

Molecular characterization and dynamic expression patterns of two types of γ -gliadin genes from *Aegilops* and *Triticum* species

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Received: 3 February 2012 / Accepted: 6 June 2012 / Published online: 30 June 2012
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Abstract Gliadins were the major components of wheat storage proteins and determine the extensibility properties of gluten dough. In this work, 19 new full-length γ -gliadin genes were isolated from various *Aegilops* and *Triticum* species. Sequence characterization showed that a specific octapeptide and celiac disease (CD)-toxic epitope Gli γ -3 (VQGQGIIQPQQAQL) were present in the rich glutamine domain and C-terminal non-repetitive domain, respectively. Based on the sequence features of both peptides, a new classification system for γ -gliadin gene family was established, in which γ -gliadins were classified into two types (types I and II) with each consisting of two groups. An uneven distribution of different types and groups of γ -gliadin genes was exhibited among 11 *Aegilops* and *Triticum* genomes. Phylogenetic analysis revealed that types I and II genes diverged at about 14 MYA while the divergence of 4 γ -gliadin group genes occurred at around 10 MYA almost simultaneously. The γ -gliadin genes from

S¹ and B genomes displayed a different transcriptional expression pattern during grain development, and rapid increasing of gliadin mRNA and proteins occurred at 15–20 DPA. In addition, genome-specific variations of CD-toxic epitopes among *Aegilops* and *Triticum* genomes were found. The A genome and its related progenitor genomes A^u and A^m had fewer CD epitopes than other genomes, suggesting that these genomes might be valuable gene resources to remove CD toxic peptides for wheat quality improvement.

Introduction

The seed storage proteins in wheat endosperm, mainly including glutenins and gliadins, contribute about 80 % to the total protein in the wheat grains (Shewry et al. 1997). Glutenins consist of high and low molecular weight glutenin subunits (HMW- and LMW-GS), which can form complex polymeric proteins by inter-chain disulphide bonds and impart dough viscoelasticity. Gliadins, the major components of wheat storage proteins (Metakovsky et al. 1984), are traditionally classified into three groups (α/β -, γ - and ω -gliadins) on the basis of their electrophoretic mobility in acidic polyacrylamide gel electrophoresis (A-PAGE). It is well known that gliadins play important roles in determining extensibility properties of gluten dough. Genetic studies confirmed that all ω -gliadins, most of the γ -gliadins and a few of β -gliadins are encoded by the *Gli-1* loci on the short arms of homoeologous chromosome 1, which is tightly linked to the *Glu-3* loci coding for LMW-GS. All α -gliadins, most of the β -gliadins and some γ -gliadins were controlled by the genes at *Gli-2* loci on the homoeologous chromosomes 6A, 6B, and 6D (Anderson et al. 1984; Redaelli et al. 1994; Bietz et al. 1997; Shewry et al. 2003).

Communicated by E. Guiderdoni.

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When compared with HMW-GS, the functions of gliadins are not easily determined due to their complex family structures. It is estimated that the copy number for γ -gliadin genes is between 15 and 40 in *Triticum aestivum* cv. Chinese Spring (Sabelli and Shewry 1991). A typical structural feature of γ -gliadins include a signal peptide of 20 amino acid residues, a short N-terminal non-repetitive domain (I), a highly variable repetitive domain (II), a non-repetitive domain containing most of the cysteine residues (III), a glutamine-rich region (IV) and the C-terminal non-repetitive domain containing the final two conserved cysteine residues (V). For the repetitive domain, there are 100–160 residues arranged as repeated sequences of one or two motifs composed of glutamine, proline, and aromatic amino acids (phenylalanine or tyrosine) (Shewry and Tatham 1990). Different classifications of γ -gliadins have been proposed according to their structural features. For example, Okita et al. (1985) classified γ -gliadins into three hybridization classes, but Pistón et al. (2006) classified γ -gliadin genes into four groups using phylogenetic analysis. More recently, two types by the length of repetitive domain and 17 subgroups by the number and placement of cysteine residues were suggested (Qi et al. 2009). Previous study indicated that there were some differences in mRNA expression between different γ -gliadin groups in different wheat cultivars (Pistón et al. 2006). However, an extensively recognized reliable classification system for γ -gliadin family has not been established, and the transcriptional and translational expression features of different gliadin groups during grain development are still not clear.

Celiac disease (CD), a widely prevalent autoimmune disease in the small intestine, is induced in susceptible individuals by exposure to dietary gluten. The ingestion of gluten-containing foods would lead to an inflammation of the small intestine (Shan et al. 2005; Loponen 2006). It is generally accepted that proline-rich peptide sequences present in gliadins and other prolamins are the causal epitope in CD, and thus gliadins are implicated in celiac disease, and can trigger an autoimmune reaction (Rocher et al. 1995; Mäki and Collin 1997; Sjöström et al. 1998; Battais et al. 2003, 2005). The peptides from repetitive domain of gliadins are involved in various celiac diseases (Loponen 2006). Particularly, γ -gliadins contain several sets of celiac disease epitopes, which mainly have five types of celiac-toxic peptide sequences: (1) FLQPQQPFPQQPQQPYPPQQPQQPFPQ, (2) LQPQQPFPQQPQQPYPPQQPQ, (3) VQGQGIIQPQQPAQL, (4) FSQPQQPFPQPQ and (5) QPQQSFPQQQ (Loponen 2006). Recent report on three types of celiac-toxic peptide sequences (types 1, 2 and 4) showed that, of 169 putatively γ -gliadin functional genes from S^1 , S^s , S^b and AABBDD genomes, seven had 3 types of CD-toxic epitopes located at the repetitive domain and each epitope appeared at most once in every sequence,

while 12 had two types of CD-toxic epitopes and four had the fourth CD-toxic epitope only (Qi et al. 2009).

In this work, 19 new γ -gliadin genes from *Aegilops* and *Triticum* species were isolated, and their molecular characterization and phylogenetic evolutionary relationships were investigated. Particularly, genome-specific variations in celiac disease epitopes of γ -gliadins were found and a new classification among γ -gliadin gene family was put forward. Furthermore, the expression patterns of different γ -gliadin types during developing grains were explored in both transcriptional and translational levels. Our work here provides new evidence for further understanding of the molecular structure, evolution and expression profiles of wheat γ -gliadin genes.

Materials and methods

Plant materials

The materials used in this work included 5 *Ae. tauschii* (D^1D^1 , $2n = 2x = 14$) accessions (AT9, AT9.1, AT25, AT48 and AT176), one *Ae. cylindrica* (CCDD, $2n = 4x = 28$) accession (PI256029), 10 *T. aestivum* (AABBDD, $2n = 6x = 42$) cultivars (Wanmai 33, Zhengmai 16, Yannong 19, Taishan 008, Xiaoyan 22, Chinese Spring (CS), Neimeng 40, Jing 411, Liangxing 99 and Sunstate), one club wheat (*T. aestivum* ssp. *compactum*, AABBDD, $2n = 6x = 42$) line (IPK58) and one CS-1S¹(1B) substitution line. In the CS-1S¹(1B) substitution line, which was developed in the Institute for Plant Breeding at Technical University of Munich (TUM), Germany, the chromosome 1S¹ from *Aegilops longissima* substituted 1B of CS (Netzle and Zeller 1984). All the materials were provided by the Institute for Plant Breeding, TUM and the Institute of Crop Science, Chinese Academy of Agricultural Sciences (CAAS). A set of CS nulli-tetrasomic lines of homologous chromosome 1 and 6, kindly provided by Prof. Z. Liu, Department of Plant Genetics and Breeding, China Agricultural University, was used for chromosomal location of cloned γ -gliadin genes.

For analyzing transcriptional expression patterns of γ -gliadin genes, CS and CS-1S¹(1B) substitution line were planted in experimental field of CAAS in 2011. Developing grains were harvested at 5, 10, 15, 17, 19, 21, 23 and 25 days post-anthesis (DPA) as described by Altenbach (1998) and Li et al. (2010). The collected grain samples were stored in liquid nitrogen.

DNA extraction and PCR amplification

Genomic DNA was extracted from leaves of single adult plants with cetyltrimethylammonium bromide (CTAB)

protocol (Murray and Thompson 1980). Based on the conserved signal peptide domain of the 5' and C-terminal non-repetitive domain of 3' sequences of γ -gliadin genes retrieved from GenBank <http://www.ncbi.nlm.nih.gov>, a pair of primers amplifying the complete open reading frame (ORF) of γ -gliadin genes were designed and used for PCR amplifications. The sequences of the specific PCR primers were 1F: 5'-ATGAAGACCTTACTCATCCT RAC-3' and 1R: 5'-TCATTGGCCACCAAT GC-3'. PCR reaction in a 30 μ l volume with genomic DNA, dNTPs and buffer was performed in a S1000TM thermal cycler (Bio-Rad, USA) with the following program: an initial step of 94 °C for 4 min, 34 cycles of 94 °C for 45 s, 57 °C for 1 min and 72 °C for 80 s, and a final step of 10 min at 72 °C. The recombinant DNA clones were sequenced by TaKaRa Biotech Inc., Japan, and 3–5 clones for each gene were sequenced to avoid possible errors.

Sequence comparison and identification of celiac-toxic peptides

Multiple alignments and analysis of complete γ -gliadin nucleotide sequences as well as deduced amino acid sequences were carried out in BioEdit 7.0 (Ibis Therapeutics, Carlsbad, California). Variations in single nucleotide polymorphisms (SNPs) and insertions/deletions (InDels) among γ -gliadin genes were detected following Wang et al. (2011). Identification of 5 γ -gliadin celiac-toxic peptides was performed according to Loponen (2006).

Phylogenetic analysis

The complete amino acid sequences of γ -gliadins were used to multiple alignment and carry out a phylogenetic analysis since the repetitive regions are considered to be related to the genomic origin of γ -gliadin genes (Qi et al. 2009). The alignment data were converted to mega format using the MEGA (Molecular Evolutionary Genetics Analysis) program (Kumar et al. 2004), and the detailed steps of phylogenetic tree construction were referred to Wang et al. (2011). Neighbor-joining tree was constructed and the divergent times of different genes or genomes were estimated using MEGA4. According to the full-ORF of γ -gliadin genes, the neighbor-joining tree was constructed using the evolutionary rate of 6.5×10^{-9} as presented by Allaby et al. (1999).

mRNA extraction, cDNA synthesis and qRT-PCR

The extraction and purification of mRNA from different grain development stages were performed according to Li

et al. (2010). A 1 μ l RNA sample was measured with NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) to verify its concentration and quality. The purified and no degraded RNA was used to synthesize cDNA with OligdT and random primer from approximately 100 ng mRNA using a superscript first-strand synthesis kit (Promega, Madison, WI, USA).

The synthesized cDNA described above was used for quantitative real-time PCR (qRT-PCR). Two pairs of gene-specific primers were designed and used for the quantification of gene expression, namely 2F + 2R (5'-GGTCAG GGCATCATCCAAC-3' + 5'-GGTGGAGCAGTCAGGT CGG-3') and 3F + 3R (5'-GATCCTGCGGCCACTATT TC-3' + 5'-CCAATGCTGGCGACT ATGCT-3'). The ADP-ribosylation factor (ADP-RF) was used as a control gene with primer ADPF + ADPR (GCTCTCCAACAACA TTGCCAAC + GCTTCTGCCTGTCACATACGC) based on Paolacci et al. (2009). The qRT-PCR reactions were performed based on Li et al. (2010) with minor revisions, following a four-step protocol with a melting curve analysis: (1) an initial incubation of 94 °C for 3 min, 40 cycles of (2) denaturation at 94 °C for 15 s, (3) hybridization at 58 °C for 15 s, and (4) extension at 72 °C for 20 s. Relative expression levels of γ -gliadin mRNA were calculated by normalizing to the level of ADP-RF mRNA according to CFX96 real-time system (Bio-Rad). Triplicates for each PCR reaction and at least three biological replicates were performed for each gene.

A-PAGE, RP-HPLC and mixograph parameters testing

The synthesis and accumulation of gliadin proteins during different grain developing stages in CS and CS-1S¹(1B) were determined using acid polyacrylamide gel electrophoresis (A-PAGE) and reversed-phase high-performance liquid chromatography (RP-HPLC). Developing kernels (0.5 g for each sample from 5, 10, 15, 20 and 25 DPA) were ground into fine powder in liquid nitrogen with a pre-cooled mortar and pestle, and was used to extract gliadins in 1 ml 70 % ethanol. Equal volume of each sample was loaded and separated through A-PAGE with a Bio-Rad PROTEAN II XL (Yan et al. 2003). RP-HPLC was performed on Agilent 1100 using a ZORBAX 300SB-C18 column (300 Å pore size 5 μ m particle size, 4.6 \times 250 mm i.d., Agilent, USA) based on the Gao et al. (2010). Gliadin subclasses were discriminated using RP-HPLC referring to Ferrante et al. (2006) and Zhang et al. (2007). Mixing properties of CS and CS-1S¹(1B) substitution line were determined in a 10 g mixograph (National Manufacturing) connected to a computer, according to the AACC method 54-40A (AACC 2000).

Results

Molecular characterization of γ -gliadin genes from *Aegilops* and *Triticum* species

Nineteen new γ -gliadin genes (16 putative functional genes and 3 pseudogenes) were amplified and cloned from diploid *Aegilops tauschii*, tetraploid *Aegilops cylindrica* and hexaploid *Triticum aestivum* species using designed AS-PCR primers. They were all deposited in GenBank, and individual accession numbers are listed in Table 1. Of 16 putative functional γ -gliadin genes isolated, 4 were from *Ae. tauschii* (HQ404659, HQ404660, HQ327440 and JQ012782), 1 from *Ae. cylindrica* (JQ012783), 1 from *T. aestivum* ssp. *compactum* (JN587178), and 10 from bread wheat (HQ404650, HQ327442, HQ404651, HQ327443, HQ286691-93, JN587179-180 and JQ012784). Three pseudogenes were from *Ae. tauschii* accession AT9 (HQ404654), Sunstate (HQ404652) and Jing 411 (HQ404658), respectively. The full ORF length of cloned functional γ -gliadin genes ranged from 873 to 984 bp, encoding 290–327 amino acid residues.

The chromosome location of cloned 19 γ -gliadin genes was determined using CS nulli-tetrasomic lines of homologous chromosome groups 1 and 6. The results of CS nulli-tetrasomic analysis showed that 11 genes were located on the chromosome 1D, 4 on 6D, 2 on 1A, and 2 on the 1B, respectively (Table 1).

The deduced amino acid sequences showed that all cloned sequences exhibited typical structural features of γ -gliadins genes, e.g., a signal peptide of 20 amino acid residues and conserved I–V domains. Eight highly conserved cysteine residues that formed four intra-chain disulphide bonds were located at non-repetitive domain (III) and C-terminal non-repetitive domain (V). To conduct a comparative analysis, the deduced amino acid sequences of two genes (HQ286692 and HQ286693) with distinct structural features were chosen for multiple alignments with other 10 typical γ -gliadin genes characterized previously. As displayed in Fig. 1, a specific octapeptide and CD-toxic epitope Gli γ -3 (VQGQGIIQPQPAQL) or variations of Gli γ -3 were present in the rich glutamine domain IV and C-terminal non-repetitive domain V (highlighted by green and red box, respectively). According to the characteristics of both consensus sequences, two types of γ -gliadins could be clearly separated, and henceforth designated as types I and II, respectively. There was no CD-toxic epitope Gli γ -3 in type I and the specific octapeptide and mutated CD-toxic epitope Gli γ -3 were VP(IQ)ILRPLFQ and A(V/I)QGL(Q)GIIQPQPAQL(Y), respectively. While in type II the CD-toxic epitope Gli γ -3 (VQGQGIIQPQ PAQL) was highly conserved and the specific octapeptide was VQILVPLS or MH(LR)IF(P) LPLS(Y). Of 19 genes isolated, 1 and 18 were involved in types I and II, respectively (Fig. 1).

Table 1 Characterization of cloned 19 γ -gliadin genes from *Triticum* and *Aegilops* species

Species	Genes	GenBank accession no.	ORF length (bp)	ORF length (amino acids)	Domain II length (amino acids)	Genome
<i>Ae. tauschii</i>	<i>Gliγ-t9a</i>	JQ012782	981	326	149	1D ^t
	<i>Gliγ-9.1t</i>	HQ404659	981	326	149	1D ^t
	<i>Gliγ-t25</i>	HQ404654*	984	327	148	1D ^t
	<i>Gliγ-t48</i>	HQ404660	984	327	149	1D ^t
	<i>Gliγ-t176</i>	HQ327440	888	295	118	1D ^t
<i>Ae. cylindrica</i>	<i>Gliγ-029</i>	JQ012783	981	326	149	1D ^t
<i>T. aes.</i> Wanmai 33 ^a	<i>Gliγ-wm33a</i>	HQ404650	984	327	149	1D
	<i>Gliγ-wm33</i>	JN587179	873	290	125	6D
<i>T. aes.</i> Zhengmai 16	<i>Gliγ-zm16</i>	HQ327442	984	327	149	1D
<i>T. aes.</i> Chinese Spring	<i>Gliγ-csa</i>	HQ404651	984	327	149	1D
	<i>Gliγ-csb</i>	HQ286691	888	295	118	6D
<i>T. aes.</i> Yannong 19	<i>Gliγ-yn19a</i>	HQ327443	984	327	149	6D
	<i>Gliγ-yn19b</i>	JN587180	876	291	114	6D
<i>T. aes.</i> Taishan 008	<i>Gliγ-ts008b</i>	HQ286693	888	295	118	1D
<i>T. aes.</i> Xiaoyan 22	<i>Gliγ-xy22</i>	HQ286692	876	291	114	1B
<i>T. aes.</i> ssp. <i>compactum</i> IPK58	<i>Gliγ-club1</i>	JN587178	897	298	121	1A
<i>T. aes.</i> Neimeng40	<i>Gliγ-nm40</i>	JQ012784	981	326	149	1B
<i>T. aes.</i> Sunstate	<i>Gliγ-susa</i>	HQ404652*	984	327	149	1D
<i>T. aes.</i> Jing 411	<i>Gliγ-j411a</i>	HQ404658*	981	327	149	1A

^a *T. aes. Triticum aestivum* cultivars, 3 pseudogenes marked with one star

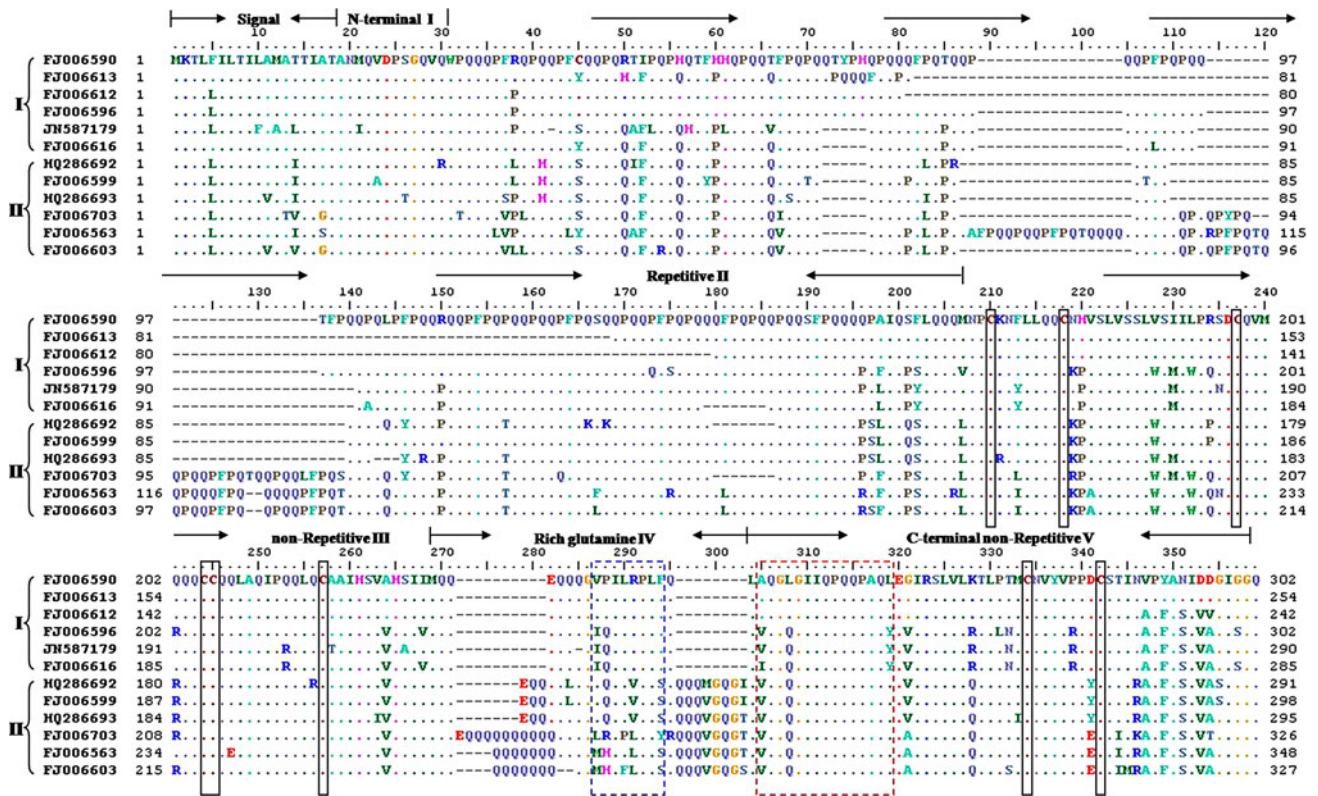


Fig. 1 Multiple alignment of the deduced amino acid sequences of 12 γ -gliadin genes. A specific octapeptide was highlighted by *green box* in the rich glutamine IV in the 12 γ -gliadins, while the celiac-toxic epitope Gli γ -3 (VQVGGIIPQ) and variations of Gli γ -3 in the 12 γ -gliadins were highlighted by *red box* in the C-terminal non-repetitive V. The conserved cysteine residues were highlighted by *black box* and two types of γ -gliadins were indicated. Signal,

N-terminal I, repetitive II, non-repetitive III, rich glutamine IV and C-terminal non-repetitive V represent signal peptide domain, a short N-terminal non-repetitive domain (I), a highly variable repetitive domain (II), a non-repetitive domain containing most of the cysteine residues (III), a glutamine-rich region (IV) and the C-terminal non-repetitive domain containing the final two conserved cysteine residues (V), respectively (color figure online)

After a multiple alignment together with 19 cloned genes and 62 γ -gliadin genes deposited in GenBank, all γ -gliadin proteins were further classified into the same two types described above. Furthermore, each type contained two groups, designated as groups 1, 2, 3 and 4. The structural features of γ -gliadins in the four groups are shown in Fig. 2. In general, the γ -gliadins in the type I had a relatively conserved octapeptide and more variable CD peptide Gli γ -3, while those in type II were of the contrary. There were different SNP variations in the octapeptide and Gli γ -3 CD sequences among the 4 γ -gliadin groups, but the locations of variation were conserved in most cases. The consensus sequences of octapeptide and Gli γ -3 were VP(Q/S)IL(R)RPLF and A(V/L)Q(E)G(Q)LGII(R)QPQPAQL in group 1, IQIL(M)RPLF and V(I)QGQGIIPQPAQY(L) in group 2, V(G)QILV(G)PLS and VQVGGIIPQPA(T)QL in group 3, and M(L/V/I)H(R/D)IF(P/L)LPLS(Y) and VQVGGIIPQ(R)QPAQL in group 4 (Fig. 2).

Variations in CD toxic epitopes among γ -gliadins from different genomes

The numerous T cell response studies have clearly indicated that T cell activation takes place in response to five types of gliadin peptides (Vader et al. 2002; Loponen 2006). Previous report showed that some prolamin gene copies with premature stop codons were still transcribed, and they appeared to be controlled at the transcriptional and/or post-transcriptional level (Xu and Messing 2009). Thus, in this work, the CD toxic epitope variations in the deduced amino acid sequences of 16 putatively functional genes together with 3 pseudogenes with premature stop codons were investigated. As shown in Table 2, all the 19 γ -gliadin proteins contained CD-toxic epitope Gli γ -5 (QPQSFPPQQ) which was located at the repetitive domain, while 5 γ -gliadins (HQ404658-59, JQ012782-84) only had Gli γ -5 without other 4 CD-toxic epitopes due to

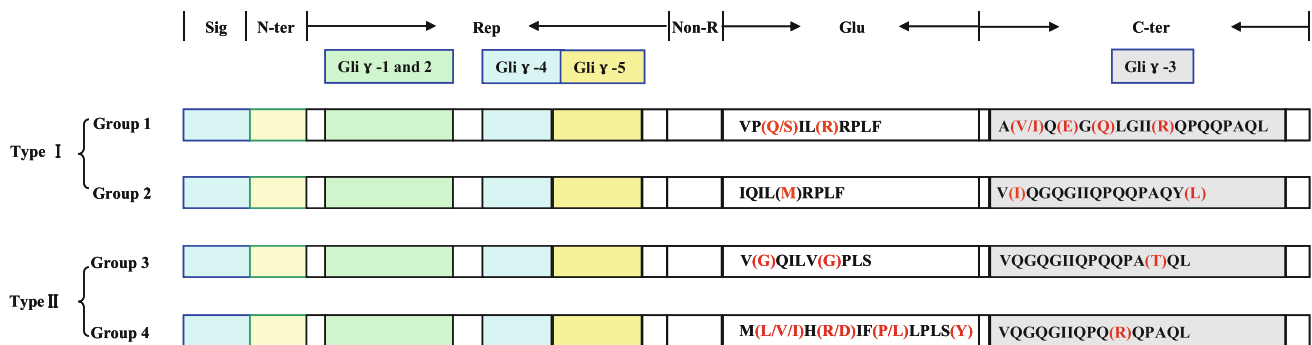


Fig. 2 Model of two types and four groups of γ -gliadin structures. *Sig* signal peptide, *N-ter* N-terminal domain, *Rep* repetitive domain, *Non-R* non-repetitive domain, *Glu* glutamine-rich region, *C-ter* the

C-terminal non-repetitive domain. The positions of specific octapeptide, five celiac-toxic epitopes and variations in Gli γ -3 (VQQQGHQPQQPAQL) were indicated

Table 2 The distribution of five celiac disease toxic peptides in 19 γ -gliadins

GenBank no.	Gli- γ 1 Rep	Gli- γ 2 Rep	Gli- γ 3 C-ter	Gli- γ 4, Rep	Gli- γ 5 Rep
HQ404659	–	–	–	–	160–169
HQ404660	–	–	273–287	–	160–169
HQ404650	–	–	273–287	–	160–169
HQ327442	–	–	273–287	–	160–169
HQ404651	–	–	273–287	–	160–169
HQ327443	–	–	273–287	–	160–169
HQ286693	–	–	129–138	–	129–138
HQ286692	–	–	125–134	–	125–134
HQ286691	–	–	129–138	–	129–138
JN587178	–	–	132–141	–	132–141
JN587179	–	–	136–145	–	136–145
JN587180	–	–	125–134	–	125–134
JQ012782	–	–	–	–	160–169
HQ327440	–	–	241–255	–	160–169
JQ012783	–	–	–	–	160–169
JQ012784	–	–	–	–	129–138
HQ404652	–	–	273–287	–	160–169
HQ404658	–	–	–	–	160–169
HQ404654	–	–	273–287	–	132–141

Horizontal dashes means there was no celiac-toxic peptide in the gliadin

Gli- γ 1-5 means the five types of CD toxic peptides: the detailed information of the five types of CD toxic peptides were as follows: *Gli- γ 1* FLQPQQPFPQQPYPQQPQQPFPQ, *Gli- γ 2* LQPQQPFPQQPQQPYPQQPQ, *Gli- γ 3* VQQQGHQPQQPAQL, *Gli- γ 4* FSQPQQPFPQ, *Gli- γ 5* QPQQSFPQQQ

SNP variations. The remaining 14 γ -gliadins only contained Gli γ -3 and Gli γ -5 without other 3 CD-toxic epitopes.

To investigate the genome-specific CD-toxic epitope variations, a total of 188 γ -gliadins (169 from GenBank and 19 from this work) from 11 *Aegilops* and *Triticum* genomes (A, A^m, A^u, S, S^s, S^b, S^l, S^{sh}, B, D and D^l), were analyzed. The results (Table 3) indicated that there existed evident

Table 3 Number of CD-toxic peptides present in 188 γ -gliadin genes from 11 *Aegilops* and *Triticum* genomes

Species and genomes	Gli- γ 1	Gli- γ 2	Gli- γ 3	Gli- γ 4	Gli- γ 5	Gene no.
<i>T. monococcum</i> (A ^m)	0	0	0	0	10	10
<i>T. urartu</i> (A ^u)	0	0	1	0	6	7
<i>Ae. speltoides</i> (S)	0	0	11	0	2	11
<i>Ae. bicornis</i> (S ^b)	4	4	5	0	9	10
<i>Ae. longissima</i> (S ^l)	5	5	8	0	10	11
<i>Ae. searsii</i> (S ^s)	1	1	1	0	1	8
<i>Ae. sharonensis</i> (S ^{sh})	0	0	8	0	9	9
<i>Ae. tauschii</i> (D ^l)	0	0	14	0	14	16
<i>T. aestivum</i> (A)	0	0	0	0	1	1
<i>T. aestivum</i> (B)	5	5	6	0	7	7
<i>T. aestivum</i> (D)	1	1	5	1	5	5
<i>T. dicoccoides</i> and <i>T. turgidum</i> (AABB)	1	1	19	0	30	31
<i>T. aestivum</i> (AABBDD)	6	7	35	10	55	62
Total	23	24	113	11	144	188

genome-specific variations of CD-toxic epitopes among *Aegilops* and *Triticum* genomes. Particularly, the Gli γ -4 CD peptide was only present in common wheat, suggesting that it occurred in the evolution after hexaploid wheat developed. Gli γ -5 existed in all 11 genomes while Gli γ -3 was present in nine of them except genomes A and A^m. In general, the A and A^m genomes had no Gli γ -1, Gli γ -2, Gli γ -3 and Gli γ -4 and the D^l, S and S^{sh} genomes did not contain Gli γ -1, Gli γ -2 and Gli γ -4. This indicated that Gli γ -5 as well as Gli γ -3 CD toxic peptide had a higher level of conservation among *Aegilops* and *Triticum* genomes.

Although the Gli γ -5 and Gli γ -3 appeared to be highly conserved compared to the other three CD toxic peptides, some SNP variations were observed among four γ -gliadin groups, and both epitopes were absent in certain γ -gliadins. For instance, a total of 8 SNP variation sites in Gli γ -3 were present in four groups (Fig. 2), four in group 1, two in

group 2, and one in groups 3 and 4, respectively. The repetitive domain of γ -gliadins contained most of the CD toxic peptides, and the type I γ -gliadins generally did not contain Gli γ -3 CD epitope because there were more SNP variation sites for the Gli γ -3 CD epitope in the two groups of type I.

Phylogenetic revolutionary relationships of γ -gliadin genes

Although 188 γ -gliadins were used to analyze CD-toxic epitope variations, most of them were very similar by sequence comparison using BioEdit 7.0. Thus, a total of 81

typical γ -gliadin genes from 11 *Aegilops* and *Triticum* genomes were used to construct a phylogenetic tree by MEGA 4.1 (Fig. 3). It is clear that 81 γ -gliadin genes were categorized into two subclasses, well corresponding to types I and II described above, respectively. Furthermore, each type was also classified into two groups, in consistency with the results indicated in Fig. 2.

The phylogenetic tree constructed (Fig. 3) contained all the 27 γ -gliadin genes (including 2 genes from this work) cloned from Chinese Spring up to date, and these genes were clustered into four different groups, suggesting that a hexaploid genotype could contain four γ -gliadin group genes. However, distributions of four groups of γ -gliadin

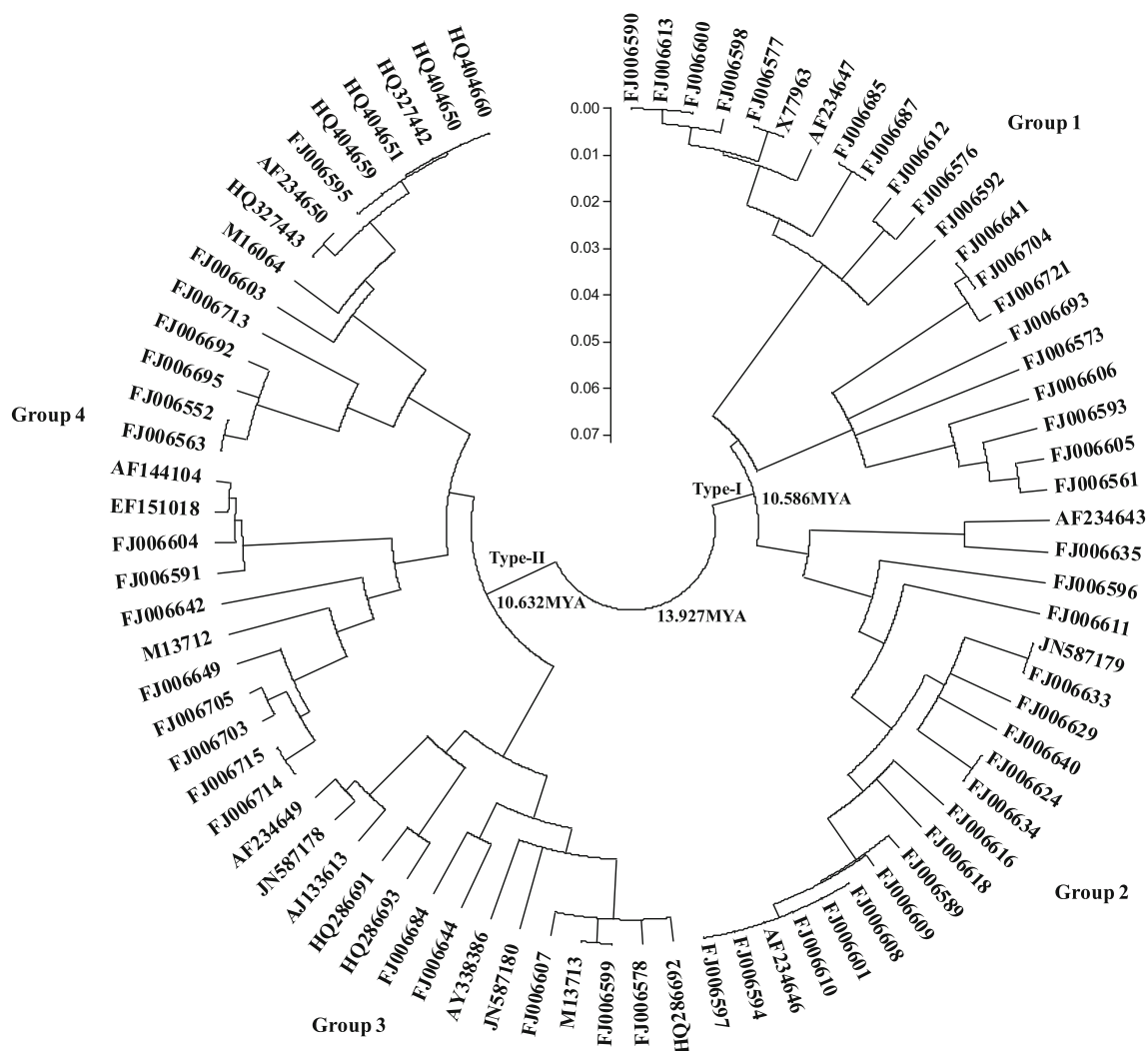


Fig. 3 The phylogenetic tree of 81 complete γ -gliadin genes constructed by MEGA4.1 software and calculated by the neighbor-joining method, including 12 new γ -gliadin genes cloned in this paper, 25 from *T. aestivum* cv. Chinese Spring (FJ006589–FJ006601, FJ006603–FJ006613, EF151018), 6 (X77963, FJ006563, FJ006573, FJ006576–FJ006578) from *T. turgidum*, 3 (FJ006684, FJ006685, FJ006687) from *Ae. searsii*, 4 (FJ006713–715, FJ006721) from *Ae.*

sharonensis, 4 (FJ006641–642, FJ006644, FJ006649) from *Ae. longissima*, 3 (FJ006703–FJ006705) from *Ae. bicornis*, 2 (FJ00692–93) from *Ae. speltoides*, 2 (FJ006552, FJ006561) from *Ae. tauschii*, 3 (FJ006634–35, FJ006635) from *T. uratu*, 3 (FJ006624, FJ006629, FJ006633) from *T. monococcum* subsp. *monococcum*, and the remaining 36 genes from *T. aestivum*. Numbers above or below branches represent divergence time MYA (million years ago)

genes in *Triticum* and *Aegilops* genomes appeared to be different. For instance, the A and B genomes only contained γ -gliadin genes of group 2 in type I and group 4 in type II, respectively. The D genome in common wheat had all four groups of γ -gliadin genes, but the D^t genome of *Ae. tauschii* had group four genes of type II only. The S, S¹, S^b and S^{sh} genomes contained group 1 genes of type I and group 4 genes of type II while S^s genome only had group 3 genes of type II. In addition, the results also demonstrated that the γ -gliadin genes from same genome were generally clustered into a closely related branch, such as six genes from D genome (HQ286693, HQ286691, HQ327443, HQ404650, HQ404651 and HQ327442). Particularly, the genes from A genome and *T. uratu*, B genome and *Ae. speltooides*, and D genome and *Ae. tauschii* exhibited closer phylogenetic evolutionary relationships, further supporting that the A and B genomes were derived from A^u and S genomes, respectively, while the D^t genome was the ancestor of D genome (Yan et al. 2003; Petersen et al. 2006).

The estimated divergence time between types I and II genes was about 13.927 MYA (Fig. 3). The groups 1 and 2 genes diverged at 10.586 MYA, while the divergence between groups 3 and 4 genes occurred at 10.632 MYA. This indicated that the γ -gliadin genes from types I and II diverged earlier than group divergence while the separation of genes from four γ -gliadin groups occurred almost simultaneously.

Transcriptional expression profiles of two types of γ -gliadin genes during grain development

Flour mixograph analysis showed that CS-1S¹(1B) substitution line had better mixing quality than Chinese Spring (Table 4). Because Chinese Spring substitution line CS-1S¹(1B) contained 1S¹-encoding storage proteins, especially some specific γ -gliadins, their expression may be related to flour quality properties. Thus, the transcriptional and translational expression patterns of γ -gliadin genes, especially those encoded by 1B and 1S¹ genomes in CS and CS-1S¹(1B) were investigated in this work.

Two pairs of primers (2F/2R and 3F/3R) were designed on the basis of the sequences of the γ -gliadin genes in each type and they can specifically amplify the encoding sequences of types I and II γ -gliadins. The results showed that each fragment had a unique melt peak, indicating that non-specific amplifications did not occur (Fig. 4a). This was also corroborated by gel electrophoresis where single products of expected lengths were obtained (Fig. 4b). Efficiency of each primer pair was determined by standard curves using serial 10 DPA cDNA dilutions in Chinese Spring (Fig. 4c, d). The unique melt peak, expected lengths separated by gel electrophoresis and efficiency of ADP-R/F primers of ADP-RF are shown in Fig. 4a–d.

Table 4 Mixograph parameters of Chinese Spring (CS) and the substitution line CS-1S¹(1B)

Lines	Mixing time (min)	Peak height (mm)	Peak integral (% tq × min)	Right of peak slope	Width at 8 min (mm)
CS	1.44 ^a	43.26a	69.2 ^a	−3.2 ^a	3.3 ^a
CS-1S ¹ (1B)	1.76 ^b	44.81b	74.2 ^b	−2.7 ^b	3.8 ^b

Different letters indicate significance at $P = 0.05$

As shown in Fig. 5, the expression of types I and II γ -gliadin genes was much lower at 5 DPA, but rapidly up-regulated at 10 DPA in both CS and the substitution line. Their maximum expression level in CS-1S¹(1B) was at 15 DPA, later than those in CS (10 DPA). Two types of γ -gliadin genes exhibited a different expression pattern in CS and the substitution line. The expression of type II genes in CS was decreasing gradually, but type I genes decreased more rapidly from 10 to 15 DPA and both type genes were down-regulated to a much lower level at 23–25 DPA. In the substitution line, however, both types of γ -gliadin genes exhibited a similar expression pattern, showing a rapid up-regulation from 5 to 15 DPA and then down-regulation until grain maturity. However, they still maintained a higher level of expression at 23–25 DPA than that in CS, suggesting that the γ -gliadin genes encoded by 1S¹ genome could keep a longer expression time than those encoded by 1B genome.

Synthesis and accumulation patterns of gliadins during grain filling

The synthesis and accumulation characteristics of gliadins at 5, 10, 15, 20 and 25 DPA in CS and the substitution line CS-1S¹(1B) revealed by A-PAGE and RP-HPLC are shown in Fig. 6. The synthesis of α/β -, γ - and ω -gliadins was synchronous during different development stages, and there were no obvious differences in the expression patterns of γ -gliadins encoded by 1B and 1S¹ chromosomes. The results also showed that grain gliadins initiated at 10 DPA and then consecutively increased until grain maturity. Their synthesis and accumulation displayed a similar pattern in both A-PAGE (Fig. 6a) and RP-HPLC (Fig. 6b). It is clear that the important stages for gliadin synthesis were 15–20 DPA where the gliadin proteins were accumulated to a considerable amount.

Discussion

Classification and evolution of γ -gliadin genes

The γ -gliadin genes were shown to be a complex family with highly repetitive sequences as glutenin genes

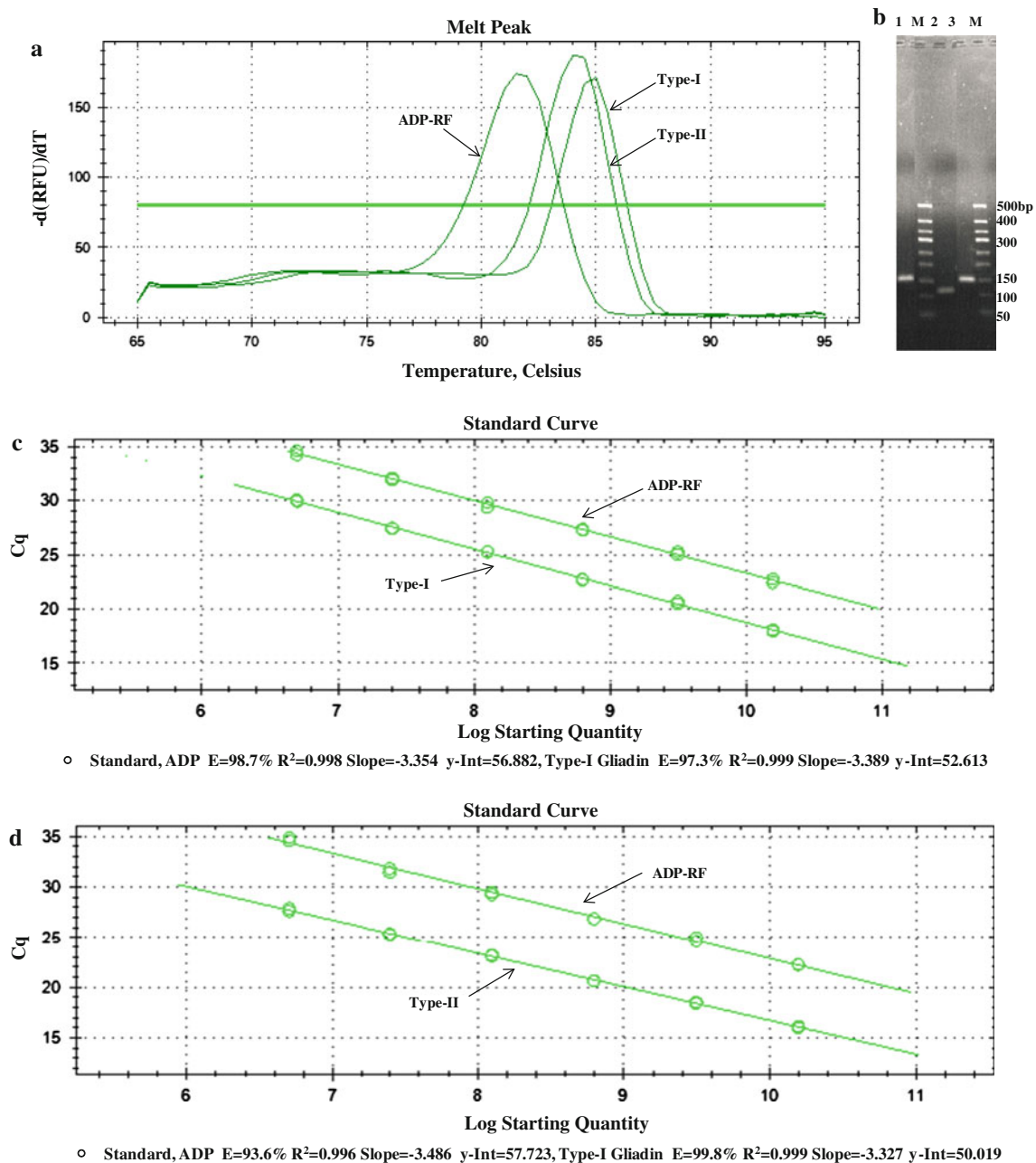


Fig. 4 Unique melt peak (a), agarose gel electrophoresis (b) and standard curves which determined efficiency of each primer pair (c, d) of the fragments amplified by qRT-PCR using three pairs of specific primers for two types of γ -gliadin genes and ADP-RF gene from Chinese Spring (CS). The above data corresponding to different

genes were marked by arrows in Fig. 4a, c and d, respectively. *Standard* standard sample, viz. a serial 10 DPA cDNA of CS dilutions (dilution factor 5), E efficiency, R^2 = correlation index, M = 50 bp DNA ladder. 1, 2 and 3 represent amplified fragments of ADP-RF, type-I gliadin, type-II gliadin genes, respectively

(Shewry and Tatham 1990; Anderson et al. 2001; Li et al. 2010). Higher diversity of γ -gliadin genes is present in different *Triticum* and related species, except that there is no obvious discrimination between *Sitopsis* and *Ae. tauschii* at the *Gli-1* loci (Okita et al. 1985; Anderson et al. 2001; Qi et al. 2009). Our study showed that sequence diversity among γ -gliadin genes from *Triticum* and related genomes was mainly attributable to SNP and InDel

variations in the repetitive region. It is estimated that there were more than 35 copies for the gene of γ -gliadins in common wheat Cheyenne and Chinese Spring (Lafiandra et al. 1984; van Herpen et al. 2006; Sabelli and Shewry 1991; Qi et al. 2009), similar to those of LMW-GS in hexaploid wheat (Harberd et al. 1985; Sabelli and Shewry 1991; Cassidy et al. 1998), but less than α -gliadin genes (Xie et al. 2010).

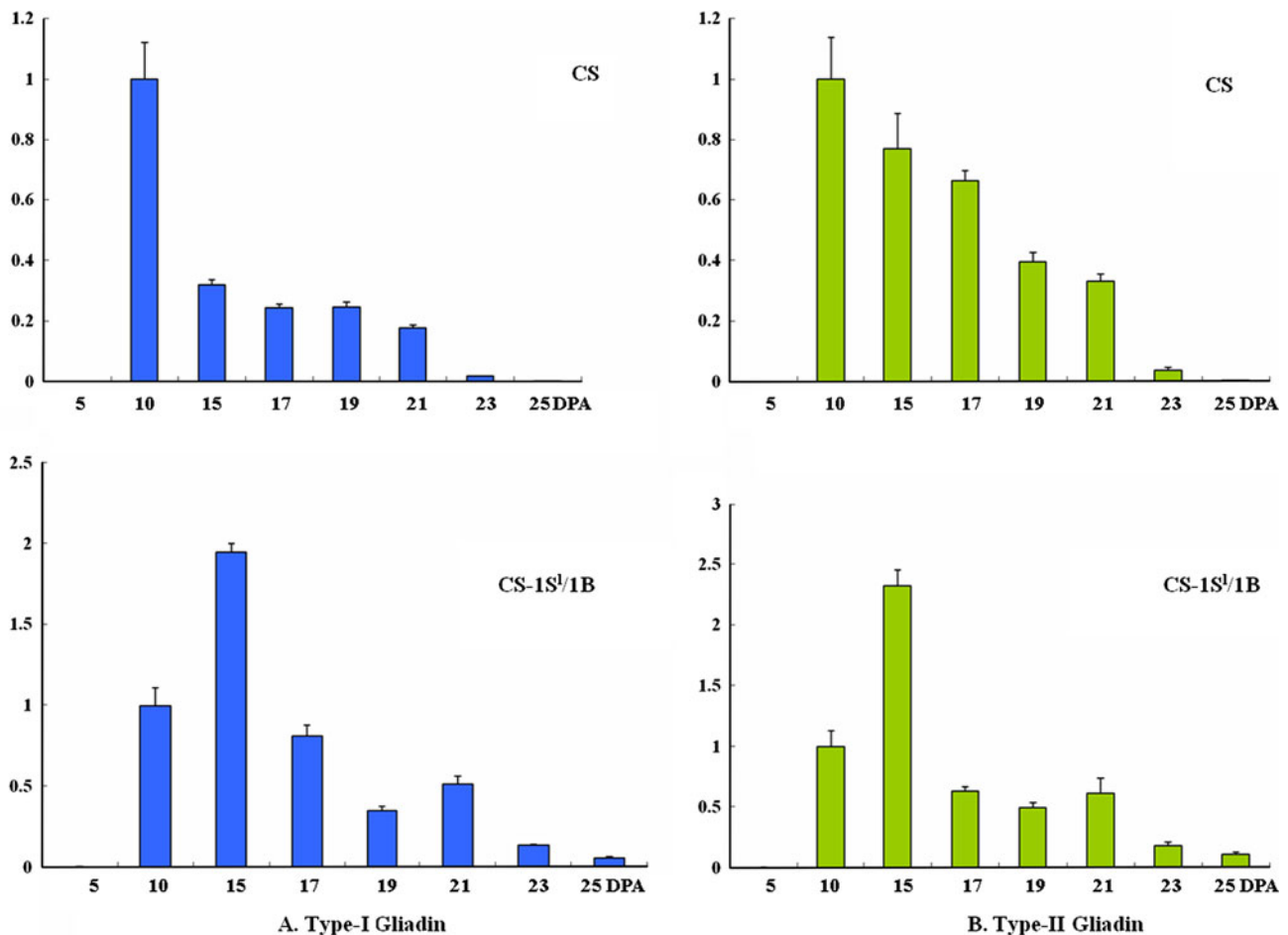


Fig. 5 The transcript expression patterns of the types I and II γ -gliadin genes during grain development in CS and the substitution line CS-1S¹(1B) by qRT-PCR. DPA day post-anthesis

Since there are extensive allelic variations for the γ -gliadin genes among *Triticum* and related genomes, their classification and phylogenetic evolutionary relationships become an important topic. Pistón et al. (2006) and Chen et al. (2009) classified the γ -gliadins into four groups by phylogenetic tree analysis. According to the number and placement of cysteine residues and phylogenetic result, Qi et al. (2009) divided γ -gliadins into 17 subgroups and they classified γ -gliadins into two types based on the length of repetitive domain. In the present study, we presented a new γ -gliadin classification based on the characterization of two specific peptides in rich glutamine domain and C-terminal non-repetitive domain as well as phylogenetic analysis. The results from 81 γ -gliadin genes from 11 *Aegilops* and *Triticum* genomes demonstrated that two types of γ -gliadins were categorized and each type contained two groups, according to the sequence features of the specific octapeptide and CD-toxic epitope Gli γ -3 and phylogenetic tree (Figs. 1, 2, 3). Furthermore, the γ -gliadins in type I

appeared to have a more conserved octapeptide and more variable CD peptide Gli γ -3 than those in type II. The divergence of γ -gliadin genes between types I and II occurred at about 14 MYA and four groups appeared to diverge at about 10 MYA simultaneously.

Although γ -gliadin is likely to be the most ancient family among prolamins (Shewry and Tatham 1990), multiplication of γ -gliadin genes might have occurred in diploid level, and a bottleneck in *Gli-1* loci was passed over to tetraploid where great changes might have happened to the *Gli-1* regions in the formation of tetraploid wheat (Qi et al. 2009). Our results showed an uneven distribution of different types and groups of γ -gliadin genes among *Triticum* and related genomes. Some ancient genomes, such as D^t genome contained fewer types and groups of γ -gliadins than D genome, suggesting that the divergence of certain groups of γ -gliadins in D genome occurred after the formation of hexaploid wheat during the evolutionary process.

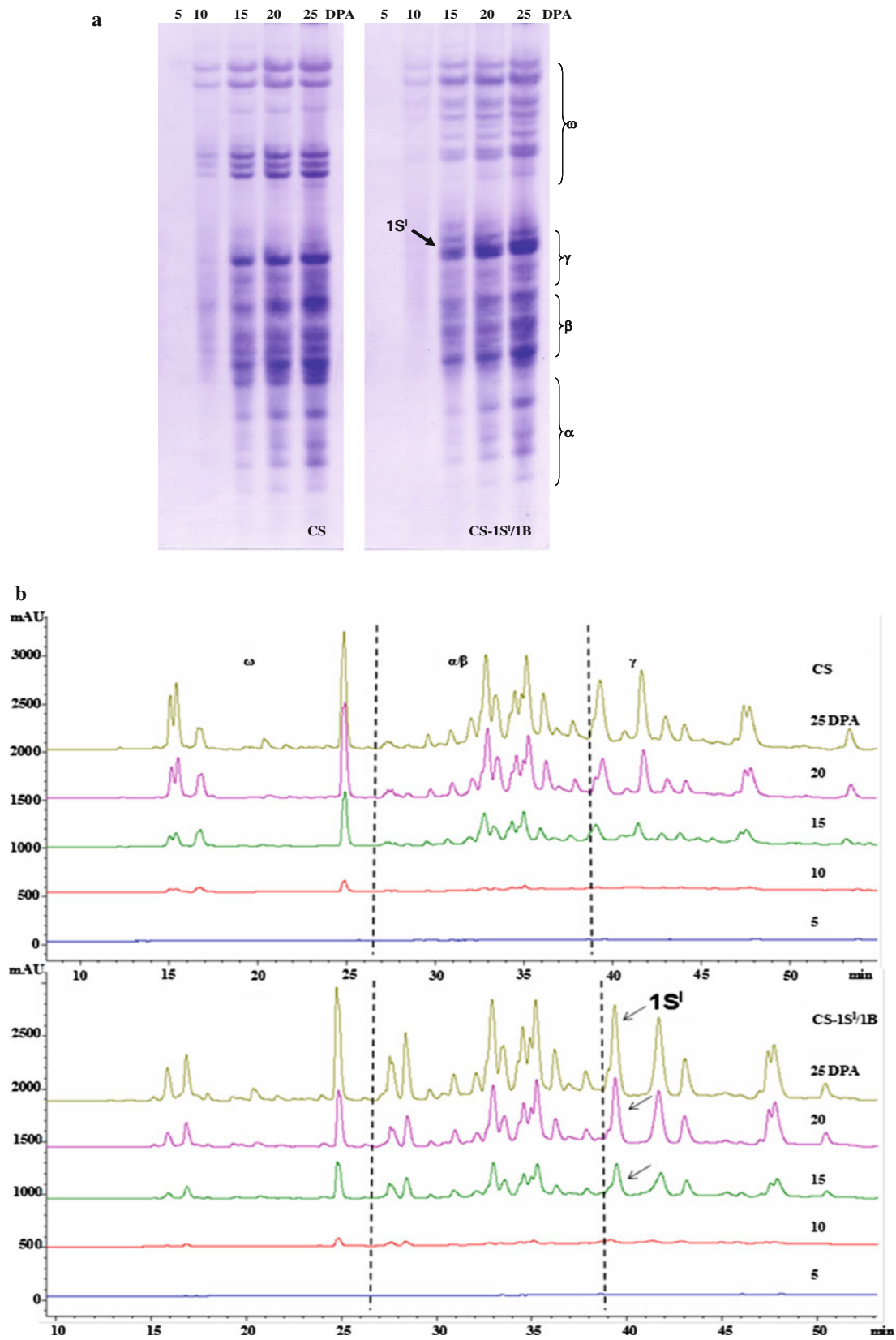


Fig. 6 Synthesis and accumulation patterns of gliadins during five grain development stages in CS and CS-1S¹(1B) by A-PAGE (a) and RP-HPLC (b). Different gliadin classes and the γ -gliadins encoded by 1S¹ genome were indicated

CD epitope variations and its application for wheat quality improvement

Gliadins play important roles in determining extensibility properties of gluten dough, but they contain various CD-related toxic peptides such as 4 T cell stimulatory toxic epitopes in α -gliadins (Xie et al. 2010) and 5 in γ -gliadins (Loponen 2006). Thus, the removal of these toxic peptides is required to minimize the safety problems/concerns of wheat to render it safe for consumption by CD patients. Van Herpen et al. (2006) found the genome-specific CD toxic peptide variations were present among α -gliadin genes and the A, B and D genomes contained different sets of CD epitopes. Therefore, α -gliadin genes cloned from hexaploid wheat could be successfully assigned to specific chromosomes based on the number of 4 T cell stimulatory epitopes (Xie et al. 2010). This would be helpful for screening gliadin genes with few or no CD epitopes from specific genomes in wheat quality improvement program.

In the present work, we found that the A genome and its related progenitor A^u and A^m genomes had fewer CD epitopes than other genomes (Table 3). Thus, these genomes would be valuable γ -gliadin gene resources with fewer CD toxic peptides. In addition, most of the CD toxic peptides were distributed at the repetitive domain, and the γ -gliadin genes in type I generally did not have Gli γ -3 CD epitope because there were more SNP variation sites for the Gli γ -3 CD epitope in two groups of type I than in other two groups of type II. Although the Gli γ -5 and Gli γ -3 were highly conserved among *Aegilops* and *Triticum* genomes, and particularly Gli γ -5 was present in all the 11 genomes investigated, both epitopes still exhibited various SNP variations. These features would be useful for selecting specific types and groups of γ -gliadin genes that contain fewer and/or even no CD epitopes by means of marker-assisted selection in wheat quality improvement.

Dynamic expression of wheat γ -gliadins during grain development

Grain development is associated with massive changes in gene expression in different genotypes or environments. The endosperm develops much faster than the embryo during the early stage of grain development (before 10 DPA) and many of the transcripts associated with the endosperm and pericarp in wheat, while storage product (starch and protein) is synthesized later (Wan et al. 2008). The gene expression in endosperm initially tended to increase, and then decrease. However, embryo transcripts tend to increase throughout the whole period of grain development (Wan et al. 2008). Comparison of genes differentially expressed revealed a dynamic transcript accumulation profile with major re-programming events that

occur at 3–7, 7–14 and 21–28 DPA, which is corresponded to distinct developmental and metabolic events occurring in the caryopsis (Laudencia-Chingcuanco et al. 2007). Differential expressions of wheat albumins and globulins showed that some proteins related to starch and storage protein synthesis, such as Rubisco small subunit, 14-3-3 and glutamine synthetase, may be expressed in a very high level at the early stage of grain development (Gao et al. 2009).

Until now, little work has been reported on dynamic transcriptional patterns of γ -gliadins during wheat grain development by qTR-PCR analysis. In this study, two types of γ -gliadin genes from CS and CS-1S¹(1B) substitution line displayed different expression patterns during grain development, indicating that there were the expression differences between the γ -gliadin genes located on 1B and 1S¹ chromosomes. This might be resulted from different copies of two types of γ -gliadin genes presented in B and S¹ genomes. Particularly, the γ -gliadin genes from CS-1S¹(1B) maintained a higher level at the late periods (Fig. 5), which might facilitate synthesis and accumulation of grain γ -gliadins. This may in part contribute to the formation of superior flour mixing property (Table 4). It was also noticed that γ -gliadin genes showed a maximum up-regulated expression during 10 or 15 DPA (Fig. 5), which is consistent with the expression patterns of storage protein genes (Laudencia-Chingcuanco et al. 2007) and LMW-GS genes (Li et al. 2010).

Different storage protein components in the grains have different synthesis and accumulation patterns during grain development, which might be relevant to wheat gluten quality (Gupta et al. 1996; Liu et al. 2011). HMW and LMW glutenin subunits and gliadins appeared to be synthesized concurrently during grain development. Previous study indicated that both gliadins and glutenins initially appeared at 10 DPA or earlier during grain development and then increased significantly throughout the grain-filling period until maturity (Zhu and Khan 1999). Similar results were obtained in this work, and the synthesis of different gliadins was synchronous and displayed similar expression patterns during grain development (Fig. 6). A rapid increase of gliadins occurred at 15–20 DPA and a dramatic up-regulated expression at both mRNA and protein levels also occurred concurrently at this stage, suggesting that this stage might be a key period for gliadin synthesis and accumulation.

Conclusion

According to a specific octapeptide in the rich glutamine domain (IV), CD-toxic epitope Gli γ -3 and its variations in C-terminal non-repetitive domain (V), γ -gliadin gene

family could be classified into two types and each consists of 2 groups. CD-toxic epitopes of γ -gliadins had genome-specific variations among *Aegilops* and *Triticum* genomes. The A genome and its related progenitor A^u and A^m genomes encoded fewer CD epitopes than other genomes. The γ -gliadin genes from S¹ and B genomes displayed a different transcriptional expression pattern during grain development. The key stage for gliadin synthesis and accumulation occur at 15–20 DPA.

Acknowledgments We are grateful to Dr. Guoliang Jiang, South Dakota State University of USA for constructive suggestions in reviewing the manuscript. This research was financially supported by grants from the National Natural Science Foundation of China (30830072, 31101145), the Chinese Ministry of Science and Technology (2009CB118303) and the National Key Projects for Transgenic Crops of China (2011ZX08009-003-004, 2011ZX08002-004).

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