# Molecular Characterization of Different
## *Triticum monococcum* ssp. *monococcum Glu-A1ᵐx* Alleles

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High-molecular-weight glutenin subunits (HMW-GSs) are important seed storage proteins associated with bread-making quality in common wheat (*Triticum aestivum* L., 2n = 6x = 42, AABBDD). Variation in the *Glu-A1x* locus in common wheat is scare. Diploid *Triticum monococcum* ssp. *monococcum* (2n = 2x = 14, AᵐAᵐ) is the first cultivated wheat. In the present study, allelic variations at the *Glu-A1ᵐx* locus were systematically investigated in 197 *T. monococcum* ssp. *monococcum* accessions. Out of the 8 detected *Glu-A1ᵐx* alleles, 5 were novel, including *Glu-A1ᵐ-b*, *Glu-A1ᵐ-c*, *Glu-A1ᵐ-d*, *Glu-A1ᵐ-g*, and *Glu-A1ᵐ-h*. This diversity is higher than that of common wheat. Compared with 1Ax1 and 1Ax2*, which are present in common wheat, these alleles contained three deletions/insertions as well as some single nucleotide polymorphism variations that might affect the elastic properties of wheat flour. New variations in *T. monococcum* probably occurred after the divergence between A and Aᵐ and are excluded in common wheat populations. These allelic variations could be used as novel resources to further improve wheat quality.

**Keywords:** genetic resources, glutenin, cultivated einkorn, quality breeding

## Introduction

Elasticity and extensibility of dough are strongly affected by allelic variation in the genes that code for high-molecular-weight glutenin subunits (HMW-GSs) and low-molecular-weight glutenin subunits (LMW-GSs) in both durum and bread wheats (Payne 1987; Gupta and Shepherd 1988; Ponga et al. 1990). HMW-GSs are components of the glutenin polymer and therefore play a key role in determining the unique visco-elastic properties of wheat dough (Payne et al. 1987). The number and composition of HMW-GSs account for the variation in bread-making quality of wheat flour. HMW-GSs are controlled by *Glu-1* loci, each locus consists of two tightly linked genes that encode one larger x-type and one smaller y-type subunit (Harberd et al. 1986). The molecular structure of HMW-GSs is composed of three distinct domains, including a central large repetitive domain flanked by short N- and C-terminal non-repetitive domains (Shewry et al. 1995). The

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N-terminal domains of the x-type subunits have 81–89 residues, whereas the C-terminal domains include 42 residues. The variable lengths of the repetitive domains are primarily responsible for the size differences of these genes (Shewry et al. 2002).

Only a small number of allelic variations in HMW-GSs exist in tetraploid wheat *Triticum turgidum* or common wheat. The 1Ay subunit encoded by the *y*-type gene at the *Glu-A1* locus has been deemed to be always silent in hexaploid wheat (Forde et al. 1985; Halford et al. 1989). However [a number of 1Ay alleles were found in diploid species of] *T. urartu* Tum. (AA, 2n = 2x = 14) and related species *T. monococcum* L. and *T. boeoticum* Boiss. (Hu et al. 2010, 2012), although the distribution of 1Ax alleles in these species has not yet been systematically investigated. The difference in genetic diversity of HMW-GSs between common wheat and its ancestral species is a result of an evolutionary bottleneck. These novel HMW-GS variations in ancestral species provide candidate genes for further wheat quality improvement.

*Triticum monococcum* ssp. *monococcum* (2n = 2x = 14, AᵐAᵐ) is the first cultivated wheat and is closely related to *T. urartu* (2n = 2x = 14, AᵘAᵘ), which is the A genome donor progenitor of hexaploid bread wheat (Dvorak et al. 1993). *Triticum monococcum* ssp. *monococcum* has high protein content and disease resistance for cultivated polyploid wheats (Lawrence et al. 1958; Fedak 1985; Tranquilli et al. 2002; Zaharieva and Monneveux 2014). Although the variability of the seed storage protein in this species has been characterized by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), only two and three 1Ax alleles of *Glu-A1ᵐx* were detected by Xu et al. (2009) and Alvarez et al. (2006), respectively.

In the present study, the allelic variations of *Glu-A1ᵐx* were systematically investigated using a large collection (197 accessions) of cultivated einkorn wheat (*T. monococcum* ssp. *monococcum*) by SDS-PAGE. Representatives of each *Glu-A1ᵐx* type were further analyzed using PCR amplification and sequencing. In addition, their evolutionary relationship with *T. urartu Glu-Aᵘ1* allelic variants and common wheat *Glu-A1x* subunits was analyzed.

## Materials and Methods

*Plant material and SDS-PAGE analysis*

In total, 197 accessions of *T. monococcum* ssp. *monococcum* were used in this study. All accessions were kindly provided by USDA-ARS germplasm bank (http://www.ars-grin. gov). These accessions were originally collected in 37 countries (Table S1*). The common wheat cultivars Chuanyu 12 (subunit 1Ax1, 7 + 8, 5 + 10), Longfumai 1 (1Ax2*, 7 + 8, 5 + 10), and Chinese Spring (null, 7+8, 2+12) were used as standards. Seed protein extraction and SDS-PAGE were conducted as described by Yan et al. (2002).

*Further details about the Electronic Supplementary Material (ESM) can be found at the end of the article.

*DNA extraction, PCR amplification and DNA sequencing analysis*

DNA isolation was carried out from young leaf tissue using the Plant Genomic DNA Kit (Tiangen, Beijing, China). A pair of primers (AxF: 5′-AGATGACTAAGCGGTTG-GTTC-3′ and AxR: 5′-CTGGCTGGCCAACAATGCGT-3′) were synthesized and used to amplify the complete coding sequence of the *Glu-A1x* gene according to D'Ovidio et al. (1995). Two new pairs of specific primers were designed based on the *Glu-A1-1b* sequence (NCBI ID: M22208) and *T. monococcum* HMW x-type gene sequence (NCBI ID: FJ44118) to amplify the two ends of the *Glu-A1ᵐx* gene. A pair of primers (P1: 5′-TGAC-TAAGCGGTTGGTTCTTTT-3′ and P2: 5′-GTACCCTGATTGCCCTTGTCCC-3′) were used to amplify fragment 1 (477 bp), including the signal peptide, N-terminal domain along with the beginning of the repetitive domain. Another pair of primers (P3: 5′-AATG-GCTGCAATCAGGGTACTA-3′) and P4: 5′-CTGGCTGGCCAACAATGCGTCGC-3′) were used to amplify fragment 2 (336 bp) including the end of the repetitive domain and C-terminal domain. PCR amplification was performed as described by D'Ovidio et al. (1995) with minor modification. The PCR products were separated on 1% agarose gels in 1× TBE buffer. Cloning and Sequencing of the expected fragments were conducted as described by Li et al. (2015).

*Data analysis*

Nucleotide sequence prediction was performed using DNAman 6.0.3 (Lynnon Biosoft, Quebec, Canada). The sequence alignments were carried out using the multiple sequence alignment software Clustal X 2.0 (Larkin et al. 2007). Alignments were further improved by visual examination and manual adjustment to maximize the similarities among the sequences. DNA analysis was conducted using DNAsp 5.0 (Librado and Rozas 2009). A neighbor-joining tree was constructed using the maximum composite likelihood method by MEGA 6.0 (Tamura et al. 2013). In the neighbor-joining (NJ) trees analysis, gaps were treated as missing data and bootstrap analysis was conducted with 1000 replicates.

## Results

*Variation of* Glu-A1ᵐx *alleles among 197 accessions detected by SDS-PAGE*

In the analyzed 197 accessions of *T. monococcum* ssp. *monococcum*, 8 *Glu-A1ᵐx* alleles were identified based on their electrophoretic mobilities in SDS-PAGE (Fig. 1a, Table S1) and named consecutively from *Glu-A1ᵐ-a* allele to *Glu-A1ᵐ-h* allele (Table S2). Out of the 8 alleles, *Glu-A1ᵐ-a*, *Glu-A1ᵐ-e*, and *Glu-A1ᵐ-f* were detected in 22 Spanish cultivated einkorn wheat (*T. monococcum* ssp. *monococcum*) by Alvarez et al. (2006). The frequency and distribution of the 8 alleles was highly variable, with the lowest for *Glu-A1ᵐ-h* (one accession, 0.5%) and the highest for *Glu-A1ᵐ-d* (132 accessions, 67%) (Table S2). *Glu-A1ᵐ-a*, which was found in two accessions, was silent, and the remainding alleles were active. The predominant allele *Glu-A1ᵐ-d* showed mobility faster than 1Ax2*.
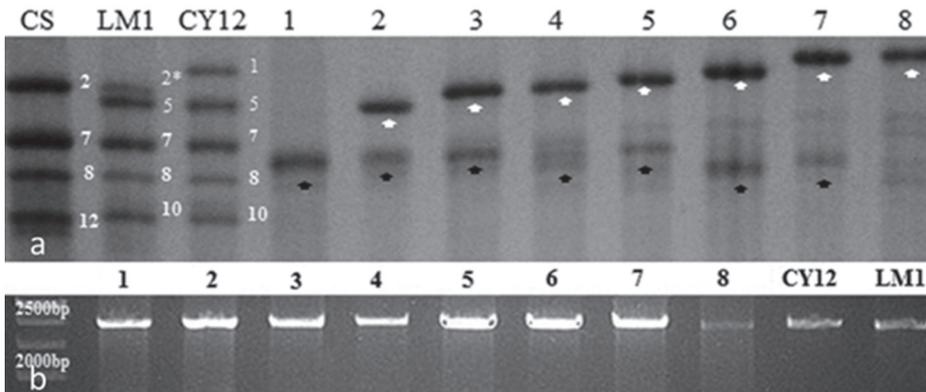
*Figure 1.* (a) SDS-PAGE of representative samples of the variability found for the *Glu-A1ᵐx* alleles Lanes; (b) PCR amplification of the complete coding region of *Glu-A1ᵐx* genes. 1. PI191146 *(Glu-A1ᵐ-a);* 2. PI355517 *(Glu-A1ᵐ-b);* 3. CItr13961 *(Glu-A1ᵐ-c);* 4. PI272558 *(Glu-A1ᵐ-d);* 5. PI272560 *(Glu-A1ᵐ-e);* 6. PI560726 *(Glu-A1ᵐ-f);* 7. PI355524 *(Glu-A1ᵐ-g);* and 8. PI355521 *(Glu-A1ᵐ-h).* The standards are CS, cv. Chinese Spring; CY12, cv. Chuanyu 12 *(Glu-A1-1a);* and LM1, cv. Longfumai 1 *(Glu-A1-1b). Glu-A1ᵐx* alleles are indicated by white arrows, whereas *Glu-A1y* alleles are marked by black arrows

### PCR analysis of the 8 Glu-A1ᵐx *alleles*

The *Glu-A1ᵐx* genes from 8 accessions that had the 8 HMW-GS alleles were amplified for their complete coding sequence of the *Glu-A1ᵐx* gene. Each analyzed line produced one amplicon that was about 2.4 kbp, but with slight differences among accessions (Fig. 1b). Two pairs of primers were further used to amplify both ends of the *Glu-A1ᵐx* gene. The first pair produced one amplicon (fragment 1), which included the signal peptide, the N-terminal domain, and the first 174 nucleotides of the repetitive domain. The second pair produced one amplicon (fragment 2), which included the last 210 nucleotides of the repetitive domain and the C-terminal domain. Sequencing analysis indicated that fragment 1 was 477 bp (Fig. S1), whereas fragment 2 was 336 bp (Fig. S2); both fragments were the same size in all sequences (Table S3). These combined sequences (fragment 1 + fragment 2) were deposited in the GenBank database (Table S3).

### DNA sequence comparison

DNA polymorphism was further evaluated using the concatenated sequence of fragments 1 and 2 (Table S4). Eighteen polymorphic sites were detected in the sequences, including 10 from fragment 1 and 8 from fragment 2 (Table S4). Of these mutations, 5 were synonymous, whereas the other 13 were non-synonymous, including 6 in the repetitive domain. The C-terminal domain (four mutations) had more variations than the N-terminal domain (two mutations; Table S4). Two statistics, $\pi$ (Nei 1987) and $\theta$ (Watterson 1975), were used to estimate nucleotide diversity (Table S4). Both values were similar for all sequences, which is consistent with a drift-mutation balance. However, the values for the

signal peptide and N-terminal domain were lower than in the other two domains, indicating lower diversity. Tajima's *D* values were not significant in any comparison, which is consistent with a neutral equilibrium (Table S4).

*Amino acid sequence comparison*

The deduced proteins were aligned with those of 1Ax1 and 1Ax2* subunits in wheat (Fig. S3). Besides single nucleotide polymorphisms (SNPs), there were differences in the presence of three deletion/insertions in the repetitive domain, one in fragment 1 (137–142 residues, Pro-Gly-Gln-Gly-Gln-Gln) and two in fragment 2 (6–10 residues; Gln-Gly-Gln-Gln-Glyl; 46–50 residues, Ser-Gly-Gln-Gly-Gln). The non-synonymous substitutions observed in all *T. monococcum* ssp. *monococcum* lines as well as comparison with the 1Ax1 and 1Ax2* subunits used as standards are shown in Table S5. Eight of these changes (five in positions 62, 63, 107, 152, and 153 of fragment 1; three in positions 4, 17, and 67 of fragment 2) were distinctive between common wheat and *T. monococcum* ssp. *monococcum* (Table S5). In fragment 1, for the residues 62, 63, 107, 152, and 153, all alleles showed the changes Gly62 → Val, Gly63 → Val, Arg107 → Lys, Ser152 → Pro, Gly153 → Glu, respectively. For residues 130, 131, eight alleles had the changes Arg130 → Gln, Pro131 → Ser, respectively, and one had no change. In fragment 2, for residues 4, 17, and 67, all alleles showed the changes Ser4 → Pro, Gln17 → Arg, Ser67 → Pro, respectively. The other changes appeared only in some alleles of *T. monococcum* ssp. *monococcum* (Table S5), with most alleles similar to the wheat subunits used as standards.

*Phylogenetic analysis of the* Glu-A1x *genes*

The 8 *Glu-A1ᵐx* sequences in this study, the *Glu-A1x* genes *Glu-A1-1af* to *Glu-A1-1aq* (JX102635 to JX102646) of 12 *Glu-Aᵘ1* allelic variants detected in *T. urartu* as well as the three main alleles *Glu-A1-1a* (X61009), *Glu-A1-1b* (M22208)), and *Glu-A1-1c* (AF145590) that were previously obtained from the A genome in tetraploid and hexaploid wheats were used to construct a neighbor-joining tree based on the maximum composite likelihood method (Fig. S4). Concatenated sequences of fragments 1 and 2 were used for all accessions. The *Glu-A1x* genes of 12 *Glu-Aᵘ1* allelic variants of *T. urartu* and three *Glu-A1x* alleles of tetraploid and hexaploid wheats were grouped in one separate cluster, which were divergent from those of *T. monococcum*. *Triticum monococcum* alleles were grouped into two clusters. One contained only the *Glu-A1ᵐ-g* allele, which was detected in three accessions from Asia Minor (1) and Germany (2). The other cluster included the other 7 alleles.

## Discussion

A high priority for practical wheat improvement worldwide is to enrich the cultivated gene pools by incorporating favorable alleles, genes, or gene complexes from a donor species (Feuillet et al. 2008). A key step is to characterize the genes of interest in alien

species before they are incorporated into common wheat. Variation in the *Glu-A1x* locus in common wheat is scare, and most modern wheat varieties have only one of the three main alleles at this locus (*Glu-A1a*, *Glu-A1b*, and *Glu-A1c*), which encoded 1Ax1, 1Ax2* and 1Ax null, respectively. These subunits were derived from limited *T. urartu* genotypes that participated in the formation of common wheat (Alvarez et al. 2013). Thus, the identification of new *Glu-A1x* alleles and their incorporation into cultivated wheat are important for quality improvement. Three *Glu-A1ᵐx* alleles were detected by analyzing 22 accessions of Spanish cultivated einkorn wheat (*T. monococcum* ssp. *monococcum*) (Alvarez et al. 2006). Here, eight *Glu-A1ᵐx* alleles were detected in 197 accessions of *T. monococcum* ssp. *monococcum*. Out of the 8 alleles, 5 were not previously reported.

It was reported that the estimated divergence time of A and Aᵐ genomes was 1.14 to 1.53 million years ago (Marcussen et al. 2014). *Triticum monococcum* ssp. *monococcum* would have produced new variations during the long divergence history. New variations in *T. monococcum* occurred after the divergence between A and Aᵐ. Therefore, these variations do not occur in common wheat populations, which are observed in the gene clusters (Fig. S4). New variations in *T. monococcum* after the divergence between A and Aᵐ is excluded in current common wheat populations, because this species did not participate in the origination and evolution of common wheat, consistent with the gene clusters (Fig. S4). The *Glu-A1x* genes of 12 *Glu-Aᵘ1* allelic variants previously detected in *T. urartu* were analyzed using PCR amplification and sequencing by Alvarez et al. (2013). The sequences of eight *Glu-A1ᵐx* alleles detected in this study were different from those of 12 *Glu-Aᵘ1* allelic variants (Fig. S4). Therefore, these allelic variations could be used as novel resources to further improve wheat quality.

Seven out of eight *Glu-A1ᵐx* alleles were active and all had the four cysteine residues that are usually present in the wheat x-type subunit. Cysteine residues provide sites for the formation of inter-chain disulphide bonds that play a significant role in determining the size and properties of the large glutenin polymers. Thus, cysteine residues affect dough strength (Shewry et al. 2001, 2002). Compared to the 1Ax1 and 1Ax2* subunits in common wheat, some variations appeared in the amino- and carboxyl-sequences, including three deletions/insertions in the repetitive domain as well as some SNP variations. Some variations might affect the elastic properties, such as Pro → Ser at position of 131 and Ser → Pro at 152 in fragment 1 as well as Ser → Pro at 4, Pro → Leu at 64, and Ser → Pro at 67 in fragment 2 (Table S5).

Besides the subunit composition, bread-making qualities are largely dependent on the number of HMW-GSs (Shewry et al. 2002). The *Glu-A1* locus consists of two tightly linked genes that encode one larger x-type and one smaller y-type subunit, i.e., *Glu-A1x* and *Glu-A1y*. The 1Ay subunit is absent from common wheat because of gene silencing (Forde et al. 1985; D'Ovidio et al. 1996; Xu et al. 2009; Hu et al. 2012). However, the 1Ay subunit is expressed in wild relatives of wheat, such as *T. urartu*, *T. monococcum* ssp. *aegilopoides*, and *T. monococcum* ssp. *monococcum* (Jiang et al. 2009; Hu et al. 2010; Gutiérrez et al. 2011; Hu et al. 2012). In this study, 1Ay subunit is expressed in 7 accessions of *T. monococcum* ssp. *monococcum*, however, the 1Ay protein was seen as a series of less prominent subunit bands with one dominating band in some accessions (Fig. 1a),

as reported by Waines and Payne (1987) and Ciaffi et al. (1998). It was suggested that the multiplicity of minor bands are also the products of the same gene and arose by proteolytic activity, either *in vitro* during endosperm development or during protein extraction (Waines and Payne 1987). Those accessions with expressed 1Ax and 1Ay subunits are potential candidate for wheat quality breeding. The replacement of the silenced 1Ay in bread wheat by an expressed 1Ay may further increase the number of HMW-GSs and thus improve flour processing quality, as shown by some reports (Ciaffi et al. 1991; Rogers et al. 1997; Alvarez et al. 2009). Therefore, during the incorporation of novel *Glu-A1ᵐx* genes from *T. monococcum* ssp. *monococcum* into common wheat, an accession with a novel *Glu-A1ᵐx* and an active *Glu-A1y* should be preferentially used. This kind of accession does exist in *T. monococcum* (Fig. 1a) and related species, such as *T. boeoticum* ssp. *thaoudar* and *T. urartu* (Rogers et al. 1997; Alvarez et al. 2013). However, we focused on the *Glu-A1ᵐx* variations in this study because of the scarcity of information on the *Glu-A1ᵐx* locus in contrast with the large number of reports on *Glu-A1y*.

## Acknowledgements

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## Electronic Supplementary Material (ESM)

Electronic Supplementary Material (ESM) associated with this article can be found at the website of CRC at http://www.akademiai.com/content/120427/

Electronic Supplementary *Table S1*. Distribution of the *Glu-A1ᵐx* alleles in the evaluated *T. monococcum* ssp. *monococcum* accessions

Electronic Supplementary *Table S2*. Allele frequencies for the *Glu-A1ᵐx* locus in the evaluated *T. monococcum* ssp. *monococcum* accessions

Electronic Supplementary *Table S3*. Origin and sequence characteristics of the evaluated *Glu-A1ᵐx* alleles

Electronic Supplementary *Table S4*. DNA polymorphism for the 8 evaluated *Glu-A1ᵐx* alleles

Electronic Supplementary *Table S5*. Amino acid (aa) comparison among common wheat (subunits 1Ax1 and 1Ax2*) and *T. monococcum* ssp. *monococcum*, and changes within *T. monococcum* ssp. *monococcum* lines

Electronic Supplementary *Figure S1*. Comparison of the nucleotide sequence of the fragment 1 of the *Glu-A1ᵐx* alleles found in the different lines of *T. monococcum* ssp. *monococcum* with the *Glu-A1-1a* allele (subunit 1Ax1) and the *Glu-A1-1b* allele (subunit 1Ax2*) of wheat

Electronic Supplementary *Figure S2*. Comparison of the nucleotide sequence of the fragment 2 of the *Glu-A1ᵐx* alleles found in the different lines of *T. monococcum* ssp. *monococcum* with the *Glu-A1-1a* allele (subunit 1Ax1) and the *Glu-A1-1b* allele (subunit 1Ax2*) of wheat

Electronic Supplementary *Figure S3*. Comparison of the amino acid sequence of fragment 1 (a) and fragment 2 (b) of the *Glu-A1ᵐx* alleles from *T. monococcum* ssp. *monococcum* with the *Glu-A1-1a* allele (subunit 1Ax1) and the *Glu-A1-1b* allele (subunit 1Ax2*) of wheat

Electronic Supplementary *Figure S4*. Neighbor-joining tree based on the maximum composite likelihood method of concatenated sequences (fragment 1 + fragment 2) of *T. monococcum* ssp. *monococcum*, *T. urartu* and wheat. Numbers in nodes indicate bootstrap estimates from 1,000 replications. The number of accessions carrying each allele is indicated between brackets