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ORIGINAL ARTICLE

Proteomics analysis of sensitive and tolerant barley genotypes under drought stress

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Abstract Drought is a severe environmental constraint to plant productivity and an important factor limiting barley yield. To investigate the initial response of barley to drought stress, changes in protein profile were analyzed using a proteomics technique. Three-day-old barley seedlings of sensitive genotype 004186 and tolerant genotype 004223 were given two treatments, one with 20 % polyethylene glycol and the second with drought induced by withholding water. After 3 days of treatments, proteins were extracted from shoots and separated by 2-dimensional polyacrylamide gel electrophoresis. Metabolism related proteins were decreased in the sensitive genotype under drought; however, they were increased in the tolerant genotype. Photosynthetic related proteins were decreased and increased among the three sensitive and three tolerant genotypes, respectively. In addition, amino acid synthesis and degradation related proteins were increased and decreased among the three tolerant genotypes. These results suggest that chloroplastic metabolism and energy related proteins might play a significant role in the adaptation process of barley seedlings under drought stress.

Keywords Barley · Proteomics · Drought · Sensitive genotype · Tolerant genotype

Abbreviations

2-DE	Two-dimensional polyacrylamide gel electrophoresis
CBB	Coomassie brilliant blue
MS	Mass spectrometry
LC	Liquid chromatography
pI	Isoelectric point
IEF	Isoelectric focusing
PEG	Polyethylene glycol
ROS	Reactive oxygen species

Introduction

In comparison to other major cereal crops, barley is a typical crop grown in low input and climatically marginal areas (Ceccarelli 1996). Drought is a great environmental constraint to plant productivity (Farooq et al. 2009) and a severe factor limiting barley yield. It can significantly reduce crop yields and restrict the latitudes and soils on which commercially important species can be cultivated (Blum 1985). Compared to other cereals like wheat, triticale and oats barley is well adapted to this condition because of its better water use and transpiration efficiency as well as faster leaf area growth rate (Lopez-Castaneda and Richards 1994). Identifying and understanding mechanisms of drought tolerance is crucial for the development of tolerant crop varieties.

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When plants were subjected to drought stress, a number of physiological and morphological responses were observed and the magnitude of the response varies among species and between varieties within a crop species (Kramer 1980). The response to drought was an increased level of abscisic acid, the accumulation of unusual metabolites such as proline and polyamines, alterations in activity of certain enzymes and the induction of a specific set of genes (Skriver and Mundy 1990). High activity of superoxide dismutase was linked with drought stress tolerance in plants that survive treatments likely to enhance the production of reactive oxygen species (ROS) (Bowler et al. 1992). High constitutive or high induced levels of antioxidants in a plant cell may provide resistance to a particular stress.

Among proteins induced by drought and low-temperature stress, dehydrins, which are late embryogenesis abundant proteins, have been the most commonly found (Close 1997). Their subcellular location is consistent with a biochemical role as an intracellular stabilizer, acting upon targets in both the nucleus and cytoplasm. Dehydrin loci are multigenic and present in clusters on more than one chromosome (Campbell and Close 1997). Different dehydrins play variable functional roles in structural protection in plants subjected to progressive water stress, associated with drought-contrasting genotypes (Qian et al. 2008). They have been reported to function in abiotic stress tolerance by minimizing the negative effects of ROS (Mowla et al. 2006).

Using proteomics technique, drought stress responsible proteins have been analyzed in rice (Ali and Komatsu 2006). The results suggested that actin depolymerizing factor was one of the target proteins expressed in leaf blades, leaf sheaths and roots under drought stress but not under cold and salt stresses and/or abscisic acid treatment. The proteomics of drought stress acclimation in sunflower has been done with the conclusion that proteins contributing to basic carbon metabolism were significantly increased (Fulda et al. 2011). In barley, the leaf apoplast was examined as a compartment, which sensitively and differentially responds to drought and salinity with consequences for plant growth (Ramanjulu et al. 1999).

Proteomics analysis of barley leaves to salinity was carried out (Rasoulnia et al. 2011) with the result that the total number of increased proteins in salt-tolerant genotype were more than the salt-sensitive one. Salt stress-induced alterations in the root proteins of barley genotypes resulted that the proteins involved in the glutathione-based detoxification of ROS were more abundant in the tolerant genotype, while proteins involved in iron uptake were expressed at a higher level in the sensitive one. Furthermore, the role of proteins in ROS detoxification and potential candidates for increasing salt tolerance in barley were identified (Witzel et al. 2009). However, there is no report regarding shoot proteins of barley genotypes under drought stress.

In response of drought stress during the reproductive stage, differentially expressed genes between two drought-tolerant and one drought-sensitive barley genotypes were studied using microarray (Guo et al. 2009). The results indicated that constitutively expressed genes in drought-tolerant genotypes were more than differentially expressed genes in all genotypes under drought. In barley drought resistant cultivar, the survival rate of seedlings was adversely affected at a severe drought condition (Volaire 2003). In semiarid and arid regions, cereals often suffer from such intense water shortage during seedling stage, which subsequently leads to major reductions in plant population and finally crop yield. To investigate the mechanism of drought stress, proteomics analysis was performed using three sensitive and three tolerant barley genotypes.

Materials and methods

Plant materials

The germplasm was obtained from Plant Genetic Resource Institute, National Agriculture Research Centre, Islamabad, Pakistan. It included 15 barley (*Hordeum vulgare* L.) genotypes named as Jau-83, Frontier-87, Jau-87, Haider-93, Sanober-96 and Soorab-96, and having accession numbers 0017655, 004186, 004201, 004222, 004223, 004325, 004360, 005130 and 005137.

Plant growth condition

Seeds of 15 barley genotypes were surface sterilized with 1 % sodium hypochlorite solution and planted into sterilized sand in pots using completely randomized design with three replications. Afterwards these pots were transferred to controlled condition of glass house. After 5 days, drought treatment with 20 % polyethylene glycol (PEG) started and continued for 20 days. Photosynthetic rate was determined using LC*pro* (ADC Bioscientific, Hertz, UK). Shoot length, root length and fresh weight were measured.

Seeds of genotypes 004186 and 004223 were sown in water for 1 day and germinated on silica sand in a growth chamber illuminated with white fluorescent light (600 μmol m^{-2} s^{-1}, 12 h light period/day) at 25 °C and 70 % relative humidity. Three-day-old seedlings were given two treatments, one with 20 % PEG and the second with drought by withholding water. After 3 days, shoots were collected. Three independent biological experiments were performed.

For the confirmatory experiment, seeds of genotypes Jau-83, Sanober-96, 004360 and Frontier-87 were sown in water for 1 day and germinated on silica sand in a growth chamber illuminated with white fluorescent light (600 μmol m^{-2} s^{-1}, 12 h light period/day) at 25 °C and 70 % relative humidity. Three-day-old seedlings were treated with drought only by withholding water. After 3 days, shoots were collected. Three independent biological experiments were performed.

Cluster analysis

The hierarchical cluster analysis was done using the data of photosynthetic rate, shoot length, root length and fresh weight of the plants grown in glass house. Ward method (Ward 1963) was chosen for this purpose and dendrogram was obtained according to the stress sensitivity and stress tolerance index for the above mentioned parameters of the various barley genotypes using statistical procedure for social sciences (SPSS-18) software (SPSS Inc., Chicago, IL, USA) (Wellman 1998).

Protein extraction

A portion (500 mg) of shoots was ground to powder in liquid nitrogen with a mortar and pestle. The powder was transferred to 10 % trichloro acetic acid and 0.07 % 2-mercaptoethanol in acetone and the mixture was vortexed. The suspension was sonicated for 5 min and then incubated for 45 min at −20 °C. After incubation, the suspension was centrifuged at 9,000×*g* for 20 min at 4 °C. The supernatant was discarded and resulting pellet was washed with 0.07 % 2-mercaptoethanol in acetone twice. The resulting pellet was dried using a Speed-Vac concentrator (Savant Instruments, Hicksville, NY, USA) and resuspended with lysis buffer consisting of 7 M urea, 2 M thiourea, 5 % CHAPS, and 2 mM tributylphosphine by vortexing for 1 h at 25 °C. The suspension was centrifuged at 20,000×*g* for 20 min at 25 °C. Supernatant was collected as protein extract and was subjected to electrophoresis. Protein contents were determined using the Bradford method (Bradford 1976) with bovine serum albumin as the standard.

Two-dimensional polyacrylamide gel electrophoresis

For 2-dimensional polyacrylamide gel electrophoresis (2-DE), protein samples in a final volume of 200 μL of lysis buffer containing 0.4 % Bio-Lyte pH 3/10 (Bio-Rad, Hercules, CA, USA) were directly loaded into a focusing tray. The immobilized pH gradient strips (3–10NL, 11 cm, Bio-Rad) were rehydrated for 14 h at 50 V. Isoelectric focusing (IEF) was carried out with the Protean IEF Cell (Bio-Rad) using following conditions: 250 V for 15 min with a linear ramp, 8,000 V for 1 h with a linear ramp, and finally 8,000 V at 35,000 V/h with a rapid ramp at 20 °C. After IEF, the strips were equilibrated with 6 M urea, 2 % SDS, 0.375 M Tris–HCl (pH 8.8), 20 % glycerol, and 130 mM dithiothreitol for 30 min. The last equilibration step was done with 6 M urea, 2 % SDS, 0.375 M Tris–HCl (pH 8.8), 20 % glycerol, and 135 mM iodoacetamide for 30 min. The equilibrated strips were placed onto 15 % SDS–polyacrylamide gel and sealed with 1 % agarose. Electrophoresis in the second dimension was performed at a constant current of 35 mA. The gels were stained with Coomassie brilliant blue (CBB).

Gel image analysis

2-DE images were obtained using a GS-800 calibrated densitometer scanner (Bio-Rad) and the position of individual proteins on gels was evaluated with PDQuest software (version 8.0.1) (Bio-Rad). Following automatic spot detection, gel images were carefully edited. Before spot matching, one of the gel images was selected as a reference gel. The amount of a protein spot was expressed as the volume of that spot, which was defined as the sum of the intensities of all the pixels that make up the spot. In order to correct the variability due to CBB staining and to reflect the quantitative variation in intensity of protein spots, the spot volumes were normalized as a percentage of the total volume in all spots in the gel. The resulting data from image analysis were transferred to PDQuest software for querying protein spots that showed quantitative and qualitative variations. The isoelectric point (pI) and molecular mass of each protein were determined using 2-DE standard marker (Bio-Rad).

Peptide preparation for mass spectrometry analysis

To identify proteins in protein spots using mass spectrometry (MS), protein spots were excised from 2-DE gels and washed with water. Proteins in the excised gel pieces were reduced with 10 mM dithiothreitol in 100 mM NH_4HCO_3 for 1 h at 60 °C and incubated with 40 mM iodoacetamide in 100 mM NH_4HCO_3 for 30 min. The proteins were digested in 100 mM NH_4HCO_3 with 1 pM trypsin (Wako, Osaka, Japan) at 37 °C overnight. The tryptic peptides were extracted from the gel grains with 0.1 % trifluoroacetic acid in 50 % acetonitrile three times. The procedure described above was performed with DigestPro 96 robotic system (Intavis AG, Cologne, Germany). The peptide solution obtained was dried and then reconstituted with 0.1 % trifluoroacetic acid in 50 % acetonitrile and desalted using C-Tip pipet tips (Nikkyo Technos, Tokyo, Japan). Desalted peptide solution was analyzed by MS.

Protein identification by matrix-assisted laser desorption ionization time-of-flight mass spectrometry

The purified peptides were added to an α-cyano-4-hydroxycinamic acid matrix and dried onto a plate for analysis using MS. Two internal markers Angiotensin I and Adrenocorticotropic hormone (clip 18–39) were present in the calibration mixture. A matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) MS (Voyager-DE RP, Applied Biosystems, Foster city, CA, USA) was used for this purpose. Calibration was manual and data were collected in the reflector mode. Data were searched on the Internet using an in-house licensed Mascot search engine (version 2.2.18) (Matrix Science, London, UK) in the NCBI database. Parameters for search were: taxonomy Viridiplantae (green plants), peptide tolerance 0.5 Da, mass values MH^+, monoisotopic and decoy. Carbamidomethylation of cysteines was set as a fixed modification and oxidation of methionines was set as a variable modification. Trypsin was specified as the proteolytic enzyme and one missed cleavage was allowed. Identified proteins with a peptide mass fingerprint were denoted as having an unambiguous identification by the following criteria: (1) the deviation between the experimental and theoretical peptide masses needed to be less than 50 ppm; (2) at least five different predicted peptide masses were needed to match the observed masses for an identification to be considered valid; (3) the matching peptides needed to cover at least 8 % of the known protein sequence; and (4) protein scores needed to have >72 identity for NCBI database (http://www.ncbi.nih.gov) ($p < 0.05$).

Protein identification by nano-liquid chromatography-tandem mass spectrometry

A nanospray LTQ XL Orbitrap MS (Thermo Fisher Science, San Jose, CA, USA) was operated in data-dependent acquisition mode with the installed XCalibur software. Using an Ultimate 3,000 nano liquid chromatography (LC) (Dionex, Germering, Germany), peptides in 0.1 % formic acid were loaded onto a 300 μm ID × 5 mm C18 PepMap trap column. The peptides were eluted from the trap column and their separation and spraying were done using 0.1 % formic acid in acetonitrile at a flow rate of 200 nL/min on a nano-capillary column (Nikkyo Technos) with a spray voltage of 1.8 kV. Full scan mass spectra were acquired in the Orbitrap over 150–2,000 m/z with a resolution of 15,000. The three most intense ions above the 1,000 threshold were selected for collision-induced fragmentation in the linear ion trap at a normalized collision energy of 35 % after accumulation to a target value of 1,000. Dynamic exclusion was employed within 30 s to prevent repetitive selection of peptides. Acquired MS/MS spectra were converted to individual DTA files using Bio Works software (version 3.3.1) (Thermo Fisher Science). The following parameters were set to create a list of peaks: parent ions in the mass range with no limitation, one grouping of MS/MS scans, and threshold at 100.

The resulting peptide sequence data were obtained in mgf format and used to search NCBI database using an in-housed MASCOT search engine. Carbamidomethylation of cysteines was set as a fixed modification and oxidation of methionine was set a variable modification. Trypsin was specified as the proteolytic enzyme, and one missed cleavage was allowed. Parameters for search were: taxonomy Viridiplantae (green plants), peptide mass tolerance 10 ppm, fragment mass tolerance 0.2 Da, maximum missed cleavage 1, peptide charges +1, +2, +3, instrument ESI-TRAP and decoy. The proteins with at least five matched peptides in the Mascot search result with more than 10 % sequence coverage were considered. In the case of peptides matching among multiple members of a protein family, the protein presented was selected based on the highest score number of matching peptides. The positive matches were searched against the NCBI database by BLASTP algorithm for updated annotation and identification of homologous proteins.

Results

Cluster analysis of barley genotypes

In the pot experiment, the growth of 20 % PEG treated plants was adversely affected as compared to the control plants (Supplementary Fig. 1). Based on the data set obtained from these plants (Supplementary Table 1) the hierarchical clustering procedure was used to construct a dendrogram using Ward method. This procedure identified relatively homogeneous groups of variables. The variables to be grouped were photosynthetic rate, shoot length, root length and fresh weight. All of these four parameters decreased in the treatment as compared to control. Using SPSS-18 software, Ward method resulted in two clearly separated sensitive and tolerant groups (Fig. 1). Among the sensitive group, the genotype 004186 came out to be the most sensitive one followed by Jau-83 and Sanober-96. Similarly, the genotype 004223 was found to be the most tolerant one among the tolerant group followed by 004360 and Frontier-87. Based on cluster analysis, the shoots of 3 sensitive (004186, Jau-83 and Sanober-96) and 3 tolerant (004223, 004360 and Frontier-87) barley genotypes were used for the proteomics experiment.

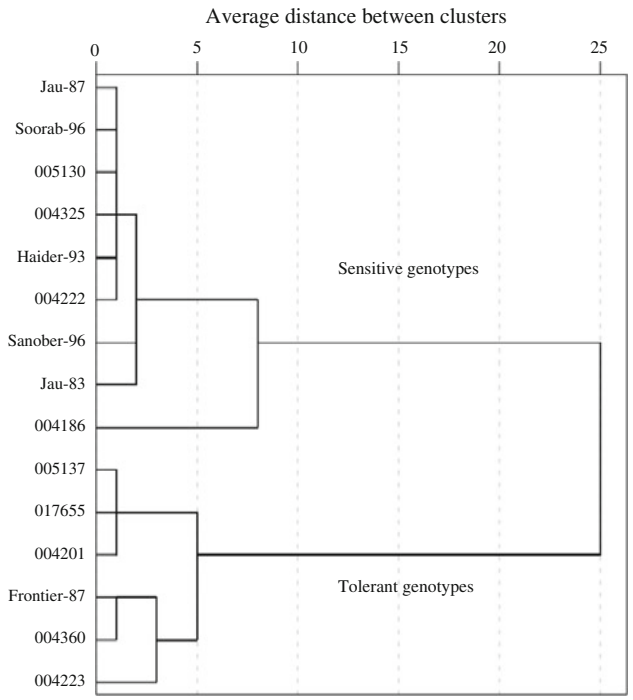

Fig. 1 Dendrogram of the phenotypic distances among barley genotypes under PEG treatment. A total of 15 barley genotypes were treated with 20 % PEG from 6-day-old seedlings until 26-day-old plantlets. Data taken for photosynthetic rate, shoot length, root length and fresh weight was used to discriminate drought sensitive genotypes from the tolerant ones. There were two completely distinguished groups with cutting dendrogram obtained from Ward method in distance ten. The experiment was repeated three times. Data used for the cluster analysis were presented in supplementary Table 1

Protein profile patterns in the shoot of sensitive and tolerant barley genotypes

Three-day-old seedlings of genotypes 004186 and 004223 were given two treatments one with drought and the second with 20 % PEG, then proteins were extracted from the shoot, separated by 2-DE, and stained with CBB to evaluate their increased and decreased level. Using PDQuest software analysis, 580 and 640 protein spots were reproducibly detected on the 2-DE gels of shoots of sensitive and tolerant genotype, respectively (Fig. 2a, b, control). In the sensitive genotype 004186, 5 and 9 protein spots were increased while 14 and 7 were decreased in response to drought and PEG, respectively (Fig. 2a). On the other hand, in the tolerant genotype 004223, 8 and 8 protein spots were increased while 6 and 14 were decreased in response to drought and PEG, respectively (Fig. 2b).

In the genotype 004186, the amount of 3 protein spots (S10, S15 and S25) changed more than two times under drought treatment. Two of them (S10 and S25) were decreased while one (S15) was increased. Similarly under PEG treatment, 4 protein spots (S6, S19, S28 and S30) changed twice. Out of them, 3 protein spots (S6, S19 and S30) were increased and one (S28) was decreased (Fig. 3a). In the genotype 004223, four protein spots were changed more than twice under drought. Out of them three protein spots (T10, T22 and T28) were increased while one (T17) was decreased. However under PEG treatment, six protein spots changed more than two times. Out of them, two protein spots (T3 and T4) were increased and four (T13, T20, T21 and T23) were decreased (Fig. 3b).

Common proteins between drought and PEG treatments in sensitive and tolerant genotypes

In the sensitive genotype 004186, a total of 19 and 16 proteins were changed in shoot by drought and PEG treatment, respectively (Fig. 4a). Three of these proteins (S2, S22 and S30) were changed both in drought and PEG, two of these common proteins (S22 and S30) were increased while one (S2) was decreased. However, only two protein spots (S15 and S29) were increased in drought while others (S4, S5, S10, S11, S12, S13, S14, S16, S17, S20, S24, S25, S26 and S31) were decreased. In PEG treatment, six protein spots (S6, S10, S18, S19, S21 and S23) were increased and six (S1, S3, S7, S8, S9 and S28) were decreased.

In the tolerant genotype 004223, a total of 14 and 22 proteins changed in shoot by drought and PEG treatment, respectively (Fig. 4b). Eight of these proteins (T3, T4, T8, T10, T23, T24, T25 and T27) were changed both in drought and PEG, four of these common proteins (T3, T4, T10 and T27) were increased while four (T8, T23, T24 and T25) were decreased. However, four protein spots (T14, T15, T22 and T28) were increased in drought while two (T16 and T17) were decreased. In PEG treatment, three (T1, T6 and T19) were increased while ten (T2, T5, T7, T9, T11, T12, T13, T20, T21 and T26) were decreased.

Common proteins between sensitive and tolerant genotype under drought and PEG

In drought, two protein pairs proton ATPase subunit E (S22/T22) and photosystem I reaction centre II (S31/T24) were found to be common between sensitive 004186 and tolerant 004223 genotype, former was increased and the later was decreased (Fig. 5a; Tables 1, 2). While S2, S4, S5, S10, S11, S12, S13, S14, S15, S16, S17, S19, S20, S22, S24, S26 and S29 were specific to the genotype 004186 and T3, T4, T8, T10, T14, T15, T16, T17, T25 and T26 were specific to the genotype 004223 (Fig. 5a). In PEG, two protein pairs Ptr ToxA-binding protein (S6/T3) and ATP synthase CF1 alpha subunit (S29/T26) were common between the two genotypes, first of them was increased and the second was decreased (Fig. 5b; Tables 1, 2). While 12 protein spots (S1, S2, S3, S7, S8, S9, S10, S19, S21, S22,

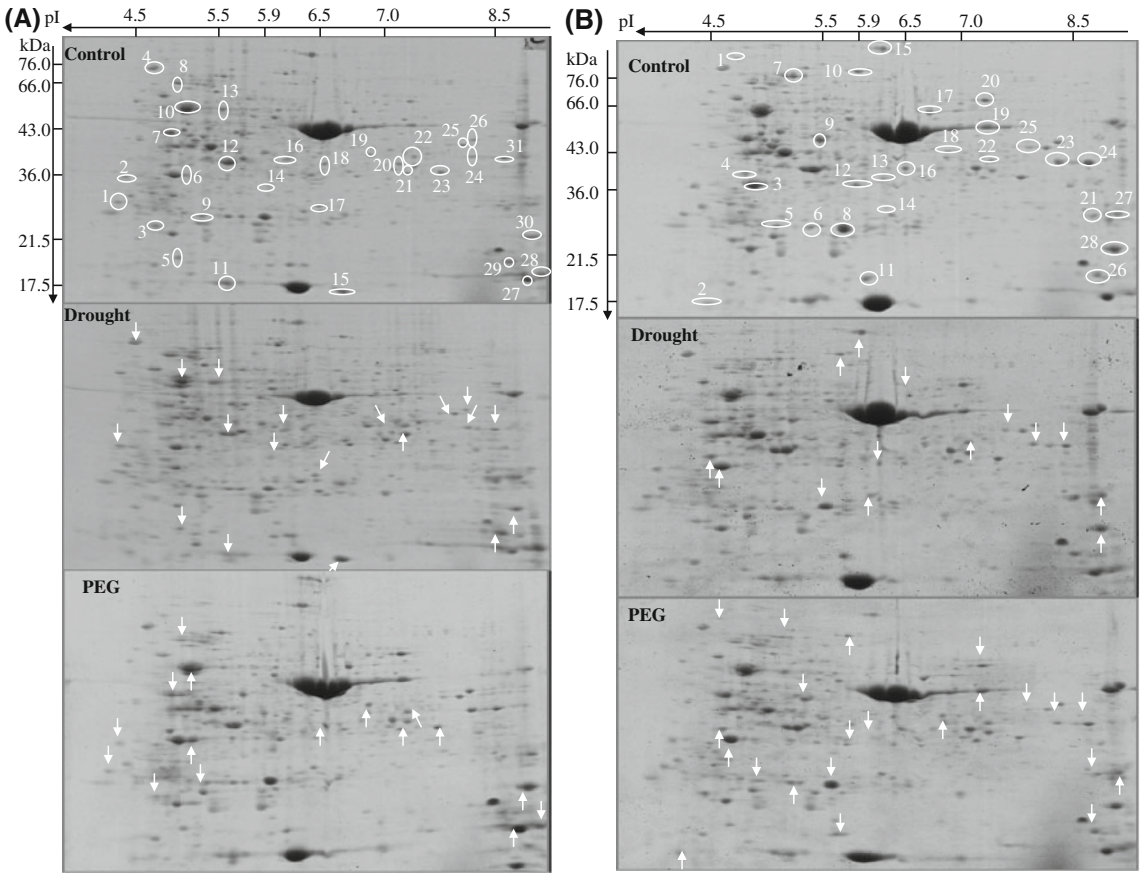

Fig. 2 Protein profile patterns in the shoots of sensitive and tolerant barley genotypes treated with drought and PEG. Seeds of sensitive barley genotype 004186 (**a**) and tolerant genotype 004223 (**b**) were sown and 3-day-old seedlings were given two treatments, one with drought and the second with 20 % PEG. After 3 days, proteins were extracted from the shoots, separated by 2-DE and visualized by CBB staining. *Arrows* indicate protein changes due to drought and PEG treatment, and *circles* mark the position of the same proteins from the control. *Upward arrows* indicate increase and *downward arrows* indicate decrease in protein intensity

S23 and S28) were specific to the genotype 004186. Similarly 16 proteins (T1, T2, T5, T6, T7, T8, T9, T10, T11, T12, T13, T19, T20, T21, T24 and T25) were specific to the genotype 004223 (Fig. 5b).

Functional categorization of identified proteins in sensitive and tolerant genotypes

In the sensitive genotype 004186, out of 31 differentially expressed proteins, 23 were classified while 8 were not identified in NCBI database. Out of 23—14, 7 and 2 were related to metabolism, energy and defense, respectively. Both under drought and PEG, six of the metabolism related proteins were increased and eight were decreased. One of the energy related proteins was increased and six were decreased. However, both of the defense related proteins were decreased (Fig. 6a; Table 1). In the tolerant genotype 004223, a total of 28 differentially expressed proteins were present. Out of them, 13, 4, 3 and 1 proteins were related to metabolism, energy, defense and transporters, respectively. Seven of the metabolism related proteins were increased and six were decreased. One of the energy related protein was increased and three were decreased both under drought and PEG. However, all of the defense and transporters related proteins were increased (Fig. 6b; Table 2).

Common proteins among 3 sensitive and 3 tolerant genotypes under drought

Differentially expressed proteins of sensitive genotype 004186 were further analyzed in genotypes Jau-83 and Sanober-96 under drought stress (Supplementary Fig. 2a). A total of nine proteins; malate dehydrogenase (S5), rubisco binding protein beta subunit (S10), rubisco activase (S12), photosystem I subunit PsaD (S13), 50S ribosomal protein L9 (S15), heat shock protein 70 (S26), ATP synthase CF1 alpha subunit (S29), malate dehydrogenase (S30) and photosystem I reaction center II (S31) were common among the 3 sensitive genotypes. While the rest were genotype specific (Fig. 7a; Table 1). Differentially

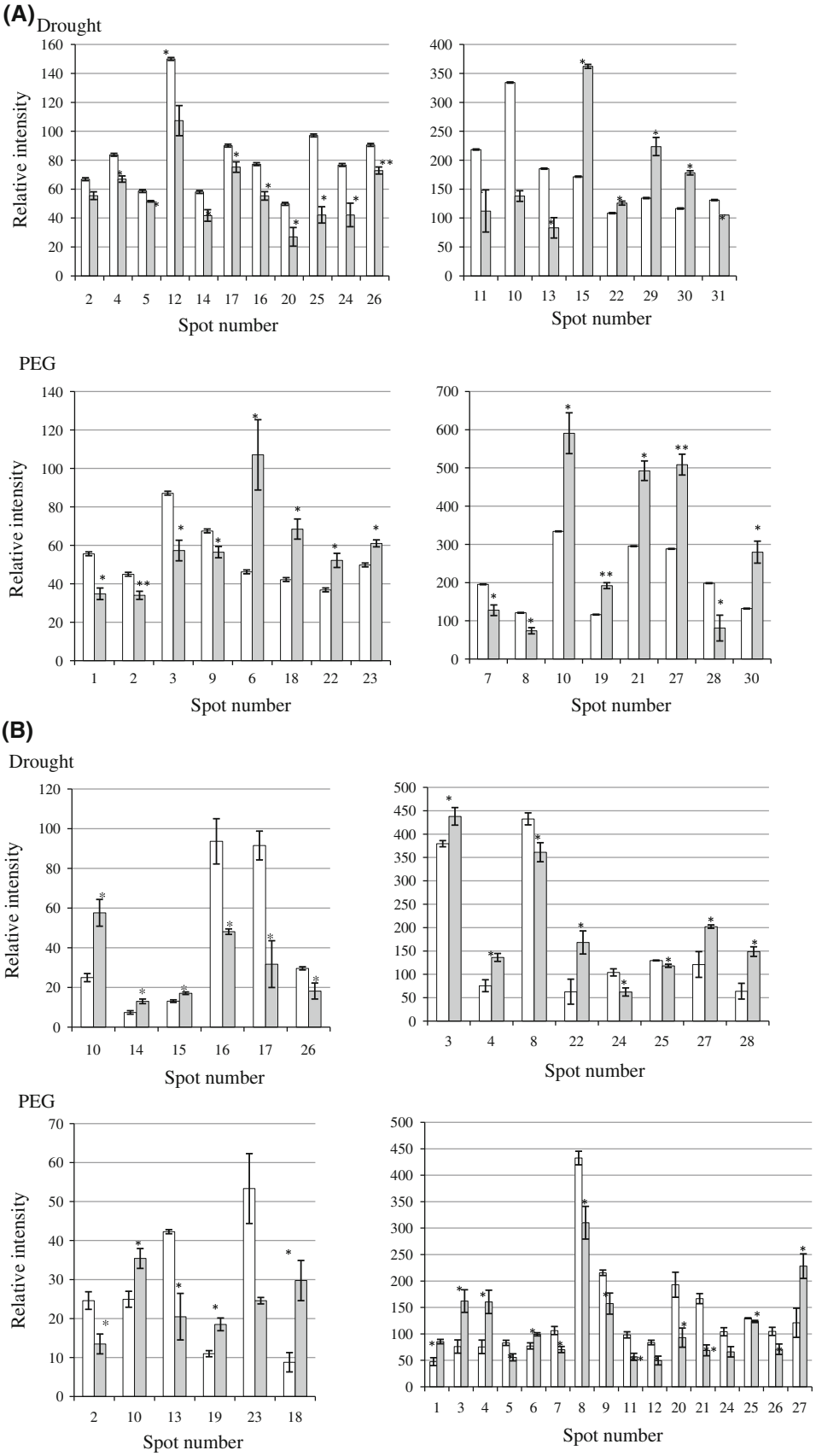

Fig. 3 Effects of drought and PEG treatments on differentially expressed proteins of sensitive and tolerant barley genotypes. Differentially expressed proteins of sensitive (**a**) and tolerant (**b**) barley genotypes without (*white columns*) and with drought and 20 % PEG (*black columns*) were analyzed. Values are the mean ± SE from three independent experiments and *asterisks* * and ** indicate significant differences at $p < 0.05$ and 0.01, respectively. Numbers correspond to the protein spots in the 2-DE patterns of the respective genotype

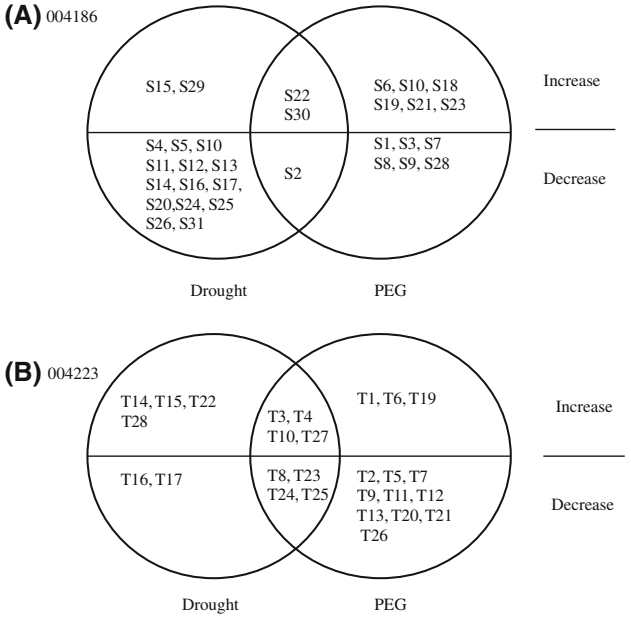

Fig. 4 Venn diagram with common proteins between drought and PEG treatments in the sensitive and tolerant barley genotypes. Effects of drought and PEG treatments on the sensitive 004186 (**a**) and tolerant 004223 (**b**) barley seedlings were analyzed. Numbers correspond to the protein spots in the 2-DE patterns of the respective genotype

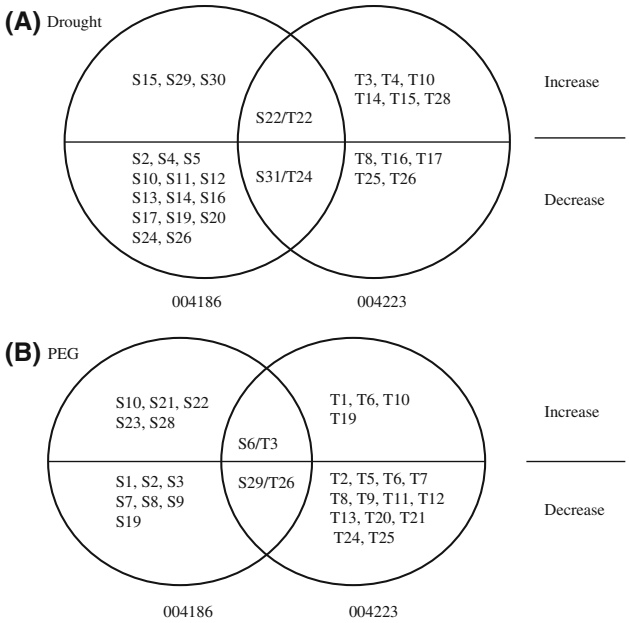

Fig. 5 Venn diagram with common proteins between sensitive and tolerant barley genotypes under drought and PEG treatments. The response of drought (**a**) and PEG (**b**) in the sensitive 004186 (*S*) and tolerant 004223 (*T*) barley seedlings were analyzed. Numbers correspond to the protein spots in the 2-DE patterns of the respective genotype

expressed proteins of tolerant genotype 004223 were further analyzed in two other tolerant genotypes 004360 and Frontier-87 under drought stress (Supplementary Fig. 2b). A total of 6 proteins alpha-SNAP (T4), methionine synthase (T10), glutathione transferase (T15), glycine decarboxylase P subunit (T16), ATP synthase CF1 alpha subunit (T17) and oxygen evolving complex precursor (T27) were commonly expressed among the 3 genotypes while the rest were genotype specific (Fig. 7b; Table 2).

Discussion

Barley growth and yield are adversely affected by drought stress in arid and semiarid regions (Volaire 2003). The total number of constitutively expressed genes in drought tolerant genotype is more than differentially expressed genes in both tolerant and sensitive genotypes under drought (Guo et al. 2009). Genotypic differences can be exploited for increased drought tolerance in barley (Forster et al. 2004). In the present study, genotypic differences at proteomics level are focused to find out the candidate proteins, which can be further analysed for improving drought tolerance in barley.

Proteomics studies of drought responses in crop plants are still in their infancy, recent significant progress illustrated the promise of proteomics projects for improving drought stress responses in crop plants (Roy et al. 2011). In maize leaves, a total of seventeen membrane protein spots were up-regulated and eighteen were down-regulated in response of 16 % PEG treatment (Yuan et al. 2011). Thirty six protein spots showed a reproducible significant change between control and stressed samples in wheat leaves (Caruso et al. 2009). A total of 75 salt and sorbitol responsive protein spots fell into ten functional categories in *Arabidopsis thaliana* using two-dimensional difference gel electrophoresis (Ndimba et al. 2005). The oxidation and S-nitrosylation patterns of leaf proteins were specific molecular signatures of citrus plant vigour under stressful conditions (Tanou et al. 2009).

Vacuolar proton ATPase subunit E increased both under drought and PEG in the present study. Plant vacuolar ATPases are large complexes composed of 7–10 different subunits. They play a central role in the growth and development of plant cells (Sze et al. 1992). The proton electrochemical gradient formed by the vacuolar ATPases provides the primary driving force for many cellular processes like osmoregulation, signal transduction and metabolic regulation (Sze et al. 1992). Under stress conditions, plant needs various osmoregulators, which accumulate inside the plant cell against the concentration gradient and the survival of the cell depends strongly on maintaining or adjusting the activity of the vacuolar-ATPase (Dietz et al. 2001). To maintain the normal cellular activity, the increased level of

Table 1 Differentially expressed proteins in sensitive barley genotype under PEG and drought treatments

Spot no.[a]	Homologous protein	Accession no.[b]	Score[c]	Cov. (%)[d]	MP[e]	Blast score[f]	Mr (kDa)/pI		FC[i]		*p* value	MS[j]	Classification	
							Theo.[g]	Exp.[h]	PEG	Drought			Func.[k]	Localization[l]
S1	ATPase beta subunit	CAA25114.1	204	63	20	966	43.8/4.11	35.0/4.3	0.62	–	0.011	MS/MS	Ene	Chlo
S2a	Triosephosphate isomerase,	P46225.1	182	45	14	522	31.9/5.00	37.0/4.4	0.75	0.68	0.007	MS/MS	Met	Chlo
S2b	Chlorophyll a–b binding protein 8	ACG28457.1	172	10	4	516	28.9/5.44	37.0/4.4				MS/MS	Met	Chlo
S3	Triosephosphate isomerase	P46225.1	225	45	14	522	31.9/5.00	28.0/4.7	0.65	–	0.011	MS/MS	Met	Cyto
S4	Chitinase	CAA55345.1	124	28	8	473	66.8/5.08	70.0/4.8	–	0.79	0.017	MS/MS	Def	Extr
S5	Malate dehydrogenase	AAS56659.1	516	31	8	645	21.5/5.5	20.0/4.9	–	0.87	0.003	MS/MS	Met	Cyto
S6	Ptr ToxA-binding protein 1	AAU82110.1	258	40	11	450	32.4/5.01	37.0/5.0	2.31	–	0.032	MS/MS	Met	Chlo
S7	ATP synthase subunit beta	NP_001056261.1	592	42	12	687	45.2/5.23	42.8/4.9	0.65	–	0.020	MS/MS	Ene	Mito
S8	Oxygen evolving enhancer protein 1	ABQ52657.1	534	50	16	646	64.7/5.08	66.0/4.9	0.61	–	0.035	MS/MS	Met	Chlo
S9	ATP synthase CF1alpha subunit	YP_538770.1	599	37	12	723	35.2/5.41	32.0/5.1	0.83	–	0.045	MS/MS	Ene	Chlo
S10	Rubisco binding-protein beta subunit	XP_002523404.1	1174	54	15	845	55.2/5.41	54.0/5.0	0.41	–	0.046	MS/MS	Met	Chlo
S11	Ribulose bisphosphate carboxylase small chain clone 512	P07398.1	91	69	9	243	13.2/5.84	17.0/5.4	–	0.51	0.045	MS	Ene	Chlo
S12	Rubisco activase	ADD60245.1	681	54	8	526	40.1/5.4	39.0/5.4	–	0.71	0.022	MS/MS	Met	Chlo
S13	Photosystem 1 subunit PsaD	ABQ63097.1	247	23	7	485	52.1/5.5	53.0/5.2	–	0.44	0.010	MS/MS	Ene	Chlo
S14	Oxygen evolving enhancer protein 1	ABQ52657.1	247	39	10	646	34.7/6.08	36.0/5.7	–	0.72	0.017	MS/MS	Met	Chlo
S15	50S ribosomal protein L9	P118994.2	212	32	10	512	19.2/6.1	16.0/6.4	–	2.11	0.048	MS/MS	Met	Chlo
S16	Glycine decarboxylase P subunit	AAB82711.1	534	45	27	1905	51.9/6.3	40.0/5.9	–	0.71	0.010	MS/MS	Met	Mito
S17	ND													
S18	ND													
S19	ND													
S20	ND													
S21	ND													
S22	Proton ATPase subunit E	ABB80135.1	622	73	18	444	36.3/6.57	40.0/6.8	1.41	1.23	0.021	MS/MS	Met	Cyto
S23	ND													
S24	ND													
S25	ND													
S26	Heat shock protein 70	ACJ11741.1	712	55	12	894	45.2/7.5	42.0/7.9	–	0.80	0.002	MS/MS	Def	Cyto

Table 1 continued

Spot no.[a]	Homologous protein	Accession no.[b]	Score[c]	Cov. (%)[d]	MP[e]	Blast score[f]	Mr (kDa)/pI		FC[i]		*p* value	MS[j]	Classification	
							Theo.[g]	Exp.[h]	PEG	Drought			Func.[k]	Localization[l]
S27	Ascorbate peroxidase 2	BAC92740	237	43	10	365	20.5/8.8	19.0/8.6	1.75	–	0.047	MS/MS	Met	Cyto
S28	Tubulin alpha-3 chain	Q9ZRR5.1	243	34	8	189	20.3/8.7	20.0/8.8	0.40	–	0.006	MS/MS	Met	Cyto
S29	ATP synthase CF1 alpha subunit	YP_874650.1	129	30	8	850	25.3/8.32	21.0/8.4	–	1.66	0.047	MS/MS	Ene	Cyto
S30	Malate dehydrogenase	AAS18241.1	401	22	7	345	25.0/8.5	30.0/8.6	1.67	1.49	0.023	MS/MS	Met	Cyto
S31	Photosystem I reaction center II	P36213.1	280	39	12	345	41.9/8.81	41.0/8.5	–	0.80	0.011	MS/MS	Ene	Chlo

p value, indicates the significance of up- or down-regulation of spots according to the *t* test through analysis of variance

[a] Spot number as given in Fig. 2a

[b] Accession number according to the NCBI database

[c] Ions score of identified protein using soybean genome sequence databases

[d] Sequence coverage, the proteins with less than 10 % sequence coverage were excluded from the result

[e] Number of query matched peptides

[f] The score of the high-scoring segment pair (HSP) from that database sequence

[g] Theoretical; Mr, molecular weight; pI, isoelectric point

[h] Experimental

[i] Fold change. The protein spots showed a significant change in abundance compared to the control analyzed by *t* test

[j] The type of mass spectrometry used in this study. MS means MALDI-TOF–MS and MS/MS means nano-LC MS/MS

[k] Category using functional classification: ProtDes, protein destination/storage; Met, metabolism; SecMet, secondary metabolism; DisDef, disease/defence; Transcr, transcription; ProtSyn, protein synthesis; SigTra, signal transduction; CellStr, cell structure; Ene, energy and Unclassi, un-classified

[l] Cyto, cytoplasm; Chlo, chloroplast; Mito, mitochondria

Table 2 Differentially expressed proteins in tolerant barley genotype under PEG and drought treatments

Spot no.[a]	Homologous protein	Accession no.[b]	Score[c]	Cov. (%)[d]	MP[e]	Blast score[f]	Mr (kDa)/pI		FC[i]		*p* value	MS[j]	Classification	
							Theo.[g]	Exp.[h]	PEG	Drought			Func.[k]	Localization[l]
T1	Heat shock cognate 70 kD protein	ACL 53191.1	168	24	17	1120	71.5/5.10	76.0/4.8	1.8	–	0.011	MS/MS	Def	Cyto
T2	Oxygen evolving enhancer protein 2	Q00434.1	717	43	10	436	27.4/4.84	22.0/4.0	0.54	–	0.031	MS/MS	Met	Chlo
T3	Ptr ToxA-binding protein 1	AAU82110.1	258	42	12	482	32.4/5.01	36.0/4.8	2.13	1.98	0.026	MS/MS	Met	Chlo
T4	Alpha-SNAP (*Hordeum vulgare* subsp. *vulgare*)	AAP79420.1	104	50	5	221	42.6/4.93	40.0/4.7	2.12	2.24	0.029	MS/MS	Trans	Mito
T5	Triosephosphate isomerase	P46225.1	172	34	29	522	31.9/6.0	30.0/5.0	0.67	–	0.028	MS/MS	Met	Chlo
T6	Heat shock protein 70	AAF23074	134	17	5	613	39.7/4.56	30.0/5.1	1.28	–	0.028	MS/MS	Def	Cyto
T7	Oxygen-evolving protein1	ABQ52657.1	106	26	6	646	74.7/5.08	70.0/5.2	0.66	–	0.020	MS/MS	Met	Chlo
T8	Oxygen-evolving enhancer protein 2	Q00434.1	90	55	9	436	27.4/5.84	30.0/5.5	0.71	0.57	0.022	MS	Met	Chlo
T9	ND													
T10	Methionine synthase (*Hordeum vulgare* subsp. *vulgare*)	BAD34660.1	99	29	17	1446	84.7/5.68	72.0/5.5	2.31	1.89	0.033	MS	Met	Cyto
T11	ND													
T12	Carbonic anhydrase,	P40880.1	112	50	17	621	35.7/5.93	38.0/5.7	1.59	–	0.022	MS/MS	Met	Chlo
T13	ND													
T14	Oxalate oxidase-like protein or germin-like protein	CAA63659.1	120	22	4	418	24.8/6.02	30.0/5.9	–	1.7	0.018	MS/MS	Def	Chlo
T15	Glutathione transferase	AAL73394.1	378	30	10	446	75.0/5.82	76.0/5.8	–	1.3	0.012	MS/MS	Met	Chlo
T16	Glycine decarboxylase P	AAB82711.1	87	21	17	1905	51.9/6.32	40.0/6.0	–	0.51	0.017	MS	Met	Mito
T17	ND													
T18	ND													
T19	ND													
T20	Os04g023800 (Oryza sativa Japonica Group)	NP_001053928.1	190	25	9	744	64.3/8.53	60.0/6.8	0.48	–	0.028	MS/MS	Met	Chlo
T21	ND													
T22	Proton ATPase subunit E	ABB80135.1	422	65	10	423	36.3/6.57	38.0/7.5	–	2.67	0.044	MS/MS	Met	Cyto
T23	ND													
T24	Photosystem I reaction centre subunit II	P36213.1	240	35	10	289	21.9/9.81		0.59	0.65	0.023	MS/MS	Ene	Chlo
T25	ND													
T26	ATP synthase CF1 alpha subunit	YP_874650.1	117	28	9	922	25.3/8.32	20.0/8.0	0.67	–	0.049	MS/MS	Ene	Cyto

Table 2 continued

Spot no.[a]	Homologous protein	Accession no.[b]	Score[c]	Cov. (%)[d]	MP[e]	Blast score[f]	Mr (kDa)/pI		FC[i]		*p* value	MS[j]	Classification	
							Theo.[g]	Exp.[h]	PEG	Drought			Func.[k]	Localization[l]
T27	Oxygen evolving complex precursor	ABO70330.1	130	46	10	275	31.1/9.72	35.0/8.8	1.67	1.59	0.041	MS/MS	Met	Chlo
T28	Photosystem I reaction center subunit II	P36213.1	270	43	13	345	21.9/9.81	22.0/9.0	2.32	–	0.013	MS/MS	Ene	Chlo

p value, indicates the significance of up- or down-regulation of spots according to the *t* test through analysis of variance

[a] Spot number as given in Fig. 2b

[b] Accession number according to the NCBI database

[c] Ions score of identified protein using soybean genome sequence databases

[d] Sequence coverage, the proteins with less than 10 % sequence coverage were excluded from the result

[e] Number of query matched peptides

[f] The score of the high-scoring segment pair from that database sequence

[g] Theoretical; Mr, molecular weight; pI, isoelectric point

[h] Experimental

[i] Fold change. The protein spots showed a significant change in abundance compared to the control analyzed by *t* test

[j] The type of mass spectrometry used in this study. MS means MALDI-TOF–MS and MS/MS means nano-LC MS/MS

[k] Category using functional classification: Met, metabolism; Def, defence; Trans, transporters; Ene, energy

[l] Cyto, cytoplasm; Chlo, chloroplast; Mito, mitochondria

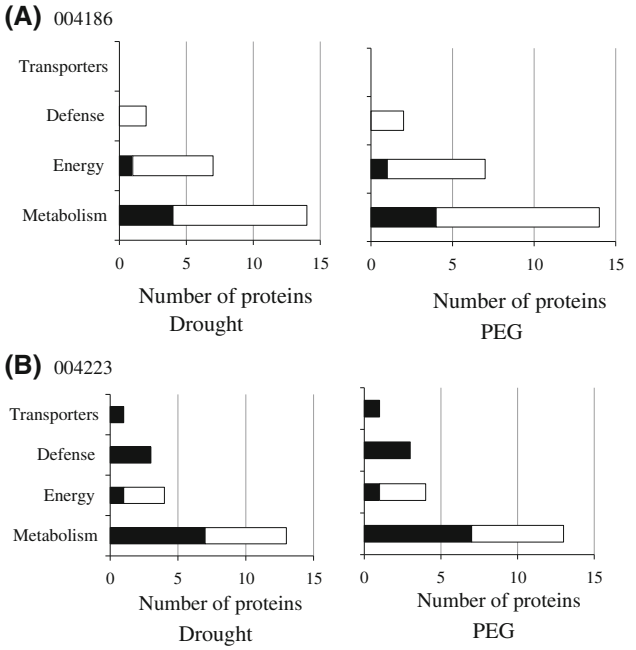

Fig. 6 Functional distribution of identified proteins expressed in sensitive and tolerant barley genotypes under drought and PEG treatments. After identification of increased (*black column*) and decreased (*white columns*) proteins under drought and PEG treatments in sensitive 004186 (**a**) and tolerant 004223 (**b**) genotypes, the proteins were classified into four functional groups, which are metabolism, energy, defense and transporters

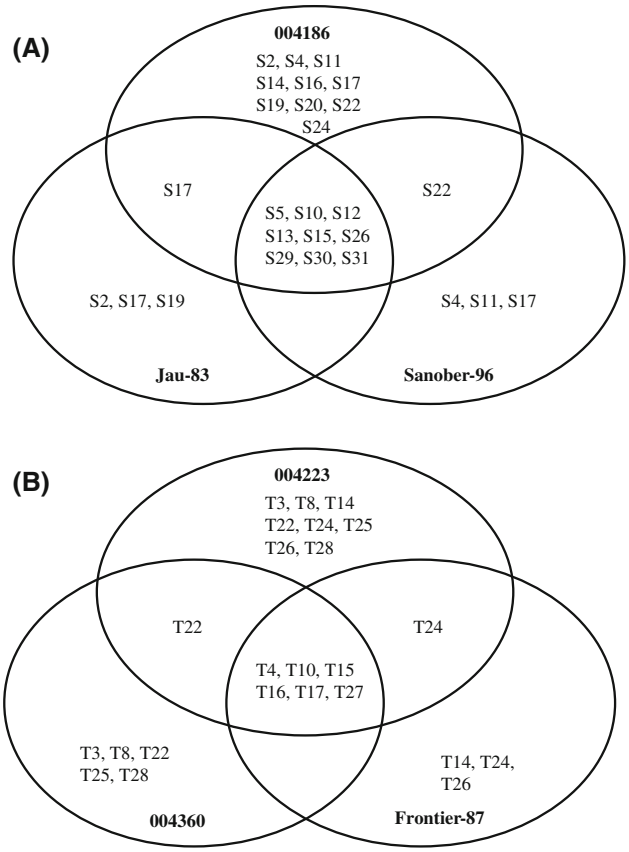

Fig. 7 Venn diagram depicting changed proteins that overlapped between 3 sensitive and 3 tolerant barley genotypes under drought. Effects of drought stress on 3 sensitive 004186, Jau-83 and Sanober-96 (**a**) and 3 tolerant 004223, 004360 and Frontier-87 (**b**) genotypes were analyzed. Numbers correspond to the protein spots of the 2-DE patterns of 004186 and 004223 genotypes

vacuolar proton ATPases is an important physiological response to cope under the stress environment.

Photosynthesis related proteins like rubisco binding protein, rubisco activase, photosystem I reaction center II decreased in sensitive genotype, however photosystem I reaction center II and oxygen evolving complex precursor were increased in the tolerant genotype. Drought stress is known to reduce photosynthetic rate and the extent of this decrease depends on osmotic adjustment and genotypic differences (Arnau et al. 1997). Drought induced the decrease in rubisco binding protein content at the leaf level in alfalfa (Aranjuelo et al. 2010). In maize leaves, the response to water deficit showed genetic variation. Some increased proteins were induced specifically in one of the two studied genotypes while others were significantly induced in both genotypes but to a different level or with different kinetics (Riccardi et al. 2004). The genotypic difference shown in the present study about the decrease in photosynthesis related proteins is similar to the previous report. The more drought tolerant genotype control stomatal function to allow carbon fixation at stress thus improving water use efficiency and photosynthetic capacity (Yordanov et al. 2000).

Malate dehydrogenase is an important enzyme of cellular metabolism and it catalyzes the conversion of oxaloacetate and malate (Musrati et al. 1998). Its activity increases under drought stress increases to cope with the high energy demand of the plant (Guicherd et al. 1997). The increased level of malate dehydrogenase in the tolerant barley genotype is also indicative of its role in drought stress acclimation in the present study. While in the sensitive genotype its level decreased as expected. Similarly, glutathione transferase also increased in the tolerant genotype to cope with the stress conditions. Glutathione transferase increased under water deficit conditions and it was also related to the suppression of ROS (Dhindsa 1991). The increased level of glutathione transferase along with malate dehydrogenase in the tolerant genotype may activate ROS scavenging cascade for better survival under drought stress.

Heat shock cognate 70 kDa protein increased in the tolerant genotype while it decreased in the sensitive genotype in the present study. The heat-shock proteins, which are chaperones, play a crucial role in protecting plants against stress by re-establishing normal protein

conformation and thus cellular homeostasis (Wang et al. 2004). They are responsible for protein folding, assembly, translocation and degradation in many normal cellular processes, stabilize proteins and membranes, and can assist in protein refolding under stress conditions. These proteins are often involved in assisting the folding of de novo synthesized polypeptides and the import/translocation of precursor proteins (Wang et al. 2004). The increased level of heat shock protein is indicative of its role in drought stress tolerance in barley in the present study.

The protein alpha SNAP increased twice in tolerant genotype in this study. This protein is involved in intracellular transport (Peter et al. 1998), which is crucial for the survival under stress conditions. Similarly, methionine synthase also increased twofold in the tolerant genotype. This enzyme catalyzes the formation of methionine by the transfer of a methyl group from 5-methyltetrahydrofolate to homocysteine. This reaction occurs in the activated methyl cycle, which is crucial under water stress to produce methylated polyols (Bohnert and Jensen 1996). These methylated polyols have been reported to have many functions, such as maintaining ion balance, chromatin protection and decreasing generation of ROS, which otherwise lead to cellular damage and ultimately to cell death during stress. In the present study, the increased level of methionine synthase indicates its role in water stress tolerance in tolerant genotype.

For improving drought tolerance in barley, genotypes are suitable target material because of the presence of difference at genomic level. This difference can be exploited at the proteomics level to find out the target proteins, which can be further utilised for such studies. Among the 3 sensitive and 3 tolerant genotypes, photosynthetic along with amino acid synthesis and degradation related proteins were differentially expressed. These results suggest that chloroplastic metabolism and energy related proteins might play a significant role in the adaptation process of barley seedlings under drought stress.

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Conflict of interest The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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