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ORIGINAL PAPER

Changes in the root proteome of *Triticosecale* grains germinating under osmotic stress

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Abstract Osmotic stress causes many adverse symptoms in plants, which include, for example, growth limitation and decrease or even absence of yield. Proteomic analyses of plant responses to stressors could lead to the introduction of crops with high resistance to osmotic stress. Such plants would be characterized by high yield even under unfavorable environmental conditions. In this article we describe changes in the protein profiles occurring in response to mild and moderate osmotic stress in triticale roots. Analysis of the protein profiles of these roots showed an increased abundance of 14 and a decreased abundance of 11 proteins under mild osmotic stress conditions while a moderate osmotic stress caused an increased abundance of 18 and a decreased abundance of 33 proteins. Twenty-five proteins, whose quantity altered under stress were identified using MALDI-TOF mass spectrometry. The identified proteins were classified into the categories of proteins associated with: defense mechanisms, metabolism, transcription, cell structure, protein synthesis, transport and signal transduction. The functions of identified proteins were discussed in relation to osmotic stress. Some of the identified proteins may be responsible for the adaptation of plants to adverse conditions.

Keywords Proteomics · Triticale · Osmotic stress · Roots

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Abbreviations

2-DE	Two-dimensional electrophoresis
ABA	Abscisic acid
ANOVA	Analysis of variance
CBB	Coomassie Brilliant Blue
HSP	Heat shock protein
IPG	Immobilized pH gradient
MALDI-TOF MS	Matrix-assisted laser desorption/ ionization time of flight mass spectrometry
NCBI	National Center for Biotechnology Information
PEG	Polyethylene glycol
PMF	Peptide mass fingerprinting
ROS	Reactive oxygen species
USF	Upstream transcription factor
yeiA	Dihydropyrimidine dehydrogenase

Introduction

Triticale is a cereal, which has been obtained artificially by cross-fertilization of rye and wheat. Through this crossing breeders obtained a cereal with higher yield than rye and higher resistance to stress than wheat (Tohver et al. 2005). Because of the high content of exogenous amino acids (mainly lysine) in grains, triticale constitutes a valuable crop plant used for animal nutrition (Jasińska and Kotecki 2003; Rakha et al. 2011). Triticale, like other crops, is exposed to many adverse environmental factors during their life. One of the most commonly occurring abiotic stressors is osmotic stress (Yokoi et al. 2002; Rao et al. 2006). Osmotic stress may be a result of diverse abiotic

factors such as drought, salinity, cold, polyethylene glycol, mannitol or sorbitol (Verslues et al. 1998; Munnik and Meijer 2001; Legocka and Kluk 2005). Osmotic stress causes many adverse symptoms, which include, among others: plant wilting, impaired growth and development, lower yield or even its absence. Other negative effects of osmotic stress include disorders in such physiological reactions as photosynthesis and formation of the reactive oxygen species (ROS) which lead to oxidation of proteins, amino and nucleic acids, lipid peroxidation, damage and even cell death (Reddy et al. 2004; Sobhanian et al. 2011). Osmotic stress also causes an ion homeostasis disorder and a reduction of chlorophyll and carotene content (Slama et al. 2007; Quados 2010; Sobhanian et al. 2011).

Osmotic stress affects up to 23 % of all arable land (Rao et al. 2006). Due to the increasing demand for food production, researchers have focused on the mechanisms of plant adaptation to water deficit. The aim of the molecular analyses is to facilitate selection of plants with higher adaptation to the conditions of water stress or to introduce new cultivars by using genetic engineering or traditional breeding techniques (Yokoi et al. 2002; Tamura et al. 2003; Rao et al. 2006). The plants' acclimation is related to the changes in gene expression and composition of the proteome and metabolome (Kosová et al. 2011; Swigonska and Weidner 2013). Proteome studies allow the examination of the actual products of gene expression and post-translational modifications of proteins in plants tissues (Jiang et al. 2007). The influence of salt or osmotic stress on the alterations in proteome was examined in tobacco (Dani et al. 2005) and rice leaves (Salekdeh et al. 2002). However, there are still only a few studies focused on the triticale proteome. In this study we used two-dimensional electrophoresis (2-DE) coupled with matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) to analyze the changes in protein composition of the roots of triticale grains germinating under osmotic stress. The aim of this experiment was to broaden the knowledge of the molecular responses of triticale to osmotic stress.

Materials and methods

Plant material and germination conditions

In these experiments, seeds of winter triticale variety 'Fredro' were used. The seeds were obtained from DANKO, The Plant Breeding Department in Choryń. The grains were collected in 2011 and their ability to germinate was determined to be 96 %. Triticale seeds were sterilized with 0.5 % NaClO solution for 10 min and washed thoroughly with tap and distilled water. Afterwards, about 120 seeds were placed on Whatman filter paper (Maidstone, Kent, England) moistened with distilled water (control—C) or with a solution of polyethylene glycol (PEG 8000) at concentrations lowering the water potential to −0.2 MPa—mild osmotic stress (S sample) and to −0.5 MPa—moderate osmotic stress (SS sample). The rolled sheets of tissue-paper with grains were placed in 250 ml glass cylinders. Approximately 30 ml of distilled water or a PEG solution was dispensed into the cylinders. The seeds were germinated in the dark at 21 °C for 72 h. The root length in all samples was measured after 24, 48 and 72 h of germination. After 72 h, the roots were cut, weighed, frozen in liquid nitrogen and stored at −80 °C for further analysis. The experiment was conducted in three independent biological replicates.

Protein extraction and purification

The extraction of proteins was carried out according to the procedure proposed by Gallardo et al. (2002). About 1 g of frozen root tissue was carefully pulverized in mortars cooled with liquid nitrogen and the material transferred to Eppendorf tubes with a suitable amount (20 µl/mg dry weight of tissue) of lysis buffer containing: 7 M urea; 2 M thiourea; 4 % CHAPS; 40 mM dithiothreitol; 2 % ampholytes pH 3–11; 60 U/ml DNase I; 5.8 Kunitz/ml RNase A; 1 pill/10 ml protease inhibitor Cocktail Tablets cOmplete Mini (Roche). The samples were shaken on ice for 45 min and then centrifuged at 18,000×*g* for 10 min at 4 °C. The supernatant was transferred to a new tube and re-centrifuged. Centrifugation was repeated until a clean solution containing the protein extract was obtained. These protein extracts were purified using Ready Prep™ 2-D Cleanup Kit (Bio-Rad), according to the manufacturer's instructions. The protein content was measured using 2 D Quant Kit (Amersham Biosciences).

2-DE

The procedure described by Görg et al. (2004) was used for protein isoelectrofocusing (first dimension). The protein pellets were dissolved in rehydration buffer (7 M urea; 2 M thiourea; 2 % CHAPS; 0.5 % ampholytes pH 3–11; 0.002 % bromophenol blue; 80 mM dithiothreitol). 450 µl of the protein solution containing 400 µg of proteins was loaded onto a 24-cm IPG gel strip with a non-linear immobilized pH gradient (3–11) (Immobiline™ DryStrip, GE Healthcare) and covered with 1 ml of mineral oil Dry Strip Cover Fluid (Amersham Biosciences). Isoelectrofocusing was performed in Ettan IPGphor 3 (GE Healthcare) apparatus. Prior to the actual separation, the rehydration process was carried out at 20 °C (30 V/12 h). The parameters of other stages of separation were as follows: 500 V/1 h, 1,000 V/1 h, 8,000 V/

3 h, 8,000 V/3 h 45 min. After isoelectrofocusing, the strips were placed in the equilibration buffer containing 6 M urea; 75 mM Tris–HCl pH 8.8; 29.3 % glycerol; 2 % sodium dodecyl sulfate; 0.002 % bromophenol blue with 100 mg dithiothreitol/10 ml buffer and were rocked gently for 15 min. The buffer was then decanted and replaced with the same amount of the equilibration solution containing 250 mg/10 ml iodoacetamide, instead of dithiothreitol and stirred for another 15 min. These IPG strips containing proteins separated according to their isoelectric point were placed onto a 12.5 % polyacrylamide denaturing gel. SDS-PAGE (second dimension) was performed according to O'Farrel (1975), using a buffer system described by Laemmli (1970). The gels were placed in Ettan DALT six (GE Healthcare) apparatus and the proteins were separated at 25 °C for 30 min at 2 W/gel and then for 3.5 h at 17 W/gel. The low-range SDS-PAGE (Bio-Rad) marker was used for the establishment of molecular weight of the proteins. After electrophoresis, gels were incubated for 30 min in the fixing solution (40 % methanol; 10 % acetic acid). In order to visualize proteins on the gels, the colloidal solution of Coomassie Brilliant Blue (CBB) G-250 staining was applied, according to the methodology described by Neuhoff et al. (1988).

Analysis of 2-DE gels

For the analysis of polyacrylamide gels, Image Scanner III (GE Healthcare) and the Image Master^{TM} 2D Platinum 6.0 (GE Healthcare) program were used. In order to determine quantitative changes between proteins, the gels were analyzed using the relative volume (%vol) of the detected proteins. The proteins, whose expression under stress changed significantly ($p < 0.05$) of at least 1.5-fold, were excised from the gels and identified by MALDI-TOF MS (Bruker Daltonik). The statistical analysis was performed using Statistica 10.0 software (StatSoft), GraphPad Prism 6 (GraphPad) and Microsoft Office Excel 2007 (Microsoft). One-way ANOVA (analysis of variance) and nonparametric Kruskal–Wallis tests were used to assess the relevance of the obtained results.

MALDI-TOF mass spectrometry

Proteins, whose expression increased or decreased at least 1.5-fold, compared with the control, were excised from the gels with a sterile scalpel blade and placed in Eppendorf tubes. Isolated proteins were destained, dehydrated and digested overnight (using 10 µl of the modified trypsin solution at a concentration of 20 ng/µl) following the procedure described by Shevchenko et al. (1996). In order to extract the peptides from the gel, 0.5 µl of acetonitrile was added to the solution with gel fragments and the tubes were placed in an ultrasonic bath for 5 min. Then, 0.5 µl of the peptide solution and the 0.5 µl of matrix (supersaturated solution of α-cyano-4-hydroxycinnamic acid in 50 % acetonitrile and 0.1 % trifluoroacetic acid) were applied on the Target Plate (Bruker Daltonik) and left to crystallize. Peptide mixtures were analyzed using the MALDI-TOF mass spectrometer (Bruker Daltonik). The *m/z* (mass to charge) ratio of the peptides appearing in mass spectra was used for protein identification by peptide mass fingerprinting (PMF) using the MASCOT program (Matrix Science) and databases of protein sequences: NCBI (National Center of Biotechnology Information) and SwissProt. The databases were screened for *Viridiplantae* (green plants) with the following parameters: 0.2 Da mass tolerance, one missed cleavage, carbamidomethylation of cysteines as fixed modifications and oxidation of methionine as variable modifications. The division of the identified proteins into groups (according to their functions) was based on the information obtained through bioinformatics tools Quick Go and UniProt and the classification method given by Bevan et al. (1998).

Results

Osmotic stress had a significant influence on elongation and alterations in protein profiles of triticale roots. The inhibitory effect of osmotic stress on root growth is shown in Fig. 1. The lengths of the roots grown for 24 h were 1.43 (±0.52) mm in the control sample (C) and 1 (±0) mm in the stress samples (S and SS). After 48 h of germination the root length reached 18.21 (±7.13) mm (C), 11.9 (±4.02) mm (S), and 6.31 (±2.78) mm (SS). The root length after 72 h of germination was 47.64 (±20.46) mm (C), 33.36 (±14.77) mm (S) and 20.71 (±9.26) mm (SS).

Analysis (qualitative and quantitative) of the proteome included separation of the extracted proteins by 2-DE, spot analysis in Image Master^{TM} 2D Platinum 6.0 and identification of the selected proteins (differing at least 1.5-fold with $p < 0.05$) using MALDI-TOF MS and MASCOT program. The electrophoregram analysis resulted in the detection of 1,698 proteins found both in the control (C) and the sample treated with a mild osmotic stress (S) and 1,870 proteins common to the control (C) and the sample under moderate osmotic stress (SS). Image analysis showed a significant quantitative increase (at least 1.5-fold) of 14 and a decrease of 11 proteins under mild osmotic stress conditions while a moderate osmotic stress caused an increase in abundance of 18 and a decrease in 33 proteins. Twenty-five of the 66 proteins which demonstrated changes in expression under conditions of osmotic stress were identified using MALDI-TOF mass spectrometry. The identified proteins are marked on the gel images in Fig. 2.

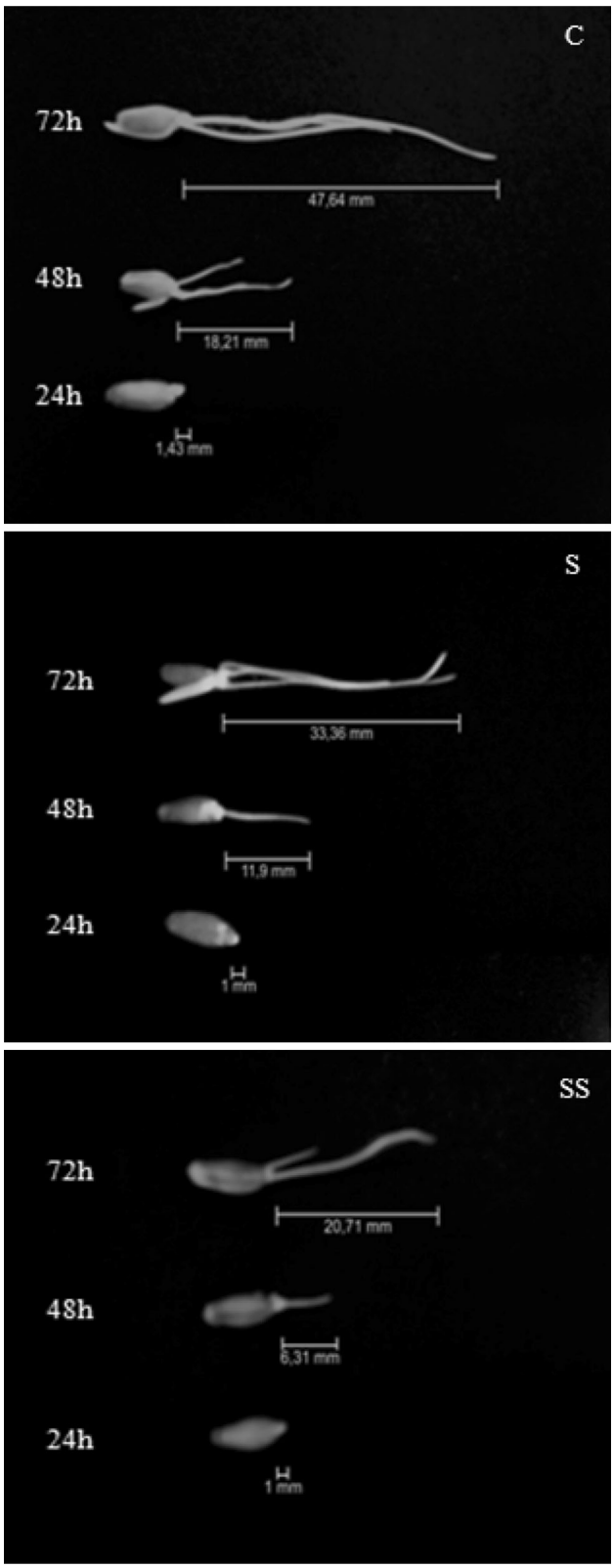

Fig. 1 Growth of triticale roots under different water stress conditions. The photographs show triticale grains after 24, 48 and 72 h of germination under: optimal conditions—*C*, mild osmotic stress conditions (−0.2 MPa)—*S* and moderate osmotic stress conditions (−0.5 MPa)—*SS*. The photographs also indicate the average root length of germinating grains

The rest of the selected proteins were not identified because of an insufficient amount of the proteins in the gel fragments or lack of the appropriate information (for triticale proteins) in the databases. Among the identified proteins, 9 showed an increased and 16 a decreased abundance as a result of 72-h osmotic stress treatment (compared with the control sample, C).

In response to both moderate and mild osmotic stress, the expression of proteins such as peroxidase 66 (spot 2), a transcription factor, protein USF (spot 19), DEAD-box ATP-dependent RNA helicase 41 (spot 20), putative phospholipase Dα1 precursor (spot 25) increased. Mild osmotic stress caused an increase in the expression of proteins such as α-1,4-glucan-protein synthase UDP-forming (spot 9), predicted protein (spot 22) (homologous to the protein from *Hordeum vulgare* subsp. *vulgare*). Increased expression under moderate osmotic stress elevated levels of small heat shock proteins—sHSP22 (spot 6), hypothetical protein TRIUR3_26221 (spot 14), and 30S ribosomal protein S3 (spot 23). Decreases in the expression in both mild and moderate osmotic stress were observed for peroxidase (spot 5) and hypothetical protein F775_28997 (spot 15). Decreased expression only under moderate osmotic stress showed the following proteins: β-glucosidase (spot 10), V-type protein ATPase subunit B1 (spot 24), actin (spot 21), hypothetical protein OsI_13043 (spot 16), peroxidase precursor (spot 1), glucan endo-1,3-β-glucosidase (spot 8), tubulin (spot 22), endoglucanase 10 (spot 12), HSP70 (spot 7), hypothetical protein TRIUR3_12251 (spot 17), *S*-adenosylmethionine synthetase (spot 13), two isoforms of ascorbate peroxidase (spot 3 and 4) and putative protein yeiA (spot 11).

The identified proteins were classified based on their biological functions (Fig. 3). The largest group of the identified proteins (32 %) whose expression was affected by the osmotic stress, were classified into the group of proteins participating in defense mechanisms. Proteins included in this category were peroxidase precursor (spot 1), peroxidase 66 (spot 2), peroxidase (spot 5), ascorbate peroxidase (spot 3 and 4), sHSP22 (spot 6), HSP70 (spot 7), and glucan endo-1,3-beta-glucosidase (spot 8). Another group of proteins (20 % of the identified polypeptides) were the proteins involved in metabolism. These include α-1,4-glucan-protein synthase UDP-forming (spot 9), β-glucosidase (spot 10), putative protein yeiA (spot 11), endoglucanase 10 (spot 12) and *S*-adenosylmethionine synthetase (spot 13). Two classes of proteins, performing functions in the process of transcription (USF protein—spot 19, DEAD-box RNA helicase 41—spot 20) and

forming cellular structures (actin—spot 21, tubulin—spot 22), constituted 8 % of the identified proteins and exhibited a much lower abundance in stress conditions. The groups with the smallest number of classified proteins were the classes of proteins involved in protein synthesis (30S ribosomal protein S3—spot 23), molecule transport (V-type ATPase subunit B1—spot 24) and signal transduction (putative phospholipase Dα1 precursor—spot 25). Five proteins (20 %) were unclassified because of their unknown function (hypothetical proteins: spots 14, 15, 16, 17, 18). The characteristics of the identified proteins assigned to the separate groups are shown in Table 1.

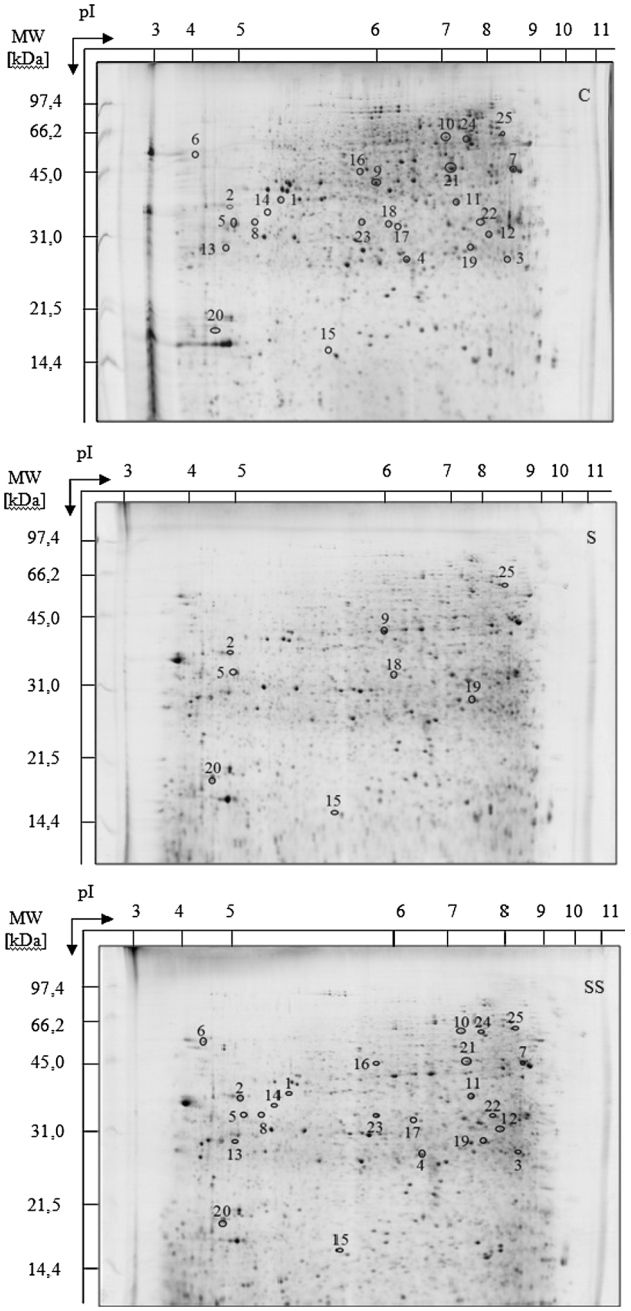

Fig. 2 Electophoregrams obtained from the separation of triticale root proteomes by 2-DE. Seeds were germinated for 72 h under optimal (*C*), mild (*S*) and moderate osmotic stress (*SS*) conditions. The proteins whose expression changed under stress conditions at least 1.5-fold compared with control (*C*) are indicated with the circles. On the *left* side of the gels are shown the molecular weights (MW) and at the *top*, the isoelectric points of the proteins (p*I*)

Discussion

Analysis of the average length of the longest triticale root indicated that significant growth inhibition was elicited by osmotic stress. Similar results were obtained by other researchers analyzing shoot elongation in bent grass (Xu and Huang 2010). It was observed that higher concentrations of PEG solutions result in more significant inhibition of root elongation. A similar relationship was also noticed in another study (El Midaoui et al. 2003).

Comparative analysis of the protein profiles of triticale roots helped to indicate proteins with expression altered by the osmotic stress. The selected proteins, whose quantity increased or decreased at least 1.5-fold, were identified by mass spectrometry (MALDI-TOF) and classified into such categories as defense mechanisms, metabolism, cell structure, protein synthesis, transport and signal transduction.

The group of proteins related to defense mechanisms is represented by peroxidases. They are involved, among other processes, in the regulation of reactive oxygen species (ROS), which are formed in excess as a result of osmotic stress (Xiong and Zhu 2002; Passardi et al. 2005; Koussevitzky et al. 2008). Our experiment showed various levels of the peroxidase family of proteins. Under moderate osmotic stress there was a decrease in the expression of the following proteins: peroxidase (lactoperoxidase), two isoforms of cytosolic ascorbate peroxidase and peroxidase

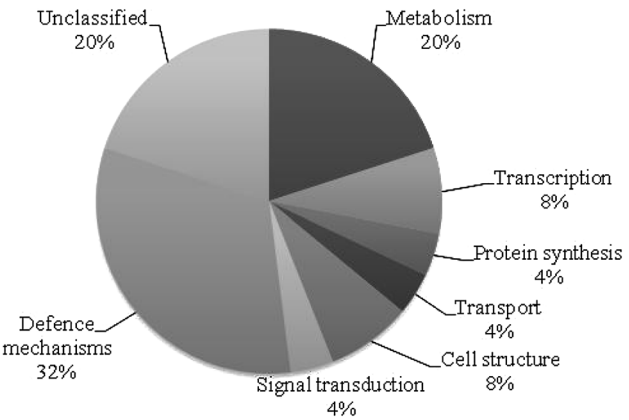

Fig. 3 The functional classification of the identified proteins whose expression altered under osmotic stress

Table 1 The proteins with altered expression (1.5-fold to control at $p < 0.05$) in the roots of triticale grains germinated for 72 h under mild (S) and moderate (SS) osmotic stress, identified using MALDI-TOF MS

ID[a]	Accession number	Identified protein (plant species)	Score	Matched peptides[b]	Cov. (%)[c]	Theor. p*I*/MW (kDa)[d]	Exp. p*I*/MW (kDa)[e]	Change in S/SS[f]
Defense mechanisms								
1	gil58334052	Peroxidase precursor (*Triticum aestivum*)	94	7	29	5.58/37.00	5.31/37.06	–/0.55
2	gil474004599	Peroxidase 66 (*Triticum urartu*)	129	11	35	5.85/46.23	4.97/36.10	1.58/2.18
3	APX2_ORYSJ	L-ascorbate peroxidase 2, cytosolic (*Oryza sativa* subsp. *japonica*)	65	6	37	5.21/27.22	8.32/27.60	–/0.66
4	gil3688398	Ascorbate peroxidase (*Hordeum vulgare* subsp. *vulgare*)	99	10	48	5.85/27.53	6.49/26.66	–/0.62
5	PER1_WHEAT	Peroxidase (*Triticum aestivum*)	70	8	34	8.37/32.87	5.02/32.87	0.41/0.15
6	HS22C_SOYBN	sHSP22, chloroplastic (*Glycine max*)	61	6	31	5.47/20.58	4.08/52.44	–/3.91
7	gil357503195	Heat shock protein (*Medicago truncatula*)	100	11	28	5.08/71.35	8.46/43.94	–/0.67
8	gil474044907	Glucan endo-1,3-beta-glucosidase GI (*Triticum urartu*)	76	7	39	7.70/33.04	5.14/32.81	–/0.63
Metabolism								
9	gil475453336	Alpha-1,4-glucan-protein synthase UDP-forming (*Aegilops tauschii*)	147	20	50	6.02/41.03	6.00/40.70	1.59/–
10	gil475505622	Beta-glucosidase, chloroplastic (*Aegilops tauschii*)	75	7	13	6.39/62.32	5.22/34.76	–/0.60
11	gil475610606	Putative protein yeiA (*Aegilops tauschii*)	75	7	22	5.99/46.76	7.50/36.28	–/0.57
12	GUN10_ARATH	Endoglucanase 10 (*Arabidopsis thaliana*)	64	7	26	6.77/58.12	5.29/30.67	–/0.46
13	gil115589746	S-adenosylmethionine synthetase 2 (*Triticum monococcum*)	95	7	40	7.34/31.55	4.85/28.47	–/0.67
Unclassified								
14	gil473965096	Hypothetical protein TRIUR3_26221 (*Triticum urartu*)	98	8	42	6.78/26.93	5.22/34.76	–/1.60
15	gil475554779	Hypothetical protein F775_28997 (*Aegilops tauschii*)	110	9	20	8.03/52.10	5.65/36.67	0.43/0.59
16	gil326505562	Hypothetical protein OsI_13043 (*Oryza sativa* Indica Group)	78	7	32	9.90/35.22	5.89/43.65	–/0.49
17	gil474401890	Hypothetical protein TRIUR3_12251 (*Triticum urartu*)	90	9	44	5.68/32.92	6.37/31.85	–/0.67
18	gil326505562	Predicted protein (*Hordeum vulgare* subsp. *vulgare*)	151	12	54	6.12/34.75	6.21/32.28	1.51/–
Transcription								
19	gil475533294	Protein USF (*Aegilops tauschii*)	80	8	49	5.23/24.70	7.79/28.45	1.99/1.68
20	RH41_ORYSJ	DEAD-box ATP-dependent RNA helicase 41 (*Oryza sativa* subsp. *japonica*)	64	6	14	7.14/59.92	4.56/18.28	1.55/1.60
Cell structure								
21	gil6103623	Actin (*Picea rubens*)	132	15	48	5.30/41.79	7.39/44.19	–/0.56
22	TBB4_WHEAT	Tubulin beta-4-chain (*Triticum aestivum*)	63	8	18	4.78/50.64	7.99/32.54	–/0.44
Protein synthesis								
23	RR3_PSINU	30S ribosomal protein S3, chloroplastic (*Psilotum nudum*)	63	5	32	9.70/25.81	5.89/32.70	–/1.72
Transport								
24	VATB1_HORVU	V-type protein ATPase subunit B 1 (*Hordeum vulgare*)	158	14	43	5.12/54.11	7.72/57.11	–/0.46
Signal transduction								
25	gil209944121	Putative phospholipase Dα1 precursor (*Triticum monococcum*)	82	8	19	5.40/62.28	8.33/59.64	2.14/1.53

[a] ID of the protein, corresponding to the number in the Fig. 1

[b] Number of matched peptides

[c] Sequence coverage (%)

[d] Theoretical p*I*/MW

[e] Experimental p*I*/MW

[f] Fold change in the roots of seeds germinating under mild (S)/moderate (SS) osmotic stress conditions

precursor. A significant decrease in expression of peroxidase was observed under mild osmotic stress conditions. Reduction of peroxidase accumulation by the action of osmotic stress was observed in other studies (Jiang and Deyholos 2006; Ma et al. 2009; Ge et al. 2012). Decrease in the abundance of this enzyme in the tissues could reduce the adaptability of the plants to stress as evidenced, for example, in the study of *Arabidopsis* mutants, deprived of the ability to synthesize ascorbate peroxidase (Koussevitzky et al. 2008). Triticale treated with mild and moderate osmotic stress, however, showed an increased expression of peroxidase 66, which indicates some defensive reaction of the plant (combating harmful excess of ROS) to the adverse environmental conditions.

Another protein whose expression significantly decreased in response to moderate osmotic stress was glucan endo-1,3-beta-glucosidase. This enzyme degrades the β-glucan—component of hemicelluloses which builds cell walls (Huber and Nevins 1981). Glucosidase produced in large quantities during the germination of seeds allows the cleavage of plant's storage substances by hydrolysis of the endosperm cell walls (Leah et al. 1995). Some studies have shown that the increase in the synthesis of beta-glucosidase is associated with a higher plant tolerance to osmotic stress (Budak et al. 2013). This enzyme stimulates tissue growth and adapts the plant to altered environmental conditions by reducing the water potential of the cells (Mohammadi et al. 2007).

Two heat shock proteins, sHSP22 and HSP70, were identified in this work. HSPs play a protective function towards other proteins during various abiotic stresses. HSPs are involved in such processes as protein–protein interactions, protein folding, secretion and transport as well as protection against protein degradation and aggregation (Timperio et al. 2008; Mohammadi et al. 2012b). The quantity of a small HSP (sHSP22) significantly increased in the sample treated with moderate osmotic stress. Small HSPs interact with HSP100/HSP70 and participate in the protection of other proteins (Sarkar et al. 2009). A high tolerance to osmotic stress of plants has been correlated with a production of large amounts of HSPs (Alvim et al. 2001; Mohammadi et al. 2012a). The small HSP identified in the roots of triticale probably prevents unfolding of other proteins and facilitates plant defense against stress. Moderate osmotic stress caused down-regulation of HSP70. A similar trend was also noticed in the studies analyzing the bentgrass leaf proteome under osmotic stress (Xu and Huang 2010) and the pea root proteome under short chilling stress (Badowiec et al. 2013). There were no significant changes in the amount of HSPs in the sample germinated under mild osmotic stress conditions. Decrease in HSP70 expression in the triticale roots suggests, therefore, some disturbances in protein metabolism, perhaps caused by weak plant adaptation to stressful conditions.

S-adenosylmethionine synthetase is an enzyme essential for DNA, RNA and protein methylation, biosynthesis of cell wall components and, indirectly, for the synthesis of polyamines and ethylene (Espartero et al. 1994; Grillo and Colombatto 2008). It was revealed that the increased synthesis of polyamines (whose production depends on this synthase) increases tolerance of some plants to the osmotic stress (Wi et al. 2006). The amount of this enzyme significantly decreased in the sample treated with moderate osmotic stress. A similar effect was also reported in other proteomic (Yan et al. 2005; Toorchi et al. 2009; Mohammadi et al. 2012b) and transcriptomic (Espartero et al. 1994) studies. This synthetase is probably involved in the control of gene expression under osmotic stress conditions due to its ability to methylate nucleic acids and, therefore, may constitute an interesting target for genetic engineering of plant resistance to this stress (Mohammadi et al. 2012b).

Under moderate osmotic stress conditions the amount of β-glucosidase decreased. Beta-glucosidases participate in lignification of the cell walls, β-glucan catabolism, activation of phytohormones, stimulation of the aromatic compounds synthesis and in other defensive processes (Cairns and Esen 2010). In response to drought stress, β-glucosidases may increase the amount of abscisic acid (ABA)—an essential signaling factor which is involved in adapting plants to stress conditions (Bargmann and Munnik 2006; Cairns and Esen 2010; Wang et al. 2011). Studies on β-glucosidase showed its important role in increasing the tolerance of plants to osmotic and drought stress (Hermosa et al. 2011; Wang et al. 2011).

Another identified protein whose expression decreased in triticale roots under moderate osmotic stress was endoglucanase 10, which is capable of degradation of cell wall components and thus involved in cell elongation (Biswas et al. 2006; Geilfus et al. 2011). Studies performed on maize showed that the decrease of cellulases in the leaves may be associated with a lower tolerance to salt stress and inhibition of plant growth (Geilfus et al. 2011).

A putative yeiA protein, also known as dihydropyrimidine dehydrogenase, exhibited a significant decrease in expression under moderate osmotic stress conditions. This enzyme is involved in the reduction of pyrimidines (uracil and thymine) to 5,6-dihydro derivatives (Hidese et al. 2011). Pyrimidine degradation contributes to the production of the substrate (β-alanine) for the synthesis of pantothenate, necessary to produce coenzyme A (Zrenner et al. 2009). Pyrimidine catabolism may be a source of nitrogen for the plants (Zrenner et al. 2009). The decrease in yeiA protein expression may be associated with a lower demand for nitrogen caused by the inhibition of growth and reduction of protein synthesis under stress conditions

(Brosowska-Arendt and Weidner 2011). Increased formation of nitrogen compounds increases the adaptability of plants to salt stress (Mansour 2000). Although the role of yeiA protein has not been so far adequately investigated, it may be assumed that the overexpression of this protein could increase triticale tolerance to osmotic stress by increasing the pool of protective amino acids in tissues.

Another protein whose expression increased under moderate osmotic stress conditions was α-1,4-glucan synthase. This enzyme is involved in synthesis of α-glucan, found in the plant's primary reserve material—starch (Hochstenbach et al. 1998; Kok-Jacon et al. 2003). It was demonstrated that osmotic stress causes changes in the biosynthesis of starch (Ge et al. 2012). A reduced demand for carbohydrates in cells may be due to the inhibition of triticale growth under osmotic stress (Brosowska-Arendt and Weidner 2011). This synthase also plays an important role in cell wall synthesis and morphogenesis of yeast (Hochstenbach et al. 1998). It is assumed that the protein may also be involved in the synthesis of hemicellulose which is a component of plant cell walls (Dhugga et al. 1997).

The increased expression under the action of both mild and moderate osmotic stress was exhibited also by the USF protein—a DNA-binding upstream transcription factor which participates in the regulation of transcription and cell proliferation inhibition (Ghosh et al. 1997; Qyang et al. 1999). An increased amount of USF protein in triticale roots could, therefore, influence the transcription of other genes.

Also the DEAD-box RNA helicase 41 (ATP-dependent) increased in abundance under the influence of both of the applied osmotic stresses. A similar trend in the expression of DEAD-box helicase was observed in barley tissues in response to salt and osmotic stress (Nakamura et al. 2004) and soybean tissues in response to salt stress (Chung et al. 2009). DEAD-box helicases affect transcription and translation, nearly all the processes related to the metabolism of RNA and ribosome synthesis (Nakamura et al. 2004; Chung et al. 2009). The work of Amin et al. (2012) shows that transgenic rice with overexpression of one of the RNA helicases exhibits an increased yield under stress conditions. Perhaps the helicase identified in our experiment influences the expression of other genes in stressed triticale tissues, responsible for plant resistance to osmotic stress (Vashisht and Tuteja 2006).

One of the cell structure proteins undergoing decreased expression due to the impact of moderate osmotic stress was β-tubulin. Similar results were also obtained in studies concerning rice stems (Song et al. 2011). As a component of microtubules, β-tubulin plays a role in cell growth and division as well as in cell wall development (Jost et al. 2004; Libusová et al. 2005). Reduced expression of β-tubulin could, therefore, affect the inhibition of triticale root growth in response to osmotic stress. Inhibition of growth in this case could be associated with impaired cell division and cell wall synthesis, arising under the action of stress. The previously reported decline of β-tubulin amount in plants susceptible to drought indicates the important role of this protein in the processes of plant adaptation to adverse conditions (Mohammadi et al. 2012a).

It was observed that the quantity of another cytoskeleton-associated protein, actin, was reduced under moderate osmotic stress. Similar results were also obtained in the study on *Arabidopsis* roots (Jiang et al. 2007). Actin is a component of the microfilaments which are involved in transport of organelles as well as in the regulation of cell division and elongation (Liu 2011). A decrease in the expression of both tubulin and actin entails a reduction in cell size which can be one of the mechanisms of plant's adaptation to osmotic stress. Reducing the volume of the cells may in fact facilitate the maintenance of cell homeostasis and turgor (Xu et al. 2010).

A protein whose expression increased under moderate osmotic stress was chloroplast 30S ribosomal protein S3—protein associated with translation. Plant roots typically contain proplastids, some of which can be changed into chloroplasts after exposure to light (Oliveira 1982). S3 protein is involved in the synthesis of small ribosomal subunit (30S) and in the repair of DNA damage (Kim et al. 2005). S3 may also function as a transcription factor (Wan et al. 2007). In connection with these functions, this protein may cause an increase in triticale stress tolerance.

Also V-type ATPase subunit B1 undergoes down-regulation under moderate osmotic stress conditions. Similar results were obtained in the roots of wheat seeds germinating under salt stress (Wang et al. 2000). V-type ATPase allows the accumulation of excess ions in the tonoplast, which is one of plant's defense mechanisms in response to salt stress (Wang et al. 2000). Increased expression of this protein in plants enables more efficient water extraction from the soil (Mohammadi et al. 2012a). It was shown that the subunit B1, apart from its role as a regulatory subunit forming the catalytic center of ATPase, may influence the glucose signaling pathway, eventually affecting gene expression and plant growth (Cho et al. 2007; Zhao et al. 2009). Studies performed on transgenic tomatoes showed that plants producing large amounts of this subunit have a greater tolerance to drought and osmotic stress than wild-type plants (Hu et al. 2012). Also wheat varieties with higher resistance to osmotic stress exhibit higher activity of V-type ATPase than susceptible cultivars (Zhao et al. 2009).

Another protein that increased in expression under osmotic stress was a putative phospholipase Dα1 precursor. Phospholipases are involved in the hydrolysis of

phospholipids, in the processes of growth and ripening of plants. Some phospholipases have the ability to regulate cell volume and turgor under osmotic stress conditions (Chapman 1998). Phospholipase D is involved in the catabolism of phospholipids, such as phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol (resulting in formation of signaling molecule—phosphatidic acid) (Bargmann and Munnik 2006). The enzymes appear to play a significant role in the control of seed germination, organ aging and trafficking (Chapman 1998; Bargmann and Munnik 2006). It was shown that phospholipase D is involved in the ABA-dependent signal transduction induced by drought and salinity stress (Bargmann and Munnik 2006). Deficiency of phospholipase Dα reduces the plant's ability to adapt to drought stress (Sang et al. 2001). Other researchers also noted that in response to osmotic stress tolerant plants show an increased activity of phospholipase D (Munnik et al. 2000). Our experiment suggests that increase in phospholipase D under osmotic stress conditions results from increasing the synthesis of a precursor of the enzyme.

An interesting result was obtained when it comes to a hypothetical protein OsI_13043. The abundance of this protein under mild osmotic stress slightly increased in triticale roots, while in the roots treated with the moderate osmotic stress the protein showed a decreased expression. It can be assumed that this protein is involved in the response to water stress of low intensity.

The putative protein homologous to *Hordeum vulgare* subsp. *vulgare* protein of unknown function showed a stronger decrease in abundance in the triticale roots treated with mild than with moderate osmotic stress.

This study shows only a few proteins involved in triticale resistance to stress. The group of proteins which probably determine some adjustment of plant includes sHSP22, ribosomal protein S3, DEAD-box RNA helicase, USF protein, hypothetical protein TRIUR3_26221, peroxidase 66 and phospholipase Dα1 precursor.

On the basis of these results, it can be concluded that osmotic stress causes a number of metabolic disorders (expressed for example in cell wall elongation disturbances) and the impairment of defense protein accumulation (greater under moderate than under mild stress conditions), which indicates the high susceptibility of germinating triticale to adverse environmental conditions (especially to the osmotic stress of higher intensity).

We suggest that the overexpression of proteins such as *S*-adenosylmethionine synthetase, ascorbate peroxidase, glucan endo-1,3-β-glucosidase, β-glucosidase, V-type ATPase B1 subunit, HSP70, yeiA protein or endoglucanase would probably provide a higher triticale tolerance to osmotic stress and should be considered as a target for genetic engineering of triticale varieties more resistant to the stress.

Author contribution Joanna Gręboszcarried out most of the research and was the main author of this manuscript. Anna Badowiec provided all the technical support during the laboratory work and helped with manuscript preparation. Stanisław Weidner was the originator and responsible for experimental design. The manuscript was approved by all of the authors, who at the same time claim no conflict of interests.

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