Acta Physiol Plant (2016) 38:146
DOI 10.1007/s11738-016-2166-y

ORIGINAL ARTICLE

Exogenous melatonin expediently modifies proteome of maize (*Zea mays* L.) embryo during seed germination

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Received: 26 November 2015 / Revised: 6 May 2016 / Accepted: 9 May 2016 / Published online: 19 May 2016
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Abstract Melatonin (*N*-acetyl-5-methoxytryptamine) has a great potential for plant biostimulation. Its role in plant physiology is intensively explored, and its important function in plant stress defence frequently underlined. Melatonin is particularly effective when applied as an additional factor of seed priming. In the presented research, hydroconditioning was chosen experimentally as optimal for maize (*Zea mays* L.) seeds. The following seed variants were compared: controlled non-treated, hydroprimed with water and hydroprimed with melatonin solutions 50 and 500 μM. To identify modifications in proteome of maize seeds caused by the applied hydroconditioning techniques, protein extracts of germinated seed embryos (24 h, 25 °C) were separated by 2D-PAGE. Next, obtained maps of proteomes were compared (statistically and graphically) using PDQuest software, and characteristic spots of proteins were analysed qualitatively by mass-spectrometric techniques and identified in the Mascot protein databases. Research helped to identify hydropriming-associated proteins, and for the first time those which were expressed only in the presence of melatonin. Study confirmed that suitably selected pre-sowing treatment with melatonin, by embryo proteome modification, effectively prepares plants to adverse environmental conditions. In melatonin treated seeds during the initial state of embryos growth, even under optimal conditions, additional antioxidative, detoxifying, anti-stresses proteins were synthesized. Moreover, the supply of energy from seed storage substances was pretty intensified. The presented results partially explain how melatonin acts in plant stress defence, and why plants with higher melatonin content have exhibited increased capacity for stress tolerance.

Keywords Germination · Melatonin · Maize · Proteomics · Seed conditioning · *Zea mays*

Communicated by M Hajduch.

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Introduction

Problems related to seed quality, their germination, seedling emergence, and development under various environmental stresses firmly influence on crop establishment and prospective yield. Seed priming/conditioning has become a common practice to obtain optimal seed performance and ultimate yield notably under adverse germination conditions (Jisha et al. 2013; Mabhaudhi and Modi 2011). Conditioning is defined as pre-sowing treatment in water that, by controlled seed hydration, leads to limited imbibition but prevents the emergence of radicle (Bradford and Bewley 2002). Seeds after priming can be redried to the initial moisture content and stored. Such conditioning can accelerate seed germination, improve seedling uniformity, and increase yields in various crops (Eskandari 2013; Rahimi, 2013). Among various conditioning techniques, e.g., osmopriming, matrix priming and hydropriming—the latter one is simple and low cost and, therefore, extremely

welcomed by farmers. Water pre-sowing treatments give the opportunity to apply biostimulators beneficial for plant. Biostimulators are different harmless substances mostly of natural origin that are able to improve plant development and stimulate life processes but in a different way than phytohormones and/or fertilizers. Their influence on plants is not the consequence of their direct ability to regulate metabolism, and their action could be multidirectional. The crucial point is that biostimulators in contrast to hormones improve plant metabolic processes without changing their natural pathway. They can facilitate the uptake of nutrients, stimulate root growth, also contribute to higher yield, and improve its quality via increase plants resistance/tolerance to unfavourable conditions, such as drought, extreme temperature, toxic pollutions, and so on (Basak 2008). Some studies indicate such a beneficial role of various substances, e.g. glycinebetaine, proline, natural nitrophenols (Asashi SL) as supplementation of irrigation into roots, with foliar fertilizers or protection spraying and also as seed treatment. Our previous research showed efficient conditioning methods for cucumber, cabbage and corn seed quality amelioration, with simultaneous melatonin application (Janas et al. 2009a; Posmyk et al. 2008, 2009a, b).

In general, results of our team suggest that melatonin (*N*-acetyl-5-methoxytryptamine) has a significant potential for biostimulation of plants (Janas and Posmyk 2013). Moreover, it is of natural origin, biodegradable, and harmless. Currently, the role of melatonin in plant physiology is being widely investigated. Solid evidence indicates melatonin as a growth promoter and plant development factor (Arnao and Hernández-Ruiz 2007; Chen et al. 2009; Hernández-Ruiz et al. 2005; Hernández-Ruiz and Arnao 2008; Sarrou et al. 2014; Zhang et al. 2014). The pre-sowing seed conditioning with melatonin protected cabbage seedlings against toxic effects of Cu^{2+} (Posmyk et al. 2008). Moreover, melatonin application into maize and cucumber seeds had a positive effect on seedling development and yield of plants that grown from them, especially those subjected to water-stress (Zhang et al. 2013) or cold (Posmyk et al. 2009a, b).

The physiological effects of seed conditioning have been extensively studied in a wide range of crops (Chen and Arora 2013; Di Girolamo and Barbanti 2012). However, until now, the performance of primed seeds can only be verified by germination tests, which provide retrospective indication of the conditioning affects (Gallardo et al. 2001). Therefore, it is necessary to develop molecular markers improved vigour of primed seeds for commercial seed lots. Proteomics become widely applicable in seed research as a novel tool for protein characterisation and function analysis (Catusse et al. 2011; Gallardo et al. 2001; Wu et al. 2011). Seeds develop differently in dicots and monocots, especially the changes induced by priming treatment. Therefore, the results obtained from *Arabidopsis* may not be applicable in monocot crops (e.g., maize). To date, the proteomic analysis of protein profiles of seed priming in maize (*Zea mays* L.)—one of the most important crops widely planted in the world—has not been done yet. There is lack of data concerning effects of melatonin applied by hydropriming on protein profiles in germinating seeds.

This study aimed to analyse the hydropriming-induced modifications in corn embryo proteome and to indicate hydropriming-associated and melatonin-associated proteins in corn seeds. Comparative proteomic analysis was applied to identify differentially expressed proteins in the untreated and hydroconditioned seeds and in those hydroprimed with melatonin. The possible roles of indicated proteins in maize seed vigour were discussed.

Materials and methods

Plant material

Grains of *Zea mays* L. var. Ambrozja were provided by TORSEED (Torun, Poland). Before experiments, seeds were stored at room temperature, in darkness, under dry conditions, in tightly closed boxes.

Hydropriming

Hydropriming was conducted by corn grains imbibing at 25 °C for 3 h, using distilled water or melatonin water solutions (50 or 500 μM). During conditioning seed, moisture content was increased from an initial 8.8 % (±0.2) to final 36.8 % (±2.3). The water quantity needed to enhance seed humidity was established experimentally. After soaking, seeds were redrying during 3 days at room temperature (20–23 °C; 40 ± 5 % RH) to obtain initial water content. Maize seed variants: non-treated (nt—gel A), hydroprimed (H—gel B), hydroprimed with melatonin 50, and 500 μM (HMel50—gel C and HMel500—gel D, respectively).

Seed germination

All investigated seed variants were germinated in darkness at 25 °C for 24 h. Afterwards, axes were isolated, treated with liquid nitrogen, and then lyophilized.

Protein preparation

Lyophilized tissue (200 mg) was homogenised (in ice bath) with 1.5 ml of extraction buffer 50-mM TrisHCl (pH 7.7) containing: 0.5-mM deoxycholate sodium salt (DOXC);

10-mM DL-dithiothreitol (DDT); and 10-mM EDTA; 1-mM phenylmethanesulfonylfluoride (PMSF). The homogenate was centrifuged: 20,000*g*/5 °C/10 min. The pellet was discarded, whereas proteins resuspended in the collected supernatant were precipitated with 0.07 % mercaptoethanol and 10 % trichloroacetic acid (TCA) in acetone, at 0 °C, during 45 min. The mixture was centrifuged at 20,000*g* for 10 min at 5 °C. The obtained pellet with precipitated proteins was then washed twice with 0.07 % mercaptoethanol in acetone. After the final centrifugation: 3000*g*/5 °C/5 min, sediment was vacuum-dried and then rediluted in buffer of Rabilloud consisting of: 65-mM DTT, 4 % CHAPS and 8 M urea. The proteins content was estimated in 96 microplates by Bradford (1976) method. The protein extracts were stored at −20 °C and then used for 2D PAGE.

2-D protein separation and image analysis

Protein separation and analysis was performed according to Kolodziejczyk et al. 2016. Samples of 300-mg proteins were applied to 11-cm IPG strips (pH 3–10, non-linear gradient ReadyStrip) in a Protean IEF cell (Bio-Rad, Hercules, CA) and in-gel rehydrated for 12 h. According to the isoelectric point, proteins were separated in the IEF-SYS (Scie-Plas Ltd. GB). Isoelectric focusing (first dimension separation) was performed at 18 °C for 72 h at 0–3000 V gradually changing (first 24 h: 0–1500 V; second: 1500–3000; third 3000 V). Before the second dimension PAGE, the gel strips were equilibrated twice with Eq solution (Eq: 50-mM Tris–HCl, pH 8.5, 6 M urea, 30 % glycerol, 10 % SDS) containing 1 % DTT for 15 min and next with Eq solution containing 2.5 % iodoacetamide. Then strips were applied to SDS–PAGE (4 % stacking gel and 12 % analytical gel) and fixed with 0.5 % agarose in 0.5 M Tris–HCl buffer (pH 6.8) and 10 % SDS. Appropriate protein markers (Thermo Scientific Page-Ruler Plus Prestained Protein Ladder No 26619) were loaded beside the strip. PAGE was performed in TV400YK Cooled Twin-Plate Maxi-Gel Electrophoresis Unit (Scie-Plas Ltd. GB) with SDS electrophoresis buffer containing: 25-mM Tris base, 192-mM glycine, 0.1 % SDS; for 2 h at 100 V and next for 2 h at 200 V, at constant temperature 25 °C. At least four independent biological replicates were performed to ensure the reliability of the results.

Separated proteins were visualised in gels using Imperial™ Protein Stain (Pierce). Then, stained gels were scanned at 300-dpi resolution using an Epson Perfection V700 Photo scanner. Obtained images were analysed with the PDQuest version 8.0.1 software (BioRad). Sample quantification was performed with four analytical gels originating from independent biological replicas. The volume of each spot was normalised to a relative volume, and mean values calculated from repeated data were compared. Qualitative and quantitative analysis sets were prepared. The cutoff for differentially expressed proteins was a fivefold change.

Protein digestion

Indicated protein spots were cut out from stained gels and subjected to in-gel tryptic (Promega, Madison, WI) digestion, as it was described previously by Kołodziejczyk et al. 2016.

Every gel piece containing the protein of interest was first decolorized in solution: 50-mM NH_4HCO_3/acetonitrile (ACN) (50:50, v/v) for 15 min at room temperature. The colourless gel pieces were washed with ACN to dehydrate the band pieces. For reducing and alkylation, gel pieces were then washed with 10-mM DTT in 100-mM NH_4HCO_3 and incubate at 56 °C for 30 min, then with 50-mM iodoacetamide in 100-mM NH_4HCO_3 and incubate at room temperature for 30 min in dark. Then, the pieces were washed twice alternately with 100-mM NH_4HCO_3 and ACN. Finally, the band pieces were dried at room temperature and rehydrated overnight at 37 °C in 25-mM NH_4HCO_3 with trypsin (Promega). After this digestion, gel bands were extracted twice with 2 % ACN/0.1 % trifluoroacetic acid (TFA) to collect the remaining peptides. The obtained supernatant was used for LC–MS/MS analysis.

LC–MS/MS analysis

LC–MS/MS analysis was performed on Eksigent Express HT Micro LC and AB Sciex QTRAP 3200 mass spectrometer with a microspray ESI ion source installed, as it was described by Kołodziejczyk et al. 2016.

Samples were ultrasonicated for 30 s, shaken on vortex, and centrifuged for 5 min. 10 µl of the protein sample was applied by injection mode directly on Eksigent C8-CL-120 column (3 µm 120 Å, 0.5 × 100 mm) at 40 °C. The mobile phase was: water with 0.1 % formic acid (A) and ACN with 0.1 % formic acid (B). Gradient profile (10 µl/min constant flow conditions) started from 95 % A for 1 min, followed by 60 % A after 40 min and 5 % in 45 min and maintained until 50 min of the run. The column equilibration under the initial gradient conditions was applied from 50.1 to 53 min and as a prerun for 1 min before subsequent injection. MS/MS detection was performed using information-dependent acquisition method (IDA) composed of mixed positive ionization scan modes and IDA criteria for dynamic *m*/*z* filtering. The process was constructed as follows: enhanced MS scan (EMS), enhanced resolution scan (ER), IDA criteria and enhanced product ion scan (EPI). Overall survey of EMS scan parameters comprehended the mass range 500–1400 Da

with scan speed 4000 Da/s and 50–70 V declustering potential (DP) values. ER scan was active in 250 Da/s scan rate (DP = 50–70 V) and was used for peptide charge determination. EPI scan was active in the range 50–1600 *m/z* (DP = 60 V and optimised rolling collision energy) and was applied for mass spectra collection and for the peptide sequence analysis. The ion source parameters were: CUR: 25.00; TEM: 400.00; GS1: 20.00; GS2: 40.00; ihe: ON; IS: 5000.00; CAD: high. The most important IDA criteria applied for selective *m/z* filtering were as follows: dynamic background subtraction—on, choose 1–3 most intense peaks from range 500–1400 *m/z* which charge state is 2–4 (include unknown) and exceeds 10,000 counts intensity, exclude former target ions for 30 s after 3 occurrences and use enhanced resolution scan to confirm charge state.

Database searching

The ProteinPilot v 4.0.8 software (AB Sciex, USA) and the MASCOT search engine v. 2.3 were applied for the database searching, as those were described previously by Kołodziejczyk et al. 2016.

The data were searched against the *Zea mays* (over 212,000 sequences) database extracted from NCBI (version 05.2013). Mascot MS/MS ion searches were conducted using trypsin as the digesting enzyme, up to two missed cleavages were tolerated, and the following modifications were applied: acetyl (N-term), carbamidomethyl (C), carbamyl (N-term), deamidated (NQ), formyl (N-term), Gln to pyro-Glu (N-term Q), Glu to pyro-Glu (N-term E) and oxidation (M). The searches were performed with a peptide mass tolerance of 0.7 Da and fragment ion mass tolerance of 0.3 Da. The proteins established by MASCOT searches were further processed using a BLAST search against the NCBI non-redundant protein sequence database (*circa* 39 million sequences) applying the algorithm of domain enhanced lookup time accelerated BLAST (DELTA-BLAST) to confirm and/or define the probable protein function.

Results and discussion

Positive effects of conditioning were not visible in corn seed germination tests performed under optimal temperature conditions. Except that hydroprimed seeds germinated more uniformly (smaller ± SEM), both the germination rate and the final germination percentage were comparable in good quality control seeds and their primed equivalents (Janas et al. 2009a). However, subsequent experiments showed that the seedlings grown from the seeds hydroconditioned with melatonin extremely well-tolerated stresses of suboptimal temperature (10 °C), and heavy metal contamination (2.5 mM Cu^{2+}) and also regenerated much better after relief of stress. It was manifested by better growth (greater weight of seedlings) and higher chlorophyll content and phenolic synthesis in the seedlings developed from the seeds hydroprimed with melatonin (Janas et al. 2009a). These experiments of our team provoked reflection that if the effects of conditioning are not visible in seed parameters investigated under the optimal conditions maybe the explanation why the seedlings grown from the seeds pretreated with melatonin tolerate stresses so well can be found at the molecular/proteomic level.

That is why proteomes of embryos isolated from particular seed variants (nt, H, HMel50 and, HMel500) germinated 24 h at optimal temperature (25 °C) were compared. The seed variants: nt and H, were the reference for the detection of melatonin-associated proteins, since it is well known that already the conditioning with water provokes modifications in seed proteome (Catusse et al. 2011; Wu et al. 2011; Gallardo et al. 2001).

The protein maps made in four replicates pointed out a significant level of reproducibility. Specialised software was able to indicate as corresponding ~95 % of the stained protein spots in the replicated gel images. PDQuest densitometric analysis of master gels was principle for qualitative (Table 1) and quantitative analyses of particular experimental seed variants (Tables 2, 3). 130 protein spots were indicated on gel A—representing proteome of axes isolated from nt seed variant, 143 on gel B—axes proteome from H seeds, 180 on gel C—axes proteome from HMel50 seeds, and 160 on gel D—axes proteome from HMel500 seeds (Figs. 1, 2). Our data suggested that hydroconditioning and especially hydroconditioning supplemented with melatonin induced new proteins biosynthesis in grain embryonic axes (Fig. 2). Previous comparative proteomic analysis by Gong et al. (2013) identified eight protein spots, which markedly differed in abundance between the primed and unprimed maize seeds, while the abundance of approximately 98 % spots was almost the same. However, the mentioned study was performed with dry—no imbibed/germinated primed and unprimed seeds. Thus, during the short hydropriming process, metabolic activities (such as protein synthesis and degradation) were quite low. We performed our study after first 24 h of germination under optimal conditions, when the metabolic processes were very intensive. Moreover, we used the relatively sensitive and effective Imperial™ Protein Stain (Pierce)—in comparison with Coomassie Brilliant Blue or Silver Stain—to visualise proteins in 2-DE gels; thereby, even the changes of low-abundance proteins might be detected.

Table 1 Identification of the proteins unique to different seed variants: control, non-treated (A), hydroprimed (B), all primed (B, C, D), all hydroprimed with melatonin (C, D) and characteristic of the seeds treated with 50-μM (C) or 500-μM (D) melatonin, determined after 24 h of germination at optimal 25 °C

Spot No	Function category	Protein family	Protein name	Accession No	Mascot score	% Cover	Peptide +/−	MW [kDa]	p*I*
Protein unique to control, non-primed seeds (1)—A									
Protein destination and storage									
0402	06.13	Phytepsin	Aspartic proteinase oryzasin-1 precursor [*Zea mays*]	gil226506070	65	10	3/1	54.90	5.41
Protein unique to hydroprimed seeds (0)—B									
Proteins unique to all primed seeds (14)—B, C, D									
Metabolism									
4503	01.01	Trp-synth-beta II	Cysteine synthase precursor [*Zea mays*]	gil295421203	321	40	13/7	34.19	5.91
1601	01.03	Ribokinase pfkB	Adenosine kinase [*Zea mays*]	gil4582787	332	29	13/6	36.01	5.23
5301	01.04	Pyrophosphatase	Inorganic pyrophosphatase [*Zea mays*]	gil414586181	59	5	1/1	24.35	5.46
Energy									
5602	02.01 02	Gp dh N	Glyceraldehyde-3-phosphate dehydrogenase 1, cytosolic isoform X1 [*Zea mays*]	gil6016075	122	16	3/2	31.97	6.4
7502	02.01 02	Gp dh N	Cytosolic glyceroldehyde-3-phosphate dehydrogenase GAPC3 [*Zea mays*]	gil6166167	76	18	5/1	36.43	7.01
2301	02.07	SugarP isomerase	6-phosphogluconolactonase isoform 1 [*Zea mays*]	gil414591367	98	22	7/3	34.76	7.71
Protein destination and storage									
1801	06.01	PDIb	Protein disulphide isomerase (PDI) [*Zea mays*]	gil145666464	447	33	25/12	56.7	5.01
2802	06.01	Chaperonin like	Chaperonin 60 [*Zea mays*]	gil257734900	79	9	4/1	61.2	5.68
8103	06.20	Cupin 2	Cupin family protein [*Zea mays*]	gil226509468	68	11	4/1	56.49	6.1
Disease/defence									
3502	11.05	SDR	General stress protein 39 [*Zea mays*]	gil414590804	79	14	5/1	38.6	9.06
4502	11.05	SDR	General stress protein 39 [*Zea mays*]	gil195659117	709	47	29/17	32.92	5.78
3901	11.05	HSP70 actin	Heat shock 70 kDa protein [*Zea mays*]	gil414589839	141	15	9/2	72.50	5.62
6902	11.05	MDR	Alcohol dehydrogenase 1 isoform X1 [*Zea mays*]	gil7262819	89	5	2/2	40.96	6.28
6001	11.06	Thioredoxin like	Thioredoxin homolog 2 protein [*Zea mays*]	gil66841004	61	8	1/1	13.03	6.19
Proteins unique to seeds hydroprimed with melatonin (14)—C, D									
Energy									
6502	02.01 02	Gp dh C	Glyceraldehyde-3-phosphate dehydrogenase, partial [*Zea mays*]	gil6016075	182	12	4/2	24.93	8.44
Cell growth/division									
7804	03.22	APP MetAP	Proliferation-associated protein 2G4 [*Zea mays*]	gil413945091	117	10	6/4	43.20	6.58
Transcryption									
2503	04.01	Ribosomal L10 P0	60S acidic ribosomal protein P0 [*Zea mays*]	gil413941828	124	9	4/3	34.47	5.2
Protein synthesis									
0301	05.04	EF1B	Elongation factor 1-delta 1 [*Zea mays*]	l414887578	199	22	6/4	24.84	4.39

Table 1 continued

Spot No	Function category	Protein family	Protein name	Accession No	Mascot score	% Cover	Peptide +/−	MW [kDa]	p*I*
Protein destination and storage									
2604	06.07	Aha1 N	Activator of 90 kDa heat shock protein ATPase [*Zea mays*]	gil414869818	89	6	2/2	38.58	5.33
2501	06.13	Ntn hydrolases	Proteasome subunit alpha type 1 [*Zea mays*]	gil226531007	277	46	11/7	30	5,19
3202	06.20	Cupin 2	Cupin family protein [*Zea mays*]	gil4139567	67	4	2/1	71	6.30
4301	06.20	Cupin 2	Globulin-1 S allele-like [*Zea mays*]	gil413956703	255	10	6/5	71.09	6.31
7401	06.20	Cupin 2	Globulin-1 S allele precursor [*Zea mays*]	gil195658011	136	11	5/2	49.93	6.16
Transporters									
2801	07.25	ABC ATPase	ATP synthase subunit beta family protein [*Zea mays*]	gil414880947	828	50	33/22	59.28	5.95
Disease/defence									
3105	11.05	α -crystallin-Hsps	Heat shock 22 kDa protein [*Zea mays*]	gil195621504	104	16	3/2	23.85	6.47
7102	11.05	α-crystallin-Hsps	Class II heat shock protein [*Zea mays*]	gil413939226	208	39	9/6	18.34	6.6
0203	11.06	PITH	Thioredoxin family Trp26 isoform 4 [*Zea mays*]	gil414881868	177	27	6/4	22.60	4.95
Secondary metabolism									
8506	20.01	DAHP synth1	2-dehydro-3-deoxyphosphooctonate aldolase [*Zea mays*]	gil414878308	64	8	1/1	15.89	5.07
Proteins unique to seeds hydroprimed with 50 µM melatonin (23)—C									
Energy									
1501	02.01	Bac FRK	Fructokinase 1 [*Zea mays*]	gil75293604	152	28	6/3	34.67	4.87
4805	02.01	alkPPc	2,3-bisphosphoglycerate-independent phosphoglycerate mutase isoform XI [*Zea mays*]	gil551288	118	10	6/3	60.58	5.29
3805	02.01	TIM phosphate binding	Triosephosphate isomerase, cytosolic [*Zea mays*]	gil257353728	64	10	3/1	60.50	5.34
7701	02.01	TIM phosphate binding	Aldolase1 [*Zea mays*] Fructose-bisphosphate aldolase cytoplasmic isozyme [*Zea mays*]	gil414879138	419	35	15/10	38.57	7.52
7603	02.01 02	Gp dh N	Glyceraldehyde-3-phosphate dehydrogenase1 isoform 1 [*Zea mays*]	gil413921396	351	45	10/7	31.92	6.45
7702	02.01 02	Gp dh N	Glyceraldehyde-3-phosphate dehydrogenase1 cytosolic isoform X1 [*Zea mays*]	gil413921395	115	13	3/1	36.47	6.46
2602	02.10	CoA-ligase	Succinyl-CoA ligase beta-chain [*Zea mays*]	gil226510248	311	29	15/8	45.17	5.99
5504	02.10	SDR	Malate dehydrogenase [*Zea mays*]	gil550576396	119	9	3/2	35.62	7.63
Cell growth/division									
2401	03.01	SMP, LEA	Late embryogeneis abundant protein D-34 [*Zea mays*]	gil414872767	226	17	4/4	27.16	5.41
0701	03.19	UBQ	DNA repair protein RAD23 [*Zea mays*]	gil525344153	62	2	1/1	42.57	4.61
Protein synthesis									
0303	05.04	EF1B	Elongation factor 1-beta [*Zea mays*]	gil414887997	141	26	6/2	23.4	4.55

Table 1 continued

Spot No	Function category	Protein family	Protein name	Accession No	Mascot score	% Cover	Peptide +/−	MW [kDa]	p*I*
Protein destination and storage									
0002	06.01	BBI plant serine protease inhibitors	Hypothetical protein ZEAMMB73_733594 [*Zea mays*] 66 % identities to Bowman-Birk trypsin inhibitor TI1 [*Coix lacryma-jobi*]	gil414876537	54	4	1/1	26.05	5.45
6301	06.01	SPFH like	Prohibitin2 [*Zea mays*]	gil7716458	79	13	2/2	30.70	6.55
8401	06.13	Ntn hydrolases	20S proteasome alpha 4 subunit [*Zea mays*]	gil414590073	201	18	6/4	27.21	8.3
Cell structure									
2001	09.04	ADF gelsolin	Actin-depolymerizing factor 3 [*Zea mays*]	gil413932602	258	65	10/6	15.9	5.47
8101	09.07	C2	Unnamed protein product [*Zea mays*] 98 % identities to ADP-ribosylation factor GTPase-activating protein AGD11 [*Zea mays*]	gil257692306	62	12	1/1	18.92	6.59
Disease/defence									
0201	11.05	α-crystallin-Hsps	Co-chaperone protein SBA1 [*Zea mays*]	gil413920965	50	3	1/1	20.80	4.43
0202	11.05	TCTP	Translationally controlled tumour protein homolog [*Zea mays*]	gil413935193	69	16	4/1	18.68	4.52
0501	11.05	USP	USP family protein [*Zea mays*]	gil413952234	81	6	1/1	27.12	4.88
2601	11.05	GAT 1	Hypothetical protein ZEAMMB73_682876 [*Zea mays*] 88 % identities to Protein DJ-1 homolog B-like [*Setaria italic*]	gil413954138	195	13	7/4	42.22	5.51
2402	11.06	Glo EDI BRP like	Glyoxalase I [*Zea mays*]	gil37932483	120	23	7/3	35.14	6.62
6201	11.06	Thioredoxin like	1-Cys peroxiredoxin antioxidant PER1 [*Zea mays*]	gil414887819	165	28	6/3	24.96	6.38
6402	11.06	Esterase lipase	Esterase D [*Zea mays*]	gil413951351	121	10	2/2	31.87	6.13
Proteins unique to seeds hydroprimed with 500 µM melatonin (3)—D									
Metabolism									
0901	01. …	Transferases	Transferase [*Zea mays*]	gil126633162	62	4	2/1	75.4	5.16
5803	01.03	TIM phosphate binding	Inosine-5-monophosphate dehydrogenase 2 [*Zea mays*]	gil226507304	81	11	4/2	52.65	5.89
Protein synthesis									
5603	05.04	ABC ATPase	Translation elongation/initiation factor family protein [*Zea mays*]	gil413932419	138	19	6/2	43.84	5.76

The table contains: spots excised from gels and numbered by PDQuest Software used for gel qualitative analysis; function category consistent with *Nature* (Bevan et al. 1998); protein family's, names and accession numbers consistent with NCBI database; Mascot score, sequence coverage (%), number of matched/unmatched (+/−) peptides, MW [kDa] and p*I* values

Influence of hydropriming on axes proteome in germinated maize grains

Proteomic analysis of extracts from particular seed variants (nt, H, HMel50 and HMel500) enabled to identify 14 proteins, which appeared after seed conditioning (presented in gels B, C, and D—Table 1; Fig. 1BCD).

There were the proteins related to: (1) cysteine biosynthesis (cysteine synthase precursor—4503)—an endogenic sulphuric amino acid with –SH group and by glutathione metabolism connected/associated with the detoxification processes and regulation of cell redox status; (2) the preservation of intracellular adenylate pools due to regulation of extracellular adenosine levels (adenosine kinase—

Table 2 Proteins showing different expressions in axes of the control nt seeds (A) germinated for 24 h at optimal 25 °C, to those from all hydroprimed seeds: H, HMel50, HMel500 (B, C, D)

Spot No	Function category	Protein name	Accession No	Mascot score	% Cover	Peptides +/−	MW [kDa]	p*I*	Fold change $p \leq 0.01$		
									A/B	A/C	A/D
Proteins up-regulated in A in comparison with B, C, D											
Metabolism											
1301	01.04	Adenylate cyclase [*Zea mays*]	gil414888163	57	8	3/0	23.51	5.18	**4.99**	1.14	**5.45**
Energy											
5601	02.10	Malate dehydrogenase, cytoplasmic [*Zea mays*]	gil320449084	498	43	16/12	35.50	5.76	3.04	**19.3**	2.65
Cell growth/division											
8803	03.01	Embryonic protein DC-8-like [*Zea mays*]	gil413936531	107	5	2/2	61.73	7.27	**34.1**	**18.6**	
Protein synthesis											
9701	05.04	Elongation factor 1 alpha [*Zea mays*]	gil7230387	162	14	7/3	49.12	9.2	1.8	7.6	
Protein destination and storage											
2903	06.01	Mitochondrial chaperonin-60 [*Zea mays*]	gil22248	1342	47	49/33	61.15	5.68	**40.8**	2.71	**5.63**
4203	06.13	Proteasome subunit beta type 6 precursor [*Zea mays*]	gil229612088	169	22	6/3	26.16	5.47	**7.63**	1.13	2.23
1702	06.13	Hypothetical protein ZEAMMB73_715164 [*Zea mays*] 96 % identity to 26S protease regulatory subunit 6A homolog [*Oryza sativa* Japonica Group]	gil413944465	77	13	6/2	47.78	4.94	**13.3**	1.29	1.51
Cell structure											
2105	09.04	Actin depolymerizing factor [*Zea mays*]	gil414873646	71	36	5/1	15.89	5.46	1.11	4.19	**5.97**
Disease/defence											
5502	11.05	General stress protein 39 [*Zea mays*]	gil414590803	829	50	24/18	38.60	5.78	1.47	**7.52**	2.44
2901	11.05	Unknown [*Zea mays*] 98 % identities to Heat shock 70 kDa protein, mitochondrial-like [*Setaria italica*]	gil194688822	289	17	11/7	72.62	5.54	**8.62**	**6.37**	3.77
1001	11.06	Glyoxalase family protein superfamily [*Zea mays*]	gil195604212	314	42	8/5	15.07	5.47	**76.6**	1.98	1.98
Unclassified											
5402	13	Secreted protein [*Zea mays*]	gil413951152	108	29	6/4	27.33	5.84	**12.6**	**17.4**	
Proteins down-regulated in A in comparison with B, C, D.											
Energy											
4201	02.01	Triose-phosphate isomerase [*Zea mays*] (EC 5.3.1.1)	gil414876338	187	25	5/5	27.01	5.52		**0.11**	**0.10**
4701	02.01	Cytosolic 3-phosphoglycerate kinase [*Zea mays*]	gil413935730	405	20	13/12	60.80	9.41	6.85	**0.15**	0.22
8601	02.01 02	Fructose-bisphosphate aldolase [*Zea mays*]	gil413951593	536	38	21/13	38.44	6.96	**0.03**	**0.17**	**0.09**
8604	02.01 02	Fructose-bisphosphate aldolase, cytoplasmic isozyme [*Zea mays*]	gil113621	115	9	2/2	38.58	7.52	**0.01**	0.39	**0.02**

Table 2 continued

Spot No	Function category	Protein name	Accession No	Mascot score	% Cover	Peptides +/−	MW [kDa]	p*I*	Fold change $p \leq 0.01$		
									A/B	A/C	A/D
8602	02.10	Succinyl-CoA ligase alpha-chain 2 [*Zea mays*]	gil414887289	61	12	3/0	34.19	8.16	0.31	**0.02**	**0.08**
Cell growth/division											
6602	03.01	Hypothetical protein ZEAMMB73_579239 [*Zea mays*] 80 % identities to LEA protein [*Setaria italica*]	gil413955864	66	11	3/1	37.89	6.6	**0.05**	**0.13**	**0.05**
Transcryption											
8902	04.19 22	Hypothetical protein ZEAMMB73_983793 [*Zea mays*] 94 % identities to Polyadenylate-binding protein 2-like [*Setaria italica*]	gil414588971	62	7	4/0	71.13	7.55	0.24	0.27	0.17
Protein destination and storage											
8202	06.01	1-Cys peroxiredoxin antioxidant [*Zea mays*]	gil87133468	115	10	4/2	24.89	6.31		**0.07**	**0.10**
3101	06.02	Globulin 2 [*Zea mays*]	gil414873301	291	14	8/6	49.89	6.16	0.21	**0.03**	**0.15**
6302	06.13	Proteasome subunit alpha type [*Zea mays*]	gil414865138	504	49	17/13	27.40	6.10	**0.03**	**0.11**	1.17
5304	06.13	Proteasome subunit alpha type [*Zea mays*]	gil414865138	330	39	13/9	27.4	6.1	0.42	**0.02**	
3702	06.20	Homogentisate 1,2-dioxygenase [*Zea mays*]	gil413953601	149	7	7/3	50.30	5.30	**0.19**	**0.17**	0.39
Signal transduction											
6003	10.0404	Nucleoside diphosphate kinase [*Zea mays*]	gil414867768	84	10	1/1	16.53	6.3	**0.013**	**0.032**	**0.064**
8001	10.0404	Nucleoside diphosphate kinase 4 [*Zea mays*]	gil413946787	64	4	1/0	25.93	9.04	**0.09**	**0.04**	**0.16**
Disease/defence											
4601	11.02	Protein Z [*Zea mays*]	gil195606550	141	16	10/5	42.16	5.52	1.12	**0.08**	0.36
5001	11.05	16.9 kDa class I heat shock protein 1 [*Zea mays*]	gil296512085	59	24	4/1	17.05	6.77	**0.10**	0.66	
7002	11.05	Stress-inducible membrane pore protein [*Zea mays*]	gil195642018	146	40	5/3	17.80	6.41		0.39	**0.02**
3301	11.05	General stress protein 39 [*Zea mays*]	gil414590803	71	6	2/1	38.6	9.06	**0.11**	**0.04**	
Unclassified											
8003	13	CBS domain protein isoform 3 [*Zea mays*]	gil414872657	359	40	11/8	22.48	9.35	0.37	**0.11**	**0.11**

The table contains: spots excised from gels and numbered by PDQuest Software used for quantitative gel analysis; function category consistent with *Nature* (Bevan et al. 1998); protein names and accession numbers consistent with NCBI database; Mascot score, sequence coverage (%), number of matched/unmatched (+/−) peptides, MW [kDa] and p*I* values; and relative abundance fold change in comparison with control nt (A) seeds ($p \leq 0.01$)—bolded number indicate ≥5-fold changes in protein expression in the compared variants

1601); and (3) phosphate metabolism (inorganic pyrophosphatase—5301).

In the axes of primed seeds, there were also a number of additional enzymes involved in cell energy metabolism: glyceraldehyde-3-phosphate dehydrogenase 1 cytosolic isoform X1—5602; cytosolic glyceraldehyde-3-phosphate dehydrogenase GAPC3—7502; 6-phosphogluconolactonase isoform 1—2301 (Table 1; Fig. 1BCD). These enzymes are involved in gluconeogenesis and glycolysis, also in the pentose phosphate pathway (the latter one). Improvement of the energy acquisition from sugar turnover is of utmost importance for growth and development of

Table 3 Proteins showing different expressions in axes of the hydroprimed seeds (B) germinated 24 h at optimal 25 °C, to those from all seeds hydroprimed with melatonin: HMel50, HMel500 (C, D)

Spot No	Function category	Protein name	Accession No	Mascot score	% Cover	Peptides +/−	MW [kDa]	p*I*	Fold change $p \leq 0.01$	
									B/C	B/D
Proteins up-regulated in B in comparison with C, D										
Energy										
8501	02.01 02	Cytosolic glyceraldehyde-3-phosphate dehydrogenase [*Zea mays*]	gi|6016075	640	61	22/11	37.16	6.67	4.32	**6.62**
8604	02.01 02	Fructose-bisphosphate aldolase, cytoplasmic isozyme [*Zea mays*]	gi|113621	115	9	2/2	38.58	7.52	**36.4**	1.60
Protein destination and storage										
8103	06.20	Cupin family protein [*Zea mays*]	gi|195606798	68	11	4/1	56.49	6.1	4.16	**11.1**
Cell structure										
2105	09.04	Actin depolymerizing factor [*Zea mays*]	gi|414873646	71	36	5/1	15.89	5.46	3.76	**5.36**
Signal transduction										
6003	10.0404	Nucleoside diphosphate kinase [*Zea mays*]	gi|414867768	84	10	1/1	16.53	6.3	2.57	**5.09**
5503	10.0410	Guanine nucleotide-binding protein β-subunit-like [*Zea mays*]	gi|413948362	176	12	5/4	36.21	6.13	**9.14**	0.88
Disease/defence										
1202	11.05	Heat shock protein 26 [*Zea mays*]	gi|453670	84	22	4/1	26.36	7.88	3.70	**60.8**
5202	11.06	1-Cys peroxiredoxin antioxidant [*Zea mays*]	gi|87133468	204	29	7/4	24.96	6.38	**5.07**	0.72
5502	11.05	General stress protein 39 SDR family protein [*Zea mays*]	gi|414590803	829	50	24/18	38.60	5.78	**5.11**	1.66
6001	11.06	Thioredoxin h2 protein [*Zea mays*]	gi|66841004	61	8	1/1	13.03	6.19	2.31	**8.09**
Proteins down-regulated in B in comparison with C, D										
Metabolism										
1601	01.03	Adenosine kinase [*Zea mays*]	gi|4582787	332	29	13/6	36.01	5.23	**0.08**	**0.08**
1602	01.01	Agmatine deiminase [*Zea mays*]	gi|226508546	133	12	4/3	41.6	4.93	**0.14**	**0.14**
3701	01.05	UTP-glucose-1-phosphate uridylyltransferase [*Zea mays*]	gi|212275097	281	21	10/6	52.06	5.3	**0.11**	0.31
4503	01.01 02	O-acetylserine (thiol) lyase [*Zea mays*]	gi|758353	321	40	13/7	34.19	5.91	0.61	**0.18**
Energy										
2301	02.07	6-phosphogluconolactonase isoform 2 [*Zea mays*]	gi|414591367	98	22	7/3	34.76	7.71	**0.14**	**0.07**
2701	02.01 02	Enolase [*Zea mays*]	gi|22273	690	34	15/11	48.03	5.2	**0.11**	
4202	02.01	Triosephosphate isomerase, cytosolic [*Zea mays*]	gi|414876339	531	42	18/11	33.04	6.96	**0.19**	**0.15**
4602	02.10	Malate dehydrogenase [*Zea mays*]	gi|414871066	148	26	7/4	35.59	5.4	0.40	**0.16**
4701	02.01	Cytosolic 3-phosphoglycerate kinase [*Zea mays*]	gi|413935730	405	20	13/12	60.80	9.41	**0.02**	**0.03**
5602	02.01 02	Glyceraldehyde-3-phosphate dehydrogenase 2, cytosolic [*Zea mays*]	gi|6016075	122	16	3/2	31.97	6.4	**0.17**	0.56
8601	02.01 02	Fructose-bisphosphate aldolase [*Zea mays*]	gi|413951593	536	38	21/13	38.44	6.96	**0.03**	**0.17**
8602	02.10	Succinyl-CoA ligase alpha-chain 2 [*Zea mays*]	gi|414887289	51	12	3/0	34.19	8.16	**0.07**	0.24
Protein destination and storage										
2802	06.01	Chaperonin 60 [*Zea mays*]	gi|309557	79	9	4/1	61.2	5.68	0.23	**0.05**
2903	06.01	Mitochondrial chaperonin-60 [*Zea mays*]	gi|22248	1342	47	49/33	61.15	5.68	**0.07**	**0.14**
3101	06.20	Globulin 2 [*Zea mays*]	gi|414873301	291	14	8/6	49.89	6.16	**0.16**	0.74
3103	06.20	Globulin-1 S allele precursor [*Zea mays*]	gi|195658	81	5	2/1	49.93	6.16	**0.03**	0.23
4003	06.20	Globulin-1 S allele precursor [*Zea mays*]	gi|195658011	99	10	4/1	49.89	6.16	**0.06**	**0.14**

Table 3 continued

Spot No	Function category	Protein name	Accession No	Mascot score	% Cover	Peptides +/−	MW [kDa]	p*I*	Fold change $p \leq 0.01$	
									B/C	B/D
5304	06.13	Proteasome subunit alpha type [*Zea mays*]	gi|414865138	330	39	13/9	27.4	6.1	**0.04**	
4203	06.13	Unnamed protein product [*Zea mays*] 97 % identities to Proteasome subunit beta type 6 precursor [*Zea mays*]	gi|229612088	169	22	6/3	26.16	5.47	**0.15**	0.29
Transporters										
1702	07.25	Hypothetical protein ZEAMMB73_715164 [*Zea mays*] 96 % identities to 26S protease regulatory subunit 6A homolog [*Oryza sativa* Japonica Group]	gi|413944465	77	13	6/2	47.78	4.94	**0.10**	**0.11**
Disease/defence										
1001	11.06	Glyoxalase family protein superfamily [*Zea mays*]	gi|195604212	314	42	8/5	15.07	5.47	**0.03**	**0.03**
3401	11.06	Glyoxalase family protein isoform 2 [*Zea mays*]	gi|413917004	579	55	19,13	32.32	5.82	**0.08**	
4502	11.05	General stress protein 39 SDR family protein [*Zea mays*]	gi|195659117	709	47	29/17	32.92	5.78	**0.15**	0.35
4601	11.02	Protein Z [*Zea mays*]	gi|195606550	141	16	10/5	42.16	5.52	**0.07**	0.33
5102	11.05	22.0 kDa class IV heat shock protein precursor [*Zea mays*]	gi|195644560	77	37	7/1	22.88	6.01	**0.10**	**0.11**
5201	11.06	Glutathione transferase III(a) [*Zea mays*]	gi|4468792	107	27	4/2	23.79	9.33	**0.04**	**0.20**
6702	11.05	Formate dehydrogenase 1 [*Zea mays*]	gi|413953926	95	14	4/2	41.39	6.32	**0.04**	**0.01**
7501	11.05	Hypothetical protein ZEAMMB73_578099 [*Zea mays*] 78 % identities to Stress responsive protein [*Zea mays*]	gi|414866592	268	22	15/10	38.37	6.3	**0.13**	**0.15**
Secondary metabolism										
1303	20.1	Caffeoyl-CoA O-methyltransferase 1 [*Zea mays*]	gi|413925228	109	19	3/0	27.3	5.09	**0.13**	
Unclassified										
4403	13	Secreted protein [*Zea mays*]	gi|413951152	238	44	12/7	27.33	5.84	**0.06**	**0.06**

The table contains: spots excised from gels and numbered by PDQuest Software used for quantitative gel analysis; function category consistent with *Nature* (Bevan et al. 1998); protein names and accession numbers consistent with NCBI database; Mascot score, sequence coverage (%), number of matched/unmatched (+/−) peptides, MW [kDa] and p*I* values; and relative abundance fold change in comparison with hydroprimed (B) seeds ($p \leq 0.01$)—bolded number indicate ≥5-fold changes in protein expression in the compared variants

seedlings, especially if starch is seed storage material, as is in the case of maize.

The appearance of new isomerases (1801), cupins (8103) and chaperonins (2802) involved in efficient protein destination and storage was also observed (Table 1; Fig. 1BCD). Cupins belong to superfamily of functionally diverse proteins, including seed storage proteins, auxin binding proteins, enzymes, transcription factors, and stress-related proteins, which are involved in seed germination and seedlings development (Dunwell et al. 2004; Gong et al. 2013). Fu et al. (2011) suggested that cupin proteins, such as native germin and germin-like proteins, protected plant cells from the oxidative stress during seed germination.

Similar to the observations by other authors (Gallardo et al. 2001; Gong et al. 2013) in the conditioned plant material, a significant synthesis of so-called stress proteins (5 new spots) was indicated. They belonged to short-chain dehydrogenases/reductases family (SDR; general stress proteins 39—3502, 4502), heat shock proteins (HSP 70 kDa—3901), thioredoxins (TRX; thioredoxin homolog 2—6001) and anaerobic proteins (ANPs; alcohol dehydrogenase 1 isoform XI—6902) (Table 1, Fig. 1BCD).

HSPs were first described as associated with heat shock, but now, it is known that they are also overexpressed during other stresses, i.a. exposure to cold, UV light, and during wound healing or tissue remodelling. They may participate in intracellular distribution and degradation of

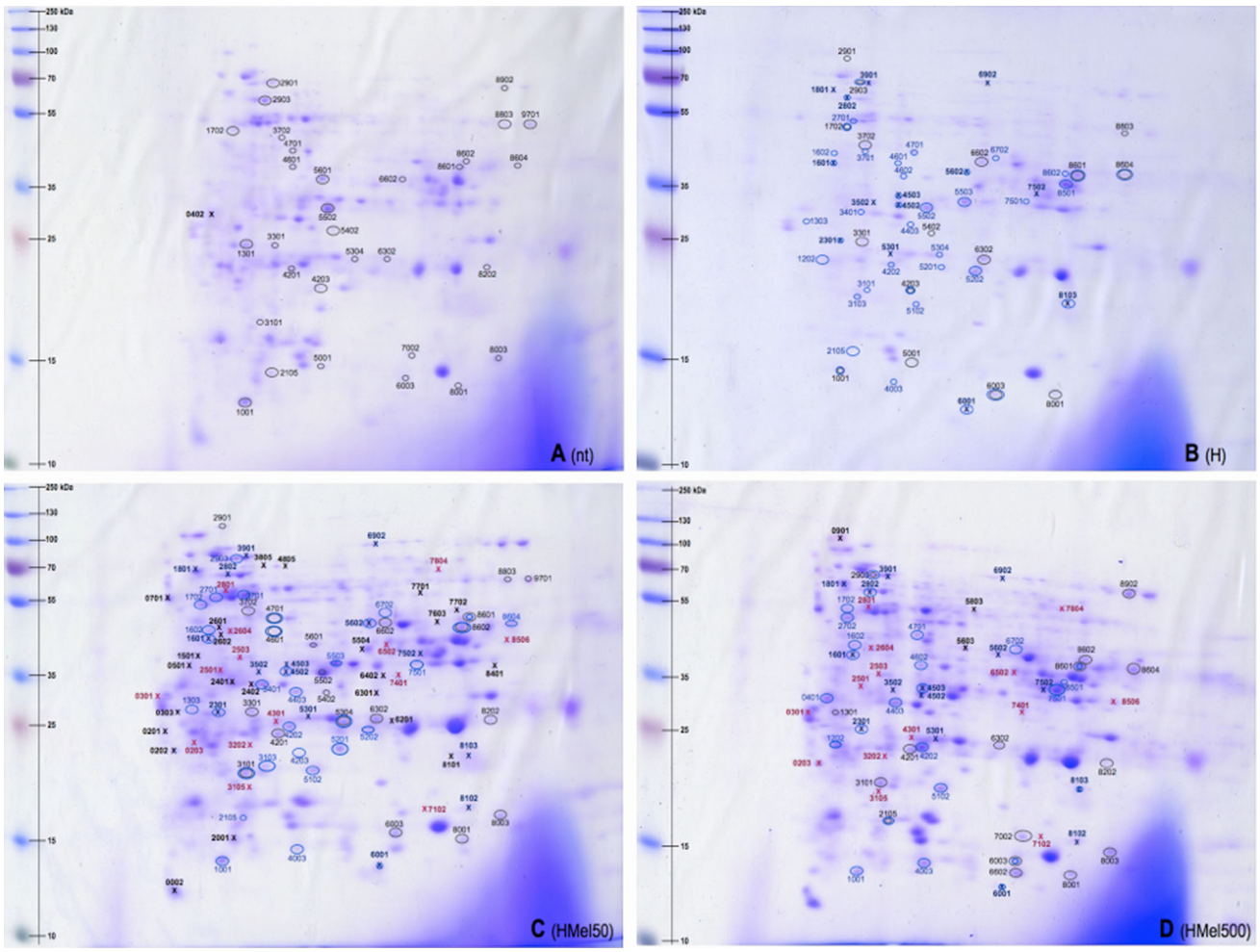

Fig. 1 Analytical gels of IEF/SDS–PAGE (2D) separation of proteins from: control nt (**A**), hydroprimed (**B**), hydroprimed with melatonin 50 μM (**C**) and 500 μM (**D**) seeds germinated in darkness at 25 °C for 24 h. The proteins were separated by first dimensional pH 3–10 non-linear IPG strips and 12 % vertical slab gels in the second dimension (ImperialTM Protein Stain). The proteins were numbered (PDQuest Software, BioRad) in a preparative 2D electrophoresis gel and excised for MS/MS analysis, corresponding to the spot/proteins in Tables 1, 2 and 3. According to the qualitative analysis, the *black X symbols* mean proteins characteristic only of particular gels (A or B or C or D), *blue X symbols* mean proteins characteristic of all hydroprimed seeds (BCD), and *red X symbols* means proteins characteristic of the seeds hydroprimed with melatonin (CD) (see Table 1). According to the quantitative analysis, ellipses are used to mark proteins five times upregulated or downregulated: *black* for A to B, C, D (see Table 2), *blue* for B to C, D (see Table 3)

proteins, thus perform chaperone activity (Timperio et al. 2008), but also act as molecular factors in signal transduction cascades, affording a protective function during seed germination (Wang et al. 2004).

TRX is a small conservative protein, which causes the changes in redox state of target proteins by reversible oxidation of di-thiol—an active site exposed at the protein's surface (at least 30 target proteins functioning are regulated this way). Some of TRXs are transcription factors and enzymes involved in plant oxidative stress response. They can also limit stress via direct hydrogen peroxide and certain radicals scavenging, and/or by acting as a reductant for peroxiredoxins (Vieira Dos Santos and Rey 2006).

ANPs mostly represent enzymes of the glycolytic and alcohol fermentation pathways. Their upregulation occurs in response to hypoxia/anoxia stress, but cross talk with other kinds of stresses is possible too (Grover et al. 2001).

Interestingly, under the influence of seed hydroconditioning aspartic proteinase oryzasin-1 precursor (0402) disappeared from embryo. It was characteristic only of the axes from control nt seeds (Table 1; Fig. 1A). This plant phytepsin is a homolog of lysosomal pepsins of mammals, and it appears in seeds but also in leaves, stems, flowers and roots. Phytepsin could be overexpressed in plant tissues undergoing apoptosis. It may influence metabolic turnover and other protein processing events.

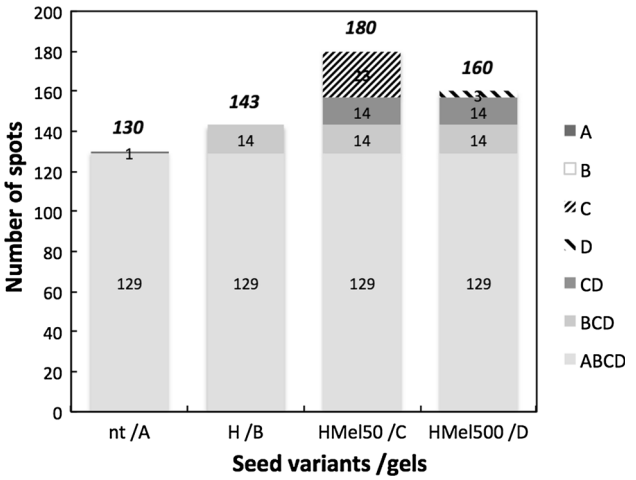

Fig. 2 Number of protein spots in particular gels: *A*, *B*, *C*, *D*, corresponding with seed variants: nt, H, HMel50, HMel500, respectively. The numbers of protein spots which occurred in certain types of gels were highlighted in different pattern: (1) common for all seeds (*ABCD*), (2) common for the hydroprimed seeds (*BCD*), (3) common for the melatonin treated seeds (*CD*) and (4) unique for the particular seed variants (*A* or *B* or *C* or *D*). *Bolded italics numbers* above the *bars* indicate the total number of protein spots in the particular gels

There were no more proteins characteristic only of the control seed axes (gel A); nevertheless, several enzymes of primary metabolism showed here overexpression comparing with the axes of conditioned seeds (gels B, C, D), i.e. adenylate cyclase—1301 and cytoplasmic maleate dehydrogenase (MDH)—5601 (Table 2; Fig. 1A). Adenylate cyclase (AC) is an enzyme that plays a crucial regulatory function in almost all cells. It facilitates the conversion of ATP to 3′ 5′- cyclic AMP (cAMP) and pyrophosphate. The resultant cAMP operates as a regulatory factor by activation of cAMP-binding proteins, transcription factors, or other enzymes (e.g. cAMP-dependent kinases). MDH acts in the Krebs cycle. It catalyses: the conversion of malate to oxaloacetate and also replenishing level of oxaloacetate via pyruvate reductive carboxylation.

An embryonic DC-8-like protein—8803 also showed significant overexpression (Table 2; Fig. 1A); generally, its content decreases during the initial phase of maize seeds imbibition (Tnani et al. 2012), and this phase, in a case of hydroconditioned seeds, took place just during priming hydration.

It should also be noted that three of the stress-related proteins that occurred in embryos of all seed variants showed overexpression in the control ones: general stress protein 39—5502; heat shock 70-kDa mitochondrial-like protein—2901; glyoxalase family protein—1001; moreover, another protein of unknown function—secreted protein—5402 also showed significant overexpression (Table 2; Fig. 1A). However, the above-described metabolism improvement in nt seed axes is not so significant compared with the effects of the aforementioned completely new stress proteins in primed seed axes (Table 1; Fig. 1BCD) as well as to a significant overexpression in the primed seed embryos of four other proteins that were present in all seed variants: protein Z—4601; 16.9-kDa class I heat shock protein 1—5001; stress-inducible membrane pore protein—7002; and other (different pI values in comparison with spot 5502) general stress protein 39—3301 (Table 2; Fig. 1).

Moreover, the enzymes most important for the energy metabolism, i.e.: triose-phosphate isomerase—4201; cytosolic 3-phosphoglycerate kinase—4701; fructose-bisphosphate aldolase—8601; fructose-bisphosphate aldolase cytoplasmic isozyme—8604; succinyl-CoA ligase alpha-chain 2—8602, occurred in much greater quantities in the embryos of conditioned seeds (Table 2, Fig. 1BCD). Triosephosphate isomerase (TPI) plays a significant role in glycolysis and is fundamental for efficacious energy generation. It makes possible interconversion of dihydroxyacetone phosphate and D-glyceraldehyde-3-phosphate. Phosphoglycerate kinase (PGK) enables the phosphate group transfer from 1,3-bisphosphoglycerate into ADP, forming ATP and 3-phosphoglycerate. This is one of the two substrate-phosphorylation reactions during glycolysis. Moreover, fructose-1,6-bisphosphate aldolase catalyses the cleavage of fructose 1,6-bisphosphate to glyceraldehyde 3-phosphate and dihydroxyacetone phosphate (DHAP). The last of mentioned above enzymes—succinyl-CoA ligase [GDP-forming]—participates in the Krebs cycle and propanoate metabolism, and thus, it also affects the improvement of energy metabolism.

In addition, some kinases crucial for signals transduction were also overexpressed in axes of primed seeds, e.g.: nucleoside diphosphate kinase—6003 and nucleoside diphosphate kinase 4—8001; (Table 2, Fig. 1BCD). Nucleoside diphosphate kinases (NPcs) are involved in the nucleoside triphosphates (NTPs) generation, and they act in various regulatory processes related to proliferation, differentiation and development. For example, they are essential for DNA and RNA synthesis—thus crucial for metabolism of macromolecules, cell division, and growth.

As it was mentioned earlier, it is difficult to compare proteomes of *Arabidopsis* seeds with those of monocotyledonous corn, especially that in research concerning *Arabidopsis,* whole seeds were usually used, while in the case of corn seeds, we were able to isolate from them the parts which were most active during germination, i.e. embryonic axes. The one important effects of hydropriming common for both plants considered in overexpression of glyceraldehyde-3-phosphate-dehydrogenase in *Arabidopsis* and appearance of its additional isoforms in corn axes (Table 1; Gallardo et al. 2001). In *Arabidopsis,* this enzyme was correlated with the secondary desiccation

(drying after seed priming hydration/imbibition) (Gallardo et al. 2001). Moreover, it was also reported to be a molecular seed vigour marker in *Beta vulgaris* seeds (Catusse et al. 2011).

In our hydroprimed plant material WD-40 proteins, aconitase and such a great amount of seed storage proteins subunits (12S) as in *Arabidopsis* (Gallardo et al. 2001) were not observed. The latter ones might not be detected because of storage tissues absence in our material (only axes). However, as concerns storage proteins, we observed that cupin translocation to the axes was much more efficient in the primed corn (especially when priming was supplemented with melatonin) than in the control.

Moreover, overexpression of tubulin and catalase (CAT) observed in *Arabidopsis* was not noted in our material. Out of energetic metabolism enzymes, other than phosphoenolopyruvate carboxykinase were overexpressed in corn. On the other hand, increase in HSP proteins was observed both in *Arabidopsis* and corn; however, in corn, not only 18 kDa but also 22- and 70-kDa HSPs were detected. (Tables 1, 2; Gallardo et al. 2001). In general, the spectrum of anti-stress and defence proteins induced by priming was wider in corn than in *Arabidopsis.*

It is difficult to find precise similarities between seed plant standard (*Arabidopsis*) and our plant material in terms of their reactions to hydropriming, but it is certainly possible to compare changes in the proteins of specific functional groups, such as: protein mobilising reserves (different enzymes depending on the seed storage material), those responsible for energy processes in cells (glycolytic enzymes, and involved in the Krebs cycle) as well as anti-stress and defence ones (e.g. various HSP, LEA, TRX, and ANP).

To sum up, significant improvement of metabolic processes involved in energy production as well as biosynthesis of anti-stress/stress-induced protective proteins before the occurrence of stress conditions are the facts that explain positive effects of hydroconditioning on seed quality.

Influence of melatonin on axes proteome in germinated maize grains

Presented results are the first that describe influences of exogenous melatonin on embryo's proteome during early stages of seed germination. Only one paper concerning proteomic modifications during leaf senescence in *Malus hupehensis* treated with exogenous melatonin has been published so far (Wang et al. 2014). Thus, those researches have been performed with completely different plant materials and focused on different developmental stages and processes (senescence).

Application of melatonin during the hydropriming treatment resulted in stronger improvement of energy metabolism in axes—compared with the only hydroprimed seed variant. Especially, the dose of 50-μM melatonin provoked a synthesis of additional six isoforms of enzymes involved in the sugar metabolism through glycolysis and gluconeogenesis processes (i.e. fructokinase 1—1501; 2,3-bisphosphoglycerate-independent phosphoglycerate mutase isoform X1—4805; triosephosphate isomerase, cytosolic—3805; aldolase1 = fructose-bisphosphate aldolase cytoplasmic isozyme—7701; glyceraldehyde-3-phosphate dehydrogenase1 isoform 1—7603 and glyceraldehyde-3-phosphate dehydrogenase1 cytosolic isoform X1—7702) and also two of the crucial enzymes in the citric acid cycle (succinyl-CoA ligase beta-chain—2602 and malate dehydrogenase—5504) (Table 1; Fig. 1C).

A very interesting result, according to Szafranska et al. (2012) research, is the fact confirming that melatonin applied to seeds stimulates polyphenol metabolism. Only in the embryos, proteome of melatonin treated seeds synthesis of 2-dehydro-3-deoxyphosphooctonate aldolase (8506) was observed (Table 1; Fig. 1CD). This enzyme is involved in the shikimate pathway. Members of its family catalyse first steps during biosynthesis of aromatic amino acids. Secondary metabolites accumulation often occurs in plants subjected to different stresses, various elicitors, and also by direct signal molecules action. It is widely known that secondary metabolites are essential for plant interaction with its environment, for adaptation and defence against stress conditions (Bennett and Wallsgrove 1994; Janas et al. 2009b; Ramakrishna and Ravishankar 2011).

In the case of embryos from seeds pretreated with melatonin, a significant improvement of the processes related to the biosynthesis, modifications, and protein destination and storage was observed. In axes of both seed variants treated with melatonin, additional transcription factor 60S acidic ribosomal eukaryotic protein P0 (2503), which composed large subunit of ribosome (it forms main structure in the large subunit, and it is believed to be linked with GTPase activities during protein biosynthesis), as well as directly involved in translations elongation factor 1-delta 1 (EF1B'—0301), occurred. Additionally, elongation factor 1-beta (EF1B—0303) was characteristic for axes from HMel50 seeds (Table 1, Fig. 1CD). Both EF1B' and EF1B catalyse the exchange of GDP bound to the G-protein, for GTP, and this is vital step in elongation processes during protein formation. EF1A—the overexpression of which was observed in all variants of conditioned seed axes (Table 2)—is related to deliver the aminoacyl-tRNA to the ribosome. Eukaryotes eEF1-alpha interacts with the cytoskeleton by actin and may thereby play important role in cellular transformation or apoptosis. This with the simultaneous occurrence of proliferating-associated protein

2G4 (7804), actin-depolymerizing factor 3 (2001), GTPase-activating protein AGD11 (8101) and of DNA repair protein RAD 23 (0701) in HMel50 seeds (Table 1; Fig. 1C) resulted in intense proliferation and better cell growth of the embryos pretreated with melatonin.

As mentioned earlier, already seed hydroconditioning treatment stimulated the biosynthesis of stress proteins in embryos (5 additional spots in B, C, D gels), while melatonin added at the same time provoked additional 3 protein spots in both melatonin seed variants (small HSP—3105, 7102 and thioredoxin TRp26 isoform 4—0203) (Table 1, Fig. 1CD) and 7 anti-stress proteins in HMel50 seeds (co-chaperone protein SBA1—0201; translationally controlled tumour protein (TCTP) homolog—0202; universal stress family protein (USP)—0501; hypothetical protein ZEAMMB73_682876—2601; 1-Cys peroxiredoxin antioxidant PER1—6201; glyoxalase I—2402; and esterase D—6402) (Table 1; Fig. 1C).

SBA1 is an alpha-crystallin-HSPs p23-like protein. TCTP is a highly conserved protein associated with a variety of cellular processes. Primarily, it acts as a molecule that prevents cell death via calcium binding and microtubule stabilization. TCTP also reduces cellular stress acting as molecular chaperone like HSP.

USP family is composed of small cytoplasmic proteins highly expressed as response to various stress factors. USP improves cell survival during prolonged exposure to harmful conditions, and may provide an overall "stress endurance". Hypothetical protein ZEAMMB73_682876 showed 88 % identities to protein DJ-1 B-like homolog [*Setaria italica*]. This DJ-1 homolog exhibits chaperone-like properties, and it is activated together with genes related to oxidative stress response in plants.

In addition, peroxiredoxins (PRXs) are involved in redox status regulation in cells. Subfamily of 1-Cys-PRX is composed of PRXs containing only one conserved cysteine. This amino acid operates as peroxidatic agent. Thus, PRXs are homodimeric thiol-specific antioxidants, which play defensive role by reduction of organic hydroperoxides, peroxynitrite, and excess of hydrogen peroxide in plant cells. While glyoxalases and esterases are typical detoxifying enzymes; glyoxalase I (other name: lactoylglutathione lyase) catalyses the hemithioacetal isomerization, formed by a glutathione and 2-oxoaldehyde, to S-D-lactoyl-glutathione. It is considered as a part of two-step system (called glyoxalase system) for detoxication of methylglyoxal (toxic side product of glycolysis).

Esterases and lipases interact with carboxylic esters. Many of them participate in the first phase of xenobiotics metabolism (especially toxins). The resulting this way carboxylates is then joined by other enzymes to increase their solubility and/or eventually for their excretion.

Quantitative analyses revealed that although the two glycolytic enzymes were overexpressed in axes of hydroprimed seeds (H) (cytosolic glycerqaldehyde-3-phosphate dehydrogenase—8501 and fructose-bisphosphate aldolase, cytoplasmic isozyme—8604) (Table 3; Fig. 1), their other isomeric forms were present in much greater quantity in axes of seeds conditioned with melatonin (glyceraldehyde-3-phosphate dehydrogenase 2, cytosolic—5602 and fructose-bisphosphate aldolase—8601) (Table 3; Fig. 1). In general, comparing the enzymes involved in energy metabolism a significant number (6) was overexpressed in the seeds hydroconditioned with melatonin compared with those hydroprimed without it. They were 6-phosphogluconolactonase isoform 2—2301, enolase—2701, triosephosphate isomerase, cytosolic—4202, malate dehydrogenase—4602, cytosolic 3-phosphoglycerate kinase—4701 and succinyl-CoA ligase alpha-chain 2—8602 (Table 3; Fig. 1).

Many proteins involved in protein destination, storage and turnover also were overexpressed under the influence of melatonin compared all hydroprimed seeds (i.e. chaperonin 60—2802, mitochondrial chaperonin-60—2903, globulin 2—3101, globulin-1 S allele precursors—3101 and 4003, proteasome subunit alpha type—5304, and unnamed protein product similar to proteasome subunit beta type 6 precursor—4203) (Table 3; Fig. 1CD). In the axes of hydroconditioned seeds in this functional protein group, only one cupin (8103) was overexpressed.

As concerns stress-related and defence proteins, 4 of them (i.e. HSP 26—1202, 1-Cys peroxiredoxin antioxidant—5202, general stress protein 39—5502, thioredoxin h2 protein—6001) were overexpressed in the hydroprimed seed axes (Table 3; Fig. 1B) and 8 in the axes from seeds hydroprimed with melatonin (i.e. glyoxalase family protein superfamily—1001, glyoxalase family protein isoform 2—3401, other general stress protein 39—4502, protein Z—4601, 22.0 kDa class IV HSP precursor—5102, glutathione transferase III(a)—5201, formate dehydrogenase 1—6702, and hypothetical protein ZEAMMB73_578099 similar to stress responsive protein—7501) (Table 3; Fig. 1CD).

Again, it was confirmed that melatonin applied to the seed affects polyphenols metabolism. In axes of HMel50 seeds, significant overexpression of caffeoyl-CoA *O*-methyltransferase 1 (1303) was observed in comparison with H seeds (Table 3; Fig. 1BC). It is an enzyme that catalyses the reaction between *S*-adenosyl-L-methionine and caffeoyl-CoA, which are transformed to *S*-adenosyl-L-homocysteine and feruloyl-CoA. A large number of secondary metabolites are generated via a step involving this enzyme. In general, it participates in phenylpropanoid biosynthesis.

Conclusions

To sum up (Fig. 2), hydropriming (H, HMel50, HMel500) provoked biosynthesis of 14 new, characteristic proteins in embryonic axes of the grains germinated at optimal temperature compared with the non-treated ones. When this pre-sowing treatment was supplemented with melatonin (HMel50, HMel500), additional 14 proteins occurred. Moreover, in the seed variant HMel50 (optimal melatonin concentration)—23 and in HMel500—3 characteristic spots were noticed. The majority of additional proteins belonged to energy metabolism enzymes, proteins involved in proteome plasticity via improving protein synthesis, folding, destination and storage, and—most importantly—defence, anti-stresses and detoxifying proteins.

Proteins belonging to these functional groups are frequently ranked as seed quality markers. Particularly, important are those considered to be anti-stress proteins, e.g. HSP, LEA, TRX, and other general stress proteins (Gallardo et al. 2001; Catusse et al. 2011; Wu et al. 2011). Especially, many of these proteins were determined in the corn seeds conditioned with melatonin.

In the literature, the beneficial effect of antioxidant enzymes, i.a. superoxide dismutase (SOD), CAT, glutathione-*S*-transferase (GST) on seed quality improvement, was underlined (Gallardo et al. 2001; Catusse et al. 2011). However, in our study, we did not observe any differences in the synthesis of these enzymes in the compared seed variants. It seems possible that supplementation of the seeds with melatonin—a highly effective, small molecule antioxidant—and overexpression of other antioxidant and detoxifying factors, such as TRXs, glioxylases, and improved polyphenol biosynthesis, were enough to regulate internal redox balance, and additional antioxidant enzymes were not needed in the conditioned (improved) seeds.

Moreover, the following factors are important for better seed vigour and quality, on the one hand, efficacious reserve mobilisation, and thus increase in the seed storage proteins, such as cupins and/or globulins—their translocation into embryonic axes of the conditioned corn seeds was noted in the presented study—and, on the other hand, the highly productive energy metabolism. With regard to that overexpression of gliceraldehyd-3-phosphate-dechydrogenase observed by us was also mentioned by other authors (Gallardo et al. 2001; Catusse et al. 2011; Wu et al. 2011), however, other glycolytic or Krebs cycle enzymes indicated as vigour markers were different depending on the seed type. In addition, Catusse et al. (2011) also pointed to the voltage-dependent anion channels (VDACs) as these markers, which were confirmed neither by other authors nor by us.

It seems that when determining markers of seed quality attention should also be paid to the proteins involved in sulphur assimilation and metabolism. Similarly, with regard to that also various proteins were indicated by scientists, i.a. phosphoserine aminotranspherase, *S*-adenosyl-L-methionine synthetase, serine hydroxymethyl transpherase (Catusse et al. 2011), but in most reports as well as in our work, cysteine synthase appeared (Gallardo et al. 2001; Catusse et al. 2011; Wu et al. 2011).

Unfortunately, it seems that the determination of specific seed quality markers will be really difficult due to the variety of seed material subjected to tests under different conditions. However, it is possible to indicate functional groups in which overexpression of different proteins could be beneficial for seed vigour improvement.

Indicating such protein groups and also specific examples for the tested plant material (Table 1), we partially explained why the hydroprimed corn seeds—especially hydroprimed with melatonin, and the seedlings grown from them were stronger in comparison with the non-treated ones, and so quickly and efficiently adapted to the changing environmental conditions (Posmyk et al. 2008, 2009a, b; Janas et al. 2009a). They could be a priori prepared to defence against potential harmful condition. In embryonic axes during the initial state of growth, even under optimal conditions, a number of antioxidative, detoxifying, and chaperon proteins were synthesized. Moreover, the supply of energy from seed storage substances was pretty intensified. The presented results for the first time direct explain how melatonin acts in seed axes as plant stress defence factor. It could be taken as a part of explanation why various plant species with higher content of melatonin have shown improved tolerance to stress (Park et al. 2013; Bajwa et al. 2014; Zhang et al. 2015).

Since melatonin is safe for environment (non-toxic, of natural origin, biodegradable) as well as being inexpensive, it is using as a supplement of various priming methods may be a reliable and cost-effective manner for beneficial seed quality modifications and could be economically favourable for organic farming (Janas and Posmyk 2013). Plant biostimulation by melatonin application is a promising tool to enhance ecological crops and to support safety-food production.

Author contributions statement Izabela Kołodziejczyk—all experiments concerning corn seeds, protein extraction, purification and 2D separation, data acquisition and PDQuest analysis/interpretation. Katarzyna Dzitko—methodological consultant and technical support, optimisation of 2D separation method for corn seed proteins. Rafał Szefczyk—LC–MS/MS and statistical analysis, database searches. Małgorzata M. Posmyk—work con-

ception, obtaining of funding, data analysis and interpretation, drafting of the manuscript, responsible for the integrity of the work.

Acknowledgments Presented research was supported by the National Science Centre of Poland as NCN NN310 111940 project.

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