RESEARCH ARTICLE

Identification of a Novel Allele of TaCKX6a02 Associated with Grain Size, Filling Rate and Weight of Common Wheat

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Abstract

Cytokinin oxidase (CKX) plays a crucial role in plant growth and development by reversibly inactivating cytokinin (CTK). Twenty-four primer pairs, designed from ESTs of the TaCKX gene family of common wheat, were used to identify their allelic variations associated with grain size, weight, and filling rate in 169 recombinant inbred lines (RIL) derived from Jing411 × Hongmangchun 21. TaCKX6a02, a member of TaCKX gene family, amplified by primer pair T31–32, showed a close association with grain traits in this RIL population. Statistical analysis indicated that allelic variation of TaCKX6a02 had significant correlation with grain size, weight, and filling rate (GFR; \(P < 0.001\)) under varied environments. The TaCKX6a02-D1a allele from Jing411 significantly increased grain size, weight and grain filling rate, compared with TaCKX6a02-D1b from Hongmangchun 21. TaCKX6a02 was located on chromosome 3DS in the interval of Xbarc1119 and Xbarc1162, with a genetic distance of 1.4 cM. The location was further confirmed using Chinese Spring nulli–tetrasomic lines. A major QTL (quantitative trait locus) tightly linked to TaCKX6a02 was detected in the RIL population, explaining 17.1~38.2% of phenotype variations for grain size, weight, GFRmax and GFRmean in different environments. In addition, significant effects of variations of TaCKX6a02 on grain weight and GFR were further validated by association analysis among 102 wheat varieties in two cropping seasons. 12.8~35.1% of phenotypic variations were estimated for these genotypes. A novel 29-bp InDel behind the stop codon was detected by DNA sequence analysis between the two alleles of TaCKX6a02-D1. The gene-specific marker, TKX3D, was designed according to the novel variation, and can be used in marker-assisted selection (MAS) for grain size, weight, and GFR in common wheat.
Introduction
As one of the key phytohormones synthesized in the root, cytokinin (CTK) regulates many important plant processes by controlling cell division and tissue differentiation [1–9]. Many researches indicate that CTK plays an important regulatory role in crop yield and related traits. Firstly, cytokinin is one key hormone in controlling grain size and weight by regulating endosperm cell numbers of crops [10–14]. Secondly, CTK also can enhance grain weight by regulating grain filling patterns of crops [15–18]. The endogenous CTK (Z+ZR) content of grain at the early grain filling stage has a close relationship with thousand-grain-weight (TGW) via regulation of starch synthase (SSase) and starch branching enzyme (Q-enzyme) activities in rice [19]. CTK also can maintain chloroplast stability of flag leaf after anthesis, significantly improving grain filling and weight [20, 21]. For other yield-related traits, such as effective tiller emergence, spikelet and floret aspects, and fertile floret and kernel numbers, CTK is generally regarded as a positive regulator [22–25].

The phytohormone, CTK, is under strict control mainly regulated by cytokinin oxidase (CKX). The CKX is considered to be only known phytohormone enzyme that can inactivate CTK irreversibly by cleaving its N6-side chain in a single enzymatic step [26]. Hence, cytokinin oxidase (CKX) genes have been proven to have a close relationship with crop yield through regulation of endogenous CTK. Ashikari et al. isolated the rice CKX gene (OsCKX2) and confirmed that reduced expression caused CTK accumulation in inflorescence meristems and increased the number of reproductive organs, resulting in enhanced grain yield [27]. As graminaceous crops, wheat, rice, barley (Hordeum vulgare L.), maize (Zea mays), and Sorghum spp. show large similarity and co-linearity between their genomes; therefore, wheat CKX genes are also involved in the regulation of grain yield. Zalewski et al. confirmed that seed numbers per plant and seed weight are improved by silencing the HvCKX1 gene in barley and the TaCKX1 gene in wheat [28]. Two putative CKX genes of wheat (TaCKX2.1 and TaCKX2.2) have been cloned and are presumed to relate to grain number per spike [29]. TaCKX6 is confirmed to have a significant association with the grain weight of common wheat [30]. Based on sequences analysis of ESTs available from the NCBI (www.ncbi.nlm.nih.gov), wheat CKX genes are proved to belong to a large gene-family containing at least 13 members [31]. So far, seven wheat CKX genes have been isolated: TaCKX1 on chromosome 3A [32], TaCKX2 on 7A or 7B [33], and TaCKX2.1, TaCKX2.2, TaCKX3 [34], TaCKX5 [35], and TaCKX6 [30] on 3DS. However, little research was carried out to investigate the effects of allelic variations of TaCKX genes on grain filling, size, and grain weight of wheat simultaneously. The objectives of this study are to: (1) identify allelic variations of CKX genes for grain filling, grain size, and grain weight based on all TaCKX ESTs, mRNA, and DNA sequences available in the NCBI; and (2) develop a gene-specific marker for TaCKX6a02 and validate its association with grain traits.

Materials and Methods

Plant Materials
One hundred and sixty-nine recombinant inbred lines (RILs) were developed from a cross between Jing 411 and Hongmangchun 21 by single-seed descent (F2:8 generation). Jing 411 is a high-yield winter wheat variety with a larger grain size; its TGW averaged 47.6 g over five cropping seasons (2006–2007, 2007–2008, 2008–2009, 2009–2010 and 2010–2011). Hongmangchun 21 is a Chinese low-yield landrace, with an average TGW of 19.7 g across the five cropping seasons (Table 1). The two parents show large differences in yield-related traits, including grain size, grain weight, and grain filling (Table 1). One hundred and two wheat
varieties from different wheat production regions of China showing large variation in grain filling, size, and weight were used for validation of the gene marker (Table 1).

Field trials
The RILs and their parents were grown in randomized complete blocks, with two replicates at the Changping Experimental Station of Chinese Academy of Agricultural Sciences (Beijing: 39°54’N, 116°28’E) in the 2006~2007 and 2007~2008 cropping seasons, and the Experimental Station of Anhui Agricultural University (Hefei; 31°58’N, 117°24’E) during the 2008~2009, 2009~2010 and 2010~2011 cropping seasons. The 102 wheat varieties were also grown in randomized complete blocks with two replicates during the 2011~2012 and 2012~2013 cropping seasons at the Experimental Station of Anhui Agricultural University.

Each plot contained three 2.0-m rows spaced 25 cm apart, with 40 plants in each row. Field management followed common practices for wheat production, and the flowering time was recorded for each line.

Recording of data for grain size, weight, and grain filling rate
Grain size and weight were determined in all seasons and locations. TGW was evaluated by weighing two samples of 1000 kernels from each genotype. Grain size, including length and width, was recorded after harvesting using the method described by Sun et al. [36], with minor modifications. Two sets of 50 grains, from each variety or RIL, were lined up length-wise along a ruler with a precision of 0.1 mm, and the average length of the samples defined as grain length (GL). The average width of the two sets of grains laid breadth-wise was defined as grain width (GW). The thickness (GT) of two sets of 20 grains was measured using vernier calipers (Kraftwelle, China) with a precision of 0.01 mm, using the method described by Dholakia et al. [37].

GFR\text{max} and GFR\text{mean} were determined for the RILs during the 2009~2010 and 2010~2011 cropping seasons, and 102 wheat varieties during the 2011~2012 and 2012~2013 cropping seasons at the Experimental Station of Anhui Agricultural University respectively, following the method described by Wang et al. (2008) [38]. Seven tagged spikes from each line were sampled at 5-day intervals from anthesis to maturity. The grains were then separated from the glumes, kept at 105°C for 10 min, and then at 70°C until reaching a constant weight. At this stage, the total number of grains was counted, and the weight recorded. Grain filling rates were calculated as described by Wang et al. [38].

Table 1. Grain traits of the two parents, RIL and natural population.

| Traits  | Parents          | RIL population (n = 169) | Natural population (n = 102) |
|---------|------------------|--------------------------|-----------------------------|
|         | Jing411          | Hongmangchun21          | Mean | C.V. % | Mean | C.V. % |
| GL (mm) | 6.9              | 5.11                     | 6.30 (4.97–7.12) | 14.15 | 6.33 (5.01–7.11) | 14.26 |
| GW (mm) | 3.82             | 2.4                      | 3.05 (2.33–3.94) | 12.86 | 3.32 (2.64–3.83) | 10.26 |
| GT (mm) | 3.81             | 2.41                     | 2.97 (2.39–3.88) | 11.13 | 3.17 (2.56–3.78) | 10.01 |
| TGW(g)  | 47.6             | 19.7                     | 36.15 (18.6–53.2) | 28.12 | 44.2 (19.7–61.2) | 26.38 |
| GFR\text{max} | 3.45             | 1.88                     | 2.49 (1.01–3.56) | 31.04 | 3.08 (2.13–3.51) | 15.79 |
| GFR\text{mean} | 2.21             | 1.04                     | 1.72 (0.97–2.37) | 22.11 | 2.03 (1.12–2.33) | 20.85 |

The means performance of grain traits were measured in five cropping seasons (RIL population), and two cropping seasons (natural population), respectively. The data in parentheses mean the range of grain traits values. GL, grain length; GW, grain width; GT, grain thickness; TGW, 1000-grain-weight; GFR\text{max} and GFR\text{mean}, maximum and mean grain filling rate, respectively; RIL, recombinant inbred line.

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Genomic DNA extraction and PCR amplification

Genomic DNA was extracted from two kernels per line in accordance with Kang et al. [39]. PCR reactions were conducted using a TC412 Thermocycler (Barloworld Scientific, UK). Twenty-four primer pairs (S1 Table) were designed to characterize allelic variations of TaCKX genes in wheat using DNAMAN (Version 6.0) software (Lynnon Biosoft, San Ramon, CA, USA) based on EST and mRNA sequences available from the NCBI.

The PCR profile was as follows: denaturation at 94°C for 5 min; followed by 40 cycles of denaturation at 95°C for 1 min, annealing at 50–60°C for 1 min 20 s, and extension at 72°C for 2 min; with a final extension for 8 min at 72°C. The annealing temperature was modulated based on the primer pair. Each 15-μl PCR reaction contained 40 ng of genomic DNA, 10 pmol of each primer, 200 mM dNTPs, 1×PCR buffer, and 1 U Taq DNA polymerase (Shanghai Sangon Biological Engineering Technology & Services, Shanghai, China). PCR products were separated by 7% PAGE containing 4 M Urea.

Sequencing of PCR fragments and statistical analysis

Purification and sequencing of PCR products, from two independent samples per wheat genotype was performed by Shanghai Sangon Biological Engineering Technology & Services. Pfu Taq DNA polymerase (Shanghai Sangon Biological Engineering Technology & Services, Shanghai, China) with high fidelity was used to avoid false priming; all TaCKX alleles were sequenced from both strands. Sequence alignment and characterization were completed using DNAMAN software. Data analysis of grain traits was conducted using SPSS software version 13.0 (IBM Software Group, Armonk, NY, USA).

Chromosomes assignment of TaCKX gene

A set of Chinese Spring nulli–tetrasomic lines was used to determine the location of TaCKX gene. PCR amplification and separation of products was as described above.

SSR marker screening and QTL analysis for grain weight and grain filling

Simple sequence repeat (SSR) markers, including the BARC, GWM, WMC, CFA, and CFD series were used to screen the two parents, and two bulks containing five high-grain-weight lines and five low-grain-weight lines, respectively [40]. Candidate polymorphic markers between the bulks were analyzed further in a subset of 40 RILs, including 20 lines with high-grain-weight and 20 with low-grain-weight, to confirm the polymorphism. The polymorphic markers were then used for genotyping the entire RIL population. The linkage map was constructed using Map Manager QTxb20 Version 3.0 [41]. Recombination fractions were converted into centimorgans (cM) using the Kosambi function [42]. Composite interval mapping (CIM) was performed for QTL analysis using Windows Cartographer 2.5 software (http://statgen.ncsu.edu/qtlcart/WQTLCart.htm) in accordance with the methods described by Zeng [43]. A QTL was declared when the LOD score was > 2.5 in at least two cropping seasons, and calculated from 2,000 permutations at a probability of 0.01.

Analysis of correlation between TaCKX alleles and grain traits

When analyzing the correlation between TaCKX alleles and grain traits in the RILs population and wheat varieties, the Jing 411 allele was scored as “1” and the Hongmangchun 21 allele as “0”. Spearman rank correlation analysis and t-test were performed to test the association significance between allelic variation and grain traits, as described in previous studies [44–46]. The effect of TaCKX variation on the variability of grain traits was estimated by R² based on the
General Linear Model [44–46]. The significance of the effect was evaluated using the GLM at 0.05, 0.01, and 0.001 levels of probability. Grain trait data analyses were conducted using SPSS software version 13.0.

Results

Correlation analysis between different grain traits

In this study, the grain traits of RILs such as GL, GW, GT, TGW, GFRmean, and GFRmax showed wide variations (Table 1). Grain size (GL, GW, and GT) had a significant correlation with grain weight ($P < 0.001$); and grain filling also showed a significant correlation with grain weight ($P < 0.001$; S2 Table). These results suggested that grain weight is not only determined by grain size but also by GFR. Grain weight showed closer relationship with grain size than grain filling.

Identification of allelic variations in *TaCKX* associated with grain traits

Of the 24 primer pairs developed from wheat *CKX* genes (Galuszka et al. 2004)[31], six pairs including T3–4 (TaCKX1), T5–6 (TaCKX2a), T13–14 (TaCKX3), T19–20 (TaCKX4), T25–26 (TaCKX5b), and T31–32 (TaCKX6a02) showed good polymorphism between the two parents. To identify *TaCKX* genes associated with grain traits, spearman rank correlation analysis and t-test were performed based on the mean performance of grain traits of RILs in the five environments. The results indicated that the allelic variation of *TaCKX6a02* had a significant correlation with grain size, grain weight, and GFR in the RIL population ($P < 0.001$; Table 2). The alleles of the A- and B-type patterns shown in Fig 1 were named TaCKX6a02-D1a and TaCKX6a02-D1b, respectively. The marker used for identifying the allelic variation of *TaCKX6a02-D1* was designated as CKX3D. RILs carrying TaCKX6a02-D1a had significantly higher values for grain size, weight and grain filling than TaCKX6a02-D1b (Table 2), indicating that the TaCKX6a02-D1a allele could improve grain traits.

Chromosome location of *TaCKX6a02*

Previous studies indicated that wheat *CKX* genes belong to a large gene-family, distributed on several chromosomes, including 3A, 3B, 3D, 7A, and 7B; hence, it was necessary to locate

| Population | Alleles/Traits | GL/mm | GW/mm | GT/mm | TGW/g | GFRmax | GFRmean |
|------------|----------------|-------|-------|-------|-------|--------|---------|
| RIL        | TaCKX6a02-D1a  | 6.89  | 3.34  | 3.20  | 41.91 | 2.94   | 2.04    |
|            | TaCKX6a02-D1b  | 5.59  | 2.70  | 2.69  | 29.26 | 1.96   | 1.33    |
| t-testa    | 11.403***      | 9.668*** | 8.15*** | 14.45*** | 8.696*** | 7.863*** |
| Correlationb | 0.585***     | 0.517*** | 0.414*** | 0.609*** | 0.471*** | 0.413*** |
| Natural    | TaCKX6a02-D1a  | 6.55  | 3.22  | 3.23  | 41.6  | 3.27   | 2.19    |
|            | TaCKX6a02-D1b  | 5.45  | 2.78  | 2.61  | 27.3  | 2.16   | 1.77    |
| t-testa    | 12.052***      | 8.464*** | 6.999*** | 8.304*** | 9.423*** | 6.712*** |
| Correlationb | 0.608***     | 0.512*** | 0.383*** | 0.556*** | 0.489*** | 0.380**  |
| Effectc    | 35.1%          | 26.2%  | 13.1%  | 31.6%  | 22.7%  | 12.8%  |

*a* t-test of the averages of grain traits between the two alleles of TaCKX6a02.

*b* Correlation between allelic variation of TaCKX6a02 and grain traits among 61 wheat varieties.

*c* Effect of allelic variation on the variability of grain traits, as estimated by $R^2$ based on the General Linear Model.

** indicates significant differences at $P < 0.01$.

*** indicates significant differences at $P < 0.001$.

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TaCKX6a02 before performing genetic linkage analysis in the RIL population. In this study, the chromosome location of TaCKX6a02 was identified using Chinese Spring nulli–tetrosomics. The PCR products were amplified in N3AT3B, N3AT3D, N3BT3A, and N3BT3D, but were not present in N3DT3A or N3DT3B (S1 Fig), indicating that the TaCKX6a02 gene is located on chromosome 3D.

**Linkage analysis of the TaCKX6a02 gene and functional marker development**

Seventy-seven SSR markers on chromosome 3D were selected based on their chromosomal location. To analyze the genetic linkage of TaCKX6a02 on 3D, and to further evaluate its effect on the phenotypic variation of grain traits, the SSR markers were screened using their two parents and two bulks. Fourteen SSR markers and the gene-marker CKX3D showed good polymorphism in the mapping population. Genetic map analysis indicated that the 14 SSR markers were assigned in the same linkage group as the CKX3D region, spanning a genetic distance of 26 cM (Fig 2).

Through the analysis of CIM, a QTL controlling grain traits was identified in the marker interval of Xbarc1119 and Xbarc1162 on chromosome 3DS, with a genetic distance of 1.4 cM (Fig 2). The QTL co-segregated with TaCKX6a02, with LOD values ranging from 9.1 to 21.1 over five environments. The six grain traits, GL, GW, GT, TGW, GFRmax, and GFRmean were all associated with the same QTL closely linked to TaCKX6a02, Xbarc1119, and Xbarc1162.

The QTL mapped at CKX3D locus was detected across all five cropping seasons, and could explain 17.4~38.2% of phenotypic variations for grain size and grain weight (Table 3). Regarding grain filling traits, the locus also accounted for 17.1% and 22.2% of phenotypic variations of GFRmax and GFRmean, respectively, in the two environments (2009~2010 and 2010~2011 cropping seasons). Therefore, TaCKX6a02 could be considered as a candidate gene for grain filling, grain size, and grain weight in this population.

**Validating effects of TaCKX6a02 variations on grain traits among 102 wheat varieties**

To further confirm effects of TaCKX6a02 on grain traits, association analysis of allelic variations with grain traits were conducted among 102 wheat genotypes. In this natural population, 68.5% of varieties had the TaCKX6a02-D1a allele, while the remainder had TaCKX6a02-D1b (S3 table).
A wide variation of grain traits was observed among these wheat varieties (Table 1). The mean values of the six grain traits all showed significant differences ($P < 0.001$) between TaCKX6a02-D1a and TaCKX6a02-D1b (Table 2). The varieties with TaCKX6a02-D1a allele had significantly higher value of grain traits than those with TaCKX6a02-D1b allele. The association analysis showed that the allelic variation of TaCKX6a02 had a significant correlation with Fig 2. Linkage map of TaCKX6a02 on chromosome 3DS of wheat. The six shaded boxes represent the six traits of TGW, GW, GL, GT, GFRmax, and GFRmean, respectively.

Table 3. Summary of QTLs for grain traits in the RILs from the Jing 411/Hongmangchun 21 cross, across different environments.

| Trait | QTL | Marker interval | LOD | $R^2$ (%) | Add. (%) | Environments observed/total |
|-------|-----|----------------|-----|-----------|----------|-----------------------------|
| GL    | QGL.ahau-3D | T31-32-Xbarc1162 | 20.4 | 34.2 | -0.6 | 5/5 |
| GW    | QGW.ahau-3D | T31-32-Xbarc1162 | 17.6 | 26.7 | -0.76 | 5/5 |
| GT    | QGT.ahau-3D | T31-32-Xbarc1162 | 9.8 | 17.4 | -11.2 | 5/5 |
| TGW   | QTGW.ahau-3D | T31-32-Xbarc1162 | 21.1 | 38.2 | -1.18 | 5/5 |
| GFRmax| QGma.ahau-3D | T31-32-Xbarc1162 | 11.9 | 22.2 | -0.49 | 2/2 |
| GFRmean| QGme.ahau-3D | T31-32-Xbarc1162 | 9.1 | 17.1 | -0.27 | 2/2 |

*All values are calculated from the overall mean dataset*
grain traits in natural population of wheat \(P < 0.01\) or \(0.001; \text{Table 2; S2 Fig}\). Phenotypic variations of grain traits explained by \(\text{TaCKX6a02}\) ranged from 12.8–35.1\% across the two cropping seasons (2011–2012, 2012–2013). Through analysis in different genetic background, RILs and natural population, \(\text{TaCKX6a02}\) could be validated to have a significant effect on grain traits in common wheat.

Sequence analysis of \(\text{TaCKX6a02}\)

A 29-bp insertion in the 3'-untranslation region (3'-UTR) behind the TGA stop codon was detected in the Jing 411 allele of \(\text{TaCKX6a02-D1a}\), compared with the Hongmangchun 21 allele \(\text{TaCKX6a02-D1b}\) (Fig 3). The sequence of \(\text{TaCKX6a02-D1a}\) was the same as the EST BQ235927 deposited at the NCBI (Galuszka et al. 2004) [31]. Other CKX genes on chromosome 3D, including \(\text{TaCKX1}\) (FJ648070.1), \(\text{TaCKX1.1}\) (GU084177.1), \(\text{TaCKX2.1}\) (JF648070.1), \(\text{TaCKX2.2}\) (JN128584), and \(\text{TaCKX6-D1b}\) (JQ797673) were also obtained by performing a BLAST search on the NCBI GenBank. Sequences between \(\text{TaCKX6a02}\) and the above \(\text{TaCKX}\) genes were analyzed using DNAMAN software. The results revealed \(\text{TaCKX6a02}\) shared a high identity with them in the open reading frame region (ORF), as shown in Fig 3. Through alignment analysis among them, \(\text{TaCKX6a02}\) had higher homology with EST BQ235927 (99%), \(\text{TaCKX2.1}\) (99%) and \(\text{TaCKX2.2}\) (99%), than with \(\text{TaCKX1}\) (92%), \(\text{TaCKX1.1}\) (92%) and \(\text{TaCKX6-D1b}\) (92%) (S3 Fig). The \(\text{TaCKX}\)s on chromosome 3D could thus be divided into two groups according to their homology.

Discussion

Homology analysis of CKX genes on chromosome 3D of wheat

Six CKX genes on chromosome 3D of wheat were identified following a BLAST search in NCBI using the \(\text{TaCKX6a02}\) sequence, including an EST BQ235927, \(\text{TaCKX2.1}\), \(\text{TaCKX2.2}\), \(\text{TaCKX2.3.1}\), \(\text{TaCKX2.3.2}\), and \(\text{TaCKX6-D1b}\). Alignment analysis revealed that \(\text{TaCKX6a02}\), \(\text{TaCKX2.3.1}\) and \(\text{TaCKX2.3.2}\) are possibly the same CKX gene because of very high identity in sequence between them. However, the function of \(\text{TaCKX2.3.1}\) and \(\text{TaCKX2.3.2}\) had remained unknown until now. \(\text{TaCKX2.1}\), \(\text{TaCKX2.2}\) and \(\text{TaCKX6-D1b}\) also shared higher sequence homology among them, but had a lower identity with \(\text{TaCKX6a02}\). For example, \(\text{TaCKX6a02}\) had a large difference in sequence at the 3'-UTR, compared with \(\text{TaCKX2.1}\) and \(\text{TaCKX2.2}\). The main variation in \(\text{TaCKX6a02}\), 29-bp InDel in the 3'-UTR, was also different from \(\text{TaCKX6-D1b}\) containing 18-bp InDel in intron 2 and 20-bp InDel in the third exon [30]. According to these analyses, these \(\text{TaCKX}\) genes were clearly divided into two groups.

Multiple CKX genes have been observed on chromosome 3DS of wheat; however, their functions varied. \(\text{TaCKX2.1}\) and \(\text{TaCKX2.2}\) are involved in the regulation of grain number per spike in wheat [29], similar to \(\text{OsCKX2}\) in rice [27]. On the other hand, \(\text{TaCKX6}\) [30] and \(\text{TaCKX6a02}\) are associated with grain weight. Interestingly, these CKX genes have a relatively high sequence identity and all located on chromosome 3DS. Furthermore, \(\text{TaCKX6}\) and \(\text{TaCKX6a02}\) share a similar linkage interval on the genetic map. Based on sequence identity and linkage analysis, \(\text{TaCKX6}\) and \(\text{TaCKX6a02}\) may be different alleles of the CKX gene. Therefore, further study is to clone full length of \(\text{TaCKX6a02}\) and compare its sequence with \(\text{TaCKX6}\).

Allelic variation of \(\text{TaCKX6a02}\)

Gene mutations occurring in coding regions often affect function as a result of amino acid changes. However, the 5'-UTR [47, 48], 3'-UTR [49–52], and intron [30, 53] also have important
Fig 3. Sequence alignment between TaCKX6a02 and TaCKX2.1, TaCKX2.2, TaCKX2.3.1, TaCKX2.3.2, and TaCKX6-D1b. Two alleles sequences of TaCKX6a02 (TaCKX6a02-D1a and TaCKX6a02-D1b) were obtained from the reverse-complement sequences of PCR fragments of Jing 411 and Hongmangchun 21. The primer pair and stop codon are marked with arrows in the figure. The InDel sequence is boxed.

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influences on gene function through regulating mRNA structure, stability, and accumulation during translation. Previous research has confirmed that an 18-bp InDel in the second intron of TaCKX6-D1b had a significant influence on the gene expression at eight days after pollination in common wheat [30]. In this study, a novel 29-bp InDel was found in the 3'-UTR of TaCKX6a02. Whether variation of TaCKX6a02 leads to enhancement of grain weight by directly increasing endogenous cytokinin levels, and what is the synergistic relationship between the two markers (C19L3/C19L4 and TKX3D) and grain weight are worthy of further study.

**TaCKX6a02 locus for grain traits**

As a complex trait, grain weight is controlled by multiple genes or loci widely distributing on the most of wheat chromosomes. On chromosome 3D, several QTLs for grain weight and related traits have been detected using varied populations, explaining 6~23% of phenotype variations in different environments [38, 54–58]. In this study, the TaCKX6a02 locus for grain traits was closely linked to Xgwm341, Xwmc533, Xbarc1119, Xbarc1162, and Xcfd62 on chromosome 3DS; the linkage intervals were similar to the previous reports [55, 56]. However, these loci explained relatively less phenotypic variation of grain traits, compared with the TaCKX6a02 locus (17.1~38.2%). Zhang et al. [30] also reported that TaCKX6 was located on chromosome 3DS and linked to Xcfd70 and Xwmc533. This is very similar to TaCKX6a02. These results revealed that varied QTLs or loci for grain weight occurred on chromosome 3D of wheat.

Identification of genes for grain traits in wheat is receiving increased attention of researchers. At present, some genes for grain size and weight are known, including TaGW2 [59–61], TaCKX6 [30], TaGS-D1 [62], 6-SFT-A2 [63]. In our study, TaCKX6a02 is also confirmed to tightly relate to grain size and weight by QTL and association analysis. Interestingly, the gene also has significant effect on grain filling rate of wheat simultaneously. The result suggests that TaCKX6a02 is likely involved both in regulating grain formation and the filling process of wheat. The influence of TaCKX6a02 on grain weight was probably due to its regulation on both grain size and filling. Therefore, findings of variations in TaCKX6a02 will help further understanding of complex mechanisms of grain weight. In addition, the TaCKX6a02-D1a is a predominant allele among modern varieties, and TaCKX6a02-D1b allele mainly distributes in local varieties or landraces of China in our study. These results show a strong selection of TaCKX6a02-D1a in breeding programs. Furthermore, the locus co-segregating with TaCKX6a02 shows good stability and reliability in varied environments and genetic backgrounds, and thus will help improve the accuracy and effectiveness of marker assisted selection for grain traits in wheat breeding.

**Conclusions**

TaCKXs belong to a big gene family, including at least 13 members in common wheat. Several TaCKX genes have been validated to relate to grain yield. In this research, TaCKX6a02 on chromosome 3DS was also identified to closely associate with grain size, filling and weight in a RIL population and natural population respectively. By sequence analysis, a novel allelic variation of TaCKX6a02 was observed, which contained a 29-bp InDel behind the stop codon. A gene-specific marker, TKX3D, was developed based on the InDel variation. The TKX3D locus could explain 12.8–38.2% of phenotypic variations in different environments.

**Supporting Information**

S1 Table. Primer pairs used in this study. (DOC)
S2 Table. Correlation between the grain traits of RILs.

S3 Table. The information of grain weight, allele of TaCKX6a02 and distributed region of 102 wheat varieties.

S1 Fig. Amplification of the TaCKX6a02 gene using the primer pair T31–32 in Chinese Spring nulli-tetrasomics.

S2 Fig. Grains of 12 wheat varieties with large difference in grain size and weight.

S3 Fig. Homology analysis of TaCKX genes.

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Author Contributions

Conceived and designed the experiments: CC JL CXM. Performed the experiments: JL CC HPZ SXW. Analyzed the data: CC JL HPZ. Contributed reagents/materials/analysis tools: HPZ CXM. Wrote the paper: CC JL GLS SHX.

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