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Proteomic analysis of common bean stem under drought stress using in-gel stable isotope labeling

Tanja Zadražnik[a,*], Wolfgang Egge-Jacobsen[b], Vladimir Meglič[a], Jelka Šuštar-Vozlič[a]

[a] Agricultural Institute of Slovenia, 1000, Ljubljana, Slovenia
[b] University of Oslo, Department of Molecular Biosciences, 0316, Oslo, Norway

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ABSTRACT

Drought is an abiotic stress that strongly influences plant growth, development and productivity. Proteome changes in the stem of the drought-tolerant common bean (*Phaseolus vulgaris* L.) cultivar Tiber have were when the plants were exposed to drought. Five-week-old plants were subjected to water deficit by withholding irrigation for 7, 12 and 17 days, whereas control plants were regularly irrigated. Relative water content (RWC) of leaves, as an indicator of the degree of cell and tissue hydration, showed the highest statistically significant differences between control and drought-stressed plants after 17 days of treatment, where RWC remained at 90% for control and declined to 45% for stressed plants. Plants exposed to drought for 17 days and control plants at the same developmental stage were included in quantitative proteomic analysis using in-gel stable isotope labeling of proteins in combination with mass spectrometry. The quantified proteins were grouped into several functional groups, mainly into energy metabolism, photosynthesis, proteolysis, protein synthesis and proteins related to defense and stress. 70 kDa heat shock protein showed the greatest increase in abundance under drought of all the proteins, suggesting its role in protecting plants against stress by re-establishing normal protein conformations and thus cellular homeostasis. The abundance of proteins involved in protein synthesis also increased under drought stress, important for recovery of damaged proteins involved in the plant cell's metabolic activities. Other important proteins in this study were related to proteolysis and folding, which are necessary for maintaining proper cellular protein homeostasis. Taken together, these results reveal the complexity of pathways involved in the drought stress response in common bean stems and enable comparison with the results of proteomic analysis of leaves, thus providing important information to further understand the biochemical and molecular mechanisms of drought response in this important legume.

1. Introduction

Common bean (*Phaseolus vulgaris* L.) is one of the most important legume species for human consumption due to its high nutritional value, including a high protein content, essential amino acids, vitamins and minerals and beneficial health properties (Broughton et al., 2003; Boschin and Arnoldi, 2011; Luna-Vital et al., 2014). Observations in the field, greenhouse and in controlled environments have shown that it is relatively sensitive to drought stress compared to other grain legumes (Costa França et al., 2000). It is estimated that 60% of the world's common bean is cultivated under rainfed conditions, and drought causes yield losses up to 80% in some regions (Cuellar-Ortiz et al., 2008). Drought stress is therefore one of the very important constraints in common bean production, due to uneven distribution and variation in the rainfall pattern from one year to another. It can have deleterious effects on common bean growth either during early establishment, vegetative expansion, flowering or grain filling (Rao, 2001; Beebe et al., 2013).

The mechanisms of drought stress response and tolerance in common bean at the molecular level are still not well characterized because of the complex response to stress with respect to physiological and molecular changes. The molecular regulatory system involved in the response to drought and its tolerance in common bean can be deciphered with different "omics" techniques, including proteomics (Gupta et al., 2013). Since proteins are directly involved in the plant stress response, proteomics can significantly contribute to unravelling the possible relationships between protein abundance and stress in plants. Important knowledge of proteomic responses to drought stress has been derived

* Corresponding author at: Agricultural Institute of Slovenia, Crop Science Department, Hacquetova ulica 17, SI–1000 Ljubljana, Slovenia.
E-mail address: tanja.zadraznik@kis.si (T. Zadražnik).

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from studies on model legumes, such as soybean (*Glycine max* L.) (Alam et al., 2010; Mohammadi et al., 2012; Hossain et al., 2013) and barrel medic (*Medicago truncatula* Gaertn.) (Larrainzar et al., 2007, 2009; Staudinger et al., 2012). Several proteomic analyses related to drought have also been performed on important crop legumes, such as chickpea (*Cicer arietinum* L.) (Bhushan et al., 2011; Subba et al., 2013; Jaiswal et al., 2014), peanut *(Arachis hypogaea* L.*)* (Kottapalli et al., 2009, 2013), pea (*Pisum sativum* L.) (Taylor et al., 2005; Irar et al., 2014) and mungbean (*Vigna radiata* L.) (Sengupta et al., 2011).

The response of common bean to abiotic stress at the proteomic level has not been widely studied. Abiotic stress research indicated a clear modulation of oxidative stress-, heat shock-, and secondary metabolism-related proteins by O_3 and has the effect in leaves of cultivated bean (Torres et al., 2007). Further, response to chilling stress was dependent on length and manner of exposure to low temperature, as determined by divergent proteomic alterations in roots of common bean in response to varying periods of low temperature stress (Badowiec and Weidner, 2014). Proteomic study of drought stress in two cultivars of common bean differing in their response to drought was analyzed by Zadražnik et al. (2013). The majority of the identified proteins from leaf proteomes in both cultivars were classified into the categories of photosynthesis and energy metabolism, ATP interconversion, protein synthesis, proteolysis, folding, defense and stress-related proteins. Of those proteins showing contrasting abundance patterns between cultivars, the most outstanding were the oxygen evolving enhancer proteins. Most recently, the osmotic stress impact has been studied on root tips of common bean using proteomic and phosphoproteomic approach (Yang et al., 2013). Analysis of total soluble proteins indicated that 22 proteins were differentially regulated by osmotic stress induced by polyethylene glycol. The expressed proteins were mainly involved in metabolic pathways, particularly of carbohydrate and amino acid metabolism. Investigation of the total soluble phosphoproteome suggested that dehydrin responded to osmotic stress with an enhanced phophosphorylation state without a change in abundance.

The aim of the present study was to increase understanding of the molecular mechanisms of the response to drought stress in common bean using a proteomic approach. A combination of one-dimensional SDS-PAGE, in-gel stable isotope labeling and liquid chromatography/mass spectrometry/mass spectrometry (LC–MS/MS) was used to identify the changes in the stem proteome affected by drought. Stems were analyzed as important vascular systems carrying water, minerals, and sugars from the roots (Pinheiro et al., 2005). In this stage of our research we focused on the cultivar Tiber, shown in a previous study to be drought tolerant (Hieng et al., 2004). Drought-related proteins in the stem were identified and possible roles of the identified proteins in drought response mechanisms are discussed.

2. Materials and methods

2.1. Plant growth and stress treatments

Plants of common bean (*P. vulgaris*, cv. Tiber) were cultivated under natural daylight conditions in a greenhouse from March until May, when day length increased gradually from 12.5 h to 15 h. No additional artificial lighting was used. Temperature ranged from 15 to 25 °C and relative humidity from 50 to 85%, on average. Seeds were sown in pots containing a mixture of fertilized substrate (Klasmann, Germany) and vermiculite (1:1, v/v). Plants were regularly irrigated with tap water. Drought treatment was initiated on 5-week-old plants. Half the plants were not watered and the other half regularly irrigated. Leaves for determination of relative water content (RWC) were collected on days 7, 12 and 17 after the beginning of the water withdrawal; stems for proteome analysis were collected on day 17. Stressed and control samples were collected at each harvesting step to ensure the same developmental stage for the determination of RWC and for the comparative proteomic analysis. Five replicates of leaves and stems from drought-stressed and from control plants were taken for each of the treatments.

2.2. Determination of relative water content (RWC)

RWC of leaves was determined by the modified method of Barrs and Weatherley (1962). The third trifoliate leaves of drought and control plants were collected and immediately weighed (fresh weight, FW). They were rehydrated in water for 24 h to regain full turgor, surface dried and weighed (turgid weight, TW). Leaves were then dried in an oven at 70 °C to constant weight and weighed again (dry weight, DW). RWC was calculated using the formula: RWC (%) = [(FW-DW)/(TW-DW)] x 100.

2.3. Preparation of protein extracts and gel electrophoresis

Stems with three internodes from the first leaf on were harvested, immediately frozen in liquid nitrogen and stored at −80 °C until further analysis. Four replicates of stressed plants were combined together in one sample; control samples were combined and prepared in the same way. Stems were ground to a fine powder in liquid nitrogen using a pestle and mortar. Protein extraction was carried out according to a modified protocol by Fasoli et al. (2011). Samples were transferred into a centrifuge tube and suspended in the extraction buffer containing 1 x PBS (pH 7.4), 2% SDS and complete protease inhibitors (Roche). Lysate was prepared by homogenization and sonication for four times for one minute, incubation on ice between each homogenization and sonication steps, and centrifugation at 16,000*g* for 15 min at 15 °C. The supernatant was collected after two centrifugation steps. Protein samples were further reduced and alkylated with tributylphosphine and iodoacetamide (ProteoPrep, Sigma) and quantified using the bicinchoninic acid assay (Sigma) according to the manufacturer's protocol.

Protein samples of equal concentrations from stressed and control plants were separated by PAGE with pre-cast 4–12% Criterion XT gel (BioRad) in MOPS buffer (BioRad), using reagents and protocols supplied by the manufacturer (BioRad). Samples were suspended with four volumes of XT sample buffer (BioRad), incubated 5 min at 96 °C and centrifuged at 16,000*g* for 5 min. Proteins were separated at 80 V, until the dye from loading buffer reached the bottom of the gel. After electrophoretic separation, the gel was fixed for 30 min in 50% methanol and 10% acetic acid, then stained for 30 min in 50% methanol, 7% acetic acid and 0.1% Coomassie Brilliant Blue R-250. Gel was destained in 20% methanol and 5% acetic acid until the background became clear. The vertical lines of stressed and control sample on the gel were cut into 16 slices and each gel piece was sliced into 1 mm^3 cubes for in-gel stable isotope labeling.

2.4. In gel stable isotope labeling and trypsin digestion

Proteins were labeled with *N*-acetoxysuccinimide reagent according to the protocol from Asara et al. (2006) with minor changes. Gel pieces were washed two times with 50% propanol and the mixtures were incubated for 45 min at 55 °C to remove dye and SDS impurities. Colorless gel pieces were totally dehydrated with 100% propanol and air dried. Proteins from stressed plants were labeled with heavy reagent (*N*-acetoxy(D_3)succinimide), whereas those from matched control plants were labeled with the light reagent (*N*-acetoxy(H_3)succinimide). *N*-acetoxysuccinimide was synthesized according to published procedures (Asara et al., 2006).