Composés bioactifs

Proteomic analysis of mature kernel aleurone layer of *Triticum spelta* and three wheat related species

Protéomique de la couche à aleurone en grain mature de *Triticum spelta* et de trois espèces apparentées aux blés

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Abstract **Introduction.** The peripheral layers (PL) of wheat, including the aleurone layer (AL), are an important source of micronutrients. AL is excluded, and mainly used for animal feed, although these tissues have significant nutritional potential as they contain most micronutrients, phytochemicals and fiber, and could improve the nutritional quality of foods. **Objective.** A proteomic approach was used to reveal major protein differences in the aleurone layer (AL) of mature grain between four wheat species: *T. Aestivum*, and *T. spelta* (6X), *T. durum* (4X), and *T. monococum* (2X). **Materiel and methods.** In each species, one representative cultivar was used for total AL protein extraction. Two-dimensional electrophoresis of AL proteins revealed 1380, 1355, 1120, and 973 Coomassie stained spots, respectively. **Results.** A total of 334 spots showed quantitative or qualitative (presence/absence) differences between the four cultivars. *T. Aestivum*, and *T. spelta* had 90.9% and 92.6% of their AL proteome, identical, respectively. Only 25 spots were significantly different between the two hexaploid cultivars. AL proteins, encoded by A genome, were mainly expressed in polyploid species. Percentages of proteins, encoded by A genome, was 85%, in tetraploid species, and 70% in the hexaploid spelt. Eighty spots proteins, that are different qualitatively and quantitatively, were identified by mass spectrometry, and data mining, and were classified in 5 biological processes, and 12 functional categories. **Conclusion.** The major differences between species are, particularly, due to storage globulins (22, 11, and 1 of Glo-3, Glo-3B, Glo-3C, respectively), stress related proteins, including antioxidant proteins, like 1-Cys peroxiredoxin, and manganese superoxide dismutase, defence proteins like xylanase, serpin3, and heat shock proteins.
**Key words**: Wheat, Species, Cultivar, Aleurone layer, Metabolic pathway

**Résumé**  *Introduction*. Les couches périphériques (PL) de blé, comprenant la couche d’aleurone (CA), sont une source importante de micronutriments. La CA est exclue et principalement utilisée pour l’alimentation animale, bien que ces tissus aient un potentiel nutritionnel important, car ils contiennent la plupart des micronutriments, des composés phytochimiques et des fibres et pourraient améliorer la qualité nutritionnelle des aliments. **Objectif**. Une approche protéomique a été utilisée pour mettre en évidence des différences protéiques majeures dans la couche d’aleurone (CA) de grains mature, entre quatre espèces de blé: *T. aestivum* et *T. spelta* (6X), *T. durum* (4X) et *T. monococcum* (2X). **Matériel et méthodes**. Dans chaque espèce, un cultivar représentatif a été utilisé pour l’extraction des protéines de la CA totale. L’électrophorèse bidimensionnelle des protéines des CA a révélé 1380, 1355, 1120 et 973 spots colorés au Coo massie, respectivement. **Résultats**. Un total de 334 spots ont montré des différences quantitatives ou qualitatives (présence/absence) entre les quatre cultivars. *T. aestivum* et *T. spelta* avaient, respectivement 90,9% et 92,6% de leur protéome CA identiques. Seuls 25 spots étaient significativement différents entre les deux cultivars hexaploïdes. Les protéines de la CA, codées par le génome A, étaient, principalement, exprimées dans des espèces polyploïdes. Les pourcentages de protéines, codées par le génome A, étaient de 85% chez les espèces tétraploïdes et de 70% dans l’hexaploïde. Quatre-vingts spots concernent des protéines spécifiques, différentes qualitativement et quantitativement, ont identifiées par spectrométrie de masse et interrogation des bases de données et ont été classées en 5 processus biologiques et 12 catégories fonctionnelles. **Conclusion**. Les principales différences entre les espèces sont dues, en particulier, aux globulines de stockage (22, 11 et 1 de Glo-3, Glo-3B, Glo-3C, respectivement), aux protéines liées au stress, notamment, des protéines antioxydantes, telles que la peroxyrédoxine 1-Cys et la superoxyde dismutase à manganèse, protéines de défense, tels que la xylanase, le serpin3 et les protéines de choc thermique.

**Mots-clés**: Blé, Espèce, Cultivar, Couche d’aleurone, Voie métabolique

**Introduction**

Triticum species other than common bread wheat, such as *Triticum spelta* (*T. Spelt*), emmer and einkorn are the subject of interest, due to the high nutritive and dietary value of their grain, and unique taste attributes [1]. The peripheral layers (PL) of wheat, including the aleurone layer (AL), are an important source of micronutrients. The AL is excluded and used mainly for animal feed, although these tissues have potential nutritional value, as they contain most micronutrients, phytochemicals, and fiber, and could greatly increase the nutritional quality of food [2,3]. The wheat AL is the only living tissue in the mature grain. The antioxidant potential of wheat grain fractions is mainly determined by aleurone content, which can be attributed to the presence of relatively large amounts of phenolic compounds, mainly ferulic acid [4]. The potential health benefit of antioxidants is associated with protection against oxidative stress [5]. The aleurone rich fractions exhibited better in vitro digestibility, and colonic ferment ability, than wheat bran [4].

Proteomic approaches have been used in cereal seeds to identify proteins, involved in seed development, and germination, like for example in barley [6,7]. In wheat, previous studies focused on the structure, and composition of the wheat grain [8]. Different markers for aleurone cell walls and aleurone cell contents were used to assess the histological composition of bran fractions to evaluate the dissociation, and accessibility of aleurone cell components [2,9]. The protein composition of the PL and AL of the hexaploid wheat cultivars Récital and Chinese Spring were described [10]. Proteomic tools (*ie*: 2DE, LC/MSMS) were also used in several approaches on wheat, like for example to describe the process occurring in the two first weeks of grain development [11], or for manually disse hand isolated AL. For *T. aestivum*, the AL was analyzed at 15 stages of grain development, allowing 327 proteins to be identified with seven different profiles of synthesis, or accumulation [12]. The AL of three varieties one only main species of wheat, *T. aestivum* (ABD), and *T. durum*...
(AB), were compared by Meziani et al (2012) [13]. A higher AL protein diversity was found within species, than between the two species. Similar result was found when AL of three cultivars from T. Aestivum, and T monococcum (genome A), were compared [14]. These both last comparisons, also, revealed the remarkable environ-mental stability of the AL proteome in the three cultivated wheat species. 

Triticum Spelt (T. spelta) is another hexaploid wheat becoming who is today more grown in European countries for food, and feed market [15]. Spelt was proposed to be an ancestor of the different hexaploid wheats [16]. Spelt is one of the oldest cultivated spe-
cies, which exhibit and hulled seeds, domestication probably thanks to the mutation that gave rise to gene conferring the widespread cultivation of bread wheat [17]. Although these two species are con-sidered to be derived, it would be interesting to know whether the absence of glume adhering to the T. aestivum wheat seed was associated to proteomic variations in its AL, as compared to the AL of T. Spelta. Currently no analysis of AL proteome of T. Spelta, and comparison with other species has been made.

The objective of this study was, thus, to identify the specific proteins of the AL in the mature grain with one cultivar of each of the following species: Triticum aetivum, and Triticum spelta (6X), Triticum durum (4X), and Triticum monococcum (2X). AL proteins were revealed qualitative (presence/absence), and quantitative variations. The interspecific comparisons allowed attributing, and discussing the genomic origin to the qualitative variation of some spots. 

Material and methods

One cultivar representative of four wheat species was used for AL characterization. The European hexaploid T. spelta Altglod (TsA), the hexaploid T. aetivum Chinese Spring (TaCS), the tetraploid T. durum Mexicali (TdM), and the diploid T. monococcum (TmDV92) by [18] were grown the same year, under normal conditions in the field with full fungicide protection at INRA, Clermont-Ferrand, France. A total of 30 mature, and homogeneous grains from each vari-
ety were harvested to separate the AL for protein extraction and characterization.

Separation of the AL

The AL was manually dissected in mature grain under a binocular microscope, as previously described by [10]. For each cultivars, 30 AL were ground with a pestle, and mortar in liquid nitrogen. The powder obtained was weighed, and separated into two biological samples, and stored at -80°C until analysis.

Obervation by electron microscopy

Microscopy imaging of AL was performed to confirm the purity of the recovered tissue, and to validate the procedure. AL samples were scanned by electron microscope (FEI Eindhoven, Netherlands) at 15 kV made with a Microvision system (Microvision Instruments, Evry, France).

Extraction of AL proteins

AL proteins were extracted from one biological sample, in 400 µL of extraction buffer (7 M urea, 4% Chaps, 2 M thiourea, 1.2 % destreak reagent, and 1% IPG buffer pH 3-10 according to [10]. The supernatant was recovered, and the protein concentration was measured, using the Bradford method [19].

Two-dimensional electrophoresis and image analysis

Four gels were made from two independent extracts for comparison with all AL extracts to obtain statistically reliable results. A total of 150 micrograms of protein were used for passive rehydration, overnight at room temperature on 24 cm immobilized pH 3-10 gradient strips. Two-dimensional electrophoresis was performed using SDS-PAGE gels (14% T, 2.1% C). The gels were stained with colloidal Coomassie Brilliant Blue (CCB) G-250, improved by [20]. The gels were scanned using a G-800 scanner, and the spots were statistically analyzed between four species using ANOVA, and the following values p, q ≤ 0.05 and fold ≥ 1.8 were used to reveal significant spot.

Identification of proteins by mass spectrometry

For identification by MALDI-TOF mass spectrometry, 334 spots with a normalized volume ≥ 0.05 were chosen among spots of interest to be excised from gels according to [11]. The subsequent identification of peptides was performed using a mass spectrometre Voyager-DE Pro MALDI-TOF (Applied Biosystems, Framingham, MA, USA). The resulting monoisotopic peptide masses were used to interrogate NCBI Viridiplantae, (Matrix Science, London, UK, [21]. Among accession numbers with a significant score, those for protein characterized in Triticaceae or in related species are listed in Table 2. Proteins were considered to have been identified if, at least, two non-redundant peptides matched a single reference in the database. Identified proteins were classified according to the KEGG database [22].

Results

Microscopic images of wheat species AL

The image of AL, observed by scanning electron microscope validated the dissection procedure and
revealed the purity of the recovered aleurone tissues, which were identified by their histological composition [2, 8] (Fig. 1). The manual isolation of the AL from testa, and hyaline layers differed according to the species. The method of dissection differed, depending on the wheat cultivars characteristics. The AL of TsA was relatively easy to dissect. TdM and TsA had wide AL cells with a thick inner pericarp (hyaline and testa), allowing easy dissection, unlike T. monococcum DV92, which has elongated cells that adhere strongly to the outer layers. Generally, the thickest (hyaline and testa) layer was the easiest to dissect.

**Fig. 1. Transverse scanning electron-microscopy of aleurone layer separated from the other layers**

(a) AL hexaploid: T. aestivum Chinese Spring, (b) AL diploid: T. monococcum DV92, (c) AL hexaploid: T. spelta Altgold, (d) AL tetraploid: Triticum Durum Mexicali. Scale 100 μm.

**Differences in AL proteins between species**

Two-dimensional electrophoresis of proteins extracted from the AL of the four different species was performed with four replicates. The scanned gels were analyzed by image analysis (Fig. 2). A total of 16 images were analysed. The AL proteins were resolved in a pH range from 4.00 to 9.1, and a molecular mass range from 11 to 110 kDa. In total, 1380, 1355, 1120 and 973 Coomassie stained spots were detected in TaCS, TsA, TdM, and TmDV92 gels, respectively (Table 1).

To analyze all quantitative, and qualitative changes in the proteome of the AL between the four species, statistical analyses on gel image comparisons were performed by pair between each species. Although all the six pair species comparisons were performed, mainly the comparisons involving TsA are here reported: A: TaCS and TsA, B: TdM and TsA, C: TmDV92 and TsA. For each pair, a gel Master was used as reference gel for image analysis.

**Fig. 2. Images of the 2D IPG strip pH (3-10) 24 cm X SDS-PAGE of T. aestivum Chinese Spring AL**

Surrounding spots are identified proteins and the five zones where major differences were found in the comparison between species are detailed in Fig. 3.

**Table 1. Comparison summary of the overall number of common spots, qualitative (presence and absence), and quantitative (more/less abundantly expressed) between hexaploid, tetraploid and diploid species (TaCS, TsA, TdM, TmDV92).**

| Comparison | Number of common spots | Number of qualitative spots | Number of quantitative spots |
|------------|-------------------------|-----------------------------|------------------------------|
| TaCS       | 1355                    | Presence/absence in TaCS    | Presence/absence in TaCS    |
| TdM vs TaCS| 1231 (92.9%)            | (1/2)                       | (3/2)                        |
| TmDV92 vs TaCS | 1064 (78.3%)       | (1/3/17)                    | (3/3/12)                     |
| TmDV92 vs TdM | 948 (70.5%)           | (6/15)                      | (6/6)                        |
| TmDV92 vs TdM | 959 (74.3%)           | (5/15)                      | (5/6)                        |
| TaCS vs TdM | 49                      | 139                         |                              |

The TaCS, TdM, and TmDV92 were used as gel Master for comparison of A, B, and C, and respectively 8, 57, and 83 spots were seen to be significantly different with TsA. Taking all comparisons involving TsA together, a total of 148 AL spots differed either quantitatively or qualitatively among the four species. The differential comparisons between species based on spots, and
spot volume percentage showed that total proteins were either absent between species (51 spots), or common (97 spots) with significant variation in quantity (Table 1). AL of the diploid cultivar TmDV92 was also compared to AL of the tetraploid TdM, in order to investigate spots that were common or different between the two geno-mes A and AB (see Comparison D, Table 1).

Protein identification between species
Of the 148 spots, 109 with percentage spot volume > 0.05 were excised from Master gels then digested, and analyzed by MALDI-TOF/MS. Finally, (84 spots identifies for comparison A, B, C, D), (77%) of excised spots were identified, and categorized in four pathways, and 12 functional categories. The largest category comprised 65% of the identified spots, and was composed of storage proteins, followed by glycolysis (10%), defence proteins (5.5%), oxidative stress, and protein folding (4%) polysaccharide catabolism, and transport mechanisms (3%), signalling and protein synthesis (1.5%), and 2% were categorized as unknown proteins.

Proteins grouped in specific zones
The majority of the 148 AL proteins that differed quantitatively, and qualitatively, between species, had spots localized in five main zones (Fig. 2). Zone I was composed of five proteins involved in environmental functions, metabolism, and defense: environmental information, and processing, such as ATP synthase (spots 619). The enzymes were involved in different metabolic pathways, including polysaccharides such as beta amylase (spot 516), glycolysis such as two enolases (spots 607 and 608), and one cytosolic 3-phosphoglycerate kinase (spot 966).

Table 2. Exemple of specific AL spots in mature wheat grain identified by MALDI-TOF

| Spot number | Name of protein | Mascot score | Protein expressed TaCS | Protein expressed TsA | Protein expressed D genome |
|-------------|----------------|--------------|------------------------|-----------------------|----------------------------|
| Comparison A
Carbohydrate metabolism
Storage proteins
| 609 | similarity to gi|170696| storage protein from wheat | 136 | - | + | AA |
| 418 | globulin 3 | 224 | - | + | AA |
| 2271 | similarity to gi|170696| storage protein from wheat | 180 | + | - |
| Stress and Defence
Oxidative stress
| 1700 | thiol peroxidin | 201 | - | + |
| 2217 | low molecular weight heat shock protein | 157 | + | - |
| Protein aminoacidase
Hexokinase
| 629 | protein disulfide isomerase | 216 | + | - | AA |

Zone II was mainly composed of storage proteins with numerous globulins Glo-3 of high molecular weight, HMW (66 kDa to 89 kDa) in the basic region of the gel. Zone III was composed of three globulins 3B of 57 kDa (spots 1130, 1246 and 1649), also classified, as storage proteins, and two xylanase inhibitors (spots 1350 and 1559). Zone IV was composed of stress and defense proteins related to folding as heat shock protein HSP 23 kDa (spot 2140) and manganese superoxide dismutase (spot 2249). The other six proteins (spots 2252, 2269, 2313, 2325, 2347, and 2435) were low molecular weight, LMW globulins (14 to 20 kDa). Zone V was composed of HSPs 21 kDa (spot 2217), and two oxidative stress proteins: 1-cys peroxiredoxin (spot 1700), and manganese superoxide dismutase (spot 1788), and three globulins: Glo-3 (spots 2363 and 2603), Glo-3B (spot 2371).

Other spots not grouped in specific zones
Many other proteins identified were located outside the five zones. In particular, we found transport proteins such as putative dihydrolipoamide dehydrogenase (spot 609), and stress inducible membrane...
pore proteins (spot 2301), and polysaccharide catabolism proteins, such as beta amylase (spot 598), glycolysis proteins, such as enolase (spot 1306), storage proteins (spots 927, 975, 1347, 1393 and 2438), and one oxidative stress disulfide isomerase (spot 329). Some proteins (spots 1445, 1765) were identified with an unknown function or with a specific role in the AL (Fig. 2).

Differences in proteins according to species
AL spots identified in each of the three species pair comparisons were classified, according to four biological processes: Environmental information processing, Carbohydrate metabolism, Stress defense and Unknown (Table 2).

Differences in proteins in the comparison (A) between TaCS and TsA
As expected, only minor differences were observed between the two TaCS, and TsA species sharing the same ABD genome. Among the eight spots that differed, six proteins were identified. Two proteins (spots 336, 488) involved in storage, and assemblage were absent in TaCS, and one protein (spot 1700) involved in oxidative stress 1-Cys peroxiredoxin was less abundant in TaCS. One HSP (spot 2217) of LMW, one protein disulfide isomerase (spot 329), and one storage protein (spot 2271) were significantly more abundant in TaCS.

Differences in proteins in the comparison (B) between TdM and TsA
Among 57 proteins that differed either qualitatively or quantitatively between TdM, and TsA, 18 proteins were identified. The majority of the identified proteins were over-expressed in the hexaploid TsA or absent in the tetraploid TdM. The cultivar TsA had 10 over-expressed globulins out of 12 that differed significantly from TdM, whereas TaCS had only nine out of 16 globulins found in TdM.
Six proteins involved in transport (spot 609), storage (spots 760, 927, 2363, and 2603), and defence (spot 1559) were also found in the comparison between TaCS and TdM.

Differences in proteins in the comparison (C) between TmDV92 and TsA
Of the 83 spots that significantly differed between the diploid TmDV92 and the hexaploid TsA, 28 were identified. All were either absent or significantly less abundant in TmDV92. Among these 28 identified spots, fourteen were also found in the comparison between TaCS, and TmDV92. For enzymes involved in glycolysis, the diploid cultivar had three spots (607, 608, and 1306), significantly, less abundant, and one absent (966), as compared to the hexaploid cultivar TsA. Twelve globulins (spots 336, 419, 425, 447, 718, 721, 730, 743, 2252, 2325, 2363 and 2371), and one transport protein (spot 2301) were also of less % volume in TmDV92 than in TsA. Five globulins (spots 393, 975, 1649, 2313 and 2347), two xylanases (spots 1350 and 1559), one HSP (spot 2140) one manganese superoxide dismutase (spot 1788) were absent in TmDV92.

Differences in proteins in the comparison (D) between TdM and TmDV92
Nineteen proteins were identified out of the 51 that differed significantly between TdM, and TmDV92. All 19 were either present in TdM, and absent or significantly less abundant in TmDV92. Other proteins, such as ATP synthase subunit beta mitochondrial (spot 619), two enolases (spot 607, 608), and one glucose/ribitol dehydrogenase (spot 1306) were less abundant in TmDV92. Four proteins (spots 425, 703, 742, and 2371) were already found in the comparison between TaCS, and TmDV92 with the same lower abundance in TmDV92. Four globulins (spots 1393, 2313, 2347 and 2438), and one manganese superoxide dismutase (spot 2249) were absent in TmDV92.

Discussion
The aim of this work was to use a proteomics approach to identify differences in the AL between the hexaploid T. spelta Altgold (TsA), and one representative cultivar of the three major cultivated wheat species T. aestivum, T. durum, and T. mono-coccum. Hand dissection enabled us to produce pure aleurone layer needed for proteomic analysis. SEM observation confirmed the purity of the AL obtained manually, which was easier when the layer was thick, this was the case for T. spelta but not for T. mono-coccum.
Depending on the species, image analysis revealed from 973 to 1380 Coomassie stained spots, of which from 0.2% to 2.4% were seen to qualitatively differ (presence/absence) between species, and from 0.3% to 6.4% differed quantitatively. Only spots that differed were selected for MS analysis, and data interrogation. The AL proteins identified were involved in nine functional processes: protein synthesis protein folding, and protein storage, oxidative stress and defense, transport, signaling molecules, and interaction.
Many proteins were extracted from the AL of the four-wheat species, as revealed by 2DE and Coomas-
sie staining. The total number of spots was 973, 1120, 1355, and 1380 for TmDV92, TdM, TsA, and TaCS respectively (Table 1). Comparison of the AL protein number, expressed in each genome, confirmed here using T spelta what was previously shown, when AL from three cultivars were compared between T aestivum, and T durum [13] or T aestivum, and T monococcum [14]: each genome did not account for the same number of AL proteins. The two hexaploid cultivars (TaCS and TsA), with the same genomic composition (AABBDD) had 1255 spots in common, representing 90.9% and 92.6% of their AL proteome, respectively. Only six spots differed significantly in percentage spot volume (3 higher and 2 lower), in TaCS than in TsA. Only one spot was present in TaCS, and absent in TsA. The two species shared a very high level of genomic sequences, and some genes involved in speltoid shape of the spike (q allele of the wheat domestication gene Q) on 5AL and some null alleles were characterized for their pleiotropic effect, like tenacious glumes, non-free-threshing seed, and elongated fragile rachis in T. spelta [17]. Recent sequence comparisons between spelt, and common wheat revealed, however, that the two species formed discrete groups, and showed a higher variability in T spelta sequences [22]. Our results showed that, at least, 90% of the AL proteome was identical between the two hexaploid species.

Comparison of the number of common spots between species that differed in genomic composition revealed that among the 973 spots of the diploid (AA) TmDV92, 951 and 948 (Table 1) were present in TdM and TsA which corresponded to 84.9% and 70%, of the proteins expressed respectively in the tetraploid (AABB) and hexaploid (AABBDD) cultivar. The AL of TdM (AABB genome) had 1064 spots in common with AL of TsA, accounting for 78.5% of the TsA AL proteins, evidencing that D genome (plus its interactions with A and B genomes) contributed to 21.5% in TsA AL gene expressions. But the A genome had the highest contribution (84.9%) of the AL proteins expressed for the tetraploid species and 70% for the hexaploid TsA (Table 1). This last percentage indicated that TsA would have less AL proteins encoded by A genome, as compared to the 93%, reported by Meziani et al. [14] for the bread wheat cultivars. Two hypotheses might explain these figures - (1) either the AL genes from A genome were predominantly expressed in tetraploid and hexaploid species, where, only some A genes (39 out of 951 (4%) or 6 out of 948 (0.6%), (Table 1) would be duplicated in genome B or B and D; or (2) either most of the common AL spots resulted from identical homoeologous genes coming from a common ances-tor to A and B genomes. The first hypothesis would imply that major differences between genomes existed specifically for genes encoding AL proteins. The second hypothesis is probably more reliable, as it has been reported for many endosperm enzymes and storage proteins, as being inherited on wheat homoeologous genes, where expression kinetic of triplets has revealed asymmetrical transcription profiles [23].

Some genes that were identical between species might also account for quantitative differences, revealed through image analysis, and ANOVA. Since the same quantity of proteins (150 µg) was loaded on the gels, the significant variations in the percentage of spot volume might result from a gene dosage effect (in the case of identical genes) or regulation of expression in response to the presence of additional genome.

Many spots, that differed qualitatively and quantitatively, were either less abundant or absent in TmDV92 compared to TaCS, TsA, and TdM. Differences, especially those in proteins involved in metabolic function (xylanase inhibitor, beta amylase, cytosolic/3phosphoglyceratekinase), might be particularly important in the wheat AL of (6X and 4X). The cytosolic/3phosphoglyceratekinase, and enolase are key enzymes in glycolysis to produce NADH and ATP, the energy necessary for pyruvate formation. This metabolite is the starting point for the synthesis of amino acids, proteins and vitamins, and is the entryway for acetyl-Co-A production for fatty acid biosynthesis, transport, and signal transduction proteins. The decrease in these proteins in the diploid species TmDV92 (AA genome), could explain the lower amount of storage proteins. These proteins were more abundant, when B and D genomes were present. The absence of defense proteins (serpin3 and xylanase inhibitor) in TmDV92 compared to respectively TaCS and TsA, indicated that they were probably encoded by B or D genomes rather than by the A genome. The presence of beta amylase (spot 516) in the AL of mature grain is in agreement with Volodymyr et al., [24], who reported the accumulation of beta amylase in the AL and sub-AL, during barley grain development. The synthesis of this enzyme during maturation means it can be rapidly mobilized for the hydrolysis required for embryo germination [25].

Globulins are important for nutrition, and also play a role in immune-activation, which is potentially antigenic, in patients with type 1 diabetes [26]. In our study, the storage protein globulins prevailed in all species. The proteomics approach helped us to identify, and characterize three globulins (Glo-3, Glo-3B and Glo-3C) of AL in wheat mature grain. These
globulins corresponded to 7S globulins, whose genes were identified in hexaploid, tetraploid, and diploid wheat species [26,27]. Glo-3 was present in all the wheat species studied; Glo-3B (HMW and LMW) were identified in all species, whereas Glo-3C of smaller MW (38 kDa) was only expressed in the AL of TaCS and TdM. The difference in pI and MW suggested that these globulins underwent post-translational modifications. Half of the globulins showed a significant decrease in intensity in TaCS than TdM, and one third of the globulins found in TdM decreased, in the comparison between TdM and TsA. Loit et al., [26] indicated that, there are, at least, two copies of Glo-3 in A, B and D diploid genomes and, at least, four homologous copies in the tetraploid, and hexaploid genome in the wheat. Their expression was observed by fluorescence, in both the AL and the embryo.

Stress protein HSP (spots 2217) with 21 kDa was absent in TmDV92 (A genome) and TdM (BB genome) and present in TaCS, TsA (ABD genome). Their expression could be controlled by the D genome. It has been reported that synthesis of HSP occurs in all wheat tissues during grain development [11], and in mature stages of grains [28]. Only one 1-Cys peroxiredoxin (1-CPR) was more abundant in TsA, TdM and TmDV92 than in TaCS. This protein (spot 1700), which was also identified in the wheat endosperm [29], conserves one Cys residue and inhibits peroxidase activity, when coupled with thiols. Mn-SOD antioxidant (spots 2249 and 1788) were present in TdM and TsA, respectively, but not in TmDV92. This would imply that these proteins are encoded by the B genome. Mn-SOD, which is localized in mitochondria and peroxisome, participates in the regulation of glucose metabolism, of fats, and in the activity of many enzymes involved in protecting cells against free radicals [30]. The two hexaploid TaCS and TsA (ABD genome) shared high similarity but with slight quantitative differences for some enzymes: the presence of two small oxidative stress proteins 1-CPR and HSP (spots 1700 and 2217) and of proteins involved in biosynthesis, like disulfide isomerase PDI (spot 329), which is absent in TsA. This absence of PDI would suggest a less ability to regulate the accumulation of storage proteins in the AL of TsA, as compared to TaCS.

**Conclusion**

Many of the AL proteins that differ between the four species remain to be identified to understand the respective gene influences, on the proteome composition of mature grain. Among the AL protein identified, the present study did not reveal qualitative (presence/absence) difference between the spelt representative, and with both of the free-threshing cultivars TaCs and TdM. Joint transcriptomics, and proteomics studies, involving several spelts, and bread wheat cultivars could be developed to know whether free-hulling has or not modified AL enzyme composition able to impact its vitamin content. Such approach also will be useful to decipher the apparent predominance of the A genome on AL proteome of the wheat species. Transcriptomics, and metabolomics studies, performed on isolated living AL also would be very profitable to better understand its protective role against oxidative stress, and attacks by pathogens, and its contribution to the nutritional value of the cultivated cereals.

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**Conflicts of interest**

Authors declare no conflict of interests.

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