Euphytica (2019) 215:66
https://doi.org/10.1007/s10681-019-2392-7

Development and utilization of functional KASP markers to improve rice eating and cooking quality through MAS breeding

Guili Yang · Siping Chen · Likai Chen · Weiwei Gao · Yuting Huang · Cuihong Huang · Danhua Zhou · Jiafeng Wang · Yongzhu Liu · Ming Huang · Wuming Xiao · Hui Wang · Tao Guo · Zhiqiang Chen

Received: 22 November 2018 / Accepted: 5 March 2019
© Springer Nature B.V. 2019

Abstract There is a steady demand for high-quality rice varieties though consumers' preference for high-quality rice varies from regions. The improvement of the quality of rice, especially its eating and cooking quality (ECQ), is an important breeding target as rice is mainly consumed in cooked form. The development and utilization of breeder-friendly and high-throughput marker systems play a pivotal role in marker-assisted breeding of rice cultivars, with pyramids of valuable genes affecting rice ECQ. In this study, we developed functional markers based on the Kompetitive Allele-Specific PCR (KASP) method for three principal genes, *Wx*, *BADH2* and *ALK*, affecting ECQ in rice. The accuracy of all KASP markers was verified and confirmed with Sanger sequencing. A diverse rice panel consisting of 38 indica cultivars, 9 japonica cultivars and 1 Javanica cultivar were used to assess the validity of these markers for genotyping. The results showed that the functional KASP markers of *Wx*, *ALK* and *BADH2* could effectively distinguish the different alleles. The genotyping results highly coincided with the phenotypic traits of rice eating and cooking quality. Eight rice entries harboring 3 favorable alleles were identified and superior rice restorers with improved ECQ were bred via functional marker genotyping. Breeders can develop rice cultivars that have desirable ECQ, customized to meet the market requirements in target regions. Hence, the development of these 3 applicable KASP functional markers based on allelic variation would be valuable for improving rice eating and cooking quality through maker-assisted selection to carter various consumer preferences especially in Asian areas.

Keywords Rice · KASP · Functional markers · Eating and cooking quality (ECQ) · Genotyping · Maker-assisted selection (MAS)

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10681-019-2392-7) contains supplementary material, which is available to authorized users.

Guili Yang and Siping Chen contributed equally to this work.

G. Yang · S. Chen · L. Chen · W. Gao · Y. Huang · C. Huang · D. Zhou · J. Wang · Y. Liu · M. Huang · W. Xiao · H. Wang · T. Guo (✉) · Z. Chen (✉)
National Engineering Research Center of Plant Space Breeding, South China Agricultural University, Guangzhou 510642, China
e-mail: guo.tao@vip.163.com

Z. Chen
e-mail: chenlin@scau.edu.cn

Introduction

Cultivated rice (*Oryza sativa* L.) is one of the most important crops and feeds approximately half of the human population of the world (Chen et al. 2014). Currently, improvement of rice eating and cooking

Published online: 08 March 2019

quality (ECQ) has become a foremost consideration for rice consumers and breeders in the main rice-producing countries and regions, with a demand for high-quality food in recent years (Pang et al. 2016; Tong et al. 2014). The rice ECQ is a very complex trait which is partially affected by environmental factors such as growing temperature and soil fertility, but mainly determined by genetic control (Sun et al. 2011). Consumers' preference for high ECQ rice varies from country to country (Bunyasiri and Sirisupluxana 2018; Musa et al. 2018). People in Lao PDR and the Isan region of Thailand prefer waxy or sticky rice, whereas consumers from Japan, Northeast of China etc., prefer low amylose rice; High AC rice is preferred in some non-Asian rice-growing countries, such as Ghana, Senegal and Suriname (Calingacion et al. 2014). Striking regional preferences drive the market and hence increase the complexity of breeding for good ECQ rice. Thus, the progress in the improvement of rice grain qualities, especially in the ECQs of commercial varieties, is relatively slow due to the above various reasons.

Given that starch comprises approximately 90% of the rice grain, the structure and composition of grain starch are two main components affecting ECQ; these components include amylose content (AC), gel consistency (GC), alkali spreading value (ASV) and gelatinization temperature (GT). Tremendous efforts have been made to identify genes involved in starch biosynthesis that are naturally expected to affect ECQ. Approximately 20 genes encoding starch synthesis enzymes that function in different stages of starch synthesis have been identified (Tian et al. 2010). The *Wx* gene is responsible for the variation in amylose content (AC) in rice grain endosperm. It encodes a granule-bound starch synthase (GBSS) and has been found to play a major role in determining AC and GC (Zhang et al. 2012). The variation in GT was mainly controlled by the *ALK* (*SSII3*) locus. Gao et al. (2003) cloned the *ALK* gene through a map-based cloning approach and found that a G264 to C264 substitution downstream of the initial codon of *ALK* is responsible for the observed variation in rice GT. Bao et al. (2006) sequenced the fragments covering introns 6 and 7, exons 6 and 8, and part of the 3′-untranslated region of the *ALK* gene from 30 varieties, and association analysis indicated that the GT variation among 30 varieties could be attributed mainly to the substitution of GC to TT in exon 8. The marker *ALK*-GC/TT was developed to distinguish two contiguous single nucleotide polymorphisms (SNPs) (GC/TT) in the *ALK* gene; these SNPs explained more than 60% of the total variation in rice thermal properties and have a very strong association with GT (Bao et al. 2006; Umemoto and Aoki 2005; Waters et al. 2006). Fragrance is another popular quality pursued by consumers worldwide. The presence of a dominant *BADH2* allele located on chromosome 8S encoding betaine aldehyde dehydrogenase (BADH2) inhibits the synthesis of 2-acetyl-1-pyrroline (2AP), a potent flavor component in rice fragrance (Chen et al. 2008). An eight-base-pair (8-bp) deletion in exon 7 of *BADH2* results in the nonfunction of *BADH2* and the accumulation of 2AP, thus causing fragrance in many of the fragrant rice varieties, including basmati and jasmine rice (Bradbury et al. 2005; Sakthivel et al. 2009).

Molecular marker-assisted selection (MAS) combined with conventional breeding approaches enables breeders to precisely identify the individual genotypes that are associated with different grain quality features, which can dramatically improve the breeding efficiency (Tian et al. 2010). Functional markers based on single-nucleotide polymorphisms (SNPs) are thought to be powerful for improving the efficiency of MAS breeding in rice due to their high throughput and well-adapted automation. The development and utilization of functional genetic markers based on SNPs play a pivotal role in maker-assisted breeding of rice cultivars during pyramiding of agronomically valuable genes (Luo et al. 2014; Ma et al. 2017; Neelam et al. 2013). However, to date, the number of functional markers used in MAS for rice ECQ improvement is still somewhat limited due to the complexity of such traits. Several SNP markers have been developed for *Wx* and *BADH2* genes via high-resolution melting (HRM) (Luo et al. 2014) and the cleaved amplified polymorphism sequences (CAPS) method (Chen et al. 2009). However, these markers are not widely utilized because of some limitations. The HRM method relies poorly on complicated and multiround PCR procedures to ensure the acquisition of unique PCR products for further detection. The CAPS method is restricted to low-throughput and special enzymes needed for specific base pair digestion. Thus, breeder-friendly and high-throughput markers are needed for large-scale selections in rice breeding programs. The Kompetitive Allele Specific

PCR (KASP) is a closed-tube, gel-free assay utilizing a unique form of competitive allele-specific PCR and suitable for high-throughput genotyping of SNPs or inserts and deletions (InDels) (He et al. 2014; Myakishev et al. 2001; Neelam et al. 2013).

The aim of this work is to develop and assess the suitability and validity of the KASP method for detecting the genotypes of the *Wx*, *BADH2* and *ALK* genes and to use these markers to breed improved rice accession via MAS. Our research showed that the development of applicable KASP molecular markers based on allelic variation would be valuable for improving rice ECQs through a MAS approach.

Materials and methods

Plant materials

Eleven rice accessions and pyramiding lines were chosen to develop and verify the KASP marker for the *Wx*, *BADH2* and *ALK* genes each (Table 1). These rice materials harboring variation alleles of 3 genes via the Sanger sequence have been identified. Another 48 rice accessions including 38 indica cultivars, 9 japonica cultivars and 1 Javanica cultivar were used to assess the validity of three KASP markers in genotyping (Table S1). 45 out of the cultivars were collected from China. One cultivar came from United States and one sourced from Pakistan. The rice genomic DNA was extracted using the CTAB method (Wang et al. 2013) and quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, USA). The OD_{260}/OD_{280} ratio was used to estimate the purity of DNA. The sterile lines, including the two-line sterile line C815S and three-line sterile line Ning A, were used for testing cross to evaluate the heterosis of the improved lines.

Primer design for KASP

We developed the allelic SNP marker using the KASP approach (http://www.lgcgroup.com/), which requires no labeling of the target-specific primers and allows greater flexibility for assay design. To design markers targeting the putative SNPs, we extracted 250 bp surrounding the candidate SNP on either side and ordered KASP primers from LGC Limited, UK. The specific primer sequences are shown in Table 2. The genotyping assays were tested in a 96-well format and set up as 10 µL reactions (4.85 µL of template (50–75 ng of DNA), 5.0 µL of 2 × Kaspar mix, and 0.15 µL of primer mix). PCR was performed on a StepOne Plus machine using the following protocol: pre-read stage at 30 °C for 1 min, hot start at 95 °C for 15 min, followed by 10 touchdown cycles (95 °C for 20 s; touchdown 65 °C, − 1 °C per cycle, for 25 s) and then by 26 cycles of amplification (95 °C for 10 s; 57 °C for 60 s). Fluorescence data were collected during the pre-read and post-read stages (30 °C for 1 min). Once the run was completed, analysis was carried out with StepOne Software ver. 2. Genotyping data were viewed as a cluster plot by SNP viewer (LGC Limited, UK).

Confirmation of genotype by Sanger sequencing

To evaluate the efficiency and accuracy of KASP assays, all the marker sites, including *Wx* (SNP), *BADH2* (InDel), and *ALK* (dinucleotide polymorphism), were amplified with the outer-primer pair, and the amplicons were sequenced using the Sanger protocol (Life Technologies, https://www.thermofisher.com/). We compared the results obtained by KASP with those determined by Sanger sequencing to confirm the genotype of all the target alleles. We used the online primer design system (http://bioinfo.ut.ee/primer3-0.4.0/) according to the genomic sequences of the *Wx*, *BADH2* and *ALK* (Table 3). PCR amplification was performed in a 50 µL reaction mixture containing 10 × PCR Buffer for KOD-Plus-Neo 5 µL, 2 mM dNTPs 5 µL, 25 mM $MgSO_4$ 3 µL, 10 µM primers each 1.5 µL, KOD-Plus-Neo (1 U/µL) 1 µL, ddH_2O 32 µL and 1 µL gDNA (approximately 100 ng). The PCR conditions were as follows: initial denaturation at 95 °C for 5 min, 32 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 5 min. The PCR products were separated on a 2% agarose gel and recycled for Sanger sequencing.

Phenotypic evaluation of grain eating and ECQ traits

We evaluated the ECQ traits of the 48 rice accessions used in the validity of the genotyping test. Dried rice grains were stored at room temperature for 1 month prior to the evaluation of rice ECQ. Samples were

Table 1 Genotypes of *Wx*, *BADH2* and *ALK*

Genes	Accessions	Genotypes
Genotypes of *Wx*	Jinhangruanzhan	T:T
	Guijingsimiao	T:T
	Huanglisimiao	T:T
	Hanghui 7	T:T
	F_1 of Peiai64S/Hanghui 7	T:G
	F_1 of Peiai64S/Hanghui 7	T:G
	F_1 of Peiai64S/Hanghui 7	T:G
	F_1 of Peiai64S/Hanghui 7	T:G
	Jinhuazhan3	G:G
	Tainongxinzhan	G:G
	Peiai 64S	G:G
Genotypes of *BADH2*	Heixiangruanmi	TATAT:TATAT
	Kexiang 2	TATAT:TATAT
	Ruanhua A	TATAT:TATAT
	Pyrimiding line H230	TATAT:TATAT
	Pyrimiding line H231	TATAT:TATAT
	Pyrimiding line H232	TATAT:TATAT
	Pyrimiding line H233	TATAT:TATAT
	Y58S	AAGATTATGGC:AAGATTATGGC
	Fengxianzhan	AAGATTATGGC:AAGATTATGGC
	Xinhuangzhan	AAGATTATGGC:AAGATTATGGC
	Fengxiusimiao	AAGATTATGGC:AAGATTATGGC
Genotypes of *ALK*	Yuemeisimiao	TT:TT
	Huangyuesimiao	TT:TT
	Baihuangzhan	TT:TT
	Guguangzhan	TT:TT
	F_1 of Guguangzhan/Huazhan	TT:GC
	F_1 of Guguangzhan/Huazhan	TT:GC
	F_1 of Guguangzhan/Huazhan	TT:GC
	F_1 of Guguangzhan/Huazhan	TT:GC
	Huazhan	GC:GC
	IRAT109	GC:GC
	Chuanhui 907	GC:GC

Table 2 Primers of KASP markers for *Wx*, *ALK* and *BADH2*

Genes	Primer names	Primer sequence
Wx	Primer_AlleleFAM	TCATCAGGAAGAACATCTGCAAGG
	Primer_AlleleHEX	GTTCATCAGGAAGAACATCTGCAAGT
	Common reverse	CGATCTGAATAAGAGGGGAAACAAAGAAT
ALK	Primer_AlleleFAM	GACATGCCGCGCACCTGGAA
	Primer_AlleleHEX	ACATGCCGCGCACCTGGAG
	Common reverse	GCCTCGAGACGTACCGCAAGTA
BADH2	Primer_AlleleFAM	TAACCATAGGAGCAGCTGAAG
	Primer_AlleleHEX	ACCTTAACCATAGGAGCAGCTGAAA
	Common reverse	TGCATTTACTGGGAGTTATGAAACTGGTA

Table 3 Primers for Sanger sequencing

Genes	Primer_Left	Primer_Right
Wx	GCTTCACTTCTCTGCTTGTG	ATGATTTAACGAGAGTTGAA
ALK	GTGGGGTTCTCGGTGAAGAT	CAAGCTTCTTCAGGGAGGCTA
BADH2	CTTGTTTGGAGCTTGCTGATG	CCAGTGAAACAGGCTGTCAAG

boiled for 10 min in volumetric flasks to completely disperse the grain powder, and the optical density of the amylose-iodine blue was measured at 620 nm using a spectrophotometer (Leng et al. 2014). The alkali spreading value (ASV) was determined by incubating six milled grains in 10 mL of 1.7% KOH at 28 °C for 23 h. The degree of spreading was rated using the following 7-point semiquantitative criteria: (1) grain not affected; (2) grain swollen; (3) grain swollen, collar incomplete and narrow; (4) grain swollen, collar complete and wide; (5) grain split, collar complete and wide; (6) grain dispersed, merging with collar; and (7) grain completely dispersed and intermingled (Su et al. 2011). It was classified as low GT for ASV 5.5–7.0, intermediate GT for ASV 3.5–5.4 and high GT for ASV B 3.4 (Tuano et al. 2016). For all the phenotypic parameters, more than two biological replicates for each genotype were assayed. The fragrance was assessed through a rice cooking test according to GB/T 15682-2008 (http://samr.cfda.gov.cn/). Correlation analysis and regression analysis of phenotype and genotype were done with Microsoft EXCEL.

Results

KASP marker developed for the Wx gene

The G/T polymorphism located at the intron 1 splice site of *Wx* is responsible for most of the variation in AC (Cai et al. 1998; Chen et al. 2008), which is also the genotyping target of the *Wx*-G/T marker. This marker can discriminate three genotypes, G/G, T/T and G/T. Four samples (Jinhangruanzhan, Guijingsimiao, Huanglisimiao and Hanghui 7) were classified as having T/T, and three samples (Jinhuazhan 3, Tainongxinzhan, Peiai 64S) were determined to have G/G. Four F_1 populations derived from the Peiai 64S cross with Hanghui 7 were classified as having both T/G alleles (Fig. 1a). Visual inspection revealed an obvious distinction between the two homozygous genotypes (red and blue) and the heterozygous genotype (green). To confirm the validity of this KASP marker, another marker specific for the *Wx*-G/T site was applied to these lines, and the PCR products were sequenced. The Sanger sequencing results are consistent with the *Wx*-G/T KASP assay (Fig. 1b–d).

KASP marker developed for the BADH2 gene

The KASP marker for the *BADH2* gene was designed based on the 8-bp deletion in exon 7. Visual inspection revealed an obvious distinction between the 8-bp deletion genotypes (red) and none of the 8-bp deletion genotypes (blue) (Fig. 2a). Heixiangruanmi, Kexiang 2, Ruanhua A and 4 pyramiding lines were classified to carry the 8-bp deletion genotype. Another four accessions, including Y58S, Fengxianzhan, Xinhuangzhan and Fengxiusimiao, did not have the 8-bp deletion. No heterozygous genotype was discovered. To validate the precision of this KASP marker, the sequence including the 8-bp polymorphism was amplified by another marker in these lines for sequencing. The Sanger sequencing results are consistent with the KASP assay (Fig. 2b–d).

KASP marker developed for the *Alk* gene

The KASP marker for *ALK* was developed to distinguish two contiguous SNPs (GC/TT) in the *ALK* gene. This marker was able to discriminate three genotypes, GC:GC, GC:TT and TT:TT. Three samples (Huazhan, IRAT109 and Chuanhui907) were classified as having TT:TT, and four samples (Yuemeisimiao, Huangyuesimiao, Baihuangzhan and Guguangzhan) were determined to carry GC:GC. Four offspring of the F1 generation from the cross between Guguangzhan and Huazhan were classified as having both alleles GC:TT (Fig. 3a). The *ALK*-GC/TT KASP genotyping results were further confirmed by Sanger sequencing (Fig. 3b–d).

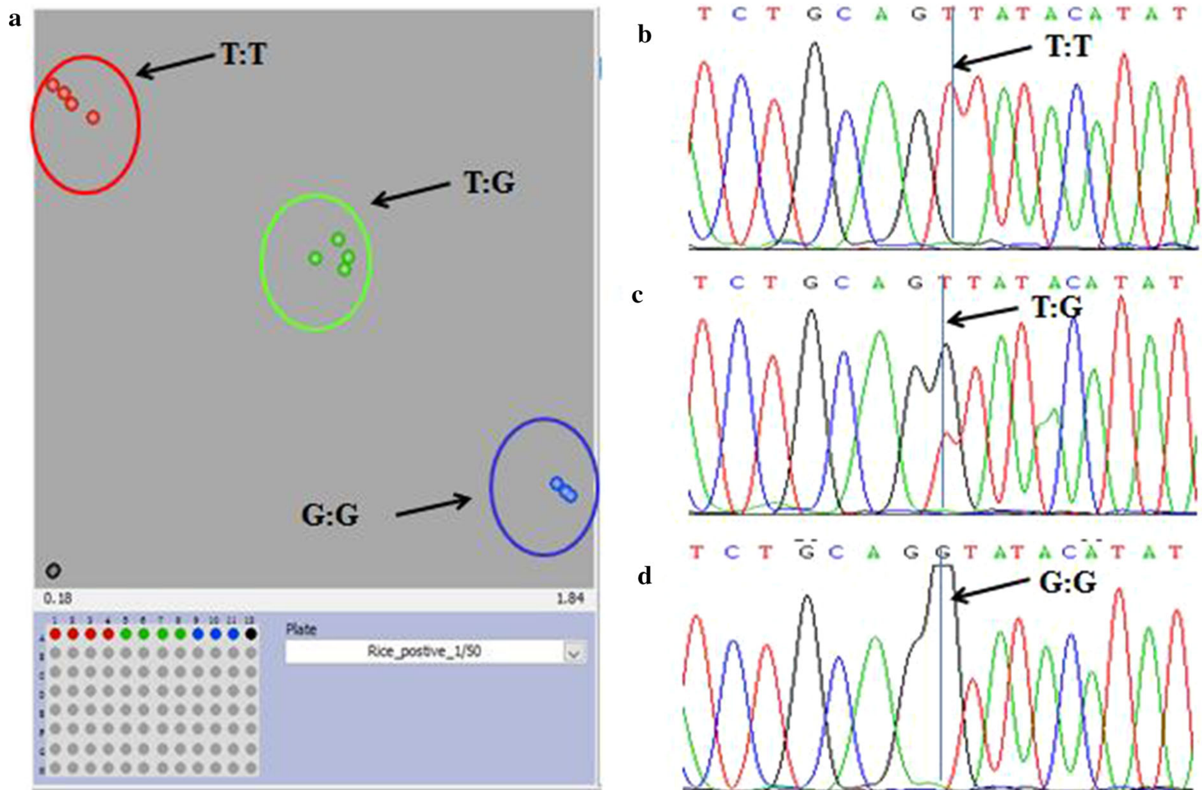


Fig. 1 KASP genotyping of *Wx* confirmed by Sanger sequencing. **a** *Wx* KASP genotyping results; **b** Sanger sequencing of SNP site T/T; **c** Sanger sequencing of SNP site T/G; **d** Sanger sequencing of SNP site G/G. Grey dot represents no template control

Molecular screening using KASP markers

To further validate the usefulness of this KASP platform in molecular screening and evaluating rice ECQ, a set of 48 rice cultivars were genotyped with the KASP markers *Wx*, *BADH2*, *and ALK*. The diverse rice panel revealed a wide range variation in AC and ASV. The highest AC was recorded as 27.22% for Kasalath while Wanlinuo had the lowest AC, 5.89%. The maximum value of ASV was 7.00 in several cultivars and the minimum value was 1.00 in Chenghui727 and HengfengB, respectively. Forty-two, 35 and 11 rice cultivars were identified to harbor the favorable allele for *Wx-TT* (T:T allele), *ALK-TT* (TT:TT allele), and *BADH2-8 bp* (8 bp-deletion allele), respectively (Fig. 4a). Eight rice cultivars, including Xiangyaxiangzhan, Basmati 370 and Meixiangzhan 2, carried all three favorable alleles (Fig. 4b). The ECQ, including AC%, ASV and fragrance, were also investigated to evaluate the phenotype of all the above accessions (Table S1). AC% varied from 5.89 to 27.47%. Four categories based on AC can be broadly classified: waxy-type (AC $< 5\%$), low-type (AC 10–14%), medium-type (AC 14–21%), and high-type (AC $> 21\%$). Almost all the accessions with AC less than 20% carried the T:T allele of *Wx*. ASV provides information about GT, and three GT grades can be classified based on the ASV. The ASV of all the accessions ranged from 1 to 7. The accessions carrying GC:GC in *ALK* had ASV values lower than 5. Fragrance existed in the accessions with TATAT:TATAT in *BADH2* when tested.

Phenotypic contribution of alleles

Because both *Wx* and *ALK* genes are key genes affecting starch synthesis, we analyzed the contribution of alleles to the phenotype of AC and ASV of the above 48 diverse rice cultivars. Correlation analysis showed that the *Wx* gene was positively correlated with AC and that the *Wx-TT* allele significantly decreased AC. In contrast, the *ALK* gene was

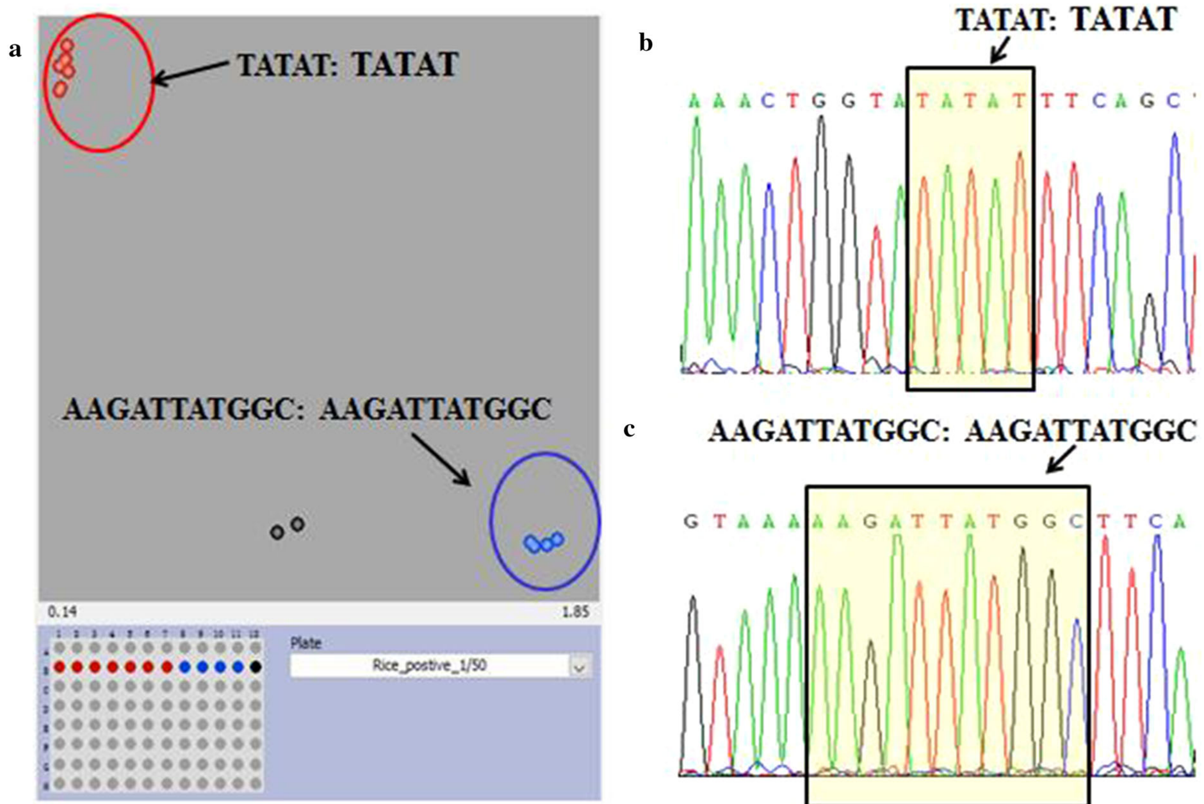


Fig. 2 KASP genotyping of *Badh2* confirmed by Sanger sequencing. **a** *Badh2* KASP genotyping results; **b** Sanger sequencing of InDel; **c** Sanger sequencing of InDel. Grey dot represents no template control

negatively correlated with the ASV phenotype, and the *ALK-TT* genotype significantly increased ASV (Table 4). Based on the correlation analysis, a regression equation between marker and trait was constructed by stepwise regression. The regression equation for *Wx* and *AC* reached a very significant level (P value 1.62×10^{-11}) and accounted for 63.1% of the phenotypic variation. The regression equation of *ALK* and ASV also achieved a very significant *P* value (*P* value 2.58×10^{-11}) and explained 62.3% of ASV phenotypic variation (Table 5). The results showed that the constructed regression equation can be used to predict phenotypes by genotype value.

Improved ECQ of rice restorer lines by genotyping

According to the molecular screening results, 9311, a mega Indica rice restorer line, harbors one favorable allele, *ALK-TT,* which is responsible for high ASV. However, 9311 also showed high AC (27.47%) that was not suitable for superior quality due to allele *Wx-GG*. To improve the quality of 9311, we selected a superior aromatic rice cultivar, Basmati 370, which has low amylose and fragrance, as favorable alleles *Wx-TT* and *BADH2-8 bp* donors, 9311 as the receptor and recurrent parent, to develop new aromatic and good quality restorer lines with a 9311 background through backcross and functional molecular marker selection. The technical route is shown in Fig. 5. Thirteen improved lines with homozygous target genes were obtained in BC_2F_2 generation by selection with functional markers. Agronomic and rice quality traits were surveyed for these improved lines. Compared with the recurrent parent 9311, the quality of 13 lines was improved to a certain extent. The AC values of 13 lines ranged from 12.06 to 16.71%, which was significantly lower than that of 9311. These lines also showed strong fragrance. The chalkiness traits of most lines were also improved, with 92.3% of the lines having lower chalkiness grain rates than 9311 (Table 6). The background recovery rate (BRR%) of the improved lines was investigated using 48 SSR

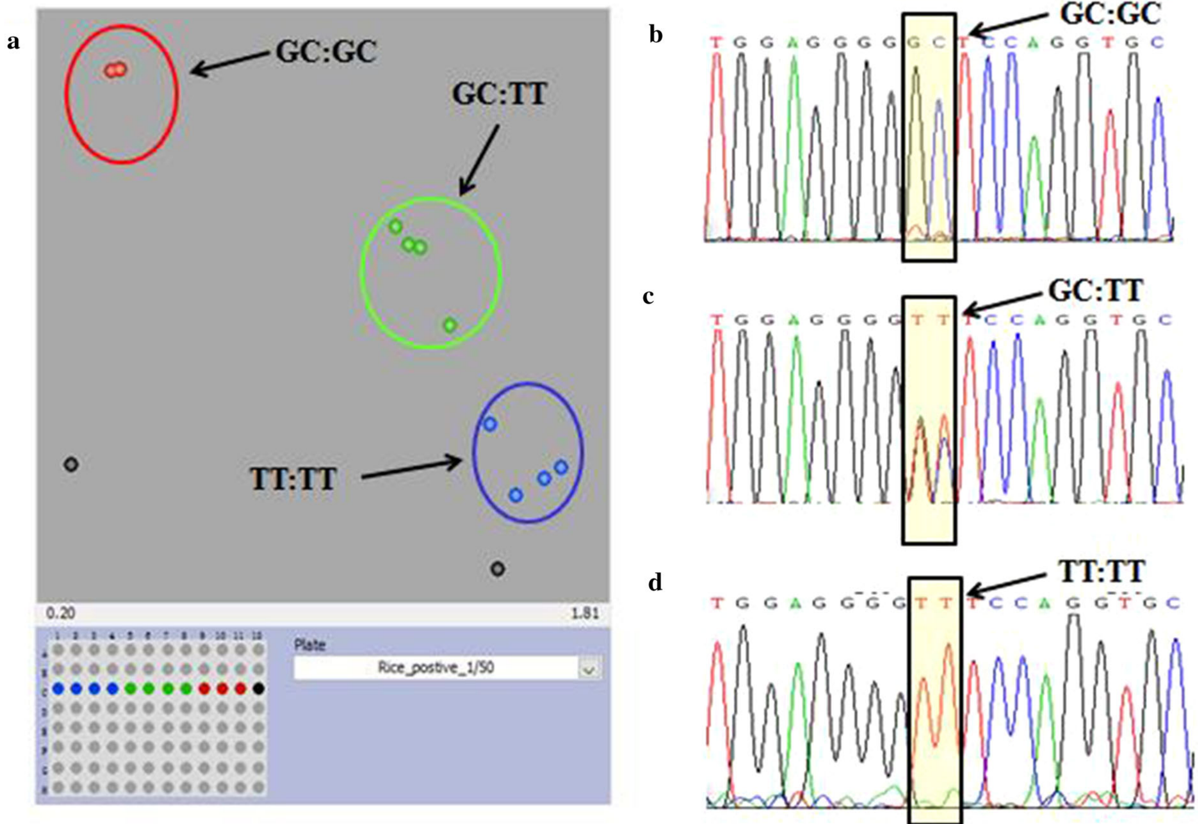
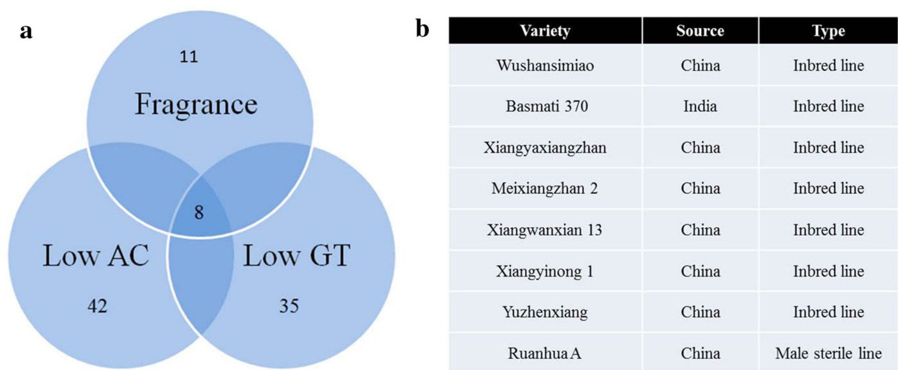


Fig. 3 KASP genotyping of *ALK* confirmed by Sanger sequencing. **a** *ALK* KASP genotyping results; **b** Sanger sequencing of SNP site GC/GC; **c** Sanger sequencing of SNP site GC/TT; **d** Sanger sequencing of SNP site TT/TT. Grey dot represents no template control



Variety	Source	Type
Wushansimiao	China	Inbred line
Basmati 370	India	Inbred line
Xiangyaxiangzhan	China	Inbred line
Meixiangzhan 2	China	Inbred line
Xiangwanxian 13	China	Inbred line
Xiangyinong 1	China	Inbred line
Yuzhenxiang	China	Inbred line
Ruanhua A	China	Male sterile line

Fig. 4 The genotyping of 48 rice accessions with KASP markers for *Wx*, *BADH2* and *ALK*. A: Classification of 48 rice accessions according to their genotypes; B: List of eight elite accessions with all three favorable genotypes of *Wx*, *ALK* and *BADH2*

markers distributed evenly on 12 chromosomes. The backgrounds of these improved lines were restored to over 80% of the recurrent parent 9311, and the agronomic characteristics of the improved lines were similar to 9311. These results indicated that molecular marker selection in early generations could effectively improve the breeding efficiency of quality.

The sterile lines, including the three-line sterile line Ning A and the two-line sterile line C815S, were used for testing cross to evaluate the heterosis of the improved lines. Compared with the control, the improved lines show the superiority in heterosis utilization. The 1000-grain quality of most crossed combination results from improved lines was

Table 4 Correlation coefficient between phenotype and genotype

	AC	ASV	*Wx*	*ALK*
AC	1			
ASV	0.132901	1		
Wx	0.794254**	0.097319	1	
ALK	0.095688	− 0.78952**	0.116335	1

AC amylose content, *ASV* alkali spreading value

**significant at 0.01 level

improved, especially for the 1000-grain weight of F_1, which was higher than that of CK (Table 7). Ning A/9311-R1, Ning A/9311-R5 and C815S/9311-R13 have significantly higher total grain weights than CK, which increased by 26.69%, 22.53% and 9.6%, respectively, indicating that these three lines had potential high yield combining ability. The AC of all hybrid combinations was lower than that of the control, indicating that the introduction of the *Wx-TT*

Table 5 Regression equation for phenotype and genotype

Trait	Gene	Allele	No. of genotypes	Minimum of trait	Maximum of trait	Regression equation models (R^2%)
AC	*Wx*	T	42	5.89	20.86	y = 4.9 + 10.63x (63.1%)
		G	6	24.15	27.47	
ASV	*ALK*	TT	37	4.67	7.00	y = 10.12 − 3.83x (62.3%)
		GC	11	1.00	4.50	

y = 4.9 + 10.63x, y indicates AC trait, x indicates *Wx* allele; y = 10.12 − 3.83x, y indicates ASV trait, x indicates *ALK* allele

AC amylose content, *ASV* alkali spreading value

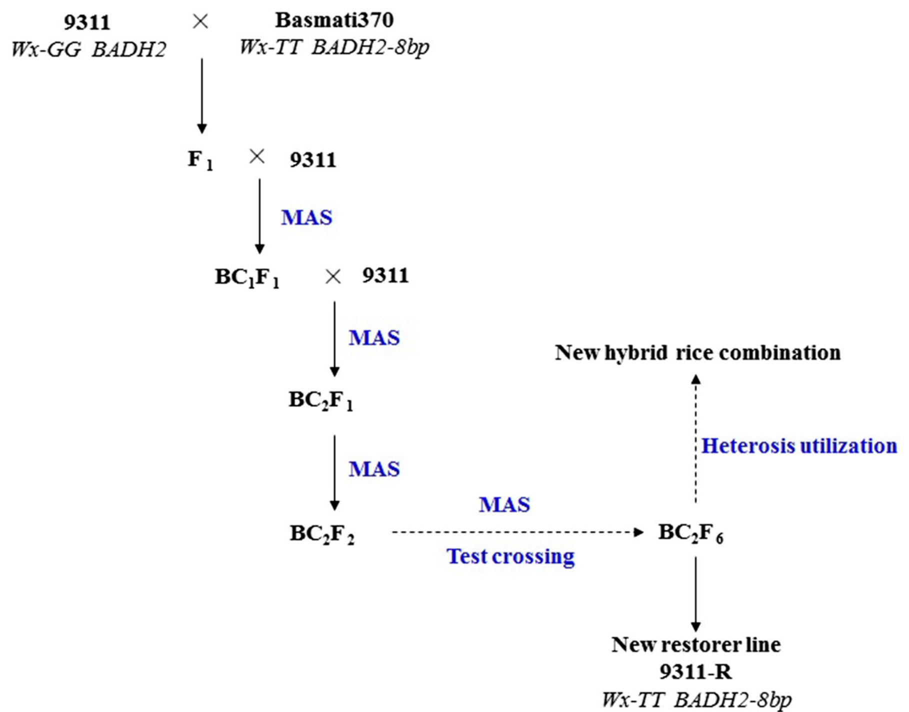


Fig. 5 Schematic working flow of molecular improvement

Table 6 Genotype and quality traits of 13 improved lines

Line	Allele genotype		AC%	Fragrance	LWR	CGR%	BRR%
	Wx	BADH2					
9311	G:G	AAAAGATTATGGC:AAAAGATTATGGC	27.47	−	3	17.65	/
Basmati370	T:T	TATAT:TATAT	15.68	+	3.4	5.67	/
9311-R1	T:T	TATAT:TATAT	12.06	+	3	10.01	80.1
9311-R2	T:T	TATAT:TATAT	13.76	+	2.9	13.06	82.6
9311-R3	T:T	TATAT:TATAT	12.54	+	3.2	7.79	83.1
9311-R4	T:T	TATAT:TATAT	16.31	+	3.1	11.26	81.9
9311-R5	T:T	TATAT:TATAT	15.09	+	3.1	8.21	85.6
9311-R6	T:T	TATAT:TATAT	14.11	+	2.8	18.01	87.3
9311-R7	T:T	TATAT:TATAT	13.27	+	3.2	8.91	84.2
9311-R8	T:T	TATAT:TATAT	14.54	+	3.1	9.26	83.9
9311-R9	T:T	TATAT:TATAT	15.09	+	3	10.15	88.1
9311-R10	T:T	TATAT:TATAT	15.23	+	3.1	12.29	81.2
9311-R11	T:T	TATAT:TATAT	16.01	+	3.2	7.62	83.7
9311-R12	T:T	TATAT:TATAT	16.71	+	3	8.31	86.2
9311-R13	T:T	TATAT:TATAT	15.62	+	2.9	9.1	87.1

AC, amylose content; +, fragrant; −, nonfragrant; LWR, length–width ratio; CGR, chalkiness grain rate; BRR, background recovery rate

allele was very effective in reducing the AC of hybrid combinations.

Discussion

Currently, rice ECQs have attracted increasing attention from breeders, especially in the main rice-producing countries and regions with economic development. In general, the genetic basis of rice ECQs is mainly determined by three physicochemical properties: AC, GC, and GT. The difficulties in measuring eating and cooking properties make the improvement of rice ECQs even slower and more difficult.

Traditional genetic markers such as SSRs (simple sequence repeats), small InDels, and CAPS markers for MAS are time-consuming, costly, labor intensive and not user-friendly (Ramkumar et al. 2015). Several high-throughput SNP genotyping platforms are commercially available today, such as Fluidigm Dynamic Arrays, fluorescence-labeled TaqMan™ are expensive, lack flexibility, and are not of practical use for small- to medium-sized laboratories (Thomson 2014). KASP is one of the uniplex SNP genotyping platforms and has evolved to be a global benchmark technology (Semagn et al. 2014). KASP genotyping can be performed in 96- or 384-well plates, giving it much higher throughput than the gel-based method; more importantly, it is a closed-tube method and therefore can significantly reduce the chance of the experimenters being exposed to hazardous chemicals. In our study, it can be very efficiently and flexibly operated according to different sample sizes. The genotyping results can be clearly displayed. The three KASP markers for the three favorable alleles of the *Wx*, *ALK* and *BADH2* genes can provide a good screening method for rice ECQs. There is a high consistency between the genotyping result and phenotypic evaluation. AC was highly associated with *Wx*, which agrees well with previous studies (Sun et al. 2005). The rice lines with GC:GC in *ALK* generally have low ASV and high GT. Our study demonstrated that the marker *Wx*-G/T accounted for 63.12% of the phenotypic variance of AC, and *ALK*-GC/TT accounted for 66.34% of the phenotypic variance for GT. Similar results were obtained by previous research (Bao et al. 2006; Kharabian-Masouleh et al. 2012; Yang et al.

Table 7 Agronomic traits of crosses combinations

Combination	PH cm	PNPP	TGW/g	SR/%	AC%
CK-NingA/9311	103.1	10.6	21.3	74.17	21.2
NingA/9311-R1	110.2	9.3	27.0	83.1	16.7
NingA/9311-R2	97.8	8.45	22.31	84.1	17.3
NingA/9311-R3	109.2	10.4	23.1	85.2	18.1
NingA/9311-R4	103.1	10.1	24.2	84.7	15.7
NingA/9311-R5	102.4	8.9	26.1	80.3	16.1
NingA/9311-R6	100.7	11.2	23.1	79.2	15.4
CK-C815S/9311	105.4	9.1	22.9	80.1	22.4
C815S/9311-R7	107.2	10.2	23.1	79.2	17.2
C815S/9311-R8	108.1	9.2	24.7	85.2	16.6
C815S/9311-R9	99.6	9.4	23.4	84.2	15.7
C815S/9311-R10	100.2	10.3	24.2	83.5	16.6
C815S/9311-R11	108.5	9.9	24.9	81.9	15.2
C815S/9311-R12	98.9	9.5	23.6	86.2	16.3
C815S/9311-R13	110.2	10.5	25.1	84.9	16.5

PH plant height, *PNPP* panicle numbers per plant, *TGW* thousand grain weight, *SR* seed setting rage, *AC* amylose content

2014) and confirmed that this marker set may be effectively utilized as a simplified tool to predict ECQs. *BADH2-8 bp* KASP array gave precise prediction for fragrance as well in our study. Through functional marker genotyping, we have effectively improved the disadvantage of high AC in restorer line 9311 and obtained a series of aromatic restorer lines with low AC. These restorers showed advantages in heterosis utilization.

Rice is by no means a 'one size fits all' crop although a combination of slender grain, low AC, low GT, and fragrance is becoming more popular (Calingacion et al. 2014). Breeders should develop rice cultivars that have desirable grain quality, customized to meet the market requirements in target regions. In the present study, we showed again that functional markers based on polymorphisms in principal genes could be used for genotyping and screening rice germplasm. The KASP markers developed in our study are reliable and may be applied in large-scale MAS breeding for rice ECQ improvement in the future.

Acknowledgements Financial support for this research was provided in part by a grant from the National Key Research and Development Project (2016YFD0101100), Science and Technology Project of Guangdong Province (No. 2015B020231011), National Key Technology Research and Development Program of China (No. 2016YFD0102102), and the earmarked fund for Modern Agro-Industry Technology Research System (No. CARS-01-12).

References

Bao JS, Corke H, Sun M (2006) Nucleotide diversity in starch synthase IIa and validation of single nucleotide polymorphisms in relation to starch gelatinization temperature and other physicochemical properties in rice (*Oryza sativa* L.). Theor Appl Genet 113:1171–1183

Bradbury LMT, Fitzgerald TL, Henry RJ, Jin QS, Waters DLE (2005) The gene for fragrance in rice. Plant Biotechnol J 3:363–370

Bunyasiri IN, Sirisupluxana P (2018) Consumer's behavior and rice attributes for Thai HomMali Rice in Sichuan Province of China. J Bus Retail Manag Res 12:242–252

Cai XL, Wang ZY, Xing YY, Zhang JL, Hong MM (1998) Aberrant splicing of intron 1 leads to the heterogeneous 5' UTR and decreased expression of waxy gene in rice cultivars of intermediate amylose content. Plant J 14:459–465

Calingacion M et al (2014) Diversity of global rice markets and the science required for consumer-targeted rice breeding. PLoS ONE 9:e85106. https://doi.org/10.1371/journal.pone.0085106

Chen SH et al (2008) Badh2, encoding betaine aldehyde dehydrogenase, inhibits the biosynthesis of 2-acetyl-1-pyrroline, a major component in rice fragrance. Plant Cell 20:1850–1861

Chen T, Zhang Y, Zhao L, Zhu Z, Lin J, Zhang S, Wang C (2009) A cleaved amplified polymorphic sequence marker to detect variation in Wx locus conditioning translucent endosperm in rice. Rice Sci 16:106–110

Chen W et al (2014) Genome-wide association analyses provide genetic and biochemical insights into natural variation in rice metabolism. Nat Genet 46:714–721

Gao Z et al (2003) Map-based cloning of the ALK gene, which controls the gelatinization temperature of rice. Sci China C Life Sci 46:661–668

He C, Holme J, Anthony J (2014) SNP genotyping: the KASP assay. Methods Mol Biol 1145:75–86. https://doi.org/10.1007/978-1-4939-0446-4_7

Kharabian-Masouleh A, Waters DLE, Reinke RF, Ward R, Henry RJ (2012) SNP in starch biosynthesis genes associated with nutritional and functional properties of rice. Sci Rep 2:557

Leng YJ et al (2014) Mapping of QTLs for eating and cooking quality-related traits in rice (*Oryza sativa* L.). Euphytica 197:99–108

Luo WL, Guo T, Yang QY, Wang H, Liu YZ, Zhu XY, Chen ZQ (2014) Stacking of five favorable alleles for amylase content, fragrance and disease resistance into elite lines in rice (*Oryza sativa*) by using four HRM-based markers and a linked gel-based marker. Mol Breed 34:805–815

Ma Y et al (2017) Development and validation of breeder-friendly KASPar markers for er1, a powdery mildew resistance gene in pea (*Pisum sativum* L.). Mol Breed 37:151. https://doi.org/10.1007/s11032-017-0740-7

Musa M, Othman N, Fatah AF (2018) Determinants of consumers purchasing behavior for rice in Malaysia. Am Int J Contemp Res 1:159–167

Myakishev MV, Khripin Y, Hu S, Hamer DH (2001) High-throughput SNP genotyping by allele-specific PCR with universal energy-transfer-labeled primers. Genome Res 11:163–169

Neelam K, Brown-Guedira G, Huang L (2013) Development and validation of a breeder-friendly KASPar marker for wheat leaf rust resistance locus Lr21. Mol Breed 31:233–237. https://doi.org/10.1007/s11032-012-9773-0

Pang YL, Ali J, Wang XQ, Franje NJ, Revilleza JE, Xu JL, Li ZK (2016) Relationship of rice grain amylose, gelatinization temperature and pasting properties for breeding better eating and cooking quality of rice varieties. PLoS ONE 11:e0168483. https://doi.org/10.1371/journal.pone.0168483

Ramkumar G, Prahalada GD, Hechanova SL, Vinarao R, Jena KK (2015) Development and validation of SNP-based functional codominant markers for two major disease resistance genes in rice (*O. sativa* L.). Mol Breed 35:129

Sakthivel K et al (2009) Development of a simple functional marker for fragrance in rice and its validation in Indian Basmati and non-Basmati fragrant rice varieties. Mol Breed 24:185–190. https://doi.org/10.1007/s11032-009-9283-x

Semagn K, Babu R, Hearne S, Olsen M (2014) Single nucleotide polymorphism genotyping using Kompetitive Allele Specific PCR (KASP): overview of the technology and its application in crop improvement. Mol Breed 33:1–14

Su Y et al (2011) Map-based cloning proves qGC-6, a major QTL for gel consistency of japonica/indica cross, responds by Waxy in rice (*Oryza sativa* L.). Theor Appl Genet 123:859–867

Sun YY, Lv Y, Dong CL, Wang PR, Huang XQ, Deng XJ (2005) Genetic relationship among Wx gene, AC, GC and GT of rice. Acta Agron Sin 31:535–539

Sun MM, Abdula SE, Lee HJ, Cho YC, Han LZ, Koh HJ, Cho YG (2011) Molecular aspect of good eating quality formation in Japonica rice. PLoS ONE 6:e18385. https://doi.org/10.1371/journal.pone.0018385

Thomson M (2014) High-throughput SNP genotyping to accelerate crop improvement. Plant Breed Biotechnol 2:195–212

Tian ZX et al (2010) Development of gene-tagged molecular markers for starch synthesis-related genes in rice. Chin Sci Bull 55:3768–3777

Tong C, Chen Y, Tang F, Xu F, Huang Y, Chen H, Bao J (2014) Genetic diversity of amylose content and RVA pasting parameters in 20 rice accessions grown in Hainan, China. Food Chem 161:239–245. https://doi.org/10.1016/j.foodchem.2014.04.011

Tuano A, Regalado M, Juliano B (2016) Grain quality of rice in selected retail stores and supermarkets in the Philippines. J Philipp Sci Techol 9:15–22

Umemoto T, Aoki N (2005) Single-nucleotide polymorphisms in rice starch synthase IIa that alter starch gelatinisation and starch association of the enzyme. Funct Plant Biol 32:763–768

Wang H, Chu Z, Ma X, Li R, Liu Y (2013) A high through-put protocol of plant genomic DNA preparation for PCR. Acta Agron Sin 39:1200–1205

Waters DLE, Henry RJ, Reinke RF, Fitzgerald MA (2006) Gelatinization temperature of rice explained by polymorphisms in starch synthase. Plant Biotechnol J 4:115–122

Yang F et al (2014) Association mapping of starch physicochemical properties with starch synthesis-related gene markers in nonwaxy rice (*Oryza sativa* L.). Mol Breed 34:1747–1763. https://doi.org/10.1007/s11032-014-0135-y

Zhang ZJ et al (2012) Diversification of the waxy gene is closely related to variations in rice eating and cooking quality. Plant Mol Biol Report 30:462–469

Publisher's Note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.