Amino Acids (2012) 43:2137–2152
DOI 10.1007/s00726-012-1299-6

ORIGINAL ARTICLE

Comparative proteome analysis of drought-sensitive and drought-tolerant rapeseed roots and their hybrid F1 line under drought stress

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Received: 13 December 2011 / Accepted: 12 April 2012 / Published online: 28 April 2012

Abstract Rapeseed (*Brassica napus* L.), which is the third leading source of vegetable oil, is sensitive to drought stress during the early vegetative growth stage. To investigate the initial response of rapeseed to drought stress, changes in the protein expression profiles of drought-sensitive (RGS-003) and drought-tolerant lines (SLM-003), and their F1 hybrid, were analyzed using a proteomics approach. Seven-day-old rapeseed seedlings were treated with drought stress by restricting water for 7 days, and proteins were extracted from roots and separated by two-dimensional polyacrylamide gel electrophoresis. In the sensitive rapeseed line, 35 protein spots were differentially expressed under drought stress, and proteins related to metabolism, energy, disease/defense, and transport were decreased. In the tolerant line, 32 protein spots were differentially expressed under drought stress, and proteins involved in metabolism, disease/defense, and transport were increased, while energy-related proteins were decreased. Six protein spots in F1 hybrid were common among expressed proteins in the drought-sensitive and -tolerant lines. Notably, tubulin beta-2 and heat shock protein 70 were decreased in the drought-sensitive line and hybrid F1 plants, while jasmonate-inducible protein and 20S proteasome subunit PAF1 were increased in the F1 hybrids and drought-tolerant line. These results indicate that (1) V-type H^+ ATPase, plasma-membrane associated cation-binding protein, HSP 90, and elongation factor EF-2 have a role in the drought tolerance of rapeseed; (2) The decreased levels of heat shock protein 70 and tubulin beta-2 in the drought-sensitive and hybrid F1 lines might explain the reduced growth of these lines in drought conditions.

Keywords Rapeseed · Proteomics · Drought · Sensitive line · Tolerant line

Abbreviations

2-DE	Two-dimensional polyacrylamide gel electrophoresis
CBB	Coomassie brilliant blue
MS	Mass spectrometry
LC	Liquid column
pI	Isoelectric point
IEF	Isoelectric focusing

Introduction

Plant growth is controlled by several factors, among which water plays a vital role. A small decrease in the availability of water immediately reduces the metabolic and physiological functions of a growing plant. Water deficit is one of the most significant stresses in agriculturally important crops, affecting growth, development, and yield. Plant responses to water stress are complex and include both morphological and biochemical changes. Under field conditions, these responses can be synergistically or antagonistically modified by the superimposition of other stresses. Plant strategies to cope with drought normally involve a

Electronic supplementary material The online version of this article (doi:10.1007/s00726-012-1299-6) contains supplementary material, which is available to authorized users.

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mixture of stress avoidance and tolerance responses that vary with genotype (Chaves et al. 2002). The most severe effect of drought was impaired germination and poor seedling stand establishment (Harris et al. 2002; Kaya et al. 2006). In pea, drought stress impaired germination and early seedling growth (Okcu et al. 2005), while in alfalfa (*Medicago sativa*), germination potential, hypocotyl length, and shoot and root fresh and dry weights were reduced by polyethylene glycol-induced water deficit, although root length increased (Zeid and Shedeed 2006). Similar effects were observed in rice, as drought stress during the vegetative stage greatly reduced plant growth and development (Tripathy et al. 2000).

Rapeseed (*Brassica napus* L.) is an important agricultural crop grown primarily for its edible oil and the meal remaining after oil extraction, which is used as a protein source for the livestock feed industry (Jensen et al. 1996). Rapeseed yields are adversely affected by numerous environmental factors, among which drought, flooding, low temperature, and salinity are considered agronomically most significant. Rapeseed was particularly sensitive to drought during the early vegetative growth stage, as many processes, including photosynthesis, stomatal conductance, transpiration, chlorophyll fluorescence, protein synthesis, and metabolite accumulation, were negatively influenced under such conditions (Jensen et al. 1996; Ohashi et al. 2006; Sangtarash et al. 2009; ShiraniRad et al. 2010; Hosseini and Hassibi 2011).

Proteomics approaches for the comparative analysis of protein abundance between untreated and stress-treated or tolerant and intolerant crops have greatly facilitated the study of plant cellular stress responses (Komatsu et al. 2003). Analysis of the proteome complement is required for a thorough understanding of the cellular processes that are associated with drought. Protein expression changes in response to drought have been studied in maize (Riccardi et al. 1998), rice (Salekdeh et al. 2002; Ali and Komatsu 2006), sugar beet (Hajheidari et al. 2005), wheat (Hajheidari et al. 2007; Peng et al. 2009), and sunflower (Castillejo et al. 2008). These analyses have identified a number of drought stress-induced proteins involved in photosynthesis (Ali and Komatsu 2006), signaling pathways (Salekdeh et al. 2002; Ali and Komatsu 2006; Zang and Komatsu 2007), oxidative stress detoxification (Salekdeh et al. 2002; Ali and Komatsu 2006; Zang and Komatsu 2007), and transport (Nouri and Komatsu 2010). However, the specific proteins induced in rapeseed under the conditions of drought stress are not known.

A proteomics approach was applied to the study of salt stress responses in rapeseed using salt-sensitive (Sarigol) and salt-tolerant (Hyola-308) lines (Bandehagh et al. 2011). Among the 75 proteins differentially expressed as a result of salt stress, most were involved in photosynthesis, energy production, and oxidative stress responses. Salt and drought stresses are types of osmotic stress, but when combined, plants endure salt-specific effects (Chaves et al. 2009).

In the present study, physiological and proteomics analyses were performed using two rapeseed genotypes, a drought-tolerant (SLM-046) and drought-sensitive line (RGS-003), to determine the proteins differentially expressed in response to drought. To confirm that the identified proteins were drought specific, F1 hybrid plants derived from crosses between drought-sensitive and tolerant lines were also examined. Rapeseed proteins induced under drought stress were separated by two-dimensional polyacrylamide gel electrophoresis (2-DE), and major differentially expressed proteins were identified by mass spectrometry (MS).

Materials and methods

Plant growth condition

Seeds of rapeseed lines, which are drought sensitive line (RGS-003), drought tolerant line (SLM-046) and their hybrid F1 line, were sterilized by sodium hypochlorite solution germinated on silica sand for 7 days under white fluorescent light (600 μmol m^{-2} s^{-1}, 12 h light period/day) at 25 °C and 70 % relative humidity in a growth chamber. Rapeseed seeds germinated on sand for 7 days were treated with drought by restricting water for 0, 1, 2, 3, 4, 5, 6 and 7 days. After treatment, physiological parameters which are root number, fresh weights and length of root, hypocotyl, and leaf were measured. Three independent biological experiments were performed with a multi time course analysis.

Protein extraction

A portion (500 mg) of root was ground to powder in liquid nitrogen with a mortar and pestle. The powder was transferred to 10 % trichloroacetic 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 the 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, consist of 8 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. Protein concentration was determined using the Bradford method (Bradford 1976) with bovine serum albumin as the standard.

Two-dimensional polyacrylamide gel electrophoresis

For 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 gels with 5 % stacking gels 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; Bio-Rad). The isoelectric point (pI) and molecular mass of each protein were determined using 2-DE standard marker (Bio-Rad). The amount of protein in a spot was estimated using the PDQuest software with local regression model normalization.

Peptide preparation for mass spectrometry analysis

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. 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. The procedure described above was performed with DigestPro (Intavis Bioanalytical Instruments AG, Cologne, Germany). The resulting peptide solutions were desalted with C-Tip pipet tips (Nikkyo Technos, Tokyo, Japan) and eluted in 0.1 % formic acid. Desalted peptide solution was analyzed by nano-liquid chromatography (LC)-MS/MS.

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 nanoLC (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 (NTTC-360/75-3, Nikkyo Technos) with a spray voltage of 1.8 kV. Fullscan 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 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 BioWorks 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 used to search the database using the MASCOT search engine (version 2.2.04, Matrix Science, London, UK). NCBInr protein database was selected for database and carbamidomethylation of cysteines was set as a fixed modification and oxidation of methionine was set as a variable modification. Trypsin was specified as the proteolytic enzyme and one missed cleavage was allowed. The search parameters were peptide mass tolerance (10 ppm), fragment mass tolerance (0.2 Da), maximum missed cleavages 1, peptide and charges +1, +2, and +3. The Mowse score of more than 100 peptides from the MS data were significant with $P < 0.05$. The first positive matches were BLASTP searched against the NCBI protein database for updated annotation and identification of homologous proteins.

Classification of expressed proteins

Identified proteins were assigned using the classification scheme described by Bevan et al. (1998) and proteins were classified into 8 functional group. Identified proteins were also classified according to the subcellular localization predicted by WOLFPSORT prediction (http://wolfpsort.org) and proteins were classified into six locational groups.

Results

Effects of drought stress on roots of sensitive, tolerant, and hybrid F1 rapeseed lines

To evaluate the effects of drought stress, 7-day-old seedlings of sensitive (RGS-003), tolerant (SLM-046), and hybrid F1 rapeseed lines received drought stress treatment for 7 days. Morphological changes, including leaf, hypocotyl, root, and plant weights, hypocotyl and root lengths, and root number, were measured daily during the 7 days of treatment (Fig. 1, Supplementary Fig. 1). After 7 days of treatment, the root lengths of both the sensitive and F1 hybrid lines were significantly decreased compared to control plants, while the root weight of the sensitive line was also markedly reduced. However, in the drought-tolerant line, no changes in root weight or length were detected between the two experimental conditions (Fig. 1). In addition, no marked changes in leaf weight, hypocotyl weight and length, and root number were observed for three rapeseed lines (Supplementary Fig. 1). As the effects of drought stress on the roots of the drought-sensitive line were first observed after 7 days of treatment, roots treated with drought for 7 days were used for subsequent proteomics analyses.

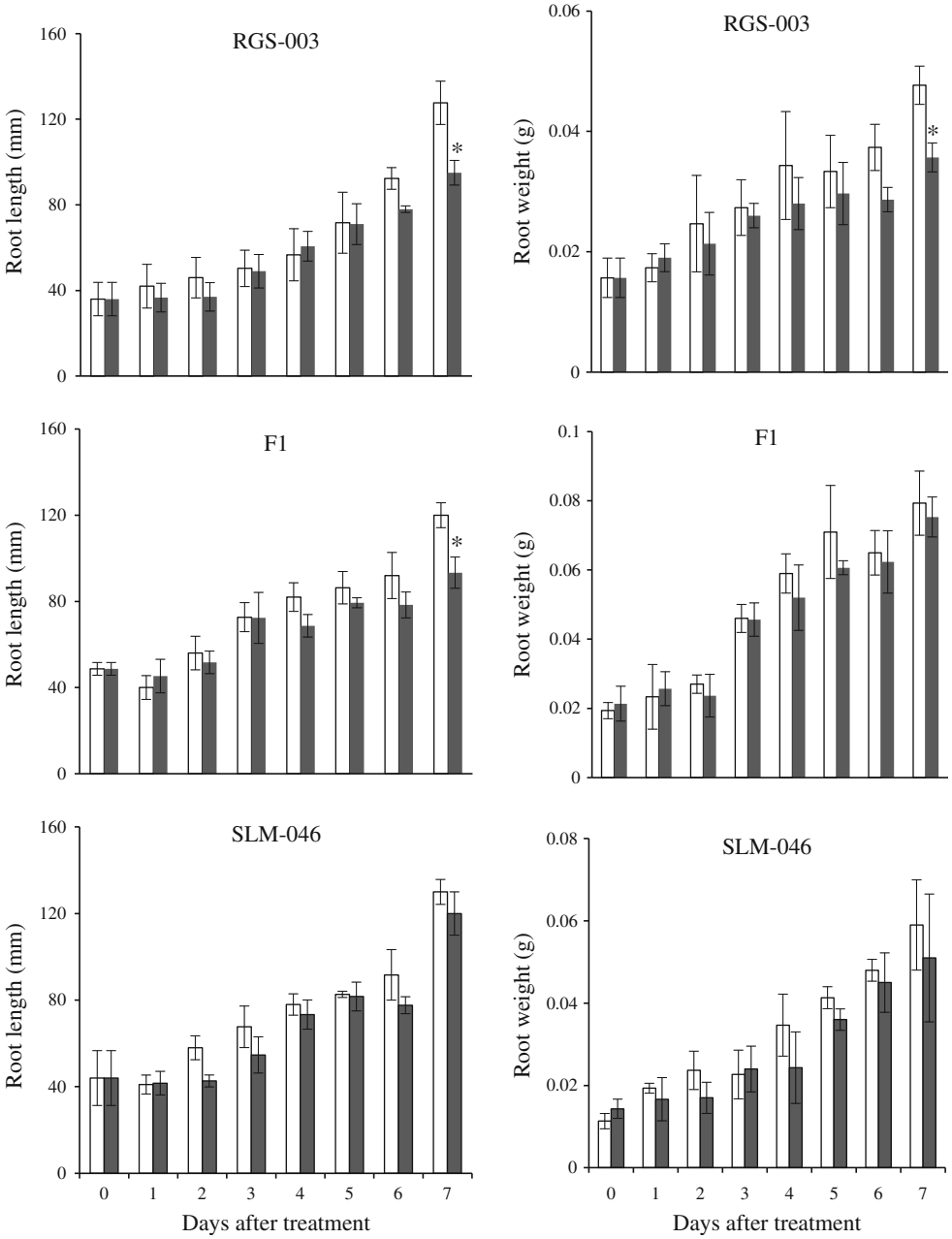

Fig. 1 Effect of drought stress on the roots of sensitive and tolerant rapeseed lines, and their F1 hybrids. Seven-day-old sensitive (RGS-003), tolerant (SLM-003), and F1 hybrid rapeseed seedlings were treated without (*white*) or with (*black*) drought stress for 1, 2, 3, 4, 5, 6, and 7 days. Root lengths (*left*) and weights (*right*) of the rapeseed seedlings were measured daily during the treatment period. Three rapeseed plants were used for each experiment and three physiologically independent experiments were performed. Values are expressed as the mean ± SE and were compared using the Student *t* test (* $P < 0.05$)

Differential expression pattern of proteins in sensitive and tolerant rapeseed lines under drought stress

A proteomics approach was used to identify proteins induced under drought stress in the sensitive and tolerant rapeseed lines at early growth stages. Seven-day-old rapeseed seedlings were treated with drought stress for 7 days, and proteins were then extracted from roots, separated by 2-DE, and stained with CBB to evaluate and compare their expression levels (Fig. 2). The relative intensities of all spots from three independent biological replicates were analyzed using PDQuest software. After separation of the extracted proteins on 2-DE gels, 687 and 681 protein spots were reproducibly detected for the sensitive and tolerant lines, respectively (Fig. 2).

Among the 687 protein spots comprising the 2-DE pattern of the sensitive line, 8 protein spots were increased by a factor of >2 and 27 protein spots were decreased in response to drought stress (Fig. 2a). Of these 35 differentially expressed protein spots, a total of 29 proteins were identified (Table 1). In the drought-tolerant line, 681 proteins were separated on the 2-DE gel, among which 21 protein spots were increased and 11 protein spots were decreased in response to drought stress (Fig. 2b). All 32

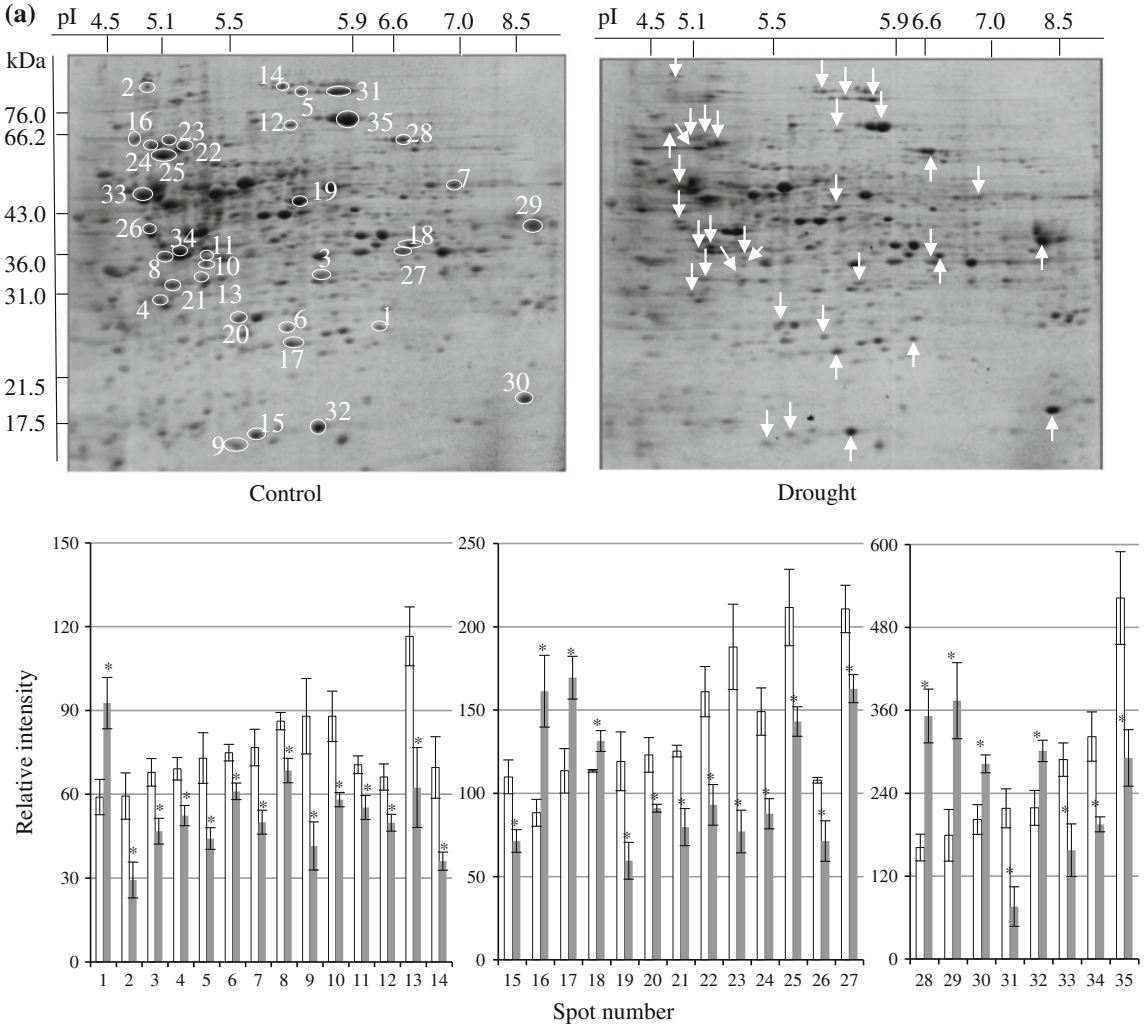

Fig. 2 Protein-expression patterns of sensitive and tolerant rapeseed lines under drought stress. Seven-day-old sensitive (**a**) and tolerant (**b**) rapeseed seedlings were exposed to drought stress or normal conditions (control) for 7 days. Following treatment, proteins were extracted from roots, separated by 2-DE, and visualized by CBB staining (*above*). The pI and Mr of each protein were determined using 2-DE markers. *Arrows* indicate the position of proteins differentially expressed in response to drought stress, and *circles* mark the position of the same proteins in the control. *Upward-* and *downward-facing arrows* indicate increased and decreased expression, respectively. Relative intensities of proteins were determined using PDQuest software. Each *value* represents the mean ± SE of relative intensity determined from gels of three biological replicates. The mean values were compared using the Student *t* test (*$P < 0.05$ or **$P < 0.01$). SE is denoted by *error bars*. *White* and *gray* columns are the control and drought stress conditions, respectively

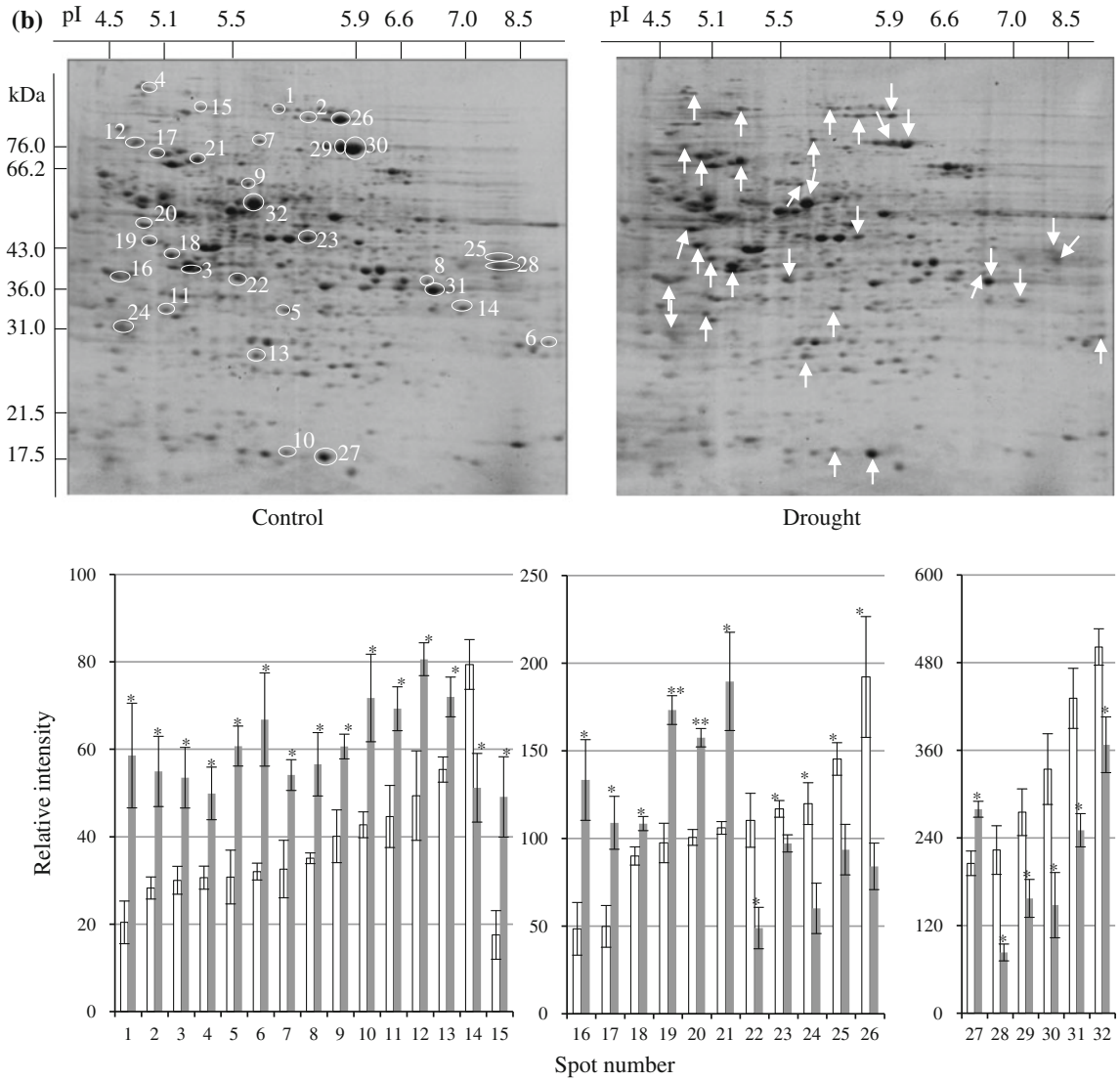

Fig. 2 continued

proteins corresponding to these 32 differentially expressed protein spots were identified (Table 2).

Functional categories and cellular localization of drought-responsive proteins

In the drought-sensitive and -tolerant lines, 35 and 32 protein spots, respectively, were differentially expressed under conditions of drought stress. The identified proteins were classified based on cellular function and subcellular location (Fig. 3). In the drought-sensitive line, proteins related to metabolism, energy, disease/defense, and transport were decreased under drought stress (Fig. 3). Approximately 70 % of the differentially expressed proteins were localized in the cytoplasm. In contrast, proteins involved in metabolism, disease/defense, and transport were increased in the drought-tolerant line under conditions of drought stress, whereas energy-related proteins were decreased. Similar to the drought-sensitive line, the majority of differentially expressed proteins (62 %) were predicted to be localized in the cytoplasm.

Differential expression pattern of proteins in the F1 hybrid rapeseed line under drought stress

To confirm the classification of the differentially expressed proteins in the sensitive and tolerant rapeseed lines under drought stress as drought responsive, a similar proteomics approach was used for the F1 hybrids generated from crosses of the two rapeseed lines. Seven-day-old F1 hybrid

Table 1 Root proteins changed in drought-sensitive rapeseed line under drought stress

Spot no[a]	Homologous protein	Accession no[b]	Score[c]	Cov. (%)[d]	M.P.[e]	Blast score[f]	Mr (kDa)/pI		F.C.[i]	*p* value[j]	Func.[k]	Local.[l]
							Theo.[g]	Exp.[h]				
1	Cobalamin-independent methionine synthase	XP_002871787.1	294	27	17	1455	86.6/6.12	27/6.3	1.57	0.039	Met	Cyto
2	Patellin-1	NP_177360	114	9	5	795	64.1/4.82	82/4.8	0.49	0.045	Tra	Cyto
3	Aconitase 2	NP_567763.2	112	7	7	1873	100.8/6.85	34/5.8	0.69	0.035	Met	Mito
4	40S ribosomal protein S3-1	NP_180719.1	164	15	3	489	27.7/9.57	30.5/5.1	0.76	0.036	ProtSyn	Cyto
5	Elongation factor EF-2	AAF02837.1	100	6	4	1666	95.1/5.89	95/5.8	0.61	0.043	ProtDes	Cyto
6	Glutathione S-transferase	ABM53759.1	226	40	7	385	24.3/5.66	25/5.7	0.82	0.030	Met	Mito
7	Heat shock protein 70	ACJ11742.1	263	22	14	1120	71.6/5.04	60/6.8	0.65	0.027	Dis/Def	Cyto
8	ATP synthase subunit beta, mitochondrial	P17614.1	100	2	1	967	59.9/5.95	36/5.2	0.79	0.030	Ene	Mito
9	ND	–	–	–	–	–	–	16/5.5	0.47	0.044	–	–
10	Phosphoglycerate kinase	NP_178073.1	119	8	2	775	42.2/5.49	36/5.4	0.66	0.034	Ene	Cyto
11a	Nitrilase	BAG72073.1	825	33	14	682	38.9/5.48	38/5.4	0.78	0.045	Met	CySk
11b	Phosphoglycerate kinase	AAM61185.1	182	21	6	775	42.2/5.49	38/5.4	0.78	0.045	Ene	Cyto
12	Cytoplasmic aconitate hydratase	AAD25640.1	203	14	10	1786	98.7/5.79	75/5.6	0.75	0.043	Met	Cyto
13	ND	–	–	–	–	–	–	33/5.3	0.54	0.038	–	–
14	ND	–	–	–	–	–	–	81/5.7	0.52	0.043	–	–
15	Triosephosphate isomerase	NP_191104.1	113	10	2	459	27.4/5.39	17/5.6	0.65	0.035	Met	Cyto
16a	Cobalamin-independent methionine synthase	XP_002871787.1	161	20	12	1455	84.6/6.12	68/4.8	1.83	0.034	Met	Cyto
16b	Heat shock protein 70	CAA52149.1	157	12	7	1093	74.5/5.14	68/4.8	1.83	0.034	Dis/Def	Cyto
17	ND	–	–	–	–	–	–	26/5.7	1.49	0.039	–	–
18	Beta-glucosidase	AAB38784	255	22	8	925	51.0/6.27	38/6.7	1.16	0.047	Met	Cyto
19	ND	–	–	–	–	–	–	46/5.7	0.50	0.046	–	–
20	ND	–	–	–	–	–	–	28/5.6	0.74	0.040	–	–
21	ND	–	–	–	–	–	–	33/5.2	0.64	0.017	–	–
22	V-type H^+ ATPase catalytic subunit A	NP_178011.1	115	6	3	1220	69.1/5.11	66/5.2	0.58	0.025	Tra	Cyto
23	ER-binding protein	AAW55475.1	101	10	6	1168	73.8/5.14	67/5.2	0.41	0.018	Dis/Def	ER
24	HSP90-2	ADC45396.1	186	15	8	1144	80.2/5.01	66/4.9	0.59	0.022	Dis/Def	Cyto
25	Heat shock cognate protein HSC70	AAB88009.1	115	10	5	1132	71.6/5.04	65/5.1	0.68	0.050	Dis/Def	Cyto
26	Plasma-membrane associated cation- binding protein 1	NP_193759.1	118	10	2	233	24.7/4.91	39/5.0	0.66	0.041	Tra	Nucl
27	Glyceraldehyde-3-phosphate dehydrogenase	P04796.2	439	50	13	638	37.0/7.70	37/6.7	0.77	0.044	Ene	Cyto
28	TCP-1/cpn60 chaperonin family protein	NP_188447.1	112	9	5	1022	58.2/7.57	66/6.7	2.18	0.012	ProtDes	Cyto
29	Peroxidase	AAM61240.1	108	14	4	647	35.4/8.74	39/8.7	2.09	0.043	Dis/Def	Cyto
30	ND	–	–	–	–	–	–	20/8.6	1.40	0.033	–	–
31	Beta-substituted ala synthase 3_1	XP_002883653.1	168	18	5	648	37.3/5.86	80/5.8	0.35	0.024	Met	Cyto

Table 1 continued

Spot no[a]	Homologous protein	Accession no[b]	Score[c]	Cov. (%)[d]	M.P.[e]	Blast score[f]	Mr (kDa)/pI		F.C.[i]	*p* value[j]	Func.[k]	Local.[l]
							Theo.[g]	Exp.[h]				
32	Rubber elongation factor protein	P15252	119	22	2	270	14.7/5.04	17.5/5.6	1.38	0.050	Met	Mito
33	Tubulin beta-2	XP_002877519.1	367	49	20	853	51.4/4.70	51/4.7	0.55	0.044	CellStr	Nucl
34	Glutamine synthetase	BAD11327.1	163	10	3	661	39.4/5.52	38/5.2	0.61	0.027	Met	Cyto
35	Glyceraldehydes-3-phosphate dehydrogenase C subunit	XP_00288233.1	142	44	14	605	37.0/6.62	76/5.9	0.56	0.042	Ene	Cyto

[a] Spot no, spot number as given in Fig. 2a

[b] Accession no, accession number according to the NCBI database

[c] Score, ions score of identified protein using soybean genome sequence database

[d] Cov., sequence coverage

[e] M.P., number of query matched peptides

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

[g] Theo., theoretical; *Mr*, molecular weight; pI, isoelectric point

[h] Exp., experimental; *Mr*, molecular weight; pI, isoelectric point

[i] F.C., fold change. The protein spots showed a significant change in abundance compared to the control analyzed by Student t-test

[j] *P* value indicates the significance of decrease or increase of abundance spots according to the Student *t*-test

[k] Func., function category using functional classification: ProtSyn, protein synthesis; ProtDes, protein destination/storage; Met, metabolism; Dis/Def, disease/defense; CellStr, cell structure; Ene, energy; Tra, transporter

[l] Local., localization category using local classification, Chlo, chloroplast; Mito, mitochondria; Nucl, nuclear; Cyto, cytoplasm; Cysk, cytoplasm skeleton; ER, endoplasmic reticulum; Extr, extracellular matrix

Table 2 Root proteins changed in drought-tolerant rapeseed line under drought stress

Spot no[a]	Homologous protein	Accession no[b]	Score[c]	Cov. (%)[d]	M.P.[e]	Blast score[f]	Mr (kDa)/pI		F.C.[i]	*p* value[j]	Func.[k]	Local[l]
							Theo.[g]	Exp.[h]				
1	Jasmonate inducible protein	CAA722271.1	102	4	2	858	69.9/5.54	90/5.7	2.87	0.042	Met	Cyto
2	Elongation factor EF-2	AAF02837.1	100	6	4	1666	95.1/5.89	89/5.7	1.94	0.034	ProtDes	Cyto
3	ND	–	–	–	–	–	–	38/5.3	1.78	0.037	–	–
4	ND	–	–	–	–	–	–	100/4.9	1.63	0.043	–	–
5	Urease accessory protein UREG	AAD16984.1	110	17	4		30.2/6.00	33/5.7	1.97	0.017	Tra	CySk
6	Methionine synthase	NP_001235794.1	210	18	13	1491	84.4/5.93	29/8.7	2.09	0.033	Met	Mito
7	Jasmonate inducible protein	CAA72271.1	226	22	10	858	69.9/5.54	78/5.7	1.66	0.045	ProtDes	Cyto
8	Glyceraldehyde-3-phosphate dehydrogenase 2	ACS68203.1	168	51	13	629	38.0/7.70	38/6.8	1.61	0.043	Ene	Cyto
9	ND	–	–	–	–	–	–	60/5.6	1.51	0.037	–	–
10	60S ribosomal protein L23	NP_180895.1	103	28	3	276	17.2/10.1	17.6/ 5.7	1.68	0.050	ProtSyn	Cyto
11	20S proteasome subunit PAF1	AAM61557	123	38	8	484	30.5/4.97	34/5.1	1.55	0.047	ProtSyn	Cyto
12a	Cobalamin-independent methionine synthase	XP_002871787.1	161	20	12	1455	84.6/6.12	79/4.8	1.63	0.046	Met	Cyto
12b	Heat shock protein 70	CAA52149.1	157	12	7	1093	74.5/5.14	79/4.8	1.63	0.046	Dis/Def	Cyto
13a	Dehydroascorbate reductase	AAQ01573	260	59	7	196	12.0/6.15	28/5.6	1.30	0.037	Dis/Def	Cyto
13b	Proteasome subunit alpha type-2-A	NP_173096.1	227	42	9	459	25.7/5.53	28/5.6	1.30	0.037	Met	Cyto
14a	Guanine nucleotide-binding protein subunit beta like protein	Q39336.1	194	46	15	664	36.2/8.05	34/7.0	0.64	0.044	CellStr	Nucl
14b	Mitochondrial malate dehydrogenase	XP_002894430.1	145	17	4	630	36.0/8.54	34/7.0	0.64	0.044	Met	Mito
15	Cell division control protein 48-A	NP_187595.1	124	16	13	1479	90.1/5.13	90/5.3	2.80	0.042	CellGr/ Di	Cyto
16	Senescence-associated cysteine protease	AAL60578.1	133	10	5	862	49.6/5.49	38/4.7	2.75	0.037	Met	Extr
17	Heat shock protein 90-2	ADC45396.1	186	15	8	1144	80.2/5.01	73/5.0	2.19	0.037	Dis/Def	Cyto
18	ND	–	–	–	–	–	–	41/5.2	1.21	0.048	–	–
19	Plasma-membrane associated cation- binding protein 1	NP_193759.1	118	10	2	233	24.7/4.91	43/4.9	1.78	0.006	Tra	Nucl
20	Plasma-membrane associated cation-binding protein 1	NP_193759.1	125	5	1	234	24.6/4.99	46/4.8	1.57	0.001	Tra	Nucl
21	V-type H^+ ATPase catalytic subunit A	NP_178011.1	115	6	3	1220	69.1/5.11	70/5.3	1.79	0.042	Tra	Cyto
22	Fructokinase	AAK62446.1	116	7	2	644	35.4/5.30	38/5.6	0.44	0.034	Ene	Extr
23	S-adenosylmethionine synthase	Q5DNB1.1	268	49	16	773	43.6/5.67	45/5.7	0.83	0.045	Met	CySk
24	Heat shock cognate protein HSC70	AAB88009.1	115	10	5	1132	71.6/5.04	31/4.6	0.50	0.033	Dis/Def	Cyto
25	GDSL esterase/lipase	NP_564647.1	124	22	6	714	43.2/6.99	41/7.5	0.64	0.039	Met	Extr
26	Beta-substituted Ala synthase 3_1	XP_002883653.1	168	18	5	648	37.3/5.86	85/5.8	0.44	0.043	Met	Cyto
27a	60S ribosomal protein L17	AAM67199.1	160	46	6	275	17.2/ 10.12	17.5/ 5.7	1.36	0.021	ProtSyn	Cyto
27b	MLP-like protein 328	NP_565264.1	126	19	4	322	17.6/5.42	17.5/ 5.7	1.36	0.021	Dis/Def	CySk

Table 2 continued

Spot no[a]	Homologous protein	Accession no[b]	Score[c]	Cov. (%)[d]	M.P.[e]	Blast score[f]	Mr (kDa)/pI		F.C.[i]	*p* value[j]	Func.[k]	Local[l]
							Theo.[g]	Exp.[h]				
27c	Ubiquitin-conjugating enzyme E2 36	NP_564011.1	118	38	3	310	17.3/6.74	17.5/5.7	1.36	0.021	Met	Mito
28	Peroxidase 32	ABO93458.1	140	6	5	694	39.4/6.30	40/7.7	0.37	0.016	Dis/Def	Extr
29	Rubber elongation factor protein	P15252.2	119	22	2	270	14.7/5.04	76/5.8	0.57	0.046	Met	Cyto
30	Glyceraldehydes-3-phosphate dehydrogenase C subunit	XP_00288233.1	142	44	14	605	37.0/6.62	76/5.9	0.44	0.047	Ene	Cyto
31	Glyceraldehydes-3-phosphate dehydrogenase	P04796.2	264	70	20	638	37.0/7.7	36/6.9	0.58	0.018	Ene	Cyto
32	ND	–	–	–	–	–	–	55/5.6	0.73	0.042	–	–

[a] Spot no, spot number as given in Fig. 2b

[b] Accession no, accession number according to the NCBI database

[c] Score, ions score of identified protein using soybean genome sequence database

[d] Cov., sequence coverage

[e] M.P., number of query matched peptides

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

[g] Theo., theoretical; *Mr*, molecular weight; pI, isoelectric point

[h] Exp., experimental; *Mr*, molecular weight; pI, isoelectric point

[i] F.C., fold change. The protein spots showed a significant change in abundance compared to the control analyzed by Student *t* test

[j] *P* value indicates the significance of decrease or increase of abundance spots according to the Student *t* test

[k] Func., function category using functional classification: ProtDes, protein destination/storage; Met, metabolism; Dis/Def, disease/defense; CellStr, cell structure; Ene, energy; Tra, transporter; CellGr/Di, Cell growth/division

[l] Local., localization category using local classification, Chlo, chloroplast; Mito, mitochondria; Nucl, nuclear; Cyto, cytoplasm; Cysk, cytoplasm skeleton; ER, endoplasmic reticulum; Extr, extracellular matrix

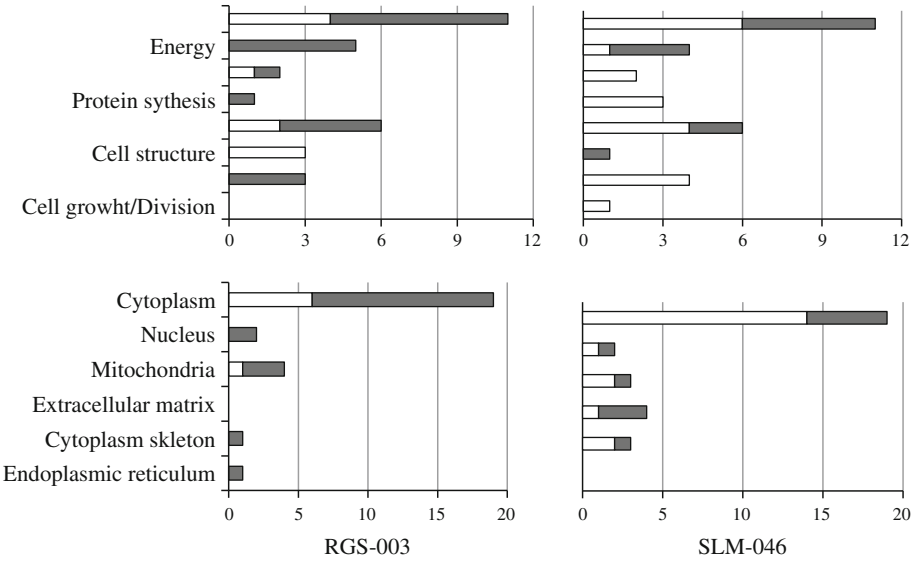

Fig. 3 Classification of proteins differentially expressed in the roots of rapeseed seedlings treated with drought stress. The functions (*upper*) of differentially expressed proteins in a drought-sensitive (RGS-003) and drought-tolerant rapeseed line (SLM-046) were assigned using the classification scheme described by Bevan et al. (1998). Identified proteins were also classified according to the subcellular localization (*lower*) predicted by the WoLF PSORT prediction program (http://wolfpsort.org). Proteins were classified into eight functional and six locational groups. *White* and *black* columns represent increased and decreased proteins, respectively

rapeseed seedlings were treated with drought stress for 7 days, and proteins were then extracted from roots, separated by 2-DE, and stained with CBB to evaluate their expression levels. A total of 607 protein spots were reproducibly detected on the 2-DE gels (Fig. 4). The relative intensities of all spots from three independent biological replicates were analyzed using PDQuest software, and differentially expressed protein spots that were common with those from both sensitive and tolerant rapeseed lines were selected (Fig. 4).

Commonly differentially expressed proteins between drought-sensitive, drought-tolerant, and F1 hybrid lines under drought stress

Among the 35 and 32 differentially expressed protein spots identified on the 2-DE gels of the drought-sensitive (S) and -tolerant (T) lines, respectively, 6 protein spots were commonly expressed under drought stress. For the hybrid F1 (F) seedlings, 2 and 3 protein spots were common with the differentially expressed proteins in the sensitive and tolerant lines, respectively, under drought stress. Only a single protein spot was common among the drought-sensitive, drought-tolerant, and F1 hybrid seedlings under drought stress (Fig. 5; Table 3).

Under conditions of drought stress, expression of cobalamin-independent methionine synthase or heat shock protein (HSP) 70 (S16 and T12) and rubber elongation factor protein (S32 and T29) was increased, while that of the glyceraldehydes-3-phosphate dehydrogenase C subunit (S35 and T30) was decreased. V-type H^+ ATPase catalytic subunit A (S22 and T21), heat shock protein 90-2 (S24 and T17), plasma-membrane associated cation-binding protein 1 (S26 and T19), and elongation factor EF-2 (S5 and R5) were decreased in drought-sensitive line, but increased in the drought-tolerant line. Tubulin beta-2 (S33 and F3) and HSP 70 (S7 and F4) were decreased in both the drought-sensitive line and F1 hybrids. Jasmonate-inducible protein (F5 and T7) and 20S proteasome subunit PAF1 (F6 and T14) were increased in F1 hybrids and the drought-tolerant line. Fructokinase (F2 and T22) was increased in F1 hybrids and decreased in the drought-tolerant line. Beta-substituted Ala synthase 3 (S31, F1, and T26) was decreased in all three types of rapeseed seedlings (Fig. 5; Table 3). Taken together, the observed expression profiles suggested that drought stress induced protein expression genotype specifically.

Discussion

Drought stress markedly decreased the root growth of rapeseed seedlings. Physiological experiments demonstrated that the drought-sensitive rapeseed line was susceptible to drought stress, while the susceptibility of F1 hybrid plants to drought sensitivity was intermediate between that of the drought-sensitive and -tolerant lines. Although a drought-induced reduction in rapeseed biomass has been reported previously (Jensen et al. 1996; Hosseini and Hassibi 2011), the present results confirm that rapeseed is relatively sensitive to drought. Several plant studies have

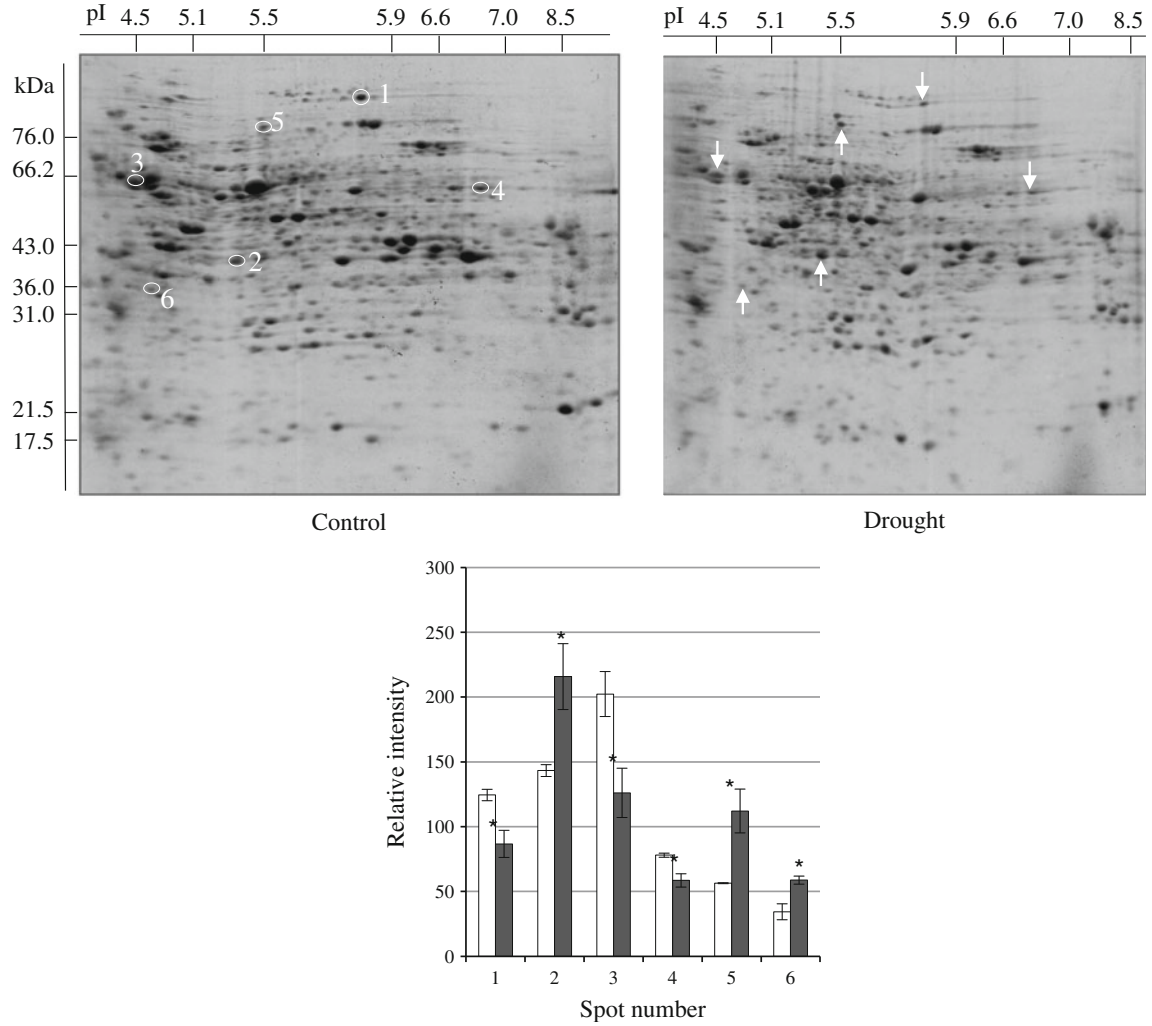

Fig. 4 Protein expression patterns in the roots of hybrid F1 seedlings under drought stress. Seven-day-old rapeseed seedlings were treated with drought stress for 7 days, and proteins were then extracted, separated by 2-DE, and visualized by CBB staining. The pI and Mr of each protein were determined using 2-DE markers. *Arrows* indicate the position of common proteins in the sensitive and tolerant lines that were altered in response to drought stress, and *circles* mark the position of the same proteins in the control. *Upward-* and *downward-facing arrows* indicate increased and decreased expression, respectively. Relative intensities of proteins were obtained using PDQuest software. Each *value* represents the mean ± SE of relative intensity determined from gels of three biological replicates. The mean values were compared using the Student *t* test (*$P < 0.05$ or **$P < 0.01$). Standard errors are denoted by *error bars*. *White* and *black* columns are the control and drought stress conditions, respectively

investigated global gene or protein expression profiles in response to drought stress; however, little attention has been paid specifically to rapeseed protein profiles in response to drought stress. Although Bandehagh et al. (2011) reported the effects of salt stress in rapeseed leaves, the present study is the first to examine the effects of drought stress on protein expression in rapeseed seedlings. The physiological analyses of rapeseed seedlings revealed that 7 days of water restriction significantly reduced root weight and length; thus, the drought-responsive proteins in rapeseed seedlings were analyzed after 7 days of drought stress.

Vacuolar-type H^+-ATPases and plasma-membrane associated cation-binding protein 1 are the enzymes responsible for the energization of membranes and acidification of compartments within eukaryotic cells via the establishment of proton and electrochemical gradients at the expense of ATP (Magnotta and Gogarten 2002). Here, both of these proteins were increased and decreased in the drought-tolerant and -sensitive rapeseed lines, respectively. Several studies have evaluated the role of vacuolar type ATPases in allowing plants to cope with environmental stresses, primarily high salt and low temperature. In cold-tolerant rapeseed and rice, increases in vacuolar type ATPase activity have been observed (Orr et al. 1995; Carystinos et al. 1995), while subjecting the facultative CAM plant *Mesembryanthemum crystallinum*, tobacco,

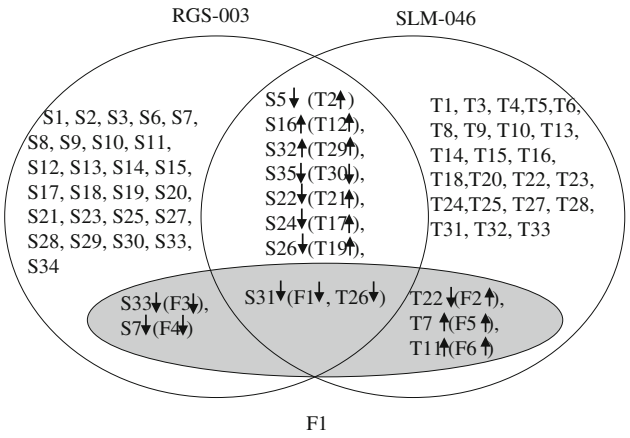

Fig. 5 Venn diagram analysis of common drought responsive proteins in the roots of sensitive (S)-, tolerant (T)- and hybrid F1 (F) rapeseed lines under drought stress. The overlapping portions denote the common protein spots among the sensitive (RGS-003), tolerant (SLM-046), and hybrid F1 seedlings exposed to drought stress. *Numbers* correspond to the protein spots in the 2-DE patterns from the roots of sensitive and tolerant lines. The *gray* area includes common protein spots of the hybrid F1 line and the sensitive and tolerant lines. *Upward-* and *downward-facing arrows* indicate increases and decreased expression, respectively

and tomato to NaCl resulted in increased transcript levels of V-ATPases (Low et al. 1996; Reuveni et al. 1990; Narasimhan et al. 1991). In addition, drought stress increased the ATPase activity of wheat (Gong et al. 2003) and resulted in increased mRNA levels of subunit A vacuolar-type H^+-ATPases in winter rapeseed (Orr et al. 1995). Here, vacuolar-type H^+-ATPases and plasma-membrane associated cation-binding protein 1 were increased in a drought-tolerant rapeseed line and decreased in a drought-sensitive line. These findings, together with our present results, suggest that increased expression of these two proteins facilitates the pumping of protons into root cell vacuoles under conditions of drought stress, allowing plants to obtain greater amounts of water from soil.

The levels of HSP 90-2 were increased and decreased in the drought-tolerant and -sensitive rapeseed lines, respectively, while that of HSP 70 was decreased in the drought-sensitive and F1 hybrid lines, but was not altered in the drought-tolerant rapeseed line, under conditions of drought stress. HSPs are involved in various intracellular processes and play important roles in protein–protein interactions, folding, assembly, intracellular localization, secretion, transport, prevention of protein aggregation and degradation, and reactivation of damaged proteins (Parsell and Lindquist 1993). HSP chaperone pathways require energy in the form of ATP hydrolysis to function. HSPs are typically induced when cells are exposed to various types of environmental stresses, such as drought, heat, cold, and oxygen deprivation (Kregel 2002). HSP was also increased by drought stress in sugar beet (Hajheidari et al. 2005), wheat (Demirevska et al. 2008), wild watermelon (Yoshimura et al. 2000), and sugarcane (Jangpromma et al. 2010). These results suggest that the increased expression of HSP under drought stress allowed the drought-tolerant

Table 3 Common changed root proteins in drought-sensitive and drought-tolerant rapeseed lines and their F1 hybrid under drought stress

No.	Protein name	Accession no.	No. in RGS-003	Regulation	No. in hybrid F1	Regulation	No. in SLM-046	Regulation
1a	Cobalamin-independent methionine synthase	XP_002871787.1	S16a	Increase	–	–	T12a	Increase
1b	Heat shock protein 70	CAA52149.1	S16b	Increase	–	–	T12b	Increase
2	Beta-substituted Ala synthase 3_1	XP_002883653.1	S31	Decrease	F1	Decrease	T26	Decrease
3	Rubber elongation factor protein	P15252.2	S32	Increase	–	–	T29	Increase
4	Glyceraldehydes-3-phosphate dehydrogenase C subunit	XP_00288233.1	S35	Decrease	–	–	T30	Decrease
5	Fructokinase	AAK62446.1	–	–	F2	Increase	T22	Decrease
6	V-type H^+ ATPase catalytic subunit A	NP_178011.1	S22	Decrease	–	–	T21	Increase
7	Heat shock protein 90-2	ADC45396.1	S24	Decrease	–	–	T17	Increase
8	Plasma-membrane associated cation-binding protein 1	NP_193759.1	S26	Decrease	–	–	T19	Increase
9	Elongation factor EF-2	AAF02837.1	S5	Decrease	–	–	T2	Increase
10	Tubulin beta-2	XP_002877519.1	S33	Decrease	F3	Decrease	–	–
11	Heat shock protein 70	ACJ11742.1	S7	Decrease	F4	Decrease	–	–
12	Jasmonate inducible protein	CAA722271.1	–	–	F5	Increase	T7	Increase
13	20S proteasome subunit PAF1	NP_180719.1	–	–	F6	Increase	T11	Increase

RGS-003 was used as drought-sensitive line and SLM-0046 was used as drought-tolerant line

rapeseed line to produce more energy through ATP hydrolysis and the degradation or reactivation of damaged proteins, and prevent protein misfolding, resulting in greater root growth.

Elongation factor EF-1 was increased in the drought-tolerant and hybrid F1 lines of rapeseed under drought stress, but was decreased in the drought-sensitive line. The elongation of polypeptide chains during translation is a conserved process among prokaryotes and eukaryotes. Elongation factors (EFs) are the critical regulators of protein synthesis, which is influenced by high temperature and drought stress (Rizhsky et al. 2004; Mittler 2006). Jain and Chattopadhyay (2010) analyzed gene expression profiles in response to drought stress for several chickpea varieties differing in drought tolerance, and found 15-fold higher expression of EFs in drought-tolerant chickpea. The increased expression of elongation factor EF-2 in the drought-tolerant and hybrid F1 rapeseed seedlings likely led to increased protein synthesis in these lines, which served to reduce the effects of drought stress.

Tubulin beta was significantly decreased in the drought-sensitive and hybrid F1 rapeseed lines under drought stress, but did not change in the drought-tolerant line. Microtubules are involved in many cellular processes in plants, including cell division and elongation. B-tubulins, which are the basic components of microtubules, are encoded by a multigene family in eukaryotes with highly conserved sequences in protein-coding regions. Changes in the levels of tubulin isoforms in plants in response to low-temperature exposure likely result from altered tubulin gene transcript levels. Spinach (Guy et al. 1985), winter rapeseed (Johnson-Flanagan and Singh 1987), alfalfa (Mohapatra et al. 1987), barley (Dunn et al. 1990), potato (Nicot et al. 2005), and *A. thaliana* (Kurkela and Franck 1990) all exhibit down-regulated tubulin gene expression in response to low temperature. Our finding that tubulin beta in the drought-sensitive and hybrid F1 lines was decreased under drought stress may explain the decreased cell division and growth induced by water restriction in these two lines.

Jasmonate-inducible protein was increased in the drought-tolerant and hybrid F1 rapeseed lines under drought stress. Jasmonates modulate many physiological events, including resistance responses to pathogens and insects, pollen development, root growth, and senescence (Sasaki et al. 2000), and can also activate the expression of several genes, leading to the accumulation of their products, which are referred to as jasmonate-induced proteins (Benedetti et al. 1998). Jasmonates activate plant defense mechanisms in response to insect-driven wounding, various pathogens, and environmental stresses, such as drought, low temperature, and salinity (Wasternack and Hause 2002). Jasmonic acid may play a role in plant responses to water deficit, because water stress induces expression of several genes that also respond to jasmonic acid (Turner et al. 2002; Hashimoto et al. 2004). For example, the expression of genes encoding the soybean vegetative storage protein acid phosphatase increased in plants when treated with either water deficit or jasmonic acid (Mason and Mullet 1990). The observed increase of jasmonate-inducible protein in the drought-tolerant and hybrid F1 lines under drought stress suggest that the induction of defense mechanisms and increased root growth in response to drought stress may be partly responsible for rapeseed drought tolerance.

20S proteasome subunit PAF1 protein was also increased in the drought-tolerant and hybrid F1 lines of rapeseed under drought stress, but did not change in the drought-sensitive line. Proteins damaged by oxidative stress are degraded by the 20S proteasome in an ubiquitin-independent manner (Grune et al. 2004; Asher et al. 2006; Voss and Grune 2007).Differential degradation of damaged proteins implies that plant cells have distinct recognition mechanisms for misfolded and oxidized proteins, and that oxidation leads to the formation of a specific degradation signal that channels the oxidized proteins directly to the 20S proteasome. Proteolysis of oxidized proteins also depends on misfolding and exposure of hydrophobic regions that serve as recognition sites for either the 20S proteasome itself or for specific chaperonins that bind the 20S proteasome (Whittier et al. 2004). Here, the increased expression of 20S proteasome subunit PAF1 in the drought-tolerant and hybrid F1 lines suggests that during drought stress, the increased degradation of proteins damaged from oxidative stress helps rapeseed seedlings to tolerate drought stress.

In conclusion, this study represents the first proteome analysis of rapeseed response to drought stress. Notably, 66 % of differentially expressed proteins in the drought-tolerant line were those increased under drought stress, whereas only 32 % of such proteins were increased in the drought-sensitive line. Based on the physiological and proteomics results, hybrid F1 displayed moderate sensitivity to water restriction between the sensitive and tolerant lines. Our findings suggest: (1) V-type H^+ ATPase, plasma-membrane associated cation-binding protein, HSP 90, and elongation factor EF-2 have a role in the drought tolerance of rapeseed; (2) The decreased levels of HSP 70 and tubulin beta-2 in the drought-sensitive and hybrid F1 lines might explain the reduced growth of these lines in drought conditions.

Acknowledgments The authors thank Dr. Yohei Nanjo and Dr. Keito Nishazawa for their valuable discussion.

Conflict of interest This work was supported by the Scholarship Section of the Ministry of Science, Research and Technology and

Tarbiat Modares University of I. R. Iran and National Institute of Crop Science of Japan.

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