The production of wheat-Aegilops sharonensis 1Ssh chromosome substitution lines harboring alien novel high-molecular-weight glutenin subunits

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The production of wheat-*Aegilops sharonensis 1S*\textsuperscript{th}

chromosome substitution lines harboring alien novel

high-molecular-weight glutenin subunits

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Abstract

In our previous work, a novel high-molecular-weight glutenin subunit (HMW-GS) with an extremely large molecular weight from *Aegilops sharonensis* was identified that may contribute to excellent wheat (*Triticum aestivum*) processing quality and increased dough strength, and we further generated HMW-GS homozygous lines by crossing. In this study, we crossed the HMW-GS homozygous line 66-17-52 with ‘Chinese Spring’ Ph1 mutant *CS ph1b* to induce chromosome recombination between wheat and *Ae. sharonensis*. SDS-PAGE was used to identify 19 derived F2 lines with the HMW-GSs of *Ae sharonensis*. The results of non-denaturing fluorescence in situ hybridization (ND-FISH) indicated that lines 6-1 and 6-7 possessed a substitution of both 5D chromosomes by a pair of 1S<sup>th</sup> chromosomes. Further verification by newly developed 1S<sup>th</sup>-specific chromosome markers showed that these two lines amplified the expected fragment. Thus, it was concluded that lines 6-1 and 6-7 are 1S<sup>th</sup>(5D) chromosome substitution lines. The 1S<sup>th</sup>(5D) chromosome substitution lines, possessing alien subunits with satisfactory quality-associated structural features of large repetitive domains and increased number of subunits, may have great potential in strengthening the viscosity and elasticity of dough made from wheat flour. Therefore, these substitution lines can be used for wheat quality improvement and further production of 1S<sup>th</sup> translocation lines.

Keywords: *Aegilops sharonensis*; substitution line; Ph gene mutant; high-molecular-weight glutenin subunits; molecular markers
**Introduction**

High-molecular-weight glutenin subunits (HMW-GSs) are essential in the determination of dough viscosity and elasticity in wheat flour processing (Shewry et al. 2003). HMW-GSs are encoded by the Glu-1 loci, located on the long arms of the homoeologous group 1 chromosomes in wheat, and each locus comprises two tightly linked genes, one encoding an x-type with larger molecular mass and the other a y-type with smaller molecular mass (Payne 1987).

In wheat and its wild related species, the typical primary structures of mature HMW-GSs are composed of the conserved signal peptides, N- and C-terminal domains, and a central repetitive region composed of tripeptide, hexapeptide, and nonapeptide motifs (Shewry et al. 1995). Molecular mass variations of HMW-GSs mainly result from the changes in the motif numbers in the repetitive domain. Some structural features of HMW-GSs are considered to be relevant to gluten polymers and their qualities during the baking of wheat dough. The number and distribution of cysteine residues decide the formation of inter- and intra-molecular disulfide bonds. A good example is the 1Dx5 subunit, which confers satisfactory quality to wheat dough due to the presence of an additional cysteine residue in its repetitive region (Lafiandra et al. 1993). Previous studies revealed the positive relationship between the length of the repetitive domains and their effects on wheat dough strength, as the subunits with long repetitive domains could form more stable interactions via inter-chain hydrogen bonds (Lee et al. 1999).
Aegilops sharonensis (Eig) Á. Löve. ($S^{sh}S^{sh}$, $2n = 2x = 14$) is an annual diploid grass species that was first discovered in a very narrow geographic range, on the coastal plains of Israel and southern Lebanon, and its genome is closely related to the B genome of common wheat (Triticum aestivum L.) (Olivera and Steffenson 2009). Ae. sharonensis is a rich source of genes that can provide resistance to important wheat diseases, such as leaf rust, stripe rust, and powdery mildew (Olivera and Steffenson 2009). Stem rust-resistance genes $Sr-1644-1Sh$ are located on the $1S^{sh}$ chromosome of Ae. sharonensis (Yu et al. 2017). The novel HMW-GS variants, $1S^{sh}x2.9$ and $1S^{sh}y2.3$, have been identified with the large molecular weight of 2.9 kb for the x-type and 2.3 kb for the y-type subunits (Jiang et al. 2012). The Glu-$1S^{sh}$ locus of Ae. sharonensis can be used for wheat quality improvement because the encoded subunits possess longer repetitive domains than most of the known subunits.

The pairing homoeologous (Ph) genes play a key role in controlling the frequency of homoeologous chromosome pairing and recombination (Qi et al. 2007; Sears 1977). In common wheat, the Ph gene is located mostly on chromosome 5BL and 3DS, but the Ph gene mutation (ph1) on 5BL locus is much more potent than the Ph gene mutation (ph2) on the 3DS locus (Riley and Chapman 1958). The ‘Chinese Spring’ Ph mutant (CS ph1b) has a deletion at the Ph1 locus, consisting of a deleted segment approximately 70 Mb long of chromosome 5BL in ph1b (Dunford et al. 1995). The Ph1-deficient genetic groups have been widely used in transferring desirable genes from wild wheat to cultivated wheat for the strengthening of resistances (Chen et al. 1994; Friebe et al. 1996; Mullan et al. 2009; Zhao et al. 2013).
As the foundation of this study, we successfully produced wheat-Ae. *sharonensis* amphidiploids by conducting a wide hybridization (Zhao et al. 2014; Jiang et al. 2014a). The wheat-Ae. *sharonensis* amphidiploids were backcrossed with hexaploid common wheat, followed by self-crosses or backcrosses for multiple years (Wei et al. 2015). Then, 24 derived lines with homozygous composition of HMW-GSs were identified using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and non-denaturing fluorescence in situ hybridization (ND-FISH) (Li et al. 2019). Based on these previous studies, we aim to induce recombination between alien *Ae. sharonensis* and hexaploid wheat (AABBDD, 2n = 6x = 42) by conducting a cross between wheat-Ae. *sharonensis*-derived lines and CS *ph1b*, and report the characterization of two 1S*sh*(5D) chromosome substitution lines using multiple methods, including SDS-PAGE, ND-FISH, and chromosome-specific molecular markers.

**Plant materials**

The diploid grass species of *Ae. sharonensis* (R7) PI584388 were kindly provided by the United States Department of Agriculture-Agricultural Research Service (USDA-ARS) ([http://www.ars-grin.gov](http://www.ars-grin.gov)). The hybrid offspring line 66-17-52 was previously generated from the progenies of self-crosses or backcrosses of the tetraploid wheat (Z636)—Ae. *sharonensis* amphidiploid with hexaploid wheat cv. Liangmai 3 (LM3), and was characterised as having alien HMW-GSs and containing six S*sh* chromosomes of *Ae. sharonensis* (Li et al. 2019; Wei et al. 2015; Zhao et al. 2014). ‘Chinese Spring’ Ph1 mutant (CS *ph1b*) was used in the cross in this study to
 induce chromosome recombination between wheat and the hybrid line. These
accessions were deposited at the Triticeae Research Institute, Sichuan Agricultural
University, China.

**Chromosome manipulation**

To further generate the carrier of $1S_{sh}$ chromosomes only, 66-17-52 (as female
parents) was crossed with CS mutant $ph1b$ (as male parents) according to a
previously described method (Liu et al. 1999). The $F_1$ plants were self-crossed, and
the derived $F_2$ seeds/plants were screened by HMW-GS composition and ND-FISH to
identify individuals with chromosome $1S_{sh}$ (Fig. 1).

**SDS-PAGE**

HMW-GSs were extracted and isolated from half of the endosperm of mature seeds
by SDS-PAGE, as previously described (Mackie et al. 1996). HMW-GSs from parent
wheat CS $ph1b$ (null, 1Bx7+1By8 and 1Dx2+1Dy12), Z636 (null, 1Bx+1By), LM3 (null,
1Bx7+1By8 and 1Dx2+1Dy12), Ae. *sharonensis* (R7) ($1S_{sh}$x2.9 and $1S_{sh}$y2.3), and
homozygous line 66-17-52 (null, 1Bx7+1By8, 1Dx2+1Dy12, and $1S_{sh}$x2.9+$1S_{sh}$y2.3)
were used as the reference.

**ND-FISH**

The methods described by (Han et al. 2006) were used to prepare chromosome
spreads. The synthetic oligonucleotide probes Oligo-pSc119.2-1 and Oligo-pTa535-1
were used for ND-FISH analysis to characterize alien chromosomes of Ae.
*sharonensis* (Tang et al. 2014). The probe (AAG)$_6$ was used to assist with identifying
chromosomes. The probes Oligo-pSc119.2-1, Oligo-pTa535-1, and (AAG)$_6$ were 5’ end-labelled with 6-carboxyfluorescein (6-FAM), 6-carboxytetramethylrhodamine (Tamra), or Cy5. The signals of probes labeled with 6-FAM, Tamra, and Cy5 appeared as the colors of green, red, and red, respectively. The ND-FISH protocol was performed according to a previously described protocol (Fu et al. 2015). Images were obtained using an epifluorescence microscope (BX51, Olympus, Tokyo, Japan).

**Molecular marker detection**

Four alien 1S$_{sh}$ chromosome-specific markers were developed according to the multiple sequence alignment between the genomic sequences from *Aegilops* (https://urgi.versailles.inra.fr/download/) and those of wheat chromosomes 1A, 1B, and 1D from Unite de Recherche Genomique Info (URGI) (ftp://ftp.ensemblgenomes.org/pub/plants/). The one pair of 5D chromosome primer sequences (wmc233F: 5’-GACGTCAAGAATCTTCGTCGGA-3’, wmc233R: 5’-ATCTGCTGAGCAGATCGTGGTT-3’) of a simple sequence repeat (SSR) marker was obtained from a previous report (Gupta et al. 2002).

The 1S$_{sh}$ and 5D chromosome primers were screened and amplified in the F$_2$ hybrids and parents. Total genomic DNA was extracted from the young leaf tissue of each one-month-old plant using the cetyltrimethylammonium bromide (CTAB) method (Murray and Thompson 1980). PCR amplification was conducted in a 25-μl reaction mixture containing 1× buffer, 200 μM of each dNTP, 0.2 μM of each primer, and 0.5 U Taq Plus DNA Polymerase (TianGen Biotech, Beijing, China). The PCR
amplification cycles consisted of denaturation for 5 min at 94°C, followed by 35 cycles at 94°C for 30 sec for the annealing of different primers at 64°C, 65°C, 67°C, 63°C, and 72°C for 65 sec, 45 sec, 45 sec, 75 sec, and 30 sec, respectively, with a final extension step at 72°C for 10 min. The PCR products were resolved in 2% agarose gel, and DNA bands were visualized with Gel Doc XR+ (Bio-Rad, Hercules, CA, USA).

Results

HMW-GS profile of F₁ and F₂ progenies of the crosses between 66-17-52 × CS ph1b

Ten seeds of F₁ were successfully obtained by conferring pollination of emasculated 66-17-52 plants with CS ph1b according to the chromosome modification scheme (Fig. 1). First, the HMW-GS composition of F₁ was identified by SDS-PAGE analysis, and the results showed that the profiles of the HMW-GSs in 10 seeds of F₁ were from Ae. sharonensis (1S<sup>sh</sup>x2.9 and 1S<sup>sh</sup>y2.3) plus those of parent LM3 (1Bx7+1By8 and 1Dx2+1Dy12) (Fig. 2a), indicating that all of those seeds were hybrids of 66-17-52 × CS ph1b. Subsequently, all 10 seeds were sown, and 4 of them (No. 1, 2, 5, and 6) successfully germinated. F₂ seeds were generated by bagging individual ears for self-pollination. The composition of the HMW-GSs of F₂ seeds was analyzed by SDS-PAGE, and the results showed that the HMW-GS composition of some F₂ seeds was identical to those of F₁ seeds, while the HMW-GSs of Ae. sharonensis (1S<sup>sh</sup>x2.9 and 1S<sup>sh</sup>y2.3) were not present in the remainder of the F₂ seeds (Fig. 2b-e).

ND-FISH observation

Based on the results of HMW-GS composition, all of the F₁ and the F₂ seeds
harboring *Ae. sharonensis* HMW-GSs (1S\textsuperscript{sh}x2.9 and 1S\textsuperscript{sh}y2.3) were selected for chromosome composition analysis by ND-FISH (Table 1). The No. 2 F\textsubscript{1} seed contained 42 chromosomes including four additional chromosomes, three alien S\textsuperscript{sh} chromosomes, and one 2D wheat chromosome as well as four lost wheat chromosomes including one of each of the 1A, 4D, 5D, and 7D chromosomes. The total number of chromosomes in the No. 5 F\textsubscript{1} seed was also 42, which contained six additional alien S\textsuperscript{sh} chromosomes, and six lost wheat chromosomes including one 1A chromosome, one 7B chromosome, and one 3D and two 5D and one 6D chromosomes. The total number of chromosomes in the No. 6 F\textsubscript{1} seed was 43, in which there were four additional alien S\textsuperscript{sh} chromosomes and three lost wheat chromosomes, including one 7B chromosome and both 5D chromosomes (Fig. 3c-d). The chromosomal composition of the No. 1 F\textsubscript{1} seed was not observed, as its root tip did not show the cell division phase.

The total number of chromosomes in the F\textsubscript{2} progenies of the No. 1 F\textsubscript{1} seed was 40 or 41, with 5 or 6 alien S\textsuperscript{sh} chromosomes that were observed (Table 1). The F\textsubscript{2} progenies of the No. 2 F\textsubscript{1} line possessed chromosome numbers varying from 40 to 42, with 2–4 alien S\textsuperscript{sh} chromosomes. Although the total number of chromosomes in the No. 5-3 F\textsubscript{2} progeny from the No. 5 F\textsubscript{1} line was 42, it contained five alien S\textsuperscript{sh} chromosomes and five lost wheat chromosomes, including one 1A chromosome and both 3D and both 5D chromosomes. The total number of chromosomes from No. 6-1 and No. 6-7 from the No. 6 F\textsubscript{1} seed was 42, and was composed of one pair of S\textsuperscript{sh} chromosomes substituting both 5D wheat chromosomes (Fig. 4), whereas the total number of
chromosomes of the lines No. 6-3, No. 6-10, and No. 6-17 was 43 and included three alien \(S^h\) chromosomes while losing both 5D wheat chromosomes. The root tip of \(F_2\) lines No. 1-5, No. 5-1, No. 5-6, No. 6-19, and No. 6-21 did not show the cell division phase. The seeds from line No. 6-20 did not germinate.

**Molecular marker analysis**

Thirty-five specific genomic DNA sites were successfully identified by the genomic sequence alignment of the *Aegilops* \(1S^h\) chromosome with those of the 1A, 1B, and 1D chromosomes. From the 19 specific genomic DNA sites, which were identified as potential sites for the development of chromosome-specific markers, four DNA markers were successfully developed that could specifically detect the *Ae. sharonensis* \(1S^h\) chromosome by PCR amplification. These four \(1S^h\) chromosome-specific DNA markers produced PCR fragments with sizes of 600, 673, 1200, and 1421 bp (Table 2). Among them, primer pairs \(1S^{sh}L2F\_2R\_1\) and \(1S^{sh}L16F\_16R\_1\) were located on the long arm of the \(1S^h\) chromosome, and homologous comparison was used to located \(1S^{sh}S5F\_2R\_2\) and \(1S^{sh}S30F\_130R\_1\) on the short arm of the \(1S^h\) chromosome (Fig. 5).

These four DNA markers were further validated by PCR, and the results indicated that the expected bands could only be amplified from *Ae. sharonensis* DNA. No amplified products were observed from DNA derived from the tetraploid wheat Z636 and the hexaploid LM3 wheat species (Fig. 6), indicating that these markers could be used for accurately tracking \(1S^h\) chromosomes. Therefore, these
DNA markers could be used to identify the presence of the *Ae. sharonensis* 1S<sup>th</sup> chromosome in the hybrid progenies.

The results of marker detection in the progenies of F<sub>2</sub> lines 66-17-52 × CS *ph1b* showed that all of the F<sub>2</sub> lines harboring the alien 1S<sup>th</sup> chromosome of *Ae. sharonensis* contained the target DNA bands, while no PCR band was amplified in the F<sub>2</sub> line without the 1S<sup>th</sup> chromosome (Fig. 6). These results were consistent with the results of SDS-PAGE and ND-FISH, and proved that the 1S<sup>th</sup> chromosomes of *Ae. sharonensis* were indeed present in the No. 6-1 and No. 6-7 F<sub>2</sub> lines. The loss of both 5D chromosomes in the No. 6-1 and No. 6-7 F<sub>2</sub> lines was validated by 5D chromosome-specific DNA markers. The results showed that the No. 6-1 and No. 6-7 F<sub>2</sub> lines could not produce DNA bands at approximately 250 bp, while CS and LM3 exhibited the target DNA band (Fig. S1).

**Discussion**

HMW-GSs are essential seed storage proteins and contribute to the dough viscosity and elasticity of wheat flour (Shewry et al. 2003). Many HMW-GS alleles with novel structural features have been identified and characterized from wild species of Triticeae (Anderson and Greene 1989; Cui et al. 2009; Forde et al. 1985; Gao et al. 2010; Jiang et al. 2012; Jiang et al. 2009; Jiang et al. 2014; bLiu et al. 2010; Liu et al. 2007; Sun et al. 2006). However, there are very few instances of successfully introducing these HMW-GS alleles from wild species into common wheat for quality improvement. The low success rate may be due to the difficulty of introducing the
alien genes into wheat by transgenics or wild crossing.

The presence of HMW-GS genes in the wheat-\textit{Dasypyrum villosum} introgression line had a positive effect on bread-making quality (Zhang et al. 2014). Bread-making quality has also been greatly improved by using wheat-\textit{Aegilops longissima} 1S\(^1\) disomic addition and substitution lines (Garg et al. 2014). (Du et al. 2018) In a previous study, a wheat-\textit{Aegilops searsii} 1S\(^1\)(1B) substitution line GL1402 was developed, and tests showed that the line could be used to make steamed bread with satisfactory quality. These results showed that alien HMW-GSs with novel structural characteristics are valuable in wheat quality improvement. However, compared to the vast numbers of cloned HMW-GS genes from wild species, the work of gene introduction and utilization has been insufficient.

In the present study, we successfully produced two 1S\(^{ph}(5D)\) chromosome substitution lines that were characterized using multiple methods, including SDS-PAGE, ND-FISH, and chromosome-specific DNA molecular markers. The results were achieved from over 10 years of research work involving gene identification and cloning, wild crossing, and backcrossing or self-crossing. The huge amount of work and great difficulty in operation were the barrier preventing gene transfer by far-distance crossing.

Homoeologous pairing is strictly restricted by Ph1 and Ph2 in wide crosses. To overcome this problem, a number of ‘Chinese Spring’ (CS) Ph1 and Ph2 mutant lines were produced as \textit{CS ph1b}, \textit{CS ph2a}, and \textit{CS ph2b}, which allow homoeologous
chromosome pairing in the hybrid of Ph mutants and related alien species and enable gene transfer from the alien species to wheat (Wall et al. 1971; Sears 1982; Martinez-Perez and Moore 2008). Of the Ph1 and Ph2 mutants, CS ph1b exhibits a greater pairing promotion effect than other mutants (Benavente et al. 1998). In our study, we successfully produced 1S<sup>th</sup>(5D) chromosome substitution lines from hybrids between 66-17-52 and CS ph1b. Our results further validated the excellent effect of CS ph1b in homoeologous pairing and supported the notion that the CS ph1b gene is valuable for alien gene transfer in distant hybridization.

Although we conducted the cross with the CS ph1b mutant to induce chromosome translocation between the alien and the wheat chromosomes, no translocations were observed in this work. We speculated that this phenomenon resulted because (1) one cross of F1 to CS ph1b was insufficient, and much more backcrossing to CS ph1b is necessary to generate translocation; and (2) the numbers of hybrid seeds were small (Table 1), which greatly reduced the probability of screening out the line with the chromosome translocation. For the two substitution lines, the alien 1S<sup>th</sup> chromosome substituted a 5D pair instead of 1S<sup>th</sup> substitution 1A or 1B or 1D. This might be due to the 1S<sup>th</sup> chromosomes possessing a higher homology to 5D chromosomes as compared to 1D of common wheat cv. LM3. Although stable expression of the alien HMW-GS locus was achieved in the 66-17-52 plants, there was uncertainty as to whether the plants were homozygous at another locus. The instability of the material 66-17-52 plants caused a difference in the gametes, and thus, the chromosome constitution of all of the F<sub>1</sub> lines was not
Previous studies have found that some chromosomes in *Ae. longissima* and *Ae. triuncialis* have the effect of "killing" gametes (Maan 1975; Endo and Tsunewaki 1975). Chromosomes with "killing" gametes function are generally called gametocidal (*Gc*) chromosomes. *Ae. sharonensis* also contains a *Gc* chromosome, and because it was mapped to chromosome 4S, it is difficult to remove the *Gc* chromosome and produce a full set of additions or substitutions (Miller et al. 1982; Olivera and Steffenson 2009). Friebe et al. (2003) produced a knockout mutation at the *Gc* locus, allowing the introgression of all S<sup>sh</sup> chromosomes (in addition to 4S<sup>sh</sup>) into wheat. However, other studies found that certain "killing" gametocidal chromosomes have no obvious preferential transmission in the context of specific common wheat, and gametogenesis seems to be partially inhibited (Endo 1988).

Although the 4S<sup>sh</sup> chromosome of *Ae. sharonensis* carries a gametocidal chromosome, preferential transmission of the 4S<sup>sh</sup> chromosomes was not found in this study, which may due to the gametocidal gene *Gc* on the 4S<sup>sh</sup> chromosomes of *Ae. sharonensis* having no obvious preferential transfer in the context of parental material (Z636, LM3, and CS *ph1b*).

The role of HMW-GSs in determining dough strength depends on their ability to create a large variation in the size of native glutenin polymers, which is a consequence of qualitative attributes (structural features) and quantitative differences (number of subunits) in the glutenin subunits (Gupta et al. 1994). Experiments incorporating 1Dx2.2 and 1Dx2.2* subunits into dough indicated that both
subunits together can lead to greater dough strength as compared to 1Dx2 alone (Belton 1999; Feeney et al. 2003). The comparison indicated that the properties and interactions of the repetitive domains are essential in determining the viscoelastic properties of wheat dough, and longer subunits can form more stable interactions within the gluten polymers. Because the 1S<sup>x</sup>2.9 and 1S<sup>y</sup>2.3 subunits of Ae. *sharonensis* are larger than almost all other known HMW-GSs, the 1S<sup>x</sup>(5D) chromosome substitution lines with two subunits of Ae. *sharonensis* may have a positive effect on dough strength. Theoretically, hexaploid wheat should contain six different HMW-GSs; however, the gene inaction among HMW-GSs results in the variable HMW-GS numbers in wheat, from three to five subunits in hexaploid bread wheat and one to three subunits in tetraploid wheat (Payne et al. 1981; Payne and Lawrence 1983).

The durum and bread wheat lines with four and six HMW-GSs, respectively, have been produced by replacing the silent genes at the *Glu-A1* locus with the allelic forms that express x-type and y-type subunits. These lines possess an increased amount of polymeric glutenin and improved flour performance (Ciaffi et al. 1995; Rogers et al. 1997; Lafiandra et al. 1993). Researchers (Wang et al. 2018) introduced the 1Ay subunit of wild emmer wheat into common wheat cv. Chuannong 16. The resulting introgression line TaAy7-40 expressed six HMW-GSs and showed higher protein content, higher sodium dodecyl sulfate (SDS) sedimentation value, higher content of wet gluten in the flour, and higher grain weight as compared to Chuannong 16.

The chromosome substitution we performed in the 6-1 and 6-7 F<sub>2</sub> lines was 5D
and not 1D through replacement by 1S<sup>th</sup>, which led to the increase in the number of HMW-GSs from four to six. Therefore, the wheat-Ae. sharonensis 1S<sup>th</sup>(5D) chromosome substitution of the 6-1 and 6-7 F<sub>2</sub> lines, possessing alien subunits with the good quality trait associated with the structural features of the large repetitive domain and increased number of subunits, may have great potential in strengthening the viscosity and elasticity of dough made from wheat flour. These chromosome substitutions can also be useful for further production of 1S<sup>th</sup> translocation lines.

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**Author contribution statement**

JQT conceived and designed research. LXY conducted experiments and prepared the manuscript. LY, KH and LY performed the crosses in the fields. THP, MJ, QPF, LW, and PZE contributed to SDS-PAGE; ZXJ, CGY, and WJR contributed to development of molecular markers; TZX assisted with ND-FISH. LXJ and DM analyzed the data, and LZY, HW, WYM, and ZYL revised the manuscript. All of the authors read and approved the manuscript.
Conflict of interest: We declare that we do not have any conflicts of interest.

Compliance with ethical standards

Ethical standards: We declare that all of the experiments in the study comply with ethical standards.
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Payne, P. I. and Lawrence, G. J. 1983. Catalogue of alleles for the complex gene loci Glu-A1, Glu-B1 and...


integrated active $Glu-1Ay$ allele in common wheat from wild Emmer and its potential role in flour improvement. International Journal of Molecular Sciences, 19, 923. doi: 10.3390/ijms19040923.


Figure Legends

Figure 1. Procedure for producing $T. aestivum$–Ae. sharonensis (R7) substitution chromosomes using the CS $ph1b$ mutant. LM3, Liangmai 3; 66-17-52, BC3F6 line from the cross between (Z636 × R7) × LM3.

Figure 2. HMW-GS composition of hybrid offspring $F_1$ and $F_2$ lines determined by
SDS-PAGE. a Lanes 3 to 10: F\textsubscript{1} seeds (labeled as 1 to 10); b Lanes 3 to 7: seeds of the F\textsubscript{2} line, F\textsubscript{2}-1 (labeled as 1-1 to 1-5); lanes 8 to 13: seeds of the F\textsubscript{2} line, F\textsubscript{2}-5 (labeled as 5-1 to 5-6); c Lanes 3 to 9: seeds of the F\textsubscript{2} line, F\textsubscript{2}-2 (labeled as 2-1 to 2-7). d, e Lanes 3 to 13: seeds of the F\textsubscript{2}-6 line (labeled as 6-1 to 6-22). CS ph1\textsubscript{b}, ‘Chinese Spring’ Ph mutant; Z636, \textit{Triticaceae durum}; LM3, Liangmai 3; R7, \textit{Ae. sharonensis}; 66-17-52, BC\textsubscript{3}F\textsubscript{6} line from the cross between (Z636 × R7) × LM3.

**Figure 3.** Non-denaturing fluorescence \textit{in situ} hybridization (ND-FISH) analysis. a, b Parent line 66-17-52; c, d F\textsubscript{1} line No. 6. Oligo-pTa535-1 (red), Oligo-pSc119.2-1 (green), and (AAG)\textsubscript{6} (red) were used as probes. Chromosomes were counterstained with DAPI (blue). 1S\textsuperscript{sh} alien chromosomes are indicated by gules S.

**Figure 4.** Non-denaturing fluorescence \textit{in situ} hybridization (ND-FISH) analysis. a, b F\textsubscript{1} line No. 6-1; c, d F\textsubscript{2} line 6-7; e 1S\textsuperscript{sh}(5D) chromosome substitution line No. 6-1. Oligo-pTa535-1 (red), oligo-pSc119.2-1 (green), and (AAG)\textsubscript{6} (red) were used as probes. Chromosomes were counterstained with DAPI (blue). 1S\textsuperscript{sh} alien chromosomes are indicated by gules 1S\textsuperscript{sh}.

**Figure 5.** Development of 1S\textsuperscript{sh} chromosome-specific DNA markers. The developed molecular DNA markers are located on the molecular linkage map for 1A, 1B, 1D, and \textit{Ae. sharonensis} 1S\textsuperscript{sh}. For each chromosome, markers are shown on the left, and marker distances are shown on the right.

**Figure 6.** Validation of 1S\textsuperscript{sh}-specific markers. a Self-cross descendants of F\textsubscript{1} line No. 1 (labeled as 1-1 to 1-5) and No. 5 (labeled as 5-1 to 5-6); b self-cross descendants of F\textsubscript{1} line No. 2 (labeled as 2-1 to 2-7); c and d self-cross descendants of F\textsubscript{1} line No. 6.
(labeled as 6-1 to 6-22). M, DNA marker; R7, *Ae. sharonensis*; Z636, *T. durum*; LM3, Liangmai 3; 66-17-52, BC$_3$F$_6$ line from the cross between (Z636 × R7) × LM3. The molecular marker images from top to bottom are 1S$_{sh}$L2F$_1$2R$_1$, 1S$_{sh}$L16F$_1$16R$_1$, 1S$_{sh}$5F$_2$5R$_2$, and 1S$_{sh}$30F$_1$30R$_1$. The seeds from No. 6-20 did not germinate, and therefore, detection experiments with DNA markers were not performed.
### Table 1. Chromosome numbers of wheat-\textit{Ae. sharonensis}-derived lines

<table>
<thead>
<tr>
<th>Line No.</th>
<th>Chromosome number</th>
<th>Alien 5\textsuperscript{th} number</th>
<th>Null chromosome</th>
<th>Glu-1S\textsuperscript{th}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parent</td>
<td>66-17-52</td>
<td>41</td>
<td>6</td>
<td>1A3D*4D6D</td>
</tr>
<tr>
<td>1</td>
<td></td>
<td></td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>F\textsubscript{1}</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2**</td>
<td>42</td>
<td>3</td>
<td>1A<em>4D</em>5D<em>7D</em></td>
<td>+</td>
</tr>
<tr>
<td>5</td>
<td>42</td>
<td>6</td>
<td>5D1A<em>7B</em>3D<em>6D</em></td>
<td>+</td>
</tr>
<tr>
<td>6</td>
<td>43</td>
<td>4</td>
<td>5D7B*</td>
<td>+</td>
</tr>
<tr>
<td>1-2</td>
<td>40</td>
<td>5</td>
<td>1A3D5D6D*</td>
<td>+</td>
</tr>
<tr>
<td>1-3</td>
<td>41</td>
<td>6</td>
<td>1A3D5D6D*</td>
<td>+</td>
</tr>
<tr>
<td>1-4</td>
<td>40</td>
<td>6</td>
<td>1A3D5D4B<em>6D</em></td>
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<tr>
<td>1-5</td>
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<td>-</td>
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<tr>
<td>2-2**</td>
<td>42</td>
<td>3</td>
<td>1A5D6D*</td>
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<td>3</td>
<td>5D1A<em>3D</em></td>
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<td>5D1A<em>3D</em></td>
<td>+</td>
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<tr>
<td>2-5</td>
<td>40</td>
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<tr>
<td>5-1</td>
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<td></td>
<td>-</td>
<td></td>
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<tr>
<td>F\textsubscript{2}</td>
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</tr>
<tr>
<td>5-3</td>
<td>42</td>
<td>5</td>
<td>3D5D1A*</td>
<td>+</td>
</tr>
<tr>
<td>5-6</td>
<td></td>
<td></td>
<td>-</td>
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<tr>
<td>6-1</td>
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<td>2</td>
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<td></td>
<td>Did not germinate</td>
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</tr>
<tr>
<td>6-21</td>
<td></td>
<td></td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

* *Indicates the null deletion of one chromosome; 2** indicates that there are three 2D chromosomes; 3 2-2** indicates that there are three 1D and three 2D chromosomes; - indicates that the chromosomal composition of the seeds was not obtained; + indicates the presence of alien HMW-GSs of \textit{Ae. sharonensis}; the two chromosome substitution lines of 1S\textsuperscript{th}(5D) are highlighted. 5 6 7
Table 2. The DNA molecular markers specific to the 1S\textsuperscript{th} chromosome arms of \textit{Ae. sharonensis}.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence (5’ to 3’)</th>
<th>Chromosome arm</th>
<th>Product size (bp)</th>
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</thead>
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<tr>
<td>1S\textsuperscript{sh}L 2F\textsubscript{1}</td>
<td>AATTGGAGATTCATGCAAAGCCG</td>
<td>1S\textsuperscript{sh} long arm</td>
<td>1200</td>
</tr>
<tr>
<td>1S\textsuperscript{sh}L 2R\textsubscript{1}</td>
<td>CGCAACTCTGAACCTTTATGTTAT</td>
<td>1S\textsuperscript{sh} long arm</td>
<td>763</td>
</tr>
<tr>
<td>1S\textsuperscript{sh}L 16F\textsubscript{1}</td>
<td>GGAAGTGGGCGATGTCAGAAG</td>
<td>1S\textsuperscript{sh} long arm</td>
<td>776</td>
</tr>
<tr>
<td>1S\textsuperscript{sh}L 16R\textsubscript{1}</td>
<td>CATAACTGAACCCCTGGCAACTGC</td>
<td>1S\textsuperscript{sh} short arm</td>
<td>600</td>
</tr>
<tr>
<td>1S\textsuperscript{sh}S 5F\textsubscript{2}</td>
<td>GTGTAACAGCAGTCTATGGT</td>
<td>1S\textsuperscript{sh} short arm</td>
<td>1421</td>
</tr>
<tr>
<td>1S\textsuperscript{sh}S 5R\textsubscript{2}</td>
<td>TACTGACCAGGCATTCCCAG</td>
<td>1S\textsuperscript{sh} short arm</td>
<td>531</td>
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<tr>
<td>1S\textsuperscript{sh}S 30F\textsubscript{2}</td>
<td>GGGAGGCAAAGATTTATGAT</td>
<td>1S\textsuperscript{sh} short arm</td>
<td>1421</td>
</tr>
<tr>
<td>1S\textsuperscript{sh}S 30R\textsubscript{2}</td>
<td>ATGCCGACCCTCCGTAGAC</td>
<td>1S\textsuperscript{sh} short arm</td>
<td>1421</td>
</tr>
</tbody>
</table>
Figure 1. Procedure for producing T. aestivum–Ae. sharonensis (R7) substitution chromosomes using the CS ph1b mutant. LM3, Liangmai 3; 66-17-52, BC3F6 line from the cross between (Z636 × R7) × LM3.
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511x655mm (72 x 72 DPI)
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335x251mm (72 x 72 DPI)