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Comparative proteome analysis of *Picrorhiza kurrooa* Royle ex Benth. in response to drought

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Abstract

Picrorhiza (*Picrorhiza kurrooa* Royle ex Benth.) is an important medicinal herb of western Himalayan region and has been used to treat various diseases and disorders. Over-harvesting and lack of cultivation has led to its entry in Red Data Book as an endangered species. Its very restrictive and narrow habitat and the lesser biomass production are the main limitations for bringing it under commercial cultivation. Both these issues necessitate deeper insights into its growth and interaction with the environmental cues. Present study aimed at analysis of molecular response of Picrorhiza to drought stress. For the purpose comparative proteomics of Picrorhiza leaf and root under drought stress was performed. Denaturing two dimensional gel electrophoresis and mass spectrometry techniques were used to detect and identify differentially expressed proteins, respectively. Thirteen proteins from leaf and 18 proteins from root showed differential expression levels under drought condition. Among the differentially expressed proteins, majority were those involved in metabolism, photosynthetic process, transcription and protein synthesis. Other differentially expressed proteins were those involved in stress and defense response, transport, signaling and cytoskeleton development indicating that many different processes work together to establish a new cellular homeostasis in response to drought stress. Proteins found to be differentially expressed under drought condition suggested a range of biochemical pathways and processes being associated with response of plant to drought condition. The identified proteins and the associated biological processes may be utilized for developing strategies for improving Picrorhiza for drought tolerance.

Keywords: *Picrorhiza kurrooa*, proteomics, drought stress, MALDI-ToF/ToF, mass spectrometry

Introduction

Picrorhiza (*Picrorhiza kurrooa* Royle ex Benth.) is a small perennial herb (Family Plantaginaceae) and grows primarily in the north-western Himalayan region at an altitude of 3000-5000 m above mean sea level. Its underground parts, rhizomes and roots are widely used in traditional system of medicine due to its antioxidative, hepatoprotective, antiproliferative, immunomodulatory, antibacterial and antiviral activities [1]. The plant is self-regenerating but unregulated over-harvesting has caused it to be threatened to near extinction and thus Picrorhiza has been listed in the Red Data Book as an endangered plant species [2]. Medicinal importance of Picrorhiza on one side and its listing in Red Data Book on the other side presses urgent need for intensive R&D interventions towards ensuring its availability for the medicinal use, its sustainability and improvement. In line with this, recently we reported the differential spatio-temporal status of various components of antioxidant system of Picrorhiza. Ascorbic acid was found to be highest in leaves and lowest in roots. Interestingly, just opposite to that, glutathione was highest in roots and lowest in leaves [3]. Also, a translation initiation factor eIF5a transcript was found to be associated with leaf senescence as well as regulated by exogenous application of ABA [4].

Narrow range habitat of Picrorhiza i.e., limited only to certain niches of Himalayan region further demands detailed studies on its interaction with environmental factors. Among various environmental cues having adverse effects on plants growth and productivity, drought stress (i.e., insufficient availability of water) has become a major deleterious factor. Drought stress disturbs cellular homeostasis and can lead to severe retardation in growth and development [5]. Drought stress can also have a devastating effect on plant metabolism. The effect of drought stress can be manifested in many ways, as varied morphological, physiological and biochemical changes in plants. In addition to the physiological and biochemical responses of plants to water stress, dissection of molecular pathways at protein level is essential for comprehensive understanding of plant stress response mechanism. In the present work, we performed comparative analysis of proteome of Picrorhiza in response to drought stress. Two-dimensional (2D) gel electrophoresis was

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firstly used to separate proteins expressed under drought condition and resulting proteomic patterns were compared. The differentially expressed proteins were identified by MALDI-ToF/ToF and searched for their biological functions.

Materials and methods

Picrorhiza (*Picrorhiza kurrooa)* plants used in the present study were collected from the naturally grown area of Rohtang pass (4100 m elevation, 32° 23′ N, 77° 15′ E, Kullu district of Himachal Pradesh, India). The plants were transplanted in plastic pots (25cm height × 24cm top diameter × 15cm bottom diameter), containing soil, sand and FYM mixture in a ratio of 2:1:1. These plants were maintained and grown under polyhouse conditions at CSIR-Institute of Himalayan Bioresource Technology (1300 m elevation; 32° 06′ N; 76° 33′ E), Palampur, Himachal Pradesh, India as described by [3,4]. Plants were allowed to acclimatize for 2 months before start of the experiment.

Imposition of drought stress and harvesting of samples

Thirty plants of Picrorhiza (approximately 2 months old) were used for drought stress related experimentation. For imposition of drought stress, plants were divided into two sets of 15 plants each set. In one set, drought stress was imposed on the plants by withholding regular watering, whereas regular watering (200 ml each pot) on alternate days was done in one set of the plants (hereinafter will be referred to as control plants) during whole course of the experimentation i.e., 10 days. Sampling for protein extraction was carried out on day 10 of the drought stress treatment. In case of control as well as drought stressed plants, leaves (without petiole) from 3 plants were excised, mixed and weighed 1g. This was referred to as one biological replicate. This way three independent biological replicates were collected for both, control and drought stressed plants. The collected samples were wrapped in labeled aluminum foil, immediately frozen in liquid nitrogen and stored at -80°C till further analysis. For sampling of roots, the plants were carefully uprooted from the pots. The roots were made soil free by gently removing soil with filter paper. Roots from 3 plants each from control and drought stressed plants were harvested, cut in small pieces and mixed thoroughly. One gram of the mixed roots were wrapped in labeled aluminum foil, immediately frozen in liquid nitrogen and stored at -80°C till further analysis. This was referred to as one biological replicate. In case of roots also, three independent biological replicates were collected for both, control and drought stressed plants. Hence, total 12 samples (6 leaf and 6 root samples) were harvested and used for 2 proteomics analysis.

Measurement of relative water content

The plants kept for drought stress experimentations were used for estimation of relative water content (RWC). Sampling of leaf tissue for RWC estimation was performed from the same plants used for measurement of photosynthesis, stomatal conductance and transpiration rate. Leaf disks of 1.5 cm diameter were cut from leaves at 3rd 4th and 5th positions (starting from the top) of 3 plants each of control and those under drought stress. The cut discs were mixed and divided into 3 sets of 5 disks in each set. These cut discs were weighed to obtain the fresh weight and put in pre-weighed empty eppendorf tubes of 1.5 ml. The tubes were filled up with deionized water and incubated inside ice for a period of 4 h; this gave the turgid weight of the leaf. The tubes were then dried inside the oven at 60°C for a period of 72 h or till the constant weight was observed to obtain the dry weight. The RWC of the leaves was calculated from the following formulae:

$$\text{RWC (\%)} = \frac{\text{Fresh weight-Dry weight}}{\text{Turgid weight-Dry weight}} \text{ X } 100$$

The data was subjected to statistical analysis for the significance using t-test as described by [6].

Measurement of photosynthesis, stomatal conductance and transpiration rate

Photosynthesis, stomatal conductance and transpiration rates were measured using a portable infra-red gas analyser (IRGA; model LI-6400; Li-Cor, Lincoln, USA). IRGA was zeroed for CO_2 and H_2O using a CO_2 scrubber and desiccant, respectively. The instrument computed the parameters by measuring the airflow rate, the incoming and chamber CO_2 concentrations, and leaf area. Observations were recorded on 3 plants both for control as well as water stressed plants.

Extraction of proteins from leaf and root samples

In leaf as well as root tissue extraction of protein was performed for the three independent biological replicates for control as well as drought stressed plants described under "Imposition of drought stress and harvesting of samples" section. Harvested leaf and root samples were put in liquid nitrogen for 10-15 min, followed by grinding to fine powder. Soluble proteins were extracted from the leaf and root samples by adding 25 ml of extraction buffer (10% trichloroacetic acid (TCA) in chilled acetone with 0.07% β-mercaptoethanol) to one gram of fine powder, followed by 1 min of vortex agitation. The homogenate was kept at -20°C for 2-3 h, and then centrifuged at 12,000xg, 4°C for 15 min. The pellet was resuspended with 80% acetone containing 0.07% β-mercaptoethanol and 2 mM EDTA and kept at -20°C overnight [7]. In case of leaf samples this step was repeated until pellet obtained was completely free from chlorophyll. After centrifugation, the pellet was resuspended with 100% acetone containing 0.07% β-mercaptoethanol, 2mM EDTA and then centrifuged at 12000xg, at 4°C for 20 min and vacuum dried. The lyophilized powder was resuspended in 1-1.5 ml rehydration buffer (7 M Urea, 2M Thiourea, 2% CHAPS, 25mM DTT added fresh) and kept at 25°C for 4h. The insoluble precipitates were removed by centrifugation at

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12,000xg for 30 min at 4°C. Protein concentration of the final supernatant was measured according to Bradford protein assay [8]. The protein concentration was expressed on tissue's (leaf/root) fresh weight (fw) as well as dry weight (dw) basis. For estimation of dry weight, 200 mg of fresh tissue sample was kept at 60°C for 72 h and subsequently weighed at regular intervals until constant weight was achieved. The observed weight was taken as dry weight.

Two-dimensional gel electrophoresis

Protein extracted from three biological replicates for both leaf and root tissue of control as well as drought stressed plants were subjected to two-dimensional gel electrophoresis. Thus, total 12 gels were run. For two-dimensional (2 D) gel electrophoresis**,** extracted protein (50 μg) was dissolved in IEF rehydration buffer, 2 μl carrier ampholytes and loaded on to Immobilized pH gradient (IPG) strips (pH, 4-7; Length, 11 cm; Bio-Rad, USA). Isoelectric focusing (IEF) was performed with the parameters as follows: 250V for 15 min, 4000 V for 1 h, 8,000 V for 35,000 Vh and finally hold at 500 V. On completion of IEF, strips were firstly equilibrated for 20 min in reducing solution [6 M urea, 375mM Tris–HCl (pH 8.8), 20% v/v glycerol, 2% w/v SDS, 130mM DTT] and subsequently placed for another 20 min in alkylating equilibration buffer containing 135mM iodoacetamide according to [9]. Second dimension SDS-PAGE was run on 12% polyacrylamide gels according to [10]. Gels were fixed overnight in 40% methanol/1% glacial acetic acid solution and after overnight fixing, the gels were stained using a modified silver-staining method that is compatible with MS [11].

Image acquisition and analysis

2D gel images were acquired by using a GS-800 calibrated densitometer scanner (Bio-Rad). The twelve 2D gels obtained from leaf as well as root samples were analysed. Protein spot quantification was performed using PDQuest software (ver.8.0.1, Bio-Rad) which allowed automatic spot detection, landmark identification and spot aligning/matching within gels and quantification of matched spots; manual gel inspection was performed to correct any error prior to final data analysis. Each spot present on the master gel met several criteria: it was present in at least two of the three gels and was qualitatively comparable in size and shape in the replicate gels. We classify "low-quality" spot as those with a quality score <30; these spots were eliminated from further analysis. The remaining high quality spot quantities were used to calculate the mean for a given spot, and this value was used as the spot quantity on the standard gel. The spot intensities were normalized against the total intensity of the gel image using the PDQuest software with local regression model normalization method. Intensities of differentially expressed proteins were determined by PDQuest software. Increasing and decreasing index (fold change) was calculated as the ratio of the spot intensities (relative volumes) in drought with respect to the control gels. Differentially expressed spots showing reproducibility were marked with PDQuest software and marked manually. The threshold of protein spot abundance ratios was set at ≥1.5-fold (treatment/control) or >−1.5 fold (control/treatment). A Student's t-test was used for drawing comparison between drought stress protein expressions with respect to controls using the statistical software. Difference in protein expression was considered statistically significant for P values of ≤0.05. The molecular mass of each protein was estimated by comparing with those of standard marker proteins (Bio-Rad), and the isoelectric point (pI) was determined by the spot positions along the immobilized pH gradient strips.

In-gel protein digestion and mass spectrometry

Differentially expressed protein spots were manually selected and excised from silver-stained polyacrylamide gels. Those spot which were up regulated/down regulated/present only in response to drought were excised from 2D gels of samples from drought stressed plants whereas the spots which were absent in 2D gels of samples from drought stressed plants were excised from 2D gels of samples from control plants. Gel pieces were destained in mixture (1:1; v/v) of 30mM potassium ferricyanide and 100mM sodium thiosulfate at room temperature for 20 min. The immersed gel pieces were intermitantly vortexed until destained, washed three times with 200μl of Milli-Q water (each time for 5 min) and dehydrated in 100μl of acetonitrile. The gel samples were swollen in a 25 μl of a trypsin (Sigma, USA) solution (20μg/ml in 25 mmol/L NH_4HCO_3) for 30 min at 37°C and further incubated overnight at 37°C. For each digest, the peptides were extracted from the gels twice with 50% trifluoroacetic acid/50% acetonitrile, at room temperature. To the extracted peptide mixture was added 0.5 μl of α-Cyano-4-hydroxy-cinnamic acid (Bruker) (20mg/ml) in 0.1% trifluoroacetic/30% (v/v) acetonitrile (1:2) and dried at room temperature. The extracted peptides were subjected to MS using MALDI-ToF/ToF- Proteomics Analyzer (UltrafleXtreme™ mass spectrometer; Bruker Daltonics Inc. Germany). A mass standard starter kit (Bruker DaltonicsInc, Germany) and a standard tryptic BSA digest (Bruker DaltonicsInc, Germany) were used for MS and MS/MS calibrations of the system. A combined MS and LIFT-MS/MS were performed using BioTools 3.0 software (Bruker Daltonics Inc, Germany). The TOF spectra were recorded in positive ion reflector mode with a mass range from 700 to 3500 Da. Five hundred shots were accumulated for each spectra. Two most abundant peptide ions were then subjected to fragmentation analysis to determine the peptide sequence. Database search was performed using MASCOT search engine (Version 2.1, Matrix Science, London, U.K) and Swiss Prot database (Release date, 5th May, 2013; version 121; 540052 sequences). All peptide masses were assumed monoisotopic and [M+H]+. The other parameters used for search were as follows: taxonomy, *Viridiaplantae* (green plants 33813 sequences); enzyme, trypsin; the fixed modification;

carbamidomethyl (C); the variable modification, Glu->pyro-Glu (N-term Q) and oxidation (M); parent ion mass tolerance at 100 ppm and MS/MS mass tolerance of 0.7 dalton; one missed cleavage allowed. The identified proteins had to meet three criteria: (1) be among the top hits on the search report; (2) individual ions scores > 44 indicate identity or extensive homology (p<0.05) as used by various workers [12-14]. Only proteins matched by a minimum of two peptide sequences were included in the results list. Also, to evaluate protein identification, the percentage of sequence coverage was considered. The confidence in the peptide mass fingerprinting matches was based on the score level and confirmed by the accurate overlapping of the matched peptides with the major peaks of the mass spectrum.

Functional categorization of identified proteins

The identified proteins were searched for their biological functions using UniProt database (http://www.uniprot.org), http://www.expasy.org. Three independent ontological sets in the *Viridiplantae* taxonomic database were used to annotate and group the proteins according to biological process, molecular function and cellular compartmentalization. Upon getting the relevant information about biological functions the identified differentially expressed proteins were classified in different groups.

Results

Protein content in Picrorhiza under drought vis-à-vis control

Total protein from leaf as well as root samples collected from plants under control and those under drought were extracted and quantified. In control plants the protein content was 1494±19 (μg/g leaf tissue) and 16444±162 (μg/g leaf tissue) on fresh weight and dry weight basis, respectively (Figure 1). In case of plants under drought stress, the protein content was 1130±127 (μg/g leaf tissue) and 10400±1192 (μg/g leaf tissue) on fresh weight and dry weight basis, respectively. In case of roots, in control plants the protein content was 789±14 (μg/g root tissue) and 7023±160 (μg/g root tissue) on fresh weight and dry weight basis, respectively (Figure 1). In case of plants under drought stress, the protein content was 881±31 (μg/g root tissue) and 6357±254 (μg/g root tissue) on fresh weight and dry weight basis, respectively. This way drought stress resulted in 24% and 37% decrease in leaf protein content on fresh weight and dry weight basis, respectively. In case of roots, drought stress resulted in 12% increase in protein content when calculated on fresh weight basis, whereas, 9% decrease in protein content was observed on dry weight basis (Figure 1).

Physiological parameters

Photosynthetic rate, stomatal conductance, transpiration and relative water content (RWC) was measured and compared. In control plants, photosynthesis rate on the 4th leaf was 8±1.0 μmolm^{-2} s^{-1}. In case of drought stressed plants 7±0.04 μmolm^{-2} s^{-1} photosynthesis rate was recorded (Table 1). Likewise, stomatal conductance in control plants and drought stressed

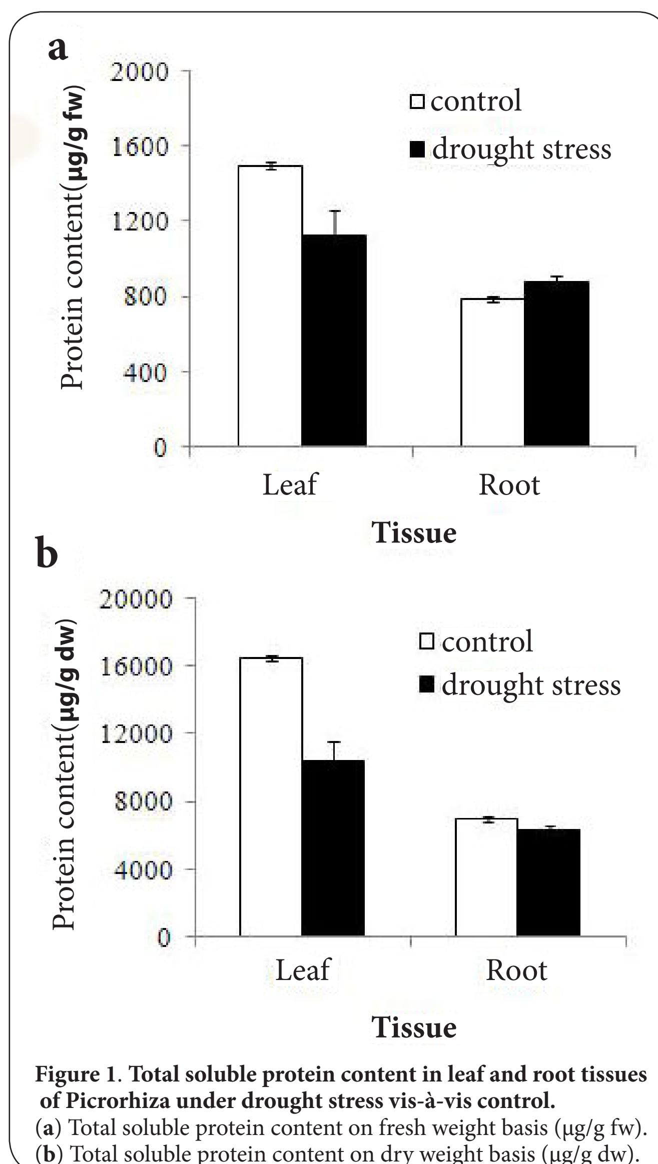

Figure 1. Total soluble protein content in leaf and root tissues of Picrorhiza under drought stress vis-à-vis control.
(**a**) Total soluble protein content on fresh weight basis (μg/g fw).
(**b**) Total soluble protein content on dry weight basis (μg/g dw).

Table 1. Physiological parameters of Picrorhiza measured during drought stress. Values are average±standard deviation (n=3).

Physiological parameter	Control	Stress	% Change *w.r.t.* control
Photosynthesis (μmolm^{-2} s^{-1})	8±1.0	7±0.04	(-)12.5*
Stomatal conductance (mmolm^{-2} s^{-1})	0.12±0.01	0.09±0.01	(-)25.0
Transpiration (nmolm^{-2} s^{-1})	4±0.4	3±0.2	(-)25.0*
Relative water content (%)	74±1.0	41±3.0	(-)44.6*

*Statistical significant difference ($p<0.05$).

plants were 0.12±0.01 mmolm^{-2} s^{-1} and 0.09±0.01 mmolm^{-2} s^{-1}, respectively. Similarly, transpiration rate was found to be 4±0.4 nmolm^{-2} s^{-1} and 3±0.2 nmolm^{-2} s^{-1}, respectively, in control plants and those under drought stress. RWC in control plants was detected to be 74±1.0%, whereas, in plants under drought, RWC was found to be only 41±3.0%. Thus, overall, drought stress resulted in 12.5%, 25.0%, 25.0% and 44.6% decrease in photosynthetic rate, stomatal conductance, transpiration rate and relative water content, respectively (**Table 1**). The decrease was found to be statistically significant for all the four measured parameters except for stomatal conductance.

Detection of differentially expressed proteins

Samples harvested on day 10 of the experimentation were analyzed for comparative proteomics. In case of leaf assembled first level matchset (master image) from three replicate 2 D gels 397 in control and 401 under drought stress were reproducibly detected (**Supplement figure S1**). The spots with quality value >30 (detected by PDQuest software) were considered as "high quality" spots. This way, 296 and 294 were classified as "high quality" spots detected in case of leaf from control and those from drought stressed plants (**Table 2**). Similarly, in roots, 398 in control and 391 under drought stress were re-producibly detected (**Supplement figure S2**). Of these, 359 and 352 were classified "high quality" spots, in case of root from control and those from drought stressed plants (**Table 2**).

Table 2. Reproducibility of 2-dimensional gels.

Sample	Average no. of spots*	Low quality spots	High quality spots**	Reproducibility (%)
Leaf (control)	397	101	296	74.55
Leaf (drought stress)	401	107	294	73.31
Root (control)	398	39	359	90.20
Root (drought stress)	391	39	352	90.02

*Average no. of spots present in three replicate gels of each sample
**Spots having quality score more than 30 assigned by PDQuest (ver.8.0.1)

In case of leaf, 13 spots were found to be differentially expressed with ≥1.5 fold differences in spot intensity and having p values <0.05 (**Figure 2**). Of these, 8 spots increased and 3 spots decreased in relative abundance in plants under drought. Two new spots were present only in plants under drought condition (**Figure 2**). Mass spectrometry analysis revealed the identities of these proteins using peptide mass fingerprinting. Identity of the 6 spots was reconfirmed by MS/MS (**Table 3**). Similarly, In case of root, 18 spots were found to be differentially expressed with ≥1.5 fold differences in spot intensity and having p values <0.05 (**Figure 3**). Of the 18, 13 spots increased and 2 spots decreased in relative abundance in plants under drought. Three new spots were detected in plants kept under drought (**Figure 3**). Mass spectrometry analysis revealed the identities of these proteins using peptide mass fingerprinting. Identity of the 5 spots was reconfirmed by MS/MS (**Table 4**).

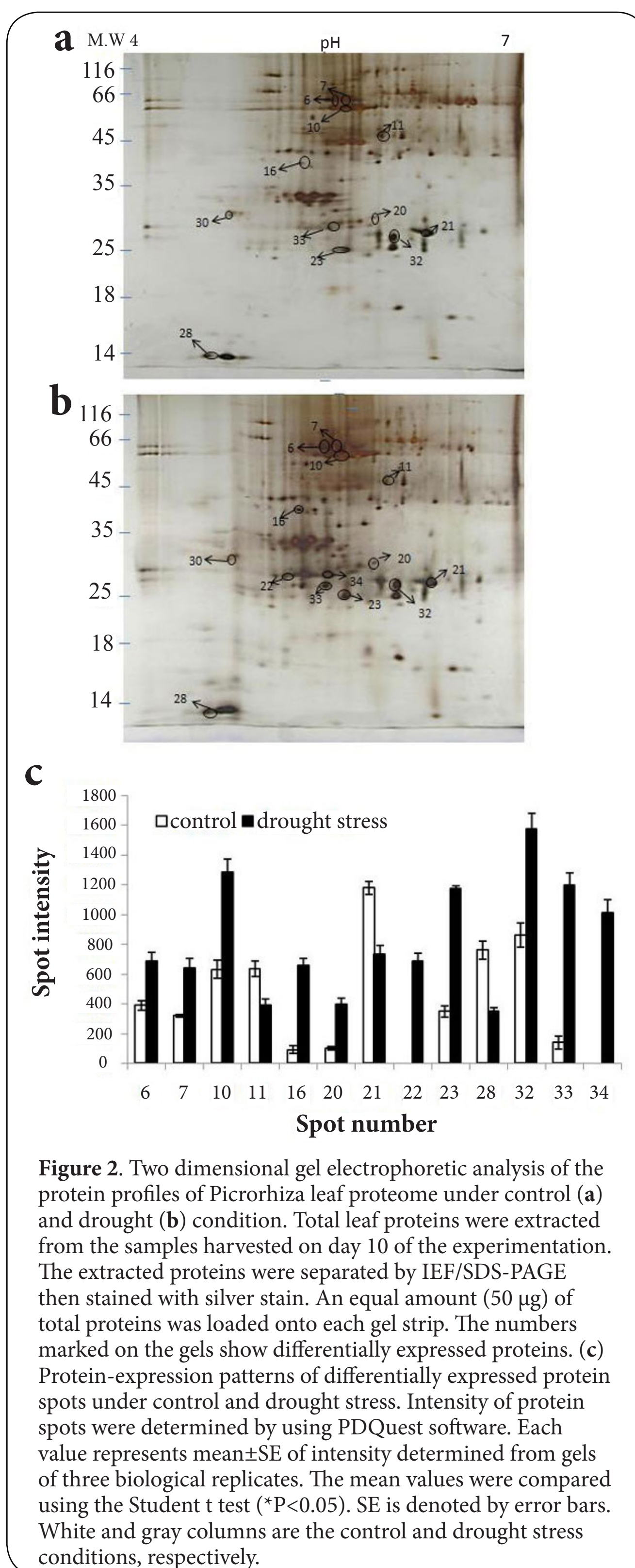

Figure 2. Two dimensional gel electrophoretic analysis of the protein profiles of Picrorhiza leaf proteome under control (**a**) and drought (**b**) condition. Total leaf proteins were extracted from the samples harvested on day 10 of the experimentation. The extracted proteins were separated by IEF/SDS-PAGE then stained with silver stain. An equal amount (50 µg) of total proteins was loaded onto each gel strip. The numbers marked on the gels show differentially expressed proteins. (**c**) Protein-expression patterns of differentially expressed protein spots under control and drought stress. Intensity of protein spots were determined by using PDQuest software. Each value represents mean±SE of intensity determined from gels of three biological replicates. The mean values were compared using the Student t test (*P<0.05). SE is denoted by error bars. White and gray columns are the control and drought stress conditions, respectively.

Table 3. Differentially expressed proteins in leaves of Picrorhiza under drought stress identified using MALDI-ToF/ToF mass spectrometry.

New spot no	Maximum homology with (protein name)	Best protein match organism	Swiss prot accession no.	Expt. MW/ Theor. MW (kDa)	Expt.pI/ Theor.pI	No. of peptides matched	MS Score	Sequence coverage (%)	Biological function	Cellular component	Molecular function	Expression status*	Relative fold change (vs control)	P value
6	Peptidyl-prolylcis-trans isomerase	*Brassica napus*	CYPH_BRANA	80/18.7	5.5/8.5	5	62	42	protein folding	cytoplasm	peptide binding	URUD	1.8	0.011
7	ATP synthase subunit beta	*Gossypium barbadense*	ATPB_GOSBA	80/53.6	5.6/5.1	7	61	24	plasma membrane ATP synthesis coupled proton transport	chloroplast thylakoid membrane	ATP binding	URUD	2.0	0.006
10	ATP synthase subunit beta	*Medicago sativa*	ATPB_MEDSA	66/52.7	5.5/5.1	13	153	38	plasma membrane ATP synthesis coupled proton transport	chloroplast thylakoid membrane	ATP binding	URUD	2.0	0.0036
11	Endoglucanase 9	*Arabidopsis thaliana*	GUN9_ARATH	40/53.6	6.2/8.7	11	68	33	carbohydrate metabolism	golgi apparatus	cellulase activity	DRUD	0.6	0.0204
16	Phenylalanine ammonia-lyase G1	*Populus kitakamiensis*	PAL1_POPKI	40/74.9	5.4/6.1	8	54	29	L-phenylalanine catabolic process,cinnamic acid biosynthetic process	cytoplasm	phenylalanine ammonia-lyase activity	URUD	7.3	0.0006
20	Cytochrome b6-f complexsubunit 6	*Scenedes-musobliquus*	PETL_SCEOB	30/34.9	6.1/9.5	2	42	84	oxidation-reduction process, photosynthesis	chloroplast thylakoid membrane	transferring electrons within cyto-chrome b6/f complex of photosystem II activity	URUD	4.0	0.003
21	NAD(P) H-quinone oxidoreductase subunit 4L	*Zea mays*	NU4LC_ MAIZE	29/11.4	6.2/9.6	5	58	52	photosynthesis	chloroplast thylakoid membrane	oxidoreduc-tase activity	DRUD	0.6	0.0042
22	Hexokinase-1	*Oryza sativa subsp. japonica*	HXK1_ORYSJ	25/52.1	6.4/4.8	10	52	22	glycolysis	unknown	hexokinase activity	POUD	0	0
23	Putative defensin-like protein 239	*Arabidopsis thaliana*	DF239_ARATH	28/11.3	5.2/8.09	4	40	44	defense response to fungus	extracellu-lar region	unknown	URUD	3.4	0.0001
28	50S ribosomal protein L2	*Scenedesmus obliquus*	RK2_SCEOB	20/30.7	6.8/11.2	5	52	24	translation	chloroplast	rRNA binding	DRUD	0.5	0.0034
32	Auxin-responsive protein IAA13	*Arabidopsis thaliana*	IAA13_ARATH	27/26.7	4.2/9.3	6	52	40	auxin-activated signaling pathway	nucleus	sequence-specific DNA binding transcription factor activity	URUD	1.8	0.0055

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Continuation of Table 3.

New spot no	Maximum homology with (protein name)	Best protein match organism	Swiss prot accession no.	Expt. MW/ Theor. MW (kDa)	Expt.pI/ Theor.pI	No. of peptides matched	MS Score	Sequence coverage (%)	Biological function	Cellular component	Molecular function	Expression status*	Relative fold change (vs control)	P value
33	Zinc finger CCCH domain-containing protein 3	*Oryza sativa*	C3H32_ORYSJ	30/80.7	4.8/5.3	5	57	12	transcription	unknown	DNA binding	URUD	8.6	0.0003
34	NAD(P) H-quinoneox-idoreductase subunit 4L	*Zea mays*	NU4LC_ MAIZE	45/11.4	6.4/9.6	4	45	52	photosynthesis	chloroplast thylakoid membrane	oxidoreduc-tase activity	POUD	0	0

Table 4. Differentially expressed proteins in root of Picrorhiza under drought stress identified using MALDI-ToF/ToF mass spectrometry.

Spot no	Maximum homology with (protein name)	Best protein match organism	Swiss prot accession no.	Expt. MW/ Theor. MW(kD)	Expt.pI/ Theor.pI	No. of peptides matched	MS Score	Sequence coverage (%)	Biological function	Cellular component	Molecular function	Expression status*	Relative fold change (Vs control)	P value
6	Phosphatidyl inositol 4-phosphate 5-kinase 1	*Arabidopsis thaliana*	PI5K1_ARATH	66.0/86.5	5.6/9.0	9	50	20	response to stress	plasma membrane	1-phosphati-dylinositol-4-phosphate 5-kinase activity	URUD	1.5	0.0372
7	Protein transport protein Sec61 subunit gamma-3	*Arabidopsis thaliana*	S61G3_ARATH	66.0/7.7	5.70/9.86	4	34	49	protein transmembrane transport	endoplasmic reticulum membrane	P-P-bond-hydrolysis-driven protein transmembrane transporter activity	URUD	3.9	0.0001
10	Clathrin light chain 3	*Oryza sativa subsp. japonica*	CLC3_ORYSJ	66.0/37.7	6.3/5.14	4	42	23	vesicle -mediated transport	clathrin coat of coated pit	structural molecule activity	DRUD	0.5	0.0224
14	Actin-42	*Solanum tuberosum*	ACT1_SOLTU	50.0/37	5.4/5.4	9	80	35	involve in cytoplasmic streaming, cell shape determination, cell division	chloroplast envelope	ATP binding	POUD	0	0
21	50S ribosomal protein L36	*Mesostigma-viride*	RK36_MESVI	44.0/4.6	6.7/11.40	2	30	39	translation	ribosome	structural constituent of ribosome	URUD	1.6	0.0206
22	H/ACA ribonucleoprotein complex subunit 4	*Arabidopsis thaliana*	CBF5_ARATH	37.0/63.3	6.4/9.1	9	58	22	rRNA processing, pseudouridine synthesis	nucleolus	RNA binding	DRUD	0.5	0.0064
23	Caffeic acid 3-O-methyltransferase	*Catharan-thus roseus*	COMT1_ CATRO	40.0/40.2	5.9/5.7	8	54	33	lignin biosynthesis	unkown	caffeate O-methyl transferase activity	URUD	2.6	0.0033
24	Pentatricopeptide repeat-containing protein	*Arabidopsis thaliana*	PP326_ARATH	32.0/12.2	4.5/8.7	10	58	17	play a role in RNA editing	mitochondria	endoribonucle-ase activity	POUD	0	0
25	Uncharacterized mitochondrial protein AtMg00240	*Arabidopsis thaliana*	M240_ARATH	33/12.46	5.2/9.62	2	20	27	unknown	mitochondria	unknown	URUD	3.2	0.0002
26	Probable E3 ubiquitin-protein ligase ARI5	*Arabidopsis thaliana*	ARI5_ARATH	31/63.90	5.3/5.12	3	23	7	protein ubiquitination	unknown	ligase activity	URUD	1.8	0.0117
28	Maturase K	*Marathrum-schiedeanum*	MATK_MARSC	30/61.5	6.0/9.5	6	43	11	RNA splicing,tRNA processing	chloroplast	RNA binding	URUD	3.6	0.009

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Continuation of Table 4.

Spot no	Maximum homology with (protein name)	Best protein match organism	Swiss prot accession no.	Expt. MW/ Theor. MW(kD)	Expt.pI/ Theor.pI	No. of peptides matched	MS Score	Sequence coverage (%)	Biological function	Cellular component	Molecular function	Expression status*	Relative fold change (Vs control)	P value
31	F-box protein At3g22350	*Arabidopsis thaliana*	FB174_ARATH	28/29.4	5.2/8.9	6	50	20	ubiquitin-mediated proteolysis, hormones signaling	unknown	unknown	URUD	1.6	0.0079
33	17.9 kDa class II heat shock protein	*Helianthus annuus*	HSP21_HELAN	28/17.9	5.8/7.7	6	59	41	response to stress	cytoplasm	unknown	URUD	1.5	0.0252
36	50S ribosomal protein L36	*Mesostigma-viride*	RK36_MESVI	20.0/4.6	5.5/11.40	2	30	39	translation	ribosome	structural constituent of ribosome	URUD	2.2	0.0190
37	Beta-D-xylosidase 1	*Arabidopsis thaliana*	BXL1_ARATH	20/84.4	5.3/8.7	9	55	17	carbohydrate metabolic process	plant-type cell wall	alpha-N-arabinofuranosidase activity	POUD	0	0
38	Pentatricopeptide repeat-containing protein At5g11310	*Arabidopsis thaliana*	PP375_ARATH	20.0/68.8	5.0/8.38	8	38	15	play a role in RNA editing	mitochondria	endoribonuclease activity	URUD	1.5	0.0805
43	Maturase K	*Aneura mirabilis*	MATK_ANEMR	14.0/61.2	6.5/9.7	8	46	22	mRNA processing,tRNA processing	chloroplast	RNA binding	URUD	8.8	0.0001
46	Protein transport protein Sec61 subunit gamma-3	*Arabidopsis thaliana*	S61G3_ARATH	30.0/7.7	4.9/9.8	4	39	43	protein transport	endoplasmic reticulum membrane	P-P-bond-hydrolysis-driven protein transmembrane transporter activity	URUD	12.2	0.0001

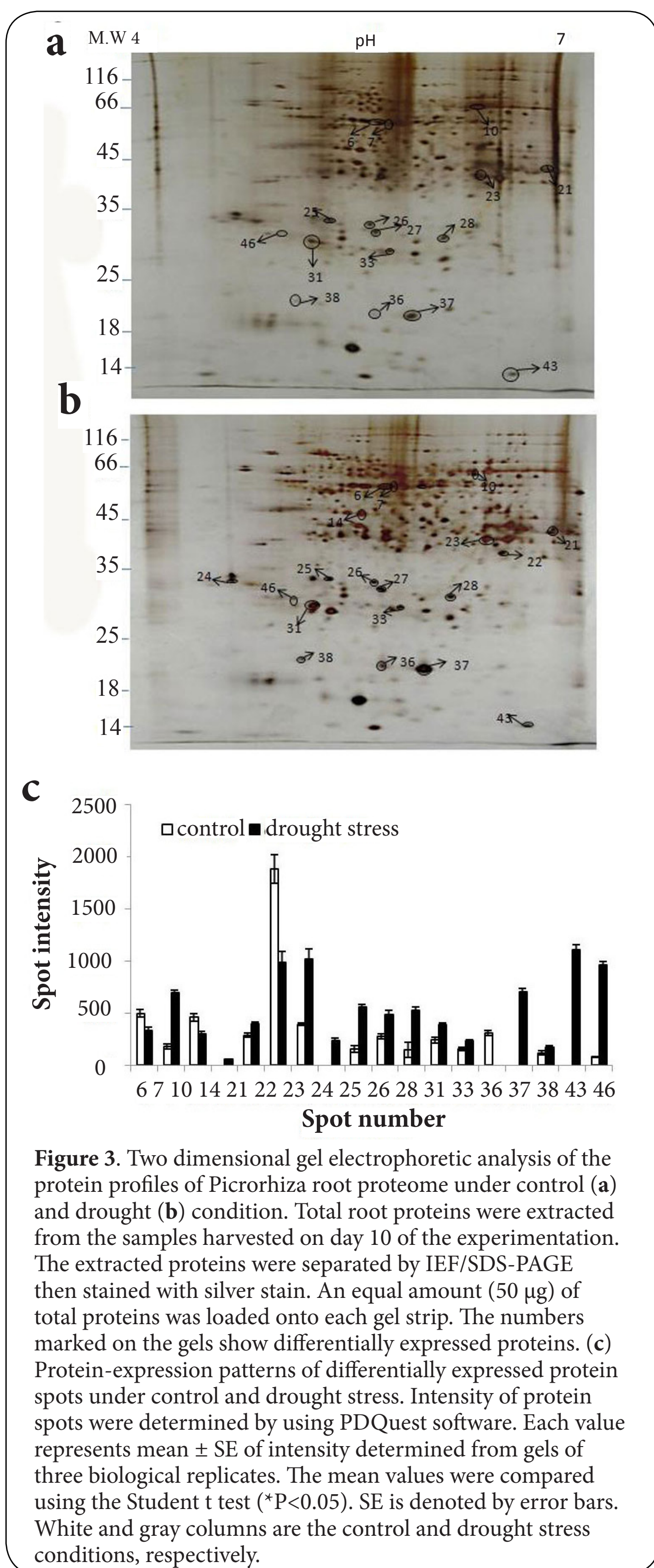

Figure 3. Two dimensional gel electrophoretic analysis of the protein profiles of Picrorhiza root proteome under control (**a**) and drought (**b**) condition. Total root proteins were extracted from the samples harvested on day 10 of the experimentation. The extracted proteins were separated by IEF/SDS-PAGE then stained with silver stain. An equal amount (50 µg) of total proteins was loaded onto each gel strip. The numbers marked on the gels show differentially expressed proteins. (**c**) Protein-expression patterns of differentially expressed protein spots under control and drought stress. Intensity of protein spots were determined by using PDQuest software. Each value represents mean ± SE of intensity determined from gels of three biological replicates. The mean values were compared using the Student t test (*P<0.05). SE is denoted by error bars. White and gray columns are the control and drought stress conditions, respectively.

Identification and functional classification of the differentially expressed proteins

Identification of the proteins that are differentially expressed is an important step towards understanding the mechanisms underlying stress responses and adaptation in plants. To understand the molecular mechanism, we combined 2 D gel electrophoresis and MALDI-TOF MS/MS to identify the differentially expressed proteins. The identified differentially expressed proteins were searched and grouped according to their biological processes, cellular locations, and molecular functions using the Gene Ontology (GO) database. The 31 identified differential proteins from leaf and root were classified into 9 groups based on their biological functions (**Figure 4**). The largest group was energy metabolism (34% in leaf and 22% in root), the following groups were stress and defense (17% in leaf and 12% in root), photosynthesis (25% in leaf), transcription (8% in leaf and 28% in root), protein synthesis (8% in leaf and 11% in root), transport-associating proteins (17% in root), signal transduction associated proteins (8% in leaf), cell structure proteins (5% in root) and function unknown proteins (5% in root). This result suggests that proteins involved in

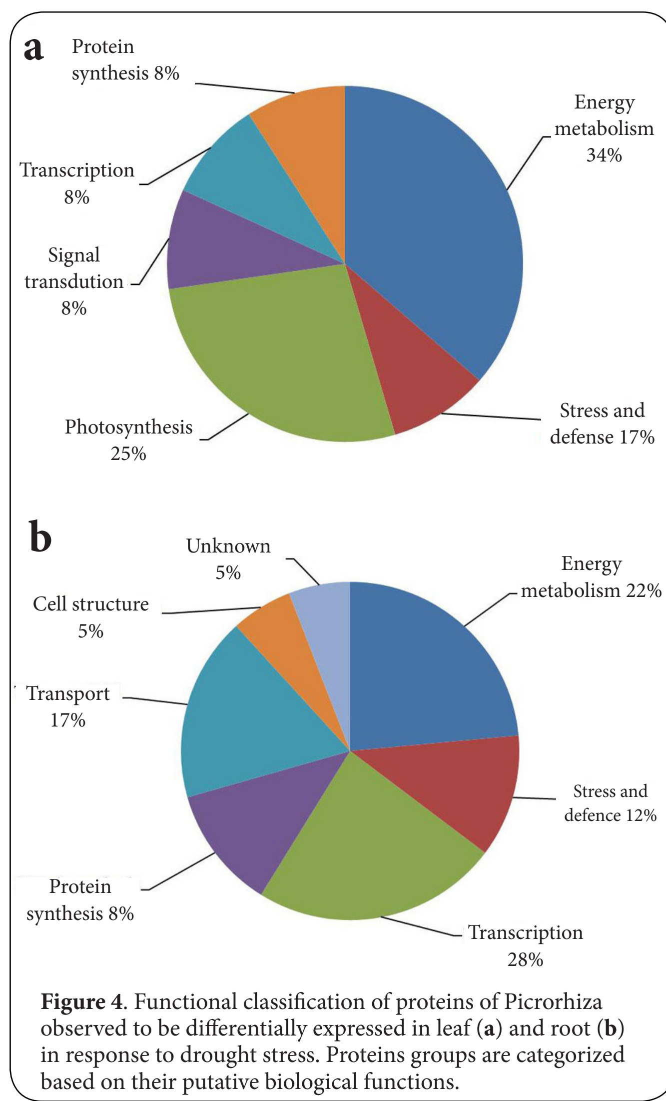

Figure 4. Functional classification of proteins of Picrorhiza observed to be differentially expressed in leaf (**a**) and root (**b**) in response to drought stress. Proteins groups are categorized based on their putative biological functions.

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metabolism, protein synthesis, photosynthesis, stress response, signal transduction, transcription, transport and cell structure play an important role in drought tolerance. The details about these identified proteins regarding experimental/theoretical molecular weights, pI, sequence coverage, peptide matched, accession numbers, subcellular localization, biological and molecular function and relative fold change values are given in **Tables 3** and **4**.

In case of leaf, 8 proteins (#6, 7, 10, 16, 20, 23, 32 and 33) were up-regulated and 3 proteins (#11, 21, 28) were down-regulated under drought condition. Two new proteins (#22, 34) were present in drought condition. In case of root, 13 proteins (#6, 7, 21, 23, 25, 26, 28, 31, 33, 36, 38, 43 and 46) were up regulated and 2 proteins (#10, 22) were down-regulated under drought condition. Three new proteins (#14, 24, 37) were present only in drought condition. The identified proteins were involved in stress and defense responses, energy metabolism, protein synthesis, photosynthesis, transcription, transport and cell structure. A comparison of differentially expressed proteins in leaf vis-à-vis root in response to drought revealed that all the differentially expressed proteins were different in root and leaf tissue.

Proteins involved in stress and defense response

In case of leaf, 2 identified proteins, phenylalanine ammonia-lyase G1 and putative defensin-like protein 239, (#16, 23) were involved in defense and stress response. Both (#16, 23) were found to be up regulated under drought condition. In case of root, proteins, phosphatidylinositol 4-phosphate 5-kinase 1 (#6) and 17.9 kDa class II heat shock protein (#33) involved in stress and defense response were up-regulated under drought stress.

Proteins involved in photosynthesis

Photosynthesis is one of the processes that is most affected by abiotic stress [**15**]. Under drought condition, identified proteins like cytochrome b6-f complex subunit 6 (#20) and NAD(P)H-quinoneoxidoreductase subunit 4L (#21, 34) in leaf were involved in photosynthesis process. Cytochrome b6-f complex subunit 6 was up regulated and NAD(P)H-quinoneoxidoreductase subunit 4L was down regulated in drought stress.

Proteins involved in energy metabolism

The identified proteins in leaf; ATP synthase subunit beta, endoglucanase 9 and hexokinase-1 (#7, 10, 11, 22) were those involved in metabolics processes like ATP synthesis, carbohydrate metabolism, amino acid catabolism. ATP synthase subunit beta was up regulated only in drought stress whereas endoglucanase 9 and hexokinase-1were found to be present in drought stress only. In case of root, proteins; caffeic acid 3-O-methyltransferase, E3 ubiquitin-protein ligase ARI5, F-box protein and beta-D-xylosidase 1 (#23, 26, 31, 37) were those involved in lignin biosynthesis, carbohydrate metabolic process, proteolysis and protein ubiquitination process. Caffeic acid 3-O-methyltransferase, E3 ubiquitin-protein ligase ARI5 and F-box protein were up regulated whereas beta-D-xylosidase was found to be present only in drought stress.

Proteins involved in transcription

The identified protein in leaf, zinc finger CCCH domain-containing protein 3 (#33) was involved in transcription process and found to be up regulated under drought stress. Other identified proteins; maturase K (#28, 43), pentatricopeptide repeat-containing protein (#24, 38) and H/ACA ribonucleoprotein complex subunit 4 (#22) in root were involved in transcription process. Maturase K and pentatricopeptide repeat-containing protein were up regulated and H/ACA ribonucleoprotein complex subunit was down regulated in drought stress.

Proteins involved in protein synthesis

Identified protein in leaf, 50S ribosomal protein L2 (#28) was involved in protein synthesis and found to be down regulated under drought stress. In case of root, 50S ribosomal protein L36 (# 21, 36) were involved in protein synthesis. 50S ribosomal proteins L36 were found to be up regulated under drought stress.

Proteins involved in signal transduction

The identified protein in leaf, auxin responsive protein IAA13 (#32) was involved in auxin mediated signaling pathway and found to be up regulated under drought stress.

Proteins involved in transport

Under drought condition, the identified proteins in root; clathrin light chain 3 (#10) and protein transport protein Sec61 subunit gamma-3 (#7, 46) were involved in transport process. Clathrin light chain 3 and transport protein Sec61 subunit gamma-3 were up regulated under drought stress.

Proteins involved in cytoskeleton development

Cytoskeleton protein was also found to be differentially expressed. Identified protein in root, actin- 42 involved in cytoplasmic streaming, cell shape determination, cell division and organelle movement was found to be present only in drought stress.

Discussion

Proteomics an important component of systems biology is contributing significantly to the understanding of complex metabolic networks of plants and their interactions with environmental factors. While understanding plants response to abiotic stresses, information at proteomics level may compliment genomics and metabolomics information. In the present study, response of leaf and root proteome of a medicinal plant Picrorhiza to drought stress was analysed. Drought stress resulted in 24% and 37% decrease in leaf protein

content on fresh weight and dry weight basis respectively, whereas in root, drought stress resulted in 12% increase in protein content when calculated on fresh weight basis and 9% decrease in protein content was observed on dry weight basis. The content of soluble proteins in roots and leaves of maize have been reported to be decreasing with increased drought stress [16]. Similarly, in chickpea decrease in soluble protein in response to drought stress has been observed [17].

Drought stress resulted in decrease in all the four measured parameters i.e., photosynthetic rate, stomatal conductance, transpiration and relative water content. These observations are in agreement with the findings of various researchers [18-20]. The decrease in photosynthesis had been attributed to the stomatal closure and the direct inhibition of photosynthetic capacity. Decrease in the transcription rate in response to drought stress has been reported in *Phaseolus vulgaris* [21]. In *Cucumis melo* decrease in stomatal conductance in response to drought stress has been observed [22]. Estimation of relative water content showed lesser relative water content in Picrorhiza plants exposed to drought stress as compared to control plants. Decrease relative water content in response to drought stress has earlier been reported in peach [23], *Matricaria chamomilla* [24] and maize [25].

2D gel electrophoresis analysis showed 13 proteins in leaf and 18 proteins in root to be differentially expressed in response to drought stress. In the present study the differentially expressed spots were derived upon assembling the first level matchset (master image) from three replicate 2 D gels. The threshold of protein spot abundance ratios was set at ≥1.5-fold (treatment/control) or >−1.5 fold (control/ treatment). For proteomic analysis of plant for which genome sequence information is not available. Using ≥1.5-fold change as threshold seems reasonable as has been used in various studies [13,14,26,27]. The spots with quality value >30 were considered as "high quality" spots. Among the "high quality" spots only those having p value <0.05 were analysed using mass spectrometry. Detection of differentially expressed spots using these parameters has been reported by many researchers [28,29].

Mass spectrometry analysis of the differentially expressed proteins revealed their identity. Functional categorization of the differentially expressed proteins put them in 8 functional groups. These proteins have been reported to influence the interaction of plants with various environmental cues. In our study, 5 energy metabolism-related proteins from leaf and 4 proteins from root were affected by drought stress. Increase in abundance of ATP synthase subunit beta in response to salt stress has been reported in wheat [30]. Identified protein endoglucanase has been reported to be involved in cell elongation [31,32]. Decrease in endoglucanase level has been suggested to be associated with lower tolerance to salt stress and inhibition of plant growth [32]. Induction of another identified protein, caffeic acid 3-O-methyltransferase caffeoyl-CoA in response to drought has been reported in watermelon and maize [33,34]. F-box proteins regulate diverse cellular processes, including in protein ubiquitination and cell cycle transition [35]. Protein beta-D-xylosidase 1 is a key enzyme involved in complete degradation of xylan present in cell wall [36]. The differentially expressed protein, cytochrome b6-f complex subunit 6 acts as the redox link between the photosynthetic reaction center complexes II and I [37]. The differentially expressed protein, NAD(P)H-quinone oxidoreductase subunit 4L has been reported to be up regulated in response to drought stress in Sunflower [27].

Transcription proteins including maturase K, pentatricopeptide repeat containing protein and zinc finger CCCH domain-containing protein 3 in root and leaf were found to be up regulated under drought stress. Maturase K catalyzes intron removal in RNA precursors and directly affects gene expression at the translation level [38]. Maturase K in response to acid rain stress in *Taxus wallichiana* has been reported [39]. Pentatricopeptide repeat containing protein (PPR) mediates organelle transcript processing and stability in a gene-specific manner through recognition by tandem arrays of degenerate 35-amino-acid repeating units, the PPR motifs [40]. Other identified protein CCCH-type zinc finger protein 3 has essential function in various developmental processes in plants [41]. Stress and defense responsive proteins, phosphatidylinositol 4-phosphate 5-kinase 1 and 17.9 kDa class II heat shock protein in root were up regulated under drought condition while in case of leaf, putative defensin-like protein 239 was found to be up regulated. In general, HSPs are involved in various intracellular processes and play important role in protein-protein interactions, folding, assembly, intracellular localization, secretion, transport, prevention of protein aggregation and degradation and reactivation of damaged protein [42,43]. Other identified protein, phenylalanine ammonia-lyase has a crucial role in secondary phenylpropanoid metabolism [44,45] and in salicylic acid -dependent defense signalling to combat microbial pathogens [46].

Transport protein Sec61 subunit gamma-3 in root which was found to be upregulated under drought stress has role in clathrin-mediated protein trafficking, auxin signaling etc [47-49], cytokinesis [50,51], cell elongation [52] etc. 50S ribosomal protein L2 in leaf and 50S ribosomal protein L36 in root were found to be up regulated under drought stress. Functional implications of up regulation of these proteins in response to drought stress need further studies. The identified signaling protein, auxin responsive protein IAA13 in leaf has been reported to be involved in auxin activated signaling pathway [53] and was found to be up regulated under drought stress. Auxin-responsive protein has been found to be down regulated under acid rain stress [39]. Cell structure related protein, actin-42 in root was found to be up regulated under drought stress. Association of actin with drought stress has been reported by various workers [54-56]. Peptidyl-prolyl cis-trans isomerase was found to be up regulated under drought stress. It has been reported to be playing roles in various

processes such as protein folding, intracellular homeostasis and response to environmental [57].

Conclusion

In the present study drought responsive differentially expressed proteins in Picrorhiza were detected and identified. Further studies on these proteins may assist understand drought associated molecular mechanisms for developing strategies for improving adaptive behavior of Picrorhiza under drought condition.

List of abbreviations

2-D: Two-dimensional
pI: Isoelectric point
IEF: Isoelectric focusing
MALDI-ToF/ToF: Matrix-assisted laser desorption/ionization-time-of-flight/time-of-flight
ABA: Abscisic acid
FYM: Farm yard manure
RWC: Relative water content

Additional files

Supplement figure S1
Supplement figure S2

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Authors' contributions	SK	JP1	PJK	MD	JP2	RJ	SD
Research concept and design	✓	✓	--	--	--	--	✓
Collection and/or assembly of data	✓	✓	✓	--	✓	--	✓
Data analysis and interpretation	✓	✓	✓	✓	--	✓	✓
Writing the article	✓	✓	--	--	--	--	✓
Critical revision of the article	✓	✓	✓	✓	✓	--	✓
Final approval of article	✓	✓	✓	✓	✓	✓	✓

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