Isolation and characterization of the TaSnRK2.10 gene and its association with agronomic traits in wheat (Triticum aestivum L.)

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Abstract

Sucrose non-fermenting 1-related protein kinases (SnRKs) comprise a major family of signaling genes in plants and are associated with metabolic regulation, nutrient utilization and stress responses. This gene family has been proposed to be involved in sucrose signaling. In the present study, we cloned three copies of the TaSnRK2.10 gene from bread wheat on chromosomes 4A, 4B and 4D. The coding sequence (CDS) is 1086 bp in length and encodes a protein of 361 amino acids that exhibits functional domains shared with SnRK2s. Based on the haplotypes of TaSnRK2.10-4A (Hap-4A-H and Hap-4A-L), a cleaved amplified polymorphic sequence (CAPS) marker designated TaSnRK2.10-4A-CAPS was developed and mapped between the markers D-1092101 and D-100014232 using a set of recombinant inbred lines (RILs). The TaSnRK2.10-4B alleles (Hap-4B-G and Hap-4B-A) were transformed into allele-specific PCR (AS-PCR) markers TaSnRK2.10-4B-AS1 and TaSnRK2.10-4B-AS2, which were located between the markers D-1281577 and S-1862758. No diversity was found for TaSnRK2.10-4D. An association analysis using a natural population consisting of 128 winter wheat varieties in multiple environments showed that the thousand grain weight (TGW) and spike length (SL) of Hap-4A-H were significantly higher than those of Hap-4A-L, but pant height (PH) was significantly lower.

Introduction

Wheat (Triticum aestivum L.) is one of the most important food crops worldwide, and obtaining higher yields is one of the primary objectives for wheat improvement. A large number of quantitative trait loci (QTLs) have been reported to control grain yield and yield components [1–7]. Recently, several yield-related genes have been cloned and transformed into functional markers (FMs), such as TaGW2 [8], TaSus2 [9], TaCwi-A1 [10], and TaGS1a [11] etc. The FMs derived from polymorphic sites in genes are important for marker-assisted selection (MAS) in breeding programs [12].

Sucrose non-fermenting 1-related protein kinases (SnRKs) form a major family of signaling proteins in plants and include three gene subfamilies, SnRK1, SnRK2 and SnRK3 [13]. SnRK1
genes play an important role in the regulation of carbon metabolism and energy status [14–15], and SnRK3 genes encode CBL-interacting protein kinases, which specifically interact with calcineurin B-like proteins (CBLs) [16]. The SnRK2 genes represent a group of plant-specific protein kinases that have been shown to be involved in abiotic stress signal transduction, nutrient utilization and growth in plants [17]. Ten members of the SnRK2 gene family have been identified [15].

In wheat, SnRK2s are involved in the response to abiotic stress and have potential functions in carbohydrate and energy metabolism [18]. PKABA1 was the first gene of the SnRK2 family cloned in wheat and is induced by abscisic acid (ABA) and hyperosmotic stress [19–20]. Overexpression of TaSnRK2.4 in Arabidopsis resulted in increased tolerance to osmotic stress, delayed seedling establishment, longer primary roots, and higher yields under both normal and stress conditions [21]. Functional analysis showed that TaSnRK2.7 is involved in carbohydrate metabolism as well as decreasing osmotic potential, enhancing photosystem II activity, and promoting root growth [18]. TaSnRK2.8 may participate in ABA-dependent signal transduction pathways, and overexpression of this gene results in enhanced tolerance to abiotic stress. Additionally, TaSnRK2.8 transgenic plants show significantly lower levels of total soluble sugar under normal growing conditions, which suggests that this gene might be involved in carbohydrate metabolism [22]. Two other members of the SnRK2s found in wheat, TaSnRK2.3 and W55a, also play important roles in the response to abiotic stress and plant growth [7, 23].

The objectives of this study were to isolate the full-length cDNA and gDNA sequences of TaSnRK2.10 in wheat, to develop and map the functional markers, and to conduct an association analysis between TaSnRK2.10 haplotypes and agronomic traits using a natural population of 128 varieties.

Materials and methods

Plant materials

Plant materials in this study came from four groups: 1) ten winter wheat varieties, including Chinese Spring, Jinan 17, Jining 17, Lumai 21, Lumai 23, Shannong 0431, Shannong 8355, Weimai 8, Xiaoyan 81, and Yannong 15, were used for the isolation of TaSnRK2.10 DNA sequences and for haplotype analysis. This material was highly polymorphic and was selected from each subgroup of the 128 natural populations of varieties (NPVs) analysed using 91 SSR and 47 functional markers; 2) a set of Chinese Spring nullisomic-tetrasomic lines (CS-N/Ts) was used for determining the special chromosomes of TaSnRK2.10; 3) a set of 179 recombinant inbred lines (RILs) derived from 'Shannong 0431 × Lumai 21’ was employed for linkage analysis. Shannong 0431 is a germplasm developed by our group with a large grain size and multi-disease resistance (wheat stripe rust, leaf rust, powdery mildew and sharp eyespot), Lumai 21 is a cultivar released by the Yantai Academy of Agricultural Science of China in 1996 and has a high yield and high drought resistance; and 4) a natural population of varieties (NPVs) was employed to validate the functional markers and analyze the relationships between TaSnRK2.10 haplotypes and agronomic traits. The population consisted of 128 winter wheat varieties released in the Huang-huai Winter Wheat Region and the Northern Winter Wheat Region of China.

DNA and RNA extraction and first-strand reverse transcription of cDNA

After sterilization for 5 min in a 10% solution of H₂O₂ and washing three times with sterilized water, wheat seeds were germinated and cultured in a growth chamber (20±1˚C with 12 h light, 12 h dark cycle). Ten days later, wheat leaves were sampled for the isolation of gDNA and total RNA. The gDNA was extracted from lyophilized mixed leaves using the CTAB method [24]. The RNA was extracted using TRIzol reagent (Invitrogen Co., Ltd., Shanghai,
China), and the first-strand synthesis was performed using M-MLV transcriptase (Invitrogen Co., Ltd., Shanghai, China) according to the manufacturer's instructions.

Cloning, sequence analysis and development of genome-specific primers

To obtain the sequence of TaSnRK2.10, the cDNA sequence of SAPK10 from rice (GenBank ID: AB125311) was used as a query sequence to screen the GenBank wheat EST database. All candidate ESTs showing high similarity to SAPK10 cDNA were obtained through BLASTN searches (http://www.ncbi.nlm.nih.gov) and assembled into a putative TaSnRK2.10 cDNA sequence using the CAP3 Sequence Assembly Program (http://doua.prabi.fr/software/cap3). The functional region and activity sites were identified with PROSITE (http://prosite.expasy.org/). The primer pairs for TaSnRK2.10-1F/R and TaSnRK2.10-2F/R (Table 1) were designed based on the putative sequence using Primer Premier Version 5.0 software (http://www.premierbiosoft.com/) and were used for isolating the cDNA and gDNA sequences of TaSnRK2.10. The genome-specific primer pairs for TaSnRK2.10-3-4AF/R, TaSnRK2.10-3-4BF/R and TaSnRK2.10-3-4DF/R (Table 1) were designed based on DNA sequence variations among the genomic sequences to identify homoeologs as well as specific alleles at individual loci.

PCR assays were performed using LA Taq polymerase (TaKaRa Biotechnology Co., Ltd., Dalian, China) in a 20 μL reaction mixture containing 80 ng of gDNA or cDNA, 5 pM of TaSnRK2.10-1F/R or TaSnRK2.10-2F/R, 200 μM of each dNTP, 1 unit of LA Taq and 2 μL of 10× PCR buffer. A touchdown PCR procedure was employed as follows: initial denaturation at 95˚C for 5 min, followed by 10 amplification cycles of 35 s at 95˚C, 35 s at 63˚C with a decrease of 0.5˚C per cycle and 2 min at 72˚C, followed by 30 amplification cycles of 30 s at 95˚C, 45 s at 59˚C and 2 min at 72˚C, and a final extension step at 72˚C for 10 min. The PCR products were separated on 1.0% agarose gels, and the target bands were recovered with the TIANgel Midi Purification kit (TianGen Biotech Co., Ltd., Beijing, China) and cloned into the pEA-SY-T1 simple vector (TransGen Biotech Co., Ltd., Beijing, China) before being transformed into competent E. coli DH5α cells via the heat shock method. Positive clones were selected for sequencing by Sangon Biotechnology Co. Ltd. (Shanghai, China). Using the software DNA-MAN (http://www.lynnon.com/), the positions of exons and introns in the TaSnRK2.10 gene

| Primer set | Primer sequence (5’-3’) | Amplified target | Size (bp) |
|------------|------------------------|-----------------|----------|
| TaSnRK2.10–1 | Forward: GCTTGCTCG GTTGCTTT GC | TaSnRK2.10cDNA | 1338, 1342, and 1284 |
| TaSnRK2.10–2 | Forward: GTCAAGTAC ATCGAGCG AGGG | TaSnRK2.10gDNA | 2127 or 2130, 2052 and 2076 |
| TaSnRK2.10-3-4A | Forward: CTTGATTCGACACCAATCTAG | A genome-specific | 1109 or 1106 |
| TaSnRK2.10-3-4B | Forward: GCTTGCGTGCTGCGAG | B genome-specific | 688 |
| TaSnRK2.10-3-4D | Forward: GCTTGCGTGCTGCGAG | D genome-specific | 1296 |
| TaSnRK2.10-4A-CAPS | Forward: CTTGATTCGACACCAATCTAG | | 1109 or 1106 |
| TaSnRK2.10-4B-AS1 | Forward: GCTTGCGTGCTGCGAG | | 688 |
| TaSnRK2.10-4B-AS2 | Forward: GCTTGCGTGCTGCGAG | | 688 |

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Table 1. Primers used in this study.
were determined by aligning the amplified gDNA and the corresponding cDNA sequences. The sequence alignment and similarity to other species were determined using the NCBI database. A phylogenetic tree was constructed based on the full-length amino acid sequences of SnRK2s using the protein sequences aligned by MAFFT7 [25]. The maximum-likelihood phylogenetic tree was reconstructed using MEGA5 [26], and the phylogenetic support for each split was evaluated with 500 bootstrap replicates.

Development and location of functional markers

We analysed the sequence of the coding region for gene TaSnRK2.10 in the ten winter wheat varieties and found two haplotypes for TaSnRK2.10-4A and TaSnRK2.10-4B, respectively. Using the Primer Premier 5.0 software, the polymorphic site for distinguishing the haplotypes of the TaSnRK2.10 gene were transformed into a cleaved amplified polymorphism sequence (CAPS) and allele-specific PCR (AS-PCR) markers [27] for TaSnRK2.10-4A-CAPS and TaSnRK2.10-4B-AS1/AS2 (Table 1), respectively. The primer pairs were used to amplify the genome-specific TaSnRK2.10 allele of CS-N/Ts, RILs and NPVs through PCR. PCR was performed using the following program: 95˚C for 5 min, followed by 30 cycles of 95˚C for 30 s, 60˚C for 30 s, and 72˚C for 1 min, and then a final extension of 72˚C for 10 min. The PCR products for the CAPS marker were digested with SalI (TaKaRa Biotechnology Co., Ltd., Dalian, China) according to the manufacturer’s directions. All segments were separated on 1.0% agarose gels with EB. For location of the functional markers a genetic map of RILs was used which was constructed using SSR markers and DArT array of Wheat PstI (TaqI) 2.6 and Wheat GBS 1.0 (Triticarte Pty. Ltd, Canberra, Australia) (Unpublished data).

Measurements of agronomic traits and association analysis

The phenotypes of the natural population of 128 wheat varieties were evaluated in field trials in three environments: Tai’an 2011 (TA11), Tai’an 2012 (TA12) and Yan’tai 2012 (YT12), in Shandong Province, China. Tai’an is part of the Huang-Huai Winter Wheat Region and Yantai is part of the Northern Winter Wheat Region of China. Each plot consisted of 3 rows that were 1.5 m long and spaced 25 cm apart; 70 seeds were planted in each row with two replicates. Plant height (PH), grain number per spike (GNS), spike length per plant (SL), sterile spikelet number per spike (SSS), fertile spikelet number per spike (FSS) and total spikelet number per spike (TSS) were determined from 10 random spikes for each line in each replicate at the grain-filling stage. The fertile spikelet number per spike (FSS) was calculated as TSS minus SSS. A 50 cm uniformed row was chosen to measure the spike number per plant (SN). The thousand grain weight (TGW) was evaluated by weighing three samples of 200 grains from each plot after harvest.

The unified mixed linear model (MLM) based on the Q + K model was used for functional markers and agronomic traits analysis in TASSEL v.2.0.1 [28–29]. The population structure matrix (Q) was obtained using STRUCTURE 2.3.1 software [30]. The relative kinship matrix (K) was obtained using TASSEL software [31]. Corrections for multiple testing were performed using the positive FDR (FDR ≤ 0.1) in QVALUE [32]. The 91 SSR and 47 functional markers were used to calculate Q and K for NPVs. A connection between functional markers and agronomic traits was determined when P ≤ 0.05.

Results

Cloning, chromosome assignment and characterization of TaSnRK2.10

Four wheat ESTs (CJ827375, CD882003, CD918384 and BJ794918) similar to the cDNA sequence of SAPK10 were selected and assembled into a putative TaSnRK2.10 cDNA sequence.
Three cDNA clones were amplified with the TaSnRK2.10-1F/R primer pair, and the corresponding gDNA sequences were amplified with the TaSnRK2.10-2F/R primer pair (S1–S3 Figs). Based on the three genome-specific primer pairs, TaSnRK2.10 genes were found to be located on chromosomes 4A, 4B and 4D using the CS-N/Ts (S4 Fig). The sequence of TaSnRK2.10 is shared high sequence similarity with the currently most updated gene model in Chinese Spring (http://plants.ensembl.org/Triticum_aestivum/Gene), including the uniformity of 99% for TaSnRK2.10-4A with TRIAE_CS2_4AL_TGACv1_291776_AA0996560, 100% for TaSnRK2.10-4B with TRIAE_CS2_4BS_TGACv1_328137_AA1083460, and 99% for TaSnRK2.10-4D with TRIAE_CS2_4DS_TGACv1_361281_AA1164930.

The cDNA sequences of TaSnRK2.10-4A, TaSnRK2.10-4B and TaSnRK2.10-4D amplified with the TaSnRK2.10-1F/R primer pair are 1339, 1342 and 1284 bp in length, respectively. Each cDNA sequence of TaSnRK2.10 on 4A, 4B and 4D contained an open reading frame (ORF) of 1086 bp through ORF finder (https://www.ncbi.nlm.nih.gov/orffinder/) which was predicted to encode a protein of 361 amino acid residues (AARs) (Fig 1) with a molecular mass of ~40.6 kDa and a pI of ~4.80. PROSITE analysis indicated that the amino acid sequence contains two conserved domains. The first conserved domain is an N-terminal catalytic domain (23–279 downstream of the Met) containing an ATP-binding site (29–52 downstream of the Met) and a serine/threonine protein kinase active site (138–150 downstream of the Met) (Fig 1). The second domain is a relatively short C-terminal domain with abundant Asp (D) residues. The amino acid sequence of TaSnRK2.10 shared high sequence similarity with counterpart monocot SnRK2s, including 95.6% with SAPK10 from rice and 94.2% with ZmSnRK2.10 from maize, and lower sequence similarity with dicotyledonous plants, including 62.5% with AtSnRK2.10 from Arabidopsis. The phylogenetic tree of the TaSnRK2.10 and SnRK2 family members from Arabidopsis, rice and maize showed that TaSnRK2.10 clustered in the same clade as OsSAPK10 and ZmSnRK2.10 (S5 Fig).

The complete gDNA sequences (from the ATG start codon to the TGA stop codon) of TaSnRK2.10-4A, TaSnRK2.10-4B and TaSnRK2.10-4D are 2322, 2244 and 2268 bp in length.

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**Fig 1. Schematic diagram of the TaSnRK2.10 gene.** (a) Known functional domains of TaSnRK2.10 protein are highlighted: ATP-binding site in diagonal stripes, serine/threonine protein kinase active site in vertical stripes. Amino acid positions of functional domains are indicated below the protein structure. (b) Exon-intron structure of TaSnRK2.10-4A gene with its eight coding exons in black boxes; the number of base pair sequence of ORF is listed above each exon; The percentage of GC content for exon 1–8 is indicated below each exon. (c) The GC content in exon 1.

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respectively (S1–S3 Figs), with eight exons and seven introns (Fig 2). The exon-intron structure of \( \text{TaSnRK2.10} \) is very similar to \( \text{SnRK2.10} \) in maize and \( \text{Arabidopsis} \), while the \( \text{SAPK10} \) gene of rice comprises seven exons and six introns. Compared with rice, the first exon of \( \text{OsSAPK10} \) could be divided into the first and second exon in \( \text{TaSnRK2.10} \) (Fig 2), but the sizes of the other exons are similar between wheat and rice, showing high sequence identity even though the introns exhibit low sequence similarity.

**Development and mapping of the functional markers**

For the \( \text{TaSnRK2.10} \) gDNA sequences among the ten wheat varieties, three SNPs (single nucleotide polymorphisms) and one indel (insertion or deletion of DNA bases) were found for \( \text{TaSnRK2.10-4A} \) with two haplotypes (named Hap-4A-H and Hap-4A-L). The SNPs were located at 1273, 1471, and 1907 bp downstream of the ATG with corresponding C-T, A-T, and G-C substitutions, respectively. The indel was 3 bp (TGA, 1982–1984 bp downstream of the ATG) (S1 Fig). For \( \text{TaSnRK2.10-4B} \), one SNP was located at 856 bp downstream of the ATG with A-G substitution, and formed two haplotypes (named Hap-4B-A and Hap-4B-G) (S2 Fig). No variation in \( \text{TaSnRK2.10-4D} \) was observed.

The sequence variations of \( \text{TaSnRK2.10-4A} \) produced a restriction enzyme \( \text{SalI} \) recognition site (GTCGAC) in Hap-4A-H at SNP-1907-C, but not in Hap-4A-L at SNP-1907-G (GTCGAG) (Fig 3A and 3B). Based on this SNP, a CAPS marker \( \text{TaSnRK2.10-4A-CAPS} \) (Table 1) was developed to distinguish the \( \text{TaSnRK2.10-4A} \) allele. The PCR product for Hap-4A-H was digested by \( \text{SalI} \) into two segments of 793 and 316 bp (Fig 3C). The RILs were genotyped using \( \text{TaSnRK2.10-4A-CAPS} \), and the mapping result using RILs showed that it had highly linked two Diversity Arrays Technology (DArT) markers, \( \text{D-1092101} \) and \( \text{D-100014232} \), with 0.80 and 0.50 cM, respectively (Fig 4).

Two complementary dominant AS-PCR markers were developed for \( \text{TaSnRK2.10-4B} \) (Table 1). The primer pair \( \text{TaSnRK2.10-4B-AS1F/R} \) yielded a 688 bp PCR fragment for Hap-4B-G, but no PCR fragment for the Hap-4B-A allele. The primer pair \( \text{TaSnRK2.10-4B-AS2F/R} \) yielded a 688 bp fragment for Hap-4B-A, but no PCR fragment for the Hap-4B-G allele (Fig 5). The mapping result using RILs showed that \( \text{TaSnRK2.10-4B-AS1/2} \) was highly linked to the DArT marker \( \text{D-1281577} \) and the SNP marker \( \text{S-1862758} \) with 0.10 and 0.10 cM, respectively (Fig 4).

**Association between haplotypes and agricultural traits**

Data on agricultural traits for the natural population of 128 wheat varieties were used in an association analysis. Using the \( \text{TaSnRK2.10-4A-CAPS} \) marker, 63 varieties harboring the Hap-
4A-H haplotype and 65 varieties with the Hap-4A-L haplotype were identified. The TGW of Hap-4A-H was significantly higher than Hap-4A-L in all three environments as well as the average value (AV) at the $p \leq 0.01$ level. The PH of Hap-4A-L was significantly higher than Hap-4A-H in TA11, YT12 and the AV ($p \leq 0.05$), but SL was lower for TA11, TA12 and the AV ($p \leq 0.05$). The TSS of Hap-4A-H was significantly higher for TA12 and the AV as well as SSS in the AV ($p \leq 0.05$). These results indicated that the environments are important in explaining the overall phenotypic variations. Using TaSnRK2.10-4B-AS1 and TaSnRK2.10-4B-AS2 markers, 93 varieties with Hap-4B-G haplotypes and 35 varieties with Hap-4B-A haplotypes were identified. However, there were no significant differences between the haplotypes except for TGW in TA11 ($p = 0.025$) (Table 2).

**Discussion**

A major hindrance to PCR amplification of GC-rich templates is the formation of secondary structures such as hairpin loops of single-stranded GC-rich sequences [33–34]. Many approaches have been developed to overcome such problems by adjusting the PCR procedure [34–39]. However, it is also difficult to get GC-rich sequence using the conventional RACE technique. In recent years, a great number of wheat ESTs have been deposited, which makes it possible to clone the full-length sequences with GC-rich sequences in combination ESTs with PCR amplification. In our present study, four EST sequences were found by performing a BLAST search with a reference sequence and were then combined to generate the tentative full-length sequence of TaSnRK2.10. The size of TaSnRK2.10 is in accordance with SnRK2s reported previously [18, 22, 40–43].

Based on the protein size and character of the acidic amino acid-enriched C-terminus, the SnRK2 family can be divided into two groups: SnRK2a and SnRK2b [44]. SnRK2a corresponds to the more recently defined subclass I, and SnRK2b includes subclasses II and III [21, 45]. Increasing evidence indicates that SnRK2s from subclass III are involved in the regulation of plant metabolism [46]. As shown in our study, TaSnRK2.10 was clustered in the subclass III
The structure of TaSnRK2.10 is similar to other SnRK2s, including two typical domains, an N-terminal highly conserved kinase domain and a regulatory C-terminal domain [46], and showed potential for serine/threonine and tyrosine kinase activities. The relatively short C-terminal domain of SnRK2.10 is abundant in Asp (D) and might play a role in activation of the kinase [47–49] and function in protein-protein interactions that are mainly involved in ABA responsiveness [40]. In rice and maize, SnRK2.10 is activated under ABA and hyperosmotic stress. In Arabidopsis, AtSnRK2.10 was found to be expressed in the vascular tissue at the base of developing lateral roots, revealing a role in root growth and architecture [50]. The ortholog in tobacco, NtOSAK, has been shown to directly interact with glyceraldehyde-3-phosphate dehydrogenase (GAPDH), linking its mode of action to metabolic processes [51]. All of the above evidence implies that TaSnRK2.10 is mainly involved in ABA responsiveness and shows a potential role in carbohydrate metabolism. In this study, we found that Hap-4A of TaSnRK2.10 was associated stably with the TGW, PH and SL, which may indicate new functions of SnRK2.10 and may be the result of carbohydrate metabolism. The Hap-4A-H varieties of TaSnRK2.10-4A showed higher TGW and SL values than the Hap-4A-L varieties, but lower PH values, indicating the Hap-4A-H is a favorable allele for the improvement of grain yield. The marker TaSnRK2.10-4A-CAPS may be useful in wheat yield breeding programs.

Exons are the regions encoding proteins in the ORFs of genes. Many studies have indicated that missense mutations can influence the function of genes. For example, Wang et al. [52]
reported that the mutant chs1-2 with a nucleotide substitution from G to A displayed defense-associated phenotypes compared with the wild type of CHS1, including extensive cell death, the accumulation of hydrogen peroxide and salicylic acid, and an increased expression of PR genes. Wang et al. [53] found a codon change from TGC to TAC in the first exon of OsCESA7, and the mutation deleteriously affected cellulose biosynthesis and plant growth. In this study, three SNPs and one indel were found in TaSnRK2.10-4A with two haplotypes. Of these, a SNP in the seventh exon in TaSnRK2.10-4A caused a missense mutation that resulted in an amino acid change from Asp to His (S1 Fig). The adjacent region is conserved between TaSnRK2.10 and the ortholog genes SAPK9 and SAPK10 in rice [54] (S5 Fig). There has been no report about the change of the function for the amino acid from Asp to His in SnRK2 gene family. The missense mutation may account for the variance in agronomic traits and this should be affirmed by more evidences in the further. The other SNPs and the indel were located in introns, which may be of little function for agronomic traits. Furthermore, only one SNP was obtained for TaSnRK2.10-4B in an intron, and it had no significant association with agronomic traits except for the TGW data in TA11. The potential functions of TaSnRK2.10-4B require further investigation for verification.

The wheat yield is affected by many factors and is a polygenic trait influenced by environmental and genetic interactions at all stages of the plant’s growth [55]. Direct cloning of yield-related genes in hexaploid wheat was difficult due to its large genome size. In our study, we hypothesized that the TaSnRK2.10 gene had functions that affect the TGW, PH and SL during the maturity stage. To date, some grain weight genes have been isolated, such as Ppd-D1 [56], CKX6-D1 [57], Gs1a [11], GW2 [8, 58–59], Gs-D1 [60], Sus [9, 61], GASR7 [62–63], TEF-7 [64], CWI [65], and 1-FEH-w3 [66–67]. Using sequence comparison and the analysis of protein.
Table 2. Association analysis between the haplotypes and agronomic traits.

| Trait | ENV  | Hap-4A-H | Hap-4A-L | p value | Hap-4B-G | Hap-4B-A | p value |
|-------|------|----------|----------|---------|----------|----------|---------|
| TGW (g) TA11 | 53.161 ± 5.505 | 51.189 ± 5.644 | 0.003** | 52.509±5.835 | 51.325±5.098 | 0.025* |
| (cm) TA12 | 46.349 ± 5.967 | 43.554 ± 4.881 | 0.007** | 45.342±6.104 | 43.816±3.875 | 0.155 |
| AV YT12 | 47.101 ± 5.140 | 45.373 ± 3.998 | 0.010** | 46.397±4.880 | 45.594±3.978 | 0.190 |
| KNS TA11 | 48.870 ± 4.989 | 46.705 ± 3.934 | 0.002** | 48.083±4.892 | 46.912±3.684 | 0.051 |
| (g) TA12 | 44.661 ± 7.009 | 42.894 ± 5.179 | 0.242 | 43.925±6.271 | 43.243±6.093 | 0.663 |
| AV YT12 | 46.347 ± 7.795 | 44.148 ± 6.025 | 0.427 | 45.513±7.423 | 44.754±5.938 | 0.739 |
| PH TA11 | 64.908 ± 7.136 | 67.194 ± 11.303 | 0.018* | 66.615±10.529 | 64.721±6.003 | 0.706 |
| (cm) TA12 | 79.985 ± 7.853 | 80.417 ± 11.612 | 0.220 | 81.292±10.810 | 77.345±6.288 | 0.722 |
| AV YT12 | 70.115 ± 8.763 | 72.936 ± 12.463 | 0.031* | 72.229±12.212 | 69.766±5.715 | 0.838 |
| SL TA11 | 71.669 ± 7.266 | 73.516 ± 11.348 | 0.041* | 73.378±10.672 | 70.611±5.559 | 0.909 |
| (cm) TA12 | 9.036 ± 1.164 | 8.565 ± 0.971 | 0.020* | 8.845±1.153 | 8.631±0.898 | 0.638 |
| AV YT12 | 9.322 ± 1.149 | 9.071 ± 1.136 | 0.411 | 9.206±1.191 | 9.183±1.038 | 0.620 |
| TSS TA11 | 9.094 ± 1.036 | 8.715 ± 0.964 | 0.045* | 8.936±1.047 | 8.803±0.940 | 0.797 |
| 19.905 ± 1.489 | 19.364 ± 1.270 | 0.081 | 19.839±1.352 | 19.063 ± 1.425 | 0.066 |
| 19.046 ± 1.693 | 19.047 ± 1.431 | 0.017* | 20.709 ± 1.679 | 19.856 ± 1.301 | 0.164 |
| YT12 | 19.705 ± 1.331 | 19.383 ± 1.143 | 0.359 | 19.646±1.312 | 19.282±1.020 | 0.397 |
| AV | 20.185 ± 1.352 | 19.598 ± 1.096 | 0.048* | 20.065±1.292 | 19.401±1.056 | 0.110 |
| FSS TA11 | 2.023 ± 0.808 | 2.007 ± 0.858 | 0.769 | 2.218±0.817 | 1.713±0.816 | 0.062 |
| 2.429 ± 1.204 | 2.305 ± 1.220 | 0.490 | 2.505±1.272 | 1.996±0.957 | 0.085 |
| YT12 | 0.457 ± 0.421 | 0.474 ± 0.474 | 0.813 | 0.477±0.465 | 0.424±0.469 | 0.872 |
| AV | 1.636 ± 0.637 | 1.595 ± 0.673 | 0.785 | 1.704±0.646 | 1.376±0.632 | 0.072 |
| SSS TA11 | 17.882 ± 1.644 | 17.357 ± 1.135 | 0.068 | 17.711±1.464 | 17.350±1.332 | 0.476 |
| 18.517 ± 1.768 | 17.742 ± 1.398 | 0.077 | 18.204±1.677 | 17.860±1.497 | 0.961 |
| YT12 | 18.248 ± 1.362 | 18.909 ± 1.165 | 0.342 | 19.169±1.364 | 18.859±1.105 | 0.355 |
| AV | 18.549 ± 1.343 | 18.003 ± 0.888 | 0.045* | 18.362±1.187 | 18.022±1.093 | 0.485 |

The natural population of 128 varieties was grouped according to their haplotypes (Hap-4B-G and Hap-4B-A). The presented values are the mean ± SD from the association analysis. * and ** designate significance differences at p<0.05 and p<0.01, respectively.

The corresponding full-length gDNA sequences of TaSnRK2.10 were 2322, 2244, and 2268 bp, comprising eight exons and seven introns and presenting an ORF of 1086 bp that...
encodes a protein of 361 amino acids with functional domains shared with SnRK2s. One SNP in an exon and two SNPs and one indel in introns were detected in TaSnRK2.10-4A alleles, resulting in two haplotypes: Hap-4A-H and Hap-4A-L. In TaSnRK2.10-4B alleles, only one SNP in an intron was detected, also resulting in two haplotypes: Hap-4B-G and Hap-4B-A. The sequences of SnRK2.10-4D were completely conserved. A CAPS marker for TaSnRK2.10-4A and two AS-PCR markers for TaSnRK2.10-4B were developed and mapped on chromosomes. The results of an association analysis provided evidence that TaSnRK2.10-4A shows an association with TGW in all of the examined environments and with PH and SL in most environments, representing new functions of SnRK2s in wheat. Hap-4A-H was found to be a favorable allele for the improvement of grain yield.

Supporting information

S1 Fig. Genome DNA sequences alignment between two haplotypes of TaSnRK2.10-4A. The exons were indicated by the gray shade and the green shaded bases are SNPs and InDels between the two haplotypes. (RAR)

S2 Fig. Genome DNA sequences alignment between two haplotypes of TaSnRK2.10-4B. The exons were indicated by the gray shade and the green shaded bases are SNPs between the two haplotypes. (RAR)

S3 Fig. Genome DNA sequences alignment of TaSnRK2.10-4D. The exons were indicated by the gray shade. (RAR)

S4 Fig. Chromosomal locations of TaSnRK2.10 homoeologs based on genome-specific primer pairs using Chinese Spring nullisomic-tetrasomic lines. M, marker; 1, Shannong 0431; 2, Lumai21; 3, N4AT4B (nullisomic 4A-tetrasomic 4B); 4, N4BT4A; 5, N4DT4B; 6, H2O. (RAR)

S5 Fig. Phylogenetic tree of TaSnRK2.10 and SnRK2s from Arabidopsis, rice, maize and wheat. Three distinct isoform groups are presented within the boxes. The phylogenetic tree was constructed based on the full-length amino acid sequences of SnRK2s using the protein sequences were aligned by MAFFT7 [25]. The Maximum-likelihood phylogenetic tree was reconstructed using MEGA5 [26], and the phylogenetic support for each split was evaluated with 500 bootstrap replicates. (RAR)

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