JOURNAL OF PROTEOMICS 91 (2013) 486–499

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Gel-free proteomics reveal potential biomarkers of priming-induced salt tolerance in durum wheat

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ARTICLE INFO

Article history:
Received 23 March 2013
Accepted 12 August 2013

Keywords:
Durum wheat
Gel-free proteomics
Wheat seed metabolic proteins
Seed priming
Salt tolerance

ABSTRACT

Seed priming has been successfully demonstrated to be an efficient method to improve crop productivity under stressful conditions. As a first step toward better understanding of the mechanisms underlying the priming-induced salt stress tolerance in durum wheat, and to overcome the limitations of the gel-based approach, a comparative gel-free proteomic analysis was conducted with durum wheat seed samples of varying vigor as generated by hydro- and ascorbate-priming treatments. Results indicate that hydro-priming was accompanied by significant changes of 72 proteins, most of which are involved in proteolysis, protein synthesis, metabolism and disease/defense response. Ascorbate-priming was, however, accompanied by significant changes of 83 proteins, which are mainly involved in protein metabolism, antioxidant protection, repair processes and, interestingly, in methionine-related metabolism. The present study provides new information for understanding how 'priming-memory' invokes seed stress tolerance.

Biological significance
The current work describes the first study in which gel-free shotgun proteomics were used to investigate the metabolic seed protein fraction in durum wheat. A combined approach of protein fractionation, hydrogel nanoparticle enrichment technique, and gel-free shotgun proteomic analysis allowed us to identify over 380 proteins exhibiting greater molecular weight diversity (ranging from 7 to 258 kDa). Accordingly, we propose that this approach could be useful to acquire a wider perspective and a better understanding of the seed proteome. In the present work, we employed this method to investigate the potential biomarkers of priming-induced salt tolerance in durum wheat. In this way, we identified several previously unrecognized proteins which were never been reported before, particularly for the ascorbate-priming treatment. These findings could provide new avenues for improving crop productivity, particularly under unfavorable environmental conditions.

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http://dx.doi.org/10.1016/j.jprot.2013.08.010

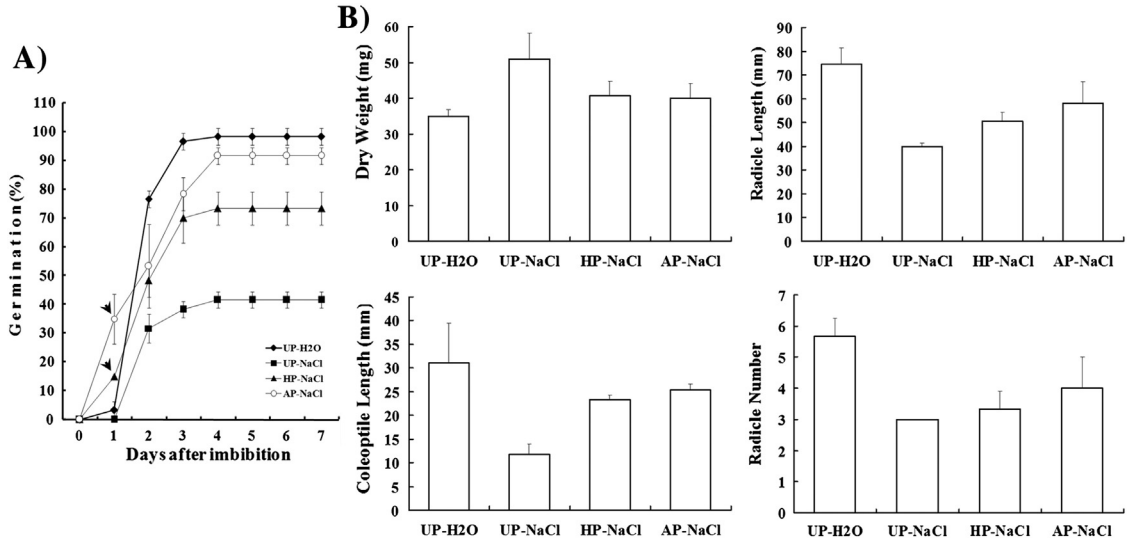

Fig. 1 – Seed germination and seedling growth of durum wheat. (A) Interactive effect of salt stress (250 mM NaCl) and priming treatments (Materials and methods) on the seed germination time-course of durum wheat. (B) Interactive effect of salt stress and priming treatments on seedling growth of durum wheat. UP, un-priming; HP, hydro-priming; AP, ascorbate-priming.

1. Introduction

Wheat is the most widely grown crop in the world, which provides about 20% of the daily protein and of the food calories for more than 4.5 billion people (http://www.wheatinitiative.org/). It is the second most important food crop in the world owing to its unique characteristics and the fact that large quantities of grain can be produced, harvested, stored, and transported in an efficient way [1]. Durum is a tetraploid species of wheat, which produces higher yield than normal wheat in areas with reduced precipitation. However, even though durum wheat is the most widely cultivated crops in the Mediterranean basin where ~75% of the world's durum grain is produced [2], its production remained low probably because of the poor seed germination and stand establishment, mainly due to drought and soil salinity [3].

Seed priming is a seed pre-sowing imbibition treatment that is widely used to improve seed performance with respect to rate and uniformity of germination [4]. This is very important under salt stress conditions, because there is general agreement that seed germination and early seedling growth are critical stages during which salt stress is especially damaging to yield [5]. Seed priming particularly with antioxidant compounds such as ascorbic acid (AsA) seems to be an efficient method to overcome seed germination problems and to improve seedling growth in the field, especially under salinity (see review in Hasanuzzaman et al. [6]). The agricultural relevance of priming in plants, as it is a simple, short-term, harmless, and low-cost technology that enhances the ability of plant to cope with stress, has motivated scientists to unravel the underlying cellular and molecular mechanisms [7]. In the past decade, several major studies, using transcriptome, metabolome and proteome approaches, have attempted to identify the molecular/biochemical mechanisms of seed priming [7–13]. The major processes potentially involved in seed priming can be described as cell cycle associated-events [9], endosperm weakening [10], mobilization of storage proteins [11] lipid and starch mobilization, protein synthesis and the methyl cycle [12,13] (see review in Rajjou et al. [8]).

The aforementioned processes suggest that primed seeds presumably possess molecular mechanisms that allow them to memorize previous priming events, which can be recruited later

Table 1 – Metabolic proteins identified according to two protocols, one comprising a standard in-solution trypsin digestion, and the other one in which an enrichment step by NIPAm/CB core VSA shell particles (HG) was added.

	Un-primed	Un-primed with HG	Combined	Overlap	Not overlap	Un-primed only	Un-primed with HG only
Proteins	292	197	323	166	157	126	31
	Hydro-primed	Hydro-primed with HG	Combined	Overlap	Not overlap	Hydro-primed only	Hydro-primed with HG only
Proteins	286	230	327	189	138	97	41
	Ascorbate-primed	Ascorbate-primed with HG	Combined	Overlap	Not overlap	Ascorbate-primed only	Ascorbate-primed with HG only
Proteins	252	225	302	175	127	77	50

when seeds are exposed to stresses during germination [14]. More recently, it has been proposed that priming increases seed vigor and stress tolerance through at least two different strategies. First, priming initiates the germination-related processes that facilitate the transition from the quiescent state into the germinating state and lead to improved germination potential. Secondly, priming allows the growth-arrested seeds to reinforce their capacity to mount adaptive defense responses useful to withstand environmental stress conditions during seedling establishment [8,14]. It is therefore interesting to apply comparative proteomic analysis in seeds treated with chemical priming agents before the imposition of abiotic stress conditions [7].

Quantitative proteomics represent an important extension to identification proteomics, enabling the comparison of changes in protein levels across different samples or treatments [15]. This requires sensitive and accurate assays for identifying proteins in complex mixtures and quantifying their abundances. The most commonly used approach for comparative proteomic analysis of plant tissues is the application of 2DE-gels, whereby differences in protein abundances were determined by comparing stained protein spot volumes followed by identification of proteins by mass spectrometry (MS). However, this method is limited in sensitivity, has a low dynamic range, and it is inefficient when analyzing proteins with very high or low molecular mass [16].

Gel-free shotgun proteomics are alternative approaches for the identification and quantification of proteins in large-scale studies [17,18]. Although it has become feasible to rapidly identify proteins from crude cell extracts using MS-based shotgun proteomics, it can be difficult to elucidate low-abundance proteins of interest in the presence of a large excess of relatively abundant proteins. Therefore, for effective proteome analysis it becomes critical to enrich the sample to be analyzed in subfractions of interest. Sequential extraction is a method particularly suited for the subfractionation of wheat endosperm proteins, because it takes advantage of the specific solubility properties of the different classes of endosperm proteins.

Moreover, a novel method suitable for high throughput MS-based proteomics that make use of hydrogel nanoparticles for selective protein enrichment in complex mixtures has been introduced recently [19]. Hydrogel nanoparticles, in particular Poly(N-isopropylacrylamide/Cibacron Blue) core vinylsulfonic acid (Poly(NIPAm/CB) core VSA) shell particles, were chosen on the basis of our previous work [20], because they can perform a further step of enrichment for less abundant proteins capable to interact with the Cibacron Blue [21].

Here we describe an LC–MS/MS platform for analysis of metabolic proteins from seeds of durum wheat using solubility-based protein fractionation [22] and protein enrichment combined with label-free quantitative tandem MS with a high-performance hybrid mass spectrometer, LTQ-Orbitrap XL. In addition, the present work aimed to investigate the possible changes in metabolic protein profiles during seed priming treatment. To the best of our knowledge, this is the first time that gel-free, label-free shotgun proteomic approach has been used to investigate the change in the metabolic proteins of durum wheat seeds in response to priming. This study could explain some of the biochemical process involved in wheat seed priming, which could be very useful for improving crop production by characterizing the genes/proteins potentially involved in enhancing seed germination and seedling vigor, particularly under stress conditions.

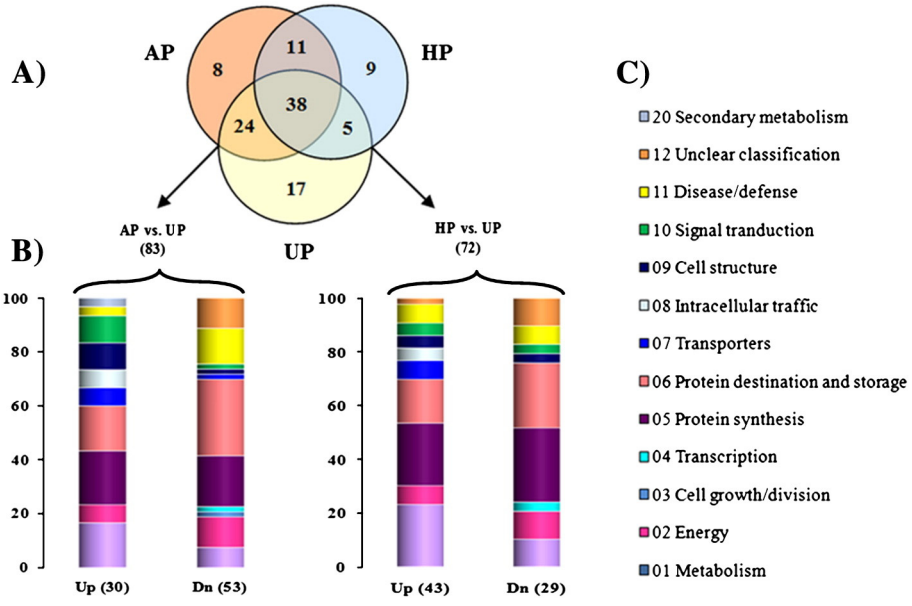

Fig. 2 – Venn diagrams and functional classification of wheat proteome in both primed seeds compared to control un-primed seeds. (A) Venn diagrams display the overlap between the proteome of primed and un-primed durum wheat seeds (see Materials and methods). (B) Functional classification of proteins shown in (A). The number of identified proteins is indicated. (C) Functional classes according to Bevan et al. [30]. Up, up-regulated; Dn, down-regulated.

2. Materials and methods

2.1. Seed treatment, germination assay and seedling growth

Seed priming treatments were performed essentially as described by Jafar et al. [23]. The ratio of seed weight to the volume of solution employed for priming was 1:5 [24]. For each seed priming treatment 50 g of wheat seeds was soaked in 250 mL aerated distilled water or ascorbate solution (0.5 mmol L^{-1}) for 12 h. During soaking period, continuous aeration was provided using a small aquarium pump. After soaking, seeds were washed three times with distilled water and then re-dried, up to almost their original weight, keeping them under shade with forced air at 27 ± 3 °C; after drying they were sealed in paper bags and stored until use. Untreated dry seeds were taken as a control.

Seeds of durum wheat (*Triticum durum* Desf. var. Waha), cultivar moderately resistant to salinity and widely cultivated in Algeria, both treated and untreated, were surface sterilized with sodium hypochlorite (5% w/v) for 3 min, washed several times with sterile water and dried in an oven. Thirty dried seeds were placed in each Petri-dish containing two layers of Whatman no. 1 filter paper initially moistened with 10 mL of saline solution (NaCl 250 mmol L^{-1}) or distilled water (control). Seeds were germinated in darkness in a temperature-controlled chamber held at 22 ± 0.5 °C. The number of germinated seeds was counted everyday until day 7. Growth measurements were taken prior to harvest (7-day-old seedlings).

2.2. Extraction and quantification of metabolic seed proteins

Metabolic seed proteins were extracted using the method developed by Hurkman and Tanaka [22] with some modifications. Briefly, wheat seeds were ground to a fine powder in a household coffee grinder, and then 75 mg of flour was suspended in 300 μL of cold KCl buffer (Tris–HCl 50 mmol L^{-1}, KCl 100 mmol L^{-1}, EDTA 5 mmol L^{-1}, pH 7.8). The suspension was incubated on ice for 5 min with intermittent mixing and then centrifuged at 14,500 *g* for 15 min at 4 °C. The KCl-soluble fraction was collected and 5 volumes of cold 100 mmol L^{-1} ammonium acetate solution in methanol were added at room temperature and incubated overnight at 20 °C. The methanol-insoluble fraction was pelleted by centrifugation at 14,500 *g* for 15 min at 4 °C. The pellet was rinsed three times with cold acetone (500 μL) to remove the ammonium acetate, and solubilized with 200 μL of urea/ammonium bicarbonate (8 mol L^{-1}/50 mmol L^{-1}). Metabolic proteins were quantified by Bradford assay using BSA standard [25]. Three experimental replicates were performed for each seed type. For each extraction, the protein mixture was divided into two aliquots and processed according to two protocols, one comprising a standard in-solution trypsin digestion, and the other one in which an enrichment step by NIPAm/CB core VSA shell particles was added, and then in-solution trypsin digestion was performed.

2.3. In solution trypsin digestion and off-line desalting

For each sample, protein aliquots, 100 μg (1 μg μL^{-1}), were reduced, alkylated, and digested with trypsin as described by Capriotti et al., with some modifications [26]. Reduction of disulphide bonds was performed with 2.5 μL of DTT (200 mmol L^{-1}), under slight agitation, in incubation at 37 °C for 1 h. Carbamidomethylation of thiol groups was performed by addition of IAA (10 μL, 200 mmol L^{-1}) and incubation for 1 h in the dark at room temperature. To consume any leftover alkylating agent and to avoid trypsin alkylation, 10 μL of DTT (200 mmol L^{-1}) was added and samples were incubated at 37 °C for 1 h, under slight agitation. The samples were then diluted with ammonium bicarbonate (50 mmol L^{-1}) to obtain a 1 mol L^{-1} final urea concentration. Sequencing grade-modified tryspin was added (1:20, enzyme:protein ratio) and the samples were incubated overnight at 37 °C. Enzymatic digestion was quenched with formic acid. Digested samples were desalted using SPE C18 cartridges conditioned with acetonitrile (ACN) and rinsed with 0.1% TFA. Peptides were eluted from the SPE column with 500 μL ACN/ddH_2O (50/50, v/v) containing 0.05% TFA and were dried in a Speed-Vac SC 250 Express (Thermo S 164 avant, Holbrook, NY, USA). Each sample was re-constituted with 0.1% HCOOH aqueous solution and stored at −80 °C until LC–MS/MS analysis.

2.4. Enrichment of proteins by hydrogel nanoparticles

200 μL aliquot of NIPAm/CB core VSA shell particles (equivalent to 2 mg dry weight) was washed by centrifugation with deionized water. Briefly the hydrogel nanoparticles were centrifuged at 15,000 *g* for 7 min, the supernatant was removed and the pellet was resuspended in 200 μL of deionized water. Following centrifugation the hydrogel nanoparticle pellet was resuspended in 400 μL protein solution (750 μg) in urea/ammonium bicarbonate (8 mol L^{-1}/50 mmol L^{-1}). The mixture was incubated under slight agitation for 15 min at room temperature. The protein–hydrogel nanoparticle complexes were then separated by centrifugation at 15,000 *g* and washed once with 200 μL of a solution NaSCN 250 mmol L^{-1}, and twice with 200 μL of ddH_2O to remove NaSCN. Proteins bound to the hydrogel nanoparticles were eluted twice with 200 μL of ACN:ddH_2O:NH_4OH (70:27:3, v/v/v) and the eluates combined in the same vial, dried in a vacuum concentrator and solubilized with urea/ammonium bicarbonate (8 mol L^{-1}/50 mmol L^{-1}) for the subsequent digestion with trypsin as described in the previous paragraph and subsequent LC–MS/MS analysis.

2.5. NanoHPLC–MS analysis

Tryptic peptides were analyzed by a Dionex Ultimate 3000 nano-HPLC system (Sunnyvale CA, USA) connected to a hybrid LTQ-Orbitrap XL mass spectrometer (Thermo Scientific, Bremen, Germany) equipped with a nanoelectrospray ion source. Peptide mixtures were separated on in-house manufactured 20 cm fritless silica microcolumn, 75 μm i.d., packed with ReproSil-Pur C18-AQ 3 μm resin. The flow rate was 250 nL min^{-1} and the LC gradient was optimized to detect the largest set of peptides, using H_2O/HCOOH (99.9/0.1, v/v) as phase A and CH_3CN/HCOOH (99.9/0.1, v/v) as phase B. After an isocratic step at 5% B for 5 min, B was linearly increased to 30% within 75 min; afterwards, B was increased to 80% within 5 min, and to 95% within the following 10 min to rinse the column.

Table 2 – Wheat metabolic proteins whose abundance varied in hydro-primed seeds.

No	Protein name	Accession no	Organism	MW	Function category	Function description	R.
212	Glutamate decarboxylase 1	DCE1_ARATH	*Arabidopsis thaliana*	57	01 metabolism	01.01 amino acid	D
147	Succinate-semialdehyde dehydrogenase.	SSDH_ORYSJ	*Oryza sativa*	56	01 metabolism	01.01 amino acid	U
302[a]	3-isopropylmalate dehydrogenase	LEU3_BRANA	*Brassica napus*	43	01 metabolism	01.01 amino acid	U
309	Arginase	ARGI1_ARATH	*Arabidopsis thaliana*	37	01 metabolism	01.01 amino acid	U
311	Methylthioribose-1-phosphate isomerase	MTNA_HORVU	*Hordeum vulgare*	39	01 metabolism	01.01 amino acid	U
332	Adenylate kinase A	KAD1_ORYSJ	*Oryza sativa*	26	01 metabolism	01.03 nucleotides	D
115	Malate dehydrogenase	MDHC1_ARATH	*Arabidopsis thaliana*	36	01 metabolism	01.05 sugars/polysaccharides	D
289	Probable sucrose-phosphate synthase 4	SPS4_ORYSJ	*Oryza sativa*	119	01 metabolism	01.05 sugars/polysaccharides	U
324[a]	Alpha-galactosidase	AGAL_ORYSJ	*Oryza sativa*	46	01 metabolism	01.05 sugars/polysaccharides	U
220[a]	Fructose-1,6-bisphosphatase,	F16P2_SACHY	*Saccharum hybrid*	37	01 metabolism	01.05 sugars/polysaccharides	U
274[a]	Acetyl-CoA carboxylase 2	ACC2_ORYSJ	*Oryza sativa*	258	01 metabolism	01.06 lipid and sterol	U
231[a]	Phospholipase D alpha 1	PLDA1_CYNCA	*Cynara cardunculus*	92	01 metabolism	01.06 lipid and sterol	U
268[a]	Pyridoxal biosynthesis protein PDX1.1	PDX11_ARATH	*Arabidopsis thaliana*	33	01 metabolism	01.07 cofactors	U
389	Triosephosphate isomerase	TPIC_SECCE	*Secale cereale*	32	02 energy	02.07 pentose phosphate	D
261[a]	Glucose-6-phosphate 1-dehydrogenase	G6PD_SOLTU	*Solanum tuberosum*	58	02 energy	02.07 pentose phosphate	U
195 [b]	Aconitate hydratase	ACOC_CUCMA	*Cucurbita maxima*	98	02 energy	02.10 TCA pathway	D
308	Isocitrate dehydrogenase [NAD] catalytic subunit 5	IDH5_ARATH	*Arabidopsis thaliana*	41	02 energy	02.10 TCA pathway	D
123	Phosphoenolpyruvate carboxylase 1	CAPP1_ARATH	*Arabidopsis thaliana*	110	02 energy	02.30 photosynthesis	U
287[a]	Phosphoenolpyruvate carboxylase 2	CAPP2_ARATH	*Arabidopsis thaliana*	110	02 energy	02.30 photosynthesis	U
239	Glycine-rich RNA-binding protein blt801	GRP_HORVU	*Hordeum vulgare*	16	04 transcription	04.22 mRNA processing	D
156	60S ribosomal protein L17-2	RL172_HORVU	*Hordeum vulgare*	20	05 protein synthesis	05.01 ribosomal proteins	U
199	40S ribosomal protein S8-2	RS82_ARATH	*Arabidopsis thaliana*	24	05 protein synthesis	05.01 ribosomal proteins	U
202	40S ribosomal protein SA	RSSA_VITVI	*Vitis vinifera*	34	05 protein synthesis	05.01 ribosomal proteins	U
203	40S ribosomal protein S16	RS16_ORYSI	*Oryza sativa*	17	05 protein synthesis	05.01 ribosomal proteins	U
341[a]	60S ribosomal protein L13-3	RL133_ARATH	*Arabidopsis thaliana*	23	05 protein synthesis	05.01 ribosomal proteins	U
313[a]	60S ribosomal protein L23	RL23_ARATH (+1)	*Arabidopsis thaliana*	15	05 protein synthesis	05.01 ribosomal proteins	U
242[a]	60S ribosomal protein L24	RL24_HORVU (+1)	*Hordeum vulgare*	18	05 protein synthesis	05.01 ribosomal proteins	U
208[a]	60S ribosomal protein L7a	RL7A_ORYSJ	*Oryza sativa*	29	05 protein synthesis	05.01 ribosomal proteins	U
291[a]	60S ribosomal protein L18a	RL18A_ORYSJ	*Oryza sativa*	21	05 protein synthesis	05.01 ribosomal proteins	D
343[a]	60S ribosomal protein L30	RL30_EUPES (+7)	*Euphorbia esula*	12	05 protein synthesis	05.01 ribosomal proteins	D
257	60S ribosomal protein L22-2	RL222_ARATH	*Arabidopsis thaliana*	14	05 protein synthesis	05.01 ribosomal proteins	D
276	60S ribosomal protein L10-1	RL101_ORYSI	*Oryza sativa*	25	05 protein synthesis	05.01 ribosomal proteins	D
281	60S ribosomal protein L23a	RL23A_DAUCA	*Daucus carota*	18	05 protein synthesis	05.01 ribosomal proteins	D
331	60S ribosomal protein L6	RL6_MESCR	*M. crystallinum*	26	05 protein synthesis	05.01 ribosomal proteins	D
280	Eukaryotic translation initiation factor 5A-1	IF5A1_ARATH (+16)	*Arabidopsis thaliana*	17	05 protein synthesis	05.04 translation factors	D
272	Eukaryotic translation initiation factor 3 subunit K	EIF3K_ORYSJ	*Oryza sativa*	26	05 protein synthesis	05.04 translation factors	U

225 [b]	Elongation factor 1-alpha	EF1A2_HORVU	*Hordeum vulgare*	49	05 protein synthesis	05.04 translation factors	D
262[a]	Elongation factor 1-delta 2	EF1D2_ORYSJ	*Oryza sativa*	25	05 protein synthesis	05.04 translation factors	U
237[a]	DNA damage-binding protein 1a	DDB1A_ARATH (+1)	*Arabidopsis thaliana*	121	06 protein destination/storage	06.01 folding and stability	U
283[a]	17.4 kDa class I heat shock protein	HSP17_ARATH	*Arabidopsis thaliana*	17	06 protein destination/storage	06.01 folding and stability	U
196	Heat shock protein 90-4	HS904_ARATH	*Arabidopsis thaliana*	80	06 protein destination/storage	06.01 folding and stability	D
358	Heat shock protein STI	STIP_SOYBN	*Glycine max*	64	06 protein destination/storage	06.01 folding and stability	D
315[a]	24.1 kDa heat shock protein, mitochondrial	HS24M_ORYSJ	*Oryza sativa*	24	06 protein destination/storage	06.01 folding and stability	D
288[a]	Chaperone protein ClpB3, mitochondrial	CLPB3_ORYSJ	*Oryza sativa*	109	06 protein destination/storage	06.01 folding and stability	D
223[a]	Proteasome subunit alpha type-4-1	PSA4A_ORYSI (+1)	*Oryza sativa*	27	06 protein destination/storage	06.13 proteolysis	D
246	Proteasome subunit alpha type-1	PSA1_ORYSJ	*Oryza sativa*	30	06 protein destination/storage	06.13 proteolysis	D
320	Ubiquitin-fold modifier-conjugating enzyme 1	UFC1_ORYSI	*Oryza sativa*	20	06 protein destination/storage	06.13 proteolysis	D
165[a]	Proteasome subunit beta type-2	PSB2_ORYSJ	*Oryza sativa*	23	06 protein destination/storage	06.13 proteolysis	U
190	Prob. 26S proteasome non-ATPase regulatory sub 3	PSMD3_DAUCA (+1)	*Daucus carota*	56	06 protein destination/storage	06.13 proteolysis	U
204[a]	26S protease regulatory subunit 10B homolog A	PS10A_ARATH	*Arabidopsis thaliana*	45	06 protein destination/storage	06.13 proteolysis	U
305[a]	26S protease regulatory subunit 6B homolog	PRS6B_ARATH (+1)	*Arabidopsis thaliana*	46	06 protein destination/storage	06.13 proteolysis	U
318	26S proteasome non-ATPase regulatory subunit 2 1A	RPN1A_ARATH	*Arabidopsis thaliana*	98	06 protein destination/storage	06.13 proteolysis	U
314	V-type proton ATPase subunit C	VATC_HORVU	*Hordeum vulgare*	40	07 transporters	07.22 transport ATPases	U
265	GTP-binding protein SAR1	SAR1_TOBAC	*Nicotiana tabacum*	23	07 transporters	07.99 others	U
264[a]	Prob. voltage-gated potassium channel sub-beta	KCAB_ORYSJ	*Oryza sativa*	36	07 transporters	07.01 ions	U
235	Coatomer subunit gamma-2	COPG2_ORYSJ	*Oryza sativa*	99	08 intracellular traffic	08.07 vesicular	U
344	Coatomer subunit beta-1	COB21_ORYSJ	*Japanese rice*	103	08 intracellular traffic	08.07 vesicular	U
159	Histone H2A.1	H2A1_WHEAT (+2)	*Triticum aestivum*	16	09 cell structure	09.13 chromosomes	D
205[a]	Prob. UDP-arabinopyranose mutase 2	RGP2_ORYSJ	*Oryza sativa*	39	09 cell structure	09.01 cell wall	U
241[a]	Actin-11	ACT11_ARATH	*Arabidopsis thaliana*	42	09 cell structure	09.04 cytoskeleton	U
161	14–3–3-like protein GF14-6	14331_MAIZE	*Zea mays*	30	10 signal transduction		D
271	14–3–3-like protein GF14-D	14334_ORYSJ	*Oryza sativa*	29	10 signal transduction		U
167	Ser/thr-prot phosphatase 2A 65 regulatory sub A beta	2AAB_ARATH	*Arabidopsis thaliana*	66	10 signal transduction	10.0407 phosphatases	U
334	Subtilisin-chymotrypsin inhibitor WSCI	ICIW_WHEAT	*Triticum aestivum*	9	11 disease/defense	11.02 defense-related	D
335	1-Cys peroxiredoxin	REHY_MEDTR	*Medicago truncatula*	24	11 disease/defense	11.04 stress responses	D
191[a]	Alcohol dehydrogenase class-3	ADHX_MAIZE	*Zea mays*	41	11 disease/defense	11.03 cell death	U
116[a]	DEAD-box ATP-dependent RNA helicase 15	RH15_ARATH	*Arabidopsis thaliana*	48	11 disease/defense	11.05 stress response	U
260[a]	NADP-dependent alkenal double bond reductase P1	P1_ARATH (+1)	*Arabidopsis thaliana*	38	11 disease/defense	11.05 stress response	U
198	Serpin-Z1B	SPZ1B_WHEAT	*Triticum aestivum*	43	12 unclear classification		U
92	Alpha-amylase inhibitor 0.53	IAA5_WHEAT	*Triticum aestivum*	13	12 unclear classification		D
245	Alpha-amylase inhibitor 0.28	IAA2_WHEAT	*Triticum aestivum*	17	12 unclear classification		D
267	HMG1/2-like protein	HMGL_WHEAT	*Triticum aestivum*	17	12 unclear classification		D

No, protein number; MW, molecular weight (kDa); R., ratio control/hydro-priming; [a]Proteins specifically affected by hydro-priming; [b]Proteins differentially affected by both treatments (up-regulated by AsA and down-regulated by water); U, up-regulated proteins; D, down-regulated proteins.

Table 3 – Wheat metabolic proteins whose abundance varies specifically in ascorbate-primed seeds.

No	Protein name	Accession no	Organism	MW	Function category	Function description	R.
163	S-adenosylmethionine synthase (AdoMet)	METK_WHEAT	*Triticum aestivum*	43	01 metabolism	01.01 amino acid	D
326	Adenylosuccinate synthetase (Fragment)	PURA_WHEAT	*Triticum aestivum*	51	01 metabolism	01.03 nucleotides	U
247	2.3-bisphosphoglycerate-independent phosphoglycerate mutase	PMGI_MAIZE	*Zea mays*	61	02 energy	02.01 glycolysis	D
354	Glucose-6-phosphate isomerase	G6PI_ARALP	*Arabidopsis thaliana*	62	02 energy	02.02 gluconeogenesis	U
259	Citrate synthase	CISY_FRAAN	*Fragaria ananassa*	52	02 energy	02.10 TCA pathway	U
207	Prob. succinyl-CoA ligase [ADP-forming] sub alpha.	SUCA_ORYSJ	*Oryza sativa*	34	02 energy	02.10 TCA pathway	D
294	Pyruvate phosphate dikinase. Chloroplastic	PPDK_FLABI (+1)	*Flaveria bidentis*	104	02 energy	02.30 photosynthesis	D
240	Cell division control protein 48 homolog D	CD48D_ARATH	*Arabidopsis thaliana*	90	03 cell growth/division	03.22 cell cycle	D
347	40S ribosomal protein S3a	RS3A_TORRU	*Tortula ruralis*	29	05 protein synthesis	05.01 ribosomal proteins	U
151	40S ribosomal protein S15a-1	R15A1_ARATH	*Arabidopsis thaliana*	15	05 protein synthesis	05.01 ribosomal proteins	D
150	60S ribosomal protein L3	RL3_ORYSJ	*Oryza sativa*	44	05 protein synthesis	05.01 ribosomal proteins	D
197	60S ribosomal protein L38	RL38_ARATH (+2)	*Arabidopsis thaliana*	8	05 protein synthesis	05.01 ribosomal proteins	D
297	Eukaryotic peptide chain release factor subunit 1-1	ERF1X_ARATH	*Arabidopsis thaliana*	49	05 protein synthesis	05.04 translation factors	D
248	Protein-L-isoaspartate O-methyltransferase	PIMT_WHEAT	*Triticum aestivum*	25	06 protein destination/storage	06.01 folding and stability	D
253	Chaperone protein ClpB2. Chloroplastic	CLPB2_ORYSJ	*Oryza sativa*	109	06 protein destination/storage	06.01 folding and stability	D
252	Protein disulfide isomerase-like 2–3	PDI23_ORYSJ	*Oryza sativa*	47	06 protein destination/storage	06.01 folding and stability	D
263	T-complex protein 1 subunit alpha	TCPA_ARATH	*Arabidopsis thaliana*	59	06 protein destination/storage	06.01 folding and stability	D
273	Stromal 70 kDa heat shock-related protein	HSP7S_SPIOL	*Spinacia oleracea*	65	06 protein destination/storage	06.01 folding and stability	D
285	70 kDa peptidyl-prolyl isomerase	FKB70_WHEAT	*Triticum aestivum*	62	06 protein destination/storage	06.01 folding and stability	D
304	Prob. protein disulfide-isomerase A6	PDIA6_MEDSA	*Medicago sativa*	40	06 protein destination/storage	06.01 folding and stability	U
348	RuBisCO large subunit-binding protein subunit alpha.	RUBA_PEA	*Pisum sativum*	62	06 protein destination/storage	06.10 complex assembly	D
227	Proteasome subunit alpha type-5	PSA5_ORYSJ	*Oryza sativa*	26	06 protein destination/storage	06.13 proteolysis	D
153	Ubiquitin-activating enzyme E1 1	UBE11_WHEAT	*Triticum aestivum*	117	06 protein destination/storage	06.13 proteolysis	D
176	Ubiquitin-NEDD8-like protein RUB1	RUB1_ARATH (+4)	*Arabidopsis thaliana*	17	06 protein destination/storage	06.13 proteolysis	D
244	Alpha/beta-gliadin clone PW1215	GDA6_WHEAT	*Triticum aestivum*	34	06 protein destination/storage	06.20 storage proteins	D
200	ADP.ATP carrier protein 1. mitochondrial	ADT1_WHEAT (+1)	*Triticum aestivum*	36	07 transporters	07.16 purine/pyrimidines	D
376	Actin-7	ACT7_ARATH	*Arabidopsis thaliana*	42	09 cell structure	09.04 cytoskeleton	U
137	Histone H2B.10	H2B10_ORYSI (+22)	*Oryza sativa*	17	09 cell structure	09.13 chromosomes	U
319	Histone H3.2	H32_ARATH (+5)	*Arabidopsis thaliana*	15	09 cell structure	09.13 chromosomes	U
333	Signal recognition particle 19 protein	SRP19_ORYSJ	*Oryza sativa*	15	10 signal transduction	10.04 mediators	D
370	Ser/thr-protein phosphatase PP2A-4 catalytic subunit	PP2A4_ORYSJ	*Oryza sativa*	36	10 signal transduction	10.0407 phosphatases	U
169	Cysteine proteinase inhibitor 12	CYT12_ORYSJ	*Oryza sativa*	27	11 disease/defense	11.02 defense-related	D
340	Selenium-binding protein 1	SEBP1_ARATH	*Arabidopsis thaliana*	54	11 disease/defense	11.04 stress responses	U
282	Dehydrin Rab15	DHR15_WHEAT	*Triticum aestivum*	16	11 disease/defense	11.04 stress responses	D
269	Em protein H5	EM4_WHEAT	*Triticum aestivum*	10	11 disease/defense	11.04 stress responses	D
350	Prob. NADPH:quinone oxidoreductase 1	NQR1_ORYSJ	*Zea mays*	21	11 disease/defense	11.06 detoxification	D
209	Alpha-amylase/trypsin inhibitor CM16	IAC16_WHEAT	*Triticum aestivum*	16	12 unclear classification		D
210	Carbonic anhydrase. Chloroplastic	CAHC_HORVU	*Hordeum vulgare*	35	12 unclear classification		D
s	Serpin-ZX	SPZX_HORVU	*Hordeum vulgare*	43	12 unclear classification		D
78	Lactoylglutathione lyase/glyoxalase	LGUL_ORYSJ	*Oryza sativa*	33	20 secondary metabolism		U

No, protein number; accession no, accession number according to the Swiss-Prot database; MW, molecular weight (kDa); R., ratio control/ascorbate-priming; U, up-regulated proteins; D, down-regulated proteins.

Finally, B was lowered to 5% over 1 min and the column re-equilibrated for 24 min (120 min total run time). MS spectra were collected over an m/z range of 400–1800 Da at 60,000 resolutions, operating in the data dependent mode to automatically switch between Orbitrap-MS and LTQ-MS/MS acquisition. MS/MS spectra were collected for the five most abundant ions in each MS scan ("TOP5 strategy") using a dynamic exclusion limit of 2 MS/MS spectra of a given mass for 30 s with exclusion duration of 100 s. CID was performed with normalized collision energy set at 35 V. In order to assess the additional variation introduced into the measurements by the technical procedure and to increase the number of identified proteins, we performed three technical replicates (LC–MS/MS runs) for each of the three experimental replicates.

2.6. Database searching and protein identification

Raw MS/MS data files from Xcalibur software (version 2.0.7 SP1, Thermo Fisher) were submitted to Proteome Discoverer software version 1.3 (Thermo Scientific) for peptide/protein identification. The searches were performed against Swiss-Prot database (version 57.15). Thermo Finnigan LCQ/DECA RAW file data import filter was used. The search was limited to proteins from species of the Viridiplantae (green plant) taxonomy entries and performed using the built-in decoy search option of Mascot. Enzymatic digestion with trypsin was selected, with maximum 2 missed cleavages, peptide charges +2 and +3, and a precursor mass tolerance of 10 ppm and 0.8 Da fragment mass tolerance; acetylation (N-term), oxidation (M) and deamidation (N, Q) were used as dynamic modifications; carbamidomethylation (C) was used as static modification.

2.7. Scaffold analysis

Scaffold software (version 3.1.2, Proteome Software Inc.) [27] was used to validate MS/MS based peptide and protein identifications and for label-free relative quantitation based on spectral counting. Spectral counting calculates the number of MS/MS scans that are attributed to the same peptide ion. The frequency of these MS/MS scans correlates with the abundance of a given peptide from a protein in the sample. The number of spectra matched to peptides from a protein is used as a surrogate measure of protein abundance. Normalization will standardize the spectral counts by multiplying some fractional amount across samples so that the total number of spectra is the same within each category and then across all categories, as from Scaffold's manual. The additional X! Tandem search engine (The GPM, Cyclone version 2010.12.01.1) was also chosen, keeping the same parameters previously used for Mascot. According to the Peptide and Protein Prophet algorithms [28,29] implemented into Scaffold, the peptide probability was set to minimum 95%, whereas the protein probability was set at 99%, with at least two identified peptides, resulting in a false discovery rate (FDR) for peptides and proteins ≤0.2% (where only 1 decoy hit was observed). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony. Fisher's exact test was used to identify statistically significant differences between unprimed seeds and primed seeds (hydro-primed or ascorbate-primed). Proteins, which had at least a two-fold difference for the mean ratio, as well as a Fisher's p-value ≤0.05, a relative standard deviation (RSD) of experimental replicates ≤40%, and appear in more than one biological replicate, were considered present in the two samples in significant different quantities.

2.8. Functional classification

Gene Ontology (GO) data about the biological processes of identified proteins were obtained by means of Scaffold's built-in option and according to Bevan et al. [30].

3. Results

3.1. Design of the experiment and proteomic approach

As a first step toward characterizing proteins that potentially associated with priming-induced salt stress tolerance in durum wheat, a comparative gel-free proteomic analysis was carried out using primed and unprimed seeds. In this work, we are also interested in examining whether the effects of seed priming can be considered as an advance in germination corresponding to the realization of germination-related processes (i.e., water uptake, cell divisions), or involve other particular mechanisms. Toward this goal, we used durum wheat seeds of varying vigor as generated by hydro- and ascorbate-priming treatments.

As expected, seed germination and seedling emergence of durum wheat are significantly ($p \leq 0.05$) improved by seed priming (Fig. 1A). The primed seeds also showed an advance in germination time as compared to untreated controls. Ascorbate-priming seems to be more effective than hydro-priming in induction of salinity tolerance in durum wheat. The results also showed that seed priming, especially with AsA, enhanced all the growth parameters of the durum wheat seedlings, particularly the coleoptile growth, which appears to be the most affected by salinity stress (Fig. 1B).

Given the importance of the sample preparation step for the analysis of MS-based proteomics, in this study, it has been decided to combine two methods. First, a fractionation method based on the specific solubility properties of different classes of wheat seed proteins, since fractionation is essential to uncovering low-abundance proteins in complex protein mixtures [31]. Second, an enrichment method for less abundant proteins using hydrogel nanoparticles, which may further increase the number of identified proteins as revealed in our previous study [20]. This approach has proved to be effective, and a large number of proteins have been identified. The combination of the two protocols, standard in-solution trypsin digestion and hydrogel nanoparticle enrichment (see Materials and methods), allows us to identify a total of 380 proteins using the average of 1830 distinct peptide sequences per sample (Supplement Table S1). Furthermore, the choice of an enrichment step, followed by an in-solution digestion, represents a fast and simple protocol allowing us to increase significantly the number of identified proteins, as shown in Table 1, providing additional information to that obtained by the standard.

To ensure the accuracy, reproducibility, and reliability of protein identification, three experimental replicates and three technical replicates were performed [32]. In order to check the correspondence among technical replicates, the unweighted spectral counts of each replicate from the same experimental sample were plotted against each other and evaluated using Scaffold's built-in option. The same assessments were applied to the experimental and control groups.

3.2. *Proteomics of durum wheat seed metabolic proteins*

Of a total of 380 identified proteins, 182 were significantly up- or down-regulated in response to priming (Supplementary material Table S1). However, applying the other conditions (i.e., at least a two-fold difference for the mean ratio; protein identified in more than one biological replicate) the number of identified proteins was reduced to 155. Of these, 72 proteins were differentially accumulated in hydro-primed seeds, among which 43 were up-regulated and 29 were down-regulated (Fig. 2 and Table 2). However, 83 proteins were found to be differentially accumulated in ascorbate-primed seeds, 30 of these were up-regulated and 53 were down-regulated (Fig. 2 and Table 3).

3.3. *Functional protein classification*

Tables 2 and 3 (summarized in Fig. 3) list 112 proteins among the 155 identified (43 are redundant proteins). These proteins were grouped into 13 functional categories according to Bevan et al. [30]. In certain cases, subcategories were devised for clarity. The graphical view highlights five biochemical processes – central metabolism (amino acids, lipids and carbohydrates), energy, protein synthesis, protein folding/assembly and storage, and stress-related proteins – that comprised ~80% of the total of proteins identified (Fig. 3). 6% of the proteins have been unclassified.

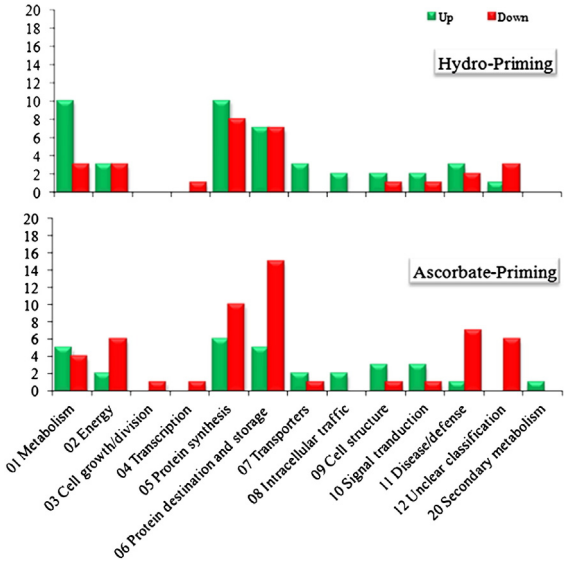

Fig. 3 – Functional distribution of the 121 proteins identified in hydro-primed and ascorbate-primed wheat seeds. Up, up-regulated proteins; Down, down-regulated proteins. Functional classes according to Bevan et al. [30].

3.4. *Comparative analysis of identified proteins*

Fig. 4 shows a comparative view of the 112 proteins whose abundance varied in response to seed priming. Among them, 43 were equally affected by both seed pretreatments (Table 2; Supplementary material Table S2). Of these, 41 proteins showed similar changes in their abundance, whereas two were differentially affected. Hydro-priming specifically affects the abundance of 29 proteins most of them belonging to the metabolism (particularly lipid metabolism) and protein synthesis categories (Figs. 2 and 4; Table 2 and Supplementary material Table S3). However, besides the 43 shared proteins, ascorbate-priming specifically affects the abundance of 40 proteins; most of them belonging to energy, protein destination and storage, and disease/defense protein categories (Figs. 2 and 4; Table 3 and Supplementary material Table S4).

4. Discussion

Salinity is probably the major abiotic stress that threatens crop productivity worldwide [5,6]. Poor seed germination and seedling emergence are among major consequences of salinity [33]. Seed priming, however, has been successfully used to improve germination and emergence in seeds of many crops [10–13,23,24,34]. This is of particular importance for wheat, since it seems to be more sensitive to salinity during the early seedling stage [23,33]. Consistent with this, priming durum wheat seeds resulted in an improved germination and seedling growth (Fig. 1). Since the improvement of seed vigor is both of academic and economical interest, a great deal of research has been done in the last few years trying to test, develop and eventually promote seed priming for improving the germination rate and uniformity of growth of many vegetables and field crops [10,13,22,23]. Nevertheless, the molecular mechanisms of priming as it relates to stress tolerance in germinating seeds remain largely unclarified. In the last decade, many comparative studies have been performed using gel-based proteomic approach to identify potential biomarkers of seed vigor under primed and non-primed conditions [11–13,35]. Although the results achieved to date are impressive, this goal remains elusive [7,14] mainly because of the high dynamic range in the abundance of particular proteins (i.e., storage proteins) [13] and the limited resolving power of gel-based approach, which only allows separation of proteins within certain isoelectric point (Ip) and molecular weight restrictions [36]. Gel-free proteomic approach is able to overcome most of these difficulties [32] and consequently, provide strong impetus to gain better understanding of the underlying mechanisms of seed vigor and priming-induced stress tolerance [7,10,11]. Here we report, for the first time, a comparative gel-free shotgun proteomic analysis of metabolic proteins extracted from durum wheat seeds of varying vigor as generated by hydro- and ascorbate-priming treatments.

4.1. *Proteomics of wheat hydro-primed seed metabolic proteins*

This study disclosed 72 proteins that were differentially accumulated in wheat seeds during hydro-priming (Table 2;

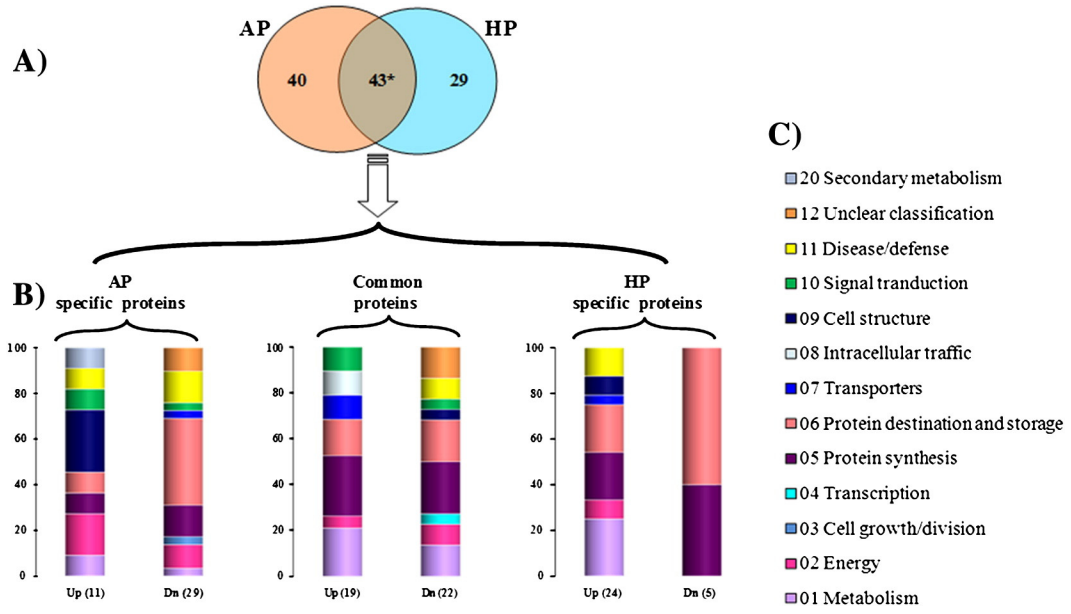

Fig. 4 – Venn diagrams and functional classification of wheat proteome in hydro-primed seeds compared to ascorbate-primed seeds. (A) Venn diagram displaying the overlap in the proteome of primed durum wheat seeds (hydro-priming vs. ascorbate-priming). (B) Functional categories of proteins shown in (A). Up, up-regulated proteins; Dn, down-regulated proteins. The number of identified proteins is indicated. (C) Functional classes according to Bevan et al. [30]. *2 proteins among them were differentially regulated by both priming treatments.

Supplementary material Table S2). Among these, proteins belonging to the functional category of "proteins destination and storage" were highly represented (Figs. 2 and 3; Table 2). In wheat, albumins and globulins constitute about 11% of total flour protein and have mainly metabolic activity or structural functions [22]. Even though wheat seed storage

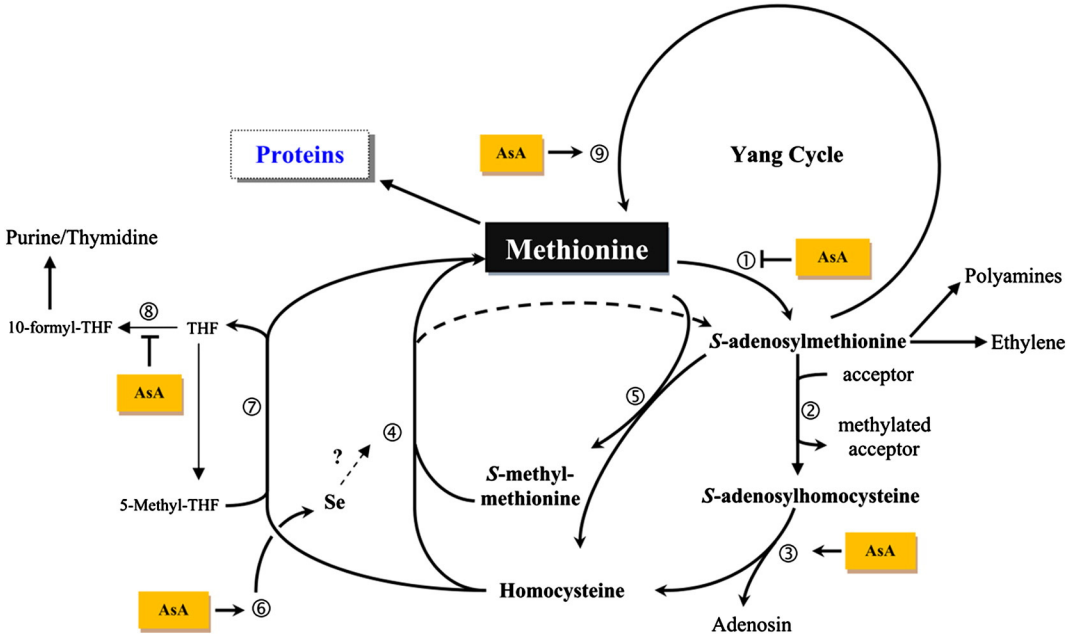

Fig. 5 – Scheme showing the possible changes in methionine metabolism induced by ascorbate-priming. Enzymes: (1) SAM synthase; (2) SAM-dependent methyltransferase; (3) S-AdoHcy hydrolase; (4) S-AdoMet:Hcy methyltransferase; (5) SMM: Hcy methyltransferase; (6) selenium binding protein 1; (7) Met synthase; (8) FTHF-ligase; (9) methylthioribose-1-phosphate isomerase. See text for details. SAM: S-adenosylmethionine; SMM: S-methyl-methionine; S-AdoHcy: S-adenosylhomocysteine; S-AdoMet: S-adenosylmethionine; Hcy: homocysteine; FTHF: formate-tetrahydrofolate.

proteins were not considered in this study, the hydro-priming treatment resulted in the up-accumulation of proteins involved in proteolysis (protein nos. 165, 190, 204, 305, 318) as well as transport, and intracellular trafficking (protein nos. 235, 264, 265, 314, 344). Interestingly, among these proteins, V-type proton ATPase (protein no. 314, identified for the first time) is described as involved in the regulation of the proteolysis of stored proteins by acidification of the protein storage vacuole [37]. This finding indicates that hydro-priming promotes the mobilization of storage proteins, as previously suggested [11–13]. This initial mobilization of the storage proteins is probably required to facilitate their further proteolytic degradation during seed germination and seedling emergence [10–13]. Furthermore, in this functional category several heat shock proteins (HSPs) and a DNA damage-binding protein were identified as changing in abundance during hydro-priming (Fig. 3; Table 2; Supplementary material Table S2). The HSPs are involved in diverse cellular processes, including regulation of protein degradation during seed germination [38] and probably during seed priming [12,13,39]. However, DNA damage-binding proteins are a component of the E3 ubiquitin–protein ligase complex, and seem to be essential for normal seed germination and post-germination growth, as recently reported in *Arabidopsis* [40]. In this study, the up-regulation of a small heat shock protein (17.4 kDa class I HSP) and a DNA damage-binding protein (DDB 1a, previously unidentified protein), along with a GTP-binding protein SAR1 (protein no. 265), suggests that mobilization of seed storage proteins during hydro-priming is likely to be under control.

In the "protein synthesis" category, several proteins were found to be up-regulated during hydro-priming (Table 2; Supplementary material Table S2). Previously, it has been reported that protein synthesis increases substantially during priming, and this including both the quantity and the type of the proteins being synthesized [41]. Also, it has been revealed that within a few minutes after rehydration, the number of single ribosomes declines, as they become recruited into polysomal protein-synthesizing complexes [42,43]. Even though initial protein synthesis is dependent on extant ribosomes, newly synthesized ribosomes are produced and used within a few hours after completion of the initial polysome assembly during germination [43,44]. Consistent with this, while six individual ribosomal proteins (protein nos. 276, 257, 281, 291, 331, 343) were down-regulated after hydro-priming, eight other ribosomal proteins (protein nos. 203, 199, 202, 156, 341, 313, 241 and 208) were found to be up-regulated by this process (Table 2 and Supplementary material Table S2). Furthermore, in this functional category, several elongation factors (EF) and eukaryotic translation initiation factors (eIF) have been found to changes in abundance in response to hydro-priming (Table 2 and Supplementary material Table S2), consistent with results obtained in *Arabidopsis* [39], sugar beet [12] and alfalfa [13] seed during priming. In this study, the abundance of eIF3K, eIF4A and many EF (protein nos. 44, 128, 225, 262) was found to be up-regulated in hydro-primed seeds (Table 2). However, it is interesting to note that eIF5A-1 was down-regulated during this treatment. This initiation factor, previously unidentified, is a highly conserved factor and thought to be necessary for selective mRNA stabilization and translation. In line with this, the stability of stored mRNAs was shown to be an important determinant of seed vigor [45]. Therefore, it will be very interesting to investigate the potential involvement of this initiation factor in seed vigor and invigoration.

In the "metabolism" category, abundance of fifteen proteins was found to be affected by hydro-priming treatment. Among them, six proteins are involved in carbohydrate metabolism (Fig. 3; Table 2; Supplementary material Table S2). In seeds, galactose-containing oligosaccharides (raffinose family, RFOs) and polysaccharides (galactomannans) are among the most prominent soluble sugars, and serve as an essential source of rapidly metabolizable carbon for early germination [46] and seed priming [13,47]. Thus, it is not surprising that the abundance of α-galactosidase and sucrose-phosphate synthase 4 (SPS4), which plays a pivotal role in the conversion of starch or fatty acids into sucrose [48], was strongly up-regulated during hydro-priming. Also, in this functional category, the abundance of five proteins involved in amino acid metabolism was found to be changed by hydro-priming (Fig. 3; Table 2; Supplementary material Table S2). Methionine (Met) is a fundamental amino acid as the building block for the biological universal methylating agent, S-adenosylmethionine (AdoMet), and as the precursor of polyamines, the plant-ripening hormone ethylene, and the vitamin biotin [8]. Previous reports suggested that the methyl cycle is activated during seed priming [12,13]. In agreement, in this study the abundance of three enzymes involved in sulfur amino acid metabolism (protein nos. 147, 212, 311) as well as a pyridoxal biosynthesis protein (PDX1.1), which is involved in biotin metabolism, was found to be up-regulated in response to hydro-priming. In addition, two enzymes involved in lipid metabolism (protein nos. 274, 231) were specifically up-regulated by hydro-priming treatment (Fig. 3; Table 2; Supplementary material Table S2). Similar results were reported in previous studies [12,13]. These findings presumably reflect an initiation of seed storage mobilization in response to hydro-priming.

In the "disease/defense" category, many proteins were found to change in abundance during hydro-priming (Table 2; Supplementary material Table S2). Previous reports revealed that priming up-regulates the abundance of several proteins associated to detoxification and stress response [12,13,39]. In the present study, three stress-related proteins (protein nos. 161, 191, 260) were found to be up-regulated during hydro-priming. Interestingly, the abundance of 1-Cys peroxiredoxin (1-CysPrx) was down-regulated. This enzyme is a peroxidase specifically and highly produced in seeds, localized in nuclei of scutellum and aleurone cells [49] and seems to be involved in the inhibition of germination particularly under salt, osmotic and oxidative stress conditions [50]. Together, these findings confirm and extend previous observations that seeds experience osmotic stress in the limiting water conditions (i.e., restricting the period of germination on water, using osmotic solution) used in priming treatments [13,39].

4.2. *Proteomics of wheat ascorbate-primed seed metabolic proteins*

Ascorbic acid is one of the most important metabolites involved in cell division, osmotic adjustment [51], and plays vital role during the onset of germination [52]. To date, only

few reports are available regarding the biochemical effects of ascorbate-priming or pretreatment on the germination of wheat seeds, most of them are in disagreements [53–55]. To further characterize the mechanisms involved in improving seed vigor by ascorbate-priming treatment as shown in this study (Fig. 1) and many others [23,55,56], we carried out for the first time a comparative analysis between the proteome of hydro-primed seeds with that of ascorbate-primed seeds.

As shown in Fig. 3 and Table 2, ascorbate-priming treatment displayed both similarities and differences compared with the hydro-priming treatment. Besides the 43 proteins whose abundance varied in common with hydro-priming treatment and for whose significance was discussed above, 40 proteins showed significant change (>2-fold; $p \leq 0.05$) in their abundance during ascorbate-priming (Table 3; Supplementary material Table S4). Among these proteins, there were 29 down-regulated proteins (Fig. 4).

Dehydration and rehydration during seed development and germination are associated with high levels of oxidative stress, resulting in DNA and protein damage [8]. These detrimental conversions can lead to the recognition, tagging, and destruction of the altered proteins [57]. In the present study, the abundance of many proteins involved in the protection, repair of damaged proteins, such as protein-L-isoaspartate O-methyltransferase (PIMT), protein disulfide isomerase-like 2–3 (PDIL2–3) and HSP7S, which are able to protect the cell from oxidative damage [13], was found to be decreased during ascorbate-priming (Fig. 3, Table 3, Supplementary material Table S4). On the contrary, the lactoylglutathione lyase (LGL) or glyoxalase I, which is involved in the glutathione-based detoxification of methylglyoxal (MG) especially during oxidative stress [58], was found to be increased in abundance in response to ascorbate-priming. In similar way, nucleoside diphosphate kinase 1 (NDK 1) was found to be up-regulated during ascorbate-priming (Table 3; Supplementary material Table S4). Interestingly, it has been reported that oxidative stress conditions strongly induced the NDK gene expression, the over-expression of which reduced the accumulation of ROS [59]. Altogether, these findings corroborate recent data [12,13,39], suggesting that seed priming initiates an oxidative stress, which is presumably attenuated by the ascorbate treatment.

The methionine biosynthesis pathway is essential for cell viability in that it provides building blocks for proteins and generates the precursor to S-adenosylmethionine (AdoMet), which is the main methyl group-donating compound in cells [8]. Previously, it has been reported that Met metabolism plays an important role during seed filling, seed germination and priming [13,39,60]. Consistent with this, in this study, the abundance of many proteins/enzymes involved in methyl cycle (protein nos. 117, 163, 169, 193, 248, 326, 340) was found to be affected by ascorbate-priming treatment (Tables 2 and 3; Supplementary material Table S2 and S4). However, it is interesting to note that adenosylhomocysteinase (AdoHcy hydrolase) was up-regulated whereas S-adenosylmethionine synthetase was down-regulated during ascorbate-priming (Table 3, Supplementary material Table S4). AdoHcy hydrolase catalyzes the production of AdoHcy (Fig. 5). AdoHcy is the product of all AdoMet-dependent biological transmethylation reactions and is a potent competitive inhibitor of S-adenosylmethionine-dependent methyl transferase reactions that are crucial to growth and development including seed germination [61]. This enzyme, presumed to play a key role in the control of DNA and other substrates methylation, may influence the expression of certain genes, such as genes related to seed germination and dormancy [62]. Thus, it will be interesting to investigate the possible role of AdoHcy hydrolase in seed priming, as previously performed in seed germination [63].

In similar way, ascorbate-priming appears to strongly enhance the abundance of selenium binding-protein 1 (>5-fold), which binds cadmium and mediates lower sensitivity to stress requiring glutathione (GSH) for tolerance [64]. In animals, selenium deficiency is shown to decrease specifically the activity of betaine homocysteine methyltransferase that catalyzes the conversion of homocysteine to methionine, suggesting the involvement of selenium binding-protein in methionine biosynthesis [65]. In plants, numerous studies reported the importance of selenium in seed germination, seedling growth, and many metabolic processes notably under stress conditions [66–68], suggesting the possible involvement of selenium binding-protein 1 in methyl-methionine cycle (Fig. 5).

Furthermore, the level of methylthioribose-1-phosphate isomerase, which catalyzes the biosynthesis of methionine via salvage pathway or Yang cycle [69], appears to up-regulate during ascorbate-priming (Fig. 5). Finally, there was a decrease in abundance of the enzyme formate-tetrahydrofolate ligase (FTHF-ligase) that catalyzes the interconversion of THF with 10-FTHF, which functions as one-carbon donor and plays a critical role in thymidine and purine biosynthesis (Supplementary material Table S4). This is consistent with a decreased abundance of FTHF-ligase during seed priming [12], and suggests that THF was used for the recycling of Met.

Altogether, these findings underpin the importance of the ROS-scavenging and the antioxidant defense system in improving germination and seedling growth of durum wheat under salt stress, and suggest a possible role of methionine in seed invigoration by seed priming with ascorbate.

5. Conclusions

Our study presents a proteomic pipeline for extensive characterization of metabolic proteins from durum wheat seeds using solubility-based protein fractionation and protein enrichment combined with gel-free, label-free quantitative tandem MS (LTQ-Orbitrap). The application of an enrichment method, such as hydrogel nanoparticle fractionation, has enhanced the identification of a large part of the metabolic proteins. Accordingly, we propose that this approach could be used to acquire a wider perspective and a better understanding of the seed proteome.

Furthermore, the above approach was successfully applied to investigate the possible biomarkers of priming-induced salt tolerance in durum wheat. The results give evidence that priming enhanced wheat seed vigor under salt stress conditions not only by advancing germination-related processes, but also by affecting the abundance of many proteins, most of which are involved in protein metabolism and stress response. More interestingly, besides the initiation of oxidative stress defense, ascorbate-priming appears to specifically affect the methionine-related metabolism.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jprot.2013.08.010.

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