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Sequence Analysis of α-gliadin Genes from *Aegilops tauschii* Native to China

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Abstract: *Aegilops tauschii* was a D-genome progenitor for cultivated wheat (*Triticum aestivum* L). The accessions of *Ae. tauschii* native to China contained novel agronomically important traits including unique seed storage proteins for modern wheat improvement. Total 19 α-gliadin gene sequences were isolated from *Ae. tauschii* accession from Henan province. Five of 19 sequences contained in-frame stop condons and were predicted to be pseudogenes, suggesting the high variation of gliadin genes in *Ae. tauschii* genome. The Open Reading Frame (ORF) lengths of these sequences encoded 281-303 residues, with the repetitive polyglutamine from 17-28 residues. There are two α-gliadin sequences present either 5 or 7 cysteine residues, which possibly related to high quality. Four peptides of T cell stimulatory epitopes in Celiac Disease (CD) patients were distributed in most *Ae. tauschii* α-gliadin gene sequences. In comparison with the reported α-gliadin sequences in wheat ancestral species, the sequences of *Ae. tauschii* displayed high haplotypes diversity and significant deviation from a neutral distribution, indicative of the fast evolution of α-gliadin genes in *Ae. tauschii* species.

Key words: *Aegilops tauschii*, α-gliadin, evolutionary analysis, wheat breeding

INTRODUCTION

Common wheat (*Triticum aestivum* L.) is an allohexaploid species, which originated from natural hybridization between tetraploid wheat (*Triticum turgidum* L.) and *Aegilops tauschii* Coss (McFadden and Sears, 1944). *Aegilops tauschii*, a wild relative of wheat, is the D-genome progenitor of hexaploid wheat (van Slageren, 1994). Recently, the synthesis allopolyploidy wheat (AABBDD) was also confirmed to be great approach to transfer the novel genes including some disease and pest resistance genes from *Ae. tauschii* germplasm to common wheat (Kerber, 1987; Ma *et al*., 1995; Mujeeb-Kazi *et al*., 1996; Zhu *et al*., 2005; Yang *et al*., 2009). It is thus to explore the natural variation in *Ae. tauschii* populations which offers potential for improving modern varieties of common wheat (Mizuno *et al*., 2010). Since the D genome of *Ae. tauschii* is considered to be the main contributor to bread-making properties of common wheat, the seed storage protein including HMW-glutenin (Yan *et al*., 2003; Johal *et al*., 2004), LMW-GS (Zhao *et al*., 2008) and ω-gliadins (Hsia and Anderson, 2001; Hassani *et al*., 2009), γ-gliadin (Qi *et al*., 2009) from the *Ae. tauschii* species have been identified. About 40 partial α-gliadin genes/pseudogenes sequences from *Ae. tauschii* have currently been registered in GenBank (van Herpen *et al*., 2006). However, the genetic divergence of *Ae. tauschii* α-gliadin genes and corresponding relationships of gliadin genes between *Ae. tauschii* and common wheat were not clear.

The distribution area of *Ae. tauschii* expanded from in central Eurasia, spreading from Turkey to western China (Lubbers *et al*., 1991; Dvorak *et al*., 1998). The studies indicated that *Ae. tauschii* accessions native to China displayed unique genetic characters including the rust and preharvest sprouting resistances (Liu *et al*., 1998, 2002, 2010). In the present study, we identified 19 complete gliadin sequences from *Ae. tauschii* accessions native to China using gene specific PCR amplification, and compared between the α-gliadin genes of common wheat retrieved from GenBank. The studies might shed light on better understanding the origin and evolution of α-gliadin gene family in bread wheat, and provide novel gene resources for wheat quality improvement.

MATERIALS AND METHODS

Plant materials: Ae. tauschii accession 82 were collected from Henan province and kindly provided by Prof. Huaren Jiang, Sichuan Agricultural University, China. Plants were grown in pots at the School of Life Science and Technology, University of Electronic Science and Technology of China, during winter season of 2009.

DNA preparation and PCR amplification: Genomic DNA was isolated from 1-2 g leaves of single plant about 4-6 weeks old, as described by Yang *et al*. (2006). Based on the published α-gliadin gene sequences, a pair of primers P1 (5'-CACTTGTAAGTAGTGCCACCA-3') and P2 (5'-TTCCCATGTTTGAACTAGTATAGGTCG-3') were designed for amplifying complete sequence of α-

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gliadin genes, in which primer P1 located at 200bp upstream of the translation start codon, whereas primer P2 located about 100bp downstream from the stop codon.

PCR was carried out using iCycle thermal cycler (Bio Rad, Hercules, CA). Each PCR reaction (100) contained 300ng template, 0.2 mM of each dNTPs, 1 of each of two primers, 10 10 CR buffer, and 5U high-fidelity Ex Taq DNA polymerase (Takara, Japan). The PCR programmed at 2 min at 95 , followed by 35 cycles of 94 for 1min, 60 1min and 72 for 2 min 30 sec. After the amplification, the final extension was kept for 10min at 72ºC. The PCR product was analyzed on 1% agarose gels.

Cloning, sequencing and comparative analyses of α-gliadin genes: The target PCR products were purified from agarose gel using QIAquick Gel Extraction Kit (QIAGEN). The fragment was ligated into a pGEMT plasmid vector (Promega, Madison, Wis.), and used to transform competent cell of *Escherichi coli* DH5α strain. The positive clones were sequenced on an automatic DNA sequencer (TaKaRa Biotech). The nucleotide sequences based on two identically independent sequences of each clone were assembled, and the sequence alignment among different α-gliadin alleles was carried out using the ClustalW program (Thompson *et al*., 1994). PlantCARE and PLACE Databases were used to determine plant cis-acting regulatory elements (Higo *et al*., 1999; Lescot *et al*., 2002). The reliabilities of each branch point were assessed by the analysis of 1,000 bootstrap replicates. The DnaSP version 4.50.3 software package was used to complete the sequences diversity analyses.

RESULTS

The nucleotide sequences of α-gliadin genes: By using the *Ae. tauschii* genomic DNA as templates, the PCR primers, P1/P2, were used to amplify the complete sequences of α-gliadin genes. The PCR give rise to the product of about 1000-1200bp, and total 19 unique clones were sequenced, and all sequences are deposited into Genbank under the accession number of HM188546 to HM188564. The nucleotide comparison of the entire sequence showed a high degree of homology with other α-gliadin sequences. Sequence prediction indicated that 14 of the 19 sequences include complete open reading frames (ORF) of genes, 216-217bp 5' upstream sequence, and 135-152bp of 3' untranslated regions.

Based on the alignment of promoter region of the 19 *Ae. tauschii* α-gliadin genes, the best matching subsequences to the TATA-box motif, several CAAT-box binding transcription factor and an Skn-1-like motif were identified (Fig. 1). The Skn-1 motif was found in a rice glutelin gene promoter (Washida *et al*., 1999), and was recently identified on several grass seed protein promoters (Fauteux and Str vik, 2009). Three CAAT-box and TATA box were conserved among the 19 sequences, however, the Skn-1 motif appeared SNP sites in 5 sequences. Therefore, it was showed that the conserved motifs enriched in the promoters of *Ae. tauschii* α-gliadin gene sequences.

Comparison of α-gliadin sequences from wheat ancestral species: Comprising the α-gliadin genes from *T. monococum*, *Ae. speltoides* and *Ae. tauschii* with *T. aestivum*, a phylogenetic tree was developed with NJ and MP analyses using the MEGA4 (Tamura *et al*., 2007) with 1,000 iterations. Each 2 partial sequences from *T. monococum* (DQ002570, DQ002571), *Ae. speltoides* (DQ002585, DQ002586) and *Ae. tauschii* (DQ002590, DQ002591) referred van Herpen *et al*. (2006) and 11 sequences of *T. aestivum* from GenBank were included. The NJ and MP tree clusters show monophyletic origin, and the sequences clustered into three subgroups (Fig. 2). Two sequences form *Ae. speltoides* clustered together as the first subgroup, and the sequences from *Ae. tauschii* and the *T. monococcum* were parallel clustered in 2 subgroups. The 9 of 11 *T. eastivum* α-gliadin sequences were clustered with *T. monococcum* subgroup and 2 *T. aestivum* α-gliadin genes were clustered with *Ae. tauschii* subgroup, which strongly suggests that the *T. aestivum* α-gliadin genes originated from diploid ancestral species. The 13 of 14 full-ORF α-gliadin sequences from *Ae tauschii* were clearly clustered together, however, only the sequence HM188550 clustered with the *T. monococcum* subgroup, indicating the variations of *Ae. tauschii* α-gliadin sequences.

The amino acid sequences of α-gliadin genes: The general structure of α- gliadin protein consists of a short N-terminal signal peptide (S) followed by a repetitive domain (R) and a longer non-repetitive domain (NR1 and NR2), separated by two polyglutamine repeats (Q1 and Q2). On the basis of the deduced amino acid sequences of the *Ae. taschii* α-gliadin genes, five of the 19 sequences with structurally similar to the full-ORF genes were pseudogenes, because they contained a typical in-frame premature stop codon resulted from the transition of T by C at the first base of glutamine codon (CAA, CAG) in either repetitive central domains or C-terminal domains. The 14 complete ORFs represented a presumptive mature protein with 281-303 residues (Fig. 2), and a calculated molecular weight of 29.45-34.27kD. A major characteristic of all α-gliadins was the presence of two polyglutamine domains encoded by microsatellite-like sequences. In the first glutamine repeat (Q1), and the second glutamine repeat (Q2) region, possessed 12-25 and 9-24, with average number of 14.3 and 18.6, respectively.

Wheat α-gliadin genes contained six conserved cysteine residues, in which four in the non-repetitive domains, and two at the end of the C-terminal region. As

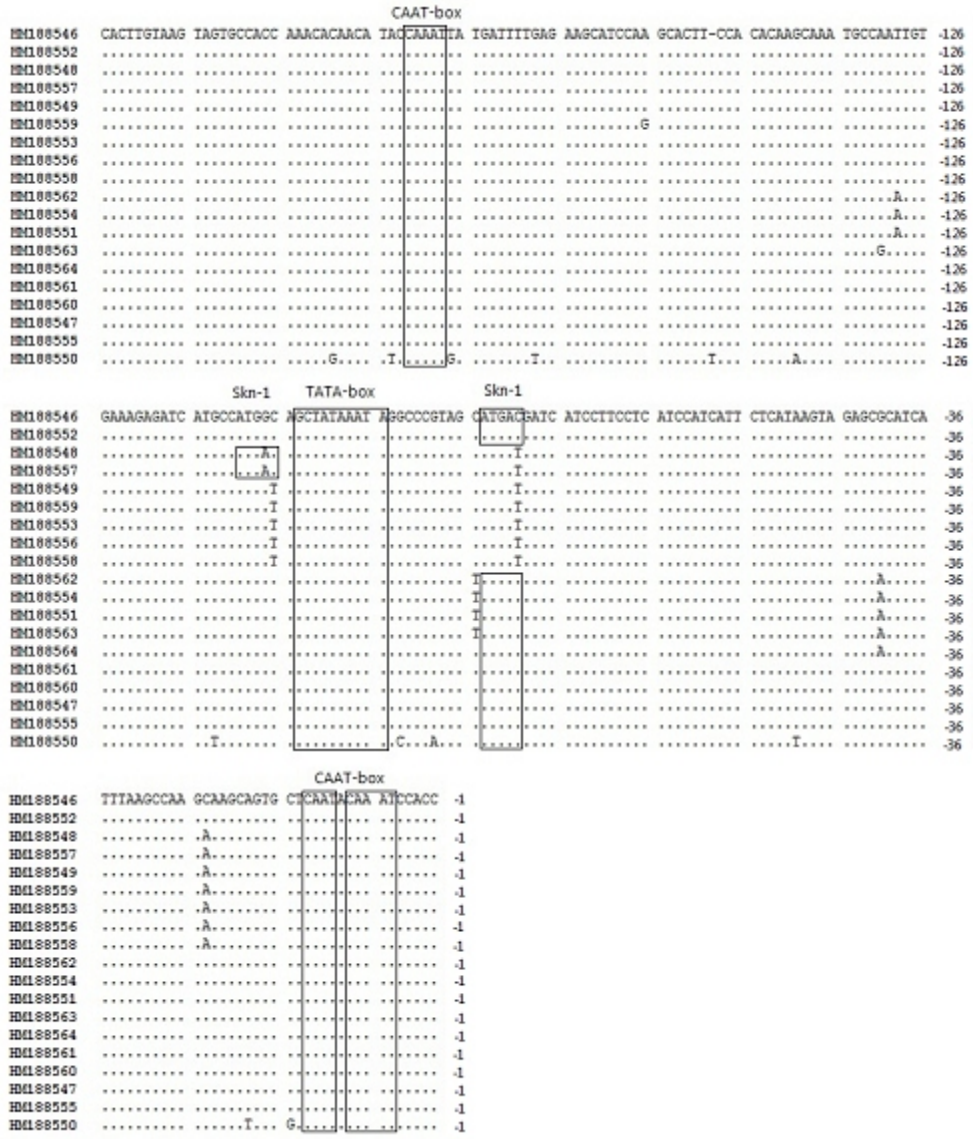

Fig. 1: Alignment of the promoter regions from the 19 α-gliadin genes sequences. The putative transcription factor banding motifs were boxed

shown in Fig. 2, 12 of 14 sequences present the six cysteine residues in the conserved region. The sequence HM188558 has an additional cysteine residue at the NR region, while the sequence HM188546 contained 2 SNPs occurred in NR region, which resulted the total of 5 cysteine residues.

The reported T cell stimulatory epitopes glia-α (QGSFQPSQQ), glia-α-2 (PQPQLYPQ), glia-α-9 (PFPQPQLPY) and glia-α-20 (FRPQQPYPQ) have their own conserved position in the wheat α-gliadin protein. Glia-α was in all cases present in the second nonrepetitive (NR2) domain, whereas glia-α-2, glia-α-9 and glia-α-20 were all found in the first repetitive (R) domain (van Herpen *et al*., 2006). We searched the perfect matches of the four epitopes in the obtained 19 sequences. As the results showed in Fig. 3, total 14, 13, 13, and 12 of 14 full-ORF sequences contained epitopes Glia-α-20, Glia-α, Glia-α-2 and Glia-α-9, respectively.

Estimates of sequence polymorphism: The nucleotide diversity and neutrality test of α-gliadin sequences from wheat and their ancestry species, *T. monococum*, *Ae. speltoides* and *Ae. tauschii* were compared (Table 1). The α-gliadin sequences from wheat, *T. monococum* and *Ae. speltoides* were obtained from Genbank. The haplotype diversity (Hd = 0.994) of *Ae. tauschii* α-gliadins is higher

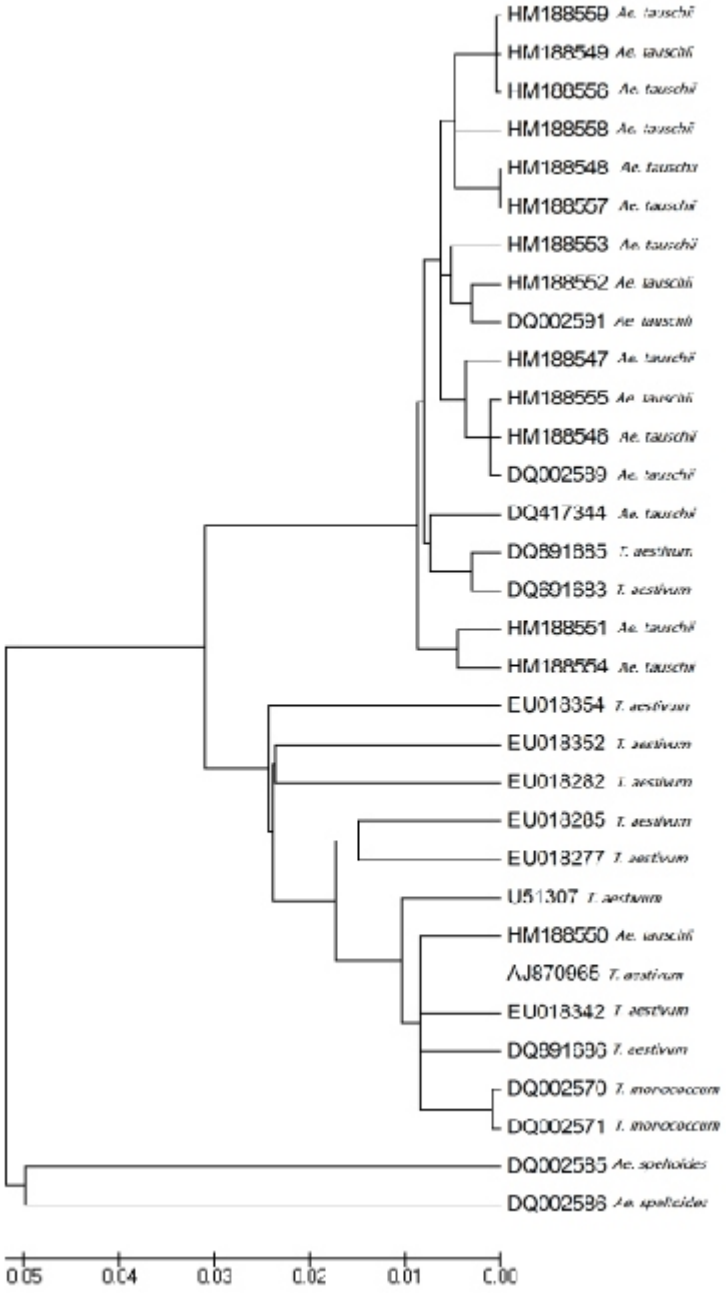

Fig. 2: A phylogenetic tree generated from the nucleotide sequence alignment of the α-gliadin genes. Scale 0.01 = 1% difference among sequences

than those of *T. monococum* (Hd = 0.984) and *Ae. speltoides* (Hd = 0.988). Nucleotide diversity for the α-gliadin sequences was estimated by Tajima's π (Tajima, 1989) and Watterson's θ (Watterson, 1975) statistics. There was a higher diversity of α-gliadin of *Ae. speltoides* ($\pi = 0.0661$/bp) than those of *Ae. tauschii* ($\pi = 0.0180$/bp) and *T. monococum* ($\pi = 0.0201$/bp). The several test statistics (*D*, *D** and *F*) indicated that the α-gliadins sequences from the *Ae. tauschii* and *T. monococum* were significant deviation from a neutral distribution (Table 1).

Table 1: Nucleotide diversity and neutrality test of α-gliadins from wheat and their ancestry species

Species	*T. aestivum*	*T. monococum*	*Ae. speltoides*	*Ae. tauschii*
No. of sequences	16	30	32	19
No. of total sites	1457	840	888	966
Polymorphic sites	321	114	192	93
Haplotypes	16	26	29	18
Hd	1.000	0.984	0.988	0.994
Nucleotide variation				
Diversity (π/bp)	0.0559	0.0201	0.0661	0.0180
Polymorphism (θ/bp)	0.0772	0.0378	0.0682	0.0327
Neutrality tests				
D	-1.3398	-1.9113*	-0.2481	-1.8694*
*D**	-1.3705	-2.9524*	-0.9727	-2.5352*
F	-1.5751	-3.0183*	-0.8603	-2.7215*

Hd = Haplotype diversity; θ = Watterson's estimate; π = Tajima's estimate; *D* = Tajima's D-test; *D** = Fu and Li's D-test without outgroup information; *F* = Fu and Li's F-test; *: 0.01<p<0.05; **: p<0.01

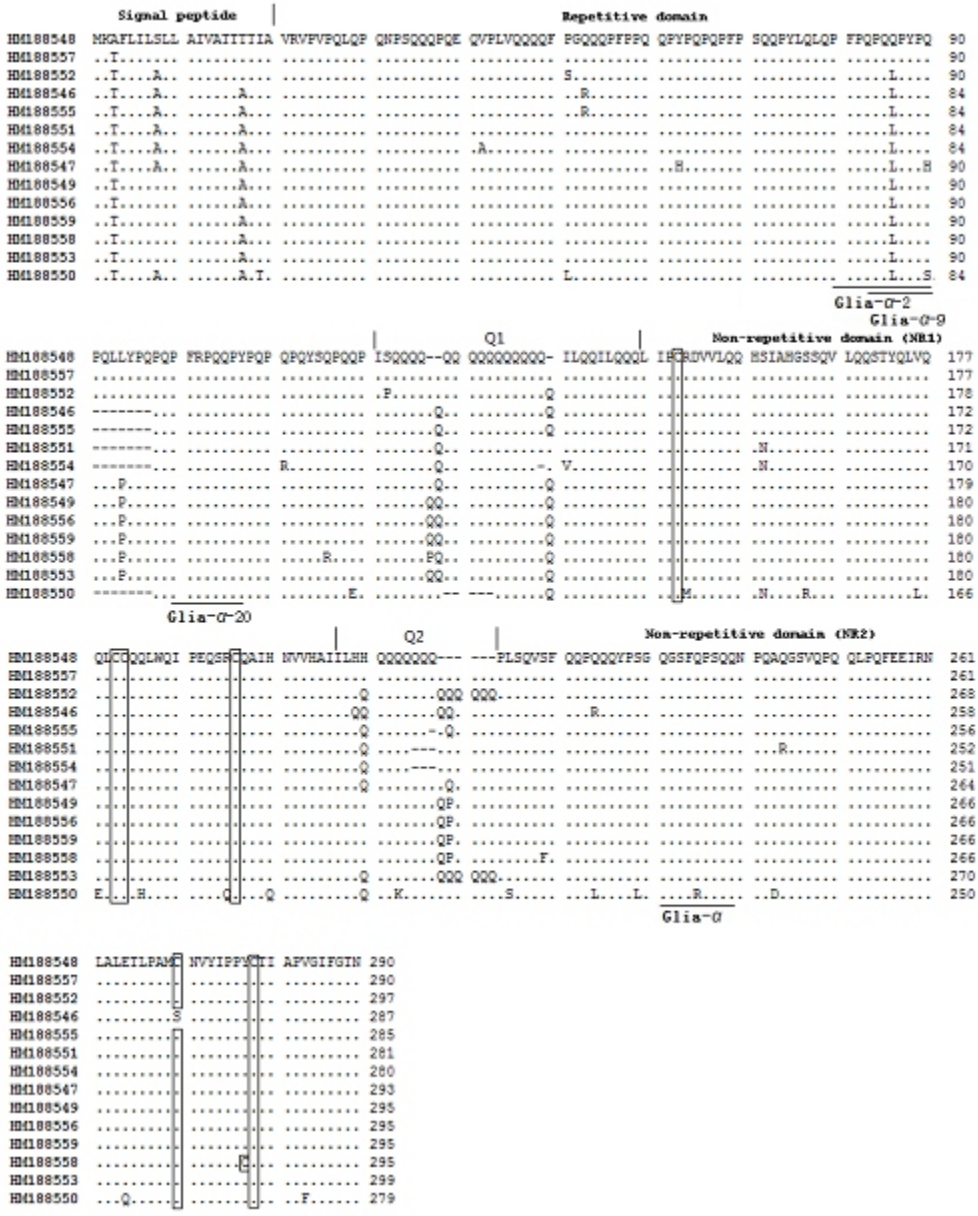

Fig. 3: Comparison of the deduced amino acid sequences of the 14 α-gliadin full ORFs. The Q1 and Q2 represent the two polyglutamine repeats. The cysteine residues are boxed. The epitopes glia-α (QGSFQPSQQ), glia-α-2 (PQPQLYPQ), glia-α-9 (PFPQPQLPY) and glia-α-20 (FRPQQPYPQ) are showed under the alignment

DISCUSSION

With respect to the prolamins storage protein genes in different subfamilies of the *Poaceae*, it is interesting to find that the great divergence seems to occur through tandem rather than dispersed amplification of gene copies (Xu and Messing, 2008). The degree of tandem amplification has increased with the formation of the most recent paralogous gene copies. Estimates for α-gliadin gene copy number range from 25-35 to perhaps even 150 copies per haploid genome. However, the number of the α-gliadin protein extracts was largely less than the estimated from genomic DNA analysis (Islam *et al*., 2003; Gu *et al*., 2004). Recently, van Herpen *et al*. (2006) found that the about 87% of distinct wheat α-gliadin gene sequences containing in-frame stop codons are pesudogenes. As these are not frequently observed in expressed α-gliadin sequences, the pseudogenes are not expressed (Anderson *et al*., 1997). In the present study, we found that the sequences of *Ae. tauschii* α-gliadin with 5 of 19 are pseudogenes, which are lower than the reports from by van Herpen *et al*. (2006). It is possible resulted from the present sequences covered from the 5' and 3' UTR region, and most α-gliadin complete sequences contained full ORF.

The α-gliadin in Triticeae species were belongs to the younger protein subfamily and exhibited large tandem gene clusters (Xu and Messing, 2009). We compared the nucleotide diversity and neutrality test of α-gliadin sequences from wheat and their ancestry species. The nucleotide diversity of α-gliadin sequences varied among the *T. monococcum*, *Ae. speltoides* and *Ae. tauschii*, and the diversity from *Ae. speltoides* was highest. The results also support the B genome was in high variation in wheat genome. The statistical test indicated that α-gliadins from the *Ae. tauschii* and *T. monococum* were significant deviation from a neutral distribution, which possibly indicated the fast evolution rate of the α-gliadin sequences in Triticeae genome. Since the large recombination of α-gliadin sequences during the genome divergence in Triticeae species, the 4 set of epitops in α-gliadin sequences were commonly distributed in the *Ae. tauschii* species. Our previous data indicated that only epitope Glia-α was observed in 18 of 20 *Dasypyrum* α-gliadin sequences (Li *et al*., 2009), and no epitope observed the α-gliadin sequences from *Astralopyrum* sp. (Li *et al*., 2010). It is thus to note that natural and artificial selection enriched the epitops sequences of α-gliadin sequences in Triticeae genomes, which may increase the probabilities to select nontoxic wheat germplasm for celiac disease patients.

Studies implied that the distribution area of subspecies *tauschii* expanded from the region of origin near the Caucasus and southern and southwestern coast of the Caspian Sea to the western habitat, Turkey, and the eastern habitat, Afghanistan, Pakistan, Turkmenistan, Tadjikistan and China (Lubbers *et al*., 1991; Dvorak *et al*., 1998). The studies on the genetic relationships between the *Ae. tauschii* genome and the common wheat D genome revealed that only a single or a few *Ae. tauschii* intraspecific lineages were involved in the evolution of common wheat (Feldman, 2001). It is thus to create synthetic hexaploid wheat (*T. turgidum Ae. tauschii*) to explore for novel genes from *Ae. tauschii* that can be used for common wheat improvement. The genotypes of *Ae. tauschii* including from China can be used to enhance the diversity of synthetic wheat. With respect to the contribution of gliadin to wheat quality, several reports demonstrated that the extra cysteines of seed storage proteins will provide opportunities to form other inter-molecular disulfide bonds, which will be related to high quality (Gianibelli *et al*., 2001). We found that either 5 or 7 cystenins were observed in the α-gliadin sequences of *Ae. tauschii*, which may support the *Ae. tauschii* contribute novel quality for the wheat breeding except for the known Glu-D1 loci. Therefore, more genotypes of *Ae. tauschii* accessions need to evaluate and the diversified gliadin genes from *Ae. tauschii* can be useful for wheat breeding of quality.

CONCLUSION

In the present study, we isolated 19 α-gliadin sequences from the *Ae. tauschii* accessions native to China. Among them, there are two α-gliadin sequences present either 5 or 7 cysteine residues, which can form the intermolecular bisulfate bonds and possibly related to good end-use quality. The identified α-gliadin gene sequences displayed high haplotypes diversity, indicating fast evolution of α-gliadin genes in *Ae. tauschii* species.

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