Global Selection on Sucrose Synthase Haplotypes during a Century of Wheat Breeding[1][C][W]

Jian Hou, Qiyan Jiang, Chenyang Hao, Yuquan Wang, Hongna Zhang, and Xueyong Zhang*

Institute of Crop Science, Chinese Academy of Agricultural Sciences, Beijing 100081, China

ORCID ID: 0000-0001-6515-1430 (J.H.).

Spike number per unit area, number of grains per spike, and thousand kernel weight (TKW) are important yield components. In China, increases in wheat (*Triticum aestivum*) yields are mainly due to increases in grain number per spike and TKW. TKW mainly depends on starch content, as starch accounts for about 70% of the grain endosperm. Sucrose synthase catalysis is the first step in the conversion of sucrose to starch, that is, the conversion of sucrose to fructose and UDP-glucose by the wheat sucrose synthase genes (*TaSus1* and *TaSus2*) that are located on chromosomes 7A/7B/7D and 2A/2B/2D, respectively. A total of 1,520 wheat accessions were genotyped at the six loci. Two, two, five, and two haplotypes were identified at the *TaSus2-2A*, *TaSus2-2B*, *TaSus1-7A*, and *TaSus1-7B* loci, respectively. Their main variations were detected within the introns. Significant differences between the haplotypes correlated with TKW differences among 348 modern Chinese cultivars from the core collection. Frequency changes for favored haplotypes showed gradual increases in cultivars released since beginning of the last century in China, Europe, and North America. Geographic distributions and time changes of favored haplotypes were characterized in six major wheat production regions worldwide. Strong selection bottlenecks to haplotype variations occurred at polyploidization and domestication and during breeding of wheat. Genetic-effect differences between haplotypes at the same locus influence the selection time and intensity. This work shows that the endosperm starch synthesis pathway is a major target of indirect selection in global wheat breeding for higher yield.

Wheat (*Triticum aestivum*) is one of the most important staple food crops in the world. The wheat cultivation area has remained more or less stable over the last 20 years. Increased production of about one-sixth above that in 1992 was mainly due to per year per unit area yield increases (http://www.ers.usda.gov/data-products/wheat-data.aspx#.UvtxFrLs64Q). Yield increases in China show a similar trend; however, in the past 5 years, per unit increases per year in yield may have declined slightly.

Yield dissection is very difficult. For a long time, quantitative trait loci mapping was commonly used in major crops such as rice (*Oryza sativa*), durum wheat (*Triticum durum*), and hexaploid wheat (Olmos et al., 2003; Mohan et al., 2009; Xing and Zhang, 2010). The disadvantage of quantitative trait loci mapping is that it takes a long time and is costly. Association analysis based on "hitchhiking" effects is an effective way to dissect important complex traits (Flint-Garcia et al., 2003; Zhang et al., 2007). Marker association, especially haplotype association analysis, accelerates the process of mapping and detection of important genomic regions and favored alleles or haplotypes for breeding (Barrero et al., 2011).

Yield in wheat is made up of three components: number of fertile spikes per unit area, grains per spike, and kernel weight usually expressed as thousand kernel weight (TKW). In China, wheat yield growth has mainly depended on increases in grain number per spike and TKW (Wang et al., 2012; Zhang et al., 2012). As starch accounts for about 70% of the dry grain weight (Dale and Housley, 1986), the rate and amount of starch synthesis should have significant effects on TKW.

Starch synthesis has been well documented in many plants. The enzyme Suc synthase (SUS) converts Suc to starch. Starch is located in the cytosol (Kleczkowski et al., 2010). SUS also plays key roles in fruit and seed maturation (Coleman et al., 2010; Jiang et al., 2011) and abiotic stress tolerance (Bologa et al., 2003; Yang et al., 2004; Bieniawska et al., 2007).

There are at least six *SUS* genes in Arabidopsis (*Arabidopsis thaliana*), rice, and *Populus tomentosa* (Bieniawska et al., 2007; Hirose et al., 2008). In maize (*Zea mays*), the paralogous genes *Shrunken1* and *SUS1* encode the SUS1 and SUS2 isozymes of Suc synthase (Chourey et al., 1998). Similar genes are present in barley (*Hordeum vulgare*) and wheat. In wheat, *TaSus2* is located on homeologous group 2 chromosomes (Jiang et al., 2011). Three single nucleotide polymorphisms (SNPs) in *TaSus2-2B* form two haplotypes, *Hap-H* and *Hap-L*. *Hap-H*, the favored haplotype associated with higher TKW, underwent strong positive selection in Chinese wheat breeding as a

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* Address correspondence to xueyongz@mail.caas.net.cn.

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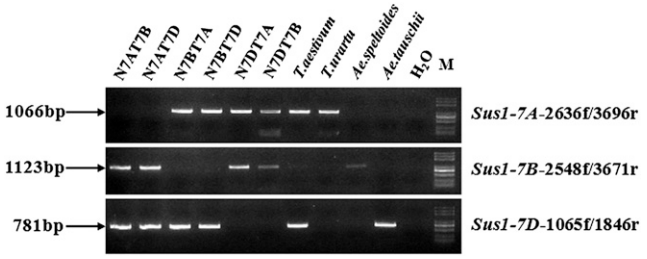


Figure 1. Chromosome locations of *TaSus1* orthologs. The sizes of PCR products are shown on the left, and the genome-specific primers are on the right. Sources of amplified DNA are at the top.

consequence of selection for higher grain yield (Jiang et al., 2011).

In this study, the loci *TaSus1* and *TaSus2* encoding wheat Suc synthases were genotyped. Two, two, five, and two haplotypes were identified at *TaSus2-2A*, *TaSus2-2B*, *TaSus1-7A*, and *TaSus1-7B*, respectively. No variation was detected at *TaSus2-2D* or *TaSus1-7D*. The influence of these haplotypes on TKW was analyzed through haplotype association, and five favored haplotypes were identified. Their frequencies of change over the last century of worldwide breeding were tracked in 1,520 common wheat cultivars. In addition, their geographic distributions were also characterized. Changes in nucleotide diversity (π) of *TaSus1-7A* from the diploid to hexaploid levels were also investigated.

RESULTS

Cloning, Chromosome Mapping, and Characterization of the *TaSus1* and *TaSus2* Sets

TaSus2 genomic DNA and complementary DNA (cDNA) sequences were amplified by primer pair *Sus2-167* and *Sus2-168* (Jiang et al., 2011), and *TaSus1* genomic DNA and cDNA sequences were amplified by primer pair *Sus1-1f* and *Sus1-3805r* from cv Chinese Spring and diploid progenitor accessions. Three different *TaSus1* genes were amplified.

Based on differences between the three genomic sequences of *TaSus1*, three genome-specific primer pairs were designed to detect the chromosome locations of the *TaSus1* orthologs (Fig. 1). Genomic DNA from the homeologous group 7 cv Chinese Spring nullitetrasomic lines, cv Chinese Spring, *Triticum urartu*, *Aegilops speltoides*, and *Aegilops tauschii* were amplified by the three primer pairs. *TaSus1* genes were located on chromosomes 7A, 7B, and 7D.

The three *TaSus1* genes each consisted of 14 exons and 13 introns (Fig. 2). The genomic sequence lengths of *TaSus1-7A*, *TaSus1-7B*, and *TaSus1-7D* were 3,805, 3,777, and 3,765 bp, respectively. The sequence homologies ranged from 94.1% to 95.2%, and the sequences of the three *TaSus1* genes had homologies of 92.1% to 92.8% with *HvSus1-7H* (barley).

The genome-specific primer pair *Sus2-2AP-302f* and *Sus2-2A-214r* was designed to map *TaSus2-2A*. Based on genetic and molecular data for the cv Hanxuan 10 × Lumai 14 double haploid (DH) population (Yang et al., 2007), *TaSus2-2A* was mapped on the short arm of 2A (2AS) and flanked by simple sequence repeat markers *Xgwm122* and *Xgwm328* (Supplemental Fig. S1) located in deletion bin 2AS-5 (Sourdille et al., 2004).

Proteins coded by *TaSus1* and *TaSus2* were matched by InterProScan (http://www.ebi.ac.uk/Tools/pfa/iprscan/). In the InterProScan database, three proteins were compared (O24301, P30298, and P49040), the first two coded by *SUS2* and the third by *SUS1*. In taxonomic coverage, the homologous proteins were distributed across bacteria, cyanobacteria, and green plants.

Haplotype Analysis at the *TaSus2-2A*, *TaSus2-2B*, *TaSus1-7A*, and *TaSus1-7B* Loci

Three SNPs earlier identified in *TaSus2-2B* formed two haplotypes (Jiang et al., 2011). Two SNPs were identified in *TaSus2-2A*, the first (20A > G) in the first exon and the second (2946A > C) in the tenth intron (Fig. 2; Supplemental Fig. S2A). Only the first SNP led to an amino acid change (GAG→Glu, GGG→Gly). These two SNPs formed two haplotypes, *Hap-A* and *Hap-G*. A cleaved amplified polymorphic site (CAPS) marker was developed based on the first SNP. Restriction endonuclease *Asc*I identified the sequence only when the SNP site was G. The PCR product of *Hap-A* amplified by the genome-specific primer pair *Sus2-2AP-302f* and *Sus2-2A-214r* was 516 bp after enzyme digestion, and the product of *Hap-G* was 322 plus 194 bp after enzyme digestion (Supplemental Fig. S2C).

Six SNPs were identified in *TaSus1-7B*, within introns 1, 3, 7, 8, and 10 and the thirteenth exon. In addition, an insertion/deletion (indel) was identified within intron 11 (Fig. 3A). These polymorphic sites formed two haplotypes, *Hap-T* and *Hap-C*. A CAPS marker was developed at the indel site using restriction endonucleases *Sph*I. The PCR product of *Hap-C* amplified by genome-specific

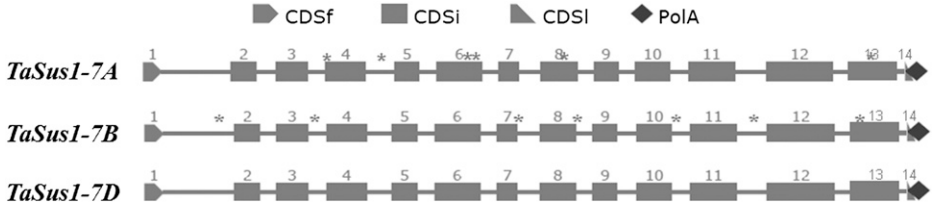


Figure 2. Coding regions of *TaSus1-7A*, *TaSus1-7B*, and *TaSus1-7D*. Asterisks indicate polymorphic sites. CDSf, First exon; CDSi, internal exons; CDSl, last coding segment.

Table I. *Comparison of mean TKW of TaSus2-2A, TaSus1-7A, and TaSus1-7B haplotypes in Chinese modern cultivars and MCC in different years*

*$P < 0.05$, **$P < 0.01$.

	TaSus2-2A TKW				*TaSus1-7A* TKW				*TaSus1-7B* TKW			
	Hap-A	*Hap-G*	*F*	*P*	*Hap-1*	*Hap-2*	*F*	*P*	*Hap-T*	*Hap-C*	*F*	*P*
	g				*g*				*g*			
348 Modern cultivars												
2002	42.89 ± 6.49	39.51 ± 7.30	2.811	0.000**	41.84 ± 6.80	45.38 ± 6.86	0.141	0.019*	43.05 ± 6.34	38.45 ± 7.68	0.652	0.000**
2006	40.42 ± 6.27	37.05 ± 7.18	2.845	0.000**	39.42 ± 6.58	41.57 ± 6.65	0.113	0.134	40.61 ± 6.23	36.03 ± 6.92	2.923	0.000**
2010	40.81 ± 6.09	38.16 ± 6.37	0.834	0.001**	39.85 ± 6.40	41.18 ± 6.68	0.822	0.328	40.84 ± 5.96	37.10 ± 7.00	2.617	0.000**
Landraces in MCC												
2002	34.05 ± 6.48	31.87 ± 4.24	4.184	0.159	32.26 ± 4.58	32.76 ± 6.99	2.921	0.090	32.32 ± 4.74	31.36 ± 3.82	0.835	0.369
2005	34.90 ± 8.03	30.09 ± 5.62	3.198	0.002*	30.48 ± 6.14	35.20 ± 6.88	0.196	0.659	30.95 ± 6.24	28.79 ± 5.37	0.643	0.146
2006	35.048 ± 7.293	32.77 ± 5.08	4.236	0.191	33.16 ± 5.40	34.89 ± 7.87	2.136	0.146	33.18 ± 5.56	32.70 ± 4.88	0.071	0.702
Modern cultivars in MCC												
2002	39.28 ± 4.74	37.26 ± 5.41	0.245	0.070	38.54 ± 5.05	39.94 ± 4.85	0.001	0.988	38.72 ± 4.84	38.42 ± 6.88	3.899	0.855
2005	38.40 ± 5.92	36.41 ± 6.73	0.560	0.185	37.50 ± 6.14	39.50 ± 6.33	0.205	0.651	37.77 ± 6.10	39.12 ± 7.70	0.724	0.559
2006	41.00 ± 5.97	37.94 ± 5.67	0.335	0.023*	39.70 ± 6.02	42.50 ± 6.00	0.001	0.991	40.14 ± 5.76	39.81 ± 8.66	4.573	0.909

primer pair *Sus1-7B-2548f* and *Sus1-7B-3671r* was 1,124 bp after restriction digestion by *Sph*I, whereas *Hap-T* produced two fragments with lengths of 730 and 394 bp, respectively (Fig. 3C).

Seven SNPs were identified in *TaSus1-7A*, within exons 4, 6, 8, and 13 and intron 4 (Supplemental Fig. S3A). SNPs in exons 6 and 13 led to amino acid changes (TCCAAT→SerAsn, AACAAA→AsnLys; GTG→Val, CTG→Leu). These seven SNPs formed five haplotypes, which were named *Hap-1*/*Hap-2*/*Hap-3*/*Hap-4*/*Hap-5*. Two CAPS markers were developed at 1,185 and 3,544 bp, using restriction endonucleases *Taq*$^{\alpha}$I and *Apa*LI, respectively (Supplemental Fig. S3, B–E). A genome-specific primer pair was developed to identify the SNPs at 1,599, 1,600, and 1,604 bp (Supplemental Fig. S3, F and G). However, no SNPs or indels were detected in *TaSus1-7D* or *TaSus2-2D* or the coding and promoter regions.

Haplotype Effects Associated with TKW

An association study of *TaSus2-2A* suggested that mean TKW of *Hap-A* was significantly higher than that of *Hap-G* ($P < 0.05$) in Chinese modern cultivars in 2002 and 2006, landraces of mini core collection (MCC) in 2006, and modern cultivars in the MCC in 2005 (Table I). In Chinese modern cultivars, the TKW difference between the two haplotypes reached a mean 3.37 g over 2 years, and *Hap-A* genotypes occupied a larger proportion of the areas in most ecological zones and provinces (Fig. 4). Among landraces in the MCC, 87.3% of genotypes were *Hap-G*, and in modern cultivars of the MCC, *Hap-A* genotypes represented 70.9% of entries. Strong positive selection for *Hap-A* suggested that it was a favored haplotype. At *TaSus2-2B*, *TaSus2-2B-Hap-H* was a favored haplotype (Jiang et al., 2011; Supplemental Fig. S4B).

At *TaSus1-7A*, *Hap-4*/*Hap-5* were present in only four accessions, so these two haplotypes were not included in subsequent statistical analyses. *Hap-3* also occurred at low frequencies in both the MCC and Chinese modern cultivars (3.8% and 2.3%, respectively). A significant difference of 5.2 g in TKW between *Hap-1* and *Hap-3* was identified in one set of near-isogenic lines (NILs; BC_3F_6 of cv Youzitou/Zhengmai 366*4) in yield trial plots (Supplemental Fig. S4A). *Hap-1* represented dominant proportions of 88.5% of the MCC and 90.6% of Chinese modern cultivars (Fig. 5). *Hap-2* was present in 7% of Chinese cultivars, but its frequency was 32% among European and American cultivars. A significant difference in TKW between *Hap-2* and *Hap-1* genotypes was only detected in Chinese modern cultivars in 2006, where the difference was 3.54 g.

At *TaSus1-7B*, a significant difference in TKW of 4.59 g was detected in Chinese modern cultivars in 2002 and 2006. In both populations, *Hap-T* was present in higher proportions of genotypes, 86.3% in the MCC and 73.9% in Chinese modern cultivars. The strong selection of *Hap-T* suggested that it was a favored haplotype.

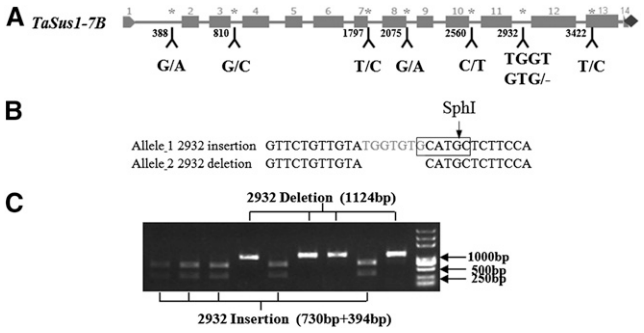


Figure 3. Polymorphisms and marker development at *TaSus1-7A*. A, Coding region of *TaSus1-7A*. Six SNPs at 388, 810, 1,797, 2,075, 2,560, and 3,422 bp and one indel at 2,932 bp are labeled. B, The indel site at 2,932 bp between different haplotypes. The rectangle and arrow represent the recognition and digestion sites of restriction endonuclease *Sph*I. C, PCR products restrictively digested by *Sph*I.

Favored Genes (Haplotypes) Have Higher Transcript and Enzyme Activity Levels

Relative quantification of mRNA expression by real-time PCR suggested that Suc synthase had a positive influence, and mRNA expression reached a peak value at 15 DPA (Fig. 6; Supplemental Table S1). At *TaSus2-2A* and *TaSus1-7A*, the relative expression levels of favored haplotypes were higher than nonfavored ones at 15 DPA. Suc synthase activity levels in developing seeds 15 DPA also demonstrated the same trends (Fig. 7; Supplemental Table S2). Transcript levels of *TaSus1-7A-Hap-1* and *TaSus1-7A-Hap-2* were not significantly different in any of the five periods. As there was no significant difference in TKW between the two *TaSus1-7A* haplotypes, they were both treated as favored haplotypes.

Changes in Haplotype Frequency during the Breeding Histories of China, Europe, and North America

Chinese modern cultivars released since the 1940s were divided into subgroups according to 10-year release intervals (Hao et al., 2006). European and American cultivars were distinguished by the same decades based on registration dates, and cultivars released earlier than 1940 were merged with the 1940s.

Frequency changes for the three favored haplotypes at *TaSus2-2A*, *TaSus2-2B*, and *TaSus1-7B* showed gradual increases in Chinese cultivars since the 1940s (Fig. 8), suggesting these favored haplotypes had undergone strong positive selection in plant breeding. The frequency changes of *TaSus1-7A-Hap-1* and *TaSus1-7A-Hap-2* showed slightly different trends in Chinese modern cultivars, with the former gradually decreasing and the latter gradually rising. At *TaSus2-2A*, *TaSus1-7A*, and *TaSus1-7B*, frequencies of favored haplotypes changed only slightly in the six decades in European and American cultivars, because they were already at very high frequencies in cultivars released in the 1940s (Fig. 8).

Geographic Distributions of Favored Haplotypes in Six Cultivar Populations

Frequencies of the five favored haplotypes at *TaSus2-2A*, *TaSus2-2B*, *TaSus1-7A*, and *TaSus1-7B* were estimated for 384 European, 447 North American, 53 International Maize and Wheat Improvement Center (CIMMYT), 82 Russian, and 51 Australian cultivars (Fig. 9). Similar frequencies of favored haplotypes were detected in European and North American cultivars (Supplemental Table S3). Similar trends were observed in Chinese and Russian cultivars. European and North American cultivars had higher proportions of favored haplotypes than the other four regions at *TaSus2-2A*, *TaSus1-7A*, and *TaSus1-7B*. CIMMYT cultivars showed high frequencies of favored haplotypes at *TaSus1-7A* and *TaSus1-7B* but relatively low frequencies at the other two loci. In Australian cultivars, except at *TaSus1-7A*, favored haplotype frequencies were not as high as in other regions (Fig. 9).

Major Haplotype Combinations at the Four Loci

Six major haplotype combinations of the four genes were detected; these represented 90.0% of European cultivars, 90.7% of American cultivars, and 80.9% of Chinese

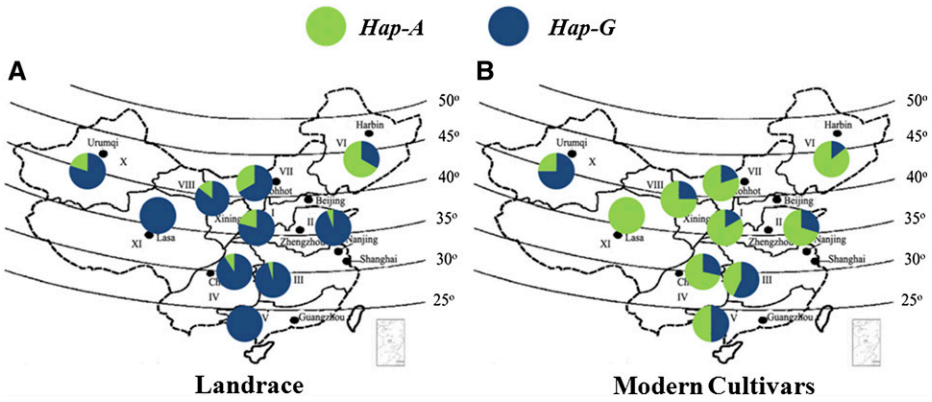


Figure 4. Distribution of *TaSus2-2A* *Hap-A* and *Hap-G* in landraces (A) and modern cultivars (B). [See online article for color version of this figure.]

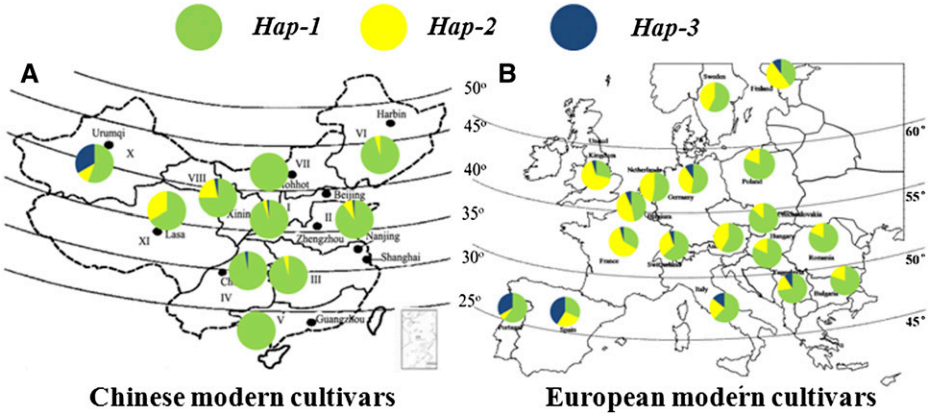


Figure 5. Distribution of *Hap-1*/*Hap-2*/*Hap-S3* at *TaSus1-7A* in Chinese (A) and European (B) cultivars. [See online article for color version of this figure.]

cultivars (Fig. 10). Combination 1 occurred in about 40% of cultivars in all three populations. The largest differences between Chinese cultivars and the other two populations were for combinations 2 and 3. *Hap-H* at *TaSus2-2B* was significantly favored in Chinese cultivars, and *Hap-2* at *TaSus1-7A* was more highly represented in European and American cultivars than in Chinese cultivars. Combinations containing nonfavored haplotypes at *TaSus2-2A* or *TaSus1-7B* only appeared in combinations 5 and 6, which accounted for very low proportions of genotypes.

π at *TaSus1-7A* in Bread Wheat and Its Diploid and Tetraploid Relatives

Sequence identities of *TaSus1-7A* compared with those of 16 diploid accessions carrying A genome ranged from 95.2% to 97.7% (Supplemental Table S4). *T. urartu* had the highest similarity. *T. urartu* could possibly be the A genome donor of common wheat. Sequence identities of *TaSus1-7B* with 23 diploid accessions carrying S genome (possible B genome donor) ranged from 89.2% to 93.6%, and *A. speltoides* had the highest similarity. However, comparing tetraploid with hexaploid wheat, sequence identities of *SUS1* were 94.6% to 99.7%. Diversities of *SUS1* were therefore significantly lower in polyploid wheat (Fig. 11, A–C). Tests of pairwise differences in *TaSus1-7A* between pairs of populations (F_{ST}, identical to the weighted average *F* statistic over loci) also suggested lower variability (Fig. 11D). F_{ST} between diploid accessions and hexaploid accessions was above 0.9, while the value between diploid and tetraploid accessions (0.542) and between tetraploid and hexaploid accessions (0.462 and 0.319) were much lower. The *P* values between populations were all below 0.05 (Supplemental Table S5), suggesting that differences between populations were significant. The F_{ST} between the wild-species *Triticum dicoccoides* and domesticated species *Triticum dicoccum*, durum wheat, and other tetraploid accessions was not significantly different; however, the F_{ST} was lower in domesticated tetraploids than in wild tetraploids (Fig. 11E), suggesting that selection at *TaSus1-7A* occurred during domestication. All these data suggested there were two strong bottlenecks at formations of polyploidy wheat.

The π and Tajima tests of haplotypes at *TaSus1-7A* were performed on tetraploid wheat collections, Chinese landraces, and modern cultivars (Fig. 11, F and G). In tetraploid accessions, π reached a peak value in the first intron, and it appeared much higher than in Chinese hexaploid cultivars at all polymorphic sites. π of tetraploid accessions was 16.6- and 10.5-fold that in Chinese landraces and modern cultivars, and 3.6-fold that of European cultivars (Table II). The dramatic decline of diversity from the tetraploid to hexaploid

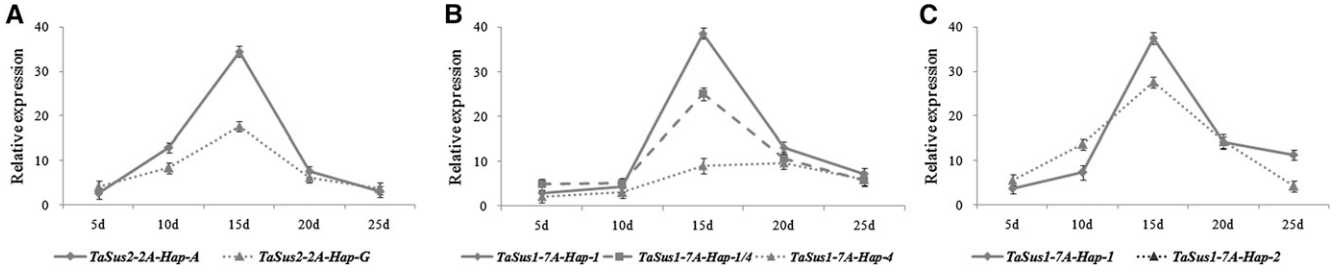


Figure 6. Relative quantification of mRNA expression by real-time PCR among different haplotypes in five periods post anthesis. The materials used in the tests were all from NIL sets BC_3F_4 population of cv Xiaoyan 6/Zhou 18*4 (A), BC_3F_3 population of cv Isengrain/Yanzhan 4110*4 (B), and F_3 population of cv Yangmai 158/Zhou 18*3//Handan 6172 (C). Two stable lines derived from the F_3 population were used for assays of *TaSus2* and *TaSus1* expression.

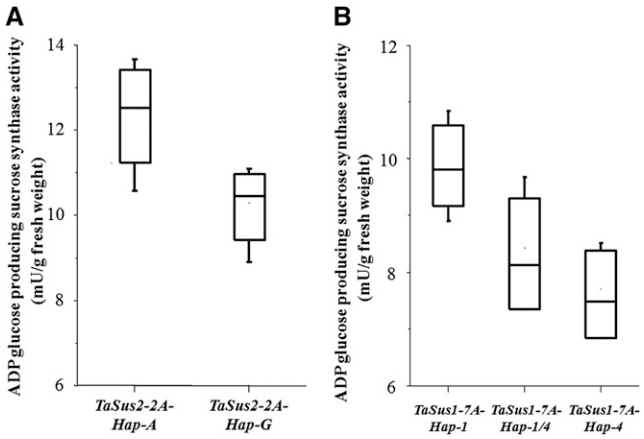


Figure 7. Boxplots of enzyme activities of Suc synthase in developing seeds among different haplotypes at 15 DPA. The bottom and top of the box are the first and third quartiles, and the band inside the box is the second quartile. The ends of the whiskers represent the minimum and maximum of all the data. The materials used in A and B were the same sets of those in Figure 6, A and B, respectively. The Suc synthase activity was measured in ADP Glc synthetic direction.

further illustrated that strong bottleneck effect occurred during the formation of common wheat. The Tajima test showed a *D* value below zero in tetraploid accessions, landraces, and Chinese cultivars and above zero in European cultivars, suggesting there was a large number of low-frequency allelic variations in tetraploid accessions, landraces, and Chinese cultivars (because *Hap-1* represented 88.2% and 89.6% of accessions, respectively), and intermediate-frequency allelic variations in European cultivars was mainly due to the population structure and selection effect.

Nucleotide substitution rate can be used for estimating times of sequence divergence. For grass genes, the average substitution rate was estimated as 6.5×10^{-9} substitutions per synonymous site per year (SanMiguel et al., 1998; Gu et al., 2006). Using this rate, divergence time was estimated for *TaSus1-7A* in diploid ancestors and tetraploid accessions (Fig. 12). The divergence time of the A genome progenitor of wheat, *T. urartu*, was estimated at 1.67 to 1.83 million years ago (*MYA*), with an average of 1.70 *MYA*. The divergence time for most tetraploid accessions occurred from 0.15 to 0.45 *MYA*.

Strong Negative Selection Occurred on Mutants within the Functional Domains of *TaSus1-7A*

Five haplotypes were detected at *TaSus1-7A* in this study; however, *Hap-4* and *Hap-5* occurred at very low frequencies in modern cultivars. Mutations at 1,599-, 1,600-, and 1,604-bp sites from the ATG initiation site could be considered rare alleles. The structure of *AtSus1* suggested *SUS* was a member of the GT-B glycosyltransferase superfamily (Zheng et al., 2011), and the active site of *AtSus1* was the *AtSus1*·UDP·Fru complex. In the complex, Fru was firmly bound within a pocket formed by residues from the GT-B_N domain. Nα1 is the first α-helix of the GT-B_N domain, and the adjacent region is very well conserved between *AtSus1* and *TaSus1* (Fig. 13A). Two nonpolar amino acids substitutions (SN→AA) at *Hap-4*/*Hap-5* might change the pocket domain binding Fru (Fig. 13B). This change might lead to reduced SUS enzyme activity and a nonbeneficial effect on yield. Therefore, it would be quickly excluded in global breeding.

DISCUSSION

Most Variation within *TaSus* Genes Occur in the Introns and Promoter Regions

As the first enzyme genes in the starch synthesis pathway in common wheat, *TaSus1* and *TaSus2* have

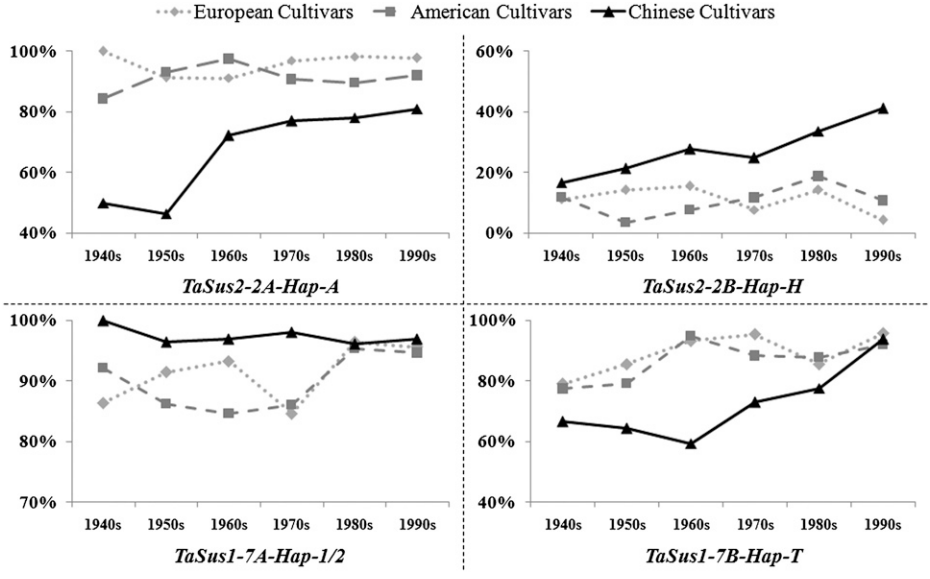


Figure 8. Changes in frequency of favored haplotypes at the *TaSus2-2A*, *TaSus2-2B*, *TaSus1-7A*, and *TaSus1-7B* loci over six decades in varietal populations released in China, Europe, and the United States.

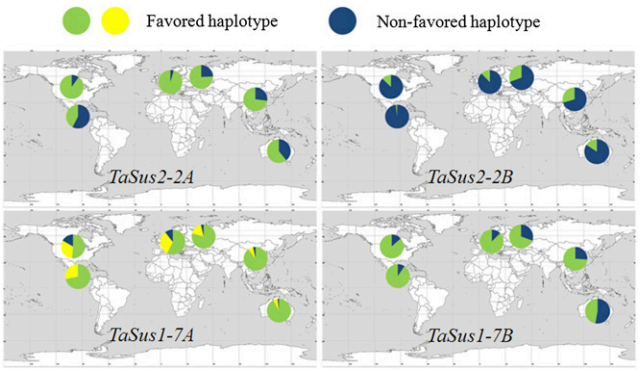


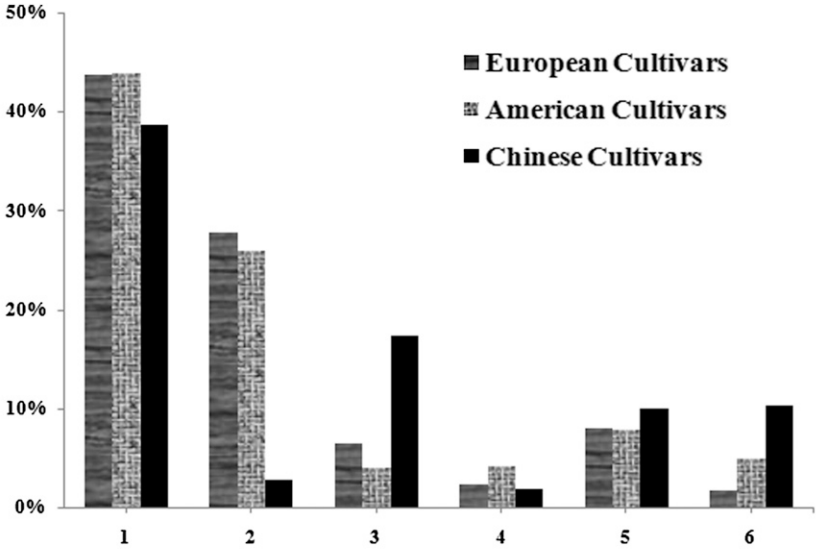
Figure 9. Geographic distribution of haplotypes at four loci in six major wheat production regions worldwide. The yellow is predominantly *TaSus1-7A-Hap-2*. [See online article for color version of this figure.]

low diversities in this study. Base substitutions in introns were more frequent than in exons. At *TaGW2-6A*, a candidate gene related to grain development, favored haplotype *Hap-A*, was associated with seed width and TKW (Su et al., 2011). No substitutions were found in the coding regions of *TaGW2-6A*, *TaGW2-6B*, and *TaGW2-6D*, but polymorphic sites were found in the promoter regions. Several downstream genes in the starch synthesis pathway show abundant SNPs in different populations (Y.M. Jiang and X.Y. Zhang, unpublished data), and the substitution sites are distributed mainly in the introns and promoter regions. This phenomenon was also detected in other crops. For example, in the carotenoid biosynthesis pathway of maize, the first enzyme gene *PSY1* (phytoene synthase gene), which converts geranylgeranyl pyrophosphate to phytoene, has less polymorphism in the coding and noncoding regions than the downstream genes encoding lycopene epsilon cyclase (*lcyε*) and *β-carotene hydroxylase1* (*crtRB1*; Yan et al., 2010; Fu et al., 2013). Therefore, it might be a general rule that that upstream genes in biosynthesis pathways have lower diversity than downstream ones.

TaSus1 and *TaSus2* Are Associated with Grain Yield of Wheat

In the starch synthesis pathway, SUS, ADP-Glc pyrophosphorylase, starch synthase, starch branching enzyme, and starch debranching enzyme are the key enzymes (Keeling and Myers, 2010). The functions and genetics

Figure 10. Proportion of haplotype combinations at four loci in three populations of released cultivars. The haplotypes below the numbers represented the combinations. Haplotypes with gray color represent nonfavored ones.



	Hap	Hap	Hap	Hap	Hap	Hap
TaSus2-2A	A	A	A	A	A	G
TaSus2-2B	L	L	H	H	L	L
TaSus1-7A	1	2	1	2	1	1
TaSus1-7B	T	T	T	T	C	T

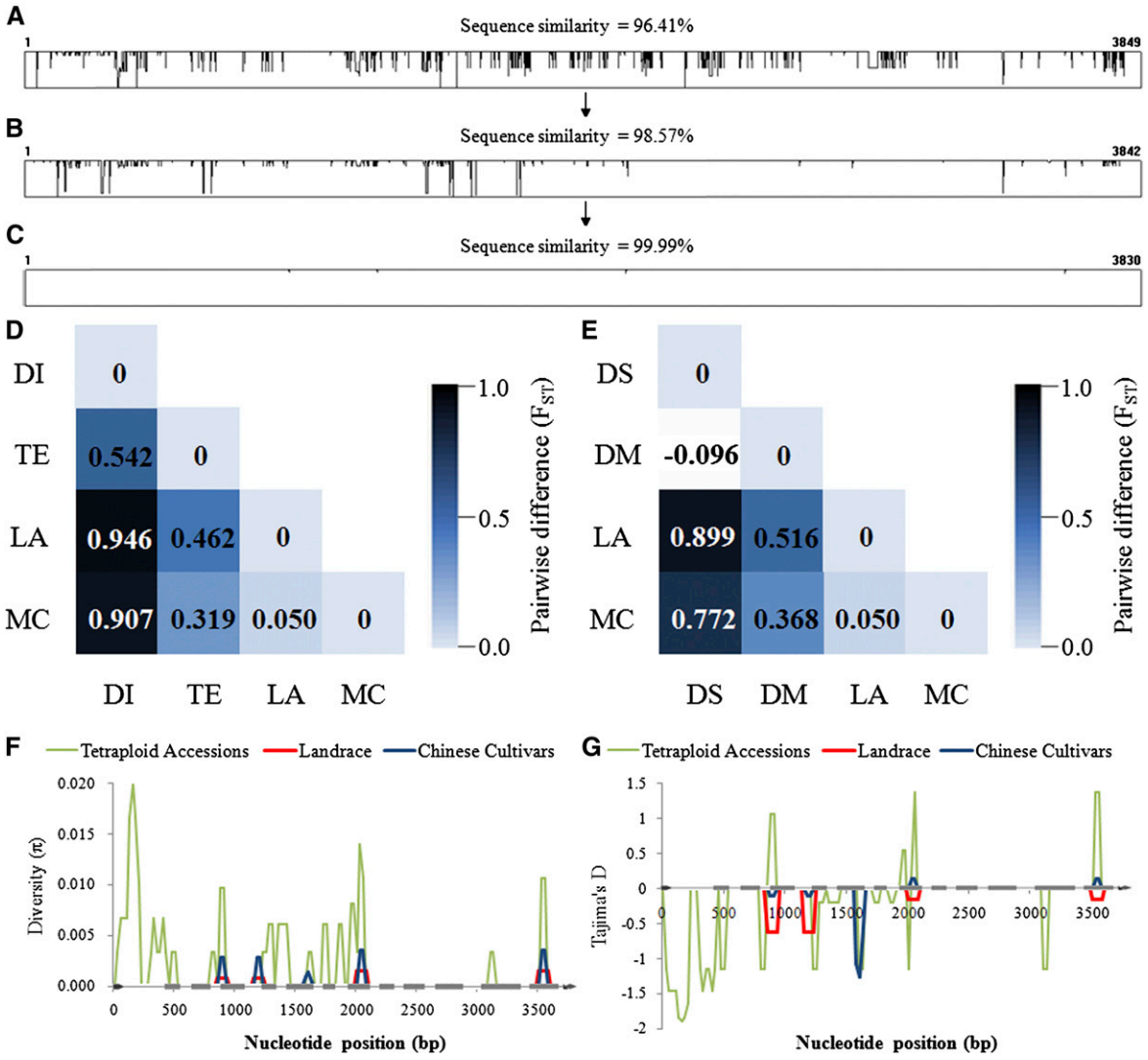


Figure 11. Diversity of the *TaSus1-7A* sequence in diploid accessions (A), tetraploid accessions (B; Supplemental Table S4), and common wheat (C; Chinese modern cultivars). Widths of rectangles represent the full lengths of *TaSus1-7A*, and lengths represent polymorphic sites and their diversity in collections. D and E, Genetic distance in *TaSus1-7A* between pairs of populations (F_{ST}). The color gradient presents F_{ST} values from dark (1.0) to light blue (0.0). D, DI, Diploid accessions; TE, tetraploid accessions; LA, landraces in MCC; MC, Chinese modern cultivars in MCC. E, DS, *T. dicoccoides*; DM, other tetraploid accessions used in this study. The πs (F) and Tajima's *D* (G) at *TaSus1-7A* between tetraploid wheat collections, landraces, and Chinese modern cultivars. The horizontal axis represents the coding region of *TaSus1-7A* (Fig. 1).

of these enzymes genes have been studied in many crops, yet associations with grain yield were rarely reported. Most previous studies focused on their influence on starch texture in wheat (Morell et al., 2003) and cooking quality in rice (Tian et al., 2009). Haplotype association revealed that *TaSus1* and *TaSus2* were associated with TKW in wheat. Favored haplotypes of four genes for higher TKW were detected based on field data of the Chinese MCC, Chinese modern cultivars, and NILs evaluated in multiple environments.

Table II. *Diversities among tetraploid accessions, Chinese landraces and cultivars, and European cultivars at TaSus1-7A*

h, Number of haplotypes; Hd, haplotype diversity; θ, nucleotide polymorphism.

Materials	*h*	Hd	π	θ	Tajima's *D*	*P*
Tetraploid accessions	8	0.924	0.00199	0.00288	–1.40251	>0.10
Landraces	3	0.156	0.00012	0.00019	–0.66614	>0.10
Chinese cultivars	5	0.193	0.00019	0.00029	–0.70361	>0.10
European cultivars	5	0.559	0.00055	0.00028	1.82542	0.10 > *P* > 0.05

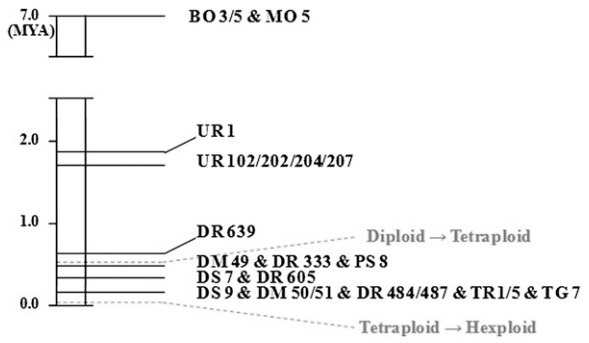


Figure 12. Estimation of divergence time at *TaSus1-7A* in *Triticum* spp. The formation of tetraploid wheat originated less than 0.5 *MYA* and that of hexaploid occurred about 6,000 to 8,000 years ago (Dubcovsky and Dvorak, 2007; Brenchley et al., 2012). BO, *Triticum boeotieum*; MO, *Triticum monococcum*; UR, *T. urartu*; DS, *T. dicoccoides*; DM, *T. dicoccum*; DR, durum wheat; PS, *Triticum persicum*; TR, *Triticum orientale*; TG, *Triticum turgidum*.

Genetic Differences on TKW between Haplotypes at the Same Locus Affect Selection Time and Intensity

The four (of six) genes all have the same function in starch synthesis and can be considered as isoenzymes but with differences between haplotypes on TKW as estimated in the MCC, Chinese modern cultivars, and NILs. The favored *Hap-1* and *Hap-2* at *TaSus1-7A* reached the highest frequencies (Fig. 9; Supplemental Table S3) among global wheat cultivars, and in some populations, one to two haplotypes (*TaSus1-7A-Hap-1* and *TaSus1-7A-Hap-2*) was almost fixed. At *TaSus2-2B*, the favored *Hap-H* was present in less than 25% of the world wheat cultivars. Analysis showed significant differences on genetic effect between haplotypes at the same locus existing among the four loci. They are 5.5 (7A), 4.6 (7B), 3.4 (2A), and 2.2g (2B), which were estimated based on the 348 Chinese modern cultivars and NILs (Table I; Supplemental Fig. S4). Therefore, genetic effect differences influenced haplotype selection time and intensity in cultivar populations. Of most interest was that at *TaSus2-2A*, *TaSus2-2B*, and *TaSus1-7B*, selection intensity for favored haplotypes in Australian cultivars was not as strong as in Chinese and European cultivars. Australian wheat is largely produced under rain-fed conditions (Turner, 2004), and yield potential of Australian cultivars is not as high as those of Europe or China. Selection intensity on *TaSus2-2A* and *TaSus2-2B* in CIMMYT cultivars is not as strong as in Chinese and former Soviet cultivars. The reason for this was not clear.

Bottleneck and Strong Selection Occurred at *TaSus1-7A* with Polyploidization and Domestication

Homologous *SUS* genes are highly diverse among different crop species, such as rice, maize, sorghum (*Sorghum bicolor*), barley, and wheat (Zhang et al., 2011).

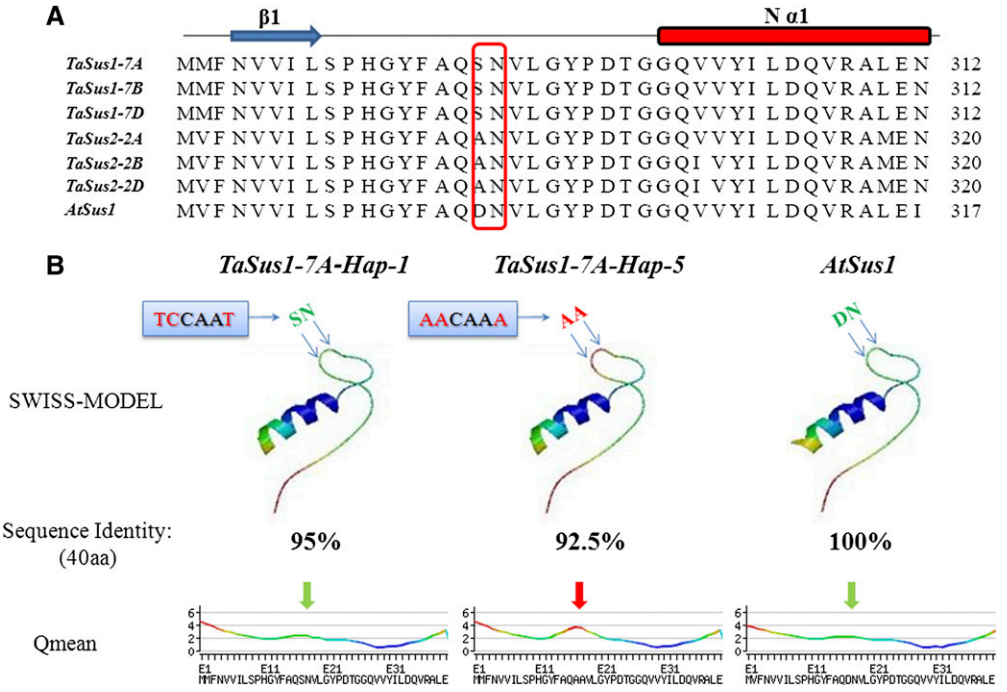


Figure 13. A, Alignment of amino acid sequences around polymorphic sites at 1,599, 1,600, and 1,604 bp at *TaSus1-7A* among *TaSus1*, *TaSus2*, and *AtSus1*. The red, hollow rectangle represents the polymorphic sites. B, Comparison of secondary protein structures of *TaSus1-7A-Hap-1* and *TaSus1-7A-Hap-5* by SWISS-MODEL (Guex and Peitsch, 1997; Schwede et al., 2003; Arnold et al., 2006) and Qmean (Benkert et al., 2011). The nucleotide sequences in blue rectangles represent the SNPs, and red letters are the SNPs. SN/AA/DN represent the amino acids coded in this region. The color of the Qmean curves represent the residue error, with gradients from blue (more reliable regions) to red (potentially unreliable regions). The downward-pointing arrows above Qmean curves represent the locations of polymorphic sites.

π in diploid wheat is significantly higher than in tetraploid and hexaploid wheat. Two dramatic declines of *SUS1* diversity occurred in *Triticum* genus evolution, one at tetraploidization and the other at hexaploidization (Fig. 11; Table II), which indicated that twice, strong bottlenecks occurred during polyploidizations. The genetic distance between wild tetraploid and hexaploid accessions was larger than that between domesticated tetraploid and hexaploid accessions (Fig. 11, D and E), suggesting that selection occurred in the process of tetraploid domestication. The extremely high frequency of *Hap-1* and *Hap-2* at *TaSus1-7A* in both landrace (95.5%) and modern cultivars (96.6%) suggested that strong selection to this locus took place in domestication of common wheat as well. This also implies that wheat originated from a rather narrow genetic base.

The significant differences in favored haplotype frequencies at *TaSus2-2A*, *TaSus2-2B*, and *TaSus1-7B* between landraces and modern cultivars suggest the occurrence of strong positive selection for superior haplotypes in breeding during the last century (Figs. 8, 9, and 11). Much of this change was likely caused by selection for larger grain size.

Favored Haplotypes Coding Key Enzymes in Starch Synthesis Pathway Were Selected Worldwide

Favored haplotypes at *TaSus2-2A*, *TaSus2-2B*, *TaSus1-7A*, and *TaSus1-7B* showed similar selection trends in six global wheat production regions (Fig. 9), covering about 51% of the harvested area and 60% of grain production worldwide (http://faostat.fao.org/site/339/default.aspx). Although favored haplotypes seem to be superior in all geographical regions, when considered across the four loci, the most favored combination has not been optimized in released cultivars (Fig. 10). Markers developed for these haplotypes should be useful in future breeding for genome selection.

MATERIALS AND METHODS

Plant Materials

Forty-seven diploid progenitor accessions and 36 tetraploid accessions were used in cloning by homology, genome-specific primer design, and evolutionary studies of two Suc synthase genes (Supplemental Table S4). Thirty-six wheat (*Triticum aestivum*) accessions, including 18 modern cultivars and 18 landraces, were used for sequencing to detect SNPs and haplotypes at the *TaSus1* and *TaSus2* loci. A DH population derived from a cross elite Chinese wheat cv Hanxuan 10 and Lumai 14 was used for mapping the haplotypes.

Two Chinese wheat populations were used in the study, namely 245 accessions from the Chinese wheat MCC representing 70% of the wheat genetic diversity of China and 348 modern cultivars from the Chinese wheat core collection (Hao et al., 2008, 2011). These two populations were selected from 23,705 accessions bred or collected in China (Zhang et al., 2002; Dong et al., 2003; Hao et al., 2008). The MCC accessions were planted at the Chinese Academy of Agricultural Sciences (CAAS) Luoyang Experiment Station in Henan Province in 2002, 2005, and 2006.

In addition, 384 European, 429 North American, 53 CIMMYT, 82 Russian, and 51 Australian cultivars were surveyed to investigate the global distribution of favored haplotypes of the *TaSus1* and *TaSus2* genes and frequency changes of the favored haplotypes during the last 100 years. Three wheat NILs derived from BC_3F_4 population of cv Xiaoyan 6/Zhou 18*4, BC_3F_3 population of cv Isengrain/Yanzhan 4110*4, and F_3 population of cv Yangmai 158/Zhou 18*3//Handan 6172 were used for detecting differences in transcript levels among cultivars with different haplotypes. One NIL pair derived from BC_3F_6 of cv Youzitou/Zhengmai 366*4 was used for detecting TKW difference between *Hap-1* and *Hap-3 at TaSus1-7A*. One NIL pair derived from BC_3F_2 of cv Shijiazhuang 8/Shi4185*4 was used for detecting TKW difference between *Hap-H* and *Hap-L at TaSus2-2B*. All hexaploid materials are listed in Supplemental Table S6.

DNA and RNA Extraction

Genomic DNA was extracted from young lyophilized leaves as described by Sharp et al. (1988). RNA isolation and reverse transcription followed Guo et al. (2010). RNA was extracted from immature embryos 5, 10, 15, 20, and 25 DAP using TRIzol reagent. cDNA was synthesized with the SuperScript II System (Invitrogen).

Haplotype Analysis of *TaSus1* and *TaSus2*

Three *TaSus2* genes were cloned by homology by Jiang et al. (2011), and three *TaSus1* genes were cloned by homology based on the sequence of barley (*Hordeum vulgare*) the *SUS1* gene (http://www.ncbi.nlm.nih.gov/nuccore/FN400939). Primer Premier 5.0 (http://www.premierbiosoft.com/) was used for designing all primers (Supplemental Table S7) used in this study. All primers were synthesized by Invitrogen and Sangon Biotech. PCRs used *LA Taq* polymerase (TaKaRa Biotechnology) in reaction volumes of 15 μL containing 50 ng DNA, 7.5 μL buffer, 1.0 μL 10 mM forward and reverse primers, 0.24 μL 25 mM deoxynucleotide triphosphates, and 0.15 μL *LA Taq* polymerase. PCR was carried out in a Veriti 96 Well Thermal Cycler (Applied Biosystems) with the following settings: denaturing at 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, annealing at 58°C to 63°C for 1 min, and 72°C for extension (1 kb min^{-1}), with a final extension of 72°C for 10 min. PCR products were purified by purification kit DP-1502 (Tiangen), then cloned by the pGEM-T Easy Cloning Vector (Tiangen), and transformed into *Escherichia coli* cells by the heat shock method. Plasmids were extracted by plasmid extraction kit DP-1002 (Tiangen). DNA was sequenced by an ABI 3730XI DNA Analyzer (Applied Biosystems). Sequence alignments were carried out by SeqMan and DNAMAN (http://www.lynnon.com/). SNPs were identified by DNASTAR (http://www.dnastar.com/). The digestion sites of restriction endonucleases were detected by Primer Premier 5.0. All the restriction endonucleases used in the study were produced by New England Biolabs.

Haplotype Transcript Analysis

Three NILs were used for detecting expression differences in relative quantification of *TaSus2-2A* and *TaSus1-7A* by real-time PCR (Fig. 6). RNA was extracted from wheat seeds at 5, 10, 15, 20, and 25 DPA and then reverse transcribed by a M-MLV Reverse Transcriptase Kit (Invitrogen). Real-time PCR was carried out by Rotor-Gene Q (Qiagen).

Enzyme Assays

The activity level of Suc synthase was assayed following Doehlert et al. (1988). One gram of powder of the lyophilized wheat seeds at 15 DPA was resuspended at 4°C in 7 mL of 50 mM HEPES (pH 7.5) and centrifugated at 10,000*g* for 10 min. The supernatant was assayed for enzymatic activity. All enzymatic reactions were performed at 30°C. One unit is defined as the amount of enzyme that catalyzes the production of 1 μmol product min^{-1}.

Statistical Analysis

Data processing were carried out by Office 2003 and SPSS Statistics 17.0 (http://www-01.ibm.com/software/analytics/spss/).

π at *TaSus1-7A*

Sequence alignments were carried out by DNASTAR. F_{ST} tests were carried out by Arlequin 3.5.1.2 (http://cmpg.unibe.ch/software/arlequin3/). Diversity analysis, Tajima *D* test, and a synonymous substitution test were carried out by DnaSP 5.10 (http://www.ub.es/dnasp). Divergence time was estimated

following Gaut and Clegg (1991). Secondary protein structures were predicted by SWISS-MODEL (http://swissmodel.expasy.org/).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. *TaSus2-2A* was mapped on 2AS flanked by simple sequence repeat markers *Xgwm122* and *Xgwm328* in DH population derived from cv Hanxuan 10 × Lumai 14.

Supplemental Figure S2. Polymorphisms and marker development at *TaSus2-2A*.

Supplemental Figure S3. Polymorphisms and marker developments at *TaSus1-7A*.

Supplemental Figure S4. Difference of TKW in two NILs in 2012 yield trial plot.

Supplemental Table S1. Haplotype transcript analysis data.

Supplemental Table S2. Enzyme assay data at 15 DPA.

Supplemental Table S3. Frequency of five favored haplotypes at TaSus2-2A, TaSus2-2B, TaSus1-7A, and TaSus1-7B in six released cultivar populations.

Supplemental Table S4. Accessions of diploid and tetraploid wheats used in this study.

Supplemental Table S5. F_{ST} between different populations and the corresponding *P* value at *TaSus1-7A*.

Supplemental Table S6. Accessions of hexaploid wheats used in this study.

Supplemental Table S7. Primers used to amplify variant regions of *TaSus1* and *TaSus2*.

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