Effects of Favorable Alleles for Water-Soluble Carbohydrates at Grain Filling on Grain Weight under Drought and Heat Stresses in Wheat

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Abstract
Drought, heat and other abiotic stresses during grain filling can result in reductions in grain weight. Conserved water-soluble carbohydrates (WSC) at early grain filling play an important role in partial compensation of reduced carbon supply. A diverse population of 262 historical winter wheat accessions was used in the present study. There were significant correlations between 1000-grain weight (TGW) and four types of WSC, viz. (1) total WSC at the mid-grain filling stage (14 days after flowering) produced by leaves and non-leaf organs; (2) WSC contributed by current leaf assimilation during the mid-grain filling; (3) WSC in non-leaf organs at the mid-grain filling, excluding the current leaf assimilation; and (4) WSC used for respiration and remobilization during the mid-grain filling. Association and favorable allele analyses of 209 genome-wide SSR markers and the four types of WSC were conducted using a mixed linear model. Seven novel favorable WSC alleles exhibited positive individual contributions to TGW, which were verified under 16 environments. Dosage effects of pyramided favorable WSC alleles and significantly linear correlations between the number of favorable WSC alleles and TGW were observed. Our results suggested that pyramiding more favorable WSC alleles was effective for improving both WSC and grain weight in future wheat breeding programs.

Introduction
Wheat (*Triticum aestivum* L.) is one of the most important crops in the world, feeding nearly half the world population [1]. High grain yield is the most important breeding objective in wheat improvement. Drought, heat and other abiotic stresses greatly affect growth and productivity of wheat, especially during grain filling stage. Grain filling in wheat depends on two major sources of carbon: current photosynthate in leaves and non-leaf organs; (2) WSC contributed by current leaf assimilation during the mid-grain filling. The latter can be important in buffering grain yields against unfavorable conditions for photosynthesis during the grain-filling period [3,4].

Water-soluble carbohydrates (WSC) accumulation and utilization depend on growing conditions and genotypes, and there may be differences between internodes [2,5,6]. Among three segments of the main stem (peduncle, penultimate internode and the remainder segments), the remainder segments are the major storage sites and the major source for WSC mobilization during the grain filling period [7]. In general, WSC accumulate until 10–20 days after anthesis, and the reserved WSC can reach more than 40% of total stem dry weight in wheat [8]. The contribution of WSC to final yield and kernel size is 10%–20% of total grain weight under normal condition [9]. Drought stress during grain filling, often involving not only water stress but also heat, inhibits current assimilation and damages photosynthetic organs, especially leaves. When photosynthetic activity is suppressed, the reserved WSC play a more important role in partial compensation of the reduced carbon supply. In addition, drought induced reserved WSC mobilization with higher efficiency, potentially contributing up to 70% of grain dry matter [8,10]. Based on a large group of genotypes with various WSC contents, the ranking of wheat lines for WSC is consistent across diverse environments. Stem WSC content shows high broad-sense heritability (*h*² = 0.9). WSC are inversely related to stem number but genotypic ranking persists when compared at similar stem densities [11,12]. In past years, selection for high WSC in stems occurred during development of drought-tolerant wheat varieties in the UK and Australia [13,14]. It has been suggested that the release of representative UK wheat cultivars from 1972 to 1995 was associated with increasing stem WSC content [15]. Therefore, high stem WSC content was suggested as a useful trait for improving grain weight in wheat breeding programs [11,14,15].
Variation in stem WSC among wheat genotypes is an important genetic factor involving grain weight and yield under drought stress conditions [16]. Thus, knowledge of stem WSC is essential for understanding yield-limiting factors and for improving yield potential in wheat. QTL associated with stem WSC have been reported in perennial ryegrass [17], rice [18], maize [19], barley [20], and wheat [21–23]. In wheat, QTL for WSC were mapped on chromosomes 1A, 2D, 4A, 4B, 5D, 6B, 7B and 7D. QTL for drought tolerance also appeared in homologous regions on the group 7 chromosomes [22]. Yang et al. [23] discovered eight, one and two additive QTL for WSC at flowering, grain-filling and maturity, respectively. However, WSC content is a complex quantitative trait controlled by polygenes, and the small effects of many independent QTL limit their direct use for marker-assisted selection in breeding programs [8,24].

Photosynthesis is the all-important metabolic process determining grain yield in wheat. When water deficit occurs during grain filling stage, photosynthetic rates significantly decrease in leaf blades and non-leaf green organs, such as leaf sheath, glume and awn [25–27]. However, non-leaf green organs are relatively more stable than leaf blades [27]. In this study, four types of WSC (Total, Leaf, Non-leaf and Remo) under drought stress (DS) and well-watered (WW) conditions in 262 winter wheat accessions were mainly used, viz. Total, total WSC at the mid-grain filling stage (14 days after flowering) produced by leaves and non-leaf organs; Leaf, WSC contributed by current leaf assimilation during the mid-grain filling; Non-leaf, WSC in non-leaf organs at the mid-grain filling, excluding the current leaf assimilation; Remo, WSC used for respiration and remobilization during the mid-grain filling. The objectives were to (1) detect the relative contributions of leaf and non-leaf organs during grain filling stage to WSC and 1000-grain weight (TGW) under two water regimes; (2) explore genetic resources with high WSC by association analysis; (3) verify the stable favorable WSC alleles with significant effect on TGW and genetic resources with high WSC by association analysis; (4) assess the positive contribution of WSC to TGW, and the results of favorable WSC allele analysis will be helpful for wheat breeders in selecting genotypes with higher TGW.

Materials and Methods

Ethics statement

Two locations, Changping (116°13′E; 40°13′N) and Shunyi (116°56′E; 40°23′N) in Beijing, are the experiment stations of the Institute of Crop Science, Chinese Academy of Agricultural Sciences. We have obtained the relevant permission for our field studies for growing our plant materials in the field from the Institute of Crop Science. We have obtained the relevant permission for our field studies for growing our plant materials in the field from the Institute of Crop Science, over 3 years for measuring TGW at maturity. The planting years were 2009 and 2010 at both Changping and Shunyi, and 2011 in Shunyi.

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Phenotyping of WSC and TGW

The methods of collecting data on WSC were reported earlier [30]. For each genotype, five main stems with the same heading date were selected as samples. The main stem was cut from the soil surface at the mid-grain filling (14 days after flowering). Leaf blades were removed, and stem samples were cut into three parts, viz. the uppermost internode (peduncle, Ped), the lower internode (the remainder segments of stem except for peduncle, Low) and the spike. The fresh samples were dehydrated until a constant dry weight. The WSC of the three sections, viz. peduncle, the lower internode and whole stem (Stc), were determined by different near-infrared reflectance spectroscopy regression models, which were developed for quantitative determination of WSC using samples of 150 doubled haploid lines (Hanxuan 10×Lumai 14) [30]. Briefly, as the first step, partial least square regression models for predicting WSC in the target parts of wheat were developed using selected wavelength regions, spectroscopy pretreatments and latent variables included in each model. The amounts of WSC (mg WSC/g dry weight, mg/g dw) in each modeling sample of 150 doubled haploid lines were also measured by chemical assay (anthrone colorimetric assay), and used for cross validation. WSC were extracted according to the modified procedure described by Wardlaw and Willenbrink [2,30]; the amounts of WSC were measured as fructose equivalents using the anthrone colorimetric assay at 620 nm by 722S spectrophotometer [31]. This showed that the near-infrared reflectance spectroscopy regression models were highly accurate in determining the true values of WSC measured by chemical assay in the wheat organs tested (coefficient of determination $R^2>0.992$ and root mean square error of prediction $RMSEP<0.228$). In addition, 40 samples per model (i.e. not included in the modeling samples) were used to assess the models. The results confirmed the high quality of the models in evaluating WSC.

We obtained four types of WSC (Total, Leaf, Non-leaf, and Remo), viz. Total, the total WSC at the mid-grain filling produced by leaves and non-leaf organs which was obtained from the treatment of cutting spikes ($WSC_{cutting}$ spikes); Leaf, WSC; Non-leaf, WSC in non-leaf organs at the mid-grain filling; Remo, WSC used for respiration and remobilization during the mid-grain filling; Non-leaf, WSC in non-leaf organs at the mid-grain filling, excluding the current leaf assimilation; Remo, WSC used for respiration and remobilization during the mid-grain filling. The objectives were to (1) detect the relative contributions of leaf and non-leaf organs during grain filling stage to WSC and 1000-grain weight (TGW) under two water regimes; (2) explore genetic resources with high WSC by association analysis; (3) verify the stable favorable WSC alleles with significant effect on TGW and genetic resources with high WSC by association analysis; (4) assess the positive contribution of WSC to TGW, and the results of favorable WSC allele analysis will be helpful for wheat breeders in selecting genotypes with higher TGW.
contributed by current leaf assimilation during the mid-grain filling, i.e. the reduction in WSC due to cutting leaves which was estimated by comparing WSC between the normal control and the treatment of cutting leaves (WSC\textsubscript{untreated} – WSC\textsubscript{removing leaves}).

Non-leaf, the WSC in non-leaf organs at the mid-grain filling (excluding the current leaf assimilation) which was estimated by the treatment of removing leaves (WSC\textsubscript{removing leaves}); Remo, WSC used for respiration and remobilization during the mid-grain filling which was obtained by comparing WSC between the normal control and the treatment of cutting spikes (WSC\textsubscript{cutting spikes} – WSC\textsubscript{untreated}).

Spikes corresponding to main stem samples were collected at maturity stage for each accession to obtain TGW. The reduction of TGW due to leaf removal was calculated for each cultivar as:

\[
\left[\frac{\text{TGW}_{\text{untreated}} - \text{TGW}_{\text{removing leaves}}}{\text{TGW}_{\text{untreated}}}\right] \times 100\%.
\]

**SSR genotyping and association mapping**

Two hundred and nine SSR markers, evenly distributed on the 21 wheat chromosomes, were selected for evaluating population structure, relative kinship, and association mapping. The genetic positions of SSR markers were obtained from the consensus map Ta-SSR-2004 [32] and the Komugi wheat genetic resources database (http://www.shigen.nig.ac.jp/wheat/komugi/?top.jsp). Fluorescent primers were synthesized by ABI (Applied Biosystems, Foster City, CA, USA). Amplification products were separated on an ABI3730 DNA Analyzer, and fragment sizes were analyzed by GeneMapper software (Applied Biosystems).

Allele number, allele frequency and polymorphism information content were calculated by PowerMarker V3.25 [33]. Population structure was estimated by STRUCTURE v2.3.2 using data from 209 SSR markers. The number of hypothetical subpopulations was set from \(k = 1\) to 10 with a burn-in period of 50,000 iterations and a run of 500,000 replications of Markov Chain Monte Carlo after burn in. The Δ\(k\) method was applied according to LnP(D) in STRUCTURE [34]. The \(Q\) data of five replicate runs were integrated by CLUMPP software [35]. Principal coordinate analysis based on genetic distances was also used to confirm the results of STRUCTURE by NTSYSpc analysis [36]. The relative kinship coefficient (\(K\)) was calculated by the SPAGeDi software package [37]. Finally, the \(Q+K\) models were performed using mixed linear model in TASSEL V2.1 for association of SWSC [38,39].

**Results**

**Contribution of leaf and non-leaf organs to 1000-grain weight during grain filling**

Figure S1 summarizes the relative contributions of leaf and non-leaf organs to final 1000-grain weight during grain filling across the 262 diverse winter wheat genotypes. Reduction in TGW due to cutting leaves (i.e. the contribution of leaves to TGW during grain filling) was 14.79% (6.26 g) at maturity under DS, compared to 19.84% (8.56 g) under WW condition (Figure S1). The lower contribution of leaves to TGW under DS condition reflected the negative effect of water deficit on photosynthetic rates in leaf blades during grain filling. Non-leaf organs contributed 85.21% (36.05 g) to TGW under DS, whereas it was 80.16% (34.60 g) under WW condition (Figure S1).

Variation in WSC of leaves and non-leaf organs at different internodes at the mid-grain filling under two water regimes

The WSC in lower internodes (the remainder segments of stem except for peduncle, Low) were higher than those in peduncles (the upmost internode, Ped; Figure 1) under both water regimes in all types of WSC. The WSC in non-leaf organs at the mid-grain filling ranged from 83.82 to 178.50 mg WSC/g dry weight (mg/g dw), and those contributed by current leaf assimilation were from 41.13 to 68.58 mg/g dw, thus showing the relative importance of stem-reserved WSC for grain filling. The WSC used for Remo at the mid-grain filling ranged from 56.88 to 98.87 mg/g dw (Figure 1). WSC in non-leaf organs were 131.51, 178.50 and 159.37 mg/g dw in peduncles, lower internodes and the whole stem under drought stress, and 83.32, 94.35 and 88.05 mg/g dw under well-watered condition, respectively; the ratios between two water regimes were 136.90%, 189.19% and 181.00%, respectively (Figure 1). This implied that long term drought stress triggered a series of metabolic reactions by increasing fructans for self-protection. At the mid-grain filling, WSC contributed by current leaf assimilation were 57.62 and 55.43 mg/g dw in the lower internode and whole stem under drought stress, compared with 68.38 and 60.05 mg/g dw under well-watered condition (Figure 1). Thus drought during grain filling greatly influences current photosynthesis and dry matter accumulation.

**Correlations between WSC at the mid-grain filling and TGW**

WSC are recognized as an important source of grain dry matter for grain filling in wheat. There were significant correlations between the four types of WSC (Total, Non-leaf, Leaf and Remo) at the mid-grain filling and TGW (Table 1, Table S2–S3). Moreover, there were higher correlations between the four types of WSC and TGW under drought stress compared to those under well-watered condition (Table 1). Under drought stress, WSC of Total was significantly correlated with TGW (\(r = 0.248^{**}, 0.386^{***}\) and 0.392***), and correlations between WSC of Non-leaf, Leaf and TGW were \(r = 0.140^{*}\) to 0.275***, 0.156 to 0.220***, respectively. Under well-watered condition, there were three instances of significant correlations between WSC of Total and TGW (\(r = 0.135^{*}, 0.146^{*}\) and 0.176**).

**Association analysis for WSC at the mid-grain filling and the search for favorable alleles**

Based on the population structure assessment using 209 markers, the 262 wheat accessions were separated into two subpopulations, comprising 126 and 136 accessions (our unpublished data). Association analysis using the 209 SSR markers and four types of WSC at the mid-grain filling was conducted using a mixed linear model, which accounted for population structure (\(Q\) and relative kinship (\(K\) matrix). Thirteen, 13, 23 and 14 novel loci were significantly \(P<0.01\) associated with WSC of Total, Leaf, Non-leaf and Remo in 17, 17, 31 and 18 instances, respectively (Table S4–S7). Variances explained by SSR markers (\(R^2\)) ranged from 0.11% to 10.51%. Twenty-two loci were identified more than once. Xgwm630 (associated with WSC of Remo under WW condition; Remo, WW), Xgwm517-7B (Non-leaf, DS) and Xgwm169-6A (Remo, WW) and Xgwm517-7B (Non-leaf, WW) were detected in all internodes (peduncle, lower internode and the whole stem). Xbbarc228-2D (Total, DS), Xgwm169-6A (Remo, WW) and Xgwm517-7B (Leaf, DS) were detected in both peduncle and lower internode. Xbarc228-2D (Non-leaf, DS) and Xgwm630 were identified in both the peduncle and whole stem; Xgwm630...
(Remo, WW), Xgwm165.1-4D (Non-leaf, WW) and Xgwm182-3D (Total, WW) were similarly identified in both the lower internode and whole stem. Xbarc125-3D was associated with WSC of Total in lower internode under both DS and WW conditions. Xgwm66, Xgwm88, Xgwm192, Xwmc470-2D, Xgwm181-3B, Xgwm358-5D, Xgwm583-5D and Xgwm428-7D were associated with more than one types of WSC (Total, Non-leaf, Leaf and Remo).

For associated loci, we explored favorable WSC alleles by assessing differences in WSC between accessions carrying favorable alleles and those with other alleles using ANOVA (SAS 8.01), i.e. the WSC of the former were significantly ($P < 0.05$) higher than those of the latter. There were 7, 10, 12 and 9 novel favorable alleles for WSC of Total, Leaf, Non-leaf and Remo, respectively (Tables S4–S7).

Xcfd17-2D (Remo, WW) had the same favorable WSC alleles (Xcfd17-2D223) in peduncle, lower internode and the whole stem estimates, i.e. 74.1 compared with 51.7 mg/g dw ($P < 0.001$), 113.5 compared with 94.3 mg/g dw ($P < 0.05$), and 89.7 compared with 69.7 mg/g dw ($P < 0.01$), respectively. Xgwm181-3B131 and 161 (Leaf, DS), Xgwm610-4A167 (Leaf, WW), Xgwm513-4B144 (Leaf, DS), Xgwm165.1-4D199 (Non-leaf, WW), Xwmc517-7B188 (Non-leaf, WW) had positive effects both in lower internode and the whole stem. Higher WSC were associated with Xgwm169-6A203 (Remo, WW) and Xgwm537-7B205 (Leaf, DS) in both the peduncle and lower internode. Xbarc125-3D147 (Total) contributed to higher WSC in lower internodes, not only under well-watered conditions but also under drought stress. Some associated loci, however, had various favorable alleles for different types of WSC; for example, accessions carrying the allele Xgwm513-4B144 exhibited higher WSC of Leaf, whereas accessions with the 142 bp allele had higher WSC of Non-leaf in lower internodes under drought-stress conditions.

Seven novel favorable WSC alleles individually exhibited positive contributions to TGW under well-watered, drought and heat stress conditions

In order to evaluate the genetic relationship between WSC and TGW, we analyzed the effects of favorable WSC alleles on final TGW by comparing differences in TGW between accessions carrying favorable WSC alleles and those with other alleles. Seven novel favorable WSC alleles exhibited significantly ($P < 0.05$) positive contributions to TGW on an individual basis. They were Xcfd17-2D223, Xcfd53-2D263, Xgwm181-3B140 and 161, Xgwm389-3B116, Xbarc125-3D147, Xgwm358-5D162 and Xgwm537-7B205 (Table 2). For Xbarc125-3D147, the higher WSC of Total (341.5 compared to 309.9 mg/g dw) in lower internodes led to a higher TGW (44.99 g compared to 41.14 g) under drought stress conditions; likewise, accessions with this allele also produced higher TGW (43.91 g) than accessions with other alleles (41.85 g) under well-watered conditions, with WSC of 284.3 and 255.9 mg/g dw, respectively. In order to verify the positive contributions of these seven favorable alleles to TGW, we used the same population (262 winter wheat accessions) planted in 16 environments (year×site×water and heat regime combinations; 5 drought stress conditions, 3 well-watered and heat stress conditions, 3 drought and heat stress conditions, 5 well-watered conditions) to confirm the above results. The average TGW of

Figure 1. WSC (mg/g dw) of different internodes at the mid-grain filling stage (14 days after flowering) under well-watered and drought stress conditions. Bars indicate 2SE. WSC, water-soluble carbohydrates; DS-Ped, peduncle under drought stress; WW-Ped, peduncle, well-watered; DS-Low, lower internode, drought stress; WW-Low, lower internode, well-watered; DS-Ste, whole stem, drought stress; WW-Ste, whole stem, well-watered; CS, cutting spikes; CL, removing leaves; CK, normal control; Non-leaf, WSC in non-leaf organs at the mid-grain filling, excluding the current leaf assimilation; Leaf, WSC contributed by current leaf assimilation during the mid-grain filling; Remo, WSC used for respiration and remobilization during the mid-grain filling.

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accessions carrying favorable WSC alleles were higher than those without the favorable alleles in all environments (Figure 2).

Pyramiding of favorable WSC alleles indicated potential application in wheat breeding

To explore whether the pyramiding of favorable WSC alleles showed additive effects, we also analyzed the mean TGW of accessions with different numbers of favorable WSC alleles in 16 environments (Table 3). The average TGW of genotypes with single favorable WSC allele were 31.75 - 42.82 g; TGW with two favorable alleles were 33.57 - 44.66 g; TGW with three ones were 35.17 - 46.76 g; TGW with more than four favorable alleles were 35.90 - 47.18 g across 16 environments. The average TGW of accessions without favorable WSC allele ranged from 30.30 to 41.01 g. In addition, a significantly linear correlation (y = 1.579x + 36.847, $R^2 = 0.369$) between TGW and number of favorable WSC alleles further confirmed the additive effect (Figure 3A). We also evaluated the distribution of combined favorable WSC alleles in modern varieties from different decades (Figure 3B). The average number of favorable WSC alleles was 0.61 before 1960, and the current average number was 2.59. The increasing number over time reveals a genomic footprint left by breeders, but the relatively low number of 2.59 alleles in current cultivars (post-2000) indicates a potential for pyramiding more favorable alleles [40].

**Table 1.** Pearson correlation coefficients of WSC at the mid-grain filling and TGW under well-watered and drought stress conditions.

| WSC Types | Internodes | TGW | DS | WW |
|-----------|------------|-----|----|----|
| Totala    | Ped        | 0.248*** | 0.135* |
|           | Low        | 0.386*** | 0.146* |
|           | Ste        | 0.392*** | 0.176** |
| Leafb     | Ped        | 0.218*** | 0.027 |
|           | Low        | 0.156*  | 0.100 |
|           | Ste        | 0.220*** | 0.071 |
| Non-leafa | Ped        | 0.011   | -0.046 |
|           | Low        | 0.177** | -0.000 |
|           | Ste        | 0.140*  | -0.011 |
| Non-leafb | Ped        | 0.207*** | 0.121 |
|           | Low        | 0.275*** | 0.011 |
|           | Ste        | 0.274*** | -0.001 |
| Remoa     | Ped        | -0.014  | 0.100 |
|           | Low        | 0.106   | 0.044 |
|           | Ste        | 0.037   | 0.105 |

*Significant at $P = 0.05$; **Significant at $P = 0.01$; ***Significant at $P = 0.001$. Total, total WSC at the mid-grain filling produced by leaves and non-leaf organs; Leaf, WSC contributed by current leaf assimilation during the mid-grain filling; Non-leaf, WSC in non-leaf organs at the mid-grain filling, excluding the current leaf assimilation; Remo, WSC used for respiration and remobilization during the mid-grain filling; Ped, peduncle; Low, lower internode; Ste, whole stem; TGW, 1000-grain weight at maturity.

*TGW was measured on the normal control; **TGW was measured with treatment with removing leaves.

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Discussion

Consistency between WSC and TGW under stress conditions

Previous studies suggested that increases in grain yield can mainly be attributed to better partitioning of photosynthetic products [41]. WSC accumulation ability and its remobilization efficiency are much higher in the internodes of drought tolerant cultivars than those of sensitive genotypes under both normal and stress conditions. On the other hand, fructans, the major components of WSC, insert between the head groups of phospholipids, acting as compatible solutes in cells to protect cell membranes and proteins from osmotic damage [42,43]. Stem samples of rainfed wheat have significantly higher average fructan than irrigated samples. In our research four types of WSC (Total, Leaf, Non-leaf and Remo) under drought stress were overall higher than those under well-watered condition (Figure 1). It has been reported that fructan synthesis is induced by drought stress, and that drought tolerant plants can manufacture more fructans. The tolerant cultivars activate their protection mechanisms faster and more efficiently than the sensitive ones to cope with stress conditions [10,44].

Drought stress during grain filling can result in reductions in grain weight, due to lower numbers of endosperm cells and a limited maximum storage capacity of the kernels [45,46]. WSC were recognized as an important source of grain dry matter for grain filling, especially when current photosynthesis is inhibited by drought stress. Water deficit during grain filling stimulates senescence of the whole plant and enhances remobilization of reserved WSC to the grains [47,48]. Thus, the reserved WSC assimilated pre-anthesis and current assimilation are critically important for grain filling. In the present study, we observed that final grain yield mainly depends on pre-anthesis assimilation by green organs and current photosynthesis of non-leaf organs during grain filling, especially under drought stress condition (Figure S1). In addition, compared with those under well-watered condition, higher correlations between the four types of WSC at the mid-grain filling and TGW under drought stress indicate that yield in...
unfavorable conditions relies more on pre-stored carbohydrates (Table 1). Association and favorable allele analyses were conducted on four types of WSC (Total, Leaf, Non-leaf and Remo) at the mid-grain filling. Seven novel favorable WSC alleles made positive individual contributions to final TGW under well-watered, drought and heat stress conditions (Table 2, Figure 2).

**Complex relationship between WSC and TGW**

WSC accumulation and remobilization are influenced by many factors, making the relationship between WSC and TGW more complex. For example, WSC remobilization is affected by N fertilizers and water deficit [6,49]. Heavy use of N fertilizers delays plant senescence and reduces the remobilization of prestored

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*Figure 2. Verification of phenotypic effects of seven novel favorable WSC alleles individually contributing to TGW in sixteen environments. E1, E3, E7, E9 and E13 were drought stress conditions, E6, E12 and E16 were well-watered and heat stress conditions, E4, E10 and E14 were drought and heat stress conditions, E2, E5, E8, E11 and E15 were well-watered conditions. Bars indicate 2SE. *, **, *** Significant at $P = 0.05$, 0.01 and 0.001, respectively.*

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### Table 2. Seven favorable WSC alleles individually contributed to significantly higher TGW.

| Locus                  | Trait        | Favorable allele (bp) | Freq. (%) | WSC Mean ± SE (mg/g dw) | P value | TGW-DS Mean ± SE (g) | P value | TGW-WW Mean ± SE (g) | P value |
|------------------------|--------------|-----------------------|-----------|-------------------------|---------|-----------------------|---------|-----------------------|---------|
| Xbarc125-3D            | Total-Low-DS | 147                   | 16.41     | 341.5 ± 6.0             | 0***    | 44.99 ± 0.61          | 0.0002***| 43.91 ± 0.62          | 0.0488* |
|                        | Others       | 83.59                 | 309.9 ± 33| 41.14 ± 0.43            |         | 41.85 ± 0.44          |         |                      |         |
|                        | Total-Low-WW | 147                   | 284.3 ± 5.5| 0***                   |         |                       |         |                      |         |
|                        | Others       | 235.9 ± 2.5           |           |                        |         |                      |         |                      |         |
| Xgwm537-7B             | Leaf-Ped-DS  | 205                   | 28.63     | 580.0 ± 3.5            | 0.0039**| 43.53 ± 0.61          | 0.0032**| 43.81 ± 0.60          | 0.0067**|
|                        | Others       | 71.37                 | 45.4 ± 2.4 | 41.06 ± 0.46            |         | 41.52 ± 0.48          |         |                      |         |
| Xgwm358-5D             | Leaf-Ste-DS  | 162                   | 30.15     | 65.4 ± 3.8             | 0.0064**| 43.43 ± 0.72          | 0.0039**| 43.94 ± 0.72          | 0.0026**|
|                        | Others       | 69.85                 | 51.6 ± 2.7 | 41.05 ± 0.44            |         | 41.43 ± 0.45          |         |                      |         |
| Xcfd5-2D               | Non-leaf-Ste-DS | 263              | 20.61     | 168.0 ± 3.4            | 0.0163**| 43.53 ± 0.75          | 0.0168**| 43.80 ± 0.76          | 0.0326* |
|                        | Others       | 79.39                 | 157.1 ± 2.1 | 41.30 ± 0.43            |         | 41.77 ± 0.44          |         |                      |         |
| Xgwm181-3B             | Non-leaf-Ste-DS | 140 and 161 | 45.42     | 163.6 ± 2.6           | 0.0377**| 43.45 ± 0.48          | 0***    | 42.91 ± 0.52          | 0.0834  |
|                        | Others       | 54.58                 | 155.9 ± 2.5 | 40.37 ± 0.54            |         | 41.58 ± 0.55          |         |                      |         |
| Xgwm389-3B             | Remo-Ste-DS  | 116                   | 19.08     | 79.4 ± 5.6             | 0.0152* | 44.10 ± 0.74          | 0.0026**| 43.95 ± 0.88          | 0.0246* |
|                        | Others       | 80.92                 | 64.5 ± 2.7 | 41.21 ± 0.43            |         | 41.76 ± 0.42          |         |                      |         |
| Xcfd17-2D              | Remo-Ped-WW  | 223                   | 24.81     | 74.1 ± 5.1             | 0.0002***| 43.31 ± 0.63          | 0.0183**| 43.75 ± 0.67          | 0.0193* |
|                        | Others       | 75.19                 | 51.7 ± 2.8 | 41.25 ± 0.46            |         | 41.67 ± 0.46          |         |                      |         |
| Remo-Low-WW            |              | 223                   | 113.3 ± 7.1| 0.0123*                |         |                       |         |                      |         |
|                        | Others       | 94.3 ± 3.6            |           |                        |         |                      |         |                      |         |
| Remo-Ste-WW            |              | 223                   | 89.7 ± 6.5| 0.0044**               |         |                       |         |                      |         |
|                        | Others       | 69.7 ± 3.3            |           |                        |         |                      |         |                      |         |

*Significant at P = 0.05; **Significant at P = 0.01; ***Significant at P = 0.001. Total, total WSC at the mid-grain filling produced by leaves and non-leaf organs; Leaf, WSC contributed by current leaf assimilation during the mid-grain filling; Non-leaf, WSC in non-leaf organs at the mid-grain filling, excluding the current leaf assimilation; Remo, WSC used for respiration and remobilization during the mid-grain filling; Ped, peduncle; Low, lower internode; Ste, whole stem; DS, drought stress; WW, well-watered; TGW, 1000-grain weight at maturity.

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assimilates; whereas water deficit performs the reverse function. Thus final TGW is often significantly increased with heavy use of N fertilizers under drought stress, even higher than that under well-watered conditions [49,50]. WSC in the stem are negatively correlated with tiller per unit area, that is, WSC accumulation is dependent on plant density [8,12]. Moreover, WSC accumulation is also affected by stem length (plant height) and stem weight. We calculated Pearson correlation coefficients between the four types of WSC at the mid-grain filling and TGW, and we also evaluated the effects of favorable WSC alleles on TGW to further understand their relationships at the genetic level. The results showed that (1) the correlations between them were significant but not robust, the highest Pearson correlation being only $r = 0.393^{***}$, and there was no relationship between WSC of Remo at the mid-grain filling and TGW; and (2) there were 7, 10, 12 and 9 favorable alleles for WSC of Total, Leaf, Non-leaf and Remo, respectively. 

**Figure 3.** Linear regressions of TGW based on seven favorable WSC alleles in sixteen environments (A), and accumulation of seven favorable WSC alleles in modern varieties released in different decades (B). There were 18, 27, 34, 39, 58 and 51 accessions released in Pre-1960, 1960s, 1970s, 1980s, 1990s and Post-2000, respectively. 15 accessions with unknown released decades were excluded. doi:10.1371/journal.pone.0102917.g003
Table 3. Pyramiding of favorable WSC alleles contributing to TGW in sixteen environments.

| Env. | No of alleles | TGW Mean ± SE (%) | Fre. (%) | Env. | No of alleles | TGW Mean ± SE (%) | Fre. (%) |
|------|---------------|--------------------|----------|------|---------------|--------------------|----------|
| E1   | ≥4            | 39.17±0.75 (A)     | 13.73    | E9   | ≥4            | 41.38±0.65 (A)     | 13.46    |
| (DS) | 3             | 38.47±0.92 (A)     | 16.08    | (DS) | 3             | 41.25±0.57 (A)     | 16.92    |
|      | 2             | 37.48±0.62 (AB)    | 27.06    |      | 2             | 40.34±0.54 (AB)    | 26.54    |
|      | 1             | 35.79±0.67 (BC)    | 23.14    |      | 1             | 38.62±0.72 (B)     | 23.08    |
|      | 0             | 33.94±0.81 (C)     | 20.00    |      | 0             | 36.69±0.71 (C)     | 20.00    |
| E2   | ≥4            | 43.67±1.09 (A)     | 12.94    | E10  | ≥4            | 35.90±0.67 (A)     | 13.46    |
| (WW) | 3             | 42.01±0.80 (AB)    | 16.86    |      | 3             | 35.17±0.62 (AB)    | 16.92    |
|      | 2             | 41.82±0.70 (AB)    | 27.06    |      | 2             | 33.57±0.54 (B)     | 26.54    |
|      | 1             | 39.72±0.92 (B)     | 23.92    |      | 1             | 31.75±0.61 (C)     | 23.08    |
|      | 0             | 36.17±1.04 (C)     | 19.22    |      | 0             | 30.30±0.67 (C)     | 20.00    |
| E3   | ≥4            | 45.17±0.70 (A)     | 13.41    | E11  | ≥4            | 42.35±0.83 (A)     | 13.51    |
| (DS) | 3             | 45.10±0.61 (A)     | 16.48    |      | 3             | 41.77±0.71 (A)     | 16.60    |
|      | 2             | 43.33±0.62 (AB)    | 26.82    |      | 2             | 40.68±0.56 (A)     | 26.64    |
|      | 1             | 41.40±0.76 (BC)    | 23.37    |      | 1             | 38.43±0.79 (B)     | 23.17    |
|      | 0             | 40.16±0.81 (C)     | 19.92    |      | 0             | 37.91±0.68 (B)     | 20.08    |
| E4   | ≥4            | 43.66±0.89 (A)     | 13.13    | E12  | ≥4            | 40.54±0.68 (A)     | 13.46    |
| (DS+HS) | 3          | 43.67±0.70 (A)     | 16.99    |      | 3             | 40.92±0.64 (A)     | 16.92    |
|      | 2             | 41.15±0.67 (B)     | 26.25    |      | 2             | 37.81±0.63 (B)     | 26.54    |
|      | 1             | 39.82±0.80 (B)     | 23.55    |      | 1             | 34.38±0.69 (C)     | 23.08    |
|      | 0             | 36.95±0.77 (C)     | 20.08    |      | 0             | 33.69±0.61 (C)     | 20.00    |
| E5   | ≥4            | 47.18±0.82 (A)     | 13.03    | E13  | ≥4            | 38.93±0.95 (A)     | 13.62    |
| (WW) | 3             | 46.76±0.63 (AB)    | 16.86    |      | 3             | 38.71±0.71 (A)     | 16.73    |
|      | 2             | 44.66±0.63 (BC)    | 26.82    |      | 2             | 36.89±0.67 (AB)    | 26.46    |
|      | 1             | 42.82±0.83 (CD)    | 23.37    |      | 1             | 35.22±0.68 (B)     | 22.96    |
|      | 0             | 41.01±0.82 (D)     | 19.92    |      | 0             | 34.91±0.73 (B)     | 20.23    |
| E6   | ≥4            | 44.21±1.00 (A)     | 12.85    | E14  | ≥4            | 38.77±0.93 (A)     | 13.62    |
| (WW+HS) | 3          | 44.44±0.71 (A)     | 16.06    |      | 3             | 38.28±0.71 (A)     | 16.73    |
|      | 2             | 42.49±0.70 (AB)    | 26.91    |      | 2             | 35.98±0.68 (B)     | 26.46    |
|      | 1             | 40.45±0.81 (B)     | 24.10    |      | 1             | 33.72±0.69 (C)     | 22.96    |
|      | 0             | 37.55±0.85 (C)     | 20.08    |      | 0             | 32.37±0.62 (C)     | 20.23    |
| E7   | ≥4            | 45.04±0.70 (A)     | 13.46    | E15  | ≥4            | 44.32±0.78 (A)     | 13.67    |
| (DS) | 3             | 44.41±0.61 (A)     | 16.92    |      | 3             | 44.26±0.67 (A)     | 16.80    |
|      | 2             | 42.36±0.59 (B)     | 26.54    |      | 2             | 42.57±0.63 (A)     | 26.17    |
|      | 1             | 39.68±0.74 (C)     | 23.08    |      | 1             | 40.21±0.70 (B)     | 23.05    |
|      | 0             | 37.22±0.82 (D)     | 20.00    |      | 0             | 39.08±0.79 (B)     | 20.31    |
respectively. However, only seven favorable WSC alleles exhibited positive individual contributions to TGW. The complex relationship between WSC and TGW due to many influential factors may help us to understand the reasons for these results. In addition, cutting spikes or removing leaves at flowering change the source-sink relationship during grain filling and therefore the four types of WSC at the mid-grain filling might not fully reflect the situation under normal condition.

Seven favorable WSC alleles will help to improve breeding progress in yield potential

Broad-sense heritability of WSC is relatively high, but shows wide fluctuations under different conditions, i.e. WSC are very sensitive to environments [11,23]. Yang et al. [23] reported that (1) QTL for WSC accumulation and remobilization could have different expression patterns at different growth stages or in different environments; and (2) 7 of 10 significantly additive QTL for WSC interacted with environment. Thus, stable molecular markers for WSC are essential to understand its genetic basis. Moreover, exploration of favorable WSC alleles in germplasm resources could be useful to plant breeders, but the effectiveness of such alleles needs to be verified [29,51]. In this study, seven favorable WSC alleles significantly ($P<0.05$) enhanced TGW on an individual basis (Table 2). An additive QTL for WSC, QSwscf.cgb-2D.1 (WMC453.1–WMC18), was detected in a Hanxuan 10×Lumai 14 doubled haploid population [23]. Xcfd17-2D was 1.5 cM from the flanking marker Xwmc18-2D. QReswc.cgb-3B, controlled WSC and remobilization efficiency, and Xgwm181-3B shares one of its flanking markers (Xgwm547–Xgwm181). Adjacent chromosome intervals, such as QAeswc.cgb-3B.1, QSwscm.cgb-3B.1 and QSwscf.cgb-3B, carry QTL for WSC and its accumulation efficiency [23]. In addition, Xcfd53-2D, Xgwm389-3B and Xgwm537-7B were associated with yield-related traits [49–54]. Xbarc125-3D was also associated with TGW (our unpublished data).

The seven favorable WSC alleles for enhancing TGW were verified under 16 environments (5 drought stress conditions, 3 well-watered and heat stress conditions, 3 drought and heat stress conditions, and 3 well-watered conditions) using a population of 262 winter wheat accessions (Table 2 and Figure 2). Many studies show that marker-based strategies of gene pyramiding are effective [27,55,56]. A dosage effect of pyramiding seven favorable WSC alleles (Table 3, Figure 3A) was also demonstrated in the study. The accumulation of favorable WSC alleles over different decades also indicated that they had been individually selected by breeders in the past and that there is potential for further improvement in the future.

Supporting Information

Figure S1 The percentage contributions of leaf and non-leaf organs to 1000-grain weight (TGW) under drought stress (DS) and well-watered (WW) conditions during grain filling. Bars indicate 2SE. The data in the columns were the absolute values of TGW (g).

Table S1 262 common wheat accessions and their origins.

Table S2 Statistic data of WSC (mg/g dw) at the mid-grain filling under well-watered and drought stress conditions.
Table S3  Statistic data of TGW under well-watered and drought stress conditions.  
(XLSX)

Table S4  Thirteen loci significantly associated with the total WSC at the mid-grain filling produced by leaves and non-leaf organs (Total) and phenotypic values of favorable marker alleles under two water regimes.  
(XLSX)

Table S5  Twenty-three loci significantly associated with the mid-grain filling (Leaf) and phenotypic values of favorable marker alleles under two water regimes.  
(XLSX)

Table S6  Twenty-three loci significantly associated with the WSC in non-leaf organs at the mid-grain filling (excluding the current leaf assimilation, Non-leaf) and phenotypic values of favorable marker alleles under two water regimes.  
(XLSX)

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