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QTL detection for grain shape and fine mapping of two novel locus *qGL4* and *qGL6 in rice*

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Abstract

Rice grain size and grain weight, which have a great influence on rice quality and yield, are complex quantitative traits that are mediated by grain length (GL), grain width (GW), length-to-width ratio (LWR), and grain thickness (GT). In this study, the BC_1F_2 and $BC_1F_{2:3}$ populations derived from a cross between two *indica* rice varieties, Guangzhan 63-4S (GZ63-4S) and Dodda, were used to locate quantitative trait loci (QTL) related to grain size. A total of 30 QTL associated with GL, GW and LWR were detected, of which six QTL were scanned repeatedly in both populations. Two QTL, *qGL4* and *qGL6*, were selected for genetic effect validation and were subsequently fine mapped to 2.359 kb and 176 kb, respectively. *LOC_Os04g52240* (known as *OsKS2/OsKSL2*), which encoding an ent-beyerene synthase and as the only gene found in 2.359 kb interval, was proposed to be the candidate for *qGL4*. Moreover, the grains of *qGL4* homozygous mutant plants generated by the CRISPR-Cas9 system became shorter and wider. In addition, the *qGL4* allele from GZ63-4S contributes to the increase of yield per plant. Our study not only laid the foundation for further functional study of *qGL4* and map-based cloning of *qGL6*, but also provided genetic resources for the development of high yield and good quality rice varieties.

Keywords Rice · Grain size · Quantitative trait loci · Fine mapping · *OsKS2/OsKSL2*

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Introduction

Rice (*Oryza sativa* L.) is one of the most important staple food crops in the world and feeds more than half of the world's population (Sasaki 2005). In the face of continuously growing population and decreasing arable land, how to further improve the grain yield of rice is a major concern of scientists and breeders. Rice yield is largely determined by three major components: grain weight, number of grains per panicle, and number of effective tillers per plant (Xing and Zhang 2010). Among them, grain weight is largely determined by grain size, which includes grain length, width, and thickness (Zuo and Li 2014). Exploring the genetic basis of grain size and grain weight will exert a crucial role in the breeding of high-yield rice varieties(Sun et al. 2024). In recent decades, many QTL associated with grain size have been cloned and shown to be involved in a variety of signaling pathways, including the guanine nucleotide-binding protein (G protein) signaling pathway, the ubiquitin–proteasome signaling pathway, the mitogen-activated protein kinase (MAPK) signaling cascade, the transcriptional regulator pathway, and the phytohormone signaling pathway (Li et al. 2018, 2019; Fan and Li 2019; Ren et al. 2023).

G proteins are guanine nucleotide-binding trimeric proteins consisting of Gα, Gβ and Gγ subunits, and are involved in transmitting signals to regulate various biological processes. *GS3*, encoding an atypical Gγ protein, is the first identified major QTL to negatively regulate grain size (Mao et al. 2010; Fan et al. 2006). DEP1, another atypical Gγ protein, positively regulates grain size by competitively binding to RGB1 (Gβ) with GS3 (Sun et al. 2018). In addition, *OsMADS1* negatively regulates grain length through directly interacting with *GS3* and *DEP1* (Liu et al. 2018).

The ubiquitin–proteasome signaling pathway is involved in the regulation of rice grain size. Large-grain rice varieties represent valuable germplasm resources, harboring novel alleles that make significant contributions to grain shape(Zhao et al. 2017). The major QTL *GW2*, responsible for grain width and weight, was identified in WY3, a japonica variety with a KGW value of 41.9 ± 1.3 g(Song et al. 2007). *GW2* encodes a RING-type E3 ubiquitin ligase and negatively regulates grain width by ubiquitinating WG1 and targeting it for degradation via the 26S proteasome pathway (Song et al. 2007; Choi et al. 2018; Hao et al. 2021). *WTG1* encodes an otubain-like protease with deubiquitination activity and controls grain size and shape mainly by affecting cell expansion in the spikelet hull(Huang et al. 2017).

The OsMKKK10-OsMKK4-OsMPK6 cascade has been revealed to positively regulate grain size by promoting cell proliferation in spikelet hulls (Duan et al. 2014; Liu et al. 2015; Guo et al. 2018, 2020; Xu et al. 2018). GSN1/OsMKP1, a MAPK phosphatase, negatively regulates grain size by directly interacting with and inhibiting the dephosphorylation of OsMPK6(Guo et al. 2018). In addition, *OsER1*, which encodes a receptor-like protein kinase, could positively regulate grain size through the MAPK signaling cascade(Guo et al. 2020).

Many transcription factors are involved in the regulation of grain size. *GLW7* encodes a plant-specific transcription factor, OSSPL13, which positively

regulates glume cell size, leading to increased rice length and weight(Si et al. 2016). *GLW7.1* (*Grain Length, Width and Weight 7.1*), which encodes the CCT motif family protein, GHD7, promotes the transcription of several cell division and expansion genes, further resulting in a larger cell size and increased cell number, and finally enhancing the grain size as well as grain weight(Liu et al. 2022). OSSPL16/GW8 is a SBP domain-containing transcription factor that regulates rice grain width and can directly bind to the promoter of *GW7* and inhibit its expression (Wang et al. 2015). GS2 has transcriptional activation activity and interacts with OsGRFs, a transcriptional coactivator, which regulates cell elongation and cell division and affects grain size and grain weight of rice(Hu et al. 2015; Duan et al. 2015). GS9 encodes a protein with an unknown conserved domain and transcription factor activity, which regulates granulation by altering cell division(Zhao et al. 2018).

Moreover, phytohormones play various roles in plant growth and development, stress responses, and metabolism. Some genes controlling grain size have been reported to be involved in the brassinosteroid (BR) signaling pathway and the auxin signaling pathway and the gibberellic acid (GA) signaling pathway. *GS5* encodes a serine carboxypeptidase, a quantitative trait gene that controls grain width, fullness and 1000-grain weight in rice(Li et al. 2011). Enhanced GS5 expression can enhance BR signaling by occupying the extracellular leucine-rich repeat (LRR) domain of OsBAK1-7 and competitive inhibition of the interaction between OsBAK1-7 and OsMSBP1, thereby preventing endocytosis induced by OsBAK1-7 interaction with OsMSBP1(Xu et al. 2015). *GW5* acts in the brassinosteroid signaling pathway to regulate grain width and weight in rice, loss of *GW5* function caused wide and heavy grains, while overexpression of *GW5* resulted in narrow grains (Liu et al. 2017; Duan et al. 2017). *qTGW3* negatively regulates grain size and weight (Hu et al. 2018; Xia et al. 2018; Ying et al. 2018). *qTGW3* encodes the GSK3/SHAGGY-like kinase OsGSK5/OsSK41 that interacts with OsARF4 to negatively regulate grain size and weight in rice(Hu et al. 2018). *Small Grain and Dwarf 2*, encoding an HD-Zip II family transcription factor, regulates plant development by modulating gibberellin biosynthesis in rice (Chen et al. 2019). *OsKS2/OsKSL2* encodes a kaurene synthase (KS), which plays a catalytic role in the early steps of GA biosynthesis(Ji et al. 2013). Ji et al. reported that a GA-responsive dwarf mutant of rice cultivar Dongjin, *dwarf2*, contains a mutation within the *OsKS2/OsKSL2* coding region that results in silencing of *OsKS2/OsKSL2* gene expression (Ji et al. 2013). However, Daisuke Tezuka et al. thought that although the study suggested the possibility that *OsKS2/OsKSL2* encodes an ent-kaurene synthase that is involved in GA biosynthesis, it lacked direct biochemical analysis for its activity and genetic complementation, in which it is therefore necessary to determine the enzymatic activity of OsKS2/OsKSL2 to clarify this point(Tezuka et al. 2015). Daisuke Tezuka et al. successfully isolated a full-length cDNA for *OsKS2/OsKSL2* and provided evidence that OsKS2/OsKSL2 mainly converts entCDP into ent-beyerene(Tezuka et al. 2015). Their data suggest that OsKS2/OsKSL2 is involved in phytoalexin biosynthesis rather than GA biosynthesis. However, it is still unclear whether the *OsKS2/OsKSL2* gene is involved in affecting rice grain size.

Although many QTL regulating grain size have been identified, the understanding of grain size regulation is still limited, so mining new QTL of grain size is important for better understanding the mechanism of grain size regulation and providing genetic resources for breeding applications. In this study, we mapped QTL for grain size using BC_1F_2 and $BC_1F_{2:3}$ populations derived from the cross between GZ63-4S and Dodda. The near-isogenic lines (NILs) for the two QTL, *qGL4* and *qGL6*, were constructed to assess the genetic effects. Subsequently, the two QTL were fine mapped to intervals of 2.359 kb and 176 kb, respectively, and *LOC_Os04g52240* (*OsKS2/OsKSL2*) was the only candidate gene in the *qGL4* interval. The knockout mutants of *LOC_Os04g52240* were obtained using CRISPR-Cas9 gene editing technology, and subsequent phenotypic studies verified the effect of this gene on rice grain size. In conclusion, our study laid a foundation for the final cloning and further functional studies of the two QTL *qGL4* and *qGL6*, which may have potential value in the breeding of new high-quality and high-yield rice varieties.

Materials and methods

Population development and field experiment

The BC_1F_2 and $BC_1F_{2:3}$ were derived from a cross between two *indica* rice varieties, GZ 63-4S (receptor parent) and Dodda (donor parent). GZ 63-4S is an *indica* photo-thermo-sensitive genic male sterile line, which was developed from the *indica* photo-thermo-sensitive genic male sterile line Guangzhan 63S by the China North Japonica Hybrid Rice Research Center and Hefei Fengle Seed Company, carrying the infertility gene of *TMS5*. Dodda is an *indica* rice variety (belonging to the core germplasms collected by our team) (Xie et al. 2015). The BC_1F_2 and its derived $BC_1F_{2:3}$ populations used for QTL mapping were developed at the Experimental Farm of Huazhong Agricultural University (Wuhan, China) during the normal rice growing seasons in year 2016 and 2018, respectively. Twelve plants were planted in each $BC_1F_{2:3}$ line. To test the genetic effects of *qGL4* and *qGL6*, we planted BC_1F_4 and BC_1F_6 populations in 2019 and 2020, respectively, at the Huazhong Agricultural University Rice Base (Wuhan, China). The progeny test was performed in the BC_1F_7 population. The schematic diagram of experimental design is shown in Fig. S1. The seeds were sown on May 15 every year, and the seedlings (25 or 30 days old) were transplanted onto the field with a single plant spacing of 16.5 cm and 26.4 cm between rows. The field was managed using customary agricultural practices.

Trait measurements

Harvested rice seeds were air-dried and stored at room temperature for three months prior to measurements. Seeds were imaged on a Canon LiDE110 office scanner, and images were saved at 300 dpi resolution. Image files were processed in the SmartGrain analysis software (Tanabata et al. 2012) for measurements of seed width, length, area, perimeter, and circularity. Phenotypic values of each line in $BC_1F_{2:3}$

population were averaged over 12 plants. GL, GW and LWR of BC_1F_4 population were obtained using Canon LiDE110 office scanner, while that of BC_1F_6 and BC_1F_7 populations were measured using yield trait scorer with more than 200 grains per plant(Yang et al. 2014).

Genetic map construction and QTL mapping

The parental varieties GZ63-4S and Dodda were sequenced using illumine HiSeq2000 (Illumina, San Diego, CA, USA), and the sequencing data were aligned and assembled according to the rice reference genome (Rice Genome Annotation Project, http://rice.uga.edu/, accessed on 6 February 2022) (Li and Durbin 2009). To locate QTL associated with GL, GW and LWR, 101 simple sequence repeat (SSR) or insert/deletion (InDel) polymorphic markers and 25 kompetitive allele-specific PCR (KASP) markers were used for the genotyping of 241 plants from a BC_1F_2 population. Rice leaf genomic DNA was extracted using the cetyltrimethylammonium bromide (CTAB) method (W.F.Thompson 1980). Genotyping was performed using 4% polyacrylamide gel (PAGE) migration as previously reported by Panaud et al.(Panaud 1996). Genotypes were obtained by silver nitrate staining and sodium hydroxide-formaldehyde solution showing DNA bands on the PAGE gel. Genetic linkage maps were constructed using the Kosambi mapping function of the MapMaker/Exp3.0 programme. QTL analysis was carried out by composite interval mapping method using Windows QTL 2.5 software (WinQTLCart 2.5) (Luciano Da Costa E. Silva 2012). Relevant primer sequences are shown in Table S2.

Effect validation and fine mapping

Disregarding the genes that had been cloned, we selected *qGL4*, which has a large LOD and Add, and *qGL6*, which was localized in two-year repeats, for the main study. Based on the two flanking markers within the localization intervals of *qGL4* and *qGL6*, we selected lines that were heterozygous for target region in the $BC_1F_{2:3}$ population and developed the BC_1F_4 population for effect validation. To fine map these two QTL, we developed two BC_1F_6 populations consisting of 1200 and 1100 individuals respectively to screen recombinants, and conduct effect validation. Furthermore, specific markers were developed for genotyping and composite interval mapping (Luciano Da Costa E. Silva 2012). The BC_1F_7 population was then used for progeny testing within the small intervals.

RNA extraction, reverse transcription, and quantitative RT-PCR

Total RNA was extracted from 8 cm, 12 cm, 14 cm and 16 cm young panicles using RNA extraction kit (TRIzol, Invitrogen, Carlsbad, CA, USA). Approximately 2 μg of total RNA was used as a template for reverse transcription to generate cDNA, total RNA was pretreated with DNase I (Invitrogen), and first-strand cDNA was synthesized using oligo (dT)18 as primer (Promega, Madison, WI, USA). Real-time PCR was carried out using Bio-Rad T100™ real-time PCR system (Bio-Rad,

Hercules, CA, USA) with the SYBR Green I mix (TaKaRa, Shiga, Japan) on the QuantStudio6Flex instrument (Applied Biosystems, Carlsbad, CA, USA). All tests were performed at least three biological repeats. *OsActin1* was used as an internal control, and relative expression level was calculated by $2^{-\Delta\Delta Ct}$. The relevant primers for this analysis are listed in Table S2.

Design of the *OsKS2/OsKSL2* target sites and construction of the CRISPR/Cas9 targeting vector

We designed target sites at bases 57–76 of the first exon of the *OsKS2/OsKSL2* gene to produce transgenic lines. The online tool CRISPR GE was used to design the target sites(Xie et al. 2017). The target sequences for the *OsKS2/OsKSL2* gene were GTGGATCCAGCGGAACCAGC. The U6-sgRNA expression cassette encoding the 20 nucleotides (nt) *OsKS2/OsKSL2* target sequence was amplified by polymerase chain reaction (PCR) and ligated into the pCXUN vector, which *Kpn I* digested to create the pCXUN-U6-sgRNA intermediate vector. The constructed CRISPR/Cas9 final vector was introduced into the NIL-GZ63-4S receptor using *Agrobacterium tumfaciens*-mediated genetic transformation(Hiei et al. 1997). The primer sequences used to construct the vector are listed in Table S2.

Statistical analysis

Correlation analysis was plotted using OmicStudioKits (v. 1.8.1) online tool at https://www.omicstudio.cn/tool. Significant differences between two sets of data were presented as the mean ± standard error and performed using the two-tailed student's *t*-test. Microsoft Excel 2016 was used to perform the preliminary processing and analysis of phenotypic data.

Results

Phenotypic variation and correlation of the B1F and $BC_1F_{2:3}$ populations

The GZ63-4S is a classic example of a photoperiod- and thermo-sensitive genic male sterile line, exhibiting male sterility during the regular growing seasons in Wuhan. Consequently, seed harvesting was not feasible, precluding any comparative analysis of grain size between the two parent lines. All the traits in BC_1F_2 and its derived $BC_1F_{2:3}$ populations were continuously variable and normally distributed in 2016 and 2018, respectively, showing typical quantitative trait characteristics (Fig. 1).

Correlation analysis showed that GL was positively correlated with LWR (the correlation coefficient between GL16 and LWR16 was 0.42, and the correlation coefficient between GL18 and LWR18 was 0.41), while GW was negatively correlated with LWR (the correlation coefficient between GW16 and LWR16 was -0.58, and the correlation coefficient between GW18 and LWR18 was -0.68) (Fig. 2).

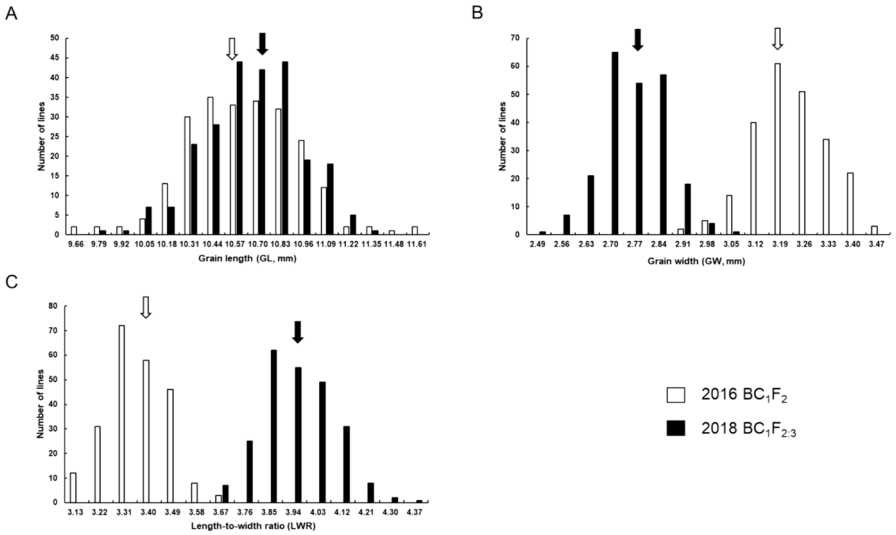

Fig. 1 Frequency distribution of GL (A), GW (B) and LWR (C) in the BC_1F_2 and $BC_1F_{2:3}$ populations in year 2016 and 2018. The vertical axis represents the number of BC_1F_2 and $BC_1F_{2:3}$ lines, with white and black bars, respectively. Arrow indicates the value of Dodda

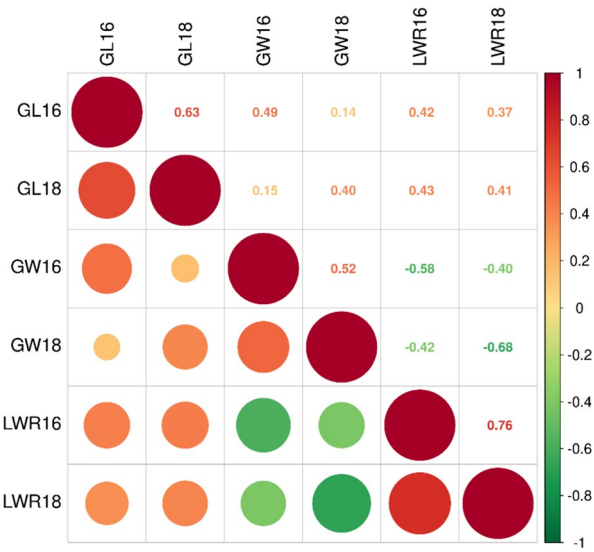

Fig. 2 Correlation coefficients among grain size-related traits in the BC_1F_2 and $BC_1F_{2:3}$ populations in year 2016 and 2018

QTL mapping and validation

QTL analysis revealed that 30 QTL related to GL, GW and LWR were detected, with 8 QTL for GL, 12 QTL for GW and 10 QTL for LWR (Fig. 3, Table 1). Eight QTL for GL were detected in two populations and distributed on chromosomes 2,

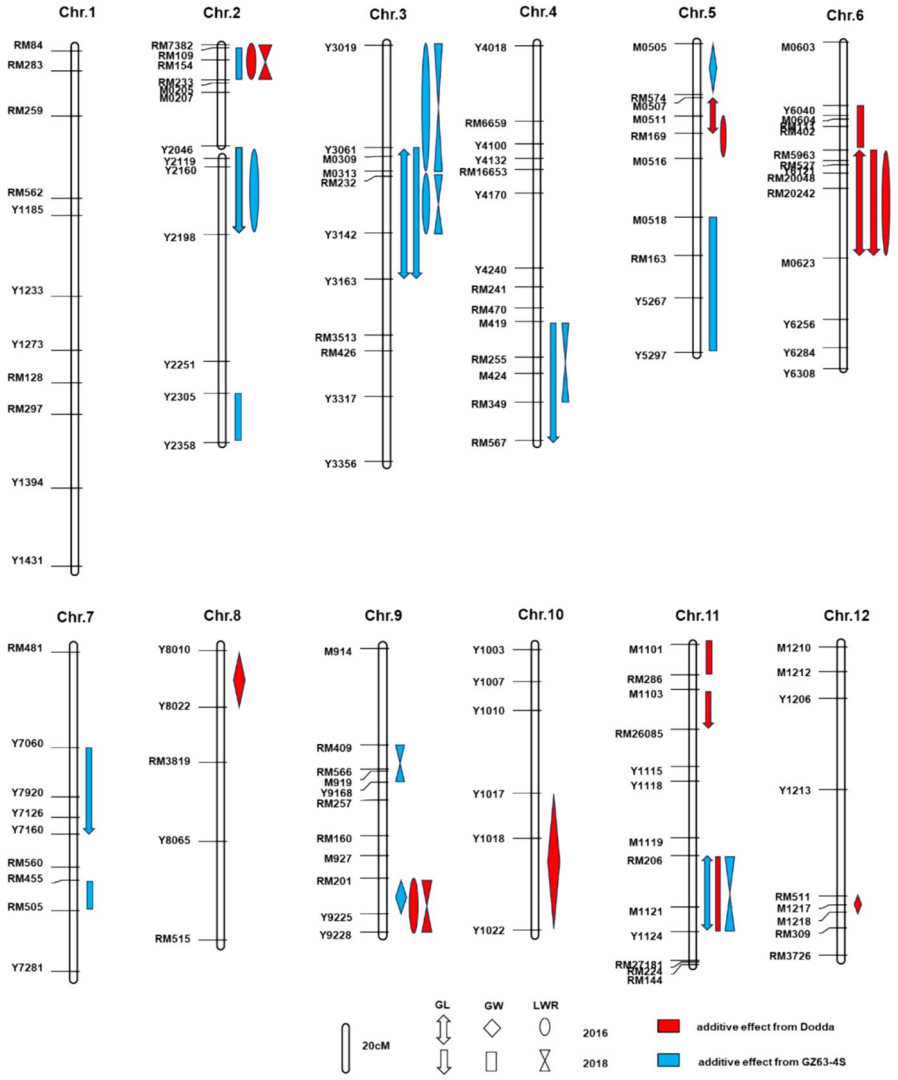

Fig. 3 Genetic linkage map of grain-size-related QTLs detected in the BC_1F_2 and $BC_1F_{2:3}$ populations

3, 4, 5, 6, 7 and 11 respectively, with phenotypic variation explained by each QTL ranging from 0.01% to 22.41% (Fig. 3, Table 1). For *qGL2*, *qGL3*, *qGL4*, *qGL7*, and *qGL11.2*, the alleles from GZ63-4S had positive effects, and the alleles showing positive effects for the remaining QTL were from Dodda. *qGL3* and *qGL6* were repeatedly detected in 2016 and 2018. In 2016, *qGL3* and *qGL6* accounted for 8.56% and 22.41% of the phenotypic variation, respectively, while in 2018, they explained 11.64% and 13.80% of the phenotypic variation, respectively. The remaining GL QTL were detected only in a single year, for example, *qGL5* and *qGL11.2* could only be detected in 2016, while the rest could only be found in 2018. Twelve QTL

Table 1 QTL detected for GL, GW and LWR in the BC_1F_2 and $BC_1F_{2:3}$ populations

QTL	Chr[a]	Interval	$16BC_1F_2$				$18BC_1F_{2:3}$				Known gene
			LOD	Add[b]	Dom[c]	R^2 (%) [d]	L0D	Add	Dom	R^2 (%)	
qGL2	2	Y2046-Y2198					3.21	-0.10	0.19	16.00	
qGL3	3	Y3061-Y3163	6.13	-0.15	-0.01	8.56	7.76	-0.15	0.00	11.64	*OsMADS1*
qGL4	4	M419-RM567					10.19	-0.12	-0.05	8.27	
qGL5	5	M507-RM169	2.72	0.10	-0.21	3.49					
qGL6	6	RM5963-M623	8.75	0.22	-0.08	22.41	12.59	0.17	0.04	13.80	
qGL7	7	Y7060-Y7160					3.56	-0.14	0.12	7.65	
qGL11.1	11	M1103-RM26085					2.82	0.07	0.09	1.89	
qGL11.2	11	RM206-Y1124	2.85	-0.03	-0.15	0.01					
qGW2.1	2	RM109-RM233					3.11	-0.03	0.04	5.48	
qGW2.2	2	Y2305-Y2358					2.52	-0.02	-0.07	0.70	*qTGW2; OsCNR1*
qGW5.1	5	M505-RM574	2.79	-0.01	-0.06	0.06					
qGW5.2	5	M518-Y5297					3.03	-0.05	0.07	21.66	
qGW6	6	Y6040-RM5963					4.62	0.04	0.00	8.38	
qGW7	7	RM455-RM505					3.09	-0.04	-0.02	2.76	
qGW8	8	Y8010-Y8022	2.94	0.02	0.07	1.02					
qGW9	9	RM201-Y9228	4.23	-0.06	0.00	8.27					
qGW10	10	Y1017-Y1022	5.62	0.04	0.05	4.01					*GW10*
qGW11.1	11	M1101-RM286					4.16	0.04	0.00	8.04	
qGW11.2	11	RM206-Y1124					5.43	0.05	0.00	11.52	
qGW12	12	RM511-M1218	2.67	0.05	0.00	4.65					
qLWR2.1	2	RM7382-RM233	3.62	0.03	-0.05	5.21	2.53	0.03	-0.10	2.00	
qLWR2.2	2	Y2119-Y2198	2.67	-0.02	0.02	4.05					
qLWR3.1	3	Y3019-M0313	11.80	-0.04	-0.06	1.94	8.11	-0.04	-0.05	2.89	*OsMADS1*
qLWR3.2	3	M0313-Y3142	8.43	-0.03	-0.07	2.60	6.34	-0.02	-0.10	1.89	
qLWR4	4	M419-RM349					3.85	-0.05	0.01	6.73	

Table 1 (continued)

QTL	Chr[a]	Interval	$16BC_1F_2$				$18BC_1F_{2:3}$				Known gene
			LOD	Add[b]	Dom[c]	R^2 (%) [d]	L0D	Add	Dom	R^2 (%)	
qLWR5	5	M511-M516	2.98	0.04	-0.03	5.10					
qLWR6	6	RM5963-M623	5.05	0.04	0.01	6.07					
qLWR9.1	9	RM409-Y9168					4.78	-0.01	0.17	8.80	
qLWR9.2	9	RM201-Y9228	8.72	0.07	0.00	13.23	4.50	0.06	0.01	6.43	
qLWR11	11	RM206-Y1124					4.72	-0.06	0.01	9.44	

[a]Chr, chromosome

[b]Add, additive effect of QTL: positive values indicate that alleles from Dodda increase the trait values, and negative values indicate that alleles from GZ63-4S increase the trait values; GL(mm); GW(mm)

[c]Dom, dominant effect of QTL

[d]R^2, variation explained by QTL

for GW were detected in two populations and distributed on chromosomes 2, 5, 6, 7, 8, 9, 10, 11 and 12, with phenotypic variation explained by each QTL ranging from 0.06% to 21.66% (Fig. 3, Table 1). Of these, the beneficial alleles of *qGW2.1*, *qGW2.2*, *qGW5.1*, *qGW5.2*, *qGW6*, *qGW7*, *qGW8*, and *qGW9* were from GZ63-4S, while that of others were from Dodda. Ten LWR-related QTL were identified in both populations, distributed on chromosomes 2, 3, 4, 5, 6, 9 and 11 (Fig. 2, Table 1). Among them, four QTL, including *qLWR2.1*, *qLWR3.1*, *qLWR3.2* and *qLWR9.2*, were repeatedly detected in both populations, while the rest LWR QTL were only detected in one population. For *qLWR2.1* and *qLWR9.2*, the alleles from Dodda had positive effects, while the alleles from GZ63-4S conferred positive effects for *qLWR3.1* and *qLWR3.2*.

To verify the authenticity of the QTL we identified, *qGL4* with a large LOD value and *qGL6*, which was repeatedly detected in two years, were selected to perform the genetic effect validation and fine mapping. The development of near-isogenic lines is common practice. Thus, plants carrying heterozygous target QTL regions were screened from the $BC_1F_{2:3}$ population and were subjected to several rounds of self-crosses for reducing the heterozygosity of non-target regions. Our strategy not only constructed near-isogenic lines for the target QTL, but also save tedious hybridisation work.

Validation and fine mapping of qGL4

Effect on grain length was evaluated in the BC_1F_4 and BC_1F_6 NIL populations of *qGL4* in Wuhan in 2019 and 2020, respectively, and the results showed that NIL-GZ63-4S^{qGL4} had significantly longer grains than NIL-Dodda^{qGL4} in both years (Fig. 4A-B). For the fine mapping of *qGL4*, a BC_1F_6 population consisting of

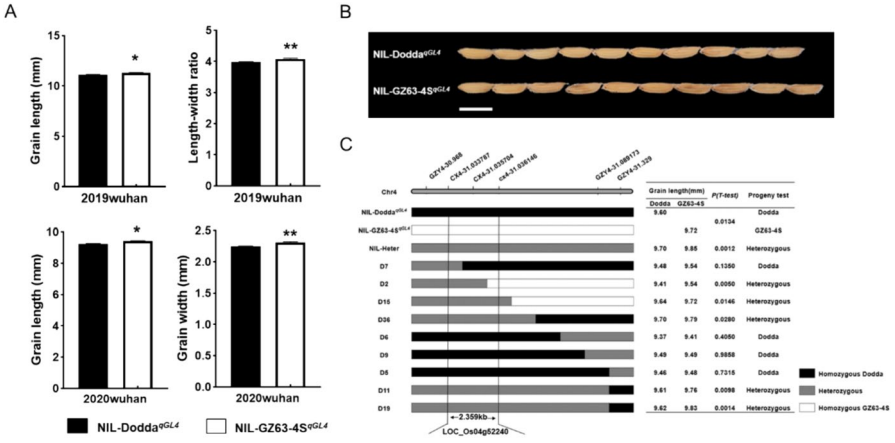

Fig. 4 Effect validation and fine mapping of *qGL4*. **A** Grain length difference of *qGL4* among near-isogenic lines in Wuhan in 2019 and 2020. **B** Seed grain morphology of NIL-Dodda^{qGL4} and NIL-GZ63-4S^{qGL4}. Scale bar:1 cm. **C** Fine mapping of *qGL4*. Data are represented as mean ± s.e.m. ($n \geq 10$). The *P* values were obtained by a two-tailed *T-test*. *and ** mean significant differences at $P < 0.05$ and $P < 0.01$ level, respectively

1200 individuals was developed, and 133 recombinants between GZY4-28.27 and GZY4-32.64 were identified. Subsequently, 14 InDel markers were developed for genotyping these recombinants according to the sequence variation of the two parents. Combining the contemporary phenotypes of these recombinants, *qGL4* was further mapped to a 361-kb genomic interval with flanking markers GZY4-30.968 and GZY4-31.329 (Fig. S4). There were also 40 recombinants between markers GZY4-30.968 and GZY4-31.329, and among them, 9 key recombinants were screened by using 4 additional polymorphic molecular markers. The progeny tests of 9 recombinants showed that *qGL4* locus was finally narrowed down to a 2.359-kb genomic interval with flanking markers CX4-31.033787 and cx4-31.036146 (Fig. 4C).

According to the Rice Annotation Project annotation, (http://rapdb.dna.afrc.go.jp/), this region contains only one annotated gene, *LOC_Os04g52240*, encoding an ent-beyerene synthase and known as *OsKS2/OsKSL2*. Previous research reported that *OsKS2/OsKSL2* gene may be involved in GA biosynthesis (Ji et al. 2013) or phytoalexin biosynthesis (Tezuka et al. 2015). However, the effect of this gene on grain shape and its regulatory mechanism are still unclear.

Comparative biparental sequencing of qGL4 candidate interval and genetic transformation and functional validation of qGL4

Comparative biparental sequencing revealed two SNP variants in the *OsKS2/OsKSL2* promoter region, one SNP variant in the first exon and nine SNP variants in the first intron (Fig. 5A). However, the SNP on the first exon was a nonsense mutation that did not result in an amino acid change. Considering that the variants in the promoter might cause differences in the relative expression levels among the near-isogenic lines, so we examined the relative expression levels of *OsKS2/OsKSL2* in 8 cm, 12 cm, 14 cm and 16 cm young spikes of the near-isogenic lines. RT-qPCR results showed that *OsKS2/OsKSL2* differed significantly between 12 and 14 cm young spikes, indicating that the differences in grain length among the near-isogenic lines caused by *OsKS2/OsKSL2* might be due to differences in its relative expression (Fig. 5B).

In order to further clarify the effect of gene *OsKS2/OsKSL2* on grain size, we performed gene editing of this gene using the CRISPR-Cas9 system in the NIL-GZ63-4S^{qGL4} genetic background for the construction of *OsKS2/OsKSL2* knockout materials. We designed the sequence in the first exon of the *OsKS2/OsKSL2* gene (GTGGATCCAGCGGAACCAGC) as a target site for sgRNA with the aim of obtaining amino acid mutations adjacent to the GGG structural domain (Fig. 5C). The gene editing experiments yielded three allelic mutant phenotypes, named KO-1, KO-2, and KO-3, respectively. We examined the grain size of the mutants, and in agreement with the expected results, the grain length of all three allelic mutant phenotypes became shorter than that of the wild type in the NIL-GZ63-4S^{qGL4} background. In contrast, it was also found that the grain width of the KO-1 and KO-2 allelic mutants became wider than that of the wild type (Fig. 5D-E).

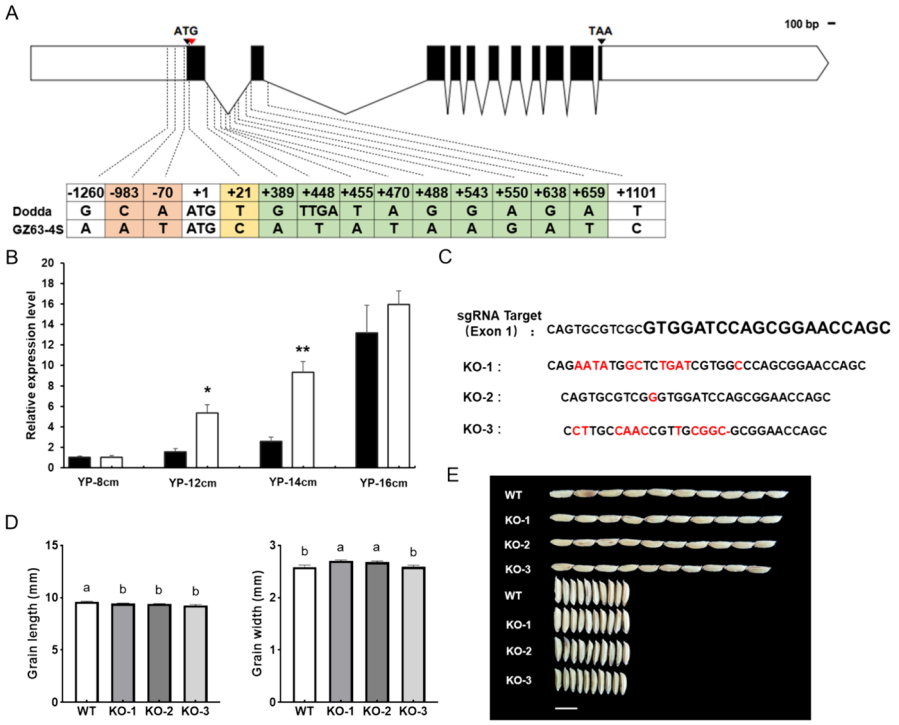

Fig. 5 Parental sequencing comparison and genetic transformation analysis of *qGL4*. **A** Localization of biparental variant sites between intervals. Red markers are sgRNA target site in the CRISPR-Cas9 system. bar:100 bp. **B** Relative expression levels of *qGL4* between near-isogenic lines. **C** Sequences of sgRNA target sites and allelic mutant sequences in the CRISPR-Cas9 system. Black bold letters for sgRNA target, red letters for base insertion, "-" for base deletion. **D** Grain length and grain width of knockout mutants and wild type. **E** Seed morphology of wild type and knockout mutants. Scale bar: 1 cm. Data are represented as mean ± s.e.m. The *P* values were obtained by a two-tailed *T-test*. * and **, indicate significant differences at $P < 0.05$ and $P < 0.01$, respectively. Different lowercase letters mean significant differences at $P < 0.05$ level in Duncan's multiple range test

qGL4 allele from GZ63-4S can improve rice yield

To assess the effect of *qGL4* on rice yield, we examined yield-related traits in NILs. Compared to NIL-Dodda^{qGL4}, NIL-GZ63-4S^{qGL4} exhibited the substantial enhancement in both grain length and grain width, as well as the thousand-grain weight (Fig. 6A-C). Although NIL-GZ63-4S^{qGL4} exhibited a reduction in panicle length and the number of primary branches than NIL-Dodda^{qGL4}, the filled grain number per panicle of NIL-GZ63-4S^{qGL4} also increased, which resulted in the improvement of yield per plant of NIL-GZ63-4S^{qGL4} (Fig. 6D-H). These results indicate that the *qGL4* allele from GZ63-4S can improve rice yield.

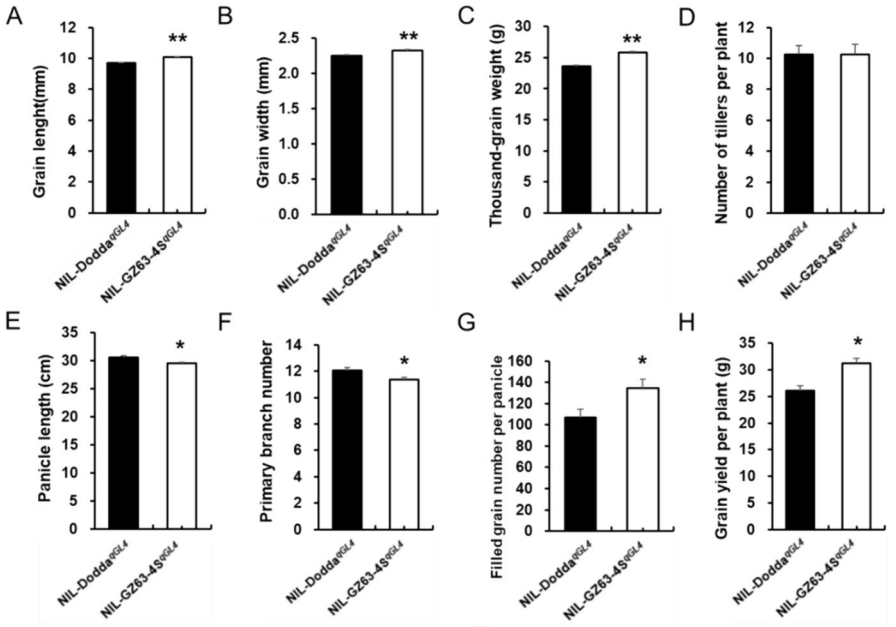

Fig. 6 The yield-related traits in the NILs of *qGL4*. **A**–**H** Grain length (mm), grain width (mm), thousand-grain weight (g), number of tillers per plant, panicle length (cm), primary branch number, filled grains number per panicle, and grain yield per plant in the NILs. All phenotypic data in (**A**–**H**) were measured from paddy-grown NIL plants grown under normal cultivation conditions. Data are represented as mean ± s.e.m. ($n = 12$). The student's *t*-test was used to produce *P* values (*, ** indicate significance at $P < 0.05$ and $P < 0.01$ level, respectively)

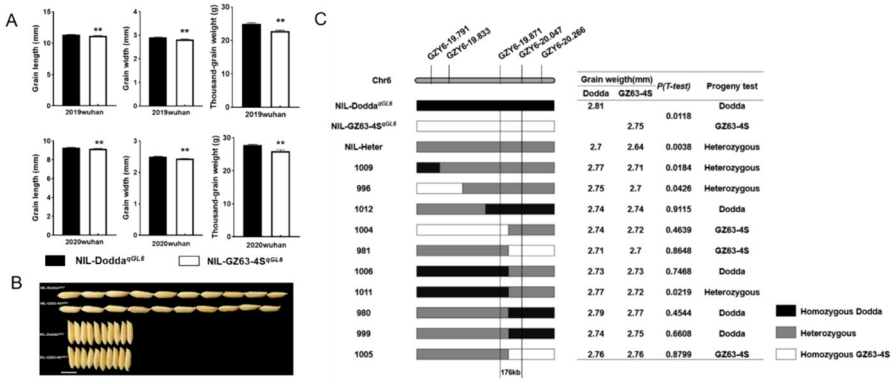

Fig. 7 Effect validation and fine mapping of *qGL6*. **A** Grain length of near-isogenic lines in Wuhan in 2019 and 2020. **B** Seed morphology of NIL-Dodda^{qGL6} and NIL-GZ63-4S^{qGL6}. Scale bar: 1 cm. **C** Fine mapping of *qGL6*. Data are represented as mean ± s.e.m. ($n \geq 13$). The *P* valueS were obtained by a two-tailed *T-test*. *and ** mean significant differences at $P < 0.05$ and $P < 0.01$ level, respectively

Validation and fine mapping of qGL6

To verify the genetic effect of *qGL6*, grain size traits were evaluated in the BC_1F_4 and BC_1F_6 NIL populations in Wuhan in 2019 and 2020, respectively. The results showed that significant differences in GL, GW and thousand-grain weight were observed between NIL-GZ63-4S^{qGL6} and NIL-Dodda ^{qGL6} in both years (Fig. 7A-B). To fine map *qGL6*, we constructed a BC_1F_6 population of 1100 plants and screened to 81 recombinants with two markers, RM20048 and GZY6-21.34. Subsequently, 13 markers were developed to genotype the recombinants and the grain size traits were examined. *qGL6* was further mapped to a 475-kb genomic interval with flanking markers GZY6-19.791 and GZY20.266 by composite interval mapping (Fig. S7). There are also 20 recombinants of *qGL6* between markers GZY6-19.791 and GZY20.266, and among them, 10 key recombinants were screened by using 3 additional polymorphic molecular markers. The progeny tests of 10 recombinants showed that *qGL6* was narrowed down to a 176-kb interval marked by GZY6-19.871 and GZY6-20.047 (Fig. 7C).

According to the annotation information of Nipponbare (http://rice.uga.edu/cgi-bin/gbrowse/rice/#search), the target region of qGL6 contains six annotated genes (*LOC_Os06g34360*, *LOC_Os06g34390*, *LOC_Os06g34400*, *LOC_Os06g34420*, *LOC_Os06g34430*, *LOC_Os06g34440*). Among these genes, *LOC_Os06g34360*, *LOC_Os06g34390* and *LOC_Os06g34400* encode a C3HC4 zinc finger protein. *LOC_Os06g34420* encodes a DEAD/DEAH box helicase domain protein. *LOC_Os06g34430* encodes zinc finger protein. *LOC_Os06g34440* encodes dnaJ domain containing protein. We further compared the sequence variations of these candidate genes including the 2 kb promoter and the coding sequence between GZ63-4S and Dodda (Table S3). The result showed that the variations occurred in the promoter, intron, and 5′ and 3′ untranslated regions (UTR).

Discussion

The QTL detected in the original population is sometimes unstable and therefore requires further effect validation. The most common and effective strategy is to use near-isogenic lines. In this study, we selected lines from the $BC_1F_{2:3}$ population that were heterozygous for each QTL in the locus interval to generate high-generation populations in case the target regions were lost in subsequent selfing. In the experimental context, the parent line GZ63-4S, being a sterile strain, necessitated a strategy to reduce heterozygosity in non-target regions. To achieve this, we employed a successive selfing process across generations with the selected germplasm. Concurrently, we utilized molecular marker-assisted screening to guarantee the maintenance of heterozygosity within the target locus intervals. This approach not only preserved the required heterozygosity but also alleviated the labor-intensive burden of re-screening for fertility in each subsequent generation following backcrossing. Zhou et al. also employed this method to precisely identify 5 QTLs influencing rice yield and quality, and accumulated

the genetic basis for the cloning of these 5 QTLs, providing valuable resources for the breeding of high-yield and high-quality rice varieties(Zhou et al. 2024).

Due to comparative biparental sequencing of the localization intervals revealed two SNPs in the *qGL4* promoter region, one SNP in the first exon and nine SNPs in the first intron (Fig. 5A). However, the SNP variants in the first exon was a nonsense mutation that did not result in an amino acid change. Difference in the relative expression levels of *qGL4* in 12 cm and 14 cm young spikes among the NILs indicated that two SNPs in the promoter region of *qGL4* may be responsible for variation in grain size among near-isogenic lines. According to the *cis*-acting element prediction website (https://www.dna.affrc.go.jp/PLACE/?action=newplace), there is a TACT *cis*-element in the Dodda promoter region of *qGL4* due to the SNP (-70 bp), but not in the GZ63-4S promoter region. Subsequently, we will focus on this SNP locus and design relevant experiments to verify whether this SNP causes differences in relative expression levels between qGL4 near-isogenic lines.

The excellent allele of *qGL4* derived from GZ63-4S can not only increase grain length but also increase single plant yield, which can be used for selection and improvement of rice grain size and yield. The genetic effect of *qGL6* could be detected in the NIL population planted for two consecutive years, which proved the stability and reliability of the QTL and was suitable for subsequent gene cloning. In short, our work laid the foundation for the functional study of *qGL4* and subsequent gene cloning of *qGL6*, and the excellent allele of *qGL4* from GZ63-4S provided genetic resources for the cultivation of high quality and high yield rice varieties.

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Author contributions Yuanyuan Zheng performed most of the experiments; Minqi Li, Ping Sun and Yanhua Li participated in part of phenotyping and genotyping; Yuanyuan Zheng and Guangming Lou performed various data analysis; Guanjun Gao and Qinglu Zhang participated in partial field experiments; Yuanyuan Zheng, Bian Wu and Yuqing He designed experiments; Yuanyuan Zheng wrote the manuscript and Guangming Lou improved it; All authors discussed and commented on the manuscript.

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Material and data availability The authors declare that all relevant data supporting the findings of this study are included in the main manuscript file or Supplementary Information or are available from the corresponding author upon reasonable request.

Declarations

Ethics approval The experiments comply with the ethical standards in the country in which they were performed.

Conflict of interest The authors declare no competing interests.

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