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Keywords: Rice; Grain shape; Chalkiness; QTL mapping; Effect verification

Communication

Mapping and Verification of QTL for Grain Shape and Grain Chalkiness in Rice Using Sequencing Technology

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Abstract: Grain shape and chalkiness are important appearance quality traits of rice, which also affect the yield and milling quality. In this study, 190 F_2 families derived from the cross between Hua 5178S and Hua 611 were sequenced to construct a genetic linkage map. Combined with F_2 and $F_{2:3}$ generations grain shape and chalkiness phenotype, a total of 15 QTL were detected. There are 8 grain shape QTL distributed on chromosomes 2, 5, 6, 8, and 10; 7 chalkiness QTL distributed on chromosomes 2, 3, and 6. A QTL cluster affecting multiple chalkiness traits was found on chromosome 6, *qWBR6.1*, *qWCR6* and *qCR6* were selected for genetic effect verification. It was found that *qWBR6.1*, *qWCR6* and *qCR6* explained 13% and 24%, and 29% of the phenotypic variations respectively, and *qWCR6* and *qCR6* were co-mapped. This study laid a foundation for further cloning of rice appearance quality genes.

Keywords: rice; grain shape; chalkiness; QTL mapping; effect verification

1. Introduction

Rice is one of the important food crops, which solves the food security problem of about half of the world's population. Grain shape and chalkiness are important appearance qualities of rice and have a significant impact on grain weight and cooking and eating quality. Rice with good appearance quality will be accepted by more consumers and increase its commodity value.

Rice grain shape, which directly affects the 1000-grain weight and the yield, is further charactered by grain length (GL), grain width (GW), length-to-width ratio (LWR) and grain thickness (GT). Moreover, the rice grain shape also has a great influence on the appearance and milling quality of rice. For more than a decade, scientists have cloned many grain shape-related genes and analyzed the corresponding molecular mechanisms. It is generally believed that rice grain shape is a complex quantitative trait regulated by multiple genes. Currently, more than 400 grain shape-related QTL have been detected [1], of which a number of grain shape-related genes have been cloned successfully [2]. Among them, *GS2*/*GL2*, *GS3*, *qGL3*, *qTGW3*, *GL3.3* were found to control grain length [3–5], and *GW2*, *GW5*/*qSW5*/*GSE5*, *GS5*, *GW8*, *GL7*/*GW7* were found to affect grain width [6–10]. These genes not only regulate grain shape, but also have varying degrees of influence on 1000-grain weight and grain yield.

Chalkiness is the white opaque part in rice endosperm. According to the opaque part in the endosperm, chalkiness can be mainly divided into white-belly, white-back, and white-core. Chalkiness is also a complex quantitative trait controlled by multiple genes. A large number of rice opaque endosperm mutants were found had similar endosperm appearance with chalkiness grains, and some of the genes controlled opaque endosperm were finally identified successfully, such as *flo4*/*OsPPDKB*, *SSIIIa*, *FLO2*, *OsPK2*, and so on [11–14]. *flo4*/*OsPPDKB* encodes a chloroplast-located pyruvate phosphate double kinase (PPDK), the *flo4* mutant showed a white-core endosperm [11]. *SSIIIa*/*FLO5* encodes a soluble starch synthase, which affects the amylose content, the structure of

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amylopectin, and the physicochemical properties of starch in rice. Compared with the wild type, the *SSIIIa/FLO5* mutant had smaller, rounder and loosely arrangement starch granule in the central part, showing a white-core endosperm phenotype [12]. So far, little progress has been made in revealing the mechanism of chalkiness formation by using natural resources, and only 2 chalkiness related genes have been successfully cloned. *Chalk5* was the first gene found to control chalkiness, it encodes a vacuolar membrane localized vacuolar membrane proton transporter pyrophosphatase. Higher *Chalk5* expression level greatly increased the gap formed between the starch granules and the protein body, led to the abnormal shape and spatial arrangement of the storage substances in endosperm, and resulted in the formation of chalkiness [15]. *WCR1* is the first gene found to negatively regulate the white-core rate of rice, it encodes a F-box protein [16]. Further experimental results showed that *OsDOF17* could up-regulate the transcription of metallothionein *MT2b* and inhibit the 26S proteasome-mediated MT2b degradation by directly activating $WCR1^{A}$ transcription, promoting the elimination of excess ROS, delaying the PCD process of endosperm cells, and finally leading to the decrease of white-core rate of rice grain.

In this study, a genetic linkage map was constructed by using GBS sequencing technology, and QTL were primary mapped based on the grain shape and chalkiness phenotypes of the F_2 and $F_{2:3}$ populations. A QTL cluster affecting chalkiness was found on chromosome 6, the genetic effect of *qWBR6.1*, *qWCR6* and *qCR6* were validated in a random segregation population. The results of this study provide a basis for cloning new quality-related genes and breeding high quality rice varieties.

2. Materials and Methods

2.1. Population development and field experiments

Two newly bred Hua 5178S and Hua 611 were used as parents to construct the genetic population, the chalkiness rate of Hua 5178S was low while Hua 611 was high. The F_1 generation was generated by crossing female parent Hua 5178S (temperature-sensitive sterility line) with male parent Hua 611. The F_2 generation population was planted under natural field conditions at the experimental station of Huazhong Agricultural University in Lingshui, Hainan Province in 2016, the fertility of plants was screened by using markers linked to the male sterile gene *TMS5*. 190 fertile plants were obtained in F_2 generation for genetic map construction and phenotypic collection, and the $F_{2:3}$ generation families of each plant were used for the second phenotypic collection. The $F_{2:3}$ generation families were planted under natural field conditions at the experimental station of Huazhong Agricultural University in Wuhan, Hubei Province in 2017.

Families with heterozygous genotypes in each QTL interval were selected for genetic effect validation, and each family consist of 190 individuals. All families were planted under natural field conditions at the experimental station of Huazhong Agricultural University in Wuhan, Hubei Province in 2019.

Seedlings of 25~30 days after sowing were transplanted with a single plant spacing of 16.5 cm and 26.4 cm between rows in the field. Field management followed local practices.

2.2. Phenotyping

Before phenotyping, harvested seeds were dried and stored at room temperature for at least three months. About 200-300 mature seeds per plant were selected and scanned on a BenQ scanner to generate high-resolution images. Then the images were analyzed by SmartGrain software to obtain the grain length, grain width, length-to-width ratio and seed number performance. At the end of the scan, all the scanned seeds were weighed and the 1000-grain weight was calculated according to the number of seeds.

After examining the grain shape of each plant, the seeds were dehulled into brown rice, and the number of chalkiness grains was recorded with the naked eye on a cold light lamp. WBR is the proportion of white-belly brown rice to the total number of brown rice, WCR is the proportion of white-core brown rice to the total number of brown rice, and the chalkiness rate (CR) is the ratio of chalkiness brown rice to the total number of brown rice.

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2.3. Genotyping and linkage map construction

Genotyping of F_2 population was carried out by GBS sequencing technology. There were 1985 polymorphisms between Hua611 and Hua5178S (Figure 2). The linkage map of this population was constructed by R Programming Language. QTL analysis was conducted by composite interval mapping using WinQTLCart V2.5. A LOD (log likelihood) value of 2.5 was used as the threshold for claiming the putative main-effect QTL. The peak points were considered to the positions of QTL.

3. Results

3.1. Phenotypic data of parents and RIL populations

Both parents have slender grains, but there are still some differences. Compared with Hua 611, Hua 5178S has shorter and wider grain shape, and heavier 1000-grain weight in 2016 (Figure 1a-d and Table 1). In the progeny populations, the grain length and grain width of F_2 population were significantly smaller than that of $F_{2:3}$, but there the 1000-grain weight showed stable in different populations (Figure 1a-d and Table 1).

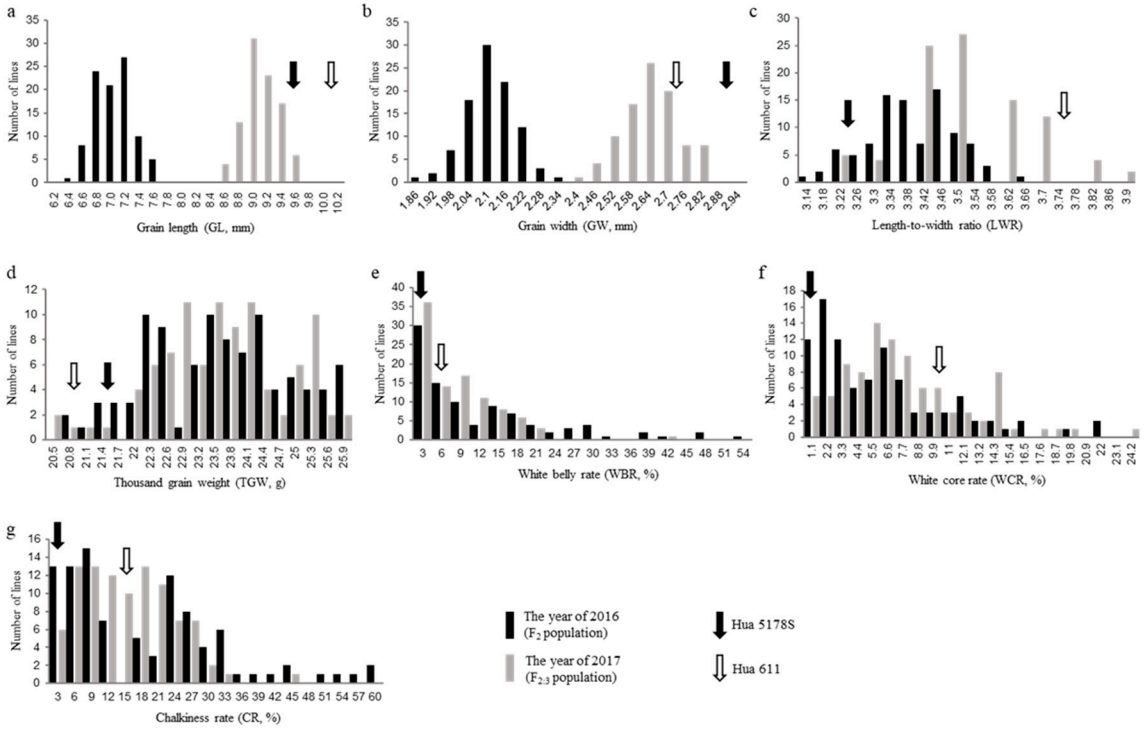

Figure 1. Phenotypic distributions of grain length (a), grain width (b), length-to-width ratio (c), thousand grain weight (d), white belly rate (e), white core rate (f) and chalkiness rate (g) in 2016 (black) and 2017 (gray).

Table 1. Phenotypes of the parental lines and frequency distributions of grain length (GL), grain width (GW), length-to-width ratio (LWR), thousand grain weight (TGW), white belly rate (WBR), white core rate (WCR) and chalkiness rate (CR) during 2016 and 2017.

Trait	Parents (2016)		F_2 (2016)				$F_{2:3}$ (2017)			
	Hua5178S	Hua611	Mean±SD	Range	Kurtosis	Skewness	Mean±SD	Range	Kurtosis	Skewness
GL (mm)	9.52	10.20	7.97±0.26	7.37-8.58	-0.55	0.22	9.02±0.24	8.54-9.60	-0.42	0.20
GW (mm)	2.91	2.71	2.50±0.12	2.18-2.70	-0.28	-0.56	2.62±0.09	2.39-2.81	-0.43	-0.05
LWR	3.27	3.76	3.21±0.12	2.94-3.50	-0.03	-0.05	3.51±0.15	3.20-3.90	0.06	0.25
TGW (g)	21.55	20.10	23.47±1.32	20.57-25.89	-0.73	-0.06	23.52±1.13	20.51-25.68	-0.43	-0.10
WBR (%)	1.45	5.56	11.46±12.09	0-53.82	1.65	1.44	6.79±6.28	0-39.92	6.69	1.89
WCR (%)	0.72	10.10	5.68±4.89	0-21.91	1.34	1.25	7.11±4.49	0-23.40	1.15	1.01
CR (%)	2.17	15.66	17.14±14.24	0.47-59.54	0.63	1.00	13.89±8.26	0.31-44.76	0.72	0.67

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The chalkiness performance of parents and progeny populations were analyzed. In the year of 2016, the white-belly rate, white-core rate and total chalkiness rate of Hua 5178S was 1.45%, 0.72% and 2.17% respectively, while those in Hua 611 was 5.56%, 10.10% and 15.66% respectively (Figure 1e-g and Table 1). The white belly rate, white core rate, and chalkiness rate of two populations showed non-normal distributions, and widely ranged than parents (Figure 1e-g and Table 1).

Correlation analysis was conduct for grain shape and chalkiness relate traits (Table 2). Both grain length and grain width displayed significantly positively correlated with 1000-grain weight in different populations. Length-to-width ratio showed significantly negatively correlation with the grain width but significantly positively correlation with the grain length. White-belly rate, white-core rate and chalkiness rate have extremely significantly positively correlation with each other. White belly rate displayed significantly positively correlate with grain width in the F_2 population while there was no significant correlation between chalkiness-related traits and grain shape-related traits in the $F_{2:3}$ population.

Table 2. Descriptive statistics of grain length (GL), grain width (GW), length-to-width ratio (LWR), thousand grain weight (TGW), white belly rate (WBR), white core rate (WCR) and chalkiness rate (CR) of the F_2 plants and their derived $F_{2:3}$ lines in 2016 and 2017, respectively.

	GL16	GL17	GW16	GW17	LWR16	LWR17	TGW16	TGW17	WCR16	WCR17	WBR16	WBR17	CR16	CR17
GL16	1.00	-	-	-	-	-	-	-	-	-	-	-	-	-
GL17	0.45**	1.00	-	-	-	-	-	-	-	-	-	-	-	-
GW16	0.66**	0.15	1.00	-	-	-	-	-	-	-	-	-	-	-
GW17	0.00	0.14	0.18	1.00	-	-	-	-	-	-	-	-	-	-
LWR16	0.36**	0.35**	-0.46**	-0.23*	1.00	-	-	-	-	-	-	-	-	-
LWR17	0.23*	0.50**	-0.12	-0.76**	0.44**	1.00	-	-	-	-	-	-	-	-
TGW16	0.65**	0.29**	0.58**	0.23*	0.04	-0.06	1.00	-	-	-	-	-	-	-
TGW17	0.32**	0.56**	0.27**	0.48**	0.03	-0.06	0.33**	1.00	-	-	-	-	-	-
WCR16	-0.04	-0.12	-0.09	0.08	0.06	-0.15	-0.15	0.05	1.00	-	-	-	-	-
WCR17	-0.17	-0.03	-0.15	-0.02	-0.02	-0.03	-0.19	-0.15	0.02	1.00	-	-	-	-
WBR16	0.18	0.02	0.29**	0.08	-0.14	-0.03	0.16	0.12	0.26*	-0.07	1.00	-	-	-
WBR17	0.02	0.06	0.13	0.11	-0.15	-0.07	0.11	0.06	0.22	0.15	0.13	1.00	-	-
CR16	0.14	-0.03	0.21	0.10	-0.10	-0.08	0.08	0.11	0.57**	-0.06	0.94**	0.19	1.00	-
CR17	-0.08	0.03	0.02	0.08	-0.12	-0.07	-0.02	-0.03	0.18	0.66**	0.06	0.84**	0.11	1.00

"16" means 2016, "17" means 2017.

3.2. QTL mapping

The genetic linkage map was constructed by using 1985 bins identified from the sequencing data of 190 F_2 individuals (Figure 2). A total of 15 QTL were detected on 6 chromosomes in two populations, and genetic variations explained by each QTL ranged from 10.7% to 46.48% (Table 3).

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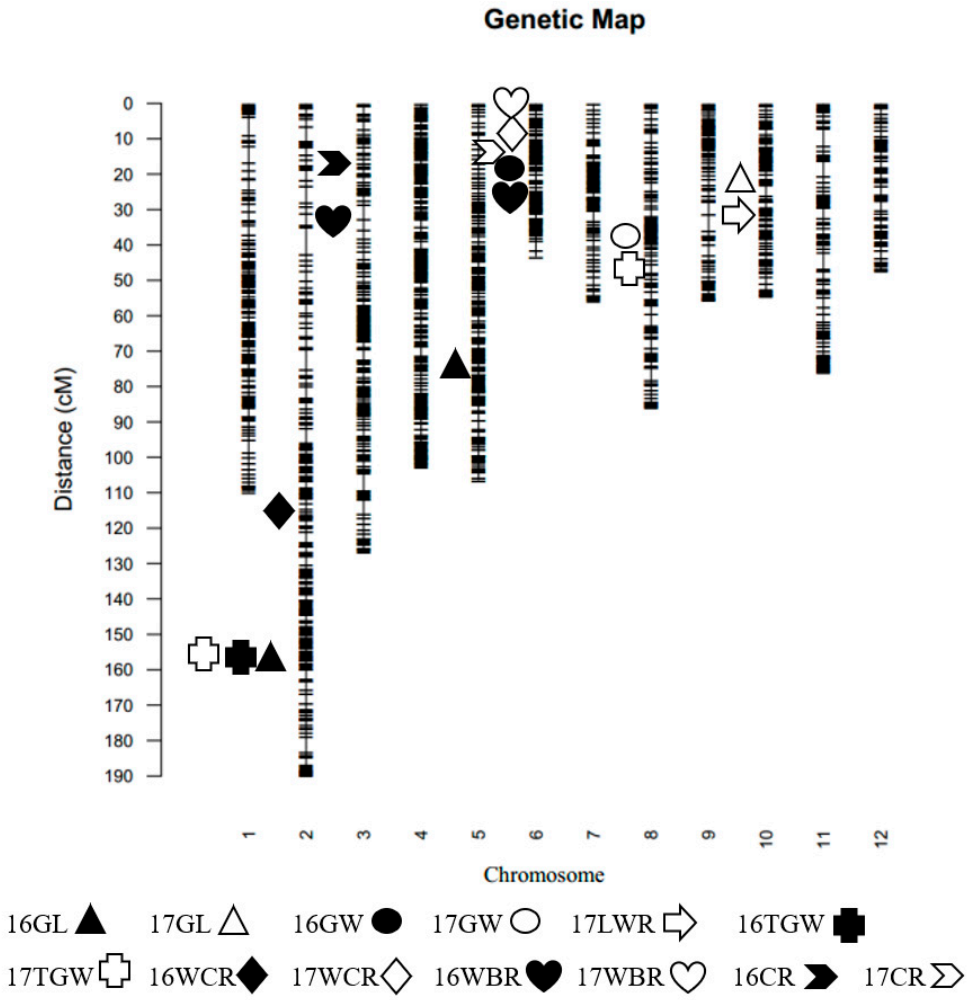

Figure 2. Genetic linkage map of F_2 population from Hua 5178S / Hua 611 constructed with SNPs markers. The ordinate represents the genetic distance of the map, and the horizontal lines represent the molecular markers on the map.

Table 3. QTL details in the F_2 and $F_{2:3}$ populations in 2016 and 2017.

Trait	QTL	Chromosome	Interval	F_2 (2016)			$F_{2:3}$ (2017)		
				LOD	Add	R^2(%)	LOD	Add	R^2(%)
Grain length	*qGL2*	2	M02259-M02344	8.40	0.18	46.48			
	qGL5	5	M05249-M05252	2.58	-0.15	14.14			
	qGL10	10	M10147-M10157				3.54	0.12	15.24
Grain width	*qGW6*	6	M0663-M0696	6.86	0.07	21.25			
	qGW8	8	M08204-M08206				5.05	-0.05	19.14
Length-to-width ratio	*qLWR10*	10	M10165-M10174				7.83	0.14	23.59
Thousand grain weight	*qTGW2*	2	M02260-M02307	3.19	0.44	17.12	7.78	0.79	20.94
	qTGW8	8	M08208-M08239				7.47	-0.69	23.96
White core rate	*qWCR2*	2	M02213-M02215	3.65	2.78	10.70			
	qWCR6	6	M0653-M0665				4.92	-1.62	24.48
White belly rate	*qWBR3*	3	M03101-M03102	3.90	5.67	25.33			
	qWBR6.1	6	M0633-M0642				5.37	-5.73	12.56
	qWBR6.2	6	M0683-M0696	3.77	4.69	17.90			
Chalkiness rate	*qCR3*	3	M0357-M0368	5.68	10.53	34.64			
	qCR6	6	M0653-M0665				7.38	-5.73	28.82

"+" additive effect comes from Hua 611; "-" additive effect comes from Hua 5178S; LOD is log of odds; Add is additive effect.

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Three QTL for grain length were detected in two populations (Figure 2, Table 3). *qGL2* located between M02259 and M02344 on chromosome 2, explained 46.48% of the phenotypic variation in 2016. *qGL5* was detected between M05249 and M05252 on chromosome 5, explained 14.14% of the phenotypic variation in 2016. *qGL10* was narrowed between M10147 and M10157 on chromosome 10, explained 15.24% of the phenotypic variation in 2017. Two grain width QTL were detected, explained 21.25% and 19.14% of phenotypic variation, respectively (Figure 2, Table 3). Two 1000-grain weight QTL were detected, and *qTGW2* was repeatedly detected in two populations, explained 17.15% and 20.94% of phenotypic variation in F_2 and $F_{2:3}$ populations, respectively (Figure 2, Table 3).

Seven QTL for chalkiness were detected in two populations and the phenotypic variation explained by each QTL ranged from 10.70% to 34.64% (Figure 2, Table 3). *qWCR2* was detected between M02213 and M02215 on chromosome 2, explained 10.70% of the phenotypic variation in 2016. Both *qWBR3* and *qCR3* located on chromosome 3, explained 25.33% and 34.64% of the phenotypic variation in 2016 respectively. *qWBR6.1* was found in the interval between M0633 and M0642 on chromosome 6, explained 12.56% of the phenotypic variation in 2017. *qWBR6.2* was detected between M0683 and M0696 on chromosome 6, explained 17.90% of the phenotypic variation in 2016. *qWCR6* and *qCR6* were found located in the same interval between M0653 and M0665 on chromosome 6, explained 24.48% and 28.82% of the phenotypic variation in 2017, respectively.

3.3. Validation of qWBR6.1, qWCR6 and qCR6

A gene cluster affecting white-belly rate, white-core rate and chalkiness rate was found on chromosome 6, and there were no related genes reported before. *qWBR6.1*, *qWCR6* and *qCR6* were selected for genetic validation. The F_4 random segregating populations of three QTL were constructed and genotyped by two flank markers, and then the phenotypic differences among different genotypes were compared. As shown in Figure 3, *qWBR6.1* locus from Hua 5178S increased white-belly rate by approximately 22.53% in the segregating population, *qWCR6* or *qCR6* locus from Hua 5178S increased white-core rate and white-belly rate by 42.75% and 41.87%, respectively. These results show that both three loci have an effect on chalkiness, and have application potential in decreasing chalkiness of rice.

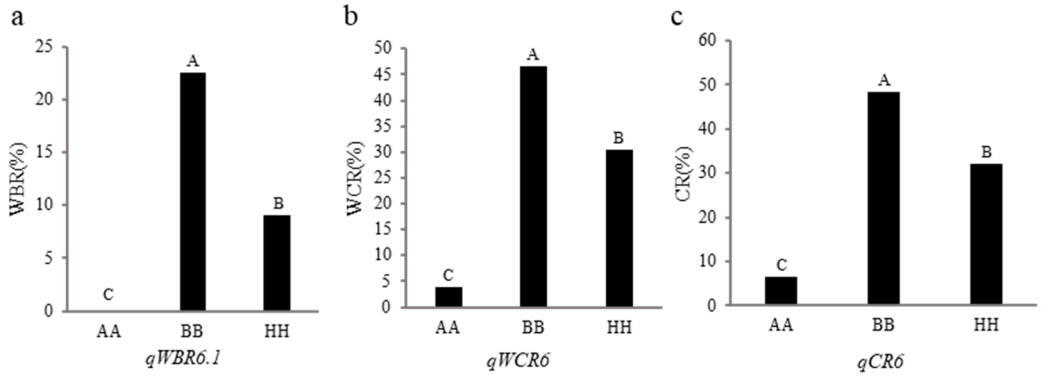

Figure 3. Genetic effects of *qWBR6.1*, *qWCR6* and *qCR6*. AA indicates that it is from the Hua 611 locus. BB indicates that it is from the Hua 5178S locus. HH indicates that it is heterozygous. Different capital letter on the black column represent extrimely significantly difference.

4. Discussion

Rice grain shape is a trait of great application value. At present, many grain shape genes have been cloned. *GS2*/*GL2* can positively regulate rice grain shape and ear length, increased expression of *GS2*/*GL2* lead to larger and more cells, result in an increase in 1000-grain weight [4]. In this study, *qGL2* and *qTGW2* were co-located at the same locus and positively regulated grain length and 1000-grain weight. The interval of *qGL2* and *qTGW2* contains the *GS2*/*GL2* locus, which indicates that the candidate genes of *qGL2* and *qTGW2* are likely to be *GS2*, but further confirmation is needed. *GS3*, *GW5*, *GW2* are the main genes affecting grain shape [3,6,7], but no grain shape-related QTL were found at these loci, which could be that the function of these genes did not differ between parents. In

addition, some new QTL that affecting grain shape and 1000-grain weight were found on chromosomes 6, 8 and 10, but the functions of these QTL need to be further verified.

Chalkiness is caused by an inhomogeneous arrangement of starch granules and proteomes in the endosperm, which has a great negative effect on rice quality. *Chalk5* controls the formation of chalkiness in the endosperm by affecting the formation of protein bodies [15]. *FLO4*, *FLO5*, *FLO2* regulate chalkiness by affecting starch synthesis [11–13]. Up to now, no chalkiness gene has been cloned on chromosome 6. In this study, some QTL with significant effect on chalkiness were found on chromosome 6, most of which were co-mapped in the interval of 3.3Mb-4.2Mb and 5.3Mb-6.5Mb. This result laid a foundation for the discovery of chalkiness genes on chromosome 6. The appearance quality of rice is very important because rice with less chalkiness has higher economic value and is more popular. Although some genes affecting grain shape and chalkiness have been found, the regulatory networks need to be further explored. In this study, 15 QTL related to grain shape and chalkiness were mapped. In addition, *qWBR6.1* and *qWCR6*/*qCR6* were found had great effect on chalkiness. Our research provides a wealth of QTL resources for rice quality improvement. Simultaneously, it also provides a foundation for fine mapping related functional genes.

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Data Availability Statement: The datasets generated during the current study are available from the corresponding author on reasonable request.

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Conflicts of Interest: Authors declare that there are no conflicts of interest.

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