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Reactivity of gluten proteins from spelt and bread wheat accessions towards A1 and G12 antibodies in the framework of celiac disease

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ABSTRACT

In the framework of celiac disease, this research aims at evaluating the reactivity of 195 wheat accessions and 240 spelt accessions to A1 and G12 monoclonal antibodies. A great variability in reactivity was found among the accessions of both subspecies. On average, spelt showed very slightly higher reactivity than wheat but accessions with low reactivity were encountered in both subspecies. In both wheat and spelt, there was no significant difference in the level of reactivity between varieties and landraces. Similarly, there was no significant difference in reactivity between old, mid and new varieties of wheat. In contrast, new spelt varieties showed lower levels of reactivity than old and mid ones. No relationship could be established between level of reactivity, protein content and the Zeleny index. This research did not establish a link between the breeding strategies for baking quality improvement and A1-G12 antibodies reactivity.

1. Introduction

Celiac disease (CD) is one of the most common food sensitivities worldwide and may affect from 1 in 100 to 1 in 300 individuals. It is an inflammatory disease of the upper small intestine in genetically susceptible individuals caused by the ingestion of gluten proteins from wheat, spelt, rye and barley and possibly oats.

Among the different proteins of gluten, gliadins have the highest clinical relevance regarding both innate and adaptive immune responses (Ribeiro et al., 2016). The role of the different gliadin types (α/β-type, γ-type and ω-type gliadins) is important and variable in the pathogenesis of celiac disease (Camarca et al., 2009; Tye-din et al., 2010) while T cell-stimulating peptides have been ranked according to immunodominance (Anderson, Degano, Godkin, Jewell, & Hill, 2000; Anderson et al., 2005; Shan et al., 2002; Shan et al., 2005; Tye-din et al., 2010). Among the gliadins, the α-gliadins have the strongest immunogenicity (Camarca et al., 2009) and four T-cell stimulatory epitopes have been identified as responsible for the strong immunogenicity of α-gliadin: two major epitopes (overlapping DQ2.5-glia-α1 and -α2) and two minor epitopes (DQ2.5-glia-α3 and DQ8-glia-α1) (Shan et al., 2002; Molberg et al., 2005). The 33-mer, the main contributor to the immunogenicity of the gluten (Shan et al., 2002), contains six copies of the two major overlapping T-cell epitopes: one copy of the DQ2.5-glia-α1a, two copies of the DQ2.5-glia-α1b and three copies of the DQ2.5-glia-α2 epitope (Sollid, Qiao, Anderson, Gianfrani, & Konig, 2012).

Several studies have demonstrated that the level of immunogenicity of different species of cereals and of different varieties within species was variable (Spaenij-Dekking et al., 2005; Van den Broeck, de Jong et al., 2010; Comino et al., 2012; Suligoj, Gregorini, Colomba, Ellis, & Ciclitira, 2013; Dubois, Bertin, & Mingeot, 2016) and some studies evaluated the impact of breeding on the immunogenicity of varieties (Van den Broeck, de Jong et al., 2010; Comino et al., 2012; Ribeiro et al., 2016; Suligoj et al., 2013; Gélinas & McKinnon, 2016; Kasarda, 2013). Most of the authors used the ELISA technique with different antibodies: Ribeiro et al. (2016) used the RIDASCREEN® based on the R5 monoclonal antibody, Comino et al. (2012) used a competitive ELISA with G12 moAb; Gélinas and McKinnon (2016) used ELISA with R5 and G12 monoclonal antibodies. ELISA kits were conceived to detect very small quantities of gluten in supposedly gluten-free food but

Abbreviations: mAb, monoclonal antibody; CD, celiac disease; LC-MS, liquid chromatography tandem mass spectrometry

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present some limitations. Indeed, some antibodies in the ELISA kit recognize more than one site. Furthermore, the specificity of the antibodies towards epitopes is low: each antibody targets a short stretch of one of the immunogenic epitopes and may detect both the non-immunogenic epitopes variants and the canonical immunogenic form together (Morón et al., 2008). Nevertheless, ELISA is a fast and easy to use tool capable of analysing a high number of accessions at a low cost. This information provides an idea of the diversity involved and allows estimation of the impact of breeding on the evolution of gliadin composition. Van den Broeck, de Jong et al. (2010) used monoclonal antibodies which target DQ2.5-glia-α1a and DQ2.5-glia-α3 to make Western-blottings on wheat and spelt without mismatching. Recently, Van den Broeck, Cordewener, Nessen, America and Van der Meer (2015) and Schalk, Lang, Wieser, Koehler and Scherf (2017) presented a liquid chromatography tandem mass spectrometry (LC-MS) method to detect and quantify the celiac disease-epitopes on several cereals, and Dubois, Bertin, Muhovski, Escarnot, and Mingeot (2017) developed TaqMan probes which specifically target the canonical form of the four major celiac disease epitopes of α-gliadin.

In addition, differences among gliadins and glutenins between spelt and wheat were observed. RP-HPLC reveals a much higher content of total gliadins and a lower content of total glutenins in spelt than in wheat. The gliadin/glutenin ratio is significantly higher in spelt, 3.5, than in wheat, 2 (Wieser, 2000; Koenig, Wieser, & Koehler, 2009). In spelt, alpha-gliadins and gamma-gliadins are predominant while LMW-GS, omega-gliadins and HMW-GS are generally minor components (Wieser, 2000). Gliadins and glutenins from spelt differ in structure from those of common wheat (Harsch, Günther, Kling, Rozynek, & Hesemann, 1997; Radic, Günther, Kling, & Hesemann, 1997; Von Büren, Lüthy, & Hübner, 2000). Spelt storage proteins form gluten with different properties and quality than that of common wheat (Schober, Clarke, & Kuhn, 2002).

As Kasarda (2013) wrote, hard wheats have been selected for higher protein content which is desirable for breadmaking. Thus, some authors analysed the correlations between protein and/or gluten contents and immunogenicity of Triticum (Gélinas & McKinnon, 2016; Ribeiro et al., 2016; Schalk et al., 2017). Indeed, for wheat improvement, end-use quality is one of the major factors. The grain protein content, completed by the Chopin alveograph and baking tests are the best tests to determine the value of the crop. However, the Chopin alveograph and baking tests require large amounts of flours, which are not available in the youngest generations of breeding, and are labour-intensive. Thus breeders have used indirect phenotypical small-scale tests like Zeleny, SDS, Pelshenke or mixograph tests (Oury et al., 2010).

Based on the knowledge displayed above, the present work relies on four questions: 1) Does spelt display a lower reactivity towards the A1 and G12 monoclonal antibodies than wheat? 2) Has breeding increased the reactivity towards the A1 and G12 monoclonal antibodies? 3) Does geographical origin or habit impact on the reactivity towards the A1 and G12 monoclonal antibodies? and 4) Do protein content and baking parameters used in breeding impact on the reactivity towards the A1 and G12 monoclonal antibodies? The aim of the present work was to evaluate the diversity of 435 accessions of spelt and wheat – plus some diploid and tetraploid wheat accessions – and to compare spelt and wheat regarding the potential celiac immunogenicity via the reactivity towards the A1 and G12 monoclonal antibodies. Furthermore, we explored the diversity through the habit (spring or winter habit), the period of release and the geographic origin of the accessions. Additionally, we defined some baking quality parameters of the accessions in relation to their potential immunogenicity.

2. Material and methods

2.1. Plant material

A collection of wheat (*Triticum aestivum* ssp. *aestivum)* and spelt (*Triticum aestivum* ssp. *spelta)* accessions was constituted with grains obtained from various sources, namely: the USDA-ARS National Small Grains Collection (USA); the Centre de Ressources génétiques Céréales à Paille INRA Clermont-Ferrand (France); the Genebank Gatersleben of the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) (Germany); the Centre for Genetics Resources, The Netherlands (CGN); the Gene Bank RICP Prague (Czech Republic); Agroscope Institut des sciences en production végétale IPV (Nyon, Switzerland); and the Centre wallon de Recherches agronomiques (Gembloux, Belgium).

The collection comprised 449 hexaploid accessions including 195 wheat and 240 spelt accessions, 13 tetraploid and 18 diploid wheats (Table 1A and B). Among the wheat and spelt accessions, there were 35 wheat landraces, 159 wheat varieties, one wheat accession with unknown status, 125 spelt landraces and 56 spelt varieties, 9 breeding material and 50 spelt accessions with unknown status. The accessions

Table 1
(A) Number of accessions per species; (B) Number of accessions per genome; (C) Number of accessions per category and per country for *Triticum aestivum* ssp. *aestivum* and *Triticum aestivum* ssp. *spelta*.

(A)

Species	Genome	Number of accessions
Triticum monococcum ssp. *aegilopoides*	AA	5
Triticum monococcum ssp. *monococcum*	AA	5
Triticum urartu	AA	4
Aegilops longissima	SS	1
Aegilops speltoides	SS	1
Aegilops tauschii	DD	2
Triticum turgidum ssp. *dicoccoides*	AA BB	3
Triticum turgidum ssp. *dicoccon*	AA BB	3
Triticum turgidum ssp. *durum*	AA BB	2
Triticum turgidum ssp. *polonicum*	AA BB	2
Triticum turgidum ssp. *turgidum*	AA BB	3
Triticum aestivum ssp *aestivum*	AA BB DD	195
Triticum aestivum ssp. *compactum*	AA BB DD	4
Triticum aestivum ssp. *macha*	AA BB DD	4
Triticum aestivum ssp. *spelta*	AA BB DD	240
Triticum aestivum ssp. *sphaerococcum*	AA BB DD	4
Triticum vavilovii	AA BB DD	2

(B)

Genome	Number of accessions
AA	14
SS	2
DD	2
AA BB	13
AA BB DD	449

(C)

Triticum aestivum ssp. *vulgare*		*Triticum aestivum* ssp. *spelta*	
Category	Number of accessions	Category	Number of accessions
Landrace	35	Landrace	125
Old variety	24	Old variety	15
Mid variety	63	Mid variety	6
New variety	72	New variety	35
Unknown	1	Unknown	50
Breeding material	–	Breeding material	9
Total	195	Total	240
Country	Number of accessions	Country	Number of accessions
Belgium	51	Belgium	66
France	41	Germany	57
Germany	39	Swiss	38
Swiss	36	Spain	12
Austria	10	France	9
United-Kingdom	6	Other	50
Other	12	Unknown	8
Total	195	Total	240

were classified according to several criteria: the category of the accessions, *i.e.* landrace vs. variety, the latter being derived from human-made crossings, and the periods: "old" for accessions cultivated before 1950; "mid" for accessions released between 1950 and 1979 and "new" for accessions cultivated from 1980 onwards. Accessions came from all over the world but most originated in Northwestern Europe, specifically Belgium, Germany, Switzerland and France (Table 1C).

All accessions were grown in the Centre wallon de Recherches agronomiques (Gembloux, Belgium) during the 2014–2015 season. The ears which were used for immunoassays were protected before and during flowering to prevent cross-pollination. After harvest, the ears were stored in a cool place for a few weeks.

2.2. ELISA immunoassay

Wholemeal flour was obtained after milling the grains through a Retsch Ultra-Centrifugal Mill ZM200 at 0.5 mm. The content in potential celiac-related immunogenic epitopes was analysed through the GlutenTox® ELISA Sandwich from Biomedal Diagnostics (Sevilla, Spain). This assay is based on the monoclonal anti-gliadin antibody A1 and the monoclonal anti-gliadin G12-HRP conjugated antibody. The A1 recognizes the sequence QPQLPY and the G12 the sequence QLPYPQP, which are present in the DQ2.5-glia-α1a, DQ2.5-glia-α1b and DQ2.5-glia-α2 immunogenic epitopes. The quantification limit of the assay is 1.56 ng/ml of gliadin. Successive dilutions were performed for better quantification of the different samples which corresponds to a dilution of 1:1,000,000. There were two replicates (n = 2). As the reactivity of the accessions to the antibodies – expressed in gluten ppm – varied from batch to batch, the Belgian spelt variety Cosmos was assayed in each batch and the A1-G12 reactivity of each accession was expressed in terms of "relative reactivity", *i.e.* the A1-G12 reactivity of the accession relative to the reactivity of the Belgian spelt variety 'Cosmos' in the same batch.

2.3. Chemical and technological analysis

Wholemeal flour was obtained after milling the grains through a Foss Tecator Cyclotec sample mill at 1 mm. The protein content was estimated by near-infrared reflectance analysis (NIR) using a NIRs Foss NIRSystems 5000 (1100–2500 nm) based on the Dumas method following the procedure ISO/TS 16634-2:2009) (International Organization for Standardization, 2009). Hardness was measured using a FOSS XDS NIR analyzer according to AACCI approved method 39-70.02 (American Association of Cereal Chemists, 1999). Zeleny index was determined following the procedure ISO 5529:2007: Wheat – Determination of the sedimentation index – Zeleny test (International Organization for standardization, 2007).

The alveograph analysis was performed on a reduced number of spelt (Cosmos, Neuegger Weisskorn, Weisser Kolbenspelz, SK3F, 69Z6,57) and wheat (Minaret, Cadenza, Nord-Desprez, Triso, Blé de Noé and Bledor) accessions, on flour obtained on a Chopin CD1 laboratory mill.

2.4. Gluten protein extraction

Gluten proteins were extracted from wheat flour according to the method of Singh, Shepherd and Cornish (1991). The wheat flour (50 mg) was suspended in 1 ml of 50% aqueous isopropanol and mixed continuously (vortex VWR VV3) during 30 min at room temperature (rt), followed by centrifugation at 2500 × *g* for 15 min (rt). The residue was re-extracted twice according to the same protocol. The third residue was re-extracted twice with 50% aqueous isopropanol/1% DTT/50 mM Tris-HCl, pH = 7.5 for 30 min at 60 °C. The solution was mixed every 5–10 min followed by centrifugation at 10,000 × *g* for 10 min (rt). The four obtained supernatants were combined and constituted the gluten protein extract. The protein content was quantified based on the Bradford dye-binding procedure. The calibration curve was established according to the equation type $y = ax + b$ with different standards: 0; 0.1; 0.2; 0.4; 0.6; 0.8; 1; 1.5 mg/ml; and the R^2 coefficient was determined.

2.5. SDS-PAGE and immunoblotting

Gluten protein extract was loaded and separated on SDS-PAGE gels at 10% (Laemmli, 1970) using a Tank Blotting Systems Bio-Rad Mini-PROTEAN® vertical electrophoresis cell. The amount of proteins loaded was 0.5 µg for A1 detection and 1 µg for G12 detection with a dilution designed to yield the same volume. The separation was carried out at 50 V for 30 min, then at 120 V until the front line reached the end of the gel. Proteins were blotted onto nitrocellulose blotting membrane (GE Healthcare Amersham™ Protran™ Premium 0,2 µm NC) with the blotting buffer (Tris-Glycine and SDS), using a Amersham Biosciences electrophoresis power supply EPS601 at 100 V during 75 min. Blots were incubated separately (Cordewener et al., 1995) using mouse monoclonal anti-gliadin 33-mer A1 antibody and mouse monoclonal anti-gliadin 33-mer G12 antibody (Biomedal, Sevilla, Spain). Antibody binding to the blots were goat anti-mouse IgG-HRP (SantaCruz, Dallas, Texas, USA). The membrane was visualized through the enhanced chemiluminescence (ECL) method (Merck Millipore Luminata Classico Western HRP Substrate) with Amersham Hyperfilm ECL and Carestream Health, Developer and Fixer Kodak® X-Ray GBX. The gluten protein extract of the spelt variety 'Cosmos' was used on each separate immunoblot as an inter-gel control.

Pictures were scanned using an HP Photosmart C4180 and saved as a Bitmap with shades of grey. Pixel intensities were calculated per lane using BIO-1D Vilber Lourmat. Relative intensities were normalized to values obtained for the inter-gel control and were expressed relatively to the reactivity of Cosmos as previously stated: A1 relative reactivity, G12 relative reactivity, and A1 + G12 relative reactivity which represents the sum of the two processes.

2.6. Statistical analyses

Data from the ELISA test were treated as follows. The normality of data was tested with Shapiro-Wilk's test and the homogeneity of variance with the Levene's test. Neither condition was met, in spite of following the square root transformation method. Consequently, the raw data were used in the statistical analysis. The difference between accessions was assessed by the non-parametric Kruskal-Wallis test. Comparisons between subspecies, habits, landraces vs. varieties, periods (old, mid and new varieties), and geographical origins were established with the linear mixed-effects model (LME4) and the Tukey's post hoc test with the criterion for significance $p < 0.05$. Correlations between A1-G12 antibody relative reactivity, Zeleny index and protein content were established through the Spearman method which produces the strength and direction of association between two ranked variables ($p < 0.05$). The Spearman coefficient was used as data were not linear. Data from quantification through Western-blotting were normal (Shapiro-Wilk). Pearson's correlation coefficient was calculated between the A1-G12 relative reactivity obtained through ELISA and the A1, G12 and A1 + G12 relative reactivities obtained through Western-blotting. All statistical analyses were performed using the statistical computing software R (R i386, version 3.0.2, 2014-10-31, CRAN, Belgium) (The R foundation for statistical computing).

3. Results and discussion

3.1. ELISA quantification

The histogram (Fig. 1) illustrates the diversity of the whole collection of accessions and the box-plots (Fig. 2) (with medians, quartiles, minimum and maximum) display the diversity of subset of accessions

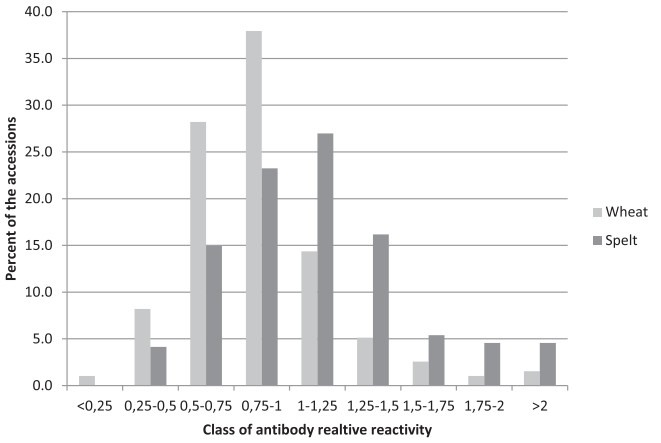

Fig. 1. Histogram of the accessions in % according to the A1-G12 antibody relative reactivity.

according to several criteria discussed below (habit, status of the accession, period of release, country of origin).

3.1.1. Evaluation of the diversity in spelt and wheat

The spelt accessions ranged between 0.25 and 4.64 A1-G12 relative reactivity with an average of 1.12 ± 0.49 and the wheat accessions between 0.23 and 4.00 A1-G12 relative reactivity with an average of 0.88 ± 0.40. The differences among the wheat accessions (p-value = 2.366e-13) and among the spelt accessions (p-value = 2.2e−16) were very highly significant (Fig. 2A). The factors between the lowest and highest accession for A1-G12 relative reactivity on spelt and wheat were 18.6 and 17.4, respectively. Among the wheat accessions, 80.5% had A1-G12 relative reactivity between 0.5 and 1.25 while among the spelt accessions, 81.3% fell within the 0.5–1.5 range (Fig. 1). The results of Ribeiro et al. (2016) also indicated that wheat varieties differed significantly in the level of the analysed T-cell-stimulatory epitopes. The researchers used the R5 competitive ELISA immunoassay and screened 53 modern varieties and 19 landraces of *Triticum aestivum* ssp. *aestivum*, and 20 varieties of *Triticum aestivum* ssp. *spelta*. They found a factor 11 between the variety Pernel and the variety Alejo among the modern wheat varieties which is in line with our factor for wheat, even somewhat higher between the variety Froment de Polders and the variety Bledor: 17.4. Gélinas and McKinnon (2016) observed statistically significant differences among the 4 *Triticum aestivum* ssp. *aestivum* and 13 *Triticum aestivum* ssp. *spelta* accessions investigated with ELISA G12. The amount of gluten reacting to G12 antibody of the spelt accessions ranged from 178 to 666.5 ppm, a factor of 3.7 which is lower than that yielded by the current study (18.6).

The comparison of spelt and wheat accessions, supported by a large spelt collection, indicated that on average spelt had a very slightly higher A1-G12 relative reactivity than wheat, 1.12 and 0.88 respectively, and the difference was highly significant (p-value = 6.44e−15). Ribeiro et al. (2016) came to the same conclusion; however their data set contained almost exclusively Spanish landraces for spelt, while modern wheat came from all over the world. In the present data set, some accessions from both species – spelt and wheat – had very low A1-G12 relative reactivity while in the spelt accessions from Ribeiro et al. (2016) none presented an R5 reactivity as low as the lowest ones found among wheat accessions. In our study, the average was higher for spelt but some accessions showed an A1-G12 relative reactivity as low as the lowest identified for wheat (Fig. 2). For Van den Broeck, de Jong et al. (2010), among the landraces, the spelt accessions did not differ systematically from wheat according to their reactivity to Glia-α9 and Glia-α20 antibodies. In the study of Schalk et al. (2017), the content of 33-mer in spelt cultivar Franckenkorn (353.9 μg/g flour) evaluated through LC-MS fell in the 200–400 μg/g flour range, and did not differ significantly from common wheat cultivars. The spelt cultivar Oberkulmer contained one of the highest amounts of the 33-mer peptide (523.4 μg/g flour). The authors could not confirm the hypothesis that spelt may be less CD-immunoreactive than modern common wheat cultivars. Gélinas and McKinnon (2016) found an average content of 9.9 ppm of R5 gluten for spelt (n = 13) and 13.8 ppm of R5 gluten for wheat (n = 4) with ELISA R5, and 380.2 ppm of G12 gluten for spelt and 396.6 ppm of G12 gluten for wheat with ELISA G12. Spelt had a lower G12 and R5 reactivity than wheat but no statistics support the analysis between both subspecies. From all these studies, no clear general trend on the epitopes reactivity to antibodies of spelt compared to those of wheat regarding celiac disease can be established. However, in our study, some particular spelt and wheat accessions presented low A1-G12 relative reactivity. Finally, the major information is that diversity among the tested accession, either spelt or wheat, was much more important than the difference between both subspecies and it is thus possible to find spelt and wheat accessions with variable levels of epitopes.

3.1.2. Study of the habit

In terms of habit of the accessions, a significant difference was observed between the spring and the winter accessions. On average, the spring accessions had higher A1-G12 relative reactivity than the winter accessions for both subspecies: 0.84 ± 0.40 for winter wheat (min = 0.23, max = 4.00); 1.09 ± 0.28 for spring wheat (min = 0.61, max = 1.81) (p < 9.8e−06); 1.05 ± 0.38 for winter spelt (min = 0.25, max = 2.47); and 1.42 ± 0.72 for spring spelt (min = 0.25, max = 4.64) (p < 5.24e−12). This difference could be ascribed to environmental conditions as the spring types did not develop under the same meteorological conditions due to the different sowing dates (October vs. March for the winter and spring habit respectively) (Fig. 2B and C).

3.1.3. Impact of breeding for spelt and wheat

Firstly, the comparison of the landraces and varieties showed that there was no significant difference between these two categories, whatever the species, regarding A1-G12 reactivity (p = 0.786 for spelt and p = 0.172 for wheat). For spelt, landraces and varieties had a mean of 1.12 ± 0.51 (min = 0.25, max = 4.64) and 1.13 ± 0.43 (min = 0.46, max = 2.88), respectively. The mean for wheat landraces and varieties was 0.82 ± 0.29 (min = 0.23, max = 1.66) and 0.89 ± 0.43 (min = 0.24, max = 4.00), respectively. This may indicate that crosses guided by breeders did not increase the A1-G12 reactivity for each improved species – wheat and spelt (Fig. 2D and E).

A deeper analysis among the wheat varieties indicated that there was no significant difference between old, mid and new varieties (0.872 < p < 0.999) (Fig. 2F). For wheat the old varieties had a mean of 0.89 ± 0.65 (min = 0.24, max = 4.00), mid varieties 0.88 ± 0.37 (min = 0.35, max = 2.65) and new varieties 0.88 ± 0.37 (min = 0.35, max = 2.65). This analysis showed that the way the breeders chose the genitors and crossed them did not have an impact on A1-G12 reactivity. The breeding process aimed at having high baking quality with dough, which translates into improved resistance to mechanical work, did not influence A1-G12 reactivity. However, Van den Broeck, de Jong et al. (2010) concluded that, in general, the immunogenicity of modern wheat varieties has increased considering the impact on CD patients of the major immunodominant DQ2.5-glia-α1 epitope. They identified one out of the 36 modern varieties and 15 out of 50 landraces with a low response against Glia-α9 mAb (targeting the immunodominant DQ2.5-glia-α1 epitope). The frequencies of high responders to this antibody was similar among modern varieties and landraces. By contrast, the opposite was found regarding Glia-α20 mAb (focusing on the DQ2.5-glia-α3 epitope) which showed a significantly higher overall antibody response in the landraces (Van den Broeck, de Jong et al., 2010). Van den Broeck, de Jong et al. (2010) identified some modern varieties and

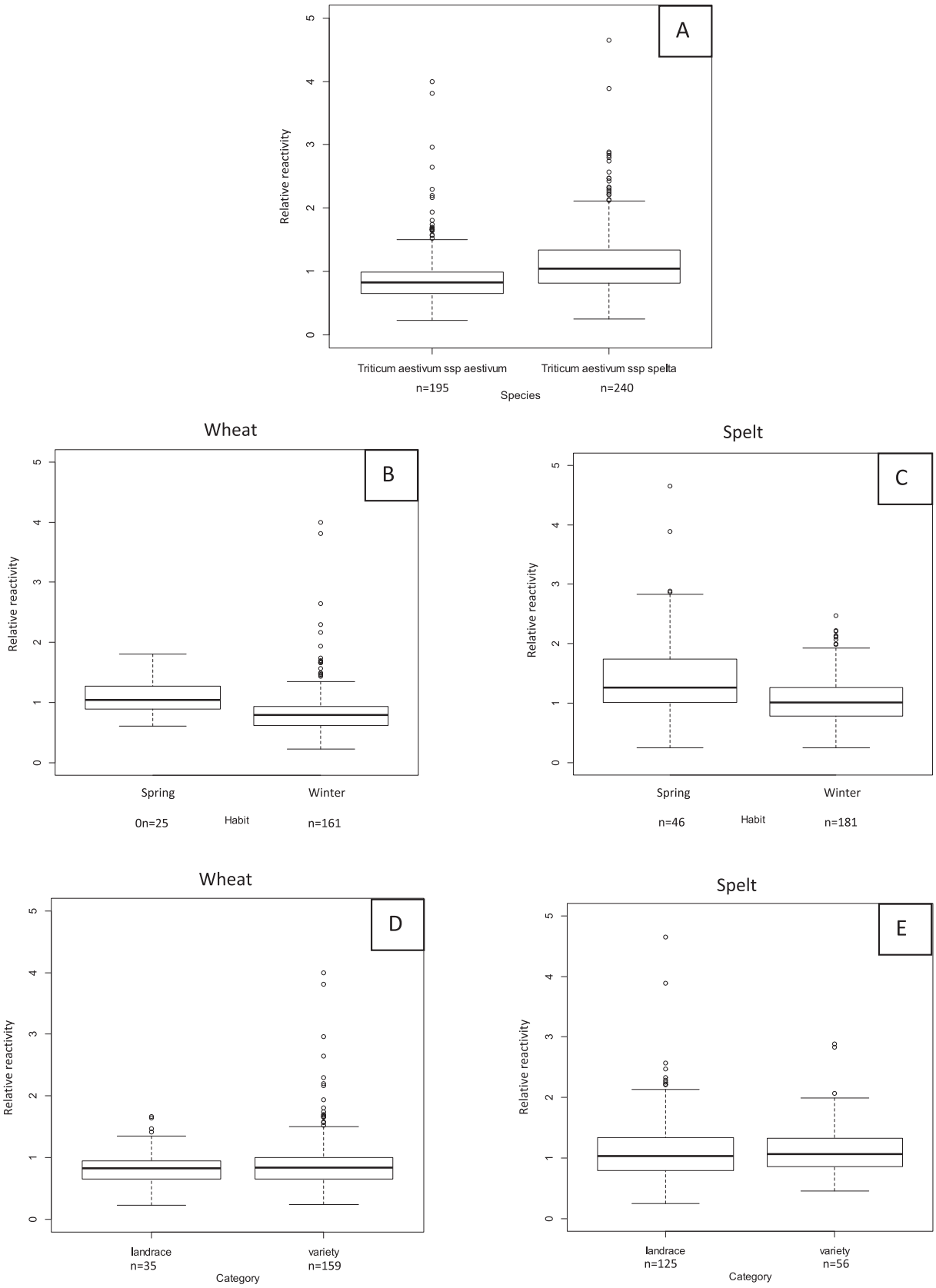

Fig. 2. Box-plots of the A1-G12 antibody relative reactivity evaluated by ELISA sandwich immunoblotting of A) *Triticum aestivum* ssp. *aestivum* and *Triticum aestivum* ssp. *spelta* B) Spring and winter accessions of *Triticum aestivum* ssp. *aestivum* C) Spring and winter accessions of *Triticum aestivum* ssp. *spelta* D) Landraces and varieties of *Triticum aestivum* ssp. *aestivum* E) Landraces and varieties of *Triticum aestivum* ssp. *spelta* F) Mid, new and old varieties of *Triticum aestivum* ssp. *aestivum* G) Mid, new and old varieties of *Triticum aestivum* ssp. *spelta* H) Countries of origin for *Triticum aestivum* ssp. *aestivum* I) Countries of origin for *Triticum aestivum* ssp. *spelta*.

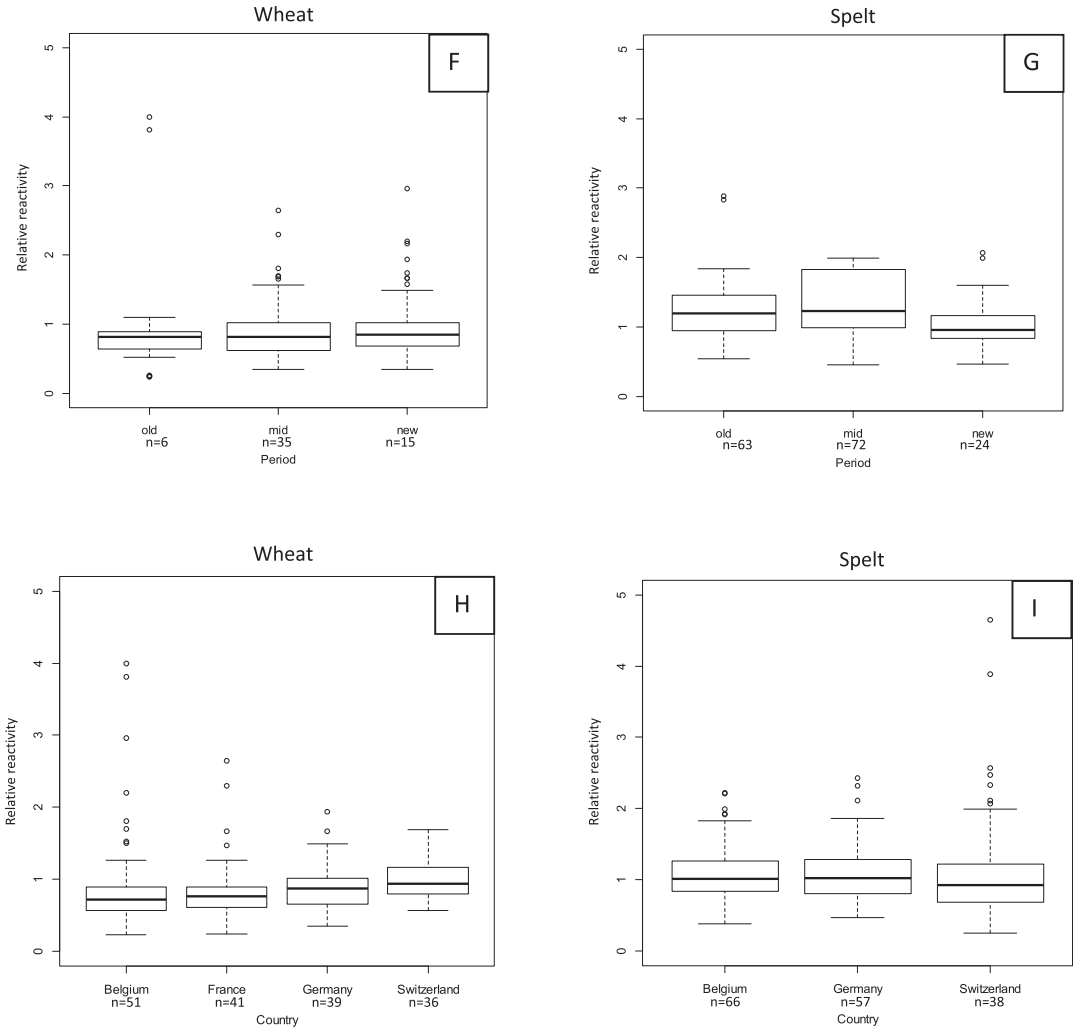

Fig. 2. (*continued*)

landraces which have relatively low content of both epitopes. Conversely, several other studies did not confirm the impact of breeding on the prevalence of celiac disease. Ribeiro et al. (2016) showed that wheat landraces had higher reactivity to R5 antibody than modern varieties and thus inferred that breeding practices did not contribute to a prevalence of celiac disease immunostimulatory epitopes. Kasarda (2013) did not find clear evidence of an increase in the gluten content in wheat in the United-States during the 20th century, and if there has been indeed an increase in celiac disease during the latter half of the century, wheat breeding for higher gluten content does not seem to be the cause. In the work of Gélinas and McKinnon (2016), the wheat with the highest G12 reactivity was the Red Fife, a traditional wheat line available in the 19th century. The data of Schalk et al. (2017) did not follow the trend that modern wheat cultivars generally contain higher amounts of 33-mer than old cultivars.

For spelt, the mean A1-G12 relative reactivity for old, mid and new verieties was 1.27 ± 0.5 (min = 0.55, max = 2.88), 1.32 ± 0.49 (min = 0.46, max = 1.99), and 1.01 ± 0.31 (min = 0.47, max = 2.07), respectively. Concerning the spelt varieties, surprisingly, the new varieties differed significantly from the old and mid varieties (for old – new $p = 0.00348$, for old – mid $p = 0.90357$ and for new – mid $p = 0.01192$) (Fig. 2G). The crossings carried out between wheat and spelt in order to improve the lodging resistance and the baking quality of spelt started in 1966 in Belgium; it can be hypothesized that it occurred in the same period in Switzerland and Germany. Given that, on average, wheat had lower A1-G12 reactivity than spelt, it may be surmised that the introduction of wheat is capable of lowering the A1-G12 reactivity of modern spelt varieties. In a study conducted by Schalk et al. (2017), the amounts of 33-mer peptide in flour for spelt cultivars Oberkulmer and Franckenkorn were 523.4 µg/g flour and 353.9 µg/g flour respectively. This example supports our data, *i.e.* the reactivity we measured follows the same trend as the measure made by Schalk et al. (2017): the oldest pure cultivar, Oberkulmer, had a higher content in 33-mer than the cultivar Franckenkorn registered in 1995 resulting from a cross between wheat and spelt.

3.1.4. Geographical origin analysis for spelt and wheat

For wheat, there was no significant difference in A1-G12 relative reactivity between the Belgian, German and French accessions ($0.3767 < p < 0.9788$) with individual means of 0.83 ± 0.58 (min = 0.23, max = 4.00), 0.88 ± 0.28 (min = 0.35, max = 1.94), 0.81 ± 0.36 (min = 0.24, max = 2.65), while those from Switzerland (mean = 0.99 ± 0.25, min = 0.57, max = 1.69) differed significantly from the French accessions ($p = 0.0367$) (Fig. 2H). This is not surprising considering that wheat breeders normally exchange material (especially between France, Belgium and Germany), as some wheat varieties can often be cultivated in several countries due to similar environments. Exchanges with Swiss lines are known to be much more uncommon. In Van den Broeck, de Jong et al. (2010), landraces could not be grouped according to their country or region of origin. They attributed this situation partly to the fact that the recorded country is the country of the first genebank collection, which often may not be the

country where it originated from. We paid attention to this point, were careful to interpret the information provided by the genebank, and complemented the profile of the accessions with specialised literature.

Concerning spelt, there was no significant difference in the A1-G12 relative reactivity between accessions coming from Belgium, Germany and Switzerland in general ($0.74 < p < 0.96$) with means of 1.06 ± 0.34 (min = 0.38, max = 2.22), 1.08 ± 0.37 (min = 0.47, max = 2.42) and 1.11 ± 0.73 (min = 0.25, max = 4.64), respectively (Fig. 2I). This is not surprising as Germany and Switzerland are important centres for spelt genetic resources and exchanges have been significant between these two countries and Belgium. Some significant differences between Belgian, Canadian and Swiss spelts were found by Gélinas and McKinnon (2016). According to G12 antibody reactivity, Belgian spelt varieties had an average gluten content of 216.5 ppm, Swiss spelt varieties 392.6 ppm and Canadian spelt varieties 563.3 ppm. However, the Canadian spelt varieties used in their study were spring varieties and we demonstrated that spring accessions had higher A1-G12 reactivity than winter ones; consequently the difference could be ascribed either to the country or the habit, or both.

3.2. Qualitative and quantitative evaluation through Western-blotting A1 and G12 antibodies

Western-blotting with A1 and G12 antibodies were performed on 58 accessions which represented a diversity regarding A1-G12 reactivity detected by ELISA, geographic origin, period of release, and some additional accessions which were analysed in other studies (Supplementary Material 1 (S.M.1) and Fig. 3). The Western-blotting test is based on the same monoclonal antibodies as those used in the Elisa test but differences in the amplitude of the results between the two techniques were observed. The range of the reactivity was lower for Western-blotting (from 0.44 to 1.63 A1 + G12 relative reactivity) than for ELISA (from 0.25 to 4.26). The factor between the minimum and the maximum of the set was 3.70 for A1 + G12 relative reactivity for Western-blot and 17.04 A1-G12 relative reactivity for ELISA. This difference may result from the different techniques used in the samples preparation and in the detection (ELISA sandwich test).

There were no correlations between quantification through ELISA and through Western-blotting. Indeed, the correlations between the A1-G12 relative reactivity of ELISA and A1 relative reactivity, G12 relative reactivity and A1 + G12 relative reactivity of Western-blotting were 0.35 (non-significant, $p > 0.001$), 0.43 (significant, $p < 0.001$) and 0.44 (significant, $p < 0.001$), respectively. Western-blotting and ELISA method used the same mAb, A1 and G12, but the ELISA test is of the sandwich type where both mAb A1 and G12 are used simultaneously.

The quantification through mAb-G12 and mAb-A1 gave similar results with a positive and highly significant correlation of 0.76 ($p < 0.001$). Van den Broeck, de Jong et al. (2010) analysed 50 landraces and 36 modern varieties from wheat with mAb Glia-α9 and mAb Glia-α20 through immunoblotting and found a relative intensity for mAb Glia-α9 that ranged from 380 to 2280 – a factor of 6 – and for mAb Glia-α20 from 55 to 160 – a factor of 2.9 – which is similar to the present results even if the antibodies were different.

The qualitative analysis of the Western-blotting (Fig. 3 and S.M.1) with A1 and G12 antibodies showed the qualitative diversity among the wheat accessions. The size and number of signals varied between accessions and showed a diversity among the accessions. Van den Broeck, de Jong et al. (2010) found that Toronto and Cadenza had different profiles than other accessions. Here, the immunoblot with mAb-G12 from these two accessions does not seem really different in the 35–40 kDa area even if the mAb-Glia-α-9 and the mAb-G12 share the QPQLPY sequence. For mAb-A1 a difference could be seen between both accessions in the 35–40 kDa zone but could not be compared with data from Van den Broeck, de Jong et al. (2010), as mAb-α-20 and mAb-A1 do not share a sequence.

3.3. Comparisons of spelt and wheat accessions in different analyses in the literature

Several accessions investigated in the present study were analysed in other studies. We know that there is a diversity of tests that target different elements (Ridascreen® ELISA with antibody R5) or the 33-mer peptide at different level of specificity (GlutenTox® ELISA Sandwich, ELISA AgraQuant® with antibody G12, mAb Glia-α9 and mAb Glia-α20 through immunoblotting, quantification through liquid chromatography tandem mass spectrometry) and we wondered if the ranking of the accessions was maintained from one technique to another.

The following paragraph will compare our results with those of other studies from literature which used different techniques (i) ELISA (Ribeiro et al., 2016; Gélinas and McKinnon, 2016) (S.M.2), (ii) Western-blotting (Van den Broeck, de Jong et al., 2010) (S.M.3), (iii) LC-MS (Schalk et al., 2017). (i) Etoile de Choisy and Soissons, analysed by Ribeiro et al. (2016), had 162.03 and 194.24 g gliadins/kg respectively in the R5 reactivity; this ranking is in line with our figures, 0.66 and 0.87 A1-G12 relative reactivity. We expressed the results of ELISA AgraQuant® with antibody G12 of several spelt varieties (Gélinas and McKinnon, 2016) relative to Cosmos (S.M.2) in order to compare this study with ours. The ranking of the varieties was not the same between both studies. (ii) Van den Broeck, de Jong et al. (2010) identified varieties that showed low response to Glia-α9 and Glia-α20 mAbs through Western-blotting. The wheat accession Cadenza showed the lowest response to both mAbs and Minaret and Rouge de la Gruyere

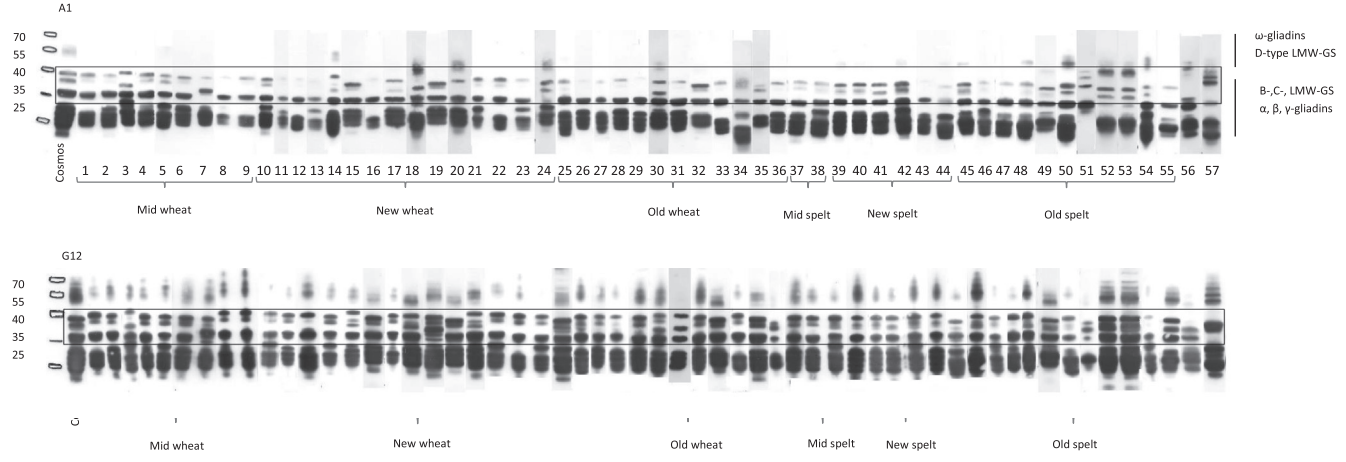

Fig. 3. Immunoblots of selected accessions using mAb-A1 and mAb-G12: mid wheat (1–9), new wheat (10–24), old wheat (25–36), mid spelt (37–38), new spelt (39–44), old spelt (45–55), *Triticum turgidum* (56) and *Triticum urartu* (57). Details on the accessions with corresponding numbers are in S.M.1.

displayed low response to the Glia-α9 mAb but medium response to the Glia-α20 mAb. Here, Cadenza showed a A1-G12 relative reactivity of 1.13 which was average; a Minaret of 0.71 which was low and a Rouge de la Gruyère of 1.31 which was high. Except for the Minaret, the position of the accessions did not conform to the results of Van den Broeck, de Jong et al. (2010). When considering the quantification resulting from the immunoblots produced by Van den Broeck, de Jong et al. (2010a) and from the present study, it is clear that the ranking of the variety was different (S.M.3). (iii) In the study of Schalk et al. (2017), the amounts of 33-mer peptide in flour for cultivars Oberkulmer and Franckenkorn were 523.4 μg/g flour and 353.9 μg/g flour, respectively. Here, Oberkulmer had a A1-G12 relative reactivity of 0.52 and Franckenkorn 1.32. Oberkulmer is in the lowest range of A1-G12 reactivity while it is among the highest in Schalk et al. (2017), and Franckenkorn in turn presented average values in both studies which points to an unstable ranking.

The variations observed between studies can be explained by the different antibodies used. Indeed, the RIDASCREEN® based on the R5 monoclonal antibody (Ribeiro et al., 2016; Gélinas & McKinnon, 2016) and AgraQuant® ELISA Gluten G12 target different epitopes than the GlutenTox® ELISA Sandwich. Furthermore, the GlutenTox® ELISA Sandwich had a less specific target in the 33-mer than other techniques such as LC-MS.

Furthermore, we wonder how big an impact did the environment have on the antibody reactivity being measured. The accessions of the different studies were grown in different environments and spring and winter accessions displayed different levels of A1-G12 reactivity in our study, which can be attributed to the genetic and/or the environmental variance. Several studies (Van den Broeck, de Jong et al., 2010; Ribeiro et al., 2016; Van den Broeck, Hongbing et al., 2010) compared the genotypic variation among accessions and species grown during the same year as in the present study while other do not mention growth conditions (Gélinas & McKinnon, 2016). Indeed, changes in gluten protein composition have been described, but are usually expected only if growth conditions are extreme (high or low temperature, dry or wet conditions). Schalk et al. (2017) recently demonstrated that "the environmental factor had a greater influence on 33-mer contents than the genetic background of the four wheat cultivars, because the results for each combination of harvest years (2011 vs. 2012, 2011 vs. 2014 and 2012 vs. 2014) were significantly different ($p \leq 0.034$)."

3.4. Baking quality parameters and A1-G12 reactivity

The correlations between the A1-G12 antibody relative reactivity and protein content was $r = 0.37$ ($p = 2.316e-14$) (Fig. 4A) when both species were mixed. The correlation was $r = 0.34$ ($p = 6.102e-06$) for wheat (Fig. 4B) and $r = 026$ ($p = 5.174e-05$) for spelt (Fig. 4C). These correlations are weak with r^2 values of only 0.14, 0.12 and 0.07 respectively but significant because of the size of the population which is big. For wheat the "old, mid and new" accessions were spread uniformly (Fig. 4B) while the new spelt accessions concentrated in the part of the low protein content on the figure contrary to the old accessions which were dispersed (Fig. 4C). Ribeiro et al. (2016) found positive significant correlations ($r = 0.24$, $p < 0.05$) for all accessions and for wheat accessions ($r = 0.54$, $p < 0.05$) but not for spelt accessions ($r = 0.16$, $p < 0.05$) between R5 reactivity and protein content. On the other hand, Schalk et al. (2017)found no correlation between 33-mer and crude protein contents ($r = 0.481$, $p < 0.001$) in 51 modern and old common wheat and spelt cultivars.

Correlations between the A1-G12 antibody relative reactivity and the Zeleny index were very weak and even lower when it came to protein content, with $r = 0.19$ for both subspecies ($p = 0.0001635$) (Fig. 4D), $r = 0.21$ for wheat accessions ($p = 0.007388$) (Fig. 4E) and $r = 0.22$ for spelt accessions ($p = 0.0007568$) (Fig. 4F) – r^2 of 0.04, 0.04 and 0.05, respectively. The old, mid and new accessions were spread uniformly for spelt and wheat on the figure. The Zeleny index reflects the behaviour of proteins in an acid environment and breeders usually use it, among other parameters, to select high baking quality lines. This practice does not seem to drag the A1-G12 antibody reactivity, given the very low correlations stated above. Gélinas and McKinnon (2016) did not find any correlation between the reactivity R5 and G12 and the amount of dry gluten of the accessions. Also, Schalk et al. (2017) found no correlation to gluten contents ($r = 0.526$, $p < 0.001$) in 51 modern and old common wheat and spelt cultivars.

The alveograph Chopin rheological characteristics – W, P/L ratio – were unrelated to A1-G12 reactivity which is in line with the correlation studied above (data not shown).

3.5. Diploids and tetraploids

A1-G12 relative reactivity was detected in *T. monocccum* and *T. urartu* (genome A), indeed *T. monococcum* ssp. *monococcum, T. monococcum* ssp. *aegilopoides* and *T. urartu* had A1-G12 relative reactivities of 0.59 ± 0.14 (min = 0.31, max = 0.80), 0.98 ± 0.3 (min = 0.72, max = 1.60) and 2.83 ± 0.61 (min = 2.04, max = 3.53), respectively (Table 2). On the Western-blots, bands were observed for *T. urartu* and A1 + G12 relative reactivity was 1.08 which is a high value. The detection of A1-G12 reactivity in species with A genome is surprising as DQ2.5-glia-α2 is muted in genome A (Molberg et al., 2005). The reactivity of species with A genome to the Elisa test implies the recognition of non-immunogenic variants by the antibodies. Molberg et al. (2005) showed that fragments identical or equivalent to the immunodominant 33-mer fragment are encoded by α-gliadin genes from wheat chromosome 6D, and are thus absent from gluten of diploid einkorn (including *T. monococcum*) which was confirmed by Schalk et al. (2017) in two diploid einkorn cultivars. However, the immunogenicity of diploids is controversial as conclusions from literature differ. Some studies demonstrated that *T. monococcum* was not immunogenic (Pizzuti et al., 2006; Vincentini et al., 2007; De Vincenzi et al., 1996) while others showed that immunogenicity of diploids was only reduced regarding hexaploids but still exist (Vaccino, Becker, Brandolini, Salamini, & Kilian, 2009; Gianfrani et al., 2012; Suligoj et al., 2013; Van Herpen et al., 2006; Ozuna et al., 2015). Concerning *Ae. tauschii* (D genome), the A1-G12 relative reactivity was 0.87 ± 0.04 (min = 0.81, max = 0.91) which is surprisingly low and contrary to what was expected (Table 2). Indeed sequences from *Ae. tauschii* and those from chromosome 6D from wheat contain all 4 epitopes in variable combinations per gene (Van Herpen et al., 2006), while results from Ozuna et al. (2015) showed that *Ae. tauschii* is among the most immunogenic species. The surprising results obtained here in regard to literature may come from the low specificity of the antibodies in the GlutenTox® ELISA Sandwich.

Concerning the tetraploids, there was no difference in A1-G12 relative reactivity between tetraploids and hexaploids in the present study with values of 1.11 ± 0.53 (min = 0.25, max = 2.13) and 1.03 ± 0.48 (min = 0.23, max = 4.64), respectively (Table 2). On the Western-blots, A1 + G12 relative reactivity was 1.04 for one tetraploid accession compared to an average value of 0.74 for hexaploid accessions (min = 0.44; max = 1.63) but fewer bands were observed than for hexaploids. In literature some studies showed that tetraploids are less immunogenic than hexaploids (Suligoj et al., 2013; Gélinas & McKinnon, 2016; Ozuna et al., 2015) and the 33-mer peptide was not detected (< limit of detection) in two durum wheat and two emmer cultivars (genome AABB) by Schalk et al. (2017). This comes from the absence of chromosome 6D which encodes this peptide but "a larger set of durum wheat, emmer cultivars would have to be analysed to conclude whether these wheat species generally lack the 33-mer peptide" (Schalk et al., 2017). Others did not see any difference and their observations are in line with our results (Ribeiro et al., 2016; Spaenij-Dekking et al., 2005; Van den Broeck, Hongbing et al., 2010; Salentijn et al., 2009). Kasarda (2013) postulated that the significance of the reduced number of immunogenic epitopes in diploids and tetraploids

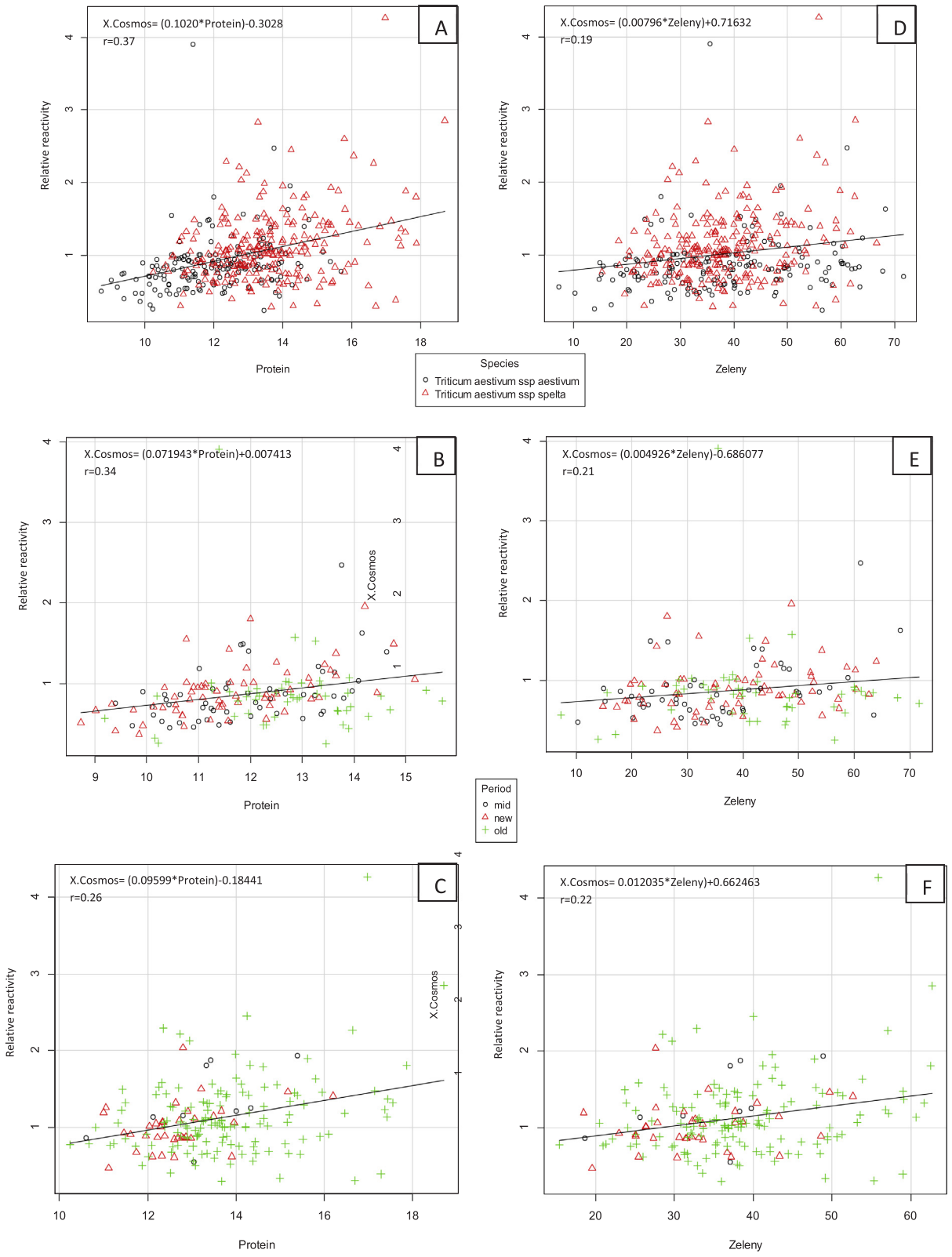

Fig. 4. Scatter-plots of the A1-G12 antibody relative reactivity and the protein content of A) All accessions of *Triticum aestivum* ssp. *aestivum* and *Triticum aestivum* ssp. *spelta* B) Old, mid and new accessions of *Triticum aestivum* ssp. *aestivum* C) Old, mid and new accessions of *Triticum aestivum* ssp. *spelta* and the Zeleny index D) All accessions of *Triticum aestivum* ssp. *aestivum* and *Triticum aestivum* ssp. *spelta* E) Old, mid and new accessions of *Triticum aestivum* ssp. *aestivum* F) Old, mid and new accessions of *Triticum aestivum* ssp. *spelta*.

Table 2
Average, standard deviation, minimum and maximum A1-G12 relative reactivity in a wide set of *Triticum* and *Aegilops* species.

Species	Genome	A1-G12 relative reactivity			
		Mean	Standard deviation	Minimum	Maximum
Triticum monococcum ssp. aegilopoides	AA	0,98	0,30	0,72	1,60
Triticum monococcum ssp. monococcum	AA	0,59	0,14	0,31	0,80
Triticum urartu	AA	2,83	0,61	2,04	3,53
Diploids	**AA**	**1,37**	**1,02**	**0,31**	**3,53**
Triticum turgidum ssp. dicoccoides	AA BB	1,76	0,28	1,42	2,13
Triticum turgidum ssp. dicoccon	AA BB	1,02	0,42	0,54	1,57
Triticum turgidum ssp. durum	AA BB	1,05	0,29	0,79	1,37
Triticum turgidum ssp. polonicum	AA BB	1,18	0,45	0,76	1,57
Triticum turgidum ssp. turgidum	AA BB	0,56	0,27	0,25	0,95
Tetraploids	**AA BB**	**1,11**	**0,53**	**0,25**	**2,13**
Triticum aestivum ssp aestivum	AA BB DD	0,88	0,41	0,23	4,00
Triticum aestivum ssp spelta	AA BB DD	1,12	0,49	0,25	4,64
Triticum aestivum ssp. compactum	AA BB DD	1,26	0,60	0,59	2,20
Triticum aestivum ssp. macha	AA BB DD	1,25	0,28	0,81	1,57
Triticum aestivum ssp. sphaerococcum	AA BB DD	2,20	0,59	1,59	3,16
Triticum vavilovii	AA BB DD	0,91	0,09	0,80	0,99
Hexaploids	**AA BB DD**	**1,03**	**0,48**	**0,23**	**4,64**
Aegilops tauschii	DD	0,87	0,04	0,81	0,91
Diploid	**DD**	**0,87**	**0,04**	**0,81**	**0,91**
Aegilops longissima	SS	2,55	0,07	2,50	2,60
Aegilops speltoides	SS	0,36	0,01	0,35	0,36
Diploids	**SS**	**1,45**	**1,27**	**0,35**	**2,60**

In bold, the information relates to the level of diploidy of the species whatever the species.

requires more investigation.

When comparing different species, the differences in results may be due to the accession chosen for the study, the number of accessions analysed and especially the method used – molecular study or immunologic methods – the second being less specific than the first. A deeper investigation with many different and well represented species is necessary to elucidate the question.

4. Conclusion

This study has been based on a wide and diverse spelt and wheat collection, thanks to which valuable comparisons could be established. The status of spelt versus wheat was again investigated regarding average values, and spelt was found to have very slight higher reactivity than wheat to A1-G12 antibodies. Variability is very high in both subspecies and accessions tested (n = 435) and low levels of reactivity have been highlighted in accessions among each species, which may be of interest for plant breeding purposes and for consumers. The breeding impact was studied in wheat which is a widely grown crop, in contrast to spelt, a minor species for which breeding is rare. At this stage, in accordance with other studies, no link between the breeding strategies for baking quality improvement and A1-G12 antibodies reactivity could be established. It is now useful to distinguish the environment and the genotype effect in the reactivity towards A1-G12 antibodies in accessions, and to investigate the difference between species with different level of ploidy.

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Compliance with ethical standards

Conflict of interest: The authors declare that they have no conflict of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.foodchem.2018.06.094.

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