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

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37 **Résumé**

38 **Contexte:** Une approche protéomique a été utilisée pour mettre en évidence des différences
39 protéiques majeures dans la couche d'aleurone (CA) en grains mature entre quatre espèces
40 de blé: *T. aestivum* et *T. spelta* (6X), *T. durum* (4X) et *T. monococum* (2X). **Matériel et**
41 **méthodes:** Dans chaque espèce, un cultivar représentatif a été utilisé pour l'extraction de la
42 protéine CA totale. L'électrophorèse bidimensionnelle des protéines des CA a révélé 1380,
43 1355, 1120 et 973 spots colorés au Coomassie, respectivement. **Résultat:** Un total de 334
44 spots ont montré des différences quantitatives ou qualitatives (présence / absence) entre les
45 quatre cultivars. *T. aestivum* et *T. spelta* avaient respectivement 90,9% et 92,6% de leur
46 protéome CA, identiques aux autres espèces. Seulement 25 spots étaient significativement
47 différents entre les deux cultivars hexaploïdes. Les protéines de la CA codées par le génome
48 A étaient principalement exprimées dans des espèces polyploïdes. Les pourcentages de
49 protéines codées par le génome A étaient de 85% chez les espèces tétraploïdes et de 70%
50 dans l' hexaploïde. Quarante-huit pour les protéines sont identifiées par spectrométrie de
51 masse et extraction de données et ont été classées en cinq processus biologiques et (12)
52 catégories fonctionnelles. **Conclusion:** Les principales différences entre les espèces étaient
53 dues en particulier aux globulines de stockage (22, 11 et 1 de Glo-3, Glo-3B, Glo-3C,
54 respectivement), à des protéines liées au stress, notamment des protéines antioxydantes
55 telles que la peroxyrédoxine 1-Cys et la superoxyde dismutase de manganèse , protéines de
56 défense telles que la xylanase, le serpin3 et les protéines de choc thermique.

57 **Mots-clés:** blé; Espèce; Cultivar; Couche d'aleurone; Voie métabolique

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61 **Abstract**

62 **Background** : A proteomics approach was used to reveal major protein differences in the
63 aleurone layer (AL) of mature grain between four wheat species: *T. aestivum* and *T. spelta*
64 (6X), *T. durum* (4X), and *T. monococum* (2X). **Material and methods**: In each species, one
65 representative cultivar was used for total AL protein extraction. Two-dimensional
66 electrophoresis of AL proteins revealed 1380, 1355, 1120 and 973 Coomassie stained spots,
67 respectively. **Result**: A total of 334 spots showed quantitative or qualitative
68 (presence/absence) differences between the four cultivars. *T. aestivum* and *T. spelta* had
69 90.9% and 92.6% of their AL proteome respectively, identical to the other species. Only 25
70 spots were significantly different between the two hexaploid cultivars. AL proteins encoded
71 by the A genome were mainly expressed in polyploid species. Percentages of proteins
72 encoded by A genome was 85%, in tetraploid species and 70% in the hexaploid spelt. Eighty
73 for proteins are identified by mass spectrometry and data mining and were classified in five
74 biological processes and (12) functional categories. **Conclusion**: The major differences
75 between species were particularly due to storage globulins (22, 11 and 1 of Glo-3, Glo-3B,
76 Glo-3C, respectively), stress related proteins including antioxidant proteins like 1-Cys
77 peroxiredoxin and manganese superoxide dismutase, defence proteins like xylanase, serpin3
78 and heat shock proteins.

79 **Keywords**: Wheat; Species; Cultivar; Aleurone layer; Metabolic pathway

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85 **1. Introduction**

86 Triticum species other than common bread wheat such as *Triticum spelta*, emmer and
87 einkorn are the subject of interest due to the high nutritive and dietary value of their grain
88 and unique taste attributes (Abdel-Aal et al., 1995). The peripheral layers (PL) of wheat,
89 including the aleurone layer (AL) are an important source of micronutrients. The AL is
90 excluded and used mainly for animal feed, although these tissues have potential nutritional
91 value, as they contain most micronutrients, phytochemicals and fiber, and could greatly
92 increase the nutritional quality of food (Hemery et al., 2007 and 2009). The AL of wheat is
93 the only living tissue in the mature grain. The antioxidant potential of wheat grain fractions
94 is said to be mainly determined by aleurone content, which can be attributed to the
95 presence of relatively large amounts of phenolic compounds, mainly ferulic acid (Mateo
96 Anson et al., 2009). The potential health benefit of antioxidants is associated with protection
97 against oxidative stress (Willcox et al., 2004). Aleurone rich fractions exhibited better in vitro
98 digestibility and colonic ferment ability than wheat bran (Mateo Anson et al., 2009).

99 Proteomics approaches have been used in cereal seeds to identify proteins involved in seed
100 development and germination like for example in barley (Finnie et al., 2006; Finnie and
101 Svensson, 2009). In wheat, previous studies focused on the structure and composition of the
102 wheat grain (Antoine et al., 2004). Different markers for aleurone cell walls and aleurone cell
103 contents were used to assess the histological composition of bran fractions to evaluate the
104 dissociation and accessibility of aleurone cell components (Barron et al., 2007; Hemery et al.,
105 2007). The protein composition of the PL and AL of the hexaploid wheat cultivars Récital and
106 Chinese Spring were described (Laubin et al., 2008). Proteomic tools (*ie*: 2DE- LC/MSMS)
107 were also used in several approaches on wheat, like for example to describe the process
108 occurring in the two first weeks of grain development (Nadaud et al, 2010) or for specifically

109 analyze the hand isolated AL. For *T. aestivum* the AL was analyzed at 15 stages of grain
110 development allowing 327 proteins to be identified with seven different profiles of synthesis
111 or accumulation (Nadaud et al, 2015). The AL of three varieties of each of the two main
112 species of wheat, *T. aestivum* (ABD) and *T. durum* (AB), were compared (Meziani et al 2012).
113 A higher AL protein diversity was found within species than between the two species. Similar
114 result was found when AL of three cultivars from *T. aestivum* and *T monococcum* (genome A)
115 were compared (Meziani et al., 2014). These two last comparisons also revealed the
116 remarkable environmental stability of the AL proteome in the three cultivated wheat
117 species.

118 Beside *T. aestivum* spelt (*T. spelta*) is another hexaploid wheat becoming today more grown
119 in European countries for food and feed market (Escarnot et al., 2012). Spelt was proposed
120 to be an ancestor of the different hexaploid wheats (McFadden and Sears 1946). Spelt is one
121 of the oldest cultivated species, which exhibit brittle rachis and hulled seeds and was
122 submitted to domestication probably thanks to the mutation that gave rise to Q gene
123 conferring free-threshing character: the key pleiotropic mutation allowing the widespread
124 cultivation of bread wheat (Simons et al., 2006). Although these two species are considered
125 to be derived from the same, it would be interesting to know whether the absence of glume
126 adhering to the *T aestivum* wheat seed was associated to proteomic variations in its AL as
127 compared to the AL of *T. spelta*. But to date, no analysis of AL proteome of *T. spelta* and
128 comparison with other species has been made.

129 The objective of this study was thus to identify the specific proteins of the AL in the mature
130 grain with one cultivar of each of the following species: *Triticum aestivum* and *Triticum*
131 *spelta* (6X), *Triticum durum* (4X) and *Triticum monococcum* (2X). AL proteins were revealed
132 on 2DE gels, and two-by-two comparison between species were performed revealing

133 qualitative (presence/absence) and quantitative variations. The AL proteome of the two
134 hexaploid species will be particularly compared. The interspecies comparisons allowed to
135 attribute and discuss the genomic origin to the qualitative variation of some spots.

136

137 **2. Materials and methods**

138 One cultivar representative of four wheat species was used for AL characterization. The
139 European hexaploid *T. spelta* Altgold (TsA), the hexaploid *T. aestivum* Chinese Spring (TaCS,
140 the tetraploid *T. durum* Mexicali (TdM), and the diploid *T. monococcum* DV92 (TmDV92) by
141 (Lijavetsky et al., 1999) were grown the same year under normal conditions in the field with
142 full fungicide protection at INRA, Clermont-Ferrand, France. A total of 30 mature and
143 homogeneous grains from each variety were harvested to separate the AL for protein
144 extraction and characterization.

145 **2.1. Separation of the AL**

146 The AL was manually dissected in mature grain under a binocular microscope as previously
147 described by Laubin et al., 2008. Thirty grains were needed from each cultivar to dissect and
148 to obtain sufficient AL proteins for three replicates. Ten grains were soaked overnight before
149 dissection in 1 ml of ultrapure water and 2 μ L protease inhibitor cocktail (Sigma, St. Louis,
150 MO). The embryo and the brush were swept from the cross section using a razor blade and
151 the outer layer of the pericarp was then removed using tweezers. An incision was made
152 along the crease in the ventral part of the seed and all around the PL containing the inner
153 pericarp, testa, hyaline and AL. The endosperm was removed by carefully scraping with the
154 back of a scalpel pushing outwards from the interior of the grain. After several washes in
155 ultrapure water containing protease inhibitor, the white cells of AL were carefully and slowly
156 removed from the hyaline with flat tweezers. Cleaned ALs were pooled in a tube containing

157 water and protease inhibitor then deposited on paper towels to remove excess water. For
158 each cultivars, 30 AL were ground with a pestle and mortar in liquid nitrogen. The powder
159 obtained was weighed and separated into two biological samples and stored at -80°C until
160 analysis.

161 **2.2. Observation by electron microscopy**

162 Microscopy imaging of AL was performed to confirm the purity of the recovered tissue and
163 to validate the procedure. Samples of AL were fixed directly on stubs, coated with gold in an
164 Emscope SC 500 (ELEXIENCE, Verrières le Buisson, France) and observed with a Philips SEM
165 505 scanning electron microscope (FEI Eindhoven, Netherlands) at 15 kV. Digital images
166 were made with a Microvision system (Microvision Instruments, Evry, France).

167 **2.3. Extraction of proteins from the AL**

168 AL proteins were extracted from one biological sample in 400 µL of extraction buffer (7 M
169 urea, 4% Chaps, 2 M thiourea, 1.2 % destreak reagent and 1% IPG buffer pH 3-10 according
170 to Laubin et al., 2008. After 1 h 30 min incubation at room temperature, the extract was
171 sonicated with a probe at 20 W for 20 s and incubated at room temperature for an
172 additional 30 min. The mixture was then centrifuged for 10 min at 10 000 rpm. The
173 supernatant was recovered and the protein concentration measured using the Bradford
174 method (Bradford., 1976).

175 **2.4. Two-dimensional electrophoresis and image analysis**

176 Four gels were made from two independent extracts for comparison with all extracts of AL
177 to obtain statistically reliable results. A total of 150 micrograms of protein were used for
178 passive rehydration overnight at room temperature on 24 cm immobilized pH 3-10 gradient
179 strips. IEF was performed to reach a total of 90 kVh according to the following program: 1 h
180 at 300 V, 4 h at 1000 V, 3h at 4000 V, 3h at 8000 V and 7h43 min at 8000 V. The strips were

181 equilibrated for 15 min in equilibration buffer (6 M urea, 50 mM Tris–HCl pH 8.8, 30%
182 glycerol, 2% SDS, 1% DTT and a trace of bromophenol blue) and 15 min with the same buffer
183 with 2.5% of iodoacetamide instead of DTT. Two-dimensional electrophoresis was
184 performed using SDS-PAGE gels (14% T, 2.1% C) at 60 W for the first 30 min and 10 W per gel
185 until exit of the migration front. The gels were stained with colloidal Coomassie Brilliant Blue
186 (CCB) G-250 improved by Rabilloud, 2000. The gels were scanned using a G-800 scanner (GE
187 Healthcare, Uppsala, Sweden). Gel images were analysed using SameSpots software version
188 3.2 using normalized spot volume for spot analysis. Spots were statistically analyzed
189 between two species using ANOVA and the following values $p, q \leq 0.05$ and $\text{fold} \geq 1.8$ were
190 used to reveal significant spot. All other spots were considered as false positives and not
191 considered.

192 **2.5. Identification of proteins by mass spectrometry**

193 For identification by MALDI-TOF mass spectrometry, 334 spots with a normalized volume \geq
194 0.05 were chosen among spots of interest to be excised from gels. For each spot, two
195 samples were taken from two gel replicates, placed in the same 0.5 mL Eppendorf and
196 destained with ammonium bicarbonate-acetonitrile buffer then digested with trypsin
197 solution (Promega, Madison WI, USA) for 4 h at 37 °C according to Nadaud et al., 2010. The
198 subsequent identification of peptides was performed using a mass spectrometer Voyager-DE
199 Pro MALDI-TOF (Applied Biosystems, Framingham, MA, USA). The resulting monoisotopic
200 peptide masses were used to interrogate NCBI Viridiplantae, Swissprot or Ta EST part CDS
201 databases using MASCOT software (Matrix Science, London, UK,
202 <http://www.matrixscience.com>). The following parameters were used for the searches: a
203 maximum mass tolerance of 25 to 30 ppm, a maximum of one missing cleavage, partial
204 methionine oxidation and partial carbamidomethylation of cysteine. If the MASCOT score

205 was significant with $p < 0.05$, protein identification was confirmed. The nucleotide sequences
206 of EST-clones with a significant Mascot score were submitted to Blastx in NCBI non-
207 redundant database. Among accession numbers with a significant score, those for protein
208 characterized in Triticeae or in related species are listed in Table 2. Proteins were considered
209 to have been identified if at least two non-redundant peptides matched a single reference in
210 the database. Identified proteins were classified according to the KEGG database
211 (<http://www.genome.jp/kegg/pathway.html>).

212 **3. Results**

213 **3.1. Microscopic images of AL of wheat species**

214 The image of AL observed by scanning electron microscope validated the dissection
215 procedure and revealed the purity of the recovered aleurone tissues, which were identified
216 by their histological composition (Antoine et al., 2004; Barron et al., 2007) (Fig. 1). The
217 manual isolation of the AL from testa and hyaline layers differed according to the species.
218 The method of dissection differed depending on the characteristics of the wheat cultivars.
219 The AL of TsA was relatively easy to dissect. TdM and TsA have wide AL cells with a thick
220 inner pericarp (hyaline and testa) allowing easy dissection, unlike *T. monococcum* DV92,
221 which has elongated cells that adhere strongly to the outer layers. Generally, the thickest
222 (hyaline and testa) layer was the easiest to dissect.

223 **3.2. Differences in AL proteins between species**

224 Two-dimensional electrophoresis of proteins extracted from the AL of the four different
225 species was performed with four replicates. The scanned gels were analyzed by image
226 analysis (Fig. 2). A total of 16 images were analysed. The AL proteins were resolved in a pI
227 range from 4.00 to 9.1 and a molecular mass range from 11 to 110 kDa. In total, 1380, 1355,
228 1120 and 973 Coomassie stained spots were detected in TaCS, TsA, TdM and TmDV92 gels,

229 respectively (Table 1). To analyze all quantitative and qualitative changes in the proteome of
230 the AL between the four species, statistical analyses on gel image comparisons were
231 performed by pair between each species. Although all the six pair species comparisons were
232 performed, mainly the comparisons involving TsA are here reported: **A:** TaCS and TsA, **B:**
233 TdM and TsA, **C:** TmDV92 and TsA. For each pair, a gel Master was used as reference gel for
234 image analysis. The TaCS, TdM and TmDV92 were used as gel Master for comparison of A, B,
235 and C, and respectively 8, 57, and 83 spots were seen to be significantly different with TsA.
236 Taking all comparisons involving TsA together, a total of 148 AL spots differed either
237 quantitatively or qualitatively among the four species. The differential comparisons between
238 species based on spots and spot volume percentage showed that total proteins were either
239 absent between species (51 spots) or common (97 spots) with significant variation in
240 quantity (Table 1). AL of the diploid cultivar TmDV92 was also compared to AL of the
241 tetraploid TdM in order to investigate spots that were common or different between the
242 two genomes A and AB (see Comparison D, Table (1))

243 **3.3. Protein identification between species**

244 Of the 148 spots, 109 with percentage spot volume > 0.05 were excised from Master gels
245 then digested and analyzed by MALDI-TOF/MS. Finally, (84 spots identifies pour les
246 comparaisons A, B, C, D), (77% of excised spots) were identified and categorized in four
247 pathways and 12 functional categories. The largest category comprised 65% of the identified
248 spots, and was composed of storage proteins, followed by glycolysis (10%), defence proteins
249 (5.5%), oxydative stress and protein folding (4%) polysaccharide catabolism and transport
250 mechanisms (3%), signalling and protein synthesis (1.5%) and 2% were categorized as
251 unknown proteins.

252

253 **3.4. Proteins grouped in specific zones**

254 The majority of the 148 AL proteins that differed quantitatively and qualitatively between
255 species had spots localized in five main zones (Fig 2).

256 Zone I was composed of five proteins involved in environmental functions, metabolism and
257 defense: environmental information and processing such as ATP synthase (spots 619). The
258 enzymes were involved in different metabolic pathways, including polysaccharides such as
259 beta amylase (spot 516), glycolysis such as two enolases (spots 607 and 608) and one
260 cytosolic 3-phosphoglycerate kinase (spot 966). Zone II was mainly composed of storage
261 proteins with numerous globulins Glo-3 of high molecular weigh, HMW (66 kDa to 89 kDa) in
262 the basic region of the gel. Zone III was composed of three globulins 3B of 57 kDa (spots
263 1130, 1246 and 1649) also classified as storage proteins, and two xylanase inhibitors (spots
264 1350 and 1559). Zone IV was composed of stress and defense proteins related to folding as
265 heat shock protein HSP 23 kDa (spot 2140) and manganese super oxide dismutase (spot
266 2249). The other six proteins (spots 2252, 2269, 2313, 2325, 2347, and 2435) were low
267 molecular weight, LMW globulins (14 to 20 kDa). Zone V was composed of HSPs 21 kDa (spot
268 2217) and two oxidative stress proteins: 1-cys peroxiredoxin (spot 1700) and manganese
269 super oxide dismutase (spot 1788), and three globulins: Glo-3 (spots 2363 and 2603), Glo-3B
270 (spot 2371).

271 **3.5. Other spots not grouped in specific zones**

272 Many other proteins we identified were located outside the five zones. In particular we
273 found transport proteins such as putative dihydrolipoamide dehydrogenase (spot 609), and
274 stress inducible membrane pore proteins (spot 2301) and polysaccharide catabolism
275 proteins such as beta amylase (spot 598), glycolysis proteins such as enolase (spot 1306),
276 storage proteins (spots 927, 975, 1347, 1393 and 2438), and one oxidative stress disulfide

277 isomerase (spot 329). Some proteins (spots 1445, 1765) were identified with an unknown
278 function or with a specific role in the AL (Fig. 2).

279 **3.6. Differences in proteins according to species**

280 The AL spots that we identified in each of the three pair comparisons of species were
281 classified according to (4) biological processes: Environmental information processing,
282 Carbohydrate metabolism, Stress defense et Unknown -(Table 2).

283 **3.6.1. Differences in proteins in the comparison (A) between TaCS and TsA**

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

290 **3.6.2. Differences in proteins in the comparison (B) between TdM and TsA**

291 Among the 57 proteins that differed either qualitatively or quantitatively between TdM and
292 TsA, 18 proteins were identified. The majority of the identified proteins were over-expressed
293 in the hexaploid TsA or absent in the tetraploid TdM. The cultivar TsA had 10 over-expressed
294 globulins out of 12 that differed significantly from TdM whereas TaCS had only nine out of 16
295 globulins found in TdM. Six proteins involved in transport (spot 609), storage (spots 760,927,
296 2363, and 2603) and defence (spot 1559) were also found in the comparison between TaCS
297 and TdM

298 **3.6.3. Differences in proteins in the comparison (C) between TmDV92 and TsA**

299 Of the 83 spots that significantly differed between the diploid TmDV92 and the hexaploid
300 TsA, 28 were identified. All were either absent or significantly less abundant in TmDV92.

301 Among these 28 identified spots fourteen were also found in the comparison between TaCS
302 and TmDV92. For enzymes involved in glycolysis, the diploid cultivar had three spots (607,
303 608 and 1306) significantly less abundant and one absent (966) as compared to the
304 hexaploid cultivar TsA. Twelve globulins (spots 336, 419, 425, 447, 718, 721, 730, 743, 2252,
305 2325, 2363 and 2371) and one transport protein (spot 2301) were also of less % volume in
306 TmDV92 than in TsA. Five globulins (spots 393, 975, 1649, 2313 and 2347), two xylanases
307 (spots 1350 and 1559), one HSP (spot 2140) one manganese superoxide dismutase (spot
308 1788) were absent in TmDV92.

309 **3.6.4. Differences in proteins in the comparison (D) between TdM and TmDV92**

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

318 **4. Discussion**

319 The aim of this work was to use a proteomics approach to identify differences in the AL
320 between the hexaploid *T. spelta* Altgold (TsA) and one representative cultivar of the three
321 major cultivated wheat species *T. aestivum*, *T. durum*, and *T. monococcum*. Hand dissection
322 enabled us to produce pure aleurone layer needed for proteomic analysis. SEM observation
323 confirmed the purity of the AL obtained manually, which was easier when the layer was
324 thick, which was the case for *T. spelta* but not for *T. monococcum*.

325 Depending on the species, image analysis revealed from 973 to 1380 Coomassie stained
326 spots, of which from 0.2% to 2.4% were seen to qualitatively differ (presence/absence)
327 between species, and from 0.3% to 6.4% differed quantitatively. Only spots that differed
328 were selected for MS analysis and data interrogation. The AL proteins identified were
329 involved in nine functional processes: protein synthesis protein folding and protein storage,
330 oxidative stress and defense, transport, signaling molecules and interaction.

331 Many proteins were extracted from the AL of the four-wheat species as revealed by 2DE and
332 Coomassie staining. The total number of spots was 973, 1120, 1355 and 1380 for TmDV92,
333 TdM, TsA and TaCS respectively (Table 1). Comparison of the AL protein number expressed
334 in each genome confirmed here using *T spelta* what was previously shown when AL from
335 three cultivars were compared between *T aestivum* and *T durum* (Meziani et al, 2012) or *T*
336 *aestivum* and *T monococcum* (Meziani et al, 2014): each genome did not account for the
337 same number of AL proteins.

338 The two hexaploid cultivars (TaCS and TsA) with the same genomic composition (AABBDD)
339 had 1255 spots in common, representing 90.9% and 92.6% of their AL proteome,
340 respectively. Only six spots differed significantly in percentage spot volume (3 higher and 2
341 lower) in TaCS than in TsA. Only one spot was present in TaCS and absent in TsA. The two
342 species share a very high level of genomic sequences and some genes involved in speltoid
343 shape of the spike (q allele of the wheat domestication gene Q) on 5AL and some null alleles
344 were characterized for their pleiotropic effect like tenacious glumes, non-free-threshing
345 seed and elongated fragile rachis in *T. spelta* (Simons et al., 2006). Recent sequence
346 comparisons between spelt and common wheat revealed however that the two species
347 formed discrete groups and showed a higher variability in *T spelta* sequences (Liu et al.,

348 2018). Our results showed that at least 90% of the AL proteome was identical between the
349 two hexaploid species.

350 Comparison of the number of common spots between species that differed in genomic
351 composition revealed that among the 973 spots of the diploid (AA) TmDV92, 951 and 948
352 (Table 1) were present in TdM and TsA which corresponds to 84.9% and 70%, of the proteins
353 expressed respectively in the tetraploid (AABB) and hexaploid (AABBDD) cultivar. The AL of
354 TdM (AABB genome) had 1064 spots in common with AL of TsA, accounting for 78.5% of the
355 TsA AL proteins, evidencing that D genome (plus its interactions with A and B genomes)
356 contribute to 21.5% in TsA AL gene expressions. But the A genome has the highest
357 contribution (84.9%) of the AL proteins expressed for the tetraploid species and 70% for the
358 hexaploid TsA (Table 1). This last percentage indicates that TsA would have less AL proteins
359 encoded by A genome as compared to the 93% reported by Meziani et al., 2014 for the
360 bread wheat cultivars. Two hypotheses may explain these figures - (1) either the AL genes
361 from A genome are predominantly expressed in tetraploid and hexaploid species where only
362 some A genes (39 out of 951 (4%) or 6 out of 948 (0.6%), (Table 1) would be duplicated in
363 genome B or B and D; -or (2) either most of the common AL spots result from identical
364 homoeologous genes coming from a common ancestor to A and B genomes. The first
365 hypothesis would imply that major differences between genomes exist specifically for genes
366 encoding AL proteins. The second hypothesis is probably more reliable as it has been
367 reported for many endosperm enzymes and storage proteins as being inherited on wheat
368 homoeologous genes where expression kinetic of triplets has revealed asymmetrical
369 transcription profiles (Pont et al., 2011).

370 Some genes that are identical between species may also account for quantitative differences
371 revealed through image analysis and ANOVA. Since the same quantity of proteins (150 µg)

372 was loaded on the gels, the significant variations in the percentage of spot volume may
373 result from a gene dosage effect (in the case of identical genes) or regulation of expression
374 in response to the presence of additional genome.

375 Many spots that differed qualitatively and quantitatively were either less abundant or
376 absent in TmDV92 compared to TaCS, TsA, and TdM. Differences especially those in proteins
377 involved in metabolic function (xylose isomerase, enolase, beta amylase, cytosolic
378 /3phosphoglyceratekinase), may be particularly important in the wheat AL of (6X and 4X).

379 The cytosolic/3phosphoglyceratekinase and enolase are key enzymes in glycolysis to
380 produce NADH, and ATP, the energy necessary for pyruvate formation. This metabolite is the
381 starting point for the synthesis of amino acids, proteins, and vitamins, and is the entryway
382 for acetyl-Co-A production for fatty acid biosynthesis, transport and signal transduction
383 proteins. The decrease in these proteins in the diploid species TmDV92 (AA genome), could
384 explain the lower amount of storage proteins. These proteins were more abundant when B
385 and D genomes were present. The absence of defense proteins (serpin3 and xylanase
386 inhibitor) in TmDV92 compared to respectively TaCS and TsA, indicate that they are probably
387 encoded by B or D genomes rather than by the A genome. The presence of beta amylase
388 (spot 516) in the AL of mature grain is in agreement with Volodymyr et al., 2009, who
389 reported the accumulation of beta amylase in the AL and sub-AL during barley grain
390 development. The synthesis of this enzyme during maturation means it can be rapidly
391 mobilized for the hydrolysis required for embryo germination (Neuberger et al., 2008).

392 Globulins are important for nutrition and also play a role in immunoactivation, which is
393 potentially antigenic in patients with type 1 diabetes (Loit et al., 2009). In our study, the
394 storage protein globulins prevailed in all species. The proteomics approach helped us to
395 identify and characterize three globulins (Glo-3, Glo-3B and Glo-3C) of AL in wheat mature

396 grain. These globulins correspond to 7S globulins, whose genes were identified in hexaploid,
397 tetraploid and diploid wheat species (Loit et al., 2009; Jerkovic et al., 2010). Glo-3 was
398 present in all the wheat species we studied; Glo-3B (HMW and LMW) were identified in all
399 species, whereas Glo-3C of smaller MW (38 kDa) was only expressed in the AL of TaCS and
400 TdM. The difference in pI and MW suggest that these globulins underwent post-translational
401 modifications. Half the globulins showed a significant decrease in intensity in TaCS than TdM
402 and one third of the globulins found in TdM decreased in the comparison between TdM and
403 TsA. Loit et al., 2009, indicated that are at least two copies of Glo-3 in A, B and D diploid
404 genomes and at least four homologous copies in the tetraploid and hexaploid genome in the
405 wheat. Their expression was observed by fluorescence both in the AL and in the embryo.

406 Stress protein HSP (spots 2217) with 21 kDa was absent in TmDV92 (A genome) and TdM (BB
407 genome) and present in TaCS, TsA (ABD genome). Their expression could be controlled by
408 the D genome. It has been reported that synthesis of HSP occurs in all wheat tissues during
409 grain development (Nadaud et al., 2010) and in mature stages of grains (Majoul et al.; 2004).
410 Only one 1-Cys peroxiredoxin (1-CPR) was more abundant in TsA, TdM and TmDV92 than in
411 TaCS. This protein (spot 1700), which was also identified in the wheat endosperm (Skylas et
412 al., 2000), conserves one Cys residue and inhibits peroxidase activity when coupled with
413 thiols. Mn-SOD antioxidant (spots 2249 and 1788) were present in TdM and TsA respectively
414 but not in TmDV92. This would imply that these proteins are encoded by the B genome. Mn-
415 SOD, which is localized in mitochondria and peroxisome, participates in the regulation of
416 glucose metabolism, of fats, and in the activity of many enzymes involved in protecting cells
417 against free radicals (Alscher et al., 2002). The two hexaploid TaCS and TsA (ABD genome)
418 shared high similarity but with slight quantitative differences for some enzymes: the
419 presence of two small oxidative stress proteins 1-CPR and HSP (spots 1700 and 2217) and of

420 proteins involved in biosynthesis, like disulfide isomerase PDI (spot 329) which is absent in
421 TsA. This absence of PDI would suggest a less ability to regulate the accumulation of storage
422 proteins in the AL of TsA as compared to TaCS.

423 **Conclusion**

424 Many of the AL proteins that differ between the four species remain to be identified to
425 understand the respective genome influences on the proteome composition of mature
426 grain. Among the AL protein identified the present study did not reveal qualitative (presence
427 / absence) difference between the spelt representative and with both of the free-threshing
428 cultivars TaCs and TdM. Joint transcriptomics and proteomics studies involving several spelts
429 and bread wheat cultivars could be developed to know whether free-hulling has or not
430 modified AL enzyme composition able to impact its vitamin content. Such approach also will
431 be useful to decipher the apparent predominance of the A genome on AL proteome of the
432 wheat species. Transcriptomics and metabolomics studies performed on isolated living AL
433 also would be very profitable to better understand (1) its protective role against oxidative
434 stress and attacks by pathogens and (2) its contribution to the nutritional value of the
435 cultivated cereals.

436

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529

530 **Figure Captions**

531 Figure 1: Transverse scanning electron-microscopy of the aleurone layer separated from the
532 other layers. (a) AL hexaploid: *T. aestivum* Chinese Spring, (b) AL diploid: *T. monococum*
533 DV92, (c) AL hexaploid: *T. spelta* Altgold, (d) AL tetraploid: *Triticum Durum* Mexicali. Scale
534 100 μ m.

535

536 Figure 2: Images of the 2D IPG strip pH (3-10) 24 cm X SDS-PAGE of *T. aestivum* Chinese
537 Spring AL. Surrounding spots are identified proteins and the five zones where major
538 differences were found in the comparison between species are detailed in figure 3.

539

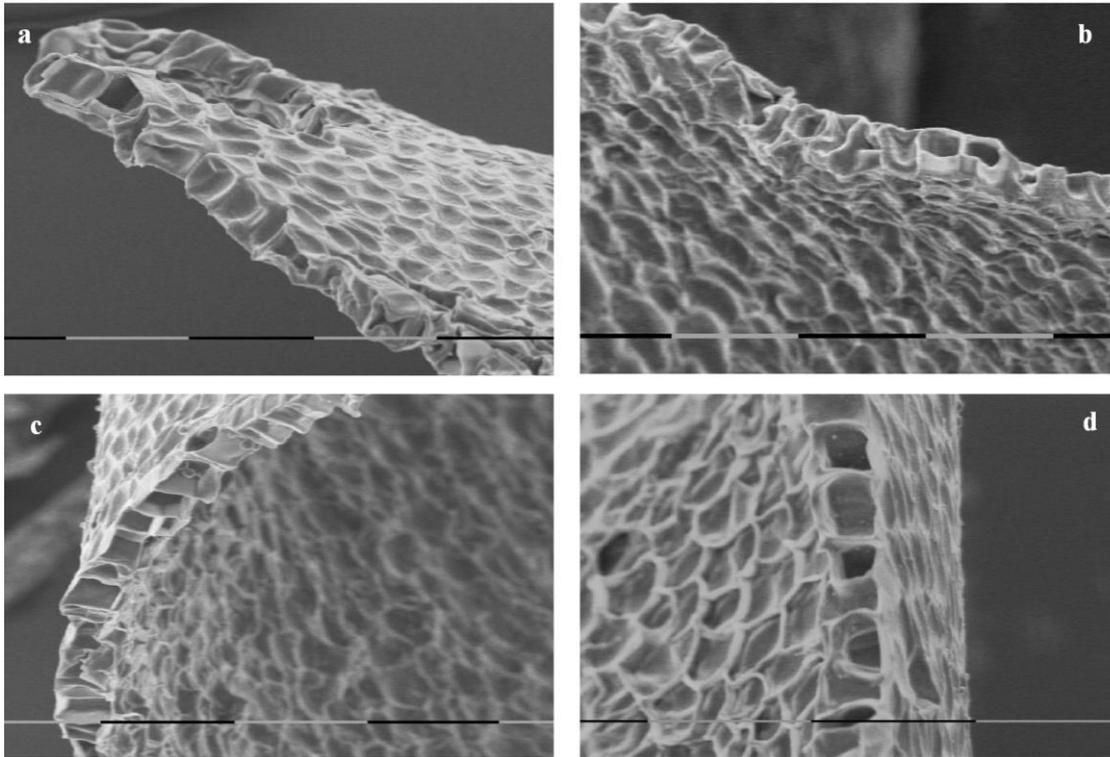
540 Figure 3: The five major zones of qualitative and quantitative differences in AL proteins in
541 mature grain of the four species TaCS, TsA, TdM, and TmDV92.

542

543 Table1: Summary of comparison of the overall number of common spots, qualitative
544 (presence and absence), and quantitative (more / less abundantly expressed) between
545 hexaploid, tetraploid and diploid species (TaCS, TsA, TdM, TmDV92). Comparison A: TaCS
546 and TsA, Comparison B: TdM and TsA, Comparison C: TmDV92 and TsA, Comparison D:
547 TmDV92 and TdM.

548

549 Table 2: Specific AL spots in mature wheat grain identified by MALDI-TOF. Spots identified by
550 MS/MS are given in (supplementary data). each spot: Spot ID, accession number in NCBI,
551 number of matched peptide, pI/MW (theoretical and experimental), over-expressed (+)
552 under-expressed (-) and absence (--) in different gels Master (TaCS, TsA, TdM, TmDV92) are
553 indicated.



555

556 Figure 1: Transverse scanning electron-microscopy of the aleurone layer separated from the
 557 other layers. (a) AL hexaploid: *T. aestivum* Chinese Spring, (b) AL diploid: *T. monococcum*
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 559 100 μm .

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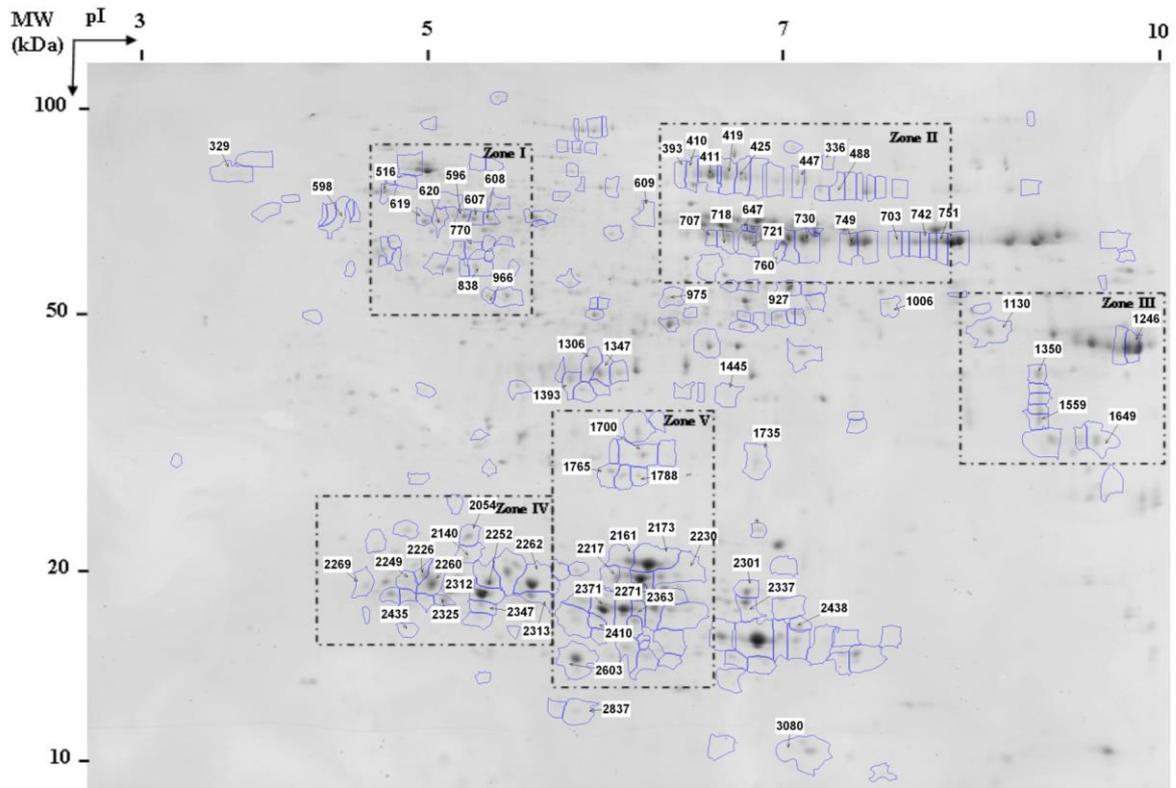
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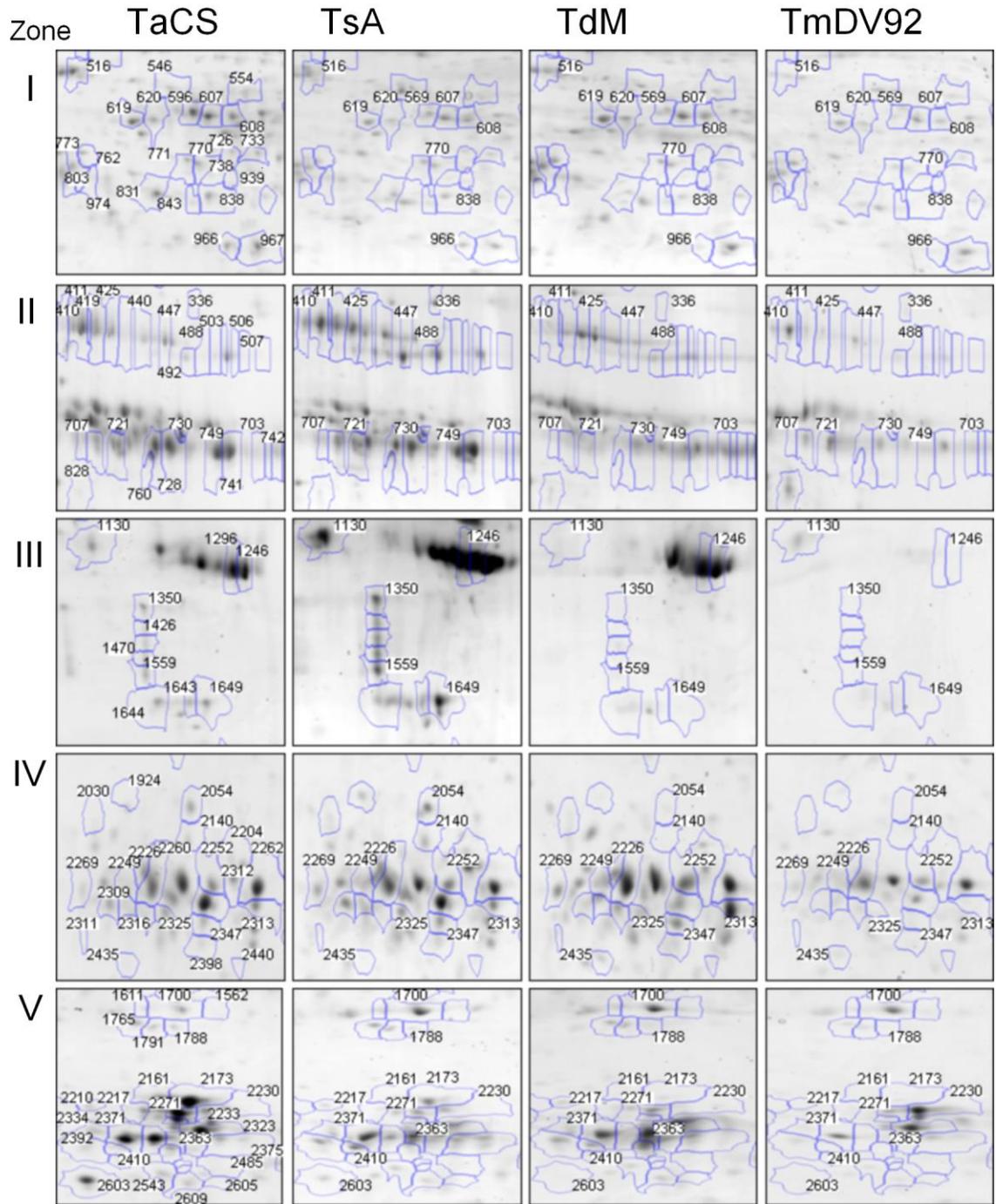
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584 Figure 3: The five major zones of qualitative and quantitative differences in AL proteins in
 585 mature grain of the four species TaCS, TsA, TdM, and TmDV92.

586

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588 Table1: Summary of comparison of the overall number of common spots, qualitative
 589 (presence and absence), and quantitative (more / less abundantly expressed) between
 590 hexaploid, tetraploid and diploid species (TaCS, TsA, TdM, TmDV92). Comparison A: TaCS
 591 and TsA, Comparison B: TdM and TsA, Comparison C: TmDV92 and TsA, Comparison D:
 592 TmDV92 and TdM.

593

594

| Number of specific spots | Number of common spots (% ID with TsA) | Number of qualitative spots | Number of quantitative spots |
|---------------------------------------|--|------------------------------------|-------------------------------------|
| TsA 1355 | | Presence/Absence in TaCS | High/Low Expressed in TaCS |
| Hexaploid TaCS : 1380 | | | |
| Comparison A (TaCS vs TsA) | 1255(92.6%) | (1/2) | (3/2) |
| | | Presence/Absence in TdM | High/Low Expressed in TdM |
| Tetraploid TdM : 1120 | | | |
| Comparison B (TdM vs TsA) | 1064 (78.5%) | (10/17) | (18/12) |
| | | Presence/Absence in TmDV92 | High/Low expressed in TmDV92 |
| Diploid TmDV92 : 973 | | | |
| Comparison C (TmDV92 vs TsA) | 948 (70%) | (6/15) | (6/56) |
| Comparison D (TmDV92 vs TdM) | 951 (84.9%) | (8/5) | (39/3) |
| Total including D comparison | | 60 | 139 |

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