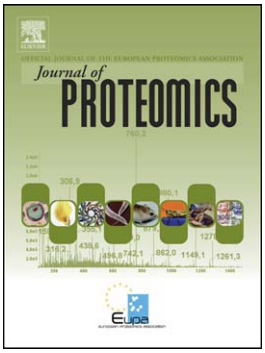


Differential proteomic analysis of drought stress response in leaves of common bean (*Phaseolus vulgaris* L.)

Tanja Zadražnik, Kristin Hollung, Wolfgang Egge-Jacobsen, Vladimir Meglič, Jelka Šuštar-Vozlič

PII: S1874-3919(12)00665-3
DOI: doi: 10.1016/j.jprot.2012.09.021
Reference: JPROT 1171

To appear in: *Journal of Proteomics*

Received date: 21 July 2012
Accepted date: 19 September 2012

Please cite this article as: Zadražnik Tanja, Hollung Kristin, Egge-Jacobsen Wolfgang, Meglič Vladimir, Šuštar-Vozlič Jelka, Differential proteomic analysis of drought stress response in leaves of common bean (*Phaseolus vulgaris* L.), *Journal of Proteomics* (2012), doi: 10.1016/j.jprot.2012.09.021

Differential proteomic analysis of drought stress response in leaves of common bean (*Phaseolus vulgaris* L.)

Tanja Zadražnik[a], Kristin Hollung[b], Wolfgang Egge-Jacobsen[c], Vladimir Meglič[a], Jelka Šuštar-Vozlič[a*]
[a] Agricultural Institute of Slovenia, SI-1000 Ljubljana, Slovenia
[b] Nofima Mat AS, Osloveien 1, NO-1430 Ås, Norway
[c] University of Oslo, Department of Molecular Biosciences, NO-0316 Oslo, Norway

[*] Corresponding author at: Agricultural Institute of Slovenia, Crop and Seed Science Department, Hacquetova ulica 17, SI-1000 Ljubljana, Slovenia; Tel: +386 (0)1 2805 188; Fax: +386 (0)1 2805 255; E-mail address: jelka.vozlic@kis.si (J. Šuštar-Vozlič)

Keywords: Phaseolus vulgaris, drought stress, proteomics, protein network

ABSTRACT

The majority of common bean plants are cultivated under drought conditions. Maintaining crop yields under drought stress is thus one of the biggest challenges facing bean production. In order to improve our understanding of the complex mechanisms involved in the response of common bean (*Phaseolus vulgaris*) to drought stress, a proteomic approach was used to identify drought-responsive proteins in leaves of two cultivars differing in their response to drought, more sensitive Starozagorski čern and Tiber. 2D-DIGE was used to compare differences in protein abundance between control and stressed plants. Fifty-eight proteins whose abundance changed significantly were identified by LC-MS/MS in Tiber and 64 in Starozagorski čern. The majority of identified proteins were classified into functional categories that include energy metabolism, photosynthesis, ATP interconversion, protein synthesis and proteolysis, stress and defence related proteins. Details of the function of the identified proteins and their abundance profiles in Tiber and Starozagorski are discussed. Interactions between identified proteins were demonstrated by bioinformatics analysis, enabling a more complete insight into biological pathways and molecular functions affected by drought stress. The results form the basis for a further understanding of the biochemical mechanisms of drought response in common bean.

1. Introduction

Drought stress is one of the main abiotic stresses limiting agricultural production of many important crops such as legumes, including common bean (*Phaseolus vulgaris* L.). Sixty per cent of the world's common bean grows under rain fed conditions, and drought causes yield losses up to 80% in some regions [1]. The response of common bean to drought has not been widely studied, so an improved understanding of drought stress response mechanisms could help in developing drought resistant lines, consequently contributing to high productivity of this important food legume [2].

The complex drought stress response in plants comprises several pathways, starting from stress perception and ending with changes in expression of specific genes, changes in composition of plant transcriptome, proteome and metabolome that result in the adjustment of metabolism and the generation of regulatory networks involved in protecting cells from the damaging effects of the stress [3]. Identifying genes responsible for drought resistance is necessary for understanding the molecular basis for regulation of the response to water deficit. Only a few studies of the common bean response to drought stress on the genetic level have been reported. Gene expression at the transcript level enabled drought-responsive genes in roots of common bean to be identified [4]. Kavar et al. [5] reported a similar study on leaves, where expression of 15 transcripts was changed significantly, mainly of those with functions in cellular and carbohydrate metabolism, transcription, stress protection and photosynthesis. It is well known that specific transcription factors play essential roles in the drought stress response [6]. Rodriguez-Uribe and O'Connell [7] identified a water deficit responsive bZIP transcription factor in tepary bean *(Phaseolus acutifolius)* and in common bean. Comparative transcript profiling of roots revealed that 64 identified drought responsive genes in common bean were grouped mainly in the functional class of stress responsive genes, while 488 identified genes in tepary bean were unannotated [8]. Cruz de Carvalho et al.

[9] reported the upregulation of an aspartic protease precursor gene in leaves under drought. Furthermore, it was shown that water stress regulates the activity of an aspartic acid protease precursor at both the transcriptional and post-transcriptional levels. This response occurs earlier and more strongly in the drought-susceptible cultivar [10]. A study of expressed sequence tags provided full length cDNA libraries, enabling *P. vulgaris* genes related to response to drought to be identified [11]. These EST tags should be useful for functional gene annotation, analysis of splice site variations, intron/exon determination and evaluation of gene homology.

Besides studies of drought stress at the gene level, proteomic studies are necessary for revealing the role of proteins in the complex mechanisms of stress response in plants. Several proteomic studies of the drought response in various plant species have recently been reviewed [3]. In legumes, the effect of drought stress on protein expression has been studied, not only in model legumes such as soybean [12,13] and *Medicago truncatula* [14,15], but also in peanut [16], chickpea [17] and forage legumes [18]. Changes of protein level in soybean roots subjected to short term drought were analyzed by Alam et al. [13]. Significant variation of the abundance of 45 protein spots was detected. Of these, 21 proteins were decreased and 5 proteins increased in abundance, while dehydrin and ferritin were detected only under drought conditions. In a study of soybean roots, performed by Yamaguchi et al. [12], two regions of primary roots were analyzed. Several proteins that increased in abundance in drought-stressed roots were related to protection from oxidative damage. Ferritin, which contributes to the reduction of highly reactive free iron and the formation of toxic hydroxyl radicals in soybean primary roots, was reported in higher abundance. The Rhizobium-legume symbioses have also been studied and examined under drought conditions in *Medicago truncatula*, where a significant reduction of symbiotic nitrogen fixation under drought was noted [14]. Moreover, a later study revealed the absence of correlation between the reduction of nitrogen fixation and various enzymes related to N assimilation, suggesting that reduction of symbiotic nitrogen fixation under drought is caused by impairment of bacteroid metabolism and N2 fixation, rather than by limitation of respiratory substrate [15]. However, compared to roots, only a few proteomic studies have been performed on the green tissues of drought stressed legumes. Analysis of the nuclear proteome from chickpea (*Cicer arietinum* L.) seedlings under dehydration revealed 205 differentially regulated proteins, while 147 identified proteins were involved in gene transcription and replication, molecular chaperones, cell signalling and chromatin remodelling [17]. Kottapalli et al. [16] analyzed the leaf proteome in three contrasting peanut genotypes subjected to drought stress, and a theoretical model of water stress tolerance in peanuts was developed.

The aim of the present study was to determine changes in the leaf proteome of common bean during drought stress. Two cultivars, previously shown to differ in their response to drought [19], were selected for the analysis. 2D-DIGE and LC/MS-MS were used to identify those proteins that exhibited changed abundance under drought stress. Their functions in drought stress mechanisms and the abundance patterns exhibited by the two cultivars are discussed.

2. Materials and methods

2.1. Plant material and growth conditions

Two cultivars of *Phaseolus vulgaris* L. (cv. Tiber from Clause Semences, France and cv. Starozagorski čern from Semenarna Ljubljana, Slovenia) were studied. The experiment was conducted in a greenhouse under natural light, temperature and moisture conditions, from the end of March till the middle of May 2010. Three seeds were sown in each of 14 cm pots containing a mixture of fertilized substrate (Klasmann, Germany) and vermiculite (1:1, v/v). Ten days after germination, seedlings were thinned to one plant per pot. Plants were regularly irrigated with tap water. Drought conditions were initiated 5 weeks after sowing. Half the plants were not watered and the rest irrigated regularly. The relative water content (RWC) of plants was measured on days 7, 12 and 17 after the beginning of water withdrawal. Plants for proteome analysis were harvested on days 12 and 17. To assess the ability of plants to recover from stress, the remaining stressed plants were re-watered and grown under controlled conditions for an additional seven days. Both stressed and control plants were collected at each harvesting step, in order to ensure the same developmental stage for comparative determinations of plant water status and for proteomic analysis. There were 5 replicates of drought-stressed plants and control plants for each of treatments.

The third trifoliate leaves were harvested, frozen immediately in liquid nitrogen and stored at -80°C until further analysis. The hydration state of leaves was defined by their RWC. Leaves for RWC were weighed immediately to determine fresh weight (FW), then placed in a petri dish to regain full turgor and weighed for turgid weight (TW). Leaves were then dried in an oven at 70°C to constant weight and their dry weight (DW) determined. The RWC was calculated according to the formula: RWC (%) = [(FW−DW)/(TW− DW)] × 100. Each plant was treated individually as a single replicate for the RWC calculation. The soil water content (SWC) was determined at each harvesting step. The following equation was used: SWC (%) = [(FW−DW)/DW] × 100, where FW is the fresh weight of a soil portion. Soil was oven dried at 105°C for 24 hours to determine its dry weight (DW).

2.2. Preparation of protein extracts

Proteins were extracted from four biological replicates of each set of treated plants, giving a total of 16 samples for each cultivar. Leaves of common bean were ground to a fine powder in liquid nitrogen using a pestle and mortar [20]. The sample was transferred to a centrifuge tube and cold 10% TCA in acetone with 1% DTT was added. Samples were kept at -20°C for at least 2 hours, then centrifuged at 13000 g for 15 min at 4°C. The resulting pellet was washed three times by suspending in acetone containing 1% DTT and centrifuged at 13000 *g* for 15 min at 4°C between each wash. The pellet was air dried and resuspended in solubilisation buffer comprising 7 M urea, 2 M thiourea, 4% CHAPS and 30 mM Tris, adjusted to pH 8.5. The solution was vortexed intensively for 30 min, then centrifuged at 20°C for 15 min at 13000 *g*. The supernatant was collected and stored at -80°C for further analysis. For the estimation of protein concentrations, equal amounts of total protein extracts were loaded on a 4-12% Bis-Tris NuPAGE Novex gel (BioRad) and stained with Coomassie Blue R-250 (CBB R-250).

2.3. Fluorescence labelling of proteins and two-dimensional gel electrophoresis

Proteins were labelled with G-Dyes from the Refraction 2D labelling kit (Dyeagnostics) developed for fluorescence 2-D DIGE technology. Each sample was labelled with 400 pmol of G-Dyes, incubated on ice for 30 min in the dark, quenched with labelling stop solution and then incubated on ice for 10 min in the dark, according to the manufacturer's protocol. Eight control and eight drought stressed samples from each cultivar were labelled separately with either G-Dye 200 or G-Dye 300, and the internal standard (a mixture of 16 samples for a single cultivar), was labelled with G-Dye 100. G-Dye 100, G-Dye 200 and G-Dye 300 labelled samples were combined for each of eight gels for a single cultivar. Samples were mixed with rehydration buffer containing 7 M urea, 2 M thiourea, 4 % CHAPS, 0.5 % IPG buffer (GE Healthcare) and a trace amount of bromphenol blue to a volume of 450 µl for loading on a 24 cm Immobiline DryStrip (BioRad) gel with pH range 5-8. Proteins were separated in the first dimension using the Ettan IPGPhor II unit (GE Healthcare), starting with a low initial voltage of 200 V, then stepwise increases to 8,000 V for a total of about 80 kVh. Following isoelectric focusing, gel strips were equilibrated for 20 min in equilibration buffer containing 50 mM Tris-HCl, pH 8.8, 6 M urea, 30 % glycerol, 2 % SDS and 1 % DTT, followed by another 20 min equilibration in which 1% DTT in equilibration buffer was replaced by 5% iodoacetamide. In the second dimension, proteins were resolved on 12.5% SDS-PAGE using the Ettan DALT twelve system (GE Healthcare Biosciences) at 2.5W/gel for 45 min, then at 25W/gel, until the bromophenol blue line reached the bottom of gels. Finally, two sets of eight gels (each containing internal standard, stressed and corresponding control samples) with fluorescence labelled proteins from common bean cultivars resulted from 2D gel electrophoresis.
Preparative gels were obtained in the same way as the analytical gels but with a higher protein load, in order to obtain adequate amounts of proteins from individual spots for identification. After electrophoretic separation, as described above, gels were stained in 50% methanol, 10% acetic acid and 0.1% CBB R-250 for one hour. The staining solution was removed and gels were destained for two hours in destaining solution consisting of 20% methanol and 7% acetic acid. Destained gels were rinsed in deionised water.

2.4. Gel imaging and data analysis

Gels were visualized using Ettan DIGE Imager (GE Healthcare) for scanning at corresponding wavelengths according to the manufacturer's instructions for the selected fluorescent dyes. Images were analyzed by

Progenesis SameSpot version 4.1 (Nonlinear Dynamics) for spot detection, gel matching and statistical analysis of spots. An internal standard was a normalization reference for calibration of the data between different samples. The separate analysis for each cultivar included alignment of gels to a reference image, whereas gel matching was performed automatically and matches were checked and corrected manually. Normalized spot volumes were used to compare different treated samples statistically and to determine fold change values. These data were imported into the Unscrambler version 10.0.1 (CAMO), which was used for an additional statistical analysis of the data by principal component analysis (PCA) and for significance testing by partial least squares (PLS) regression analysis. The selection of protein spots of interest for analysis by MS was based on fold change >1.3, ANOVA ($p < 0.05$ and $q < 0.05$) from Progenesis SameSpot software, and significance testing by PLS using the Jack-knife uncertainty test.

2.5. Preparation of peptide samples

Preparative gels stained with CBB R-250 were compared with the DIGE gels in order to match and localize spots of interest on Coomassie stained gels. Protein spots that differed significantly in abundance between control and stressed samples were excised manually, using pipette tips, and subjected to in-gel trypsin digestion according to Shevchenko et al. [21]. Picked gel plugs were washed extensively with 50% (v/v) acetonitrile to remove dye and SDS impurities. Colourless gel plugs were totally dried out with 100% acetonitrile and then rehydrated with sequencing-grade modified trypsin (Promega, Madison, WI, USA) in 50mM ammonium bicarbonate. Samples were kept on ice for 30 min; the remaining solution was then removed and replaced by 50mM ammonium bicarbonate; digestion was carried out overnight at 37°C. Digested peptides were extracted with 5% formic acid in 100% acetonitrile (1:1 (v/v)) and supernatant was collected. Gel pieces were washed once again with the same solution and once with 100% acetonitrile. Supernatants were combined, dried down in a speed-vac bench-top centrifuge and resuspended in 0.1% formic acid. Extracted peptides were analyzed immediately by MS or frozen at -80°C.

2.6. MS analysis

LC-MS/MS analysis of tryptic digested peptides was performed with an Agilent 1200 nanoflow HPLC system consisting of binary pumps coupled to an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a nanoelectrospray ion source. Typically, 6 µl of sample was injected onto the extraction column (5 by 0.3 mm) filled with Zorbax 300 SB-C18 of 0.5 µm particle size (Agilent). Samples were washed with a mobile aqueous phase of 0.1% formic acid and 3% acetonitrile. The flow rate provided by the capillary pump was 4µl/min. After 7 min, the integrated switching valve was activated, and the peptides eluted in the back-flush mode from the extraction column onto a 150 × 0.075-mm C18 column (GlycproSIL C18-80Å, Glycpromass, Stove, Germany), with 3-µm resin. The mobile phase consisted of acetonitrile and MS grade water, both containing 0.1% formic acid. Chromatographic separation was achieved using a binary gradient from 5 to 55% acetonitrile in 60 min. The nanoflow pump flow rate was 0.2 µl/min.
Mass spectra were acquired in the positive-ion mode by applying a data dependent automatic switch between the survey scan and tandem mass spectrum (MS/MS) acquisition. Peptide samples were analyzed by the high-energy collision dissociation (HCD) fragmentation method by acquiring one Orbitrap survey scan in the mass range from *m/z* 300 to 2000, followed by MS/MS of the three most intense ions in the Orbitrap. The target value in the LTQ-Orbitrap was 1,000,000 for a survey scan at a resolution of 30,000 at *m/z* 400 using lock masses for recalibration to improve the mass accuracy of precursor ions. Fragmentation in the C-trap was performed by collision-induced dissociation with a target value of 5,000 ions. The ion selection threshold was 500 counts.

2.7. Data analysis and database searching

Raw data files produced in Xcalibur software (Thermo Fisher Scientific, Bremen, Germany) were converted to *.mgf files using BioWorks 3.2 software. These files were submitted to MASCOT searches within the Matrix Science web server (www.matrixscience.com). Searches were performed using the NCBInr database with a taxonomy parameter set to green plants. For the database search, parameters such as one missed cleavage site by trypsin, an MS tolerance of 10 ppm and an MS/MS tolerance of 0.05 Da, peptide charge of 2+, 3+ and 4+,

carbamidomethylation of cysteine and oxidation of methionine as fixed and variable modification were used. Expected cut off value was set to 0.05 for removing low scoring matches. The MASCOT score, number of peptide matches, sequence coverage, pI, and molecular weight were used to evaluate the database search results. The MS/MS spectra from all protein spots, with special attention to those proteins whose identification was based on one or two peptide sequences, were inspected manually. In addition, primary database searching was confirmed with "Plant_EST" database queries, with parameters set as before. Sequences of proteins identified as unknown, hypothetical or proteins with an uncharacterized function were used as queries for searching their homologues with BLASTP algorithm (www.ncbi.nlm.nih.gov/BLAST).

2.8. Bioinformatic analysis

The function of the identified proteins was elucidated by using the gene index accompanied Uniprot accession number as input for the Uniprot database (http://www. uniprot.org). To better understand functions and interactions of identified proteins, a protein-protein interaction network (PPI) was predicted. Since protein identification was based upon different organisms listed in the NCBI Viridiplantae database, all identified proteins were blasted against the *Arabidopsis thaliana* TAIR10 (The Arabidopsis Information Resource) protein database (http://www.arabidopsis.org/) with the intention of obtaining annotated protein entries for PPI tools. Results with the highest score and lowest E value were considered as relevant for each identified protein (Supplementary Table 1). A PPI was constructed with the online analysis tool STRING 9.0 [22]. Biological processes and molecular functions were predicted by BiNGO 2.44 [23], a plugin for Cytoscape. A hypergeometric test with Benjamini and Hochberg correction, p-value of 0.001 and *Arabidopsis thaliana* taxonomy were selected for search parameters.

3. Results

Two cultivars of common bean were included in the leaf proteome analysis. Leaves of plants in two stages of drought stress were studied, 12 days and 17 days after the withdrawal of water. These points were chosen on the basis of relative changes of leaf RWC. After 12 days without watering, RWC in the third trifoliate leaves dropped to 70.4±3.6 % for Starozagorski and 75.6±4.3 % for Tiber cultivar. After 17 days following withdrawal of water, leaf RWC was 44.8±8.2 % for Starozagorski and 45.0±3.5 % for Tiber. RWC in leaves of well-watered plants changed little during the treatment period for both cultivars (above 80 % during all stages). In addition, the SWC was measured at these two time points and was between 25.5±1.6 % (Starozagorski) and 29.6±4.8 % (Tiber) at day 12, reaching 17.5±1.2 % for Starozagorski and 18.3±0.7 % for Tiber at day 17. Control plants were irrigated to maximum retention capacity. The remaining stressed plants were re-watered after 17 days of water deficit. Under growth conditions in the greenhouse these plants regained the RWC of control plants, showing that the chosen duration of drought stress did not bring plants to the point where they were unable to overcome the stress upon re-watering.

3.1. Analysis of proteins by 2D-DIGE and protein identification

To determine the proteins whose abundance changed during drought stress, a proteomic study using 2D-DIGE followed by LC-MS/MS was performed. Based on the preliminary separation of leaf protein extract by 2D-PAGE in the 3-10 pH range, it was observed that only a small number of protein spots were located in the highly acidic and basic area (data not shown). For this reason, a pH range of 5 to 8 was selected for further experiments. 2D-DIGE analysis of leaf protein extracts was performed with 4 biological replicates for the two treatments in each cultivar. A total of 543 protein spots were matched across all the gels in the proteomic analysis of cultivar Starozagorski and 400 spots in the case of Tiber. The number of spots matched between cultivars differs due to separate labelling and electrophoretic separation for the two cultivars. One gel was excluded from the analysis in Tiber, due to contamination of the strip. All matched protein spots across the gels were included in the PCA testing to identify sample outliers and to group samples from different stages of treatment for each cultivar (Fig. 1). PCA distinguishes well-watered plants from drought stressed plants in both cultivars, with one mis-grouped control sample in the analysis of Starozagorski and Tiber. Component 1 explains 32% (Tiber) and 38% (Starozagorski) of the total variation and component 2 explains 17% and 11% of the variation. Sixty eight

protein spots changed significantly in abundance between stressed and control plants in Starozagorski and 62 were determined for cultivar Tiber. All these spots could also be localized and excised from the preparative gels (Fig. 2). Of the 68 protein spots, 36 were more abundant and 32 less abundant in drought-stressed plants than in control plants in Starozagorski. Of the identified proteins in Tiber, 42 spots were more abundant and 20 less abundant. A total of 58 proteins were successfully identified in Tiber and 64 in Starozagorski. Eight protein spots were not identified, due to their very low abundance and hence the inability to extract them successfully from the preparative gels, or to lack of sequence similarity in the database. To investigate the influence of different stages of drought stress on the leaf proteome, protein abundance profiles of plants at days 12 and 17 were compared. The main differences in normalized volumes of protein spots between drought stressed and control were observed in samples taken at day 17. Samples subjected to drought for 12 days also differed from control samples but to a lesser extent (data not shown), indicating an early influence of drought on the leaf proteome. Only significant changes in abundance of proteins between untreated and drought-treated samples were considered in our study.

Fig. 1 - PCA score plots of Tiber (A) and Starozagorski (B). Plants were harvested on days 12 and 17 after the beginning of withholding water. S1, S2, S3 and S4 represent the stressed plants corresponding to the 4 biological replicates. C1, C2, C3 and C4 refer to control plants, representing 4 biological replicates. Clustering of drought-stressed and control samples is evident. One sample from Tiber and the corresponding control sample were excluded from the analysis.

3.2. Functional distribution of identified proteins

In order to determine which types of proteins are involved in the drought stress response, the identified proteins were categorized into seven major groups based on their putative biological functions (Table 1). Fig. 3 shows the functional classification of the identified proteins. The majority are classified into the categories of photosynthesis and energy metabolism, while approximately one third are categorized into groups such as ATP interconversion, protein synthesis, proteolysis, folding, defence and stress-related proteins. These protein groups are common to both cultivars while, in Starozagorski, three proteins were classified into two additional categories, secondary metabolism and signal transduction. The proteins for which no known function could be assigned accounted for 19% of the identified proteins in Tiber and 25% in Starozagorski. Protein sequences of proteins with unknown function were searched for their homologues (Table 2). In Tiber, functional information was obtained for all protein homologues while, in Starozagorski, no information about biological function was available for five unknown proteins. Protein homologues could be classified into the above mentioned protein groups. Details about the function of identified proteins and their abundance profiles in Tiber and Starozagorski are discussed later.

Table 1- Proteins in common bean leaves with changed abundance following drought stress.

Table 2 - Homologues of the unknown, hypothetical or uncharacterized proteins. The NCBI non-redundant protein database within BLASTP was used to search homologue proteins.

Fig. 2 - 2D electrophoresis gels of extracts of leaves under drought in Tiber (A) and Starozagorski (B). Proteins were visualized by CBB staining. Identified proteins are numbered on the gels.

Fig. 3 - Functional classification of proteins identified from cultivars Tiber and Starozagorski. Protein groups are categorized based on their putative functions.

3.3. Protein-protein interaction analysis of identified proteins

Identified proteins were grouped into functional classes according to the biological processes in which they are involved. STRING and BiNGO, which offer an upgrade of the functional analysis, were used to visualize the protein-protein interaction, biological pathways and molecular functions.

The protein interaction network generated with STRING revealed the functional links between different proteins. In Tiber, two major clusters of interacting proteins are highlighted with circles as marked on Fig. 4 − proteins involved in photosynthesis and those involved in energy metabolism. Fructose-biphosphate aldolase (AT4G38970) is the central core protein of the interacting network, due to its interactions with many other proteins involved in photosynthesis and energy metabolism. To obtain statistically over- or under-represented categories of biological pathways and molecular functions related to drought stress, BiNGO was used to analyze identified proteins (Fig. 5). A complete list of the enriched Gene Ontology (GO) biological pathway and molecular function terms for the proteins is presented in Supplementary Table 2. One of the most significantly overrepresented biological pathways in Tiber includes the response to abiotic stimulus (p=2.90e-13). Two other major groups can be observed − regulation of protein metabolic processes and regulation of photosynthesis. The most highly enriched molecular functions are copper ion binding (p= 9.82e-08), catalytic activity (p= 1.07E-05) and antioxidant activity (p= 4.01E-05). A similar interaction network was built for Starozagorski (Supplementary Fig. 1).

Fig. 4 - Analysis of a functional network by STRING 9.0 (http://string-db.org). PPI is presented for the tolerant cultivar Tiber. *Arabidopsis thaliana* and confidence level of 0.4 were used for analysis parameters. Different line colours represent the types of evidence used in predicting the associations: gene fusion (red), neighbourhood (green), co-occurrence across genomes (blue), co-expression (black), experimental (purple), association in curated databases (light blue) or co-mentioned in PubMed abstracts (yellow). Two clusters of highly interacting protein nodes are marked with circles and include proteins involved in photosynthesis and energy metabolism.

Fig. 5 - Biological pathway (A) and molecular function (B) networks generated by BiNGO. GO categories of TAIR homologous proteins are presented for cultivar Tiber. The size of the node is related to the number of proteins and the colour represents the p-value for the statistical significance of the overrepresented GO term. For Starozagorski, a similar hierarchy of GO categories with the addition of pathways for secondary metabolism was obtained (Supplementary Fig. 1).

4. Discussion

Proteins whose abundance changes during drought stress in the leaves of common bean have been identified, using a proteomic approach. Cultivars Tiber and Starozagorski were chosen on the basis of the previous study of Hieng et al. [19], where it was shown that they differ at the levels of water potential and water content of leaves under drought conditions and of the integrity of their cellular membranes following osmotic stress. Based on these indicators it was concluded that Tiber was more tolerant to drought than Starozagorski. That study was carried out in a growth chamber under controlled conditions and was confirmed in the greenhouse. Our study was conducted only in the greenhouse in a different period of the year. RWC was used as a key indicator of the degree of cell and tissue hydration, which is crucial for optimum physiological functioning and growth processes. No significant differences in RWC between Tiber and Starozagorski were observed at any stage of water deficit. This could be explained by the growth conditions that differed from those used by Hieng et al. [19]. RWC was used as the basis for determining appropriate sampling times. Although at the end of our experiment plants were visibly stressed, they were able to revive on rehydration.

Identification of proteins whose abundances differed in stressed and control plants has revealed groups of proteins that differ with respect to their roles in response to drought conditions. About one third of the identified proteins were detected in multiple spots with different pIs or molecular masses, implying the existence of isoforms and posttranslational modification. The putative implications for identified proteins in drought stress are discussed below. The network clusters obtained can provide a broader insight into the different roles of identified proteins. These proteins are included in a wide range of biological pathways that are involved, either directly or indirectly, in plant protection.

4.1. Proteins involved in photosynthesis

A large proportion of the proteins whose abundance changed significantly under drought are associated with photosynthesis. Rubisco appeared in multiple spots differing in pI and Mr, as has been observed in other species

under drought [24,25]. The presence of the same protein in different spots could indicate greater rates of protein degradation, as suggested by Hajheidari et al. [24]. Luo et al. [26] showed that Rubisco large subunit can be cleaved by reactive oxygen species (ROS) generated at the metal-binding site. In addition to Rubisco, phosphoribulokinase is also involved in the Calvin cycle. In our study, it was found to decrease in abundance under drought and, together with the reduced abundance of Rubisco, it suggests that the efficiency of $C0_2$ fixation is decreased under drought stress. Besides the identification of Rubisco large subunit fragments and Rubisco small subunit − which had lower abundances under drought in both cultivars − Rubisco subunit-binding protein beta subunit was identified only in Tiber.

Oxygen evolving enhancer proteins were identified among the proteins that exhibited changes in abundance profile and showed contrasting patterns between cultivars. In Tiber, their abundance was increased and, in Starozagorski, reduced. Both oxygen evolving enhancer proteins 1 and 2 (OEE1 and OEE2) belong to the oxygen-evolving complex in photosystem II. OEE2 is responsible for catalysing the splitting of water and OEE1 is a manganese-stabilizing protein required for PSII core assembly/stability [27,28]. Gazanchian et al. [29] have reported increased expression levels of OEE2 in response to severe drought stress on wheatgrass, concluding its requirement for repairing protein damage caused by dissociation and for retaining the formation of oxygen. In contrast, a few reports have shown decreased abundance of OEE proteins during salt stress in potato [30] and wheat [31].

Another protein involved in photosynthesis is carbonic anhydrase. This protein is involved in CO_2 fixation [32] and was identified in both cultivars. In Tiber, it showed increased and reduced abundance during stress while, in Starozagorski, abundance upon stress was reduced. As evidenced in our study, the same carbonic anhydrase identified in different spots could be related to the presence of different isoforms, indicating that carbonic anhydrase isoforms may have complex overlapping roles during drought stress.

The abundance of chlorophyll a/b binding proteins, as important components of the light harvesting complex [33], increased under drought in Tiber but was reduced in Starozagorski. Expression of genes for the light-harvesting chlorophyll a/b-binding proteins was reported to be downregulated by oxidative stress [34]. Decreased abundance of ferredoxin NADP-reductase was detected only in Starozagorski. In the study on *Arabidopsis thaliana* the overall expression level of genes encoding this enzyme was found to increase under drought stress [35].

The decreased abundance of the identified proteins involved in photosynthesis in Starozagorski and their mixed abundance profiles in Tiber indicate that drought negatively affects the key proteins of the photosynthetic apparatus and, to a greater extent, those in Starozagorski. Earlier results of the influence of drought on gene expression in *P. vulgaris* [5] indicate that photosynthesis is inhibited later in Tiber than in Starozagorski. The fact that some of the identified proteins show opposite changes in abundance profiles for Tiber and Starozagorski is worthy of further investigation. Such studies should include direct proteomic analysis of the two cultivars and thus take into account possible differences in the abundance of proteins, since the levels of specific proteins may differ between cultivars.

4.2. Energy metabolism proteins

Plants also respond to stress conditions by triggering a network of events linked to energy metabolism. As expected, energy metabolism is affected by drought in both cultivars. Plastidic and cytoplasmic aldolase were identified in each cultivar. In the study of *Nicotiana* species, Yamada et al. [36] showed salt induced expression of the plastidic aldolase gene AldP2, whereas expression of AldP1 was decreased. In the analysis of cultivar Starozagorski, the levels of two other proteins involved in glycolysis, glyceraldehyde-3-phosphate dehydrogenase and glucose-6-phosphate 1-epimerase, were shown to change during stress. In Tiber, enolase increased in abundance. The greater abundance of enzymes such as triosephosphate isomerase, glyceraldehyde 3-phosphate dehydrogenase and enolase could be related to the need of cells for extra energy in order to deal with stress and repair damage. Furthermore, an increase in abundance of malate dehydrogenase was observed in both cultivars. Malate dehydrogenase is a component of the TCA cycle, an important source of energy for the cell. Further to energy metabolism, transketolase and ribulose phosphate 3-epimerase were decreased in abundance under drought in both cultivars. These enzymes catalyze reactions of the Calvin cycle and pentose phosphate pathway, thus influencing plant productivity under drought stress. Datko et al. [37] suggested that the downregulation of transketolase in heat stressed leaves of barley may induce inhibition of photosynthesis and

metabolism of aromatic acids. In addition to the above set of proteins involved in energy metabolism, two uncharacterized proteins found in spots 700 and 707 in Tiber show strong similarity to NAD dependent epimerase/dehydratase and the unknown protein detected in spot 324 Starozagorski was similar to alcohol dehydrogenase. Both proteins are associated with carbohydrate metabolism and their levels were increased slightly under drought conditions.

All the proteins described are important in metabolism. It is not surprising that proteins with different abundance profiles were identified in our study due to the wide range of proteins involved in the complex regulation and production of different metabolites within energy metabolism.

4.3. Stress response related proteins

A few of the proteins involved in defence against stress show significant changes in abundance in both cultivars. Drought stress is known to inhibit photosynthetic activity in tissues due to the imbalance between light capture and its utilization [38]. As a result, ROS, with the potential to cause cellular damage, are generated. Plants have evolved protective mechanisms to eliminate or reduce ROS levels. These protective mechanisms include the activity of antioxidative enzymes. Not surprisingly, enzymes involved in the oxidative stress response were also identified in our study. All the identified proteins related to ROS scavenging, defence and stress showed higher abundance under drought in Tiber and Starozagorski, where only quinone oxidoreductase-like protein and thioredoxin had reduced abundance.

Superoxide dismutase is very efficient at scavenging superoxide radicals by converting them to molecular oxygen and hydrogen peroxide. The latter is then reduced to water by peroxidase. Ascorbate peroxidase is well known to play an essential role in scavenging ROS, with ascorbate serving as the electron donor [39]. In addition to ascorbate peroxidase, a peroxiredoxin also catalyzes the detoxification of H_2O_2 and other peroxides. Peroxiredoxins are localized in different cellular compartments [40], but in this study only mitochondrial peroxiredoxins were found through BLASTP searching of unknown proteins. Another important antioxidant enzyme identified in our study is thioredoxin. It maintains the thiol redox balance through involvement in the redox regulation cycle and has a role in the regeneration of oxidized peroxiredoxin to the active, reduced form [39]. It showed slightly higher abundance in Tiber, but a homologue of the hypothetical protein identified in spot 836 in Starozagorski was found to be thioredoxin M4, which had reduced abundance under drought.

Another enzyme with a highlighted role under various stress conditions was identified as glutathione S transferase (GST). GST catalyses the conjugation of glutathione with a wide variety of hydrophobic and electrophilic compounds to form non-toxic peptide derivates [41]. Upregulation of GSTs has been associated with the response of wheat to drought [42] and of rice subjected to osmotic stress [43].

Besides excessive production of ROS in stressed plants, there is also an increased accumulation of cytotoxic methylglyoxal which is regulated through the glyoxalase system. The glyoxalase system consists of two enzymes; glyoxalase I, or lactoylglutathione lyase, and glyoxalase II or hydroxacylglutathione hydrolase [44]. These enzymes act coordinately to convert methylglyoxal and other 2-oxoaldehydes to their 2-hydroxyacids using glutathione as a cofactor.

A formate dehydrogenase, with increased abundance during stress in both cultivars, was identified. It is an NAD-dependent enzyme that catalyzes the oxidation of formate to CO_2. Formate is one of the stress signals in plants, thus formate dehydrogenase contributes to a detoxification process in plants under stress [45].

Two proteins, one belonging to the dehydrins and the other to heat shock proteins, were more abundant under drought conditions only in Starozagorski. The same proteins were not identified in Tiber although they are well known stress related proteins in plants. Dehydrins form a complex group of different proteins. It could be that dehydrins, whose accumulation was observed in Starozagorski, are not present or are modified in Tiber. The accumulation of dehydrin transcript was associated with a tolerance mechanism in the study of drought stressed sunflower [46], while three genes coding for dehydrin-like proteins were downregulated in common bean under chilling stress [47]. Heat shock proteins (HSP) have been induced under various stress conditions [48]. Hajheidari et al. [24] reported the induction of two small HSP under drought stress in sugar beet and strong induction of a small HSP is documented in the study of Vaseva et al. [18].

Another protein showing contrasting abundance between cultivars was identified as quinone oxidoreductase-like protein. The importance of this enzyme in defence against oxidative stress caused by salt stress was shown in the study of Nohzadeh Malakshah et al. [49]. Notably, Sobhanian et al. [50] reported downregulation of quinone

oxidoreductase, but its function is probably not related to soybean salt tolerance. However, based on the contrasting abundance of this protein between cultivars it is not possible to speculate on its role in drought tolerance in common bean.

4.4. Proteins involved in ATP interconversion

Nucleoside diphosphate kinase (NDPK) and ATP synthase were found to change significantly in abundance under drought stress in both cultivars. NDPK has a housekeeping role to maintain the balance between cellular ATP and other NTPs. Its differential expression occurs in response to heat [51], oxidative [52], cold [53], salt [54,55] and drought [24,56] stresses. In our study, it was more abundant under drought, which could associate NDPK with a general stress response mechanism. ATP synthase CF1 alpha subunit was reduced in abundance during stress in both cultivars, while the ATPase subunit 1 from mitochondria was increased in Starozagorski. ATP synthase is related to energy metabolism through synthesis of ATP which is further utilized in the reactions of photosynthesis. Thus, the decrease in abundance during stress is in accordance with the reduced photosynthesis during drought and decreased consumption of ATP [38]. Similarly, in drought stress, the transport of protons into the vacuole with vacuolar H+ ATPase may be slowed down due to the reduced energy metabolism. The different abundance patterns could be associated with the difference between ATP synthase from chloroplasts and that from mitochondria, and with their environments, which could be connected to the different responses of photosynthesis and respiration to stress [57].

4.5. Proteins related to synthesis, folding and proteolysis

Proteins involved in protein synthesis, such as glutamine synthetase, ribosomal proteins, cysteine synthetase and acetohydroxyacid synthase were increased in abundance under drought. Glutamine synthetase is essential for ammonium assimilation and the biosynthesis of glutamine, and cysteine synthase is the key enzyme in the synthesis of cysteine, which is required for biosynthesis of glutathione, a major factor in plant stress defence [58]. Acetohydroxyacid synthase catalyzes the first reaction in the synthesis of the branched-chain amino acids such as valine, leucine and isoleucine [59]. Under drought, together with the ribosomal proteins, they contribute to the synthesis of stress-defence proteins and other proteins as a strategy of cells to cope with stress.

Proteolysis-related proteins, which are necessary for maintaining cellular protein homeostasis, were also more abundant in stressed samples. This group includes cysteine proteinase precursors and beta and alpha type proteasome subunits. Proteins damaged by cell stress are degraded by proteasomes and proteolytic enzymes [60]. In our study, a protein involved in refolding misfolded proteins was identified as a 20 kDa chaperonin. In general, chaperones and chaperonins play a crucial role in protecting plants against stress by establishing normal protein conformations for cell function [48]. Another protein involved in protein folding is peptidyl-prolyl cis-trans isomerase or cyclophilin. In our study it had higher abundance in Tiber and reduced abundance in Starozagorski. Similar behaviour in abundance profile was observed in the study of peptidyl-prolyl cis-trans isomerase activity in two contrasting cultivars of sorghum under drought [61]. The authors suggested that this effect can be connected to different regulatory pathways in those cultivars.

4.6. Other protein groups

In addition to the functional categories described above, three important proteins identified in Starozagorski are classified under the group of secondary metabolism and signal transduction pathways. 1-deoxy-D-xylulose 5-phosphate reductoisomerase and the protein in spot 669, homologous to 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase, are both involved in the 2-C-methyl-D-erythritol-4- phosphate pathway of isoprenoid biosynthesis [62]. Isoprenoid compounds are involved in the response to different stress conditions [63,64]. They have diverse roles under environmental stress, including antioxidative functions and protection of membranes and photosynthetic apparatus against scavenging ROS. Since proteins involved in isoprenoid synthesis were identified only in Starozagorski, it is not clear whether this implies a relationship between isoprenoid biosynthesis and drought tolerance, as well as changed expression of isoprenoid biosynthesis.

Additionally, an increase in abundance of annexin-like protein, which is involved in signalling pathways, was observed. Again, increased abundance of the protein was observed only in Starozagorski. The connection of

certain annexins with plant tolerance to salt and drought stresses has been reported. In the proteome analysis of soybean hypocotyl, Sobhanian et al. [50] reported the upregulation of annexin, which indicates its important role in salt tolerance. Similarly, Konopka-Postupolska et al. [65] investigated the relationship between annexin and drought tolerance in *Arabidopsis thaliana*. Since our results do not fit well with the general concept that increased abundance of annexin proteins may contribute to more drought tolerant plants, further work is needed to clarify this point.

5. Conclusion

Our study has revealed that the levels of a number of proteins involved in various cellular pathways are affected during drought stress in common bean. Proteins that exhibited changes in abundance during drought in Tiber and Starozagorski cultivars were identified. These proteins are involved in known mechanisms associated with the general stress response in plants and the results provide new details of their involvement in drought stress in common bean. Our conclusions about the tolerance mechanisms to drought are based on the results of water deficit on the leaf proteome rather than on differences between cultivars. The results nevertheless suggest that certain identified proteins could be used as markers in the selection process for drought tolerance in common bean. Of those proteins showing contrasting abundance patterns between cultivars, the most outstanding are the oxygen evolving enhancer proteins, OEE1 and OEE2, and proteins that were identified in either of the two cultivars could be candidates. This needs to be confirmed by a proteomic comparison of the two cultivars, which will be the aim of our further study. This research provided the basic insight needed to further investigate the molecular regulatory mechanism of drought response in common bean.

Acknowledgements

The authors thank Anders Moen for assistance with mass spectrometry and Dr Marjetka Kidrič and Professor Roger H. Pain for critically reviewing the manuscript. This work was supported by the Slovenian Research Agency (Grants J4-4126 and P4-0072). Tanja Zadražnik gratefully acknowledges funding provided by the Slovenian Fellowship Programme and the Norwegian Financial Mechanism for mobility of researchers.

REFERENCES

[1] Cuellar-Ortiz SM, De La Paz Arrieta-Montiel M, Acosta-Gallegos J, Covarrubias AA. Relationship between carbohydrate partitioning and drought resistance in common bean. Plant Cell Environ 2008;31:1399–409.

[2] Beaver J, Osorno J. Achievements and limitations of contemporary common bean breeding using conventional and molecular approaches. Euphytica 2009;168:145–75.

[3] Kosová K, Vítámvás P, Prásil IT, Renaut J. Plant proteome changes under abiotic stress - Contribution of proteomics studies to understanding plant stress response. J Proteomics 2011;74:1301–22.

[4] Torres GAM, Pflieger S, Corre-Menguy F, Mazubert C, Hartmann C, Lelandais-Brière C. Identification of novel drought-related mRNAs in common bean roots by differential display RT-PCR. Plant Sci 2006;171:300–7.

[5] Kavar T, Maras M, Kidrič M, Šuštar-Vozlič J, Meglič V. Identification of genes involved in the response of leaves of *Phaseolus vulgaris* to drought stress. Mol Breeding 2008;21:159–72.

[6] Hussain SS, Kayani MA, Amjad M. Transcription factors as tools to engineer enhanced drought stress tolerance in plants. Biotechnol Progr 2011;27:297–306.

[7] Rodriguez-Uribe L, O'Connell MA. A root-specific bZIP transcription factor is responsive to water deficit stress in tepary bean (*Phaseolus acutifolius*) and common bean (*P. vulgaris*). J Exp Bot 2006;57:1391–8.

[8] Micheletto S, Rodriguez-Uribe L, Hernandez R, Richins RD, Curry J, O'Connell MA. Comparative transcript profiling in roots of *Phaseolus acutifolius* and *P. vulgaris* under water deficit stress. Plant Sci 2007;173:510–20.

[9] Cruz de Carvalho MH, d' Arcy-Lameta A, Roy-Macauley H, Gareil M, El Maarouf H, Pham-Thi A-T, Zuily-Fodil Y. Aspartic protease in leaves of common bean (*Phaseolus vulgaris* L.) and cowpea (*Vigna unguiculata* L. Walp): enzymatic activity, gene expression and relation to drought susceptibility. FEBS Lett 2001;492:242–6.

[10] Contour-Ansel D, Torres-Franklin ML, Zuily-Fodil Y, de Carvalho MHC. An aspartic acid protease from common bean is expressed "on call" during water stress and early recovery. J Plant Physiol 2010;167:1606–12.

[11] Blair MW, Fernandez AC, Ishitani M, Moreta D, Seki M, Ayling S, Shinozaki K. Construction and EST sequencing of full-length, drought stress cDNA libraries for common beans (*Phaseolus vulgaris* L.). BMC Plant Biol 2011;11:171.

[12] Yamaguchi M, Valliyodan B, Zhang J, Lenoble ME, Yu O, Rogers EE, Nguyen HT, Sharp RE. Regulation of growth response to water stress in the soybean primary root. I. Proteomic analysis reveals region-specific regulation of phenylpropanoid metabolism and control of free iron in the elongation zone. Plant Cell Environ 2010;33:223–43.

[13] Alam I, Sharmin SA, Kim K-H, Yang JK, Choi MS, Lee B-H. Proteome analysis of soybean roots subjected to short-term drought stress. Plant Soil 2010;333:491–505.

[14] Larrainzar E, Wienkoop S, Weckwerth W, Ladrera R, Arrese-Igor C, González EM. *Medicago truncatula* root nodule proteome analysis reveals differential plant and bacteroid responses to drought stress. Plant Physiol 2007;144:1495–1507.

[15] Larrainzar E, Wienkoop S, Scherling C, Kempa S, Ladrera R, Arrese-Igor C, Weckwerth W, González EM. Carbon metabolism and bacteroid functioning are involved in the regulation of nitrogen fixation in Medicago truncatula under drought and recovery. Mol Plant Microbe In 2009;22:1565–76.

[16] Kottapalli KR, Rakwal R, Shibato J, Burow G, Tissue D, Burke J, Puppala N, Burow M, Payton P. Physiology and proteomics of the water-deficit stress response in three contrasting peanut genotypes. Plant Cell Environ 2009;32:380–407.

[17] Pandey A, Chakraborty S, Datta A, Chakraborty N. Proteomics approach to identify dehydration responsive nuclear proteins from chickpea (*Cicer arietinum* L.). Mol Cell Proteomics 2008;7:88–107.

[18] Vaseva I, Akiscan Y, Demirevska K, Anders I, Feller U. Drought stress tolerance of red and white clover–comparative analysis of some chaperonins and dehydrins. Sci Hortic 2011;130:653–9.

[19] Hieng B, Ugrinović K, Šuštar-Vozlič J, Kidrič M. Different classes of proteases are involved in the response to drought of *Phaseolus vulgaris* L. cultivars differing in sensitivity. J Plant Physiol 2004;161:519–30.

[20] Méchin V, Damerval C, Zivy M. Total protein extraction with TCA-acetone. In: Thiellement H, Zivy M, Damerval C, Méchin V, editors. Plant Proteomics: Methods and Protocols, vol 335: Methods in Molecular Biology. Totowa NJ: Humana Press Inc; 2007, p.1-8
[21] Shevchenko A, Tomas H, Havlis J, Olsen JV, Mann M. In-gel digestion for mass spectrometric characterization of proteins and proteomes. Nat Protoc 2007;1:2856–60.
[22] Szklarczyk D, Franceschini A, Kuhn M, Simonovic M, Roth A, Minguez P, Doerks T, Stark M, Muller J, Bork P, Jensen LJ, von Mering C. The STRING database in 2011: functional interaction networks of proteins, globally integrated and scored. Nucleic Acids Res 2011;39:D561–568.
[23] Maere S, Heymans K, Kuiper M. BiNGO: a Cytoscape plugin to assess overrepresentation of Gene Ontology categories in Biological Networks. Bioinformatics 2005;21:3448 –3449.
[24] Hajheidari M, Abdollahian-Noghabi M, Askari H, Heidari M, Sadeghian SY, Ober ES, Salekdeh GH. Proteome analysis of sugar beet leaves under drought stress. Proteomics 2005;5:950–60.
[25] Durand TC, Sergeant K, Renaut J, Planchon S, Hoffmann L, Carpin S, Label P, Morabito D, Hausman JF. Poplar under drought: Comparison of leaf and cambial proteomic responses. J Proteomics 2011;74:1396–410.
[26] Luo S, Ishida H, Makino A, Mae T. Fe2+-catalyzed site-specific cleavage of the large subunit of ribulose 1,5-bisphosphate carboxylase close to the active site. J Biol Chem 2002;277:12382–12387.
[27] Yang EJ, Oh YA, Lee ES, Park AR, Cho SK, Yoo YJ, Park OK. Oxygen-evolving enhancer protein 2 is phosphorylated by glycine-rich protein 3/wall-associated kinase 1 in Arabidopsis. Biochem Bioph Res Co 2003;305:862–8.
[28] Yi X, McChargue M, Laborde S, Frankel LK, Bricker TM. The manganese-stabilizing protein is required for photosystem II assembly/stability and photoautotrophy in higher plants. J Biol Chem 2005;280:16170–4.
[29] Gazanchian A, Hajheidari M, Sima NK, Salekdeh GH. Proteome response of *Elymus elongatum* to severe water stress and recovery. J Exp Bot 2007;58:291–300.
[30] Aghaei K, Ehsanpour AA, Komatsu S. Proteome analysis of potato under salt stress. J Proteome Res 2008;7:4858–68.
[31] Gao L, Yan X, Li X, Guo G, Hu Y, Ma W, Yan Y. Proteome analysis of wheat leaf under salt stress by two-dimensional difference gel electrophoresis (2D-DIGE). Phytochemistry 2011;72:1180–91.
[32] Tripp BC, Smith K, Ferry JG. Carbonic anhydrase: new insights for an ancient enzyme. J Biol Chem 2001;276:48615–8.
[33] Xu Y-H, Liu R, Yan L, Liu Z-Q, Jiang S-C, Shen Y-Y, Wang X-F, Zhang D-P. Light-harvesting chlorophyll a/b-binding proteins are required for stomatal response to abscisic acid in *Arabidopsis*. J Exp Bot 2011;63:1095-1106.
[34] Staneloni RJ, Rodriguez-Batiller MJ, Casal JJ. Abscisic acid, high-light, and oxidative stress down-regulate a photosynthetic gene via a promoter motif not involved in phytochrome-mediated transcriptional regulation. Mol Plant 2008;1:75–83.
[35] Lehtimäki N, Lintala M, Allahverdiyeva Y, Aro E-M, Mulo P. Drought stress-induced upregulation of components involved in ferredoxin-dependent cyclic electron transfer. J Plant Physiol 2010;167:1018–22.
[36] Yamada S, Komori T, Hashimoto A, Kuwata S, Imaseki H, Kubo T. Differential expression of plastidic aldolase genes in *Nicotiana* plants under salt stress. Plant Sci 2000;154:61–69.
[37] Datko M, Zivcak M, Brestic M. Proteomic analysis of barley (*Hordeum vulgare* L.) leaves as affected by high temperature treatment. In: Allen JF, Gantt E, Golbeck JH, Osmond B, editors. Photosynthesis. Energy from the sun. Dordrecht: Springer Netherlands; 2008, p.1523-1527.
[38] Ramachandra Reddy A, Chaitanya KV, Vivekanandan M. Drought-induced responses of photosynthesis and antioxidant metabolism in higher plants. J Plant Physiol 2004;161:1189–202.
[39] Navrot N, Finnie C, Svensson B, Hägglund P. Plant redox proteomics. J Proteomics 2011;74:1450–62.
[40] Bhatt I, Tripathi BN. Plant peroxiredoxins: Catalytic mechanisms, functional significance and future perspectives. Biotechnol Adv 2011;29:850–9.
[41] Dixon DP, Skipsey M, Edwards R. Roles for glutathione transferases in plant secondary metabolism. Phytochemistry 2010;71:338–50.
[42] Bazargani MM, Sarhadi E, Bushehri A-AS, Matros A, Mock H-P, Naghavi M-R, Hajihoseini V, Mardi M, Hajirezaei M-R, Moradi F, Ehdaie B, Salekdeh GH. A proteomics view on the role of drought-induced

senescence and oxidative stress defense in enhanced stem reserves remobilization in wheat. J Proteomics 2011;74:1959–73.

[43] Zang X, Komatsu S. A proteomics approach for identifying osmotic-stress-related proteins in rice. Phytochemistry 2007;68:426–37.

[44] Thornalley PJ. The glyoxalase system: new developments towards functional characterization of a metabolic pathway fundamental to biological life. Biochem J 1990;269:1–11.

[45] Igamberdiev AU, Bykova NV, Kleczkowski LA. Origins and metabolism of formate in higher plants. Plant Physiol Bioch 1999;37:503–13.

[46] Cellier F, Conéjéro G, Breitler J-C, Casse F. Molecular and physiological responses to water deficit in drought-tolerant and drought-sensitive lines of sunflower. Plant Physiol 1998;116:319–28.

[47] Woronuk G, Vijayan P, Laberge S, Vandenberg B, Bett K. Transcriptomic analysis of chilling stress in *Phaseolus* spp. Environ Exp Bot 2010;69:95–104.

[48] Wang W, Vinocur B, Shoseyov O, Altman A. Role of plant heat-shock proteins and molecular chaperones in the abiotic stress response. Trends Plant Sci 2004;9:244–52.

[49] Nohzadeh Malakshah S, Habibi Rezaei M, Heidari M, Salekdeh GH. Proteomics reveals new salt responsive proteins associated with rice plasma membrane. Biosci Biotech Bioch 2007;71:2144–54.

[50] Sobhanian H, Razavizadeh R, Nanjo Y, Ehsanpour AA, Jazii FR, Motamed N, Komatsu S. Proteome analysis of soybean leaves, hypocotyls and roots under salt stress. Proteome Sci 2010;8:19.

[51] Escobar Galvis ML, Marttila S, Håkansson G, Forsberg J, Knorpp C. Heat stress response in pea involves interaction of mitochondrial nucleoside diphosphate kinase with a novel 86-kilodalton protein. Plant Physiol 2001;126:69–77.

[52] Moon H, Lee B, Choi G, Shin D, Prasad DT, Lee O, Kwak S-S, Kim DH, Nam J, Bahk J, Hong JC, Lee SY, Cho MJ, Lim CO, Yun D-J. NDP kinase 2 interacts with two oxidative stress-activated MAPKs to regulate cellular redox state and enhances multiple stress tolerance in transgenic plants. Proc Natl Acad Sci U S A 2003;100:358–363.

[53] Imin N, Kerim T, Rolfe BG, Weinman JJ. Effect of early cold stress on the maturation of rice anthers. Proteomics 2004;4:1873–82.

[54] Kawasaki S, Borchert C, Deyholos M, Wang H, Brazille S, Kawai K, Galbraith D, Bohnert HJ. Gene expression profiles during the initial phase of salt stress in rice. Plant Cell 2001;13:889–905.

[55] Dooki AD, Mayer-Posner FJ, Askari H, Zaiee A, Salekdeh GH. Proteomic responses of rice young panicles to salinity. Proteomics 2006;6:6498–507.

[56] Salekdeh GH, Siopongco J, Wade LJ, Ghareyazie B, Bennett J. Proteomic analysis of rice leaves during drought stress and recovery. Proteomics 2002;2:1131–45.

[57] Tezara W, Mitchell VJ, Driscoll SD, Lawlor DW. Water stress inhibits plant photosynthesis by decreasing coupling factor and ATP. Nature 1999;401:914–7.

[58] Kopriva S, Rennenberg H. Control of sulphate assimilation and glutathione synthesis: interaction with N and C metabolism. J Exp Bot 2004;55:1831–42.

[59] Belenky I, Steinmetz A, Vyazmensky M, Barak Z, Tittmann K, Chipman DM. Many of the functional differences between acetohydroxyacid Synthase isozyme I and other AHASs are a result of the rapid formation and breakdown of the covalent acetolactate-ThDP adduct in AHAS I. FEBS J 2012;279:1967-79.

[60] Kurepa J, Wang S, Li Y, Smalle J. Proteasome regulation, plant growth and stress tolerance. Plant Signal Behav 2009;4:924–7.

[61] Sharma AD, Singh P. Comparative studies on drought-induced changes in peptidyl prolyl cis-trans isomerase activity in drought-tolerant and susceptible cultivars of *Sorghum bicolor*. Curr Sci 2003;87:911-918

[62] Lichtenthaler HK. The 1-deoxy-D-xylulose-5-phosphate pathway of isoprenoid biosynthesis in plants. Annu Rev Plant Physiol Plant Mol Biol 1999;50:47–65.

[63] Gill SS, Tuteja N. Reactive oxygen species and antioxidant machinery in abiotic stress tolerance in crop plants. Plant Physiol Bioch 2010;48:909–30.

[64] Loyola J, Verdugo I, González E, Casaretto JA, Ruiz-Lara S. Plastidic isoprenoid biosynthesis in tomato: physiological and molecular analysis in genotypes resistant and sensitive to drought stress. Plant Biol 2012;14:149–56.

[65] Konopka-Postupolska D, Clark G, Goch G, Debski J, Floras K, Cantero A, Fijolek B, Roux S, Hennig J. The role of annexin 1 in drought stress in *Arabidopsis*. Plant Physiol 2009;150:1394–1410.

Table 1- Proteins in common bean leaves with changed abundance following drought stress.

Spot no.[a]	Identified protein (species)	NCBI accession number	Score[b]	matched peptides[c]	% sequence coverage[d]	Teoretical pI/Mw (kDa)	Experimental pI/Mw (kDa)	Fold change[e]
	ATP interconversion							
	Tiber							
1098	Nucleoside diphosphate kinase [Arabidopsis thaliana]	gi|16396	117	2	9	7.0/16.3	7.2/15.9	-3.4
1235	ATP synthase CF1 alpha subunit [Phaseolus vulgaris]	gi|139387459	1406	20	42	5.2/55.7	5.4/66.3	3.0
230	ATP synthase CF1 alpha subunit [Phaseolus vulgaris]	gi|139387459	1092	20	48	5.2/55.7	5.5/66.0	2.1
231	ATP synthase CF1 alpha subunit [Phaseolus vulgaris]	gi|139387459	1150	19	45	5.2/55.7	5.6/66.0	3.1
161	V-H(+)-ATPase subunit A [Glycine max]	gi|156616913	933	14	32	5.4/69.0	5.6/75.9	1.4
	Starozagorski							
804	Nucleoside diphosphate kinase 1 [Pisum sativum]☐	gi|1346672	150	2	11	5.9/16.5	7.3/16.4	-2.0
805	Nucleoside diphosphate kinase [Arabidopsis thaliana]	gi|16396	177	3	18	7.0/16.3	6.7/16.3	-1.5
162	ATP synthase CF1 alpha subunit [Phaseolus vulgaris]	gi|139387459	940	14	51	5.2/55.7	5.5/66.7	1.9
164	ATP synthase CF1 alpha subunit [Phaseolus vulgaris]	gi|139387459	1247	20	43	5.2/55.7	5.4/66.0	1.5
171	ATPase subunit 1 [Vigna radiata]	gi|323149044	836	14	33	6.2/55.6	6.9/62.9	-2.0
	Protein synthesis							
	Tiber							
473	Glutamine synthetase PR-2 [Phaseolus vulgaris]☐	gi|121345	478	9	35	5.4/39.4	5.6/42.8	-2.5
619	30S ribosomal protein S5 [Arabidopsis thaliana]	gi|15226167	172	3	11	9.0/32.7	6.6/36.5	-2.5
	Starozagorski							
343	Glutamine synthetase PR-2 [Phaseolus vulgaris]☐	gi|121345	401	9	28	5.4/39.4	5.5/43.0	-2.7
394	Cysteine synthase-like [Glycine max]	gi|356573072	605	10	37	5.5/34.4	5.7/40.6	-1.3
157	Acetohydroxyacid synthase [Phaseolus vulgaris]	gi|258618634	358	6	13	6.8/70.7	6.8/68.8	-2.5
	Energy metabolism							
	Tiber							
523	Malate dehydrogenase [Plantago major]	gi|52851186	451	8	26	6.1/36.0	6.3/41.0	-1.7
536	Malate dehydrogenase [Glycine max]	gi|5929964	240	5	15	8.2/36.3	7.0/40.4	-1.7
749	Triosephosphate isomerase. putative [Ricinus communis]	gi|255576721	258	4	16	6.6/34.1	5.6/28.7	-1.9
725	Triose-phosphate isomerase [Phaseolus vulgaris var. nanus]	gi|57283985	829	12	53	5.9/27.4	6.2/30.4	-2.1
1195	Plastidic aldolase NPALDP1 [Nicotiana paniculata]	gi|4827251	692	9	20	6.9/42.8	6.0/42.2	2.6
1213	Plastidic aldolase NPALDP1 [Nicotiana paniculata]	gi|4827251376	376	5	20	6.9/42.8	6.8/41.5	1.8
458	Fructose-bisphosphate aldolase. cytoplasmic isozyme-like [Glycine max]	gi|356500825	757	11	32	7.1/38.5	7.5/43.4	-2.3
1193	Enolase [Glycine max]	gi|351724891	939	15	43	5.3/48.0	5.7/59.2	-2.2
136	Transketolase. chloroplastic-like [Glycine max]	gi|356576867	510	10	17	6.0/80.5	6.0/78.9	2.2
141	Transketolase. chloroplastic-like [Glycine max]	gi|356536526	581	9	20	6.2/80.7	6.1/78.8	1.8
774	Ribulose-phosphate 3-epimerase. chloroplastic-like [Glycine max]	gi|356511994	267	5	25	8.2/30.1	5.8/27.0	1.6
	Starozagorski							
885	Malate dehydrogenase [Glycine max]	gi|5929964	368	7	19	8.2/36.3	7.1/40.0	-1.7
546	Triose-phosphate isomerase [Phaseolus vulgaris var. nanus]	gi|57283985	604	13	54	5.9/27.4	6.5/28.8	-1.5
339	Fructose-bisphosphate aldolase. cytoplasmic isozyme 1 [Pisum sativum]☐	gi|1168408	532	6	18	6.4/38.7	5.9/43.3	2.4
373	Probable fructose-bisphosphate aldolase 2. chloroplastic-like [Glycine max]	gi|356538694	738	11	25	8.2/43.1	5.9/42.0	2.1
381	Plastidic aldolase NPALDP1 [Nicotiana paniculata]	gi|4827251	568	9	21	6.9/42.8	6.1/41.8	1.9
347	Fructose-bisphosphate aldolase. cytoplasmic isozyme-like [Glycine max]	gi|356500825	377	6	19	7.1/38.5	7.4/43.0	-2.3
357	Glyceraldehyde-3-phosphate dehydrogenase. cytosolic [Antirrhinum majus]☐	gi|120666	311	5	16	8.3/36.8	7.3/42.4	-1.7
442	Putative glucose-6-phosphate 1-epimerase-like [Glycine max]	gi|356540771	84	2	6	5.1/30.7	5.5/37.9	1.7
98	Transketolase. chloroplastic-like [Glycine max]	gi|356576867	503	10	12	6.0/80.5	6.1/78.8	1.5
101	Transketolase. chloroplastic-like [Glycine max]	gi|356576867	489	9	11	6.0/81.1	5.9/78.7	2.5
102	Transketolase. chloroplastic-like [Glycine max]	gi|356576867	533	10	14	6.0/80.5	6.0/78.4	2.0
105	Transketolase. chloroplastic-like [Glycine max]	gi|356576867	664	12	15	6.0/80.5	6.1/78.3	1.4
573	Ribulose-phosphate 3-epimerase. chloroplastic-like [Glycine max]	gi|356562858	315	5	28	7.7/30.1	5.7/27.5	1.6
	Photosynthesis							
	Tiber							
413	Ribulose bisphosphate carboxylase/oxygenase activase. chloroplastic [Phaseolus vulgaris]☐	gi|10720248	1341	21	56	8.2/48.3	5.6/44.4	2.3
506	Ribulose bisphosphate carboxylase/oxygenase activase. chloroplastic [Phaseolus vulgaris]☐	gi|10720248	376	6	19	8.2/48.3	6.2/41.8	1.7
1184	Ribulose bisphosphate carboxylase [Phaseolus vulgaris]	gi|809069	388	6	45	8.6/15.9	7.2/14.2	2.6
419	Ribulose bisphosphate carboxylase/oxygenase activase. chloroplastic [Phaseolus vulgaris]☐	gi|10720248	540	7	27	8.2/48.3	5.8/44.4	1.4
350	Rubisco activase [Glycine max]	gi|290766483	725	12	29	5.7/52.7	5.2/50.6	-2.3
187	RuBisCO large subunit-binding protein subunit beta. chloroplastic-like [Glycine max]	gi|356525839	1281	19	39	5.9/63.1	5.4/72.5	1.9
647	Oxygen-evolving enhancer protein 1. chloroplastic [Solanum tuberosum]☐	gi|131385	339	5	17	5.8/35.6	5.4/34.6	-1.7
812	Oxygen-evolving enhancer protein 2. chloroplastic [Pisum sativum]	gi|131390	164	3	12	8.3/28.2	6.5/25.3	-2.4
644	Oxygen-evolving enhancer protein 1 [Zea mays]	gi|195619530	399	6	19	5.6/34.8	5.3/34.7	-1.7
1197	Oxygen-evolving enhancer protein 1. chloroplastic-like [Glycine max]	gi|356559442	141	2	11	6.7/35.3	5.8/29.8	-1.8
645	Oxygen-evolving enhancer protein 1. chloroplastic-like [Glycine max]	gi|356559442	416	7	25	6.7/35.3	5.3/34.6	-1.4
804	Oxygen-evolving enhancer protein [Medicago truncatula]	gi|357494079	268	3	15	9.1/29.4	7.3/16.4	-2.0
832	Chlorophyll a-b binding protein 6A. chloroplastic	gi|115764	211	4	17	5.8/26.8	5.7/23.1	-1.6
714	Carbonic anhydrase [Phaseolus vulgaris]	gi|270342124	778	11	46	8.1/35.9	6.6/30.7	-2.2
763	Carbonic anhydrase [Phaseolus vulgaris]	gi|270342124	288	5	26	8.1/35.9	7.0/27.6	1.7
767	Carbonic anhydrase [Phaseolus vulgaris]	gi|270342124	274	6	24	8.1/35.9	6.6/27.3	2.0
784	Carbonic anhydrase [Phaseolus vulgaris]	gi|270342124	891	14	49	8.1/35.9	7.2/26.8	1.3
793	Carbonic anhydrase [Phaseolus vulgaris]	gi|270342124	725	11	45	8.1/35.9	7.7/26.6	-2.0
	Starozagorski							
464	Oxygen-evolving enhancer protein 1. chloroplastic-like [Glycine max]	gi|356559442	799	13	43	6.7/35.5	5.2/34.4	2.6
465	Oxygen-evolving enhancer protein 1. chloroplastic-like [Glycine max]	gi|356559442	851	15	46	6.7/35.5	5.3/34.4	1.8
877	Oxygen-evolving enhancer protein 2. chloroplastic-like [Glycine max]	gi|356567470	313	5	20	7.7/28.6	5.6/26.9	2.4
890	Oxygen-evolving enhancer protein 2. chloroplastic-like [Glycine max]	gi|356567470	374	6	20	7.7/28.6	6.0/26.7	1.9
557	Carbonic anhydrase [Phaseolus vulgaris]	gi|270342124	215	4	16	8.1/35.9	6.2/28.2	1.9
438	Chloroplast ferredoxin-NADP+ reductase [Pisum sativum]	gi|141448056	407	6	20	8.6/40.4	6.1/38.1	1.6
302	Ribulose bisphosphate carboxylase/oxygenase activase. chloroplastic [Phaseolus vulgaris]☐	gi|10720248	1401	21	52	8.2/48.3	5.4/46.1	2.9
851	Ribulose bisphosphate carboxylase [Phaseolus vulgaris]	gi|21050	213	4	28	9.2/20.4	7.2/14.2	3.8
919	Ribulose bisphosphate carboxylase/oxygenase activase. chloroplastic [Phaseolus vulgaris]☐	gi|10720248	1390	20	54	8.2/48.3	5.5/45.0	2.7
325	Ribulose bisphosphate carboxylase/oxygenase activase. chloroplastic [Phaseolus vulgaris]☐	gi|10720248	1441	20	55	8.2/48.3	5.7/44.3	2.2
320	Ribulose bisphosphate carboxylase/oxygenase activase. chloroplastic [Phaseolus vulgaris]☐	gi|10720248	759	13	34	8.2/48.3	5.7/44.6	1.8
321	Ribulose bisphosphate carboxylase/oxygenase activase. chloroplastic [Phaseolus vulgaris]☐	gi|10720248	578	8	25	8.2/48.3	5.9/44.6	1.4

	Proteolysis and folding							
	Tiber							
815	Proteasome subunit beta type [Medicago truncatula]	gi|357466571	265	5	29	5.3/24.9	5.5/24.8	-1.8
867	Peptidyl-prolyl cis-trans isomerase [Medicago truncatula]	gi|357511613	146	3	12	7.6/28.1	6.0/20.2	-2.8
892	Peptidyl-prolyl cis-trans isomerase [Medicago truncatula]	gi|357511613	152	3	12	7.6/28.1	6.0/19.5	-2.2
800	20 kDa chaperonin. chloroplastic-like [Glycine max]	gi|356547960	221	4	19	8.6/26.5	5.9/25.9	-1.9
679	cysteine proteinase precursor [Phaseolus vulgaris]	gi|2511691	152	3	12	6.4/40.4	5.7/33.0	-2.3
672	Cysteine proteinase CP2 [Phaseolus vulgaris]	gi|113208365	299	6	27	6.2/40.4	5.9/33.3	-2.2
	Starozagorski							
545	Proteasome subunit alpha type-2-A-like [Glycine max]	gi|356525754	430	8	35	5.5/25.6	6.0/28.9	-1.8
497	Cysteine proteinase precursor [Phaseolus vulgaris]	gi|2511691	262	5	16	6.4/40.4	5.9/32.4	-1.9
499	Cysteine proteinase precursor [Phaseolus vulgaris]	gi|2511691	155	3	10	6.4/40.4	5.5/32.3	-2.5
711	Putative peptidylprolyl isomerase [Oryza sativa Japonica Group]	gi|46359893	161	2	12	9.4/26.7	6.9/21.3	1.5
	Secondary metabolism							
	Starozagorski							
279	1-deoxy-D-xylulose 5-phosphate reductoisomerase [Glycine max]	gi|329402648	615	10	23	5.9/50.8	5.9/49.4	-1.6
1x								
	Signal transduction							
	Starozagorski							
448	Annexin-like protein RJ4-like [Glycine max]	gi|356556839	280	6	13	7.1/35.8	7.3/37.1	-3.6
	ROS scavering. defence. stress related							
	Tiber							
404	Formate dehydrogenase [Phaseolus vulgaris]	gi|270342112	261	5	20	6.5/41.5	6.8/44.5	-2.6
820	Manganese-superoxide dismutase [Glycine max]	gi|27526758	130	2	18	6.1/15.4	6.7/23.9	-2.3
730	Cytosolic ascorbate peroxidase [Vigna unguiculata]	gi|1420938	203	3	16	5.6/27.1	5.9/30.0	-1.7
753	Cytosolic ascorbate peroxidase [Vigna unguiculata]	gi|1420938	655	9	42	5.6/27.1	6.0/28.4	-1.5
959	Thioredoxin fold [Arachis hypogaea]	gi|115187464	205	4	18	5.6/17.5	5.8/18.3	-1.5
	Starozagorski							
626	Manganese-superoxide dismutase [Glycine max]	gi|27526758	178	2	18	6.1/15.4	6.7/25.0	-2.4
267	Peroxidase 1 precursor [Phaseolus vulgaris]	gi|5002342	421	6	19	5.8/37.1	6.8/52.3	-4.6
737	17.7 kDa class I small heat shock protein [Vigna unguiculata]	gi|154293473	237	5	39	6.8/17.7	7.0/20.3	-10.0
327	Formate dehydrogenase [Phaseolus vulgaris]	gi|270342112	878	15	67	6.5/41.5	6.8/44.2	-2.1
521	Dehydrin [Vigna unguiculata]	gi|6358640	121	2	16	6.0/26.5	7.0/30.1	-2.6
602	Putative glutathione S-transferase [Phaseolus acutifolius]	gi|21217741	195	3	23	5.6/24.8	6.2/26.6	-1.9
495	Hydroxyacylglutathione hydrolase [Medicago sativa]	gi|71534880	97	2	11	5.5/18.9	6.2/32.5	-2.1
400	Quinone oxidoreductase-like protein At1g23740. chloroplastic-like [Glycine max]	gi|356567630	631	10	31	9.0/42.0	6.2/40.3	1.5
396	Quinone oxidoreductase-like protein At1g23740. chloroplastic-like [Glycine max]	gi|356567630	423	7	28	9.0/42.0	5.9/40.5	1.7
	Unclear classification							
	Tiber							
431	unknown [Glycine max]	gi|255646270	407	8	28	5.9/45.8	5.2/44.1	1.8
794	unknown [Glycine max]	gi|255635846	148	3	17	7.7/28.6	5.4/26.3	2.2
779	unknown [Glycine max]	gi|255640167	177	2	32	8.6/29.8	5.9/26.9	-1.5
809	unknown [Glycine max]	gi|255627415	120	2	12	5.8/23.5	6.2/25.5	-2.3
831	hypothetical protein VITISV_028610 [Vitis vinifera]	gi|147787657	112	2	9	6.9/26.9	5.8/23.2	-2.3
673	hypothetical protein LOC100527131 [Glycine max]	gi|351727317	144	2	12	5.4/27.9	6.2/33.3	-3.0
700	PREDICTED: uncharacterized protein At2g37660. chloroplastic-like [Glycine max]	gi|356567949	547	10	36	5.7/27.7	5.5/31.4	-1.3
707	PREDICTED: uncharacterized protein At2g37660. chloroplastic-like [Glycine max]	gi|356567949	450	8	34	5.7/27.7	5.7/31.1	-1.5
888	hypothetical protein LOC100500096 [Glycine max]	gi|351722815	517	9	49	8.7/21.3	5.9/19.7	-2.2
1058	hypothetical protein LOC100500093 [Glycine max]	gi|351721369	446	7	50	5.5/15.3	5.8/16.6	-2.4
1234	unknown [Glycine max]	gi|255635896	255	4	10	6.7/34.5	6.7/39.9	-1.9
	Starozagorski							
385	unknown [Medicago truncatula]	gi|217072508	149	3	7	8.8/44.8	6.6/41.5	1.8
390	PREDICTED: uncharacterized protein At1g09340. chloroplastic-like [Glycine max]	gi|356572914	209	4	14	7.7/42.4	7.0/41.3	1.5
580	unknown [Glycine max]	gi|255635846	280	4	18	7.7/28.6	5.3/27.3	3.5
612	uncharacterized protein LOC100305513 [Glycine max]	gi|351721274	400	7	35	5.3/25.2	5.5/26.1	-1.4
623	unknown [Medicago truncatula]	gi|217071344	242	5	17	6.6/31.2	5.4/25.4	1.8
634	unknown [Glycine max]	gi|255636441	219	5	23	8.6/29.7	7.7/24.8	-2.5
669	unknown [Glycine max]	gi|255628349	267	5	26	8.4/24.5	7.7/23.5	-2.9
694	hypothetical protein LOC100500096 [Glycine max]	gi|351722815	368	7	35	8.7/21.3	5.8/21.9	-1.7
746	hypothetical protein LOC100499771 [Glycine max]	gi|351724985	339	6	23	5.4/17.5	5.7/19.6	-1.5
756	hypothetical protein LOC100500325 [Glycine max]	gi|351727066	73	1	8	6.0/17.5	6.4/18.7	-4.8
759	unknown [Glycine max]	gi|255630026	72	1	8	6.0/17.5	6.5/18.4	-3.4
781	hypothetical protein LOC100500093 [Glycine max]	gi|351721369	215	3	46	5.5/15.3	5.7/17.4	-1.7
836	hypothetical protein LOC100526924 [Glycine max]	gi|351725393	118	3	17	9.1/20.1	5.8/14.3	1.5
875	g5bf [Arabidopsis thaliana]	gi|2765081	245	5	11	8.2/42.8	6.8/41.0	2.6
449	unknown [Glycine max];	gi|255637721	328	5	19	5.4/31.7	5.6/37.0	-2.4
324	unknown [Glycine max]	gi|255640955	108	3	22	6.5/41.2	6.6/44.5	-1.5

Spots of interest were identified by LC/MS-MS as described under Material and methods.

[a] Protein spot refers to the numbered spots in Fig. 3.

[b] MASCOT protein score from MS analysis.

[c] The number of peptides identified for each protein.

[d] Percentage of amino acids in reference proteins covered by matching peptides from MS analysis.

[e] Fold change values from Progenesis SameSpot (negative values - increased in abundance under drought; positive values - decreased in abundance under drought).

Table 2 - Homologues of the unknown, hypothetical or uncharacterized proteins. The NCBI non-redundant protein database within BLASTP was used to search homologue proteins.

Spot no.	NCBI accession number of unknown protein	Name of the homologous protein (species)	NCBI accession number of homologue	Score	Identities (%)	Positives (%)
	Tibor					
431	gi|255646270	Phosphoribulokinase. chloroplastic-like [Glycine max]	gi|356495988	817	99	99
794	gi|255635846	Oxygen-evolving enhancer protein 2. chloroplastic-like [Glycine max]□	gi|356567470	523	99	99
779	gi|255640167	Chlorophyll a-b binding protein 3. chloroplastic-like [Glycine max]□	gi|356501755	469	99	99
809	gi|255627415	DHAR class glutathione transferase DHAR2 [Populus trichocarpa]	gi|283135906	352	82	88
831	gi|147787657	Thylakoid lumenal 19 kDa protein. chloroplast precursor. putative [Ricinus communis]	gi|255571642	257	85	93
673	gi|351727317	Hydroxyacylglutathione hydrolase [Medicago truncatula]	gi|355516798	431	83	92
700	gi|255642211	NAD-dependent epimerase/dehydratase [Zea mays]	gi|226499246	426	82	91
707	gi|255642211	NAD-dependent epimerase/dehydratase [Zea mays]	gi|226499246	426	82	91
888	gi|351722815	Mitochondrial peroxiredoxin [Pisum sativum]	gi|47775654	330	83	91
1058	gi|351721369	40S ribosomal protein S12 [Medicago truncatula]	gi|355512670	244	99	99
1234	gi|255635896	Quinone oxidoreductase-like protein At1g23740. chloroplastic-like [Glycine max]	gi|356571369	435	73	88
	Starozagorski					
580	gi|255635846	Oxygen-evolving enhancer protein 2. chloroplastic-like [Glycine max]□	gi|356567470	523	99	99
612	gi|255625747	Proteasome subunit beta type-6-like [Glycine max]	gi|356507848	447	97	99
623	gi|217071344	Chlorophyll A/B binding protein. putative [Ricinus communis]	gi|255585090	398	91	95
634	gi|255636441	ATP-dependent Clp protease proteolytic subunit 6. chloroplastic-like [Glycine max]	gi|356538797	511	99	99
669	gi|255628349	2-C-methyl-D-erythritol 2 4-cyclodiphosphate synthase [Medicago truncatula]	gi|357481553	369	82	88
694	gi|351722815	Mitochondrial peroxiredoxin [Pisum sativum]	gi|47775654	330	83	91
746	gi|351724985	Peroxiredoxin [Pisum sativum]	gi|189094613	305	93	98
781	gi|351721369	40S ribosomal protein S12 [Medicago truncatula]	gi|357482099	244	99	99
836	gi|351725393	Thioredoxin M4. chloroplastic-like [Glycine max]	gi|356546877	341	92	96
449	gi|255637721	Putative lactoylglutathione lyase-like [Glycine max]	gi|356520071	572	99	100
324	gi|255640955	Alcohol dehydrogenase class-3-like [Glycine max]	gi|356572303	775	99	99

Fig. 1 - PCA score plots of Tiber (A) and Starozagorski (B). Plants were harvested on days 12 and 17 after the beginning of withholding water. S1, S2, S3 and S4 represent the stressed plants corresponding to the 4 biological replicates. C1, C2, C3 and C4 refer to control plants, representing 4 biological replicates. Clustering of drought-stressed and control samples is evident. One sample from Tiber and the corresponding control sample were excluded from the analysis.

Fig. 2 - 2D electrophoresis gels of extracts of leaves under drought in Tiber (A) and Starozagorski (B). Proteins were visualized by CBB staining. Identified proteins are numbered on the gels.

Fig. 3 - Functional classification of proteins identified from cultivars Tiber and Starozagorski. Protein groups are categorized based on their putative functions.

Fig. 4 - Analysis of a functional network by STRING 9.0 (http://string-db.org). PPI is presented for the tolerant cultivar Tiber. *Arabidopsis thaliana* and confidence level of 0.4 were used for analysis parameters. Different line colours represent the types of evidence used in predicting the associations: gene fusion (red), neighbourhood (green), co-occurrence across genomes (blue), co-expression (black), experimental (purple), association in curated databases (light blue) or co-mentioned in PubMed abstracts (yellow). Two clusters of highly interacting protein nodes are marked with circles and include proteins involved in photosynthesis and energy metabolism.

Fig. 5 - Biological pathway (A) and molecular function (B) networks generated by BiNGO. GO categories of TAIR homologous proteins are presented for cultivar Tiber. The size of the node is related to the number of proteins and the colour represents the p-value for the statistical significance of the overrepresented GO term. For Starozagorski, a similar hierarchy of GO categories with the addition of pathways for secondary metabolism was obtained (Supplementary Fig. 1).

A

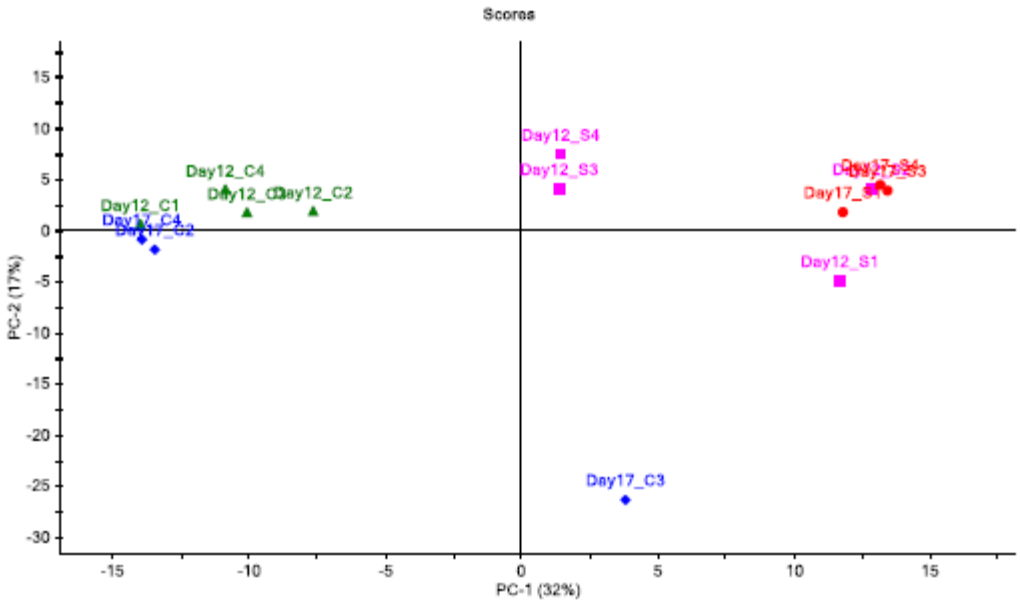


Figure 1A

B

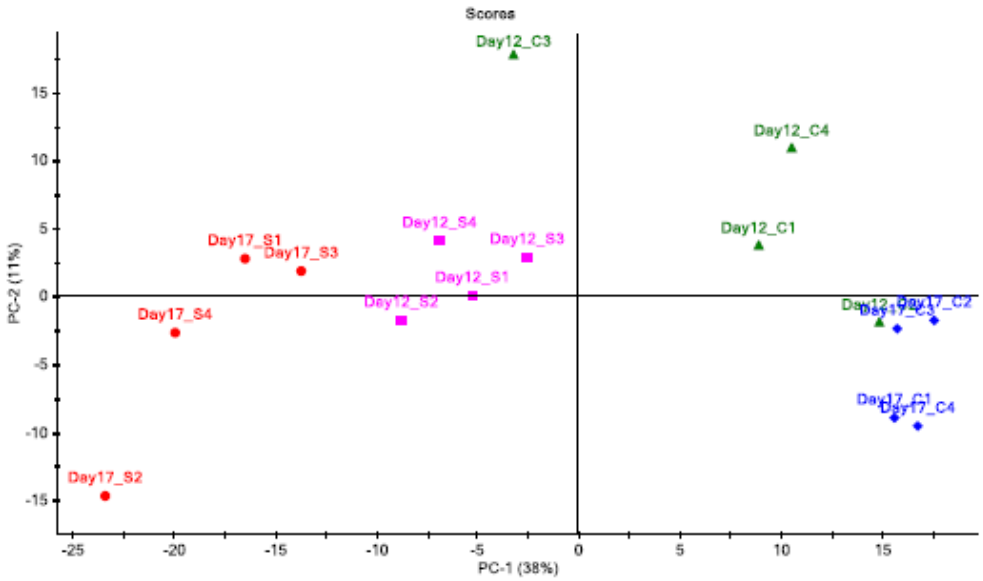


Figure 1B

A

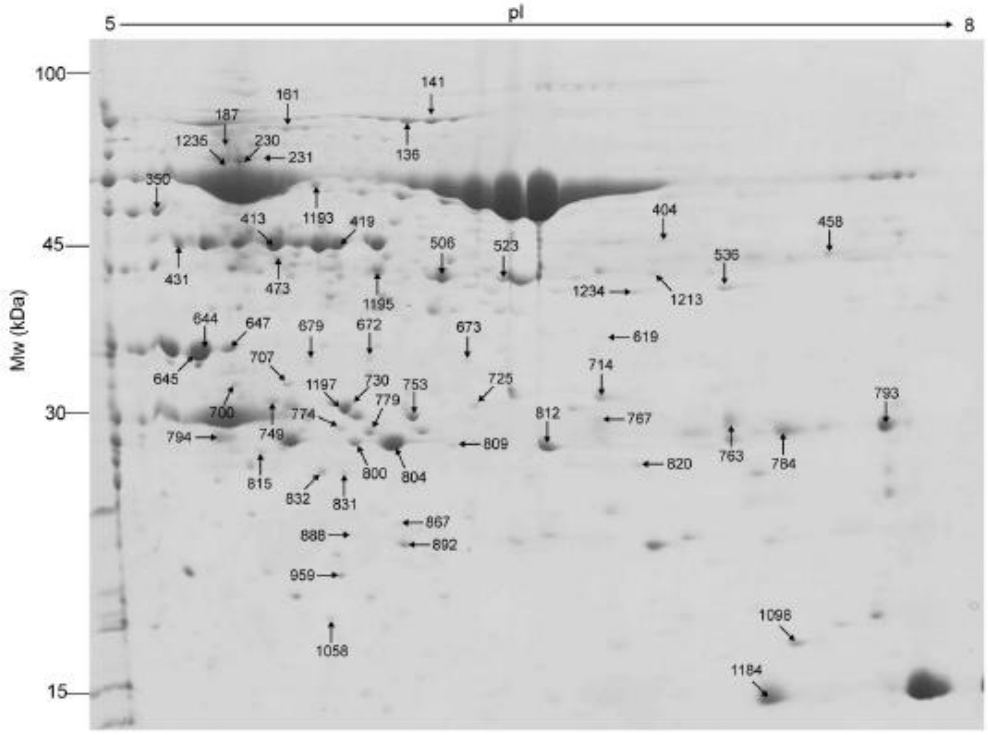


Figure 2A

B

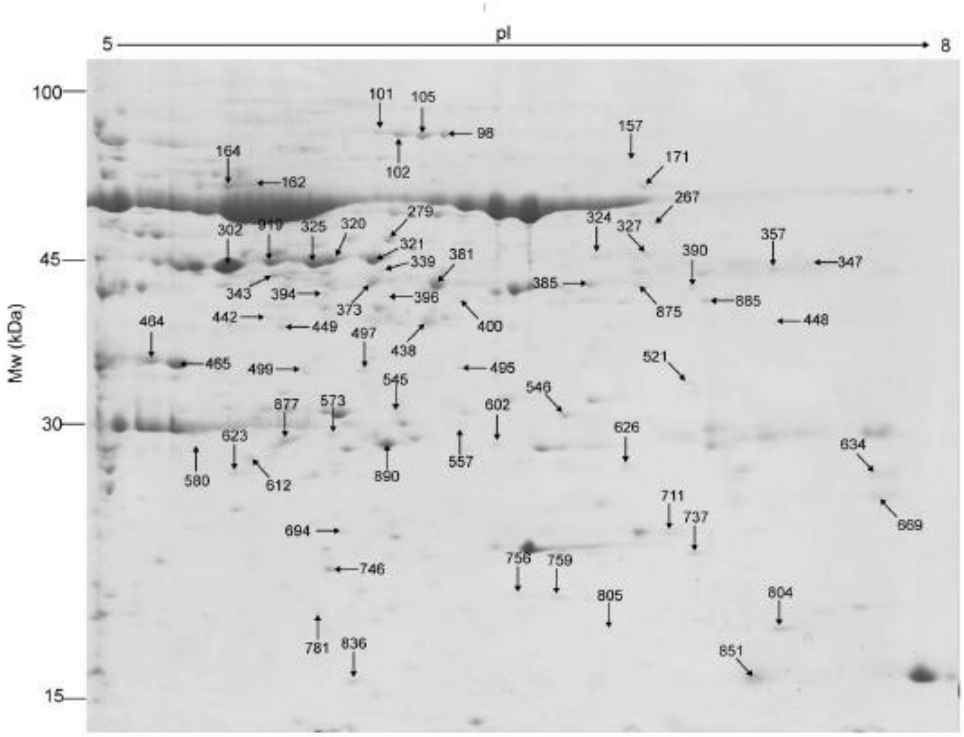


Figure 2B

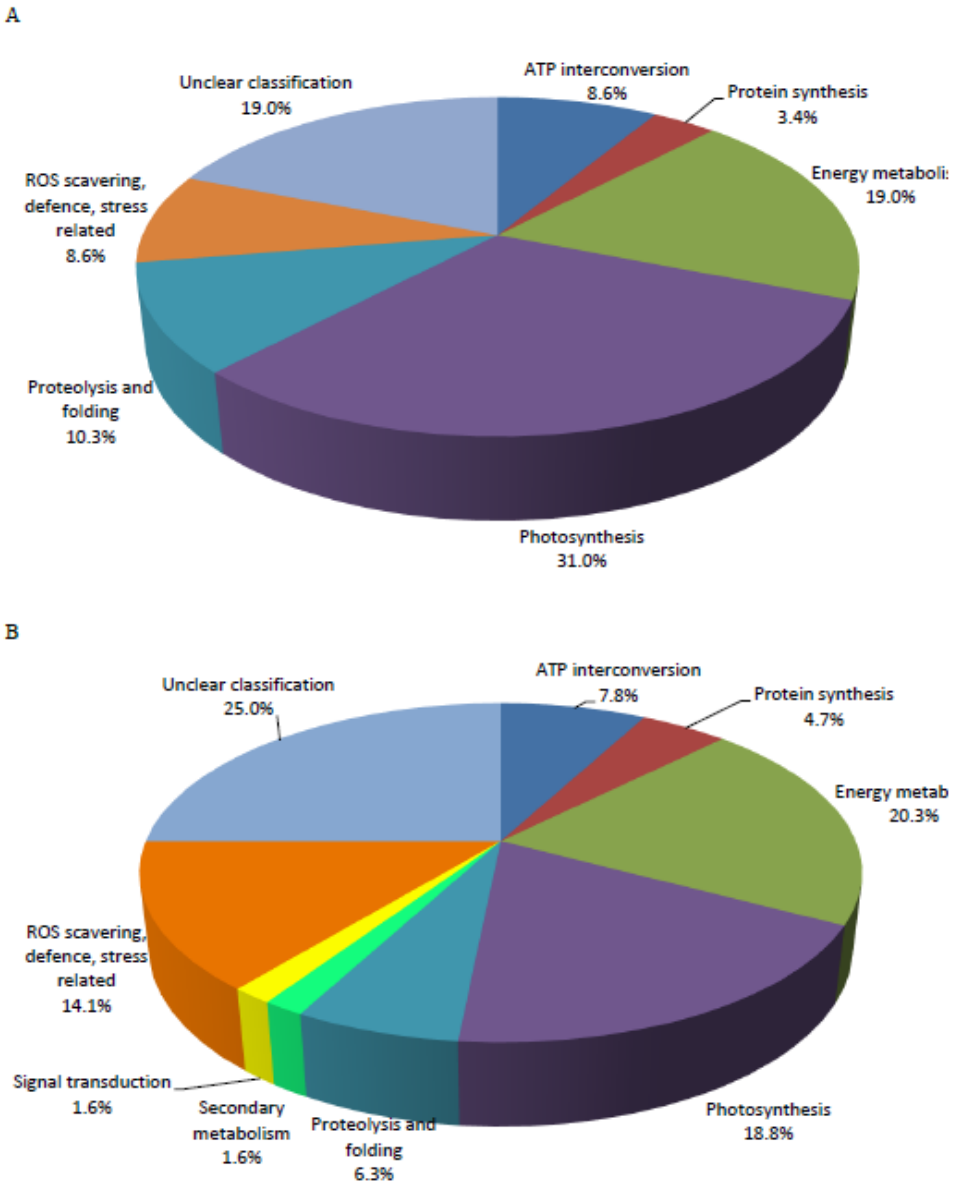


Figure 3

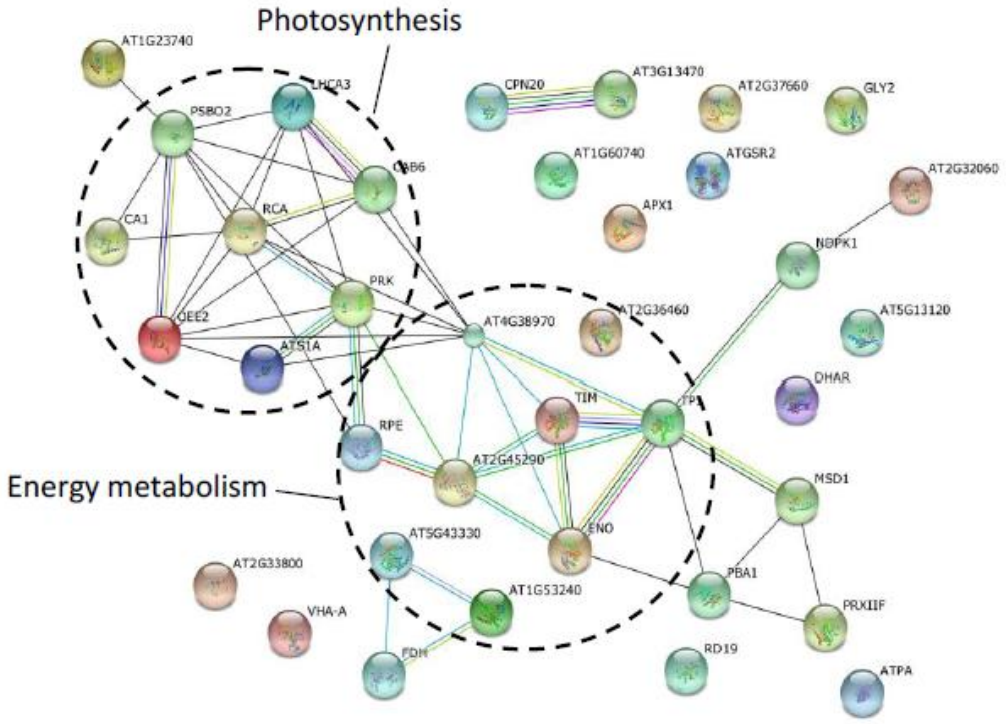


Figure 4

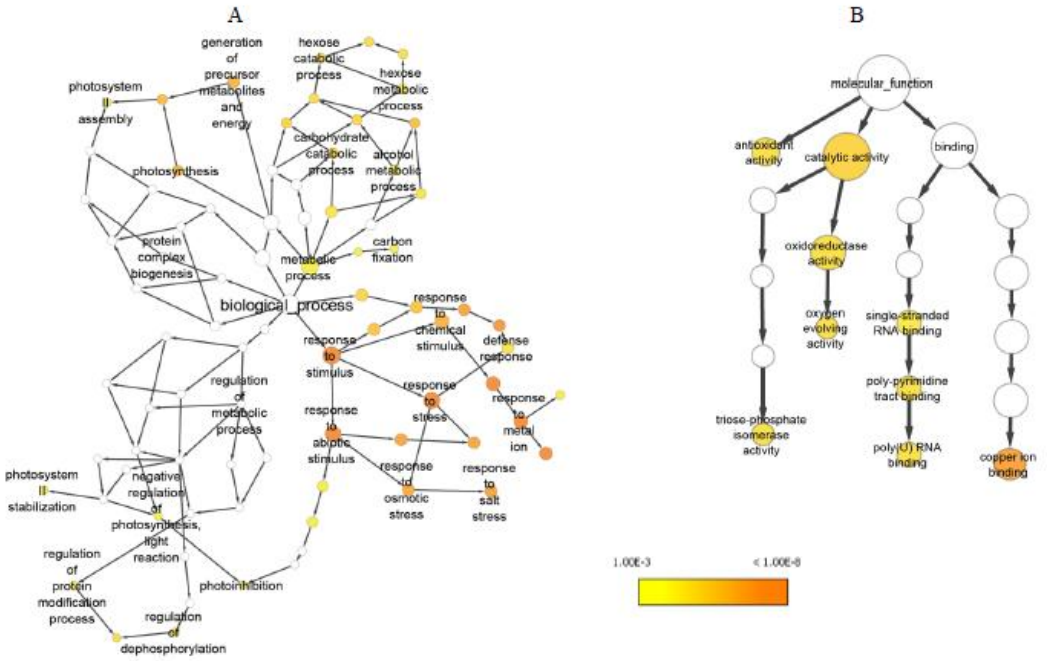
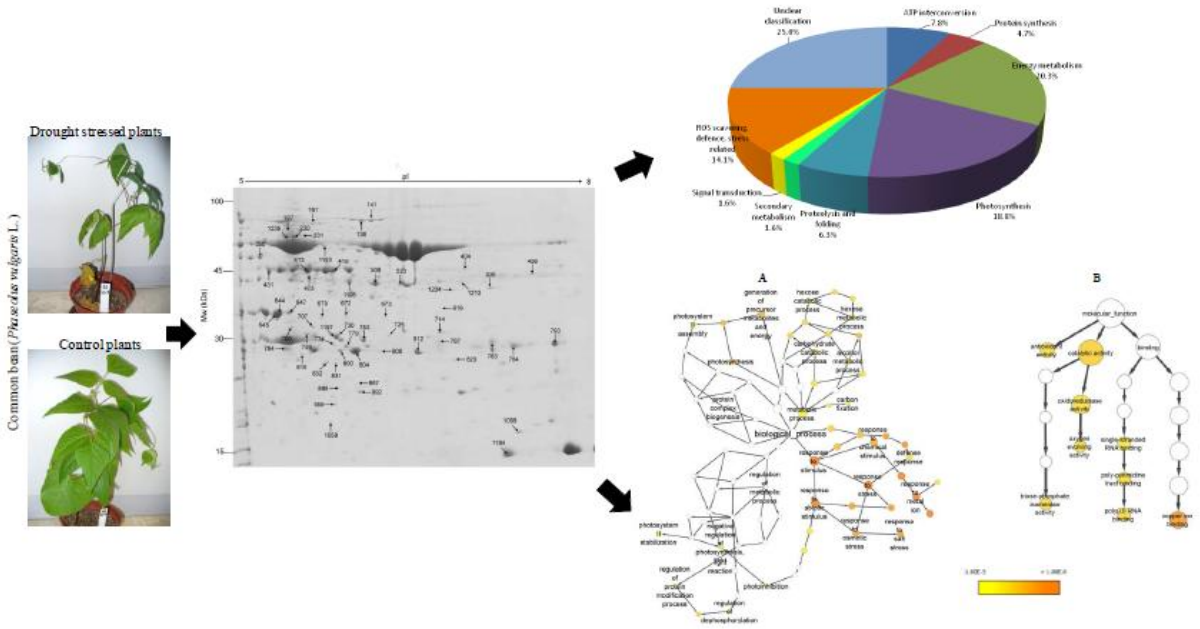


Figure 5



Graphical Abstract

Highlights

2D-DIGE approach is used to reveal change in protein levels of common bean under drought

Leaves of two cultivars with contrasting response to drought are analyzed

We identified proteins with different biological functions

A protein-protein interaction network is proposed for both cultivars