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Changes in the proteome of pea (*Pisum sativum* L.) seeds germinating under optimal and osmotic stress conditions and subjected to post-stress recovery

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Abstract Plants growing under natural conditions are exposed to a variety of stresses, which can lead to undesirable changes in the physiological processes and yielding. These changes can be regulated at different levels, resulting in the synthesis of specific proteins which participate in the plant's response to stress. The purpose of this study was to determine changes in the accumulation of proteins in germinating pea (*Pisum sativum* L.) seeds under optimal and osmotic (short- and long-term) stress conditions as well as recovery following a short-term stress. For identification of the proteins, two-dimensional electrophoresis and mass spectrometry (MALDI-TOF) were employed. Germination in optimal conditions increased the accumulation of several proteins involved in glycolysis, Krebs cycle, synthesis of fatty acids, cell growth, cellular transport and detoxification. Osmotic stress, in turn, depressed the accumulation of proteins involved in glycolysis, synthesis of fatty acids, detoxication, methionine conversions, cellular transport, translation, growth control and of cytoskeletal proteins, but

raised the accumulation of enzymes of the tricarboxylic acid cycle as well as proteins participating in signal transduction and protection (chaperones). One protein, 6-hydroxymaackian-3-O-methyltransferase, which is involved in the synthesis of pisatin, was present only under osmotic stress conditions and recovery. Pisatin is synthesized mainly in response to microbiological infections and under stress conditions, indicating its key role in the acquisition of stress tolerance by plants.

Keywords *Pisum sativum* L. · Pea seeds · Germination · Proteome · Osmotic stress · Recovery

Introduction

Seeds are an important element in the life cycle of higher plants, which ensure the survival of plant species. Among the processes indispensable for the proper seed germination are the generation of metabolic energy and production of substrates, later used for the synthesis of components necessary to sustain the growth of a seedling (Bewley and Black 1994). The development and life cycle of plants are influenced by environmental stresses, both short- and long-term ones, which determine the acreage and yields of various plants, including agricultural crops (Howarth and Ougham 1993). Pea is among economically viable crops (Gepts et al. 2005). At present, it is a major legume grown in Europe (www.grainlegumes.com), and the fourth dominant leguminous crop, after soybean, peanuts and bean (Vidal-Valverde et al. 2003). Pea seeds are a rich source of proteins, carbohydrates, fiber, vitamins and minerals, thus playing an important role in diets of animals and humans (Wang et al. 2003; Urbano et al. 2005). The demand for plant protein sources raises the importance of pea as a cultivated crop.

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All metabolic processes in a cell take place in the protoplasm. Therefore, good hydration of the protoplasm determines the right course of all cellular processes. Among the most significant environmental factors which affect the hydration of tissue and, consequently, the course of cellular processes is osmotic stress, which can be studied in a laboratory through the application of polyethylene glycol (PEG) (Couper and Eley 1984). Osmotic stresses within -0.2 and -0.5 MPa often occur in soils across Europe. The response of plants to water deficit differs, depending on the plant species as well as the intensity and duration of the stress. Water deficit can lead to changes such as a depressed seed germination rate and capacity as well as a worse growth and development of sprouts, seedlings and plants. Thus, it can reduce plants' productivity (Boyer 1982; Bensen et al. 1988; Xu and Bewley 1991; Gill et al. 2002; Murillo-Amador et al. 2002).

The response of plants to environmental stresses includes alterations in the expression of genes as well as physiological, biochemical and molecular adaptation processes, for example changes in the synthesis of proteins (synthesis of adaptation and protection proteins), accumulation of soluble protection substances and in the cellular metabolism (Hasegawa et al. 2000; Kato-Noguchi 2000b; Popova et al. 2001; Kawaguchi et al. 2004). All such modifications are controlled at the transcriptional, post-transcriptional and post-translation levels (Kawaguchi et al. 2004). Experiments simulating conditions which appear in the nature enable us to describe interactions between plants and environmental changes, to recognize mechanisms involved in the response of plants to stress factors and to identify the physiological and molecular basis of the resistance to stresses, using more and more up-to-date tools applied in proteomics, genomics or metabolomics (Hasegawa et al. 2000; Liu et al. 2000; Chaves et al. 2003; Kmiec et al. 2005; Shao et al. 2005). Such studies may facilitate the development of cultivars of improved quality, with enhanced tolerance to specific stress factors, which is of great importance in agricultural practice because it can help expand acreage of cropped land and to increase yields of crops (Liu et al. 2000; Postel 2000; Bazzaz 2001; Graves and Haystead 2002; Xiong et al. 2002; Chaves et al. 2003; Graham and Vance 2003; Shao et al. 2005). Our understanding of the mechanisms through which plants receive environmental signals and transmit them to cells, in which the mechanisms responsible for plant adaptation are activated, is important for continuing rational plant breeding strategies towards the improvement of plant tolerance to environmental stresses, both abiotic and biotic ones (Xiong et al. 2002).

The objective of this study was to analyse changes in the accumulation of proteins in pea (*Pisum sativum* L.) seeds germinated under optimal and osmotic stress (-0.5 MPa) conditions and after post-stress recovery.

Materials and methods

Plant material and germination experiments

The experiments were conducted on the pea (*Pisum sativum* L. cultivar TOR) 6-week seeds, supplied by Torseed S. A. (Toruń, Poland). Seeds were surface disinfected in 1 % sodium hypochloride for 3 min and then washed with tap and sterilized water. Intact seeds were placed on Petri dishes with two layers of Whatman 2 filter paper No. 1 (Whatman, Maidstone, Kent, UK) wetted with 40 ml distilled water and germinated in the dark at $+20$ °C for 48 (C48), 72 (C72) and 96 (C96) h. After 48 h, some of the seeds which germinated under optimal conditions, i.e. in distilled water, were transferred when the roots were ≥ 1 mm for another 24 h to osmotic stress conditions with PEG, which decreased the osmotic potential to -0.5 MPa (S72). After short-term osmotic stress, early seedlings of pea were transferred again to optimal germination conditions for the next 24 h, which was a recovery period (SR96). Simultaneously, another batch of pea seeds was germinated under long-term osmotic stress conditions (-0.5 MPa) at 20 °C for 96 h (S96). After the set germination time, sprouts and early seedlings (according to Gong et al. 2001) were isolated from seeds and used for further tests. All determinations were repeated three times. The design of the experiment is presented in detail in Fig. 1. For interpretation of the results of pea seeds' growth, the STATISTICA programme (one-factor analysis of variance, ANOVA, multiple comparison test and Tukey test) was used; control trials after 24, 48, 72 and 96 h of pea seeds germination were compared with osmotic stress trials after 24, 48, 72 and 96 h, respectively.

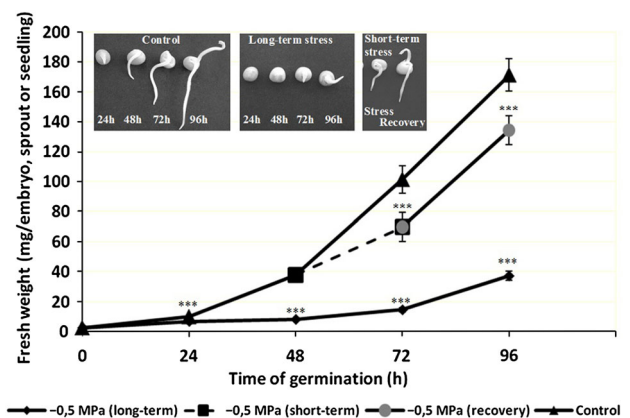


Fig. 1 Growth conditions and the course of the experiment. Statistically significant differences between control trials after 24, 48, 72, and 96 h of pea seeds germination and osmotic stress trials after 24, 48, 72, and 96 h, respectively, were indicated: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (ANOVA)

Preparation of total protein extracts

Total protein extracts were prepared from seeds at different stages of germination in optimal and osmotic stress conditions. Sprouts and early seedlings were ground in liquid nitrogen using a mortar and a pestle. Subsequently, total proteins were extracted at 4 °C in the thiourea/urea lysing buffer (Harder et al. 1999; Gallardo et al. 2002b) containing 7 M urea (Amersham Biosciences), 2 M thiourea (Merc), 21 mM Tris–HCl (Trizma HCl, Sigma), 16.5 mM Trizma base (Sigma), 4 % (w/v) CHAPS (Amersham Biosciences), 14 % protease inhibitor (cocktail “complete Mini”, Roche), 60 U/ml DNase I (Roche), 5.8 Kunitz units/ml RNase A (Sigma), 0.2 % (v/v) Triton X-100 (Sigma) and 1 % (v/v) pharmalyte pH 3–10 carrier ampholytes (Amersham Biosciences). After 10 min at 4 °C, 8 mM dithiothreitol (DTT, Amersham Biosciences) was added. Proteins were extracted from germinating seeds in 20 µl/mg of plant tissue dry matter. The protein extract was stirred for 45 min at 4 °C and then centrifuged for 10 min at 35,000g at 4 °C. The supernatant was submitted to another clarifying centrifugation as above. The final supernatant corresponded to the total protein extract. Protein concentration was measured according to Bradford (1976); bovine serum albumin was used as a standard.

Two-dimensional electrophoresis

Proteins were first separated by electrophoresis according to charge. Isoelectric focusing was carried out with 200 µg of proteins of the various extracts. Proteins were separated using gel strips forming an immobilized nonlinear pH gradient from 3 to 10 (Immobiline DryStrip, pH 3–10 NL, 24 cm, Amersham Bioscience). Strips were rehydrated in the IPG-Phor system (Amersham Biosciences) for 7 h at 50 V, at 20 °C with the rehydration buffer containing 7 M urea (Amersham Biosciences), 2 M thiourea (Merck), 65 mM CHAPS (Amersham Biosciences), 20 mM DTT (Amersham Biosciences), 0.5 % pharmalyte pH 3–10, 2 % (v/v) Triton X-100 (Sigma) and the protein extracts (Schiltz et al. 2004). Afterwards, isoelectrofocusing was performed at 20 °C in the IPG-Phor system for 1 h at 350 V, 3 h at 3,500 V and 7 h at 8,000 V. Proteins were then separated according to size. Prior to the second dimension, the gel strips were equilibrated at room temperature for 2 × 15 min in 2 × 100 ml equilibration solution containing 6 M urea (Amersham Biosciences), 30 % (v/v) glycerol, 87 mM sodium dodecyl sulphate (SDS, Amersham Biosciences), 150 mM BisTris (Sigma), 0.1 M HCl (Görg et al. 1987; Harder et al. 1999). DTT (26 mM) was added to the first equilibration solution and iodoacetamide (0.22 M, Sigma) to the second one (Harder et al. 1999). Equilibrated gel strips were placed on the top

of vertical polyacrylamide gels composed of 10 % (v/v) acrylamide (Amersham Biosciences), 0.33 % (w/v) piperazine diacrylamide (PDA, Bio-Rad), 16 mM Tris–HCl (Trizma HCl, Sigma), 0.07 % (w/v) ammonium persulphate (APS, Sigma), 0.03 % (v/v) Temed (BioRad). A denaturing solution [1 % (w/v) low-melting agarose (Gibco BRL), 0.4 % (w/v) SDS, 0.15 M BisTris (Sigma), 0.1 M HCl] was loaded on gel strips. After agarose solidification, electrophoresis was performed at 16 °C in a buffer (pH 8.3) containing 25 mM Trizma base (Sigma), 200 mM taurine (Sigma) and 0.1 % (w/v) SDS (Amersham Biosciences), for 1 h at 35 V and 14 h at 110 V (Gallardo et al. 2002b). Twelve gels (200 × 250 × 1.0 mm) were run in parallel (Isodalt system, Amersham Biosciences). For each sample, 2D gels were made in triplicate and from two independent protein extractions.

Protein staining and analysis of 2D gels

Gels were stained with Coomassie Brilliant Blue G-250 (Bio-Rad) according to the protocol of Mathesius et al. (2001) or with silver nitrate according to a modified method of Blum et al. (1987) using the Hoefer Processor Plus system (Amersham Biosciences). Coomassie Brilliant Blue staining was conducted with the buffer containing 10 % (w/v) ammonium sulphate (Sigma), 2 % (w/v) phosphoric acid (pH 6.5) and 0.1 % (w/v) CBB G-250 (Bio-Rad) for 3 days. Gels were washed for 3 min in 0.1 M Tris/Phosphoric acid (pH 6.5), for 30 s in 25 % (v/v) methanol and then for 48 h in 20 % (w/v) ammonium sulphate. Gels were stored at 4 °C in 20 % ammonium sulphate. Silver staining consisted of 14 steps. At the beginning, gels were rinsed in deionized water (MilliQ water) for 4 × 10 min (steps 1–4), next in 0.02 % (w/v) sodium thiosulphate (Fluka) for 1 min (step 5) and again in deionized water for 2 × 1 min (steps 6–7). Gels were coloured by 1.2 % (v/v) silver nitrate (Fluka) in the presence of 0.1 % (v/v) formaldehyde 37 % (Sigma) for 10 min (step 8). After colouration, gels were rinsed in deionized water for 2 × 1 min (steps 9–10) and then underwent revelation using 3.2 % (w/v) potassium carbonate (Sigma), 0.1 % (v/v) formaldehyde 37 % (Sigma) and 0.01 % (w/v) sodium thiosulphate (Fluka) (step 11). When the proteins (spots) appeared, the reaction was stopped by rinsing gels in 1.5 % (w/v) ethylenediamine-tetraacetic acid disodium salt (EDTA, Sigma) for 10 min (step 12). Subsequently, gels were rinsed in deionized water for 2 × 5 min (steps 13–14) and conserved in buffer of 30 % (v/v) ethanol with 5 % (v/v) glycerol. Stained gels were scanned with a Sharp JX-330 scanner equipped with the Labscan, version 3.0 (Amersham Biosciences) of the resolution of 300 dpi. Image analysis was carried out from the silver-stained gels with the ImageMaster™ 2-D

Platinum version 5.0 software (GE Healthcare/Amersham Biosciences) according to the instruction manual. After spot detection and background subtraction, a synthetic gel was created, allowing the visualization of all the polypeptides. This composite reference map was then used for protein pattern comparison during the time course and for matching with 2-D gels from sprouts and early seedlings tissue. An attempt was made to exclude spots where overlap with other spots was readily apparent. The quantitative determination of spot volumes was performed using ImageMaster™ 2D Platinum (version 5.0) software and normalization performed according to ‘total spot volume normalization’ (Gallardo et al. 2002b). For interpretation of the results, apart from ImageMaster™ 2D Platinum 5.0, other programmes such as STATISTICA (one-factor analysis of variance (ANOVA) and multiple comparison test, Tukey test) were used. Trial C48 was compared with C72, C72 with C96, C96 with S96, C72 with S72 and S72 with SR96. For each analysis, statistical data showed a high level of reproducibility between normalized spot volumes of gels produced in triplicate from the two independent protein extractions (Gallardo et al. 2002b). It was assumed that protein expression was stimulated/repressed when normalized spot volume increased/decreased at least 1.5-fold compared to the control sample.

Protein identification by mass spectrometry

Proteins which showed the most significant changes in their abundance between the different samples were subjected to mass spectrometry analysis. Spots were excised from Coomassie stained 2-D gels and digested by sequence grade trypsin (Promega, Madison, USA). After digestion, the supernatant containing peptides was concentrated on POROS 50 R2 beads (Roche Molecular Biochemicals, Switzerland) and used for MALDI-mass spectrometry analysis on a Bruker Reflex II MALDI-TOF spectrometer after on-target desorption with matrix solution (Gevaert et al. 1998). Before each analysis, the instrument was externally calibrated using two synthetic peptides spotted as close as possible to the biological sample. Proteins were identified using peptide mass fingerprinting (Pappin et al. 1993) and available protein databases like MASCOT PMF (<http://www.matrixscience.com>) (Schiltz et al. 2004; Gallardo et al. 2002b). To denote a protein as positively identified, the following criteria were used: protein scores should be above 75, coverage of the protein by the matching peptides must reach a minimum of 10 %, and at least four independent peptides should match within a stringent 0.001 % (10 ppm) maximum deviation of mass accuracy. Carbamidomethylation of cysteine residues and oxidation of methionine were considered as variable modifications (Gallardo et al. 2002a; Schiltz et al. 2004;

Dihazi et al. 2005). Theoretical masses and isoelectric points of identified proteins were predicted by entering the sequence at <http://www.expasy.ch/tools/peptide-mass.html> and were compared with experimental masses and pI.

Results

Physiological changes in growth of seedlings

Under osmotic stress, seed germination and growth of early pea seedlings were evidently inhibited (data not shown), an event which was accompanied by a reduced increase in fresh weight. In the control sample, fresh weight of an early seedling after 72 h of germination was about 101.34 mg, whereas in the samples subjected to short osmotic stress it reached 69.83 mg, falling down to 14.52 mg when long osmotic stress was applied (Fig. 1). When early seedlings were transferred from water stress to optimal conditions, recovery processes occur. They were accompanied by an increase in fresh weight of about 61.61 mg (from 69.83 to 131.44 mg). This increase, however, was smaller than in the control sample after 96 h of germination (171.20 mg). These results were drawn by comparing the sample subjected to short osmotic stress (S72) with the control sample after 72 h (C72) and the sample subjected to long osmotic stress (S96) with the control sample after 96 h of germination (C96). In turn, the recovery capacity of plant cells previously subjected to osmotic stress was assessed by comparing the samples: recovery (SR96) and stress (S72). In addition, the samples mentioned above (C72, C96, S72, S96, SR96) were subjected to proteomic analysis.

Global proteomic changes in optimal conditions

Proteomic analyses enabled us to detect 1,630 different proteins (over 1,300 proteins on silver-stained 2D gels, within pH 3–10). All the proteins were arranged into four classes (Table 1). Under optimal conditions (C), 149 proteins were up-regulated. Synthesis of these proteins was associated with a specific time of seed germination: 47 proteins were accumulated after 72 h (C72) of germination, 71 after 96 h (C96) and another 31 proteins after 72 and 96 h (C72, C96). Moreover, during the germination of pea seeds under optimal conditions, 81 proteins were down-regulated after 72 h (C72), 93 proteins after 96 h (C96) and 45 proteins after 72 and 96 h (C72, C96) (Table 1).

Global proteomic changes under osmotic stress conditions and recovery

Forty-four proteins were accumulated in pea seeds germinating under long-term osmotic stress (S96), whereas 50

Table 1 Classification of proteins which were present in the proteome of pea sprouts and early seedlings grown under: optimal (C), long-term osmotic stress (S96) and short-term osmotic stress (S72) conditions, and during recovery after short-term stress (SR96)

Class	Description	Number of proteins	
		Proteins increased at specific stages	Proteins decreased at specific stages
I	Proteins regulated under osmotic stress (S72, S96)		
	S72	50	97
	S96	44	76
	S72 and S96	53	21
II	Proteins regulated during recovery (SR96)		
	SR96	85	69
III	Proteins regulated under optimal conditions (C)		
	C72	47	81
	C96	71	93
	C72 and C96	31	45
IV	Other proteins whose level does not vary significantly between the different samples	767	
Total		1,630	

Number of detected proteins whose abundance varied or remained constant was based on statistical evaluation of the data (quantitative comparison). Statistically significant differences were determined between samples C48 and C72, C72 and C96, C96 and S96, C72 and S72, S72 and SR96

other proteins were detected under short-term osmotic stress (S72) (Table 1). Long-term osmotic stress (S96) also depressed 76 proteins, while short-term osmotic stress (S72) lowered other 97 proteins. Under the influence of

both analysed stresses (S72, S96), the accumulation of other 53 proteins was observed, while other 21 proteins were down-regulated (Table 1). During the recovery after short-term stress (SR96), 85 proteins were accumulated and 69 proteins were depressed (Table 1).

Identified proteins and their functional categories

Among all the proteins discovered in 2-D gels, 28 proteins which showed the most significant changes in their abundance between the different samples were excised and identified by MS (MALDI-TOF). These proteins are shown in Fig. 2. All the identified proteins were divided into functional categories proposed by Bevan et al. (1998). Among all the identified proteins, 3 were engaged in the primary metabolism, especially in amino acid and lipid metabolism (cat. A), and 5 proteins were involved in energy reactions like glycolysis/gluconeogenesis, pentose phosphate and in the Krebs cycle (cat. B). The ‘protein translocation and storage’ category (cat. E) contained 5 proteins (4 folding and stability/chaperons, 1 storage protein) and the ‘cell structure’ category (cat. H) was composed of 3 proteins (2 included in the cytoskeleton and 1 in mitochondria). The most numerous was the group of proteins connected with defence mechanisms (cat. J), as it consisted of six of the identified proteins (half active in stress responses and the other half involved in detoxification reactions). The other categories (cat. C, D, F, G, I and K) comprised one protein each. All the identified proteins are presented in Table 2. Among the identified proteins, 27 were accumulated in all samples, while one protein—6a-hydroxymaackian methyltransferase—was accumulated

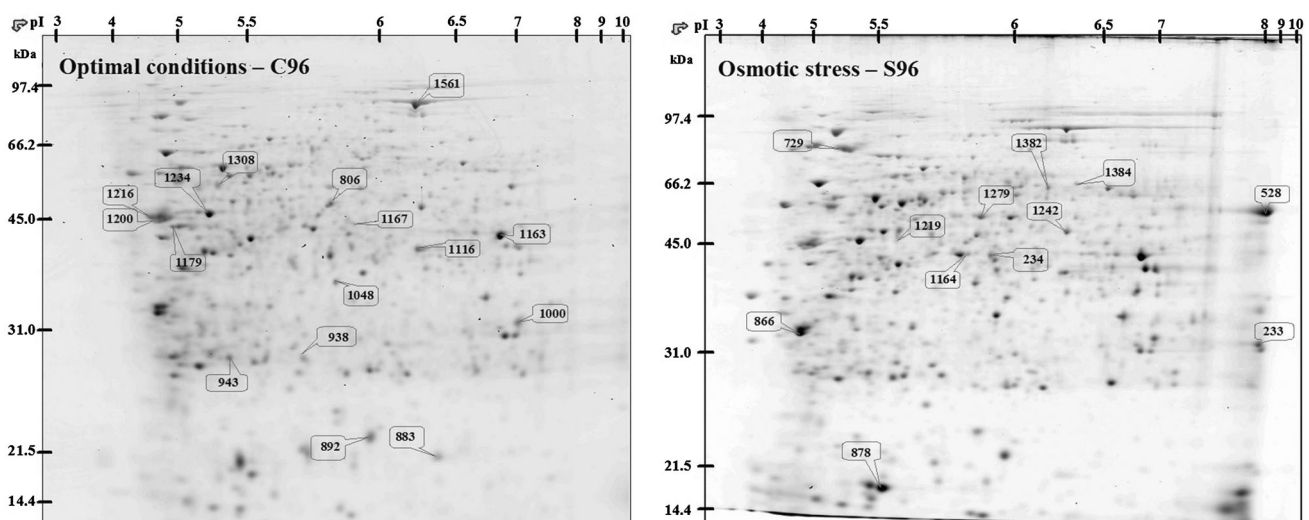
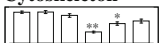
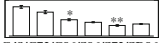






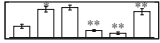

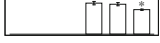


Fig. 2 Proteome of germinating pea seeds under optimal (C96) and osmotic stress (S96) conditions. Green colour indicates 28 proteins which were excised from 2DE gels and identified by mass spectrometry (MALDI-TOF) (colour figure online)

Table 2 Changes in the accumulation of the 28 identified proteins under optimal germination conditions (C48, C72, C96), long-term (S96) and short-term (S72) osmotic stress conditions, and also during recovery after short-term stress (SR96)

No. of protein groups	Expression level	Identified protein	Seq. Cov. (%)	No. of matched peptides	No. of unmatched peptides	Organism/ GenBank Acc. No.	pI	MW (kDa)
1	2	3	4	5	6	5	6	7
A. METABOLISM								
Amino acid								
1561		methionine synthase	13	9	21	<i>Gly</i> /AAQ08403	5.93	84.40
1219		S-adenosylmethionine synthetase-2 (SAM2)	28	7	2	<i>Ps</i> /BAC81655	6.27	37.84
Lipid (lipid acid) and sterols								
1048		3-ketoacyl-acyl carrier protein synthase III (KAS III)	16	5	4	<i>At</i> /AAA61348	6.70	43.49
B. ENERGY								
Glycolysis/ Gluconeogenesis								
1116		glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (phosphorylating)	23	5	4	<i>Ps</i> /AAA33667	6.55	36.70
1163		fructose-bisphosphate aldolase	39	10	7	<i>Ps</i> /CAA61947	6.77	38.64
Pentose phosphate								
1279		6-phosphogluconate dehydrogenase (6PGDH) (decarboxylating)	22	10	10	<i>Ms</i> /AAB41553	5.33	53.86
TCA pathway (Kreb's cycle)								
1242		NADP-dependent isocitrate dehydrogenase (NADP-IDH)	33	13	4	<i>Ps</i> /AAU44341	6.20	46.26
1384		dihydroliipoamide dehydrogenase (DLDH)	52	19	7	<i>Ps</i> /CAA45066	6.66	53.54
C. CELL GROWTH/DIVISION								
Cell growth								
892		profucosidase precursor	48	10	6	<i>Ps</i> /CAA09607	5.78	23.91
D. PROTEIN SYNTHESIS								
Translation factor								
528		elongation factor-1 alpha (EF1 alpha)	29	9	13	<i>Nc</i> /BAA34348	9.20	49.63
E. PROTEIN TRANSLOCATION AND STORAGE								
Folding and stability/ Chaperons								
1200		heat shock protein 70 (HSP70)	28	8	8	<i>Nc</i> /BAD22854	4.68	43.11
1216		heat shock cognate protein 70	20	9	15	<i>Pm</i> /AAN86275	4.68	44.02
1179		heat shock protein 81-2 (HSP81-2)	13	9	8	<i>At</i> /AAN31859	4.95	40.33
729		binding protein (BiP)	27	15	7	<i>At</i> /BAA12348	5.08	73.73
Storage proteins								
943		albumin 2	61	12	8	<i>Ps</i> /AAA02981	5.16	26.39
F. TRANSPORTERS								
Transport ATPases								
1382		H ⁺ -transporting ATPase	57	25	7	<i>Ps</i> /BAA03524	5.84	55.33
G. INTRACELLULAR TRAFFIC								
Vesicular (or ER/Golgi)								
883		ADP-ribosylation factor 1 (ARF)	51	8	4	<i>Pp</i> /AAO63779	5.82	20.62

Table 2 continued

H. CELL STRUCTURE							
Cytoskeleton							
1234		actin 7	31	8	10	<i>At/AAB52506</i>	5.31 41.93
1308		tubulin (alpha-5 chain)	22	7	5	<i>At/AAN31860</i>	4.95 50.25
Mitochondria							
233		porin por1	74	14	15	<i>Ps/CAA80988</i>	9.11 29.58
I. SIGNAL TRANSDUCTION							
866		14-3-3-like protein	36	6	11	<i>Ps/CAB42546</i>	4.68 29.45
J. DISEASE/ DEFENCE							
Stress responses							
1000		chitinase	27	6	6	<i>Ps/AAA75196</i>	7.34 35.74
878		lectin	40	6	4	<i>Ps/AAA33676</i>	5.31 19.97
938		ferritin precursor	24	6	6	<i>Ps/CAA45763</i>	6.14 28.77
Detoxification							
234		alcohol dehydrogenase (ADH)	27	9	10	<i>Ps/CAA29609</i>	6.08 41.87
806		monodehydroascorbate reductase (NADH ₂) (MDHAR)	48	15	17	<i>Ps/AAA60979</i>	5.79 47.39
1167		glutathione-disulphide reductase (GR)	26	9	8	<i>Ps/CAA66924</i>	6.09 54.20
K. SECONDARY METABOLISM							
1164		6a-hydroxymaackian methyltransferase (HMM)	29	8	7	<i>Ps/AAC49856</i>	5.70 40.55

Identification was performed by mass spectrometry (MALDI-TOF). Proteins were divided into functional categories according to Bevan et al. (1998). Statistically significant differences between C48 and C72, C72 and C96, C96 and S96, C72 and S72, S72 and SR96 trials were indicated: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (ANOVA)

GenBank Accession No. NCBI (<http://www.ncbi.nlm.nih.gov/protein>), *At Arabidopsis thaliana*, *Gly glycine max*, *Ms Medicago sativa*, *Nc Nicotiana paniculatalbenthamiana*, *Ps Pisum sativum*, *Pm Pumpkin (Cucurbita maxima)*, *Pp Populus tremuloides*

only during osmotic stress conditions (S72, S96) and post-stress recovery (SR96).

Changes in accumulation of identified proteins under optimal conditions

Among the proteins whose accumulation increased during the germination period under optimal conditions were proteins which participated in metabolism (lipid acid synthesis—3-ketoacyl-acyl carrier protein synthase III), energy reactions (e.g. glycolysis/gluconeogenesis—glyceraldehyde-3-phosphate dehydrogenase and the Krebs cycle—NADP-dependent isocitrate dehydrogenase), cell growth (profucosidase precursor), transport (H⁺-transporting ATPase, ADP-ribosylation factor 1), detoxification (monodehydroascorbate and glutathione-disulphide reductase) and in stress responses (chitinase). In contrast, the accumulation of storage proteins (albumin 2), chaperons (binding protein), proteins participating in metabolism of amino acids (methionine synthase, S-adenosylmethionine

synthetase-2), translation (elongation factor-1 alpha), cytoskeleton (tubulin), signal transduction (14-3-3-like protein) and in defence mechanism (e.g. stress responses—lectin, ferritin precursor, detoxification—alcohol dehydrogenase) became considerably depressed during the same period (Table 2).

Changes in accumulation of identified proteins under osmotic stress conditions

Among the proteins whose abundance decreased under osmotic stress conditions are 17 proteins associated with defence mechanisms—4 (stress responses—chitinase, ferritin precursor and detoxification—monodehydroascorbate and glutathione-disulphide reductase), translocation—3 (HSP70, HSPc70, HSP81-2), metabolism—2 (S-adenosylmethionine synthetase-2, 3-ketoacyl-acyl carrier protein synthase III), glycolysis/gluconeogenesis—2 (glyceraldehyde-3-phosphate dehydrogenase, fructose-bisphosphate aldolase), cell growth—1 (profucosidase precursor),

protein synthesis—1 (elongation factor-1 alpha), intracellular traffic—1 (ADP-ribosylation factor 1) and structural components of cells—3 (cytoskeleton—actin 7, tubulin and mitochondria—porin por 1). Also, for six of the identified proteins, considerably increased accumulation was observed. These were proteins involved in the Krebs cycle (dihydrolipoamide dehydrogenase), chaperons (BiP), transport ATPases (H^+ -transporting ATPase), signal transduction (14-3-3-like protein) and detoxification (alcohol dehydrogenase). Besides, under the influence of osmotic stress (S72, S96), the accumulation of one identified protein participating in secondary metabolism increased. This enzyme, 6a-hydroxymaackian methyltransferase (HMM), is involved in the synthesis of pisatin. It can be assumed that these proteins whose accumulation increased under osmotic stress conditions play a key role in the acquisition of stress tolerance by plants.

Changes in accumulation of identified proteins under recovery after stress

Among the proteins whose accumulation increased under the recovery period after short-term stress (SR96) were proteins involved in lipid biosynthesis (KASIII), energy (GADPH), cell structure (actin 7, tubulin, porin por 1) and defence mechanism (chitinase, alcohol dehydrogenase, monodehydroascorbate, glutathione-disulphide reductase). Among the proteins whose accumulation was considerably depressed during recovery were proteins involved, for example, in the Krebs cycle (NADP-dependent isocitrate and dihydrolipoamide dehydrogenase), pentose phosphate (6-phosphogluconate dehydrogenase), amino acid synthesis (methionine synthase), signal transduction (14-3-3-like protein), stress responses (lectin, ferritin precursor) and storage protein (albumin 2) (Table 2). Another protein whose expression was lower during recovery was the enzyme HMM, participating in secondary metabolism in plants, which may indicate that this enzyme is accumulated only during unfavourable environmental conditions.

Discussion

Growth and development during osmotic stress

Under osmotic stress, the germination of pea seeds as well as the growth and development of early seedlings are inhibited (Xu and Bewley 1991; Gill et al. 2002; Murillo-Amador et al. 2002). Here, we found that the accumulation of the following proteins involved in cellular divisions and growth is depressed under stress conditions: SAM (Espartero et al. 1994; Schröder et al. 1997), AFR, profucosidase, chitinase, actin 7 (McDowell et al. 1996), tubulin (Dihazi

et al. 2005), EF1 α (Singh et al. 2004), porin por 1 (Kirch et al. 2000; Smart et al. 2001; Vera-Estrella et al. 2004) or ferritin (Petit et al. 2001). Ado-Met synthase is associated with high metabolic activity of embryos, which occurs during germination and during the growth and development of plants by catalysing the synthesis of Ado-Met (Gallardo et al. 2002a). Reduction of SAM under stress conditions could be related to growth inhibition. Porin por proteins are embedded in the outer membrane of mitochondria (Benz 1985), where they enable plants to store more water in tissues during drought, thus reinforcing plant tolerance to this stress (Kirch et al. 2000; Smart et al. 2001). Depressed accumulation of ferritin in the embryonic tissue of pea in response to osmotic stress can be attributed to the activation of iron regulatory proteins (IRPs). As evidenced by Pantopoulos et al. (1997), H_2O_2 activates these proteins and, according to Swenson et al. (1991) or Harrison and Arosio (1996), it suppresses the synthesis of ferritin at the translation level.

Cellular energy reactions and metabolism during osmotic stress

Under osmotic stress, the accumulation of proteins involved in cellular metabolism, like GAPDH, fructose-bisphosphate aldolase and KASIII, is depressed (Ricard et al. 1989; Yamada et al. 2000). Reduction in the accumulation of fructose-bisphosphate aldolase leads to a depressed rate of photosynthesis, synthesis of carbohydrates and plant growth. Depressed accumulation of aldolase is accompanied by an increase in the content of 3-phosphoglyceric aldehyde and glycerophosphate in plant tissues (Haake et al. 1998). Accumulated phosphate trioses are transported to the cytosol, where they are transformed to hexoses and function as osmoprotectants (Yamada et al. 2000). Depressed accumulation of GAPDH most probably inhibits the process of glycolysis and consequently the synthesis of fatty acids. Accordingly, the level of KASIII, which participates in condensation of acylmalonyl-ACP from acetyl-ACP and malonyl-ACP, decreased during osmotic stress. According to Dehesh et al. (2001), a factor which may limit the content of fats could be the absence of malonyl-CoA, produced from acetyl-CoA. The accumulation of 6-phosphogluconate dehydrogenase (6PGDH) or NADP-IDH increased in response to short-term stress but decreased under long-term stress (Popova et al. 2001; Huang et al. 2003; Hou et al. 2007). The role of NADP-IDH, activated during stress, most probably consists in accelerating α -ketoglutarate synthesis, which is needed for reorganization of the cell's metabolism and induction of metabolic pathways, important for adaptation processes, especially when proline and γ -aminobutyric acid (GABA) are accumulated during stress; 2-ketoglutarate is a

nitrogen-free precursor for proline and GABA (Popova et al. 2001). Another important element in plants' response to abiotic stresses is the pentose phosphate pathway, in which 6PGDH may function as a factor regulating the efficiency of this pathway (Huang et al. 2003; Hou et al. 2007).

Detoxification under osmotic stress

Under osmotic stress, the concentration of ABA in cells increases (Taylor et al. 2000; Starck 2002), which induces alcohol dehydrogenase (ADH) gene expression (De Bruxelles et al. 1996). Stimulating the expression of ADH under the influence of a variety of stresses has been demonstrated (Jarillo et al. 1993; Conley et al. 1999; Kato-Noguchi 2000a). ADH is probably essential for removing toxic acetic aldehydes and regulating cytoplasmic pH during stress conditions (Kato-Noguchi 2000a). In turn, accumulation and activity of antioxidant enzymes, e.g. monodehydroascorbate reductase (Huang et al. 2005; Shohael et al. 2006) and glutathione reductase (Van Der Mescht et al. 1998) are depressed under stress conditions. These proteins play a key role in the acquisition of the ability to detoxify reactive oxygen species (ROS) by plants, especially during germination and growth (Cakmak et al. 1993).

Plant adaptation to osmotic stress: defence and tolerance mechanism

Plants are protected from the negative effects of stress by dihydrolipoamide dehydrogenase—DLDH (Banzai et al. 2002), which, through its nitrogen reductase activity, depresses high level of nitrogen oxide (NO) in a cell by transforming it into nitrate (Igamberdiev et al. 2004). An increase in the level of NO, which occurs in response to unfavourable environmental conditions, is connected to an increase in the accumulation of DLDH. Tolerance of plants to water deficit is also improved by BiP (Cascardo et al. 2000; Alvim et al. 2001), which maintains the proper structure of proteins and membranes during stress (Gallardo et al. 2001). Moreover, BiP is engaged in the binding or transfer of water as well as accumulation of ions and also supports high secretion activity of proteins during water deficit, controlling proper folding and maturation of proteins (Ingram and Bartels 1996; Cascardo et al. 2000). Accumulation of this protein significantly increased during stress (Kalinski et al. 1995; Cascardo et al. 2000; Alvim et al. 2001), for example to protect plants from endogenous oxidative stress caused by water deficit in cells (Alvim et al. 2001). In turn, the accumulation of lectin is depressed under the influence of short-term stress but rises during long-term stress (Esteban et al. 2002; Shakirova et al.

2003). This means that lectins protect plants from harmful effects of long-term water stress, which inhibits the mitotic activity in cells of a growing root (Shakirova et al. 2003).

Functioning of signal transduction and cell transport system during osmotic stress

Osmotic stress improves the hydrolysis of ATP and transport of H^+ through membranes owing to the formation of a membrane complex 14-3-3-ATPase (Babakov et al. 2000; Kerkeb et al. 2002; Shanko et al. 2003). By joining the autoinhibitory domain in the C-terminal region of this enzyme with 14-3-3-like protein, an active form of H^+ -transporting ATPase is created, which stimulates the uptake of nutrients by the cell (Jahn et al. 1997; Borch et al. 2002). Increased transport of H^+ is necessary for the cell to restore ionic homeostasis under new environmental conditions and to activate the signal transduction cascade (Kerkeb et al. 2002). Proper functioning of H^+ -transporting ATPase also enables plants to maintain the right turgor pressure in cells during stress (Yan et al. 2004). The results of our experiment indicate that an increased H^+ transport through ATPase, phosphorylated and connected with 14-3-3 protein, is part of an early response of cells to disorders caused by osmotic stress (Babakov et al. 2000; Kerkeb et al. 2002). Moreover, the content of the 14-3-3 protein connected with the plasmatic membrane increases under osmotic stress alongside the stimulation of the flow of H^+ through cells (Babakov et al. 2000; Kerkeb et al. 2002; Shanko et al. 2003). Plants with elevated expression of 14-3-3-like protein encoding gene (GF14 λ) demonstrate higher tolerance to water stress by maintaining the proper turgor pressure and a higher rate of photosynthesis when shortage of water appears (Yan et al. 2004). It is probable that 14-3-3 is also needed for activation of Ca^{2+} -dependent protein kinase (CDPK) and other enzymes during water stress (Camoni et al. 1998). It is worth mentioning that limited participation of the ADP-ribosylation factor (ARF) also leads to the activation of the 14-3-3 protein gene (Žuk et al. 2003).

Functioning of secondary metabolism during osmotic stress

Under the influence of changeable environmental conditions, enzymes of the secondary metabolic pathways, which lead to the synthesis of various protective substances, e.g. phytoalexins, are activated (Soylu et al. 2002; Kuniga 2004; Zhao et al. 2005; Liu et al. 2006). It needs to be emphasized that phytoalexins are not synthesized in healthy plants growing under optimal conditions (Mert-Türk 2002). Among the products of the biosynthesis of

phytoalexins is pisatin. An enzyme which participates in synthesis of pisatin is 6a-hydroxymaackian methyltransferase (HMM) (Wu et al. 1997; Liu et al. 2006). According to Sweigard et al. (1986), accumulation of phytoalexin isoflavonoids in leguminous plants is preceded by an increased level and activity of enzymes participating in their synthesis. This hypothesis has been verified by the experimental results obtained by Preisig et al. (1989), who observed that an increase in the content of pisatin was preceded by an enhanced activity of HMM, which means that HMM and its product, pisatin, can play an important role in the formation of plants' tolerance to osmotic stress. It is worth mentioning that HMM decreased under recovery after stress.

Accumulation of proteins under recovery after osmotic stress

Recovery following osmotic stress caused the resumed intensive growth of early pea seedlings and accumulation of fresh matter (Bensen et al. 1988; Gill et al. 2002). This means that during the recovery period, cellular processes were activated, such as cell division and metabolic pathways in seedlings as well as the accumulation of accompanying proteins, which had been depressed under stress (e.g. GAPDH, KASIII, MDHAR, GSH reductase, ADH, chitinase, actin7, ADP-ribosylation factor). Higher accumulation of enzymes such as GAPDH occurs in cells undergoing intensive growth and divisions (McAlister and Holland 1985). MDHAR in elongating plant cells under recovery plays an important role by mediating the transport of electrons through the plasma membranes (Morre et al. 1986). It is noteworthy that the accumulation of porin por 1 increased during recovery. These proteins are necessary for the proper growth and functions of cells, ensuring fast inflow of water to vacuoles (Ludevid et al. 1992; Chaumont et al. 1998). This could imply that regeneration processes after stress occur very slowly because, as reported by Martre et al. (2002), a higher content of porins indicates faster recovery process and growth of plants after water stress. The fact that plants underwent recovery was also indicated by the decreased accumulation of proteins whose synthesis had been considerably raised under stress conditions, e.g. H⁺-transporting ATPase, NADPH-IDH, HM 14-3-3-like protein, or albumin 2. Albumins, which function as a reserve of nitrogen amino acids and skeletal carbons during the growth of plants, were degraded under recovery and used to synthesize new proteins (Murray 1979). Moreover, processes which are regulated by 14-3-3-like protein such as primary metabolism, intermembrane transport and cellular transduction signal (Aitken et al. 1992; Ferl 1996; Comparot et al. 2003) were also depressed under recovery.

Conclusions

The identified protein, 6a-hydroxymaackian methyltransferase, can serve as an object for an engineering strategy, involved in the creation of new plants which will be more resistant to unfavourable environmental conditions.

Author contribution W. Brosowska-Arendt carried most of the research and was the main author of this manuscript. K. Galardo provided all the technical support during the laboratory work and helped with the manuscript preparation. N. Sommerer was responsible for analysis of the proteomic data. Stanisław Weidner was the founder and a person responsible for experimental design.

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