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ORIGINAL ARTICLE

ABA pretreatment can limit salinity-induced proteome changes in growing barley sprouts

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Abstract A proteomic study was conducted on caryopses of barley cv. Stratus (*Hordeum vulgare*). The caryopses were germinated in darkness at 20 °C, according to one of the three experimental setups: (a) in distilled water for 24 h, followed by 100 mM NaCl for another 24 h (salinity stress, SS); (b) in 100 μM abscisic acid for 24 h, with rinsing in distilled water to remove residual ABA, followed by 100 mM NaCl for another 24 h (pretreatment with ABA + salinity stress, ABAS); and (c) only in distilled water, for comparison with the tested samples (control, C). After 48 h, proteomes were extracted from barley sprouts in each treatment, according to the method by Gallardo et al. (2002a, b, modified). Proteins were then separated by 2DE and analyzed by MALDI–TOF/MS (Bruker). Under salinity stress, we observed changes in the pattern of proteins involved in: (a) energy metabolism, (b) one-carbon metabolism, (c) cell wall biogenesis, lignification, and adhesion, (d) response to oxidative stress, desiccation stress, and other defense responses, (e) structure and organization of the cytoskeleton, (f) signal transduction, transcription and translation processing, and (g) protein turnover. Our results revealed that ABA can alter the response of barley seedlings to salinity stress. In general, ABA pretreatment limited stress-induced proteomic changes. Alterations in energy and one-carbon metabolism indicate the importance of ABA in glycolytic/gluconeogenic pathways and the SMM cycle during salinity stress. Other observations demonstrated the role of ABA in hemicellulose, cellulose and lignin biosynthesis, ROS scavenging, actin CK formation, control of PI signaling, chromatin-mediated mechanism of stress tolerance, mRNA stability, and protection against dehydration.

Abbreviations

2D PAGE	Two-dimensional electrophoresis in polyacrylamide gel
ABA	Abscisic acid
ACTH	Adrenocorticotrophic hormone
CHAPS	3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate
CHCA	α-Cyano-4-hydroxycinnamic acid
CK	Cytoskeleton
DHN	Dehydrins
DMSP	3-Dimethylsulfoniopropionate
DTT	Dithiothreitol
DUBs	Deubiquitinating enzymes
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GPX	Glutathione peroxidase
GST	Glutathione *S*-transferase
HAC	Histone acetyltransferase
HATs	Histone acetyltransferases
HGA	Homogalacturonan
HMT	Homocysteine methyltransferase
HSC	Heat shock protein cognate
HSP	Heat shock protein

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IEF	Isoelectrofocusing
JIP60	60 kDa jasmonate-induced protein
LEA proteins	Late embryogenesis-abundant proteins
MALDI– TOF/MS	Matrix-assisted laser desorption/ionization time-of-flight/mass spectrometry
MMT	Methionine methyltransferase
MFs	Microfilaments
PKC	Protein kinase C
PMF	Peptide mass fingerprinting
PMT	Pectin methyltransferase
ROS	Reactive oxygen species
SAM	S-Adenosylmethionine
SMM	S-Methylmethionine
SHMT	Serine hydroxymethyltransferase
TFA	Trifluoroacetic acid
UDPGDH	UDP-D-glucose dehydrogenase
USPs	Ubiquitin-specific proteases

Introduction

Abscisic acid (ABA) is a hormone that is essential for the growth and development of plants. It is also a key mediator of physiological responses to environmental stressors. Plants have to cope with various abiotic stressors, including drought, cold, freezing, heat, and salinity. Crop productivity is considerably thwarted by salinity stress. An excess of mineral salts in soil could result from intensive bedrock weathering or scant precipitation, which prevents leaching of soluble mineral compounds from soil. In recent years, rapid climate changes, including higher temperatures and lower rainfalls, have exacerbated the problem of soil salinity in Europe. Unskillful fertilization can also cause transient soil salinity which is harmful to emerging plants. Soil salinity is induced by higher concentrations of potassium, magnesium and calcium salts, sulfuric acid, and salts of carbonic acid, which leads to excessive alkalization of the substrate and prevents iron uptake. However, soil salinity (sodicity) is largely determined by sodium chloride (NaCl), a soluble salt. Plants growing in saline environments are exposed to two main stressors: the osmotic effect (1) and salt ions which are accumulated in the cytoplasm (2). Under saline conditions, the osmotic potential of soil water decreases, which induces osmotic stress and a decrease in water uptake by plants. Therefore, salinity stress leads to dehydration of plant cells. This non-specific process is also observed in response to other abiotic stressors, such as drought, freezing, and mechanical damage. By contrast, the concentration of salt ions increases only in salinity stress. Moreover, salinity stress is always accompanied by non-specific, oxidative stress.

ABA is a plant stress hormone and a major endogenous messenger that induces high salinity responses and activates protective mechanisms against dehydration, toxic ions, and oxidation (Fahad et al. 2015). ABA is largely responsible for activating genes responsible for osmotic adjustment, ion compartmentalization and homeostasis, root hydraulic conductivity, membrane stability, shoot and root growth regulation, inhibition of transpiration rate, and wilting, thus contributing to the maintenance of the water and osmotic balance in plants (Lata and Prasad 2011). The discussed phytohormone regulates stomatal closure, thus minimizing transpiration and loss of water (Wilkinson and Davies 2010). Under abiotic stress, stomatal closure and gene expression which are induced by ABA involve Ca^{2+} and/or H_2O_2 as second messengers (Leung and Giraudat 1998; Chinnusamy et al. 2005; Zhang et al. 2007). The group of salt-responsive genes upregulated by ABA includes genes encoding: (1) dehydrins and late embryogenesis-abundant proteins (e.g., HVA1, RAB18, RD22, and AtADH), (2) vacuolar H^+-inorganic pyrophosphatase (HVP1 and HVP10), tonoplast Na^+/H^+ antiporter (NHX1), the catalytic subunit (subunit A) of vacuolar H^+-ATPase (HvVHA-A), and γ-tonoplast intrinsic protein-1 (TIP1), (3) mitogen-activated protein kinases (e.g., MAPK4 and OsMAPK5), germin-like protein-1 (GLP1), radical-scavenging antioxidant enzymes, including catalase (CAT), superoxide dismutase (SOD), glutathione reductase (GR), and ascorbate peroxidase (APX), and (4) enzymes that participate in the biosynthesis of osmoprotectants, such as proline and trehalose (Shi and Zhu 2002; Jiang and Zhang 2003; Chinnusamy et al. 2005; Fukuda and Tanaka 2006; Gomez et al. 2010; Keskin et al. 2010; Bakht et al. 2012; Sripinyowanich et al. 2013; Iqbal et al. 2014). Halophytes are plants that tolerate salt, whereas glycophytes such as wheat, rice, barley, soybeans, and potatoes are sensitive to saline conditions. Despite the above, barley is relatively resistant to saline environments (Mahmood 2011).

This study set out to determine the effect of salt stress on changes in the proteome of barley sprouts in an early growth stage. Abscisic acid (ABA) mediates the responses and/or adaptive changes to external stressors in the environment, therefore, the responses of ABA-pretreated barley sprouts to salinity stress were also monitored.

Materials and methods

Plant material and treatment conditions

The experiments were conducted on caryopses of barley cv. Stratus (Hordeum vulgare) supplied by the Plant Cultivation Station in Strzelce. Seeds were surface disinfected in 0.5% sodium hypochlorite for 20 min and washed with sterilized water. Twenty intact grains were placed on two layers of Whatman paper No. 1 (Whatman, Midstone,

Kent, UK) in a Petri dish (ø 9 cm) and germinated in the dark at 20 °C under different conditions. Caryopses were germinated in distilled water for 24 h and immersed in 100 mM NaCl for another 24 h (salinity stress, SS). The remaining seeds were incubated in 100 µM abscisic acid for the first 24 h, rinsed with distilled water to remove residual ABA, and immersed in 100 mM NaCl for another day (ABA pretreatment + salinity stress, ABAS). Seeds germinated in distilled water for 48 h were the control (C). The examined material was composed of barley sprouts with a coleoptile emerged and a leaf at the tip. In agricultural practice, the above could be indicative of the germination phase, whereas the growth of a seedling starts with the emergence of the first leaf through the coleoptile and its unfolding. The phenotypes of the analyzed barley sprouts grown under different conditions are shown in Fig. 1.

Determination of sprout length, fresh and dry weight content

The length (coleoptiles, roots), fresh and dry weight of sprouts were determined in approximately 100 plants from at least three independent biological replicates ($n = 3$) of each sample (C, SS, and ABAS). Sprouts were separated from caryopses, surface drained on filter paper, measured, and weighed. To estimate dry weight content, sprouts were additionally dried at 85 °C for 48 h and weighed again. Root length was estimated for the longest root. The data were analyzed using Duncan's test (Statistica 12) and expressed as mean ± SD. Differences in the length, fresh and dry weight of SS and C samples, and ABAS and SS samples were considered statistically significant at $p \leq 0.01$.

Proteome extraction and 2D gel electrophoresis

Proteomes were extracted from the sprouts according to the method described by Gallardo et al. (2002a, b,

modified). Young plants were isolated from 100 caryopses in each treatment and powdered in liquid nitrogen. The powdered tissue of each sample was suspended in chilled lysate buffer (10 µl per 1 mg of calculated DW) containing 7.5 M urea, 2.2 M thiourea, 63 mM CHAPS, 0.25% Triton X-100 (Bio-Rad), 5 mM Trizma base, 21 mM Trizma hydrochloride, 1.1% ampholytes (pH 3–10), 60 U/ml DNase I, 72 lg/ml RNase A (5.8 Kunitz units/ml), 14% protease inhibitors (Cocktail complete mini, Roche), and 8 mM DTT (Bio-Rad). Each sample was stirred, shaken on ice for 45 min and centrifuged several times at $12,000 \times g$ for 10 min to obtain clear supernatant. The protein extract was purified using the 2D Clean-Up Kit (GE Healthcare). Protein concentration was measured at 280 nm using a NanoDrop 1000 spectrophotometer (Thermo Scientific). 600 µg of the protein sample was solubilized in rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS, 0.5% ampholytes (pH 3–10) (Amersham), 10 mM DTT, and 0.002% bromophenol blue) to a total volume 450 µl. The mixture was loaded onto a 24 cm Immobiline DryStrip Gel strip with a linear pH gradient of 3–10 (GE Healthcare). IEF was performed in the Ettan IPGphor 3 system (GE Healthcare) according to the manufacturer's instructions. Immediately prior to the second-dimensional run, the strip (containing separated proteins) was equilibrated in a glass tube in an equilibration buffer containing 6 M urea, 75 mM Tris–HCl (pH 6.8), 30% glycerol, and 26 mM DTT (in the first step) or 220 mM iodoacetamide (in the second step) for 15 min each. After equilibration, the strip was transferred to 12.5% SDS-PAGE gel (1 mm) and sealed with 10% agarose solubilized in an electrode buffer containing 25 mM Trizma base, 195 mM glycine, and 0.1% SDS (Laemmli 1970). Electrophoresis was carried in the EttanDalt Six electrophoresis unit (GE Healthcare) using the Laemmli buffer system, first at 2 W/gel for 0.5 h and then at 17 W/gel for the next 3 h. Low Molecular Mass Standards (Bio-Rad) were used to estimate the molecular masses of proteins.

Fig. 1 Phenotypes of 2-day-old barley sprouts isolated from caryopses germinating under different conditions: control (C), salinity stress (SS), and ABA pretreatment (ABAS). A detailed description of germination conditions can be found in the text

Image and data analysis

After 2D-PAGE, the gel was stained with Coomassie Brilliant Blue G-250 according to Neuhoff et al. (1988) and scanned in an Image Scanner using the Labscan 3.0 software (GE Healthcare). Three images representing three independent biological replicates for each treatment (C, SS, and ABAS) were grouped and analyzed in the ImageMaster 2D Platinum 6.0 program. After spot detection, the quantitative determination of spot intensity, including data normalization before principal component analysis (Wibig 2007), was performed based on the average values of background optical density (OD) and spot intensity on each gel. The spot volumes in SS were compared with those in C, and the spot volumes in ABAS were compared with those in SS. Proteins exhibiting at least 1.5-fold reproducible changes in abundance between the compared samples were subjected to statistical analysis using Duncan's parametric test ($p \leq 0.01$). Proteins whose abundance changed significantly were excised from the gels manually and identified by mass spectrometry.

Protein identification

The spots which were cut out from the gels were washed and destained with 25 mM ammonium bicarbonate in 50% ethanol for 1 h at 37 °C. Before in-gel digestion, proteins were reduced with 10 mM DTT in 25 mM ammonium bicarbonate for 30′ at 60 °C, and alkylated with 10 mM iodoacetamide in 25 mM ammonium bicarbonate for 45′ at 37 °C in the dark. Proteins were dried in a vacuum centrifuge and digested using sequencing grade trypsin solution (Promega, Madison, USA) containing 10 ng/μl trypsin in 25 mM ammonium bicarbonate. Digestion was carried out at 37 °C overnight. After in-gel digestion, gels were washed three times with 0.1% TFA in 50% acetonitrile to collect protein peptides. After the extraction of peptide fragments from the gel, samples were desalted and cleaned with Zip-Tip C18 (Millipore) according to the manufacturer's instructions. Tryptic mixed peptides in the amount of 1 μl were loaded onto an AnchorChip (Bruker) target plate, one volume of saturated CHCA (Sigma) in 50% v/v acetonitrile with 0.1% TFA was added, and the peptides were cocrystallized. The loaded target was analyzed in the MALDI–TOF/MS system (Bruker) equipped with a nitrogen laser and operated in a reflector/delayed extraction mode for MALDI–TOF PMF with the FlexControl software (Bruker). The instrument was calibrated externally with $(M + H)^+$ ions of Bradykinin 1, Angiotensin II, Angiotensin I, Substance P, Bombesin, Renin Substrate, ACTH clip 1, ACTH clip 18, and Somatostatin 28 (Peptide Calibration Standard II, Bruker). The MS spectra were searched against the National Center for Biotechnology Information database (NCBI). The MASCOT search engine (http://www.matrixscience.com) was used for PMF search. Database queries were carried out using the following parameters: trypsin cleavage, taxonomy of "Viridiplantae (Green Plants)", monoisotopic mass accuracy with 80 ppm tolerance, one missed cleavage site, carbamidomethylation of cysteine as fixed modification, and oxidation of methionine as variable modification. A protein was positively identified based on the following criteria: protein scores higher than 75 and minimum 10% protein coverage by the matching peptides, including at least three independent peptides with mass deviation of less than 50 ppm. Theoretical pI and MW values were calculated from the identified sequence in NCBInr using the ExPASy tool (http://www.expasy.org).

Results

Changes in the length, fresh and dry weight of barley sprouts

The effect of salinity on barley growth and the changes induced by ABA pretreatment were investigated based on differences in the length of coleoptiles and roots, as well as fresh and dry weight of sprouts. The investigated parameters were altered by the applied treatments, both in SS and in ABAS probes (Fig. 2a, b). In SS probes, the length, fresh and dry weight of stressed plants were reduced by around 45% (both coleoptile and root), 51 and 33%, respectively. These results indicate that the growth, dry weight accumulation, and hydration of seedling tissue were significantly inhibited under salinity stress. All of the examined parameters were low in SS, but the stressed sprouts had a characteristic morphology. The roots of stressed seedlings had more root hairs than control roots (Fig. 1). The inhibition of seedling growth under ABA pretreatment was manifested by primary root growth (by around 48%). Despite the above, changes in the length of coleoptiles or dry weight were not observed in the ABAS sample. However, the average fresh weight of sprouts in ABAS was approximately 28% higher than in SS.

Changes in the proteome under different treatments

Our results revealed proteomic changes in barley seedlings treated with NaCl, indicating that ABA pretreatment altered the salinity response. To separate and identify differentially expressed proteins, IEF/SDS-PAGE coupled with MALDI TOF mass spectrometry was performed. In the group of matched spots, the abundance levels (% vol) of 52 protein spots were changed under saline conditions.

Fig. 2 Effect of different treatments on the growth parameters of barley sprouts: **a** fresh and dry weight and **b** length of the coleoptile and the root. The results are presented as mean ± SD ($n = 3$). Statistically significant differences are marked with *different letters*
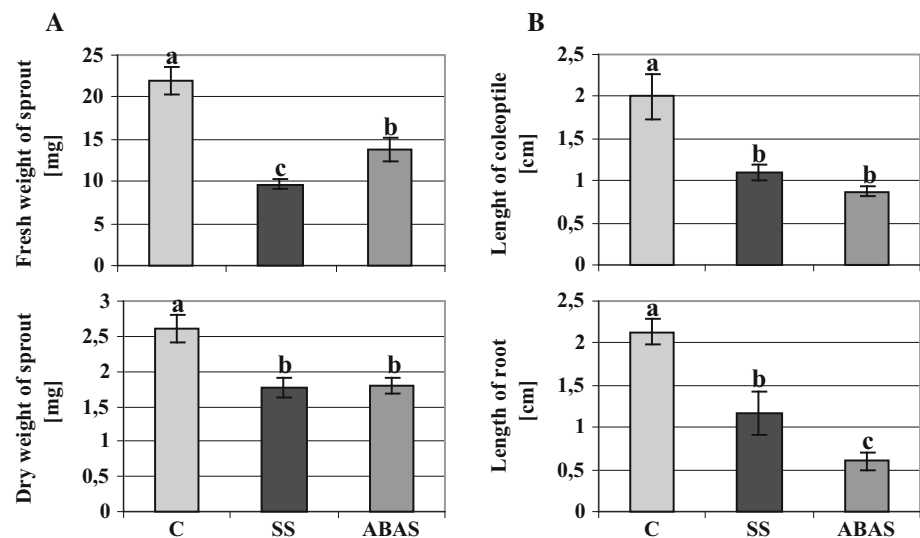
However, 44 of these spots were successfully identified by spectrometric analysis (Fig. 3; Table 1). Based on their function, the identified salt-responsive proteins were classified as proteins involved in: (a) energy metabolism, (b) one-carbon metabolism, (c) cell wall biogenesis, lignification, and adhesion, (d) response to oxidative stress, desiccation stress, and other defense responses, (e) structure and organization of the cytoskeleton, (f) signal transduction, transcription, and translation processing, and (g) protein turnover (Table 1; Fig. 4).

In the group of 44 proteins regulated by salinity, 24 proteins were altered by ABA pretreatment. Exogenously applied ABA minimizes salinity-induced changes. Moreover, the expression of five proteins (spots 13, 14, 15, 26, and 35) in the ABAS probe was silenced to the extent comparable to that noted in the control treatment (Fig. 3; Table 1). The highest percentage of proteins altered by ABA was observed in the group of proteins involved in: (1) biogenesis and lignification of the cell wall and (2) responses to oxidative and desiccation stress. By contrast, ABA did not influence salt-responsive proteins classified as proteins that are involved in other defense responses and protein turnover (Fig. 4).

Discussion

High salt concentrations inhibit plant growth, development, and survival. Exposure to high salt concentrations influences major life processes in plants (Batool et al. 2014). Our research demonstrated that salinity stress significantly inhibited the growth of barley sprouts. However, the characteristic morphology of roots (with a higher number of root hairs) in stressed plants points to the initiation of protective processes against salinity. The aim of our work was to estimate processes that play a key role in salinity response, including the role of ABA in salt stress prevention. Our proteomic study revealed salinity-induced changes which can explain the morphological adaptation of sprouts to saline conditions. Incubation in 100 µM ABA which inhibits plant growth and development, followed by salinity stress, led to even greater inhibition of root growth, but not the growth or density of root hairs. However, the fresh weight of stressed sprouts preincubated in ABA increased. Jones et al. (1987) demonstrated that the growth of wheat roots was inhibited at ABA concentrations that increased turgor pressure. The above findings suggest that the physiological effect of salt stress was alleviated by ABA pretreatment, mainly by the regulation of the water balance in analyzed barley sprouts. In our study, ABA pretreatment limited stress-induced changes in the barley proteome. Our independent research (data not shown) demonstrated that the treatment of barley caryopses with 100 µM ABA induced proteomic changes similar to those caused by salinity, excluding the JIP protein. The observed reduction in the salt-responsive proteome could indicate that ABA pretreatment plays a role in the acquisition of tolerance to salinity stress. The metabolic functions of proteins affected by both salt and ABA are discussed below.

Energy metabolism

Under NaCl stress, plants decrease their metabolic rates to conserve energy and limit ROS production (Möller 2001; Golldack et al. 2014). Two enzymes involved in energy metabolism were downregulated: putative cytochrome c oxidase subunit II PS17 (spot 4) and ATP synthase beta
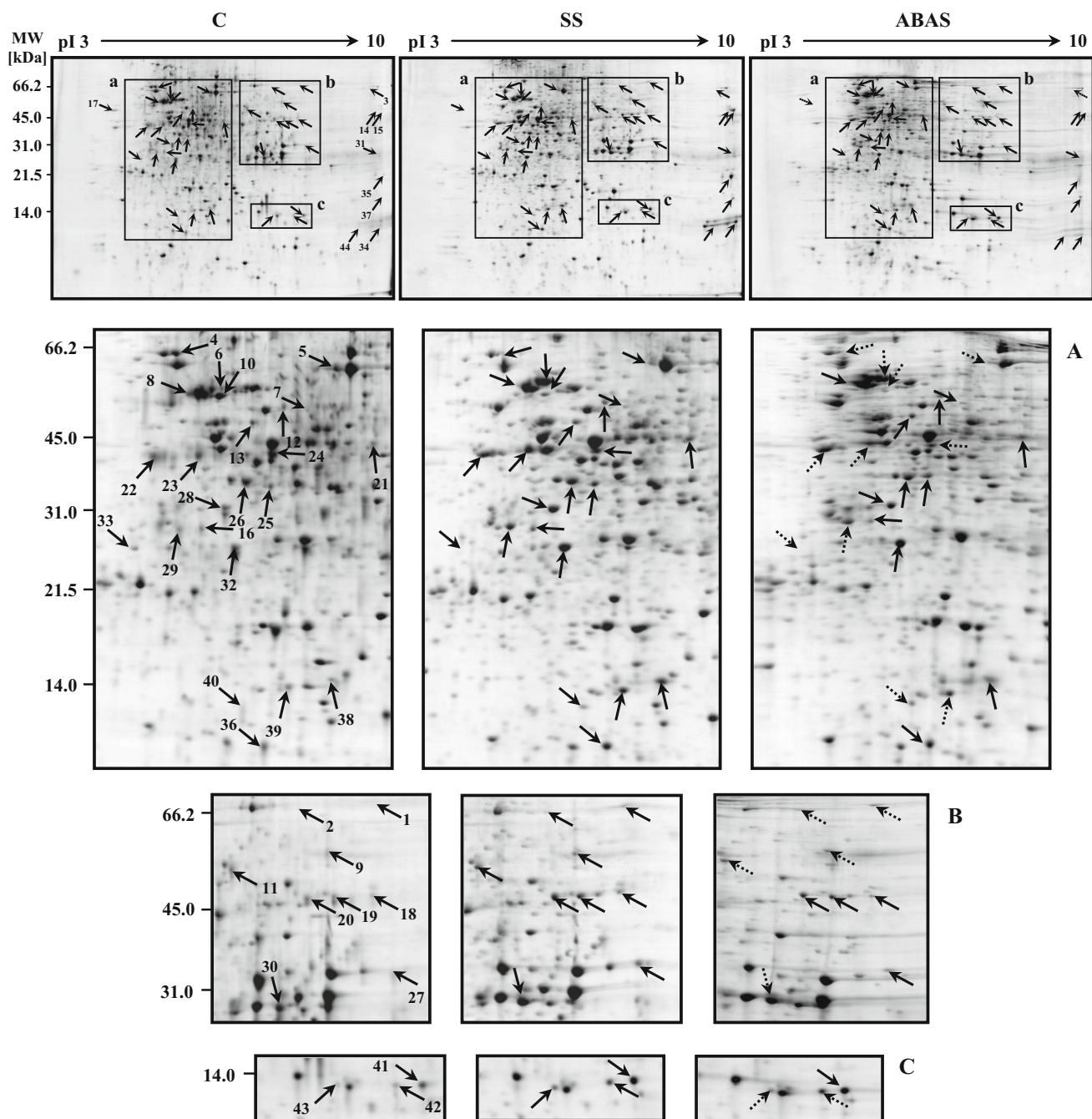
Fig. 3 2DE profiles of proteins from barley sprouts grown under control conditions (C), salt stress conditions (SS), and pretreated with ABA before exposure to salt stress (ABAS). Proteins were separated from each sample in the amount of 600 μg by 2DE and visualized by CBB G-250 staining. They were separated in pH 3–10 (24 cm, linear) in the first dimension and 12.5% SDS-PAGE in the second dimension. Significantly altered protein spots ($p \leq 0.01$), whose volume% changed more than 1.5-fold, are marked with *arrows* (unchanged spots in ABAS are marked with *dotted arrows*). Altered spots were analyzed by MALDI–TOF/MS. The list of the identified proteins is shown in Table 1
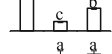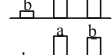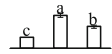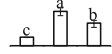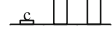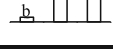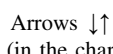
subunit (spot 10). Cytochrome c oxidase is a terminal enzyme in the mitochondrial respiratory chain, whereas the ATP synthase beta subunit is a mitochondrial proton-transporting ATP synthase complex. The downregulation of these proteins could result from the disruptive influence of salinity stress on membranes.

However, not all proteins involved in catabolism were downregulated. For example, we observed the upregulation of the cytosolic enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (spot 25) which plays a major role in glycolytic and gluconeogenic pathways. GAPDH catalyzes the reversible oxidation and phosphorylation of

Table 1 Salt stress-responsive proteins from barley seedlings (SS) and the effect of ABA pretreatment on changes in salt response (ABAS)

Spot No	Protein name	Species	Accession No	Mw (kDa)/pI Exp.	Theo.	Peptides/ Coverage (%)	Expression C SS ABAS	ABA effect
Energy metabolism								
4	Putative cytochrome c oxidase subunit II PS17	*P. strobus*	P84733	64.19/4.70	1.71/9.31 (83.10/7.40)	2/90		→
10	ATP synthase beta subunit, EC 3.6.3.14	*T. aestivum*	Q41534	54.27/4.98	59.25/5.56	12/30		→
25	**Glyceraldehyde-3-phosphate dehydrogenase 1, cytosolic, EC 1.2.1.12**	***H. vulgare***	**P26517**	**36.35/5.38**	**36.51/6.67**	**9/32**		↓
One-carbon metabolism, methylation processes								
1	Dehydration-responsive protein-like	*O. sativa*	Q8H636	69.62/7.82	68.44/7.86	7/12		→
5	Methionine synthase, EC 2.1.1.13	*H. vulgare*	Q6BCT3	62.20/5.87	84.51/5.67	11/21		→
19	**Serine hydroxymethyltransferase (SHMT), EC 2.1.2.1**	***T. monococcum***	**A6XMY5**	**47.08/7.46**	**56.12/8.18**	**5/11**		↓
24	S-adenosylmethionine synthase 1 (AdoMet synthase 1), EC 2.5.1.6	*H. vulgare*	P50299	39.91/5.40	42.84/5.49	12/45		→
28	**Homocysteine S-methyltransferase 2, EC 2.1.1.10**	***Z. mays***	**Q9FUM9**	**33.37/5.00**	**36.96/5.17**	**7/41**		↓
Cell wall biogenesis and adhesion, lignin accumulation								
2	Probable methyltransferase PMT10, EC 2.1.1.-	*A. thaliana*	Q94KE1	67.58/7.20	74.45/6.27	19/31		→
12	**Putative anthocyanidin-3-glucoside rhamnosyltransferase, EC 2.4.1.-**	***O. sativa***	**Q8S7R8**	**52.309/5.47**	**53.43/6.32**	**12/20**		↓
13	**Xylose isomerase, EC 5.3.1.5**	***H. vulgare***	**Q40082**	**51.21/4.6853**	**53.61/5.31**	**6/15**		↓
21	**UDP-glucose 6-dehydrogenase 1 (UDPGDH 1), EC 1.1.1.22**	***G. max***	**Q96558**	**45.86/6.18**	**52.94/5.74**	**11/19**		↓
31	**Glucan endo-1-3-beta-glucosidase GIII, EC 3.2.1.39**	***H. vulgare***	**Q02126**	**27.02/9.61**	**34.89/9.19**	**4/13**		↓
Structure and organization of the cytoskeleton								
23	Tubulin alfa 1 chain	*H. vulgare*	Q43473	41.05/4.85	49.60/4.95	16/58		→
26	**Actin-58**	***S. tuberosum***	**P30167**	**36.35/5.21**	**41.79/5.46**	**5/13**		↓
36	**Putative formin-like protein 15b (AtFH15b)**	***A. thaliana***	**P0C5K3**	**11.03/5.49**	**39.47/6.37**	**12/39**		↓
40	Profilin1	*H. vulgare*	P52184	13.98/5.22	14.30/5.04	5/64		→
Signal transduction, transcription and translation processing								
7	**60 kDa jasmonate-induced protein (rRNA N-glycosidase), EC 3.2.2.22**	***H. vulgare***	**Q00531**	**58.87/5.83**	**60.36/6.54**	**9/20**		↓
15	**Elongation factor 1 alfa (EF1α)**	***H. vulgare***	**Q40034**	**49.36/9.54**	**49.14/9.15**	**15/32**		↓
17	Calreticulin	*H. vulgare*	Q40041	48.33/4.00	47.36/4.48	17/37		→
22	Putative calcium-binding protein annexin 6	*O. sativa*	Q5VNH3	42.25/4.57	34.97/6.08	11/16		→
32	**Histone acetyltransferase HAC2, EC 2.3.1.48**	***A. thaliana***	**Q9FYH1**	**26.18/5.15**	**155.77/8.16**	**13/12**		↓
33	14-3-3-like protein A (14-3-3A)	*H. vulgare*	P29305	25.56/4.48	29.35/4.83	4/13		→
Protein turnover								
3	Ubiquitin carboxyl-terminal hydrolase 25 (AtUBP 25), EC 3.4.19.12	*A. thaliana*	Q9FPs2	64.19/9.28	73.29/9.21	23/26		→

Table 1 continued

	Response to desiccation stress							
6	Heat Shock 70 kDa protein	*Z. mays*	P11143	62.03/4.90	70.57/5.22	20/23	b a a	→
8	**Heat shock cognate 70 kDa protein 2 (AtHsc70-2)**	***A. thaliana***	**P22954**	**56.30/4.81**	**71.39/5.03**	**20/25**	a c b	↑
9	Dehydrin	*H. vulgare*	Q9SPA7	55.09/7.03	58.50/6.55	8/14	b a a	→
16	**Late embryogenesis abundant protein, group 3 (LEA-3)**	***O. sativa***	**P0C5A4**	**28.32/4.83**	**20.51/5.89**	**15/36**	c a b	↓
18	**Dehydrin 6**	***H. vulgare***	**Q9ZTR5**	**47.70/7.78**	**47.65/8.09**	**5/11**	c a b	↓
34	**Dehydrin DHN1 (B8)**	***H. vulgare***	**P12951**	**12.05/9.66**	**14.24/8.81**	**6/55**	c a b	↓
35	**ABA-inducible protein PHV A1**	***H. vulgare***	**P14928**	**20.58/9.59**	**21.82/9.02**	**7/30**	b a b	↓
38	**Salt stress-induced protein**	***O. sativa***	**A2WPN7**	**14.90/5.51**	**15.20/5.19**	**5/58**	b a b	↓
39	Late embryogenesis abundant protein B19.3 (LEA B19.3)	*H. vulgare*	Q02400	14.04/5.54	14.60/5.38	9/47	b a a	→
41	**Protein HVA22**	***H. vulgare***	**Q07764**	**13.97/7.83**	**15.06/8.67**	**6/60**	a a b	↓
42	Dehydrin DHN3 (B17)	*H. vulgare*	P12948	13.93/7.68	16.16/8.08	4/51	b a a	→
44	**Dehydrin DHN2 (B9)**	***H. vulgare***	**P12952**	**12.27/9.08**	**14.44/8.00**	**5/39**	c a b	↓
	Response to oxidative stress							
11	Catalase, EC 1.11.1.6	*H. vulgare*	F2CPL2	52.72/6.67	56.59/6.68	22/48	b a a	→
14	**Glutathione peroxidase, EC 1.11.1.9**	***H. vulgare***	**F2CZQ2**	**49.62/9.59**	**24.94/9.57**	**5/11**	b a b	↓
20	**Catalase 1, EC 1.11.1.6**	***H. vulgare***	**Q71VA1**	**46.83/7.28**	**46.11/6.59**	**14/52**	c a b	↓
27	**Peroxidase BP1, EC 1.11.1.7**	***H. vulgare***	**Q40069**	**33.66/7.91**	**38.82/7.57**	**14/46**	c a b	↓
37	**Peroxidase 2 (Atperox P2), EC 1.11.1.7**	***A. thaliana***	**Q67Z07**	**15.88/9.61**	**35.62/9.35**	**6/19**	a a b c	↓
	Other defense response							
29	RNase S-like protein	*H. vulgare*	Q8L827	28.76/4.76	27.65/6.58	10/33	b a a	→
30	Putative endonuclease	*O. sativa*	Q8S642	27.09/7.10	56.10/5.74	9/14	b a a	→
43	Trypsin inhibitor CMe (BTI-CMe1)	*H. vulgare*	P01086	13.55/7.24	16.14/7.50	7/20	b a a	→

The proteins changed by ABA by more than 1.5-fold at the significance level of $p \leq 0.01$ are marked in bold letters. Arrows ↓↑ indicate downregulation or upregulation induced by ABA, respectively. Different letters denote statistically significant differences (in the charts). Spot numbers correspond to the spots in Fig. 3

glyceraldehyde 3-phosphate into 3-phospho-D-glyceroyl phosphate. This enzyme is essential for the maintenance of cellular ATP levels and carbohydrate metabolism. GAPDH is synthesized in roots during anoxic conditions, and it belongs to the group of anaerobic polypeptides (Manjunath and Sachs 1997). It appears that GAPDH responds to NAD:NADH and ATP:ADP ratios in the cell to accommodate the cell's specific requirements (Velasco et al. 1994). In addition to its catalytic role, GAPDH is also involved in responses to various abiotic stressors in plants (Yang Zhai 2017). Under stress, GAPDH plays a pivotal role in redox signaling by interacting with F-actin. Cytosolic GAPDH also interacts with the plasma membrane-associated phospholipase D (PLDδ) in H_2O_2-mediated signal transduction. Genetic ablation of PLDδ impeded stomatal response to abscisic acid and hydrogen peroxide, placing PLDδ downstream of H_2O_2 in mediating ABA-induced stomatal closure (Guo et al. 2012). In our study, GAPDH was the only energy metabolism enzyme to be affected by ABA treatment.
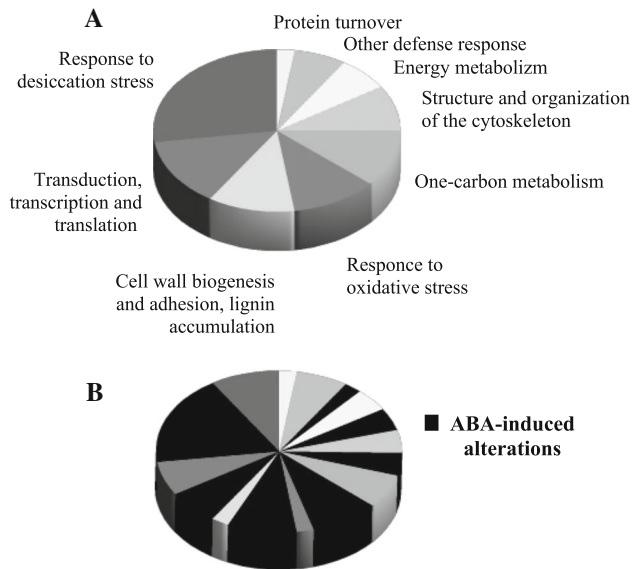
Fig. 4 Percentage of **a** identified proteins with various metabolic functions in the salinity stress response and **b** ABA-regulated proteins in each functional group

One-carbon metabolism

Methionine (Met) is metabolized in two ways: by (1) the activated methyl cycle or (2) the *S*-methylmethionine (SMM) cycle in one-carbon (C1) metabolism. In the first metabolic process, Met is transformed to *S*-adenosylmethionine (SAM) which participates in the synthesis of selected metabolites, such as glycine betaine, methylated polyols, and polyamines. Glycine betaine and polyols are osmoprotectants which are accumulated in response to water/salinity stress (Bohnert and Jensen 1996; Fujiwara et al. 2010). Polyamines participate in various processes at the cellular level, including DNA replication, transcription, translation, cell proliferation, modulation of enzymatic activity, membrane stability, cation–anion balance in cells, and wall lignification (Gill and Tuteja 2010; Ogawa and Mitsuya 2012).

In the second metabolic process, Met is converted to *S*-methylmethionine in the SMM cycle. SMM is synthesized when a methyl group is transferred from *S*-adenosylmethionine (SAM) to methionine (Met) in a reaction mediated by methionine methyltransferase (MMT), and it is reconverted to Met with homocysteine (Hcy) in a reaction catalyzed by homocysteine methyltransferase (HMT). Ranocha et al. (2001) suggested that the SMM cycle uses half of the synthesized SAM (1) to inhibit the accumulation of SAM. However, SMM is converted to 3-dimethylsulfoniopropionate (DMSP), a compatible solute in selected salt-tolerant plants (Hanson et al. 1994).

In our work, salinity induced the downregulation of two enzymes involved in the activated methyl cycle—methionine synthase (spot 5) and *S*-adenosylmethionine synthase (spot 24), and the upregulation of homocysteine *S*-methyltransferase (spot 28) involved in MMT. In this group of enzymes, only HMT was altered by ABA. This observation revealed changes in one-carbon metabolism, and it emphasized the importance of SMM in salinity response and ABA action. Our findings differ from the results reported in other studies, where the activated methyl cycle played the main role in responses to ABA and salt treatment. Espartero et al. (1994) suggested that *SAM* genes are responsive to salt stress and that ABA could participate in the upregulation of *SAM* transcripts.

Serine hydroxymethyltransferase (SHMT), an enzyme that catalyzes the reversible conversion of serine and tetrahydrofolate (THF) to glycine and N^5,N^{10}-methylene-THF, is also involved in the pathway for the interconversion of C-1 compounds. We identified SHMT (spot 19) as a salinity-upregulated protein which was also altered by ABA treatment. We also observed the upregulation of dehydration-responsive protein (spot 1), a predicted protein with *S*-adenosylmethionine-dependent activity. The increase in both proteins in the analyzed tissue emphasizes the significance of methylation processes during salinity stress. Less is known about the direct influence of ABA on SHMT, but Verslues et al. (2007) and Kaur et al. (2009) suggested links between sensitivity to ABA and glycolate metabolism, in particular the activity of peroxisomal glu:glyoxylate aminotransferase (GGT). GGT converts glycolate to glycine, the substrate for SHMT. GGT is essential for ABA-induced accumulation of proline and gene expression, and accumulation of ABA under exposure to stress and pretreatment with ABA. The results of this study validate the assumption that hydrogen peroxide participates in ABA signaling in proline accumulation.

Cell wall biogenesis, lignification, and adhesion

The cell walls of monocots, including wheat and barley, are composed of a cellulosic network embedded in a matrix of hemicellulose fibers and pectin polysaccharides. The major polysaccharides include glucuronoarabinoxylans and (1,3;1,4)-β-D-glucans, whereas the levels of glucomannans, xyloglucans, and pectic polysaccharides are relatively low (Farrokhi et al. 2006).

In our study, selected enzymes responsible for wall synthesis were upregulated under salinity stress. We identified two enzymes involved in hemicellulose synthesis: UDP-D-glucose dehydrogenase (spot 21) and xylose isomerase (spot 13). UDP-D-glucose dehydrogenase (UDPGDH) oxidizes UDP-D-glucose (UDP-Glc) to UDP-D-glucuronate (UDP-GlcA), the precursor of UDP-D-xylose and UDP-L-arabinose, and the main cell wall polysaccharide precursors. Xylose isomerase catalyzes the synthesis

of D-xylose from D-xylulose, a precursor of xyloglucan, and hemicellulose.

Another detected enzyme, glucan endo-1-3-β-glucosidase GIII (spot 31), could play a role in the synthesis of cellulose. Several authors have demonstrated that hydrolytic enzymes are involved in the reorganization of newly synthesized cellulose chains (Szyjanowicz et al. 2004; Farrokhi et al. 2006). Moreover, the key quantitative trait locus (QTL) associated with the content of (1,3;1,4)-β-D-glucan in barley grain co-segregates with the gene encoding (1,3;1,4)-β-D-glucanase (Han et al. 1995).

We also identified pectin methyltransferase, probable methyltransferase PMT10 (spot 2) which could control cell wall adhesion by incorporating methyl groups into pectin in their synthesis site (Vannier et al. 1992; Goubet and Mohnen 1999). Pectic polysaccharides such as homogalacturonan (HGA) are produced in the Golgi apparatus. HGA comprises a linear chain of α-1,4-linked D-galacturonic acid (GalA) that undergoes different methyl esterification reactions at C6 (Bacic et al. 1988). The degree of pectin methyl esterification is a key determinant of pectin's adhesive properties (Krupková et al. 2007).

We also observed the upregulation of three enzymes involved in lignin biosynthesis: putative anthocyanidin-3-glucoside rhamnosyltransferase (spot 12) and two peroxidases—peroxidase BP1 (spot 27) and peroxidase 2 (spot 37).

In the walls of grass cells, lignin is a polymer composed of phenylpropanoid monolignol units: hydroxycinnamic, ferulic, and p-coumaric acids. Ferulic acid and, partly, p-coumaric acid are esterified to arabinose molecules of arabinoxylans. Arabinoxylan polymers are also cross-linked through diferulates (Jung and Deetz 1993).

Oxidative stress caused by pathogenic infections, wounding, and other environmental stressors leads to oxidative burst and the release of ROS. Peroxidases catalyze the reduction of H_2O_2 by relying on cell wall molecules as substrates such as lignin precursors, phenolic compounds, or auxin, which leads to polymerization reactions, including suberization, lignification, and cell wall protein cross-linking (Kristensen et al. 1999; Passardi et al. 2004a, b). An increase in peroxidase levels could suggest that the cell wall is undergoing modification to reduce the influx of ions under saline conditions. Moreover, lignification is intensified by putative anthocyanidin-3-glucoside rhamnosyltransferase which is involved in the biosynthesis of flavonoids, stilbene and lignin. Peroxidases play an important role in the metabolism of apoplastic ROS, and they contribute to root growth and development. Hydrogen peroxide appears to be involved in root growth restriction and root hair formation (Dunand et al. 2007). In their oxygenated form (oxyperoxidases), peroxidases can increase H_2O_2 levels in the zone of differentiation in roots.

The catalytic versatility of peroxidases can explain the morphological changes observed in barley roots in our study. Hydrogen peroxide could inhibit root growth and stimulate root hair formation under salt stress. The observed significant inhibition of root growth, accompanied by rapid formation of root hairs, indicates that ABA pretreatment enhances the mechanisms of resistance to environmental stress.

Our results suggest that the maintenance of cell wall architecture is crucial under stress and that ABA plays an important role in this process. The only protein that was not affected by abscisic acid was probable methyltransferase PMT10 (spot 2). These findings emphasize the importance of ABA in cellulose and hemicellulose synthesis and in lignification processes which increase the thickness of cell walls. Park et al. (2011) suggested a possible connection between the involvement of MYB52 genes in ABA response and cell wall biosynthesis. Other authors noted that ABA inhibits seed germination by preventing the loosening and expansion of cell walls (Gimeno-Gilles et al. 2009). Our results suggest that ABA induces cell wall thickening by increasing the biosynthesis of cell wall components, which could restrict elongation and enhance protective mechanisms against stress.

Response to oxidative stress

Other enzymes that catalyze ROS scavenging, including catalase (spot 11, 20) and glutathione peroxidase (spot 14), were also identified in this study. Roxas et al. (2000) reported that overexpression of glutathione S-transferase with glutathione peroxidase activity (GST/GPX) in transgenic plants promoted the growth of seedlings under exposure to various stressors. They demonstrated that seedlings expressing GST/GPX (GST+) were characterized by a high growth rate under exposure to low temperatures and saline stress, whereas the growth of control seedlings was stunted under the same conditions. In other studies, transgenic GST+ seedlings were significantly more abundant in oxidized glutathione (GSSG) under exposure to stress in comparison with wild-type seedlings (Roxas et al. 1997). High levels of GSSG could point to intensified scavenging activity of GPX-dependent peroxide in seedlings. The above could prevent cellular damage, promote metabolic activity, and stimulate growth in seedlings (Roxas et al. 2000). According to Miao et al. (2006), GPX3 plays a dual role: first, as a scavenger of H_2O_2, and second, as a transducer in ABA and H_2O_2 signaling pathways, where it regulates stomatal movement during drought.

In plant cells, the activity of ABA could be related to oxidative stress. This phytohormone could stimulate the

production of H_2O_2 (Zhang et al. 2001; Jiang and Zhang 2001, 2003) and induce the expression of selected antioxidant genes encoding, for example, catalase (CAT) and peroxidase (POX) (Guan et al. 2000; Jiang and Zhang 2003; Agarwal et al. 2005). Low doses of ABA (10–100 μM) triggered an antioxidant defense mechanism to prevent oxidative damage to membrane lipids and proteins (Jiang and Zhang 2001). A minor, ABA-induced increase in H_2O_2 levels could be required to build stress tolerance and enhance survival in plants exposed to environmental stressors.

Structure and organization of the cytoskeleton

We identified two cytoskeletal proteins: tubulin alpha-1 chain (spot 23) and actin-58 (spot 26), as well as proteins that affect cytoskeleton (CK) organization: profilin1 (spot 40) and putative formin-like protein 15b (AtFH15b) (spot 36). All proteins were upregulated under saline conditions. Tubulin is a building block of the cytoskeleton, and it is involved in the intracellular transport of organelles, including chromosomes and mitotic spindles. The first group of compounds that are targeted by oxidative stress includes tubulin (Apraiz et al. 2006; Wu et al. 2013). In animal cells, β-tubulin can promote phosphorylation during microtubule formation, and it protects the cytoskeleton against oxidative stress (Howard and Hyman 2003). The cytoskeleton is also composed of microfilaments (MFs) which could play a role during salt stress. According to Zhao et al. (2013), stress signaling relies on the interactions between microfilaments and Ca^{2+} in plants. In bacteria, the actin cytoskeleton controls immune cell signaling and the production of cytokines and reactive O^{2-} (Aktories et al. 2011).

Profilin is a ubiquitous protein which binds to actin and modifies cytoskeletal structure by polymerizing or depolymerizing actin filaments. It also participates in plant cell responses to internal and external signals (Fatehi et al. 2012). Profilin is involved in signaling pathways, which promotes rapid formation of actin filaments (Ridley et al. 2003; Lindberg et al. 2008; Yu 2008). Actin polymerization also involves formins which belong to a different family of multimodular proteins (Carlier and Pantaloni 2007). Formins associate with the fast-growing end of actin filaments and incorporate actin from profilin:actin structures (Romero et al. 2007; Sarmiento et al. 2008; Yu 2008).

In the present study, ABA affected two cytoskeletal proteins: actin and putative formin-like protein, which suggests that ABA influences the organization of actin filaments. The cytoskeleton is involved in signaling, and the potential significance of ABA-induced actin reorganization is described below.

Signal transduction, transcription, and translation processing

The signal transduction pathway starts with signal perception by different receptors, such as ion channels. In plant cells, the transient influx of Ca^{2+} ions is one of the earliest responses to salinity stress. The initial increase in cytosolic Ca^{2+} is followed by the generation of secondary signaling molecules such as inositol phosphates or reactive oxygen species. In turn, secondary signaling molecules can provoke the release of other receptor-mediated Ca^{2+}. The influx of Ca^{2+} ions initiates the protein phosphorylation cascade, followed by the activation of proteins that are directly involved in cellular protection or the regulation of stress-responsive genes (Xiong et al. 2002).

In our study, we identified four proteins that are directly or implicitly involved in PI signaling: EF1α (spot 15), calreticulin (spot 17), putative calcium-binding protein annexin 6 (spot 22), and 14-3-3-like protein A (spot 33), which were responsive to salinity. EF1α was also altered by ABA pretreatment.

EF1α is a common protein elongation factor, but there is evidence to suggest that it also plays an important role in controlling phosphatidylinositol signaling, which leads to F-actin remodeling (Amiri et al. 2007) and increases the amount of CK surrounding ER and Lys content in maize endosperm (Azama et al. 2003). eEF1α has the ability to bind and bundle F-actin. During PI signaling, PI is converted via PIP and PIP_2 forms to IP_3, which triggers the release of Ca^{2+} from cellular stores. Actin MFs could regulate the activity of Ca^{2+} channels (Wang et al. 2011). It has been reported that PIK-A49, an eEF1A-like protein isolated from carrots, binds and activates carrot phosphatidylinositol-4 kinase (PI4K) in response to an increase in cytosolic calcium. In turn, PI4K catalyzes the biosynthesis of phosphatidylinositol-4 phosphate (PI4P) (Yang and Boss 1994). According to Choi et al. (2008), PIP (PI3P and PI4P) participates in the reorganization of ABA-induced actin filament and stomatal closing in guard cells. They suggested that ABA increases the content of PI3P and PI4P which are involved in the generation of ROS. This triggers an influx of Ca^{2+}, the activation of actin-binding proteins (ABPs), such as profilin, and facilitates the reorganization of actin.

In the presence of calcium, membrane binding proteins, annexins, interact with membrane phospholipids and activate PI signaling. Two proteins were identified in this study: putative calcium-binding protein annexin 6 (spot 22) and 14-3-3A (spot 33) as positive and negative regulators of protein kinase C (PKC), respectively. Annexin A6 acts as a scaffold for recruiting PKCα to the plasma membrane, without its phosphorylation (Lizarbe et al. 2013). 14.3.3A inhibits PKC and activates H^+-ATPase (Chen et al. 1994;

Chen et al. 2006a, b; Schoonheim et al. 2007). The downregulation of 14.3.3 during salinity stress could result in: (1) reduction of H^+-ATPase activity, followed by stomatal closure to prevent water loss and (2) phosphorylation of PKC and activation of Ca^{2+}-ATPase by receptor-mediated hydrolysis of inositol phospholipids.

The Ca^{2+}-storage protein calreticulin is an ER chaperone protein which is also found in Golgi bodies and plasma membrane. Cytosolic Ca^{2+} levels increase under salt stress when they are released from calcium storage sources. Typically, calreticulin is activated under stress conditions (Tuteja and Sopory 2008). In our study, salinity reduced calreticulin levels, which can be attributed to the release of free Ca^{2+}.

Our study also demonstrated changes in translation and transcription processing in response to salinity. Histone acetyltransferase HAC2 (spot 32) and 60 kDa jasmonate-induced proteins (spot 7) were upregulated during salinity stress. Both HAC2 and 60 kD JIP were ABA-modulated. Acetylation of histones leads decreases transcriptional activation by: (1) DNA relaxation and (2) changes in the interactions between chromatin and chromatin-associated proteins (Fang et al. 2014). Histone acetyltransferases (HATs) are involved in the chromatin-mediated mechanism of stress tolerance in plants exposed to ABA and salt stress (Chen et al. 2006a, b; Sokol et al. 2007; Zhou et al. 2009). 60 kDa jasmonate-induced protein (JIP60) is a ribosome-inactivating protein (RIP). JIP60 exhibited N-glycosidase activity and hydrolyzed the adenine residue from a conserved loop of 25S rRNA (α-sarcin/ricin loop). The α-sarcin/ricin loop binds elongation factors (EFs) to ribosomes. Therefore, any modifications in this fragment of 25S rRNA lead to the inactivation of the large ribosomal subunit and cessation of protein synthesis during the elongation step (Dunaeva et al. 1999; Sawasaki et al. 2008). JIP60 also plays a key role in inhibiting cytoplasmic protein synthesis via reversible ribosome dissociation into small and large subunits (Reinbothe et al. 1994; Rustgi et al. 2014). Some authors have suggested that the upregulation of JIP60 during salinity stress inhibits growth (Görschen et al. 1995) and protects plants against stress. Our results confirm the correlation between salinity and higher levels of 60 kDa jasmonate-induced protein. Surprisingly, exogenously applied ABA inhibited the expression of JIP60 (independent experiment, data not shown). Our findings differ from the observation that ABA, similar to jasmonic acid (JA), can induce JIPs (Lehmann et al. 1995). According to some reports, ABA promotes JA accumulation (Fan et al. 2009). Furthermore, the presence of MYC and NAC transcription factors suggests the existence of cross-talk between JA and ABA signaling (Singh and Laxmi 2015). It should also be noted that ABA and JA antagonistically regulate the expression of salt stress-inducible proteins (Moons et al. 1997). In our previous study (Szypulska and Weidner 2016), the following responses were observed in salt-stressed barley sprouts: (1) increase in the content of free cytosolic polysomes resulting from damage to cell membranes and (2) increase in the concentrations of ribosomal units and free monosomes. The results of the present study suggest that the above could be attributed to the action of JIP60. ABA's antagonistic effect on JIP60 expression could explain the inhibition of ribosome dissociation and, consequently, the stabilization of polysomes, which was observed in our previous study.

Response to desiccation stress

We identified two main groups of proteins involved in responses to desiccation stress: HSP and LEA proteins. These proteins were upregulated mainly under salinity stress, excluding the HSC70-2 protein (spot 8) which was strongly downregulated.

In general, HSP70 chaperones are involved in the folding of newly synthesized protein chains, prevention of aggregation and refolding of proteins denatured by stress, separation of protein aggregates, translocation of proteins across membranes, and disposal of misfolded proteins (Yong et al. 2003; Cazale et al. 2009; Song et al. 2014). The above explains the high levels of 70 kDa HSPs (spot 6) in stress conditions. The negative effect of salinity on HSC70 (heat-shock protein cognate) expression is unclear, and it could result from low protein yield under stress (Badowiec et al. 2012). HSC70-2 was the only protein whose expression increased under exposure to abscisic acid. The upregulation of HSC70 protein in response to ABA treatment provides additional evidence that translation processes are more effective in response to ABA treatment.

Late embryogenesis-abundant (LEA) proteins and members of this group, such as RAB (responsive to ABA) proteins, also known as dehydrins (DHN), are thought to be involved in protein repair during water stress. Due to their chaperone-like properties, LEA proteins assist proteins that were denatured or misfolded by water stress in recovering their native conformation (Campbell and Close 1997; Brini et al. 2007). In our study, the following LEA proteins were upregulated due to salinity stress: dehydrin (spot 9), dehydrin 6 (spot 18), group 3 LEA proteins (spot 16), late embryogenesis-abundant protein B19.3 (spot 39), protein HVA22 (spot 41), dehydrin DHN1 (spot 34), dehydrin DHN2 (spot 44) and dehydrin DHN3 (spot 42). In this group, HVA22, dehydrin 6, dehydrin DHN2, and dehydrin DHN3 were altered under ABA pretreatment.

Exposure to salinity stress also increased the levels of ABA-inducible protein PHV A1 (spot 35) and salt stress-induced protein (spot 38), both of which were altered by

ABA. Hong et al. (1988) demonstrated that the amino acid sequence of PHV A1 shares homology with the LEA7 protein in cotton. Claes et al. (1990) observed that the organ-specific response of sa/T (transcripts of salt stress-induced protein) was correlated with the pattern of Na$^+$ accumulation during salt stress.

Protein turnover

The ubiquitin–proteasome pathway is largely responsible for the degradation of specific cellular proteins during various processes, including exposure to salinity stress (Kornitzer and Ciechanover 2000; Wang et al. 2011;
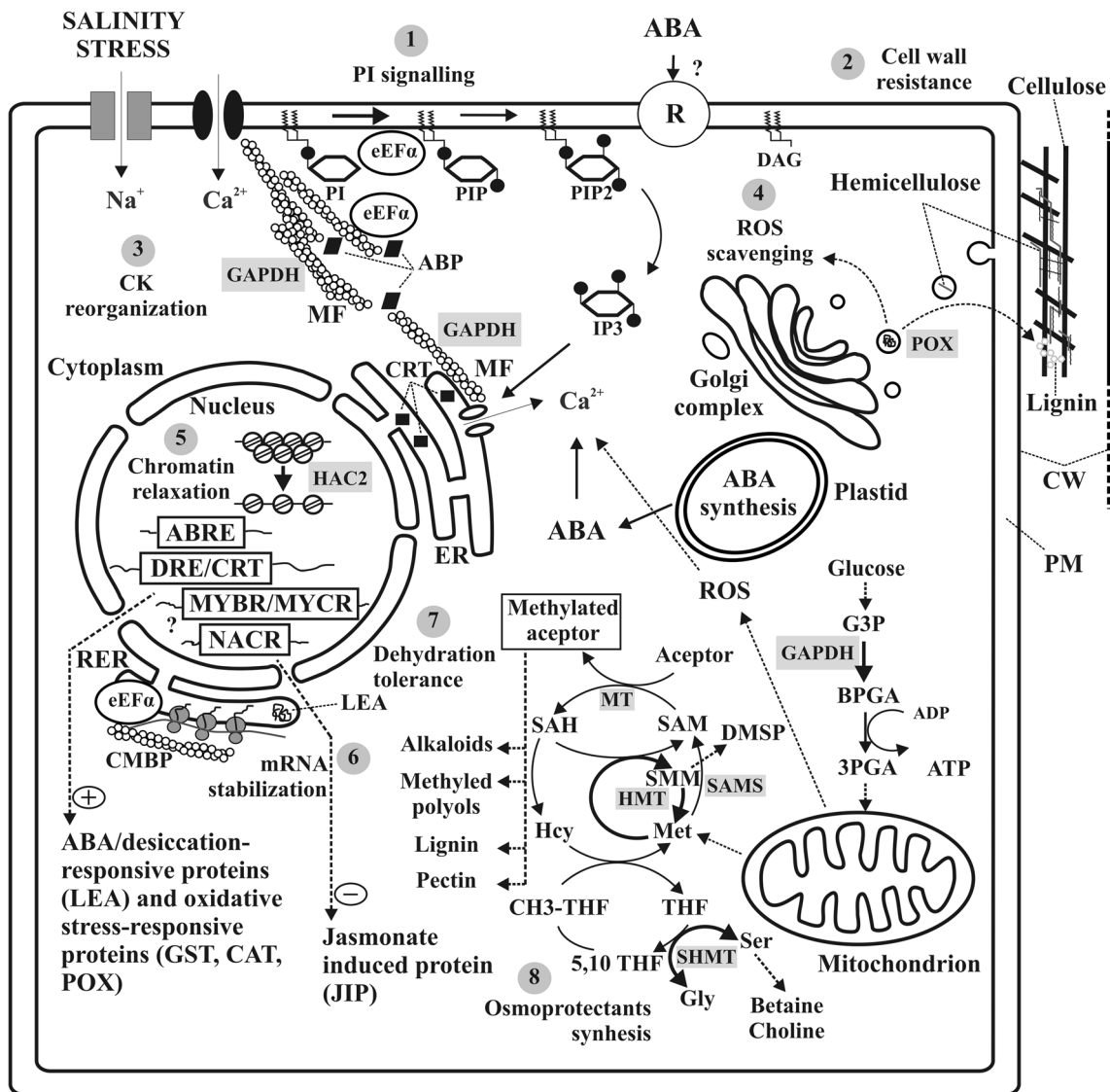
Fig. 5 Hypothetical model of ABA's influence on salt tolerance in higher plants. The role of ABA in salt response may consist in: (*1*) PI signaling and Ca^{2+} release due to EF1α regulation and cytoskeletal reorganization, (*2*) increase in cell wall resistance due to changes in hemicellulose, cellulose, and lignin synthesis, (*3*) cytoskeleton remodeling due to interactions with ABP and GAPDH, (*4*) ROS scavenging by antioxidant enzymes (GST, CAT, and POX), (*5*) chromatin relaxation due to changes in HAC2, (*6*) translation regulation due to improved mRNA stabilization, (*7*) dehydration tolerance, mainly due to synthesis of LEA proteins in RER, and (*8*) osmoprotectant synthesis (DMSP, betaine). The regulated metabolic pathways are marked with *bold arrows*. *ABP* actin-binding protein (e.g., formin), *cis*-regulatory elements: *ABRE* ABA-responsive element, *DRE/CRT* dehydration-responsive element/C-repeat, *MYBR/MYCR* myeloblastosis oncogene/myelocytomatosis oncogene recognition element, *NACR* non-aggressive challenge responsive element, *CK* cytoskeleton, *CMBP* cytoskeleton-membrane-bound polysomes (cytomatrix-bound polysomes), *CRT* calreticulin, *CW* cell wall, *DAG* diacylglycerol, *ER* endoplasmic reticulum, *MF* actin microfilament, *PM* plasma membrane, *R* potential ABA receptor, and *RER* rough endoplasmic reticulum. A detailed description can be found in the text

Lyzenga and Stone 2012; Zhang et al. 2014). According to Yan et al. (2000), ubiquitin-specific proteases (USPs)) play a host of important roles in the ubiquitin/26S proteasome pathway by: (1) releasing of free ubiquitins from their translation products, (2) recycling of ubiquitin during degradation of the target compound, which involves the removal of ubiquitin from peptide fragments and splitting of multiubiquitin chains, and/or (3) removing ubiquitins from conjugates before the degradation of the target compound. The latter role indicates that USPs prevent target degradation. These deubiquitinating enzymes (DUBs) are a group of ubiquitin-specific thiol proteases which break the bond between the Gly C-terminus of ubiquitin and covalently attached polypeptides (Yan et al. 2000). In our study, ubiquitin-specific protease 25 (spot 3) was upregulated under salinity stress. The described processes explain the role of protease which was identified in this study in response to salinity stress.

Other defense responses

The following salt-responsive proteins were identified in this study: RNase S-like protein (spot 29), putative endonuclease (spot 30) and trypsin inhibitor CMe (spot 43). All of the above proteins were upregulated in response to salinity and were not modified by ABA pretreatment.

It has been reported that S-like RNase genes are induced in response to various biotic and abiotic factors (Ye and Droste 1996; Galiana et al. 1997; Lee et al. 2009; Zheng et al. 2014). Although the amino acid sequence of S-like RNases is closely related to that of S-RNase, it has no RNase activity (Van Damme et al. 2000; Lee et al. 2009). S-like RNases play an important role in phosphate recycling during senescence and are induced by inorganic phosphate starvation, which is associated with the accumulation of anthocyanins (Ticconi et al. 2001; Lee et al. 2009). Salinity stress induces the accumulation of anthocyanins (Borghesi et al. 2011). Pi starvation could also be associated with salinity stress.

During oxidative stress, the influx of ROS can damage DNA structure (Saha et al. 2015). Efficient DNA repair mechanisms remove oxidized bases. For example, endonuclease III (NTH protein) and endonuclease VIII (NEI protein) from *Escherichia coli* cells excise many oxidized pyrimidines (Laval 1996; Kumari et al. 2009). The observed upregulation of endonuclease (spot 30) could play a role in DNA repair during salinity stress accompanied by oxidative stress.

Specific protease inhibitors are currently being overexpressed in certain transgenic plants to protect them against invaders (Habib and Fazili 2007) rather than to induce stress tolerance. It has been reported that the sequence of the maize α-amylase/trypsin inhibitor resembles the sequence of osmotin (Claes et al. 1990), a salt-responsive protein. However, protease inhibitors could inhibit the degradation of storage proteins and stilt the growth of salt-stressed seedlings.

Influence of ABA pretreatment on salinity response—summary

In general, ABA pretreatment limited stress-induced changes in the proteome. Interestingly, ABA-inducible protein PHV A1 (spot 35) was one of the five strongly downregulated proteins (to the level comparable with that noted in the control treatment). This finding suggests that the influence of ABA pretreatment on salinity response results from a regulation process, but not downregulation in the strict sense. It implies that ABA plays a major role in plant responses to salinity stress.

The observed changes in GAPDH point to the importance of glycolytic/gluconeogenic pathways in the ABA-modulated salinity response. The regulation of HMT suggests that ABA influences the SMM cycle. The noted changes in the expression of HMT and SHMT indicate that ABA is implicated in the synthesis of osmoprotectants. Other observations demonstrate the important role of ABA in hemicellulose, cellulose, lignin biosynthesis, and ROS scavenging. ABA's influence on cytoskeletal structure and formation was manifested in the reorganization of actin microfilaments (MFs). We allow for the possibility that GAPDH, in addition to formins the proteins classified to ABPs (actin-binding proteins), can take part in this process. The alterations in EF1α and HAC2 could indicate that ABA participates in the control of PI signaling and chromatin-mediated mechanism of stress tolerance. The number of proteins responsive to desiccation stress was altered by ABA pretreatment (mainly dehydrins), which suggests that ABA contributes to protection against dehydration. Our previous research revealed that ABA treatment increases the concentration of cytomatrix-bound polysomes, a fraction of polysomes that are attached to the membrane and the cytoskeleton, mainly actin microfilaments (Szypulska and Weidner 2011). In that study, we suggested the role of cytomatrix-bound polysomes in LEA proteins synthesis. Moreover, our previous study demonstrated that exogenous abscisic acid stabilizes polysomes, which could be attributed to the high concentration of cytomatrix-bound structures (Weidner et al. 2006). The present study demonstrated the role of ABA in JIP regulation, which expands our knowledge of other factors implicated in mRNA stabilization. In the light of our present and previous findings, we have proposed a hypothetical model of ABA's role in the induction of salt tolerance in barley sprouts (Fig. 5).

Author contribution statement ES: idea, realization, laboratory work, and description of results. KJ: laboratory work. SW: financial support and consultancy.

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