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Molecular Characterization of Seven Novel *Glu-A1^{m}x* Alleles from *Triticum monococcum* ssp. *monococcum*

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Seven *Glu-A1^m* allelic variants of the *Glu-A1^{m}x* genes in *Triticum monococcum* ssp. *monococcum*, designated as *1Ax2.1^a*, *1Ax2.1^b*, *1Ax2.1^c*, *1Ax2.1^d*, *1Ax2.1^e*, *1Ax2.1^f*, and *1Ax2.1^g* were characterized. Their authenticity was confirmed by successful expression of the coding regions in *E. coli*, and except for the *1Ax2.1^a* with the presence of internal stop codons at position of 313 aa, all correspond to the subunit in seeds. However, all the active six genes had a same DNA size although their encoding subunits showed different molecular weight. Our study indicated that amino acid residue substitutions rather than previously frequently reported insertions/deletions played an important role on the subunit evolution of these *Glu-A1^{m}x* alleles. Since variation in the *Glu-A1x* locus in common wheat is rare, these novel genes at the *Glu-A1^{m}x* can be used as candidate genes for further wheat quality improvement.

Keywords: cultivated einkorn, glutenin, gene expression, quality breeding

Abbreviations: aa, amino acid(s); bp, base pair(s); BLAST, basic local alignment search tool; DTT, DL-Dithiothreitol; HMW-GS, high molecular weight glutenin subunit; IPTG, isopropyl β-Δ-thiogalactopyranoside; NCBI, National Center for Biotechnology Information; ORF, open reading frame; PCR, polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SNP, single nucleotide polymorphism.

Introduction

In common wheat (*Triticum aestivum* L., AABBDD, $2n = 6x = 42$), HMW-GSs (high-molecular-weight glutenins) are an important group of seed storage proteins affecting the processing quality (Payne 1987; Shewry et al. 1995, 2002, 2003). HMW-GSs are encoded by three *Glu-1* loci (*Glu-A1*, *Glu-B1,* and *Glu-D1*), located on the long arms of homoeologous group 1 chromosomes, which encode an x-type subunit with higher molecular weight and a y-type subunit with lower molecular weight, respectively. The x- and y-type subunits have similar primary structures, consisting of a signal peptide, a non-repetitive N-terminal domain, a repetitive central domain, and a non-repetitive C-terminal domain

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(Shewry et al. 1995). The size differences of HMW-GS are mainly resulted from repetitive domain variations (Shewry et al. 1992).

The constitution of HMW-GSs affects the processing quality of wheat flour. For examples, 1Dx5 + 1Dy10 and 1Ax1 are positively related to good bread-making quality (Altpeter et al. 1996; Barro et al. 1997; Shewry et al. 2006). Searching and characterizing new HMW-GSs genes are valuable further improving wheat quality (Wang et al. 2012; Alvarez et al. 2013). *T. monococcum* ssp. *monococcum* was considered as a valuable resource for wheat bread-making quality improvement (Tranquilli et al. 2002). This species has abundant allele variations of HMW-GSs (Waines and Payne 1987; Ciaffi et al. 1998; Ma et al. 2007; Hu et al. 2012). In our previous study, eight different variants of the *Glu-A1^{m}x* subunits were detected in 197 accessions of this species (Li et al. 2016). Among them, the gene encoding subunit 1Ax2.1 from the accession CItr13961 has been characterized (http://www.ncbi.nlm.nih.gov/nuccore/HQ834308). Here, we characterized the genes encoding the rest seven subunits and analyzed their evolutionary relationships with other *Glu-A1x* alleles present in diploid, tetraploid, and hexaploid wheats.

Materials and Methods

Plant material

T. monococcum ssp. *monococcum* accessions PI191146, PI355517, PI272558, PI272560, PI560726, PI355524, and PI355521 were used in this study. They have different *Glu-A1^{m}x* subunits (Li et al. 2016). These accessions were kindly provided by USDA-ARS germplasm Bank (http://www.ars-grin.gov).

Molecular cloning and sequencing

DNA isolation was carried out from young leaf tissue using the Plant Genomic DNA Kit (TIANGEN, Beijing, China). To amplify the complete coding sequence of the *Glu-A1x* gene, a pair of primers, P1 (5'-AGATGACTAAGCGGTTGGTTC-3') and P2 (5'-CTGGCTGGCCAACAATGCGT-3') were synthesized according to D'Ovidio et al. (1995). PCR (polymerase chain reaction) amplification was performed using a high fidelity DNA polymerase LA Taq polymerase (TaKaRa, Dalian, China) in GC buffer for GC-rich templates. The reaction volume of 50 µl containing 5 µl of 10×LA PCR buffer, 0.2 mM of dNTPs, 1 µM of each primer and 2.5 U of LA Taq polymerase. The PCR cycling parameters were 94 °C for 5 min, followed by one step at 62 °C for 2 min then for 30 cycles at 94 °C for 30 s, 62 °C for 1 min and 72 °C for 2 min and 30 s, final incubation at 72 °C for 10 min. The PCR products were separated on 1.5% agarose gels and all DNA fragments were recovered, purified, and ligated into the pMD19-T vector (TaKaRa, Dalian, China). The ligated mixtures were transformed into *E. coli* DH10B competent cells. DNA sequencing was performed by Sangon Biotechnology Company (Shanghai, China). Both cloning and sequencing were repeated at least three times to exclude errors.

Expression of the cloned Glu-A1^{m}x *alleles in* E. coli

To express mature HMW-GS proteins, a pair of primers AxB1F (5′-CTCATCCATATG-GAAGGTGAGGCCTCTGGGCA-3′) and AxB1R (5′-GGCAATGAATTCGCTGGC-CAACAATGCGTCGC-3′) were designed to amplify the mature *Glu-A1^{m}x* ORF (open reading frame) without the signal peptide region sequence while restriction enzyme sites for *NdeI* and *EcoRI* were introduced into the ORF for subsequent cloning. PCR conditions for amplifying the mutant ORF were identical to those described above except that the template was plasmid DNA purified from the identified clones. The resulting fragments were cloned into the pET-30a expression vector (Novagen). The recombinant constructs were transformed into the *E. coli* strain BL21 (DE3) pLysS. Bacterial expression was induced with 1 mM IPTG (isopropyl β-Δ-thiogalactopyranoside) for 3–5 h. The expressed proteins were purified by extraction with 50% (v/v) propanol containing 2% (w/v) DTT (DL-Dithiothreitol) and then separated by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) (Wan et al. 2002). The common wheat cultivar Chuanyu 12 (subunit 1Ax1, 7 + 8, 5 + 10) was used as standards.

Sequence alignments and phylogenetic analysis

The prediction of nucleotide sequences and conversion to amino acids were performed using the DNAMAN software (Version6.0.3, Lynnon Biosoft, Canada). Sequence similarity searching was performed using NCBI (the National Center for Biotechnology information) standard nucleotide BLAST (basic local alignment search tool) program against the GenBank nucleotide database (http://www.ncbi.nlm.nih.gov/BLAST). Multiple sequence alignments of nucleotide and the deduced amino acid sequences were completed by ClustalX2.0 (Larkin et al. 2007). Alignment was further improved by visual examination and manual adjustment with the aim of maximizing the similarities among the sequences of central repetitive regions. The deduced complete amino acid sequences were used for phylogenetic analysis. NJ (Neighbor-joining) tree was constructed by the software MEGA6.0 (Tamura et al. 2013). In the NJ trees analysis, the gaps were treated as missing data and the boot-strap analysis was conducted with 1000 replicates.

Results

Molecular characterization of Glu-A1^{m}x *alleles*

The *Glu-A1^{m}x* genes from seven accessions were amplified for their complete coding sequences and produced amplicons with similar size (Fig. S1*). They had a sequence length of 2430 bp, same as the previously reported gene *1Ax2.1* from *T. monococcum* ssp. *monococcum* (HQ834308) but shorter than that of *1Ax1, 1Ax1.1*, and *1Ax2** from common wheat. These genes were designated as *1Ax2.1^{a}, 1Ax2.1^{b}, 1Ax2.1^{c}, 1Ax2.1^{d}, 1Ax2.1^{e}, 1Ax2.1^{f},* and *1Ax2.1^{g}* (Table S1). Except that *1Ax2.1^{a}* was silent because of the presence

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

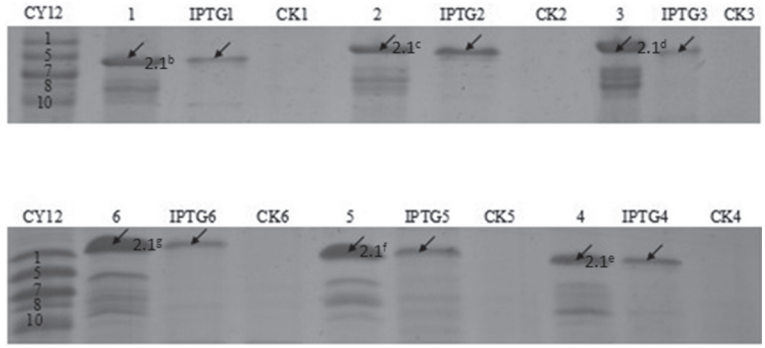

Figure 1. Bacterial expression of the cloned *Glu-A1^{m}x* alleles in *E. coli* BL21 (DE3). Lanes 1–6 show the 1Ax subunits (arrows) from seeds, corresponding IPTG1-6 are the expression of mature protein detected in the IPTG-induced bacterial cells and CK1-6 are not induced by IPTG. The standard is CY12, cv. Chuanyu 12 (*Glu-A1-1a*)

of internal stop codons at position of 313 aa (amino acid), the rest six active *Glu-A1^{m}x* alleles were successfully expressed in *E. coli* (Fig. 1).

Amino acid sequence comparison of Glu-A1^{m}x *alleles*

These x-type subunits had similar structural characteristics, including a signal peptide with 21 aa, a conserved N-terminal domain with 86 aa, a central repetitive domain with 660 aa and a conserved C-terminal domain with 42 aa. Four cysteine residues were present, including three at the N-terminal domain and one at the C-terminal domain (Table S1). The deduced proteins of *Glu-A1^{m}x* alleles were aligned with those of 1Ax1 and 1Ax2* subunits in wheat (Fig. S2). Compared with 1Ax1 subunit, seven *Glu-A1^{m}x* alleles had six Indels and 58 SNPs (single nucleotide polymorphism). Compared with 1Ax2* subunit, seven *Glu-A1^{m}x* alleles had four Indels and 58 SNPs.

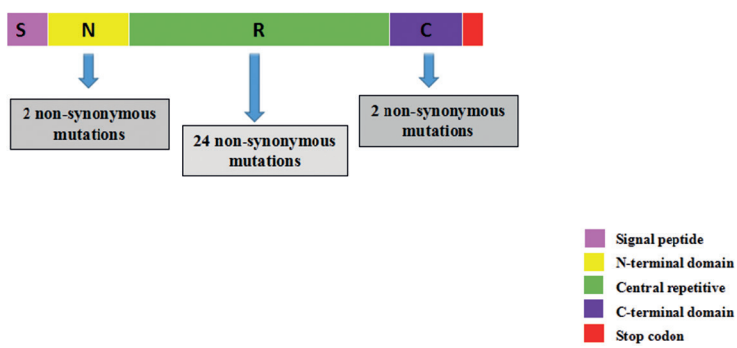

Figure 2. The distribution of 28 non-synonymous mutations resulted from 35 non-synonymous SNPs in the coding sequences of the seven *Glu-A1^{m}x* alleles, with 2 at the N-terminal, 24 at the central repetitive, and 2 at the C-terminal domains

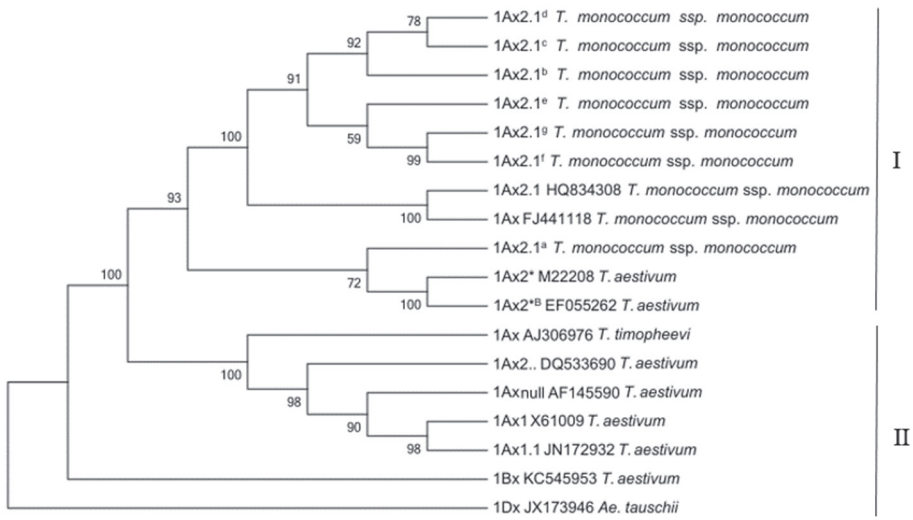

Figure 3. Neighbor-joining tree based on based on the maximum composite likelihood method of complete amino acid sequences deduced from 18 x-type alleles including 14 active *Glu-A1-1* alleles, 2 pseudogenes 1Axnull and 1Ax2.1^{a}, and 2 other HMW-GS genes (outgroups) reported. Numbers in nodes indicate bootstrap estimates from 1,000 replications

There were 46 SNPs without Indels among the coding sequences of the seven *Glu-A1^{m}x* alleles, including 28 (61%) transitions (A–G and C–T) and 18 transversions (A–T, C–G, A–C and G–T). Out of the 46 SNPs, 35 were of non-synonymous SNPs that produced 28 amino acid residue substitutions, with 2 at the N-terminal, 24 at the central repetitive, and 2 at the C-terminal domains (Fig. 2, Table S2).

Phylogenetic analysis

To further investigate the evolution of the *Glu-A1^{m}x* alleles, a NJ tree was constructed using 18 *1Ax* alleles with 1Bx7 and 1Dx2 as outgroups (Fig. 3). The results showed that *1Ax* alleles were clustered together and separated from the clades of *1Bx* and *1Dx* alleles. In the clade of *1Ax* alleles, there were clearly two subclades with a very high bootstrap value of 100 (Fig. 3). The subgroup I included all the *Glu-A1^{m}x* alleles from *T. monococcum* ssp. *monococcum*, suggesting their close phylogenetic relationship. The subgroup II included *1Ax* genes from *T. timopheevi* and *1Ax1*, *1Ax2**, *1Ax2*^{B}*, *1Axnull*, *1Ax1.1*, and *1Ax2..* from *T. aestivum* (Fig. 3).

Discussion

A lot of HMW-GS genes from wheat and its wild relatives have been isolated and characterized (e.g. Anderson and Greene 1989; Shewry et al. 1995; Yan et al. 2002; Liu et al. 2008; Dai et al. 2013; Jiang et al. 2014). These studies have demonstrated that the differ-

ence of molecular weight among HMW-GSs is mainly resulted from variation in the number of repeat motifs in the repetitive domain. However, all the analyzed seven genes had a same DNA size although their encoding subunits showed different molecular weight (Li et al. 2016). Our investigated seven genes had 46 SNPs leading to 28 non-synonymous mutations. Therefore, these non-synonymous mutations are the reason for the variation of subunit size. Our study indicated that amino acid residue substitutions rather than insertions/deletions played an important role on the subunit evolution of *Glu-A1^{m}x* alleles.

HMW-GSs are critical in determining the composition of wheat gluten and dough elasticity, which promote the formation of larger glutenin polymers (Shewry et al. 1995). The number and composition of HMW-GSs have been reported to account for the variation in bread-making quality of wheat flour. Variation in the *Glu-A1x* locus in common wheat is rare, and most wheat varieties have only one of the three main alleles *1Ax1*, *1Ax2** and *1Axnull* at this locus. 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. This study provided seven novel genes at the *Glu-A1^{m}x* (Fig. 3). Except that 1Ax2.1^a was close to 1Ax2* subunit in wheat, the rest six *Glu-A1^{m}x* alleles in this study were separated from those genes in wheat (Fig. 3). These *Glu-A1^{m}x* alleles can be used as candidate genes for further wheat quality improvement.

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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*. Comparison of properties of the 1Ax subunits in *T. monococcum* ssp. *monococcum* with those in *T. aestivum*

Electronic Supplementary *Table S2*. The mutation sites and information of 28 non-synonymous mutations in the *Glu-A1^{m}x* alleles

Electronic Supplementary *Figure S1*. PCR amplification of *Glu-A1^{m}x* alleles. Lane M is DNA marker and lanes 1–7 show the PCR bands from seven *Triticum monococcum* ssp. *monococcum* accessions

Electronic Supplementary *Figure S2*. Comparison of the deduced amino acid sequences of the *Glu-A1^{m}x* alleles from *T. monococcum* ssp. *monococcum* with the *Glu-A1-1a* allele (subunit 1Ax1) and the *Glu-A1-1b* allele (subunit 1Ax2*) of wheat