

RESEARCH ARTICLE

Comparative proteome analysis of metabolic proteins from seeds of durum wheat (cv. Svevo) subjected to heat stress

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In Central and Southern Italy, where durum wheat represents one of the most widely cultivated crops, grain filling occurs during Spring, a period characterized by sudden increases in temperature. Wheat grain proteins are classified into albumins, globulins, and prolamins. The nonprolamin fractions include proteins with metabolic activity or structural function. In order to investigate the consequences of heat stress on the accumulation of nonprolamin proteins in mature durum wheat kernels, the Italian cultivar Svevo was subjected to two thermal regimes (heat stress *versus* control). The 2-D patterns of nonprolamin proteins were monitored to identify polypeptides affected by heat stress during grain fill. This study shows that heat stress alters significantly the durum wheat seed proteome, although the changes range is only between 1.2- and 2.2-fold. This analysis revealed 132 differentially expressed polypeptides, 47 of which were identified by MALDI-TOF and MALDI-TOF-TOF MS and included HSPs, proteins involved in the glycolysis and carbohydrate metabolism, as well as stress-related proteins. Many of the heat-induced polypeptides are considered to be allergenic for sensitive individuals.

Received: December 4, 2009

Revised: March 10, 2010

Accepted: March 24, 2010

Keywords:

Durum wheat / Heat stress / Plant proteomics / Wheat kernel metabolic proteins / Wheat kernel proteome

1 Introduction

Wheat is a widely used cereal for human consumption. The two most important cultivated wheat species are *Triticum aestivum* L. (bread wheat) and *T. durum* Desf. (durum wheat). *T. aestivum* is a hexaploid species possessing the

genome AABBDD, whereas *T. durum* is tetraploid with genome composition AABB. This last species is extremely important, in the Mediterranean areas, where it represents the most widespread crop, being extensively used in the production of a wide range of end products, such as pasta, leavened and unleavened breads, couscous, *etc.* Genes present on D-chromosomes, such as those determining flour texture [1] or those encoding specific high-molecular-weight glutenin subunits [2], missing in durum wheat, confer the typical bread-making properties to bread wheat flour. In addition, the absence of the D-genome in durum

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Abbreviations: DFR, dihydroflavonol reductase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IFR, isoflavone reductase; LEA, late embryogenesis abundant; NDPK, nucleoside diphosphate kinase

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wheat may also affect the response to biotic and abiotic stresses, because it has been shown that several stress-related genes are located on the D-genome [3, 4].

Abiotic stresses, such as drought, salinity, extreme temperatures, chemical toxicity, and oxidative stress are serious threats to agriculture because they affect quality properties, including yield, and the characteristics of the final product. These effects stem from altered synthesis and functionality of specific proteins [5]. Proteome analysis is an effective tool for investigation of changes in protein accumulation in wheat kernel in response to heat stress [6].

The seed protein content is the most important factor determining wheat quality. Wheat kernel proteins are divided according to their solubility properties into prolamins (gliadins and glutenins, collectively known as gluten proteins), soluble in dilute acid or alkali or alcohol–water mixtures, and albumins and globulins, which are water and salt soluble, respectively [7]. Gluten proteins represent about 80% of wheat seed proteins, and are the most important determinant of the dough properties.

High temperature during the grain filling has been reported to alter the yield and the dough quality of bread wheat [8, 9]. Heat stress, in fact, modifies the ratio between the different gluten proteins in the seed by affecting mainly the composition of the polymeric fraction (soluble/insoluble polypeptides) [10]. High temperatures thus favor synthesis of gliadins, whereas glutenin synthesis decreases. An explanation of the gliadin increases after heat stress might be related to the presence of heat stress elements in the upstream regions of some gliadin genes [11]. As glutenins, however, are the most important polypeptides in determining quality properties, their decrease, coupled to the relative increase of gliadins, might explain the negative performance of bread wheat exposed to high temperatures.

Water-soluble albumins and salt-soluble globulins constitute about 20% of total flour protein [12, 13]. The nonprolamin proteins have mainly metabolic activity or structural functions [14]. Certain wheat globulins, however, are reported to be storage proteins [15, 16]. In contrast to gliadins and glutenins, albumins and globulins have been less thoroughly characterized, most probably due to their minor role in wheat quality compared with gluten proteins, although the ratio of albumin to globulin has been reported to correlate with bread-making quality [12]. A relationship between the water-soluble α -amylase/trypsin inhibitors and pasta quality has also been suggested [17, 18]. The soluble proteins from wheat seeds are now receiving increasing attention because several polypeptides from this fraction have been identified as human allergens [19].

Effects of heat stress on metabolic proteins in wheat kernel were previously studied in bread wheat kernels. One of the consequences was a reduction in starch accumulation and activity of soluble starch synthase [20–22]. More recently, proteome analysis of bread wheat showed the involvement of enzymes in starch biosynthesis, *e.g.* glucose-1-phosphate adenylyltransferase and the granule bound starch

synthase in heat stress response. In addition, increased accumulation of a large number of cytoplasmic HSPs was confirmed [6].

Very recent investigations have shown that environmental changes, such as heat, cold, drought, and water-logging mainly affect storage proteins of the 7S globulin (vicilin-like) and R-globulin families, along with defense proteins such as serpins and chitinases [23].

Durum wheat is mostly grown in the Mediterranean region where heat stress during grain fill is a likely event. In order to understand the response of the metabolic protein fraction to heat stress and make a comparison with bread wheat, we have used proteome analysis to identify the durum wheat-soluble seed proteins that are influenced by heat stress and which may affect the nutritional and technological quality of the derived products.

2 Materials and methods

2.1 Plant material and heat treatment

Durum wheat (cv. Svevo) was grown in a climate chamber in a medium composed of soil, sand, peat (6:3:1) at 10°C (9 h day)/7°C (15 h night) with 60% relative humidity and photon flux of 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ until appearance of the third leaf. At this point, conditions were gradually (according to the development stage) switched to 20°C (13 h day)/17°C (11 h night) with 55% relative humidity and photon flux of 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$. These conditions were maintained until 5 days after anthesis. Then, while control plants were maintained in the same conditions, stressed plants were subject to heat-shock treatment, carried out at 37/17°C (13 h day/11 h night) with 55% relative humidity for 5 days. Following the heat shock, the temperature was decreased to 28°C for 4 h, and then the growing cycle was set up at 20°C (13 h day)/17°C (11 h night) with 55% relative humidity and 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photon flux. Starting from the milk maturity stage, both control and stressed plants were brought to complete maturity at 25°C (16 h day)/20°C (8 h night) with 45% relative humidity. In both control and stress treatments, seeds were collected from four biological replicates.

2.2 Protein content analysis and selective extraction

The seed protein content was determined by Kjeldahl nitrogen analysis (Nx5.7) on total flour, according to the standard procedure (NMKL method no. 6). Metabolic seed proteins were extracted according to [24], but amounts were scaled up in order to perform larger 2-D gels. Briefly flour (150 mg) was suspended in 600 μL of cold KCl buffer (50 mM Tris-HCl, 100 mM KCl, 5 mM EDTA, pH 7.8). The suspension was incubated on ice for 5 min with intermittent mixing and centrifuged (14 500 $\times g$, 15 min, 4°C).

The KCl-soluble fraction was collected and five volumes v/v of cold 0.1 M ammonium acetate in methanol were added at room temperature. Following incubation overnight at -20°C , the methanol-insoluble fraction was pelleted by centrifugation as above. The pellet (containing metabolic proteins) was rinsed with cold acetone, dried down, and stored at -20°C until further use.

2.3 2-DE

Metabolic proteins were dissolved in 800 μL of strip rehydration buffer containing 7 M Urea, 2 M thiourea, 2% w/v CHAPS, 2% v/v Triton X-100, 1.2% v/v Destreak reagent (GE Healthcare), and 0.5% v/v IPG buffer pH 3–10. IEF linear IPG strips (18 cm, GE Healthcare) pH 3–10 were used as first dimension. Strips were rehydrated overnight at 20°C with 200 μL of dissolved proteins (about 300 μg protein) and 150 μL of rehydration buffer was added. The same extract volume (200 μL) was loaded. Focusing was performed at 20°C for 80 kVh (200 V 4 h, 500 V 2.5 h, 1000 V 3 h, 5000 V 2 h, 8000 V 9 h). The gel strips were subsequently equilibrated for 25 min in 0.1 M Tris-HCl, pH 8.8, containing 6 M Urea, 30% w/v glycerol, 2% w/v SDS, 1% w/v DTT, and 0.01% w/v bromophenol blue as tracking dye. For the second dimension, the strips were transferred onto 18×20 cm polyacrylamide gels (15%T, 1.28%C) (Protean II X-Cell, BioRad) and run at 40 mA *per* gel for 3–4 h at 11°C until the dye front left the gel.

After electrophoresis, proteins were visualized by CBB staining according to [25]. All gels were stained for 16 h and destained for 1 h with distilled water before image acquisition. Three technical replicas were performed for each of the four biological replicas from each treatment, giving a total of 24 gels. Destained gels were scanned with Image Master LabScan (GE Healthcare) and analyzed using the software SameSpots Progenesis (version 1.0.2602.33289, Nonlinear Dynamics, UK). This software includes statistical analyses such as ANOVA ($p \leq 0.05$), determination of false discovery rate ($q \leq 0.05$), and PCA calculated according to [26].

2.4 Protein identification by MALDI-TOF-TOF

Protein identification was done by picking gel spots from two independent gels to minimize technical variability. CBB-stained spots were excised from the gel, cut into pieces, and washed twice with 40% ethanol until colorless. The destained gel pieces were dehydrated with ACN, treated with 10 mM DTT in 50 mM NH_4HCO_3 for 1 h at 56°C , and finally alkylated with 55 mM iodoacetamide in 50 mM NH_4HCO_3 for 30 min in the dark. Gel pieces were dehydrated with ACN, rehydrated with 1 pmol trypsin (Sigma) solution in 50 mM NH_4HCO_3 , and incubated at 37°C overnight for digestion. The peptides were extracted twice from gel slices

with 5% formic acid in 50% ACN. The peptide solution was applied to an Anchorchip targetTM (Bruker Daltonics) (that also allows desalting and concentration of the sample) using CHCA as matrix, according to the manufacturer's instructions and [27]. MS analysis was performed on a MALDI-TOF-TOF Ultraflex II in positive ion reflector mode and spectra were processed and analyzed using the software FlexAnalysis and BioTools (Bruker Daltonics). Database searching was carried out individually using an in-house MASCOT server (Matrix Science, London, UK) to search NCBI nr (<ftp://ftp.ncbi.nih.gov/blast/db/>) (Viridiplantae database) and the Wheat Gene Index (<http://compbio.dfci.harvard.edu/tgi/tgipage.html>) release 10.

Proteins were identified by PMF. Matrix contaminants and predominant keratin peaks were removed from peak lists using PeakErazor (<http://www.protein.sdu.dk/gpmaw/Help/PeakErazor/peakerazor.html>). In order to identify a protein unambiguously, the following criteria were used: MASCOT score with $p \leq 0.05$; protein sequence coverage $>15\%$; at least five independent peptides matching with a mass tolerance of 50 ppm and maximum one trypsin miscleavage site. The Oxidation (M) and the Gln->pyro-Glu were selected as variable fragment modifications. Where the PMF-based identification was uncertain, fragment ion spectra were obtained for at least three peaks with a signal:noise ratio >5 and $m/z >1400$. Each fragment ion spectrum was checked against the same database as used for PMF and the identification was confirmed if correspondence was found.

3 Results and discussion

Heat stress can be an important factor affecting yield and quality of durum wheat. Although quality is mostly determined by gluten proteins, that are the major protein components of wheat seeds, the soluble metabolic protein fraction (albumins and globulins) also plays a role, especially in terms of nutritional/antinutritional properties. Most of these polypeptides, in fact, show allergenic properties in sensitive individuals [19]. Albumins and globulins are distributed mainly in the outer layers of wheat kernels, and thus are important components in whole-wheat flours and semolina, toward which there is an increasing consumer interest, because of the higher content of fiber, protein, and other functional constituents [28].

In this article, the durum wheat cultivar Svevo, widely grown in Italy and moderately resistant to cold stress, was subjected to two thermal regimes, *i.e.* heat stress and control conditions during grain fill. The effect on the metabolic protein fraction was analyzed by comparison of the proteomes from the control and heat-treated plants.

Protein content, when compared between stressed and control samples, revealed that heat-stressed samples accumulated significantly higher amounts of protein, the protein content being 19.30% (± 1.43) in stressed

and 13.85% (± 1.87) in control samples ($p = 0.004$). A correlation between heat stress and increase in protein content was previously reported in bread wheat [29]. In our case, the increase in protein content was due mostly to gluten proteins, that were about 50% more abundant in heat-stressed samples with respect to control samples, whereas the metabolic fraction increased by about 20% (data not shown).

The highly reproducible 2-D gels showed about 1000 spots in the pI range 3–10 and comparison revealed a range of protein abundance ($1.2 < \text{fold change} < 2.5$) in 132 spots (Fig. 1), 65% were increased in amount in heat-treated samples (Fig. 2). We are confident that the observed differences are significant as only those spots satisfying ANOVA ($p < 0.05$) and false discovery rate ($q < 0.05$) values were chosen, and the PCA performed on the differentially changed spots [26] indicated the presence of two separate groups of gels corresponding to the two thermal regimes (Fig. 3).

3.1 Protein identification

MALDI-TOF analysis identified 47 proteins from the picked spots that varied in intensity (Table 1; Fig. 4). Among the identified proteins, 85% were increased in abundance and 15% decreased in abundance. Proteins identified by MS were classified based on their main activity although most are involved in different pathways or signaling.

Noteworthy is the observation that the magnitude of the changes is in a narrow range ($1.2 < \text{fold change} < 2.5$). This is in agreement with the previous findings relative to the bread wheat cultivar Butte 86 [30].

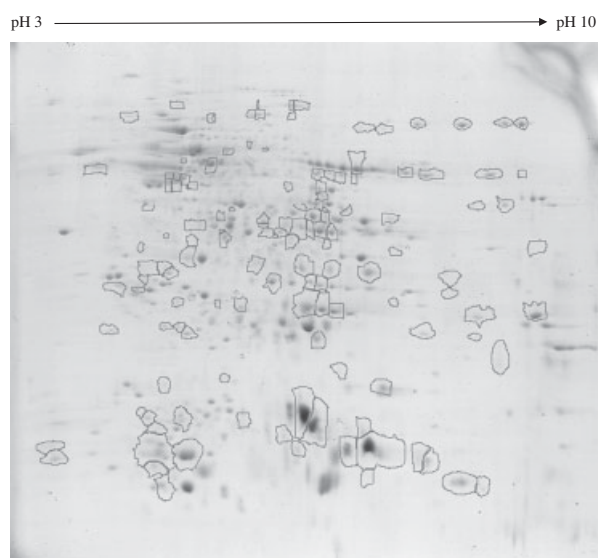


Figure 1. 2-D PAGE map of metabolic proteins in the pH range 3–10: the spots with differential volumes in the samples submitted to heat shock are indicated.

As expected, heat stress increased the spot volume of many proteins related to desiccation and oxidation stress, e.g. late embryogenesis abundant (LEA) proteins, and HSP70 and HSP26 [5, 31–33]. These proteins, through binding or interaction with other proteins, prevent damage of proteins and cell membranes (reviewed in [34]). Also glyoxalase I, one of the enzymes of the glyoxalase pathway related to the detoxification pathway of methylglyoxal in plants [35] showed an increased spot volume.

3.1.1 Carbohydrate metabolism or energy-related proteins

Nucleoside diphosphate kinase (NDPK), which is required for the synthesis of nucleotide triphosphate precursors of DNA and RNA, was increased in abundance along with some housekeeping enzymes involved in glycolysis and the pentose phosphate pathway (glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and glucose and ribitol dehydrogenase), also in agreement with that reported in [30], in which the effect of high temperature on soluble proteins of developing bread wheat grains has been studied. Altered levels of abundance of NDPK were previously found in response to abiotic (including heat stress) and biotic stresses in rice and other plant species [36, 37], suggesting that NDPK plays a regulatory role in addition to its primary metabolic functions.

Five spots (427, 444, 460, 503, and 514) which were increased in amount by heat stress contained GAPDH,

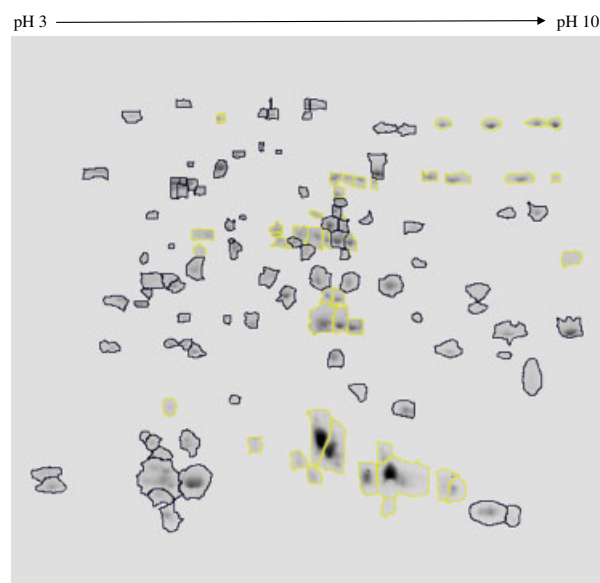


Figure 2. 2-D PAGE of the metabolic proteins that show changes in spot volumes after the heat treatment: the yellow selection refers to spots that show decreased spot volumes, whereas the black selection refers to spot that increased in volumes (fold change between 1.2 and 2.5). In the map, all other proteins not involved in the heat response have been removed.

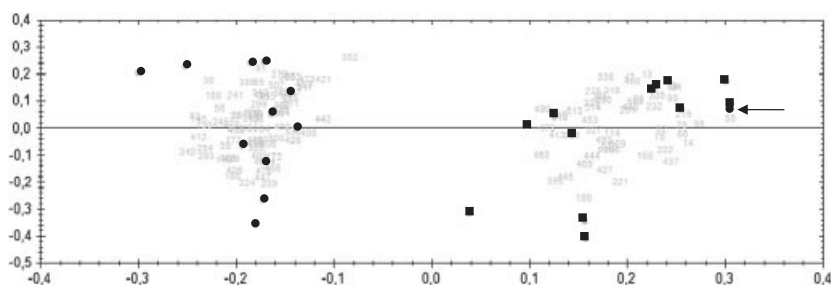


Figure 3. PCA representation in which differential spots relative to each gel analyzed are reported. Circles correspond to gels where heat-stressed samples were run; rectangles indicate the gels corresponding to the separation of control samples. One of the heat stress gels is an outlier (arrow), whereas all the other gels form two separate clusters, on the basis of treatment. The cluster of spots (indicated by numbers) close to each gel cluster indicates those spots showing differential volumes in the particular gel group.

which is involved in glycolysis and has been identified as one of the allergens implicated in bakers' asthma [19]. Bustos and Iglesias [38] reported that wheat endosperm and shoot GAPDH undergo posttranslational phosphorylation, enabling interaction with 14-3-3 family proteins and thus exerting a regulation aimed at maintaining the levels of energy and reductants in the cytoplasm. Recently, it was established using a proteomic approach that GAPDH activity in *Arabidopsis* was inhibited by H_2O_2 , suggesting that GAPDH is a direct target of H_2O_2 and might have a role in mediating ROS signaling in plants [39].

Three upregulated spots (436, 470, and 502) belonged to the glucose and ribitol dehydrogenase protein family.

Among the polypeptides showing decreased spot volumes after heat shock, ATP synthase β -subunit (spot 110) was identified, in agreement with the observations in bread wheat [6]. The decrease in amount of ATP synthase may affect energy-dependent processes involved in heat stress resistance. It has been reported that energy-consuming processes are the primary cellular targets for a decreasing of ATP demand in response to stresses, such as anoxia [40], and it is known that heat shock and anoxia are abiotic stresses eliciting similar cellular responses [41].

In addition, phosphoglycerate kinase (spot 109), glycoside hydrolase family 85 (spot 173), and fructose 6 phosphate-phosphotransferase (spot 299), all involved in glycolysis, showed decreased protein abundance levels. The glycoside hydrolase family is present essentially in all living organisms and has been implicated in a diversity of roles, such as biomass conversion in microorganisms and activation of defense compounds, phytohormones, lignin precursors, aromatic volatiles, and metabolic intermediates [42]. Phosphoglycerate kinase was previously found to be over-expressed in the nuclear proteome of *Arabidopsis* in the cold stress response [43].

3.1.2 Stress related

Two members of the HSP family, HSP70 (spots 70 and 426) and HSP26 (spot 497) showed increased protein abundance

after the heat stress. HSP70s have been linked to the development of acquired thermotolerance in heat stress, although they also seem to correlate with tolerance to low-temperature stress [44]. HSP26 belongs to the family of small HSPs, exhibiting chaperone activity and thought to protect proteins from irreversible aggregation [45].

A polypeptide of the 14-3-3 protein family was slightly decreased in abundance by heat stress (spot 341). Also Hurkman *et al.* [30] observed decreased spot volume for members of this protein family after heat stress in bread wheat. Transcripts encoding proteins belonging to the 14-3-3 family accumulate in barley after biotic stresses [46, 47] and in wheat a region of chromosome 4AL containing genes coding for 14-3-3 is associated with a resistance Quantitative Trait Locus (QTL) against specific fungal diseases [48]. The increased accumulated polypeptides after heat treatment moreover included flavonoid O-methyltransferase (spot 445), a 14-3-3-binding protein involved in production of antimicrobial secondary metabolites, thus showing a potential role in response to a pathogen attack in barley [49]. Furthermore, these compounds have antioxidants properties and could thus be involved in oxidative stress.

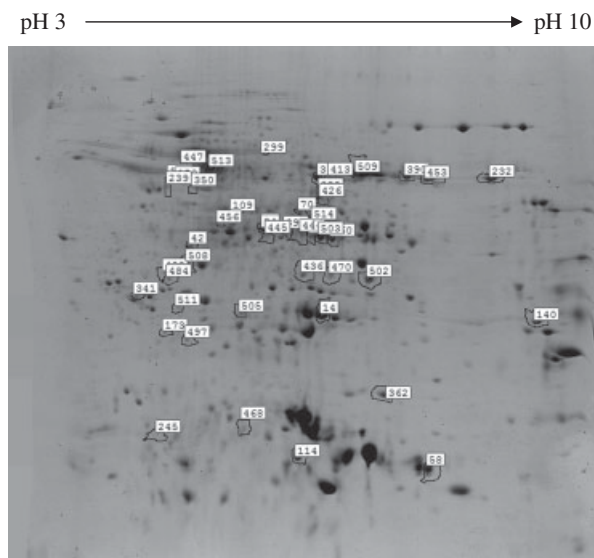
Serpins (serine protease inhibitors) were identified in multiple spots (89, 94, 395, and 456) as increased in amount in response to high temperature. The same observation was made by Hurkman *et al.* [30]. Serpins, found in the soluble fraction of wheat seeds, undergo differential regulation in response to environmental stress [23]. The serpins are widespread in the plant kingdom and represent up to 4% of the total protein in the mature endosperm of cereal grains [50, 51]. Although the precise physiological role of serpins remains unclear, their activity suggests that they are involved in inhibition of endogenous proteases, or proteases from grain pests. Because of their high concentration in the endosperm, serpins have potential to influence grain quality traits [52–54].

Other proteins with a defense role change in abundance in response to heat shock. Such proteins are typically identified in multiple forms on 2-D gels [55] and include: tritins (spots 140 and 222), α -amylase inhibitors (spot 362),

Table 1. Proteins responsive to heat shock identified by PMF and MS-MS analysis

Spot	ANOVA	Fold change	Protein	Accession	Species	Score/coverage%	Theoretical Mw (kDa)
Proteins showing increased spot volumes							
14	1.58E–05	1.7	1-Cys peroxiredoxin PER 1	gi/1710077	<i>T. durum</i>	87/35	24.1
42	0.004	1.2	IFR homolog	TC235506		148/30	42.8
58	1.21E–08	2.5	Homologue to embryo globulin	TC234172		135/27	71.3
70	3.58E–05	1.6	HSP70	gi/2827002	<i>T. aestivum</i>	83/21	71.4
89	0.001	2.2	Serpin	gi/5734506	<i>T. aestivum</i>	96/21	43.3
94	4.61E–04	2	Serpin	gi/5734506	<i>T. aestivum</i>	109/33	43.3
114	0.035	1.3	NDPK	TC262718		96/26	19
222	2.45E–04	1.4	Tritin	gi/147744620	<i>T. aestivum</i>	111/38	29.5
232	0.002	1.9	Globulin-like protein	TC246874		356/45	71.1
336	0.015	1.3	Globulin-like protein 57%	TC246703		133/22	68.2
350	0.002	1.4	Globulin-like protein	TC246874		76/18	71.1
362	0.008	1.5	Endogenous α -amylase inhibitor (WASI)	gi/123975	<i>H. vulgare</i>	232/85	14.7
390	0.033	1.2	Globulin-like protein	TC246703		257/27	68.2
395	0.033	1.3	Serpin	gi/5734506	<i>T. aestivum</i>	74/29	43.3
413	0.008	1.4	Globulin-like protein	TC246703		133/19	68.2
426	0.014	1.2	HSP70	gi/476003	<i>H. vulgare</i>	87/30	71.4
427	0.002	1.2	GAPDH cytosolic	gi/32478662		103/53	18.2
436	0.023	1.5	Glucose and ribitol dehydrogenase	TC233140		113/17	31.6
444	0.007	1.3	GAPDH	gi/148508784	<i>T. aestivum</i>	125/42	36.6
445	0.027	1.5	Flavonoid 7-O-methyltransferase-like (52%)	TC252404		141/26	49.7
447	0.026	1.3	Rubisco large subunit binding protein	gi/2493650	<i>Secale</i>	141/38	53.7
453	0.014	1.2	Globulin-like protein	TC246874	<i>T. aestivum</i>	383/47	71.1
456	0.049	1.2	Serpin	TC236181		87/34	34.8
460	0.01	1.2	GAPDH cytosolic	gi/32478662	<i>T. aestivum</i>	103/53	18.2
468	0.047	1.4	Embryo globulin	TC234134		94/16	76.5
470	0.022	1.3	Glucose and ribitol dehydrogenase	gi/7431022	<i>H. vulgare</i>	89/28	31.6
483	0.032	1.3	LEA	TC268629		97/32	30.5
484	0.003	1.3	LEA	TC268629		126/34	30.5
497	0.004	1.6	HSP26	gi/147225072	<i>T. aestivum</i>	110/45	26.6
502	0.006	1.2	Glucose and ribitol dehydrogenase homologue	gi/7431022	<i>H. vulgare</i>	95/31	31.6
503	0.005	1.2	GAPDH	gi/148508784	<i>T. aestivum</i>	123/61	36.6
505	0.01	1.4	Embryo-specific protein	TC235043		98/14	37.6
508	0.012	1.4	Glyoxalase I	TC264636		91/36	44
509	0.006	1.7	Globulin-like protein	TC246874		290/50	71.1
511	0.009	1.3	Globulin-like protein	TC246703		112/26	68.2
513	0.033	1.3	Hypothetical protein Oryza with enolase Domain	gi/115451911	<i>O. sativa</i>	69/22	51.1
514	0.046	1.2	GAPDH cytosolic	gi/120680 TC264316		119/28	36.6
Proteins showing decreased spot volumes							
109	0.018	–1.3	Phosphoglycerate Kinase	gi/129916	<i>T. aestivum</i>	176/43	42.1
110	0.016	–1.6	ATP synthase β -subunit	gi/525291 and TC264886	<i>T. aestivum</i>	123/21	77.5
110	0.016	–1.6	Globulin-like protein	TC246874		104/23	71.1
140	8.82E–04	–1.6	Tritin	gi/391929	<i>T. aestivum</i>	112/24	29.5
158	0.002	–1.4	ATP syntase β -subunit	gi/525291	<i>T. aestivum</i>	81/13	59.3
173	0.007	–1.3	Glycosyl hydrolase 85	gi/30695320	<i>A. thaliana</i>	85/29	40.7
239	0.022	–1.2	Homologue to embryo globulin	TC234045		163/41	77.3
245	0.008	–1.3	Single-stranded nucleic acid-binding protein	gi/974605, TC249148		100/19	19.3
299	0.003	–1.2	Fructose-6-P 1-phosphotransferase	TC248170		71/13	82.3
341	0.007	–1.4	14-3-3 Homologue	gi/22607 and TC233195	<i>H. vulgare</i>	172/47	29.4

The following criteria were used: MASCOT score ($p < 0.05$), minimal coverage 13%, at least six independent peptides should match with a mass tolerance of 50 ppm and one missed cleavage site. The database search was carried out using the sequences in the NCBI nr and TIGR wheat databases. For all the identifications, three peptide fragmentations were done (MS-MS) and used in combination in the database search. The ANOVA value for each spot, the fold change of the normalized volumes between the spots in the control and heat-stressed maps, the proteins names, accession numbers, the MASCOT scores, the protein coverage, and the theoretical molecular weight of the proteins identified are reported.



in abundance by heat shock. The changes in abundance ranged between 1.2 and 2.2, which was also in agreement with [30].

In agreement with the previous studies performed in bread wheat cultivars [6, 30, 70], both HSP70 and LEA proteins were found in increased abundance in response to heat stress. Other proteins, such as ATP synthase β -subunit and NDPK related to the energy metabolism also responded to heat treatment. This also agrees with the findings of Hurkman *et al.* [30]. Interestingly, we did not find any difference in the level of any starch-related enzymes, although this was a finding of Majoul *et al.* [6] in bread wheat. This might be a result either of the different protein extraction procedures, or of a differential protein turnover in the plant material, or the stress conditions. Another possibility is that the enzyme forms that are regulated in bread wheat may be coded by genes present on the D-genome, that is absent in durum wheat.

GAPDH, a housekeeping enzyme involved in glycolysis, was found to be increased in abundance. Others recently suggested a relationship between this enzyme and certain abiotic stresses [71]. Proteins reported to be influenced by abiotic stresses, *e.g.* oxidative and drought stresses, and also found to have changing levels of abundance in this study, include 14-3-3 proteins, serpins, LEA proteins, 1-Cys peroxiredoxin, glyoxalase I, and proteins with a storage function (*e.g.* globulin-like proteins).

It is noteworthy that some of the proteins that changed in abundance are considered to be allergens (α -amylase inhibitors, serpins, tritins, GAPDH, and globulins in general) and were found to be upregulated after heat stress, suggesting that individuals sensitive to these proteins might be adversely affected by wheat products exposed to stressors.

These results, in conjunction with those of others, suggest that there is a common network “response” to different types of abiotic stress, such as drought, oxidative, cold and heat stress.

This work was supported by the National project “Proteine e geni per la protezione delle piante dagli stress biotici e abiotici (PROTEO-STRESS)”, MIPAF. Paolo Laino was the recipient of a Ph.D. fellowship in Plant Biotechnology funded by MIUR, “Fondo per il sostegno dei giovani e per favorire la mobilità degli studenti” in the framework “Valorizzazione dei prodotti tipici dell’agroalimentare e sicurezza alimentare attraverso nuovi sistemi di caratterizzazione e garanzia di qualità”, and the recipient of an STMS award from the COST Action FA0603 Plant Proteomics. Birgit Andersen (Technical University of Denmark) is thanked for assistance with MS. Dale Shelton was funded by a Large Multi-Disciplinary Research Grant from the Danish Research Council. The authors wish to acknowledge Dr. Federico Scossa (ENEA-Casaccia, Rome, Italy) for having performed protein content measurements.

The authors have declared no conflict of interest.

5 References

- [1] Morris, C. F., Puroindolines: the molecular genetic basis of wheat grain hardness. *Plant Mol. Biol.* 2002, **48**, 633–647.
- [2] Shewry, P. R., Halford, N. G., Tatham, A. S., Popineau, Y. *et al.*, The high molecular weight subunits of wheat glutenin and their role in determining wheat processing properties. *Adv. Food Nutr. Res.* 2003, **45**, 219–302.
- [3] Boyko, E. V., Gill, K. S., Mickelson-Young, L., Nasuda, S. *et al.*, A high-density genetic linkage map of *Aegilops tauschii*, the D-genome progenitor of bread wheat. *Theor. Appl. Genet.* 1999, **99**, 16–26.
- [4] Aprile, A., Mastrangelo, A. M., De Leonardis, A. M., Galiba, G. *et al.*, Transcriptional profiling in response to terminal drought stress reveals differential responses along the wheat genome. *Biomed. Chromatogr. Genomics* 2009, **10**, art. no. 279.
- [5] Timperio, A. M., Egidi, M. G., Zolla, L., Proteomics applied on plant abiotic stresses: Role of heat shock proteins (HSP). *J. Proteomics* 2008, **71**, 391–411.
- [6] Majoul, T., Bancel, E., Triboui, E., Hamida, J. B., Branlard, G., Proteomic analysis of the effect of heat stress on hexaploid wheat grain: characterization of heat-responsive proteins from non-prolamins fraction. *Proteomics* 2004, **4**, 505–513.
- [7] Osborne, T. B., *The Vegetable Proteins*, Longmans, Green & Co., London 1924.
- [8] Stone, P. J., Nicolas, M. E., Wheat cultivars vary widely in their responses of grain yield and quality to short periods of post-anthesis heat stress. *Aust. J. Plant Physiol.* 1994, **21**, 887–900.
- [9] Wardlaw, I. F., Blumenthal, C., Larroque, O., Wrigley, C. W., Contrasting effects of chronic heat stress and heat shock on kernel weight and flour quality in wheat. *Funct. Plant Biol.* 2002, **29**, 25–34.
- [10] Blumenthal, C. S., Bekes, F., Batey, I. L., Wrigley, C. W. *et al.*, Interpretation of grain quality results from wheat variety trials with reference to high temperature stress. *Aust. J. Agric. Res.* 1991, **42**, 325–334.
- [11] Blumenthal, C. S., Batey, I. L., Wrigley, C. W., Barlow, E. W. R., Involvement of a novel peptide in the heat shock response of Australian wheats. *Aust. J. Plant Physiol.* 1990, **17**, 441–449.
- [12] Pence, J. W., Weinstein, N. E., Mecham, D., The albumin and globulin contents of wheat flour and their relationship to protein quality. *Cereal Chem.* 1954, **31**, 303–311.
- [13] Singh, J., Skerritt, J. H., Chromosomal control of albumins and globulins in wheat grain assessed using different fractionation procedures. *J. Cereal Sci.* 2001, **33**, 163–181.
- [14] Goesaert, H., Gebruers, K., Courtin, C. M., Brijs, K., Delcour, J. A., Enzymes in breadmaking. in: Hui, Y. H. (Ed.), *Bakery Products: Science and Technology*, 2006, pp. 337–364.
- [15] Singh, N. K., Shepherd, K. W., The structure and genetic control of a new class of disulphide-linked proteins in wheat endosperm. *Theor. Appl. Genet.* 1985, **71**, 79–92.
- [16] Singh, N. K., Shepherd, K. W., Solubility behavior synthesis, degradation and subcellular location of a new class of

- disulfide linked proteins in wheat endosperm. *Aust. J. Plant Physiol.* 1987, **14**, 245–252.
- [17] Kobrehel, K., Alary, R., The role of a low molecular weight glutenin fraction in the cooking quality of durum wheat pasta. *J. Sci. Food Agric.* 1989, **47**, 487–500.
- [18] Kobrehel, K., Alary, R., Isolation and partial characterization of two low molecular weight durum wheat (*Triticum durum*) glutenins. *J. Sci. Food Agric.* 1989, **48**, 441–452.
- [19] Tatham, A. S., Shewry, P. R., Allergens to wheat and related cereals. *Clin. Exp. Allergy* 2008, **38**, 1712–1726.
- [20] Shy, Y. C., Seib, P. A., Bernardin, J. E., Effects of temperature during grain-filling on starches from six wheat cultivar. *Cereal Chem.* 1994, **71**, 369–383.
- [21] Hawker, J. S., Jenner, C. F., High temperature affects the activity of enzymes in the committed pathway of starch synthesis in developing wheat endosperm. *Aust. J. Plant Physiol.* 1993, **20**, 197–209.
- [22] Keeling, P. L., Bacon, P. J., Holt, D. C., Elevated temperature reduces starch deposition in wheat endosperm by reducing the activity of soluble starch syntase. *Planta* 1993, **191**, 342–348.
- [23] Sancho, A. I., Gillabert, M., Tapp, H., Shewry, P. R. *et al.*, Effect of environmental stress during grain filling on the soluble proteome of wheat (*Triticum aestivum*) dough liquor. *Agric. Food Chem.* 2008, **56**, 5386–5393.
- [24] Hurkman, W. J., Tanaka, C. K., Improved methods for separation of wheat endosperm proteins and analysis by two-dimensional gel electrophoresis. *J. Cereal Sci.* 2004, **40**, 295–299.
- [25] Neuhoﬀ, V., Arold, N., Taube, D., Ehrhardt, W., Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with a clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. *Electrophoresis* 1988, **9**, 255–262.
- [26] O’Gorman, M., Beauvallet, C., Lepercq, P. David, O. *et al.*, An investigation into Crohn’s disease using the Progenesis SameSpots analysis platform. *24th Journée Françaises de Spectrométrie de Masse*, PAU 2007.
- [27] Zhang, X., Shi, L., Shu, S., Whang, Y. *et al.*, An improved method of sample preparation on AnchorChip™ targets for MALDI-MS and MS/MS and its application in the liver proteome projects. *Proteomics* 2007, **7**, 2340–2349.
- [28] Dewettinck, K., Van Bockstaele, F., Kühne, B., Van de Walle, D. *et al.*, Nutritional value of bread: influence of processing, food interaction and consumer perception. *J. Cereal Sci.* 2008, **48**, 243–257.
- [29] Stone, P. J., Gras, P. W., Nicolas, M. E., The influence of recovery temperature on the effects of a brief heat shock on wheat. III. Grain protein composition and dough properties. *J. Cereal Sci.* 1997, **25**, 129–141.
- [30] Hurkman, W. J., Vensel, W. H., Tanaka, C. K., Whitehand, L., Altenbach, S. B., Effect of high temperature on albumin and globulin accumulation in the endosperm proteome of the developing wheat grain. *J. Cereal Sci.* 2009, **49**, 12–23.
- [31] Treglia, A., Spano, G., Rampino, P., Giangrande, E. *et al.*, Identification by *in vitro* translation and Northern blot analysis of heat shock mRNAs isolated from wheat seeds exposed to different temperatures during ripening. *J. Cereal Sci.* 1999, **30**, 33–38.
- [32] Renaut, J., Hausman, J.-F., Wisniewski, M. E., Proteomics and low-temperature studies: bridging the gap between gene expression and metabolism. *Physiol. Plant.* 2006, **126**, 97–109.
- [33] Qureshi, M. I., Qadir, S., Zolla, L., Proteomics-based dissection of stress-responsive pathways in plants. *J. Plant Physiol.* 2007, **164**, 1239–1260.
- [34] Buitink, J., Hoekstra, F. A., Leprince, O., in: Black, M., Pritchard, H. W. (Eds.), *Biochemistry and Biophysics of Tolerance Systems*. CAB International, Wallingford, UK 2002.
- [35] Hossain, M. A., Hossain, M. Z., Fujita, M., Stress-induced changes of methylglyoxal level and glyoxalase I activity in pumpkin seedlings and cDNA cloning of glyoxalase I gene. *Aust. J. Crop Sci.* 2009, **3**, 53–64.
- [36] Salekdeh, Gh. H., Siopongco, J., Wade, L. J., Ghareyazie, B., Bennett, J., Proteomic analysis of rice leaves during drought stress and recovery. *Proteomics* 2002, **2**, 1131–1145.
- [37] Hajheidari, M., Abdollahian-Noghabi, M., Askari, H., Heidari, M. *et al.*, Proteome analysis of sugar beet leaves under drought stress. *Proteomics* 2005, **5**, 950–960.
- [38] Bustos, D. M., Iglesias, A. A., Phosphorylated non-phosphorylating glyceraldehyde-3-phosphate dehydrogenase from heterotrophic cells of wheat interacts with 14-3-3 proteins. *Plant Physiol.* 2003, **133**, 2081–2088.
- [39] Hancock, J. T., Henson, D., Nvirenda, M., Desikan, R. *et al.*, Proteomic identification of glyceraldehyde 3-phosphate dehydrogenase as an inhibitory target of hydrogen peroxide in *Arabidopsis*. *Plant Physiol. Biochem.* 2005, **43**, 828–835.
- [40] Boutilier, R. G., St-Pierre, J., Surviving hypoxia without really dying. *Comp. Biochem. Physiol.* 2000, **126A**, 481–490.
- [41] Wu, B. S., Lee, J. K., Thompson, K. M., Walker, V. K. *et al.*, Anoxia induces thermotolerance in the locust flight system. *J. Exp. Biol.* 2002, **205**, 815–827.
- [42] Opasiri, R., Pomthong, B., Onkoksoong, T., Akiyama, T. *et al.*, Analysis of rice glycosyl hydrolase family 1 and expression of Os4bglu12 β -glucosidase. *Biomed. Chromatogr. Plant Biol.* 2006, **6**, art. no. 33.
- [43] Bae, M. S., Cho, E. J., Choi, E. Y., Park, O. K., Analysis of the *Arabidopsis* nuclear proteome and its response to cold stress. *Plant J.* 2003, **36**, 652–663.
- [44] Guy, C. L., Freezing tolerance of plants: current understanding and selected emerging concepts. *Can. J. Bot.* 2003, **81**, 1216–1223.
- [45] Nguyen, H. T., Weng, J., Joshi, C. P., A wheat (*Triticum aestivum*) cDNA clone encoding a plastid-localized heat-shock protein. *Plant Physiol.* 1993, **103**, 675–676.
- [46] Brandt, J., Thorald-Christensen, H., Vad, K., Gregersen, P. L., Collinge, D. B., A pathogen-induced gene of barley encodes a protein showing high similarity to a protein kinase regulator. *Plant J.* 1992, **2**, 815–820.

- [47] Gregersen, P. L., Thordal-Christensen, H., Forster, H., Collinge, D. B., Differential gene transcript accumulation in barley leaf epidermis and mesophyll in response to attack by *Blumeria graminis* f.sp. *hordei* (syn. *Erysiphe graminis* f.sp. *hordei*). *Physiol. Mol. Plant Pathol.* 1997, 51, 85–97.
- [48] Faris, J. D., Li, W. L., Liu, D. J., Chen, P. D., Gill, B. S., Candidate gene analysis of quantitative disease resistance in wheat. *Theor. Appl. Genet.* 1999, 98, 219–225.
- [49] Christensen, A. B., Gregersen, P. L., Olsen, C. E., Collinge, D. B., A flavonoid 7-O-methyltransferase is expressed in barley leaves in response to pathogen attack. *Plant Mol. Biol.* 1998, 36, 219–227.
- [50] Rasmussen, S. K., Klausen, J., Hejgaard, J., Svensson, B., Svendsen, I., Primary structure of the plant serpin BSZ7 having the capacity of chymotrypsin inhibition. *Biochim. Biophys. Acta* 1996, 1297, 127–130.
- [51] Østergaard, H., Rasmussen, S. K., Roberts, T. H., Hejgaard, J., Inhibitory serpins from wheat grain with reactive centers resembling glutamine-rich repeats of prolamin storage proteins. Cloning and characterization of five major molecular forms. *J. Biol. Chem.* 2000, 275, 33272–33279.
- [52] Roberts, T. H., Marttila, S., Rasmussen, S. K., Hejgaard, J., Differential gene expression for suicide-substrate serine proteinase inhibitors (serpins) in vegetative and grain tissues of barley. *J. Exp. Bot.* 2003, 54, 2251–2263.
- [53] Salt, L. J., Robertson, J. A., Jenkins, J. A., Mulholland, F., Mills, E. N. C., The identification of foam-forming soluble proteins from wheat (*Triticum aestivum*) dough. *Proteomics* 2005, 5, 1612–1623.
- [54] Roberts, T. H., Hejgaard, J., Serpins in plants and green algae. *Funct. Integr. Genomics* 2008, 1, 1–27.
- [55] Østergaard, O., Finnie, C., Laugesen, S., Roepstorff, P., Svensson, B., Proteome analysis of barley seeds: identification of major proteins from two-dimensional gels (pI 4–7). *Proteomics* 2004, 4, 2437–2447.
- [56] Šotkovský, P., Hubálek, M., Hernychová, L., Novák, P. *et al.*, Proteomic analysis of wheat proteins recognized by IgE antibodies of allergic patients. *Proteomics* 2008, 8, 1677–1691.
- [57] Veena, R. V. S., Sopory, S. K., Glyoxalase I from *Brassica juncea*: molecular cloning, regulation and its over-expression confer tolerance in transgenic tobacco under stress. *Plant J.* 1999, 17, 385–395.
- [58] Singla-Pareek, S. L., Reddy, M. K., Sopory, S. K., Genetic engineering of the glyoxalase pathway in tobacco leads to enhanced salinity tolerance. *Proc. Natl. Acad. Sci. USA* 2003, 100, 14672–14677.
- [59] Yadav, S. K., Singla-Pareek, S. L., Reddy, M. K., Sopory, S. K., Transgenic tobacco plants overexpressing glyoxalase enzymes resist an increase in methylglyoxal and maintain higher reduced glutathione levels under salinity stress. *FEBS Lett.* 2005, 579, 6265–6271.
- [60] Rivero, R. M., Ruiz, J. M., García, P. C., López-Lefebvre, L. R. *et al.*, Resistance to cold and heat stress: accumulation of phenolic compounds in tomato and watermelon plants. *Plant Sci.* 2001, 160, 315–321.
- [61] Demirevska, K., Simova-Stoilova, L., Vassileva, V., Feller, U., Rubisco and some chaperone protein responses to water stress and rewatering at early seedling growth of drought sensitive and tolerant wheat varieties. *Plant Growth Regul.* 2008, 56, 97–106.
- [62] Riccardi, F., Gazeau, P., de Vienne, D., Zivy, M., Protein changes in response to progressive water deficit in maize, quantitative variation and polypeptide identification. *Plant Physiol.* 1998, 11, 1253–1263.
- [63] Lal, S. K., Lee, C., Sachs, M. M., Differential regulation of enolase during anaerobiosis in maize. *Plant Physiol.* 1998, 118, 1285–1293.
- [64] Forsthoefel, N. R., Cushman, M. A., Cushman, J. C., Post-transcriptional and posttranslational control of enolase expression in the facultative Crassulacean acid metabolism plant *Mesembryanthemum crystallinum* L. *Plant Physiol.* 1995, 108, 1185–1195.
- [65] Umeda, M., Hara, C., Matsubayashi, Y., Li, H. H. *et al.*, Expressed sequence tags from cultured cells of rice (*Oryza sativa* L.) under stressed conditions: analysis of transcripts of genes engaged in ATP generating pathways. *Plant Mol. Biol.* 1994, 25, 469–478.
- [66] Tunnacliffe, A., Wise, M. J., The continuing conundrum of the LEA proteins. *Naturwissenschaften* 2007, 94, 791–812.
- [67] Bystrova, M. F., Budanova, E. N., Novoselov, V. I., Fesenko, E. E., Analysis of the oligomeric state of rat 1-Cys peroxidoredoxin. *Biophysics* 2007, 52, 277–281.
- [68] Karlson, D., Imai, R., Conservation of the cold shock domain protein family in plants. *Plant Physiol.* 2003, 131, 12–15.
- [69] Karlson, D., Nakaminami, K., Toyomasu, T., Imai, R., Cold-regulated nucleic acid-binding protein of winter wheat shares a domain with bacterial cold shock proteins. *J. Biol. Chem.* 2002, 277, 35248A–35256A.
- [70] Majoul, T., Bancel, E., Triboï, E., Ben Hamida, J., Branlard, G., Proteomic analysis of the effect of heat stress on hexaploid wheat grain: characterization of heat-responsive proteins from total endosperm. *Proteomics* 2003, 3, 175–183.
- [71] Bustos, D. M., Bustamante, C. A., Iglesias, A. A., Involvement of non-phosphorylating glyceraldehyde-3 phosphate dehydrogenase in response to oxidative stress. *J. Plant Physiol.* 2008, 165, 456–461.