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In search of tetraploid wheat accessions reduced in celiac disease-related gluten epitopes

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Tetraploid wheat (durum wheat) is mainly used for the preparation of pasta. As a result of breeding, thousands of tetraploid wheat varieties exist, but also tetraploid landraces are still maintained and used for local food preparations. Gluten proteins present in wheat can induce celiac disease, a T-cell mediated auto-immune disorder, in genetically predisposed individuals after ingestion. Compared to hexaploid wheat, tetraploid wheat might be reduced in T-cell stimulatory epitopes that cause celiac disease because of the absence of the D-genome. We tested gluten protein extracts from 103 tetraploid wheat accessions (obtained from the Dutch CGN genebank and from the French INRA collection) including landraces, old, modern, and domesticated accessions of various tetraploid species and subspecies from many geographic origins. Those accessions were typed for their level of T-cell stimulatory epitopes by immunoblotting with monoclonal antibodies against the α -gliadin epitopes Gli α -9 and Gli α -20. In the first selection, we found 8 CGN and 6 INRA accessions with reduced epitope staining. Fourteen of the 57 CGN accessions turned out to be mixed with hexaploid wheat, and 5 out of the 8 selected CGN accessions were mixtures of two or more different gluten protein chemotypes. Based on single seed analysis, lines from two CGN accessions and one INRA accession were obtained with significantly reduced levels of Gli α -9 and Gli α -20 epitopes. These lines will be further tested for industrial quality and may contribute to the development of safer foods for celiac patients.

Introduction

Tetraploid wheat is a so-called hard wheat and is generally used for the preparation of pasta, but in Mediterranean countries it is also used for the preparation of various types of breads.¹ Tetraploid wheat has originated from a hybridization between *Triticum urartu* (an A-genome wheat species) and most likely *Aegilops speltoides* (a B-genome wheat species) resulting in a tetraploid species having the AB-genome.^{2–5} Tetraploid wheat domestication occurred in Southeastern Turkey.^{6–10} During thousands of years, cultivated tetraploid species spread throughout Europe and Asia because of human migration. Further cultivation and natural selection resulted in an enormous variation of tetraploid wheats, many of which have been collected in genebank collections as landraces. The features of the landraces depend on the local environmental conditions and on the local food traditions and food preparations. Many landraces are still maintained in some regions because the properties of their flour are considered important for traditional food applications. Landraces are adapted to local climatic conditions, cultural practices, and (partly) to disease and pests.⁷ A landrace may be a mixture of genotypes, which

evolved under the environmental conditions where they were grown because of natural selection and selection by the farmer. Tetraploid wheat can mix up with hexaploid bread wheat very easily under agricultural conditions and care should be taken if the tetraploid wheat should be maintained as a pure genotype.¹¹ As a result, many commercial lots, currently sold as durum wheat, nearly always contain some hexaploid bread wheat.

The typical food-technological characteristics of wheat are related to the presence of gluten proteins. These proteins are water-insoluble, making them especially useful in a wide and still rapidly increasing variety of food applications. Gluten proteins are comprised of gliadins and glutenins, which are present in approximately equal amounts and form 80% of the total storage protein content in the wheat kernel.¹² In tetraploid wheat, the genes encoding gliadins are located on the short arm of chromosomes 1A and 1B (*Gli-1* loci) and on the short arm of chromosomes 6A and 6B (*Gli-2* loci). The *Gli-1* loci represent genes that are tightly linked and mainly encode the γ - and ω -gliadins. The *Gli-2* loci represent tightly clustered genes encoding the α/β - and some γ -gliadins.^{13,14}

Gluten proteins from wheat varieties used for preparation of pasta or bread (but also from rye and barley) can induce celiac disease (CD) in genetically susceptible individuals.¹⁵ CD is characterized by an inflammation of the small intestine. The prevalence is about 1% in the Western (Caucasian) population. The vast majority (~95%) of this group is unaware of having CD because of large-scale underdiagnosis and wrong diagnosis. The incidence of CD appears to be increasing.^{16,17} The only

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cure today is a strict life-long gluten-free diet. However, it is difficult to avoid wheat gluten, because of its rapidly increasing application in an increasing number of food products.¹⁸ The starting point of our research is that any reduction in the total CD-stimulating gluten consumption will contribute to an overall reduction of the prevalence and symptom severity of CD.

Overall, tetraploid wheats contain less T-cell stimulatory α -gliadin epitopes than hexaploid bread wheat because of the absence of the D-genome.^{19,20} The highly immunodominant T-cell stimulating 33-mer^{21,22} is exclusively present in α -gliaadins encoded by the D-genome. In addition, the levels of T-cell stimulatory epitopes have been shown to vary among varieties (van den Broeck *et al.* in press).^{23–26} This opens possibilities to select for wheat varieties with significantly reduced α -gliadin epitope levels, aiming at direct use or to apply in breeding programs directed towards large-scale reduction or even total elimination of CD-stimulating gluten-elements from wheat.

The aim of this study is to analyze, as a first step, the genetic diversity and heterogeneity regarding the presence of T-cell stimulatory epitopes of old and modern tetraploid wheat accessions collected from many geographic origins worldwide.

Results

The gluten protein extracts from all accessions were analyzed by immunoblotting using mAbs against the T-cell stimulatory epitopes GliA- α 9 and GliA- α 20 to identify the epitope sequence-containing gluten proteins (Fig. 1). In 14 out of 57 CGN accessions, an additional set of bands at ~55–60 kDa was observed reacting with both mAbs GliA- α 9 and GliA- α 20 (Fig. 1, proteins indicated by arrows). These proteins are also present in the hexaploid wheat variety Bovictus (Fig. 1, lane B). It was shown before²⁷ that the genes encoding these two proteins, being ω -gliadin/D-type LMW-GS, are located on the short arm of chromosome 1D in 'Chinese Spring' deletion lines. These proteins are also detected in diploid *Triticum tauschii*, containing the D-genome, and in the immunoblot analysis of CIMMYT synthetic hexaploid wheat lines, but are not stained in their tetraploid parent lines (results not shown). Therefore, the accessions showing the two chromosome 1D encoded proteins

are, at least, admixed with hexaploid wheats. Single seeds from accession CGN08006 were used in cDNA sequencing as described by Salentijn *et al.*²⁰ and this confirmed its general tetraploidy by the absence of the D-genome sequences. Such admixtures were detected in wheat accessions originating from Ethiopia, Germany, USSR, Iran, France, and the USA (Fig. 1, lanes 18, 20, 23, 26, 28, 34, 35, 36, 45, 46, 51, 52, 55, and 56, Table 1). Two different seeds present in landrace accession CGN8360 (Fig. 1, lane 34) were confirmed in flow cytometric ploidy level determination to be hexaploid.

Five accessions originating from Egypt shared the same gluten protein pattern for mAb GliA- α 20 but differed in one protein band for mAb GliA- α 9 (Fig. 1, lanes 6, 7, 8, 11, and 12). These accessions probably share the same progenitors, or they may be independently propagated accessions in which small differences have been evolved *e.g.* because of mixtures with (related) genotypes. Accessions CGN16069 from Algeria and CGN16069 from Egypt seemed very similar to each other, as they showed the same patterns on immunoblots for both mAbs and are both mixed with hexaploid wheat (Fig. 1, lanes 55 and 56). For all other accessions, the gluten protein patterns on immunoblots were quite different, even if accessions originate from the same country.

In order to evaluate the presence and the level of T-cell stimulatory epitopes in tetraploid wheat accessions, eight accessions were selected that showed to contain a lower amount of T-cell stimulatory gluten proteins in the overall (seed mixture) protein pattern (Fig. 1, indicated with ▼). The seeds of accessions CGN08006, CGN07975, CGN08173, CGN08339, CGN08379, CGN08397, CGN08407, and CGN16074 were subsequently examined for the presence of different seed phenotypes. The phenotypes of the seeds of accession CGN08006 were diverse in seed color and shape and when the gluten proteins from nine phenotypically different seeds (A to I) were analyzed by immunoblotting, six different gluten protein chemotypes could be detected (Fig. 2). Accession CGN08006 is a landrace collected from Ethiopia which was originally classified as a hexaploid wheat. Another ten landraces from Ethiopia have been analyzed by immunoblotting and six of these were mixed with hexaploid wheat. In contrast, seeds from accessions CGN07975, CGN08339, and CGN08407 showed only one phenotype. Seeds from

Fig. 1 Immunoblot analysis of gluten protein extracts from 57 tetraploid wheat accessions compared to the gluten protein extract of modern hexaploid variety Bovictus. (A) mAb GliA- α 9, (B) mAb GliA- α 20. Brackets group accessions with similar protein patterns. E is emmer wheat. ▼ indicates accessions selected for further analysis. All gluten proteins reacting to the mAbs are stained.

Table 1 Details of tetraploid accessions obtained from CGN Wageningen, The Netherlands

	Acc nr	Acc name	Species	Subspecies	Population type	Type	Collection date	Collection site
1	CGN06223	Lugaymi	<i>T. aestivum</i>	<i>aestivum</i>	L		1968	Saudi Arabia
2	CGN08006	Dibillik Sinde	<i>T. aestivum</i>	<i>aestivum</i>	L		1972	Ethiopia
3	CGN12037	Mate Sinde	<i>T. aestivum</i>	<i>aestivum</i>	L		1972	Ethiopia
4	CGN04221	Black Persian	<i>T. turgidum</i>	<i>carthlicum</i>	L	Spring	1962	Iran
5	CGN06550	Dniepopetrovsk	<i>T. turgidum</i>	<i>durum</i>	B		1965	USSR
6	CGN06551	Gaza1	<i>T. turgidum</i>	<i>durum</i>		Spring		Egypt
7	CGN06552	Gaza 2; Gaya W 277	<i>T. turgidum</i>	<i>durum</i>		Spring	1937	Egypt
8	CGN06553	Gaza 8; Gaza W 277	<i>T. turgidum</i>	<i>durum</i>		Spring	1950	Egypt
9	CGN06555	Kaza kylek	<i>T. turgidum</i>	<i>durum</i>	L			Azerbaijan
10	CGN06567	Hansia Broach	<i>T. turgidum</i>	<i>durum</i>	L	Spring	1915	India
11	CGN06576	Beladi	<i>T. turgidum</i>	<i>durum</i>	L	Spring		Egypt
12	CGN06578	Beladi dakar	<i>T. turgidum</i>	<i>durum</i>			1948	Egypt
13	CGN06582	Gaurani	<i>T. turgidum</i>	<i>durum</i>	L	Spring	1949	India
14	CGN06590	Eretria	<i>T. turgidum</i>	<i>durum</i>	B	Spring	1955	Greece
15	CGN07975	Abessinischer Emmer	<i>T. turgidum</i>	<i>dicoccon</i>		Spring	1965	Ethiopia
16	CGN07989	Beladi	<i>T. turgidum</i>	<i>durum</i>	L	Spring	1939	Egypt
17	CGN07991	St. 472 Ethiopia	<i>T. turgidum</i>	<i>durum</i>	L	Spring	1950	Ethiopia
18	CGN08143	Hongrie 2883	<i>T. turgidum</i>	<i>durum</i>	B	Spring	1965	Portugal
19	CGN08145	Durazio Molar 918	<i>T. turgidum</i>	<i>durum</i>	B	Spring	1965	Portugal
20	CGN08146	Candial	<i>T. turgidum</i>	<i>durum</i>	B	Spring	1949	Portugal
21	CGN08154	Akmalinka 5	<i>T. turgidum</i>	<i>durum</i>	B	Spring	1959	USSR
22	CGN08173	Gharnovka	<i>T. turgidum</i>	<i>durum</i>			1900	USSR
23	CGN08190	Deves; Kamboyra	<i>T. turgidum</i>	<i>durum</i>	L	Spring	1916	Greece
24	CGN08202	Gandum Andojird	<i>T. turgidum</i>	<i>durum</i>			1940	Iran
25	CGN08232	Psathas	<i>T. turgidum</i>	<i>durum</i>	L	Spring	1957	Cyprus
26	CGN08235	Hebda 3	<i>T. turgidum</i>	<i>durum</i>	B	Spring	1960	Algeria
27	CGN08239	Etrusco	<i>T. turgidum</i>	<i>durum</i>		Spring	1961	Italy
28	CGN08240	Capeiti	<i>T. turgidum</i>	<i>durum</i>	B	Spring	1961	Italy
29	CGN08245	Mokhtar II	<i>T. turgidum</i>	<i>durum</i>	L	Spring		Egypt
30	CGN08262	Probstdorfer Pandur; Pandur	<i>T. turgidum</i>	<i>durum</i>	B	Spring	1972	Austria
31	CGN08339	Weisser Sommer	<i>T. turgidum</i>	<i>dicoccon</i>	L	Spring	<1945	Germany
32	CGN08341	Yaroslav Spring Emmer	<i>T. turgidum</i>	<i>dicoccon</i>	L	Spring		USSR
33	CGN08357	Diha	<i>T. turgidum</i>	<i>carthlicum</i>	L	Spring	1964	USSR
34	CGN08360	Diha Dzavakhetskaja	<i>T. turgidum</i>	<i>carthlicum</i>	L	Spring	1959	USSR
35	CGN08361		<i>T. turgidum</i>	<i>carthlicum</i>	L	Spring	1958	USSR
36	CGN08367		<i>T. turgidum</i>	<i>turgidum</i>	L?	Winter		
37	CGN08379	Asa de Corvo 5279	<i>T. turgidum</i>	<i>turgidum</i>	L?	Spring		Portugal
38	CGN08390	LHTAXO 549/65	<i>T. turgidum</i>	<i>polonicum</i>	L?	Spring	1965	
39	CGN08396	Acme	<i>T. turgidum</i>	<i>durum</i>	B	Spring	1963	USA
40	CGN08397	Arnoutka	<i>T. turgidum</i>	<i>durum</i>	B	Spring	1954	USA
41	CGN08398	Golden Ball	<i>T. turgidum</i>	<i>durum</i>	B	Spring	1961	South Africa
42	CGN08407		<i>T. timopheevi</i>	<i>timopheevi</i>		Spring		USSR
43	CGN10468	Vernal	<i>T. turgidum</i>	<i>dicoccon</i>	B	Spring		USA
44	CGN10495		<i>T. timopheevi</i>	<i>timopheevi</i>	L?	Spring	1964	USSR
45	CGN11494	Brkulja	<i>T. turgidum</i>	<i>durum</i>	L	Winter	1949	Yugoslavia
46	CGN12275	Vernal Emmer	<i>T. turgidum</i>	<i>dicoccon</i>	L	Spring	1962	USA
47	CGN12282		<i>T. turgidum</i>	<i>dicoccon</i>			1952	Iran
48	CGN12283	Polba 3	<i>T. turgidum</i>	<i>dicoccon</i>	B	Spring		USSR
49	CGN12287	Tripsiro	<i>T. turgidum</i>	<i>durum</i>		Spring	1921	Libya
50	CGN12293	PI 294478	<i>T. turgidum</i>	<i>polonicum</i>	L?	Spring	1964	France
51	CGN12296	Biskri × Bruteille	<i>T. turgidum</i>	<i>durum</i>	B	Spring	1965	Morocco
52	CGN14995	Sari Bugday	<i>T. turgidum</i>	<i>durum</i>	B	Winter		Turkey
53	CGN16051		<i>T. turgidum</i>	<i>dicoccon</i>		Spring	1964	France
54	CGN16057	Gaza 6; Gaza 277	<i>T. turgidum</i>	<i>durum</i>		Spring	1941	Egypt
55	CGN16068	Saba 22738	<i>T. turgidum</i>	<i>durum</i>	B	Spring	1960	Algeria
56	CGN16069	Durum H-2	<i>T. turgidum</i>	<i>durum</i>		Spring	1939	Egypt
57	CGN16074	WTC 1229/65	<i>T. timopheevi</i>	<i>timopheevi</i>	L?	Spring	1964	USSR

Passport details (CGN, Wageningen, The Netherlands): B is breeder line; L is landrace/traditional cultivar; empty cell means unknown information.

accessions CGN08173, CGN08379, and CGN16074 showed two different phenotypes, but immunoblot protein patterns were the same using both mAbs. Seeds from accession CGN08397 showed four different phenotypes and two different immunoblot protein patterns were detected.

To compare the levels of T-cell stimulatory epitopes present in tetraploid accessions to those in hexaploid wheat varieties,

relative intensities were calculated from the obtained immunoblots of the eight tetraploid CGN accessions (Fig. 3). The calculation of relative intensities is our method to measure the amount of epitopes in the different wheat samples and is the method we use to compare the presence and levels of epitopes in wheat, as it takes into account the fact that there are differences in relative expression levels of proteins and that

Fig. 2 Immunoblot analysis of gluten protein extracts of nine single seeds (A to I) from accession CGN08006. (A) mAb Glia- α 9, (B) mAb Glia- α 20. All gluten proteins reacting to the mAbs are stained.

some may contain multiple copies of an epitope. Intensities of single seed gluten protein extracts were compared to gluten protein extracts of the modern bread wheat variety Toronto and old bread wheat variety Minaret. Results presented in Fig. 3 showed that when using the mAb against Glia- α 9, five of the eight accessions contained lower amounts of T-cell stimulatory epitopes than the hexaploid variety Toronto. Two of these five accessions, CGN08006 and CGN08339, also contained a lower amount of T-cell stimulatory epitopes than the relatively low amount in hexaploid variety Minaret. When using the mAb against Glia- α 20, all eight accessions showed

more than three-fold reduced amounts of T-cell stimulatory epitopes than the hexaploid variety Toronto. Seven accessions contained lower amounts of T-cell stimulatory epitopes than the hexaploid variety Minaret. The lowest value was obtained for accession CGN08006, which was about two-fold lower than the variety Minaret.

Using the same methods as for the 57 CGN accessions, the 46 tetraploid wheats from INRA (Montpellier) were subsequently analyzed by immunoblotting using mAbs against the same T-cell stimulatory epitopes Glia- α 9 and Glia- α 20 to identify the number of proteins present containing these epitope sequences. Lines 45963, 117887, 46516, 84866, 95920, and Neodur contained the lowest amount of T-cell stimulatory epitopes and were therefore compared to CGN08006 and CGN08339 and compared to the hexaploid variety Minaret (Fig. 4). Although the gluten protein patterns on the immunoblot are different, these lines overall do not contain less T-cell stimulatory epitopes than the accessions selected from CGN. A calculation of the relative intensities on the immunoblots (Fig. 4) showed that accessions 84866 and Neodur are comparable to accessions CGN08006 and CGN08339 regarding Glia- α 9 epitopes. Analysis of the Glia- α 20 epitopes showed that Neodur together with CGN08006 are the lowest responders.

Discussion

In this study we analyzed the genetic diversity and heterogeneity regarding the presence of T-cell stimulatory epitopes of old and modern tetraploid wheat accessions collected from many geographic origins world-wide. The most striking result is that even true tetraploid wheat accessions have a level of CD-epitopes that is not orders of magnitude lower than

Fig. 3 Immunoblot analysis and calculated intensities of single seed gluten protein extracts from accessions CGN08006, CGN07975, CGN08173, CGN08339, CGN08379, CGN08397, CGN08407, and CGN16074 compared to hexaploid varieties Toronto and Minaret. (A) mAb Glia- α 9, (B) mAb Glia- α 20. All gluten proteins reacting to the mAbs are stained on the immunoblots.

Fig. 4 Immunoblot analysis and calculated intensities of single seed gluten protein extracts from accessions Minaret, CGN08006, CGN08339, INRA45963, INRA117887, INRA46516, INRA84866, INRA95920, and Neodur compared to the hexaploid variety Minaret. (A) mAb Glia- α 9, (B) mAb Glia- α 20, (C) SDS-PAGE and PageBlue staining. All gluten proteins reacting to the mAbs are stained on the immunoblots.

observed for hexaploid wheat. This may seem surprising, as the D-genome contains many α -gliadins with all epitopes present.^{19,20} On the other hand, the antibodies we used in this study (mAbs against Glia- α 9 and Glia- α 20) detect epitopes that are also present in the A-genome α -gliadins, which may form the majority of the gliadins being expressed in tetraploid wheat varieties.²⁰ There are no antibodies that specifically detect D-genome α -gliadin specific epitopes, such as the 33-mer (Salentijn *et al.* submitted). As a result, the actual differences may be larger than what we detect in this study using immunoblotting with mAbs against T-cell stimulatory epitopes Glia- α 9 and Glia- α 20.

Differences among wheat varieties in gluten proteins occur because of allelic variation (genotype) that determines the gluten protein composition. The approach we used in this study analyzes this genotypic variation by comparing the same amount of gluten protein per accession. Changes in gluten protein composition have been described, but are mainly expected if growth conditions are extreme (high or low temperature, dry or wet conditions).²⁸ The varieties and accessions we have analyzed were grown under normal wheat growth conditions and therefore, their influence on the gluten protein composition is not expected.

Accessions showing different gluten protein patterns with low CD-epitope content can be selected for making crosses to

obtain varieties with different combinations of gluten proteins and reduced numbers of T-cell stimulatory epitopes. To improve dough and baking quality, tetraploid wheats low in T-cell stimulatory epitopes may be highly useful for the construction of synthetic hexaploid bread wheats by hybridization with D-genome species low in T-cell stimulatory epitopes. Baking quality and dough properties of tetraploid wheat can be improved by introduction of or substitution with chromosome 1D^{1,29–33} or by breeding. In addition, baking quality of tetraploid wheat can also be improved by sexual transfer of the HMW-GS from the D-genome (the *Glu-D1* locus) to chromosome 1A without adding the gliadins^{34,35} or by genetic transformation using both the *1Dx5* and *1Dy10* HMW-GS genes.³⁶

The occurrence of different genotypes and even different ploidy levels in a single genebank accession is a complicating phenomenon for genebank managers to accurately characterize landraces. Many landraces often result from maintenance and selection practices by local farmers directed towards optimizations to local agronomic and food applications. As a consequence, genebank passport data turned out to be poor predictors of the real genetic composition of landrace accessions that may be mixtures of genotypes of tetraploid and even hexaploid wheat species. Salentijn *et al.*²⁰ analyzed cDNA sequences of α -gliadins and observed that some accessions described as

tetraploid contained *Gli-D2* transcripts and in one accession described as hexaploid no *Gli-D2* transcript could be found.

It is to be expected that the reduced levels of CD-epitopes, as found among some of the accessions, will not directly result in safe foods to (all) CD-patients. However, we believe that an overall lowering of the 'CD-epitope load' of gluten that is daily consumed by many millions of people world-wide may contribute to delay or even prevent the onset of CD and its symptom development in that part of the population that is genetically susceptible, especially in children³⁷ (van den Broeck *et al.* in press), because the quantity of consumed CD-epitopes is a major factor that may influence the clinical representation of CD, along with some other recognized factors such as the type of cow's milk formulas, omission of breast feeding, and age at gluten introduction.^{38–41} A reduction in overall 'CD-epitope load' might especially help to release the constraint of the still undiagnosed CD-patients (approximately 95% of all CD-patients) who are daily consuming CD-stimulating gluten proteins without realizing its effect on their health and well being. With the present results we show a first and relevant contribution into this direction.

Experimental

Grain samples

A set of 57 tetraploid wheats was obtained from the Centre for Genetic Resources (CGN), the Netherlands (<http://www.cgn.wur.nl/uk/>) (Table 1). The accessions were selected based on collection period and diversity of geographic origin. Some CGN accessions showed heterogeneous seed compositions. In this study, the taxonomy of *Triticum* of Morris and Sears⁴² is followed. A second set of 46 tetraploid wheat accessions was obtained from INRA (Montpellier) that was selected by genetic diversity based on 10 microsatellites

polymorphism and resulted in a core collection as described by David *et al.*⁴³ and Thuillet *et al.*⁴⁴ (Table 2). Accessions not present at INRA were provided by USDA or ICARDA. Seed compositions of INRA accessions were homogeneous. Both sets included *T. turgidum* ssp. *dicoccoides* accessions from the Near East (wild ancestors), domesticated *T. turgidum* ssp. *dicoccon* accessions (domesticated ancestors), domesticated *T. turgidum* ssp. *polonicum* accessions, old *T. turgidum* ssp. *durum* varieties, and elite varieties from the French catalog. For comparison, three hexaploid bread wheat varieties (*T. aestivum* 'Bovictus', 'Toronto', and 'Minaret') have been included.

Accessions CGN06223, CGN08006, and CGN12037 were selected from a previous study on hexaploid wheat accessions (van den Broeck *et al.* in press). Based on the banding pattern, these accessions seemed to be tetraploids instead of hexaploids. Their tetraploidy was confirmed in flow cytometric ploidy level determination of young leaf material.

Gluten protein extraction

Multiple (~10–30) wheat grains of 57 tetraploid landraces obtained from CGN, and single grains of selected landraces were ground in an analytical mill (A 11 Basic, IKA-Werke) and sieved through a mesh (0.5 mm). From 46 tetraploid wheat accessions obtained from INRA (Montpellier) single seeds were used for gluten protein extraction. Gluten proteins were extracted according to van den Broeck *et al.* (2009)⁴⁵ which describes extraction of most of the gluten proteins. Gluten proteins were extracted from 50 mg wheat flour or ground single seeds by addition of 0.5 ml of 50% aqueous iso-propanol with continuous mixing (MS1 Minishaker, IKA Works, Inc.) at 1,000 rpm for 30 min at room temperature, followed by centrifugation at 10,000 rpm for 10 min at room temperature. The residue was re-extracted twice with 50% aqueous iso-propanol/1% DTT/50 mM Tris-HCl, pH 7.5,

Table 2 Details of tetraploid wheat accessions obtained from INRA—Montpellier, France

	Acc nr	Subspecies	Collection site	Type		Acc nr	Subspecies	Collection site	Type
1	45383	<i>dicoccon</i>	Bulgaria	D	24	84866	<i>durum</i>	Syria	O
2	45351	<i>dicoccon</i>	Iran	D	25	95920	<i>durum</i>	Syria	O
3	45239	<i>dicoccon</i>	Italy	D	26	82697	<i>durum</i>	Turkey	O
4	45354	<i>dicoccon</i>	Russia	D	27	82726	<i>durum</i>	Turkey	O
5	45280	<i>dicoccon</i>	Slovakia	D	28	82715	<i>durum</i>	Turkey	O
6	45309	<i>dicoccon</i>	Slovakia	D	29	Brumaire	<i>durum</i>	France	O
7	352365	<i>dicoccon</i>	Germany	D	30	Primadur	<i>durum</i>	France	E
8	355484	<i>dicoccon</i>	Spain	D	31	Neodur	<i>durum</i>	France	E
9	94635	<i>dicoccon</i>	Iran	D	32	IXOs9442	<i>durum</i>	France	E
10	415152	<i>dicoccon</i>	Israel	D	33	Villemur	<i>durum</i>	France	E
11	94648	<i>dicoccon</i>	Italy	D	34	Armet910	<i>durum</i>	France	E
12	113302	<i>dicoccoides</i>	Iran	W	35	Durental	<i>durum</i>	France	E
13	45963	<i>dicoccoides</i>	Jordan	W	36	Lloyd945	<i>durum</i>	France	E
14	46391	<i>dicoccoides</i>	Jordan	W	37	B6Rtchir	<i>durum</i>	Bulgaria	O
15	117887	<i>dicoccoides</i>	Syria	W	38	LRB6R595	<i>durum</i>	Bulgaria	O
16	46470	<i>dicoccoides</i>	Syria	W	39	68405	<i>polonicum</i>	—	D
17	46253	<i>dicoccoides</i>	Turkey	W	40	585015	<i>polonicum</i>	China	D
18	116172	<i>dicoccoides</i>	Turkey	W	41	384266	<i>polonicum</i>	Ethiopia	D
19	467014	<i>dicoccoides</i>	Israel	W	42	14140	<i>polonicum</i>	—	D
20	428133	<i>dicoccoides</i>	Lebanon	W	43	81285	<i>turgidum</i>	—	D
21	46516	<i>dicoccoides</i>	Syria	W	44	7786	<i>turgidum</i>	Ethiopia	D
22	487255	<i>dicoccoides</i>	Syria	W	45	387456	<i>turgidum</i>	Ethiopia	D
23	Kubanka	<i>durum</i>	USSR	O	46	134952	<i>turgidum</i>	Portugal	D

D is domesticated; W is wild; O is old; E is elite.

for 30 min at 60 °C with mixing every 5 to 10 min followed by centrifugation at 10,000 rpm for 10 min at room temperature. After addition of each next extraction solution, the residue was resuspended by shaking in a Fastprep[®] FP220A Instrument for 10 s at a speed of 6.5 m s⁻¹ followed by sonification for 10 min in an ultrasonic bath (Branson 3510, Branson Ultrasonics Corporation). The three obtained supernatants were combined and considered as the total gluten protein extract. The protein content was quantified using Bio-Rad Protein Assay (Bio-Rad Laboratories), based on the Bradford dye-binding procedure, according to the manufacturer's instruction.

SDS-PAGE and immunoblotting

Equal amounts of gluten proteins were separated on SDS-PAGE gels (10%)⁴⁶ using a Hoefer SE 260 mighty small II system (GE Healthcare) followed by staining with PageBlue[™] (Fermentas). Proteins were blotted onto nitrocellulose (0.2 µm, Bio-Rad Laboratories), omitting methanol from the blotting buffer, using a Mini Trans-Blot Cell (Bio-Rad Laboratories) at 100 V for 1 h. Blots were stained using a MemCode[™] Reversible Protein Stain Kit for Nitrocellulose Membranes (Fisher Scientific) prior to incubation with mAbs. Incubation with mAbs specific for T-cell stimulatory epitopes GliA- α 9^{47,48} and GliA- α 20^{47,49} was performed as described.⁵⁰ Antibody binding to the blots was visualized by staining for alkaline phosphatase conjugated secondary antibody, using Nitro Blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Sigma). Blots were scanned using a Bio-Rad GS-710 Calibrated Imaging Densitometer (Bio-Rad Laboratories) and saved as TIFF images. Pixel intensities were calculated per lane using Quantity One software (Bio-Rad Laboratories). The gluten protein extracts of either the modern hexaploid wheat varieties Bovictus or Toronto (having high amounts of T-cell stimulatory epitopes, as analyzed before) and/or the hexaploid wheat variety Minaret (with relatively low amounts of T-cell stimulatory epitopes) were used on each separate immunoblot as an 'inter-gel' control as in van den Broeck *et al.* (in press).

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