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Research Paper

Allelic variation of LMW-GS composition in Chinese wheat landraces of the Yangtze-River region detected by MALDI-TOF-MS

Yanchun Peng[1,2,3], Zitong Yu[2,3], Shahidul Islam[2,3], Yujuan Zhang[2,3], Xiaolong Wang[2,3], Zhensheng Lei[4], Kan Yu[1], Dongfa Sun*[1,5] and Wujun Ma[2,3]

[1] *College of Plant Science and Technology, Huazhong Agricultural University*, Wuhan 430070, China
[2] *State Agriculture Biotechnology Centre, Murdoch Unievrsity*, WA 6150, Australia
[3] *Australian Export Grain Innovation Centre*, Perth, WA 6150, Australia
[4] *Wheat Research Institute, Henan Academy of Agricultural Sciences*, Zhengzhou 450002, China
[5] *Hubei Collaborative Innovation Center for Grain Industry*, Jingzhou 434025, China

Low molecular weight glutenin subunits are important components of wheat storage proteins, which play an important role in determining end-use quality of common wheat. A newly established matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) procedure was used to analyze 478 landraces of bread wheat collected from the Yangtze-River region in China. Results indicated that 17 alleles at three loci: *Glu-A3*, *Glu-B3* and *Glu-D3* were identified, resulting in 87 different allele combinations. Of the 17 alleles detected at all the *Glu-3* loci, five belonged to *Glu-A3*, seven to *Glu-B3* and five to *Glu-D3* locus. MALDI-TOF-MS indicated *Glu-A3a/c* was present in 72.8%, *Glu-A3b* in 8.4%, *Glu-A3d* in 8.4%, *Glu-A3f* in 5.2% and *Glu-A3e* in 3.6% lines. Seven types of alleles were identified at the *Glu-B3* locus: *Glu-B3d/i* (25.5%), *Glu-B3b* (21.3%), *Glu-B3c* (16.9%), *Glu-B3h* (13.8%), *Glu-B3f* (8.4%), *Glu-B3a* (8.2%), and *Glu-B3g* (5.2%). Five types of *Glu-D3* alleles were detected: *Glu-D3a* (58.4%), *Glu-D3c* (22.6%), *Glu-D3d* (15.5%), *Glu-D3b* (3.3%) and *Glu-D3f* (0.2%). Four new alleles that showed abnormal MALDI-TOF spectrum patterns were identified at the *Glu-A3* and *Glu-B3* loci. A detailed study is needed to further characterize these alleles and their potential usage for wheat improvement.

Key Words: LMW-GS alleles, wheat bread-making, MALDI-TOF-MS, Chinese landraces.

Introduction

Gluten proteins, responsible for the functional characteristics of wheat flour products, are conventionally divided into monomeric gliadins and polymeric glutenins, which determine the rheological characteristics (strength and extensibility) of flour dough (Békés *et al.* 2001, Butow *et al.* 2003, Fu and Kovacs 1999, Ma *et al.* 2005, Maucher *et al.* 2009). The glutenins are divided into high molecular weight glutenin subunits (HMW-GSs) and low molecular weight glutenin subunits (LMW-GSs) based on their mobility during sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

The LMW-GSs, which are further subdivided into B, C and D groups on the basis of their mobility in SDS-PAGE and their isoelectric points (Jackson *et al.* 1983), ranging in molecular mass from 25 to 43 kDa (Liu *et al.* 2010). They represent about one-third of the total seed protein, account for approximately 60% of total glutenins (Bietz and Wall 1973), and are essential contributors that determine dough properties in wheat, such as dough extensibility (Cornish *et al.* 2001) and gluten strength (Gianibelli *et al.* 2001). Different allelic forms of LMW-GSs play different roles in determining different quality parameters (Appelbee 2007, He *et al.* 2005, Luo *et al.* 2001). Therefore, it is essential to identify the allelic composition of LMW-GSs in different wheat varieties in order to efficiently use the allele in wheat breeding.

LMW-GSs exhibit high polymorphic protein complexes encoded by a multigene family (D'Ovidio and Masci 2004). Generally, the genes encoding LMW-GSs are located at the *Glu-A3*, *Glu-B3* and *Glu-D3* loci on the short arms of chromosomes 1A, 1B and 1D, respectively (Pogna *et al.* 1990, Singh and Shepherd 1988). The LMW-GSs are not as well assessed as the HMW-GSs by SDS-PAGE because of their large numbers of subunits and the complexity of the LMW-GS patterns (Huang and Cloutier 2008, Zhang *et al.* 2011). Multiple analytical procedures have been established to

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*Corresponding author (e-mail: sundongfa1@mail.hzau.edu.cn)

accurately analyze the HMW-GS in bread wheat, including one-dimensional SDS-PAGE (Branlard *et al.* 2003), 2-DE (An *et al.* 2005), PCR (Ma *et al.* 2003) and MALDI-TOF (Liu *et al.* 2009). Due to the complexity, accurate identification of LMW-GS alleles has been proven to be a difficult task. The SDS-PAGE procedure often results in errors. The 2-DE analytical process can display much more information than SDS-PAGE, but is not generally recommended in breeding programs due to its time consuming and high cost nature. Recently, PCR approach has become a powerful tool for characterizing LMW-GS composition in common wheat (Appelbee *et al.* 2009, Ram *et al.* 2011, Wang *et al.* 2009, 2010, Zhang *et al.* 2011). However, allele specific PCR markers are only available for a proportion of LMW-GS alleles, which limited its usage in wheat breeding. Recently, MALDI-TOF-MS has been put into use for analyzing LMW-GS alleles (Liu 2008, Wang 2008). It is the most efficient method to characterize wheat gluten proteins and requires only a few minutes per sample to perform the analysis (Liu *et al.* 2009, Wang 2008, Zheng *et al.* 2011). The standard allele specific MALDI-TOF spectrum patterns have been established for most known LMW-GS alleles by Wang *et al.* (2015), which enables fast and reliable identification of LMW-GS alleles.

China is considered as a secondary centre of origin for common wheat (Dong and Zheng 2000) according to its history of wheat production and the extensive genetic variation that is identified in the middle and low branches of the Yellow river (i.e. in Henan and Shandong provinces). There are a mass of wheat landraces that have been accumulated. The Chinese National Germplasm Bank stores more than ten thousand Chinese wheat landrace accessions collected from various wheat production regions. Some novel HMW-GS alleles have been found in the Chinese wheat landraces (Fang *et al.* 2009, Guo *et al.* 2010, Liu *et al.* 2007, Zheng *et al.* 2011). Unfortunately, little is known about the LMW-GS allelic compositions due to lack of efficient analytical procedures. This has hampered the usage of Chinese landraces LMW-GS variations in wheat breeding. The objective of this study was to detect the LMW-GS allelic compositions of Chinese wheat landraces from the Yangtze-River region.

Materials and Methods

Plant material

Four hundred and seventy-eight accessions of bread wheat (*Triticum aestivum* L.) landraces were collected from the Yangtze-River region (389 landraces from Hubei province, and 89 landraces from Tibet Autonomous Region) in China, including most accessions collected by Huazhong Agricultural University Wuhan, Hubei province in the past fifty years (**Supplemental Table 1**).

Protein extraction

Proteins were extracted from whole meal based on the procedure reported by Wang *et al.* (2015). Whole meal (100 mg) was suspended in 1.0 ml of 50% propanol-1-ol (v/v) for 5 min continuous vortexing, followed by incubation at 65°C for 20 min, vortexing for 5 min, and centrifugation at 10,000 × g for 5 min. This step was repeated three times to remove the majority of the gliadins. The glutenin in the pellet was reduced with 50 mM Tris-HCl buffer containing 50% propanol-1-ol and 1% w/v dithiothreitol, then 1.4% v/v of 4-vinylpyridine was added, and alkylation was carried out overnight at room temperature.

MALDI-TOF-MS protocol and nomenclature

MALDI-TOF-MS was carried out at the State Agriculture Biotechnology Center, Murdoch University, Australia. Acetone (80%) was used to precipitate the glutenin fraction, and the resulting pellets were dissolved in 60 µL acetonitrile/H_2O (50:50 v/v) with 0.05% v/v trifluoroacetic acid (60 min at room temperature). Sample preparation was performed on the basis of the dried droplet method (Kussmann *et al.* 1997), employing sinapinic acid as matrix. The sinapinic acid was dissolved in acetonitrile/H_2O (50:50 v/v) with 0.05% v/v trifluoroacetic acid at a concentration of 10 mg/ml.

A sandwich matrix/sample/matrix 1:1:1 (0.7 µL) was placed to a 100-sample MALDI probe tip, and dried at room temperature.

MALDI-TOF-MS was carried out on a Voyager DE-PRO TOF mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with nitrogen laser (337 nm) and delayed extraction. Analyses were carried out on a positive linear ion mode with the following parameters: mass range 10000–50000 Da, acceleration voltage 25 kV, and delay time 900 ns. Ten thousands Da was selected as the low mass gate value for analysis to avoid saturation of the detector. The allele specific MALDI-TOF spectrum patterns followed the newly established system (Wang *et al.* 2015). The LMW-GS nomenclature system of Ikeda *et al.* (2008) was used.

Result

By following the new method of Wang *et al.* (2015), the mass spectra of the LMW-GSs displayed distinct, well-separated spectrum peaks for all wheat lines, with the molecular masses ranging from 25,000 to 44,000 Da in the spectra. Representative spectra are shown in **Fig. 1** to **Fig. 8**. The allele combinations and variants at the *Glu-3* loci identified by MALDI-TOF-MS are shown in **Table 1**. All scored alleles matched well with the reported allele specific spectrum peak patterns (Wang *et al.* 2015). Low level of peak distortion or position shifting in a specific pattern was observed.

At the *Glu-A3* locus, five alleles were identified, with the most frequent one being *Glu-A3a/c* present in 72.8% of the lines. *Glu-A3b* (8.4%) and *Glu-A3d* (8.4%) were less common. The less frequent alleles were the *Glu-A3f* (5.2%) and *Glu-A3e* (3.6%). Three novel alleles were detected which were represented by three unrecognized peak patterns. A

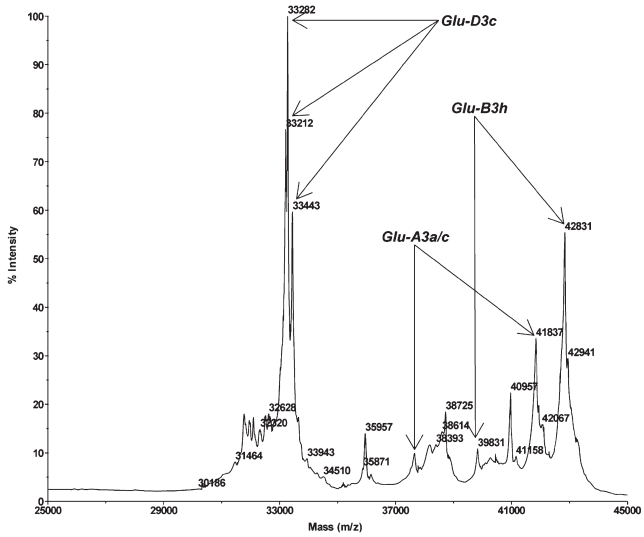

Fig. 1. Representative MALDI-TOF spectrum: Huazhong 9

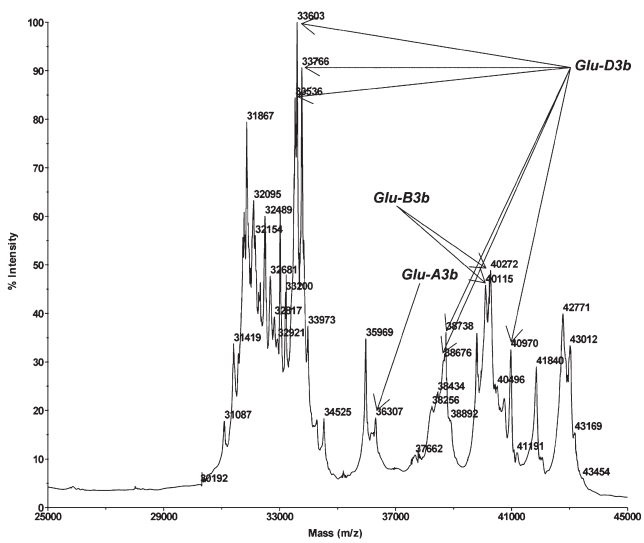

Fig. 2. Representative MALDI-TOF spectrum: Huazhong 815

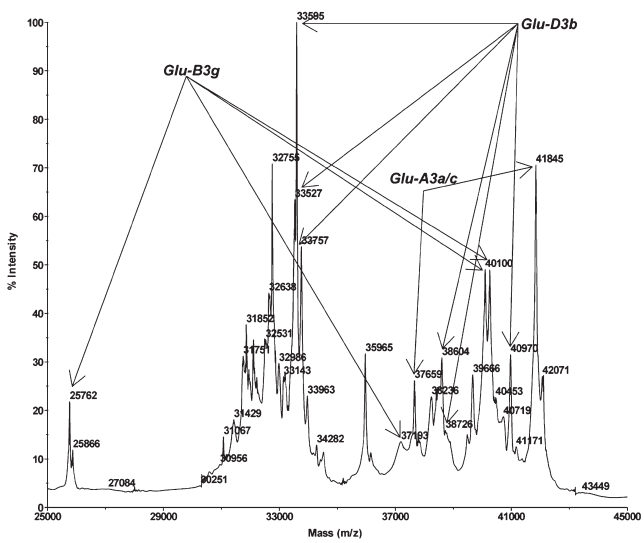

Fig. 3. Representative MALDI-TOF spectrum: Huazhong 790

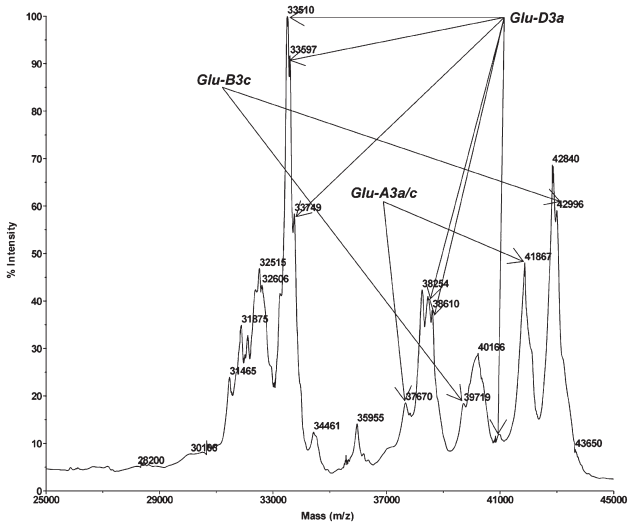

Fig. 4. Representative MALDI-TOF spectrum: Huazhong 175

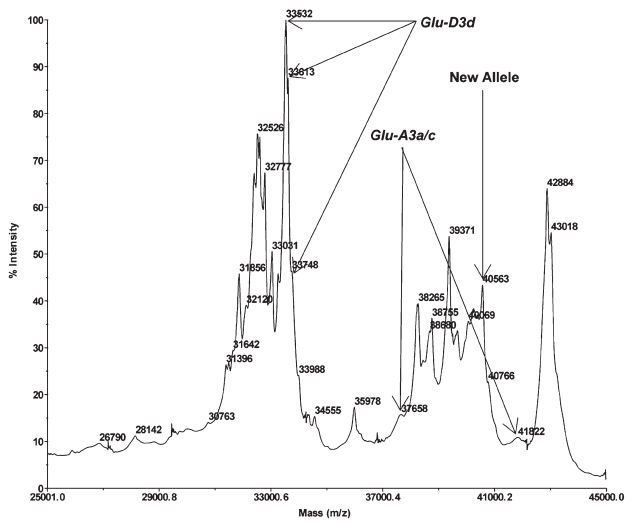

Fig. 5. Representative MALDI-TOF spectrum: Huazhong 628

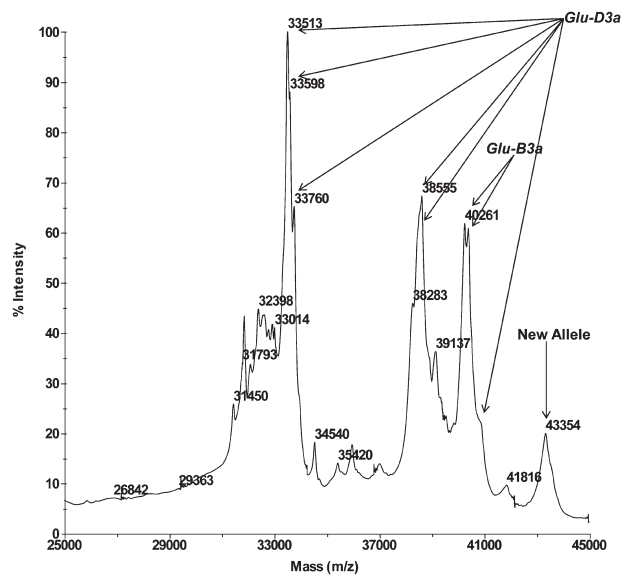

Fig. 6. Representative MALDI-TOF spectrum: Huazhong 693

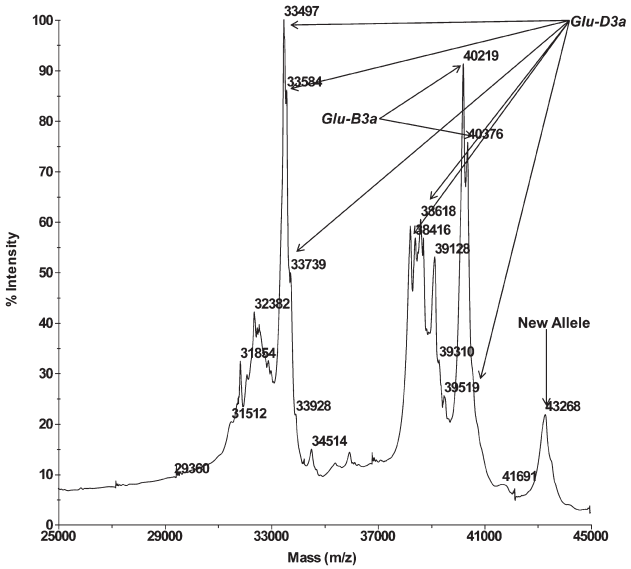

Fig. 7. Representative MALDI-TOF spectrum: Huazhong 657

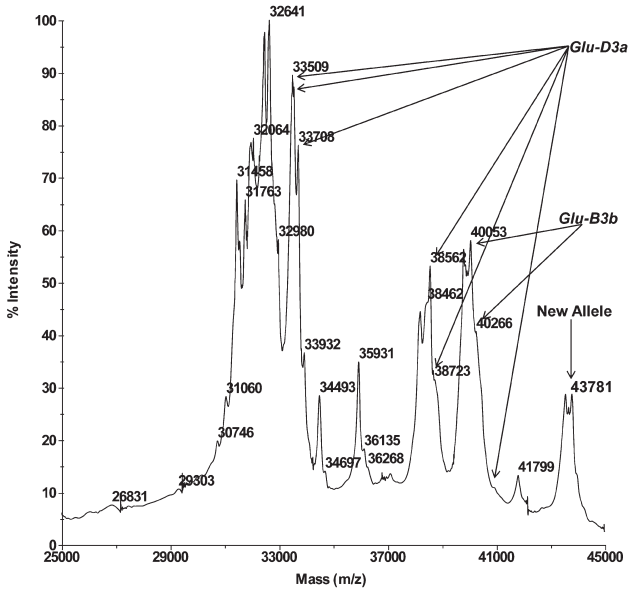

Fig. 8. Representative MALDI-TOF spectrum: Huazhong 793

Table 1. Allele combinations and variants at the *Glu-3* loci in Chinese wheat landraces of the Yangtze-River region

	Glu-A3	*Glu-B3*	*Glu-D3*	Varieties	Frequency (%)		*Glu-A3*	*Glu-B3*	*Glu-D3*	Varieties	Frequency (%)
1	43268Da	*a*	*a*	1	0.2	45	*b*	*c*	*d*	6	1.3
2	43338Da	*d/i*	*a*	1	0.2	46	*b*	*d/i*	*a*	1	0.2
3	43354Da	*a*	*a*	1	0.2	47	*b*	*d/i*	*c*	1	0.2
4	43384Da	*h*	*a*	1	0.2	48	*b*	*d/i*	*d*	2	0.4
5	43385Da	*h*	*d*	1	0.2	49	*b*	*f*	*d*	1	0.2
6	43754Da	*b*	*b*	1	0.2	50	*b*	*g*	*a*	3	0.6
7	43770Da	*a*	*a*	1	0.2	51	*b*	*h*	*a*	3	0.6
8	43781Da	*b*	*a*	1	0.2	52	*b*	*h*	*c*	1	0.2
9	*a/c*	40563Da	*d*	1	0.2	53	*b*	*h*	*d*	4	0.8
10	*a/c*	40576Da	*b*	1	0.2	54	*d*	*b*	*a*	6	1.3
11	*a/c*	40642Da	*a*	1	0.2	55	*d*	*b*	*c*	3	0.6
12	*a/c*	*a*	*a*	19	4.0	56	*d*	*b*	*d*	2	0.4
13	*a/c*	*a*	*b*	1	0.2	57	*d*	*c*	*a*	3	0.6
14	*a/c*	*a*	*c*	4	0.8	58	*d*	*c*	*d*	2	0.4
15	*a/c*	*a*	*d*	4	0.8	59	*d*	*f*	*a*	6	1.3
16	*a/c*	*b*	*a*	45	9.4	60	*d*	*f*	*c*	2	0.4
17	*a/c*	*b*	*b*	1	0.2	61	*d*	*f*	*d*	2	0.4
18	*a/c*	*b*	*c*	19	4.0	62	*d*	*g*	*b*	1	0.2
19	*a/c*	*b*	*d*	11	2.3	63	*d*	*g*	*c*	3	0.6
20	*a/c*	*c*	*a*	30	6.3	64	*d*	*g*	*d*	1	0.2
21	*a/c*	*c*	*b*	2	0.4	65	*d*	*h*	*a*	3	0.6
22	*a/c*	*c*	*c*	12	2.5	66	*d*	*h*	*b*	2	0.4
23	*a/c*	*c*	*d*	10	2.1	67	*d*	*h*	*c*	2	0.4
24	*a/c*	*c*	*f*	1	0.2	68	*d*	*h*	*d*	2	0.4
25	*a/c*	*d/i*	*a*	66	13.8	69	*e*	*a*	*a*	1	0.2
26	*a/c*	*d/i*	*b*	3	0.6	70	*e*	*a*	*c*	1	0.2
27	*a/c*	*d/i*	*c*	21	4.4	71	*e*	*b*	*a*	2	0.4
28	*a/c*	*d/i*	*d*	10	2.1	72	*e*	*c*	*a*	3	0.6
29	*a/c*	*f*	*a*	17	3.6	73	*e*	*c*	*c*	1	0.2
30	*a/c*	*f*	*c*	7	1.5	74	*e*	*d/i*	*a*	3	0.6
31	*a/c*	*f*	*d*	2	0.4	75	*e*	*d/i*	*c*	1	0.2
32	*a/c*	*g*	*a*	6	1.3	76	*e*	*f*	*a*	2	0.4
33	*a/c*	*g*	*b*	1	0.2	77	*e*	*g*	*a*	1	0.2
34	*a/c*	*g*	*c*	9	1.9	78	*e*	*h*	*a*	2	0.4
35	*a/c*	*h*	*a*	30	6.3	79	*f*	*a*	*a*	2	0.4
36	*a/c*	*h*	*b*	1	0.2	80	*f*	*b*	*c*	4	0.8
37	*a/c*	*h*	*c*	5	1.0	81	*f*	*b*	*d*	3	0.6
38	*a/c*	*h*	*d*	8	1.7	82	*f*	*c*	*a*	1	0.2
39	*b*	*a*	*a*	3	0.6	83	*f*	*d/i*	*a*	9	1.9
40	*b*	*a*	*b*	1	0.2	84	*f*	*d/i*	*c*	2	0.4
41	*b*	*b*	*b*	1	0.2	85	*f*	*d/i*	*d*	2	0.4
42	*b*	*b*	*c*	3	0.6	86	*f*	*f*	*a*	1	0.2
43	*b*	*c*	*a*	4	0.8	87	*f*	*h*	*a*	1	0.2
44	*b*	*c*	*c*	6	1.3						

new subunit with molecular weight of 43,754 Da was found in three lines (66, 778, 793). Line 657 contained a new subunit of 43,268 Da, and three lines (158, 170, 693) had a new *Glu-A3* type subunit of 43,350 Da. These new *Glu-A3* subunits are located around the *Glu-A3d* position in the spectra.

Seven alleles were identified at the *Glu-B3* locus. Overall, the *Glu-B3* locus had four predominant allele compositions including *Glu-B3d/i* (25.5%), *Glu-B3b* (21.3%), *Glu-B3c* (16.9%) and *Glu-B3h* (13.8%). The rest three alleles, namely *Glu-B3f*, *Glu-B3a* and *Glu-B3g*, existed in 8.4%, 8.2% and 5.2% of the tested landraces, respectively. Only one new LMW-GS was identified at the *Glu-B3* locus which was present in three lines (241, 628, 649), with the molecular weight of 40,600 Da.

Glu-D3 allele usually consists of high number of spectrum peaks. However, accurate identification of all known *Glu-D3* alleles was achieved. Five alleles were observed at the *Glu-D3* locus. The frequencies of *Glu-D3a* and *Glu-D3c* were 58.4% and 22.6%, respectively. *Glu-D3d* was found in 15.5% of the landraces and *Glu-D3b* was present in 3.3% of the landraces. *Glu-D3f* was observed only in one landrace (line155).

Discussion

Allele identification of glutenins is important for promoting wheat quality. Many favorable alleles with positive effects on dough characteristics and bread-making quality are encoded by *Glu-1* and *Glu-3* loci. Accurate identification of these alleles is essential for selecting parents in crossing and accumulating them by pyramidal breeding. Meanwhile, discovery of new allele will further aid the wheat quality improvement efforts. Chinese wheat landraces are well known to harbor novel genes. Characterization of glutenin compositions of Chinese landraces will make it possible to utilize these old wheat lines in modern wheat breeding.

In this study, the allelic compositions of LMW-GSs in 478 wheat landraces collected from the Yangtze-River region of China were identified using the newly established MALDI-TOF procedure (Wang *et al.* 2015). The landraces were collected from Hubei province (Huazhong1–Huazhong700), and Tibet Autonomous Region (Huazhong740–Huazhong833) (**Supplemental Table 1**). Concerning the landraces from Hubei province, the most frequent alleles at *Glu-3* were *Glu-A3a/c* (74.6%), *Glu-A3b* (7.7%), *Glu-A3d* (7.2%); *Glu-B3d/i* (30.6%), *Glu-B3c* (20.3%), *Glu-B3b* (15.9%), *Glu-B3h* (13.4%); *Glu-D3a* (59.4%), *Glu-D3c* (20.1%), *Glu-D3d* (17.0%). As for the landraces from Tibet Autonomous Region, the most frequent alleles at *Glu-3* were *Glu-A3a/c* (65.2%), *Glu-A3d* (13.5%), *Glu-A3b* (11.2%); *Glu-B3b* (44.9%), *Glu-B3f* (19.1%), *Glu-B3h* (15.7%), *Glu-B3g* (12.4%); *Glu-D3a* (53.9%), *Glu-D3c* (33.7%). Some landraces with the same name were collected from different regions of the Yangtze-River; however, they are phenotypical different in many agronomic traits, and published results identified that they are different landraces (Zheng *et al.* 2011), but farmers called them the same name.

For the *Glu-A3* locus, the most frequent alleles were *Glu-A3a* and *Glu-A3c*. Bellil *et al.* (2010, 2012), Bradová and Štočková (2010), Branlard *et al.* (2003) and Igrejas *et al.* (1999) also reported a similar conclusion in that *Glu-A3a* was the predominant allele in wheat cultivars with the frequency of 49.3% among 69 cultivars grown in France, 60.0% among 40 cultivars of Saharan wheats originating from Algerian oases, 47.0% in a collection of 86 Czech registered winter wheat varieties, 44.5% among a set of 200 hexaploid wheat cultivars grown commonly in France and 57.1% among 63 bread wheats primarily grown in Portugal, respectively. The frequency of *Glu-A3c* allele among 65 accessions representing a historical trend in the cultivars released or introduced in Iran from the year 1940 to 1990 was 40.3% (Izadi-Darbandi *et al.* 2010), and a higher frequency (40.8%) was also identified in a diverse set of 103 cultivars of common wheat collected from 12 countries including 21 cultivars from China, 19 from Argentina, 15 from Australia, 14 from France, 10 from Japan, 8 from Mexico, 7 from Canada, 3 from the USA, two from the Netherlands, two from Italy, one from Germany and one from Finland (Liu *et al.* 2010). Thus, these results indicated that the alleles *Glu-A3a* and *Glu-A3c* are worldwide predominance among bread wheat. It is worthy of noting that the *Glu-A3d* is a desirable allele for gluten quality and pan bread quality (He *et al.* 2005) and was present in 40 landraces. Moreover, the four newly identified *Glu-A3* alleles are all located around the *Glu-A3d* location in the spectra, suggesting that it is novel *glu-A3d* sub-alleles. The previously discovered unfavorable *Glu-A3e* allele that reduced the maximum resistance and extensibility of dough (Appelbee 2007) was found to be the least frequent allele (3.5%) in the studied Chinese wheat landraces, suggesting the high value of Chinese wheat landraces for modern wheat quality improvement.

The number of alleles identified at the *Glu-B3* (7 alleles) was the same to that reported by Bradová and Štočková (2010) in the Czech winter wheat cultivars. The predominant alleles in our study were *Glu-B3d/i* and *Glu-B3b*. The same result was observed in Saharan bread wheat cultivars and French cultivars for *Glu-B3d* allele, but *Glu-B3b* was rare in both collections (Bellil *et al.* 2010, 2012). On the contrary, Bradová and Štočková (2010), Branlard *et al.* (2003), and Shan *et al.* (2007) reported that *Glu-B3g* was the most frequent allele in their collections.

In China, *Glu-D3* alleles were typically classified as *Glu-D3a*, *Glu-D3b*, *Glu-D3c*, *Glu-D3d* and *Glu-D3f* (Liu 2008). The frequency of *Glu-D3* alleles from our study was primarily agreeable with previous report (Liu 2008). In comparison with *Glu-D3* allele frequencies previously found in 233 Chinese bread wheats (Liu 2008), both *Glu-D3a* and *Glu-D3c* accounted for a high proportion, while the rare allele *Glu-D3f* was observed only in six lines in Liu's study (Liu 2008) and one line in our study. Some research discovered only minor *Glu-D3* effects on end-use quality traits

(Branlard *et al.* 2003, Eagles *et al.* 2002, Gupta *et al.* 1994, He *et al.* 2005), while other researchers found more important effects (Appelbee 2007, Dong *et al.* 2010, Flaete and Uhlen 2003, Jin *et al.* 2013, Luo *et al.* 2001, Maucher *et al.* 2009, Park *et al.* 2011). For example, *Glu-D3h* was found to display a significantly positive effect on dough rheological quality. Unfortunately, this allele was not discovered in the Chinese landraces used here.

Based on the bread wheat nomenclature reported by Ikeda *et al.* (2008) for LMW-GSs, we were able to identify the LMW-GS allele compositions of 467 out of the 478 landraces. The other 11 lines contained four novel alleles with each expressing a subunit with molecular weight of about 40,600 Da, 43,268 Da, 43,385 Da, or 43,754 Da. The four novel subunits may play a positive role in determining the viscoelastic properties of wheat, and meeting new end-product requirement. A more detailed study is required to characterize the four novel alleles. Recently, numerous LMW-GS genes have been cloned and characterized (Jiang *et al.* 2008, Li *et al.* 2008, Zhang *et al.* 2010, Zhao *et al.* 2006, 2008). Based on the available knowledge, the newly identified *Glu-3* alleles should be readily cloned and analyzed.

In conclusion, Seventeen known LMW-GS alleles and four novel alleles were found in a collection of 478 landraces from the Yangtze-River region of China. The information obtained in this study is useful for wheat breeders to make decisions on crossing and selection strategies to improve wheat quality, especially to breed new cultivars to meet specific end-product requirements. The results also add to our understanding of genetic effects of LMW-GSs.

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