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DEVELOPMENT OF FUNCTIONAL MARKERS FOR QUANTITATIVE TRAIT LOCUS UNDERLYING RICE QUALITY COMPONENTS

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Abstract

Three locus (*GS3*, *Chalk5* and *Wx*) controlling grain size, grain chalkiness and amylose content (AC) have been cloned and can be used to improve rice quality by molecular marker-assisted selection (MAS). At present, identification of the above-mentioned alleles need DNA sequencing or restriction enzyme digestion, which were laborious, time-consuming tasks. Developing functional markers harboring the advantage of rapid, simple and low cost will contribute to rice quality improvement by MAS. Tetra-primer ARMS-PCR technology is an economical method for single nucleotide polymorphisms (SNP) scoring, however, its utilization is limited due to low amplification efficiency and inaccuracy extension caused by distinct melting temperature (*Tm*) difference existing between four primers in one PCR system. In this paper, we developed a set of functional markers using tetra-primer ARMS-PCR technology, and we also put forward corresponding solving schemes on low amplification efficiency and inaccuracy extension caused by *Tm* difference. The results showed that five markers were developed through adjusting the location of deliberate mismatch bases introduced, three markers ($GS3^{ac}$-ARMS, $Chalk5^{tc}$-ARMS, $Waxy^{gt}$-ARMS) could identify different alleles for each loci holding the advantage of rapid, simple and low cost. Further, we analyzed the alleles distribution on rice varieties derived from Northeast China. All of these works could provide foundations for rice quality improvement utilizing the suitable genes (QTLs) by MAS.

Key words: Rice quality**,** *GS3*, *Chalk5*, *Wx*, Functional markers, Tetra-primer ARMS-PCR.

Abbreviations: AC: Amylose content; MAS: Molecular marker-assisted selection; SNP: Single nucleotide polymorphisms; *Tm*: Melting temperature.

Introduction

Rice (*Oryza sativa* L.) is an essential food crop, providing a carbohydrate source for nearly half of the world's population (Peng *et al*., 2000). In order to gain more commercial value, superior quality was expected and aroused more and more concerns (Cheng *et al*., 2002; Luo *et al*., 2015). Rice quality mainly include milled quality, appearance quality, nutrient quality and cooking quality (Tian *et al*., 2009). Grain chalkiness is an optical character caused by the air-gap of loose arrangement between proteinoplast and amyloplast, which is an undesirable trait owing to negatively affecting rice milled and appearance quality (Cheng *et al*., 2002; Septiningsih *et al*., 2003; Gao *et al*., 2016). AC is an important factor impacting rice eating quality (Cameron & Wang *et al*., 2005; Naoko *et al*., 2012), rice varieties harboring intermediate AC have the advantage of tender, glossy and cohesive after cooking (Luo *et al*., 2015). Long grain shape rice varieties often have superior appearance quality and further improve the commercial value of rice (Song *et al*., 2007; Shomura *et al*., 2008). In conclusion, low grain chalkiness and intermediate AC are always the expected targets in rice breeding process (Miura *et al*., 2011). Improving the appearance of rice varieties towards a long grain shape also attracted much concerns (Fan *et al*., 2006; Wang *et al*., 2012).

With the development of molecular biology, many genes involved in rice quality have been successfully cloned, such as *GS5, GW5, GS3, GW8, Chalk5, Wx, SSII-3* and *NRT1.1B* (Wang *et al*., 1995; Fan *et al*., 2006; Wan *et al*., 2008; Tian *et al*., 2009; Li *et al*., 2011; Wang *et al*., 2012; Li *et al*., 2014; Hu *et al*., 2015), providing a solid theoretical foundation for rice quality improvement. The *Wx* gene, which is the major regulatory gene for AC, one G-T SNP at the first base of the splice donor in the first intron produced two mainstream alleles, Wx^a (*indica* rice) and Wx^b (*japonica* rice), corresponding to high AC and intermediate AC (Wang *et al*., 1995). *Chalk5*, as the major QTL controlling grain chalkiness in *indica* rice, was successfully cloned by Li *et al*. (2014). Two consensus SNPs might partly account for the difference in *Chalk5* mRNA levels among different *indica* rice varieties, resulting in diversity in the white belly phenotype (Li *et al*., 2014; Qiu *et al*., 2015). *GS3*, a major QTL for grain length and weight and minor QTL for grain width and thickness in rice, was mapped on chromosome 3, one SNP responded for the genetic variation between the two grain-length groups, in which C (short-grain type) was substituted by A (long-grain type) in the second exon (Fan *et al*., 2006). Multiple studies have been focused on improving rice quality utilizing the above-mentioned favorable alleles, and produced great successes (Cai *et al*., 2002; Mao *et al*., 2010; Naoko *et al*., 2012; Chen *et al*., 2013). However, some papers utilized non-functional primers nearby the genes to distinguish different alleles (Huang *et al*., 1997; Tian *et al*., 2009), which were easy to implement. However, the false positive results were produced owning to genetic recombination (Jeppe & Thomas, 2003; Yan *et al*., 2009; Ameer *et al*., 2016). Enzyme digestion and sequencing technology could provide accurate identification results,

but these methods were laborious, time-consuming tasks owning to lot of materials needs to be tested in MAS (Shu *et al*., 2001; Chen *et al*., 2013).

The concept of functional marker was first proposed by Jeppe & Thomas, (2003). It derives from functionally characterized sequence motifs and can reflect a difference of functional base. Functional markers were superior to random DNA markers such as SSR, AFLP and RFLP owing to complete linkage with trait locus alleles, and have been widely used in MAS (Tian *et al*., 2009; Jiang *et al*., 2012; Chen *et al*., 2013; Siraj *et al*., 2016). As many genes were characterized by SNP diversity, the method that could be used affordably on lot of materials had aroused more concerns (Shu *et al*., 2001; Jeppe & Thomas. 2003). Tetra-primer ARMS-PCR, an efficient SNP analytical technique invented by (Shu *et al*., 2001) had been used in SNP analysis from medical science and plant sciences (Shu *et al*., 2001; Chen *et al*., 2013). In the design process, **owning to the inner primers was a location constant,** the *Tm* difference between four primers was difficult to eliminate, which caused low amplification efficiency and inaccuracy extension and limited its further application. (Shu *et al*., 2001) proposed that screening optimal annealing temperature or utilizing touchdown PCR technology should partly eliminate this dilemma, however, this solving scheme was only suitable to the minor *Tm* difference existing. The corresponding primer design strategy need to be deeply explored.

Materials and Methods

In this paper, we developed a set of functional markers using tetra-primer ARMS-PCR technology, and we also put forward corresponding solving schemes on low amplification efficiency and inaccuracy extension caused by significant *Tm* difference. Further, we analyzed the alleles distribution on rice varieties derived from Northeast China on the locus of *GS3*, *Chalk5* and *Wx*.

Plant materials: Two types of plant materials were used in this study: (i). The control varieties containing 'Minghui 63', 'Chuan 7', 'Zhenshan 97', 'H 94', 'Teqing' and 'Akihikari' were shown in Table 1. (ii). The tested cultivated varieties derived from the Northeast China. All the varieties were planted for two growing seasons during 2012–2013 in a trial field of the Research Institute of saline alkali land use in Liaoning Province, located in Panjin, China (41.07 °N; 122.03°E).

DNA isolation, genotype testing and sequencing the PCR production: The total genomic DNA was extracted using a plant genomic isolation kit (Tiangen, China). PCR was carried out in a total volume of 30 µl containing 0.6 µl of each outer primer, 0.4 µl of each inner primer (4 pmol/µL); 4ng of genomic DNA, 15 µl of 2×*Tap* PCR Master Mix (Tiangen, China), diluted with ddH_2O to 30 µl. Thermal cycler parameters included the following: an initial denaturation at 95°C for 1 min; 35 cycles of 95°C for 30 s, 55.1–58.4°C for 30 s and 72°C for 30s, followed by a final extension at 72°C for 10 min (Tables 2-4). The PCR products were analyzed on 3%–5% agarose gels stained with ethidium bromide and photographed with a GEL DOC 1,000 system (BioRad, America). The sequencing of the PCR production was performed at BGI Corporation (China).

Primer Design

Design strategies for *GS3* marker: The sequence information of *GS3* can be obtained from RAP-DB (Os03g0407400), the markers were developed to reveal an A-C SNP in the second codon of the exon (Fig. 1). We designed two candidate markers named $GS3^{ac}$-ARMS-1 and $GS3^{ac}$-ARMS, the design strategies were shown as following:

In the design process of $GS3^{ac}$-ARMS-1, we utilized the online primer design software Primer 1 (http://cedar.genetics.soton.ac.uk/public_html/primer1; Shu *et al*., 2001) to get four primers: one pair of outer primers were obtained to amplify a short stretch of genomic DNA containing the A-C mutation; one forward inner primer and one reverse inner primer were obtained, with the 3′-end of the extension primer located on the SNP site of the wild type or mutant type respectively. Each inner primer was introduced one deliberate mismatch base on the -3 site of the 3′-end of the extension (Table 2).

In the design process of $GS3^{ac}$-ARMS, the outer primers were the same as $GS3^{ac}$-ARMS-1, the location of deliberate mismatch bases introduced was adjusted for two inner primers (Table 2).

Design strategies for *Chalk5* marker: The sequence information of *Chalk5* can be obtained from NCBI (KJ363317), the marker was developed to reveal a T-C SNP located on -721 of the promoter region (Fig. 2). We utilized the online primer design software Primer 1 to get the marker named $Chalk5^{tc}$-ARMS (Table 3).

Design strategies for *Wx* marker: The sequence information of *Wx* can be obtained from NCBI (AF141954), the markers were developed to reveal a G-T SNP located on +1 of *Wx* intron 1 (Fig. 3). We designed two candidate markers named $Waxy^{gt}$-ARMS-1 and $Waxy^{gt}$-ARMS, the design strategies were shown as following:

$Waxy^{gt}$-ARMS-1 was obtained from the online primer design software Primer 1 (Table 4). In the design process of the $Waxy^{gt}$-ARMS, the outer primers were the same as $Waxy^{gt}$-ARMS-1; the location of deliberate mismatch bases introduced was adjusted (Table 4).

Table 1. List of control varieties.

Number	Varieties name	Subspecies	Origin	Genotype
1.	Minghui 63	*indica*	Fujian Province, China	Long-grain type *gs3*
2.	Chuan 7	*indica*	Fujian Province, China	Short-grain type *GS3*
3.	Zhenshan 97	*indica*	Zhejiang Province, China	High chalkiness *Chalk5*
4.	H 94	*indica*	Fujian Province, China	Low chalkiness *chalk5*
5.	Teqing	*indica*	Guangdong Province, China	High AC Wx^a
6.	Akihikari	*japonica*	Japan	Intermediate AC Wx^b

Table 2. Molecular markers designed for *GS3*.

Gene	Marker name	Primer sequence (5'-3')	Melting temperature (°C)	Annealing temperature (°C)	Extension time (s)	Gel	Produce size (bp)	Problems
GS3	GS3^{ac}-ARMS-1	Forward inner primer (A) GGATCCACGCTGCCTCCAGATGCCGA	79 69	-	-	-	-	Inaccuracy extension
		Reverse inner primer (C) AAAGAAACAGCAGGCTGGCTTACTCTTTG	72					
		Forward outer primer CCTCAGACATCACCTGAAAAGTTGACAGGC	72					
		Reverse outer primer CGGTCAAAGTTCATGATCAAAAACTGGGG						
	GS3^{ac}-ARMS	Forward inner primer (A) GGATCCACGCTGCCTCCAGATGCTTA	79 69	55.1	30	3% Agarose	147 bp (A allele) 177 bp (C allele) 270 bp (from two outer primers)	-
		Reverse inner primer (C) AAAGAAACAGCAGGCTGGCTTACTCTCGG	72					
		Forward outer primer CCTCAGACATCACCTGAAAAGTTGACAGGC	72					
		Reverse outer primer CGGTCAAAGTTCATGATCAAAAACTGGGG						

The deliberate introduction of mismatch position is shown in red

Table 3. Molecular markers designed for *Chalk5*.

Gene	Marker name	Primer sequence (5'-3')	Melting temperature (°C)	Annealing temperature (°C)	Extension time (s)	Gel	Produce size (bp)	Problems
Chalk5	Chalk5^{tc}-ARMS	Forward inner primer (T) AGAAGAGAGAAGTGCCAAGGATCGGT	67 67	58.4	30	5% Agarose	225 bp (T allele) 194 bp (C allele) 364 bp (from two outer primers)	-
		Reverse inner primer (C) GGTTTTTGAATAAAACAACTCTGGGTCCTG	67					
		Forward outer primer GATTGCATGCATCTTAACAGCAAAGAGA	67					
		Reverse outer primer CAAATTAGGTGTATCTGACGCATGAGCA						

Legend as in Table 2

Table 4. Molecular markers designed for *Wx*.

Gene	Marker name	Primer sequence (5'-3')	Melting temperature (°C)	Annealing temperature (°C)	Extension time (s)	Gel	Produce size (bp)	Produce size (bp)
Wx	Waxy^{gt}-ARMS-1	Forward inner primer (T)	65	-	-	-	-	Low amplification efficiency and inaccuracy extension
		GTTCATCAGGAAGAACATCTGCACGT	54					
		Reverse inner primer (G)	59					
		AAACAAAGAATTATAAACATATATGTAGAC						
		Forward outer primer						
		GTTCTTTGTCTATCTCAAGACACAAATAA	59					
		Reverse outer primer						
		ATATATATGGATCTTGGCAAGTCAATTA						
	Waxy^{gt}-ARMS	Forward inner primer (T)	65	55.8	30	3% Agarose	198 bp (T allele) 143 bp (G allele)	-
		GTTCATCAGGAAGAACATCTGCAATT	54					
		Reverse inner primer (G)	59					
		AAACAAAGAATTATAAACATATATGTATAC					297 bp/ 287 bp (from two outer primers)	
		Forward outer primer						
		GTTCTTTGTCTATCTCAAGACACAAATAA	59					
		Reverse outer primer						
		ATATATATGGATCTTGGCAAGTCAATTA						

Results

***GS3* marker:** 'Minghui 63' and 'Chuan 7' were used as the control varieties (Fan *et al*., 2006; Table 1), corresponding to long-grain type *gs3* allele and short-grain type *GS3* allele. Two markers named GS3^{ac}-ARMS-1 and GS3^{ac}-ARMS were developed to reveal the A-C SNP in the second exon region (Fig. 1).

Initially, we established the optimum PCR system through screening for the optimum annealing temperature. For GS3^{ac}-ARMS-1, screening for the annealing temperature between 55.1°C–59.1°C (Fig. 4A), we cannot differentiate alleles owning to inaccuracy extension. Further, we utilized touch-down technology described by Shu *et al*. (2001). We also cannot get the accurate result as the same is shown in Fig. 4A (date not shown). We speculated that this phenomenon was caused by the large *Tm* difference between two inner primers (Table 2).

Through adjusting the location of deliberate mismatch bases introduced, we developed another marker GS3^{ac}-ARMS (Table 2), screening for the annealing temperature between 55.1°C–59.1°C. PCR fragment was the most clearest with an annealing temperature of 55.1°C (Fig. 4B), we verified this result through multiple repetitions. Corresponding to long-grain type variety 'Minghui 63' (the SNP is A), the marker GS3^{ac}-ARMS can amplify 270 bp and 147 bp DNA fragments. Corresponding to short-grain type variety 'Chuan 7' (the SNP is C), the marker GS3^{ac}-ARMS can amplify 270 bp and 177 bp DNA fragments. The grain length of 'Minghui 63' and 'Chuan 7' were 9.8mm and 6.4mm respectively, we contrasted the phenotypic date, and further confirmed the accuracy of the marker GS3^{ac}-ARMS. Afterwards, PCR products were sequenced to verify the amplification results.

***Chalk5* marker:** 'H 94' and 'Zhenshan 97' were used as the control varieties, corresponding to low chalkiness type *chalk5* allele and high chalkiness type *Chalk5* allele (Li *et al*., 2014; Table 1). One marker named Chalk5^{tc}-ARMS was developed to reveal the T-C SNP located on the promoter region (Fig. 2). Screening for annealing temperature between 55.1°C–59.1°C, PCR fragment was the most clearest with an annealing temperature of 58.4°C (Fig. 5). We verified this result through multiple repetitions. The detailed primer information was shown on Table 3.

The PCR assay utilizing Chalk5^{ac}-ARMS was demonstrated in Fig. 5. Corresponding to low chalkiness type variety 'H 94' (the SNP is T), the marker Chalk5^{tc}-ARMS can amplify 364 bp and 225 bp DNA fragments. Corresponding to high chalkiness type variety 'Zhenshan 97 (the SNP is C)', the marker Chalk5^{ac}-ARMS could amplify 364 bp and 194 bp DNA fragments. The chalkiness degree of 'H 94' and 'Zhenshan 97' are 0.3% and 15% respectively, we contrasted the phenotypic date, and further confirmed the accuracy of the marker Chalk5^{tc}-ARMS. Afterwards, PCR products were sequenced to verify the amplification results.

Fig. 1. Design strategy for *GS3* markers
Functional SNPs are shown in red, gray background shows primers' binding site, the green full arrows indicates the primers' amplification direction, the red short dash arrows indicates that the primer could not amplify effectively.

Fig. 2. Design strategy for *Chalk5* marker
Legend as in Fig. 1.

Fig. 3. Design strategy for *Wx* marker
Legend as in Fig. 1.

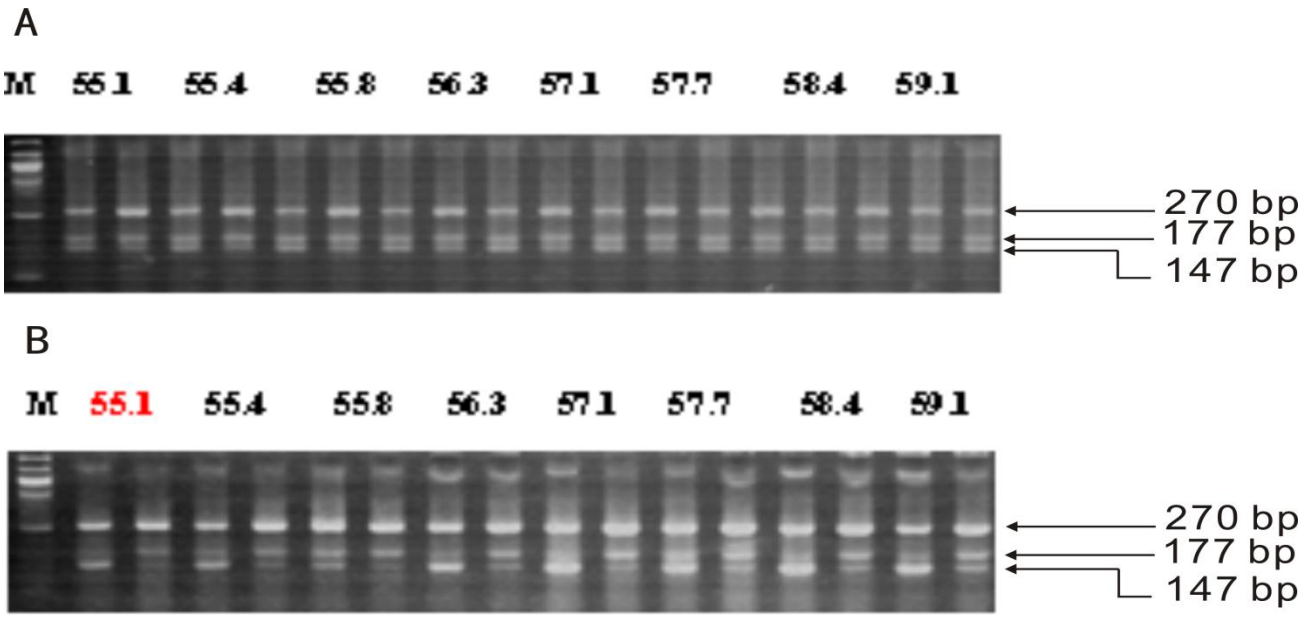

Fig. 4. PCR assay of *GS3* alleles utilizing markersGS3^{ac}-ARMS-1 and GS3^{ac}-ARMS between different annealing temperatures
M indicate Marker 2000 and display the DNA fragments corresponding to 2000 bp, 1500 bp, 750 bp, 500 bp, 250 bp, 100 bp.
A, The PCR results on different annealing temperature (^{o}C) using the marker of GS3^{ac}-ARMS-1, on every groups of annealing temperatures, the former line is 'Minghui 63', later line is 'Chuan 7'.
B, The PCR results on different annealing temperature (^{o}C) using the marker of GS3^{ac}-ARMS, on every groups of annealing temperatures, the former line is 'Minghui 63', later line is 'Chuan 7'.

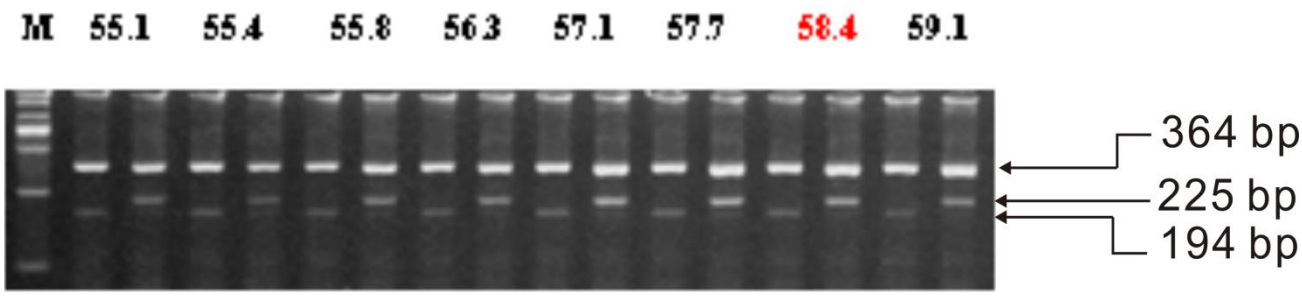

Fig. 5. PCR assay of *Chalk5* alleles utilizing Chalk5^{ac}-ARMS marker between different annealing temperatures
Legend as in Fig. 4.
On every groups of annealing temperatures, the former line is 'Zhenshan 97', later line is 'H 94'.

***Wx* marker:** 'Teqing' and 'Akihikari' were used as the control varieties (Cai *et al*., 2002; Table 1), corresponding to *Wx*^{a} allele (high AC type) and *Wx*^{b} allele (intermediate AC type). Two markers named Waxy^{gt}-ARMS-1 and Waxy^{gt}-ARMS were developed to reveal the G-T SNP in the second exon region (Fig. 3).

For Waxy^{gt}-ARMS-1, screening for the annealing temperature between 55.1°C–59.1°C (Fig. 6A), we cannot get accurate result: with the tested variety 'Teqing', the desired DNA fragment product length was not very clear and a DNA fragment product length corresponding to 'Akihikari' was also found. Low amplification efficiency and inaccuracy extension both occurred owning to unsuitable deliberate mismatch bases introduced.

Through adjusting the location of deliberate mismatch bases introduced, We developed another marker Waxy^{gt}-ARMS (Table 4), screening for the annealing temperature between 55.1°C–59.1°C, PCR fragment is the most clearest with an annealing temperature of 55.8°C (Fig. 6B), we verified this result through multiple repetitions. Sequencing on control varieties 'Teqing' and 'Akihikari' utilizing the primer composed of Waxy^{gt}-ARMS-O-F and Waxy^{gt}-ARMS-O-R, we found that there also existed another 10bp InDel polymorphism outside of the G-T polymorphism (Fig. 3). The PCR product derived from 'Akihikari' amplified by the primer composed of Waxy^{gt}-ARMS-O-F and Waxy^{gt}-ARMS-O-R was 297 bp; the corresponding PCR product derived from 'Teqing' was 287 bp (Fig. 6B).

The PCR assay utilizing Waxy^{gt}-ARMS was shown in Fig. 6B. Corresponding to high AC type variety 'Teqing' (the SNP is G, *Wx*^{a}), the marker Waxy^{gt}-ARMS can amplify 287 bp and 143 bp DNA fragments. Corresponding to intermediate AC type variety 'Akihikari' (the SNP is T, *Wx*^{b}), the marker Waxy^{gt}-ARMS can amplify 297 bp and 198 bp DNA fragments. The amylose content of 'Teqing' and 'Akihikari' were 24% and 17% respectively, we contrasted the phenotypic date, and further confirmed the accuracy of the marker Waxy^{gt}-ARMS. The amplification results were further verified by PCR products sequenced.

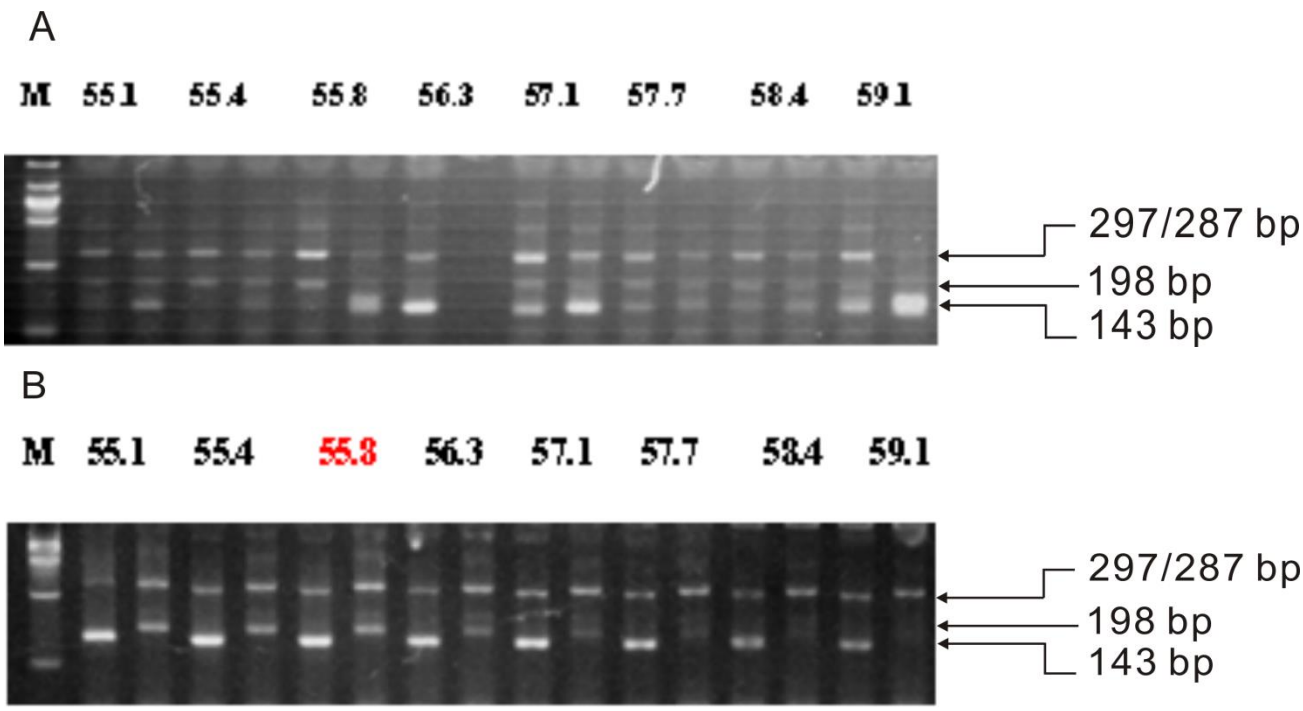

Fig. 6. PCR assay of *Wx* alleles utilizing markers Waxy^{gt}-ARMS-1 and Waxy^{gt}-ARMS between different annealing temperatures
Legend as in Fig. 4.
A, The PCR results on different annealing temperature (^{o}C) using the marker of Waxy^{gt}-ARMS-1, on every groups of annealing temperatures, the former line is 'Teqing', later line is 'Akihikari'.
B, The PCR results on different annealing temperature (^{o}C) using the marker of Waxy^{gt}-ARMS, on every groups of annealing temperatures, the former line is 'Teqing', later line is 'Akihikari'.

Analysis of alleles distribution on cultivated varieties in Northeast China: Utilizing these new markers, a survey of the alleles distribution on cultivated varieties in Northeast China was surveyed. All the cultivated varieties carried the *Wx^{b}* allele, which was the wild-type allele in *japonica* rice. None of the cultivated varieties carried the *chalk5* allele corresponding to 'H 94'. Two of the 110 tested varieties carried the long-grain type *gs3* allele, with all others carrying theshort-grain type *GS3* allele.

Discussion

MAS is a more efficient breeding approach which can offer an opportunity to select a targeted gene rapidly and precisely, and has been widely applied to the selection of rice yield, quality and resistance (Tian *et al*., 2009; Miura *et al*., 2011; Jiang *et al*., 2012; Chen *et al*., 2013; Wasim & Tariq, 2017). Aimed at improving rice quality, many papers focused on utilizing *Wx*, *GS3* and *Chalk5* favorable alleles to improve AC and grain appearance (Miura *et al*., 2011). AC is the most important influence factor on rice eating quality, multiple *indica*varieties were introduced *Wx^{b}* allele to replace the origin *Wx^{a}* allele, and their eating quality improved significantly (Tian *et al*., 2009; Luo *et al*., 2015). Through introducing the long grain shape *gs3* allele derived from "Minhui63", Fan *et al*. (2006) successfully improved the grain shape and appearance of "Chuan 7" (short grain shape type). Zeng *et al*. (2017) improved taste and appearance quality of "Teqing" utilizing the *Wx^{b}* and *gs3* allele derived from "Nipponbare" and "9311" respectively, and successfully achieved breeding superior quality rice by rational design. Li *et al*. (2014) successfully lowered the grain chalkiness degree and improved appearance quality of "Zhenshan97" utilizing the *chalk5* allele derived from "H 94". Due to these locus possessing great significant on rice quality improvement, many studies have put emphasis on developing functional markers to differentiate alleles. However, all these markers based on DNA sequencing or restriction enzyme digestion limited their further application (Cai *et al*., 2002; Tian *et al*., 2009, Yan *et al*., 2009; Li *et al*., 2014).

Shu *et al*. (2001) introduced a procedure named tetra-primer ARMS-PCR that can be used to identify SNPs with feasible, easy and affordable features. Owing to its high throughput and low cost, this technology has been used for SNP detection in the fields of medicine and plant sciences (Shu *et al*., 2001; Chen *et al*., 2013, Kim *et al*., 2016). However, the design strategy analysis of tetra-primer ARMS-PCR is limited. Shu *et al*. (2001) defined two rules on the design strategy, one was that a deliberate mismatch should be introduced on the -3 site of the 3′ -end of the extension inner primer, the other is that the *Tm* between four primers in one PCR system should not differ significantly. In this paper, we found that the position of the deliberate mismatch was the most important factor impacting RCR results. The deliberate mismatch base should be far away from 3'-terminus or not be introduced under the circumstance of low amplification efficiency, and when the inaccuracy extension emerge. Deliberate mismatch base should be introduced near to 3'-terminus. Once the introduction of deliberate mismatch was optimal, we could successfully design markers and develop an optimum PCR system through screening the

optimum annealing temperature. This provided an important theoretical foundation on primer designing by tetra-primer ARMS-PCR.

In northeast China, all the registered rice varieties carried a certain amount of *indica* background (Sun *et al*., 2013). However, the distribution frequency of alleles underlying rice quality components was unclear. The cultivated varieties used in this study carried the Wx^b allele to maintain good eating quality. The minor *gs3* allele corresponding to 'Minghui 63' was introduced to add grain length and a better appearance (data not shown). Previous research results have shown that different genotypes of *Chalk5* impact natural grain chalkiness in *indica*, but may have no function in *japonica* (Li *et al*., 2014). In this study, none of the cultivated varieties in Northeast China carried the *chalk5* allele corresponding to 'H 94', but the grain chalkiness had a relatively large diversity (data not shown), so we concluded that *Chalk5* loci was not the QTL regulating chalkiness in *japonica*. Further studies were needed to verify if the *Chalk5* allele corresponding to 'H 94' could be used to improve grain chalkiness in *japonica* rice.

Conclusions

To date, multiple crucial regulatory genes in rice were illustrated caused by SNP diversity. Thus, cost-effective identification method on SNP diversity is a crucial foundation in current rice-breeding programs by MAS. In this study, three markers were developed to identify alleles on the locus of *GS3*, *Chalk5* and *Wx*, that could be used as a useful tool for rice quality improvement. The design strategy mentioned in this study had the reference value for SNP scoring. According to a survey of these alleles distribution in Northeast China, all these works had the practical valuable for employing MAS to breeding superior quality rice varieties.

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