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ORIGINAL PAPER

Mapping and proteomic analysis of albumin and globulin proteins in hexaploid wheat kernels (*Triticum aestivum* L.)

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Abstract Albumins and globulins of wheat endosperm represent 20% of total kernel protein. They are soluble proteins, mainly enzymes and proteins involved in cell functions. Two-dimensional gel immobiline electrophoresis (2DE) (pH 4-7) × SDS-Page revealed around 2,250 spots. Ninety percent of the spots were common between the very distantly related cultivars 'Opata 85' and 'Synthetic W7984', the two parents of the International Triticeae Mapping Initiative (ITMI) progeny. 'Opata' had 130 specific spots while 'Synthetic' had 96. 2DE and image analysis of the soluble proteins present in 112 recombinant inbred lines of the F9-mapped ITMI progeny enabled 120 unbiased segregating spots to be mapped on 21 wheat (*Triticum aestivum* L. em. Thell) chromosomes. After trypsic digestion, mapped spots were subjected to MALDI-Tof or tandem mass spectrometry for protein identification by database mining. Among the 'Opata' and 'Synthetic' spots identified, many enzymes have already been mapped in the barley and rice genomes. Multigene families of Heat Shock Proteins, beta-amylases, UDP-glucose pyrophosphorylases, peroxydases and thioredoxins were successfully identified. Although other proteins remain to be identified, some differences were found in the number of segregating proteins involved in response to stress: 11 proteins found in the modern selected cultivar 'Opata 85' as compared to 4 in the new hexaploid 'Synthetic W7984'. In addition, 'Opata' and 'Synthetic' differed in the number of proteins involved in protein folding (2 and 10, respectively). The usefulness of the mapped enzymes for future research on seed composition and characteristics is discussed.

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Introduction

Wheat kernel, a staple human food, is mainly eaten in the form of baked products. Hence, analysis of all the kernel components is important for human health. Many studies have been devoted to the analysis of kernel composition and to the identification of genes involved in its two major components, starch and storage proteins. The genetic inheritance of gliadins and glutenins, the two major storage protein fractions of the kernel endosperm has also been the subject of many studies (for review see Lafiandra et al. 2004). In addition to storage proteins, like other cereals, wheat kernel contains albumins and globulins, also called soluble proteins because they are easily extracted from flour using a water and sodium chloride solution. Albumins and globulins, which each account for approximately 10% of total flour proteins, are known to be easily soluble enzymes and proteins soluble in polar solution, respectively. Powerful protein separation techniques such as Two-dimensional gel electrophoresis (2DE) have shown that more than 2,000 spots can easily be extracted from flour with salt solution. Several attempts have been made to identify these wheat endosperm proteins using proteomic

approaches (Skylas et al. 2000, 2005; Islam et al. 2003). Studies on kernel responses to heat stress have enabled identification of many of the enzymes in kernel of wheat (Majoul et al. 2003, 2004; Skylas et al. 2000; Dupont et al. 2006), of barley (Østergaard et al. 2002) and of rice (Komatsu et al. 1993). However, the majority of soluble proteins still need to be identified and mapped. The use of aneuploid lines has enabled to assign on chromosome arms genes encoding some major wheat endosperm enzymes, and Mendelian genetic analyses has enabled to calculate genetic distances between these genes (McIntosh et al. 2003). The genotyped inbred progenies often used in quantitative trait loci (QTL) analyses of morphology, agronomy and quality characters may also greatly help protein mapping (Nelson et al. 2006). To map the proteins involved in kernel composition it is therefore important to use very densely mapped progeny of unrelated progenitors. International Triticeae Mapping Initiative (ITMI), recombinant inbred lines (RILs) are suitable for this objective. ITMI progeny, which was mapped jointly worldwide (Gupta et al. 2002; Marino et al. 1996; Mingeot and Jacquemin 1999; Nelson et al. 1995a, b, c; Röder et al. 1998; Van Deynze et al. 1995), was used to search for QTLs of agronomical importance (Börner et al. 2002), for quality traits (Nelson et al. 2006), and was also used to map amphiphilic kernel proteins identified using the proteomic approach (Amiour et al. 2002, 2003). We used this densely genotyped international standard ITMI progeny in the present study to map the segregating soluble wheat kernel proteins revealed by 2DE. Some of the proteins were then identified using mass spectrometry and by interrogating international public sequence data banks.

Materials and methods

Plant material

One hundred and twelve RILs of the ITMI population derived from the cross between the synthetic hexaploid wheat 'W7984' also named 'Synthetic' (generated via a cross between *Triticum tauschii* accession 'CI 18' = 'WPI 219 (PR88-89) and the tetraploid wheat *T. turgidum* cv durum named 'Altar 84' used as the female parent) and the spring wheat 'Opata 85' (Marino et al. 1996; Nelson et al. 1995b) were used in this study. Plants of the F9 generation were grown in the field at the INRA station in Clermont-Ferrand (France), in normal conditions with full pesticide protection. Only bagged spikes were used for analysis. For each RIL, 15 kernels were randomly sampled and ground into wholemeal flour using a Cyclotec mill 14920 (ASN Foss Electric, Hilleröd, Denmark).

Methods

Extraction and quantification

One hundred milligrams of wholemeal flour was used to extract the albumins and globulins (agl for wheat 2D protein markers) according to the procedure proposed by Marion et al. 1994. The agl were extracted with a salt solution (Phosphate 50 mM, NaCl 0.1 M, pH 7.8) added to a cocktail of plant protease inhibitors (Sigma, St Louis, MO, USA) with continuous mixing at 4°C for 2 h. The mixture was centrifuged (8,000*g*, 20 min) and the proteins in the supernatant were precipitated with acetone at −20°C, the pellet was then washed several times with acetone before being dried at room temperature.

The protein content in the dry pellet was measured using the BCA method (Uptima, Interchim, Montluçon, France). The precipitate was dissolved in 250 µl of a solution (4% CHAPS, 9 M Urea) compatible with BCA quantification. The protein solution was then added with a second solution (4% CHAPS, 6 M Urea, 3 M Thiourea, 1% Pharmalytes (pH 3–10), 1% Resolytes (pH 4–6.5), 2% DTT). Finally, the protein solution was added with 2% Pharmalytes (pH 3–10), 2% Resolytes (pH 4–6.5), 0.7% 4-vinyl-pyridine, and 60% glycerol (Amiour et al. 2002). The concentration of the proteins was approximately 10 µg/µl in the final protein solution.

2D gel electrophoresis

Eighteen centimetres immobiline pH gel strips (pH 4–7) (GE Healthcare, Uppsala, Sweden) were first rehydrated for 16 h at room temperature with 340 µl of rehydration solution (4% CHAPS, 7 M Urea, 2 M Thiourea, 1% Pharmalytes (pH 3–10), 1% Resolytes (pH 4–6.5), 2% DTT) added with 40 µg of protein extract. Immobiline pH Gel electrophoresis (IPGE) was performed at 20°C for a total of 30 kV-hours [400 v (0.25 h); 600 v (0.33 h); 1,200 v (0.25 h); 2,500 v (0.24 h); 5,000 v (5.76 h)] with a Multiphor II unit (GE Healthcare, Uppsala, Sweden).

After IEF, strip equilibration was performed by steeping the strips for 15 min in the Tris–Urea solution (6 M Urea, 50 mM Tris–HCl (pH 8.8), 30% glycerol and 2% SDS) added with 2% of DTT. The proteins were then alkylated for 15 min using the same Tris–Urea solution added with 1.4% of 4-vinyl-pyridine. The strips were deposited on second dimension gel for SDS-Page (T: 14%, C: 2.1%). The gels were then silver-stained according to the method of the silver stain PlusOne kit (GE Healthcare, Uppsala, Sweden). Three replicates of IPGE × SDS-Page were performed for each of the 112 RILs. MW and the pI were estimated by running 2D SDS-PAGE standards (Bio-Rad,

Richmond, VA, USA) covering a mass range of 17.5–76 kDa and a pI range of 4.5–8.5.

Image analysis and mapping

Gels were scanned and images analyzed using Melanie-3 software (GE Healthcare, Uppsala, Sweden). Chromosome assignment of each protein spot to a given locus was carried out using Mapmaker/exp version 3.0 b software (Lincoln et al. 1992). Chromosome assignment of the agl spots was carried out using the ITMI framework map comprising 2,000 molecular markers (Leroy et al. 1997). These agl spots were assigned between the anchor markers using the Mapmaker 'assign' command at Lod 3.0 and the recombination fraction of 0.35. After chromosome assignment, the spots were placed at Lod 3.0. Segregation distortion was calculated using an in-house S + programme. Spots showing a segregation distortion higher than 1/1,000 were discarded. Spots below the threshold of Lod 3.0 and above a recombination fraction of 0.35 were left unassigned.

Protein identification by MALDI-Tof mass spectrometry

The first series of protein spots (105) was excised from six gels (60 µg of protein extract/gel) after staining with silver nitrate according to the modified Blum's technique (Blum et al. 1987), compatible with the mass spectrometry. The spots were distained with a solution of 30 mM potassium ferricyanide, 100 mM sodium thiosulphate (1/1) for 2–3 min. The solution was then eliminated by washing in water twice for 15 min. Excised gels were washed a second time with solutions (25 mM NH_4HCO_3, 5% acetonitrile for 30 min and 25 mM NH_4HCO_3, 50%, acetonitrile twice for 30 min). After dehydration in 100% acetonitrile and drying, 100–200 ng of trypsin (V511, Promega, Madison, WI, USA), depending of the volume of the spot, in solution in 25 mM NH_4HCO_3 was added to the spots and digestion was performed at 37°C for 16–18 h. After centrifugation, peptides were extracted by adding 8–15 µl of acetonitrile. The mixture was sonicated for 5 min and centrifuged. For MALDI-Tof mass spectrometry, 1 µl of peptides was loaded directly onto the MALDI target. The matrix solution (5 mg ml^{-1} α-cyano-4-hydroxycinnamic acid in 50% acetonitrile/0.1% trifluoroacetic acid) was added immediately and allowed to dry at room temperature. A Voyager DE-Pro model of MALDI-Tof mass spectrometer (Perseptive BioSystems, Farmingham, MA, USA) was used in positive-ion reflector mode for peptide mass fingerprinting. External calibration was performed with a standard peptide solution (Proteomix C002, LaserBio Labs, Sophia-Antipolis, France). Internal calibration was performed using peptides resulting from auto-digestion of porcine trypsin.

To increase the efficiency of identification, a second series of protein spots (86) was excised from four gels (200 µg of protein extract/gel) stained with the Coomassie Brilliant Blue G250 according to the method of Neuhoff et al. 1988, modified by Rabilloud 2000. These spots were washed with solutions of NH_4HCO_3 and acetonitrile (as for silver-stained spots) then prepared for MALDI-Tof mass spectrometry as described above.

Monoisotopic peptide masses were compared to those from NCBInr (2008/09/03) and SwissProt (2008/04/08) databases using 'Mascot' and/or 'Profound' softwares (http://www.matrixscience.com and http://prowl.rockefeller.edu). The following parameters were considered for the searches: a maximum fragment ion mass tolerance of ±30 ppm, a maximum of one missed cleavage, partial methionine oxidation and partial pyridylethylation of cysteine. If the Mascot or Profound score was highly significant ($P < 0.05$) protein was valid.

When the best match in the NCBInr database was to a protein from another species, a further search for a homologue protein in the *T. aestivum* species was carried out using the powerful and well-documented search engine of the Gramene database (http://www.gramene.org/db/protein/protein-search). In that database similarity with other proteins can be visualized through blast2 at NCBI (National Center for Biotechnology Information, USA).

Protein identification by ion trap-MS

To confirm the initial MALDI-Tof identification and for further identification, 76 spots of the second series were analyzed using an ion trap mass spectrometer HPLC-MS/MS. Nano HPLC was performed with an Ultimate LC system combined with Famos autosampler and Switchos II microcolumn switching for preconcentration (LC Packings, Amsterdam, The Netherlands). The peptide samples were loaded on the column (PEPMAP C18, 5 µm, 75 µm ID, 15 cm; LC Packings) using a preconcentration step in a micro-precolumn cartridge (300 µm ID, 1 mm). Six microlitres of the sample were loaded on the precolumn at 40 µl/min. After 3 min, the precolumn was connected with the separating column and the gradient was started at 200 nl/min. The solvents used with 0.5% formic acid were 95% water/5% acetonitrile (A) and 95% acetonitrile/5% water (B). A linear gradient from 10 to 90% of B was applied for 45 min. For ion trap-MS, a LCQ^{DECA} with a nano-electrospray interface (ThermoElectron, Les Ulis, France) was used. Ionization (1.8 kV ionization potential) was performed with a liquid junction and a noncoated capillary probe (New Objective, Cambridge, USA). Peptide ions were analyzed using the data-dependant "triple-play" method as follows: (i) full MS scan (m/z 400–2,000), (ii) Zoom Scan (scan of the major ion with higher resolution), (iii) MS/MS of this ion.

Identification of peptides was performed with SEQUEST (Bioworks 3.1, ThermoElectron) or MASCOT (v2.2), using the query settings "a maximum of two missed cleavage, partial methionine oxidation and partial pyridylethylation of cysteine" and "mass deviation lower than 1.5 and 0.8 Da for ions parents and fragments, respectively", within NCBInr (2008/09/03). The candidate database peptide with the highest score was retained if the following identification criteria were met: Xcorr (SEQUEST) of at least 2.0, 2.2 and 3.5 for singly, doubly and triply charged peptides, respectively, and/or probability based Mowse score (MASCOT, v2.2) significant ($P < 0.05$). The validation was confirmed by visual inspection proving a satisfactory correlation between experimental and theoretical MS/MS spectra. Thus, when two or more valid peptides were obtained for one protein, the protein was validated.

Like for protein identification with MALDI-Tof, we tried to find the homologue protein in *T. aestivum* and when this was not the case, we used the search engine of the Gramene database (http://www.gramene.org/db/protein/protein-search).

If identification failed, the MS/MS sequence was used to search the EST Poaceae database (Release96 2008/08/28) from EMBL-EBI (http://srs.ebi.ac.uk/srsbin/) using the Mascot search engine with the same criteria as described above. The protein corresponding to the EST sequence was identified using the MS Blast search of EMBL (European Molecular Biology Laboratory) (http://dove.embl-heidelberg.de/Blast2/msblast.html), where a BLAST-based protocol is available for identification of proteins by sequence similarity searches using peptide sequences produced by the interpretation of tandem mass spectra (Shevchenko et al. 2001).

Results

Albumins and globulins

Many alg spots observed on the 2D gels were common to the two parents. To count spots that were specific to each of the two parents, a reference gel called 'SICOP' made from a 50/50 mixture of 'Synthetic' and 'Opata' was used as control. This SICOP two-dimensional gel revealed a total of 2,250 silver-stained spots; and subsequent comparison with the parental gels revealed that 130 were specific to 'Opata' and 96 spots to 'Synthetic'. These 226 proteins had a molecular mass ranging from 13 and 100 kDa (Mw) and an isoelectric point (pI) between 4 and 7 (Fig. 1).

Chromosome assignment

Image analysis of each of the 112 RILs enabled us to characterize the 226 spots that segregated in each specific RIL, by registering their presence or absence. Among the 226 spots, 24 (10%) that showed segregation distortion were removed from the analysis. The majority of these 24 spots were very small and not easily detected on the gels. The other spots that displayed segregation distortion corresponded to spots that were not distinguished on some segregating RILs although a slight difference was detected in the SICOP reference gel. Of the 202 spots remaining which had a 1:1 segregation (presence/absence) ratio, 164 were assigned, and 120 (53%) which had a positive relevant statistical threshold were successfully mapped. Among these 120 spots 59 and 61 belong to Opata and Synthetic, respectively. Loci related to these 120 spots were located on the whole wheat genome (Fig. 2a, b). Examination of the genetic mapping of many known enzymes (GrainGenes, http://wheat.pw.usda.gov), clearly show that they are located on all chromosomes.

Spot mapping on the different chromosomes

The distribution of the wheat 2D spot loci on the different chromosomes was globally homogenous. From 13 to 19 spots were assigned per chromosome group excepted for group 5 which had 25 loci (Table 1). The distribution per genome was in favour of the B genome, with 50 loci, whereas A and D genomes had 37 and 33 loci, respectively. The most wheat 2D markers were assigned to chromosome 5B and the least on chromosome 1D. The number of spots assigned per chromosome was not related to chromosome length and was also independent of the density of the markers per chromosome. No significant difference was detected between the chromosome arms: 50% of the spots were assigned on short and long arms. This finding differed from the chromosome mapping of the amphiphilic proteins which was carried out on the same ITMI progeny: 70% of the amphiphilic proteins loci were located on the short arms of the group 1 chromosome (Amiour et al. 2002).

Identification of spots

Out of the 111 spots that we attempted to identify, only 48.6% were successfully identified either through peptide mass fingerprinting (PMF) (35 spots, Table 2) or MS/MS peptide sequencing (19 spots, Tables 3, 4). The percentage (48.6%) of spots identified is lower to the 56% of total wheat endosperm soluble protein identified by Vensel et al. 2005, where 80% were identified using MS/MS. There are several possible explanations for why 51.3% of the trypsic digested

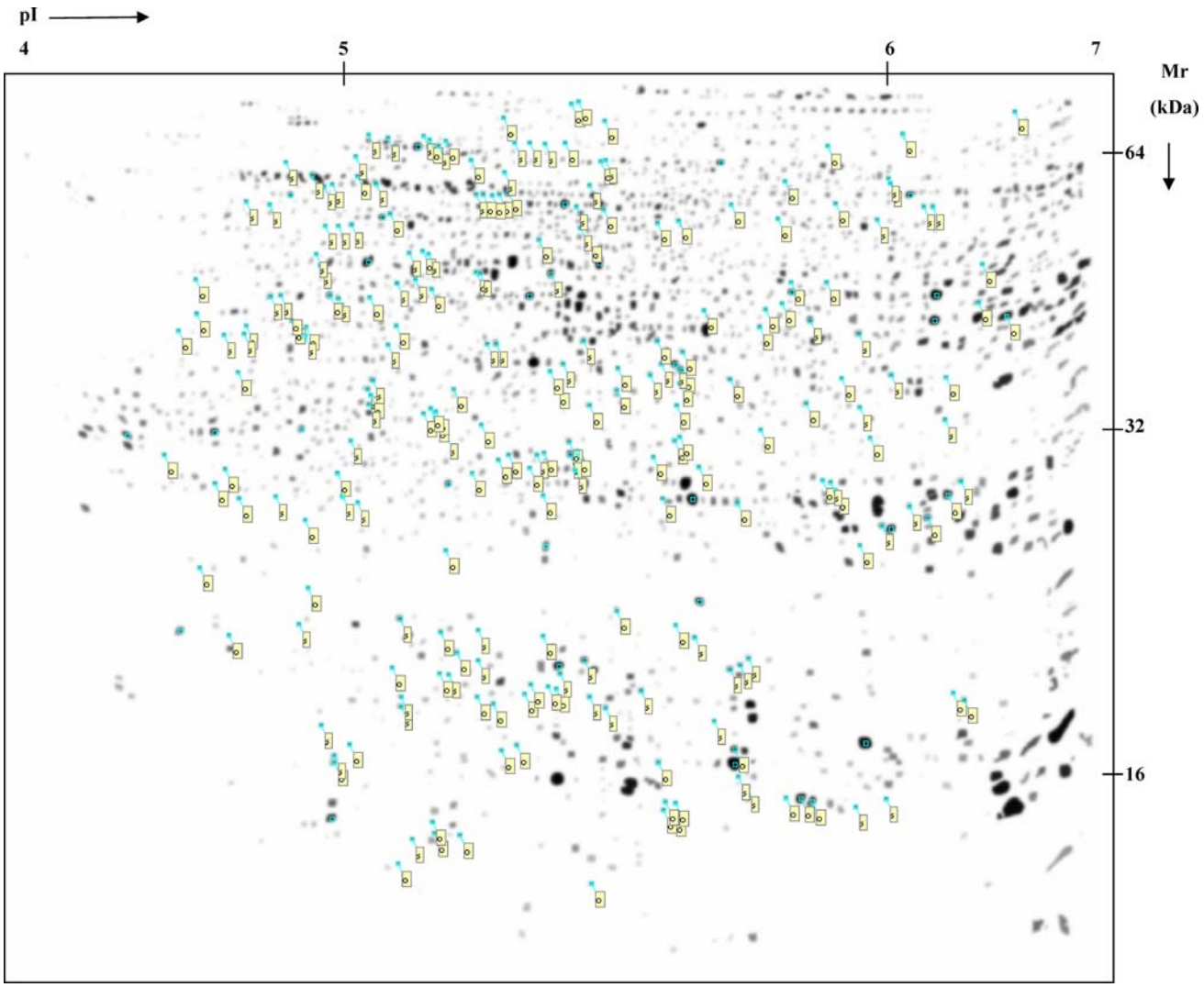

Fig. 1 Image gel (IPG × SDS-Page) of albumins–globulins from a mixture (50/50) of 'synthetic' and 'opata' (SICOP). Annotations 'O' and 'S' show the specific spots of each parent

spots were not identified: (1) the fact that the wheat genome is not yet sequenced may be one of the major causes, (2) protein sequences in data bases were mainly from *Arabidopsis*, *Oryza* or *Hordeum* genus's rather than from the *Triticum* genus, (3) several other spots could have been successfully identified if it had been possible to analyze them using an up-to-date spectrometer enabling post-translational modifications to be detected, (4) only a low number of proteins were identified, in our conditions, using EST databases.

The agl spots we identified can be classified in the three following major biological processes (GO: 0008150): carbohydrate metabolism (22%), protein folding (22%) and response to stress (28%) (Fig. 3). These major biological processes do not appear to be specific to any of the seven chromosomes groups, and the genes appear to be randomly located on the chromosomes. Although we recognize that not the all proteins were identified and only 25 and 29 proteins were identified for Synthetic and Opata, respectively, varietal differences were revealed concerning the number of spots identified in protein folding and response to stress (Fig. 4). 'Synthetic' and 'Opata', had, respectively, 10 spots (40% of specific identified proteins) and 2 spots (7%) involved in protein folding process; whereas 'Opata' had more spots 11 (38%) than 'Synthetic' 4 (16%) involved in response to stress process. These variety differences must be confirmed by further identifications for the remaining segregating spots (30 for 'Opata' and 36 for 'Synthetic') whose identifications were not successful.

Chromosome mapping of albumin–globulin spots identified on two-dimensional gel

The identification of the agl spots segregating in the ITMI progeny enabled us to distinguish seven different

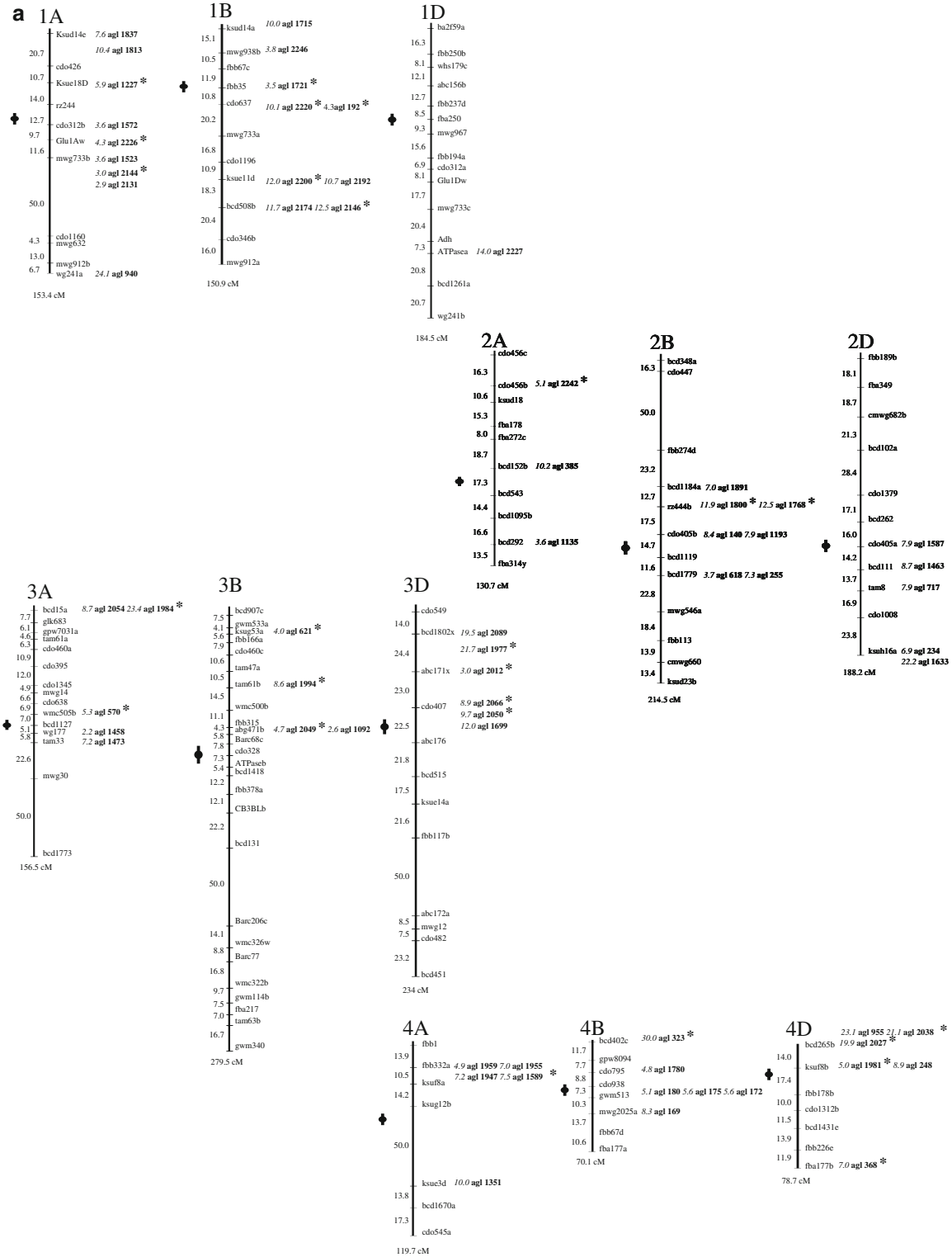

Fig. 2 **a**, **b** Molecular linkage map of the 120 wheat 2D markers (agl). The agl markers with a * are proteins which have been identified. For each chromosome, markers are shown on the *right* (anchor and wheat 2D markers) and genetic distances in cM are shown on the *left*. *Ellipse* indicate the approximate position of centromere

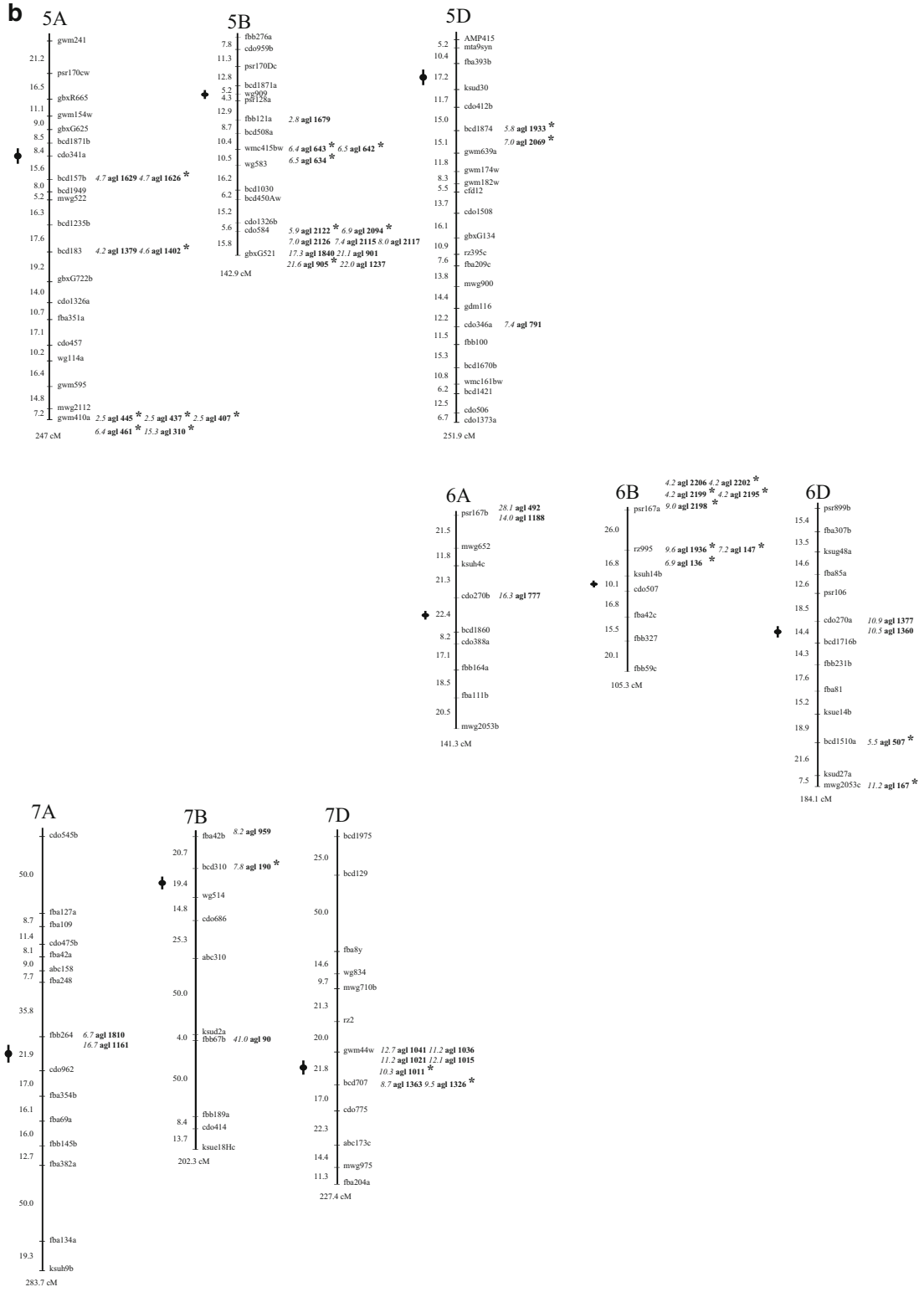

Fig. 2 continued

zones on the two-dimensional gel of SICOP where several spots belonged to the same protein family (Fig. 5).

Zone I was composed of three Heat Shock Proteins 70 kDa (HSP70) which are ATP dependant and are involved in protein folding.

Table 1 Number of wheat 2D markers per group of chromosomes and per genome

Homoeologous group	A genome	B genome	D genome	Total
Group 1	9	9	1	19
Group 2	3	7	5	15
Group 3	5	4	7	16
Group 4	5	6	6	17
Group 5	9	13	3	25
Group 6	4	8	4	16
Group 7	2	3	7	12
TOTAL	37	50	33	120

The agl136 and agl147 spots inherited from 'Synthetic', both mapped on the 6BS, were similar to HSP70 found in barley. It is interesting to note that the barley chromosome 6 was reported to carry an ADNc specific to HSP70 (Chen et al. 1994), and that co linearity was reported between wheat and barley chromosomes (Seungho et al. 2006). However, this situation does not mean that the loci are syntenic.

The agl192 inherited from 'Synthetic', and also identified as HSP70, was mapped on the centromeric zone of Chr1B. This finding is related to previous molecular analyses in which the molecular marker psr161 was reported to be very similar to genes encoding HSP70 and located near the centromeric zone of Chr1B (Francki et al. 2002).

Zone II was composed of several enzymes belonging to glycoside hydrolase, family 14.

Five spots inherited from 'Opata' (agl407, agl437, agl445, agl461 and agl310) were all identified as beta-amylase. The first four formed a chain of regularly spaced spots indicating they had possibly resulted from post-translational modifications. The five spots were mapped at the extremity of chromosome arm 5AL which could correspond to the *beta-Amy-1* gene mapped on chromosome arm 5AL (Wheat Composite 2004, GrainGenes, http://wheat.pw.usda.gov).

The agl368 spot that was specific to 'Synthetic' and that we identified as being similar to the same beta-amylase, was mapped on 4DL and could be a possible orthologue to the *beta-Amy-1* gene. We know that the *beta-amy-D1* gene was mapped on wheat 4DL (Graingenes, http://wheat.pw.usda.gov).

The agl167 spot from 'Opata' that we also identified as being similar to a barley beta-amylase (whose gene *Bmy1* was mapped on chromosome 4H), was mapped on 6DL, indicating that agl167 is probably paralogous to agl368.

Zone III was composed of three spots (agl642, agl643 and agl634) inherited from 'Synthetic', which we identified as being similar to barley UDP-Glucose pyrophosphorylase. They were all mapped on 5BL. In barley, three loci (*Ugp1*, *Ugp2* and *Ugp3*) were mapped on 3H, 3H and 5H, respectively (Barley, BinMap 2005, Kleinhofs, http://wheat.pw.usda.gov). This may indicates that our three (agl642, agl643 and agl634) spots could be orthologous to the *Upg3* locus.

Zone IV was composed of two spots inherited from 'Opata' (agl1768 and agl1800) which we identified as 1-cys peroxiredoxin. Several tissue-specific genes encoding peroxydases at loci named *Per1* (for coleoptile), *Per2* (for root), and *Per3* (for embryo) were located on chromosomes of wheat groups 1, 2 and 3, respectively, whereas *Per4* (for endosperm) were located on 7AS, 4AL and 7DS (Liu et al. 1990). In our case, the two spots were mapped on 2BS, suggesting that agl1768 and agl1800 are 1-cys peroxiredoxins homologous to those encoded at the *Per2* locus.

Zone V was composed of nine spots: four (agl1977 and agl2012 from 'Opata', agl1984 and agl1994 from 'Synthetic') were identified as HSP16.9 kDa, and three from 'Synthetic' (agl2049, agl2050 and agl2066) were identified as HSP 17.5 kDa or HSP17.8 kDa. Except agl1981 from 'Synthetic', identified as HSP18 from *orysa sativa*, which was mapped on 4DS, they were all mapped on group 3 chromosome. This is in agreement with previous chromosomal assignment where some 17 and 18 kDa HSPs were reported to be encoded at 3AL, 3BS and 3DS but also on chromosome arms 4BS and 4DL (Porter et al. 1989).

One spot (agl1936 from 'Synthetic'), with a molecular weight slightly higher than the previous spots, was identified as HSP23.5 kDa and mapped on 6BS. This protein is similar to the rice protein encoded at *Os02g0758000* gene (http://www.ncbi.nlm.nih.gov/UniGene/) located on rice chromosome 2.

Zone VI was composed of three spots (agl2144, agl2220 and agl2226 from 'Opata'). We identified these spots as being similar to type H thioredoxin. These 12–14 kDa proteins, which are abundant in cereal seeds, have been shown to be involved in many cytosolic redox reactions involving dithiol-disulphide exchange. The proteins were mapped on 1AL and 1BL which is in good agreement with previous mapping of thioredoxin H (Holton et al. 2002).

Zone VII was composed of four spots from 'Opata' (agl2195, agl2198, agl2199 and agl2202) identified as monomeric alpha-amylase inhibitor. These four proteins, which belong to the big protease inhibitor family, were all mapped on 6BS. This is in total agreement with previous mapping where Gomez et al. 1991, using RP-HPLC and 2DE, mapped the two homoeologous *WMAI-1* and *WMAI-2* genes on 6DS and 6BS, respectively.

Another spot in this zone, agl2122 from 'Opata', matched the dimeric alpha-amylase inhibitor 0.19 and was mapped on chromosome 5BL. Thirteen genes encoding dimeric alpha-amylase inhibitors were found in the cv Chinese Spring (Wang et al. 2006), and the use of

Table 2 Specific spots of Opata (O) and Synthetic (S) identified by MALDI-Tof mass spectrometry

agl no.	Spot from O or S	Chromosomal location	Protein name	UniProtKB accession no.	Species	mW theo (mW exp)/ pI theo (pI exp)	Significant score (Mascot >55 or Profound >1.65)	No. of peptides matched (% coverage)	Biological process/molecular function	Homologue protein in wheat[a]
136	S	6BS	HSP70	Q40058	*Hordeum vulgare*	67.3 (74.5)/5.76 (5.1)	81	16 (29)	Protein folding	No
147	S	6BS	HSP70	Q40058	*Hordeum vulgare*	67.3 (73.9)/5.76 (5.1)	84	13 (26)	Protein folding	No
190	O	7BS	Putative glycyl-tRNA synthetase	Q6ZD35	*Oryza sativa*	78.2 (71.4)/5.9 (5.8)	2.26	8 (12)	Translation	No
192	S	1BL	HSP70	Q9SAU8	*Triticum aestivum*	71.7 (70.6)/5.1 (5.2)	119	17 (33)	Protein folding	–
310	O	5AL	Beta-amylase (fragment)	Q7X9M2	*Triticum aestivum*	31.2 (63.8)/8.6 (5.3)	90	13 (42)	Carbohydrate metabolism	–
323	S	4BS	Protein disulphide isomerase 2	Q93XQ8	*Triticum aestivum*	56.9 (63.3)/5.0 (4.9)	101	14 (31)	Electron transport	–
368	S	4DL	Beta-amylase (fragment)	Q7X9M2	*Triticum aestivum*	31.2 (61.1)/8.6 (5.1)	107	13 (54)	Carbohydrate metabolism	–
407	O	5AL	Beta-amylase (fragment)	Q7X9M2	*Triticum aestivum*	31.2 (58.8)/8.6 (5.3)	156	16 (67)	Carbohydrate metabolism	–
437	O	5AL	Beta-amylase (fragment)	Q7X9M2	*Triticum aestivum*	31.2 (58.8)/8.6 (5.3)	146	15 (67)	Carbohydrate metabolism	–
445	O	5AL	Beta-amylase (fragment)	Q7X9M2	*Triticum aestivum*	31.2 (58.4)/8.6 (5.3)	75	9 (39)	Carbohydrate metabolism	–
461	O	5AL	Beta-amylase (fragment)	Q7X9M2	*Triticum aestivum*	31.2 (58)/8.6 (5.3)	158	15 (67)	Carbohydrate metabolism	–
507	O	6DL	Cytosolic glutathione reductase	Q6UQ06	*Triticum monococcum*	53.7 (56.1)/5.9 (5.8)	86	13 (32)	Electron transport	No
570	O	3AS	ATP synthase beta subunit	Q41534	*Triticum aestivum*	59.4 (52.9)/5.6 (5.1)	147	21 (43)	ATP biosynthetic	–
621	O	3BS	GAD1	Q7XZU7	*Hordeum vulgare*	55 (52)/5.3 (5.6)	69	9 (19)	Glutamate metabolism	No
634	S	5BL	UDP-glucose pyrophosphorylase	Q43772	*Hordeum vulgare*	51.951.1)/5.2 (5.0)	106	14 (37)	Carbohydrate metabolism	No
642	S	5BL	UDP-glucose pyrophosphorylase	Q43772	*Hordeum vulgare*	51.9 (51.1)/5.2 (5.0)	122	14 (41)	Carbohydrate metabolism	No
643	S	5BL	UDP-glucose pyrophosphorylase	Q43772	*Hordeum vulgare*	51.9 (51.1)/5.2 (5.0)	81	11 (32)	Carbohydrate metabolism	No
905	S	5BL	Serpin	Q9ST57	*Triticum aestivum*	43.3 (43)/5.4 (5.3)	125	13 (49)	Response to stress	–

Table 2 continued

agl no.	Spot from O or S	Chromosomal location	Protein name	UniProtKB accession no.	Species	mW theo (mW exp)/ pI theo (pI exp)	Significant score (Mascot >55 or Profound >1.65)	No. of peptides matched (% coverage)	Biological process/molecular function	Homologue protein in wheat[a]
1402	S	5AL	Serpin	Q9ST58	*Triticum aestivum*	43 (34.5)/5.6 (5.0)	104	7 (28)	Response to stress	–
1721	S	1BS	Glutathione-S-transferase 19E50	Q8LGN5	*Triticum aestivum*	25.1 (28.1)/6.0 (5.8)	75	5 (24)	Response to stress	–
1768	O	2BS	1-Cys peroxiredoxin	Q9AXH7	*Triticum turgidum subsp. durum*	24.3 (27.8)/6.3 (5.7)	143	14 (46)	Response to stress	Q6W8Q2 *Triticum aestivum*
1800	O	2BS	1-Cys peroxiredoxin	Q6W8Q2	*Triticum aestivum*	24.3 (27.8)/6.1 (5.7)	67	7 (30)	Response to stress	–
1936	S	6BS	Small heat shock protein Hsp23.5	Q9ZP25	*Triticum aestivum*	23.4 (21.6)/6.2 (4.9)	102	11 (44)	Protein folding	–
1977	O	3DS	16.9 kDa Class I heat shock protein	P12810	*Triticum aestivum*	16.8 (20.2)/5.8 (5.2)	73	6 (49)	Protein folding	–
1984	S	3AS	16.9 kDa Class I heat shock protein	P12810	*Triticum aestivum*	16.8 (20.1)/5.8 (5.5)	86	7 (58)	Protein folding	–
1994	S	3BS	16.9 kDa Class I heat shock protein	P12810	*Triticum aestivum*	16.8 (19.9)/5.8 (5.7)	90	8 (62)	Protein folding	–
2012	O	3DS	16.9 kDa Class I heat shock protein	P12810	*Triticum aestivum*	16.8 (19.5)/5.8 (5.2)	62	7 (50)	Protein folding	–
2094	S	5BL	Single-stranded nucleic acid binding protein	Q41518	*Triticum aestivum*	16.3 (17.9)/5.2 (4.9)	94	7 (62)	Translation	–
2122	O	5BL	0.19 Dimeric alpha-amylase inhibitor	Q5MD68	*Triticum aestivum*	14.2 (16.6)/5.2 (5.7)	76	6 (69)	Response to stress	–
2146	O	1BL	Alpha-1-purothionin	P01544	*Triticum aestivum*	15 (16)/4.7 (5.6)	58	5 (45)	Defence response	–
2195	O	6BS	Monomeric alpha-amylase inhibitor	A4ZIZ0	*Triticum aestivum*	14 (15.1)/5.3 (5.8)	111	9 (71)	Response to stress	–
2198	O	6BS	Monomeric alpha-amylase inhibitor	A4ZIY9	*Triticum aestivum*	17.8 (15.2)/7.4 (5.8)	69	6 (40)	Response to stress	–
2199	O	6BS	Monomeric alpha-amylase inhibitor	A4ZIZ0	*Triticum aestivum*	14.1 (15)/5.3 (5.8)	100	8 (59)	Response to stress	–
2200	S	1BL	Wheatwin1	O64392	*Triticum aestivum*	16.3 (15.1)/7.9 (6.0)	2.16	5 (54)	Defence response	–
2202	O	6BS	Monomeric alpha-amylase inhibitor	A4ZIZ0	*Triticum aestivum*	14.1 (15.1)/5.3 (5.6)	114	9 (62)	Response to stress	–

[a] Similarity with *Triticum aestivum* through Blast2 at NCBI

Table 3 Specific spots of Opata (O) and Synthetic (S) identified by MS/MS

agl no.	Spot from O or S	Chromosomal location	Protein name	UniProtKB accession no.	Species	mW theo (mW exp)/ pI theo (pI exp)	Mascot score	Matched peptides	Biological process/ molecular function	Homologue protein in wheat[a]
167	O	6DL	Beta-amylase	P16098	*Hordeum vulgare*	59.6 (73)/5.6 (5.2)	138	(LFGFTYLR) (LSNQLVEGQNYVNFK)	Carbohydrate metabolism	P93594 *Triticum aestivum*
1011	O	7DS	Serpin	Q43492	*Hordeum vulgare*	42.7 (40.3)/ 5.5 (4.9)	94	(LVLGNALYFK) (GLWTKKFDESK)	Response to stress	Q9ST57 *Triticum aestivum*
1227	O	1AS	Legumin-like protein	Q84TL6	*Zea mays*	38 (37.4)/6.2 (5.6)	129	(EVGLGADLVR) (IEGGSLFIVPR)	Nutrient reservoir activity	No
1326	S	7DL	Lactoylglutathione lyase	Q948T6	*Oryza sativa*	32.5 (36)/5.5 (5.6)	126	(SAEAVELVTK) (QPGPLPGLNTK)	Carbohydrate metabolism	Q9XGF2 *Triticum aestivum*
1626	O	5AL	Putative uncharacterized protein Ose731	Q7G156	*Oryza sativa*	26.3 (30.8)/ 5.6 (5.5)	186	(GGVLFMPGVPGVVER + Oxidation (M)) (GDALPLGLPQIMMALTR + Oxidation (M)) (GDALPLGLPQIMMALTR + 2 Oxidation (M))	Protein of unknown function (PUF)	No
1933	S	5DL	Putative uncharacterized protein P0022B05.116	Q8LHY4	*Oryza sativa*	15.7 (21.8)/ 0.7 (5.1)	177	(SIELLEELGLPK) (KSIELLEELGLPK)	Protein of Unknown Function (PUF)	No
1946	S	6AS	EM4_WHEAT Em protein H5	P42755	*Triticum aestivum*	10 (21.3)/5.3 (5.1)	159	(EGIEIDESK) (EGETVVPGGTGGK) (KEQLGEEGYR)	Response to stress	–
2049	S	3BS	Small heat shock protein HSP17.5	A5A8T4	*Aegilops longissima*	17.5 (18.5)/ 5.6 (5.5)	95	(VLVISGER) (DGVLTVTVEK)	Protein folding	Q94KM0 *Triticum aestivum*
2050	S	3DS	Small heat shock protein HSP17.8	Q94KM0	*Triticum aestivum*	17.7 (18.5)/ 5.9 (5.1)	134	(VLVISGER) (ELPGAYAFVVDMPGLGSGDIK + Oxidation (M))	Protein folding	–
2066	S	3DS	Small heat shock protein HSP17.8	Q94KM0	*Triticum aestivum*	17.7 (18)/5.9 (5.1)	147	(VLVISGER) (ELPGAYAFVVDMPGLGSGDIK + Oxidation (M))	Protein folding	–
2144	O	1AL	Thioredoxin H-type	O64394	*Triticum aestivum*	13.5 (16.2)/ 5.12 (5)	229	(IMAPIFADLAK) (IMAPIFADLAK + Oxidation (M)) (VVGAIKEELTTK)	Electron transport	–
2220	O	1BL	Thioredoxin H-type	O64394	*Triticum aestivum*	13.5 (14.5)/ 5.1 (5.2)	204	(IMAPIFADLAK) (IMAPIFADLAK + Oxidation (M)) (VVGAIKEELTTK)	Electron transport	–
2226	O	1AL	Thioredoxin H	O64394	*Triticum aestivum*	13.5 (14.2)/ 5.1 (5.2)	111	(KFPAAVFLK) (IMAPIFADLAK + Oxidation (M))	Electron transport	–

[a] Similarity with *Triticum aestivum* through Blast2 at NCBI

Table 4 Specific spots of Opata (O) and Synthetic (S) identified by MS/MS using EST Poaceae database

agl no.	Spot from O or S	Chromosomal location	EST name	Clone accession no.	Species	Mascot score	Matched peptides	MS blast search	Biological process/ Molecular function	Homologue protein in wheat[a]
1589	O	4AS	cDNA clone:whe2l03	BJ238596	*Triticum aestivum*	390	(LDPQFLLQHTK) (YIDSNFDGPALLPDDSAK) (KQFAEELLVYTDEFNK) (GDVAEETVAALDKIEAALGK)	Q8H8U5 Oryza sativa Putative glutathione S-transferase	Response to stress	O82071 Triticum aestivum Putative In2.1 protein
1981	S	4DS	cDNA clone:wh37c17	BJ210957	*Triticum aestivum*	146	(TSSDTAAFAGAR) (ASMENGVLTVTVPKEEAKKPEVK + Oxidation (M))	Q84Q77 Oryza sativa Heat shock protein 18	Protein folding	No
2027	O	4DS	cDNA clone:whsl15n07	BJ288672	*Triticum aestivum*	255	(GSGSESESESEEQQDQQR) (AKDQQDEGFVAGPEQQEQER) (AKDQQDEGFVAGPEQQEQERGDR)	Q7DMU0 *Triticum aestivum* Storage protein	Nutrient reservoir activity	–
2038	S	4DS	Clone rwhem9c11 3'	CJ544573	*Triticum aestivum*	176	(LDNPAQELTFGR) (AKDQQDEGFVAGPEQQEQER)	Q7DMU0 *Triticum aestivum* Storage protein	Nutrient reservoir activity	–
2069	S	5DL	cDNA clone:whsl9g03	BJ290200	*Triticum aestivum*	280	(TVELLEELGLPK) (TNTGLSDTHDAAVFALGE) (LNQTVSYDTEVTAFVEK)	Q8LHX8 Oryza sativa Hypothetical protein	Protein of unknown function (PUF)	No
2242	O	2AS	cDNA clone:wh21i01	BJ213239	*Triticum aestivum*	169	(VLIIDFKPTAGN) (EYVYHPAHVEFATDFLGSTEK)	Q8LQD2 Oryza sativa putative stress-responsive protein	Response to stress	No

[a] Similarity with *Triticum aestivum* through Blast2 at NCBI

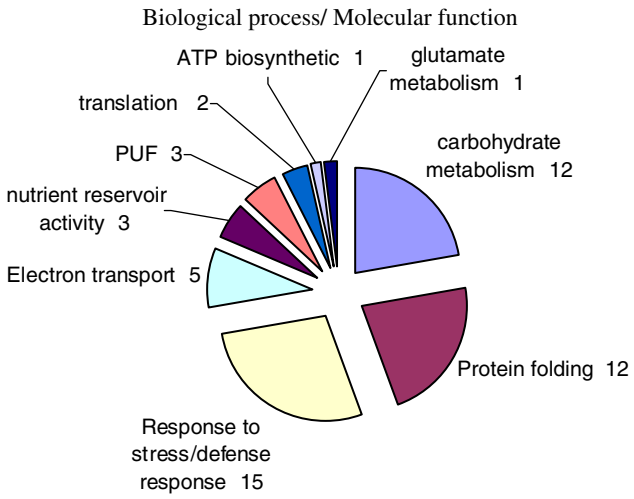

Fig. 3 Classification of all specific proteins identified in both 'Opata' and 'Synthetic' according to their biological process or molecular function. PUF: protein of unknown function

ditelosomic lines enabled them to assign the alpha-amylase inhibitor 0.19 to 3DS and not to 5BL. This indicates that among the multigene family encoding alpha-amylase inhibitor, at least one gene, not previously mapped, would be located, with the ITMI population, on 5BL. We can also hypothesis that a possible post-translational modification of the agl2122 could be caused by a gene located on 5BL.

Other spots not grouped in specific zones

One spot, agl2146, from 'Opata', identified as alpha-1-purothionin, was mapped on 1BL. In a previous work, Castagnaro et al. (1992) described this protein involved in plant defence mechanisms as being encoded at gene clusters on the long arm of the group 1 chromosome.

The agl2200 spot from 'Synthetic' which was identified as a wheatwin 1 protein belonging to the PR4 family, homologous to the barley barwin protein, has been shown to be involved in plant defence. Transcript profiles and expression profiles of the unmapped wheatwin proteins have previously been reported with respect to wheat kernel development (Altenbach et al. 2007). We mapped it on 1BL.

The agl1721 spot from 'Synthetic', which we identified as a glutathione-S-transferase (GST), was mapped on 1BS. GSTs are known to be a multigene family of enzymes associated with stress response in plants (Edwards et al. 2000; McGonigle et al. 2000). Genes encoding GST were located on 6AS, 6BS and 6DS (Riechers et al. 1998). Recently, genetic mapping of wheat progeny enabled the cDNA of one GST to be located on 1BS (Paillard et al. 2003), which is in agreement with our mapping. Another spot, agl1589 from 'Opata', was found to be similar to a putative rice GST and was mapped on 4AS. In the latter species, the gene (*Os03g0283100*) encoding this GST was located on chromosome 3 (TIGR Rice Genome Annotation-release 5: http://www.tigr.org), which is a chromosome known to have segments close to wheat chromosome 4 (Sorrels et al. 2003; Salse et al. 2007).

The agl507 spot from 'Opata', identified as a cytosolic glutathione reductase belonging to the pyridine nucleotide-disulphide oxidoreductase family known to be involved in stress defence, was mapped on 6DL. A rice gene (Os02g081350), coding for a cytosolic glutathione reductase, was located on rice chromosome 2 (http://www.ncbi.nlm.nih.gov/sites/entrez), also known to show synteny with wheat chromosome 6.

Three other spots involved in stress defence agl905 and agl1402, from 'Synthetic', were identified as wheat serpins (SERine Proteinase INhibitors) whereas agl1011, from 'Opata', was seen to be similar to a barley serpin, which is very similar to that of agl905. Serpins that are active against some peptidases have been shown to be involved in allergy to wheat flour (Akagawa et al. 2007). The two first spots were mapped on 5BL and 5AL and agl1011 was mapped on 7DS.

The agl621 spot from 'Opata' and mapped on 3BS was identified as being similar to the glutamate ascorbate

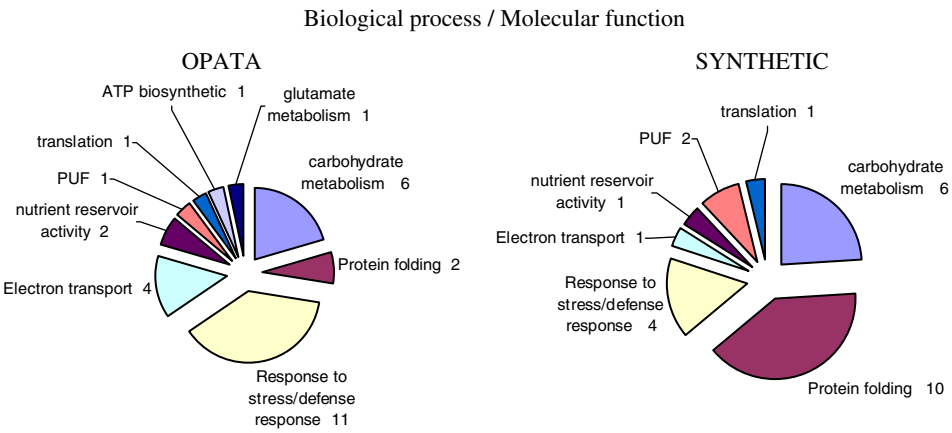

Fig. 4 Classification of specific protein from 'Opata' and 'Synthetic' according to their biological process or molecular function. PUF: protein of unknown function

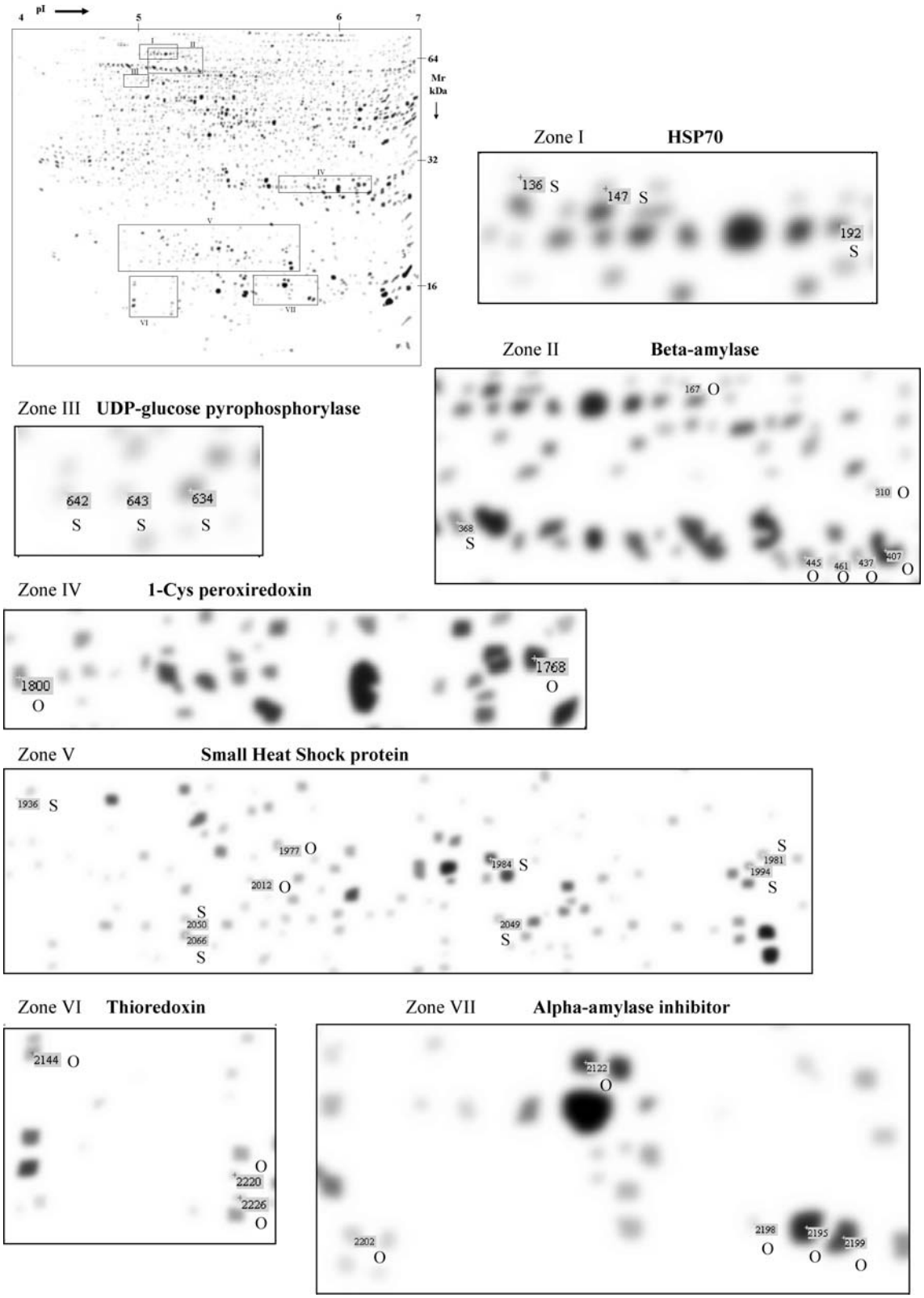

Fig. 5 Major zones of the two-dimensional gel of SICOP showing segregating protein family and their chromosomal location reported in text

deshydrogenase (GAD1) of barley. In barley, the *Hvgad1* gene was mapped near the *Rph7* locus on 3HS (Brunner et al. 2003).

The agl323 spot from 'Synthetic', identified as a protein disulphide isomerase 2 (PDI2) was mapped on 4BS. This result is in total agreement with those described by Ciaffi et al. 1999, who assigned *Pdi* genes on 4BS, 4DS and 4AL.

Two spots, agl2027 from 'Opata' and agl2038 from 'Synthetic', showed homology with proteins of the cupin family involving 11S and 7S seed-storage globulins. Millan et al. (1992) identified the loci of a 7S storage globulin sequence (X*Glo*) on Chr 4AL, 4BS and 4DS, and we mapped our two spots on 4DS.

The agl1946 spot from 'Synthetic' was identified as Em protein H5 which belongs to the small hydrophilic plant seed protein family involved in stress response. This agl1946 was mapped on the chromosome 6AS. Futers et al. (1990), in analyzing the wheat embryo shoot, reported that the Em polypeptide was the product of a small multigene family in which the copies were located on each of the long arms of the homoeologous group 1 chromosomes.

Finally, the agl570 spot from 'Opata', mapped on 3AS, was identified as the beta subunit of an ATP synthase. A gene coding this enzyme was mapped on rice chromosome 1 which is collinear to chromosome 3 of wheat (http://compbio.dfci.harvard.edu/tgi).

Discussions and conclusions

The analysis was performed on the all kernel proteins and consequently some of these proteins may be originated from embryo, endosperm or peripheral layers as it was revealed for barley (Finnie and Svensson 2003). Our proteomic analysis of the albumins–globulins wheat kernel revealed that 'Opata' had more segregating spots than 'Synthetic' (130 vs. 96). The 226 segregating spots represented almost exactly 10% of the 2,250 silver-stained spots. Consequently, 90% of the albumin–globulin proteins were identical, based on the 2DE resolution power, in the modern cultivar 'Opata' and the inter-specific 'Synthetic' wheat. Knowing the genetic origin of the two wheat parents, we would have expected many more differences between proteins. Can we conclude that, as was the case for 'Opata' ancestors and for 'Opata' itself, continuous wheat breeding programs cumulated more different genes and proteins than those that could be detected in a new inter-specific hexaploid wheat like 'Synthetic'? The latter, which resulted from a cross between *T. turgidum* (namely Altar 84) with a *T. tauschii* accession, might add many genes not yet found in the current wheat genome. Since only 96 (vs. 130) were inherited from Synthetic (vs. Opata) in the segregating progeny, we could hypothesis that Synthetic's genes were eliminated after inter-specific crossing. The answer is not so simple. First, it may depend on the functions of the protein: for the protein involved in response to stress, more spots were inherited from 'Opata' than from 'Synthetic' (11 and 4, respectively). Conversely, 'Synthetic' had more protein involved in protein folding than 'Opata' (10 and 2, respectively). Secondly, not all albumins–globulins were analyzed since isofocussing was performed between pH 4 and pH 7, where the majority of the proteins are separated. Other albumins–globulins could also have been revealed if protein extract had been focussed between pH 7 and pH 11, which would have revealed additional segregating proteins. Some of these basic proteins (e.g. amphiphilic proteins) were previously analyzed in the ITMI progeny (Amiour et al. 2002). Amphiphilic proteins, which are soluble in Triton X-114, a non-ionic detergent used for extracting lipid-binding proteins, were less numerous than albumins–globulins on 2D electrophoresis gel. Out of a total of 446 amphiphilic silver-stained proteins between pH 6 and pH 11, comparison of the parental profiles revealed that 'Synthetic' possessed almost two times more amphiphilic proteins than 'Opata': 111 and 59, respectively. Only 62 of these segregating proteins have already been mapped (Amiour et al. 2002, 2003). Identification of the amphiphilic proteins evidenced that many of them were associated with plant defence mechanisms. Further analyses are required, first to identify all seed proteins specific to the two parents and, of particular interest today, to identify any plant defence proteins among them. To this end, it will be useful to analyze these proteins in the different seed tissues and particularly in the peripheral layers and in the aleurone layer (Laubin et al. 2008).

Among the albumins–globulins we identified, several were enzymes belonging to families like HSPs, beta-amylases, UDP-glucose pyrophosphorylases, peroxydases and thioredoxins. Indeed several groups or chain of spots of the same protein family (like beta-amylases, UDP-Glucose pyrophosphorylase) were mapped at a single locus since no recombination was detected in the 112 RILs. Each of these groups of enzymes may result either from the expression of gene clusters or post-translational modifications. Further analyses using genome sequencing and new mass spectrometry tools in parallel would be very useful to identify the genetic and biochemical factors responsible for the diversity of these proteins.

Our study enabled us to map 120 out of 226 spots on 21 chromosomes. Analysis of additional RILs (or the use of aneuploid lines) is now needed to unambiguously locate the remaining unmapped wheat 2D markers. The present results together with those of amphiphilic proteins are a starting point for the analysis of genetic factors as well as genetic × environmental interactions involved in seed characteristics. Using ITMI progeny, several QTL analyses have already been achieved on kernel characteristics such as

size of starch granules (Igrejas et al. 2002a), grain hardness and puroindoline content (Igrejas et al. 2002b), grain protein content and thousand grain weight (Börner et al. 2002), grain protein content and quality traits (Nelson et al. 2006). Some of the above QTLs should now be reassessed using the results of the present protein mapping to identify candidate genes. The variations in spot volumes of the albumins–globulins both for segregating and common spots also deserve to be analyzed for different RILs. Quantitative analysis of the relative amount of protein, also named protein quantity loci (PQL) (Damerval et al. 1994), which may provide specific QTLs, has already been computed for amphiphilic proteins (Amiour et al. 2003). These PQLs may be associated with gene regulation and consequently be very useful for understanding quantitative variations in the traits analyzed. The above proteomic approach can also be used to analyze kernel tissues to track genetic factors associated with nutritional and health compounds.

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