinguish the ploidy of the regeneration plants, and this method did not destroy the plants. Our 40 regeneration lines were divided into three types based on plant height, stomatal perimeter on the lower epidermis and setting seed characteristics. Type 2 plants were similar to the female parent 'Zhaxima' in plant height and stomatal perimeter on the lower epidermis, but sterile with abnormally hooked anthers. Previous studies have suggested that plants with small stomatal perimeters might be haploid (Liang 1979; Choe et al. 2012), which is similar to our results. The acarpous plants belonged to the type 3 with the smallest stomatal size might be caused by haploidy. Only 30.0% of the plants were dihaploid and produced seeds. There were still 22.5% regeneration plants that were not doubled. In addition to these sterile plants, there were 47.5% sterile plants with similar characteristics of plant height and stomatal sizes as their parents. The results indicated that the reason on low rate of producing RS rice plants from anthers of crosses between *indica* and *japonica* might be other intrinsic factors, in addition to the undouble obstacle.

The current focus of breeders is mainly how to obtain the crop high in RS. In this work, we used an efficient and safe regeneration system using F1 hybrids of 'Zhaxima' × 'Nanjing 46' to get the rice plants with high RS content (7.6 g/100 g dry weight). Based on genetic differentiation of the *indica* and *japonica* types using 35 indel sites (Lu et al. 2009), 'Zhaxima' was a typical *indica* variety and 'Nanjing 46' was a typical *japonica* plant. Using the S_{5-136} primers, we determined that two parents did not contain the wide compatibility gene (S_5). Regeneration plants of hybrids of *indica* and *japonica* may exhibit morphological traits that differ from those of their parents as a result of random recombination and assortment of chromosomes as well as genetic isolation. Among them, the regeneration plants of type 1 classified in this work can be easy to obtain the fertile green plant, and their subspecies characteristics were partial *japonica*, like their male parent, Nanjing 46.

Regeneration plants of type 1 all produced seeds, and the RS content of these plants were normally distributed. According to the results of Sun et al. (2012) on the genetic traits of hybrid rice seeds, different combinations of parents produce offspring with different RS contents. The variance analysis of RS content indicated that RS quality might be regulated by different genes and RS level is mainly controlled by additive effects in the hybrid offspring. Thus, the RS contents of offspring are generally distributed between the levels of the parents, also in this experiment they were skewed toward the male parent, just like our results here. The authors did present the amylose content, which is closely related to RS content (Zeng et al. 2016). The high amylose content in rice grain is positively correlated with RS content (Hu et al. 2004). Our results suggest that the high RS in some regenerated plants might be due to *Wx-a* allele of *Wx* gene coding for the enzyme for amylose synthesis, which is incorporated from the female *indica* parent. Through map-based cloning of a RS locus in *indica* rice, Zhou et al. (2016) have identified a defective soluble starch synthase gene (*SSIIIa*) responsible for RS production and further showed that RS production is dependent on the high expression of the $Waxy^a$ (Wx^a) allele, which is prevalent in *indica* varieties. Although the same *ssIIIa* mutation could be used in *japonica* rice together with introduction of a *Wxa* gene, the resulting rice would have higher amylose content than what is normally preferred by consumers of japonica varieties (Zhou et al. 2016). In this study, Nanjing 46 with low amylose and good eating taste was used as male parent to increase RS in *japonica* rice lines by anther culture, adding a new way of using *indica* rice to improve *japonia*. The different alleles of the *SSIIa* gene are responsible for differences in

amylopectin structure between the *indica* and *japonica* rice varieties (Umemoto et al. 2002). This suggests that the interactions with different soluble starch synthase genes (*SSIIIa* and *SSIIa*) in *japonica* rice variety (Nanjing 46) and *Wxa* in *indica* rice (Zaxima) can be helpful to obtain high RS content and improve the taste quality of rice. Further analysis of the regenerative plants with different partial-*japonica* or partial-*indica* genetic background with high RS may provide new clues for improving RS in rice. It is believed that the strategies to increase RS in rice, including *indica* and *japonica*, will be developed in the future with the discovery of the molecular basis underlying RS production in rice.

Conclusions

In this study, we established an efficient technology for regenerating stable rice lines high in RS using anthers, which not only shorten the breeding period and improve breeding efficiency, but also avoids having to identify multiple generations. The higher culture ability of hybrid rice cross *indica* and *japonica* seemed partial male such as *japonic*a, Nanjing 46, while the equal distribution of *indica* and *japonic*a characteristics was not suitable for regenerating green plants and setting seeds. Furthermore, the rice materials carrying the genetic components of RS by anther culture will be the basis for further studies on the genetic basis of the control of resistant starch in rice.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s43014-019-0010-7.

Additional file 1: Table S1. Information on 35 indel markers (Shen et al., 2003).

Additional file 2: Table S2. Indel classification standard of dividing the *indica* and the *japonica* variety by (F_i) or (F_j) gene frequency.

Abbreviations

2,4-D: 2,4-dichlorophenoxyacetic acid; 6-BA: 6-benzylaminopurine; ABA: Abscisic acid; Am: Amylose; AMG: Amyloglucosidase; Ap: Amylopectin; CAPS: Codominant cleaved amplified polymorphic sequence; DH: Doubled haploid; DM: Differentiation medium; GABA: r-aminobutyric acid; GI: Glycemic index; GOPO: Glucose oxidase/peroxidase; IM: Induction medium; InDel: Insertion–deletion; KT: Kinetin; NAA: 1-naphthaleneacetic acid; RDS: Rapidly digestible starch; RM: Rooting medium; RS: Resistant starch; SBE: Starch branching enzymes; SDS: Slowly digestible starch; SM: Seedling medium; TRS: Teqing Resistant Starch; WCVs: Wide compatibility varieties

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Authors' contributions

LX supervised the project, designed and guided the experiment, and wrote the manuscript. TQQ conducted most of the experiments. LCM helped with some experiments and revised the manuscript. FXW guided some experiments. All authors have read and approved the final manuscript.

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Availability of data and materials

All the data and materials were kept in Dr. Li Xia's lab, Institute of Food Crops, Jiangsu Academy of Agricultural Sciences, Jiangsu High Quality Rice R&D Center, Nanjing Branch, China National Center for Rice Improvement, Nanjing 210014, P.R. China. Data sharing is not applicable to this article as no data sets were analyzed during the current study. If the readers wish to understand these data and material in detail, please contact the corresponding author for data requests.

Competing interests

The authors declare that they have no competing interests.

Author details

[1]Institute of Food Crops, Jiangsu Academy of Agricultural Sciences, Jiangsu High Quality Rice R&D Center, Nanjing Branch, China National Center for Rice Improvement, Nanjing 210014, China. [2]Jiangsu Collaborative Innovation Center for Modern Crop Production, Yangzhou 225009, China. [3]College of Life Science, Nanjing Normal University, Nanjing 210023, China.

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