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GLU-A3 AND *GLU-B3* ALLELES FOR LMW PROTEIN IN LOCAL WHEAT IDENTIFICATION OF GLU-A3 AND GLU-B3 ALLELES FOR LMW PROTEIN SUBUNITS IN SOME WHEAT GENOTYPES OF PAKISTAN

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Identification of alleles responsible for low molecular weight protein subunits (LMW-GS) is of immediate concern due to their large effect on wheat processing qualities, especially gluten elasticity. Allele-specific PCR (AS-PCR) molecular markers have been developed to screen wheat genotypes for appropriate LMW-GS alleles. In this study sequence tagged site AS-PCR markers were used for identification of alleles at *Glu-A3* and *Glu-B3* loci for LMW protein subunits in twenty-six wheat cultivars developed in Pakistan to assess their potential contribution to enhance bread-making quality. Three alleles at *Glu-A3* and four alleles at *Glu-B3* loci were explored in the wheat genotypes under study. Allelic richness (4) and genetic diversity (*H*) (0.7) was comparatively higher for *Glu-B3* locus as compared to *Glu-A3* locus. *Glu-A3b* and *Glu-A3d* alleles were observed in 11 (64%) cultivars, which were known to encode high sedimentation and protein contents, which made them the important sources for wheat genetic improvement with special emphasis on quality of wheat.
Keywords: Allelic frequency, AS-PCR, glutenins, LMW-GS, STS markers, wheat

INTRODUCTION

Wheat is one of the major food crops and virtually provides nutrition for 35% of the world population. Wheat (*Triticum aestivum* L.) endosperm contains a major class of storage proteins, where glutenin is considered to be playing a major role in bread making quality. Glutenin proteins are the foremost cause for the unique viscoelastic properties of wheat flour and dough. Glutenin possess the rheological characteristics that are vital for a wide range of food products (He *et al.,* 2005; Shewry *et al.,* 1995). End use quality of wheat is influenced by the composition of storage proteins (Dessalegn *et al.,* 2011), its quality and quantity confers elasticity and extensibility necessary for bread making. It contributes 80-85% of the total flour protein (Shewry *et al.,* 1995). This endosperm protein consists of two manly prolamine groups namely monomeric gliadins and polymeric glutenin (An *et al.,* 2006). The polymeric glutenin proteins are separated into two group of subunits, low molecular weight glutenin subunits (LMW-GS and high molecular weight glutenin subunits (HMW-GS) and), according to their mobility's in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) (Payne *et al.,* 1979). Glutenins (LMW and HMW) are long polypeptide chains which are linked together by disulfide bonds to form gluten macro polymers (Gras *et al.,* 2000) that are thought to be mostly accountable for the visco elasticity and extensibility of dough. LMW-GS have a very strong relationship with gluten elasticity and are controlled by the genes *Glu-A3, Glu-B3,* and *Glu-D3*present on short arms of chromosomes 1A, 1B and 1D, respectively (Payne, 1987). Conventional classification of LMW-GS was divided into three sub groups i.e., LMW-i, LMW-s & LMW-m (D'Ovidio and Masci, 2004). The gene LMW-I encode on the A genome whereas band D genomes encode LMW-m and LMW-s type subunits respectively (An *et al.,* 2006; Huang and Cloutier 2008). Different experiments various studies also demonstrated that LMW-GS contribute importantly to dough properties (Jin *et al.,* 2012). Therefore, it has been an important target since their discovery to identify the alleles for LMW-GS to devise a breeding strategy to enhance the bread-making quality in wheat (Jin *et al.,* 2012; Rasheed *et al.,* 2014)

Functional markers developed from gene sequences help in the determination of allelic compositions in breeding materials (Liu *et al.,* 2012). These are the most valuable markers that can be exploited in breeding for gene identification, marker-assisted selection and gene pyramiding. Allele-specific PCR (AS-PCR) markers have been developed for determining the alleles at *Glu-A3*and *Glu-B3* loci (Wang *et al.,* 2010), which have been validated on the wheat germplasm for the pinpoint LMW-GS allelic identification. Previously, these marker systems have been used to identify the allelic variations for LMW-GS in various germplasm pools (Rasheed *et al.,* 2014). The objective of the study was to determine the allelic variations at *Glu-A3*and *Glu-B3*loci in diverse Pakistani wheat cultivars using AS-PCR markers with the aim to identify cultivars having desirable end-use quality encoding alleles.

MATRIALS AND METHODS

Table 1. List of different wheat genotypes.

S. No	Variety	Institution	S. No	Variety	Institution
1	Pirsabak 2005	CCRI. Pirsabak	14	Bahawalpur97	-do-
2	Zam 2009	-do-	15	Panjnad 2001	-do-
3	Khyber 87	-do-	16	Fareed 2006	-do-
4	Daman 98	-do-	17	Chakwal 50	BARI, Chakwal
5	Fakhr-e-Sarhad	-do-	18	GA 2002	-do-
6	Pirsabak 2004	-do-	19	Chakwal 97	-do-
7	Tatara	-do-	20	BARS 2009	BARS, Fateh Jhang
8	Wafaq 2001	NARC, Islamabad	21	Pasban	AARI, Faisalabad
9	NARC 2009	-do-	22	Shafaq	AARI,
10	Bahawalpur 2000	RARI, Bahawalpur	23	Inqlab 91	AARI
11	Bakhtawer 92	-do-	24	Sehar 2006	AARI
12	Manthar 2003	-do-	25	Faisalabad 2009	AARI, Faisalabad
13	Miraj 2008	-do-	26	Lasani 2008	-do-

Table 2. List of STS primers used for characterization of alleles at Glu-A3 and Glu-B3 loci.

	Locus	Allele	Sequence (5	Size	Conditions	Reference
LMW-GS	Glu-A3	Glu-A3a	F: AAACAGAATTATTAAAGCCGG R: GGTTGTTGTTGTTGCAGCA	529	94°C/35s-60°C/45s-72°C/90s	Wang *et al.* (2010)
		Glu-A3b	F: TTCAGATGCAGCCAAACAA R: GCTGTGCTTGGATGATACTCTA	894	94˚C/35s-60°C/45s-72°C/90s	
		Glu-A3ac	F: AAACAGAATTATTAAAGCCGG R: GTGGCTGTTGTGAAAACGA	573	94°C/35s-60°C/45s-72°C/90s	
		Glu-A3d	F: TTCAGATGCAGCCAAACAA R: TGGGGTTGGGAGACACATA	967	94°C/35s-60°C/45s-72°C/90s	
		Glu-A3e	F: AAACAGAATTATTAAAGCCGG R: GGCACAGACGAGGAAGGTT	158	94°C/35s-60°C/45s-72°C/90s	
		Glu-A3f	F: AAACAGAATTATTAAAGCCGG R: GCTGCTGCTGCTGTGTAAA	552	94°C/35s-60°C/45s-72°C/90s	
		Glu-A3g	F: AAACAGAATTATTAAAGCCGG R: AAACAACGGTGATCCAACTAA	1345	94°C/35s-60°C/45s-72°C/90s	
	Glu-B3	Glu-B3a*	F: CACAAGCATCAAAACCAAGA R: TGGCACACTAGTGGTGGTC	1095	94°C/35s-56°C/35s-72°C/90s	Wang *et al.* (2009)
		Glu-B3b	F: ATCAGGTGTAAAAGTGATAG R: TGCTACATCGACATATCCA	1570	94°C/35s-56°C/35s-72°C/90s	
		Glu-B3c	F: CAAATGTTGCAGCAGAGA R: CATATCCATCGACTAAACAAA	472	94°C/35s-56°C/35s-72°C/90s	
		Glu-B3d	F: CACCATGAAGACCTTCCTCA R: GTTGTTGCAGTAGAACTGGA	662	94°C/35s-58°C/35s-72°C/90s	
		Glu-B3e	F: GACCTTCCTCATCTTCGCA R: GCAAGACTTTGTGGCATT	669	94°C/35s-58°C/35s-72°C/90s	
		Glu-B3fg	F: TATAGCTAGTGCAACCTACCAT R: CAACTACTCTGCCACAACG	812	94°C/35s-63°C/35s-72°C/90s	
		Glu-B3g	F: CCAAGAAATACTAGTTAACACTAGTC R: GTTGGGGTTGGGAAACA	853	94°C/35s-61°C/35s-72°C/90s	
		Glu-B3h	F: CCACCACAACAAACATTAA R: GTGGTGGTTCTATACAACGA	1022	94°C/35s-60°C/35s-72°C/90s	
		Glu-B3i	F: TATAGCTAGTGCAACCTACCAT R: TGGTTGTTGCGGTATAATTT	621	94°C/35s-58°C/35s-72°C/90s	
		Glu-B3bef	F: GCATCAACAACAAATAGTACTAGAA R: GGCGGGTCACACATGACA	750	94°C/35s-60°C/35s-72°C/90s	

***DNA isolation and PCR amplification*:** The research material consists of twenty six diverse wheat varieties collected from different research institutes of Pakistan (Table 1). Phenol-chloroform method was used for genomic DNA extraction (Pagnotta *et al.,* 1995). In summary, 10 cm long pieces of fresh leaf material were collected, frozen in liquid nitrogen and ground to a fine powder. A total of 500µl DNA extraction buffer was added and mixed well, and 500µl of phenol: chloroform: isoamylalcohol (25:24:1) was added and well shaken. The eppendorf tubes were centrifuged for 3 min and the supernatant was transferred to a new tube. The 500µl of cold chloroform was added and mixed gently. It was centrifuged for 1 min and the supernatant was transferred to a fresh tube. Then 50µl 3M sodium acetate (pH = 4.8) and 500µl of isopropanol were added, mixed gently and centrifuged for 5 min. The supernatant was poured off and the pellet washed with 70% ethanol. The pellet was dried and re-suspended in 50µl TE or double distilled water. After treatment with RNase the DNA concentration was measured by spectrophotometer DNA Quant TM 200. The total genomic DNA was diluted in TE buffer to a concentration of 50 ng/µl for PCR analysis.

Allele-specific PCR of *Glu-A3* and *Glu-B3* loci was conducted with the help of primers as mentioned in Table 2 (Wang *et al.*, 2009; 2010). The PCR reactions were carried out in 25µl volume containing 100ng genomic DNA-, Primer (0.25 µM of each), dNTPs (200 µM of each), KCl (50 mM), Tris (10 mM), MgCl2 (1.5 mM) and *Taq* DNA polymerase (2.5 units) (Dweikat *et al.*, 1993). The detail of temperatures required for PCR reaction i.e. annealing (94°C), denaturation (60°C) and final primer extension (72°C) are given in Table 2. These PCR reactions were repeated twice for all primers to confirm the results of the amplified products. Amplified products were resolved on2% agarose gel. DNA ladder M-2000 (TAKARA, Co. China) was used to detect the band size of DNA amplicon of wheat varieties.

RESULTS AND DISCUSSION

The wheat varieties collected from different research institutes (Table 1) were subjected to molecular characterization using AS-PCR markers specific to LMW-GS. Seven markers for *Glu-A3* and ten for *Glu-B3* loci were used to determine the particular alleles. AS-PCR markers

Table 3. Frequency of alleles at *Glu-A3* and *Glu-B3* loci in bread wheat genotypes from Pakistan.

Locus	Allele	Allele size (bp)	Frequency	Relative Frequency (pi) = Freq./T. Ferq.	$(pi)^2$	Genetic Diversity (H)= $1 - \Sigma(pi)^2$
Glu-A3	*Glu A3b*	894	9	0.5294	0.0280	0.8337
	Glu-A3d	967	2	0.1176	0.0138	
	Glu-A3g	1345	6	0.3529	0.1245	
Total			17	0.9999	0.1663	
Glu-B3	*Glu-B3b*	1570	2	0.0760	0.0058	0.6971
	Glu-B3d	662	4	0.1538	0.0236	
	Glu-B3e	669	11	0.4230	0.1789	
	Glu-B3i	621	8	0.3076	0.0946	
Total			25	0.9604	0.3029	

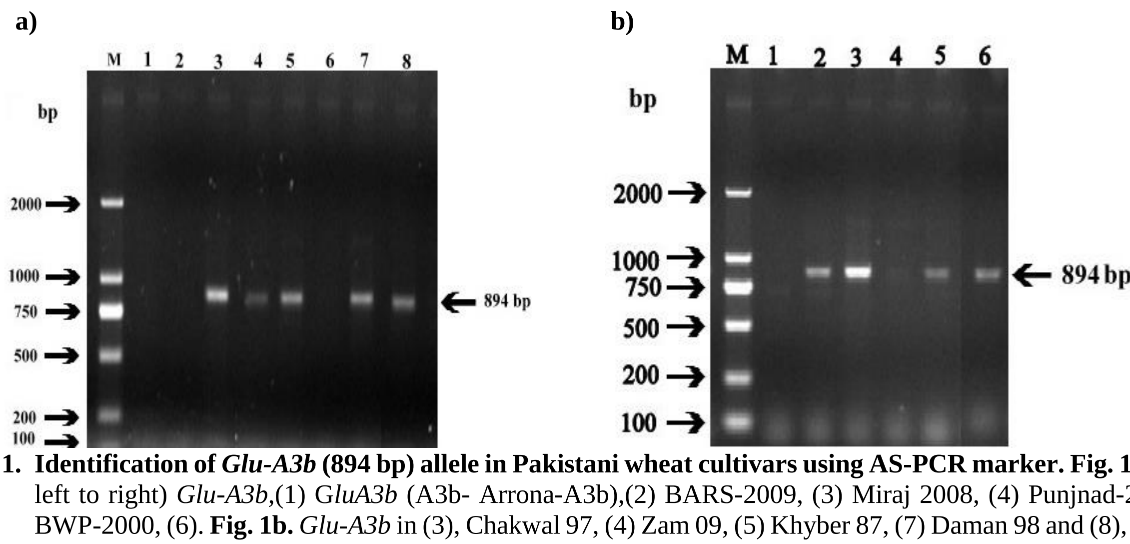


Figure 1. Identification of *Glu-A3b* (894 bp) allele in Pakistani wheat cultivars using AS-PCR marker. Fig. 1a. (From left to right) *Glu-A3b*,(1) G*luA3b* (A3b- Arrona-A3b),(2) BARS-2009, (3) Miraj 2008, (4) Punjnad-2001, (5) BWP-2000, (6). **Fig. 1b.** *Glu-A3b* in (3), Chakwal 97, (4) Zam 09, (5) Khyber 87, (7) Daman 98 and (8), Pirsabak 04.

amplified 03 alleles at *Glu-A3* locus and 04 alleles at *Glu-B3* locus (Table 3). At *Glu-A3* locus, 09 varieties possessed *Glu-A3b* allele (894 bp) i.e., BARS-2009, Miraj-2008, Punjnad-2001, Bahawalpur-2000, Chakwal-97, Zam-2009, Khyber-87, Daman-98 and Pirsabak-04 (Fig. 1a and 1b); 02 varieties amplified *Glu-A3d* allele (967 bp) i.e., Inqlab-91 and Faisalabad-2008, whereas 06 varieties contained *Glu-A3g* allele (1345 bp) viz., Fakhr-e-Sarhad, Seher-06, Bahawalpur-97, Bakhtawer-92, Tatara and Pasban (Fig. 2a,b); the remaining genotypes could not be amplified by any primers used in this study. The genetic diversity (H) of these 17 varieties was 0.83 (Table 3).

a)

b)

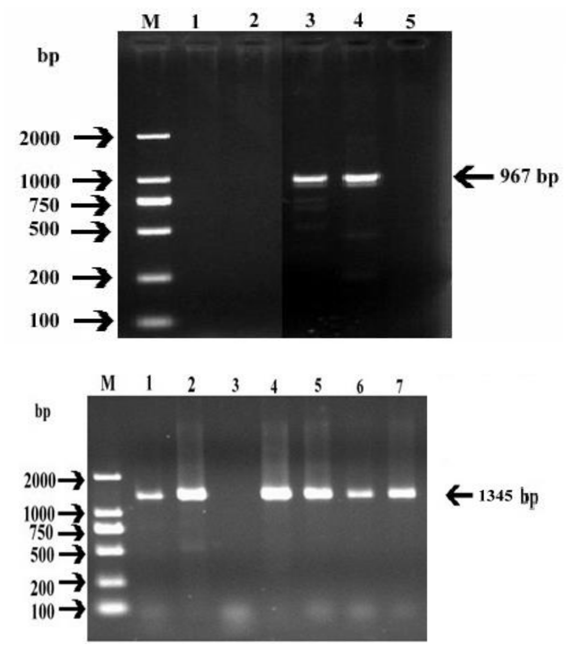


Figure 2. Identification of *Glu-A3d* (967 bp) and *Glu-A3g* (1345 bp) allele in Pakistani wheat cultivars. Fig. 2a. (From left to right) *Glu-A3d* allele in (3) Inqlab-91, (4) Faisalabad-2008. **Fig. 2b.** (From left to right) *Glu-A3g*allele in (1) Fakhr-e-Sarhad, (2) Seher-06, (4) Bahawalpur 97, (5) Bakhtawer 92, (6) Tatara, and (7), Pasban

At *Glu-B3* locus, 04 varieties namely BARS-2009, Miraj-2008, Punjnad-2001 and Inqlab-91 had Glu-*B3d* allele (662 bp), and the remaining 02 varieties viz., Chakwal-50 and GA-2002 possessed *Glu-B3b* allele (1570 bp), (Fig. 3a,b). Similarly, 08 varieties amplified *Glu-B3i* allele (621 bp), which included Bahawalpur-2000, Fakhr-e-Sarhad, Seher-2006, Pirsabak-05, Wafaq-2001, NARC-2009, Shafaq and Minthar-2003 (Fig. 4a) whereas 11 varieties had *Glu-B3e* allele (669 bp) i.e., Chakwal-97, Zam-09, Khyber-87, Daman-98, Pirsabak-04, Bakhtawer-92, Tatara, Pasban, Lasani-08, GA-2002 and Fareed-2006 (Fig. 4b). The average genetic diversity at *Glu-B3* was 0.69 (Table 3).

a)

b)

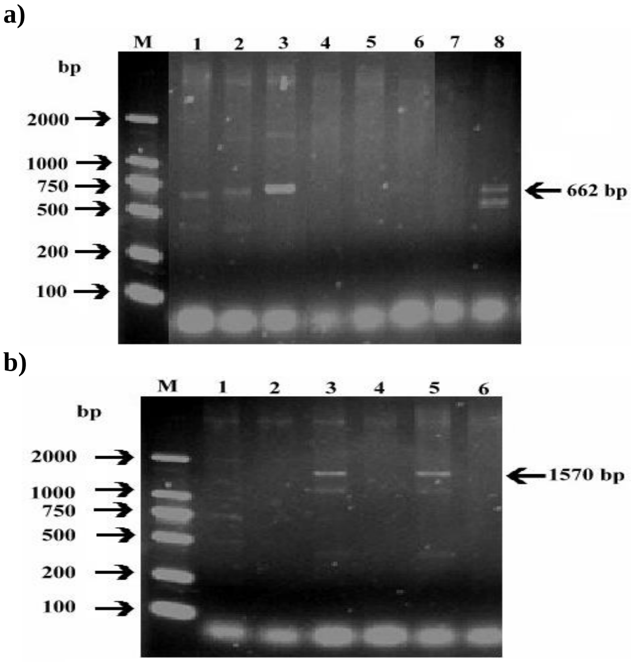


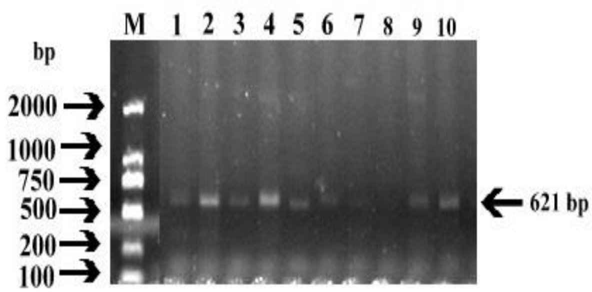
Figure 3. Identification of *Glu-B3d* (662 bp) and *Glu-B3b* (1570 bp) alleles in Pakistani wheat cultivars. (3a) (From left to right) *GluB3d* allele in (1) BARS-2009, (2) Miraj-08, (3) Punjnad-2001, (8) Inqilab-91. (**3b)** (From left to right) *Glu-B3b* allele in (3), Chakwal-50, (5) GA-2002.

Table 4. Quality characteristics of *Glu-A3* and *Glu-B3* alleles identified in some Pakistani wheat cultivars.

Allele	Functional characteristics	Reference
Glu-A3b	High sedimentation; better RWCN color b*	Hernandez *et al.* (2012); Jin *et al.* (2012)
Glu-A3c	High sedimentation	Jin *et al.* (2012)
Glu-A3d	High protein content; Sedimentation volume	Liu *et al.* (2005)
Glu-B3b	High Zeleny sedimentation volume; High quality parameters	Zhang *et al.* (2012); Jin *et al.* (2012)
Glu-B3d	High sedimentation; Superior mixograph properties; Strong gluten strength	Hernandez *et al.* (2012); Jin *et al.* (2012)
Glu-B3g	High sedimentation; Superior mixograph properties; Strong gluten strength	Hernandez *et al.* (2012); Jin *et al.* (2012); He *et al.* (2005)
Glu-B3h	High sedimentation	Hernandez *et al.* (2012); Jin *et al.* (2012)

The quality features of each LMW-GS have been mentioned in the Table 4. Allelic diversity at the *Glu-3* loci encoding LMW-GS has a remarkable effect on the dough elastic properties (Gupta *et al.,* 1994; However, difficulties have been reported to correctly identify the LMW-GS using SDS-PAGE (Impiglia *et al.,* 2005). The current advancement in the development of AS-PCR markers for *Glu-A3* (Wang *et al.,* 2010) and *Glu-B3* (Wang *et al.,* 2009) increased the efficiency, accuracy and lowered the cost for allelic characterization in wheat germplasm (Liu *et al.,* 2012). The study of LMW-GS is needed for exploring their relationship with dough properties and as a result their use in breeding. However, no markers were developed for the *Glu-D3* locus due to the very little variation among the alleles (Liu *et al.,* 2012). Moreover, the impact of *Glu-D3* on dough quality is small as compared with the *Glu-A3* and *Glu-B3* loci (Gupta *et al.,* 1991).

a)



(b)

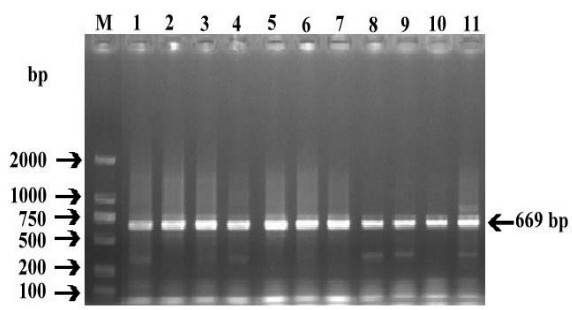


Figure 4. Identification of Glu-B3i (621 bp) and Glu-B3e (669 bp) alleles in Pakistani wheat cultivars. Fig. 4a. (From left to right) *Glu-B3i* allele in (1) Bahawalpur 2000, (2) Fakhr-e-Sarhad, (3) Seher-2006, (4) Pirsabak-05, (5) Wafaq-2001, (6) NARC-2009, (9) Shafaq, (10) Manthar-2003. **Fig. 4b**. (From left to right) *Glu-B3e* allele in (1) Chakwal-97, (2) Zam-09, (3) Khyber-87, (4) Daman-98, (5) Pirsabak-04, (6) Bakhtawar-92, (7) Tatara, (8) Pasban, (9) Lasani-08, (10) GA-2002, (11) Fareed-06.

In our study, 3 alleles at *Glu-A3* locus and 4 at *Glu-B3* locus were identified by AS-PCR markers (Table 3). Previously, the dominance of *Glu-A3c* allele (64.6%) was reported in 182 bread wheat cultivars developed in India (Ram *et al.,* 2011) and other studies also showed its presence in diverse wheat genotypes representing different regions (Wang *et al.,* 2010; Zhang *et al.,* 2004), which justify the higher frequency of this allele in Pakistani wheat cultivars. There are many reports indicating different allelic frequencies representing the *Glu-B3* locus in genotypes from different regions. Among Indian cultivars, frequency of *Glu-B3b* was highest (29.3%) followed by *Glu-B3j* (27.1%) and *Glu-B3h* (13.8%). Similarly, Branlard *et al.* (2001) reported *Glu-B3b* in 10.0% of cultivars in France, *Glu-B3g* in 49.0% and Glu-B3d in 3.5%. Other reports have also indicated the presence of *Glu-B3b* alleles in large numbers of cultivars (Wang *et al.,* 2009). *Glu-B3b* has been shown to have a positive effect on gluten strength.Later on, Jin *et al.* (2012) evaluated the properties of LMW-GS alleles for bread quality in isogenic lines, which improved our understanding for the functional aspects of LMW-GS alleles. Varietal candidate selection based on LMW-GS for bread-making quality trait is reliable and is considered as a crucial analysis of the germplasm.

***Conclusion*:** *Glu-A3* and *Glu-B3* alleles controlling low molecular weight glutenin sub units (LMW-GS) are of immediate concern due to their large effect on wheat processing qualities, especially gluten elasticity. These alleles are responsible for high sedimentation and protein contents, which made them an important source for wheat genetic improvement with special emphasis on quality. The selected wheat genotypes of Pakistan in the current study possessed better bread making alleles of *Glu-A3* and *Glu-B3* loci, which make them important candidates to exploit for grain yield and quality collectively.

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