# The development and validation of new DNA markers linked to the thousand-grain weight QTL in bread wheat (*Triticum aestivum* L.)

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**Abstract:** Thousand-grain weight (TGW) is an important trait affecting wheat production. We previously identified a major quantitative trait loci (QTL) controlling the TGW on the 2D chromosome of wheat using a recombinant inbred line (RIL) population constructed by the cross between Tibetan semi-wild wheat Q1028 (Q1028) and Zhengmai 9023 (ZM9023). The positive allele at this QTL is from ZM9023. To further characterise this QTL, here, we try to develop and validate the high-resolution melting (HRM) and sequence-characterised amplified region (SCAR) markers. One HRM marker (0C98-411) and two SCAR markers (E301-700 and B0BB-10470) were developed and integrated into the genetic map. All of these three markers were validated in three populations with different genetic backgrounds. 0C98-411 is the most closely linked marker that could trace *QTgw.sau-2D* in molecular marker assisted breeding.

**Keywords:** high-resolution melting marker; marker development; *QTgw.sau-2D*; sequence-characterised amplified region marker

The common wheat (*Triticum aestivum* L., 2n = 6x = 42, AABBDD genome) is one of the three major food crops (Tahmasebi et al. 2017). The acceleration of urbanisation has been reducing the area of cultivated land, posing a huge challenge to wheat production in China. Therefore, the development of a high yield wheat is an important target for wheat breeding in China. The wheat yield per unit area mainly depends on the number of effective panicles per unit area, the number of grains per spike and the thousand-grain weight (TGW). Compared with

other quantitative traits of wheat, such as tillering and quality-related traits, the heritability of TGW is the largest and most stable, and it is least affected by the environment (Chen et al. 2014).

It is believed that the isolation and application of TGW quantitative trait loci (QTL) could greatly improve the wheat yield. With the continuous development of molecular biology, genomics and molecular quantitative genetics, various molecular markers and molecular genetic maps have been developed and provide chances for studying the genetic mechanism of

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wheat quantitative traits. Thus far, numerous studies have been conducted to identify the QTL associated with TGW on almost each of the 21 chromosomes in wheat. For example, Wu *et al.* (2015) detected the QTL of TGW on chromosomes 1A, 1B, 2A, 2B, 3A, 3B, 3D, 4A, 4D, 5A, 5B, and 6B.

In rice, many TGW QTL have been cloned and characterised, such as OsGW2 and OsGW7/GL7, (Song et al. 2007; Wang et al. 2015a, b). However, few TGW QTL have been accurately located or even cloned in wheat. The publication of the draft genome sequences of the common wheat cultivar Chinese Spring (IWGSC 2014) and its two progenitors, T. uratu (AA genome, 2n = 14) (Ling et al. 2013) and Aegilops tauschii (DD genome, 2n = 14) (Jia et al. 2013) enables an informed analysis of wheat on a genome wide scale (Saintenac et al. 2013) and so it is convenient to develop molecular markers.

Zhengmai 9023 (ZM9023) is a commercial wheat cultivar bred in Henan, China. Given its desirable characteristics including strong gluten, compact plant

type, early maturity, high yield, multi-resistance, and wide adaptability, it was one of the main cultivars in China.

Luo et al. (2016) have identified the TGW QTL on 2BS, 2DL, 6BL, and 7AL chromosomes using a RIL population constructed by the cross between Tibetan semi-wild wheat Q1028 (Q1028) and ZM9023. Among them, *QTgw.sau-2D* was a major and stable QTL detected in three environments and its hereditary effect was from ZM9023 (Luo et al. 2016). In this study, we are aiming at developing and validating effective molecular markers that are tightly linked to *QTgw.sau-2D*.

# MATERIAL AND METHODS

**Plant material.** A RIL population containing 186 lines developed by crossing Q1028 and ZM9023 (Figure 1) (QZ population) was used to reconstruct a genetic map with newly developed markers. Three  $F_{2:3}$  populations were developed to further verify

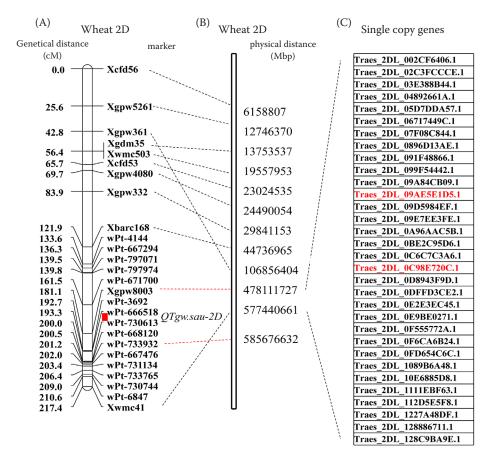


Figure 1. The genetic and physical maps of QTgw.sau-2D: the previously constructed genetic map of QTgw.sau-2D on 2D (A), the physical positions of the markers (B) and the single copy genes located in the target interval where QTgw.sau-2D was seated (C)

the newly developed markers and they were QZ36  $\times$ QZ203 (150 lines), QZ82 × QZ270 (150 lines) and QZ36 × Chuannong 16 (CN16; 150 lines), respectively. Among them, QZ36 and QZ82 contain QTgw. sau-2D allele only, while QZ203 and QZ270 do not contain any TGW QTL. CN16 is a native wheat cultivar with a lower TGW compared to ZM9023 in Sichuan, China. All the populations were evaluated at the experimental farm of the Triticeae Research Institute, Sichuan Agricultural University, China. Each line of a given population was a single-seed planted with 10 cm between the plants in a 2 m long bed and 30 cm between the rows (Ma et al. 2019). Nitrogen and superphosphate fertilisers were applied at a rate of 80 and 100 kg/ha, respectively, at sowing (Yu et al. 2018). The field management followed that commonly practiced in wheat production. All the uniform and plump seeds from each line were weighed and converted into a TGW.

Data collection and screening for gene sequences. The shotgun genome sequences and gene models of the wheat cultivar Chinese Spring (CS) were downloaded from http://wheat-urgi.versailles.inra.fr/ hosted by Unité de Recherche Génomique Info (URGI; IWGSC 2014, http://www.wheatgenome.org/). The coding sequences (CDS) and assembled whole genome sequences of Brachypodium distachyon v2.0 and rice (Oryza sativa) were obtained from Phytozome v10 and v9.0, respectively (http://www.phytozome. org/). The Ae. tauschii (Aet V4.0) (https://www. ncbi.nlm.nih.gov/assembly/GCA\_002575655.1/#/ def\_asm\_Primary\_Assembly) (Luo et al. 2017) genome sequences were downloaded as well. We downloaded the genome shotgun sequences and the CDS data of T. urartu from the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) and GIGA\_DB (http://gigadb.org/), respectively. The wild emmer (T. turgidum ssp., dicoccoides) genome was downloaded from https://www.dropbox.com/ sh/3dm05grokhl0nbv/AAC3wvlYmAher8fY0srX 3gX9a?dl=0%22 (Avni et al. 2017). To design the chromosome 2D specific primers, the homeologous sequences from 2A, 2B and 2D in CS and the orthologs on 2A from T. urartu, 2D from Ae. tauschii, as well as 2A and 2B from *T. turgidum* ssp. *dicoccoides* were aligned for detecting the polymorphism. We used the available markers flanking the major QTL *QTgw.sau-2D* from the originally constructed genetic map (Luo et al. 2016) to blast against the CS contig sequences using the BLAST++ BLASTN algorithm with the E value cut-off of  $0-10^{-5}$  (Ma et al. 2018). Furthermore, the CS contigs between the anchored ones (2DL\_478111860 and 2DL\_585677005) that flanked QTgw.sau-2D were retrieved for searching for the CS gene models (Figure 1). Given the rich polymorphism of the promoter sequences, we isolated the sequences (1~3kbp) located at 5' upstream of the start codon of the single copy genes for the parents of Q1028 and ZM9023. In addition, the coding sequences of several genes located in the target interval were isolated as well for developing the sequence-characterised amplified region (SCAR) markers. The homeologous sequences from 2A, 2B and 2D for a given gene were compared by DNA-MAN (Ver. 7.0, 2005) to design the primers specific to chromosome 2D.

Sequence isolation and sequencing. We used the cetyltrimethylammonium bromide (CTAB) method to extract the genomic DNA (MURRAY & THOMPSON 1980). The PCR reaction was performed as follows: pre-denaturation at 94 °C for 5 min, then 40 cycles of 94 °C for 1 min, annealing at 58~65 °C for 40 s, extension at 72 °C for 50 s, and a final extension at 72 °C for 7 min (Tables 1 and 2). The PCR amplification was performed with the Gene Amp® PCR System 9700 (Applied Biosystems, Germany). The PCR reaction mixtures (20 µl) contain 10 µl mix (Vazyme Biotech Co., Ltd., China), 100 ng of genomic DNA, 0.5 μM of each primer and 7.5 μl ddH<sub>2</sub>O. The amplification products were separated on 1.0% agarose gels, and the fragments of interest were purified using a Gel Extraction Kit D2500 (Omega Bio-Tek, USA). The

Table 1. The sequences, the single nucleotide polymorphism (SNP), and annealing temperature of the developed high-resolution melting (HRM) markers

Marker	SNP (Q1028/ZM9023)	Forward primer (5'-3')	Reverse primer(5'-3')	Product length (bp)	Annealing temperature (°C)
09AE-590	A/G	AAGCAGACAGCA- GACTTTG	GTCTTCAATC TC- TGCCTGC	1482	59.1
0C98-411	A/G	CTCTTTCGTGCT- CTTGAC	GGATACCTTC AC- CCTGAG	721	60.2

Table 2. The sequences and annealing temperature of the developed sequence-characterised amplified region (SCAR) markers

Marker	Forward primer (5'-3')	Reverse primer(5'-3')	Product length (bp)	Annealing temperature (°C)
B0BB-10470	TCTAACGCTGCTCTGACTT	CTCTAGGGTGTATCAGGCCG	700	60
E301-700	TAGTACCACCTGGGTTGTT	ATGGTGTATGCGTGTGGA	779	58.6

recovered PCR products were cloned and sent to Tsingke Biotech Co., Ltd. for sequencing. At least three independent clones for each amplicon were sequenced in both directions.

Sequence analysis and markers development. To detect the single nucleotide polymorphisms (SNPs), we used DNAMAN 7.0 to compare the cloned sequences (Figure 2A). The identified SNPs were further converted into high-resolution melting<sup>TM</sup> (HRM) markers as described previously (Wittwer et al. 2003) for further analysis. We designed the HRM primers with Beacon Designer 7.9 and evaluated them with Oligo 6.0 26 (Zhang & Gao 2004). The parameters were set as follows: an inner product size of 60-100 bp, a melting temperature of  $55 \pm 5$  °C, a primer length of  $20 \pm 3$  bp, and 3'- end stability to avoid self-complementarity and primer dimer formation (Ma et al. 2018). Again, we used DNAMAN 7.0 to design the specific primers on 2D based on the alignments of the homeologous sequences of 2A, 2B and 2D.

Firstly, we used the parental DNA (Q1028 and ZM9023) to detect the efficiency of the developed markers (Figure 2B). Then we used these confirmed

markers to genotype the QZ mapping populations (Figure 2C). For the HRM markers, the amplification reactions were performed in a total volume of 10 µl containing 100 ng of template DNA, 5µl of Fast Super EvaGreen mixture, and 5 pmol of each forward and reverse primer, and DNase/RNase-free water up to the final value. The PCR reaction procedure was as follows: 5 min at 94 °C, 40 cycles of 1 s at 94 °C, and 30 s at 60 °C. SYBR Premix Ex TaqTM II (TaKaRa, Shiga, Japan) was used in the amplification system. For the SCAR markers, the PCR system was the same as that used for the sequence isolation as described previously. The amplification products of the SCAR markers were separated on 1.5% agarose gels (Figure 3). The newly developed markers were integrated in the previously constructed genetic map based on the QZ mapping population by Joinmap (Ver. 4.0., 2007)

**QTL validation.** We randomly selected 60 lines from three validation populations, QZ36  $\times$  QZ203, QZ82  $\times$  QZ270, and QZ36  $\times$  CN16 to further validate the efficiency of the developed markers and measure the QTL effects. According to the HRM and SCAR

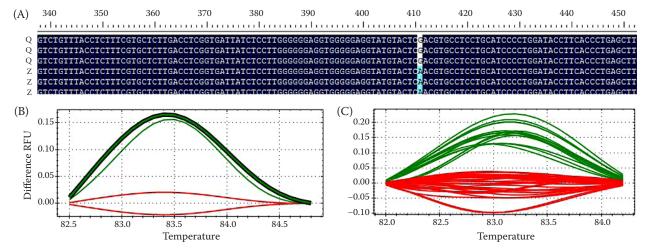


Figure 2. The process of the high-resolution melting (HRM) marker development: the single nucleotide polymorphism between the Q1028 and ZM9023 identification (A), the HRM marker test between the parents of Q1028 (the green line) and ZM9023 (the red line) (B) and the HRM analysis in the QZ RIL population (C)

The red lines represent the offspring consistent with the genotype of ZM9023; the green lines represent the offspring consistent with the genotype of Q1028

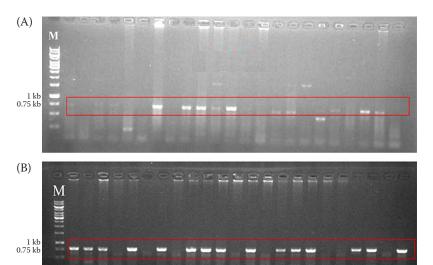


Figure 3. The development and analysis of the sequence-characterised amplified region (SCAR) markers B0BB-10470 (A) and E301-700 (B) in the QZ recombinant inbred line (RIL) population (only the partial lines were shown here)

The bands in the red box represent the lines having the same genotype with Q1028 and no bands represent those having the same genotype with ZM9023

analyses, we divided the lines for a given validation population into two groups: lines with homozygous alleles from non-ZM9023 (designated as 'a') and lines with homozygous alleles from ZM9023 (designated as 'b'). Student's t-test (P < 0.05) was used to assess the differences in the TGW between the above two groups.

## RESULTS AND DISCUSSION

Based on the preliminary QTL mapping results (Luo et al. 2016) and the CS genome sequence, we anchored the target interval between 478.11–585.68 Mbp on 2D. A total of 100 genes were detected in this interval (Table S1 in the Electronic Supplementary Material (ESM)). Of them, 32 single copy genes were further screened for sequence isolation as suggested in Ma et al. (2015a, b) (Figure 2, Table S2 in the ESM). As non-coding sequences usually possess more polymorphism than coding sequences (Ma et al. 2018), we attempted to select the sequences located at the upstream of the start codon for a given gene to isolate for these 32 single copy genes.

It is well known that the sequences of numerous orthologs among bread wheat and its ancestor species *T. urartu*, *Ae. tauschii*, and *T. turgidum* are conserved (MA *et al.* 2015a, b). To design the 2D specific primers, the homeologous sequences from 2A, 2B and 2D in the CS and the orthologs on 2A from *T. urartu*, 2D from *Ae. tauschii*, as well as 2A and 2B from *T. turgidum* ssp *dicoccoides* were aligned for detecting the polymorphism among 2A, 2B, and 2D. Multiple sequence alignment indicated that the 2D specific primers can be designed

for only 11 of these 32 single copy genes (Table S3 in the ESM).

Out of these 11 genes, 6 were successfully amplified and sequenced, namely  $Traes\_2DL\_0C98E720C.1$ ,  $Traes\_2DL\_10E6885D8.1$ ,  $Traes\_2DL\_1089B6A48.1$ ,  $Traes\_2DL\_09AE5E1D5.1$ ,  $Traes\_2DL\_07F08C844.1$ ,  $Traes\_2DL\_0896D13AE.1$ . Of them, 11 SNPs between the parents were detected for three genes ( $Traes\_2DL\_0C98E720C.1$ ,  $Traes\_2DL\_0C98E720C.1$  and  $Traes\_2DL\_0896D13AE.1$ ) only (Table S4 in the ESM).

We developed 12 HRM markers for these SNPs (Table S4 in the ESM), and only two markers could detect the polymorphism (i.e., the different colours of the curves) between the parents (Table 1). Although the HRM analysis technique has the advantages of a higher resolution and detection ability (Wittwer et al. 2003), we only successfully developed two HRM markers here. Previous studies have shown that the accuracy of the HRM analysis was affected by the fluorescent dye, amplified fragment length and instrument resolution (Pirulli et al. 2000; Lipsky et al. 2001; Dufresne et al. 2006; Herrmann et al. 2006; Farrar & Wittwer 2017). Since wheat is a polyploidy plant, it is difficult to design homologous-specific primers (Botticella et al. 2011), most likely resulting in the amplification of non-target chromosome fragments, thereby interfering with the HRM analysis and lowering the development efficiency of the HRM markers.

Then we found that only 0C98-411 was successfully integrated into the genetic map (Figure 4). The reason is most likely that we used the sequence of the CS wheat as a reference (IWGSC 2014). As chromo-

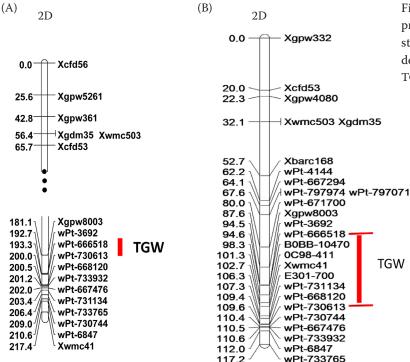


Figure 4. The comparison between the previous genetic map (A) and the re-constructed one with the integrated, newly developed markers (B)

TGW - the thousand-grain weight

some rearrangements are widely distributed in the different wheat genotypes (Badaeva et al. 2007; Ma et al. 2014, 2015a, b; Huang et al. 2018), we cannot rule out the possibility that an inconsistency in the physical maps may exist among ZM9023, Q1028 and CS. Additionally, the relative lower polymorphism of the wheat D genome has made it difficult to develop markers (Röder *et al.* 2008).

We also attempted to isolate and sequence three genes,  $Traes\_2DL\_458CEA548.1$ ,  $Traes\_2DL\_B0BBEE64.1$  and  $Traes\_2DL\_E301FB6B9.1$  flanking the QTgw.sau-2D to detect the polymorphism (Table S5 in ESM). Unexpectedly, we found that

primer BOBB-10470 and E301-700 showed the characteristics of SCAR markers between ZM9023 andQ1028 (Figure 3). We, thus, directly used them as the dominant markers to the genotype QZ RIL population (Figure 3), and both of them (B0BB-10470 and E301-700) were successfully integrated to the re-constructed genetic map (Figure 4).

Based on the genotypes of the 7 markers close to *QTgw.sau-2D*, 78 of the QZ RILs were classified into 6 different groups (Figure 5). As shown in Figure 5, the TGW of all the groups with both the B0BB-10470 and 0C98-411 markers having the ZM9023-like allele was significantly higher than the group with both

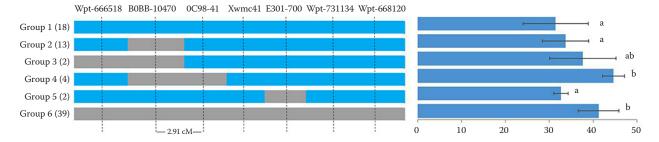


Figure 5. The thousand-grain weight (TGW) quantitative trait loci (QTL), QTgw.sau-2D was mapped to a 2.91 cM interval on 2D; left: the graphical genotypes of Q1028 × ZM9023 recombinant inbred line (RIL) groups with the number of lines in each group shown in parentheses; the RIL were grouped based on their genotypes defined by having either the Q1028-like (blue) or the ZM9023-like (grey) allele at each marker shown across the interval; right: the ANOVA (analysis of variance) adjusted mean TGW of the RIL groups across all the experiments

The error bars represent the standard error of the mean (SEM)

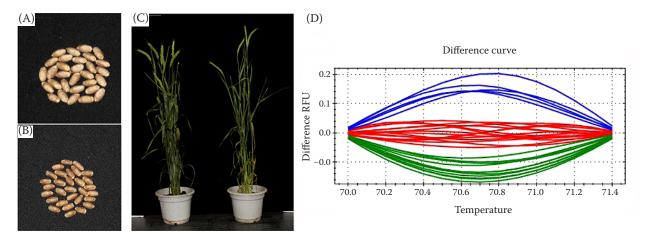


Figure 6. The QZ82 and QZ270 phenotype and validation of the high-resolution melting (HRM) markers: the seeds of QZ82 with the contributed allele of QTgw.sau-2D QTL only (A) seeds of QZ270 without QTgw.sau-2D or another TGW locus (B), plants of QZ82 (left) and QZ270 (right) (C), the HRM analysis of the QZ82 × QZ270  $F_{2:3}$  population (D) The different colours represent the different genotypes, the green lines represent the offspring consistent with the ZM9023 genotype; the blue lines represent the offspring consistent with Q1028; the red lines represent the heterozygote

B0BB-10470 and 0C98-411 having the Q1028-like allele. Thus, it is likely that the target QTL *QTgw. sau-2D* was mapped to a 2.91cM interval between B0BB-10470 and 0C98-411 on the wheat chromosome 2D (Figure 5).

The newly developed markers were further detected in three validation populations. Since 09AE-590 was not successfully integrated into the genetic map, we used 0C98-411, B0BB-10470 and 0C98-411 for the validation only (Figures 6 and 7). The marker 0C98-411 could distinguish all of the three populations and the average TGW of the genotypes with the homozygous alleles from ZM9023 was significantly higher (P < 0.01) than that of the genotypes with the homozygous alleles from the non-ZM9023

parents. The marker B0BB-10470 could distinguish all of the three populations and the average TGW of the genotypes with the homozygous alleles from ZM9023 was significantly higher (P < 0.05) than that of the genotypes with the homozygous alleles from the non-ZM9023 parents. The marker E301-700 could only distinguish the QZ36/QZ203 population and the average TGW of the genotypes with the homozygous alleles from ZM9023 was significantly higher (P < 0.05) than that of the genotypes with the homozygous alleles from the non-ZM9023 parents (Table 3). These results further indicate that 0C98-411 may be the closest marker to QTgw.sau-2D.

A higher TGW was usually negatively correlated with the quality characteristics (Xu et al. 2015).

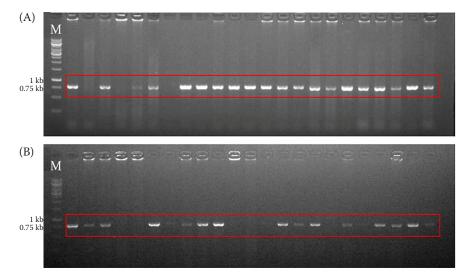


Figure 7. The validation of the SCAR makers: marker E301-700 in the hybrid progeny of QZ36 and QZ203 (A) and marker B0BB-10470 in the hybrid progeny of CN16 and QZ36 (B) the partial lines are shown here

The bands in the red box represent the lines having the same genotype with Q1028 and no bands represent those having the same genotype with ZM9023

Table 3. Validation of the developed markers

Marker	Population	Genotype	Mean (g/1000 grain)	SD	<i>P</i> -value
	CN16/QZ36 F <sub>3</sub>	a	37.41	3.08	0.006
		b	49.34	0.91	
0.600 411	QZ36/QZ203 F <sub>3</sub>	a	35.23	0.42	0.000
0C98-411		b	46.48	1.00	
	QZ82/QZ270 F <sub>3</sub>	a	38.68	0.10	0.002
		b	48.91	2.29	
	CN16/QZ36 F <sub>3</sub>	a	43.46	4.74	0.105
		b	49.34	1.12	
E201 E00	QZ36/QZ203 F <sub>3</sub>	a	32.34	4.92	0.016
E301-700		b	44.03	0.89	
	QZ82/QZ270 F <sub>3</sub>	a	38.58	0.13	0.082
		b	42.86	3.2	
	CN16/QZ36 F <sub>3</sub>	a	36.69	0.95	0.034
		b	48.66	2.73	
DODD 10470	QZ36/QZ203 F <sub>3</sub>	a	33.98	6.17	0.013
B0BB-10470		b	44.43	2.07	
	QZ82/QZ270 F <sub>3</sub>	a	37.19	5.95	0.004
		b	47.88	2.72	

a – the genotypes with the homozygous alleles from non-ZM9023; b – the genotypes with the homozygous alleles from ZM9023; SD – the standard deviation

However, in this study, ZM9023, a commercial cultivar, performed well with both a high yield and high quality with the characteristics of a strong gluten, compact plant type, and early maturity. We developed the genetically linked markers flanking TWG QTL, QTgw.sau-2D, the hereditary effect of which was from ZM9023. QTgw.sau-2D may have a synergistic positive effect on both the yield and quality traits. This suggests that it is theoretically feasible for pyramiding genes controlling both the yield and quality traits being aimed at improving wheat yield and quality.

Here, we developed markers linked to the major TGW QTL, *QTgw.sau-2D*. These markers are helpful for developing the near-isogenic lines for further fine mapping work. It is suggested that QTL mapping has a limited resolution ability (Paterson et al. 1988) and the molecular markers developed from such a method may not be adequate to trace the phenotype for a given trait. Thus, it is essential to develop tightly linked or diagnostic markers by further fine mapping and even cloning work (Ma et al. 2019).

In conclusion, we have successfully developed one HRM marker (0C98-411) and two SCAR markers (E301-700 and B0BB-10470), and integrated them

into the genetic map. Among them, *0C98-411* was the most closely linked marker that could follow *QTgw.sau-2D*. This marker provides a reference for the molecularly assisted breeding.

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