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Effects of 1Dy12 subunit silencing on seed storage protein accumulation and flour-processing quality in a common wheat somatic variation line

Haiqiang Chen[a,1], Shijin Li[a,1], Yongwei Liu[b,1], Junxian Liu[c], Xiaolan Ma[a], Lipu Du[a], Ke Wang[a,*], Xingguo Ye[a,*]

[a] Institute of Crop Sciences, Chinese Academy of Agricultural Sciences, Beijing 100081, China
[b] Institute of Genetics and Physiology, Hebei Academy of Agriculture and Forestry Sciences, Shijiazhuang 050051, China
[c] College of Life Science, Capital Normal University, Beijing 100048, China

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ABSTRACT

Dissecting the functions of high molecular weight glutenin subunits (HMW-GSs) is helpful for improving wheat quality via breeding. In this study, we used a wheat mutant AS273 in which HMW-GS 1Dy12 was silenced to investigate the silencing mechanism of *1Dy12* and its effects on gluten accumulation and flour-processing quality. Results suggested that the expression of *1Dy12* in AS273 was decreased by one fifth during grain development; a stop codon produced by a base mutation (C/T) led to truncated translation; the absence of 1Dy12 stimulated the accumulation of low molecular weight glutenin subunits (LMW-GSs), gliadins, and glutenin macropolymers, and was resulted in larger protein bodies; AS273 had an inferior flour-processing performance. Based on the outputs achieved in this study it is concluded that 1Dy12 makes important contributions to bread, sponge cake and biscuit-processing quality.

1. Introduction

Wheat (*Triticum aestivum* L., 2n = 6x = 42, AABBDD) is one of the most important cereal crops planted globally and plays an enormous role in energy and nutrition supply for humans (Shewry, 2009). In comparison with other major cereals such as rice and maize, wheat has unique end-use traits and can be used to make a series of food products such as breads, noodles, cakes, and cookies. These special properties are mainly conferred by the gluten subunits in wheat grains (Wang et al., 2013). Gluten proteins are commonly classified into polymeric glutenins and monomeric gliadins according to their solubility in various solvents, which are regarded to be the major determinants of dough elasticity and viscosity, respectively (Lambourne et al., 2010; Shewry & Halford, 2002). The monomeric gliadins related to dough extensibility and ductility can be further divided into four subfamilies, α/β-, γ-, δ- and ω-gliadins (Veraverbeke & Delcour, 2002). The polymeric glutenins, including two major subfamily types of high molecular weight glutenin subunits (HMW-GSs) and low molecular weight glutenin subunits (LMW-GSs), are the major determinants of gluten elasticity and strength for bread- processing (Rasheed et al., 2014). Although content of HMW-GSs only accounts for 5–10% of the storage proteins and is at relatively low levels in wheat grains, it greatly impacts the bread-processing quality of wheat flour (Branlard & Dardevet, 1985; He, Liu, Xia, Liu, & Pena, 2005). Thus, the HMW-GSs are considered as the major

* Corresponding authors.
E-mail addresses: wangke03@caas.cn (K. Wang), yexingguo@caas.cn (X. Ye).
[1] These authors contributed equally to this work.

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factors affecting the end-use quality of wheat.

It is well known that HMW-GSs in wheat are encoded by three homoeologous alleles *Glu-A1*, *Glu-B1*, and *Glu-D1* at the *Glu-1* loci located on the long arms of group 1 chromosomes (Payne, Holt, & Law, 1981). At each locus the two HMW-GS genes encoding an x-type (80–88 kDa) and a y-type (67–73 kDa) subunit are tightly linked. Thereby, wheat theoretically contains up to six different HMW-GSs, while, in fact only three to five subunits were expressed in most wheat cultivars because of gene silencing and allelic variation (Anjum et al., 2007; Shewry, Halford, & Tatham, 1992). Normally, the genes belonging to 1Bx, 1Dx, and 1Dy are expressed, whereas the genes for 1Ay are often silent (Roy et al., 2018; Yu et al., 2019). Currently, more than 30 *HMW-GS* alleles have been cloned by various strategies. The *HMW-GS* genes typically hold highly conserved sequences, including long DNA repeats (Ma, Yu, She, Zhao, & Islam, 2019; Rasheed et al., 2014). It was demonstrated that *1Dx5*, *1Dx10*, *1Ax1*, *1By8*, *1Bx13*, and *1By16* may have relatively more important roles in conferring dough elasticity and strength than other alleles of *HMW-GSs* (Li et al., 2015; Shewry et al., 1992). Some subunit combinations such as *1Dx5* + *1Dy10*, *1Bx7* + *1By8*, and *1Bx17* + *1By18*, display a strong positive effect on the bread-processing quality through contributing superior dough strength, while other subunits combinations, such as *1Dx2* + *1Dy12*, *1Bx20*, and *1Bx7* + *1By9*, have weak effects on gluten quality (Guo, Wu, Lu, & Yan, 2019; Jiang et al., 2019; Liu et al., 2016; Shewry et al., 1992; Yan et al., 2009).

The x- and y-type HMW-GSs consist of a signal peptide, an *N*-terminal domain (ND), a central repetitive domain (RD), and a C-terminal domain (CD). The non-repetitive highly conserved N- and C-terminal domains are rich in cysteine residues (Tatham, Marsh, Wieser, & Shewry, 1990). The x-type subunits generally have four conserved cysteine residues in which three are placed in ND and one in CD, while the y-type subunits contain seven conserved cysteine residues (five in ND, one in RD, and one in CD). These cysteine residues are involved in intermolecular disulfide bonding in the formation of glutenin macropolymers (GMPs), and affect the structure and function of glutenins (Delcour et al., 2012). Many studies have confirmed that the RD constitutes β-turns, while the ND and CD are rich in α-helices by molecular modeling and secondary structural analyses (Wieser, 2007). There are many repeat units such as nona- (GYYPTSL/PQQ), hexa- (PGQGQQ) and tri-peptides (GQQ) in the RD. HMW-GS allelic variants are mainly attributed to variations in the size of the RD, in which different numbers of repeated peptide motifs are contained. These are crucial features associated with the physical properties of wheat dough. Additionally, the *HMW-GS* genes possessing longer repetitive domains, higher expression level, and additional cysteines may have relatively more positive impacts on wheat dough quality (Wang et al., 2013).

Generally, developing wheat variation mutants and near-allelic variation lines at the *Glu-1* locus is an ideal strategy to dissect the effects of HMW-GSs on the flour-processing quality. Normally, silencing or variation at the *Glu-1* locus greatly affects wheat bread-processing quality. The loaf volumes of the five knockout mutants (ma1-1, ma1-1mb14-1, mb14-1, ma1-2, and mb14-2) for the complete set of five *HMW-GS* genes (*1Ax1*, *1Bx14*, *1By15*, *1Dx2*, and *1Dy12*) developed by ethyl methane sulfonate (EMS) induction, in which one or more of those aforementioned HMW-GSs was missing in a wheat variety Xiaoyan54, were significantly reduced in comparison with the wild-type, demonstrating that 1A1 and 1Bx14 had positive effects on dough or bread-processing quality (Li et al., 2015). Comparative analysis on single and double deletion mutants suggested that the *Glu-D1* locus has more effect on bread-processing quality than the loci *Glu-A1* and *Glu-B1* (Wang et al., 2017). Similar studies showed that the x-type HMW-GSs belonging to strong effect subunits play more important roles in processing quality than the y-type HMW-GSs belonging to weak effect subunits (Blechl et al., 2007). The examination of the mutants missing 1Dx2 or 1Dy12 showed that 1Dx2 has a stronger function than 1Dy12 in promoting functional glutenin macropolymers (FGMPs) by enabling the binding of HMW-GSs and LMW-GSs onto FGMPs, combining the tests for gluten, dough and bread-processing quality parameters (Wang et al., 2017). Additionally, it was found that the absence of 1Ax1 or 1Dx2 significantly decreased the dough development time and dough stability, but increased the uniformity of microstructure using two sets of wheat near-isogenic lines (Gao et al., 2018). Silencing of HMW-GSs in the transgenic line LH-11 significantly reduced the dough properties, glutenin macropolymers (GMP) content, wet gluten content, sedimentation value, and dough development and stability time (Zhang et al., 2018).

Using the wheat deletion lines DLGluA1, DLGluB1, and DLGluD1 missing loci *Glu-A1*, *Glu-B1*, and *Glu-D1*, respectively, which were created by the ion beam methods, the contribution of each of the HMW-GSs to bread-processing quality was precisely dissected in the same genetic background; results indicated that the genetic effects of the *Glu-1* locus on gluten functionality was *Glu-D1* > *Glu-B1* > *Glu-A1*. In addition, the *Glu-1* locus was proved to affect gluten functionality by promoting the formation of GMP and balancing the ratios of HMW-GSs, LMW-GSs, and gliadins (Yang et al., 2014). A study using the near-isogenic lines (NILs) lacking the HMW-GSs at the *Glu-1* locus created from a hybrid cross revealed that the lacking of 1Ax1 or 1Dx2 significantly decreased the dough development time and dough stability time, but increased the uniformity of micro structure; the absence also decreased the accumulation of GMP and thus delayed the rapid accumulation period for GMP by 10 d at least, which led to a lower percentage of polymeric protein in mature wheat grains (Gao et al., 2018). Recently, a *Glu-1Ay* allele was integrated into wheat from wild emmer, and the introgressed lines showed positive effects on flour-processing quality and yield traits (Roy et al., 2020; Wang et al., 2018).

Although many *HMW-GSs* genes have been identified in wheat grains, the contribution of individual HMW-GSs (including 1Dy12) to gluten composition and functionality and flour-processing quality in seeds is not completely clear. To understand the role of 1Dy12 in the flour-processing products and the accumulations of the main seed storage proteins, we investigated the primary accumulation of HMW-GSs during different seed developmental stages by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in a wheat somatic variation line AS273 in which *1Dy12* was silenced. Then, gene sequencing and expression analysis were performed to explore the possible reason for the silencing of *1Dy12*. The relative content of glutens including HMW-GSs, LMW-GSs, gliadins, and GMPs were also determined in the mutant. Additionally, dynamic changes of PBs were performed in AS273 during grain development. Further, AS273 was evaluated for flour-processing quality traits. The results achieved in this study will be valuable for wheat quality breeding and flour food production.

2. Materials and methods

2.1. Plant materials

Wheat cultivar Kenong199 (KN199) and its somatic variation line AS273 were used in this study. KN199 was obtained from the National Germplasm Bank of China at the Institute of Crop Sciences (ICS), Chinese Academy of Agricultural Sciences (CAAS), Beijing, China. AS273 was developed from the immature embryos culture of KN199. The HMW-GS composition of KN199 consists of 1Ax1, 1Dx2, 1Dy12, 1Bx7, and 1By9 (Fig. S1). In AS273 the HMW-GS encoding gene *1Dy12* at the *Glu-D1* locus was silenced (Fig. S1). AS273 and KN199 were planted at the Experimental Station of CAAS (Beijing, China) in the autumn of 2017 in 20 rows with a length of 1.5 m and a width of 20.0 cm. Six immature grains were collected from AS273 and KN199 in the middle part of the ears after flowering for 7, 10, 13, 16, 19, 22, 25, and 28 d, and immediately frozen in liquid nitrogen. Three immature grains from each sampled time-point were used to extract RNA for gene expression analysis; the remaining three immature grains at each time-

point were used for glutenin extraction for the HMW-GS composition investigation at different developmental stages.

2.2. Investigation of main agronomic characters

At the maturity stage, ten plants were randomly picked from the two materials to examine agronomic traits including plant height, spike length, spikelets per ear, grains per spike, thousand kernels weight (TKW), seed length, seed width, and seed diameter (mm). The length, width, area, and chlorophyll content of flag leaves were also measured at the grain filling stage using a CI-203 AREA METER and a SPAD-502Plus chlorophyll meter.

2.3. Extraction of seed storage proteins and SDS-PAGE assay

Glutenin was extracted from the sampled grains following a previously described method with some modifications (Liu et al., 2016). In brief, grain samples collected from AS273 and KN199 at different developmental stages were ground in a high flux tissue grinder and then transferred to a 1.5 mL Eppendorf tube with 1 mL 70% ethanol. The samples were kept overnight at room temperature, and then vortexed for 20 min and centrifuged at 13,000 rpm for 10 min. The sample pellet was washed with 1 mL 55% isopropanol, maintained in a 65 ˚C water bath for 40 min, and subsequently centrifuged at 13,000 rpm for 10 min. Glutenin was purified from the clean pellet by adding extraction buffer consisting of 50% isopropanol, 200 mM Tris-HCl (pH 8.0), and 1% DTT. For SDS-PAGE analysis, each 60 μL of the extracted supernatant was mixed in an Eppendorf tube with 60 μL of loading buffer [2% (w/v) SDS, 80 mM Tris-HCl (pH 8.0), 40% (v/v) glycerol, and 0.02% (w/v) bromophenol blue) following incubation in a water bath at 65 ˚C for 20 min and centrifugation at 13,000 rpm for 5 min. Electrophoresis was performed in a 12% PAGE gel on a Bio-Rad PROTEAN II XL apparatus at 30 mA for 4 h.

2.4. Determination of glutens and GMP contents

Glutenins and gliadins in AS273 and its wild type were extracted and quantitated subsequently from three biological replicates by reversed-phase ultra-performance liquid chromatography (RP-UPLC) according to a previously described method (Han et al., 2015; Yan et al., 2014; Yu et al., 2013) with minor modifications. Flour (30 mg) was taken from each sample and mixed in 1 mL 70% ethanol by vortexing. After centrifugation the sample pellet was washed with 1 mL 55% isopropanol and incubated in a 65 ˚C water bath for 30 min. Glutenins were extracted from the samples using extraction buffer consisting of 50% isopropanol, 0.08 M Tris-HCl (pH 8.0), and 1% dithiothreitol (DTT), in the water bath, followed by an alkylated reaction in a new extraction buffer in which 1% DTT was replaced by 1.4% 4-vinylpyridine (v/v). After centrifuging, the supernatant was precipitated with 100% analytical pure acetone, and the precipitated glutenins were dissolved in a solution containing 21% v/v acetonitrile (ACN) and 0.1% m/v trifluoracetic acid (TFA). The final solution was filtered with a 0.22 μm membrane for RP-UPLC.

To extract gliadins, the same amount of flour was treated by vortexing in a 500 μL 0.04 M NaCl solution. After centrifugation, the pellet was dissolved in ddH_2O and the gliadins were extracted with 70% ethanol combined with vortexing. The supernatant was collected by centrifuging and then filtered with a 0.22 μm membrane for RP-UPLC.

Glutenin macropolymers were extracted and quantitated by size-exclusion ultra-performance liquid chromatography (SE-UPLC) based on a previous method (Zhou et al., 2018) with minor modifications. Flour (20 mg) was dissolved in a 1800 μL extraction buffer consisting of 0.05 M phosphate buffer solution (PBS) with 2% SDS by vortexing combined with ultrasonic treatment, and kept on oscillation for 1 h. After centrifugation, the supernatant was filtered with a 0.45 μm membrane for SE-UPLC.

The analyses for RP-UPLC and SE-UPLC were respectively carried out on a Thermo DGLC UPLC system with a Waters BEH C18 column (100 × 2.1 mm, 1.7 μm) and an Agilent *SEC*-5 column (500 Å, 4.6 × 300 mm; Agilent Tech). The mobile phases were 5 mM trifluoroacetic acid (TFA) and acetonitrile with 5 mM TFA for RP-UPLC, and 0.05 M PBS with 0.1% SDS and ddH_2O for SE-UPLC. In RP-UPLC analysis, a volume of 5 μL glutens for each sample was injected, the flow rate was set to 0.35 mL/min, and the elution and washing time was 25 min. During the process, the column temperature was set to 45 ˚C. The protein peaks were detected under UV light with absorbance areas at 210 nm. The peak area of chromatogram was used to represent the relative content of each HMW-GS. In the SE-UPLC analysis, a volume of 20 μL GMPs for each sample was injected and GMPs were determined using the same flow rate and UV wavelength to those aforementioned. For RP-UPLC and SE-UPLC analysis, the peak area (mAU*min) was used to measure content of gluten proteins and GMPs.

2.5. DNA extraction and PCR amplification

Genomic DNA was extracted from the leaves of one-month-old seedlings using a NuClean Plant Genomic DNA Kit (CW Bio Inc., China). One pair of primers specific to *1Dy12*, named F1 (5′- ATGGCTAAGCG GCTGGTCCTC-3′) and R1 (5′-CTATCACTGGCTAGCCGACAATG-3′), was designed according to published DNA sequence (GenBank accession X03041.1) to amplify the complete coding sequence (CDSs) in AS273 and KN199. KOD-Plus-Neo (TOYOBO, Osaka, Japan) was used for the amplification. Each reaction volume (50 μL) was composed of 10 × KOD PCR buffer, 0.2 mM dNTPs, 1.5 mM $MgSO_4$, 0.2 μM of each primer, 1 μL KOD-Plus-Neo, and 200 ng of genomic DNA. The PCR program started at 94 ˚C for 5 min, followed by 35 cycles of 95 ˚C for 30 s, 65 ˚C for 30 s and 72 ˚C for 2 min, with a final extension at 72 ˚C for 8 min. Agarose gels (1.0%) were used to separate the PCR products.

2.6. Sequencing of the amplified 1Dy12 gene

The target DNA fragments were purified from the agarose gel using the TIANgel Midi Purification Kit (TIANGEN BIOTECH, Beijing, China) and then ligated by the Lethal Based Fast Cloning Kit (TIANGEN BIOTECH, Beijing, China). The positive clones were sequenced by Sangon Biotech, Shanghai, China. The gene sequences were assembled and aligned using SnapGene Version 3.2.1 software (GSL Biotech). The coding sequences obtained were translated into amino acid sequences by ExPASy (http://web.expasy.org/translate/).

2.7. RNA extraction and reverse transcription and qRT-PCR assays

Immature wheat seeds collected from AS273 and KN199 at different grain development stages were used for total RNA extraction with the TransZol Up Plus RNA Kit (TransGen Biotech, China). Complementary DNA (cDNA) was synthesized using a reverse transcription kit (HiScript III RT SuperMix for qPCR, Vazyme). Traditional reverse transcription PCR (RT-PCR) and real-time quantitative PCR (qRT-PCR) were both used to evaluate the expression level of *1Dy12* in AS273 and KN199 at different grain developmental stages.

For traditional RT-PCR, the PCR reaction mixture was composed of 7.5 μL PCR Mix buffer (2 ×), 1 μL cDNA (200 ng·μL^{-1}), 0.6 μL primer mix (10 μM), and 5.9 μL ddH_2O. The specific primers (KS1) for *1Dy12* (Table S1) was used to analyze its expression. PCR was performed on a Bio-Rad C1000 Thermal Cycler (Bio-Rad, USA) at 95 ˚C for 5 min followed by 35 cycles of 95 ˚C for 30 *sec*, 60 ˚C for 30 *sec*, 72 ˚C for 30 *sec*, and a final extension at 72 ˚C for 8 min.

For qRT-PCR, a 20 μL reaction volume contained 10 μL 2 × RealStar Green Fast Mixture with ROX II, 1 μL first-stand cDNA, 0.6 μL primer mix (10 μM) each for *1Dy12*, and 8.2 μL ddH_2O. The amplification was performed on an ABI PRISM 7500 Real-Time PCR System (ABI, USA) with a thermal cycling program of 95 ˚C for 5 min, followed by 40

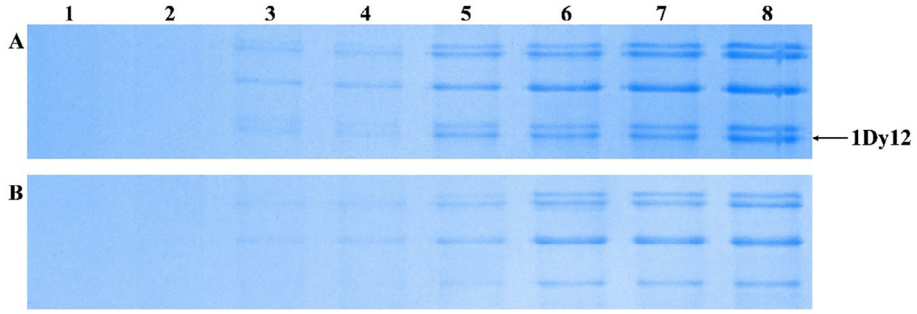

Fig. 1. Dynamic accumulations of HMW-GSs in KN199 and AS273 during the grain development based on SDS-PAGE. A: the dynamic accumulations of HMW-GSs in KN199; B: the dynamic accumulations of HMW-GSs in AS273. 1–8: samples at different grain developmental stages. 1: 7 DPA; 2: 10 DPA; 3: 13 DPA; 4: 16 DPA; 5: 19 DPA; 6: 22 DPA; 7: 25 DPA; and 8: 28 DPA.

cycles of amplification (95 °C for 5 *sec*, 60 °C for 30 *sec*, and 72 °C for 30 *sec*).

2.8. Observation of protein bodies by transmission electron microscopy

The developmental grain samples at 7, 10, 13, 16, 19, 22, 25, and 28 DPA were collected from AS273 and KN199 for transmission electron microscopy (TEM) observation. The main steps including sample fixation, rinsing, dehydration, and ultrathin section preparation were conducted as in a previous report with some modifications (Zhu et al., 2018). All samples were fixed in the dark at room temperature for 48 h in 3% (v/v) glutaraldehyde dissolved in 50 mM potassium phosphate buffer (pH 7.2). Observation of TEM was carried out using an H7500 transmission electron microscope (Hitachi, Tokyo, Japan).

2.9. Flour-processing quality evaluation for bread, sponge cake and biscuit

Mixograph analysis and bread baking experiments of AS273 and KN199 were carried out at the Wheat Quality Laboratory at the Institute of Crop Sciences, CAAS, Beijing, China. Some main parameters related to wheat dough quality, such as crude protein content, water absorption, dough development time and stability time, and mixing tolerance index were examined to evaluate the differences between AS273 and KN199 on bread-processing quality according to a previously described method (Yan et al., 2009). The baking procedure was manipulated by the standard rapid-mix-test using 1 kg flour containing 14% moisture. Bread size and loaf score were calculated according to a published method (Darlington et al., 2003).

Sponge cake and biscuit processing experiments were carried out at the Academy of State Administration of Grain, Beijing, China. The qualities for sponge cake and biscuit were evaluated according to the National Standards of China GB/T 24303–2009 and GB/T 20980–2007, respectively. Briefly, sponge cake quality was assessed by the coarser texture and structure; and biscuit quality was assessed by the width/thickness, texture, and surface characteristics.

2.10. Statistical analysis

Data analysis was performed using SPSS version 20 in Windows (SPSS, USA). qRT-PCR results were analyzed by ABI 7500 software and Microsoft Excel 2016. The criterion for statistical significance was set at $P < 0.01$.

3. Results

3.1. Comparison of the main agronomic and botanic traits between AS273 and KN199

In an offspring population derived from the immature culture of KN199, we identified a somatic variation plant, designated AS273, in which HMW-GS 1Dy12 was absent based on the SDS-PAGE analysis (Fig. S1). This somatic variation line was sown in the autumn of 2017 in a field. To examine the morphological changes caused by silencing the 1Dy12 subunit, AS273 was compared with KN199 for several important agronomic and botanic traits. Line AS273 was highly similar to KN199 in plant height (80.8 cm for KN199 and 80.9 cm for AS273), spikelet number, seed length, seed width, and seed diameter (Fig. S2, Table S2). However, differences were identified in spike length, kernel number per spike, grain weight per spike, and TKW (35.6 g for KN199 and 34.2 g for AS273) between the two wheat materials (Table S2). In particular, AS273 had significantly higher leaf length, width, and area than KN199, while it was lower in chlorophyll content than KN199 (Table S3). These results indicate that AS273 possesses significant differences from KN199 in a few agronomic and botanic traits.

3.2. HMW-GS dynamic accumulations in AS273 at different seed developmental stages

In order to study the dynamic accumulation of HMW-GSs, especially 1Dy12 in AS273, glutenin was extracted from the immature grains of AS273 and KN199 at increasing days post anthesis (DPA), and then analyzed by SDS-PAGE. The HMW-GSs in the grains of AS273 and KN199 started to accumulate at 13 DPA, and the accumulative amount of HMW-GSs was increased in AS273 and KN199 as the grain-filling period extended (Fig. 1). It was clear that four HMW-GSs (1Ax1, 1Dx2, 1Bx7, and 1By9) accumulated normally in AS273 starting from 11 DPA, while 1Dy12 was not detected over all grain developmental stages (Fig. 1). In contrast, 1Dy12, as well as 1Ax1, 1Dx2, 1Bx7, and 1By9, were normally accumulated in KN199. These results indicated that the 1Dy12 subunit in AS273 was silenced during all grain developmental stages (Fig. 1).

3.3. Expression profiling of 1Dy12 in AS273 at different grain developmental stages

The 1Dy12 protein was completely missing in AS273 according to the above results. To clarify if the *1Dy12* gene at the *Glu-D1* locus was normally expressed in transcriptional and translational levels in AS273 during the course of seed development, especially at the early grain-filling stage, total RNA was extracted and reverse-transcribed to cDNA from the immature grains of AS273 and KN199 at different DPAs. Then, RT-PCR and qRT-PCR were employed to determine the expression profiles of *1Dy12* in AS273 and KN199. As a result, *1Dy12* was completely expressed in AS273 and KN199 at all stages of grain development (Fig. 2). However, the expression level of *1Dy12* in KN199 was stronger than that of AS273 throughout all grain development stages, indicating that the expression of *1Dy12* was significantly suppressed in AS273 (Fig. 2). Moreover, the reference gene *ADP* was normally expressed at the same level across all samples in the two wheat materials, indicating that the cDNAs from all the samples were almost equal in both quality and concentration (Fig. 2).

In contrast to the results by RT-PCR, qRT-PCR analysis showed similar but more reliable results. It was found that the transcriptional expression level of *1Dy12* in KN199 was about five times higher than that of AS273 at most stages, except that it was 12 times higher at 22 DPA and two times higher at 10 and 25 DPA. The expression of *1Dy12*

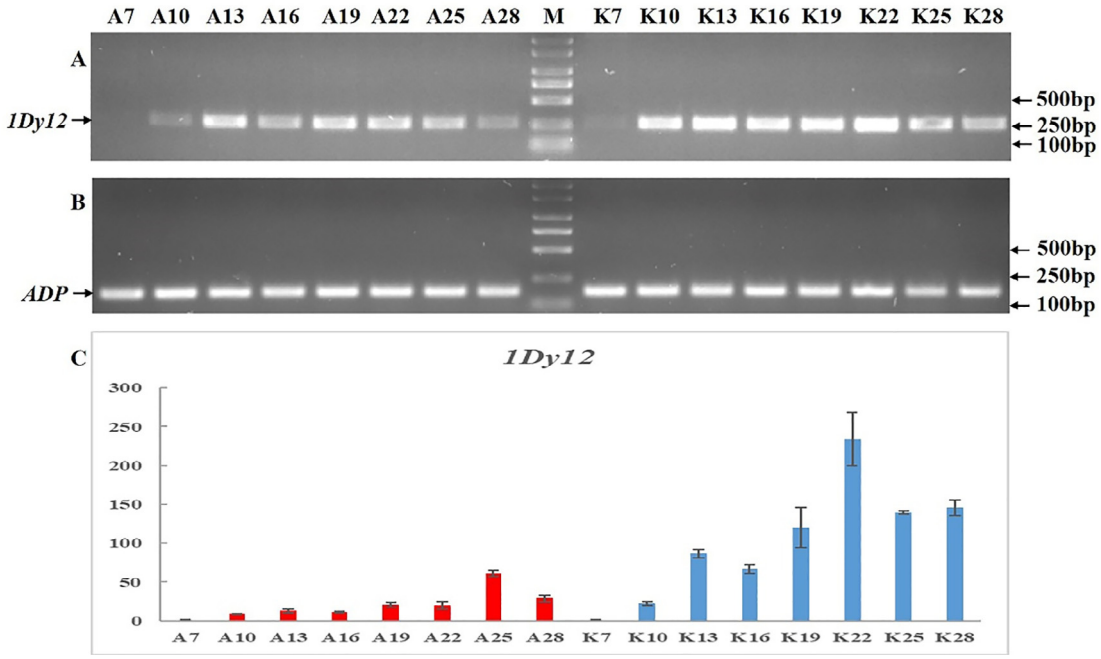

Fig. 2. Expression profiling of the *1Dy12* gene at different developmental stages in AS273 and KN199 by RT-PCR and qRT-PCR. A and C: *1Dy12* gene; B: *ADP* gene. A and B: RT-PCR; C: qRT-PCR. A7-A28: cDNA from AS273 at 7, 10, 13, 16, 19, 22, 25, and 28 DPA, respectively; K7-K28: cDNA from KN199 at 7, 10, 13, 16, 19, 22, 25, and 28 DPA, respectively.

started from 7 DPA, reached the highest level at 22 DPA, and then declined as grain-filling progressed in KN199, while it peaked at 25 DPA, and then declined as grain-filling extended in AS273 (Fig. 2). The expression of *1Dy12* slightly decreased at 16 DPA in AS273 and KN199, implying that the expression of the *HMW-GS* was also affected by the environment. Similarly, the expression level of *1Dy12* in KN199 was much higher than in AS273 across all developmental stages, demonstrating that the expression of *1Dy12* was significantly suppressed in AS273 (Fig. 2). These results were also verified by the brightness of the bands by RT-PCR. Based on these results, it could be concluded that *1Dy12* in AS273 had lower and longer expression at the mRNA level but HMW-GS 1Dy12 was not synthesized.

3.4. Molecular mechanism of 1Dy12 silencing in AS273

Analysis of SDS-PAGE showed that HMW-GS 1Dy12 was not detected in all grain samples of AS273 at different grain developmental stages, but RT-PCR and qRT-PCR analyses showed that *1Dy12* was expressed during the grain-filling period in AS273. We speculated that *1Dy12* in AS273 was silenced after transcription. To dissect the molecular mechanism of *1Dy12* silencing in AS273, the complete coding sequences of *1Dy12* in AS273 and KN199 were amplified, sequenced, and aligned with each other. Consequently, similar bands of approximately 2000 bp in size were amplified in AS273 and KN199, using the specific primers to 1Dy12 (Table S1, Fig. S3). Then, the four complete coding sequences of *1Dy12* in AS273 and one complete coding sequence of *1Dy12* in KN199 were selected for sequence comparison. After sequencing the amplified products, a complete open reading frame (ORF) was obtained, which was 1980 bp for AS273 and 1986 bp for KN199 (Fig. S3).

Alignment analysis found three variants in the *1Dy12* sequences of AS273 and KN199 (Fig. S4). The first mutation was a SNP at the 84th nucleotide with a G to A conversion in AS273, but the encoded amino acid was not changed (Fig. S4). The second mutation was a deletion of six bases “ggacag” from 1426 to 1431 nucleotides in AS273 (Fig. S4), leading to a lack of two amino acids (G and Q) and a frameshift (Fig. S4). The third mutation was another SNP at nucleotide 1528 with a conversion of C to T in AS273 (Fig. S4), causing a stop codon “TAG” and leading to truncated translation of 1Dy12 in AS273 (Fig. S4). The complete coding sequence of *1Dy12* could normally translate into 660 amino acids in KN199, while it was only 507 amino acids in AS273 (Fig. S4). Additionally, *1Dy12* has seven conserved cysteine residues (five in *N*-terminal domain, one in central repetitive domain, and one in C-terminal domain) (Fig. S4).

3.5. Contents of glutenins, gliadins, and GMPs in the mutant line

RP-UPLC analysis showed that 1Dy12 was completely missing in the mutant line AS273 (Fig. 3), consistent with the results from SDS-PAGE (Fig. 1). In addition, the 1By9 content in AS273 (10.25 mAU*min) increased by 4.70% compared with that in KN199 (9.79 mAU*min), while 1Dx2 content in AS273 (25.34 mAU*min) decreased by 3.06% in comparison with that in KN199 (26.14 mAU*min) (Table S4, Fig. 3). The contents for the other two HMW-GSs (1Ax1 and 1Bx7) did not differ between the two materials. Furthermore, the content for the total HMW-GSs in AS273 (80.47 mAU*min) was lower than that in KN199 (90.36 mAU*min), while the content for the total LMW-GSs in AS273 (89.22 mAU*min) was higher than that in KN199 (82.04 mAU*min). Thus, the total glutenins content in AS273 (169.69 mAU*min) was lower than that in KN199 (172.40 mAU*min) (Table S4, Fig. 3). The contents for ω, α/β, and γ-gliadins in AS273 were all higher than those in KN199, leading to an increase in total gliadins in AS273 (1093.89 mAU*min) compared to that in KN199 (1012.25 mAU*min) (Table S4, Fig. 3). The ratio of glutenins/gliadins was reduced in AS273 based on the above results. SE-UPLC analysis revealed that the GMP content in both the 22 DPA immature grains and the mature grains of AS273 was higher than that of KN199 (Table S4, Fig. 3).

3.6. Protein body differences during grain development between AS273 and KN199

Wheat seed storage proteins are synthesized and folded in the lumen of the ER and then deposited into the organelles of the PB (Loussert, Popineau, & Mangavel, 2008). In particular, the synthesis and folding

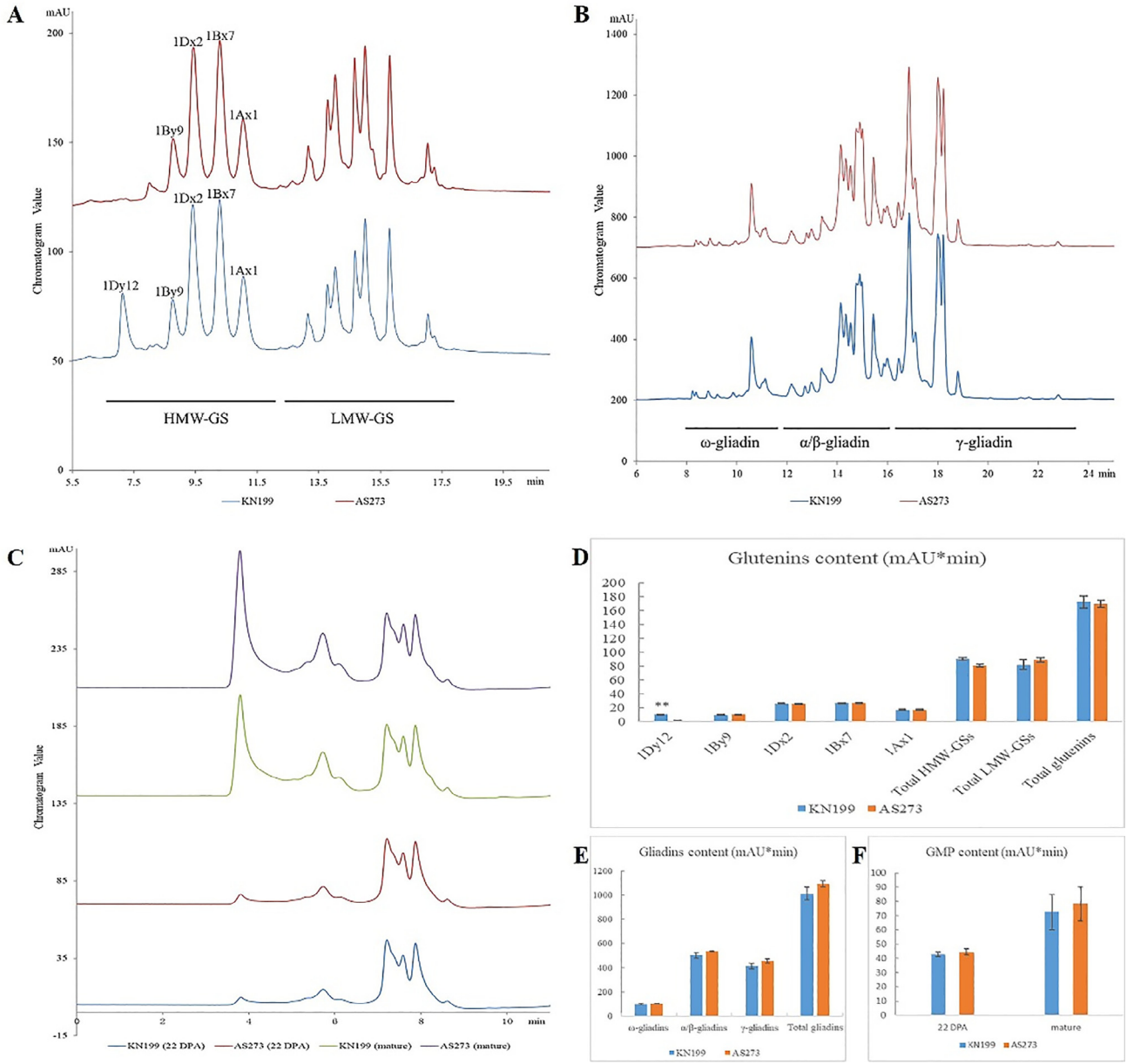

Fig. 3. Contents of glutenins, gliadins, and GMPs in the grains of AS273 and KN199. A: contents of glutenin subunits by RP-UPLC analysis; B: contents of gliadins by RP-UPLC analysis; C: contents of GMPs by SE-UPLC analysis; D: comparison of relative contents of different glutenins; E: comparison of relative contents of different gliadins; F: comparison of relative contents of GMPs in the immature and mature seeds.

pathways of HMW-GSs affect the formation PB. In order to observe the morphological differences and further dissect the formation mechanism of PBs between AS273 and KN199, TEM was used to observe changes in PBs at different grain developmental stages. Line AS273 had more and larger PBs than KN199 during the grain developmental period in general, except for those at 7 DPA (1 μm for AS273 and 1.5 μm for KN199, respectively) and 28 DPA (lamellar structure in AS273 and KN199) (Fig. 4). It was generally found that the accumulation of PBs increased at a rate of about 4 μm every 3 d from 7 DPA to 25 DPA in AS273, while the increase was approximately 3 μm every 3 d from 13 DPA to 25 DPA in KN199. These results indicate that the accumulation rate of PBs in AS273 is faster than that in KN199, and the absence of 1Dy12 stimulated the accumulation rate of PBs (Fig. 4). Moreover, both AS273 and KN199 formed lamellar structure of PBs at 28 DPA, which basically completed the formation of the PB networks (Fig. 4).

3.7. Effect of 1Dy12 silencing on flour-processing quality in AS273

The test for bread-processing quality clearly showed that most of the important quality-related parameters, such as water absorption, dough development time, and stability time in AS273, were significantly lower than those in KN199, while the tolerance index in AS273 was higher than that in KN199, and the crude protein content was nearly similar between the two materials (Table 1, Fig. 5). These properties resulted in a loaf volume of 820 cm^3 in AS273 which was significantly decreased by 10.0% compared to that of KN199 (910 cm^3) (Table 1, Fig. 5). The bread sensory evaluation score was also decreased in AS273 (74.3) compared to that of KN199 (83.5). Results suggested that silencing *1Dy12* in AS273 led to significantly decreased dough rheological properties and bread-processing quality, demonstrating that *1Dy12* is essential in bread-processing quality.

Line AS273 showed a volume reduction in sponge cake compared to KN199 (Fig. 5). The transverse slice of AS273 had coarser texture and

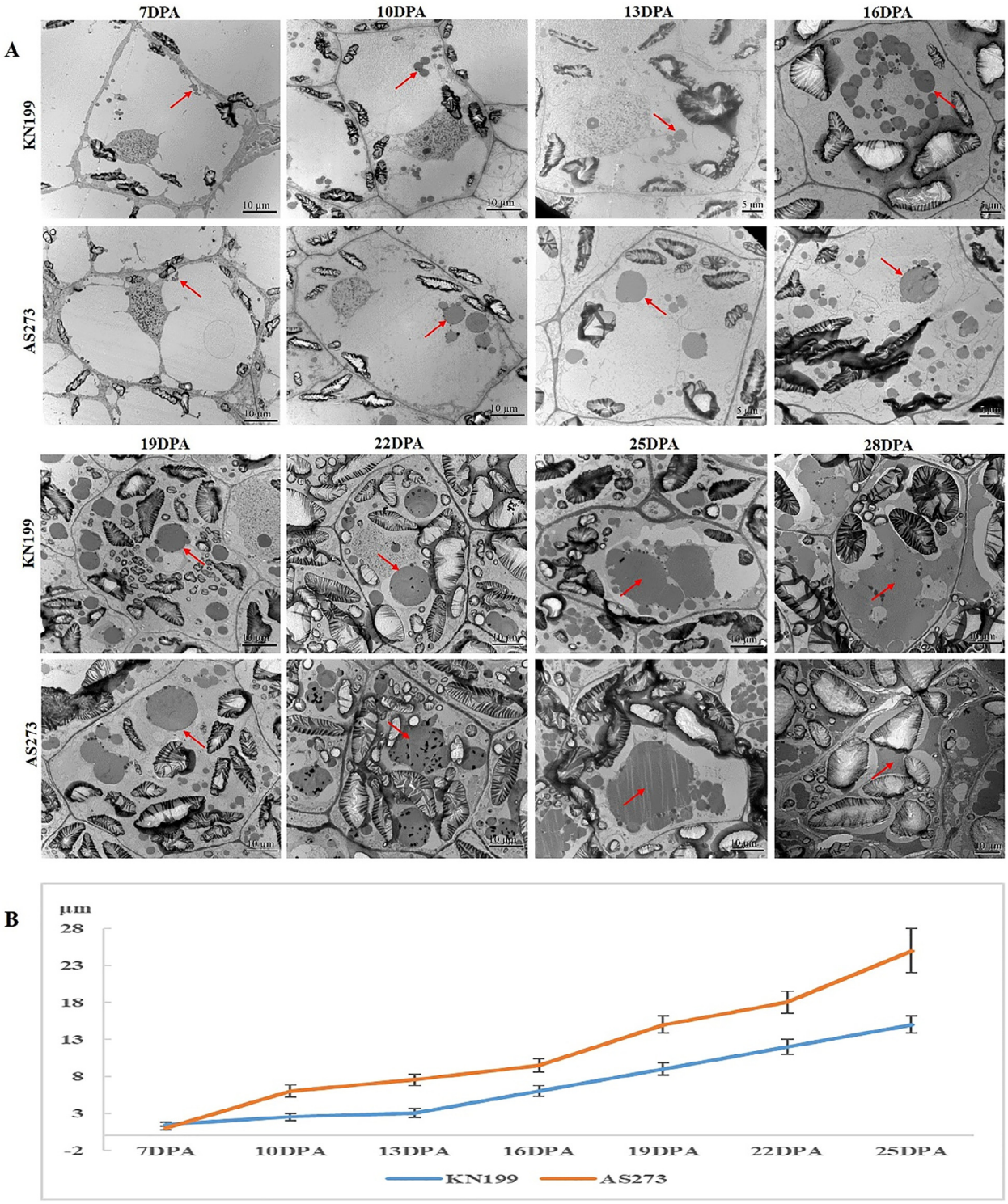

Fig. 4. Dynamic observation of development of protein bodies (PBs) during the grain-filling period in AS273 and KN199. A: the dynamic development course of PBs. Red arrows point to PBs; all bars stand for 10 μm in size except those for 5 μm in the four photos at 13 and 16 DPA in AS273 and KN199. B: statistical analysis of the size of PBs. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

sunken tops with greater collapse and shrinkage seriously, while the transverse slice of KN199 exhibited uniform and smooth crumb structure except for a few irregular air bubbles (Table 1, Fig. 5). In addition, AS273 in texture was slightly rougher than KN199 in regarding to the sensory evaluation of sponge cakes (Table 1). These results indicate that the lack of 1Dy12 in AS273 leads to inferior sponge cake quality, and 1Dy12 is an essential component in the formation of sponge cake structure.

The biscuits made from AS273 had a less width and a decreased spreading ability than those from KN199, while they had an increased thickness compared to those from KN199 (Table 1, Fig. 5). Moreover, the processed biscuits from KN199 was more uniform and fine in texture (Fig. 5). Taken together, our results suggested that AS273 displayed an inferior biscuit quality, indicating that 1Dy12 might also play a positive role in biscuits quality.

Table 1
Parameters related to flour-processing quality of AS273 and its wild-type parent KN199.

Materials	Bread parameters						Sponge cake parameters						Biscuit parameters		
	Crude protein content (%)	Water absorption (500 fu)	Formation time (min)	Stabilization time (min)	Mixing tolerance	Bread size (cm^3)	Loaf score	Specific volume score	surface appearance score	Internal structure score	Elastic flexibility score	Mouthfeel score	Quality score	Width (cm)	Thickness (mm)
AS273	14.9	57.8	2.3	2.6	90	820	74.3	26	5	20	5	12	68	23	7.5
KN199	14.6	58.6	3.3	3.8	80	910	83.5	25	5	22	6	15	73	23.5	6.4

4. Discussion

Wheat HMW-GSs consist of various subunits, which individually and cooperatively affect the end-use quality of the dough (Delcour et al., 2012). It is important to thoroughly clarify the contributions of individual HMW-GSs in flour-processing quality so that their effects can be efficiently utilized in breeding. In the present study, we found that the absence of 1Dy12 in AS273 led to inferior flour-processing quality. Nevertheless, previous studies have confirmed that the absence of other HMW-GSs, such as 1Ax1 at the *Glu-A1* locus, *1Dx2* at the *Glu-D1* locus, and *1Bx14*, *1By15*, *1Bx20*, and *1By20* at the *Glu-B1* locus all had negative effects on bread-processing quality (Gao et al., 2018; Li et al., 2015; Liu et al., 2016; Wang et al., 2017). Taken the aforementioned results together, we thought that all HMW-GSs contribute positive impacts to dough or bread-processing quality; some HMW-GSs belong to strong effect subunits, and some ones to weak effect subunits; there is no absolute inferior subunits; the bread-processing feature of wheat is mainly decided by the number, combination, and superior member of HMW-GSs. It was reported recently that the absence of *1Bx7* or *1By9* in wheat mutants led to weaker dough strength and inferior sponge cake performance, demonstrating that 1Bx7 and 1By9 make important contributions to gluten functionality and sponge cake processing in wheat industry (Chen et al., 2019).

Gene silencing can be divided into two major mechanistic classes, post-transcriptional gene silencing (PTGS) and transcriptional gene silencing (TGS) (Hammond, Caudy, & Hannon, 2001). Silenced *1Dy12* in AS273 is not TGS because RT-PCR and qRT-PCR results on *1Dy12* implied that this gene was expressed normally in AS273 at all the grain developmental stages (Fig. 2). Therefore, we speculated that the gene silencing in our mutant was caused by PTGS. To further determine if the lack of 1Dy12 accumulation in AS273 resulted from PTGS, the primers specific to *1Dy12* were used to amplify the sequence of *1Dy12* from AS273 and KN199. *1Dy12* was amplified from both AS273 and KN199, and three SNPs or deletion variants were found in AS273 according to the sequence alignment (Fig. S4). A stop codon produced by a base conversion mutation (C/T) led to truncated translation of *1Dy12* in AS273. In previous reports, the silencing of the genes encoding HMW-GSs was confirmed to be caused by the deletion of small chromosomal fragments and alteration of promoter regions (Beshkova, Ivanov, & Ivanova, 1998; Beshkova et al., 1998; Liu et al., 2016; Upelniek, Novoselskaya, Sutka, Galiba, & Metakovsky, 1995).

We found that the PBs in the endosperm of AS273 during the grain developmental period were larger and accumulated faster than those of KN199 (Fig. 4). Moreover, the contents for LMW-GSs, gliadins, and GMPs in AS273 were higher than those in KN199. These results demonstrate that silencing of wheat *1Dy12* stimulated the expression and secretion of other storage proteins, especially LMW-GSs and gliadins, and ensured the fusion of PBs in the endosperm. The grain protein content in AS273 and KN199 was similar, which also provided strong evidence for these phenomena (Table 1). Therefore, these results indicated that silencing of *1Dy12* in AS273 greatly promote the formation and fusion of PBs in seeds, which might stimulate the expression of some molecular chaperones and TFs to regulate the formation and fusion of wheat storage proteins for complementing the absence of HMW-GS 1Dy12 during grain development. The negative feedback regulation effect greatly increased the size of PBs during grain development.

Wheat flours can be used to make many products such as bread, noodles, cookies, and cakes. To make bread or noodles, the flours are required to have strong gluten and high protein content. For processing cookies or cake, the flours should possess weak gluten and low protein level (Rasheed et al., 2014). We found that AS273 had an inferior flour-processing quality including bread, sponge cake, and biscuit. The absence of HMW-GS 1Dy12 led to a decreased HMW-GS content and an increased gliadin content, which might cause bread-processing quality inferior. We speculated that the increased LMW-GS content could compensate the decrease of HMW-GSs, thus the gluten is not weak and

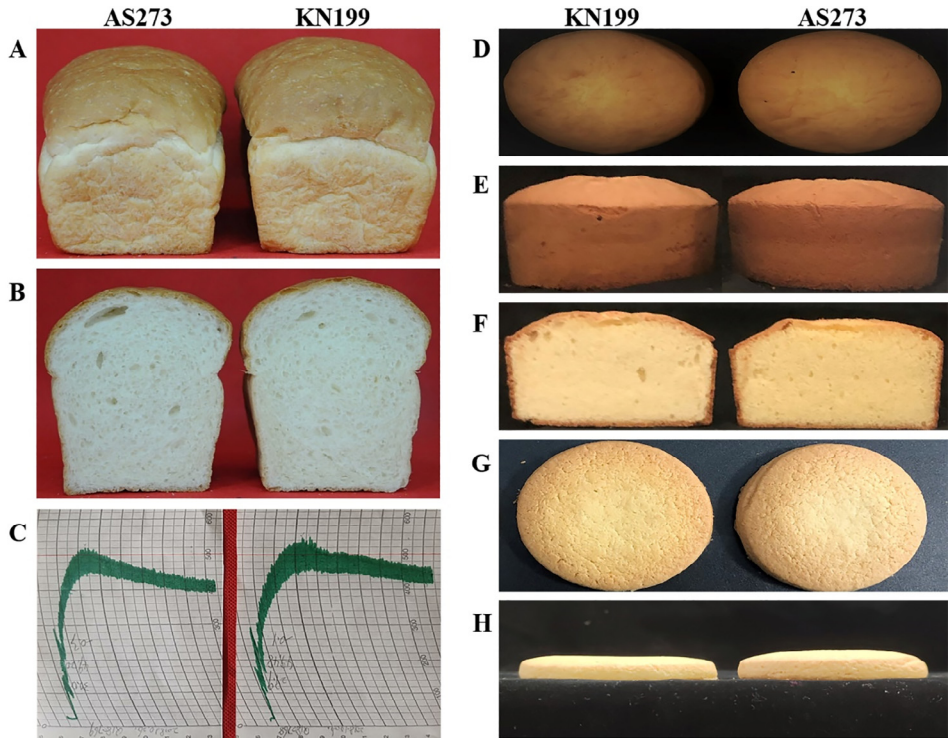

Fig. 5. Flour-processing evaluation from AS273 and KN199. A: bread shapes; B: bread slices; C: Farinograph of bread; D: top view of sponge cakes; E: lateral view of sponge cakes; F: vertical sectional view of sponge cakes; G: top view of biscuits; H: lateral view of biscuits. The bread processed from AS273 was smaller than that from KN199. The dough formation time and stabilization time for AS273 (left) were both shorter than those for KN199 (right) for dough processing. The sponge cake baked from AS273 was also smaller than that from KN199. The biscuit from KN199 had a greater width, a decreased spreading ability, and a thinner thickness than that from AS273.

the protein level is not reduced in the flour of AS273. Therefore, AS273 also showed an inferior sponge cake and biscuit-processing quality, in which subunit 1Dy12 is very important not only in bread-processing but also in sponge cake and biscuit-processing.

5. Conclusions

A wheat somatic variation line AS273 was produced in which *1Dy12* encoding an HMW-GS at the *Glu-D1* locus was silenced. The agronomic and botanic traits, *1Dy12* silencing mechanism, gene expression profiling, glutens and GMPs determination and bread-processing quality were studied. Some agronomic and botanic traits differences between AS273 and its wild-type KN199 were observed, indicating that other genes in AS273 might vary in addition to *1Dy12*. The expression of *1Dy12* in AS273 was expressed at different grain developmental stages, but was much lower than that in KN199. There was a stop codon in the *1Dy12* coding sequence, which leads to the truncated translation of *1Dy12*. The absence of 1Dy12 also led to larger PBs compared to KN199 during grain development and stimulated the accumulation of LMW-GSs, ω, α/β and γ-gliadins, and GMPs. The lack of 1Dy12 in AS273 negatively affected its dough traits and flour-processing quality.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author contributions

X.Y. and K.W. designed this research and supervised the study. H. C. and S. L. performed most of the experiments. K. W, Y. L. and L. D. developed the somatic variation line. H. C., Y. L. and L. D. investigated the agronomic traits. H. C., J. L and X. M. did the gene and protein expression analysis. H. C. and X. Y. conducted the data analysis and prepared the manuscript. All the authors read and agreed on the final manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2020.127663.

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