SCIENTIFIC REPORTS

OPEN

Genome-wide analysis of complex wheat gliadins, the dominant carriers of celiac disease epitopes

Da-Wei Wang^{1,2,3,*}, Da Li^{1,2,*}, Junjun Wang^{1,2,*}, Yue Zhao¹, Zhaojun Wang^{1,2}, Guidong Yue¹, Xin Liu¹, Huanju Qin¹, Kunpu Zhang¹, Lingli Dong¹ & Daowen Wang^{1,2,4}

Received: 21 October 2016

Accepted: 09 February 2017

Published: 16 March 2017

Gliadins, specified by six compound chromosomal loci (*Gli-A1/B1/D1* and *Gli-A2/B2/D2*) in hexaploid bread wheat, are the dominant carriers of celiac disease (CD) epitopes. Because of their complexity, genome-wide characterization of gliadins is a strong challenge. Here, we approached this challenge by combining transcriptomic, proteomic and bioinformatic investigations. Through third-generation RNA sequencing, full-length transcripts were identified for 52 gliadin genes in the bread wheat cultivar Xiaoyan 81. Of them, 42 were active and predicted to encode 25 α -, 11 γ -, one δ - and five ω -gliadins. Comparative proteomic analysis between Xiaoyan 81 and six newly-developed mutants each lacking one *Gli* locus indicated the accumulation of 38 gliadins in the mature grains. A novel group of α -gliadins (the CSTT group) was recognized to contain very few or no CD epitopes. The δ -gliadins identified here or previously did not carry CD epitopes. Finally, the mutant lacking *Gli-D2* showed significant reductions in the most celiac-toxic α -gliadins and derivative CD epitopes. The insights and resources generated here should aid further studies on gliadin functions in CD and the breeding of healthier wheat.

Wheat is a major staple crop in the world, with a total of 728.967 million metric tons of grains produced in 2014¹. Wheat foods are consumed globally and supply approximately 20% of the calories and protein that humans consume². However, despite intensive breeding efforts in the past, there still exist many undesirable substances in wheat grains and derived foods, whose consumption can induce severe illnesses in sensitive human individuals^{3,4}. Comprehensive characterization of these compounds is a prerequisite for effectively decreasing their contents in the development of healthier wheat foods through innovative plant breeding⁵.

Gliadins, a distinct family of gluten proteins, are strongly accumulated in wheat grains^{6–8}. In hexaploid bread wheat (*Triticum aestivum*, $2n = 6x = 42$, AABBDD), which accounts for over 95% of global wheat production⁹, gliadins constitute approximately 40–50% of total grain proteins^{6–8}. Gliadins have been divided into α -, γ -, δ - and ω -types based on differences in the primary structure and the presence and number of conserved cysteine residues in the protein^{6–8}. During wheat food processing, gliadins and two other families of gluten proteins, i.e., high-molecular-weight glutenin subunits (HMW-GSs) and low-molecular-weight glutenin subunits (LMW-GSs), form a gluten complex^{6–8}. Thus, gliadins have traditionally and more frequently been studied for their effects on the functional properties of gluten complex and the end-use quality of wheat grains^{6–8}. However, in the human gastrointestinal track, many gluten proteins are poorly digested, and the resultant peptides, especially those derived from gliadins, have been linked with the elicitation of celiac disease (CD)¹⁰. CD has been considered as one of the most important food related disorders in the world, because 1) it affects 1–3% of human population, and 2) the incidence of CD is rising in many developing countries in association with increased consumption of wheat based foods^{5,10,11}. Furthermore, due to the involvement of similar genetic predispositions, the incidence of CD is substantially higher among the individuals with type 1 diabetes, and CD can exacerbate the detrimental effects of type 1 diabetes¹². Together, these factors have prompted extensive international studies on CD, which have revealed the central importance of gliadins in the cause and pathogenesis of CD¹³.

¹The State Key Laboratory of Plant cell and Chromosome Engineering, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China. ²University of Chinese Academy of Sciences, Beijing, China.

³Tobacco Research Institute, Chinese Academy of Agricultural Sciences, Qingdao, China. ⁴The Collaborative Innovation Center for Grain Crops, Henan Agricultural University, Zhengzhou, China. *These authors contributed equally to this work. Correspondence and requests for materials should be addressed to K.Z. (email: kpzhang@genetics.ac.cn) or L.D. (email: lldong@genetics.ac.cn) or D.W. (email: dwwang@genetics.ac.cn)

In bread wheat, the genes encoding gliadins are contained mainly in six compound loci, with *Gli-A1*, *-B1* and *-D1* located on the short arms of group 1 chromosomes (1AS, 1BS and 1DS) and *Gli-A2*, *-B2* and *-D2* on the short arms of group 6 chromosomes (6AS, 6BS and 6DS)^{6–8}. Moreover, *Gli-A1*, *-B1* and *-D1* are, respectively, linked with the *Glu-A3*, *-B3* and *-D3* loci that specify LMW-GSs^{6–8}. Orthologous *Gli* loci are present in the diploid grasses *T. urartu* and *Aegilops tauschii*, which donated the A and D genomes to bread wheat, respectively, and the tetraploid wheat *T. turgidum*, which is involved in the hexaploidization event yielding hexaploid wheat^{6–8,14}. The genes coding for γ -, ω - or δ -gliadins are carried in *Gli-1* loci, whereas those for α -gliadins are in *Gli-2* loci^{6–8}. There are frequently highly similar homoeologous and paralogous gliadin members, and many gliadins have multiple allelic variants among different wheat cultivars^{6–8}. Apart from the six major *Gli* loci outlined above, several minor gliadin loci have been reported to locate on 1AS (*Gli-A3*, *-A5* and *-A6*), 1BS (*Gli-B3* and *-B5*) and 1DS (*Gli-D4* and *-D6*)⁶. In addition to carrying the genes for α -gliadins, *Gli-2* loci have also been suggested to harbor the genes encoding β -gliadins that migrated slower than α -gliadins in the electrophoresis under low pH⁷. But later studies revealed that the two types of gliadins were very similar in amino acid sequence and could be collectively named as α -gliadins⁷.

To date, none of the six *Gli* loci of bread wheat has been completely sequenced. A partial sequencing of bread wheat *Gli-A2*, *-B2* and *-D2* loci, approximately 200 kb for each locus, has been reported, with 12 active and four inactive α -gliadin genes identified¹⁵. A recent study has sequenced a chromosomal region harboring the entire *Gli-D1* locus of *Aegilops tauschii*, which is orthologous to *Gli-D1*¹⁶. In *Gli-D1*, six ω -gliadin, four γ -gliadin and two δ -gliadin genes are found in a genomic DNA segment of about 888.5 kb, and for all three types of gliadin genes, there are both active and pseudogene members. A proteomic approach, involving digestion of excised protein spots separated by two-dimensional gel electrophoresis (2-DE) with multiple proteases, has been proposed for identifying and differentiating similar gliadins accumulated in bread wheat grains^{17,18}. Attempts have also been made to match the gliadins identified by tandem mass spectrometry (MS/MS) to gliadin gene coding sequences constructed with expressed sequence tags (ESTs)^{17,18}. But many of the gliadin gene coding sequences assembled using ESTs were incomplete, which compromised accurate matching between gliadin proteins and transcripts present in the grains^{17,18}. A transcriptomic analysis of the genes expressed in developing wheat seeds using second generation sequencing (SGS) technology has identified multiple gliadin transcripts and genes¹⁹. However, the gliadin gene sequences constructed with SGS reads may be error-prone because the presence of highly repetitive nucleotide sequence elements in these genes can cause misassembly of the reads from similar homoeologous or paralogous members. In the recently published draft genome sequence for the spring wheat line Chinese Spring (CS), the chromosomal regions carrying *Gli* loci are largely covered by short contigs²⁰. Moreover, CS is unlikely useful for genome-wide analysis of gliadin genes because of lacking *Gli-D2* locus due to a natural deletion on chromosome 6D^{19,20}. Clearly, gliadins, especially those in bread wheat, are highly heterogeneous and complex. The progress in genome-wide characterization of bread wheat gliadins and their corresponding genes has been slow. Comprehensive characterization of gliadin proteins and their corresponding genes expressed in bread wheat varieties remains a strong challenge.

The gliadin peptides that promote CD generally harbor one or more epitopes capable of binding to human T cells^{21–23}. Such epitopes are also present in HMW-GSs and LMW-GSs but in a reduced amount^{5,10}. The epitopes are generally rich in proline and glutamine, and the high content of proline renders them resistant to protease digestion^{21–23}. These epitopes bind to specific haplotypes of human leucocyte antigen (HLA) class II proteins, i.e., HLA-DQ2.2, HLA-DQ2.5, HLA-DQ8 and HLA-DQ8.5, expressed on the surface of CD4⁺ T cells. This elicits complex biochemical and cell biological events, leading to epithelial cell destruction and villous atrophy in the small intestine^{21–23}. So far, a total of 31 epitopes involved in CD have been compiled based on available *in vitro* and *in vivo* evidence, the majority of which are derived from gliadins^{5,10}. Bioinformatic analysis of protein sequences has frequently been employed to detect the presence of CD epitopes in different gliadins. The results show that different types of gliadins differ substantially in the CD epitopes contained, with the α -gliadins specified by *Gli-B2* containing very few CD epitopes^{5,10,24–27}. However, this type of analysis has not been reported for δ -gliadins, which are recognized only recently^{28,29}, nor has it been conducted for all of the gliadins accumulated in a single bread wheat variety.

Based on the information presented above, we aimed to develop an experimental model for comprehensively characterizing the gliadins expressed in bread wheat. Because of the high complexity of gliadins in bread wheat, multidisciplinary investigations combining transcriptomic, proteomic, mutagenetic and bioinformatic approaches were used. Important to our efforts was the preparation of six unique mutants each lacking one of the six *Gli* loci in our model variety Xiaoyan 81, an elite winter type bread wheat cultivar³⁰. To efficiently identify the mRNAs of transcribed gliadin genes, the newly emerged third generation sequencing platform PacBio RSII, which is effective for obtaining full-length transcript sequences^{31–35}, was adopted. For differentiating closely related gliadin proteins, the 2-DE-MS/MS based proteomic approach, previously recommended for gliadin identification^{17,18}, was employed. The data generated allowed us to identify the spectrum of gliadin genes expressed through matching full-length transcripts to their protein products, to assign the expressed gliadin genes to individual *Gli* loci, and to bioinformatically assess the presence of CD epitopes in all of the gliadin proteins accumulated in the grains of a bread wheat cultivar.

Results

Analysis of full-length transcripts of gliadins and their corresponding genes. Three RNA sequencing experiments (RSE1, 2 and 3) were conducted to investigate the full-length transcripts of gliadins. RSE1 used total RNAs extracted from a pooled sample of the grains collected at 0, 5, 15 and 25 days after flowering (DAF), and was accomplished with PacBio RSII platform. RSE2 was conducted with Illumina HiSeq 2000 platform, and sequenced RNAs in the grains at 0, 5, 15 and 25 DAF, respectively. RSE3 was carried out with PacBio RSII, and used total RNAs from the grains collected at 25 DAF. The high-quality reads produced in RSE2 were

α -gliadin				γ -, δ - and ω -gliadin			
Full-length transcript ^a	Cognate gene	Protein (aa) ^b	Chromosomal location ^c	Full-length transcript ^a	Cognate gene	Protein (aa) ^b	Chromosomal location ^c
$\alpha 1$ (31)	<i>Gli-$\alpha 1$</i>	293	6A	$\gamma 1$ (38)	<i>Gli-$\gamma 1$</i>	339	1A
$\alpha 2$ (45)	<i>Gli-$\alpha 2$</i>	293	6A	$\gamma 2$ (28)	<i>Gli-$\gamma 2$</i>	285	1A
$\alpha 3$ (69)	<i>Gli-$\alpha 3$</i>	287	6A	$\gamma 3$ (19)	<i>Gli-$\gamma 3$</i>	285	1A
$\alpha 4$ (62)	<i>Gli-$\alpha 4$</i>	287	6A	$\gamma 4$ (67)	<i>Gli-$\gamma 4$</i>	357	1B
$\alpha 5$ (24)	<i>Gli-$\alpha 5$</i>	286	6A	$\gamma 5$ (14)	<i>Gli-$\gamma 5$</i>	302 (9 cys)	1B
$\alpha 6$ (32)	<i>Gli-$\alpha 6$</i>	289	6A	$\gamma 6$ (16)	<i>Gli-$\gamma 6$</i>	297	1B
$\alpha 7$ (10)	<i>Gli-$\alpha 7$</i>	287	6A	$\gamma 7$ (72)	<i>Gli-$\gamma 7$</i>	291	1B
$\alpha 8$ (7)	<i>Gli-$\alpha 8$</i>	296	6A	$\gamma 8$ (83)	<i>Gli-$\gamma 8$</i>	327	1D
$\alpha 9$ (10)	<i>Gli-$\alpha 9$</i>	325	6B	$\gamma 9$ (60)	<i>Gli-$\gamma 9$</i>	298	1D
$\alpha 10$ (75)	<i>Gli-$\alpha 10$</i>	299	6B	$\gamma 10$ (36)	<i>Gli-$\gamma 10$</i>	295 (9 cys)	1D
$\alpha 11$ (19)	<i>Gli-$\alpha 11$</i>	293	6B	$\gamma 11$ (86)	<i>Gli-$\gamma 11$</i>	298	1D
$\alpha 12$ (23)	<i>Gli-$\alpha 12$</i>	293	6B				
$\alpha 13$ (17)	<i>Gli-$\alpha 13$</i>	296	6B	$\delta 1$ (4)	<i>Gli-$\delta 1$</i>	327	1D
$\alpha 14$ (9)	<i>Gli-$\alpha 14$</i>	296	6B				
$\alpha 15$ (84)	<i>Gli-$\alpha 15$</i>	313	6B	$\omega 1$ (10)	<i>Gli-$\omega 1$</i>	359	1A
$\alpha 16$ (83)	<i>Gli-$\alpha 16$</i>	313	6B	$\omega 2$ (12)	<i>Gli-$\omega 2$</i>	406 (1 cys)	1B
$\alpha 17$ (58)	<i>Gli-$\alpha 17$</i>	297	6B	$\omega 3$ (18)	<i>Gli-$\omega 3$</i>	439	1B
$\alpha 18$ (2)	<i>Gli-$\alpha 18$</i>	312	6B	$\omega 4$ (9)	<i>Gli-$\omega 4$</i>	387	1D
$\alpha 19$ (24)	<i>Gli-$\alpha 19$</i>	282	6D	$\omega 5$ (7)	<i>Gli-$\omega 5$</i>	377 (1 cys)	1D
$\alpha 20$ (15)	<i>Gli-$\alpha 20$</i>	286	6D				
$\alpha 21$ (57)	<i>Gli-$\alpha 21$</i>	293	6D				
$\alpha 22$ (54)	<i>Gli-$\alpha 22$</i>	308	6D				
$\alpha 23$ (44)	<i>Gli-$\alpha 23$</i>	309	6D				
$\alpha 24$ (6)	<i>Gli-$\alpha 24$</i>	291	6D				
$\alpha 25$ (9)	<i>Gli-$\alpha 25$</i>	299 (7 cys)	6D				

Table 1. List of 42 unique and active full-length transcripts found for different types of gliadins through RNA sequencing. ^aThe value in brackets indicates the total number of FLNC reads found in RSE1 and RSE3 for the given full-length transcript. ^bDeduced from the listed full-length transcripts. The gliadin having variant number of conserved cysteine (cys) residues is shown in bold. ^cChromosomal location of the gene yielding the given transcript.

employed to correct nucleotide errors in the PacBio transcriptomic reads obtained in RSE1 and RSE3 as described previously^{36,37}. A foregoing analysis of the RSE1 data showed that the majority of the full-length non-chimeric (FLNC) reads, approximately 74.6%, were likely to contain full-length open reading frame (ORF), and by searching RSE1 FLNC reads, 72 unique full-length transcripts were identified for wheat gluten genes, including six for HMW-GSs, 14 for LMW-GSs and 52 for gliadins³⁸. Among the full-length transcripts of gliadins, 32 were transcribed from α -gliadin genes, 13 from γ -gliadin genes, one from δ -gliadin gene, and six from ω -gliadin genes.

To verify the number of full-length transcripts found for gluten genes by RSE1, a total of 187,279 FLNC reads, produced in RSE3 (Table S1), were analyzed. After removal of redundancy, 41,611 unique transcripts were obtained, 35,380 of which could be mapped to the draft genome sequence of CS; the 35,380 transcripts covered 14,747 extant chromosomal loci and 5,449 new loci (not annotated by the draft genome sequence of CS) (Table S1). Searching the RSE3 transcriptomic data yielded an identical set of full-length transcripts for gluten genes as that based on RSE1 data. Thus, the 52 unique full-length transcripts for gliadins were supported by two independent PacBio RNA sequencing experiments. Of the 52 transcripts, 42 had intact coding sequence, and the remaining 10 had defective coding region owing to the presence of premature stop codon or frame shift mutations (Table S2). Here, we focused on studying the gliadins specified by the 42 full-length transcripts having intact coding region, with their corresponding genes tentatively named to facilitate more detailed analysis (Table 1). The chromosomal locations of the genes yielding the 42 gliadin transcripts were determined with the aid of six deletion lines lacking each of the six *Gli* loci and by PCR mapping (see below). For the 10 transcripts with disrupted coding sequence, they were transcribed from seven α -, two γ - and one ω -gliadin pseudogenes, respectively (Table S2).

Among the 25 transcribed, intact α -gliadin genes, eight members came from chromosome 6A, 10 from chromosome 6B, and seven from chromosome 6D (Table 1). In general, their transcript levels were upregulated during grain development, although those of *Gli- $\alpha 7$* , *Gli- $\alpha 8$* , *Gli- $\alpha 18$* and *Gli- $\alpha 25$* were comparatively lower than their respective counterparts from the same chromosome (Figure S1). The length of the proteins deduced from the 25 members ranged from 286 to 325 residues (Figure S2), and their sequence identities varied from 75.7% (between *Gli- $\alpha 9$* and *Gli- $\alpha 20$*) to 99.7% (between *Gli- $\alpha 1$* and *Gli- $\alpha 2$* , *Gli- $\alpha 11$* and *Gli- $\alpha 12$* , *Gli- $\alpha 13$* and *Gli- $\alpha 14$* , or *Gli- $\alpha 15$* and *Gli- $\alpha 16$*). Their primary structure was identical to that previously defined for α -gliadins³⁹, being composed of a signal peptide, a N-terminal repetitive region, two regions containing polyglutamine, and two unique

regions carrying conserved cysteine residues (Figure S2). The number of conserved cysteine residues was six, with one additional cysteine found in Gli- α 25 (Figure S2). Interestingly, the 25 α -gliadins could be divided into two groups based on amino acid variation downstream of the last conserved cysteine residue. The CSTT group had 12 members, including Gli- α 8 specified by chromosome 6A, Gli- α 9 to Gli- α 18 by chromosome 6B, and Gli- α 19 by chromosome 6D, whereas the CT group contained the remaining 13 members by either 6A (Gli- α 1 to Gli- α 7) or 6D (Gli- α 20 to Gli- α 25) chromosomes (Figure S2). Members in the CSTT group also exhibited unique amino acid substitutions in the first polyglutamine region (PQR1) and the first unique region (UR1) (Figure S2).

The 11 transcribed, intact γ -gliadin genes resided on chromosomes 1A (*Gli- γ 1 to Gli- γ 3*), 1B (*Gli- γ 4 to Gli- γ 7*) or 1D (*Gli- γ 8 to Gli- γ 11*) (Table 1). Their transcript levels were generally increased as grain development progressed (Figure S3). The primary structure of the 11 deduced γ -gliadins was identical to that previously reported for this type of proteins^{40,41}, which was consisted of a signal peptide (SP), a short N-terminal region (NR), a repetitive region (RR), a region with polyglutamine (PQR), and two unique regions (UR1 and UR2) containing the conserved cysteine residues (Figure S4). The number of conserved cysteine residues was eight, with one extra cysteine found in Gli- γ 5 and Gli- γ 10, respectively (Figure S4). The length of the 11 deduced γ -gliadins ranged from 285 to 357 residues (Figure S4), and their sequence identities varied from 66.7% to 100%. For the two members (Gli- γ 2 and Gli- γ 3) that were 100% identical, their coding regions (from the start to stop codons) differed by two synonymous nucleotide substitutions.

The δ -gliadins are recognized very recently, and one to two active genes have been identified for δ -gliadins in two common wheat varieties (CS and Hereward)^{28,29}. In this work, we found the full-length transcript for only one unique and intact δ -gliadin gene (*Gli- δ 1*, Table 1) in our experimental variety Xiaoyan 81. The transcript level of *Gli- δ 1* was up-regulated during grain development (Figure S5a), and its deduced protein was highly similar to the two δ -gliadins of CS (Figure S5b). The main primary structural features, including a signal peptide, a short N-terminal region, a repetitive region, and a unique region with eight cysteine residues, were shared among the three compared δ -gliadins.

The ω -gliadins have been designated according to the first four amino acid residues of the N-terminal region^{42,43}. The ω -gliadins specified by chromosomes 1A or 1D are generally AREL- and ARQL-types, while those by chromosomes 1B are usually SRL- and ARQL-types^{42,43}. There is also an alternative classification scheme that divides these gliadins into two subgroups, ω 5-gliadins and ω 1, 2-gliadins⁴³. In general, the ω 5-gliadins have a N-terminus starting with SRL and carry the repetitive motifs FPQQQ and QQIPQQ, while the ω 1, 2-gliadins possess a N-terminus starting with ARE, ARQ or KEL and have the repetitive element PQQPFP. Here, we identified five transcribed, intact ω -gliadin genes, coming from chromosomes 1A (*Gli- ω 1*), 1B (*Gli- ω 2* and *Gli- ω 3*) and 1D (*Gli- ω 4* and *Gli- ω 5*), respectively (Table 1). The transcript levels of the five ω -gliadin genes were generally increased as grain development proceeded (Figure S6). Consistent with previous studies, we noticed that the ω -gliadins by 1A or 1D were either ARQL-type (Gli- ω 1 and -5) or AREL-type (Gli- ω 4), and that one of the 1B ω -gliadin (Gli- ω 3) belonged to the SRL- and ARQL-type (Figure S7a,b). In contrast, the other 1B ω -gliadin, Gli- ω 2, had a novel N-terminus starting with ARPL, and exhibited high similarity to a previously reported D-type glutenin protein (Figure S7c). The deduced Gli- ω 2 and Gli- ω 5 proteins each contained a cysteine residue in the repetitive region, but no such residue was observed in Gli- ω 1, - ω 3 or - ω 4 (Figure S7). Gli- ω 1, -4 and -5 belonged to ω 1, 2-gliadins because their N-terminus started with ARQ or ARE and they carried the repetitive motif PQQPFP; Gli- ω 3 was a typical ω 5-gliadin because its N-terminus began with SRL and it carried the repetitive motifs FPQQQ and QQIPQQ (Figure S7a,b). Gli- ω 2 was likely a ω 1, 2-gliadin because the first three residues of its N-terminus (ARP) resembled ARE and ARQ, and it had the repetitive motif PQQPFP (rather than the FPQQQ or QQIPQQ elements normally found in ω 5-gliadins) (Figure S7c).

Among the four types of genes analyzed above, the transcript levels of γ -gliadin genes were comparatively high, followed by those of α -gliadin genes; the transcript levels of δ - and ω -gliadin genes were relatively low except for *Gli- ω 4* (Figures S1, S3, S5 and S6). This gene was substantially more highly transcribed than other ω -gliadin gene members (Figure S6).

Development of six deletion lines lacking individual *Gli* loci. Our previous study has shown that a mutant population of Xiaoyan 81 created by ion beam mutagenesis is useful for isolating the deletion mutants of important wheat chromosomal loci³⁰. Thus, in this work, we screened this mutant population for isolating the deletion mutants lacking individual *Gli* loci (*Gli-A1*, -*B1*, -*D1*, -*A2*, -*B2* or -*D2*) by MALDI-TOF-MS analysis. For Xiaoyan 81, 10 compound gliadin peaks ($1^{\#}$ to $10^{\#}$) in the mass range of 28–40 kD were consistently obtained (Figure S8a). A similar set of MS peaks was observed for the gliadin extract of CS (Figure S8a). Because it is known that *Gli-A1*, -*B1*, -*D1*, -*A2*, -*B2* and -*D2* are located on chromosomes 1A, 1B, 1D, 6A, 6B and 6D, respectively^{6–8}, we examined the gliadin MS peaks of the six nulli-tetrasomic lines of CS each lacking one of the six chromosomes. This permitted us to link the 10 gliadin MS peaks with the *Gli* locus on 1A, 1B, 1D, 6A, 6B or 6D chromosomes (Figure S8b). Subsequently, we screened the gliadin extracts of 10,100 M2 seeds (five from each of the 2020 M2 families), and a total of 78 M2 families were found to miss one or more of the 10 gliadin MS peaks. Eventually, six unique and homozygous deletion lines (DLGliA1, DLGliB1, DLGliD1, DLGliA2, DLGliB2 and DLGliD2), lacking *Gli-A1*, -*B1*, -*D1*, -*A2*, -*B2* and -*D2*, respectively, were developed (Table 2). Examination with chromosome specific microsatellite markers revealed that the missing of individual *Gli* loci in the six mutant lines was generally caused by large genomic deletion in 1A, 1B, 1D, 6A, 6B or 6D chromosomes (Figure S9). The microsatellite markers missed in the six mutant lines provided a convenient means for their identification (Table 2). DLGliA1, DLGliB1, DLGliD1, DLGliA2 and DLGliD2 were fertile with normal seed development; DLGliB2 was partially infertile but the selfed seeds developed and germinated normally (Figure S10).

Proteomic analysis of gliadins in Xiaoyan 81 and *Gli* locus deletion lines. As the first step in this series of experiments, the gliadin extract prepared from the mature grains of Xiaoyan 81 was separated using

Deletion line	MALDI-TOF-MS peak missed ^a	<i>Gli</i> locus deleted	Size of the deletion (cM) ^b	Location of the deletion	Diagnostic microsatellite marker (missed in the deletion line)
DLGliA1	9 [#]	<i>Gli-A1</i>	>40.4	1AS	<i>Xgdm33</i> , <i>Xcfa2153</i> , <i>Xgpw7072</i> , <i>Xgpw2276</i> , and <i>Xwmc24</i>
DLGliB1	10 [#]	<i>Gli-B1</i>	>23.2	1BS	<i>Xpsp3000</i> , <i>Xwmc406</i> , <i>Xwmc230</i> , and <i>Xgpw4069</i>
DLGliD1	4 [#] , 8 [#]	<i>Gli-D1</i>	>12.5	1DS	<i>Xwmc147</i> , <i>Xgpw7082</i> , <i>Xwmc432</i> , <i>Xcfd15</i> , and <i>Xwmc336</i>
DLGliA2	2 [#]	<i>Gli-A2</i>	>51.5	6AS	<i>Xgwm334</i> , <i>Xgpw2082</i> , <i>Xgpw7073</i> , <i>Xgpw7076</i> , and <i>Xgpw7592</i>
DLGliB2	5 [#] , 7 [#]	<i>Gli-B2</i>	>7	6BS	<i>Xgdm113</i> , <i>Xbarc14</i> , <i>Xpsp3009</i> , <i>Xwmc494</i> , and <i>Xgwm508</i>
DLGliD2	1 [#] , 3 [#] , 6 [#]	<i>Gli-D2</i>	>23.5	6DS	<i>Xcfd132</i> , <i>Xcfd33</i> , and <i>Xgdm127</i>

Table 2. Characteristics of the six deletion lines lacking individual *Gli* loci. ^aThe ten compound peaks are shown in Figure S8a. ^bDetailed in Figure S9.

Figure 1. A schematic representation of the 2-DE spots resolved for the gliadin extract of Xiaoyan 81 mature grains. The spots were numbered (1 to 84) and the types of gliadins (α , γ , δ or ω) to which they belonged were indicated. They were also color-coded to indicate the control by individual *Gli* loci (i.e., *Gli-A1*, *-B1*, *-D1*, *-A2*, *-B2* and *-D2*), which was determined by comparing the 2-DE data of Xiaoyan 81 with those of the six *Gli* locus deletion lines (DLGliA1, DLGliB1, DLGliD1, DLGliA2, DLGliB2 and DLGliD2). The majority of the spots were well separated by IEF under pH 6–11, but those shown in insets a and b were better resolved by IEF under pH 3–10 (Figure S12). Two spots (28 and 84) could not be resolved because both were mixtures of different types of gliadins. The image was prepared based on the data of 25 independent 2-DE runs (seven for Xiaoyan 81 and three for each of the six deletion lines) and the MS/MS analysis results of the 2-DE spots.

2-DE, which involved isoelectric focusing (IEF) and SDS-PAGE in the first and second dimensions, respectively. To maximize the resolving of gliadins, IEF was performed under two pH regimes, one under pH 6–11 and the other under pH 3–10. The majority of the gliadin spots were well separated under pH 6–11 (Figure S11), but IEF under pH 3–10 enabled better separation of approximately a dozen of protein spots with the isoelectric point (pI) ranging from 3 to 6 or 6 to 7 (Figure S12). A total of 97 protein spots were consistently found for Xiaoyan 81 gliadin extract in seven independent 2-DE runs. These spots were excised from the gel, digested by chymotrypsin and thermolysin separately, and examined by nano LC-LTQ-MS/MS, with the resultant mass spectral data analyzed bioinformatically (see Methods). Of the 97 spots, 95 corresponded to individual gliadin (82 spots) or LMW-GS (13 spots) proteins, with the remaining 2 (spots 28 and 84) being mixtures of different gliadins. For the 82 well resolved gliadin spots, they were identified to be α -gliadin (35 spots), γ -gliadin (37 spots), ω -gliadin (9 spots) or δ -gliadin (1 spot) (Fig. 1). To stick to the main objective of this work, the MS data of the 82 gliadin spots are described in Table S3.

Subsequently, the gliadin extracts of the six deletion lines were investigated following the experimental scheme outlined above. The lack of individual *Gli* loci in these lines decreased the number of gliadin spots present in 2-DE. Specifically, for DLGliA1, DLGliB1 and DLGliD1, the numbers of gliadin spots missed were 10, 17 and 20, respectively; in DLGliA2, DLGliB2 and DLGliD2, the numbers of gliadin spots lacked were 15, 13 and 7, respectively (Fig. 1, Table 3). The total number of gliadin spots lacked in the six deletion lines (i.e., 82) equaled to the well resolved gliadin spots found for Xiaoyan 81 (Fig. 1, Table 3). Thus, the 82 gliadin spots could all be assigned

Deletion line (<i>Gli</i> locus deleted)	Gliadin spot lacked		<i>Gli</i> locus assignment of the spot
	2-DE spot ^a	Total	
DLGliA1 (<i>Gli-A1</i>)	1–10	10	<i>Gli-A1</i>
DLGliB1 (<i>Gli-B1</i>)	11–27	17	<i>Gli-B1</i>
DLGliD1 (<i>Gli-D1</i>)	29–48	20	<i>Gli-D1</i>
DLGliA2 (<i>Gli-A2</i>)	49–63	15	<i>Gli-A2</i>
DLGliB2 (<i>Gli-B2</i>)	64–76	13	<i>Gli-B2</i>
DLGliD2 (<i>Gli-D2</i>)	77–83	7	<i>Gli-D2</i>

Table 3. The 2-DE gliadin spots lacked in individual *Gli* locus deletion lines. ^aSpots 28 and 84 were not included in the table because both were mixtures of different types of gliadins.

to individual *Gli* loci (Table 3). In addition to gliadin spots, DLGliA1, DLGliB1 and DLGliD1 also lacked two, six and five LMW-GS spots, respectively, indicating that the *Glu-A3*, *-B3* and *-D3* loci specifying LMW-GSs were also deleted in these three mutant lines.

Matching gliadin spots to active gliadin genes. The MS peptide data of the 82 gliadin spots were searched against the Xiaoyan 81_Gluten database in order to match the 2-DE spots to the 42 intact and transcribed gliadin genes (detailed in Methods). As shown in Table 4 and Table S3, positive match was obtained for 38 active gliadin genes, but not for *Gli-α7*, *Gli-α8*, *Gli-α18* and *Gli-α25*. The match was most satisfactory for the single δ -gliadin and 11 γ -gliadin genes, followed by the five ω -gliadin genes. For δ - and γ -gliadin genes, the match was generally supported by unique peptides; *Gli-γ2* and *Gli-γ3* were matched to the same set of 2-DE spots (4–9, Table 4 and Table S3), which was in agreement with the fact that the deduced proteins of the two genes were 100% identical (see above). In the case of ω -gliadin genes, specific matching (supported by unique peptides) was obtained for *Gli-ω1*, *Gli-ω2* and *Gli-ω3*, with *Gli-ω4* and *Gli-ω5* being matched to the same two spots (43 and 44) and *Gli-ω4* being additionally matched to the spots 45–48 (Table 4 and Table S3). For the 21 α -gliadin genes, specific matching (supported by unique peptides) was achieved for 13 members (i.e., *Gli-α1*, *-α2*, *-α3*, *-α4*, *-α5*, *-α6*, *-α9*, *-α19*, *-α20*, *-α21*, *-α22*, *-α23* and *-α24*); for the remaining eight members (*Gli-α10*, *-α11*, *-α12*, *-α13*, *-α14*, *-α15*, *-α16* and *-α17*), the matching was deduced based upon maximal protein coverage by the peptides derived from individual spots (Table 4 and Table S3). *Gli-α1* and *Gli-α2* were matched to the same two spots (50 and 51, Table 4 and Table S3), consistent with the finding that the deduced proteins of the two genes were 99.7% identical (see above). This was also true for *Gli-α11* and *Gli-α12*, with both being matched to the 2-DE spot 66 (Table 4 and Table S3).

For the 38 active gliadin genes with positive 2-DE spot matching (Table 4), they were assigned to different *Gli* loci based on *Gli* locus information of the matched 2-DE spots (Table 3). For *Gli-α7*, *Gli-α8*, *Gli-α18* and *Gli-α25*, which did not have positive matching, PCR mapping, aided with gene specific primers and the six *Gli* locus deletion lines, was conducted to assign them to specific *Gli* loci. The results showed that both *Gli-α7* and *Gli-α8* were from *Gli-A2*, with *Gli-α18* and *Gli-α25* being from *Gli-B2* and *Gli-D2*, respectively (Table 4).

PCR mapping was also undertaken to validate *Gli* locus assignment of the active genes based on matched 2-DE spots. We selected 10 α -gliadin (*Gli-α1*, *-α4*, *-α5*, *-α9*, *-α10*, *-α11*, *-α16*, *-α17*, *-α20* and *-α24*), two γ -gliadin (*Gli-γ1* and *-γ11*) and one ω -gliadin (*Gli-ω2*) genes as representatives for this validation test. As anticipated, PCR mapping results for the 13 representative genes agreed with their *Gli* locus assignment based on matching 2-DE spots (Table 4). A typical result of the validation test is shown in Figure S13.

Computation of CD epitopes in gliadin proteins. The protein sequences of the 38 gliadins accumulated in Xiaoyan 81 mature grains were each searched for the presence of 24 CD epitopes previously found in different gliadins⁵. The results are summarized in Tables S4 and S5, and schematically presented in Fig. 2. The 11 γ -gliadins were found to have more diverse and numerous CD epitopes, followed by the five α -gliadins (*Gli-α20* to *Gli-α24*) specified by *Gli-D2* and four ω -gliadins by *Gli-A1* (*Gli-ω1*), *Gli-B1* (*Gli-ω2*) or *Gli-D1* (*Gli-ω4* and *Gli-ω5*); the number of CD epitopes in these 20 gliadins ranged from three to 23. Previous studies have characterized a major celiac-toxic α -gliadin peptide composed of 33 residues and carrying the three epitopes DQ2.5-glia-α1a, DQ2.5-glia-α1b and DQ2.5-glia-α2^{44,45}. Among the α -gliadins by *Gli-D2*, *Gli-α20*, *-α22* and *-α23* carried all of the three epitopes, and *Gli-α21* and *Gli-α24* harbored two of the three epitopes (Fig. 2 and Tables S4). The remaining 18 gliadins contained fewer or no CD epitopes. Specifically, two epitopes were found in each of the eight α -gliadins by *Gli-A2* (*Gli-α1* to *Gli-α6*) or *Gli-B2* (*Gli-α9* and *Gli-α10*); no epitope was present in the seven α -gliadins (*Gli-α11* and *Gli-α17*) by *Gli-B2*, *Gli-α19* by *Gli-D2*, *Gli-δ1* by *Gli-D1*, and *Gli-ω3* by *Gli-B1* (Fig. 2 and Tables S4 and S5).

To sum up, of the 38 gliadins accumulated in Xiaoyan 81 mature grains, 10 did not carry any CD epitope, eight had one or two epitopes in their proteins, and 20 contained more than three epitopes in their proteins. Interestingly, eight of the 10 accumulated CSTT α -gliadins did not contain CD epitopes, whereas the 11 expressed CT α -gliadins all harbored CD epitopes (Fig. 2 and Table S4). Finally, we noticed that the five δ -gliadins, previously reported for bread wheat and *Ae. tauschii*^{28,29}, did not contain CD epitope either (Figure S14).

Investigating the levels of α -gliadins and CD epitopes in DLGliD2. Because the majority of the α -gliadins encoded by *Gli-D2* carried the highly celiac-toxic epitopes (Fig. 2 and Table S4), the deletion line DLGliD2, which lacked *Gli-D2* and the entire suite of α -gliadins specified by it (Table 3), may be useful for

α -gliadin			γ -, δ -, and ω -gliadin		
Active gene	2-DE spot	Assignment to <i>Gli</i> locus ^b	Active gene	2-DE spot	Assignment to <i>Gli</i> locus ^b
<i>Gli-α1</i>	50, 51	<i>Gli-A2</i> (SA + PM)	<i>Gli-γ1</i>	1–3	<i>Gli-A1</i> (SA + PM)
<i>Gli-α2</i>	50, 51	<i>Gli-A2</i> (SA)	<i>Gli-γ2</i>	4–9	<i>Gli-A1</i> (SA)
<i>Gli-α3</i>	61–63	<i>Gli-A2</i> (SA)	<i>Gli-γ3</i>	4–9	<i>Gli-A1</i> (SA)
<i>Gli-α4</i>	52, 57	<i>Gli-A2</i> (SA + PM)	<i>Gli-γ4</i>	12–16, 22, 23	<i>Gli-B1</i> (SA)
<i>Gli-α5</i>	54–56, 60	<i>Gli-A2</i> (SA + PM)	<i>Gli-γ5</i>	17–20	<i>Gli-B1</i> (SA)
<i>Gli-α6</i>	49, 53, 58, 59	<i>Gli-A2</i> (SA)	<i>Gli-γ6</i>	21	<i>Gli-B1</i> (SA)
<i>Gli-α7</i>	NMF ^a	<i>Gli-A2</i> (PM)	<i>Gli-γ7</i>	24–26	<i>Gli-B1</i> (SA)
<i>Gli-α8</i>	NMF	<i>Gli-A2</i> (PM)	<i>Gli-γ8</i>	30–33	<i>Gli-D1</i> (SA)
<i>Gli-α9</i>	64	<i>Gli-B2</i> (SA + PM)	<i>Gli-γ9</i>	34–37	<i>Gli-D1</i> (SA)
<i>Gli-α10</i>	65, 76	<i>Gli-B2</i> (SA + PM)	<i>Gli-γ10</i>	38–39	<i>Gli-D1</i> (SA)
<i>Gli-α11</i>	66	<i>Gli-B2</i> (SA + PM)	<i>Gli-γ11</i>	40–42	<i>Gli-D1</i> (SA + PM)
<i>Gli-α12</i>	66	<i>Gli-B2</i> (SA)			
<i>Gli-α13</i>	67	<i>Gli-B2</i> (SA)	<i>Gli-δ1</i>	29	<i>Gli-D1</i> (SA)
<i>Gli-α14</i>	73	<i>Gli-B2</i> (SA)			
<i>Gli-α15</i>	68, 71, 72	<i>Gli-B2</i> (SA)	<i>Gli-ω1</i>	10	<i>Gli-A1</i> (SA)
<i>Gli-α16</i>	70	<i>Gli-B2</i> (SA + PM)	<i>Gli-ω2</i>	27	<i>Gli-B1</i> (SA + PM)
<i>Gli-α17</i>	69, 74, 75	<i>Gli-B2</i> (SA + PM)	<i>Gli-ω3</i>	11	<i>Gli-B1</i> (SA)
<i>Gli-α18</i>	NMF	<i>Gli-B2</i> (PM)	<i>Gli-ω4</i>	43–48	<i>Gli-D1</i> (SA)
<i>Gli-α19</i>	77	<i>Gli-D2</i> (SA)	<i>Gli-ω5</i>	43, 44	<i>Gli-D1</i> (PM)
<i>Gli-α20</i>	83	<i>Gli-D2</i> (SA + PM)			
<i>Gli-α21</i>	80	<i>Gli-D2</i> (SA)			
<i>Gli-α22</i>	78	<i>Gli-D2</i> (SA)			
<i>Gli-α23</i>	79	<i>Gli-D2</i> (SA)			
<i>Gli-α24</i>	81, 82	<i>Gli-D2</i> (SA + PM)			
<i>Gli-α25</i>	NMF	<i>Gli-D2</i> (PM)			

Table 4. Matching between active gliadin genes and 2-DE spots and *Gli* locus assignment of the active genes. ^aNMF, no match found. ^bBased on 2-DE spot assignment (SA) (Table 3), PCR mapping (PM) or both (SA + PM).

developing wheat lines with reduced celiac toxicity. This led us to examine the levels of α -gliadins and CD epitopes in DLG1D2. The flour samples, milled from the grains of DLG1D2 and its progenitor Xiaoyan 81 harvested from two years (2014 and 2015), were used in this set of experiments. As shown in Fig. 3, for both Xiaoyan 81 and DLG1D2 and in both years, the content of α -gliadins was significantly higher than that of γ -gliadins or ω -gliadins, with the level of ω -gliadins being the lowest. The content of α -gliadins was significantly reduced in DLG1D2 in both years compared to that of Xiaoyan 81, but the levels of γ - and ω -gliadins did not differ considerably between the two lines. The total amount of gliadins in DLG1D2 was also decreased relative to that of Xiaoyan 81, with the decrease reaching the significance level in 2015 (Fig. 3).

The monoclonal antibody G12, raised against the highly celiac-toxic 33-mer peptide, offers an effective means for assessing the level of CD epitopes^{46,47}. We therefore used a G12 based immunoassay (see Methods) to investigate the level of CD epitopes in DLG1D2. In both 2014 and 2015, the level of CD epitopes was significantly decreased in DLG1D2 relative to that in Xiaoyan 81, with the reduction exhibited by DLG1D2 exceeding 20% in 2015 (Fig. 4).

Discussion

The high multiplicity and similarity of gliadins and the complexity of bread wheat genome have imposed many difficulties for efficiently dissecting the expression and functionalities of different gliadins. Here, we conducted a genome-wide characterization of bread wheat gliadins and derivative CD epitopes by combining RNA sequencing, proteomic and bioinformatic approaches. The new insights gathered and their implications for future research are discussed below.

Efficient identification of gliadin genes expressed in bread wheat. Two outstanding questions in the genetic and genomic studies of gliadins are (1) how many gliadin genes exist in the bread wheat genome and (2) how many of them are expressed during grain development. To date, neither of the two questions has been addressed satisfactorily. None of the six *Gli* loci was adequately sequenced and annotated in the draft genome sequence of CS²⁰. Although the transcription of multiple gliadin genes in developing wheat grains was noted in a recent transcriptomic study¹⁹, the *Gli* locus location and protein products of the transcribed gliadin genes were not systematically determined. The combination of EST sequencing and proteomic analysis has improved the understanding on the gliadins expressed in the bread wheat cultivar Butte 86, but the cDNA (mRNA) sequences

Figure 2. A diagram illustrating the CD epitopes computed for the 38 gliadins accumulated in Xiaoyan 81 mature grains. The six *Gli* loci (*Gli-A1*, *-B1*, *-D1*, *-A2*, *-B2* and *-D2*) specifying the different gliadins are presented. The four filled red boxes with varying width indicate one to four copies of CD epitopes, respectively. The CSTT α -gliadins (Gli- α 9 to α 17 and Gli- α 19) are marked in blue. The CD epitopes examined are listed in Tables S4 and S5.

Figure 3. Comparison of gliadin accumulation level in the grains between Xiaoyan 81 and the deletion line DLGliD2 harvested from two different years (2014 and 2015). The gliadins were separated and quantified using reverse phase high performance liquid chromatography, with the resultant values shown as means $\pm$ SD. The columns marked by different letters are statistically different ($P < 0.05$, one-way ANOVA with Bonferroni correction).

constructed for different gliadins are still largely incomplete^{17,18}. None of the eight ω -gliadin gene sequences constructed had a complete coding region; eight α -gliadin and two γ -gliadin gene sequences were also incomplete¹⁸.

In this work, we achieved a genome-wide characterization of the gliadins accumulated in bread wheat grains by combining transcriptomic and proteomic approaches and with the aid of six newly prepared *Gli* locus deletion lines. Our transcriptomic analysis made use of the advantages of both second generation RNA sequencing (i.e., high throughput and accurate nucleotide sequence data) and third generation RNA sequencing (long read capacity and efficient construction of full-length transcripts) platforms. Furthermore, to maximize the chance to catch the whole set of gliadin transcripts, we conducted two separate PacBio RNA sequencing experiments using the RNAs extracted from pooled developing grains or those at 25 DAF in which the prolamins genes were strongly

Figure 4. Analysis of CD epitope level in the grains of Xiaoyan 81 and the deletion line DLGliD2 harvested from two different years (2014 and 2015). The level of CD epitopes was determined using the G12 based immunoassay. The values shown are means $\pm$ SD. The asterisk indicates statistical significance ($P < 0.05$, independent samples t-test) from the mean of Xiaoyan 81.

transcribed (Figures S1, S3, S5 and S6). This led to the identification of 52 unique full-length transcripts for four types of gliadins (i.e., α -, γ -, δ - and ω -gliadins), with 42 of them being active members. To our knowledge, this has not been achieved in previous studies on wheat gliadins.

Finding the protein products encoded by active gliadin genes from individual *Gli* loci has been a difficult task, because 1) multiple gliadin members from different loci are expressing in the same endospermic tissue, and 2) gliadins of the same type are often highly similar in both molecular mass and amino acid composition^{17,18,39–43}. Here, this problem was dealt with by comparative proteomic analysis between Xiaoyan 81 and six *Gli* locus deletion mutants. In agreement with past studies^{17,18}, we found that analysis of the 2-DE spots digested with chymotrypsin and thermolysin separately generated more informative peptides for gliadin identification. But unique from previous studies, we employed a set of deletion lines each lacking one of the six *Gli* loci, which permitted assignment of the identified gliadin spots to *Gli-A1*, *-B1*, *-D1*, *-A2*, *-B2*, or *-D2*. These *Gli* locus assigned 2-DE spots were then matched to 38 of the 42 active gliadin genes (full-length transcripts) revealed by PacBio RNA sequencing. And as such, the majority of the active gliadin genes were duly assigned to individual *Gli* loci. This assignment was verified to be correct by PCR mapping of 13 representative gliadin genes. The procedure outlined above was not successful for the four α -gliadin genes (*Gli- α 7*, *Gli- α 8*, *Gli- α 18* and *Gli- α 25*) without matching 2-DE spots. But with the availability of full-length transcript sequence information, the four genes were readily assigned to their hosting loci through PCR mapping.

The applications of full-length transcripts and *Gli* locus deletion lines are of central importance in promoting the efficient identification of gliadin genes expressed in our experimental variety. Apart from the active genes, we also identified full-length transcripts for 10 gliadin pseudogenes. Considering recent findings on the important roles of pseudogenes in regulating active gene expression^{48,49}, the 10 pseudogenes may be valuable for future studies of the mechanisms controlling gliadin gene expression.

A genome-wide view of the gliadins accumulated in bread wheat grains. Based on the data obtained in this work, we suggest that, during the grain development of Xiaoyan 81, 52 gliadin genes are transcribed, 42 of them carry intact coding sequence, and at least 38 of them have their proteins accumulated in the mature grains. The 38 expressed members include 21 α -, 11 γ -, 1 δ -, and 5 ω -gliadins. Clearly, the number of genes expressed is highest for α -gliadins, intermediate for γ -gliadins and relatively low for δ - and ω -gliadins. This is consistent with the finding that the content of α -gliadins in the flour is highest compared with that of γ - or ω -gliadins, and that the content of γ -gliadins is higher than that of ω -gliadins (Fig. 3). Although the transcript levels of the 11 γ -gliadin genes were generally higher than those of the 25 α -gliadin genes (Figures S1 and S3), this transcriptional difference was not sufficiently large to raise the content of γ -gliadins to that of α -gliadins. Clearly, the expression of more gene members is an important contributor to the very high accumulation level of α -gliadins in Xiaoyan 81 grains.

At the locus level, the number of gliadins accumulated in mature grains is four (*Gli-A1*), six (*Gli-B1*), seven (*Gli-D1*), six (*Gli-A2*), nine (*Gli-B2*) and six (*Gli-D2*), respectively. For the three subgenomes, the number of accumulated gliadins is highest for subgenome B (15), followed by subgenomes D (13) and A (10). Subgenome D expresses all four types of gliadins (α , γ , δ and ω), whereas subgenomes A and B express only α -, γ - and ω -gliadins. For the four α -gliadin genes (*Gli- α 7*, *Gli- α 8*, *Gli- α 18* and *Gli- α 25*) that did not have matching 2-DE spots, their protein products may not be present in the mature grains, or accumulated to a very low level if present. This is possible because the transcript levels of the four genes were generally and comparatively lower than other active α -gliadin genes (Figure S1).

Previously, Dupont and coauthors identified the presence of 23 α -gliadins, 13 γ -gliadins and seven ω -gliadins in the flour of the spring wheat variety Butte 86 by integrating EST analysis with proteomic approach¹⁸. However, their efforts to match gliadin genes with 2-DE spots might be complicated by the fact that many of the gliadin coding sequences constructed with ESTs were incomplete. Inclusion of the polypeptides deduced from incomplete coding sequences may compromise the matching of 2-DE spots to active gliadin genes because some of the partial coding sequences may be transcribed from gliadin pseudogenes. Many past studies have found the transcripts derived from gliadin pseudogenes^{39–42}. According to our data, 19.2% of the gliadin full-length transcripts (i.e., 10 out of 52) were transcribed from pseudogenes. Consequently, in this work, we matched 2-DE spots to only the proteins deduced from 42 active gliadin genes, thus increasing the accuracy in matching active gliadin genes to their protein products.

It is interesting to note that α -gliadins can be divided into CT and CSTT groups (Figure S2). Remarkably, the α -gliadins specified by *Gli-B2* all belonged to the CSTT group, whereas the majority of the α -gliadins by *Gli-A2* or *-D2* belonged to the CT group. The CSTT α -gliadins are also present in other wheat genotypes and Triticeae species based on previous studies^{25,39,50,51}. Consequently, further work is warranted to investigate the evolutionary and functional differences between CT and CSTT α -gliadins (see also below).

Similar to past studies^{39,41,52,53}, this work identified one α - (Gli- α 25) and two γ -gliadins (Gli- γ 5 and Gli- γ 10) carrying additional cysteine residue apart from the conserved ones, and two ω -gliadins (Gli- ω 2 and Gli- ω 5) each containing a cysteine residue (Table 1). Moreover, we found that Gli- γ 5, Gli- γ 10, Gli- ω 2 and Gli- ω 5 accumulated in the mature grains (Table 4), thus paving the way for their functional analysis in further research.

Systematic mapping of CD epitopes in the gliadins accumulated in an elite bread wheat variety. Consistent with previous reports^{5,10,24–27}, more numerous and diverse CD epitopes were detected in all γ -gliadins and many ω -gliadins, with the most celiac-toxic epitopes (DQ2.5-glia- α 1a, DQ2.5-glia- α 1b and DQ2.5-glia- α 2) found in the majority of the α -gliadins specified by *Gli-D2* (Fig. 2 and Tables S4 and S5). The finding of less CD epitopes in the α -gliadins by *Gli-A2* was also in line with what was reported in the past^{24,25}. However, we made two new observations. First, the 10 accumulated CSTT α -gliadins, including nine specified by *Gli-B2*, carried very few or no CD epitopes (Fig. 2 and Table S4). Second, the δ -gliadins identified in this work and by previous studies harbored no CD epitopes (Fig. 2, Table S5 and Figure S14). So for future development of the wheat lines beneficial to celiac patients, γ -gliadins, ω -gliadins and the CT group of α -gliadins (especially those by *Gli-D2*) should be removed (see also below). The CSTT α -gliadins and δ -gliadins may be kept to aid the likely function of gliadins in the end-use traits of wheat grains.

Implications for future research. The data gathered in this work and the points discussed above have several implications for further research on wheat gliadins. First, the strategy for genome-wide characterization of gliadins detailed in this work should also be applicable for analyzing gliadin gene expression in other wheat genotypes. This is possible because PacBio transcriptome sequencing is becoming more and more effective^{54,55}, and the method we used for isolating *Gli* locus deletion mutants is relatively easy to adopt.

Second, the 52 unique full-length transcripts and the 38 expressed gliadins identified by this work encompass the different types of gliadins known to exist in different wheat species and genotypes. They may be used as basic reference materials for future studies on (1) gliadin gene transcription and protein expression in different wheat materials, and (2) the roles of individual gliadins in CD and other gliadin related illnesses. As multiple wheat types are cultivated and consumed in different regions of the world^{1,2}, and gliadin composition differs widely among wheat genotypes^{7,8}, systematic studies of gliadin gene expression and functionalities will be important for continuously optimizing the health promotion effects of wheat grains. These studies will benefit from the sets of gliadin transcript and protein sequences reported by this work. Moreover, the six *Gli* locus deletion lines prepared by us may also aid studies on the minor gliadin loci (i.e., *Gli-A3*, *-A5*, *-A6*, *-B3*, *-B5*, *-D4* and *-D6*)⁶, for which there is still little structural and functional formation available.

Third, our insight on the distribution of CD epitopes in different gliadins and the significant reduction of celiac-toxic α -gliadins in the deletion line lacking *Gli-D2* may stimulate more efforts for developing the wheat lines with enhanced health benefits. A number of transgenic studies have shown that silencing gliadin gene expression may facilitate the development of wheat lines beneficial to the individuals affected by CD and other gliadin related illnesses^{56–58}. An alternative, non-transgenic approach in this direction is to selectively remove the undesirable gliadin genes through mutagenesis. Our analysis reveals that many gliadins (i.e., 18 out of the 38 accumulated gliadins) do not carry, or contain very few, CD epitopes (Fig. 2 and Tables S4 and S5). Furthermore, the *Gli-D2* locus specifying the highly celiac-toxic gliadins can be removed without affecting the overall agronomic performance of bread wheat. Thus, the number of genes encoding harmful gliadin species is limited, and the development of non-transgenic wheat lines lacking undesirable gliadins but with improved health benefits is possible through mutagenesis and molecular breeding. In this context, the *Gli* locus deletion lines created by us may find useful applications because optimization of gliadin composition in bread wheat may best start from a genotype lacking one or more of the six *Gli* loci. However, to develop a wheat line completely free of CD epitopes is still difficult at present, because HMW-GSs and LMW-GSs, which are indispensable for wheat end-use quality control, also carry some CD epitopes^{5,10}. In the long term, as gene editing technologies become more and more powerful⁵⁹, it may be possible to eliminate CD epitopes from both gliadins and glutenins through genome engineering. Thus, the resources generated in this work may also help future genome editing studies of complex gliadins.

Finally, aside from eliciting CD, some gliadins also contribute to the occurrence of wheat allergy (WA)¹⁰. For example, ω 5-gliadins have been found to be an important cause of the serious food allergy wheat-dependent excise-induced anaphylaxis^{60,61}. There is also emerging evidence for the involvement of gliadins in a newly recognized gluten-dependent disorder termed nonceliac gluten sensitivity (NCGS) that affects 0.6 to 6% of the human

population^{10,62}. Consequently, the gliadin resources generated in this work may additionally help future studies on WA and NCGS related disorders.

In summary, this work shows that the combination of third generation of RNA sequencing, proteomic analysis and bioinformatic investigation is highly effective for resolving complex gliadins and derivative CD epitopes in bread wheat. The new insights and resources generated here may stimulate and facilitate more systematic studies of gliadin functions in CD and other wheat food related disorders and the development of healthier wheat in the future.

Methods

Plant materials. Xiaoyan 81 and the six *Gli* locus deletion lines were cultivated in the field^{30,38}. At 25 DAF, the grains of Xiaoyan 81 were collected, and used for total RNA purification as described previously³⁸. The mature grains of the seven lines were harvested, cleaned, and stored at 4 °C until use. The ion beam mutant population of Xiaoyan 81 used for isolating *Gli* locus deletion lines was reported in our previous study³⁰. The six NT lines (N1AT1D, N1BT1D, N1DT1A, N6AT6D, N6BT6A and N6DT6B) and its WT progenitor CS were also described previously^{63,64}.

PacBio RNA sequencing and data analysis. We performed the third RNA sequencing experiment (RSE3) of Xiaoyan 81 developing grains collected at 25 DAF using PacBio RSII following the protocol detailed before³⁸. In brief, three cDNA libraries (1–2 kb, 2–3 kb and >3 kb) were constructed and sequenced (Table S1). The subreads were processed, followed by FLNC reads identification and error correction using Illumina HiSeq transcriptomic reads generated in RSE2. Non-redundant transcripts were identified from the FLNC reads, and then mapped against the draft genome sequence of CS, with the corresponding loci divided into two types (extant and newly identified, Table S1). Finally, full-length transcripts of gluten genes in the FLNC reads were searched using local BlastN as reported previously³⁸.

Examining the transcriptional profile of gliadin genes. The transcriptional profile of the 42 active gliadin genes at 0, 10, 15 and 25 DAF was examined using the HiSeq transcriptomic data obtained in RSE2. HTSeq-count was employed to obtain the reads mapped to individual gliadin genes, with the transcript level expressed as reads per kilobase per million mapped reads (RPKM)⁶⁵.

Screening mutant population by MALDI-TOF-MS. Each gliadin extract was prepared using one half of the grain (lacking the embryo end). It was crushed into powder, followed by extraction in 1 ml of 70% ethanol for 40 min at room temperature (RT). The suspension was centrifuged (at 13,500 g) for 5 min at RT, and 200 µl supernatant was kept. The supernatant was centrifuged again for 5 min at RT, with 5 µl of the extract used for MALDI-TOF-MS analysis in an Autoflex MALDI-TOF mass spectrometer (Bruker Daltonics, Billerica, MA, USA). The processing of gliadin extract for MS analysis and calibration of mass spectra were conducted following the manufacturer's instructions. The gliadin MS peaks of Xiaoyan 81 were compared to those of CS and six NT lines to infer their control by specific chromosomes (Figure S8). During the screening of M2 seeds, a positive sample (derived from the non-embryo half of a cut grain) was judged by missing one or more gliadin MS peaks. The corresponding embryo half of the positive grain was germinated and the resultant plant was raised to produce M3 seeds. The M3 seeds were again screened by MALDI-TOF-MS to validate inheritance of the desired mutation. Six non-redundant mutants, collectively lacking all 10 gliadin MS peaks of Xiaoyan 81, were used for developing the six *Gli* locus deletion lines (i.e., DLGliA1, DLGliB1, DLGliD1, DLGliA2, DLGliB2 and DLGliD1, Table 2). Three cycles of backcrossing (with Xiaoyan 81 as recurrent parent) were carried out to reduce background mutations. The *Gli* locus lacked in each of the six deletion lines was confirmed by examination using chromosome specific microsatellite markers (see below).

Examination of six *Gli* locus deletion lines using microsatellite markers. Genomic DNA samples were prepared from the leaf tissues of Xiaoyan 81 and the six deletion lines, and used for microsatellite marker analysis following the methods described previously⁶⁴. Because it is known that the six *Gli* loci are located on 1AS, 1BS, 1DS, 6AS, 6BS and 6DS, respectively^{6–8}, we selected only the microsatellite markers located on these chromosomal arms for our analysis. Specifically, the microsatellite markers selected from the six arms were 5, 4, 6, 8, 7 and 6, respectively. The chromosomal positions of these markers and the nucleotide primers used for amplifying them are listed in Table S6.

Proteomic analysis of gliadins. The gliadin samples prepared from the mature grains of Xiaoyan 81 and those of the six *Gli* locus deletion lines were comparatively analyzed in this series of experiments. The manipulations were mostly conducted at RT unless otherwise stated. Each gliadin sample was prepared using 5 half grains (embryo end removed), which were ground into a fine powder in liquid nitrogen. The powder was extracted 3 times with the RAG solution (0.4 M NaCl in 0.067 M KNaHPO₄ buffer, pH 7.6) to remove albumins and globulins. For each extraction, 1 ml of RAG solution was used, the suspension was shaken for 10 min followed by centrifugation for 10 min at 13,500 g, and the supernatant was discarded. The pellet was then extracted twice using 70% ethanol to prepare gliadins. In each gliadin extraction, 500 µl of 70% ethanol was used, the suspension was agitated for 1 h followed by centrifugation for 10 min at 13,500 g, and the supernatant was kept. The combined supernatant was freeze-dried, with the resultant protein sample being redissolved in 250 µl lysis solution [7 M urea, 2 M thiourea, 40 mM DTT, 4% (w/v) CHAPS, and 2% (v/v) immobilized pH gradient buffer]. The protein solution was kept for 1 h with vortexing every 10 minutes, followed by centrifugation for 20 min at 15,800 g. The supernatant was transferred to a new tube, and its protein concentration was determined using the 2-D Quant Kit (GE Life Sciences, Beijing, China). Subsequently, the gliadin samples were subjected to 2-DE separation following the instructions detailed in the Handbook of 2-D Electrophoresis (Amersham Biosciences UK Ltd,

Buckinghamshire, UK). The first dimension was conducted using Immobiline DryStrip gels (IPG strips) with a linear pH gradient of 3–10 (24 cm) or 6–11 (18 cm). The second dimension was accomplished in 12% SDS-PAGE. For each gliadin sample, at least 5 different 2-DE separations were executed, with the amount of gliadins used varying from 150–350 µg in order to maximize the resolution of individual protein spots.

The protein spots were excised from 2-DE gels, and digested by chymotrypsin or thermolysin following the methods detailed previously^{17,18}. MS/MS analysis of the digests was conducted on a nano LC-LTQ-MS/MS platform (Thermo Scientific, Waltham, MA). The resulting MS/MS data were analyzed in two ways. First, for investigating the protein identity of each excised spot, a Wheat_Gluten database was constructed with the protein sequences obtained by searching NCBI nr database using the key words ‘prolamin’, ‘gliadin’, ‘gluten’, ‘glutenin’, ‘storage’, ‘avenin’, ‘hordein’ and ‘secalin’ according to a previous study⁶⁶. Redundant sequences in the resultant database were removed using the program Cd-hit⁶⁷. The MS spectra obtained in this work were interrogated against the Wheat_Gluten database using both MASCOT and SEQUEST search engines in the Proteome Discoverer software (Version 1.4, Thermo Scientific, Bremen, Germany). Carbamidomethylation of cysteine and oxidation of methionine were used as variable residue modifications. The following parameters were used during the search. MS/MS extraction: 300–6000 Da; Peak count > 3; Intensity > 50; Maximum missed cleavage: 2; q value < 0.01; Precursor mass tolerance: 3 Da; Fragment mass tolerance: 0.8 Da. This analysis identified the excised 2-DE spots as different types of gliadins or LMW-GSs. Second, for finding correspondence between the active gliadin genes of Xiaoyan 81 and the 2-DE gliadin spots, another database (Xiaoyan 81_Gluten) was assembled by combining the 58 gluten proteins of Xiaoyan 81 and the common contaminant proteins provided by the software MaxQuant documentation (<http://www.coxdocs.org/doku.php?id=:maxquant:start>). The 58 proteins of Xiaoyan 81 were deduced from the active gluten gene transcripts (5 coding for HMW-GSs, 11 for LMW-GSs and 42 for gliadins) uncovered by our previous study³⁸. The MS spectra of the gliadin spots were searched against Xiaoyan 81_Gluten database as described above. Positive correspondence was based on the presence of unique peptide as well as coverage of the target protein by the MS peptides.

PCR mapping of gliadin genes. Gene specific primers (listed in Table S7) were designed for each of the 17 gliadin genes subjected to PCR mapping. The DNA templates used for the mapping experiment were prepared from Xiaoyan 81 and the six *Gli* locus deletion lines. The method for extracting genomic DNA and the conditions of PCR were described previously^{63,64}.

Bioinformatic analysis of CD epitopes. The deduced proteins of the 38 gliadins accumulated in Xiaoyan 81 mature grains were each examined for the presence of the 24 CD epitopes compiled for gliadins⁵. The examination was carried out using the “search for sequence” function of the DNAMAN software (Lynnon Biosoft, Lynnon Corporation, <http://www.lynnon.com>).

Measuring gliadin and CD epitope levels in Xiaoyan 81 and DLGliD2. The grains of Xiaoyan 81 and DLGliD2, harvested from the experimental farm in 2014 and 2015, respectively, were milled, with the resulting flour samples used for assaying the gliadin and CD epitope levels. The assessment of gliadins was accomplished with reverse phase high performance liquid chromatography (RP-HPLC) following the method described in our previous work³⁰. The levels of CD epitopes in the flour samples were determined using an AgraQuant® Gluten G12 Assay kit (Romer Labs UK Ltd., Cheshire, UK), with some modifications in preparing the gluten sample. Briefly, each gluten sample was prepared by suspending 100 mg flour in 2.5 ml extraction buffer (supplied with the kit). The suspension was shaken vigorously, followed by incubation at 50 °C for 40 minutes in a laboratory vortex. After the incubation, the suspension was cooled to RT, and 7.5 ml of 80% ethanol was added. The suspension was again shaken for 60 minutes at RT with a laboratory rotator. Afterwards, a centrifugation (5 min, 12,000 g) was applied, and the supernatant was kept. The supernatant was diluted with the sample dilution buffer supplied in the kit before being used in the assay. The remaining steps of the assay were finished following the instructions of the supplier. In these experiments, three separate samples were analyzed for both Xiaoyan 81 and DLGliD2 and for each year, with the resultant data expressed as means ± SD. Statistical analysis of the data was accomplished by one-way ANOVA (with genotype as factor and Bonferroni correction) or independent samples t-test in the SPSS program (SPSS Inc., Chicago, IL, USA).

Data availability. The 197,709 FLNC reads produced in this study have been deposited in the BioProject database of National Center for Biotechnology Information (accession number SRR4279819).

References

1. Food and Agriculture Organization of the United Nations Statistics Division. *FAOSTAT*, <http://faostat3.fao.org/download/Q/QC/E> (2016).
2. International Maize and Wheat Improvement Center (CIMMYT). *Wheat Research*, <http://www.cimmyt.org/global-wheat-research/> (2016).
3. Inomata, N. Wheat allergy. *Curr. Opin. Allergy Clin. Immunol.* **9**, 238–243 (2009).
4. Sapone, A. *et al.* Spectrum of gluten-related disorders: consensus on new nomenclature and classification. *BMC Med.* **10**, 13 (2012).
5. Shewry, P. R. & Tatham, A. S. Improving wheat to remove celiac epitopes but retain functionality. *J. Cereal Sci.* **67**, 12–21 (2016).
6. Shewry, P. R., Halford, N. G. & Lafiandra, D. Genetics of wheat gluten proteins. *Adv. Genet.* **49**, 111–184 (2003).
7. Qi, P. F., Wei, Y. M., Yue, Y. W., Yan, Z. H. & Zheng, Y. L. Biochemical and molecular characterization of gliadins. *Mol Biol (Mosk)* **40**, 796–807 (2006).
8. Juhasz, A., Bekes, F. & Wrigley, C. W. Wheat proteins. In *Applied Food Protein Chemistry* (ed Ustunol, Z.) 219–303 (John Wiley & Sons, Ltd., 2015).

9. Shewry, P. R. Wheat. *J. Exp. Bot.* **60**, 1537–1553 (2009).
10. Scherf, K. A., Koehler, P. & Wieser, H. Gluten and wheat sensitivities - An overview. *J. Cereal Sci.* **67**, 2–11 (2016).
11. Lionetti, E., Gatti, S., Pulvirenti, A. & Catassi, C. Celiac disease from a global perspective. *Best Pract. Res. Clin. Gastroenterol.* **29**, 365–379 (2015).
12. Antvorskov, J. C., Josefsen, K., Engkilde, K., Funda, D. P. & Buschard, K. Dietary gluten and the development of type 1 diabetes. *Diabetologia* **57**, 1770–1780 (2014).
13. Barone, M. V. & Zimmer, K. P. Endocytosis and transcytosis of gliadin peptides. *Mol. Cell Pediatr.* **3**, 8 (2016).
14. Marcussen, T. *et al.* Ancient hybridizations among the ancestral genomes of bread wheat. *Science* **345**, 1250092 (2014).
15. Kawaura, K. *et al.* Genome change in wheat observed through the structure and expression of α/β -gliadin genes. *Funct. Integr. Genomics* **12**, 341–355 (2012).
16. Dong, L. *et al.* Rapid evolutionary dynamics in a 2.8-Mb chromosomal region containing multiple prolamin and resistance gene families in *Aegilops tauschii*. *Plant J.* **87**, 495–506 (2016).
17. Altenbach, S. B., Vensel, W. H. & Dupont, F. M. Analysis of expressed sequence tags from a single wheat cultivar facilitates interpretation of tandem mass spectrometry data and discrimination of gamma gliadin proteins that may play different functional roles in flour. *BMC Plant Biol.* **10**, 7 (2010).
18. Dupont, F. M., Vensel, W. H., Tanaka, C. K., Hurkman, W. J. & Altenbach, S. B. Deciphering the complexities of the wheat flour proteome using quantitative two-dimensional electrophoresis, three proteases and tandem mass spectrometry. *Proteome Sci.* **9**, 10 (2011).
19. Pfeifer, M. *et al.* Genome interplay in the grain transcriptome of hexaploid bread wheat. *Science* **345**, 1250091 (2014).
20. International Wheat Genome Sequencing Consortium. A chromosome-based draft sequence of the hexaploid bread wheat (*Triticum aestivum*) genome. *Science* **345**, 1251788 (2014).
21. Sollid, L. M. Molecular basis of celiac disease. *Annu. Rev. Immunol.* **18**, 53–81 (2000).
22. Stamnaes, J. & Sollid, L. M. Celiac disease: Autoimmunity in response to food antigen. *Semin. Immunol.* **27**, 343–352 (2015).
23. du Pré, M. F. & Sollid, L. M. T-cell and B-cell immunity in celiac disease. *Best Pract. Res. Clin. Gastroenterol.* **29**, 413–423 (2015).
24. Van Herpen, T. *et al.* Alpha-gliadin genes from the A, B, and D genomes of wheat contain different sets of celiac disease epitopes. *BMC Genomics* **7**, 1–13 (2006).
25. Xie, Z. *et al.* Molecular characterization of the celiac disease epitope domains in α -gliadin genes in *Aegilops tauschii* and hexaploid wheats (*Triticum aestivum* L.). *Theor. Appl. Genet.* **121**, 1239–1251 (2010).
26. Wang, S. *et al.* Molecular characterization and dynamic expression patterns of two types of γ -gliadin genes from *Aegilops* and *Triticum* species. *Theor. Appl. Genet.* **125**, 1371–1384 (2012).
27. Salentijn, E. M. *et al.* Celiac disease T-cell epitopes from gamma-gliadins: immunoreactivity depends on the genome of origin, transcript frequency, and flanking protein variation. *BMC Genomics* **13**, 277 (2012).
28. Wan, Y., Shewry, P. R. & Hawkesford, M. J. A novel family of γ -gliadin genes are highly regulated by nitrogen supply in developing wheat grain. *J. Exp. Bot.* **64**, 161–168 (2013).
29. Anderson, O. D., Dong, L., Huo, N. & Gu, Y. Q. A new class of wheat gliadin genes and proteins. *PLoS One* **7**, e52139 (2012).
30. Yang, Y. *et al.* Efficient isolation of ion beam-induced mutants for homoeologous loci in common wheat and comparison of the contributions of *Glu-1* loci to gluten functionality. *Theor. Appl. Genet.* **127**, 359–372 (2014).
31. Roberts, R. J., Carneiro, M. O. & Schatz, M. C. The advantages of SMRT sequencing. *Genome Biol.* **14**, 405 (2013).
32. Sharon, D., Tilgner, H., Grubert, F. & Snyder, M. A single-molecule long-read survey of the human transcriptome. *Nat. Biotechnol.* **31**, 1009–1014 (2013).
33. Zhang, W., Ciclitira, P. & Messing, J. PacBio sequencing of gene families – A case study with wheat gluten genes. *Gene* **533**, 541–546 (2014).
34. Xu, Z. *et al.* Full-length transcriptome sequences and splice variants obtained by a combination of sequencing platforms applied to different root tissues of *Salvia miltiorrhiza* and tanshinone biosynthesis. *Plant J.* **82**, 951–961 (2015).
35. Minoche, A. E. *et al.* Exploiting single-molecule transcript sequencing for eukaryotic gene prediction. *Genome Biol.* **16**, 184 (2015).
36. Koren, S. *et al.* Hybrid error correction and *de novo* assembly of single-molecule sequencing reads. *Nat. Biotechnol.* **30**, 693–700 (2012).
37. Salmela, L. & Rivals, E. LoRDEC: accurate and efficient long read error correction. *Bioinformatics* **30**, 3506–3514 (2014).
38. Dong, L. *et al.* Single-molecule real-time transcript sequencing facilitates common wheat genome annotation and grain transcriptome research. *BMC Genomics* **16**, 1039 (2015).
39. Anderson, O. D. & Greene, F. C. The α -gliadin gene family. II. DNA and protein sequence variation, subfamily structure, and origins of pseudogenes. *Theor. Appl. Genet.* **95**, 59–65 (1997).
40. Anderson, O. D., Hsia, C. C. & Torres, V. The wheat γ -gliadin genes: characterization of ten new sequences and further understanding of γ -gliadin gene family structure. *Theor. Appl. Genet.* **103**, 323–330 (2001).
41. Anderson, O. D., Huo, N. & Gu, Y. Q. The gene space in wheat: the complete γ -gliadin gene family from the wheat cultivar Chinese Spring. *Funct. Integr. Genomics* **13**, 261–273 (2013).
42. Anderson, O. D., Gu, Y. Q., Kong, X., Lazo, G. R. & Wu, J. The wheat ω -gliadin genes: structure and EST analysis. *Funct. Integr. Genomics* **9**, 397–410 (2009).
43. Tatham, A. S. & Shewry, P. R. The S-poor prolamins of wheat, barley and rye: revisited. *J. Cereal Sci.* **55**, 79–99 (2012).
44. Shan, L. *et al.* Structural basis for gluten intolerance in celiac spruce. *Science* **297**, 2275–2279 (2002).
45. Tye-Din, J. A. *et al.* Comprehensive, quantitative mapping of T cell epitopes in gluten in celiac disease. *Sci. Transl. Med.* **2**, 41ra51 (2010).
46. Morón, B. *et al.* Toward the assessment of food toxicity for celiac patients: characterization of monoclonal antibodies to a main immunogenic gluten peptide. *PLoS One* **3**, e2294 (2008).
47. Halbmayr-Jech, E. *et al.* Characterization of G12 sandwich ELISA, a next-generation immunoassay for gluten toxicity. *J. AOAC Int.* **95**, 372–376 (2012).
48. Polisenio, L. *et al.* A coding-independent function of gene and pseudogene mRNAs regulates tumor biology. *Nature* **465**, 1033–1038 (2010).
49. Groen, J. N., Capraro, D. & Morris, K. V. The emerging role of pseudogene expressed non-coding RNAs in cellular functions. *Int. J. Biochem. Cell Biol.* **54**, 350–355 (2014).
50. Altenbach, S. B., Vensel, W. H. & Dupont, F. M. Integration of proteomic and transcriptomic data from a single wheat cultivar provides new tools for understanding the roles of individual alpha gliadin proteins in flour quality and celiac disease. *J. Cereal Sci.* **52**, 143–151 (2010).
51. Li, G. R., Lang, T., Yang, E. N., Liu, C. & Yang, Z. J. Characterization and phylogenetic analysis of α -gliadin gene sequences reveals significant genomic divergence in Triticeae species. *J. Genet.* **93**, 725–731 (2014).
52. Ferrante, P. *et al.* proteomic approach to verify *in vivo* expression of a novel γ -gliadin containing an extra cysteine residue. *Proteomics* **6**, 1908–1914 (2006).
53. Altenbach, S. B. & Kothari, K. M. Omega gliadin genes expressed in *Triticum aestivum* cv. Butte 86: Effects of post-anthesis fertilizer on transcript accumulation during grain development. *J. Cereal Sci.* **46**, 169–177 (2007).
54. Wang, B. *et al.* Unveiling the complexity of the maize transcriptome by single-molecule long-read sequencing. *Nat. Commun.* **7**, 11708 (2016).

55. Abdel-Ghany, S. E. *et al.* A survey of the sorghum transcriptome using single-molecule long reads. *Nat. Commun.* **7**, 11706 (2016).
56. Gil-Humanes, J., Pistón, F., Tollefsen, S., Sollid, L. M. & Barro, F. Effective shutdown in the expression of celiac disease-related wheat gliadin T-cell epitopes by RNA interference. *Proc. Natl. Acad. Sci. USA* **107**, 17023–17028 (2010).
57. Pistón, F., Gil-Humanes, J. & Barro, F. Integration of promoters, inverted repeat sequences and proteomic data into a model for high silencing efficiency of celiac disease related gliadins in bread wheat. *BMC Plant Biol.* **13**, 136 (2013).
58. Altenbach, S. B., Tanaka, C. K. & Seabourn, B. W. Silencing of omega-5 gliadins in transgenic wheat eliminates a major source of environmental variability and improves dough mixing properties of flour. *BMC Plant Biol.* **14**, 393 (2014).
59. Hilscher, J., Bürstmayr, H. & Stoger, E. Targeted modification of plant genomes for precision crop breeding. *Biotechnol. J.* doi: 10.1002/biot.201600173 (2016).
60. Morita, E. *et al.* Fast ω -gliadin is a major allergen in wheat-dependent exercise-induced anaphylaxis. *J. Derm. Sci.* **33**, 99–104 (2003).
61. Palosuo, K. *et al.* A novel wheat gliadin as a cause of exercise-induced anaphylaxis. *J. Allergy Clin. Immunol.* **103**, 912–917 (1999).
62. Fasano, A., Sapone, A., Zevallos, V. & Schuppan, D. Nonceliac gluten sensitivity. *Gastroenterology* **148**, 1195–204 (2015).
63. Yu, C. *et al.* Molecular analysis of phosphomannomutase (PMM) genes reveals a unique PMM duplication event in diverse Triticeae species and the main PMM isozymes in bread wheat tissues. *BMC Plant Biol.* **10**, 214 (2010).
64. Wang, G. F. *et al.* Molecular analysis of common wheat genes encoding three types of cytosolic heat shock protein 90 (Hsp90): functional involvement of cytosolic Hsp90s in the control of wheat seedling growth and disease resistance. *New Phytol.* **191**, 418–431 (2011).
65. Anders, S., Pyl, P. T. & Huber, W. HTSeq - A Python framework to work with high-throughput sequencing data. *Bioinformatics* **31**, 166–169 (2015).
66. Vensel, W. H., Dupont, F. M., Sloane, S. & Altenbach, S. B. Effect of cleavage enzyme, search algorithm and decoy database on mass spectrometric identification of wheat gluten proteins. *Phytochemistry* **72**, 1154–1161 (2011).
67. Li, W. Z. & Godzik, A. Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences. *Bioinformatics* **22**, 1658–1659 (2006).

Acknowledgements

This study was supported by the Ministry of Science and Technology of China (grant 2016YFD0100500), the National Natural Science Foundation of China (grant 31471483) and Chinese Academy of Sciences (grant XDA08020302). The authors are grateful to Drs Susan Altenbach and William Vensel (USDA-ARS, Western Regional Research Center, USA) for constructive advice on proteomic analysis of gliadins.

Author Contributions

D.W., L.D. and K.Z. designed the research. D.-W.W., D.L., J.W., Y.Z., Z.W. and G.Y. performed the experiments. X.L. and H.Q. contributed reagents and computing facility to the work. D.-W.W., D.L., J.W., L.D., K.Z. and D.W. analyzed the data. D.W. and D.-W.W. wrote the paper. All authors read and approved the final manuscript.

Additional Information

Supplementary information accompanies this paper at <http://www.nature.com/srep>

Competing Interests: The authors declare no competing financial interests.

How to cite this article: Wang, D.-W. *et al.* Genome-wide analysis of complex wheat gliadins, the dominant carriers of celiac disease epitopes. *Sci. Rep.* **7**, 44609; doi: 10.1038/srep44609 (2017).

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit <http://creativecommons.org/licenses/by/4.0/>

© The Author(s) 2017