



Functional assessment and SNP-based molecular marker development of two 1S¹-encoded HMW glutenin subunits in *Aegilops longissima* L.

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Abstract We investigated the functional properties of two 1S¹-encoded high molecular weight glutenin subunits (HMW-GS), 1S¹x2.3* and 1S¹y16*, from *Aegilops longissima* L., and developed single-nucleotide polymorphism (SNP)-based molecular markers. Two HMW-GS near-isogenic lines (NILs), HMW-NIL1 (1Ax1, 1S¹x2.3* + 1S¹y16*, and 1Dx2 + 1Dy12) and HMW-NIL2 (1Ax1, 1Bx17 + 1By18, and 1Dx2 + 1Dy12), were developed by crossing the ‘Chinese Spring’ (CS)-*Ae. longissima* 1S¹/1B substitution line (CS-1S¹/1B) with the spring wheat cultivar CB037A, and consecutive selfing combined with selection and identification. Quality analysis of the two NILs showed that the 1S¹x2.3* + 1S¹y16* subunits conferred better dough rheological properties and breadmaking quality than the 1Bx17 + 1By18 subunits; in particular, they significantly improved dough strength, loaf volume and texture, and final bread score. Allele-specific polymerase chain reaction markers for both genes were

developed based on SNP variations and successfully amplified 290-bp and 283-bp specific fragments from the 1S¹x2.3* and 1S¹y16* genes, respectively. Both SNP-based molecular markers were further validated using 38 wheat varieties, two recombination inbred lines, and four F₂ generations and showed high specificity and accuracy. Thus, these markers can be used for molecular marker-assisted selection in the early generations of wheat quality improvement programs.

Keywords *Aegilops longissima* L. · HMW-GS · Breadmaking quality · SNP marker · Quality improvement

Abbreviations

AS-PCR	allelic-specific polymerase chain reaction
HMW-GS	High molecular weight glutenin subunits
HPCE	high-performance capillary electrophoresis
LMW-GS	Low molecular weight glutenin subunits
MAS	Marker-assisted selection
MS	mass spectrometry
NILs	near-isogenic lines
RILs	recombination inbred lines
RP-HPLC	reversed-phase high-performance liquid chromatography
RP-UPLC	reversed-phase ultra-performance liquid chromatography
RVA	Rapid Visco Analyzer
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SNPs	Single-nucleotide polymorphisms

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Introduction

Wheat (*Triticum aestivum* L.), one of the three main cereal crops cultivated worldwide, represents a major source of energy and nutrients for humans. To support a growing global population and economy, it is vitally important to increase wheat production and enhance grain quality (Tilman et al. 2002; Pfeifer et al. 2014). The wheat endosperm contains gluten proteins, which account for 80–85% of total flour protein and are the major seed storage proteins, found as monomeric gliadins and polymeric glutenins.

Glutenins are formed from high and low molecular weight glutenin subunits (HMW-GS and LMW-GS, respectively) and are vital in dough viscoelasticity (Payne 1987; Payne et al. 1988; Weegels et al. 1996). Although HMW-GS only account for about 5–10% of total flour protein, they are particularly important in conferring dough strength and breadmaking quality (Payne et al. 1988; Ma et al. 2005). HMW-GS are encoded by *Glu-A1*, *Glu-B1*, and *Glu-D1* loci and each locus consists of two tightly linked genes, named x- and y-type genes according to their size and the sequence of the conserved N-terminal domain (Payne and Lawrence 1983; Halford et al. 1987). Differences among HMW-GS are mainly the result of repeat motif variations in the central repeat domain (Gianibelli et al. 2001).

Considerable work has confirmed the extensive allelic variations present at *Glu-1* loci in wheat and related species, which are closely related to dough properties and breadmaking quality (Rasheed et al. 2014). Early work identified and classified 20 alleles at *Glu-A1*, *Glu-B1*, and *Glu-D1* in around 300 bread wheat cultivars (Payne and Lawrence 1983). Among them, 1Dx5 + 1Dy10 (*Glu-D1d*), 1Bx7 + 1By8 (*Glu-B1b*), and 1Bx17 + 1By18 (*Glu-B1i*) have higher quality scores, whereas 1Dx2 + 1Dy12 (*Glu-D1a*), 1Bx20 (*Glu-B1e*), and 1Bx7 + 1By9 (*Glu-B1c*) are related to poor quality performance (Shewry et al. 1992; Weegels et al. 1996; Gianibelli et al. 2001; Jiang et al. 2018; Guo et al. 2019). However, studies have shown that the allelic variations at *Glu-1* loci in the A, B, and D genomes of bread wheat are limited. Thus, discovery of new HMW-GS variations in species related to wheat is important for wheat quality improvement programs.

Among 20 Triticeae groups, *Aegilops* is the closest relative to *Triticum*. The genus *Aegilops* includes 22 species with S, M, C, U, N, D, and T genomes. Among them, *Aegilops longissima* L. (S¹S¹, 2n = 2x = 14) is a

diploid *Aegilops* species that has potential genetic resources for wheat genetic improvement, such as resistance genes against powdery mildew (Ceoloni et al. 1992; Cenci et al. 1999; Alberto et al. 2003), eyespot (Sheng et al. 2012; Sheng and Murray 2013), and drought stress (Zhou et al. 2016). In addition, hybridization of *Ae. longissima* and common wheat yielded hybrid offspring with higher trace element contents (e.g., iron, zinc, copper, manganese, calcium, magnesium, and potassium) (Neelam et al. 2013).

Furthermore, extensive allelic variation in storage protein composition has been found in *Aegilops* species, including *Ae. tauschii* (Xie et al. 2001; Yan et al. 2003a; Jiang et al. 2008; Zhang et al. 2008a), *Ae. longissima* (Wang et al. 2013; Zhu et al. 2015; Hu et al. 2019), *Ae. speltoides* (Wang et al. 2018), *Ae. umbellulata* (Wang et al. 2017), *Ae. caudata* (Wang et al. 2016), *Ae. kotschyi*, and *Ae. juvenalis* (Li et al. 2008). In particular, 1S¹-encoded HMW-GS in the 1S¹/1B substitution line had positive effects on breadmaking quality (Wang et al. 2013). Through 1S¹/1A substitution, the grain hardness was reduced, and the gluten strength, formation time, stability time, dough bread volume, and bread score were improved (Garg et al. 2014). These results indicate that *Ae. longissima* contains potential genetic resources for wheat quality improvement.

In this work, we used two *Glu-1* near-isogenic lines (NILs), HMW-NIL1 and HMW-NIL2, to investigate the functional properties of two 1S¹-encoded HMW-GS, 1S¹x2.3* and 1S¹y16*, and developed single-nucleotide polymorphism (SNP)-based molecular markers. Our results demonstrate that the 1S¹x2.3* and 1S¹y16* subunits from *Ae. longissima* confer superior breadmaking quality through improved mixing properties, bread texture, and loaf score and have potential value for wheat quality improvement. The developed SNP-based molecular markers for the 1S¹x2.3* and 1S¹y16* subunit genes show high specificity and reliability, making them useful for molecular marker-assisted selection in the early generations of wheat quality improvement programs.

Materials and methods

Plant materials and field trial

The experimental materials used in this study included a pair of *Glu-B1* NILs (HMW-NIL1 and HMW-NIL2), 38

bread wheat cultivars, two recombination inbred lines (NILs) derived from the crossing between CB037C and the ‘Chinese Spring’ (CS) substitution line (CS-1S¹/1B), and four F₂ generation populations from CS-1S¹/1B × ‘Ningchun 4’, CS-1S¹/1B × ‘Wanmai 33’, CS-1S¹/1B × ‘Yumai 34’, and CS-1S¹/1B × ‘Zhongmai 8601’ (Table 1). The NILs HMW-NIL1 (1Ax1, 1Slx2.3* + 1Sly16*, and 1Dx2 + 1Dy12) and HMW-NIL2 (1Ax1, 1Bx17 + 1By18, and 1Dx2 + 1Dy12) were developed by our group through crossing CS-1S¹/1B and the spring wheat cultivar CB037A and consecutive selfing for seven times combined with selection and identification. CS-1S¹/1B with a HMW-GS composition of 1Ax1, 1S¹x2.3* + 1S¹y16*, and 1Dx2 + 1Dy12 was developed by the Institute for Plant Breeding, Technical University Munich, Germany, in which the chromosome 1S¹ from *Ae. longissima* was substituted by 1B from CS (Wang et al. 2013). CB037A is a wheat-*Haynaldia villosa* 6VS/6AL translocation line developed at the Capital Normal University and Institute of Crop Science, Chinese Academy of Agricultural Science, China, which carries the *Pm21* gene with a HMW-GS composition of 1Ax1, 1Bx17 + 1By18, and 1Dx2 + 1Dy12 and has high resistance to powdery mildew and superior gluten quality (Wu et al. 2017).

Both HMW-NIL1 and HMW-NIL2 were planted in the experimental fields of the China Agricultural University during the 2017–2018 wheat growing season. Field experiments were performed in a randomized block design with three biological replicates (each plot: 20 m²). The crops were cultivated and managed following the same procedures as local field cultivation practices.

Agronomic trait assessment

The mature plants of HMW-NIL1 and HMW-NIL2 from the three biological replicates were harvested, and their main agronomic traits were measured, including plant height, ear length, sturdy spikelet number per spike, spikelet number per spike, grain number per spike, and 1000-grain weight.

HMW-GS extraction and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

We used an improved procedure for seed HMW-GS extraction based on the method of Mackie et al.

(1996). The extracted glutenin proteins (0.02 mL of the supernatant) were subjected to SDS-PAGE, as described previously Yan et al. (2003a).

Quality testing

The following grain quality parameters were tested in three biological replicates: ash, protein, and gluten contents; grain hardness; water absorption; Rapid Visco Analyzer (RVA) parameters; Farinograph parameters; C-cell parameters related to bread inner structure; loaf volume; and appearance score. Student’s *t* test was used for data evaluation. The quality parameters were tested according to Sun et al. (2010) with minor modifications. Grain hardness was measured using the Perten Single Kernel Characterization System 4100 (Perten Instruments, Springfield, IL, USA) according to American Association of Cereal Chemists (AACC) method 55-31. Flour moisture and ash content (% dry basis) were determined according to AACC (2000) methods 44-15A and 08-02, respectively. The flour protein content was determined using a near-infrared analyzer (Instalab 610 NIR Analyzer; DICKEY-john Co. Ltd., Auburn, IL, USA) according to AACC method 39-10A. The RVA profile was obtained using AACC (2000) method 76-21 using a RVA (Series 3; Newport Scientific, Jessup, MD, USA). AACC (2000) methods 54-21 and 54-10 were followed to determine the Farinograph parameters (Farinograph-E; Brabender GmbH & Co. KG, Duisburg, Germany) and tensile strength (Brabender GmbH & Co. KG). Bread baking and evaluation were performed based on AACC method 10-10B according to Jongh (1953). Bread crumb image analysis was performed using C-cell image analysis software and equipment (Calibre Control International Ltd., Warrington, UK) according to Zhen et al. (2016).

Development and validation of SNP-based molecular markers

BioEdit 7.0 was used to compare the sequences of HMW-GS genes cloned from *Ae. longissima* with previously cloned HMW-GS genes from common wheat varieties. Based on SNP variations, specific primers were designed to develop SNP-based molecular markers. The materials used for marker development and verification included 40 bread wheat varieties and related species with different HMW-GS compositions, four F₂ generations, and two RILs (Table 1). In total,

Table 1 Wheat materials and their *Glu-1* compositions used in the current study

No.	Wheat variety name	<i>Glu-A1</i>	<i>Glu-B1</i>	<i>Glu-D1</i>
1	CS-1S ¹ /1B	N	1S ¹ x2.3* + 1Sly16*	2 + 12
2	HMW-NIL1	1	1S ¹ x2.3* + 1Sly16*	2 + 12
3	CS	N	7 + 8	2 + 12
4	HMW-NIL2	1	17 + 18	2 + 12
5	Ningchun 4	1	17 + 18	5 + 10
6	Ningchun 47	1	7 + 8	5 + 10
7	Zhoumai 22	1	7 + 9	5 + 10
8	Longmai 26	2*	7 + 9	5 + 10
9	Zhongmai 8601	1	7 + 9	2 + 12
10	Wanmai 33	1	13 + 19	5 + 10
11	Yumai 34	1	7 + 8	5 + 10
12	CB037A	1	17 + 18	2 + 12
13	RochbergersfruherDinke	N	13* + 19*	2 + 12
14	Schwabenkom	1	6.1 + 22.1	2 + 12
15	Spelt137	2.1*	13 + 22*	2 + 12
16	Spelt146	1	13 + 22*	2 + 12
17	CB037B	1	17 + 18	2 + 12
18	CB037C	1	17 + 18	5 + 10
19	Imbros	1	14 + 15	2 + 12
20	Xiaoyan 6	1	20x + 20y	2 + 12
21	Jimai 20	1	13 + 16	5 + 10
22	Zhengyou 63	1	7 + 9	2 + 12
23	Yangmai 10	1	7 + 8	2 + 12
24	Yangmai 158	N	7 + 8	2 + 12
25	Lumai 21	1	7 + 8	5 + 10
26	Pandas	1	7 + 9	2 + 12
27	Kontrast	N	17 + 18	5 + 10
28	Shan229	1	14 + 15	5 + 10
29	Shannong 215953	N	6 + 8	2 + 12
30	Lumai 18	N	7 + 8	2 + 12
31	Zhengzhou 992	N	17 + 18	2 + 12
32	Yannong 15	1	7 + 9	5 + 12
33	Linfen 127	1	17 + 18	2 + 12
34	Yin11-12	2*	17 + 18	5 + 10
35	Lumai 17	1	7 + 8	2 + 12
36	Lumai 19	1	7 + 8	2 + 12
37	Yangmai 5	N	7 + 9	5 + 10
38	Yanyou 361	1	17 + 18	5 + 10
39	HMW-RIL1	1	7 + 9	5 + 10
40	HMW-RIL2	1	7 + 8	2 + 12
41	CS-1S ¹ /1B × Ningchun 4	1	17 + 18	5 + 10
42	CS-1S ¹ /1B × Wanmai 33		13 + 19	5 + 10
43	CS-1S ¹ /1B × Yumai 34	1	7 + 8	5 + 10
44	CS-1S ¹ /1B × Zhongmai 8601	1	7 + 9	2 + 12

50–80 grains from each variety or RIL and 250–300 grains from each F₂ generation were detected.

Genomic DNA was extracted using the Phanta Super-Fidelity DNA Polymerase system (Vazyme Biotech, Nanjing, China). The PCR protocol was as follows: initial denaturation at 95 °C for 3 min, 30 cycles at 94 °C for 15 s, 61 °C for 15 s, and 72 °C for 10 s, and a final extension at 72 °C for 5 min. PCR products were analyzed by 1% agarose electrophoresis in Tris-acetic acid-EDTA buffer.

The amplified fragments were purified from the gel using a Gel Extraction Kit (Omega Bio-Tek, Norcross, GA, USA). Purified products were ligated to the CE Vector (Vazyme) and transformed into trans 2-blue receptor cells (TransGen Biotech, Beijing, China). DNA sequencing from five positive clones for each PCR fragment was carried out by TaKaRa Biotechnology (Dalian, China).

Results

Development and characterization of HMW-NIL1 and HMW-NIL2

Two HMW-GS NILs, HMW-NIL1 and HMW-NIL2, were developed via hybridization of the CS-1S¹/1B substitution line with common wheat CB037A and consecutive selection. Phenotype and main agronomic trait analysis showed clear differences between HMW-NIL1 and HMW-NIL2. The phenotypes of plants and ears (with awns) of HMW-NIL1 were similar to those of CB037A, whereas the phenotypes of HMW-NIL2 (without awns) were more similar to those of CS-1S¹/1B (Fig. 1a, b). Compared with HMW-NIL2, spike length, grain number per spike, and 1000-grain weight were highly significantly reduced in HMW-NIL1. Plant height, sturdy spikelet number per spike, and spikelet number per spike showed no significant differences between the NILs (Table 2).

SDS-PAGE analysis of the HMW-GS showed that both HMW-NIL1 and HMW-NIL2 had the same *Glu-A1a* allele-encoded 1Ax1 subunit and *Glu-D1a* allele-encoded 1Dx2 + 1Dy12 subunits, but the *Glu-B1* locus had different HMW-GS compositions. HMW-NIL1 contained a pair of 1S¹-encoding HMW-GS subunits, 1S¹x2.3* + 1S¹y16*, whereas HMW-NIL2 had the *Glu-B1i*-encoded 1Bx17 + 1By18 subunit (Fig. 1c). These

results indicate that HMW-NIL1 and HMW-NIL2 are a pair of typical *Glu-B1* NILs.

Functional properties of 1S¹-encoded 1S¹x2.3* and 1S¹y16* subunits

A comprehensive quality analysis of HMW-NIL1 and HMW-NIL2 was performed to assess the functional properties of the 1S¹-encoded 1S¹x2.3* and 1S¹y16* subunits, including basic quality, RVA, Farinograph, C-cell, and loaf parameters. The basic quality parameters included protein, moisture, and ash contents, gluten over sieves, total gluten, gluten index, and hardness. The results showed that HMW-NIL1 had significantly higher hardness (+ 5%) and highly significantly higher gluten sieving and gluten index (each + 18%) compared to HMW-NIL2. The NILs showed no significant differences in protein, moisture, ash, and total gluten contents (Table 3).

The RVA parameters included peak viscosity, final viscosity, and setback, which are closely associated with starch pasting and dispersion during heating/stirring and cooling/stirring (Qiao et al. 2008). The results revealed significantly lower peak viscosity (− 22%) and final viscosity (− 22%) and significantly increased setback (+ 6%) in HMW-NIL1 compared to HMW-NIL2, indicating inferior starch pasting properties of HMW-NIL1. Farinograph parameters, including water absorption, development time, stability time, and softening degree, reflect the flour mixing properties and dough strength (Liu et al. 2004). Compared to HMW-NIL2, HMW-NIL1 had significantly higher development and stability times, which are positively related to dough strength, but a significantly lower softening degree (− 60%), which is negatively correlated with dough strength. Water absorption did not differ between the NILs (Table 3).

C-cell image and parameter analyses were performed to evaluate the inner structural features of bread slices from HMW-NIL1 and HMW-NIL2, including slice brightness, slice area, wrapper length, attenuation ratio, cell contrast, volume of course cells, average cell elongation, cell diameter, number of cells, and cell density (Table 4). HMW-NIL1 had a significantly higher cell diameter (+ 12%) and significantly lower cell number (− 9%) and density (− 17%). Although the differences in the other parameters between HMW-NIL1 and HMW-NIL2 were not significant, several important indicators related to loaf volume (e.g., slice area, wrapper length,

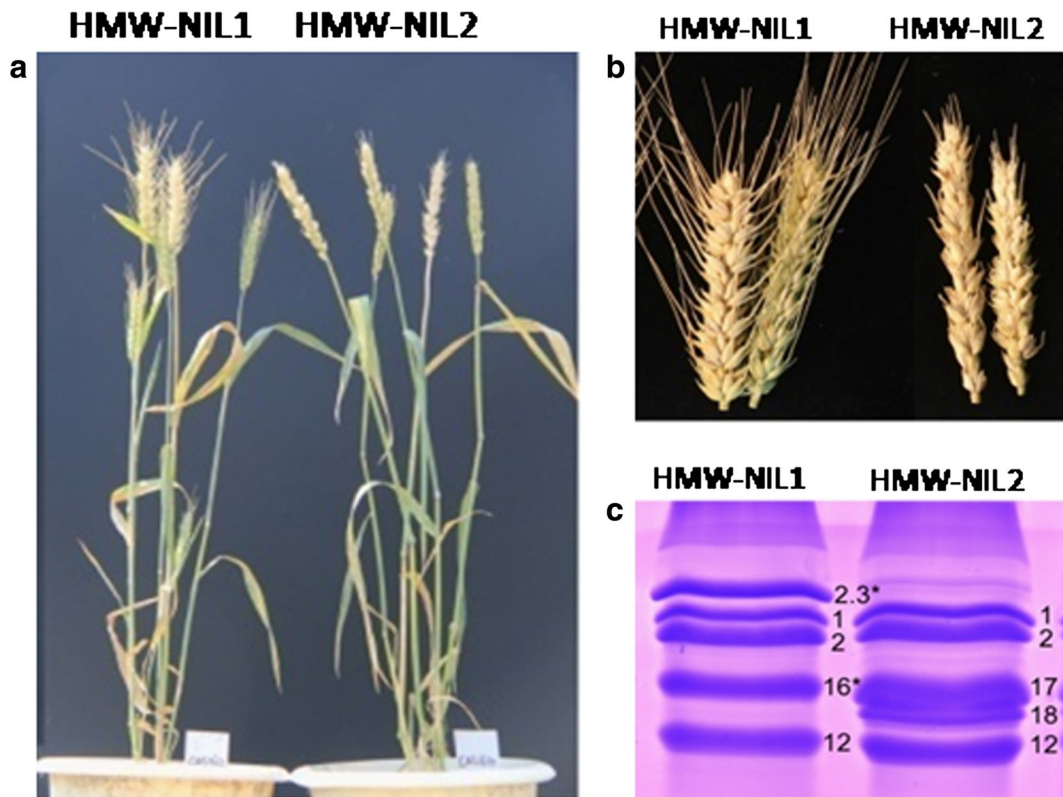


Fig. 1 Comparison of HMW-NIL1 and HMW-NIL2 **a** plant and **b** ear phenotypes and **c** identification of HMW-GS from HMW-NIL1 and HMW-NIL2 by SDS-PAGE

and attenuation ratio) were non-significantly increased in HMW-NIL2 (Fig. 2a, Table 4). Loaf parameters, determined according to the evaluation standard of wheat flour bread baking quality in China (GB/T16411-2008), showed that the loaf volume of HMW-NIL1 (963 mL³) was significantly higher than that of HMW-NIL2 (918 mL³) (+12%). Ultimately, the loaf inner structure scores and total loaf scores of HMW-NIL1 were 34 (+6%) and 96 (+5%), respectively, both of which significantly exceeded those of HMW-NIL2 (Fig. 2b, Table 4). These results demonstrate that the

1S^lx2.3* + 1S^ly16* subunits result in better quality performance than the 1Bx17 + 1By18 subunits, particularly in terms of dough strength, cell size, loaf volume, texture, and score.

Development and validation of SNP-based molecular markers for 1S^lx2.3* and 1S^ly16* genes

To develop SNP-based molecular markers for the 1S^lx2.3* and 1S^ly16* genes, BioEdit 7.0 was used to compare different HMW-GS gene sequences to

Table 2 Comparison of main agronomic traits between near-isogenic lines HMW-HIL1 and HMW-HIL2

Materials	Plant height (cm)	Spike length (cm)	Sturdy spikelet number per spike	Spikelet number per spike	Grain number per spike	Thousand-grain weight (g)
HMW-HIL1	67.73 ± 1.91	9.61 ± 0.07**	18.81 ± 1.19	20.21 ± 2.53	37.08 ± 1.31**	24.82 ± 0.1**
HMW-HIL2	64.95 ± 0.69	10.82 ± 0.08	18.82 ± 1.82	19.82 ± 2.82	48.91 ± 1.56	29.84 ± 0.14

*Significant level at 5%

**Significant level at 10%

Table 3 Comparative analysis of basic quality parameters and mixing parameters between near-isogenic lines HMW-HIL1 and HMW-HIL2

Materials	Basic quality parameters				Mixing parameters				Softening degree (FU)
	Protein content (%)	Hardness	Moisture (%)	Ash	Gluten over sieves	Total gluten (%)	Gluten index (%)		
HMW-NIL1	14.12 ± 0.09	99 ± 1.6*	14.97 ± 0.01	0.65 ± 0.01	3.34 ± 0.04**	3.77 ± 0.01	88.75 ± 0.89**		
HMW-NIL2	14.43 ± 0.05	94 ± 1.8	15.26 ± 0.02	0.64 ± 0.02	2.82 ± 0.01	3.75 ± 0.03	75.19 ± 0.67		
Materials	RVA parameters				Farinograph parameters				
	Peak viscosity	Final viscosity	Setback	Water absorption (500 FU)	Development time (min)	Stability time (min)	Softening degree (FU)		
HMW-NIL1	2504.5 ± 48.5**	3343 ± 26*	1449 ± 36*	57.05 ± 0.15	4.95 ± 0.25**	9.35 ± 0.35**	51.2 ± 2**		
HMW-NIL2	3071.5 ± 37.5	3526 ± 36	1369 ± 27	58.65 ± 0.25	4.51 ± 0.21	5.71 ± 0.37	82.5 ± 3		

*Significant level at 5%

**Significant level at 10%

Table 4 Comparative analysis of C-cell and bread parameters between near-isogenic lines HMW-HIL1 and HMW-HIL2

Materials	C-cell parameters			Bread parameters			
	Slice brightness	Slice area/px	Wrapper length/px	Attenuation ratio	Cell contrast	Volume of course cell	Average cell elongation
HMW-NIL1	151.05 ± 0.25	305161 ± 2471	2094.5 ± 2.5	69.37 ± 0.08	0.765 ± 0.001	10.21 ± 0.37	1.73 ± 0.02
HMW-NIL2	152.2 ± 0.8	301354 ± 1798	2074.5 ± 3.5	66.89 ± 0.67	0.767 ± 0.005	10.26 ± 0.71	1.77 ± 0.02
Materials	C-cell parameters			Loaf parameters			
	Cell diameter/px	Number of cells	Cell density	Wall thickness/px	Loaf volume (mL ³)	Inner structures (35)	Score (100)
HMW-NIL1	15.98 ± 0.67**	3617 ± 102**	0.012 ± 0.0001**	3.18 ± 0.01	963 ± 3.8*	34 ± 1*	96 ± 2*
HMW-NIL2	14.26 ± 0.41	3927 ± 101	0.014 ± 0.0004	3.08 ± 0.005	918 ± 2.0	32 ± 1	91 ± 1

*Significant level at 5%

**Significant level at 10%

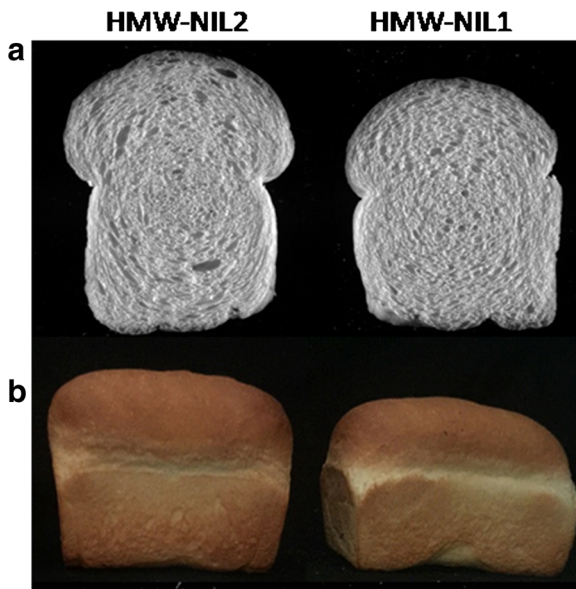


Fig. 2 **a** C-cell and **b** baked loaf images of bread made from HMW-NIL1 and HMW-NIL2 flour

identify specific SNP variations. Then, specific primers were designed and used to amplify the targeted genes. Sequence alignment was performed for the *ISLx2.3** gene and 14 other x-type HMW-GS genes deposited in GenBank (Supplementary Fig. S1): X61009 (*IAx1*), M22208 (*IAx2**), X13927 (*IBx7*), DQ119142 (*IBx7OE*), EF540764 (*IBx13*), KF733216 (*IBx14*), AB263219 (*IBx17*), AJ437000 (*IBx20*), AY553933 (*IBx23*), X03346 (*IDx2*), AY517724 (*IDx2.1*), AY159367 (*IDx2.2*), AJ893508 (*IDx2.2**), and JX12928 (*IDx5*). Two specific SNP sites (A-G and A-G) in *ISLx2.3** were identified, which were located at the signal peptide region (11th base) and the N-terminal non-repeat region (285th base), respectively. A pair of specific primers (*ISLx2.3*F*: 5'-GGTTAGTCCTCTTTGTGGCGA-3'; *ISLx2.3*R*: 5'-CCAAATATACTTTGTTG GAGTTGT-3') for amplifying *ISLx2.3** (fragment from bases 11–309) was designed based on the SNP variations. In addition, sequence alignment was performed for the *ISLy16** gene and eight other y-type HMW-GS genes: FJ404595 (*IAy*), AY245797 (*IBy8*), X61026 (*IBy9*), KF733215 (*IBy15*), EF540765 (*IBy16*), KF430649 (*IBy18*), X12929 (*IDy10*), and BK006459 (*IDy12*). Similarly, two specific SNP sites (C-T and T-C) present in *ISLy16** were identified, which were located at the signal peptide region

(38th base) and the N-terminal non-repeat region (281th base), respectively (Supplementary Fig. S2). Based on the SNP variations, a pair of specific primers (*ISLy16*F*: 5'-GTCCTCTTTGCGACAGTAGTAAC-3'; *ISLy16*R*: 5'-AGGATCCGCCTTGGGCA-3') for amplifying *ISLy16** (fragment from bases 16–298) was designed.

The designed primers were used to amplify the specific fragments of *ISLx2.3** and *ISLy16**, and the SNP-based molecular markers were developed in combination with SDS-PAGE, allele-specific polymerase chain reaction (AS-PCR) amplification, and sequencing. First, the HMW-GS compositions of 38 wheat varieties and two RILs were identified by SDS-PAGE (Fig. 3a), and then, AS-PCR was used to amplify the *ISLx2.3** and *ISLy16** genes (Fig. 3b, c). The results showed that a 290-bp specific fragment was amplified using the *ISLx2.3*F/ISLx2.3*R* primer pair from the varieties containing the *ISLx2.3** subunit, whereas no amplified products were obtained in wheat varieties lacking the *ISLx2.3** subunit. Similarly, a 283-bp specific fragment was amplified by the *ISLy16*F/ISLy16*R* primer pair from varieties containing the *ISLy16** subunit, but no amplified fragments were obtained from varieties lacking the *ISLy16** subunit. These results were consistent with those from the SDS-PAGE identification. Furthermore, both amplified fragments were collected and sequenced, and the results were consistent with their amplified regions.

To further validate the developed molecular markers, two pairs of specific primers were used to amplify the target genes in four F_2 generations derived from CS-1S¹/1B × 'Ningchun 4', CS-1S¹/1B × 'Wanmai 33', CS-1S¹/1B × 'Yumai 34', and CS-1S¹/1B × 'Zhongmai 8601' crosses. SDS-PAGE was also used to verify the AS-PCR results (Figs. 4 and 5). The results showed that the same 290-bp and 283-bp specific fragments were amplified by the *ISLx2.3*F/ISLx2.3*R* and *ISLy16*F/ISLy16*R* primer pairs from the four F_2 generations containing the *ISLx2.3** and *ISLy16** subunits, respectively. Similarly, no amplified fragments were obtained from the four F_2 generations lacking the respective subunits. These results were consistent with the SDS-PAGE identification. Overall, these findings indicate that the developed SNP-based molecular markers have high specificity and accuracy and can effectively identify the *ISLx2.3** and *ISLy16** genes.



Fig. 3 Development of SNP-based molecular markers for the $IS^I_x2.3^*$ and $IS^I_y16^*$ genes. **a** Identification of HMW-GSs from different wheat cultivars and RILs by SDS-PAGE. Lanes 1–40 correspond to material numbers 1–40 in Table 1. **b** AS-PCR amplification of the $IS^I_x2.3^*$ gene from wheat material numbers

1–40. The 290-bp specific amplified fragment is indicated. **c** AS-PCR amplification of the $IS^I_y16^*$ gene from wheat material numbers 1–40. The 283-bp specific amplified fragment is indicated. M: DNA size markers at 10 kb, 8 kb, 6 kb, 5 kb, 4 kb, 3 kb, 2 kb, 1.5 kb, 1 kb, 750 bp, 500 bp, 250 bp, and 100 bp

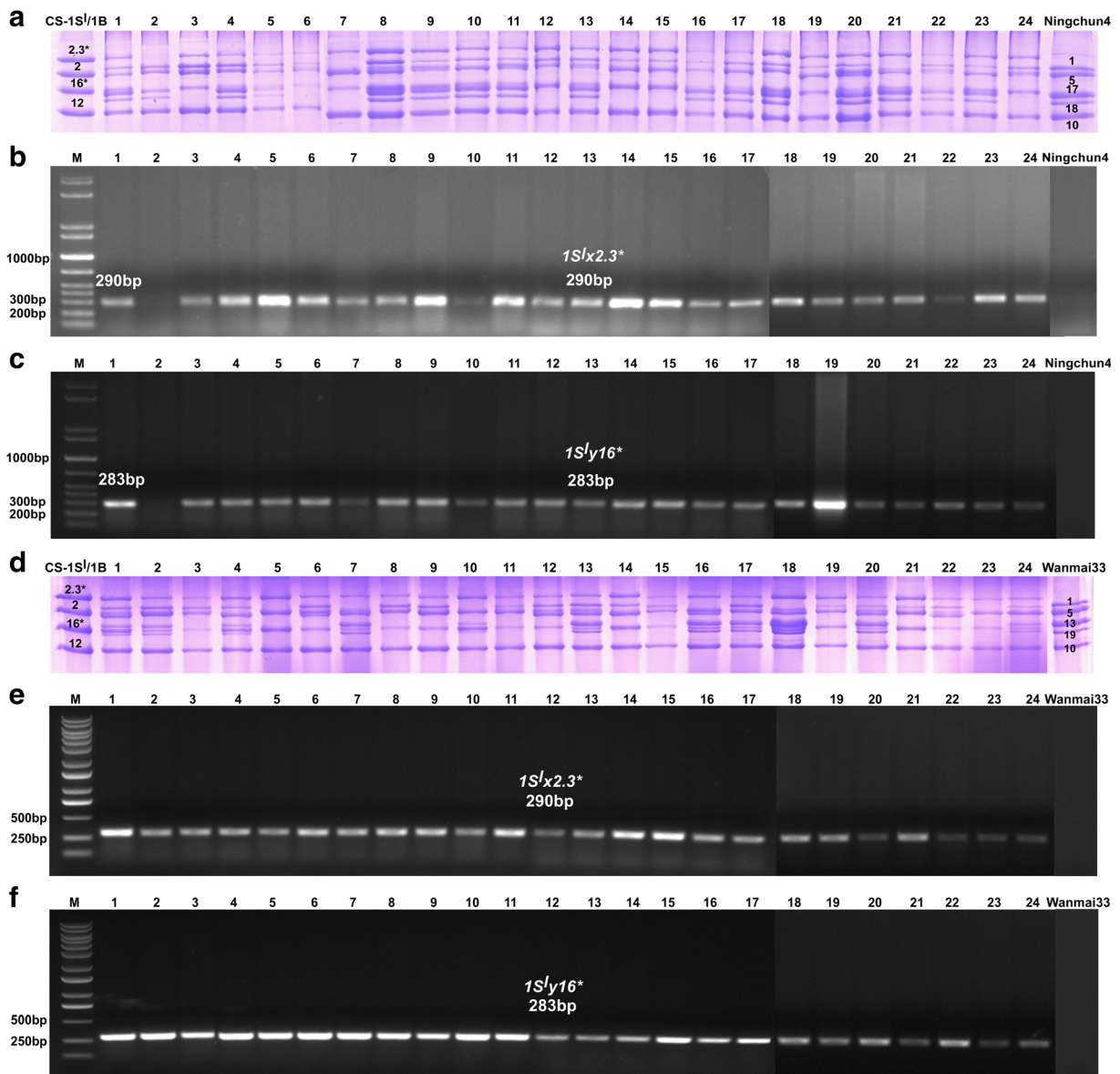


Fig. 4 Validation of SNP-based molecular marker for *IS^x2.3** and *IS^y16** genes in two F₂ generations of CS-1S¹/1B × Ningchun 4 and CS-1S¹/1B × Wanmai 33. **a** Identification of HMW-GS from the F₂ generations (lanes 1–24) of CS-1S¹/1B × Ningchun 4 by SDS-PAGE. **b** AS-PCR amplification of *IS^x2.3** gene from the F₂ generations of CS-1S¹/1B × Ningchun 4 (lane No. 1–24 are same as **a**). **c** AS-PCR amplification of *IS^y16** gene from F₂ generations of CS-1S¹/1B × Ningchun 4 (Lane No. 1–24 are same as **a** and **b**). M: DNA markers with 10 kb, 8 kb, 5 kb, 2 kb, 1.6 kb,

1 kb, 700 bp, 500 bp, 400 bp, 300 bp, 200 bp, and 100 bp. **d** Identification of HMW-GS from the F₂ generations (lanes 1–24) of CS-1S¹/1B × Wanmai 33 by SDS-PAGE. **e** AS-PCR amplification of *IS^x2.3** gene from the F₂ generations of CS-1S¹/1B × Wanmai 33 (lane Nos. 1–24 are same as **d**). **f** AS-PCR amplification of *IS^y16** gene from the F₂ generations of CS-1S¹/1B × Wanmai 33 (lane Nos. 1–24 are same as **d** and **e**). M: DNA markers with 10 kb, 8 kb, 5 kb, 2 kb, 1.5 kb, 1 kb, 750 bp, 500 bp, 250 bp, and 100 bp

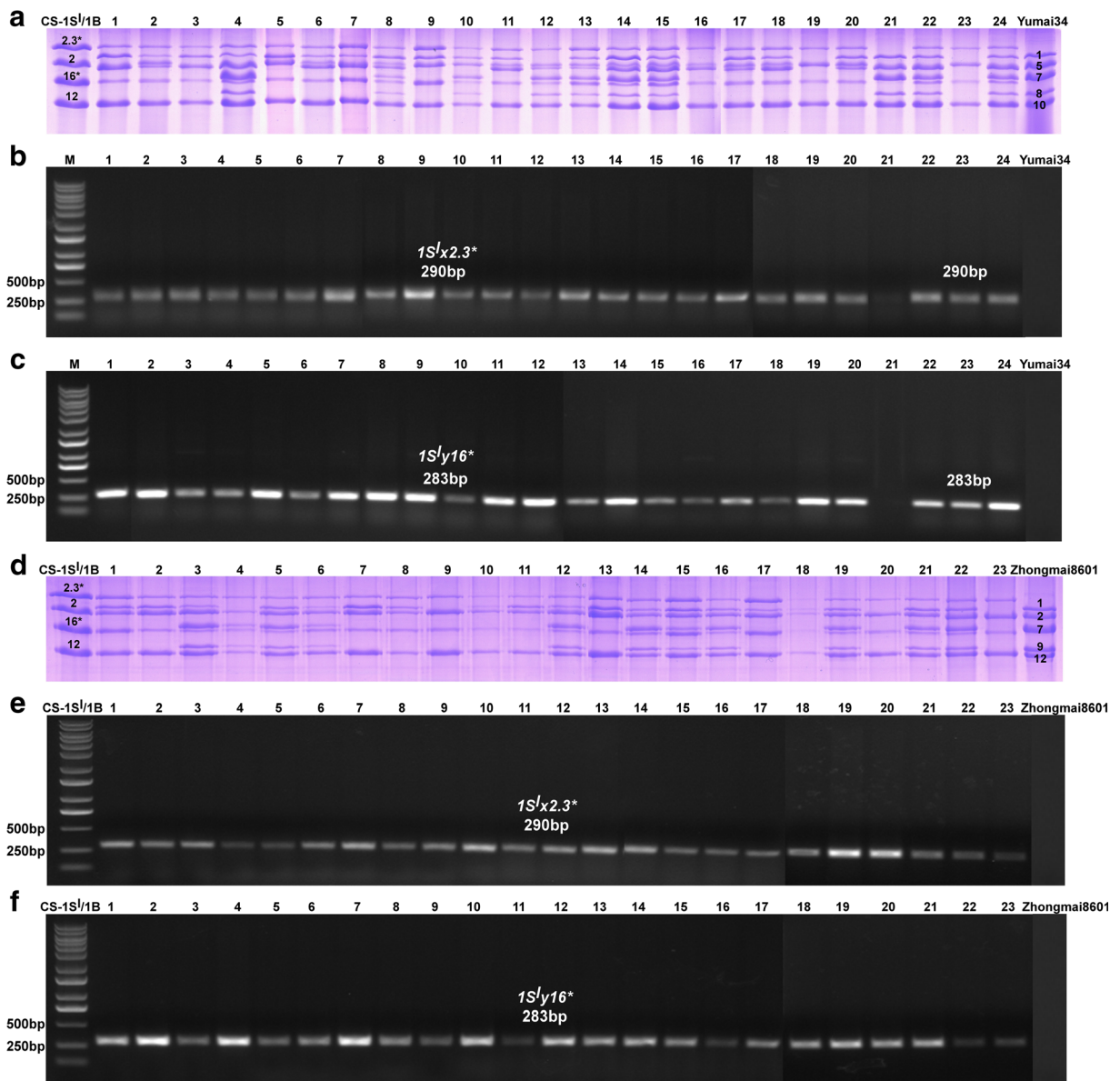


Fig. 5 Validation of the SNP-based molecular markers for *IS^x2.3** and *IS^y16** genes in two F₂ generations of CS-1S¹/1B × Yumai 34 and CS-1S¹/1B × Zhongmai 8601. **a** Identification of HMW-GS from the F₂ generations (lanes 1–24) of CS-1S¹/1B × Yumai 34 by SDS-PAGE. **b** AS-PCR amplification of *IS^x2.3** gene from the F₂ generations of CS-1S¹/1B × Yumai 34 (lane Nos. 1–24 are same as **a**). **c** AS-PCR amplification of *IS^y16** gene from F₂ generations of CS-1S¹/1B × Yumai 34 (lane Nos. 1–24 are same as **a** and **b**). M: DNA markers with 10 kb, 8 kb, 5 kb, 2 kb,

1.6 kb, 1 kb, 700 bp, 500 bp, 400 bp, 300 bp, 200 bp, and 100 bp. **d** Identification of HMW-GS from the F₂ generations (lanes 1–24) of CS-1S¹/1B × Zhongmai 8601 by SDS-PAGE. **e** AS-PCR amplification of *IS^x2.3** gene from the F₂ generations of CS-1S¹/1B × Zhongmai 8601 (lane Nos. 1–24 are same as **d**). **f** AS-PCR amplification of *IS^y16** gene from the F₂ generations of CS-1S¹/1B × Zhongmai 8601 (lane Nos. 1–24 are same as **d** and **e**). M: DNA markers with 10 kb, 8 kb, 6 kb, 5 kb, 4 kb, 3 kb, 2 kb, 1.5 kb, 1 kb, 750 bp, 500 bp, 250 bp, and 100 bp

Discussion

Function of the 1S^lx2.3* and 1S^ly16* subunits and their potential value in wheat quality improvement

HMW-GS have been shown to be more important than gliadins and LMW-GS in determining dough rheological properties (Ma et al. 2005). Breadmaking quality largely depends on the number and composition of HMW-GS (Burnouf and Bouriquet 1980; Altpeter et al. 1996; Yan et al. 2004). Furthermore, the yield factors such as spike grain number and grain weight have no significant effects on processing quality (Zhao et al. 1994; Wang et al. 1995). Our results from a pair of *Glu-B1* NILs indicated that the 1S^l-encoded HMW-GS 1S^lx2.3* and 1S^ly16* from *Ae. longissima* conferred superior dough rheological and bread qualities, including significantly higher grain hardness, gluten index, development and stability times, loaf cell size, volume, inner structure, and score compared to the 1Bx17 + 1By18 subunits (Fig. 2, Tables 3 and 4). Thus, although two NILs have significant differences in main yield traits (Table 2), the allelic variations at *Glu-B1* locus could be responsible for their quality differences.

Grain hardness is closely related to the end-use quality of wheat flour. According to endosperm texture, common wheat is usually classified as soft wheat, hard wheat, or mixed wheat. Soft wheat generally has a lower damaged starch content and smaller flour granules, whereas hard wheat has a higher damaged starch content and larger flour granules due to the smaller intercellular space of the starch granules. Therefore, flour made from hard wheat is generally more suitable for making bread (Ma et al. 2010). The gluten-sieving parameter is determined via centrifugation of a gluten solution (total gluten), which forces gluten through a sieve plate, where a lower value of sieved gluten indicates higher gluten strength. The related gluten index is the ratio of gluten on the sieve to the total gluten content, and a higher gluten index represents stronger gluten (Wang 2013). Development time and stability time are two important parameters for estimating dough strength (Wang et al. 1997; Kari et al. 2003). Development time refers to the mixing time from the start of adding water to the maximum consistency of the dough. A higher gluten content in dough results in greater elasticity, increased mechanical energy consumption, and a prolonged time for final dough development (Wang et al. 1997). Dough stability

time refers to the time from the initial time until reaching 500 BU, which reflects dough resistance. Longer stability times represent better dough toughness and greater gluten strength (Wang et al. 1997). The significant promotion of grain hardness, gluten index, and dough rheological properties by the 1S^l-encoded 1S^lx2.3* and 1S^ly16* subunits led to significant improvements in bread texture, loaf volume, and score (Table 4).

The allelic variations at the *Glu-1* loci are closely related to flour quality, and individual subunits and subunit combinations have different contributions to quality formation. Previous studies found that the 1Dx5 + 1Dy10 subunit combination encoded by *Glu-D1d* was associated with the highest quality scores, whereas the 1Bx17 + 1By18 subunit combination encoded by *Glu-B1i* ranked second (Shewry et al. 1992). A recent report from our group based on analysis of a set of *Glu-1* NILs confirmed that the 1Bx17 + 1By18 subunit combination is second only to the Dx5 + Dy10 subunit combination (Jiang et al. 2018). In the present study, the 1S^l-encoded 1S^lx2.3* + 1S^ly16* subunits endowed better dough rheological properties and breadmaking quality than the 1Bx17 + 1By18 subunits (Table 4), indicating that 1S^lx2.3* + 1S^ly16* may represent a new superior set of subunits for the improvement of breadmaking quality.

Molecular characterization revealed that both 1S^lx2.3* and 1S^ly16* subunits have relatively long repeat domains. In particular, 1S^lx2.3* has an additional 102-amino acid residue insertion in the central repeat domain, and 1S^ly16*, as one of the largest y-type HMW subunits characterized to date, contains 749 amino acid residues (Wang et al. 2013). The long repeat domain is thought to have a positive effect on wheat flour quality (Masci et al. 1998, 2000; Belton 1999), which can form more β -turn structures, imparting protein molecular elasticity (Tatham et al. 1985; Gianibelli et al. 2001). Insertions in the central repeat domain can directly affect functional properties (Hassani et al. 2005). Thus, the additional 102-amino acid residue insertion in the central repeat domain of the 1S^lx2.3* subunit may have a positive effect on the viscoelastic properties of dough. The y-type HMW-GSs are also considered to be main components of the technical characteristics of flour (D'Ovidio and Anderson 1994). These structural features may reflect the important roles of 1S^lx2.3* and 1S^ly16* in determining gluten quality for improving wheat breadmaking quality.

SNP-based molecular markers for $1S^Lx2.3^*$ and $1S^Ly16^*$ genes and wheat quality improvement applications

Various methods have been developed and used to separate and identify HMW-GS, including SDS-PAGE, reversed-phase high-performance liquid chromatography, high-performance capillary electrophoresis, reversed-phase ultra-performance liquid chromatography, and mass spectrometry (Yan et al. 2003a, b; Zhang et al. 2008b; Gao et al. 2010; Yan et al. 2014). However, these methods for identifying individual protein subunits are based on protein physicochemical properties, such as isoelectric point and molecular mass, and present various disadvantages. For example, the widely used SDS-PAGE cannot accurately distinguish subunits with similar molecular weights and electrophoretic mobilities because the electrophoretic mobility of the radical is not always consistent with its molecular weight. It is also time-consuming, requires the use of toxic reagents, and is destructive. Meanwhile, liquid chromatography, capillary electrophoresis, and mass spectrometry require expensive analytical instruments, restricting their use.

SNPs are considered third-generation molecular markers and are a powerful tool in marker-assisted breeding (Rafalski 2002). SNP-based AS-PCR markers appear to be capable of effectively identifying HMW-GS genes. In general, PCR-based molecular markers are fast, cost-efficient, and subject to few restrictions, allowing for high-throughput selection during marker-assisted selection for wheat quality improvement. Various molecular markers for HMW-GS genes have been developed (Ahmad 2000; Liang et al. 2015; Shiri et al. 2015). These markers can effectively screen and identify the target HMW-GS genes from different varieties and isolated populations and have potential value for marker-assisted selection in wheat quality improvement programs.

In this study, we developed and validated two specific SNP-based markers for the $1S^Lx2.3^*$ and $1S^Ly16^*$ genes using different wheat cultivars, RILs, and F_2 generations (Figs. 3, 4, and 5). Both SNP-based markers showed high specificity and accuracy and can be used for marker-assisted selection of the superior $1S^Lx2.3^*$ and $1S^Ly16^*$ genes in wheat quality improvement programs. Such molecular markers are expected to accelerate breeding processes and promote gluten quality improvement.

Conclusion

Analysis of two NILs, HMW-NIL1 (1Ax1, $1S^Lx2.3^*$ + $1S^Ly16^*$, and 1Dx2 + 1Dy12) and HMW-NIL2 (1Ax1, 1Bx17 + 1By18, and 1Dx2 + 1Dy12), showed that the $1S^L$ -encoded $1S^Lx2.3^*$ + $1S^Ly16^*$ subunits confer better dough rheological properties and breadmaking quality than 1Bx17 + 1By18 subunits via improvements in dough strength, cell size, loaf volume, texture, and score. Subsequently, two specific SNP-based molecular markers for $1S^Lx2.3^*$ (290 bp) and $1S^Ly16^*$ (283 bp) were developed and validated. These markers can be used for molecular marker-assisted selection in early generations for wheat quality improvement to improve breeding efficiency.

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