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Journal:	*PROTEOMICS*
Manuscript ID:	Draft
Wiley - Manuscript type:	Research Article
Date Submitted by the Author:	
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Key Words:	Durum wheat, Heat stress, Wheat kernel proteome, Wheat kernel metabolic proteins

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

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Abbreviations: FDR, false discovery rate; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; LEA, Late Embryogenesis Abundant; NDPK, Nucleoside diphosphate kinase

Keywords: Durum Wheat; Heat Stress; Wheat Kernel Proteome; Wheat Kernel Metabolic Proteins

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ABSTRACT

In Central and Southern Italy, where durum wheat represents one of the most widely cultivated crops, grain filling occurs during Spring, a period characterised by sudden increases in temperature. Wheat grain proteins are classified into albumins, globulins, and prolamins. The non-prolamin fractions include proteins with metabolic activity or structural function.

In order to investigate the consequences of heat stress on the accumulation of non-prolamin proteins in mature durum wheat kernels, the Italian cultivar Svevo was subjected to two thermal regimes (heat stress *vs.* control) during grain filling. The 2D patterns of non-prolamin proteins were monitored to identify polypeptides affected by heat stress. This study shows that heat stress alters significantly the durum wheat seed proteome, although the fold changes range only between 1.2 and 2.2. This analysis revealed 132 differentially expressed polypeptides, 47 of which were identified by MALDI TOF and MALDI-TOF-TOF MS and included heat shock proteins, 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.

The differences observed with previously reported data regarding bread wheat may be explained by the absence of the D genome in durum wheat.

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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 genomes AABBDD, whereas *T. durum* is tetraploid with genome composition AABB. This last species is extremely important, in the Mediterranean areas, where represents the most widespread crop, finding large utilization in the production of a wide range of end products, such as pasta, leavened and unleavened breads, cous cous 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 wheat may affect also 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 protein [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 diluted 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.

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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 increase after heat stress might be related to the presence of heat stress elements (HSE) in the upstream regions of some gliadin genes [10]. Because 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 [11,12]. The non-prolamin proteins have mainly metabolic activity or structural functions [13]. Certain wheat globulins, however, are reported to be storage proteins [14,15]. 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 to gluten proteins, although the ratio of albumin to globulin has been reported to correlate with bread-making quality [11]. A relationship between the water-soluble α-amylase/trypsin inhibitors and pasta quality has also been suggested [16,17]. The soluble proteins from wheat seeds are now receiving increasing attention because several polypeptides from this fraction have been identified as human allergens [18].

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 [19-21]. More recently, proteome analysis of bread wheat showed the involvement of enzymes in starch biosynthesis, e.g. glucose-1-phosphate adenyltransferase and the granule bound starch synthase in heat stress response. In addition, up-regulation of a large number of cytoplasmic heat shock proteins (HSP) was confirmed [6].

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Very recent investigations have shown that environmental changes, such as heat, cold, drought, and waterlogging mainly affect storage proteins of the 7S globulin (vicilin-like) and R-globulin families, along with defense proteins such as serpins and chitinases [22].

Durum wheat is mostly grown in the Mediterranean region where heat stress during grain filling 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.

MATERIALS AND METHODS

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 µmol m^{-2} 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 µmol m^{-2} s^{-1}. These conditions were maintained until five 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 µmol m^{-2} 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 replicas.

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Protein content analysis and selective extraction

The seed protein content was determined by Kjeldahl nitrogen analysis (Nx5.7). Metabolic seed proteins were extracted according to [23], but amounts were scaled up in order to perform larger 2D 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*g*, 15 min, 4 °C). The KCl-soluble fraction was collected and 5 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-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 X100, 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 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.

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After electrophoresis, proteins were visualized by CBB staining according to [24]. 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 (vers. 1.0.2602.33289, Nonlinear Dynamics, UK). This software includes statistical analyses such as ANOVA ($p \leq 0.05$), determination of False Discovery Rate (FDR, $q \leq 0.05$), and Principle Component Analysis (PCA) calculated according to [25].

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 to 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 then desalted, concentrated and applied to an Anchorchip target™ (Bruker Daltonic) using α-cyano-4-hydroxycinnamic acid as matrix, according to the manufacturer's instructions and [26]. Mass spectrometric analysis was performed on a MALDI TOF-TOF Ultraflex II in positive ion reflector mode and spectra were processed and analysed using the software FlexAnalysis and BioTools (Bruker Daltonics). Database searching was carried out using an in-house MASCOT server (Matrix Science, London, UK) to search NCBInr (ftp://ftp.ncbi.nih.gov/blast/db/) and the Wheat Gene Index (http://compbio.dfci.harvard.edu/tgi/tgipage.html) release 10.

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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.

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, also the soluble metabolic protein fraction (albumins and globulins) plays a role, especially in terms of nutritional/antinutritional properties. Most of these polypeptides, in fact, show allergenic properties in sensitive individuals [18]. Albumins and globulins are distributed mainly in the outer layers of wheat kernels, and thus are important components in whole-wheat flours and semolina, towards which there is an increasing consumer interest, because they have a higher amount of fibers, proteins and functional components [27].

In the present paper 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

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grain filling. The effect on the metabolic protein fraction was analysed by comparison of the corresponding proteomes.

Firstly, protein contents were compared between stressed and control samples, which revealed that heat stressed samples accumulated significatively 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 [28]. 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 differential expression of proteins ($1.2<$fold change<2.2) in 132 spots (Fig. 1), 65% were up-regulated in heat-treated samples (Fig. 2). Because only those spots satisfying ANOVA ($p<0.05$) and FDR ($q<0.05$) values were chosen, and the Principal Component Analysis (PCA) performed on the differentially expressed spots [25] indicated two separate groups corresponding to the two thermal regimes (Fig. 3), we are confident that the observed differences are significant.

Protein identification

MALDI TOF analysis identified 47 proteins from the picked varying spots (Table 1; Fig. 4). Among the identified proteins, 85% were up-regulated and 15% down-regulated. Proteins identified by mass spectrometry were classified based on to their main activity although most are involved in different pathways or signaling.

Noteworthy is the observation that the fold changes observed, are in a narrow range ($1.2>$fold change>2.2). This is in agreement with previous findings relative to the bread wheat cultivar Butte 86 [29].

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As expected, heat stress increased expression of many proteins related to desiccation and oxidation stress, e.g. Late Embryogenesis Abundant (LEA)-proteins, the heat shock proteins HSP70 and HSP26 [5, 30-32]. These proteins, through binding or interaction with other proteins, prevent damage of proteins and cell membranes (reviewed in [33]). Also glyoxalase I, one of the enzymes of the glyoxalase pathway related to the detoxification pathway of methylglyoxal in plants [34] was up-regulated.

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 up-regulated along with some housekeeping enzymes involved in glycolysis and the pentose phosphate pathway (glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase, and glucose and ribitol dehydrogenase), in agreement also with that reported in [29], in which the effect of high temperature on soluble proteins of developing bread wheat grains has been studied. Altered expression pattern of NDPK was previously found in response to abiotic (including heat stress) and biotic stresses in rice and other plant species [35-36], suggesting that NDPK plays a regulatory role in addition to its primary metabolic functions.

Five spots (427, 444, 460, 503, 514) which were up-regulated by heat stress contained glyceraldeyde 3-phosphate dehydrogenase (GADPH), which is involved in glycolysis and has been identified as one of the allergens implicated in bakers' asthma [18]. Bustos and Iglesias [37] reported that wheat endosperm and shoot GAPDH undergoes posttranslational phosphorylation enabling interaction with 14-3-3 family proteins, thus exerting a regulation aimed at maintaining the levels of energy and reductants in the cytoplasm. Recently, it was established using a proteomics 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 [38].

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

Among the polypeptides down-regulated after heat shock, ATP synthase β subunit (spot 110) was identified, in agreement with 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 [39], and it is known that heat shock and anoxia are abiotic stresses eliciting similar cellular responses [40].

Also phosphoglycerate kinase (spot 109), glycoside hydrolase family 85 (spot 173), and fructose 6 phosphate-phosphotransferase (spot 299), all involved in glycolysis, were down-regulated. The glycoside hydrolase family is present in essentially 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 [41]. Phoshoglycerate kinase was previously found to be over-expressed in the nuclear proteome of Arabidopsis in the cold stress response [42].

Stress related

Two members of the heat shock protein family, HSP70 (spots 70, 426) and HSP26 (spot 497) were up-regulated after the heat stress. HSP70s have been linked to the development of acquired thermotolerance in heat stress, although they seem also to correlate with tolerance to low temperature stress [43]. HSP26 belongs to the family of small heat shock proteins (sHSP) exhibiting chaperone activity and thought to protect proteins from irreversible aggregation [44].

A polypeptide of the 14-3-3 protein family was slightly down-regulated by heat stress (spot 341). Also Hurkman *et al* [29] observed down-regulation of members of this protein family after heat stress in bread wheat. Transcripts encoding proteins belonging to the 14-3-3 family accumulate in

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barley after biotic stresses [45,46] and in wheat a region of chromosome 4AL containing genes coding for 14-3-3 is associated with a resistance QTL against specific fungal diseases [47]. The up-regulated 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 [48]. Furthermore these compounds have antioxidants properties and could thus be involved in oxidative stress.

Serpins (*ser*ine *pr*otease *in*hibitors) were identified in multiple spots (89, 94, 395, 456) as up-regulated in response to high temperature. The same observation was made by Hurkman *et al* [29]. Serpins, found in the soluble fraction of wheat seeds, undergo differential regulation in response to environmental stress [49]. 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]. While 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 have a modified expression profile in response to heat shock. Such proteins are typically identified in multiple forms on 2-D gels [55] and include: tritins (spots 140, 222), α-amylase inhibitors (spot 362), and some 14-3-3 related or binding proteins. Most of these proteins besides having a metabolic role, are also considered as storage proteins. Furthermore, some are also considered wheat grain allergens along with the serpins [56].
Another enzyme found in response to the heat treatment is glyoxalase I (spot 508). The glyoxalase system is a set of enzymes that carry out detoxification of methylglyoxal and other reactive aldehydes that are produced during normal metabolism. In plants it has been demonstrated that different kinds of stress, such as salt and metal stress, elicits enhance the expression of glyoxalase I [57-59].

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LEA proteins (spots 483 and 484) were up-regulated and are involved in stress responses. They are typically correlated with cellular dehydration in response to cold stress. Other LEA functions include roles as antioxidants and membrane and protein stabilisers during water stress [60]. Finally, 1-Cys peroxiredoxin (spot 14), also observed to be up-regulated, has antioxidant and chaperone activity [61].

Storage proteins

Up-regulated proteins with a storage role here identified in the heat response mainly belong to the globulin-like protein family (spots 110, 232 350, 509, 511) or protein homologous to embryo globulin (spots 58, 239, 468, 505). Spot 239 (Homologue to Embryo Globulin) was found to be down-regulated by heat stress. A role for these proteins in thermotolerance is not known. They may be directly related to heat stress response by an unknown mechanism or, indirectly, be a target for other proteins involved in the heat shock response. This is in agreement with [29].

CONCLUDING REMARKS

The results here reported offer a picture of the consequences of heat stress occurring during grain filling on the accumulation of the soluble proteins in the mature seeds of the widely grown Italian durum wheat *cv* Svevo. The results provide a basis for understanding how this environmental change influences on protein synthesis and consequently the metabolic and quality traits of the durum wheat kernel. Moreover, since the durum wheat kernel is of primary interest because the semolina obtained by crushing mature seeds is the basis of many products of common use, among which pasta is the most important, it is critical to understand if the different types of stresses that wheat plants can potentially undergo, may alter protein composition, and, consequently, qualitative and nutritional properties of the derived products.

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In the present study 47 metabolic proteins were identified from the soluble seed fraction of durum wheat which were induced or repressed by heat shock. The fold changes observed were between 1.2 and 2.2, and this was also in agreement with [29].

In agreement with previous studies performed in bread wheat cultivars [6,29,62] both HSP70 and LEA proteins were up-regulated in response to heat stress. Other proteins, such as ATP synthase β subunit and nucleoside diphosphate kinase (NDPK) related to the energy metabolism also responded to heat treatment. This agrees also with the findings of Hurkman *et al* [29]. Interestingly, we did not find any difference in the level of expression of 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 different protein turnover in the plant material, or 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 enzymes involved in glycolysis, was found to be up-regulated. Others recently suggested a relationship between this enzyme and certain abiotic stresses [63]. Proteins reported to be influenced by abiotic stresses, e.g. oxidative and drought stresses, and also found as differentially regulated in the present work 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 differentially regulated proteins are considered as allergens (α-amylase inhibitors, serpins, tritins, GAPDH) and were found to be up-regulated after heat stress, which obviously represents a disadvantage for sensitive individuals.

These results illustrate that there is a common network “response” to different types of abiotic stress, such as drought, oxidative, cold and heat stress.

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ACKNOWLEDGMENTS

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 PhD 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 a STMS award from the COST Action FA0603 Plant Proteomics. Birgit Andersen (Technical University of Denmark) is thanked for assistance with mass spectrometry. 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.

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Conflict of interest statement

The authors declare that there is no financial/commercial conflict of interest

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Tab.1 Proteins responsive to heat shock identified by PMF and MS-MS analysis. The following criteria were used: MASCOT score ($p<0.05$), minimal coverage 13%, at least 6 independent peptides should match with a mass tolerance of 50 ppm and 1 miss cleavage site. The database search was applied in NCBInr and TIGR wheat. 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.

Spot	Anova	Fold Change	Protein	Accession	Species	Score/coverage%	Theor Mw (kDa)
Up-regulated proteins							
14	1.58E-05	1.7	1-Cys peroxiredoxin PER 1	gi/1710077	*T. durum*	87/35	24.1
42	0.004	1.2	Isoflavone reductase 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	*T. aestivum*	83/21	71.4
89	0.001	2.2	Serpin	gi/5734506	*T. aestivum*	96/21	43.3
94	4.61E-04	2	Serpin	gi/5734506	*T. aestivum*	109/33	43.3
114	0.035	1.3	Nucleoside diphosphate Kinase	TC262718		96/26	19
222	2.45E-04	1.4	Tritin	gi/147744620	*T. aestivum*	111/38	29.5
232	0.002	1.9	Globulin-like protein	TC246874		356/45	71.1

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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	*H. vulgare*	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	*T. aestivum*	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	*H. vulgare*	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	*T. aestivum*	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	*Secale*	141/38	53.7
453	0.014	1.2	Globulin-like protein	TC246874	*T. aestivum*	383/47	71.1
456	0.049	1.2	Serpin	TC236181		87/34	34.8
460	0.01	1.2	GAPDH cytosolic	gi/32478662	*T. aestivum*	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	*H. vulgare*	89/28	31.6

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483	0.032	1.3	Late embryogenesis abundant (LEA)	TC268629		97/32	30.5
484	0.003	1.3	Late embryogenesis abundant (LEA)	TC268629		126/34	30.5
497	0.004	1.6	HSP26	gi/147225072	*T. aestivum*	110/45	26.6
502	0.006	1.2	Glucose and ribitol dehydrogenase homologue	gi/7431022	*H. vulgare*	95/31	31.6
503	0.005	1.2	GAPDH	gi/148508784	*T. aestivum*	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	*O .sativa*	69/22	51.1
514	0.046	1.2	GAPDH cytosolic	gi/120680 TC264316		119/28	36.6
Down regulated proteins							
109	0.018	-1.3	Phosphoglycerate Kinase	gi/129916	*T. aestivum*	176/43	42.1
110	0.016	-1.6	ATP Synthase β subunit	gi/525291 and TC264886	*T. aestivum*	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	*T. aestivum*	112/24	29.5

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158	0.002	-1.4	ATP Syntase β subunit	gi/525291	*T. aestivum*	81/13	59.3
173	0.007	-1.3	Glycosyl hydrolase 85	gi/30695320	*A. thailiana*	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	*H. vulgare*	172/47	29.4

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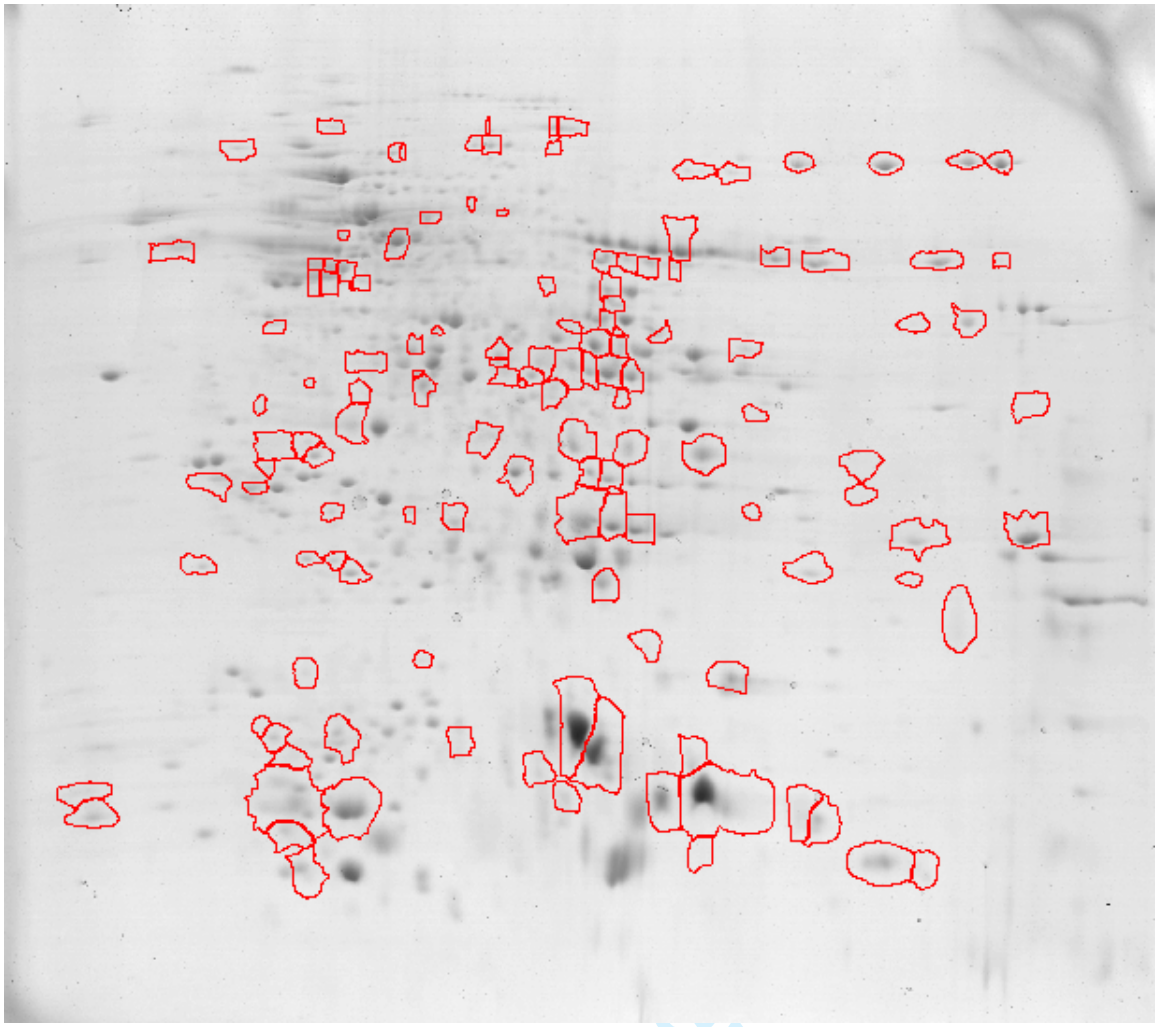

Fig. 1. 2-D PAGE map of metabolic proteins in the 3–10 pH range: circles indicate the differential spots

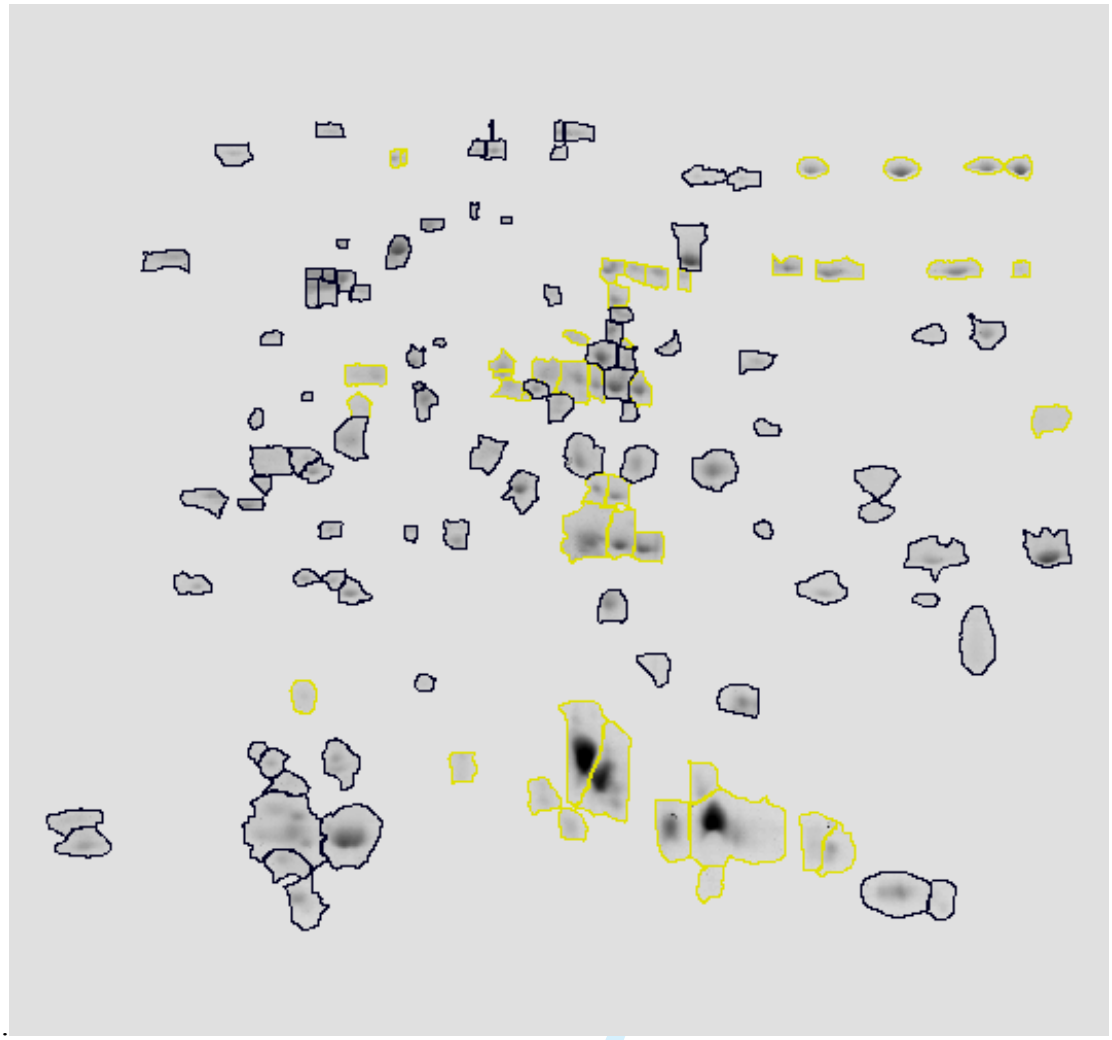

FIG 2 2D-PAGE of the metabolic proteins that are differentially regulated after the heat treatment: the yellow selection is referred to spots down-regulated, whereas the black one to up-regulated (fold change between 1.2 and 2.2). In the map all other proteins not involved in the heat response have been removed.

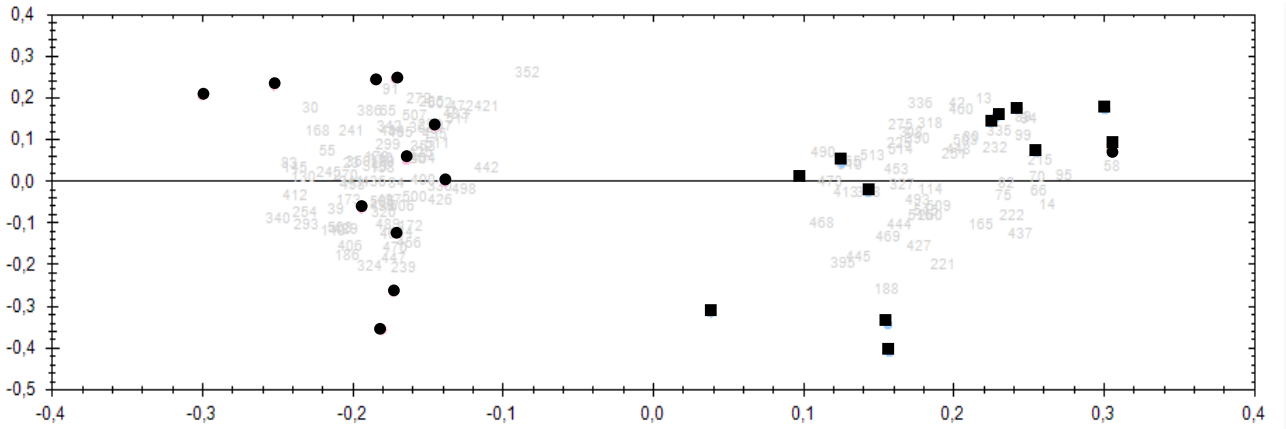

Fig. 3. PCA representation in which differential spots relative to each gel analysed are reported. Circles: heat stressed samples; squares: control samples. Numbers represent the differentially expressed spots.

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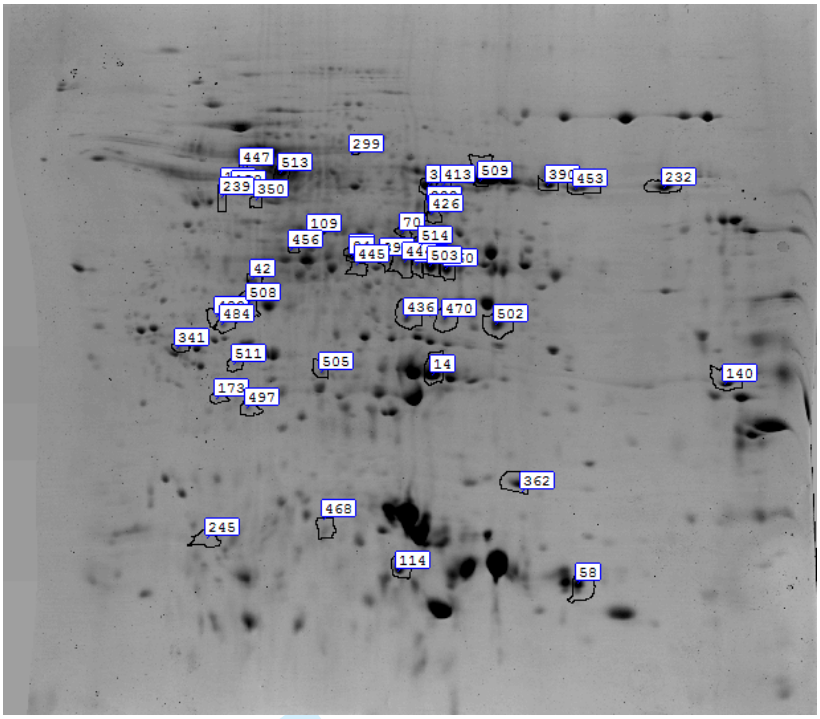

Fig. 4. 2-D PAGE map of metabolic proteins in the 3-10 pH range: circles indicate the identified proteins by MALDI-TOF and MALDI-TOF-TOF

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