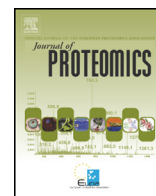




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Differential representation of albumins and globulins during grain development in durum wheat and its possible functional consequences

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ABSTRACT

Durum wheat (*Triticum turgidum* ssp. *durum* (Desf.) Husn.) is an economically important crop used for the production of semolina, which is the basis of pasta and other food products. Its grains provide proteins and starch for human consumption. Grain development is a key process in wheat physiology; it is highly affected by a number of enzymes that control the metabolic processes governing accumulation of starch and storage proteins and ultimately grain weight. Most of these enzymes are present in the albumin/globulin grain fraction, which represents about a quarter of total seed proteins. With the aim to describe the dynamic profile of the albumin/globulin fraction during durum wheat grain development, we performed a proteomic analysis of this subproteome using a two-dimensional differential gel electrophoresis (2D-DIGE)-based approach and compared six developmental stages. A total of 285 differentially (237 over- and 48 under-) represented spots was identified by nanoLC-ESI-LIT-MS/MS, which were associated with 217 non-redundant *Triticum* sequence entries. Quantitative protein dynamics demonstrated that carbon metabolism, energy, protein destination/storage, disease/defense and cell growth/division functional categories were highly affected during grain development, concomitantly with progressive grain size increase and starch/protein reserve accumulation. Bioinformatic interaction prediction revealed a complex network of differentially represented proteins mainly centered at enzymes involved in carbon and protein metabolism. A description of 18 proteins associated with wheat flour human allergies was also obtained; these components showed augmented levels at the last developmental stages. By providing a comprehensive understanding of the molecular basis of durum wheat grain development, yield and quality formation, this study provides the foundation and reveals potential biomarkers for further investigations of durum wheat breeding and semolina quality.

Biological significance: A 2D-DIGE-based comparative analysis of the albumin/globulin fraction from durum wheat caryopses at six developmental stages was performed to describe the dynamic subproteomic changes associated with grain development. Quantitative variations of 217 differentially proteins demonstrated that highly affected are the functional categories of carbon metabolism, energy, protein destination/storage, disease/defense and cell growth/division, which displayed a general over-representation, consistently with concomitant occurrence of grain size increase and starch/protein reserve accumulation. Bioinformatics revealed a complex protein network centered mainly at enzymes involved in carbon and protein metabolism. Differentially represented proteins and corresponding functional categories highly resembled those previously identified as variable in developing bread wheat grain. This suggests that the main differences in kernel hardness between durum and bread wheat probably do not depend on proteomic changes in corresponding albumins/globulins, but on other specific factors affecting the interaction between the starch granules and the endosperm protein matrix in the kernel.

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1. Introduction

Durum wheat [*Triticum turgidum* ssp. *durum* (Desf.) Husn.] is an economically important crop, which is used for the production of semolina. Its cultivation involves 17 M ha of territory (mainly in the Mediterranean basin) and yields about 35.6 Mt. of desiccated grain (International Grain Council, IGC Grain Market reports, 2013). Semolina is fundamental for the industrial chain making pasta (13.5 Mt. per year) [1], couscous, pudding and breakfast products, which provide proteins (10–15% dry weight) and starch (70–80% dry weight) for human consumption. In particular, durum wheat grain proteins are well-adjusted for human nutrition because of their optimal content with respect to essential amino acids [2].

Knowledge of grain development, composition and structure is important for generating high-quality products [3]. Wheat grain formation involves three main phases: i) early development (0–3 days post-anthesis, DPA), where endosperm cell division/expansion occurs; ii) filling and maturation (4–21 DPA), which corresponds to the accumulation of storage compounds; iii) desiccation (22–30 DPA) [4,5]. Carbohydrates and proteins stored in the grain during its development are important reserves to fuel further germination and early seedling growth. Since the quality of the final seed is largely affected by molecular events occurring during grain development, understanding the corresponding enzyme activities and regulation is essential for improving final product quality.

Wheat grain proteins are grouped into two major families: i) salt-/alcohol-soluble globulins and prolamins, e.g. gliadins and glutenins, which are the major storage components in wheat endosperm, influence the viscoelastic properties of products, and affect the corresponding processing and rheological properties [6]; ii) water-soluble non-prolamins, e.g. albumins and globulins, which include different enzymes and polypeptide inhibitors playing important functions in grain metabolism, development and response to environmental cues [7]. In durum wheat, information on the changes associated with these major protein families during grain development has been obtained according to a single protein-centered perspective, losing the holistic view deriving from a -omic approach. Conversely, proteomic investigations on bread wheat (*T. aestivum* L.) have already provided a comprehensive description of the accumulation of reserve and metabolic proteins in the course of grain development [5,8–16]. These studies integrated transcriptomic [4,17] and metabolomics [4] analyses that often did not account for the accumulation of specific components. Indeed, comparative proteomic studies of different grain developing stages in *T. aestivum* provided valuable information for the interpretation of the biochemical processes of grain development in different cultivars; they have been performed by two-dimensional electrophoresis (2-DE) [5,8–11], 2D-DIGE [12–14], and gel-free isobaric tag for relative and absolute quantitation (iTRAQ) approaches [15–16]. In particular, proteomic analysis covered all grain formation phases [9–15] or focused on the early development phase to unveil metabolic processes associated with active cell enlargement [8,16]. Conversely, similar studies are lacking in durum wheat, where proteomics was used only to compare grains from plants subjected to heat [18], salinity [19–21] or drought [22] challenge, identifying potential markers of stress tolerance in specific cultivars or seed treatments.

In order to obtain a comprehensive description of reserve and metabolic proteins during grain development in durum wheat, a comparative proteomic study was accomplished on Svevo, a widely cultivated variety in the Central-Southern Italy. In particular, protein extracts corresponding to different grain developing stages were differentially fractionated to obtain albumin/globulin and gliadin/glutenin subproteomes [23], which were further investigated to obtain quantitative information on the highest number of molecular species. This study describes the proteomic investigation based on 2D-DIGE of the albumin/globulin fraction, which contains major metabolic enzymes essential for grain development.

2. Materials and methods

2.1. Plant material and growth conditions

Durum wheat cv. Svevo was planted in the early spring 2016 and grown as reported previously [24] and described in detail in Supplementary Material. According to the method of Majoul and coworkers [25], grain samples were harvested at 6 key developmental stages during the post-anthesis period, namely 3 DPA (embryo increase three-fold in size) (T0), 5 DPA (kernel development at one-third of full developmental size, water-ripening stage) (T1), 11 DPA (kernel development at half of full size, water-ripening stage) (T2), 16 DPA (kernel full development, water-ripening stage) (T3), 21 DPA (milk-ripening stage) (T4) and 30 DPA (dough stage) (T5). Morphological details at each developmental stage are reported in Supplementary Table S1. Collected samples at each stage were pooled to make three biological replicates, which were stored at -80°C prior to proteomic analysis.

2.2. Protein extraction, fractionation and quantification

Grain samples were ground to a powder using a mortar and pestle, and then dried. Extraction and fractionation of grain proteins was carried out as reported by Hukman and Tanaka [23]. For each sample, 100 mg of grain powder was suspended in 1000 μL of cold 50 mM Tris-HCl, 100 mM KCl, 5 mM EDTA, pH 7.8, incubated on ice for 15 min with intermittent mixing, and finally centrifuged at $14,500 \times g$ for 10 min, at 4°C . Extraction was carried out twice. The pellet was further analyzed for gliadin and glutenin content [26]; the supernatant from both extractions was collected, combined and 5 vol of 0.1 M ammonium acetate in methanol were then added at room temperature. Following incubation overnight at -20°C , each sample was centrifuged at $14,500 \times g$ for 15 min, at 4°C , discarding the supernatant. Resulting pellet (albumins and globulins) was rinsed with cold acetone, mixed with 9:1 v/v acetone/methanol for 16 h, at -20°C , recovered by centrifugation at $16,000 \times g$ for 30 min, at 4°C , dried, and stored at -80°C until use. Then, it was solubilized in 7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris-HCl. Protein concentration in each sample was determined using the Bradford assay (Bio-Rad, USA), after adjusting the pH value to pH 8.5.

2.3. 2D-DIGE

Three biological replicates of each experimental condition were subjected to 2D-DIGE analysis. Fifty micrograms of proteins were labeled with 400 pmol of Cy2-, Cy3- or Cy5-dyes (GE Healthcare, UK), using a dye-swapping strategy [27]. A mixture of all the samples was labeled with Cy2 dye and was used as the internal standard required for 2D-DIGE. Each labelling reaction was performed in the dark for 30 min, at 0°C , and was quenched with 1 mM lysine. Appropriate Cy3- and Cy5-labeled pairs and a Cy2-labeled reference were used to generate mixtures. Each mixture was supplemented with 1% v/v IPG buffer, pH 3–10 NL (GE Healthcare, UK), 1.4% v/v DeStreak reagent (GE Healthcare, UK) and 0.2% w/v DTT to reach a final volume of 450 μL in 7 M urea, 2 M thiourea, and 4% w/v CHAPS. The mixtures (150 μg of total protein content) were used for passive hydration of immobilized pH gradient IPG gel strips (24 cm, pH 3–10 NL) in the dark, for 16 h, at 20°C . Isoelectric focusing (IEF) was carried out with an IPGphor II apparatus (GE Healthcare) up to 80,000 V/h at 20°C . After IEF, each strip was equilibrated in 6 M urea, 2% w/v SDS, 20% w/v glycerol, 0.375 M Tris-HCl pH 8.8, in the presence of 0.5% w/v DTT, for 15 min, in the dark. Then, it was equilibrated in the same buffer containing 4.5% w/v iodoacetamide for additional 15 min. Equilibrated IPG strips were transferred onto 12% polyacrilamide gels to perform the second-dimension SDS-PAGE, using an ETTAN DALT twelve electrophoresis system (GE Healthcare, UK). Gels were scanned with a Typhoon 9400 variable mode imager (GE Healthcare, UK) using a proper excitation/emission wavelength for Cy2 (488/520 nm), Cy3 (532/

580 nm), and Cy5 (633/670 nm). Gel images were visualized with the Image-Quant software (GE Healthcare, UK) and analyzed using the DeCyder 5.0 software (GE Healthcare, UK). A DeCyder differential In-gel-Analysis (DIA) module was used for spot detection and pairwise comparison of each sample (Cy3 and Cy5) to the Cy2 mixed standard present in each gel. Then, the DeCyder Biological Variation Analysis (BVA) module was used to simultaneously match all of the protein-spot maps from the gels, and to calculate average abundance ratios and statistical parameters (Student's *t*-test). Differentially represented spots were identified as those having a relative expression ratio > 1.50 or < 1.50 , with a *P* value ≤ 0.05 .

Preparative 2-DE was performed using 500 μ g of protein extracts from all samples. Electrophoresis was performed as reported above. Resulting gel was stained with Sypro Ruby protein gel staining (Thermo Fisher Scientific, USA), according to the manufacturer's instructions. After spot matching with the master gel from the analytical assay in the BVA module of DeCyder software, a pick list was generated for spot picking by a robotic picker (Ettan spot picker, GE Healthcare, UK).

2.4. Protein identification

Differentially represented spots (DRSs) from preparative 2-DE were excised from gels and washed with water. Proteins were, *in-gel* reduced with dithiothreitol, S-alkylated with iodoacetamide, and then digested with trypsin [28]. Resulting peptide mixtures were subjected to a desalting/concentration step on μ Zip-TipC₁₈ devices (Millipore Corp., Bedford, MA, USA) before MS analysis. Recovered peptides were then analyzed for protein identification by nanoLC-ESI-LIT-MS/MS, using an LTQ XL mass spectrometer (Thermo Fisher Scientific, USA) equipped with a Proxeon nanospray source connected to an Easy-nanoLC (Proxeon, Denmark). Peptides were resolved on an Easy C₁₈ column (100 mm $\times$ 0.075 mm, 3 μ m) (Proxeon) [28]. Mobile phases were 0.1% v/v formic acid (solvent A) and 0.1% v/v formic acid in acetonitrile (solvent B), running at a total flow rate of 300 nL/min. Linear gradient was initiated 20 min after sample loading; solvent B ramped from 5 to 35% over 45 min, from 35 to 60% over 10 min, and from 60 to 95% over 20 min. Spectra were acquired in the range *m/z* 400–2000. Each peptide mixture was analyzed under CID-MS/MS data-dependent product ion scanning procedure, enabling dynamic exclusion (repeat count 1 and exclusion duration 60 s), over the three most abundant ions. Mass isolation window and collision energy were set to *m/z* 3 and 35%, respectively.

Raw nanoLC-ESI-LIT-MS/MS data were searched with v.2.2.06 MASCOT software (Matrix Science, UK) against an updated (11/2015), non-redundant UniProt database (taxonomy *Triticum*) to identify protein(s) present within each gel spot. Database searching was performed by using Cys carbamidomethylation and Met oxidation as fixed and variable protein modifications, respectively, a mass tolerance value of 1.8 Da for precursor ion and 0.8 Da for MS/MS fragments, trypsin as proteolytic enzyme, and a missed cleavage maximum value of 2. Other MASCOT parameters were kept as default. Protein candidates assigned on the basis of at least two sequenced peptides with an individual peptide expectation value < 0.05 (corresponding to a confidence level for peptide identification $> 95\%$) were considered confidently identified. Definitive peptide assignment was always associated with manual spectra visualization and verification.

2.5. Bioinformatics

Hierarchical cluster analysis of DRSs in six development stages was performed using Genesis 1.7.7 software, which allowed the visualization and analysis of gene expression dataset (http://genome.tugraz.at/genesisclient/genesisclient_description.shtml). To assign protein nomenclature and functional annotation, identified sequence entries were compared for similarity to Viridiplantae and *Oryza sativa* database using Blast (<http://www.uniprot.org/blast>) and CloudBlast software,

respectively; the latter occurs within the bioinformatic platform Blast2Go (<https://www.blast2go.com>). Functional annotation of each protein entry according to Bevan classification [29] was obtained using the Mercator pipeline (<http://mapman.gabipd.org/web/guest/app/mercator>). Molecular function and assignment of each protein entry to specific pathways were further derived from dedicated analysis of UniProt and KEGG pathway database. Protein interaction networks were obtained with STRING (<http://string-db.org>) and were visualized with Cytoscape software (<http://www.cytoscape.org>). Allergens in durum wheat grain were derived from data present in the Allergome database (<http://www.allergome.org>) and literature information.

3. Results and discussion

3.1. Protein representation dynamics during grain developmental stages

In order to investigate the metabolic proteins involved in the development of *T. turgidum* ssp. *durum* cv. Svevo grain, whole caryopses sampled at 3 (T0), 5 (T1), 11 (T2), 16 (T3), 21 (T4), and 30 (T5) DPA were extracted for proteins and further fractionated [23]. This procedure yielded the gliadin/glutenin subproteomes that were compared separately [26], while the albumin/globulin counterparts were subjected to comparative 2D-DIGE experiments. In particular, albumin/globulin samples were labeled with either Cy3 or Cy5 fluorescent dyes according to the scheme shown in Supplementary Table S2. Each Cy3/Cy5-labeled sample pair was comixed with a Cy2-labeled pooled standard sample containing an equal amount of 18 samples; Cy2/Cy3/Cy5-labeled samples run together on the same gel. For each gel, the Cy3, Cy5, and Cy2 images were imported and analyzed to reveal constant and differentially represented spots. Approximately, 3200 spots were detected in the durum wheat proteomic map, which migrated within pH 3–10 and Mr. 10–120 kDa ranges (Fig. 1). They were then filtered for fold changes > 1.5 or < 1.5 (T1, T2, T3, T4 and T5 vs T0) and *p* value < 0.05 (Student's paired *t*-test); this revealed 310 statistically relevant DRSs. A hierarchical cluster analysis of the normalized volumes of these DRSs provided information on the dynamic spot representation profile in all grain development stages (Fig. 2A). Four distinct clusters were identified; the first one corresponds to the first two stages (T0 and T1), the second one to T2, the third one to T3, and the fourth one to the last two stages (T4 and T5).

2D-DIGE software modules were also used to match the analytical and preparative gel images. DRSs from the preparative gel were excised, *in-gel* digested with trypsin, and subjected to nanoLC-ESI-LIT-MS/MS analysis for protein identification. MS analysis identified proteins in 285 of the 310 DRSs, among which 237 and 48 showed over- or under-representation, thus suggesting that a higher number of molecular processes/metabolic pathways are activated during grain development, with respect to the deactivated ones. On the whole, 217 non-redundant sequence entries from the *Triticum* database were associated with 285 DRSs (Supplementary Table S3). Identification details are reported in Supplementary Table S4. Electrophoretic spot trains corresponding to the same sequence entry were often observed; they were associated with the possible occurrence of protein post-translational modifications. On the other hand, a variable number of sequence entries was sometimes associated with the same protein name. This phenomenon may depend on the concomitant occurrence of different sequence isoforms in the *T. turgidum* ssp. *durum* genome that matched with different entries in the *Triticum* database, but also on the number/nature of the detected peptides that, depending on the case, matched with specific portions of the sequence entries present in this database. Thus, it is conceivable that most observations of redundancy reported below may depend on the actual lack of information on durum wheat genome. In order to limit sequence redundancy, *Triticum* entries were associated with non-redundant counterparts in *O. sativa* genome (Supplementary Table S3).

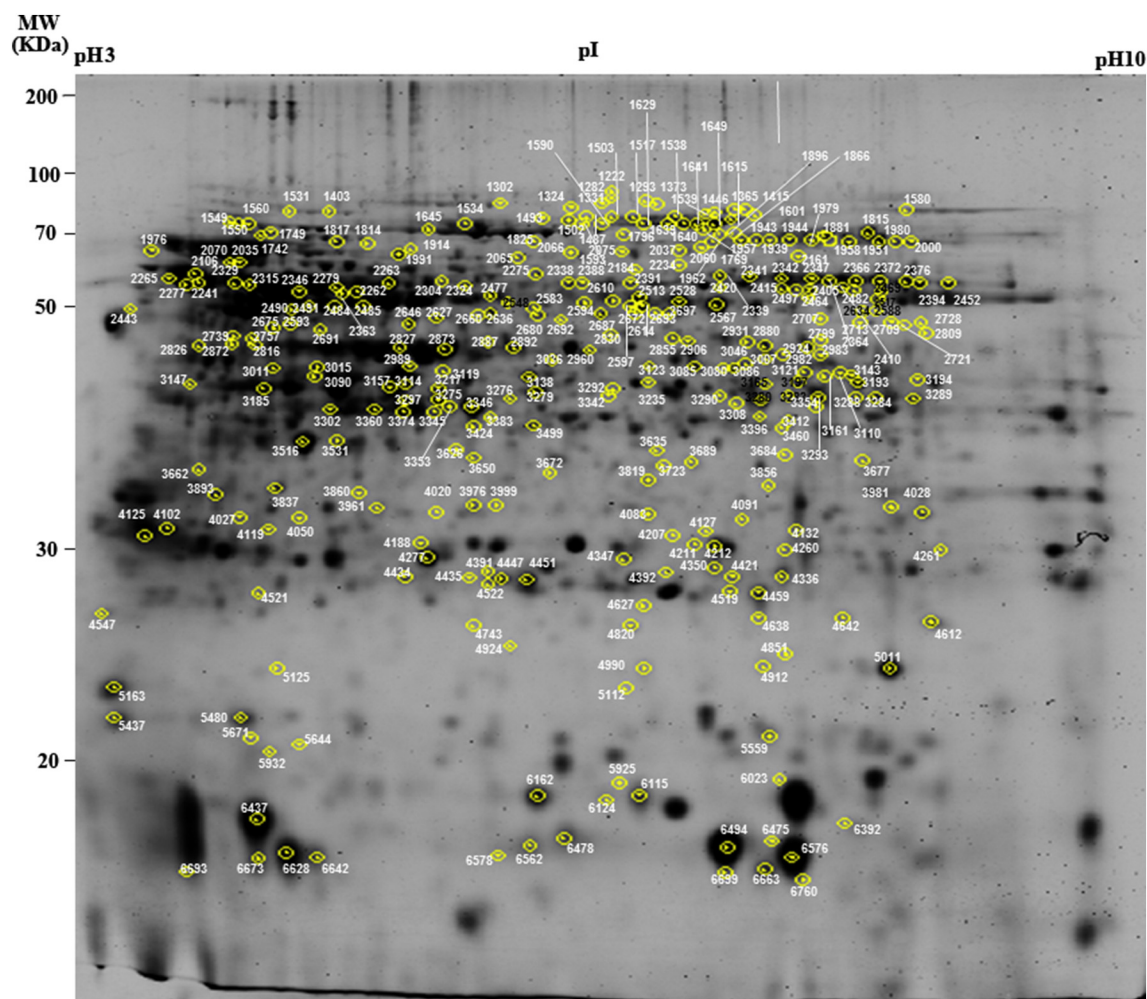


Fig. 1. Preparative two-dimensional gel electrophoresis of the albumin/globulin fraction extracted from durum wheat grain. The figure reports the results of a Sypro Ruby-stained gel loaded with a sample containing equimolar amounts of protein extracts from grains at 3, 5, 11, 16, 21 and 30 DPA, here named T0, T1, T2, T3, T4 and T5, respectively. Preparative and analytical 2D-DIGE was performed as reported in the experimental section. Differentially represented spots are numbered on the gel image. Protein identification results are reported in Supplementary Tables S3 and S4.

Differentially represented proteins (DRPs) were then classified according to the functional catalogue of plant genes made according to Bevan and coworkers [29]. Based on their nature (and frequency in 285 DRPs), DRPs were associated with the functional categories of metabolism (24%), energy (19%), protein destination/storage (19%), disease/defense (16%), cell growth/division (7%), protein synthesis (5%), cell structure (4%), unclassified (3%), signal transduction (2%) and secondary metabolism (1%) (Fig. 2B), thus indicating the most important molecular processes affected in durum wheat during grain development. The differential representation of the single proteins and of the corresponding functional categories was in very good agreement with cumulative data reported for *T. aestivum* [8–10,14–16]. The number of DRPs present at T1, T2, T3, T4 and T5 stages is reported in Supplementary Table S5; this information was used to report the differential representation of the most important functional categories during grain development (Fig. 2C). DRPs progressively reached the apex at T4, and then declined. Hierarchical cluster analysis of the identified species reported in Fig. 2A evidenced that: i) the first cluster (T0 plus T1) was highly populated by proteins associated with disease/defense and cell growth/division; ii) the second one (T2) from species related to disease/defense and protein destination/storage; iii) the third one (T3) from proteins associated with metabolism and energy; iv) the last one (T4 plus T5) from species related to metabolism and protein destination/storage. In particular, over-represented proteins devoted to metabolism, energy, protein destination/storage, disease/defense, and cell

growth/division were those having the highest percentages (Fig. 2C and Supplementary Fig. S1); generally, they did not show significant percentage differences between T2–T5 stages. Unique exceptions were proteins devoted to cell growth/division that peaked at T2, and then showed a progressive, decreasing representation at T3, T4 and T5. On the other hand, under-represented proteins belonging to the same category showed an increased representation moving from T2 to T5 (Supplementary Fig. S2). Since Bevan's classification dates almost 20 years ago [29] and further functional information accumulated later, identified proteins were further analyzed according to updated descriptors for biological processes and molecular pathways.

A heat-map and hierarchical clustering representation of the normalized volumes of the DRPs within each functional category is reported in Figs. 3 and 4 and Supplementary Fig. S3, which show the dynamic expression profiles of the various protein species among the different grain developmental stages. They are discussed in details in dedicated sections in this manuscript and Supplementary Material, which deal with each functional group and where individual DRPs are also discussed.

3.2. Predictive interaction network of differentially represented proteins involved in durum wheat grain development

A bioinformatic analysis of protein-protein interaction networks involving DRPs predicted the involvement of 78 sequence entries (Table

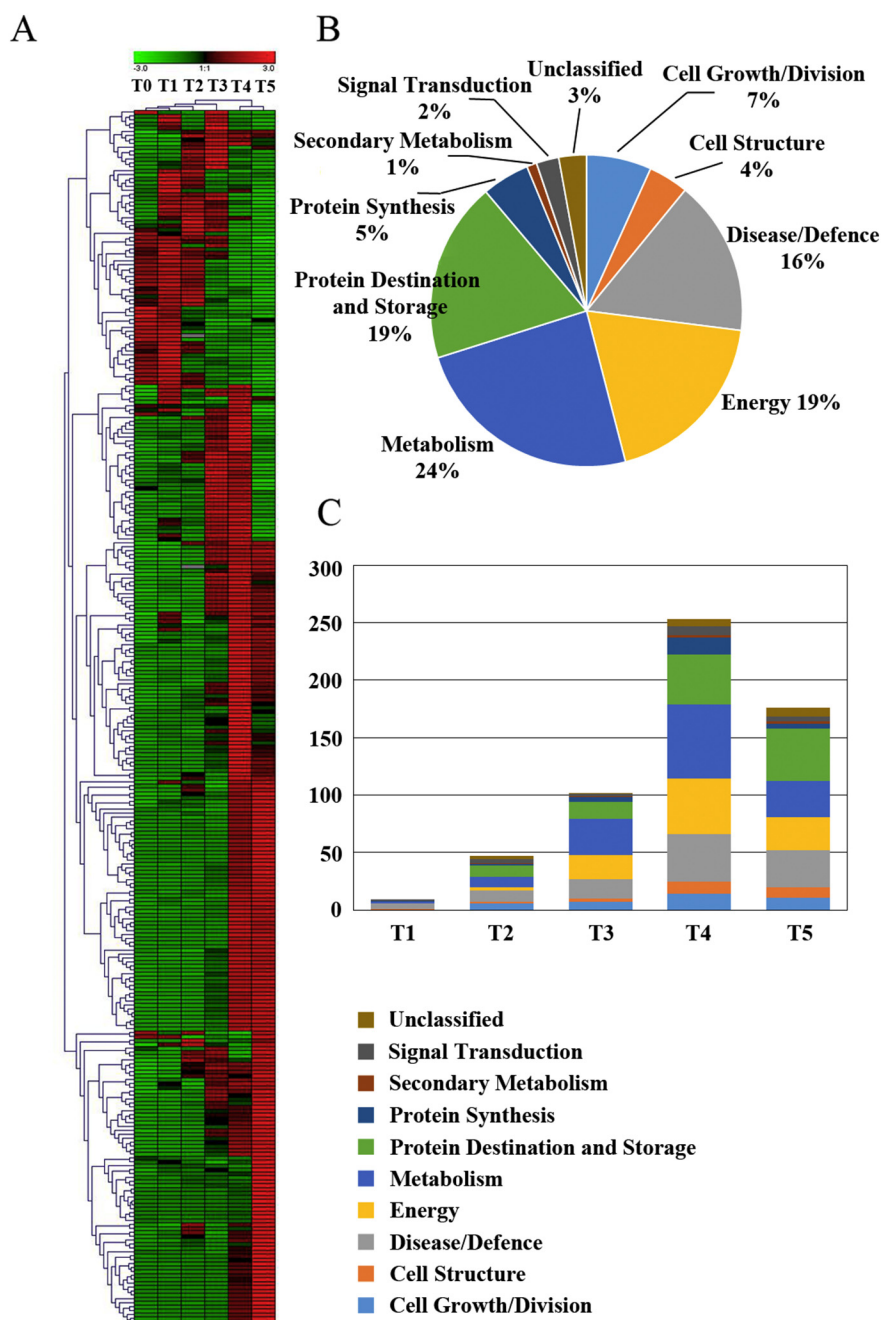


Fig. 2. Quantitative representation of differentially represented spots (DRSs) and proteins (DRPs) in the albumin/globulin fraction of durum wheat grain during development. **A.** Hierarchical clustering analysis of DRSs. An average value of spot volume is reported according to a color-based representation. **B.** Functional distribution of the identified DRPs during grain development according to a pie chart representation. Proteins were divided in functional groups according to the Bevan classification [29]. Reported is the percentage of the number of spots associated with a protein belonging to a certain functional group with respect to the total number of identified spots. **C.** Functional distribution of the identified DRPs in the different stages of grain development. T0, T1, T2, T3, T4 and T5 correspond to 3, 5, 11, 16, 21 and 30 DPA, respectively. Reported are the number of identified proteins belonging to a certain functional group.

S5), mainly related to carbohydrate, nitrogen, cell and energy metabolism, signal transduction, transcription/translation, and stress/defense. The groups of carbohydrate metabolism and stress/defense included a higher number of members (Fig. 5), which were concentrated along the enolase-malate dehydrogenase-ATP citrate synthase β chain-aspartate aminotransferase-centered interaction axis and the RuBisCO large subunit-binding protein α subunit knot, respectively. Other important knots were serine hydroxymethyltransferase, elongation factor 1 γ 2 and UTP-glucose-1-phosphate uridylyltransferase. A general, progressive appearance of proteins involved in above-mentioned protein interaction networks was observed over the time of grain maturation; they provided the large amounts of energy, metabolites and metabolic

processes needed for seed development. These results indicate that all functional groups interact with each other, through a single main network, thus suggesting that various metabolic processes act synergistically during grain maturation. This phenomenon was also observed in *T. aestivum* [14–16], where identical or different components of the same functional groups were identified within the reported interaction maps.

3.3. Sucrose metabolism and starch biosynthesis

Starch comprises about 70–80% of wheat seed dry weight; it is the major determinant for grain yield formation and also plays a prominent

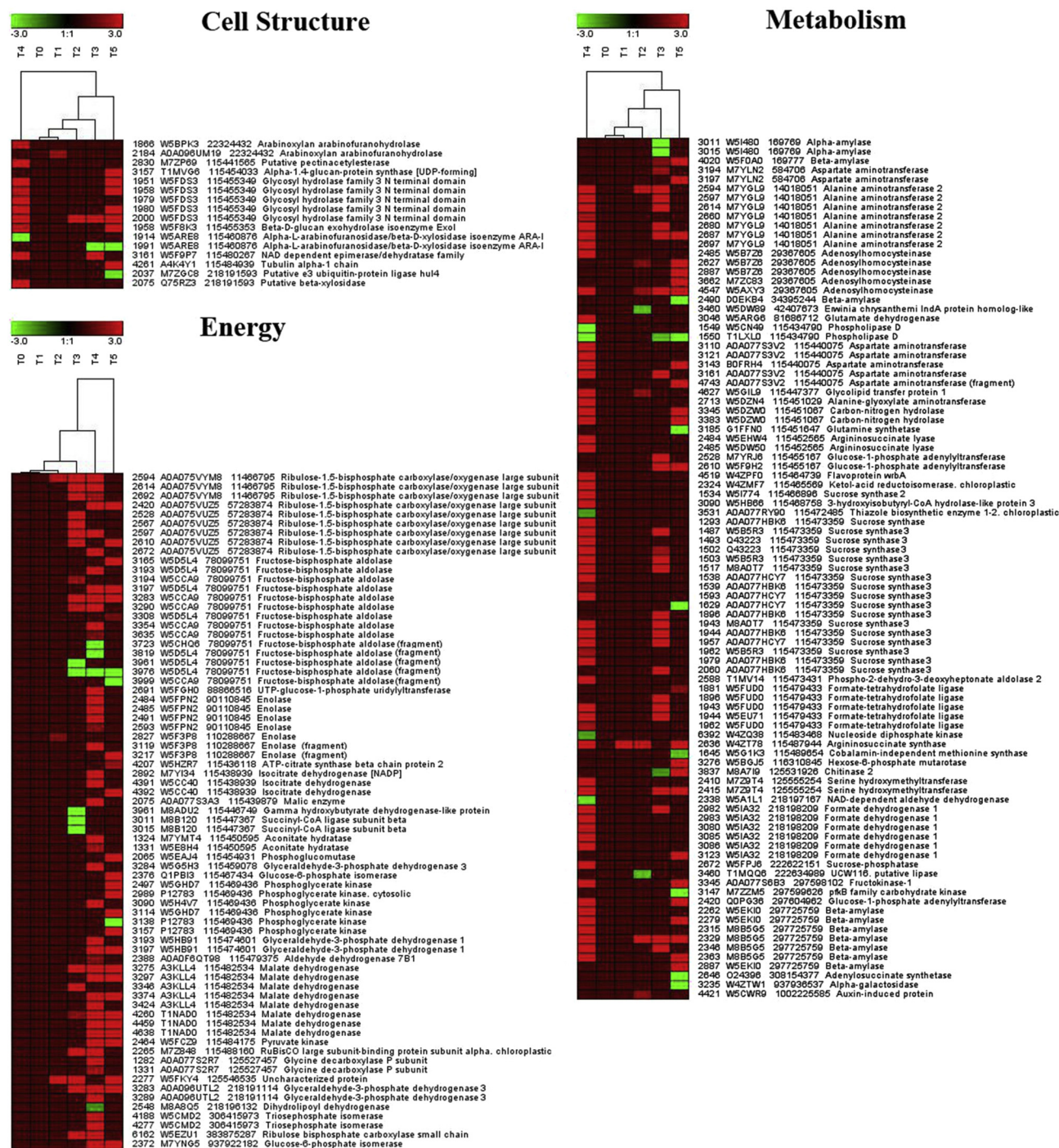


Fig. 3. Heat-map representation of the differentially represented, energy-, carbon metabolism- and cell structure-related proteins in the albumin/globulin fraction of durum wheat grain during development. Relative quantitative results with respect to T0 are highlighted according to a color-based representation. T0, T1, T2, T3, T4 and T5 correspond to 3, 5, 11, 16, 21 and 30 DPA, respectively.

role for semolina quality. Starch is synthesized and accumulated by the synchronized action of a group of enzymes (Fig. 6) [8,30]; sucrose decomposition is the initial event of starch biosynthesis, and sucrose synthases catalyze the reversible conversion of sucrose and UDP to UDP-glucose and fructose [31]. UDP-glucose is then transformed into α -glucose-1P by the action of UTP-glucose-1-phosphate uridylyltransferase. This intermediate is finally converted into ADP-glucose, which is a key compound for starch accumulation in the grain; glucose-1-phosphate adenyltransferase catalyzes this reaction and acts a major rate-limiting factor in starch biosynthesis [32]. Other hexoses, i.e. fructose and hexose-phosphates, determining the hexoses-P pool in the cytosol,

contribute to generate α -glucose-1P through the sequential action of various enzymes, including fructokinase 1, glucose-6-phosphate isomerase and phosphoglucosomate [33,34]. The latter protein regulates the activity of starch-branching enzymes and their protein-protein interactions [35]. ADP-glucose allows for the elongation of amylose and amylopectin, and various proteins catalyze the formation of α -(1,6)-glucosides within the polymers and the hydrolysis of α -(1,6)-linkages within the polyglucan to stabilize biopolymer structure [36]. Our proteomic study demonstrated that a number of enzymes essential for sucrose metabolism and starch biosynthesis were over-represented at T3, T4 and/or T5 in *T. turgidum* ssp. *durum* (Figs. 3 and 6); they include sucrose synthase 2 and 3 (18

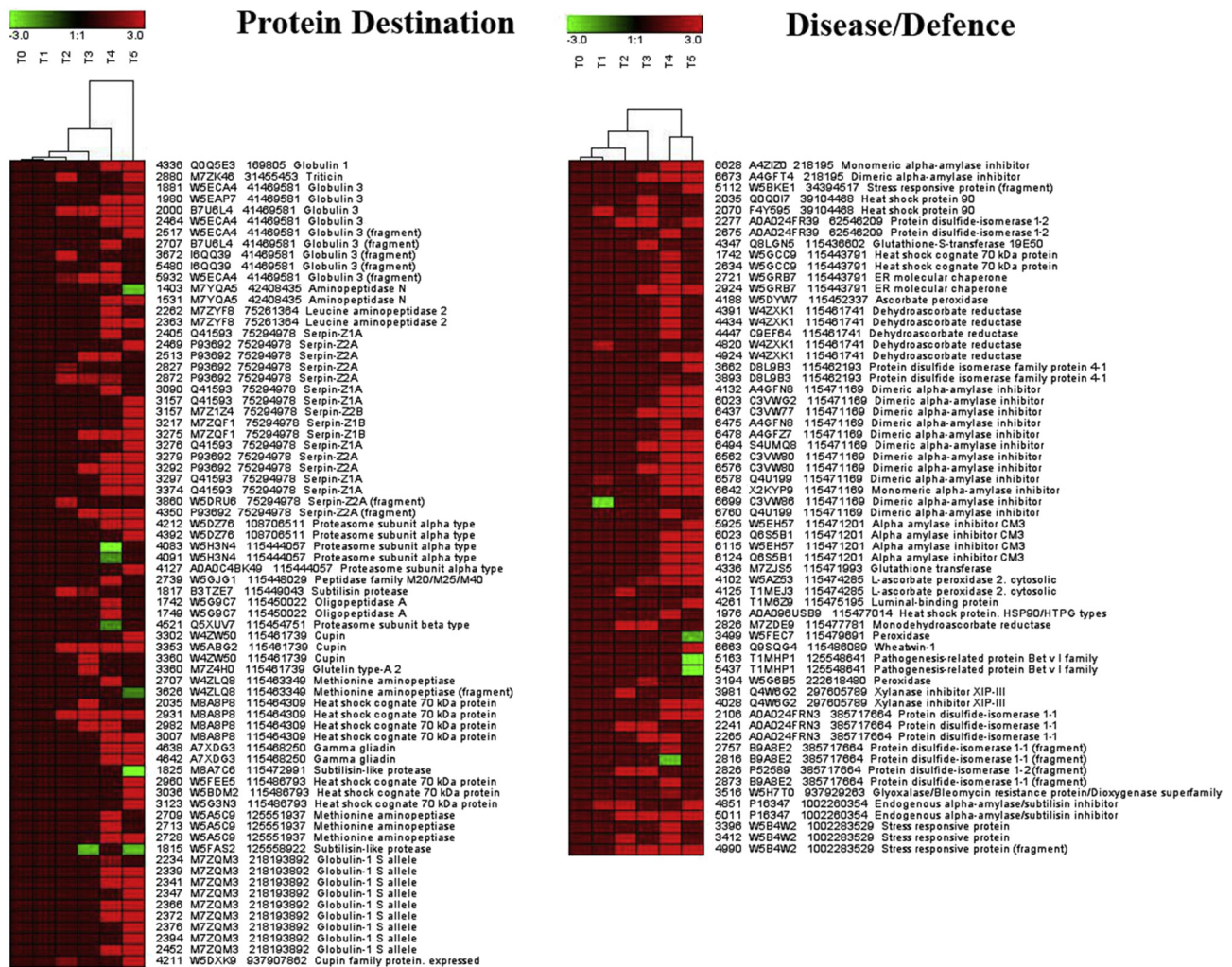


Fig. 4. Heat-map representation of the differentially represented, protein destination/storage- and disease/defense-related proteins in the albumin/globulin fraction of durum wheat grain during development. Relative quantitative results with respect to T0 are highlighted according to a color-based representation. T0, T1, T2, T3, T4 and T5 correspond to 3, 5, 11, 16, 21 and 30 DPA, respectively.

spots), sucrose-phosphatase (1 spot), UTP-glucose-1-phosphate uridylyltransferase (1 spot), glycosyl hydrolase family 3 N-terminal domain (5 spots), β -D-glucan exohydrolase isoenzyme ExoI (1 spot), fructokinase 1 (1 spot), glucose-6-phosphate isomerase (2 spots), phosphoglucomutase (1 spot) and glucose-1-phosphate adenylyltransferase (3 spots). This is consistent with the rapid increase of grain weight and starch granules. In some cases, those having a representation apex at T3/T4 returned at basal levels at T5. Notably, UTP-glucose-1-phosphate uridylyltransferase was present as a node in the network reported in Fig. 5, also including 4 augmented DRPs.

Enzymes involved in starch degradation were either under-represented, i.e. α -amylase (2 spots), or showed a mixed quantitative behavior, i.e. β -amylase (9 spots) (Fig. 3). The latter phenomenon was associated with the occurrence of different sequence isoforms of β -amylase having a variable expression, or of phosphorylation events that can modulate the enzyme activity and ultimately alter relative spot quantitative distribution [37]. In this context, recent studies have shown the prominent role of phosphorylation in regulating starch biosynthesis [38]. Nevertheless, inhibition of α -amylase activities in durum wheat grain was ensured by a significant over-representation of corresponding monomeric (8 spots) and dimeric (12 spots) inhibitor isoforms preventing starch degradation (Fig. 4). Their inhibitory function toward α -amylase has also been reported to have important roles in plant defense (see below) [39] and pre-harvest sprouting resistance [40]. β -Amylase as well as α -amylase inhibitors have been reported

among the most active allergenic proteins associated with wheat flour food and inhalation human allergies [41,42] (Table 1).

3.4. Additional processes of carbon and energy metabolism

Previous studies in *T. aestivum* showed that both central carbon and energy metabolism play key roles in grain development [8,9,14–16,43–45]. In fact, the respiratory pathways of glycolysis, tricarboxylic acid (TCA) cycle and photosynthesis, together with those of anaerobic alcoholic fermentation, provide energy, cofactor regeneration, and building blocks for metabolite interconversion and final biosynthesis of sucrose, starch and storage proteins, with compound concentration gradients generally acting as signals for grain development. In agreement with this observation, we identified various DRPs involved in glycolysis showing a higher representation at T4 and T5 stages, i.e. enolase (7 spots), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) 1 and 3 (5 spots), phosphoglycerate kinase (5 spots), triosephosphate isomerase (TPI) (2 spots), pyruvate kinase (1 spot), glucose-6-phosphate isomerase (2 spots) and fructose-bisphosphate aldolase (FBA) (9 spots) (Fig. 3 and Supplementary Fig. S4). Enolase is a central node in the network reported in Fig. 5, which bridges other functional knots and makes a subnetwork including 7 over-represented glycolytic DRPs. Among the latter enzymes, some play a multiple role in different catabolic pathways, which is the case of FBA, a key glycolytic/gluconeogenic enzyme in plants that is also involved in TCA in

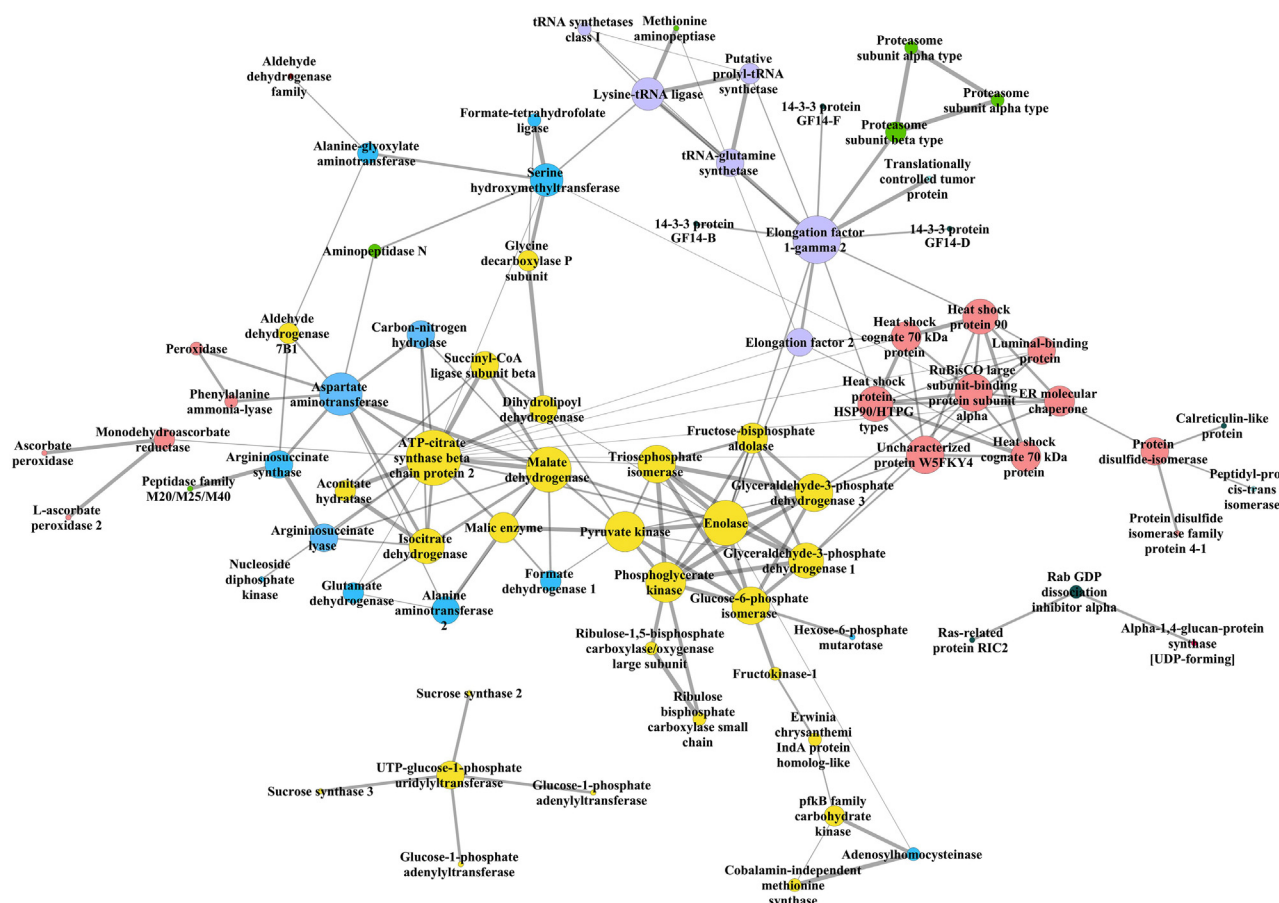


Fig. 5. Interaction network of differentially represented proteins in durum wheat grain during development. Differentially represented proteins were comparatively analyzed with BLAST against *O. sativa* genome to identify corresponding rice counterparts. The latter ones were then searched in the STRING database using a confidence cut-off of 0.700. The interaction network was reconstructed by using the Cytoscape software. Proteins belonging to the same functional group [29] are highlighted with the same color. Pink, disease/defense; light green, protein destination; green, signal transduction; violet, protein synthesis; light blue, cell growth; blue, metabolism; yellow, energy; red, secondary metabolism.

plastids, where it stimulates photosynthetic yield [46]. Our proteomic observation was in line with the idea that T4-T5 stages correspond to cell expansion and the beginning of dry matter accumulation, during which much energy is needed [47,48]. GAPDH and TPI have also been reported among wheat protein allergens [41,42] (Table 1).

An over-representation profile was also observed for some *T. turgidum* ssp. *durum* enzymes related to the TCA cycle, i.e. ATP citrate synthase β chain (1 spot), aconitate hydratase (2 spots), malate dehydrogenase (MDH) (8 spots), isocitrate dehydrogenase (3 spots), malic enzyme (1 spot), and aldehyde dehydrogenase 7B1 (1 spot), while succinyl-CoA ligase β chain (2 spots), adenylosuccinate synthase (1 spot) and dihydrolipoyl dehydrogenase (1 spot) showed reduced levels during grain development. These results support the conclusion that this metabolic pathway is sustained by relative enzyme representation in providing energy for durum wheat grain development. In this context, worth mentioning is the role of MDH, which catalyzes the production of oxalacetic acid to be further converted in aspartate for protein biosynthesis. This enzyme was predicted to interact with many other over-represented DPRs in the grain (Fig. 5). Together with other enolase-, citrate synthase β chain- and aspartate aminotransferase-centered knots, MDH constitutes the most populated network axis, whose interaction with other DRPs underlines its pivotal role in the energy supply for grain development.

In line with augmented photosynthetic and alcoholic fermentation activities in the developing wheat grain [8–10,14–16,43–45] was the observation in *T. turgidum* ssp. *durum* of over-represented levels of

RuBisCO large (9 spots) and small (1 spot) subunits, RuBisCO large subunit-binding protein α subunit (1 spot) and glycine decarboxylase P subunit (2 spots) at T3-T4, and of formate dehydrogenase (6 spots) and formate-tetrahydrofolate ligase (5 spots) at T4-T5 (Fig. 3). At the early grain developmental stages, photosynthesis provides the raw material triose-phosphate for starch biosynthesis, and plays a role in maintaining the endogenous O_2 balance and in the grain filling stages [15,49,50]. Our results suggest that photosynthesis should be one of the earliest activated energetic processes in durum wheat, whereas alcoholic fermentation may provide supplementary energy at the late developmental stages. Formate dehydrogenase was predicted to be located close to the central protein-protein network involved in carbohydrate metabolism (Fig. 5), suggesting its important role for carbohydrate metabolism and energy supply during grain development.

Finally, a number of *T. turgidum* ssp. *durum* enzymes involved in amino acid and nitrogen metabolism were observed as over-represented in T3-T5 stages (Fig. 3). They include glutamate dehydrogenase (1 spot), aspartate aminotransferases (7 spots), carbon-nitrogen hydrolase (2 spots), alanine aminotransferase (7 spots), alanine-glyoxylate aminotransferase (1 spot), serine hydroxymethyltransferase (2 spots), argininosuccinate lyase (2 spots), argininosuccinate synthase (1 spot), adenosylhomocysteinase (5 spots), phospho-2-dehydro-3-deoxyheptonate aldolase 2 (1 spot) and ketol-acid reductoisomerase (1 spot). The latter three enzymes are involved in Met, Phe/Tyr/Trp and Val/Leu/Ile biosynthesis, respectively. Most of these proteins have also been reported to show a beneficial action for gluten quantity and

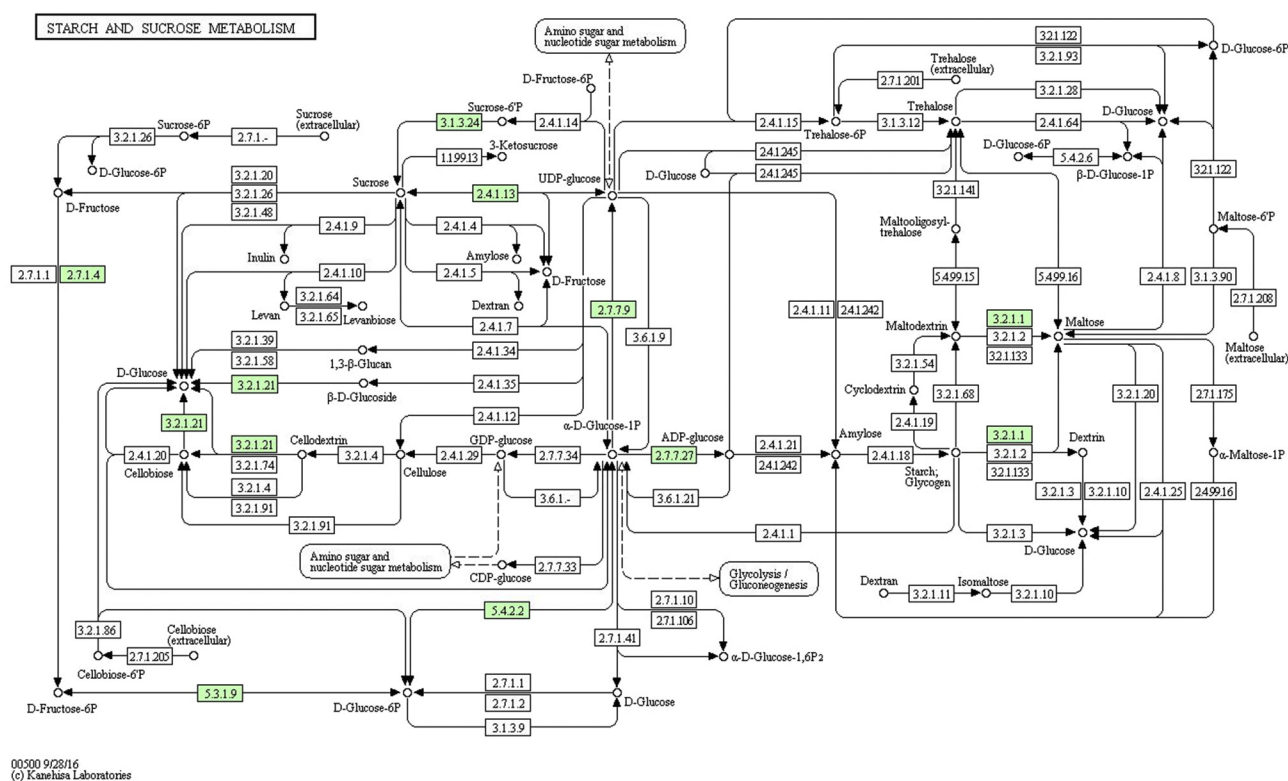


Fig. 6. Proteins with differential representation involved in starch and sucrose metabolism as mapped with KEGG. Sucrose synthase [EC:2.4.1.13 – synthase]; glucose-1-phosphate adenylyltransferase [EC:2.7.7.27 – adenylyltransferase]; fructokinase 1 [EC:2.7.1.4 – fructokinase]; glucose-6-phosphate isomerase [EC:5.3.1.9 – isomerase]; phosphoglucomutase [EC:5.4.2.2 – phosphoglucomutase]; UTP-glucose-1-phosphate uridylyltransferase [EC:2.7.7.9 – uridylyltransferase]; glycosyl hydrolase family 3 N-terminal domain and β -D-glucan exohydrolase isoform ExoI [EC:3.2.1.21 – betaglucohydrolase]; sucrose-phosphatase [EC:3.1.3.24 – phosphatase]; α -amylase and β -amylase [EC:3.2.1.1 – glycogenase]. Proteins mentioned above are highlighted in color.

quality in the grains [8–10,14–16]. In fact, a number of them may favor catabolic pathways providing intermediate metabolites to be used in the TCA cycle and ultimately energy to growing grains. In this context, the importance of amino acids as energy donors to fulfill the requirements of developing seeds has already been established [50].

3.5. Protein synthesis, destination and storage

Cereal grains ensure almost a half of the dietary proteins for humans [51]; this is based on storage proteins, i.e. glutenins, gliadins, albumins

and globulins, among which the first two have a prominent role, contributing about 70% of total grain proteins. All these proteins highly affect the nutritional and technological properties of mature seeds. Information on the first two protein classes during durum wheat grain development was achieved by an independent dedicated investigation on this subproteome [26]. Conversely, this proteomic study provided information on the remaining two families as well as on those enzymes assisting biosynthesis of storage proteins in the developing grains. In particular, a progressive accumulation of *T. turgidum* ssp. *durum* albumins and globulins was ascertained by the detection of increasing levels of globulin 1 (1 spot), globulin 3 (9 spots), globulin 1S (9 spots), triticin (1 spot), cupin (3 spots) and cupin family protein (1 spot) at T3-T5 (Fig. 4); at these stages, augmented levels of residual γ -gliadin (2 spots) and glutelin type A2 (1 spot) were also detected. These results were in perfect agreement with single protein-centered studies on durum wheat grain [22,26].

An augmented representation of storage proteins found a counterpart in increased levels of gene products associated with protein biosynthetic machineries. In fact, various ribosomal proteins resulted concomitantly over-represented in developing durum wheat grains (Supplementary Fig. S3); they include tRNA-glutamine synthetase (5 spots), tRNA synthetase class I (2 spots), prolyl-tRNA synthetase (1 spot), lysine-tRNA ligase (1 spot), eukaryotic initiation factor 4A (EIF4A) (4 spots), and elongation factor (EF) 1 γ 2 (1 spot) and EF2 (3 spots). A reduced proteolysis of EF2 was also ascertained, as deduced from the corresponding fragments (2 spots). EIF1 γ is a component of the elongation factor complex involved in protein biosynthesis and also participates in NF- κ B signaling [52]; Fig. 5 shows its localization in the network of DRPs present in developing durum wheat grain, bridging various other interaction knots.

In parallel, an increased representation of methionine aminopeptidase (4 spots) was also detected in developing *T. turgidum* ssp. *durum*

Table 1

Durum wheat allergens identified in this study by combined 2D-DIGE and nanoLC-ESI-LIT-MS/MS analysis. Proteins were associated with specific *Triticum* entries in the Allergome database (<http://www.allergome.org>) or with data from dedicated literature [41,42].

Protein	Allergome code	References
α -Amylase/trypsin inhibitor CM3 precursor	1051, 8191	[41,42]
α -Amylase inhibitor 0.19	8186	[41,42]
Monomeric α -amylase inhibitor	8725	
Serpin-Z1B	5724	[41,42]
Serpin-Z2B	5724	[41]
27 k Protein	6148	
Glutathione S-transferase	6326	
Xylanase inhibitor XIP-III	9596	
Wheatwin 1		[41]
Peroxidase		[41,42]
Glyceraldehyde 3-phosphate dehydrogenase		[41]
Triosephosphate isomerase		[41]
Endogenous α -amylase/subtilisin inhibitor		[41]
Chitinase 2		[41]
β -Amylase		[41]
Serpin-Z1A		[41]
Serpin-Z2A		[41]
β -Glucosidase		[41]

grains (Fig. 4); this enzyme ensures a proper N-terminal processing of the nascent polypeptide chain to yield proteins in their final form [53]. Augmented levels of this and other endopeptidases, namely oligopeptidase A (2 spots), peptidase family M20/M25/M40 (1 spot) and leucine aminopeptidase (2 spots), were already detected in *T. aestivum* by dedicated enzymatic assays [54]; these enzymes were suggested not affecting storage proteins through hormonal and inhibitor control mechanisms. In line with this hypothesis was the observed over-representation of protease inhibitors serpin Z1A (6 spots), Z2A (8 spots), Z1B (2 spots) and Z2B (1 spot). The serpin family acts through irreversible inhibition of endogenous/exogenous proteinases, playing important roles in plant growth, development, stress response and defense against insects/pathogens (see below) [55]. Its massive over-representation has also been detected in developing bread wheat grain, where it represents up to 4% of the total protein in the mature endosperm. Members of this family generate a strong allergenic response to wheat in humans [41,42] (Table 1). Protection against degradation of storage proteins was also guaranteed by a reduced representation of subtilisin-like protease (2 spots). Finally, a development-dependent putative regulation of the activity of additional proteases and the proteasome machinery was hypothesized based on the mixed quantitative representation of aminopeptidase N (2 spots), and proteasome subunit α (4 spots) and β (1 spot). These proteins have been reported to be phosphorylated in *O. sativa* and *A. thaliana* [56,57], where this modification was associated with regulative events.

Augmented synthesis of storage proteins has to be related also with increasing activities of enzymes assisting proper polypeptide folding, preventing aggregation and regulating a correct arrangement of the corresponding disulfide bonds. In this context, intra- and inter-molecular S—S bonds have been reported to play an essential role for albumins, globulins, gliadins, and glutenins, respectively [58,59]. In this study, different *T. turgidum* ssp. *durum* chaperones involved in stabilizing protein conformation were detected as over-represented at T3–T5 (Fig. 4); they include ER molecular chaperone (2 spots), heat shock protein (HSP) 90 (2 spots), and different isoforms of HSP cognate 70 kDa (10 spots). A coherent quantitative trend of disulfide isomerase (PDI)-like 1-1 (6 spots), 1-2 (3 spots), PDI 4-1 (2 spots) and peptidyl-prolyl *cis-trans* isomerase (1 spot) was also observed. Most of the above-mentioned proteins constitutes an interaction network centered at RuBisCO large subunit-binding protein α subunit (Fig. 5). HSPs have been suggested to affect the aggregation of gluten proteins during grain filling, thereby modulating their dough-forming potential [60]. PDIs have been reported to play important roles in controlling proper assembly/maturation of glutenin macropolymer through intramolecular S—S bonds [61]. Selected members of both protein families were reported to interact with molecules involved in cell signaling and transcription regulation pathways [62]. Their functional role in the development of durum wheat grain was confirmed here through the observed, augmented representation of the corresponding levels.

3.6. Stress/defense proteins

To protect themselves against a possible adverse environment due to abiotic and biotic stresses, plants produce various defensive proteins to selectively adapt and resist to drought, temperature, salinity, pathogens and insect challenges. Exposure to abiotic and biotic stresses often associates with increased concentrations of reactive oxygen species [63]; thus, various antioxidant enzymes are induced in plant tissues to counteract the detrimental activity of these toxic oxygen species [64]. Indeed, we verified that ascorbate peroxidase (1 spot), ascorbate peroxidase 2 (2 spots), dehydroascorbate reductase (5 spots), monodehydroascorbate reductase (1 spot), glutathione S-transferase (GST) (1 spot), GST 19E50 (1 spot), peroxidase (1 spot), glyoxalase (1 spot) and quinone oxidoreductase wrbA (1 spot) showed increased levels at the late stages of durum wheat grain development (Fig. 4). Some of these enzymes are integral parts of the glutathione-ascorbate

cycle [65], which detoxifies H_2O_2 and hydrogen peroxides using ascorbate as substrate. Ascorbate has been reported to play a vital role in controlling cell division, osmotic adjustment and seed germination [66]. Similarly, glyoxalase is involved in glutathione-based detoxification of methylglyoxal, especially during oxidative and salinity stresses [67, 68]; this enzyme is over-represented in durum wheat seeds treated with ascorbate to prevent undesired germination during storage and improved seedling growth under salinity [19]. On the other hand, GSTs are important detoxification enzymes that catalyze the conjugation of redox stress-related xenobiotics or their metabolites to glutathione [63,69]; in plants, they are over-represented in a number of abiotic and biotic stresses. Finally, quinone oxidoreductase wrbA has been already reported as over-represented in response to xenobiotics or oxidative challenging conditions [70].

A number of proteins involved in defense processes against the attack of pathogens and insects were also observed as over-represented during *T. turgidum* ssp. *durum* grain development (Fig. 4); they include members of the stress-responsive family of ricin B (4 spots), xylanase inhibitor protein III (2 spots) and wheatwin 1 (1 spot), which all showed augmented levels at T2–T5. After an initial quantitative decrease, chitinase 2 (1 spot) also resulted as over-represented. Ricins are toxic proteins acting on ribosome to inhibit protein synthesis; they are among the most lethal substances known [71]. Although through different molecular mechanisms, xylanase inhibitor proteins, wheatwins and chitinases are well known to exert their biological activity against several pathogenic fungi and bacteria [72–74]. Conversely, pathogenesis-related protein Bet v I family (2 spots) was under-represented at T5. Various stress-responsive proteins reported above constitute the largest group of allergenic molecules present in durum wheat [41,42] (Table 1).

The inventory of augmented stress responsive/defensive proteins in the developing durum wheat grain was completed by various proteins already discussed above that, acting through different processes, contribute to face possible adverse environmental conditions. They include HSPs and PDIs that, assisting protein folding and correct disulfide pairing, have been already reported to prevent protein misfolding and aggregation; they are very highly induced in wheat grain exposed to heat, drought and salinity stresses [75,76]. Comparative proteomics on near-isogenic *T. aestivum* lines showing hard or soft kernel phenotype demonstrated that these proteins are synthesized in high amounts in the hard line missing the puroindoline-a gene, with respect to the soft counterpart containing this gene [11]. These results and the absence of puroindoline genes in durum wheat genome strengthened the importance of HSP and PDI functions in hard grain-producing wheat plants in compensating this molecular lack; in this context, puroindolines are known to induce the soft phenotype by affecting the interaction between the starch granules and the endosperm protein matrix in the kernel [77]. An analogous defensive role can be hypothesized for α -amylase inhibitors and serpins, which have been reported to protect plants against insect wounding or to inhibit fungal growth [39,55,78–80]. Worth mentioning is also the fact that previous studies classified about 25% of wheat globulins and albumins as proteins belonging to the stress/defense family [13,15].

3.7. Cell growth and cell structure remodeling

Wheat grain development has been associated with an increased expression of genes involved in plant cell growth and division [4,17]. This study confirmed this finding; indeed, various DRPs seemed to contribute to the dynamic picture of cellular growth and division events involved in developing durum wheat grain. In particular, an over-representation of translationally controlled tumor protein (TCTP) (1 spot), pfkB family carbohydrate kinase (also known as phosphofructokinase-like 2 or adenosine kinase 2) (1 spot), translin protein family (1 spot) and some auxin-induced aldo-keto reductase proteins (3 spots) occurred at T2–T5 stages (Fig. 3 and Supplementary Fig. S3);

conversely, lipoxygenases were over-represented at T2, T3 and T4, and then under-represented at T5. TCTP acts as a guanine nucleotide dissociation inhibitor on the translation elongation factor eEF1A [81]; it is an important regulator of growth in plants, and has many critical functions, such as maintenance of heat stability, calcium binding, and prevention of H₂O₂ build-up, and induced cell death. It was reported that the expression of TCTP is increased in physiologically active and proliferating tissues, and under a variety of stress conditions [82,83]. Phosphofructokinase-like 2 protein is a constituent of the membrane-associated plastid-encoded polymerase complex [84]. It is essential for plant cell growth and development, especially at the chloroplast site [85], where it regulates plastid gene expression. Translin is a conserved protein in eukaryotic organisms that has a nucleic acid-binding activity and acts as enhancer component of an RNAi (RNA interference) pathway [86]; it has been implicated in a broad spectrum of biological activities, including cell growth regulation, mRNA processing, and genome stability regulation. Finally, lipoxygenases have been associated with several aspects of plant physiology, including growth and development, protein storage, and response to abiotic and biotic stresses [87]. They catalyze hydroxyperoxidation of polyunsaturated fatty acids to yield oxylipins, which regulate cell growth and various defensive processes. Increased levels of lipoxygenases in mature grains have also been positively associated with the decrease of carotenoids content specifically observed in *T. aestivum* [88].

Plant cell wall provides a physical barrier against chemical challenges, osmotic stress and pathogen attack. This polysaccharide-made compartment must reshape its structure in growing plant cells to allow corresponding physical expansion, duplication and division phases. Thus, a number of enzymes involved in continuous remodeling of cell wall and cytoskeleton compartments have to be induced in developing wheat grains in order to catalyze the molecular processes associated with these necessary structural modifications [89–91]. In line with this consideration, this study revealed at T3–T5 an over-representation of *T. turgidum* ssp. *durum* enzymes classified as related to the cell structure (Fig. 2); they include glycosyl hydrolase family 3 N-terminal domain (5 spots), β -xylosidase (1 spot), NAD-dependent epimerase/dehydratase (also known as UDP-glucose 4-epimerase 3) (1 spot), α -1,4-glucan-protein synthase (UDP-forming), arabinoxylan arabinofuranohydrolase (2 spots), pectinacetyltransferase (1 spot) and β -D-glucan exohydrolase isozyme ExoI (1 spot). Conversely, under-represented enzymes were α -L-arabinofuranosidase/ β -D-xylosidase isoenzyme ARA-I (2 spots) and α -galactosidase (1 spot). A part of these enzymes is involved in the metabolism of arabinoxylan and pectin polysaccharides that reshape cell wall during cell growth and development [91,92]. Due to their possible involvement in starch biosynthesis, some of these enzymes have already been discussed in a previous section. On the other hand, tubulin α -1 chain (1 spot) was the unique cytoskeletal protein differentially represented during durum wheat grain development.

4. Conclusions

In this study, a 2D-DIGE-based comparative analysis of the albumin/globulin fraction from durum wheat caryopses at 6 developmental stages was performed to describe the dynamic subproteomic changes associated with grain development. Quantitative variations of 217 DRPs demonstrated that highly affected are the functional categories of carbon metabolism, energy, protein destination/storage, disease/defense and cell growth/division, which displayed a general over-representation, consistently with the occurrence of grain size increase and starch/protein reserve accumulation during seed development. Bioinformatic interaction predictions revealed a complex protein network centered mainly at enzymes involved in carbon and protein metabolism. Specific DRPs and differentially represented protein functional categories highly resembled those already demonstrated as modified in developing bread wheat grain [8–10,14–16], suggesting that the main differences in kernel hardness between *T. aestivum* and *T. turgidum*

ssp. *durum* are not ascribable to proteomic changes in the corresponding albumin/globulin fraction during organ development, but are related to other grain subproteomes or to other specific factors affecting the interaction between the starch granules and the endosperm protein matrix, i.e. puroindoline and polar lipids (and related biosynthetic enzymes) [77,93], which are absent in durum wheat or whose quantitative levels therein may eventually need dedicated analytical procedures.

In addition to the gluten proteins associated with celiac disease that will be described elsewhere [26], this study also described the dynamic representation of a number of proteins associated with wheat flour food and inhalation human allergies. In particular, durum wheat grain showed an over-representation of allergenic albumins/globulins in the last stages of its development. Notably, 18 allergens were identified in a single two-dimensional electrophoretic run by combining proteomics with information from Allergome database or scientific literature [41, 42]; they often escape detection by conventional broad-range assays. Accordingly, this 2D-DIGE-based approach will allow simultaneous quantitative detection of these allergens in comparative studies on durum wheat cultivars used for pasta preparations. In conclusion, by providing a comprehensive understanding for the molecular basis of durum wheat grain development, yield and quality formation, this study provides potential markers for further investigations on durum wheat breeding and semolina quality assessment.

Transparency document

The Transparency document associated with this article can be found, in online version.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jprot.2017.05.004>.

References

- [1] IPO International Pasta Organization, The World Pasta Industry Status Report, <http://www.internationalpasta.org/index.aspx> 2013.
- [2] H. Wiesner, W. Seilmeier, H.D. Belitz, Vergleichende untersuchungen über partielle aminosäuresequenzen von prolaminen und glutelinen verschiedener getreidearten, Z. Lebensm. Unters. Forsch. 170 (1980) 17–26.
- [3] T. Evers, S. Millar, Cereal grain structure and development: some implications for quality, J. Cereal Sci. 36 (2002) 261–284.
- [4] P.R. Shewry, R.A.C. Mitchell, P. Tosi, Y. Wan, C. Toscano-Underwood, A. Lovegrove, J. Freeman, G.A. Toole, E.N.C. Mills, J.L. Ward, An integrated study of grain development of wheat (cv. Hereward), J. Cereal Sci. 56 (2012) 21–30.
- [5] D.J. Skylas, J.A. Mackintosh, S.J. Cordwell, D.J. Basseal, B.J. Walsh, J. Harry, C. Blumenthal, L. Copeland, C.W. Wrigley, W. Rathmell, Proteome approach to the characterization of protein composition in the developing and mature wheat grain endosperm, J. Cereal Sci. 32 (2000) 169–188.
- [6] Y. Song, Q. Zheng, Dynamic rheological properties of wheat flour dough and proteins, Trends Food Sci. Technol. 18 (2007) 132–138.
- [7] N.K. Singh, G.R. Donovan, I.L. Batey, F. MacRitchie, Use of sonication and size-exclusion high-performance liquid chromatography in the study of wheat flour proteins. I. Dissolution of total proteins in the absence of reducing agents, Cereal Chem. 67 (1990) 150–161.
- [8] I. Nadaud, C. Grousse, C. Debiton, C. Chambon, M.F. Bouzidi, P. Martre, G. Branlard, Proteomic and morphological analysis of early stages of wheat grain development, Proteomics 10 (2010) 2901–2910.
- [9] A. Tasleem-Tahir, I. Nadaud, C. Grousse, P. Martre, D. Marion, G. Branlard, Proteomic analysis of peripheral layers during wheat (*Triticum aestivum* L.) grain development, Proteomics 11 (2011) 371–379.

- [10] P. Ge, C. Ma, S. Wang, L. Gao, X. Li, G. Guo, W. Ma, Y. Yan, Comparative proteomic analysis of grain development in two spring wheat varieties under drought stress, *Anal. Bioanal. Chem.* 402 (2012) 1297–1313.
- [11] V.S. Lesage, M. Merlino, C. Chambon, B. Bouchet, D. Marion, G. Branlard, Proteomes of hard and soft near-isogenic wheat lines reveal that kernel hardness is related to the amplification of a stress response during endosperm development, *J. Exp. Bot.* 63 (2012) 1001–1011.
- [12] L. Gao, A. Wang, X. Li, K. Dong, K. Wang, R. Appels, W. Ma, Y. Yan, Wheat quality related differential expressions of albumins and globulins revealed by two-dimensional difference gel electrophoresis (2-D DIGE), *J. Proteome* 73 (2009) 279–296.
- [13] K. Dong, P. Ge, C. Ma, K. Wang, X. Yan, L. Gao, X. Li, J. Liu, W. Ma, Y. Yan, Albumin and globulin dynamics during grain development of elite Chinese wheat cultivar Xiaoyan 6, *J. Cereal Sci.* 56 (2012) 615–622.
- [14] H. Cao, M. He, C. Zhu, L. Yuan, L. Dong, Y. Bian, W. Zhang, Y. Yan, Distinct metabolic changes between wheat embryo and endosperm during grain development revealed by 2D-DIGE-based integrative proteome analysis, *Proteomics* 16 (2016) 1515–1536.
- [15] C. Ma, J. Zhou, G. Chen, Y. Bian, D. Lv, X. Li, Z. Wang, Y. Yan, iTRAQ-based quantitative proteome and phosphoprotein characterization reveals the central metabolism changes involved in wheat grain development, *BMC Genomics* 15 (2014) 1029.
- [16] M. Yang, J. Dong, W. Zhao, X. Gao, Characterization of proteins involved in early stage of wheat grain development by iTRAQ, *J. Proteome* 136 (2016) 157–166.
- [17] Y. Wan, R.L. Poole, A.K. Huttly, C. Toscano-Underwood, K. Feeney, S. Welham, M.J. Gooding, C. Mills, K.J. Edwards, P.R. Shewry, R.A.C. Mitchell, Transcriptome analysis of grain development in hexaploid wheat, *BMC Genomics* 9 (2008) 121.
- [18] P. Laino, D. Shelton, C. Finnie, A.M. De Leonardi, A.M. Mastrangelo, B. Svensson, D. Lafiandra, S. Masci, Comparative proteome analysis of metabolic proteins from seeds of durum wheat (cv. Svevo) subjected to heat stress, *Proteomics* 10 (2010) 2359–2368.
- [19] A. Fercha, A.L. Capriotti, G. Caruso, C. Cavaliere, H. Gherroucha, R. Samperi, S. Stampachiachiere, A. Laganà, Gel-free proteomics reveal potential biomarkers of priming-induced salt tolerance in durum wheat, *J. Proteome* 91 (2013) 486–499.
- [20] A.L. Capriotti, G.M. Borrelli, V. Colapicchioni, R. Papa, S. Piovesana, R. Samperi, S. Stampachiachiere, A. Laganà, Proteomic study of a tolerant genotype of durum wheat under salt-stress conditions, *Anal. Bioanal. Chem.* 5 (2014) 1423–1435.
- [21] A. Fercha, A.L. Capriotti, G. Caruso, C. Cavaliere, R. Samperi, S. Stampachiachiere, A. Laganà, Comparative analysis of metabolic proteome variation in ascorbate-primed and unprimed wheat seeds during germination under salt stress, *J. Proteome* 108 (2014) 238–257.
- [22] M.M. Giuliani, C. Palermo, M.A. De Santis, A. Mentana, M. Pompa, L. Gluzio, S. Masci, D. Centonze, Z. Flagella, Differential expression of durum wheat gluten proteome under water stress during grain filling, *J. Agric. Food Chem.* 63 (2015) 6501–6512.
- [23] W.J. Hurlkman, C.K. Tanaka, Extraction of wheat endosperm proteins for proteome analysis, *J. Chromatogr. B Anal. Technol. Biomed. Life Sci.* 849 (2007) 344–350.
- [24] M. Maccaferri, M.C. Sanguineti, S. Corneti, J.L. Araus Ortega, M. Ben Salem, J. Bort, E. DeAmbrogio, L.F. Garcia del Moral, A. Demontis, A. El-Ahmed, F. Maalouf, H. Machlab, V. Martos, M. Moragues, J. Motawaj, M. Nachit, N. Nserallah, H. Ouabbou, C. Royo, A. Slama, R. Tuberosa, Quantitative trait loci for grain yield and adaptation of durum wheat (*Triticum durum* Desf.) across a wide range of water availability, *Genetics* 178 (2008) 489–511.
- [25] T. Majoul, E. Bancel, E. Tribou, J. Ben Hamida, G. Branlard, Proteomic analysis of the effect of heat stress on hexaploid wheat grain: characterization of heat-responsive proteins from non-prolamins fraction, *Proteomics* 4 (2004) 505–513.
- [26] F. Mazzeo, L. Di Stasio, C. D'Ambrosio, S. Arena, A. Scaloni, S. Corneti, A. Ceriotti, R.A. Siciliano, G. Picariello, G. Mamone, *J. Agric. Food Chem.* 65 (2017) 3242–3250.
- [27] M. Di Carli, M.E. Villani, G. Renzone, L. Nardi, A. Pasquo, R. Franconi, A. Scaloni, E. Benvenuto, A. Desiderio, Leaf proteome analysis of transgenic plants expressing anti-viral antibodies, *J. Proteome Res.* 8 (2009) 838–848.
- [28] G.S. Scippa, M. Rocco, M. Iallicco, D. Trupiano, V. Viscosi, M. Di Michele, S. Arena, D. Chiatante, A. Scaloni, The proteome of lentil (*Lens culinaris* Medik.) seeds: discriminating between landraces, *Electrophoresis* 31 (2010) 497–506.
- [29] M. Bevan, I. Bancroft, E. Bent, K. Love, H. Goodman, C. Dean, R. Bergkamp, W. Dirkse, M. Van Staveren, W. Stiekema, L. Drost, P. Ridley, S.A. Hudson, K. Patel, G. Murphy, P. Piffanelli, H. Wedler, E. Wedler, R. Wambutt, T. Weitzenegger, T.M. Pohl, N. Terry, J. Gielen, R. Villarroel, R. De Clerck, M. Van Montagu, A. Lecharny, S. Auborg, I. Gy, M. Kreis, N. Lao, T. Kavanagh, S. Hempel, P. Kotter, K.D. Entian, M. Rieger, M. Schaeffer, B. Funk, S. Mueller-Auer, M. Silvey, R. James, A. Montfort, A. Pons, P. Puigdomenech, A. Douka, E. Voukelatou, D. Milioni, P. Hatzopoulos, E. Piravandi, B. Obermaier, H. Hilbert, A. Dusterhöft, T. Moores, J.D. Jones, T. Eneva, K. Palme, V. Benes, S. Rechman, W. Ansoorge, R. Cooke, C. Berger, M. Delseny, M. Voet, G. Volckaert, H.W. Mewes, S. Klosterman, C. Schueller, N. Chalwatzi, Analysis of 1.9 Mb of contiguous sequence from chromosome 4 of *Arabidopsis thaliana*, *Nature* 391 (1998) 485–488.
- [30] P.L. Keeling, J.R. Wood, R.H. Tyson, I.G. Bridges, Starch biosynthesis in developing wheat grain: evidence against the direct involvement of triose phosphates in the metabolic pathway, *Plant Physiol.* 87 (1988) 311–319.
- [31] J.G. Núñez, J. Kronenberger, S. Wuilleme, L. Lepiniec, C. Rochat, Study of AtSUS2 localization in seeds reveals a strong association with plastids, *Plant Cell Physiol.* 49 (2008) 1621–1626.
- [32] E.D. Smidansky, F.D. Meyer, B. Blakeslee, T.E. Weglarz, T.W. Greene, M.J. Giroux, Expression of a modified ADP-glucose pyrophosphorylase large subunit in wheat seeds stimulates photosynthesis and carbon metabolism, *Planta* 225 (2007) 965–976.
- [33] J.Z. Angeles-Núñez, A. Tiessen, Regulation of AtSUS2 and AtSUS3 by glucose and the transcription factor LEC2 in different tissues and at different stages of *Arabidopsis* seed development, *Plant Mol. Biol.* 78 (2012) 377–392.
- [34] E.J. Davies, I.J. Tetlow, C.G. Bowsher, M.J. Emes, Molecular and biochemical characterization of cytosolic phosphoglucotransferase in wheat endosperm (*Triticum aestivum* L. cv. Axona), *J. Exp. Bot.* 386 (2003) 1351–1360.
- [35] F. Grimaud, H. Rogniaux, M.G. James, A.M. Myers, V. Planchot, Proteome and phosphoproteome analysis of starch granule-associated proteins from normal maize and mutants affected in starch biosynthesis, *J. Exp. Bot.* 59 (2008) 3395–3406.
- [36] P. Geigenberger, Regulation of starch biosynthesis in response to a fluctuating environment, *Plant Physiol.* 155 (2011) 1566–1577.
- [37] K. Dong, S. Zhen, Z. Cheng, H. Cao, P. Ge, Y. Yan, Proteomic analysis reveals key proteins and phosphoproteins upon seed germination of wheat (*Triticum aestivum* L.), *Front. Plant Sci.* 6 (2015) 1017.
- [38] I.J. Tetlow, K.G. Keisel, S. Cameron, A. Makhmoudova, F. Liu, N.S. Bresolin, R. Wait, M.K. Morell, M.J. Emes, Analysis of protein complexes in wheat amyloplasts reveals functional interactions among starch biosynthetic enzymes, *Plant Physiol.* 146 (2008) 1878–1891.
- [39] E.A. MacGregor, S.L. Bazin, E.W. Ens, J. Lahnstein, L.J. Macri, N.J. Shirley, A.W. MacGregor, Structural models of limit dextrinase inhibitors from barley, *J. Cereal Sci.* 31 (2000) 79–90.
- [40] M.D. Gale, J.E. Flinham, K.M. Devos, Cereal comparative genetics and preharvest sprouting, *Euphytica* 126 (2002) 21–25.
- [41] P. Šotkovský, J. Sklenář, P. Halada, J. Cinová, I. Šetinová, A. Kainarová, J. Goliáš, K. Pavlásková, S. Honzová, L. Tučková, A new approach to the isolation and characterization of wheat flour allergens, *Clin. Exp. Allergy* 41 (2011) 1031–1043.
- [42] I. Sander, P. Rozynek, H.P. Rihs, V. Van Kampen, F.T. Chew, W.S. Lee, N. Kotschy-Lang, R. Merget, T. Brüning, M. Raulf-Heimsoth, Multiple wheat flour allergens and cross-reactive carbohydrate determinants bind IgE in baker's asthma, *Allergy* 66 (2011) 1208–1215.
- [43] A.R. Fernie, F. Carrari, L.J. Sweetlove, Respiratory metabolism: glycolysis, the TCA cycle and mitochondrial electron transport, *Curr. Opin. Plant Biol.* 7 (2004) 254–261.
- [44] L. Gutierrez, O. Van Wuytswinkel, M. Castelain, C. Bellini, Combined networks regulating seed maturation, *Trends Plant Sci.* 12 (2007) 294–300.
- [45] S.B. Xu, T. Li, Z.Y. Deng, K. Chong, Y. Xue, T. Wang, Dynamic proteomic analysis reveals a switch between central carbon metabolism and alcoholic fermentation in rice filling grains, *Plant Physiol.* 148 (2008) 908–925.
- [46] K. Uematsu, N. Suzuki, T. Iwamae, M. Inui, H. Yukawa, Increased fructose 1,6-bisphosphate aldolase in plastids enhances growth and photosynthesis of tobacco plants, *J. Exp. Bot.* 63 (2012) 3001–3009.
- [47] T. Evers, S. Millar, Cereal grain structure and development: some implications for quality, *J. Cereal Sci.* 36 (2002) 261–284.
- [48] C. Debiton, M. Merlino, C. Chambon, E. Bancel, M. Decourteix, V. Planchot, G. Branlard, Analyses of albumins, globulins and amphiphilic proteins by proteomic approach give new insights on waxy wheat starch metabolism, *J. Cereal Sci.* 53 (2011) 160–169.
- [49] H. Tschiersch, L. Borisjuk, T. Rutten, H. Rolletschek, Gradients of seed photosynthesis and its role for oxygen balancing, *Biosystems* 103 (2011) 302–308.
- [50] G. Galili, T. Avin-Wittenberg, R. Angelovici, A.R. Fernie, The role of photosynthesis and amino acid metabolism in the energy status during seed development, *Front. Plant Sci.* 5 (2014) 447.
- [51] D. Deutscher, The current status of breeding for protein quality in corn, *Adv. Exp. Med. Biol.* 105 (1978) 281–300.
- [52] D. Liu, C. Sheng, S. Gao, W. Jiang, J. Li, C. Yao, H. Chen, J. Wu, S. Chen, W. Huang, eEF1B is a positive regulator of NF- κ B signaling pathway, *Biochem. Biophys. Res. Commun.* 446 (2014) 523–528.
- [53] S. Ross, C. Giglione, M. Pierre, C. Espagne, T. Meinell, Functional and developmental impact of cytosolic protein N-terminal methionine excision in *Arabidopsis*, *Plant Physiol.* 137 (2005) 623–637.
- [54] F. Dominguez, J. Cejudo, Characterization of the endoproteases appearing during wheat grain development, *Plant Physiol.* 112 (1996) 1211–1217.
- [55] T.H. Roberts, J. Hejgaard, Serpins in plants and green algae, *Funct. Integr. Genomics* 8 (2008) 1–27.
- [56] H. Nakagami, N. Sugiyama, K. Mochida, A. Daudi, Y. Yoshida, T. Toyoda, M. Tomita, Y. Ishihama, K. Shirasu, Large-scale comparative phosphoproteomics identifies conserved phosphorylation sites in plants, *Plant Physiol.* 153 (2010) 1161–1174.
- [57] N. Sugiyama, H. Nakagami, K. Mochida, A. Daudi, M. Tomita, K. Shirasu, Y. Ishihama, Large-scale phosphorylation mapping reveals the extent of tyrosine phosphorylation in *Arabidopsis*, *Mol. Syst. Biol.* 4 (2008) 193.
- [58] T.A. Egorov, T.I. Odintsova, A.K. Musolyamov, R. Fido, A.S. Tatham, P.R. Shewry, Disulphide structure of a sunflower seed albumin: conserved and variant disulphide bonds in the cereal prolamin superfamily, *FEBS Lett.* 396 (1996) 285–288.
- [59] P.R. Shewry, A.S. Tatham, Disulphide bonds in wheat gluten proteins, *J. Cereal Sci.* 25 (1997) 207–227.
- [60] F.M. Dupont, W.J. Hurlkman, W.H. Vensel, C. Tanaka, K.M. Kothari, O.K. Chung, S.B. Altenbach, Protein accumulation and composition in wheat grains: effects of mineral nutrients and high temperature, *Eur. J. Agron.* 25 (2006) 96–107.
- [61] N.L. Houston, C. Fan, Q. Xiang, J.M. Schulze, R. Jung, R.S. Boston, Phylogenetic analyses identify 10 classes of the protein disulfide isomerase family in plants, including single-domain protein disulfide isomerase-related proteins, *Plant Physiol.* 137 (2005) 762–778.
- [62] Y.G. Zhao, R. Gilmore, G. Leone, M.C. Coffey, B. Weber, P.W.K. Lee, Hsp90 phosphorylation is linked to its chaperoning function: assembly of the reovirus cell attachment protein, *J. Biol. Chem.* 276 (2001) 32822–32827.
- [63] A. Bachi, I. Dalle-Donne, A. Scaloni, Redox proteomics: chemical principles, methodological approaches and biological/biomedical promises, *Chem. Rev.* 113 (2013) 596–698.
- [64] S. Rinalducci, L. Murgiano, L. Zolla, Redox proteomics: basic principles and future perspectives for the detection of protein oxidation in plants, *J. Exp. Bot.* 59 (2008) 3781–3801.

- [65] G. Nector, C.H. Foyer, Ascorbate and glutathione: keeping active oxygen under control, *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 49 (1998) 249–279.
- [66] M.C. De Tullio, O. Arrigoni, The ascorbic acid system in seeds: to protect and to serve, *Seed Sci. Res.* 13 (2003) 249–260.
- [67] S.K. Yadav, S.L. Singla-Pareek, M.K. Reddy, S.K. Sopory, Transgenic tobacco plants overexpressing glyoxalase enzymes resist an increase in methylglyoxal and maintain higher reduced glutathione levels under salinity stress, *FEBS Lett.* 579 (2005) 6265–6271.
- [68] S.L. Singla-Pareek, M.K. Reddy, S.K. Sopory, Genetic engineering of the glyoxalase pathway in tobacco leads to enhanced salinity tolerance, *Proc. Natl. Acad. Sci. U. S. A.* 100 (2003) 14672–14677.
- [69] C. Frova, The plant glutathione transferase gene family: genomic structure, functions, expression and evolution, *Physiol. Plant.* 119 (2003) 469–479.
- [70] J. Carey, J. Brynda, J. Wolfová, R. Grandori, T. Gustavsson, R. Ettrich, I.K. Smatanová, WrbA bridges bacterial flavodoxins and eukaryotic NAD(P)H:quinone oxidoreductases, *Protein Sci.* 16 (2007) 2301–2305.
- [71] M.R. Hartley, J.M. Lord, Cytotoxic ribosome-inactivating lectins from plants, *Biochim. Biophys. Acta, Proteins Proteomics* 1701 (2004) 1–14.
- [72] F. Payan, P. Leone, S. Porciero, C. Furniss, T. Tahir, G. Williamson, A. Durand, P. Manzanera, H.J. Gilbert, N. Juge, A. Roussel, The dual nature of the wheat xylanase protein inhibitor XIP-I: structural basis for the inhibition of family 10 and family 11 xylanases, *J. Biol. Chem.* 279 (2004) 36029–36037.
- [73] L. Bertini, C. Caporale, M. Testa, S. Proietti, C. Caruso, Structural basis of the antifungal activity of wheat PR4 proteins, *FEBS Lett.* 583 (2009) 2865–2871.
- [74] F. Mauch, L.A. Staehelin, Functional implications of the subcellular localization of ethylene-induced chitinase and β -1,3-glucanases in bean leaves, *Plant Cell* 1 (1989) 447–457.
- [75] A.M. Timperio, M.G. Egidi, L. Zolla, Proteomics applied on plant abiotic stresses: role of heat shock proteins (HSP), *J. Proteome* 71 (2008) 391–411.
- [76] A. Mostek, A. Börner, A. Badowiec, S. Weidner, Alterations in root proteome of salt-sensitive and tolerant barley lines under salt stress conditions, *J. Plant Physiol.* 174 (2015) 166–176.
- [77] A. Pauly, B. Pareyt, E. Fierens, J.A. Delcour, Wheat (*Triticum aestivum* L. and *T. turgidum* L. ssp. *durum*) kernel hardness: I. Current view on the role of puroindolines and polar lipids, *Compr. Rev. Food Sci. Food Saf.* 12 (2013) 413–426.
- [78] F. Garcia-Olmedo, G. Salcedo-Duran, R. Sanchez-Monge Laguna de Rins, L. Gomez, J. Royo, P. Carbonero-Zaldegui, Plant proteinaceous inhibitors of proteinases and alpha-amylase, *Surv. Plant Mol. Cell Biol. Oxf.* 4 (1987) 275–334.
- [79] W.H. Vensel, C.K. Janaka, N. Cai, J.H. Wong, B.B. Buchanan, W.J. Hurkman, Developmental changes in the metabolic protein profiles of wheat endosperm, *Proteomics* 5 (2005) 1594–1611.
- [80] A.I. Pekkarinen, C. Longstaff, B.L. Jones, Kinetics of the inhibition of *Fusarium* serine proteinases by barley (*Hordeum vulgare* L.) inhibitors, *J. Agric. Food Chem.* 55 (2007) 2736–2742.
- [81] C. Cans, B.J. Passer, V. Shalak, V. Nancy-Portebois, V. Crible, N. Amzallag, D. Allan, R. Tufino, M. Argenti, D. Moras, G. Fiucci, B. Goud, M. Mirande, R. Amson, A. Telerman, Translationally controlled tumor protein acts as a guanine nucleotide dissociation inhibitor on the translation elongation factor eEF1A, *Proc. Natl. Acad. Sci. U. S. A.* 100 (2003) 13892–13897.
- [82] M. Liao, Y. Li, Z. Wang, Identification of elicitor-responsive proteins in rice leaves by a proteomic approach, *Proteomics* 9 (2009) 2809–2819.
- [83] O. Berkowitz, R. Jost, S. Pollmann, J. Masle, Characterization of TCTP, the translationally controlled tumor protein, from *Arabidopsis thaliana*, *Plant Cell* 20 (2008) 3430–3447.
- [84] B. Arsova, U. Hoja, M. Wimmelsbacher, E. Greiner, S. Ustün, M. Melzer, K. Petersen, W. Lein, F. Börnke, Plastidial thioredoxin z interacts with two fructokinase-like proteins in a thiol-dependent manner: evidence for an essential role in chloroplast development in *Arabidopsis* and *Nicotiana benthamiana*, *Plant Cell* 22 (2010) 1498–1515.
- [85] T. Ogawa, K. Nishimura, T. Aoki, H. Takase, K. Tomizawa, H. Ashida, A. Yokota, A phosphofructokinase B-type carbohydrate kinase family protein, NARA5, for massive expressions of plastid-encoded photosynthetic genes in *Arabidopsis*, *Plant Physiol.* 151 (2009) 114–128.
- [86] A. Jaendling, R.J. McFarlane, Biological roles of translin and translin-associated factor-X: RNA metabolism comes to the fore, *Biochem. J.* 429 (2010) 225–234.
- [87] H. Porta, M. Rocha-Sosa, Plant lipoxygenases. Physiological and molecular features, *Plant Physiol.* 130 (2002) 15–21.
- [88] F. Leenhardt, B. Lyan, E. Rock, A. Boussard, J. Potus, E. Chanliaud, C. Remesy, Wheat lipoxygenase activity induces greater loss of carotenoids than vitamin E during breadmaking, *J. Agric. Food Chem.* 54 (2006) 1710–1715.
- [89] I. Levy, Z. Shani, O. Shoseyov, Modification of polysaccharides and plant cell wall by endo-1,4- β -glucanase and cellulose-binding domains, *Biomol. Eng.* 19 (2002) 17–30.
- [90] H. Konno, Y. Yamasaki, M. Sugimoto, K. Takeda, Differential changes in cell wall matrix polysaccharides and glycoside-hydrolyzing enzymes in developing wheat seedlings differing in drought tolerance, *J. Plant Physiol.* 165 (2008) 745–754.
- [91] N. Obel, A.C. Porchia, H.V. Scheller, Dynamic changes in cell wall polysaccharides during wheat seedling development, *Phytochemistry* 60 (2002) 603–610.
- [92] T. Stolle-Smits, J.G. Beekhuizen, M.T. Kok, M. Pijnburg, K. Recourt, J. Derksen, A.G. Voragen, Changes in cell wall polysaccharides of green bean pods during development, *Plant Physiol.* 121 (1999) 363–372.
- [93] K. Heinze, A.M. Kiszona, J.C. Murray, C.F. Morris, V. Lullien-Pellerin, Puroindoline genes introduced into durum wheat reduce milling energy and change milling behavior similar to soft common wheats, *J. Cereal Sci.* 71 (2016) 183–189.

**DIFFERENTIAL REPRESENTATION OF ALBUMINS AND GLOBULINS DURING
GRAIN DEVELOPMENT IN DURUM WHEAT AND ITS POSSIBLE
FUNCTIONAL CONSEQUENCES**

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SUPPLEMENTARY MATERIAL

Materials and methods

Plant material and growth conditions

The Italian pasta-quality reference cv. Svevo was released by Società Produttori Sementi Bologna. It was obtained from the cross between a CIMMYT line (pedigree rok/fg//stil/3/dur1/4/sapi/teal//hui), related to the widely utilized Yavaros79 genetic background (Jori/Anhinga//Flamingo), and the cv. Zenit originating from a cross between Italian and American accessions (Valriccardo/Vic); it was registered in 1996. Svevo is a medium to early photoperiod insensitive durum cultivar well adapted to the whole Mediterranean Basin cultivation area. In rainfed and irrigated Mediterranean environments, Svevo combines high grain production levels to overall high pasta quality value. It is characterized by higher-than-average grain yellow pigment and grain protein contents, the latter mainly due to Svevo's highly efficient nitrogen remobilization at grain filling and maturity.

In early spring 2016, Svevo was grown in a growth chamber under completely controlled conditions of photoperiod, air temperature and humidity, under horizontal laminar air flux. The growing cycle lasted for three months. Initially the plants were grown at 14/10 h day/night photoperiod and 18/14 °C temperature up to the first node elongation stage. The plants were then grown under long days conditions (16/8 h day/night photoperiod) at 20/16 °C. Temperatures were increased to 24/18 °C from flowering to maturity. The experimental unit consisted of 4-l cylindrical pots, filled with a mix of 6 l peat moss, 3 l silt-loam soil mix, 3 l perlite, 4 l vermiculite, 4 l sand, 300 mg of complex mineral fertilizer. Eight petri-dish germinated Svevo's seedlings were transplanted per pot. Subsequently, at the two-leaf stage the growing seedlings were thinned to allow four uniform adult plants to grow to maturity per pot. A complex fertilizer with N:P:K at ratio 15:9:15 and microelements (NPK[®] Original Gold[®], Compo Expert, Italy) was applied at the following consecutive stages: sowing (incorporated into the soil), three leaf stage, tillering, first and second elongated nodes and booting (50 g m⁻² for the

whole growing cycle). Soil moisture was kept at optimal water availability conditions throughout the whole plant cycle by manually irrigating the pots to full field capacity every two days.

Each replicate consisted of six pots in total (24 adult plants in total) that were used for subsequent destructive grain sampling at the chosen grain developmental phases. The experiment consisted of three replicates in total. Svevo's kernels were harvested at key developmental stages, namely at 3 d (embryo increase three-fold in size), 5 d (kernel development at 1/3 of full developmental size, water-ripening stage), 11 d (kernel development at 1/2 full size, water-ripening stage), 16 d (kernel full development, water-ripening stage), 21 d (milk-ripening stage) and 30 d (dough stage) post-anthesis, according to the reference kernel developmental stages reported in the CerealDB (<http://bio-gromit.bio.bris.ac.uk/cerealgenomics/cgi-bin/grain3.pl>). Kernel's development in growth chamber was daily inspected to ensure that samplings were conducted at the right developmental stage. Triplicate samples were harvested according to the method of Majoul and coworkers [1] at the above mentioned six developed stages: 3 days post-anthesis (DPA) (T0), 5 DPA (T1), 11 DPA (T2), 16 DPA (T3), 21 DPA (T4) and 30 DPA (T5). Immediately after harvesting, kernels were stored at -80 °C, until their use for proteomic analysis. Morphological details at each developmental stage are reported in Supplementary Tab. S1. Collected samples at each developmental stage were pooled to make three biological replicates, which were stored at -80 °C prior to comparative proteomic analysis.

Results and Discussion

Cell signaling

Although key signal pathways and networks remain to be fully elucidated during seed development, different signaling effectors are involved in the various phases of grain development [2]. In analogy with the dynamics of changes observed for the metabolic pathways reported above, various components involved in cell signaling in *T. turgidum* ssp. *durum* were also over-represented; they

include three different 14-3-3 proteins (4 spots), calreticulin (CRT)-like protein (1 spot) and Rab GDP dissociation inhibitor α (3 spots); conversely, Ras-related protein RIC2 (1 spot) and nucleoside diphosphate kinase (NDPK) (1 spot) were both down-represented at T4. Generally, signal transduction proteins were down-regulated during seed development in *T. aestivum* [3], but the expression profile of 14-3-3 proteins was different and in line to what detected in the present study. 14-3-3 proteins have been reported to bind to the specific sites of protein kinases to make them active/inactive through its phosphorylation specificity [4]. Plant 14-3-3 proteins are involved in many cellular events, including enzyme regulation, gene expression, and protein translocation [5]. On the other hand, CRT has been reported to play a significant role in intracellular Ca^{2+} homeostasis and Ca^{2+} signaling pathways [6]; this exerts a positive functional role in the plant response to cold, drought and disease stresses [7,8]. Rab GDP dissociation inhibitor was shown to play an essential role in regulating the state of bound nucleotides and subcellular localizations of Rab proteins [9]. It was shown to inhibit MAPK2 activity through physical interaction. Its differential representation, together with that of Ras-related protein RIC2, suggested the activation of signaling pathways regulating exocytic and endocytic vesicular trafficking during durum wheat grain development, as already demonstrated in *T. aestivum* [10]. Finally, NDPK helps plant cells maintaining the nucleoside triphosphate pool, which is essential for plant survival [11]. It has been reported that NDPK plays a regulatory role in *Arabidopsis* tolerance to multiple abiotic stresses. Yeast two-hybrid and *in vitro* protein pull-down assays revealed that NDPK specifically interacts with H_2O_2 -mediated mitogen-activated protein kinase (MAPK) signaling [12]. These data suggest that NDPK down-representation mediates plant tolerance of multiple stressors through inactivation of the MAPK cascade.

Secondary metabolism

Few enzymes involved in secondary metabolism were also differentially represented in developing durum wheat grain. 4-Hydroxy-7-methoxy-3-oxo-3, 4-dihydro-2H-1, 4-benzoxazin-2-yl glucoside β -D-glucosidase 1 (GLU1A) and phenylalanine ammonia-lyase (PAL) were down-represented at T2-T4 and T5, respectively; conversely, O-methyltransferase COMT type was over-represented at T5. GLU1A acts in defense of young plant parts against pests through the production of hydroxamic acids from corresponding hydroxamic acid glucosides [13,14]; its reduced levels were related to the accumulation of hydroxamic acid glucosides in grain tissues. Its enzymatic activity has been highly correlated with plant growth. PAL is involved in *trans*-cinnamate biosynthesis [15] and plays a key role in mediating carbon flux from primary metabolism into the phenylpropanoid pathway, which contributes to the hardness, color, taste, and odor of plant tissues. Finally, O-methyltransferase COMT type is involved in the biosynthesis of aromatic secondary metabolites.

References

1. T. Majoul, E. Bancel, E. Triboï, J. Ben Hamida, G. Branlard, Proteomic analysis of the effect of heat stress on hexaploid wheat grain: characterization of heat-responsive proteins from non-prolamins fraction, *Proteomics* 4 (2004) 505-513.
2. L. Kong, H. Guo, M. Sun, Signal transduction during wheat grain development, *Planta* 241 (2015) 789–801.
3. L. Gao, A. Wang, X. Li, K. Dong, K. Wang, R. Appels, W. Ma, Y. Yan, Wheat quality related differential expressions of albumins and globulins revealed by two-dimensional difference gel electrophoresis (2-D DIGE), *J. Proteomics* 73 (2009) 279–296.

4. Y. Ikeda, N. Koizumi, T. Kusano, H. Sano, Specific binding of a 14-3-3 protein to autophosphorylated WPK4, a SNF1-related wheat protein kinase, and to WPK4-phosphorylated nitrate reductase, *J. Biol. Chem.* 275 (2000) 31695–31700.
5. C. Finnie, J. Borch, D.B. Collinge, 14-3-3 Proteins: Eukaryotic regulatory proteins with many functions, *Plant Mol. Biol.* 40 (1999) 545–554.
6. K. Nakamura, A. Zuppin, S. Arnaudeau, J. Lynch, I. Ahsan, R. Krause, S. Papp, H. De Smedt, J.B. Parys, W. Müller-Esterl, D.P. Lew, K.H. Krause, N. Demaurex, M. Opas, M. Michalak, Functional specialization of calreticulin domains, *J. Cell Biol.* 154 (2001) 961–972.
7. M.-H. Chen, G.-W. Tian, Y. Gafni, V. Citovsky, Effects of calreticulin on viral cell-to-cell movement, *Plant Physiol.* 138 (2005) 1866–1876.
8. X.Y. Jia, C.Y. Xu, R.L. Jing, R.Z. Li, X.G. Mao, J.P. Wang, X.P. Chang, Molecular cloning and characterization of wheat calreticulin (CRT) gene involved in drought-stressed responses, *J. Exp. Bot.* 59 (2008) 739–751.
9. J.B. Heo, Y.B. Yi, J.D. Bahk, Rice GDP dissociation inhibitor 3 inhibits OsMAPK2 activity through physical interaction, *Biochem. Biophys. Res. Commun.* 414 (2011) 814–819.
10. I. Nadaud, C. Grousse, C. Debiton, C. Chambon, M.F. Bouzidi, P. Martre, G. Branlard, Proteomic and morphological analysis of early stages of wheat grain development, *Proteomics* 10 (2010) 2901–10.
11. S. Moisyadi, S. Dharmasiri, H.M. Harrington, T.J. Lukas, Characterization of a low-molecular-mass autophosphorylating protein in cultured sugarcane cells and its identification as a nucleoside diphosphate kinase, *Plant Physiol.* 104 (1994) 1401–1409.
12. H. Moon, B. Lee, G. Choi, D. Shin, D.T. Prasad, O. Lee, S.S. Kwak, D.H. Kim, J. Nam, J. Bahk, J.C. Hong, S.Y. Lee, M.J. Cho, C.O. Lim, D.J. Yun, NDP kinase 2 interacts with two oxidative

stress-activated MAPKs to regulate cellular redox state and enhances multiple stress tolerance in transgenic plants, *Proc. Natl. Acad. Sci. USA* 100 (2003) 358–363.

13. H.M. Niemeyer, Hydroxamic acids (4-hydroxy-1,4-benzoxazin-3-ones), defence chemicals in the gramineae, *Phytochemistry* 27 (1988) 3349–3358.
14. M. Sue, K. Yamazaki, S. Yajima, T. Nomura, T. Matsukawa, H. Iwamura, T. Miyamoto, Molecular and structural characterization of hexameric β -D-glucosidases in wheat and rye, *Plant Physiol.* 141 (2006) 1237–1247.
15. X. Zhang, C.J. Liu, Multifaceted regulations of gateway enzyme phenylalanine ammonia-lyase in the biosynthesis of phenylpropanoids, *Mol. Plant* 8 (2015) 17-27.

Supplementary Table S1. Morphological details of the samples taken at various grain developmental stages for proteomic analysis.

Sample	Day post-anthesis (DPA)	Phenotypic characteristics
T0	3	Seed increases three-fold in size. Rapid growth phase after fertilization, associated to rapid swelling of the tissues of the carpel, both the pericarp and the embryo sac; these tissues surround the fertilised embryo. The zygote has divided only once or twice.
T1	5	Kernel development; water-ripe stage. Embryo has still a globular structure with no clear cell differentiation. The nuclei of the endosperm continue to divide rapidly and corresponding cell wall grow near the inner nucellar epidermis.
T2	11	Kernel development; water-ripe stage. The densely cytoplasmic cellular endosperm that nourish the embryo at the bottom of the embryo sac starts to degenerate.
T3	16	Kernel full development; water-ripe stage. The meristematic cells of the endosperm continue to divide and compartments are starting to form within the endosperm. Typical aleurone cells are first visible. Densely cytoplasmic cellular endosperm, still present at eleven days after flowering, has been completely consumed by the embryo. Scutellum is clearly defined and the embryo now uses the endosperm starch reserves near the scutellum for its own development.
T4	21	Milk-ripe stage. Grains reach their maximum fresh weight. Cell division stopped in the endosperm and during this stage two types of starch grains, type 'A' and the smaller type 'B', are packed. The embryo has grown to half its final size. Protein and starch are accumulating in the endosperm at a maximum rate.
T5	30	Dough stage. Death of the endosperm cells in preparation for dry down. The embryo is fully developed. Scutellum, which is essentially a massive storage organ for the support of the embryo during germination, has started to adhere to the endosperm.

Supplementary Table S2. Experimental design of 2D-DIGE for the analysis of the albumin/globulin fraction at different developmental stages (T0, T1, T2, T3, T4 and T5). Scheme of labeling for protein extracts. Reference mixture was a pool of all biological replicates from all developmental stages. Biological replicate number is reported in parenthesis.

Gel	Cy2	Cy3	Cy5
1	Reference mixture	T0 (1)	T1 (1)
2	Reference mixture	T2 (1)	T3 (1)
3	Reference mixture	T1 (2)	T2 (2)
4	Reference mixture	T3 (2)	T0 (2)
5	Reference mixture	T5 (1)	T2 (3)
6	Reference mixture	T4 (2)	T5 (3)
7	Reference mixture	T1 (3)	T4 (3)
8	Reference mixture	T4 (1)	T3 (3)
9	Reference mixture	T0 (3)	T5 (2)

Supplementary Table S3. Differentially represented albumins/globulins in durum wheat grain during seed development as deduced by 2D-DIGE analysis of the corresponding extracts at T0, T1, T2, T3, T4 and T5 stages and corresponding nanoLC-ESI-LIT-MS/MS. See the experimental section for plant sampling, protein extraction and proteomic analysis. Proteins are reported according to their functional classification [29]. Spot number, UniProt code, organism, protein name, GI code of the corresponding rice counterpart, Uniprot code of the plant protein with the highest sequence similarity, corresponding sequence identity (%), Mascot score, number of amino acids (AA), theoretical Mr and pI values, number of peptides identified, number of unique peptides identified, sequence coverage (%), and emPAI values are listed, together the corresponding fold changes in the different stage (T1, T2, T3, T4 and T5) with respect to T0. T.u., *Triticum urartu*; T.a., *Triticum aestivum*; T.t., *Triticum turgidum*; A.s., *Aegilops speltoides*; T.d., *Triticum dicoccoides*; A.se., *Aegilops searsii*; A.u., *Aegilops umbellulata*; A.l., *Aegilops longissima*; T.p., *Triticum polonicum*; S.c. x T.t., *Secale cereale* x *Triticum turgidum*.

Spot	Uniprot Code	Species	Protein Name	GI Oryza	BLAST Code	Identity (%)	Mascot score	Mw _{th}	pI _{th}	AA	Σ Pept	Σ Pept Unique	Coverage (%)	emPAI	T1vsT0	T2vsT0	T3vsT0	T4vsT0	T5vsT0
Cell growth/division																			
1415	M8AUY3	T.u.	Lipoxygenase	115454785	Q42847	>90	580	85752	6.07	769	12	11	19	0.53					-3.01
1446	W5F9D7	T.a.	Lipoxygenase	115454785	Q42847	>90	457	96632	6.1	864	10	10	15	0.4					-5.63
1487	X2D2D5	T.a.	Lipoxygenase	115454785	Q42847	>90	523	96121	5.85	861	11	11	16	0.35			4.84	8.44	
1493	W5EBH5	T.a.	Lipoxygenase	115454785	Q42847	>90	545	90172	5.68	805	12	11	17	0.5				4.68	
1502	X2D2V6	T.a.	Lipoxygenase	115454785	Q42847	>90	813	96120	5.71	861	16	15	21	0.76			2.28	3.00	
1503	E5F115	T.t.	Lipoxygenase	115454785	Q42847	>90	607	96080	5.8	861	12	12	17	0.46			5.56	10.82	
1517	E5F115	T.t.	Lipoxygenase	115454785	Q42847	>90	776	96080	5.8	861	17	15	25	0.9			5.27	11.71	
1534	W5EBH5	T.a.	Lipoxygenase	115454785	Q42847	>90	646	90172	5.68	805	16	13	16	0.5				4.6	
1538	M8AUY3	T.u.	Lipoxygenase	115454785	Q42847	>90	377	85752	6.07	769	8	8	14	0.4				6.96	
1539	W5F9D7	T.a.	Lipoxygenase	115454785	Q42847	>90	258	96632	6.1	864	7	6	10	0.21				1.95	
1593	C6K7G4	T.a.	Lipoxygenase	115454785	Q42847	>90	207	96072	5.9	861	4	4	6	0.16				4.26	
1601	M8AUY3	T.u.	Lipoxygenase	115454785	Q42847	>90	330	85752	6.07	769	8	8	12	0.34		2.01	1.54	2.58	
1615	W5F9D7	T.a.	Lipoxygenase	115454785	Q42847	>90	815	96632	6.1	864	23	18	28	0.82		1.5		-2.27	-7.03
1629	W5F9D7	T.a.	Lipoxygenase	115454785	Q42847	>90	714	96632	6.1	864	18	14	21	0.69					-16.47
1639	W5F9D7	T.a.	Lipoxygenase	115454785	Q42847	>90	925	96632	6.1	864	31	20	28	0.97					-86.31
1640	W5F9D7	T.a.	Lipoxygenase	115454785	Q42847	>90	914	96632	6.1	864	29	20	29	1.37				-6.97	-59.27
1641	W5F9D7	T.a.	Lipoxygenase	115454785	Q42847	>90	809	96632	6.1	864	25	18	28	1.2					-69.06

1645	W5EB58	T.a.	Lipoxygenase	115454785	Q42847	>90	380	90178	5.82	805	8	8	11	0.33					-2.35
1649	W5F9D7	T.a.	Lipoxygenase	115454785	Q42847	>90	1104	96632	6.1	864	40	24	34	1.37					-37.01
1769	M8AU Y3	T.u.	Lipoxygenase	115454785	Q42847	>90	354	85752	6.07	769	12	8	13	0.53		2.97			
1796	W5F9D7	T.a.	Lipoxygenase	115454785	Q42847	>90	641	96632	6.1	864	15	13	20	0.57		2.5			
2391	M8AU Y3	T.u.	Lipoxygenase	115454785	Q42847	>90	558	85752	6.07	769	11	10	20	0.53				-2.03	
4392	W5ASQ5	T.a.	Probable aldo-keto reductase 2	115457788	Q7XT99	88.7	232	26400	6.66	241	4	4	19	0.71					3.36
4421	W5ASQ5	T.a.	Probable aldo-keto reductase 2	115457788	Q7XT99	88.7	275	26400	6.66	241	4	4	20	0.71		2.04			
4912	T1LG78	T.u.	Peptidyl-prolyl <i>cis-trans</i> isomerase	115461585	T1LG78	>90	233	27231	9.49	256	5	5	22	0.69			2.4		
4547	A0A0A7C089	T.a.	Translationally controlled tumor protein	115486523	Q9MSG3	>90	318	18851	4.55	168	6	6	38	1.53		2.14	5.83	15.38	8.57
Cell Structure																			
1866	W5BPK3	T.a.	Arabinoxylan arabinofuranohydrolase	22324432	H9NHF9	>90	417	73407	6.89	669	7	7	14	0.41				2.61	
2184	A0A096UM19	T.a.	Arabinoxylan arabinofuranohydrolase	22324432	H9NHF9	>90	275	73154	6.68	666	5	5	10	0.28	2.15				
2830	M7ZP69	T.u.	Putative pectinacetylsterase	115441565	Q5JLW7	66.1	215	47681	6.2	425	4	4	13	0.35				4.92	
3157	T1MVG6	T.u.	Alpha-1,4-glucan-protein synthase [UDP-forming]	115454033	Q8H8T0	>90	762	41988	5.64	368	24	15	51	5.03					10.36
1951	W5FDS3	T.a.	Glycosyl hydrolase family 3 N-terminal domain	115455349	I1GNA2	>90	523	67686	7.57	624	18	10	23	0.9				3.89	5.26
1958	W5FDS3	T.a.	Glycosyl hydrolase family 3 N-terminal domain	115455349	I1GNA2	>90	624	67686	7.57	624	20	12	25	1				2.74	3.51
1979	W5FDS3	T.a.	Glycosyl hydrolase family 3 N-terminal domain	115455349	I1GNA2	>90	425	67686	7.57	624	8	8	16	0.45				3.64	
1980	W5FDS3	T.a.	Glycosyl hydrolase family 3 N-terminal domain	115455349	I1GNA2	>90	372	67686	7.57	624	8	7	18	0.53				4.47	5.1
2000	W5FDS3	T.a.	Glycosyl hydrolase family 3 N-terminal domain	115455349	I1GNA2	>90	168	67686	7.57	624	3	3	7	0.17		6.22	2.9	10.2	9.07
1958	W5F8K3	T.a.	Beta-D-glucan exohydrolase isoenzyme ExoI	115455353	Q9XEI3	>90	423	67266	6.39	617	13	9	17	0.54				2.74	3.51
1914	W5ARE8	T.a.	Alpha-L-arabinofuranosidase/beta-D-xylosidase isoenzyme ARA-I	115460876	Q8W012	>90	397	83042	5.6	776	5	5	11	0.24				-3.45	
1991	W5ARE8	T.a.	Alpha-L-arabinofuranosidase/beta-D-xylosidase isoenzyme ARA-I	115460876	Q8W012	>90	887	83042	5.6	776	30	15	26	1.5			-3		-4.22
3161	W5F9P7	T.a.	NAD dependent epimerase/dehydratase family	115480267	F2DEC3	>90	243	40742	6.61	368	5	5	17	0.55			4.1	3.63	
4261	A4K4Y1	T.a.	Tubulin alpha-1 chain	115484939	Q22347	>90	117	50368	4.89	451	2	2	6	0.15					4.05
2037	M7ZGC8	T.u.	Putative e3 ubiquitin-protein ligase hu14	218191593	A0A0B0P4J1	60.8	382	56096	5.52	517	8	8	21	0.67					-2.69
2075	Q75RZ3	T.a.	Putative beta-xylosidase	218191593	M0XR X2	>90	382	62561	5.9	573	7	7	19	0.41				10.19	
Disease/defence																			
6628	A4ZIZ0	T.a.	Monomeric alpha-amylase inhibitor	218195	A4ZIZ0	>90	89	13617	5.35	121	2	2	17	0.66				34.44	48.1
6673	A4GFT4	A.s.	Dimeric alpha-amylase inhibitor	218195	P01085	>90	304	13948	5.71	124	5	5	40	1.69			10.24	46.2	98.3
5112	W5BKE1	T.a.	Stress responsive protein (fragment)	34394517	I6V2S3	72.5	113	34006	6.3	306	2	2	8	0.23					11.3
2035	Q0Q0I7	T.a.	Heat shock protein 90	39104468	Q0Q0I7	>90	920	80695	4.96	700	24	17	27	1.15			5.56		
2070	F4Y595	T.a.	Heat shock protein 90	39104468	Q0Q0I7	>90	637	80668	4.96	700	14	13	20	0.64	3.64		3.24		

2277	A0A024FR39	T.a.	Protein disulfide-isomerase 1-2	62546209	P80284	>90	1318	53988	5.1	485	33	23	55	5.5		5.13	9.82		6.85
2675	A0A024FR39	T.a.	Protein disulfide-isomerase 1-2	62546209	P80284	>90	514	53988	5.1	485	12	11	26	0.95				3.47	
4347	Q8LGN5	T.a.	Glutathione-S-transferase 19E50	115436602	Q8VWW3	>90	305	25015	5.99	219	15	6	24	1.03			3.04		
1742	W5GCC9	T.a.	Heat shock cognate 70 kDa protein	115443791	P09189	>90	581	73036	5.06	661	12	12	22	0.64				2.76	
2634	W5GCC9	T.a.	Heat shock cognate 70 kDa protein	115443791	P09189	>90	517	73036	5.06	661	9	9	17	0.41				2.38	
2721	W5GRB7	T.a.	ER molecular chaperone	115443791	R9W6A6	>90	395	65687	5.9	603	8	8	13	0.39				5.35	
2924	W5GRB7	T.a.	ER molecular chaperone	115443791	R9W6A6	>90	1228	65687	5.9	603	45	21	31	3.67			7.43	7.85	36.81
4188	W5DYW7	T.a.	Ascorbate peroxidase	115452337	O23983	>90	433	27579	5.85	250	7	6	36	0.91				2.8	
4391	W4ZXX1	T.a.	Dehydroascorbate reductase	115461741	Q84UH6	>90	357	33911	8.91	311	7	6	31	0.88				8.87	
4434	W4ZXX1	T.a.	Dehydroascorbate reductase	115461741	Q84UH6	>90	614	33911	8.91	311	15	11	44	2.93				3.96	
4447	C9EF64	T.a.	Dehydroascorbate reductase	115461741	Q84UH6	>90	601	23457	5.88	212	16	9	59	2.89				9.38	
4820	W4ZXX1	T.a.	Dehydroascorbate reductase	115461741	Q84UH6	>90	451	33911	8.91	311	7	7	33	1.09	2.34				
4924	W4ZXX1	T.a.	Dehydroascorbate reductase	115461741	Q84UH6	>90	435	33911	8.91	311	7	6	29	1.09				18.17	6.87
3662	D8L9B3	T.a.	Protein disulfide isomerase family protein 4-1	115462193	D8L9B3	>90	423	40578	6.17	367	8	7	28	1.03					9.44
3893	D8L9B3	T.a.	Protein disulfide isomerase family protein 4-1	115462193	D8L9B3	>90	495	40578	6.17	367	12	8	32	1.64				1.59	
4132	A4GFN8	T.d.	Dimeric alpha-amylase inhibitor	115471169	P01085	>90	492	13743	6.49	124	11	8	86	8.57				5.66	7.39
6023	C3VWG2	T.d.	Dimeric alpha-amylase inhibitor	115471169	P01085	>90	208	15558	5.28	141	4	4	42	1.45				49.3	53.8
6437	C3VW77	T.d.	Dimeric alpha-amylase inhibitor	115471169	P01085	>90	577	15578	4.99	141	20	9	70	10.72			8.45	32.53	62.73
6475	A4GFN8	T.d.	Dimeric alpha-amylase inhibitor	115471169	P01085	>90	227	13743	6.49	124	4	4	37	1.73				6.04	
6478	A4GFZ7	A.se.	Dimeric alpha-amylase inhibitor	115471169	P01085	>90	112	13963	5.23	124	2	2	31	0.64				8.61	14.98
6494	S4UMQ8	A.u.	Dimeric alpha-amylase inhibitor	115471169	P01085	>90	247	15553	5.26	139	4	4	35	0.96			16.88	49.98	68.38
6562	C3VW80	T.d.	Dimeric alpha-amylase inhibitor	115471169	P01085	>90	400	15588	5.28	141	6	6	60	2.83				11.85	11.72
6576	C3VW80	T.d.	Dimeric alpha-amylase inhibitor	115471169	P01085	>90	342	15588	5.28	141	7	5	52	2.06			11.83	49.18	78.16
6578	Q4U199	T.a.	Dimeric alpha-amylase inhibitor	115471169	P01085	>90	350	15558	5.3	141	6	4	42	2.06	1.76			15.1	16.61
6642	X2KYP9	T.a.	Monomeric alpha-amylase inhibitor	115471169	X2KYP9	>90	185	17187	7.45	151	6	4	27	1.25				28.74	75.11
6699	C3VW86	T.d.	Dimeric alpha-amylase inhibitor	115471169	P01085	>90	122	15919	8.04	139	2	2	12	0.55	-2.53				
6760	Q4U199	T.a.	Dimeric alpha-amylase inhibitor	115471169	P01085	>90	348	15558	5.3	141	5	5	50	2.06				11.62	
5925	W5EH57	T.a.	Alpha amylase inhibitor CM3	115471201	P17314	>90	182	18990	7.44	168	3	3	29	0.74					22.52
6023	Q6S5B1	T.t.	Alpha amylase inhibitor CM3	115471201	P17314	>90	294	18893	7.44	168	4	4	38	1.1				49.3	53.8
6115	W5EH57	T.a.	Alpha amylase inhibitor CM3	115471201	P17314	>90	179	18990	7.44	168	3	3	29	0.74				16.86	26.03
6124	Q6S5B1	T.t.	Alpha amylase inhibitor CM3	115471201	P17314	>90	173	18893	7.44	168	3	3	29	0.75				7.74	8.36
4336	M7ZJS5	T.u.	Glutathione transferase	115471993	Q8RW02	>90	194	25112	6.36	222	4	4	23	0.53				11.71	26.9

4102	W5AZ53	T.a.	L-ascorbate peroxidase 2, cytosolic	115474285	M8C1W9	>90	413	44540	6.45	406	11	9	29	1.24			2.05	2.52	2.94
4125	T1MEJ3	T.u.	L-ascorbate peroxidase 2, cytosolic	115474285	M8C1W9	>90	308	25953	5.16	238	7	6	32	1.6		2.56			
4261	T1M6Z9	T.u.	Luminal-binding protein	115475195	N1QT88	86.5	99	72976	5.2	665	2	2	4	0.1					4.05
1976	A0A096USB9	T.a.	Heat shock protein, HSP90/HTPG types	115477014	W5FTM6	>90	889	79657	4.81	695	17	15	31	1.17				2.95	
2826	M7ZDE9	T.u.	Monodehydroascorbate reductase	115477781	M8AQW3	>90	339	46657	5.18	435	7	6	16	0.59		3.78	3.44		
3499	W5FEC7	T.a.	Peroxidase	115479691	W5FEC7	>90	576	37898	5.7	343	11	10	34	1.83					-1.98
6663	Q9SQG4	T.a.	Wheatwin-1	115486089	O64392	>90	236	13430	6.28	120	4	3	48	1.16					17.03
5163	T1MHP1	T.u.	Pathogenesis-related protein Bet v I family	125548641	Q7XUY5	>90	479	17023	4.71	153	19	10	59	10.79					-14.32
5437	T1MHP1	T.u.	Pathogenesis-related protein Bet v I family	125548641	Q7XUY5	>90	645	17023	4.71	153	20	12	65	31.95					-12.92
3194	W5G6B5	T.a.	Peroxidase	222618480	M0V135	>90	421	36312	8.33	334	6	6	26	0.81			2.18		
3981	Q4W6G2	T.a.	Xylanase inhibitor XIP-III	297605789	Q4W6G2	>90	182	33554	7.14	305	4	4	13	0.53		2.88			
4028	Q4W6G2	T.a.	Xylanase inhibitor XIP-III	297605789	Q4W6G2	>90	508	33554	7.14	305	15	10	35	2.23				4.97	17.9
2106	A0A024FRN3	T.a.	Protein disulfide-isomerase 1-1	385717664	P80284	>90	1006	56618	5.03	512	40	20	34	3.92				134.71	52.43
2241	A0A024FRN3	T.a.	Protein disulfide-isomerase 1-1	385717664	P80284	>90	1283	56618	5.03	512	42	23	49	5.77		18.16	34.37		
2265	A0A024FRN3	T.a.	Protein disulfide-isomerase 1-1	385717664	P80284	>90	967	56618	5.03	512	21	18	36	2.15			6.59	8.02	
2757	B9A8E2	T.a.	Protein disulfide-isomerase 1-1 (fragment)	385717664	P80284	>90	699	56921	4.96	515	13	13	25	1.14				2.58	
2816	B9A8E2	T.a.	Protein disulfide-isomerase 1-1 (fragment)	385717664	P80284	>90	868	56921	4.96	515	17	16	33	1.94				-2.16	
2826	P52589	T.a.	Protein disulfide-isomerase 1-2 (fragment)	385717664	P80284	>90	682	56726	4.99	515	16	14	31	1.77		3.78	3.44		
2873	B9A8E2	T.a.	Protein disulfide-isomerase 1-1 (fragment)	385717664	P80284	>90	761	56921	4.96	515	14	14	26	1.28				44.08	
3516	W5H7T0	T.a.	Glyoxalase/Bleomycin resistance protein/Dioxygenase superfamily	937929263	I110X2	>90	699	33053	5.34	292	36	13	47	5.98				1.92	
4851	P16347	T.a.	Endogenous alpha-amylase/subtilisin inhibitor	1002260354	P07596	>90	213	19849	6.77	180	4	4	28	1.03	2.45	2.75			4.20
5011	P16347	T.a.	Endogenous alpha-amylase/subtilisin inhibitor	1002260354	P07596	>90	616	19849	6.77	180	33	12	79	33.4		3.5	5.58	12.98	26.97
3396	W5B4W2	T.a.	Stress responsive protein	1002283529	I6V2S3	71.4	485	43300	7.62	382	11	10	34	0.94				3.13	
3412	W5B4W2	T.a.	Stress responsive protein	1002283529	I6V2S3	71.4	502	43300	7.62	382	12	9	30	1.29		2.62		5.64	
4990	W5B4W2	T.a.	Stress responsive protein (fragment)	1002283529	I6V2S3	71.4	312	43300	7.62	382	8	5	18	0.65		2.63	3.44	5.41	14.71
Energy																			
2594	A0A075VYM8	A.l.	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	11466795	A1E9T2	>90	368	53344	6.04	476	7	7	15	0.5		2.69	8.76	33.2	
2614	A0A075VYM8	A.l.	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	11466795	A1E9T2	>90	704	53344	6.04	476	20	12	28	2.37			4.67	15.65	
2692	A0A075VYM8	A.l.	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	11466795	A1E9T2	>90	304	53344	6.04	476	6	6	15	0.5			2.96	2.3	
2420	A0A075VUZ5	T.u.	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	57283874	P11383	>90	378	53445	6.22	477	8	8	17	0.6				5.29	7.3
2528	A0A075VUZ5	T.u.	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	57283874	P11383	>90	824	53445	6.22	477	43	16	30	3.72			7.82		
2567	A0A075VUZ5	T.u.	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	57283874	P11383	>90	503	53445	6.22	477	18	9	21	1.25			8.81		

2597	A0A075VUZ5	T.u.	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	57283874	P11383	>90	282	53445	6.22	477	6	6	15	0.31			4.81	3.3	
2610	A0A075VUZ5	T.u.	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	57283874	P11383	>90	412	53445	6.22	477	10	9	19	0.84			3.25		4.19
2672	A0A075VUZ5	T.u.	Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	57283874	P11383	>90	477	53445	6.22	477	10	9	18	0.84				6.17	
3165	W5D5L4	T.a.	Fructose-bisphosphate aldolase	78099751	F2CXT7	>90	848	39185	6.85	358	20	15	55	3.32					88.01
3193	W5D5L4	T.a.	Fructose-bisphosphate aldolase	78099751	F2CXT7	>90	886	39185	6.85	358	29	17	55	5.83				34.35	
3194	W5CCA9	T.a.	Fructose-bisphosphate aldolase	78099751	F2CXT7	>90	259	39179	6.09	357	5	5	20	0.32			2.18		
3197	W5D5L4	T.a.	Fructose-bisphosphate aldolase	78099751	F2CXT7	>90	453	39185	6.85	358	7	7	21	0.9				16.80	23.08
3283	W5CCA9	T.a.	Fructose-bisphosphate aldolase	78099751	F2CXT7	>90	401	39179	6.09	357	8	7	31	1.08			2.96	8.77	11.64
3290	W5CCA9	T.a.	Fructose-bisphosphate aldolase	78099751	F2CXT7	>90	235	39179	6.09	357	4	4	18	0.44			2.68	3.55	
3308	W5D5L4	T.a.	Fructose-bisphosphate aldolase	78099751	F2CXT7	>90	234	39185	6.85	358	5	4	16	0.58					1.67
3354	W5CCA9	T.a.	Fructose-bisphosphate aldolase	78099751	F2CXT7	>90	491	39179	6.09	357	9	9	37	1.28				3.8	
3635	W5CCA9	T.a.	Fructose-bisphosphate aldolase	78099751	F2CXT7	>90	462	39179	6.09	357	10	8	29	1.08				1.81	
3723	W5CHQ6	T.a.	Fructose-bisphosphate aldolase	78099751	F2CXT7	>90	359	39507	5.77	362	6	6	26	0.57				-2.86	
3819	W5D5L4	T.a.	Fructose-bisphosphate aldolase	78099751	F2CXT7	>90	291	39185	6.85	358	9	6	26	0.58				-4.7	
3961	W5D5L4	T.a.	Fructose-bisphosphate aldolase	78099751	F2CXT7	>90	441	39185	6.85	358	11	9	31	1.08			-6.12		
3976	W5D5L4	T.a.	Fructose-bisphosphate aldolase	78099751	F2CXT7	>90	420	39185	6.85	358	14	8	33	1.28			-2.95	-4.11	-13.53
3999	W5CCA9	T.a.	Fructose-bisphosphate aldolase	78099751	F2CXT7	>90	271	39179	6.09	357	5	4	15	0.44					-5.53
2691	W5FGH0	T.a.	UTP-glucose-1-phosphate uridylyltransferase	88866516	Q43772	>90	484	51819	5.2	473	9	8	24	0.74				5.15	
2484	W5FPN2	T.a.	Enolase	90110845	Q42971	>90	1506	48456	5.49	446	34	23	68	4.93				7.5	
2485	W5FPN2	T.a.	Enolase	90110845	Q42971	>90	1276	48456	5.49	446	28	20	57	4.51				2.98	
2491	W5FPN2	T.a.	Enolase	90110845	Q42971	>90	1224	48456	5.49	446	29	19	58	4.51				4.16	
2593	W5FPN2	T.a.	Enolase	90110845	Q42971	>90	709	48456	5.49	446	10	10	35	0.95				1.94	
2827	W5F3P8	T.a.	Enolase	110288667	Q42971	>90	1277	47093	5.36	434	35	20	66	5.26		1.87			
3119	W5F3P8	T.a.	Enolase (fragment)	110288667	Q42971	>90	735	47093	5.36	434	12	11	31	1.32				3.26	
3217	W5F3P8	T.a.	Enolase (fragment)	110288667	Q42971	>90	799	47093	5.36	434	20	13	36	2.67					12.58
4207	W5HZR7	T.a.	ATP-citrate synthase beta chain protein 2	115436118	Q9FGX1	>90	395	66429	6.87	608	6	6	14	0.39					2.20
2892	M7YI34	T.u.	Isocitrate dehydrogenase [NADP]	115438939	A5BX54	>90	1069	47717	5.99	422	29	18	46	3.52				2.92	
4391	W5CC40	T.a.	Isocitrate dehydrogenase	115438939	R7WAR0	>90	265	35510	7.05	317	4	4	16	0.5				8.87	
4392	W5CC40	T.a.	Isocitrate dehydrogenase	115438939	R7WAR0	>90	206	35510	7.05	317	4	4	16	0.5					3.36
2075	A0A077S3A3	T.a.	Malic enzyme	115439879	J3L3Y9	>90	357	64313	6.14	582	7	7	16	0.4				10.19	
3961	M8ADU2	T.u.	Gamma hydroxybutyrate dehydrogenase-like protein	115446749	Q84VC8	81.1	340	27649	7.68	259	7	6	29	1.46			-6.12		
3011	M8B120	T.u.	Succinyl-CoA ligase subunit beta	115447367	Q6K9N6	>90	762	45498	6.5	422	19	14	39	2.27			-3.34		

3015	M8B120	T.u.	Succinyl-CoA ligase subunit beta	115447367	Q6K9N6	>90	500	45498	6.5	422	10	10	27	0.74			-3.38		
1324	M7YMT4	T.u.	Aconitate hydratase	115450595	M7YMT4	>90	452	93850	6.04	860	7	7	10	0.31				3.41	
1331	W5E8H4	T.a.	Aconitate hydratase	115450595	Q1KSB0	>90	359	86830	6.08	796	7	7	12	0.28				2.36	
2065	W5EAJ4	T.a.	Phosphoglucomutase	115454931	Q9SNX2	>90	628	70213	7.21	648	15	13	24	0.67					16.29
3284	W5G5H3	T.a.	Glyceraldehyde-3-phosphate dehydrogenase	115459078	Q7FAH2	>90	478	36799	7.05	337	10	9	39	1.4				17.22	
2376	Q1PBI3	T.a.	Glucose-6-phosphate isomerase	115467434	K4A7S6	>90	407	62389	6.98	567	7	7	19	0.34					45.39
2497	W5GHD7	T.a.	Phosphoglycerate kinase	115469436	P12783	>90	171	43284	6.21	409	2	2	7	0.18					6.32
2989	P12783	T.a.	Phosphoglycerate kinase, cytosolic	115469436	P12783	>90	644	42153	5.64	401	18	13	39	1.78					18.23
3090	W5H4V7	T.a.	Phosphoglycerate kinase	115469436	P12783	>90	581	42154	5.52	401	13	10	36	1.78				4.73	
3114	W5GHD7	T.a.	Phosphoglycerate kinase	115469436	P12783	>90	1313	43284	6.21	409	75	20	66	9.21					4.23
3138	P12783	T.a.	Phosphoglycerate kinase	115469436	P12783	>90	1221	42153	5.64	401	53	24	61	11.85					-5.51
3157	P12783	T.a.	Phosphoglycerate kinase	115469436	P12783	>90	653	42153	5.64	401	12	12	48	1.78					10.36
3193	W5HB91	T.a.	Glyceraldehyde-3-phosphate dehydrogenase 1	115474601	D9YMC4	>90	628	38561	7.09	353	15	12	47	1.54				34.35	
3197	W5HB91	T.a.	Glyceraldehyde-3-phosphate dehydrogenase 1	115474601	D9YMC4	>90	300	38561	7.09	353	9	7	18	0.45				16.80	23.08
2388	A0A0F6QT98	T.a.	Aldehyde dehydrogenase 7B1	115479375	I1IQG6	>90	460	55092	6.12	509	10	9	29	0.8				1.95	
3275	A3KLL4	T.a.	Malate dehydrogenase	115482534	P57106	>90	620	35805	5.75	333	13	10	34	2.66			6.69	24.47	27.25
3297	A3KLL4	T.a.	Malate dehydrogenase	115482534	P57106	>90	837	35805	5.75	333	24	13	43	5.03				6.61	9.16
3346	A3KLL4	T.a.	Malate dehydrogenase	115482534	P57106	>90	1042	35805	5.75	333	71	16	49	12.39				5.70	
3374	A3KLL4	T.a.	Malate dehydrogenase	115482534	P57106	>90	906	35805	5.75	333	24	13	47	5.66				17.9	11.81
3424	A3KLL4	T.a.	Malate dehydrogenase	115482534	P57106	>90	561	35805	5.75	333	11	10	33	1.71				3.4	
4260	T1NAD0	T.u.	Malate dehydrogenase	115482534	P57106	>90	557	34279	5.6	318	17	9	33	2.15			2.01	8.56	21.55
4459	T1NAD0	T.u.	Malate dehydrogenase	115482534	P57106	>90	436	34279	5.6	318	8	8	31	1.07				13.07	19.47
4638	T1NAD0	T.u.	Malate dehydrogenase	115482534	P57106	>90	159	34279	5.6	318	3	3	14	0.37				6.49	11.52
2464	W5FCZ9	T.a.	Pyruvate kinase	115484175	W5FCZ9	>90	267	42774	5.31	390	5	5	20	0.52				5.11	32.41
2265	M7Z848	T.u.	RuBisCO large subunit-binding protein subunit alpha, chloroplastic	115488160	P08823	>90	1106	65626	5.17	613	28	17	33	1.85			6.59	8.02	
1282	A0A077S2R7	T.a.	Glycine decarboxylase P subunit	125527457	C5YS41	>90	289	1,00E+05	6.42	1031	5	5	6	0.18				7.41	
1331	A0A077S2R7	T.a.	Glycine decarboxylase P subunit	125527457	C5YS41	>90	272	1,00E+05	6.42	1031	6	5	6	0.22				2.36	
2277	W5FKY4	T.a.	Uncharacterized protein	125546535	W5FKY4	>90	713	61779	5.3	588	11	11	25	0.9		5.13	9.82		6.85
3283	A0A096UTL2	T.a.	Glyceraldehyde-3-phosphate dehydrogenase 3	218191114	Q7FAH2	>90	384	36840	6.4	337	8	7	26	0.79			2.96	8.77	11.64
3289	A0A096UTL2	T.a.	Glyceraldehyde-3-phosphate dehydrogenase 3	218191114	Q7FAH2	>90	609	36840	6.4	337	17	13	54	1.91				10.1	
2548	M8A8Q5	T.u.	Dihydrolipoyl dehydrogenase	218196132	W4ZZ26	>90	654	56512	6.07	525	12	10	25	1.15				-1.79	
4188	W5CMD2	T.a.	Triosephosphate isomerase	306415973	P46226	>90	472	26986	5.57	253	12	8	34	2.27				2.8	

4277	W5CMD2	T.a.	Triosephosphate isomerase	306415973	P46226	>90	744	26986	5.57	253	62	11	50	12.93				3.74	
6162	W5EZU1	T.a.	Ribulose bisphosphate carboxylase small chain	383875287	P26667	>90	421	16088	5.4	136	11	9	57	7.7			7.25	4.12	
2372	M7YNG5	T.u.	Glucose-6-phosphate isomerase	937922182	K4A7S6	>90	436	62467	6.96	567	9	8	19	0.59				5.21	29.03
Metabolism																			
3011	W5I480	T.a.	Alpha-amylase	169769	P00693	>90	464	49075	5.41	446	12	9	27	0.94			-3.34		
3015	W5I480	T.a.	Alpha-amylase	169769	P00693	>90	623	49075	5.41	446	15	10	30	1.08			-3.38		
4020	W5F0A0	T.a.	Beta-amylase	169777	M8B5G5	>90	191	34253	5.94	304	4	4	17	0.52			1.91		6.44
3194	M7YLN2	T.u.	Aspartate aminotransferase	584706	M7YLN2	>90	508	48454	7.23	439	8	8	24	0.68			2.18		
3197	M7YLN2	T.u.	Aspartate aminotransferase	584706	M7YLN2	>90	444	48454	7.23	439	7	7	20	0.68				16.80	23.08
2594	M7YGL9	T.u.	Alanine aminotransferase 2	14018051	M8BFC4	>90	793	57751	6.77	519	21	13	29	1.72		2.69	8.76	33.2	
2597	M7YGL9	T.u.	Alanine aminotransferase 2	14018051	M8BFC4	>90	302	57751	6.77	519	6	6	16	0.46			4.81	3.3	
2614	M7YGL9	T.u.	Alanine aminotransferase 2	14018051	M8BFC4	>90	988	57751	6.77	519	30	19	42	2.49			4.67	15.65	
2660	M7YGL9	T.u.	Alanine aminotransferase 2	14018051	M8BFC4	>90	725	57751	6.77	519	16	13	30	1.55				2.4	
2680	M7YGL9	T.u.	Alanine aminotransferase 2	14018051	M8BFC4	>90	719	57751	6.77	519	19	14	26	1.89			11.55	14.71	
2687	M7YGL9	T.u.	Alanine aminotransferase 2	14018051	M8BFC4	>90	636	57751	6.77	519	14	12	28	0.99			28.44	46.55	42.39
2697	M7YGL9	T.u.	Alanine aminotransferase 2	14018051	M8BFC4	>90	748	57751	6.77	519	16	15	31	1.4				8.11	
2485	W5B7Z6	T.a.	Adenosylhomocysteinase	29367605	O23255	>90	828	53897	5.56	485	23	16	35	2.11				2.98	
2627	W5B7Z6	T.a.	Adenosylhomocysteinase	29367605	O23255	>90	390	53897	5.56	485	9	9	18	0.82		1.87			3.54
2887	W5B7Z6	T.a.	Adenosylhomocysteinase	29367605	O23255	>90	439	53897	5.56	485	9	9	17	0.6					21.98
3662	M7ZC83	T.u.	Adenosylhomocysteinase	29367605	O23255	81.9	415	83796	6.45	752	8	8	11	0.35					9.44
4547	W5AXY3	T.a.	Adenosylhomocysteinase	29367605	N1QRC7	>90	204	26551	4.95	239	4	4	24	0.71		2.14	5.83	15.38	8.57
2490	D0EKB4	T.u.	Beta-amylase	34395244	M7YQB5	>90	133	25204	5.35	221	2	2	13	0.32					-3.4
3460	W5DW89	T.a.	Erwinia chrysanthemi IndA protein homolog-like	42407673	Q6ZK88	87.8	201	35543	8.77	335	4	4	14	0.5		-2.28			
3046	W5ARG6	T.a.	Glutamate dehydrogenase	81686712	C5YD28	>90	447	44861	6.2	411	15	9	26	1.06				3.43	
1549	W5CN49	T.a.	Phospholipase D	115434790	W5D4Q6	>90	830	92605	5.24	812	21	17	25	0.95				-3.68	
1550	T1LXL0	T.u.	Phospholipase D	115434790	W5D4Q6	>90	928	92719	4.96	812	21	17	31	1.1			-2.27	-4.55	-8.91
3110	A0A077S3V2	T.a.	Aspartate aminotransferase	115440075	I1HRT4	>90	964	49658	8.29	454	24	16	43	2.19				12.56	
3121	A0A077S3V2	T.a.	Aspartate aminotransferase	115440075	I1HRT4	>90	724	49658	8.29	454	17	13	34	1.57				12.61	
3143	B0FRH4	T.a.	Aspartate aminotransferase	115440075	I1HRT4	>90	667	42060	6.77	380	19	12	39	1.35				137.29	77.82
3161	A0A077S3V2	T.a.	Aspartate aminotransferase	115440075	I1HRT4	>90	313	49658	8.29	454	5	5	14	0.44			4.1	3.63	
4743	A0A077S3V2	T.a.	Aspartate aminotransferase (fragment)	115440075	I1HRT4	>90	132	49658	8.29	454	2	2	5	0.16					4.86
4627	W5GIL9	T.a.	Glycolipid transfer protein 1	115447377	O22797	74.3	311	22899	6.21	202	8	6	26	1.53		2.05		3.04	

2713	W5DZN4	T.a.	Alanine-glyoxylate aminotransferase	115451029	F2D6C1	>90	843	52146	8.02	479	14	13	32	1.63				10.75	
3345	W5DZW0	T.a.	Carbon-nitrogen hydrolase	115451067	I1H946	>90	689	37898	8.18	348	15	11	39	2.11				21.2	20.24
3383	W5DZW0	T.a.	Carbon-nitrogen hydrolase	115451067	I1H946	>90	483	37898	8.18	348	9	7	26	0.94				4.76	3.68
3185	G1FFN0	S.c. x T.t.	Glutamine synthetase	115451647	I1H7X9	>90	578	38990	5.35	354	14	10	43	2.02					-9.54
2484	W5EHW4	T.a.	Argininosuccinate lyase	115452565	M8CD44	>90	543	57741	5.96	516	8	8	21	0.65				7.5	
2485	W5DW50	T.a.	Argininosuccinate lyase	115452565	M8CD44	>90	753	57858	6.07	516	14	12	28	1.11				2.98	
2528	M7YRJ6	T.u.	Glucose-1-phosphate adenyltransferase	115455167	I1GNL6	>90	748	50969	6.22	463	11	11	34	1.18			7.82		
2610	W5F9H2	T.a.	Glucose-1-phosphate adenyltransferase	115455167	I1GNL6	>90	258	44162	5.88	396	5	5	14	0.28			3.25		4.19
4519	W4ZPF0	T.a.	Flavoprotein wrbA	115464739	M8C0I8	>90	338	30760	8.73	287	6	6	26	1				4.23	
2324	W4ZMF7	T.a.	Ketol-acid reductoisomerase, chloroplastic	115465569	M8BE45	>90	614	42212	5.67	381	10	10	21	1.34				5.14	
1534	W5I774	T.a.	Sucrose synthase 2	115466896	P31922	>90	547	92740	5.72	808	13	12	16	0.54				4.60	
3090	W5HB66	T.a.	3-hydroxyisobutyryl-CoA hydrolase-like protein 3	115468758	Q9T0K7	67.5	782	37899	5.25	348	14	14	43	2.11				4.73	
3531	A0A077RY90	T.a.	Thiazole biosynthetic enzyme 1-2, chloroplastic	115472485	M8D2G6	78.7	286	36513	6	338	5	5	19	0.63				-1.92	
1293	A0A077HBK6	T.p.	Sucrose synthase 3	115473359	P31923	>90	320	93120	6.03	816	7	7	9	0.22				10.72	
1487	W5B5R3	T.a.	Sucrose synthase 3	115473359	P31923	>90	597	93055	6.06	816	15	15	21	0.54			4.84	8.44	
1493	Q43223	T.a.	Sucrose synthase 3	115473359	P31923	>90	309	93062	6.17	815	7	7	10	0.12				4.68	
1502	Q43223	T.a.	Sucrose synthase 3	115473359	P31923	>90	483	93062	6.17	815	10	10	17	0.31			2.28	3	
1503	W5B5R3	T.a.	Sucrose synthase 3	115473359	P31923	>90	853	93055	6.06	816	18	17	24	0.87			5.56	10.82	
1517	M8A0T7	T.u.	Sucrose synthase 3	115473359	P31923	>90	1325	93127	6.01	816	33	27	37	1.98			5.27	11.71	
1538	A0A077HCY7	T.p.	Sucrose synthase 3	115473359	P31923	>90	705	93169	6.06	816	17	16	23	0.87				6.96	
1539	A0A077HBK6	T.p.	Sucrose synthase 3	115473359	P31923	>90	331	93120	6.03	816	8	6	9	0.26				1.95	
1593	A0A077HCY7	T.p.	Sucrose synthase 3	115473359	P31923	>90	398	93169	6.06	816	10	9	13	0.42				4.26	
1629	A0A077HCY7	T.p.	Sucrose synthase 3	115473359	P31923	>90	496	93169	6.06	816	11	10	15	0.42					-16.47
1896	A0A077HBK6	T.p.	Sucrose synthase 3	115473359	P31923	>90	388	93120	6.03	816	9	8	11	0.26			4.83		
1943	M8A0T7	T.u.	Sucrose synthase 3	115473359	P31923	>90	469	93127	6.01	816	12	11	17	0.48			4.98	6.6	
1944	A0A077HBK6	T.p.	Sucrose synthase 3	115473359	P31923	>90	606	93120	6.03	816	14	13	17	0.6			7.45	9.8	
1957	A0A077HCY7	T.p.	Sucrose synthase 3	115473359	P31923	>90	430	93169	6.06	816	8	8	13	0.26			3	4.89	
1962	W5B5R3	T.a.	Sucrose synthase 3	115473359	P31923	>90	583	93055	6.06	816	13	12	20	0.6				2.31	
1979	A0A077HBK6	T.p.	Sucrose synthase 3	115473359	P31923	>90	360	93120	6.03	816	8	8	10	0.22				3.64	
2060	A0A077HBK6	T.p.	Sucrose synthase 3	115473359	P31923	>90	518	93120	6.03	816	12	11	18	0.48			2.92		
2588	T1MV14	T.u.	Phospho-2-dehydro-3-deoxyheptonate aldolase 2	115473431	Q75W16	>90	228	51817	6.44	460	4	4	9	0.23				9.79	
1881	W5FUD0	T.a.	Formate-tetrahydrofolate ligase	115479433	R7W2G4	>90	487	70372	6.42	655	8	8	15	0.43					1.91

1896	W5FUD0	T.a.	Formate-tetrahydrofolate ligase	115479433	R7W2G4	>90	485	70372	6.42	655	9	7	14	0.59			4.83		
1943	W5FUD0	T.a.	Formate-tetrahydrofolate ligase	115479433	R7W2G4	>90	600	70372	6.42	655	13	10	18	0.85			4.98	6.6	
1944	W5EU71	T.a.	Formate-tetrahydrofolate ligase	115479433	R7W2G4	>90	464	54226	6.51	502	8	8	18	0.7			7.45	9.8	
1962	W5FUD0	T.a.	Formate-tetrahydrofolate ligase	115479433	R7W2G4	>90	627	70372	6.42	655	10	9	18	0.67				2.31	
6392	W4ZQ38	T.a.	Nucleoside diphosphate kinase	115483468	W4ZQ38	>90	223	26064	10.03	233	4	4	17	0.72				-1.99	
2636	W4ZT78	T.a.	Argininosuccinate synthase	115487944	M8C9Y0	>90	715	52387	6.78	475	19	16	31	1.28	3.28	2.81		7.85	8.81
1645	W5G1K3	T.a.	Cobalamin-independent methionine synthase	115489654	B6UF55	>90	689	84841	5.74	765	11	10	21	0.53					-2.35
3276	W5BGJ5	T.a.	Hexose-6-phosphate mutarotase	116310845	I1J2U2	>90	631	37261	5.39	334	11	9	37	1.37					3.76
3837	M8A7I9	T.u.	Chitinase 2	125531926	M8BM62	>90	430	24930	4.95	225	16	7	39	3.76			-1.82		3.13
2410	M7Z9T4	T.u.	Serine hydroxymethyltransferase	125555254	A0A096QEP1	>90	543	52065	6.62	471	10	9	22	1				3.26	
2415	M7Z9T4	T.u.	Serine hydroxymethyltransferase	125555254	A0A096QEP1	>90	268	52065	6.62	471	5	5	10	0.41			4.54	6.82	15.64
2338	W5A1L1	T.a.	NAD-dependent aldehyde dehydrogenase	218197167	Q8W0Q2	>90	617	61492	7.21	551	19	13	32	1.15				-2.68	
2982	W5IA32	T.a.	Formate dehydrogenase 1	218198209	Q9SXP2	>90	426	41639	6.51	377	10	10	23	0.68			8.39	27.1	
2983	W5IA32	T.a.	Formate dehydrogenase 1	218198209	Q9SXP2	>90	548	41639	6.51	377	12	11	29	1.58				5.02	
3080	W5IA32	T.a.	Formate dehydrogenase 1	218198209	Q9SXP2	>90	451	41639	6.51	377	10	9	22	0.54				2.89	
3085	W5IA32	T.a.	Formate dehydrogenase 1	218198209	Q9SXP2	>90	772	41639	6.51	377	26	16	36	3.33				7.93	
3086	W5IA32	T.a.	Formate dehydrogenase 1	218198209	Q9SXP2	>90	500	41639	6.51	377	20	10	26	1.17				34.23	
3123	W5IA32	T.a.	Formate dehydrogenase 1	218198209	Q9SXP2	>90	494	41639	6.51	377	11	10	24	1.17					14.3
2672	W5FPJ6	T.a.	Sucrose-phosphatase	222622151	Q84ZX7	>90	374	57202	8.23	510	10	8	16	0.56				6.17	
3460	T1MQQ6	T.u.	UCW116, putative lipase	222634989	A1C0L3	>90	323	37290	6.9	343	6	5	20	0.62		-2.28			
3345	A0A077S6B3	T.a.	Fructokinase-1	297598102	Q0JGZ6	89.8	826	34890	5.8	323	24	15	56	4.72				21.2	20.24
3147	M7ZZM5	T.u.	pfkB family carbohydrate kinase	297599626	I1IBR3	>90	408	37680	5.01	344	6	6	24	0.77					-3.65
2420	Q0PG36	T.a.	Glucose-1-phosphate adenyllyltransferase	297604962	P12299	>90	518	58399	6.12	522	11	10	26	0.97				5.29	7.3
2262	W5EKI0	T.a.	Beta-amylase	297725759	M8B5G5	>90	656	61360	5	550	25	13	33	1.72				3.21	
2279	W5EKI0	T.a.	Beta-amylase	297725759	M8B5G5	>90	852	61360	5	550	28	15	33	1.56				8.41	15.67
2315	M8B5G5	T.u.	Beta-amylase	297725759	M8B5G5	>90	772	58995	5.34	525	29	14	36	2.01				42.31	
2329	M8B5G5	T.u.	Beta-amylase	297725759	M8B5G5	>90	623	58995	5.34	525	15	11	29	1.22		9.84	16.74	43.55	54.79
2346	M8B5G5	T.u.	Beta-amylase	297725759	M8B5G5	>90	997	58995	5.34	525	31	18	45	2.01			36.9	123.95	
2363	M8B5G5	T.u.	Beta-amylase	297725759	M8B5G5	>90	927	58995	5.34	525	30	16	40	2.4				3.91	35.99
2887	W5EKI0	T.a.	Beta-amylase	297725759	M8B5G5	>90	497	61360	5	550	11	9	20	0.51					21.98
2646	O24396	T.a.	Adenylosuccinate synthetase	308154377	I1H951	>90	595	51399	5.93	476	19	11	28	1.16					-4.14
3235	W4ZTW1	T.a.	Alpha-galactosidase	937936537	M0UZ48	>90	540	41769	6.07	373	9	9	32	1.17					-3.62

4421	W5CWR9	T.a.	Auxin-induced protein	1002225585	M8BD63	89.4	201	38029	6.38	341	4	4	16	0.46		2.04			
Protein destination and storage																			
4336	Q0Q5E3	T.a.	Globulin 1	169805	Q8LKV8	>90	339	25553	8.72	229	8	5	38	1.64				11.71	26.9
2880	M7ZK46	T.u.	Triticin	31455453	Q08837	>90	536	64230	6.62	567	21	9	22	0.75		3.6			76.35
1881	W5ECA4	T.a.	Globulin 3	41469581	B7U6L4	87.7	764	69750	6.59	616	19	14	27	1.29					1.91
1980	W5EAP7	T.a.	Globulin 3	41469581	B7U6L4	89.2	362	69207	7.17	611	10	8	17	0.37				4.47	5.1
2000	B7U6L4	T.a.	Globulin 3	41469581	I6QQ39	>90	245	66652	7.78	588	5	5	11	0.18		2.91	2.9	10.2	9.07
2464	W5ECA4	T.a.	Globulin 3	41469581	B7U6L4	87.7	401	69750	6.59	616	9	8	19	0.37				5.11	32.41
2517	W5ECA4	T.a.	Globulin 3 (fragment)	41469581	B7U6L4	87.7	603	69750	6.59	616	15	13	23	0.86					12.81
2707	B7U6L4	T.a.	Globulin 3 (fragment)	41469581	I6QQ39	>90	390	66652	7.78	588	8	8	17	0.38				9.84	
3672	I6QQ39	T.a.	Globulin 3 (fragment)	41469581	B7U6L4	>90	267	66627	8.48	588	5	5	12	0.24		3.25			
5480	I6QQ39	T.a.	Globulin 3 (fragment)	41469581	B7U6L4	>90	239	66627	8.48	588	5	4	8	0.24				3.82	
5932	W5ECA4	T.a.	Globulin 3 (fragment)	41469581	B7U6L4	87.7	210	69750	6.59	616	5	5	14	0.23		1.88	4.54	5.62	
1403	M7YQA5	T.u.	Aminopeptidase N	42408435	M8CXX4	>90	788	100785	5.71	893	19	17	22	0.72					-2.41
1531	M7YQA5	T.u.	Aminopeptidase N	42408435	M8CXX4	>90	335	100785	5.71	893	7	6	7	0.24				3.34	2.65
2262	M7ZYF8	T.u.	Leucine aminopeptidase 2	75261364	Q6K669	>90	975	55804	5.57	533	15	15	41	1.64				3.21	
2363	M7ZYF8	T.u.	Leucine aminopeptidase 2	75261364	Q6K669	>90	1006	55804	5.57	533	17	15	44	1.81				3.91	35.99
2405	Q41593	T.a.	Serpin-Z1A	75294978	P93693	>90	511	43262	5.6	398	14	9	41	0.94				4.78	
2469	P93692	T.a.	Serpin-Z2B	75294978	P93692	>90	395	43341	5.46	398	15	8	23	0.65					14.09
2513	P93692	T.a.	Serpin-Z2B	75294978	P93692	>90	269	43341	5.46	398	7	6	17	0.39			5.69	3.58	
2827	P93692	T.a.	Serpin-Z2B	75294978	P93692	>90	503	43341	5.46	398	10	8	30	1.11		1.87			
2872	P93692	T.a.	Serpin-Z2B	75294978	P93692	>90	691	43341	5.46	398	49	12	37	3.1		2.7	2.4	2.27	
3090	Q41593	T.a.	Serpin-Z1A	75294978	P93693	>90	389	43262	5.6	398	8	8	25	0.79				4.73	
3157	Q41593	T.a.	Serpin-Z1A	75294978	P93693	>90	1201	43262	5.6	398	63	18	61	12.09					10.36
3157	M7Z1Z4	T.u.	Serpin-Z2B	75294978	P93692	>90	628	45226	6.03	415	16	9	25	1.05					10.36
3217	M7ZQF1	T.u.	Serpin-Z1B	75294978	P93693	>90	718	42956	5.62	398	25	12	39	2.81					12.58
3275	M7ZQF1	T.u.	Serpin-Z1B	75294978	P93693	>90	759	42956	5.62	398	42	12	34	3.9			6.69	24.47	27.25
3276	Q41593	T.a.	Serpin-Z1A	75294978	P93693	>90	403	43262	5.6	398	10	7	25	1.11					3.76
3279	P93692	T.a.	Serpin-Z2B	75294978	P93692	>90	839	43341	5.46	398	49	13	45	4.71				44.4	64.36
3292	P93692	T.a.	Serpin-Z2B	75294978	P93692	>90	383	43341	5.46	398	11	8	30	0.94			7.66	11.73	20.77
3297	Q41593	T.a.	Serpin-Z1A	75294978	P93693	>90	681	43262	5.6	398	33	12	39	2.77				6.61	9.16
3374	Q41593	T.a.	Serpin-Z1A	75294978	P93693	>90	595	43262	5.6	398	32	11	27	2.47				17.9	11.81

3860	W5DRU6	T.a.	Serpin-Z2B (fragment)	75294978	P93692	>90	262	33343	5.64	304	5	5	20	0.71		2.68			
4350	P93692	T.a.	Serpin-Z2B (fragment)	75294978	P93692	>90	279	43341	5.46	398	10	4	18	0.39			3.83	14.06	23.54
4212	W5DZ76	T.a.	Proteasome subunit alpha type	108706511	W5DZ76	>90	544	32455	8.48	287	17	10	37	2.75				30.28	47.19
4392	W5DZ76	T.a.	Proteasome subunit alpha type	108706511	W5DZ76	>90	192	32455	8.48	287	4	3	12	0.39					3.36
4083	W5H3N4	T.a.	Proteasome subunit alpha type	115444057	P52428	>90	698	31577	5.25	288	18	11	46	3.88				-4.09	
4091	W5H3N4	T.a.	Proteasome subunit alpha type	115444057	P52428	>90	492	31577	5.25	288	14	8	39	2.1				-1.82	
4127	A0A0C4BK49	T.a.	Proteasome subunit alpha type	115444057	P52428	>90	160	31422	5.06	288	3	3	14	0.41					16.16
2739	W5GJG1	T.a.	Peptidase family M20/M25/M40	115448029	I1ICS4	>90	664	49170	5.5	445	12	11	34	1.24				3.34	
1817	B3TZE7	T.a.	Subtilisin protease	115449043	B3TZE7	>90	461	59593	5.12	571	15	8	18	0.62		3.27	2.01		
1742	W5G9C7	T.a.	Oligopeptidase A	115450022	R7WFW5	>90	761	88233	5.69	789	17	15	25	0.85				2.76	
1749	W5G9C7	T.a.	Oligopeptidase A	115450022	R7WFW5	>90	993	88233	5.69	789	25	17	24	0.93				3.78	
4521	Q5XUV7	T.a.	Proteasome subunit beta type	115454751	I1GPC9	>90	296	23485	5.57	213	6	5	25	1.12				-1.71	
3302	W4ZW50	T.a.	Cupin	115461739	I1HMD5	>90	561	31861	6.01	295	24	12	51	4.37					4.76
3353	W5ABG2	T.a.	Cupin	115461739	I1HMD5	>90	396	32011	6.01	295	15	8	32	1.73		3.57	7.07	12.25	7.76
3360	W4ZW50	T.a.	Cupin	115461739	I1HMD5	>90	610	31861	6.01	295	19	13	42	3.29			2.86		
3360	M7Z4H0	T.u.	Glutelin type-A 2	115461739	N1QVK3	86.3	475	35787	6.46	337	12	9	29	1.02			2.86		
2707	W4ZLQ8	T.a.	Methionine aminopeptidase	115463349	E0A9F0	>90	524	43715	7.14	394	11	10	30	1.09				9.84	
3626	W4ZLQ8	T.a.	Methionine aminopeptidase (fragment)	115463349	E0A9F0	>90	538	43715	7.14	394	18	10	29	1.68					-1.67
2035	M8A8P8	T.u.	Heat shock cognate 70 kDa protein	115464309	P09189	>90	513	71399	5.14	648	9	9	18	0.58			5.56		
2931	M8A8P8	T.u.	Heat shock cognate 70 kDa protein	115464309	P09189	>90	786	71399	5.14	648	14	14	24	0.93		5.34	10.54	13.46	32.41
2982	M8A8P8	T.u.	Heat shock cognate 70 kDa protein	115464309	P09189	>90	595	71399	5.14	648	10	10	20	0.66			8.39	27.1	
3007	M8A8P8	T.u.	Heat shock cognate 70 kDa protein	115464309	P09189	>90	906	71399	5.14	648	20	15	27	1.25				5.33	22.16
4638	A7XDG3	T.a.	Gamma gliadin	115468250	A7XDG3	>90	160	16537	8.88	145	2	2	21	0.52				6.49	11.52
4642	A7XDG3	T.a.	Gamma gliadin	115468250	A7XDG3	>90	176	16537	8.88	145	2	2	21	0.52				8.8	16.34
1825	M8A7C6	T.u.	Subtilisin-like protease	115472991	N1QPD5	>90	409	65472	5.93	614	10	7	19	0.39					-5.12
2960	W5FEE5	T.a.	Heat shock cognate 70 kDa protein	115486793	P09189	>90	435	71491	5.12	648	8	8	17	0.5					5.65
3036	W5BDM2	T.a.	Heat shock cognate 70 kDa protein	115486793	P09189	>90	1052	71492	5.07	647	30	17	32	2.54					3.95
3123	W5G3N3	T.a.	Heat shock cognate 70 kDa protein	115486793	P09189	>90	587	71462	5.07	647	13	11	22	0.75					14.3
2709	W5A5C9	T.a.	Methionine aminopeptidase	125551937	E0A9F0	>90	646	43799	7.59	394	15	13	33	1.9				8.9	
2713	W5A5C9	T.a.	Methionine aminopeptidase	125551937	E0A9F0	>90	520	43799	7.59	394	11	11	30	1.09				10.75	
2728	W5A5C9	T.a.	Methionine aminopeptidase	125551937	E0A9F0	>90	815	43799	7.59	394	16	16	45	2.42				6.73	8
1815	W5FAS2	T.a.	Subtilisin-like protease	125558922	N1QPD5	88.5	670	42070	8.74	392	21	11	47	2.3			-2.31		-2.33

2234	M7ZQM3	T.u.	Globulin-1 S allele	218193892	M8B8C6	84.5	229	55586	7.77	493	5	5	12	0.38				1.94	
2339	M7ZQM3	T.u.	Globulin-1 S allele	218193892	M8B8C6	84.5	302	55586	7.77	493	7	6	16	0.48				8.54	16.58
2341	M7ZQM3	T.u.	Globulin-1 S allele	218193892	M8B8C6	84.5	434	55586	7.77	493	10	7	15	0.57				7.28	13.26
2347	M7ZQM3	T.u.	Globulin-1 S allele	218193892	M8B8C6	84.5	731	55586	7.77	493	19	13	28	1.48					18.25
2366	M7ZQM3	T.u.	Globulin-1 S allele	218193892	M8B8C6	84.5	569	55586	7.77	493	13	10	19	0.79				7.86	33.5
2372	M7ZQM3	T.u.	Globulin-1 S allele	218193892	M8B8C6	84.5	717	55586	7.77	493	16	12	25	1.18				5.21	29.03
2376	M7ZQM3	T.u.	Globulin-1 S allele	218193892	M8B8C6	84.5	574	55586	7.77	493	18	10	23	1.32					45.39
2394	M7ZQM3	T.u.	Globulin-1 S allele	218193892	M8B8C6	84.5	611	55586	7.77	493	17	10	25	1.04					52.04
2452	M7ZQM3	T.u.	Globulin-1 S allele	218193892	M8B8C6	84.5	605	55586	7.77	493	18	10	21	1.32				22.08	56.15
4211	W5DXK9	T.a.	Cupin family protein, expressed	937907862	Q10QG3	64.6	608	66681	6.04	594	15	9	20	0.82		1.9			22.82
Protein synthesis																			
2855	W5H8Z0	T.a.	Elongation factor 1-gamma 2	90101285	R7WG39	>90	647	47060	6.18	413	11	10	32	1.32	4.69	3.89	10.77	34.02	14.77
1939	W5CNM6	T.a.	tRNA-glutamine synthetase	115434960	A0A067Y2M0	>90	330	75595	7.06	655	6	6	9	0.27				7.24	
1944	W5CNM6	T.a.	tRNA-glutamine synthetase	115434960	A0A067Y2M0	>90	453	75595	7.06	655	10	9	16	0.4			7.45	9.8	
1957	W5CNM6	T.a.	tRNA-glutamine synthetase	115434960	A0A067Y2M0	>90	597	75595	7.06	655	13	11	17	0.47			3	4.89	
1962	W5CNM6	T.a.	tRNA-glutamine synthetase	115434960	A0A067Y2M0	>90	500	75595	7.06	655	10	10	16	0.61				2.31	
1979	W5DJM5	T.a.	tRNA-glutamine synthetase	115434960	A0A067Y2M0	>90	356	75683	7.33	655	8	7	12	0.47				3.64	
1293	W5G125	T.a.	Elongation factor 2	115446385	O23755	>90	298	94952	5.85	843	6	6	8	0.26				10.72	
1324	M8A571	T.u.	Elongation factor 2	115446385	O23755	>90	228	97317	5.96	864	5	5	9	0.16				3.41	
2799	W5G125	T.a.	Elongation factor 2 (fragment)	115446385	O23755	>90	320	94952	5.85	843	6	6	12	0.26				3.33	
3531	W5DR17	T.a.	Elongation factor 2 (fragment)	115446385	O23755	>90	305	45593	5.4	408	7	6	21	0.6				-1.92	
3650	M8A571	T.u.	Elongation factor 2 (fragment)	115446385	O23755	>90	391	97317	5.96	864	9	7	11	0.3					-1.89
1814	W5FB69	T.a.	Lysine-tRNA ligase	115453943	I1GQJ1	>90	614	69237	5.49	612	11	10	21	0.69				2.56	
2593	A0A0C4BKH5	T.a.	Eukaryotic initiation factor 4A	115469764	P41377	>90	518	47227	5.31	414	9	8	24	0.99				1.94	
2675	P41378	T.a.	Eukaryotic initiation factor 4A	115469764	P41377	>90	719	47183	5.31	414	15	14	31	0.99				3.47	
2691	P41378	T.a.	Eukaryotic initiation factor 4A	115469764	P41377	>90	479	47183	5.31	414	9	9	25	0.71				5.15	
4050	P41378	T.a.	Eukaryotic initiation factor 4A (fragment)	115469764	P41377	>90	193	47183	5.31	414	4	4	12	0.36			1.82		
2906	W5G4H5	T.a.	tRNA synthetases class I	115488928	I1IHT0	>90	531	45954	6.3	401	11	10	28	1.02				1.87	
3036	W5G4H5	T.a.	tRNA synthetases class I	115488928	I1IHT0	>90	643	45954	6.3	401	11	11	35	1.19					3.95
2477	A0A077RYX7	T.a.	Putative prolyl-tRNA synthetase	297613111	M8BBJ8	>90	627	57954	6.6	505	13	12	30	1.11					7.29
Secondary metabolism																			
2275	Q1XIR9	T.a.	4-hydroxy-7-methoxy-3-oxo-3,4-dihydro-2H-1,4-benzoxazin-2-yl glucoside beta-D-glucosidase 1a, chloroplastic precursor	75296357	Q9FYS3	>90	633	64980	6.55	569	14	13	25	1.06		-1.62	-2.07	-1.78	

1825	W5GUI9	T.a.	Phenylalanine ammonia-lyase	115447403	P14717	>90	297	76199	5.89	704	7	6	11	0.27					-5.12
2304	W5G6L7	T.a.	Aldehyde dehydrogenase family	115476474	I1I751	>90	481	55110	5.46	502	10	9	28	0.8				3.26	
3342	W5API6	T.a.	O-methyltransferase, eukaryotic COMT type	115478675	M8A393	>90	490	39138	6.02	350	10	9	31	1.08					16.46
Signal transduction																			
5644	L0GED8	T.a.	14-3-3 protein 14-F	115454901	Q01526	>90	385	29361	4.83	261	9	7	29	1.63				5.94	12.49
5671	L0GED8	T.a.	14-3-3 protein 14-F	115454901	Q01526	>90	274	29361	4.83	261	5	5	23	0.62				4.82	7.29
2443	W5DPI4	T.a.	Calreticulin-like protein	115456397	Q9SP22	>90	554	48437	4.59	428	11	10	26	0.95				5.17	
4125	A0A096UL77	T.a.	14-3-3 protein 14-B	115458806	Q01526	>90	577	30043	4.69	263	13	11	39	2.68		2.56			
2583	W5A376	T.a.	Rab GDP dissociation inhibitor alpha	115463085	R7W728	>90	534	47463	6.13	421	15	11	26	1.68		1.82	3.79	8.74	
2636	W4ZUD0	T.a.	Rab GDP dissociation inhibitor alpha	115463085	R7W728	>90	658	50241	5.53	446	12	12	32	1.36	3.28	2.81		7.85	8.81
2693	W5A376	T.a.	Rab GDP dissociation inhibitor alpha	115463085	R7W728	>90	307	47463	6.13	421	6	6	15	0.58				6.56	
4521	W5I0S7	T.a.	Ras-related protein RIC2	115468476	M8C7T6	>90	207	31884	8.35	291	4	4	14	0.57				-1.71	
4102	Q9FXQ9	T.a.	14-3-3 protein 14-D	297611974	Q01526	>90	485	29491	4.75	266	11	10	43	2.34			2.05	2.52	2.94
Unclassified																			
4435	W5A364	T.a.	Translin family protein, expressed	115482220	Q337S9	80.5	281	32218	7.7	291	5	5	21	0.74				1.91	
2066	W5HZS1	T.a.	Uncharacterized protein	125556053	W5HZS1	>90	204	61172	5.54	548	3	3	8	0.19					3.29
5559	A0A0C4B2Z2	T.a.	Uncharacterized protein	125568690	A0A0C4B2Z2	>90	296	36865	5.88	339	6	5	20	0.79			1.7		
1580	M7YRC9	T.u.	Uncharacterized protein	125586595	M7YRC9	>90	627	62371	6.92	568	12	10	22	0.78					-4.77
4451	W5DWP8	T.a.	27k protein	218192616	D3KVP5	87.3	411	25599	5.9	226	12	8	42	2.47				10.24	
4211	W5DXK9	T.a.	Cupin family protein, expressed	937907862	Q10QG3	64.6	608	66681	6.04	594	15	9	20	0.82		1.9			22.82
4119	M8ARL1	T.u.	Lipoprotein	1002266326	B4FFZ9	77.9	383	29228	5.54	261	12	8	40	2		1.83		2.51	5.29
4521	W5G7V8	T.a.	Uncharacterized protein	1002287767	W5G7V8	>90	208	29595	6.67	267	4	4	15	0.62				-1.71	
3499	M7YDJ6	T.u.	Epoxide hydrolase 2	115462939	A0A0B0PAJ8	62.7	565	24035	6.12	212	23	11	60	8.06					-1.98
3677	W4ZUK2	T.a.	Glucose and ribitol dehydrogenase homolog	125550788	Q75KH3	78	533	37921	9.33	347	17	9	34	1.57					15.88
3684	W5AB71	T.a.	Glucose and ribitol dehydrogenase homolog	125550788	Q75KH3	78	469	32116	6.25	294	11	8	29	2.04				4.19	8.37
3689	W5AB71	T.a.	Glucose and ribitol dehydrogenase homolog	125550788	Q75KH3	78	816	32116	6.25	294	22	14	62	5.63		3.12		4.43	18.09

Supplementary Table S5. Over- and down-represented spots according to 2D-DIGE analysis of albumins/glubulins from durum wheat grain at different (T1, T2, T3, T4 and T5) developmental stages. Data are referred to T0. These spots were further characterized by nanoLC-ESI-LIT-MS/MS analysis.

Stage	Over-represented spots	Down-represented spots
T1	7	1
T2	35	2
T3	62	9
T4	171	18
T5	107	29

Legend to Supplementary Figures

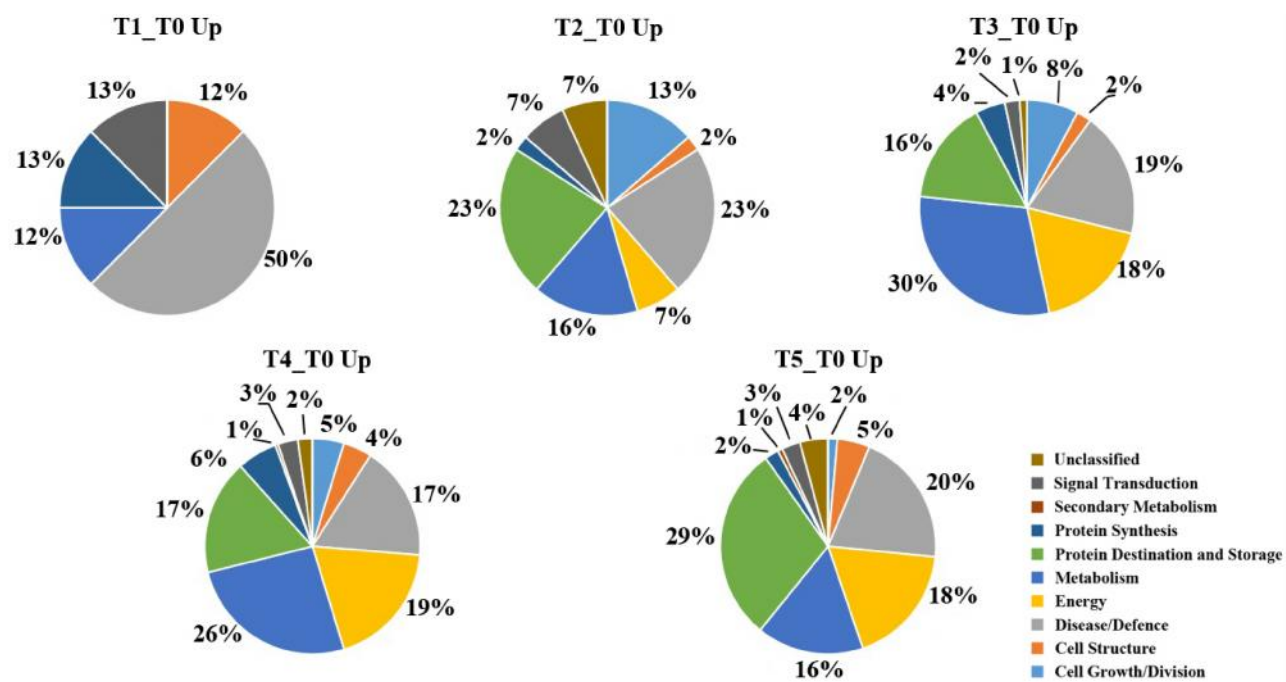
Supplementary Figure S1. Functional distribution of the identified over-represented proteins during each stage (T1, T2, T3, T4 and T5) of grain development according to a pie chart representation. Proteins were divided in functional groups according to Bevan classification [29]. Reported is the percentage of the number of protein identifications determined at a certain developmental stage and associated with a certain functional group, with respect to the total number of protein identification having a coherent quantitative trend at that stage. Data are referred to T0. T0, T1, T2, T3, T4 and T5 correspond to 3, 5, 11, 16, 21 and 30 DPA, respectively.

Supplementary Figure S2. Functional distribution of the identified down-represented proteins during each stage (T1, T2, T3, T4 and T5) of grain development according to a pie chart representation. Proteins were divided in functional groups according to Bevan classification [29]. Reported is the percentage of the number of protein identifications determined at a certain developmental stage and associated with a certain functional group, with respect to the total number of protein identification a coherent quantitative trend at that stage. Data are referred to T0. T0, T1, T2, T3, T4 and T5 correspond to 3, 5, 11, 16, 21 and 30 DPA, respectively.

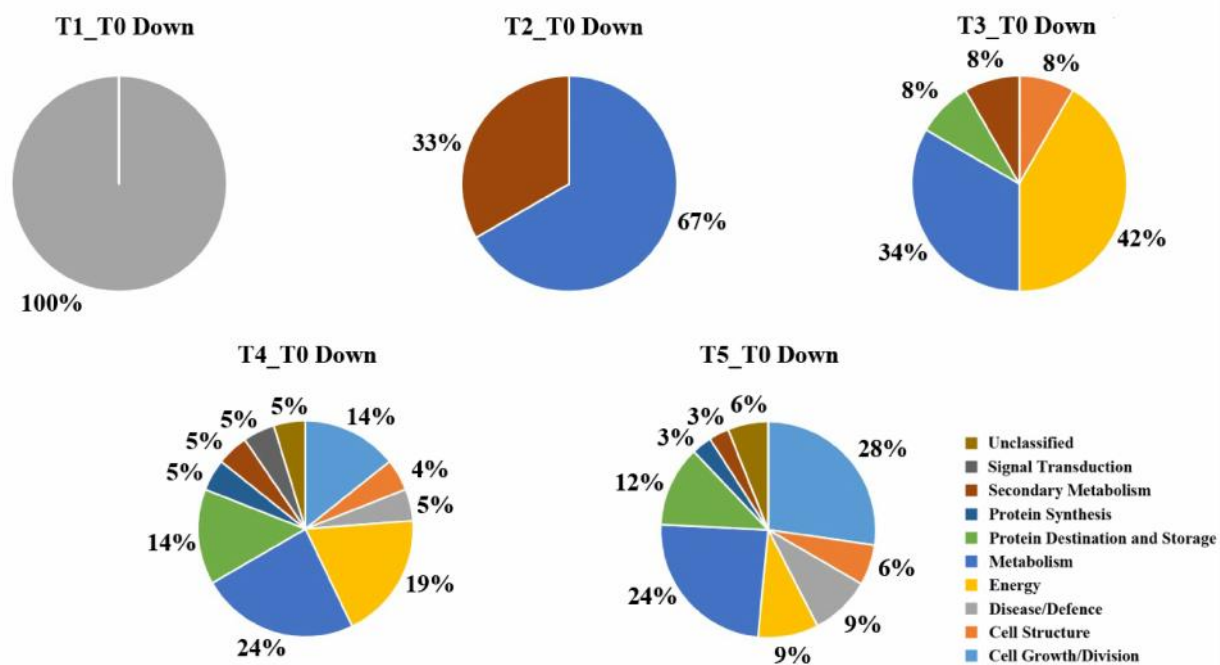
Supplementary Figure S3. Heat-map representation of the differentially represented, cell growth-, protein synthesis-, signal transduction-, unclassified- and secondary metabolism-related proteins in the albumin/globulin fraction of durum wheat grain during development. Relative quantitative results with respect to T0 are highlighted according to a color-based representation. T0, T1, T2, T3, T4 and T5 correspond to 3, 5, 11, 16, 21 and 30 DPA, respectively.

Supplementary Figure S4. Proteins with differential representation involved in carbon and

energy metabolism as mapped with KEGG. Malate dehydrogenase [EC:1.1.1.37- malate dehydrogenase]; isocitrate dehydrogenase [EC:1.1.1.42 - isocitrate dehydrogenase]; glyceraldehyde 3-phosphate dehydrogenase [EC:1.2.1.12 - glyceraldehyde 3-phosphate dehydrogenase]; malic enzyme [EC:1.1.1.40 - malate dehydrogenase (oxaloacetate-decarboxylating) (NADP⁺)]; formate dehydrogenase 1 [EC:1.2.1.2 - formate dehydrogenase]; glutamate dehydrogenase [EC:1.4.1.3 - glutamate dehydrogenase (NADP⁺)]; glycine dehydrogenase P subunit [EC:1.4.4.2 - glycine dehydrogenase]; dihydrolipoyl dehydrogenase [EC:1.8.1.4 - dihydrolipoamide dehydrogenase]; serine hydroxymethyltransferase [EC:2.1.2.1 - glycine hydroxymethyltransferase]; alanine aminotransferase 2 [EC:2.6.1.2 - alanine transaminase]; pyruvate kinase [EC:2.7.1.40 - pyruvate kinase]; phosphoglycerate kinase [EC:2.7.2.3 - phosphoglycerate kinase]; ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit [EC:4.1.1.39 - ribulose-bisphosphate carboxylase large chain]; ribulose-bisphosphate carboxylase small chain [EC:4.1.1.39 - ribulose-bisphosphate carboxylase small chain]; fructose-bisphosphate aldolase [EC:4.1.2.13 - fructose-bisphosphate aldolase, class I]; aconitate hydratase [EC:4.2.1.3 - aconitate hydratase]; enolase [EC:4.2.1.11 - enolase]; triosephosphate isomerase [EC:5.3.1.1 - triosephosphate isomerase]; glucose-6-phosphate isomerase [EC:5.3.1.9 - glucose-6-phosphate isomerase]; succinyl-CoA ligase subunit beta [EC:6.2.1.4/6.2.1.5 - succinyl-CoA synthetase beta subunit]; formate--tetrahydrofolate ligase [EC:6.3.4.3 - formate--tetrahydrofolate ligase]; 3-hydroxyisobutyryl-CoA hydrolase – like protein 3 [EC:3.1.2.4 - 3-hydroxyisobutyryl-CoA hydrolase]; aspartate aminotransferase [EC:2.6.1.1 - aspartate aminotransferase, cytoplasmic]; γ -hydroxybutyrate dehydrogenase-like protein [EC:1.1.1.79 - glyoxylate/succinic semialdehyde reductase].

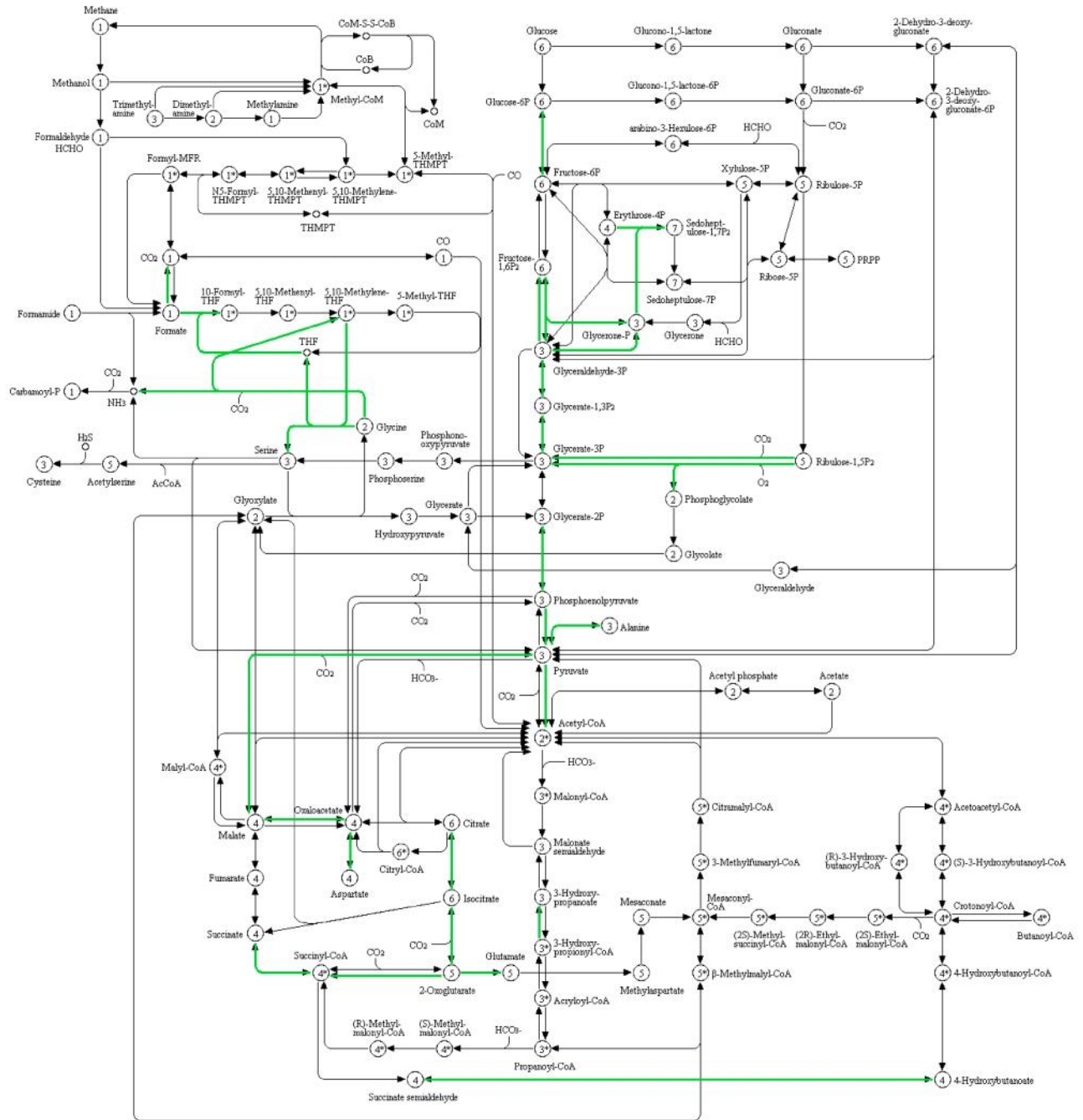


Arena et al., Supplementary Fig. S1



Arena et al., Supplementary Fig. S2

CARBON METABOLISM



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