Gene editing of the wheat homologs of TONNEAU1-recruiting motif encoding gene affects grain shape and weight in wheat

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Received 1 April 2019; revised 2 June 2019; accepted 10 June 2019; published online 20 June 2019.

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SUMMARY

Grain size and weight are important components of a suite of yield-related traits in crops. Here, we showed that the CRISPR-Cas9 gene editing of TaGW7, a homolog of rice OsGW7 encoding a TONNEAU1-recruiting motif (TRM) protein, affects grain shape and weight in allohexaploid wheat. By editing the TaGW7 homoeologs in the B and D genomes, we showed that mutations in either of the two or both genomes increased the grain width and weight but reduced the grain length. The effect sizes of mutations in the TaGW7 gene homoeologs coincided with the relative levels of their expression in the B and D genomes. The effects of gene editing on grain morphology and weight traits were dosage dependent with the double-copy mutant showing larger effect than the respective single copy mutants. The TaGW7-centered gene co-expression network indicated that this gene is involved in the pathways regulating cell division and organ growth, also confirmed by the cellular co-localization of TaGW7 with α- and β-tubulin proteins, the building blocks of microtubule arrays. The analyses of exome capture data in tetraploid domesticated and wild emmer, and hexaploid wheat revealed the loss of diversity around TaGW7-associated with domestication selection, suggesting that TaGW7 is likely to play an important role in the evolution of yield component traits in wheat. Our study showed how integrating CRISPR-Cas9 system with cross-species comparison can help to uncover the function of a gene fixed in wheat for allelic variants targeted by domestication selection and select targets for engineering new gene variants for crop improvement.

Keywords: CRISPR-Cas9, gene editing, grain size and weight, TaGW7, Triticum aestivum, wheat yield component.

INTRODUCTION

Understanding the genetic basis of yield component traits in major crops holds great promise to improve yield potential by allowing breeders to make informed decisions while assembling beneficial alleles to create improved varieties. Yield component traits affecting grain size, shape and weight have been the subject of extensive genetic studies in a number of crops aimed to identify genes controlling these traits (Song et al., 2007; Takano-Kai et al., 2009; Segami et al., 2012; Wang et al., 2012; Wang et al., 2015a; Xie et al., 2018; Wang et al., 2018b). Recently, several genes contributing to grain size and/or weight including TaGW2 (Wang et al., 2018a,b), TaCKX2 (Zhang et al., 2012a), TaGASR7 (Zhang et al., 2016), TaTGW-7A (Hu et al., 2016), and TaGW8 (Yan et al., 2019) have been identified providing insights into the complexity of biological pathways that underlie regulation of grain size and weight in wheat. Many genes identified in wheat have rice homologs with similar function indicating that these developmental pathways are conserved among related grass species (Li and Li, 2016; Li et al., 2017).

The natural variation in the promoter of the OsGW7/OsGL7 gene or its copy number in rice was shown to be associated with changes in grain morphology, grain quality and yield (Wang et al., 2015a,b). The highly expressed variant of this gene was linked with more slender grain, improved quality, reduced endosperm chalkiness, and higher yield, while the lowly expressed variant resulted in lower quality and shorter grain. OsGW7 encodes a
homolog of the TONNEAU1-recruiting motif (TRM1) protein, a part of the complex involved in pre-prophase band formation evolved to control oriented cell division during the formation of complex tissues (Hashimoto, 2015). Developmental pathways that affect morphological diversity of plant organs through the interaction with TRM appear to be common in plants. For example, it was shown that the interaction of the OVATE family proteins with TRM regulates cell division and alters fruit shape in tomato, and possibly in melon and cucumber (Wu et al., 2018). The high level of cross-species conservation of pathways controlling grain morphology and weight (Li et al., 2019) suggests that the homologs of TRM in wheat might also be involved in biological pathways defining similar traits. Currently, the roles played by the OsGW7 gene homologs in defining grain morphology and weight traits in wheat are not known.

In wheat, CRISPR-Cas9 gene editing was successfully applied to modify such traits of agronomic value as disease resistance (Wang et al., 2014), gluten content (Sánchez-León et al., 2018), protein content (Zhang et al., 2018), male sterility (Okada et al., 2019) and grain size and weight (Wang et al., 2018b; Zhang et al., 2018, 2019). These studies also demonstrated that the editing of either individual copies of duplicated genes on homoeologous chromosomes or all three homoeologous copies of genes is possible (Wang et al., 2014, 2018a). For relatively low gene editing efficiency, mutants carrying editing events in multiple homoeologous gene targets can be obtained in the later generations of transgenic plants (Wang et al., 2018a). Here, we used CRISPR-Cas9 gene editing to produce single- and double-gene knockouts of TaGW7, and to study the effects of these mutations on grain morphometric and weight traits. To better understand the role of TaGW7 in the regulation of biological pathways controlling these traits in wheat, we constructed the TaGW7-centered gene co-expression network. The evolutionary dynamic of the TaGW7 gene region during wheat domestication and improvement was studied by analyzing the SNP diversity in a diverse panel of 818 accessions of hexaploid and tetraploid wheat (He et al., 2019).

RESULTS

Identification of the OsGW7 gene orthologs in wheat

We identified three wheat orthologs of the OsGW7 gene, TraesCS2A01G176000, TraesCS2B01G202300, and TraesCS2D01G183400, that will be referred to as TaGW7-A1, TaGW7-B1, and TaGW7-D1, respectively. The OsGW7 orthologs in the ancestral species, tetraploid wild emmer Triticum turgidum ssp. dicoccoides (TRIDC2AG022470, TRIDC2BG025740) and diploid goatgrass Aegilops tauschii (AET2Gv20367100) will be referred to as TtGW7-A1, TtGW7-B1, and AetGW7-D1. The TaGW7-A1, TaGW7-B1, and TaGW7-D1 genes each has five exons (Figure 1a) and encode proteins of 958, 950, and 954 amino acids in length, respectively (Figure S1). Compared with TaGW7-A1, a protein sequence encoded by TaGW7-B1 has six amino acids deleted between position 48 and 53, and two amino acids deleted between positions 506 and 507. TaGW7-D1 differs from TaGW7-A1 by one deletion, four amino acids long, between positions 50 and 53. In addition to these deletions, we found 32 amino acid differences (3.4%) between TaGW7-A1 and TaGW7-B1, 25 amino acid differences (2.6%) between TaGW7-A1 and TaGW7-D1, and 23 amino acid differences (2.4%) between TaGW7-B1 and TaGW7-D1. The phylogenetic analysis of the OsGW7 gene orthologs from hexaploid wheat, wild emmer, and Ae. tauschii showed clustering of sequences into three groups, each including the TaGW7 homoeologs along with the homoeologs from the ancestral species (Figure 1b).

We have re-sequenced the TaGW7 gene homoeologs in cv. Bobwhite, which was used for the CRISPR-Cas9 gene editing. Intercultivar comparison revealed several single-base mutations differentiating the TaGW7 gene homoeologs between cultivars Chinese Spring and Bobwhite. The TaGW7-A1 had one synonymous mutation T735C, and TaGW7-B1 had one synonymous mutation A411G and three nonsynonymous mutations T217A, C1594T, and G2418T. These nonsynonymous mutations result in amino acid replacements C73S, R532C, and K806N (Figures 1a and S1). The TaGW7-D1 gene sequence was identical in both cultivars.

CRISPR-Cas9 editing of the TaGW7 genes in wheat

In total, eight CRISPR-Cas9 target sites were selected within the first three exons of the TaGW7 gene (Figure 1a). Seven target sites were conserved among the three homoeologs, whereas the GW7T4 target was conserved only between TaGW7-B1 and TaGW7-D1. The gene editing efficiency of each gRNA target was assessed by the transient expression of the pA9Cas9sg constructs in the wheat protoplasts and next generation sequencing (NGS) of each target site (Wang et al., 2018a). These analyses showed that most of the targets could be edited by the designed CRISPR-Cas9 constructs. With the protoplast transformation efficiency of ~30%, target site GW7T6 showed the highest level of editing efficiency of 8.3%, followed by GW7T9 and GW7T2, which showed the editing efficiency of 1.2% and 1.1%, respectively. The gRNA target sites GW7T2 and GW7T6 were selected for TaGW7 gene editing (Figure 1a, Tables S1 and S2).

Two constructs, pA9Cas9sg-GW7T2 and pA9Cas9sg-GW7T6, were pooled in equimolar proportions and co-transformed with pAH20 into the immature embryos of cv. Bobwhite. Out of 15 regenerated plants, nine plants carrying both Cas9 and at least one of the two gRNAs were further analyzed by NGS. Out of these CRISPR-Cas9
positive plants, target site mutations were detected only in plant YLD2-5-1. The line YLD2-5-1 contained low frequency mutated reads at target site GW7T6 in all three homoeologs of TaGW7 (Figure 1c). By analyzing 70 plants from the T1 generation of YLD2-5-1 line, two plants heterozygous for the edited variants of the TaGW7 gene were detected; the remaining plants were null for the mutations. The T1 line YLD2-5-1-41 carried mutations in the TaGW7-B1 gene, and line YLD2-5-1-52 had mutations in both the B and D genome copies of TaGW7 (Figure 1c). All detected mutations caused frame shifts in the coding sequence (Figure 1c).

Effects of the TaGW7 gene editing on grain size and weight

The effects of CRISPR-Cas9-induced mutations in the TaGW7 gene on grain morphometric traits (grain width, grain length, and grain area) and thousand grain weight (TGW) were evaluated in the T2 generation plants from YLD2-5-1-41 and YLD2-5-1-52. Preliminary evaluation was performed using a small sample of plants including seven and 37 plants of YLD2-5-1-41 and YLD2-5-1-52 T2 lines, respectively. The comparison of TGW and grain morphometric traits between Bobwhite and mutant lines with a homozygous mutation in the TaGW7-B1 gene (genotype AABBDD vs. genotype AAbbDD, where ’b’ corresponds to the mutated allele in B genome), showed significant increase in grain width in the mutants (Figure S2). Because of the limited number of plants with genotypes AABBdd and AAbbdd, we compared phenotyping data collected for plants with the AABbdd and AAbbDD genotypes to estimate the effects of mutations in TaGW7-D1. This comparison showed significant decrease in grain length in the plants homozygous for mutation in TaGW7-D1 (Figure S2). The phenotypic effects of mutations in TaGW7-B1 and TaGW7-D1 were further evaluated in a small population of plants from the T3 generation of YLD2-5-1-52 (Figure S3). In this population, the homozygous TaGW7-D1 mutants showed significantly increased grain width compared with that of the homozygous and heterozygous TaGW7-B1 mutants (Figure S3). The grain length in the TaGW7-B1 and TaGW7-D1 double-gene mutants (genotype AABBdd) was significantly decreased compared with that of the TaGW7-B1 mutants (genotype AAbbDD) (Figure S3).

To validate these results, larger populations from the T3 generation of YLD2-5-1-41 and YLD2-5-1-52 were evaluated for the kernel size and weight traits. To minimize the possible effects of plant regeneration from the immature
embryos on the grain size and weight traits, the phenotypic comparisons between the wild-type and mutant genotypes were performed only between those T3 plants that were derived from the same T1 plant. From this point forward, plants with a wild-type genotype derived from transgenic plants will be referred to as ‘t-wild-type’. Among the T3 plants derived from both YLD2-5-1-41 and YLD2-5-1-52 lines, the grains were significantly wider (1.9–2.2%) in the TaGW7-B1 mutants than in the nonmutated T3 plants (Tables 1 and 2, Figures 2 and S4). Comparison of grain width between the heterozygous and homozygous TaGW7-B1 mutants appears to show the dosage effect of mutations on grain width in the YLD2-5-1-41 family (Table 1, Figure S4). The lack of changes in grain length in the TaGW7-B1 mutants suggested that the grain width contributed most to the grain area increase in the YLD2-5-1-41 progeny.

Among YLD2-5-1-52 T3 plants, compared with t-wild-type, mutations in the TaGW7-D1 gene resulted in significantly increased grain width (2.2%) and significantly decreased grain length (2.1%) (Table 2 and Figure 2) without impact on grain area. While statistically nonsignificant, mean TGW was increased in the single genome mutants of both TaGW7-B1 and TaGW7-D1 compared with t-wild-type plants (Table 2). However, statistically significant increases observed for TGW in the TaGW7-B1 mutants from YLD2-5-1-41 family suggested that the lack significant TGW change in the YLD2-5-1-52-derived T3 mutants is likely to be associated with insufficient statistical power (Table 1). In the two independent experiments including the YLD2-5-1-52 T3 lines, the TaGW7-D1 mutants always had wider and shorter grains, and higher TGW compared with the TaGW7-B1 mutants, indicating that the TaGW7 gene mutations in the D genome have stronger phenotypic effects than mutations in the B genome (Table 2, Figures 2 and S3).

The dosage-dependent effects of mutations in TaGW7-B1 and TaGW7-D1 were supported by a more substantial increase in grain width and decrease in grain length in the double-genome mutants than in the single genome mutants (Figures 2 and S3, Table 2). The higher TGW in the double-gene mutants (3.16%) than in the single gene mutants (1.78% and 2.9%) also supported the dosage effect of mutations in the TaGW7 gene homoeologs (Table 2). To further confirm these results, 116 T3 plants were grouped based on the number of nonmutated copies of the TaGW7 gene. With the increase in the functional TaGW7 copy number, the grain width and TGW decreased and grain length increased (Figure 3), but change in grain area was not obvious.

**TaGW7 gene expression pattern in wild-type and mutant plants**

To better understand the phenotypic effects of the TaGW7 mutants, the expression levels of TaGW7 homoeologs in Bobwhite were investigated using quantitative RT-PCR (Figure S5). The expression of the TaGW7 homoeologs was substantially higher in nonpollinated young spikes than in other tissues. The D genome copy of the gene expressed at the higher levels than other two homoeologs in nonpollinated young spikes and grain (Figure 4a). These results are consistent with the expression levels of the TaGW7 homoeologs in cultivars Chinese Spring and Azhurnaya assessed by RNA-seq analyses (Figure S6) (Ramirez-González et al., 2018). The expression levels of the TaGW7 homoeologs in the other tissues were lower and did not show significant differences among the genomes. These results suggested the likely association between the higher expression level and the stronger phenotypic effect for the TaGW7 gene homoeologous copies.

The expression level of TaGW7 was also evaluated in the plants carrying gene editing mutations in the B and D genomes (Figure 4b). These analyses revealed a substantial reduction of transcripts generated from the edited copies of the TaGW7 gene, which agrees with TaGW2 gene expression pattern from our previous study (Wang et al., 2018b).

**Gene co-expression networks associated with TaGW7**

To better characterize the TaGW7-associated biological pathways in wheat, we have constructed TaGW7-centered gene networks (Figure 5a). The expression data from 14

### Table 1 The TGW and grain morphometric traits in the T3 generation plants derived from YLD2-5-1-41

| Genotype       | N  | Width (mm)       | Length (mm)      | Area (mm²)     | TGW (g)      |
|----------------|----|-----------------|-----------------|----------------|--------------|
| AABBDD         | 9  | 3.62 ± 0.033    | 6.16 ± 0.056    | 16.09 ± 0.27   | 40.32 ± 0.88 |
| AAbbDD         | 21 | 3.65 ± 0.031    | 6.19 ± 0.030    | 16.37 ± 0.10   | 41.33 ± 0.76 |
| AAbbDD         | 13 | 3.7 ± 0.023     | 6.23 ± 0.038    | 16.64 ± 0.17   | 42.31 ± 0.69 |

*The TGW and grain morphometric measurements for T3 plants derived from YLD2-5-1-41 are shown as mean ± standard error. N is number of plants. The phenotype data of each genotype were compared with that of genotype AABBDD using two-tailed Student’s t-test; *significant at 0.01 < P < 0.05. The value increase compared with the t-wild-type genotype ‘AABBDD’ is shown as a percentage.*

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RNA-seq transcriptome profiling experiments (Ramírez-González et al., 2018), were used to measure pair-wise expression level correlation between TaGW7 and other genes in the expression datasets (Table S3). Genes showing a Pearson correlation coefficient (PCC) above 0.6 and in the top 1% of PCC value distribution for all pair-wise comparisons, were analyzed for functional enrichment in different GO terms. Enriched GO terms include microtubule-based movement (GO:0007018), regulation of mitotic cell cycle (GO:0007346), regulation of mitotic nuclear division (GO:0007088), auxin transport (GO:0060918), regulation of developmental growth (GO:0040008), and cell wall biogenesis (GO:0042546) (Figure 5a, Tables 3 and S4). Co-expression of TaGW7 along with these genes is suggestive of its involvement in the control of cell division and developmentally regulated organ growth in response to auxin signaling. These findings are consistent with the previous studies that showed the association of TONNEAU1-recruiting motif proteins with protein phosphatase 2A and TONNEAU1 forming the pre-prophase band (Spinner et al., 2013).

The validity of TaGW7-associated co-expression network is corroborated by the substantial overlap of the networked genes with the protein interacting partners identified for OsGW7 in rice (Wang et al., 2015a). Out of 18 rice proteins that were shown to interact with OsGW7 in yeast control of cell division and developmentally regulated organ growth in response to auxin signaling. These findings are consistent with the previous studies that showed the association of TONNEAU1-recruiting motif proteins with protein phosphatase 2A and TONNEAU1 forming the pre-prophase band (Spinner et al., 2013).

The validity of TaGW7-associated co-expression network is corroborated by the substantial overlap of the networked genes with the protein interacting partners identified for OsGW7 in rice (Wang et al., 2015a). Out of 18 rice proteins that were shown to interact with OsGW7 in yeast

Table 2 The TGW and grain morphometric traits in the T3 generation plants derived from YLD2-5-1-52

| Genotype   | N  | Width (mm) | Length (mm) | Area (mm²) | TGW (g)  |
|------------|----|------------|-------------|------------|----------|
| AABBDD     | 26 | 3.67 ± 0.017 | 6.27 ± 0.033 | 16.67 ± 0.14 | 42.72 ± 0.48 |
| AABBdd     | 7  | 3.74 ± 0.028 | 6.29 ± 0.066 | 16.92 ± 0.24 | 43.48 ± 1.03 |
| AABBdd     | 6  | 3.75 ± 0.032 | 6.14 ± 0.057 | 16.73 ± 0.25 | 43.96 ± 0.72 |
| AABBdd     | 47 | 3.76 ± 0.012 | 6.12 ± 0.021 | 16.86 ± 0.09 | 44.07 ± 0.35 |
| ANOVA (P-value) | | 0.00032 | 1.88e-05 | 0.32 | 0.13 |

All data are shown as mean ± standard error. N is the number of plants. The phenotype data of each genotype were compared with that of genotype AABBDD using the Student’s t-test; *significant at 0.01 < P < 0.05, ****significant at P < 0.0001. The value increase relative to the t-wild-type genotype ‘AABBDD’ is shown as a percentage.
two-hybrid assay, seven had wheat orthologs that were part of the TaGW7-associated network (Table S5).

To further investigate the involvement of TaGW7 into the control of cell division, we determined its cellular localization relative to α- and β-tubulin, the building blocks of microtubule arrays (Hashimoto, 2015). We found that the TaGW7 gene co-expresses with the β-tubulin gene (PCC ≥ 0.6) (Figure S7, Table S3). The TaGW7-A1, TaGW7-B1, and TaGW7-D1 proteins fused with yellow fluorescent protein (eYFP) (Figure S7), were transiently co-expressed in the wheat protoplasts along with α- and β-tubulin genes fused with mRFP (Figure 5b). The fluorescence signals from the TaGW7 fusion proteins co-localized with α- and β-tubulin-eYFP signals suggest that these proteins occupy the same cellular compartments (Figure 5b).

Evolutionary history of the TaGW7 genes in wheat

To better understand the evolution of the TaGW7 gene locus, we performed the analyses of SNP diversity in a set of 818 hexaploid and tetraploid wheat accessions including 34 accessions of wild emmer, 40 accessions of domesticated emmer, 286 landraces and 458 cultivars of hexaploid wheat

Figure 4. Expression levels of the TaGW7 gene homoeologs in (a) cv. Bobwhite, and (b) gene-edited mutants. The quantitative RT-PCR of the TaGW7 gene homoeologs was conducted using genome-specific primers (Figure S1) using actin as reference. Expression data for the A, B and D genome gene copies is represented by different shades of gray. Relative expression levels are shown as means ± SEM based on five (a) or three (b) biological replicates. In (a), different letters above the bars indicate significant differences (P < 0.05) using Tukey’s multiple-comparison test following one-way ANOVA. In (b), spikes at the stage when half of the spike emerged from the flag leaf sheath were sampled from the T1 generation TaGW7 mutants or t-wild-type plants segregated from the same T2 mutant line. The expression levels in mutants was compared with t-wild-type plants using t-test; ‘*’ and ‘**’ stand for significance levels of 0.05 and 0.01, respectively.

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Population differentiation XP-CLR test (Chen et al., 2010) applied to tetraploid wild and domesticated emmer wheat showed that the TtGW7-A1 gene, the ortholog of OsGW7 in wild emmer, is located in the middle of domestication selective sweep region on chromosome 2A (Figure 6). This region also showed the reduction of genetic diversity \( (p) \) in domesticated emmer and bread wheat and increased genetic differentiation \( (F_{ST}) \) between wild and domesticated wheat (Figure 6). These results are consistent with strong selection at the TtGW7-A1 gene region during domestication. While we did observe some increase in the level of \( F_{ST} \) between wild and domesticated emmer near the TtGW7-B1 gene on homoeologous chromosome 2B, this genic region did not show elevated XP-CLR values consistent with strong selective sweep (Figure 6).

Using the same exome capture data, we further evaluated the haplotypic diversity of the TaGW7 genes in the populations of hexaploid wheat landraces and cultivars, and tetraploid wild and domesticated emmer (He et al., 2019). We have extracted SNPs from the genic regions (introns-exons) of the TaGW7 gene homologs in the A, B, and D genomes (Table S6). In the A genome of hexaploid wheat, we have identified five haplotypes (Ha1–Ha5) of the TaGW7 gene (Table 4, Table S6).

Both cultivars Bobwhite and Chinese Spring had haplotype H1a. The analysis of hexaploid wheat showed that H1a is the most abundant haplotype present at the frequency of 84.3% and 85.0% in cultivars and landraces, respectively. The frequency of remaining haplotypes was different between wheat landraces and cultivars. Compared with landraces, the H2a haplotype in cultivars increased in frequency from 1.2% to 10.7%, whereas haplotype H3a decreased in frequency from 12.6% to 4.7%. Haplotype H4a was not identified in landraces, whereas haplotype H5a was present at a frequency of 1.2% in landraces, but was not detected in cultivars. The analysis of domesticated emmer wheat population, the direct ancestor of the hexaploid wheat's A and B genomes (Avni et al., 2017; He et al., 2019), also identified only one haplotype, H1a. In the wild emmer wheat population, haplotype H1a was present at significantly lower frequency (19.4%) than in domesticated emmer (Fisher’s exact two-tailed \( P \)-value <0.0001). The most common haplotype H5a (45.2%) from wild emmer was not detected in the analyzed sample of...
domesticated emmer, but was found at low frequency (1.2%) in wheat landraces (Table 4). These findings suggested that the H1a haplotype was one of the likely targets of domestication selection and was inherited by bread wheat from domesticated emmer.

The analysis of the TaGW7-B1 gene diversity in cultivated bread wheat identified four haplotypes H1b, H2b, H3b, and H4b with the frequencies of 57.3%, 38.4%, 1.1% and 3.1%, respectively (Tables 4 and S6). The reference cultivar Chinese Spring and cultivar Bobwhite had common haplotypes H1b and H2b, respectively. In the population of landraces, the H4b haplotype was missing, and H1b, H2b, and H3b were present at the frequencies of 70.9%, 26.5%, and 2.6%, respectively. Even though both common H1b and H2b haplotypes were detected in the wild and domesticated emmer wheat (Table 4), we observed significant differences in their frequency (Fisher’s exact two-tailed P-value < 0.0001). The H1b haplotype found in 13.3% of wild emmer accessions was detected in nearly 70% of domesticated emmer accessions, suggesting that H1b was the possible target of domestication selection.

The fact that both H1b and H2b haplotypes are found in both wild and domesticated emmer populations might be one of the reasons why XP-CLR and FST tests did not show the evidence of substantial allele frequency differentiation in the TaGW7-B1 region (Figure 6 and Table S6).

Out of two haplotypes identified for the TaGW7-D1 gene, the H1d haplotype was nearly fixed and found in 99.1% of

| GO accession | GO description                        | Adjusted P-valuea | Fold changeb |
|--------------|---------------------------------------|-------------------|--------------|
| GO:0007018   | Microtubule-based movement            | 7.91E-35          | 14.9         |
| GO:0006918   | Auxin transport                       | 1.26E-19          | 26.8         |
| GO:00040008  | Regulation of growth                  | 2.40E-19          | 20.3         |
| GO:0000226   | Microtubule cytoskeleton organization | 3.74E-19          | 7.4          |
| GO:0010411   | Xyloglucan metabolic process          | 1.65E-19          | 16.7         |
| GO:0049638   | Regulation of developmental growth     | 5.44E-16          | 20.5         |
| GO:0042546   | Cell wall biogenesis                  | 5.20E-15          | 10.4         |
| GO:0007346   | Regulation of mitotic cell cycle       | 1.68E-14          | 6.8          |
| GO:0010109   | Regulation of photosynthesis           | 1.94E-11          | 13.2         |
| GO:0045489   | Pectin biosynthetic process            | 4.81E-11          | 28.0         |
| GO:0051783   | Regulation of nuclear division         | 5.10E-11          | 11.0         |
| GO:0010052   | Guard cell differentiation             | 3.58E-10          | 230.7        |
| GO:0009958   | Positive gravitropism                 | 1.69E-09          | 24.3         |
| GO:0034219   | Carbohydrate transmembrane transport   | 2.27E-09          | 5.0          |

aFisher’s exact test P-values were adjusted using the Benjamini–Hochberg correction.
bFold change in the representation of genes from a given GO term in the TaGW7-associated network compared with the genome-wide estimates.

Figure 6. Patterns of genetic diversity and differentiation on chromosomes 2A and 2B. Selective sweeps on chromosomes 2A and 2B associated with wheat domestication were identified using the XP-CLR scans performed using wild emmer (WE) as the reference population and domesticated emmer (DE) as the target population. Average pair-wise genetic differentiation (FST) and average pair-wise diversity per SNP site (π) in the corresponding regions in the populations of wheat, WE and DE was estimated using SNPs in 2-Mb sliding windows. FST was measured between wild and domesticated emmer (FST (WE-DE)) and between WE and wheat (FST (WE-Wheat)). The positions of the OsGW7 gene orthologs are shown by the vertical dashed red lines. [Colour figure can be viewed at wileyonlinelibrary.com]
accessions, including cv. Chinese Spring and cv. Bobwhite (Table 4, Table S6). This major haplotype was identical to the one in the reference genome of Ae. tauschii, the diploid wild relative of the D genome (Luo et al., 2017).

**DISCUSSION**

Early comparative analyses of the related plant species revealed parallelism in the variation of their morphological and physiological traits (Vavilov, 1922). Numerous genetic studies have shown that many of these traits have a common genetic basis. With the high-density genetic maps and reference genome sequences available for multiple crops, this strategy of using comparative genomics for understanding the genetic basis of trait control in related crops appeared to be effective and allowed to reduce time and efforts needed to identify causal genes. During the last several years, several genes controlling grain size and weight identified in rice were shown to underlie similar traits in wheat (Zhang et al., 2012a; Dong et al., 2014; Wang et al., 2018b; Zhang et al., 2019). Here, we characterized the wheat TaGW7 gene, the ortholog of the OsGW7 gene in rice. Its downregulation in rice correlated with the production of shorter and wider grains (Wang et al., 2015a). Our study demonstrated that the gene-edited alleles of TaGW7 showed phenotypic effects similar to that observed in rice, and produce wider and shorter wheat grain. However, mutants with two copies of edited TaGW7 in wheat increased TGW (~3.2%), which was not observed in a rice line with the down-regulated OsGW7 (Wang et al., 2015a). It is possible that even though the genetic architecture of grain dimension traits in wheat and rice tends to be conserved, the phenotypic effects of gene perturbations in related species can sometimes be different due to interactions with the divergent species-specific pathways.

Variation among genes in the relative contribution of individual homoeologs to the total expression level of a homoeologous gene set is a common phenomenon in wheat (Akhunova et al., 2010; Ramírez-González et al., 2018). However, until recently the role of this factor in the contribution of gene homoelogs to trait variation received little attention. Our earlier study showed that the effect sizes of mutations in the individual TaGW2 gene homoeologs correlated well with their levels of expression relative to other gene copies (Wang et al., 2018b). While we did not investigate the phenotypic effects of mutations in the TaGW7-A1 gene, it appears that TaGW7, similar to TaGW2, also showed similar relationships between the effect sizes of mutations and the expression levels of individual homoeologs. Interestingly, in both genes, the edited copy in the D genome of cultivar Bobwhite contributed the most to trait variation and gene expression, indicating that the merger with the D genome ancestor during bread

| Gene in wheat | Species | Improvement status | Number of accessions | Haplotype | Frequency |
|---------------|---------|--------------------|---------------------|-----------|-----------|
| TaGW7-A1      | T. aestivum ssp. aestivum | Cultivars | 306 | H1a | 0.843 |
|               |         |                    | 39 | H2a | 0.107 |
|               |         |                    | 17 | H3a | 0.047 |
|               |         |                    | 1 | H4a | 0.003 |
|               |         | Landraces | 210 | H1a | 0.050 |
|               |         |                    | 3 | H2a | 0.012 |
|               |         |                    | 31 | H3a | 0.126 |
|               |         |                    | 3 | H5a | 0.012 |
|               | T. turgidum ssp. dicoccoides | Wild | 6 | H1a | 0.194 |
|               |         |                    | 14 | H5a | 0.452 |
|               |         |                    | 11 | H6a | 0.355 |
| TaGW7-B1      | T. aestivum ssp. aestivum | Cultivars | 203 | H1b | 0.573 |
|               |         |                    | 136 | H2b | 0.384 |
|               |         |                    | 4 | H3b | 0.011 |
|               |         |                    | 11 | H4b | 0.031 |
|               |         | Landraces | 163 | H1b | 0.709 |
|               |         |                    | 61 | H2b | 0.265 |
|               |         |                    | 6 | H3b | 0.026 |
|               |         |                    | 6 | H4b | 0.026 |
|               | T. turgidum ssp. dicoccoides | Wild | 14 | H1b | 0.700 |
|               |         |                    | 6 | H2b | 0.300 |
| TaGW7-D1      | T. aestivum ssp. aestivum | Cultivars | 341 | H1d | 0.991 |
|               |         |                    | 3 | H2d | 0.009 |
|               |         | Landraces | 242 | H1d | 0.992 |
|               |         |                    | 2 | H2d | 0.008 |

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wheat origin could have played important roles in the evolution of yield component traits. In addition to homoeolog-specific effects of mutations, the gene editing of both TaGW2 (Wang et al., 2018b) and TaGW7 revealed dosage-dependent effects on grain morphometric and weight traits. Responsiveness of the TaGW7-connected developmental pathway to gene dosage variation is also confirmed in rice, in which the number of the OsGW7 gene copies had a strong effect on grain shape (Wang et al., 2015b). Our observations are consistent with the hypothesis suggesting that many duplicated homoeologs in allopolyploid wheat retain their functionality and may impose dosage-mediated effects on trait variation (Akhunova et al., 2010; Diaz et al., 2012; Krasileva et al., 2017; He et al., 2019).

The involvement of microtubules in embryo and endosperm morphogenesis has been previously demonstrated (Brown et al., 1994; Pignocchi et al., 2009; Hashimoto, 2015; Luptovciak et al., 2017). Microtubule topology during the three major stages of cereal endosperm development (syncytial, cellularization and differentiation) showed close relationships with the cell shape, cell wall development and positioning of cell organelles (Brown et al., 1994). Consistent with these observations, the enrichment analysis of the TaGW7-centered networks showed significant over-representation of genes involved in biological processes connected with microtubule-based movement (GO:0007018), regulation of mitotic cell cycle (GO:0007346), regulation of mitotic nuclear division (GO:0007088) and regulation of developmental growth (GO:0040008), and cell wall biogenesis (GO:0042546). Recent findings suggest that the ability of microtubule arrays to interact with multiple proteins plays a critical role in the regulation of these biological processes linked with organ morphogenesis. The OsGW7 and TaGW7 genes are homologs of TRM1 that are likely to affect grain dimension traits by influencing cell elongation and division (Wang et al., 2015a). The TRM1 protein was shown to interact with TON1 (Drevenské et al., 2012), which in turn interacts with microtubule arrays involved in the formation of preprophase band (Hashimoto, 2015). An interaction between rice homologs of TRM1 and TON1 was confirmed using the yeast two-hybrid assay (Wang et al., 2015a). Another interacting partner of TRM protein in both rice and Arabidopsis is a protein phosphatase 2A (PP2A) encoded by the FASS/TON2 gene (Drevenské et al., 2012; Wang et al., 2015a), which together with TRM and TON1 was shown to form PPB and be involved in the spatial control of cell division in Arabidopsis (Spinner et al., 2013). Our finding that TaGW7 co-localizes with α- and β-tubulins, the main building blocks of microtubule arrays, is consistent with the co-localization and interaction studies in Arabidopsis demonstrating that TRM1 can be recruited to microtubule arrays through a positively charged basic domain, one of the main features of the TRM proteins (Drevenské et al., 2012). These results suggested that protein–protein interactions including microtubule arrays and TRM are important part of the pathways that control grain or fruit dimension traits in wheat and other crops. Consistent with this conclusion is the finding that, in rice, a mutation in an α-tubulin gene (Sr5s mutant) affected grain dimension traits (Segami et al., 2012). Similarly, the interaction between the OVATE family proteins and TRM were shown to regulate cell division and alter fruit shape in tomato (Wu et al., 2018).

Wheat domestication was accompanied by an increase in kernel size and weight (Peleg et al., 2011). The analyses of selective sweeps, genetic differentiation and haplotypic diversity suggested that the TaGW7 gene was one of the likely targets of domestication selection. The shorter and wider grain characteristics of the domesticated forms of tetraploid wheat compared with its wild tetraploid ancestor (Nave et al., 2016) could have resulted from selection acting on grain dimension traits modulated by TaGW7. Strong selection on the H1a haplotype of the TaGW7-A1 gene was consistent with its relatively low frequency in wild emmer (19.4%), fixation in domesticated emmer and high frequency in wheat cultivars (84.3%) and landraces (85%). At the same time, the most frequent haplotype H5a from wild emmer was not detected in domesticated emmer and wheat cultivars, and present at low frequency (1.2%) in wheat landraces. The latter was consistent with the evidence of higher rates of gene flow from wild emmer into wheat landraces than into cultivars (He et al., 2019). Similar changes in the haplotype frequency accompanying wheat domestication and improvement were found for the TaGW7-B1 gene on chromosome 2B. Our data suggested that only one of the TaGW7 haplotypes or its close derivatives reached high frequency in domesticated and cultivated wheats. This observation can potentially explain the lack of overlap between the TaGW7 gene and previously identified QTL for yield component traits (Rasheed et al., 2014; Zanke et al., 2015; Yan et al., 2019). It is possible that wheat populations have been nearly fixed for the beneficial alleles of TaGW7 precluding the identification of their phenotypic effects using genome-wide association mapping. This finding is consistent with studies in rice demonstrating that some alleles of yield component genes with strong effect are rare in population (Zhang et al., 2012b; Hu et al., 2015; Song et al., 2015).

The phenotypic effects of genes that lost diversity during domestication and selection are not easily detectable in genetic mapping studies based on the analyses of only domesticated forms of crops. Our results suggested that the CRISPR-Cas9-based editing of the wheat orthologs of genes with the known phenotypic effects in other crops can help to overcome some of these limitations and select targets for engineering new variants of genes with
beneficial effect on agronomic traits, broadening genetic diversity available for wheat improvement.

EXPERIMENTAL PROCEDURES

Analysis of the TaGW7 gene sequences

The coding sequence (CDS) of the OsGW7 gene from rice (Wang et al., 2015a) was used to perform BLASTN search using the genomes of allohexaploid wheat Triticum aestivum ssp. aestivum (genome formula AABBDD) (The International Wheat Genome Sequencing Consortium (IWGSC), 2018), and its tetraploid ancestor T. turgidum ssp. dicoccoides or wild emmer (genome formula AABB) (Avni et al., 2017) and diploid ancestor Aegilops tauschii (genome formula DD) (Luo et al., 2017) from the Ensembl Plants database (plants.ensembl.org). The orthologous genes were identified based on the BLASTN hits showing sequence identity higher than 70%, e-value less than 1e-5 and located on the wheat chromosomes 2A, 2B, and 2D, which are syntenic to rice chromosome 7 (Sorrells et al., 2003). Applying these sequence similarity thresholds revealed no hits on other chromosomes were detected.

The gene sequences including both introns and exons were used to build the phylogenetic tree. Alignment was performed using MUSCLE (Edgar, 2004). Pair-wise distances between the aligned gene sequences after removal of regions carrying insertions and deletions were calculated using the Kimura-2-parameter model implemented in program MEGA v.6.06. The gene tree was constructed using the Neighbor-Joining method followed by bootstrap testing with 1000 replications. The cDNA was prepared from the total RNA isolated from the spikes of cv. Bobwhite before anthesis. The CDS of the TaGW7 gene in cv. Bobwhite was amplified using the genome-specific primers designed to the 5’ and 3’ UTRs of the TaGW7 gene homoeologs (Table S1). PCR was performed using NEBNext Ultra II QS Master Mix (New England Biolabs, Catalog #M0654S, Ipswich, MA, USA) following the manufacturer’s protocol. The PCR amplimers were subcloned into pCR-Blunt vector using the Zero Blunt™ PCR Cloning Kit (Thermo Fisher Scientific, Waltham, MA, USA, Catalog #K270020). Plasmid DNA was extracted from three to five bacterial colonies for each PCR product using QIAprep Spin Miniprep Kit (QIAGEN, Catalog #27106, Hilden, Germany). The sequence of each clone was determined using the Sanger method and used to infer the consensus sequence of each gene.

Design and validation of CRISPR-Cas9 targets in the TaGW7 gene

The CIRSPR-Cas9 targets within the TaGW7 gene were designed as described previously (Wang et al., 2016, 2018a). Briefly, the CDS of TaGW7-A1 was analyzed using sgrNAScorer 1.0 (http://crispr.med.harvard.edu/sgrNAScorer). The highest scoring gRNA targets were compared with the wheat reference genome RefSeq (IWGSC, 2018) using the BLASTN program. The gRNA target sequences conserved between at least two wheat genomes and not showing similarity outside of the target region were used to create the gRNA constructs.

Each gRNA was synthesized as two complementary oligonucleotides with four-nucleotide overhangs at both termini (Table S1). The oligonucleotides were annealed and subcloned into CRISPR-Cas9 pA9Cas9sg as described (Wang et al., 2018a). The genome editing efficiency of each gRNA construct was tested by transient expression in the wheat protoplasts (Table S2), and the NGS of gRNA target region (Wang et al., 2016).

Regeneration of transgenic plants and genotyping of the TaGW7 gene mutants

The pA9Cas9sg constructs targeting two sites, referred as GW7T2 and GW7T6, were mixed in equimolar amounts with the bar-gene carrying pAHC20 construct and biolistically transformed into the embryos of wheat cultivar Bobwhite. The T2 plants were regenerated as described in our previous study (Wang et al., 2018b). Three primer pairs spanning the CRISPR-Cas9 construct were used to identify CRISPR-Cas9 positive plants using PCR (Table S1). The target sites of the CRISPR-Cas9 positive T3 plants and plants from the T1, T2 and T3 generations were analyzed by NGS of pooled barcoded PCR amplicons, as described previously (Wang et al., 2018a). The primers used for NGS are listed in Table S1.

Plant growth conditions

The plants from the T3 generation of YLD2-5-1-52 line were grown in a growth chamber under 16 h light/8 h dark with day and night temperature set to 24°C and 21°C, respectively. All plants in other experiments were grown in a greenhouse under 16 h light/8 h dark treatments at 21–24°C. The T1 generation plants were grown in the 0.2 L square pots filled with SunGro soil (Sun Gro Horticulture, Agawam, MA, USA). All other plants were grown in 1 L square pots filled with three parts of soil mix (volume ratio was 20 soil:20 peat moss:10 perlite:1 CaSO4) and topped with one part of the SunGro soil mix (Sun Gro Horticulture, Agawam, MA, USA). Plants in the greenhouse were arranged according to the complete randomized design.

The expression analysis of TaGW7 in cv. Bobwhite and its mutants

To analyze the TaGW7 gene expression in cv. Bobwhite, total RNA was isolated from the plants at anthesis stage from the following tissues on the main spike tiller: 1-week-old grain, spike without the grain, flag leaf, flag leaf sheath, and stem. In addition, one nonpollinated spike on another tiller and roots were sampled from the same plants. RNA was extracted using TRIzol (Thermo Fisher Scientific, catalog #15596018) following the manufacturer’s protocol. The first-strand cDNA was synthesized using ‘SuperScript™ III First-Strand Synthesis SuperMix’ (Thermo Fisher Scientific, Catalog #11752-250) according to Thermo Fisher’s recommendations. The TaGW7-A1, -B1 and -D1 homoeolog-specific RT-PCR primers (Table S1) were designed and validated by PCR using the DNA isolated from the Chinese Spring null-tetrasomic lines (Figure S5). Quantitative RT-PCR was performed using the IQ™ SYBR Green Supermix (Bio-Rad, Catalog #170-8882, Hercules, CA, USA) following the manufacturer’s instructions with the actin gene as reference.

To investigate the possible effects of mutations in one or two copies of the TaGW7 gene on the expression of unmutated gene copies, the relative expression levels of each gene copy in the single- and double-gene mutant lines were characterized. For this purpose, we used total RNA isolated from the spikes at the stage when half of the spike emerged from the flag leaf sheath. At least three biological replicates were used for expression analyses.

Intracellular localization of TaGW7

To study the cellular localization of TaGW7, the CDS of each gene homoeolog was fused to the 3’-ends of the eYFP gene from plasmid pABReYFP (Figure S7). The CDS was amplified using primers GW7AD/B_CDS_F-9RY and GW7_CDS_R-9RY (Table S1) and

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inserted into pA9ReYFP between the KpnI and MfeI cut sites. The vector constructs were validated by Sanger sequencing.

The constructs expressing microtubule-localized mRFP were created by fusing γ- and β-TUBULIN-6 with the C-terminus of mRFP in plasmid pA9mRFP (Wang et al., 2018a). For this purpose, the coding regions of the γ- and β-TUBULIN-6 gene orthologs (TracesCS4A02G257800 and TracesCS1B02G269400) were amplified using PCR from the cDNA prepared using RNA isolated from the Bobwhite spikes. The 18 bp-long glycine–serine linker was inserted between γ- and β-TUBULIN-6 and mRFP by adding tails to the 5’-ends of PCR primers. The PCR products generated using NEBNext Ultra II Q5® Master Mix (New England Biolabs, Catalog #M0544S) were subcloned into pA9mRFP plasmid using NEBuilder® HiFi DNA Assembly Cloning Kit (New England Biolabs, Catalog #E5520S). These constructs, from this point forward, will be referred to as pA9mRFP-γ-TUBULIN-6 and pA9mRFP-β-TUBULIN-6. The constructs expressing the TaGW7-eYFP fusion proteins were co-transformed into Bobwhite protoplasts along with pA9mRFP-γ-TUBULIN-6 and pA9mRFP-β-TUBULIN-6. After 18 h of incubation at room temperature, the fluorescent signals were analyzed using the LSM 700 Confocal Laser Scanning Microscope (Zeiss Inc, Jena, Germany).

Collection of grain shape, size and thousand grain weight data

The three main spikes were collected from each plant. The grain width, grain length, grain area and TGW were measured separately for each spike, and used to calculate the mean values for each plant. Information about the sample sizes for each phenotyping experiment is provided in the respective sections in the Results. Trait measurements were conducted using a MARVIN seed analyzer (GTA Sensork GmbH, Germany), as described previously (Wang et al., 2018b).

Co-expression analysis of the TaGW7 gene

The expression data (transcripts per million, TPM) of 209 RNA-seq samples from the developmental time-course of Azhurnaya were downloaded from the Wheat Expression Browser (http://www.wheat-expression.com/download) (Ramirez-González et al., 2018). We used gene expression levels estimated from the sum of all isoforms. A quantile robust normalization was performed followed by the log2 transformation of the TPM values. Our co-expression network was comprised of two parts: (i) a global network based on top 10 000 genes that had the highest levels of gene expression variability assessed based on the standard deviation; and (ii) a local network build using the top 1% genes that showed the strongest correlation with the levels of the TaGW7 gene expression. We used Gephi (https://gephi.org) to visualize the network. The gene pairs with the PCC less than –0.90 or larger than 0.98 in the global network were visualized. The gene pairs with absolute PCC larger than 0.60 in the local network were visualized along with the global network.

GO enrichment of TaGW7 co-expressed neighbors

In addition to the Gene Ontology data released by the IWGSC, we used BLAST2GO to generate more detailed functional annotations for both high-quality (HC) and low-quality (LC) gene models reported for the wheat genome assembly RefSeq v.1.0 (The IWGSC, 2018). All protein sequences were compared with the NCBI non-dundant sequence database using the BLAST tool. The BLAST output was processed using blast2go (blast2go cli_v1.3.3) to extract GO terms for each gene. Fisher’s exact test was used to determine the significance of the enrichment for every GO term, followed by Benjamini–Hochberg correction (Benjamini and Hochberg, 1995).

Genetic diversity analyses

We used XP-CLR statistic (Chen et al., 2010) to identify the genomic regions showing the signatures of selective sweeps associated with wheat domestication. For this purpose, we used exome capture data generated for the accessions of tetraploid wild and domesticated emmer wheat (Avni et al., 2017). In the XP-CLR scan, 34 accessions of wild emmer were used as a reference population and 40 accessions of domesticated emmer were used as a test population. The XP-CLR scan was run with the grid size of 50 kb, the window size of 1 cM, the maximum number of SNP within a window set to 200 and the inter-variant linkage disequilibrium (r²) less than 0.95.

The genetic diversity analyses of the TaGW7 gene in the populations of wheat 286 landraces and 485 cultivars were performed using previously described whole exome capture SNP data collected for a geographically and genetically diverse panel of accessions (He et al., 2019). The SNP variants were extracted from the TaGW7 gene regions in the A, B, and D genomes of wheat (http://wheatgenomics.plantpath.ksu.edu/1000EC).

Statistical analysis of data

The distribution of grain length, grain width, grain area and TGW data was visualized using the box and whisker plots. One-way ANOVA was applied to compare the significance of inter-group differences followed by post-hoc Tukey’s test. The Student’s t-test was applied to assess the significance of difference between the two groups of data.

AVAILABILITY OF DATA AND MATERIAL

CRISPR-Cas9 gene editing constructs and wheat lines are available upon request.

MATERIALS REQUESTS

Materials requests should be addressed to Eduard Akhunov (eakhunov@ksu.edu).

ACKNOWLEDGMENTS

This project was supported by the Agriculture and Food Research Initiative Competitive Grants 2017–67007–25932 and 2017–67007–25939 (Wheat-CAP) from the USDA National Institute of Food and Agriculture, and grants from the Bill and Melinda Gates Foundation and Kansas Wheat Commission. The authors would like to thank D. Davidson for taking care of the plants and K. Jordan for help with assessing the gene diversity in emmer wheat.

AUTHOR CONTRIBUTIONS

WW conducted gene editing experiments, generated and analyzed gene expression data, drafted the manuscript; QP analyzed gene editing events using NGS and conducted gene expression analysis; BT conducted plant transformation experiments; FH constructed gene expression networks and performed diversity data analyses; YC performed biolistic transformation of wheat embryos; AA designed experiments for NGS analysis of editing events.
and performed NGS; GB validated constructs by Sanger sequencing; HT performed biologic transformation of wheat embryos with the gene editing constructs and coordinated project; EA conceived idea, designed gene editing experiments, coordinated project, analyzed data and wrote the manuscript.

**CONFLICT OF INTEREST**

The authors declare that they have no conflict of interests.

**SUPPORTING INFORMATION**

Additional Supporting Information may be found in the online version of this article.

**Figure S1.** The CDS and predicted protein sequence of the TaGW7 gene in cultivars Chinese Spring and Bobwhite.

**Figure S2.** Phenotypic effects of mutations induced by CRISPR-Cas9 in the TaGW7 gene in T1 generation plants.

**Figure S3.** Phenotypic effects of mutations induced by CRISPR-Cas9 in the TaGW7 gene in the T3 generation plants derived from YLD2-5-1-52.

**Figure S4.** Phenotypic effects of mutations induced by CRISPR-Cas9 in the TaGW7 gene in the T3 generation plants derived from YLD2-5-1-41.

**Figure S5.** Validation of the TaGW7 gene homoelog-specific primers for RT-PCR.

**Figure S6.** The expression levels of the TaGW7 gene in T2 generation plants.

**Table S1.** List of primers and oligonucleotides used in the study.

**Table S2.** Assessment of gRNA editing efficiency in the wheat protoplasts.

**Table S3.** List of genes co-expressed with the TaGW7 gene homoelogues.

**Table S4.** GO terms significantly enriched in the TaGW7-associated gene networks.

**Table S5.** Wheat genes orthologous to genes in rice that encode proteins interacting with rice OsGW7 in the yeast two-hybrid (Y2H) assay.

**Table S6.** Haplotypes of the TaGW7 genes identified in hexaploid and tetraploid wheat.

**REFERENCES**

Akhunova, A.R., Matniyazov, R.T., Liang, H. and Akhunov, E.D. (2010) Homoeolog-specific transcriptional bias in allopolyploid wheat. BMC Genom. 11, 1–16.

Avni, R., Nave, M., Barad, O., et al. (2017) Wild emmer genome architecture and diversity elucidate wheat evolution and domestication. Science, 97, 93–97.

Benjamini, Y. and Hochberg, Y. (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. J. R. Stat. Soc. Ser. B Stat. Methodol. 57, 289–300.

Brown, R.C., Lemmon, B.E. and Olsen, O.-A. (1994) Endosperm development in barley: microtubule involvement in the morphogenetic pathway. Plant Cell, 6, 1241–1252.

Chen, H., Patterson, N. and Reich, D. (2010) Population differentiation as a test for selective sweeps. Genome Res. 20, 393–402.

Diaz, A., Zikhali, M., Turner, A.S., Isaac, P. and Laurie, D.A. (2012) Copy number variation affecting the Photoperiod-B1 and Vernalization-A1 genes is associated with altered flowering time in wheat (Triticum aestivum). PLoS ONE, 7, e33234.

Dong, L., Wang, F., Liu, T., et al. (2014) Natural variation of TaGASRT-A1 affects grain length in common wheat under multiple cultivation conditions. Mol. Breed. 34, 937–947.

Drevenská, S., Gousset, M., Duroy, Y., et al. (2012) The Arabidopsis TRM1–TON1 interaction reveals a recruitment network common to plant cortical microtubule arrays and eukaryotic centrosomes. Plant Cell, 24, 178–191.

Edgar, R.C. (2004) Muscle: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32, 1792–1797.

Hashimoto, T. (2015) Microtubules in plants. Arabidopsis Book 13, e0179.

He, F., Pasam, R., Shi, F., et al. (2019) Exome sequencing highlights the role of wild relative introgression in shaping the adaptive landscape of the wheat genome. Nat. Genet. 51, 896–904.

Hu, J., Wang, Y., Fang, Y., et al. (2015) A Rare Allele of GS2 enhances grain size and grain yield in rice. Mol. Plant, 8, 1455–1466.

Hu, M.-J., Zhang, H.-P., Liu, K., et al. (2016) Cloning and characterization of TaGW7-A gene associated with grain weight in wheat via SLAF-seq-BSA. Front. Plant Sci. 7, 1902.

Krasileva, K.V., Vasquez-Gross, H.A., Howell, T., et al. (2017) Uncovering hidden variation in polyloid wheat. Proc. Natl Acad. Sci. USA, 114, E913–E921.

Li, N. and Li, Y. (2016) Signaling pathways of seed size control in plants. Curr. Opin. Plant Biol. 33, 23–32.

Li, W., Liu, N., Liu, H., et al. (2017) The conserved and unique genetic architecture of kernel size and weight in maize and rice. Plant Physiol. 175, 774–785.

Luo, M.-C., Gu, Y.Q., Puiu, D., et al. (2017) Genome sequence of the progenitor of the wheat D genome Aegilops tauschii. Nature, 551, 498–502.

Luptovicka, I., Samakovli, D., Komis, G. and Samaj, J. (2017) KATANIN 1 is essential for embryogenesis and seed formation in Arabidopsis. Front. Plant Sci. 8, 728.

Nave, M., Avni, R., Ben-Zvi, B., Hale, I. and Distelfeld, A. (2016) QTLs for uniform grain dimensions and germination selected during wheat domestication are co-located on chromosome 4B. Theor. Appl. Genet. 129, 1303–1315.

Okada, A., Arndell, T., Borisjuk, N., Sharma, N., Watson-Haigh, N.S., Tucker, E.J., Baumann, U., Langridge, P. and Whitford, R. (2018) CRISPR/Cas9-mediated knockout of Md1 enhances the rapid generation of male-sterile hexaploid wheat lines for use in hybrid seed production. Plant Biotechnol. J. https://doi.org/10.1111/pbi.13106.

Peleg, Z., Fahima, T., Korol, A.B., Abbo, S. and Saranga, Y. (2011) Genetic analysis of wheat domestication and evolution under domestication. J. Exp. Bot. 62, 5051–5061.

Pignocchi, C., Minns, G.E., Nesi, N., Kouprouglou, R., Kitsios, G., Benning, C., Lloyd, C.W., Doonan, J.H. and Hills, M.J. (2009) ENDOSPERM DEFECTIVE1 is a novel microtubule-associated protein essential for seed development in Arabidopsis. Plant Cell, 21, 90–105.

Ramirez-Gonzalez, R.H., Borris, P., Lang, D., et al. (2018) The transcriptional landscape of polyloid wheat. Science, 361, eaar8089.

Rasheed, A., Xia, X., Ogbonnaya, F., Mahmood, T., Zhang, Z., Mujeeb-Kazi, A. and He, Z. (2014) Genome-wide association for grain morphology in synthetic hexaploid wheats using digital imaging analysis. BMC Plant Biol. 14, 128.

Sánchez-León, S., Gil-Humanes, J., Ovina, C.V., Gimenez, M.J., Sousa, C. and Voyeras, D.F. and Barro, F. (2018) Low-gluten, nontransgenic wheat engineered with CRISPR/Cas9. Plant Biotechnol. J. 16, 902–910.

Segami, S., Kono, I., Ando, T., Yano, M., Kitano, H., Miura, K. and Iwasaki, Y. (2012) Small and round seed 5 gene encodes alpha-tubulin regulating seed cell elongation in rice. Rice, 5, 4.

Song, X.-J., Huang, W., Shi, M., Zhu, M.-Z. and Lin, H.-X. (2007) A QTL for rice grain width and weight encodes a previously unknown RING-type E3 ubiquitin ligase. Nat. Genet. 39, 623–630.

Song, X.-J., Kuroha, T., Ayano, M., et al. (2015) Rare allele of a previously unidentified histone H4 acetyltransferase enhances grain weight, yield, and plant biomass in rice. Proc. Natl Acad. Sci. USA, 112, 76–81.

Sorrells, M.E., Rota, M.La, Bermudez-kandianis, C.E., et al. (2003) Comparative DNA sequence analysis of wheat and rice genomes, Genome Res. 13, 1818–1827.

Spinner, L., Gadeyne, A., Belcrum, K., et al. (2013) A protein phosphatase 2A complex spatially controls plant cell division. Nat. Commun. 4, 1–13.

Takano-Kai, N., Hui, J., Kubo, T., et al. (2009) Evolutionary history of GS3, a gene conferring grain length in rice. Genetics, 182, 1323–1334.

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The International Wheat Genome Sequencing Consortium (IWGSC). (2018) Shifting the limits in wheat research and breeding using a fully annotated reference genome. Science, 361, eaar7191.

Vavilov, N.I. (1922) The law of homologous series in variation. J. Genet. 12, 47–90.

Wang, S., Wu, K., Yuan, Q., et al. (2012) Control of grain size, shape and quality by OsSPL16 in rice. Nat. Genet. 44, 950–954.

Wang, Y., Cheng, X., Shan, Q., Zhang, Y., Liu, J., Gao, C. and Gao, J-L. (2014) Simultaneous editing of three homoeoalleles in hexaploid bread wheat confers heritable resistance to powdery mildew. Nat. Biotechnol. 32, 947–951.

Wang, S., Li, S., Liu, Q., et al. (2015a) The OsSPL16-GW7 regulatory module determines grain shape and simultaneously improves rice yield and grain quality. Nat. Genet. 47, 949–954.

Wang, Y., Xiong, G., Hu, J., et al. (2015b) Copy number variation at the GL7 locus contributes to grain size diversity in rice. Nat. Genet. 47, 944–948.

Wang, W., Akhunova, A., Chao, S. and Akhunov, E. (2018a) Transgenerational CRISPR/Cas9 activity facilitates multiplex gene editing in allopolyploid wheat. CRISPR J. 1, 65–74.

Wang, W., Simmonds, J., Pan, Q., et al. (2018b) Gene editing and mutagenesis reveal inter-cultivar differences and additivity in the contribution of TaGW2 homoeologues to grain size and weight in wheat. Theor. Appl. Genet. 131, 2463–2475.

Wu, S., Zhang, B., Keyhaninejad, N., et al. (2018) A common genetic mechanism underlies morphological diversity in fruits and other plant organs. Nat. Commun. 9, 4734.

Xie, G., Li, Z., Ran, Q., Wang, H. and Zhang, J. (2018) Over-expression of mutated ZmDA1 or ZmDAR1 gene improves maize kernel yield by enhancing starch synthesis. Plant Biotechnol. J. 16, 234–244.

Yan, X., Zhao, L., Ren, Y., Dong, Z., Cui, D. and Chen, F. (2019) Genome-wide association study revealed that the TaGW8 gene was associated with kernel size in Chinese bread wheat. Sci. Rep. 9, 1–10.

Zanke, C.D., Ling, J., Plieske, J., et al. (2015) Analysis of main effect QTL for thousand grain weight in European winter wheat (Triticum aestivum L.) by genome-wide association mapping. Front. Plant Sci. 6, 1–14.

Zhang, L., Zhao, Y.-L., Gao, L.-F., Zhao, G.-Y., Zhou, R.-H., Zhang, B.-S. and Jia, J.-Z. (2012a) TaCKX6-D1, the orthologue of rice OsCKX2, is associated with grain weight in hexaploid wheat. New Phytol. 195, 574–584.

Zhang, X., Wang, J., Huang, J., et al. (2012b) Rare allele of OsPPKL1 associated with grain length causes extra-large grain and a significant yield increase in rice. Proc. Natl Acad. Sci. USA, 109, 21534–21539.

Zhang, Y., Liang, Z., Zong, Y., Wang, Y., Liu, J., Chen, K., Gao, J.L. and Gao, C. (2016) Efficient and transgene-free genome editing in wheat through transient expression of CRISPR/Cas9 DNA or RNA. Nat. Commun. 7, 1–8.

Zhang, Y., Li, D., Zhang, D., et al. (2018) Analysis of the functions of TaGW2 homoeologs in wheat grain weight and protein content traits. Plant J. 94, 857–866.

Zhang, Z., Hua, L., Gupta, A., Tricoli, D., Edwards, K.J., Yang, B. and Li, W. (2019) Development of an Agrobacterium-delivered CRISPR/Cas9 system for wheat genome editing. Plant Biotechnol. J. https://doi.org/10.1111/pbi.13088