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Comprehensive tissue-specific proteome analysis of drought stress responses in *Pennisetum glaucum* (L.) R. Br. (Pearl millet)☆

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ABSTRACT

Pearl millet is the fifth most important cereal crop worldwide and cultivated especially by small holder farmers in arid and semi-arid regions because of its drought and salt tolerance. The molecular mechanisms of drought stress tolerance in *Pennisetum* remain elusive. We have used a shotgun proteomics approach to investigate protein signatures from different tissues under drought and control conditions. Drought stressed plants showed significant changes in stomatal conductance and increased root growth compared to the control plants. Root, leaf and seed tissues were harvested and 2281 proteins were identified and quantified in total. Leaf tissue showed the largest number of significant changes (120), followed by roots (25) and seeds (10). Increased levels of root proteins involved in cell wall-, lipid-, secondary- and signaling metabolism and the concomitantly observed increased root length point to an impaired shoot–root communication under drought stress. The harvest index (HI) showed a significant reduction under drought stress. Proteins with a high correlation to the HI were identified using sparse partial least square (sPLS) analysis. Considering the importance of Pearl millet as a stress tolerant food crop, this study provides a first reference data set for future investigations of the underlying molecular mechanisms.

Biological significance: Drought stress is the most limiting factor for plant growth and crop production worldwide. At the same time drought susceptible cereal crops are among the largest producers worldwide. In contrast, Pearl millet is a drought and salt tolerant cereal crop especially used in arid and semi-arid regions by small farmers. The multifactorial molecular mechanisms of this unique drought tolerance are not known. Here, we employ shotgun proteomics for a first characterization of the Pearl millet drought stress proteome. The experimental setup and the data set generated from this study reveal comprehensive physiological and proteomic responses of the drought stressed Pearl millet plants. Our study reveals statistically significant tissue-specific protein signatures during the adaptation to drought conditions. Thus, the work provides a first reference study of the drought stress proteome and related drought responsive proteins (DRP's) in Pearl millet.

1. Introduction

Drought stress is one of the major abiotic plant stresses worldwide and will increase due to global climate change, increasing temperatures and fluctuating weather conditions [1–5]. It is one of the major limiting factors for agricultural production impairing growth and productivity of plants [6]. Drought stress is usually marked by loss of water content, stomata closure, decreased cell elongation and growth, reduced leaf and water potential and loss of turgor [7]. Cellular homeostasis is maintained when root absorbs water and nutrients from the soil and supplies them throughout the plant body. However, this balanced system is altered during the stress duration when roots are forced to adopt several functional and structural modifications. Disruption of metabolism, slow termination of photosynthesis and energy production processes, and ultimately cell death occur due to severe water loss [8]. Drought stress in plants results in increased abundance of reactive oxygen species (ROS) and subsequent lipid peroxidation [9]. The intensity of drought majorly depends upon distribution and occurrence of rainfall, evaporation and water retention capacity of the soil [10]. Therefore, understanding the

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responses of plants to drought and breeding plants for increased drought tolerance are two major goals for plant biologists and crop breeders in the present changing scenario of water deficiency across the globe.

Pennisetum glaucum (L.) R. Br., commonly known as Pearl millet, is one of the widely cultivated cereals (http://www.ceg.icrisat.org/ipgsc.html) and the fifth most important cereal crop next to rice, wheat, maize, and sorghum. This crop is majorly grown in arid and semi-arid regions of the Sahel zone, India and Latin America. It is well adapted to low soil fertility, high salinity, pH, and high temperature. Around 90% of Pearl millet cultivated is consumed as food crop. Pearl millet is a highly cross-pollinated (more than 85% outcrossing) diploid annual ($2n = 2\times = 14$) with a large genome size (2450 Mbp). The crop contains a high nutritional value of about 22–25 g of proteins/200 g grain in addition to iron, zinc and other minerals.

Proteomic studies in drought stressed plants are particularly important because proteins are the main drivers of all cellular events and will reveal major processes of drought stress adaptation. Proteome analysis is required for the understanding of the cellular processes associated with drought [6]. Drought sensing signals and their effects on physical, chemical, morphological and metabolic features have been well documented in plants [11,12]. One major signal is the stress hormone abscisic acid (ABA) emphasized as a key drought stress signal from root to shoot [13,14], but also highly varying in different plant species [12,15]. Thus, processes of ABA signaling are highly crop and environment specific. Another strong effect is decreased photosynthetic electron transport rate during drought stress due to less availability of CO_2 [16]. This in turn increases oxidative stress resulting in higher production of reactive oxygen species (ROS). Chaperone synthesis and oxidative stress tolerance mechanisms allow plants to survive water deficit [17]. Here, in this study we apply a highly sensitive shotgun proteomics approach for identification and quantification of the proteomes in different tissues like root, seed and leaf under drought stress and well irrigated condition. We have observed pronounced changes in the proteome of the specific tissues under these conditions (Drought vs. Control). Hence on this basis we have identified tissue specific drought responsive proteins (DRPs) which potentially explain the protective mechanism of the plant in order to survive under harsh water deficit condition. Our study represents a comprehensive reference data set of the drought stress proteome. Potential drought stress markers for the specific different tissues of Pearl millet are discussed.

2. Experimental procedures

2.1. Experimental design

P. glaucum (L.) R. Br. (Pearl millet, genotype ICTP 8203) plants were grown under controlled greenhouse conditions in high temperature tubes (Fig. 1; HT pipe made up of polypropylene) (Approximately, 150 cm deep and 10 cm in diameter) in a soil mixture consisting of 3 parts of potground (peat, humus) known as Kranzinger Blumenerde, 2 parts of Quarzsand and 1 part of Styromull. In order to avoid any leakage of soil and allow water to drain, the tubes were having small perforations at the bottom. The soil moisture content and temperature of soil were continuously monitored with sensors (ML3 ThetaProbe provided by Delta — T Devices Ltd). The control plants were watered routinely with calcium reduced tap water (5–10 Ca μg L^{-1}) at a temperature of 18 °C on a daily basis, maintaining soil humidity at a level of 25% ± 2.5% of soil volume. This was ensured by continuous monitoring of soil moisture content every 30 min employing Delta-T ML3 theta probes (1% accuracy) connected to a Delta-T GP2 data logger from the beginning to end of the experiment. Plants exposed to drought stress were 12 weeks old. Watering was withheld in the stress plants throughout the experiment till considerable difference in soil moisture content was observed and a significant response was measured in stomatal conductance (Fig. 2). For harvest at least three plants in three tubes from both conditions (control and drought) were selected as biological replicates. Roots were removed from the tubes, washed in order to remove soil particles and grinded in chilled mortar-pestle and frozen for proteomic analysis. Washing was performed in order to remove soil particles and avoid contamination of root tissue during protein extraction. The time duration between washing step and grinding of the root tissue was kept extremely short (~4 to 10 s, in order to avoid prolonged exposure of water to the roots). In order to see the effect of drought stress on crop productivity seeds were harvested from both conditions. Furthermore young leaves were also harvested. All samples were frozen in liquid nitrogen to stop any enzymatic activity and subjected to proteomic analysis (see below).

Fig. 1. Application of drought stress to *Pennisetum glaucum*. Plants are grown under controlled glasshouse conditions in high temperature tubes (HT pipe made of polypropylene, commonly known as PP pipes). Water content is monitored with sensors in the upper and bottom part of the tube.

2.2. Measurement of stomatal conductance

Stomatal conductance (gs) was measured using a PWMR-4 Porometer according to Parkinson [18]. Based on the principle of the instrument, a fast estimation of transpiration rate and stomata conductivity is possible. Two humidity sensors measured the humidity of incoming and outgoing air, transpiration rate is determined by inlet and outlet humidities; air flow through the chamber is measured using mass flow meter and the leaf surface area. Fully developed leaves from each replicate of control and stressed plants were selected for the measurement in five time points (10:30 am, 2:00 pm, 5:00 pm, 10:00 pm and 6:30 am Central European Time (CET)). In order to prevent the influence of real transpiration and conductance, measurement was not longer than 20–30 s. Recorded data are available in Supporting Information Table 1. Measurement of conductance (gs) was performed using following calculation:

$$\text{Stomatal conductance (gs)} = {}^{1}/_{\text{rs}} (\text{mmol m}^{-2}\ \text{s}^{-1})$$

$$\text{rs} = {}^{\text{U}}/_{\text{I}}$$

$$\text{U} = \Delta\ \text{Humidity}, \text{I} = \text{Transpiration rate (E)}$$

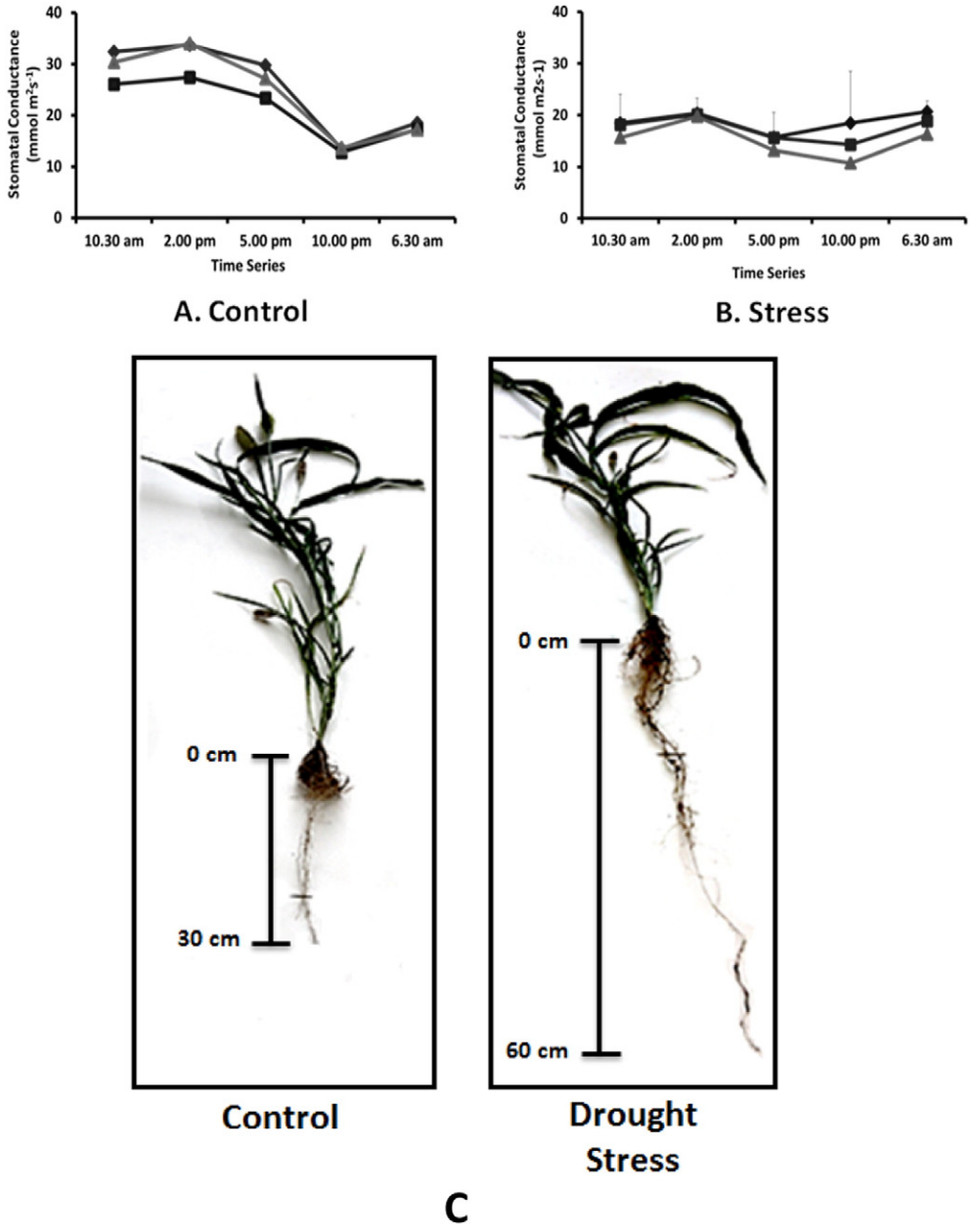

Fig. 2. Phenotypic responses to drought stress. (A) Stomatal conductance of the plants in control condition, showing normal diurnal rhythm with the conductance ranging approximately between 10 and 40 mmol m^2 s^{-1}. (B) Stomatal conductance of the plants in stress condition, showing decrease in conductance ranging approximately between 10 and 20 mmol m^2 s^{-1} compare to control. (C) Elongated root growth under drought stress condition compared to control.

2.3. Calculation of harvest index

Plant weight and yield (seed weight) from each replicate was measured and HI was calculated according to the following equation:

$$\text{Harvest index} = \text{Total yield}/(\text{Total yield} + \text{Plant weight})$$

2.4. Protein extraction, pre-fractionation, digestion, and LC–MS/MS

Protein extraction and analysis was performed according to Chaturvedi et al. [19]. Proteins from the harvested root, seed and leaf were extracted by grinding them for 2 min in a shaking mill using steel balls (2 mm). The homogenized samples were suspended in 200 μl of protein extraction buffer (100 mM Tris–HCl, pH 8.0; 5% SDS, 10% glycerol; 10 mM DTT; 1% plant protease inhibitor cocktail (Sigma P9599)) and incubated at room temperature for 5 min followed by incubation for 2.5 min at 95 °C and centrifugation at 21,000 ×*g* for 5 min at room temperature. The supernatant was carefully transferred in a new tube. Two hundred microliters of 1.4 M sucrose was added to the supernatant and proteins were extracted twice with 200 μl TE buffer equilibrated phenol followed by counter extraction with 200 μl of 1.4 M sucrose and 200 μl distilled water. Phenol phases were combined and subsequently mixed with 2.5 volumes of 0.1 M ammonium acetate in methanol for precipitation of proteins. After 16 h of incubation at −20 °C, samples were centrifuged for 5 min at 5000 ×*g*. The pellet was washed twice with 0.1 M ammonium acetate, one time with acetone and air dried at room temperature. The pellet was re-dissolved in 6 M Urea and 5% SDS and protein concentration was determined using the bicinchoninic acid assay (BCA method).

Pre-fractionation of proteins was carried out by SDS-PAGE [20]. Fourty micrograms of total protein was loaded onto a gel and run

for 1.5 cm. Gels were fixed and stained with in Methanol:Acetic Acid: Water:Coomassie Brilliant Blue R-250 (40:10:50:0.001), destained in methanol:water (40:60) and then each lane was divided into two fractions. Gel pieces were destained, equilibrated and digested with trypsin, desalted and concentrated. Prior to mass spectrometric measurement, the tryptic peptide pellets were dissolved in 4% (v/v) acetonitrile, 0.1% (v/v) formic acid. 10 μg of digested protein was injected into a one dimensional (1D) nano-flow LC–MS/MS system equipped with a pre-column (C18, Eksigent, Redwood City, CA, USA). Peptides were eluted using a Ascentis column (Ascentis Express, peptide ES-C18 HPLC column (SUPELCO Analytical, USA), dimension 15 cm × 100 μm, pore size 2.7 μm) during a 80 min gradient from 5% to 50% (v/v) acetonitrile, 0.1% (v/v) formic acid. MS analysis was performed on an Orbitrap LTQ XL mass spectrometer (Thermo, Germany) with a controlled flow rate of 500 nl per minute. Specific tune settings for the MS were as follows: spray voltage was set to 1.8 kV; temperature of the heated transfer capillary was set to 180 °C. Each full MS scan was followed by ten MS/MS scans, in which the ten most abundant peptide molecular ions were dynamically selected, with a dynamic exclusion window set to 90 s. Dependent fragmentation was performed in CID mode, with a normalized collision energy of 35, isolation window of 1.0, activation Q of 0.250 and 30 ms activation time. Ions with a +1 or unidentified charge state in the full MS were omitted from MS/MS analysis.

2.5. Peptide and protein identification and quantification

Raw data were searched with SEQUEST algorithm present in Proteome Discoverer version 1.3 (Thermo, Germany) as described in Valledor & Weckwerth [20]. In brief, identification confidence was set to a 5% FDR and the variable modifications were set to: acetylation of N-terminus and oxidation of methionine, with a mass tolerance of 10 ppm for the parent ion and 0.8 Da for the fragment ion.

A recently assembled Pearl millet genome sequence (http://www.ceg.icrisat.org/ipgsc.html) was employed for protein identification (will be published elsewhere). Peptides were matched against this database and decoys, considering a significant hit when the peptide was tryptic (with two miscleavages) and confidence was at medium or high and an Xcorr threshold was established at 1 per charge (2 for +2 ions 3 for +3 ions). All the raw- and result-files are uploaded to proteomexchange (http://www.proteomexchange.org) and the proteomics database PROMEX (http://promex.pph.univie.ac.at/promex/).

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The identified proteins were quantitated by a label-free approach based on total ion count

followed by a NSAF normalization strategy [21]:

$$(\mathrm{NSAF})_k = (\mathrm{PSM}/\mathrm{L})_k \Big/ \sum_{i=1}^{N} (\mathrm{PSM}/\mathrm{L})_i$$

in which the total number of spectra-counts for the matching peptides from protein k (PSM) was divided by the protein length (L), then divided by the sum of PSM/L for all N proteins. Three biological replicates were measured per tissue, control versus drought treated plants.

2.6. Multivariate statistical and bioinformatic data analysis

Principal component analysis (PCA), bi-cluster plot and ANOVA were performed using the statistical tool box COVAIN [22]. This software can be accessed online at http://www.univie.ac.at/mosys/software.html. The obtained data were log transformed before PCA analysis. K means cluster analysis was performed in Matlab and only proteins were chosen, if they were present in all the three biological replicates of at least one tissue. Biclustering was performed as follows: All identified proteins were categorized into functional groups to allow for a functional view of the tissue-specific proteome. The sum of the normalized spectral abundance factor for each functional category was then analyzed by bi-clustering using the statistical toolbox COVAIN. The bi-clustering uses average linkage of Euclidean distance between groups as the metric. Sparse partial least squares (sPLS) regression analysis was performed using the mixOmics package [23–25] for the statistical software environment R [R Core 26].

All the identified proteins were blasted for the closest *Arabidopsis* (Tair 10), rice and sorghum orthologs using stand-alone BLAST v2.2.28+ (using the default matrix) in conjunction with an unpublished Python script used for the following homology searches. The top three hits with an e-value below the threshold of 10^{-3} were selected from the results for further comparison. This way, most *Pennisetum* protein accessions could be assigned to a functional bin of an *Arabidopsis*, rice and sorghum ortholog (see Tables 1 and 2). The Venn diagram was produced by using Venny (http://bioinfogp.cnb.csic.es/tools/venny/index.html).

3. Results and discussion

3.1. Plant growth, stomatal conductance and soil moisture content under control and drought stress conditions

Pearl millet is a widely cultivated crop in semi-arid regions (drought- and high-temperature conditions). In order to provide a suitable experimental setup to simulate drought conditions, plants were transferred in high temperature tubes (HT pipe) made of polypropylene (Fig. 1). The use of polypropylene (PP pipes) was also recently described to provide a suitable and reproducible system in order to study drought stress responses of plants growing under controlled glass house conditions [27,28]. This system also facilitates the measurement of soil moisture content and soil water potential capacity. Compared to field conditions it induces more rapid and severe drought effects. In the present study the experimental setup consisted of plants which were watered routinely (Controls) and plants without watering (Stress) (see Experimental procedures). Soil moisture content and temperature were continuously measured with sensors including the soil surface as well as the bottom layer of the invidual pipes (see Experimental procedures) (Fig. 1). The temperature of the soil in control and drought conditions remained constant at 30 °C throughout the experiment. Drought stress of the plants was monitored by measuring stomatal conductance. Under water deficit conditions the first response of the plants is the closure of stomata. The major role of stomata in plants includes the uptake of CO_2 for photosynthesis and controlling detrimental water loss through transpiration. Environmental stimuli such as light, humidity and CO_2 concentration can subtly control opening and closing of the stomata [29]. The effect of drought stress in plants is commonly characterized by loss of water content, reduction in leaf water potential, reduction in cell elongation and stomata closure [7]. We measured stomatal conductance (gs) at five time points (10.30 am, 2.00 pm, 5.00 pm, 10.00 pm and 6.30 am), considering fully grown leaves using a PWMR-4 Porometer (see Experimental procedures). It was observed that stomatal conductance of the plants in control condition showed a normal diurnal rhythm with the conductance ranging approximately between 10 and 40 mmol m^2 s^{-1} (Fig. 2A), whereas in the stressed plants the conductance decreased to approximately 10–20 mmol m^2 s^{-1} 1 (Fig. 2B) (Supporting Information Table 1). This decrease in the stomatal conductance is an indication of stomata closure to avoid further dehydration of leafs under drought stress [30]. A similar effect was observed in wheat (*Triticum aestivum*), as well as in drought-sensitive and drought-tolerant cultivars of maize and soybean [31,32]. A study performed by Kusaka et al. determined stomatal conductance in seedlings of Pearl millet considering different cultivars under different stress conditions [33]. Stomatal closure was linked to a leaf turgor potential without drought stress, but long term stress conditions led to low

stomatal conductance controlling osmotic adjustments and reducing water loss under water deficit condition in Pearl millet. An intriguing observation for Pearl millet is described by Winkel et al. where stomata regulation minimizes water usage in pre-anthesis water deficit conditions [34]. Further, elongated root length was observed in the plants growing under water deficit condition compared to the plants in control condition (Fig. 2C).

3.2. Tissue and drought specific proteome analysis of root, seed and leaf

Comparative proteomic analysis was performed using a LC–MS/MS technique [20], in order to identify putative proteome signatures which are associated with drought stress response in Pearl millet. Protein abundances were quantified by NSAF normalization analysis [35]. Only proteins were chosen for quantification when they were present in all the three biological replicates of at least one tissue.

From all the detected peptides, 1095 proteins were identified in root (Supporting Information Tables 2 and 3), 1299 proteins were identified from seed (Supporting Information Tables 4 and 5), 1208 proteins were identified in young leaf of Pearl millet (Supporting Information Tables 6 and 7). There is a pronounced change in the proteome of the stressed plants compared to control conditions as revealed by Venn analysis. In root, 670 proteins are common in both the conditions (root stress and root control). Seed proteome analysis led to the identification of 984 proteins in common and similarly 681 proteins were in common from leaf stress and leaf control. From the Venn analysis all the putative drought responsive proteins (DRPs) in root (271 proteins), seed (159 proteins) and leaf (292 proteins) were identified (Supplementary Fig. 1), these DRPs are detailed in Supporting Information Table 8 along with their nearest orthologs in rice and sorghum.

We also compared the stress proteome of Pearl millet with 545 heat and drought responsive genes of *Arabidopsis* and found 34 genes were present under drought stress in root, seed and leaf (see Table 1), (Supporting Information Table 9).

Furthermore, we performed correlation analysis using sparse partial least square (sPLS) regression analysis according to Valledor et al. [36]. In this analysis the identified proteomes of root, seed and leaf were defined as predictors and the harvest index (HI) as the response vector. As a result several protein predictors were identified with high correlation scores, 12 protein candidates in roots (Fig. 3A, only correlation scores higher than 0.9 are shown), 15 in seeds (Fig. 3B, only correlation scores higher than 0.9 are shown) and 91 in leaf (Fig. 3C, only correlation scores higher than 0.9 are shown). All the information regarding the predictors can be viewed in Supporting Information Table 10. Furthermore we performed tissue-specific box-whisker plot analysis for those markers (seen in Supplementary Fig. 2). In Supplementary Fig. 3 a

Table 1
Details of 34 identified proteins under drought stress in root, seed and leaf of Pearl millet matched to 545 heat and drought responsive genes of *Arabidopsis*.

Pearl millet accession	*Arabidopsis* orthologs	AGI description	Rice orthologs	Sorghum orthologs
Pgl_GLEAN_10002391	AT1G10370	Encodes GSTU17 (Glutathione S-Transferase U17)	LOC_Os10g38740	Sb01g030780
Pgl_GLEAN_10002651	AT5G49910	Stromal heat shock protein involved in protein import into chloroplast.	LOC_Os12g14070	Sb08g009580
Pgl_GLEAN_10002939	AT1G07890	Encodes a cytosolic ascorbate peroxidase APX1		
Pgl_GLEAN_10005520	AT5G02500	Encodes a member of heat shock protein 70 family.		
Pgl_GLEAN_10006025	AT4G16660	Heat shock protein 70 (Hsp 70) family protein	LOC_Os02g48110	Sb04g030160
Pgl_GLEAN_10007489	AT3G07770	Heat shock protein 89.1 (Hsp89.1)	LOC_Os12g32986	Sb08g016560
Pgl_GLEAN_10009107	AT2G32120	Heat-shock protein 70T-2 (HSP70T-2)		
Pgl_GLEAN_10010309	AT5G12030	Encodes a cytosolic small heat shock protein with chaperone activity that is induced by heat and osmotic stress and is also expressed late in seed development.		
Pgl_GLEAN_10010682	AT2G04030	Encodes a chloroplast-targeted 90-kDa heat shock protein located in the stroma involved in red-light mediated response and crucial for protein import into the chloroplast stroma	LOC_Os08g38086	Sb07g028940
Pgl_GLEAN_10013122	AT5G56010	A member of heat shock protein 90 (HSP90) gene family		
Pgl_GLEAN_10014398	AT5G59720	Expression is induced in response to heat shock.		
Pgl_GLEAN_10014399	AT2G33380	Encodes a calcium binding protein whose mRNA is induced upon treatment with NaCl, ABA and in response to dessication		
Pgl_GLEAN_10016697	AT2G25140	Encodes ClpB4, which belongs to the Casein lytic proteinase/heat shock protein 100 (Clp/Hsp100) family	LOC_Os02g08490	Sb04g005570
Pgl_GLEAN_10017209	AT5G14420	Encodes RGLG2 (RING domain ligase 2), a RING domain ubiquitin E3 ligase that negatively regulates the drought stress response by mediating ERF53 transcriptional activity.	LOC_Os01g68060	Sb03g043290
Pgl_GLEAN_10017414	AT5G53400	Encodes BOBBER1 (BOB1), a non-canonical small heat shock protein required for both development and thermotolerance		
Pgl_GLEAN_10018470	AT5G52640	Encodes a cytosolic heat shock protein AtHSP90.1. AtHSP90.1 interacts with disease resistance signaling components SGT1b and RAR1 and is required for RPS2-mediated resistance.	LOC_Os04g01740	Sb06g000660
Pgl_GLEAN_10018509	AT2G18960	Encodes a plasma membrane proton ATPase		
Pgl_GLEAN_10019941	AT1G74310	Encodes ClpB1, which belongs to the Casein lytic proteinase/heat shock protein 100 (Clp/Hsp100) family		
Pgl_GLEAN_10021526	AT4G24190	Encodes an ortholog of GRP94, an ER-resident HSP90-like protein and is involved in regulation of meristem size and organization	LOC_Os06g50300	Sb10g030240
Pgl_GLEAN_10022408	AT5G56000	HEAT SHOCK PROTEIN 81.4 (Hsp81.4); FUNCTIONS IN: unfolded protein binding, ATP binding	LOC_Os09g30412	Sb07g028270
Pgl_GLEAN_10022665	AT5G12020	17.6 kDa class II heat shock protein (HSP17.6II)	LOC_Os01g08860	Sb03g003530
Pgl_GLEAN_10023437	AT4G27670	Encodes Hsp21, a chloroplast located small heat shock protein	LOC_Os03g14180	Sb01g041180
Pgl_GLEAN_10024005	AT1G54050	HSP20-like chaperones superfamily protein	LOC_Os02g54140	Sb04g035130
Pgl_GLEAN_10024324	AT1G48130	Encodes a protein similar to the 1-cysteine (1-Cys) peroxiredoxin family of antioxidants. Expression is limited to seed (aleurone and embryo) and is not induced by ABA or drought.	LOC_Os07g44430	Sb02g040650
Pgl_GLEAN_10024605	AT3G13860	Heat shock protein 60-3A (HSP60-3A)		
Pgl_GLEAN_10024877	AT5G15450	Encodes a chloroplast-targeted Hsp101 homologue	LOC_Os03g31300	Sb01g032210
Pgl_GLEAN_10028496	AT3G12580	Heat shock protein 70 (HSP70)		
Pgl_GLEAN_10030401	AT1G76080	Encodes a thioredoxin localized in chloroplast stroma	LOC_Os07g29410	Sb02g033120
Pgl_GLEAN_10030863	AT2G22360	DNAJ heat shock family protein	LOC_Os05g26926	Sb07g000660
Pgl_GLEAN_10031251	AT5G21430	Chaperone DnaJ-domain superfamily protein		
Pgl_GLEAN_10032289	AT3G23990	Mitochondrial chaperonin HSP	LOC_Os10g32550	Sb01g020010
Pgl_GLEAN_10032763	AT1G55490	Encodes the beta subunit of the chloroplast chaperonin 60, a homologue of bacterial GroEL		
Pgl_GLEAN_10032990	AT5G50920	Encodes a protein that is similar to ATP-dependent Clp protease ATP-binding subunit/ClpC	LOC_Os12g12850	Sb08g007750
Pgl_GLEAN_10033371	AT5G09590	Heat shock protein 70 (Hsc70-5)	LOC_Os02g53420	Sb01g017050

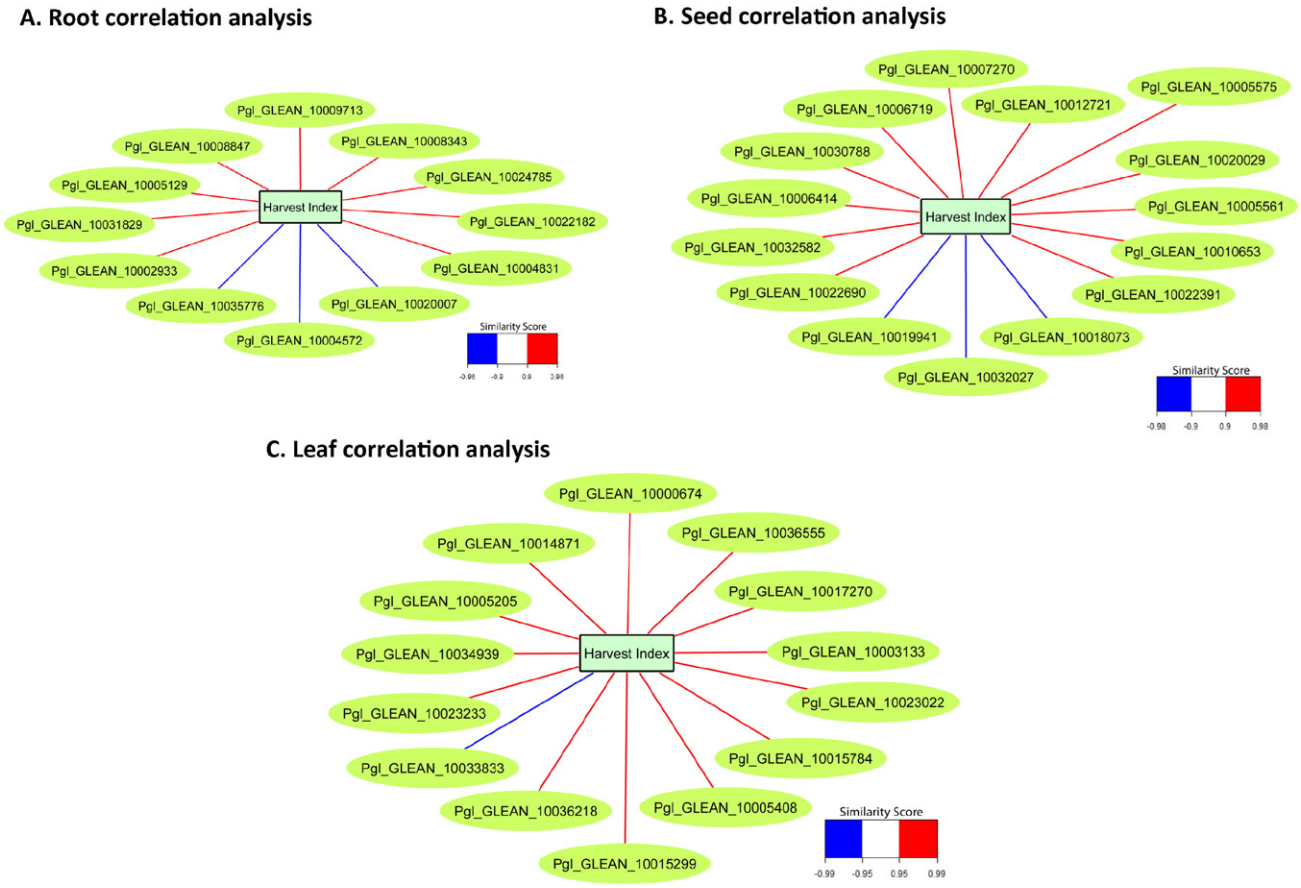

Fig. 3. Sparse partial least square analysis of proteins and harvest index (HI). (A) Proteins of the root proteome which predict the harvest index. (B) Proteins of the seed proteome which predict the harvest index. (C) Proteins of the leaf proteome which predict the harvest index.

comparison of the significantly changed proteins under drought stress and the predictors of harvest index (HI) is shown. The protein candidates are further compared with high loadings of a Principal component analysis (PCA) and discussed in the following sections. A Principal component analysis (PCA) was performed using COVAIN. The proteins were classified by identifying the nearest *Arabidopsis* orthologs and assigning them a function according to *Arabidopsis* mapping file of Mapman. In Fig. 4A a PCA plot is shown with all the tissues together in control and stress conditions (Supporting Information Table 11). The leaf proteome showed the strongest variation in response to drought stress compared to root and seed. The second strongest effect was observed in roots and the weakest response in the seed proteome. This observation is also in agreement with sPLS analysis where most protein candidates with a high correlation score to harvest index were detected in leafs compared to roots and seeds (Fig. 3A, B and C). In summary, these data indicate that the drought stress – although applied in the root system – has a severe impact on leaf metabolism. Therefore it is interesting to reveal the relationships between the alterations in the root system in conjunction with changes in the leaf proteome. This analysis is discussed in the last section "Systemic analysis of the Pearl millet drought stress proteome".

In a next step, tissue specific grouping of proteins in different condition (Control and Stress) was performed using K means cluster analysis with k = 25 (Supporting Information Table 12, Fig. 4B). Cluster analysis revealed specific groups of proteins either changing concentrations in tissue or drought stress treatment. Furthermore ANOVA analysis was performed to determine statistically significant proteins associated with specific tissue and specific condition (Supporting Information Table 13). Table 2 provides a detailed list of significantly changed proteins in roots (25), seeds (10) and leaf (120). These proteins are also analyzed by using box-whisher-plots (Supplementary Fig. 4). In the following chapters proteins which showed significant tissue and drought stress responses are described.

3.3. Pearl millet root proteome under drought stress

We revealed significant changes in the root proteome under drought conditions by multi- and uni-variate statistics. A principal component analysis of the root clearly separates stress and control condition. The proteins with highest positive loadings showed comparatively high abundance in control samples (Supporting Information Tables 14 and 3 including fold-changes and t-test), including proteins like UDP-glucosyl transferase, ribosomal L4/L1 family protein, profilin and Cytochrome C1 family. In comparison, the proteins with highest negative loadings showed high abundance in stressed samples (Supporting Information Table 14), including proteins such as peroxidases, germin like protein (GLP 5), Annexin, heat shock proteins 70 (Hsp 70) family protein. Considering the negative loadings which have shown high abundance in stress condition, some of the protein candidates like peroxidases were identified which are actively involved in plant cell metabolism and mostly localized in the vacuole and cell wall [37]. These proteins are mainly involved in defense against pathogen and adjustment of water balance; hence they are characteristic markers for stress conditions [38]. Abundance of these proteins has been reported in wheat as well as in wheat genotypes SERI M 82 (SE) and SW89.5193/kAu2 (SW) in response to water stress [8,39]. Similarly, germin like proteins were also identified in stress conditions. These proteins are abundant during drought stress condition. They have two subgroups: oxalate oxidases (OXOs) and germin like proteins (GLPs). These proteins are involved in the processes like germination, development, and responses to biotic and abiotic stresses [40]. GLPs have shown high abundance in

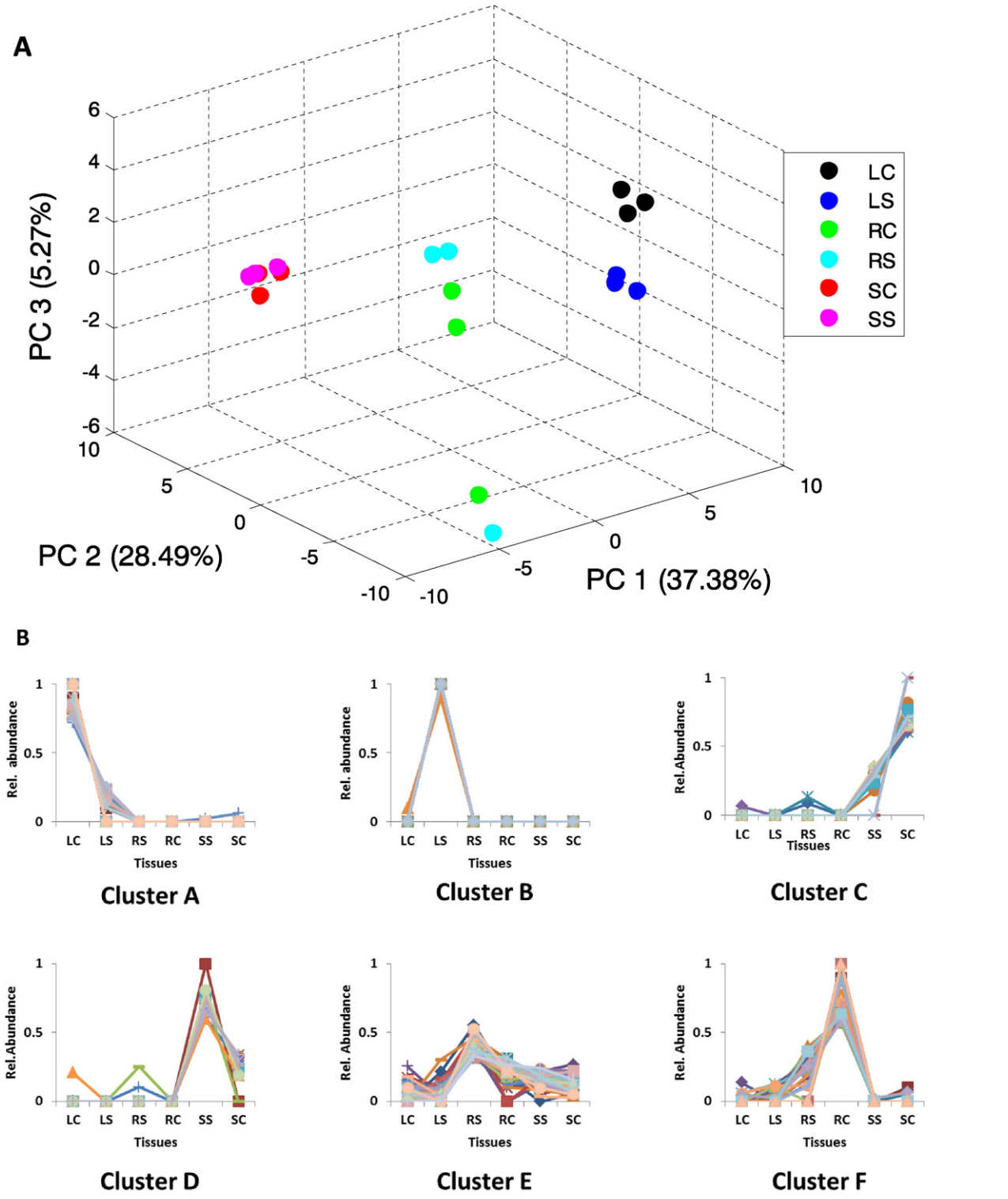

Fig. 4. (A) Principal component analysis (PCA) considering all the tissues in both the condition (Drought stress and Control). (B) Cluster analysis, tissue specific drought stress responsive proteins, determining the levels of proteins increased in a specific tissue in a specific condition present in all the biological replicates. (LC: Leaf control; LS: Leaf stress; RC: Root control; RS: Root stress; SC: seed control; SS; Seed stress).

barley and wheat in response to drought stress [41]. Differential gene expression of GLPs in response to abiotic stress such as drought, salt and cold in rice highlights their major integrative role in stress conditions [42].

Several heat shock proteins (HSP 70) were also identified; HSPs play a very important role in intracellular processes like protein–protein interactions, protein folding, protein assembly, transport and reactivation of damaged proteins [43]. Similar studies were performed with roots of the seedlings from rapeseed where HSP 70 was identified and showed decreased levels in the drought sensitive and F1 hybrid lines. No alteration of the protein isoform was observed in drought tolerant rapeseed lines under drought stress condition [44]. HSPs are typically induced when cells are exposed to stress conditions like drought, heat, cold and other environmental stresses. However, there is also evidence

Table 2
Details of significantly changed protein candidates in roots, seeds and leaf. Significant tissue-specific changes are visualised in Box-Whisker-plots in supplementary information Fig. 4.

Pearl millet accession	AGI orthologs	Rice orthologs	Sorghum orthologs	AGI description
Roots				
Pgl_GLEAN_10006975	AT5G15490	LOC_Os12g25690.1	Sb01g007580.2	Encodes one of four UDP-glucose dehydrogenase UGD) genes.
Pgl_GLEAN_10013122				
Pgl_GLEAN_10030607				
Pgl_GLEAN_10030129	AT3G16910	LOC_Os03g19240.1	Sb01g037610.1	Encodes a peroxisomal protein with acetyl-CoA synthetase activity that is responsible for the activation of acetate for entry into the glyoxylate cycle.
Pgl_GLEAN_10038571	AT1G60690	LOC_Os04g26910.1	Sb10g001900.1	NAD(P)-linked oxidoreductase superfamily protein
Pgl_GLEAN_10031278	AT3G19450	LOC_Os02g09490.1	Sb04g005950.1	Encodes a catalytically active cinnamyl alcohol dehydrogenase which uses p-coumaryl aldehyde as a preferred substrate.
Pgl_GLEAN_10031829	AT4G35450	LOC_Os09g33810.1	Sb07g025610.1	Involved in targeting of chloroplast outer membrane proteins to the chloroplast.
Pgl_GLEAN_10002933				
Pgl_GLEAN_10022182	AT2G15480	LOC_Os01g45110.1	Sb03g029070.1	UDP-glucosyl transferase 73B5 (UGT73B5)
Pgl_GLEAN_10032396	AT1G55020	LOC_Os03g52860.1	Sb03g042450.1	Lipoxygenase
Pgl_GLEAN_10007781	AT5G09650	LOC_Os02g52940.1	Sb04g034340.1	Encodes a protein with inorganic pyrophosphatase activity.
Pgl_GLEAN_10037166	AT2G36530	LOC_Os06g04510.1	Sb10g002460.1	Involved in light-dependent cold tolerance and encodes an enolase. Protein is tyrosine-phosphorylated and its phosphorylation state is modulated in response to ABA in *Arabidopsis thaliana* seeds.
Pgl_GLEAN_10027847	AT1G54320			LEM3 (ligand-effect modulator 3) family protein/CDC50 family protein
Pgl_GLEAN_10020577	AT3G04880	LOC_Os01g36090.1	Sb03g024040.1	Encodes a novel protein involved in DNA repair from UV damage
Pgl_GLEAN_10022809	AT4G10540			Subtilase family protein
Pgl_GLEAN_10002649	AT2G05790			O-glycosyl hydrolases family 17 protein
Pgl_GLEAN_10010682	AT2G04030	LOC_Os08g38086.3	Sb07g028940.1	Encodes a chloroplast-targeted 90-kDa heat shock protein
Pgl_GLEAN_10010310				
Pgl_GLEAN_10010809				
Pgl_GLEAN_10016081	AT5G40810	LOC_Os05g23620.2	Sb07g024370.1	Cytochrome C1 family
Pgl_GLEAN_10033988	AT3G13580	LOC_Os04g51630.2	Sb07g006970.2	Ribosomal protein L30/L7 family protein
Pgl_GLEAN_10038412	AT5G65720	LOC_Os09g16910.1	Sb02g022360.2	Encodes a cysteine desulfurase whose activity is dependent on AtSufE activation.
Pgl_GLEAN_10007586	AT2G38810	LOC_Os03g53190.1	Sb04g025140.1	Encodes HTA8, a histone H2A protein.
Pgl_GLEAN_10021024	AT3G08510	LOC_Os05g03610.1	Sb09g002320.1	Phosphoinositide-specific phospholipase C (PI-PLC).
Seeds				
Pgl_GLEAN_10022988	AT5G42790	LOC_Os02g04100.1	Sb04g002770.1	Encodes a protein with extensive homology to the largest subunit of the multicatalytic proteinase complex (proteasome).
Pgl_GLEAN_10030219	AT3G06580	LOC_Os03g61710.1	Sb01g002480.1	Encodes a protein with galactose kinase activity.
Pgl_GLEAN_10015508	AT5G16150	LOC_Os01g04190.2	Sb03g007080.1	Encodes a putative plastidic glucose transporter.
Pgl_GLEAN_10000489	AT1G07750	LOC_Os05g02520.1	Sb09g001680.1	RmlC-like cupins superfamily protein
Pgl_GLEAN_10028490				
Pgl_GLEAN_10004666	AT2G21660	LOC_Os03g46770.1	Sb01g012300.1	Encodes a small glycine-rich RNA binding protein
Pgl_GLEAN_10026551	AT1G48030	LOC_Os05g06750.1	Sb09g004610.1	Encodes a mitochondrial lipoamide dehydrogenase whose expression is induced by light.
Pgl_GLEAN_10030769				
Pgl_GLEAN_10001639				
Pgl_GLEAN_10007218	AT4G03200	LOC_Os01g70950.1	Sb03g045190.1	Catalytics
Leaves				
Pgl_GLEAN_10014726	AT5G62740	LOC_Os05g51420.1	Sb09g030530.5	Hypersensitive-induced response protein 1 (HIR1)
Pgl_GLEAN_10030783				
Pgl_GLEAN_10013725	AT2G34420	LOC_Os09g17740.1	Sb09g028720.1	Photosystem II type I chlorophyll-a/b-binding protein
Pgl_GLEAN_10013085	AT3G27690	LOC_Os03g39610.1	Sb01g015400.1	Encodes Lhcb2.4
Pgl_GLEAN_10037327	AT2G34420	LOC_Os01g41710.1	Sb03g027040.1	Photosystem II type I chlorophyll-a/b-binding protein
Pgl_GLEAN_10019779	AT1G26220	LOC_Os08g01170.1	Sb07g000310.1	Acyl-CoA N-acyltransferases (NAT) superfamily protein
Pgl_GLEAN_10028876	AT2G37170	LOC_Os02g41860.1	Sb04g026650.1	A member of the plasma membrane intrinsic protein subfamily PIP2
Pgl_GLEAN_10028121				#N/A
Pgl_GLEAN_10037787	AT5G52100		Sb01g049480.1	Is essential for chloroplast NAD(P)H dehydrogenase activity, which is involved in electron transfer between PSII and PSI.
Pgl_GLEAN_10005143	AT4G39710	LOC_Os02g51570.1	Sb04g027730.2	FK506-binding protein 16-2 (FKBP16-2)
Pgl_GLEAN_10028064				
Pgl_GLEAN_10008353				
Pgl_GLEAN_10019292	AT5G16450	LOC_Os01g52460.2	Sb09g025235.1	Ribonuclease E inhibitor RraA/dimethylmenaquinone methyltransferase
Pgl_GLEAN_10004798				
Pgl_GLEAN_10010145				
Pgl_GLEAN_10015411	AT2G14170	LOC_Os07g09060.1	Sb02g005200.1	*Arabidopsis thaliana* methylmalonate-semialdehyde dehydrogenase
Pgl_GLEAN_10005779	AT3G46100	LOC_Os02g51830.1	Sb03g003570.1	Histidyl-tRNA synthetase
Pgl_GLEAN_10015409	AT5G03795	LOC_Os07g09050.1	Sb02g005180.1	Exostosin family protein
Pgl_GLEAN_10002792	AT5G04710	LOC_Os01g73680.1	Sb03g047100.1	Zn-dependent exopeptidases superfamily protein
Pgl_GLEAN_10030018	AT5G59890	LOC_Os04g46910.1	Sb01g003250.1	Actin depolymerizing factor 4 (ADF4) mRNA, complete cds
Pgl_GLEAN_10026635				
Pgl_GLEAN_10013187	AT2G30620	LOC_Os07g08710.1	Sb01g005010.1	Winged-helix DNA-binding transcription factor family protein
Pgl_GLEAN_10027569	AT2G34420	LOC_Os01g41710.1	Sb03g027040.1	Photosystem II type I chlorophyll-a/b-binding protein
Pgl_GLEAN_10001175				
Pgl_GLEAN_10017437	AT3G10920	LOC_Os05g25850.1	Sb09g011450.1	Manganese superoxide dismutase (MSD1)
Pgl_GLEAN_10033321	AT1G17290	LOC_Os10g25130.1	Sb01g023750.1	Encodes for alanine aminotransferase (ALAAT1), involved in alanine catabolism during plants recovery from hypoxia
Pgl_GLEAN_10030168	AT1G56500	LOC_Os03g19760.1	Sb01g037270.1	Haloacid dehalogenase-like hydrolase family protein
Pgl_GLEAN_10028793		LOC_Os01g73250.1	Sb06g016530.1	
Pgl_GLEAN_10030289	AT1G57770	LOC_Os03g62510.1	Sb01g001750.1	FAD/NAD(P)-binding oxidoreductase family protein

(continued on next page)

Table 2 (*continued*)

Pearl millet accession	AGI orthologs	Rice orthologs	Sorghum orthologs	AGI description
Pgl_GLEAN_10004901	AT5G06460	LOC_Os11g01510.2	Sb05g000520.1	Encodes a ubiquitin-activating enzyme (E1), involved in the first step in conjugating multiple ubiquitins to proteins targeted for degradation.
Pgl_GLEAN_10008425	AT1G53850			Encodes alpha5 subunit of 20s proteosome involved in protein degradation and RNA degradation.
Pgl_GLEAN_10025482				
Pgl_GLEAN_10007878	AT3G58140			Phenylalanyl-tRNA synthetase class IIc family protein
Pgl_GLEAN_10015784	AT5G01530	LOC_Os07g37240.1	Sb02g036260.1	Light harvesting complex photosystem II (LHCB4.1)
Pgl_GLEAN_10000674	AT5G08680	LOC_Os10g21266.1	Sb09g027790.1	Encodes the mitochondrial ATP synthase beta-subunit.
Pgl_GLEAN_10006086	AT5G15490	LOC_Os03g40720.1	Sb01g014970.1	Encodes one of four UDP-glucose dehydrogenase UGD) genes.
Pgl_GLEAN_10014986	AT4G31180	LOC_Os02g46130.1	Sb03g001240.1	Class II aminoacyl-tRNA and biotin synthetases superfamily protein
Pgl_GLEAN_10033667	AT4G36130	LOC_Os12g38000.1	Sb08g018650.1	Ribosomal protein L2 family
Pgl_GLEAN_10020225				
Pgl_GLEAN_10012602				
Pgl_GLEAN_10033485				
Pgl_GLEAN_10016313				
Pgl_GLEAN_10030401	AT1G76080	LOC_Os07g29410.1	Sb02g033120.1	Encodes a thioredoxin localized in chloroplast stroma. Known as CDSP32 (Chloroplastic drought-induced stress protein of 32 kDa).
Pgl_GLEAN_10026969	AT4G09040	LOC_Os08g37700.1	Sb07g029220.1	RNA-binding (RRM/RBD/RNP motifs) family protein
Pgl_GLEAN_10014734	AT3G24420	LOC_Os05g51240.1	Sb09g030500.1	Alpha/beta-hydrolases superfamily protein
Pgl_GLEAN_10021406	AT2G35500	LOC_Os10g42700.1	Sb01g027930.1	Encodes a protein with some sequence similarity to shikimate kinases
Pgl_GLEAN_10003206				
Pgl_GLEAN_10021430	AT2G35490	LOC_Os10g42500.1	Sb01g028150.1	Plastid-lipid associated protein PAP/fibrillin family protein
Pgl_GLEAN_10008923				
Pgl_GLEAN_10016566	AT1G23740	LOC_Os08g29170.1	Sb07g019260.1	AOR is an alkenal/one oxidoreductase that acts on compounds with unsaturated alpha, beta-carbonyls.
Pgl_GLEAN_10000606	AT3G16640			Translationally controlled tumor protein (TCTP)
Pgl_GLEAN_10019203				
Pgl_GLEAN_10009112	AT3G50820	LOC_Os01g31690.1	Sb10g028120.1	Unknown protein
Pgl_GLEAN_10013567	AT3G61470	LOC_Os07g38960.1	Sb02g037410.1	Encodes a component of the light harvesting antenna complex of photosystem I.
Pgl_GLEAN_10006857	AT5G42960	LOC_Os03g63860.1	Sb01g000680.1	Unknown protein
Pgl_GLEAN_10028069				
Pgl_GLEAN_10014847	AT1G65970	LOC_Os01g48420.1	Sb03g030950.1	Thioredoxin-dependent peroxidase 2
Pgl_GLEAN_10001520				
Pgl_GLEAN_10021964	AT1G15820	LOC_Os04g38410.1	Sb06g032690.1	Lhcb6 protein (Lhcb6), light harvesting complex of photosystem II.
Pgl_GLEAN_10000489	AT1G07750	LOC_Os05g02520.1	Sb09g001680.1	RmlC-like cupins superfamily protein
Pgl_GLEAN_10034841	AT5G62740	LOC_Os08g30790.1	Sb07g019760.1	Hypersensitive-induced response protein 1 (HIR1)
Pgl_GLEAN_10002667	AT5G38660	LOC_Os08g27010.1	Sb07g015170.1	Unknown protein
Pgl_GLEAN_10010310				
Pgl_GLEAN_10022888				
Pgl_GLEAN_10034711	AT3G47470	LOC_Os08g33820.1	Sb07g021260.1	Encodes a chlorophyll a/b-binding protein that is more similar to the PSI Cab proteins than the PSII cab proteins. The predicted protein is about 20 amino acids shorter than most known Cab proteins.
Pgl_GLEAN_10013653	AT1G68830	LOC_Os05g47560.1	Sb09g027380.1	STN7 protein kinase; required for state transitions, phosphorylation of the major antenna complex (LHCII) between PSII and PSI, and light adaptation
Pgl_GLEAN_10017620	AT5G06720	LOC_Os10g02040.2	Sb01g027330.1	Encodes a peroxidase with diverse roles in the wound response, flower development, and syncytium formation.
Pgl_GLEAN_10036281	AT3G14940	LOC_Os01g11054.1	Sb03g002220.1	Encodes a cytosolic phosphoenolpyruvate carboxylase (PEPC) that has activity when expressed in *E. coli*.
Pgl_GLEAN_10023186		LOC_Os05g33290.1	Sb04g010390.1	
Pgl_GLEAN_10000605	AT5G44650			Encodes a nucleus-encoded thylakoid protein
Pgl_GLEAN_10006840	AT3G60810	LOC_Os03g64140.1	Sb01g000446.1	Unknown protein
Pgl_GLEAN_10023966	AT5G11720	LOC_Os06g46284.1	Sb10g027110.1	Glycosyl hydrolases family 31 protein
Pgl_GLEAN_10028447				
Pgl_GLEAN_10026937	AT4G01050	LOC_Os02g15750.1	Sb04g009380.1	Hydroxyproline-rich glycoprotein family protein, contains a rhodanese homology domain. Required for anchoring the FNR flavoenzyme to the thylakoid membranes and sustaining high efficiency photosynthetic linear electron flow.
Pgl_GLEAN_10028323	AT3G04870	LOC_Os07g10490.1	Sb02g006100.1	Involved in the biosynthesis of carotenes and xanthophylls, reduces zeta-carotene to lycopene.
Pgl_GLEAN_10021055				
Pgl_GLEAN_10026105				
Pgl_GLEAN_10003133	AT5G05340	LOC_Os06g35520.1	Sb04g008590.1	Peroxidase superfamily protein
Pgl_GLEAN_10023233	AT2G04039		Sb02g038940.1	Unknown protein
Pgl_GLEAN_10005205	AT5G20720	LOC_Os09g26730.1	Sb02g025700.1	Encodes a chloroplast co-chaperonin with similarity to CPN21 from spinach, *E. coli* GroES.
Pgl_GLEAN_10014066	AT1G07700	LOC_Os02g35900.1	Sb04g023500.1	Thioredoxin superfamily protein
Pgl_GLEAN_10018665				
Pgl_GLEAN_10024612				
Pgl_GLEAN_10035773				
Pgl_GLEAN_10017685				
Pgl_GLEAN_10016932	AT5G09810	LOC_Os05g01600.1	Sb05g003880.1	Member of Actin gene family. Mutants are defective in germination and root growth.
Pgl_GLEAN_10008934				
Pgl_GLEAN_10010788	AT1G61520	LOC_Os02g10390.1	Sb10g023930.1	PSI type III chlorophyll a/b-binding protein (Lhca3*1)
Pgl_GLEAN_10032990	AT5G50920	LOC_Os12g12850.1	Sb08g007750.1	Encodes a protein that is similar to ATP-dependent Clp protease ATP-binding subunit/ClpC.
Pgl_GLEAN_10001595	AT1G36280	LOC_Os03g19930.1	Sb06g023390.1	L-Aspartase-like family protein
Pgl_GLEAN_10025556	AT1G06680		Sb02g002690.1	Encodes a 23 kDa extrinsic protein that is part of photosystem II and participates in the regulation of oxygen evolution
Pgl_GLEAN_10001789	AT2G21870	LOC_Os02g03860.1	Sb04g002620.1	Encodes the FAd subunit of mitochondrial F1F0-ATP synthase.
Pgl_GLEAN_10029347	AT3G54050	LOC_Os03g16050.1	Sb01g039980.1	Encodes a chloroplastic fructose 1,6-bisphosphate phosphatase.

Table 2 (*continued*)

Pearl millet accession	AGI orthologs	Rice orthologs	Sorghum orthologs	AGI description
Pgl_GLEAN_10004517				
Pgl_GLEAN_10014871	AT1G14540	LOC_Os06g35490.1	Sb10g021630.1	Peroxidase superfamily protein
Pgl_GLEAN_10002809	AT5G09810	LOC_Os05g01600.1	Sb09g000750.1	Member of Actin gene family.
Pgl_GLEAN_10007875	AT3G06350			Encodes a bi-functional dehydroquinate-shikimate dehydrogenase enzyme that catalyzes two steps in the chorismate biosynthesis pathway.
Pgl_GLEAN_10034557	AT5G01410	LOC_Os07g01020.1	Sb02g000720.1	Encodes a protein predicted to function in tandem with PDX2 to form glutamine amidotransferase complex with involved in vitamin B6 biosynthesis.
Pgl_GLEAN_10010568	AT1G12900	LOC_Os04g38600.1	Sb06g018880.1	Glyceraldehyde 3-phosphate dehydrogenase A subunit 2 (GAPA-2)
Pgl_GLEAN_10023896	AT1G17160	LOC_Os01g47550.1	Sb03g030470.1	pfkB-like carbohydrate kinase family protein
Pgl_GLEAN_10019743	AT1G03860	LOC_Os07g15880.1	Sb02g008640.1	Prohibitin 2
Pgl_GLEAN_10000891				
Pgl_GLEAN_10006042	AT1G77380		Sb03g041840.1	Amino acid permease which transports basic amino acids.
Pgl_GLEAN_10029289	AT3G15360			Encodes a prokaryotic thioredoxin
Pgl_GLEAN_10018660				
Pgl_GLEAN_10012084	AT2G24820	LOC_Os02g54980.1	Sb04g035660.1	Translocon at the inner envelope membrane of chloroplasts 55-II (TIC55-II)
Pgl_GLEAN_10033184	AT3G10740			Encodes a bifunctional alpha-l-arabinofuranosidase/beta-d-xylosidase that belongs to family 51 of glycoside hydrolases. It may be involved in cell wall modification.
Pgl_GLEAN_10006836	AT2G28000	LOC_Os03g64210.1	Sb01g000380.1	Encodes chaperonin-60 alpha
Pgl_GLEAN_10025601	AT5G35170			Adenylate kinase family protein
Pgl_GLEAN_10006755	AT1G77490	LOC_Os02g34810.1	Sb04g022560.1	Encodes a chloroplastic thylakoid ascorbate peroxidase tAPX
Pgl_GLEAN_10034809	AT5G27560	LOC_Os06g05390.1	Sb10g003190.1	Unknown
Pgl_GLEAN_10015796	AT5G54270	LOC_Os07g37550.1	Sb02g036380.1	Lhcb3 protein is a component of the main light harvesting chlorophyll a/b-protein complex of Photosystem II (LHC II).
Pgl_GLEAN_10037666	AT3G01440		Sb02g000550.1	Encodes a subunit of the NAD(P)H complex located in the chloroplast thylakoid lumen.
Pgl_GLEAN_10011348				
Pgl_GLEAN_10031436				
Pgl_GLEAN_10016086	AT5G03630	LOC_Os08g44340.1	Sb07g024320.1	ATMDAR2
Pgl_GLEAN_10038288				
Pgl_GLEAN_10018367				
Pgl_GLEAN_10032833	AT2G20260	LOC_Os07g25430.1	Sb02g010190.1	Encodes subunit E of photosystem I.
Pgl_GLEAN_10033833	AT3G14420	LOC_Os03g57220.2	Sb01g005960.1	Aldolase-type TIM barrel family protein

that HSPs are developmentally primed [19]. They were also identified in the drought stress condition of sugar beet [45] and wheat [46].

Further, annexin was also identified in higher abundance under stress condition. Annexins are proteins that bind to phospholipids in a calcium dependent manner. They typically contain about 4–8 repeats of ~70 amino acids. Annexins are majorly involved in the organization and function of biological membranes [47]. It is also observed that annexin takes part in several secretory pathways which involve ATPase and peroxidase activities [47]. Górecka et al. [48] demonstrated the role of Annexin At1 of *Arabidopsis thaliana* in pH mediated cellular response to environmental stimuli and also suggested that AnnAt1 might also play major role in intracellular ion homeostasis.

3.4. Pearl millet seed proteome under drought stress

We revealed significant changes in the seed proteome under drought conditions by multi- and uni-variate statistics. Principal component analysis of seeds showed very weak separation between control and stress condition (Supporting Information Tables 15 and 5 including fold-changes and t-test). Considering the loadings, proteins like LEA, seed maturation protein, threonine synthase, heat shock proteins 70 and 21 were identified in higher abundances under stress condition.

LEA proteins were first identified in seeds by Dure [49]. They are located in various cellular compartments which determine their possible roles in protecting or regulating the biochemical processes such as replication and respiration. These proteins play a very important role in seed maturation and osmotic stress. They are related to the desiccation tolerance [50]. LEA proteins are hydrophilic in nature and in drought tolerant seeds they play a role in preferential hydration using polar groups which are on the surface of the LEA proteins. During drought condition these proteins interact with other groups and replace water from the cell which can be a key protective role of LEA proteins. Farrant et al. [51] investigated recalcitrant seeds of *Avicennia marina* and *Podocarpus henkelii* and revealed the absence of LEA proteins in drought sensitive seeds. Further studies also postulated that LEA proteins are present throughout developmental stages with different expression levels in all tissues. E.g. Em, RAB21 and dehydrins can also be found in root, stem, leaf, callus and suspension cultures of higher plants under ABA or/and NaCl treatment [52].

Also heat shock proteins are not confined to a specific tissue but also found in roots, seeds and leafs. In seeds we identified Hsp 21 and Hsp 70 as stress responsive. HSPs protect seeds from dehydration by regulating protein misfolding and maintaining membrane integrity. Many small heat shock proteins (sHSPs) play a major role in protection of seeds from desiccation.

3.5. Pearl millet leaf proteome under drought stress

We revealed significant changes in the seed proteome under drought conditions by multi- and uni-variate statistics. Principal component analysis of leaf under control and stressed condition determined a clear separation on PC1. Considering the highest loadings of PC1, protein candidates like peroxidase superfamily protein, L-aspartase-like family protein, thioredoxin-dependent peroxidase, ribosomal proteins and prohibitin 3 were identified with higher abundance under stress condition (Supporting Information Tables 16 and 7 with fold-changes and t-test). Some of the proteins which are important components of the light harvesting complex were identified under drought stress for e.g. chlorophyll a/b-binding protein. Thioredoxin was also identified. Thioredoxin is involved in redox regulation of many important enzymes in primary metabolism and also has an important role in the regeneration of oxidized peroxiredoxin in an active or reduced form [53]. Higher concentrations of these proteins were also identified under drought stress in the leaves of common bean (*Phaseolus vulgaris* L.) [54]. Prohibitin 3 is mainly involved in the function of cell division and plays also an important role in mitochondrial electron transport chain. In transgenic lines of *Arabidopsis* (AtPHBs) mitochondrial functions and cell division and differentiation of apical tissues were affected [55].

Another protein responsive to drought encodes a subunit of the NAD(P)H complex located in the chloroplast thylakoid lumen

(Pgl_GLEAN_10037666). Under drought stress we have observed a pronounced closure of stomata (see Fig. 2). This leads to less CO_2 available for the Calvin cycle which in turn increases the NADPH/$NADP^+$-ratio. As a consequence, the electron transport chain in the thylakoid membrane becomes highly reduced and cannot be balanced because of less $NADP^+$ availability. Instead electrons are captured by water resulting in the generation of ROS (reactive oxygen species) [56]. This leads to photoinhibition and increased photorespiration. Hence under water deficit condition metabolic pathway which converts NADPH to $NADP^+$ is stimulated [57]. Accordingly, we found proteins involved in NADPH metabolism. Also proteins involved in photorespiration showed increased abundance under water deficit condition in leaf (see Figs. 5 and 6, further discussion of these figures below).

3.6. Systemic analysis of Pearl millet drought stress proteome

Functional annotation of proteins was performed by matching identified proteins with the Mapman file of *Arabidopsis* (Tair 10) and grouping them according to their predicted functions (Supporting Information Table 17). The total NSAF scores were summed up for different functional categories according to Chaturvedi et al. and Ischebeck et al. [19,58]. Bicluster analysis was performed with the COVAIN toolbox in Matlab [59] (Fig. 5). A schematic summary of the changes is shown in Fig. 6. Proteins with putative function in cell wall synthesis showed increased concentrations in root under stress conditions compared to control while in leaf and seed they were downregulated under both the condition. Roots play an important role in the drought response. Under stress conditions roots have to enhance the cellular activity in order to absorb water from the deep layer of the soil to prevent water stress and turgor loss [60]. Increased levels of cell wall proteins are most probably involved in the enhanced growth rate which we have observed (see Fig. 2C). This response allows the plant to reach deeper regions belowground. *Pennisetum* seems to have a pronounced enhancement of root growth in response to drought stress and we will investigate this phenomenon in more detail in the future. Deep penetrating roots can provide a chance of survival under harsh condition. Furthermore, proteins involved in the function of lipid metabolism, peroxidases, and transport were upregulated in the root under drought stress condition. Furthermore, the abundance of proteins associated with a variety of cellular functions, including nucleotide metabolism and N-metabolism, cell division and cell cycle was found to be highly affected in *Pennisetum* root under drought stress (Figs. 5 and 6).

Signaling plays an important role for the adaptation of the plant to water stress [61]. Sensing of water stress provokes a range of signal molecules which are transported via the xylem into leafs. As a response leaf transpiration and leaf growth are reduced. These molecular signals are different from hydraulic signals [62]. Both of these processes are important as they reduce the stomatal conductance and leaf growth under drought stress condition. Reduction of stomatal conductance and reduced leaf growth was also observed in our study, hence we can postulate similar effects under water deficit condition in *Pennisetum*. In the functional category *signaling* proteins such as GTP binding protein, leucine rich transmembrane protein kinase, calreticulin, calnexin, 14-3-3 protein, and phosphoinositides-specific phospholipase C (PI-PLC) were binned together and showed increased levels under stress conditions.

Calcium is an important macro-nutrient that can be readily taken up by roots and delivered to the shoots in order to regulate several physiological processes [47]. In plant cells several vesicular compartments store calcium which can be released in the cytoplasm when required. Activation of PLC (phospholipase C) which hydrolyse PIP_2 to IP_3 allows the release of calcium from the intracellular storage [55]. The levels of

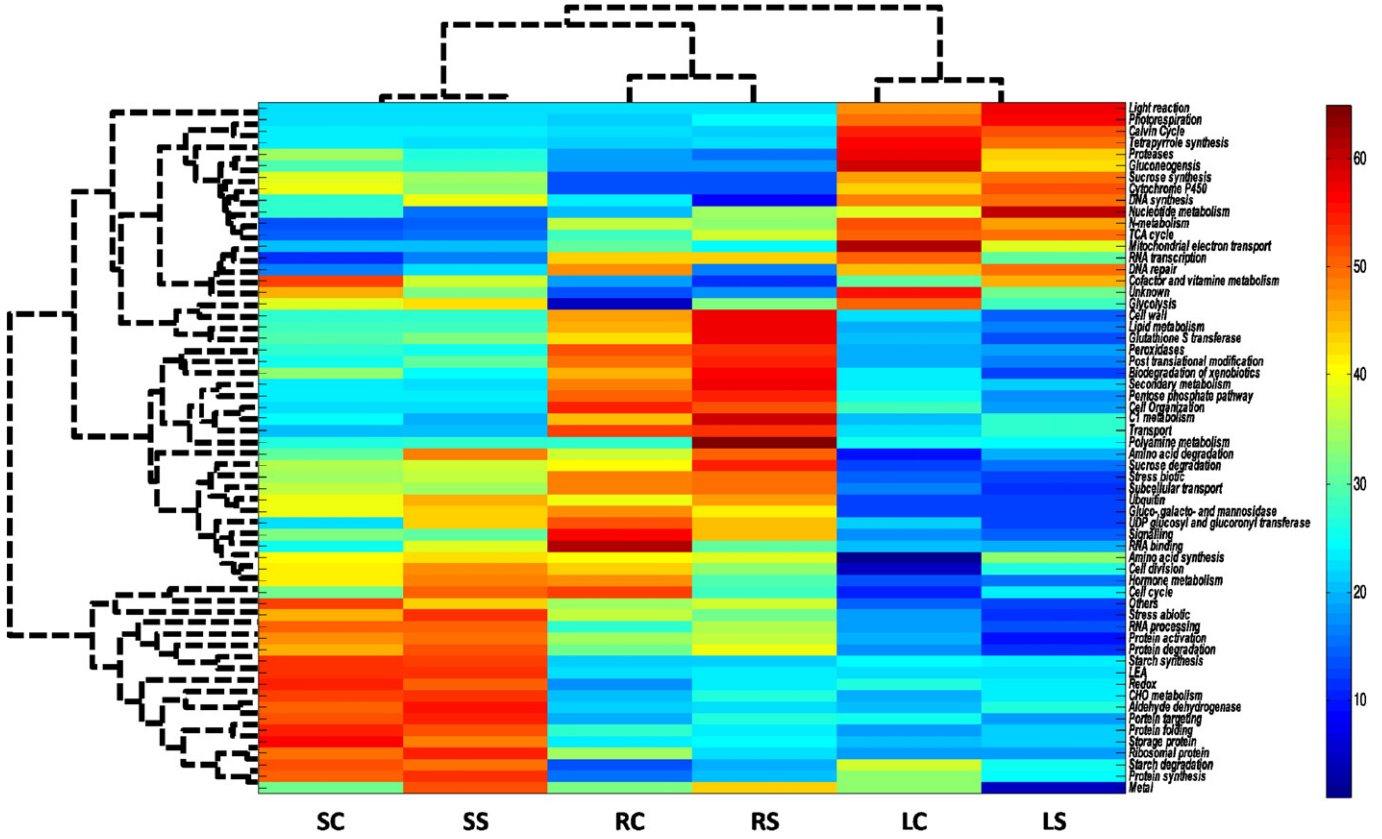

Fig. 5. Bicluster analysis of functional protein categories in root, seed and leaf under control and drought stress conditions. All identified proteins were categorized into functional groups to allow for a functional view of the tissue-specific proteome. The sum of the normalized spectral abundance factor for each functional category was analyzed by bi-clustering using the statistical toolbox COVAIN. The bi-clustering uses average linkage of Euclidean distance between groups as the metric. (SC: Seed control; SS: Seed stress; RC: Root control; Root stress; LC: Leaf control; LS: Leaf stress).

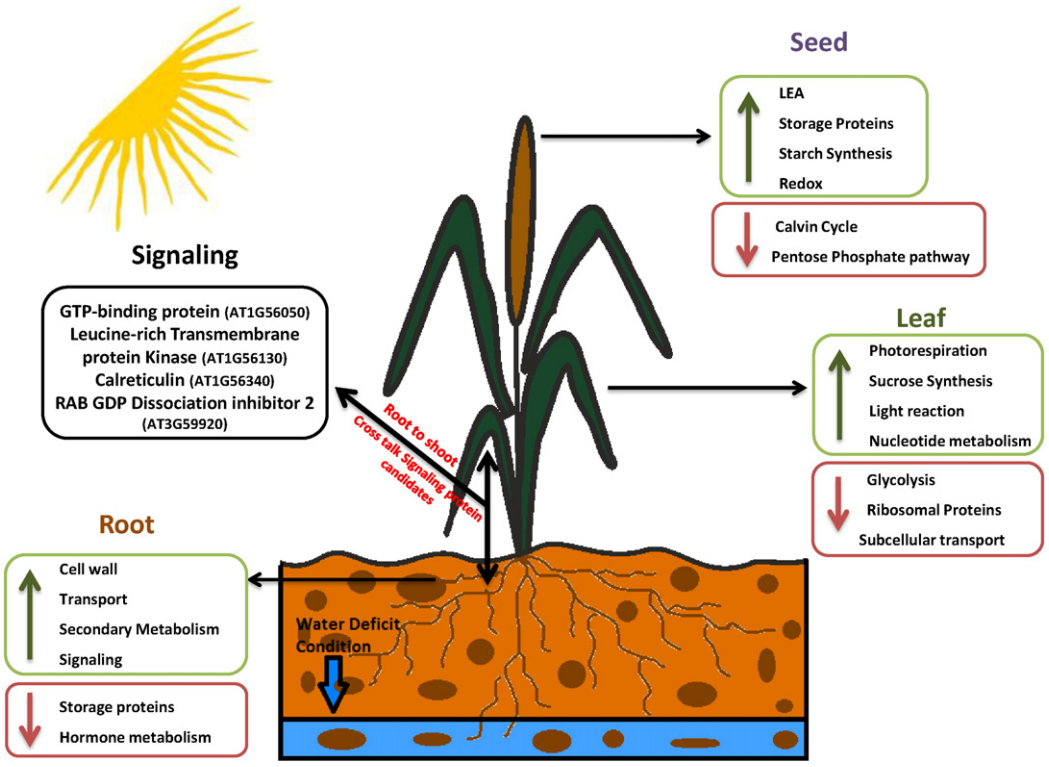

Fig. 6. Systemic analysis and overview of the Pearl millet drought stress proteome.

calcium are altered under stress conditions. In the present study, calreticulin and calnexin were identified which are important proteins of the ER chaperone system. Calnexin is a 90 kDa integral membrane protein type 1 which processes newly synthesized N-linked glycoprotein's and also interacts with the soluble proteins of secretory pathways. Calreticulin plays a major role in cellular functioning which includes calcium storage and signaling. Several studies suggest that the wheat genome contains three copies of TaCrt thereby enhancing drought resistance under water deficit condition [63]. It is also demonstrated that calreticulin is involved in the response to water stress in *Arabidopsis* [64]. In developing soybean roots, calnexin shows high accumulation under abiotic stress [65].

Further, we also identified a 14-3-3 protein. These proteins play major roles as regulator in signaling pathways which are elicited in response to stress in plants. For example, CPK-1 of *Arabidopsis*, an isoform of CDPK (calcium dependent protein kinase), is bound by *Arabidopsis* 14-3-3 isoform (Ω, ψ and φ) thereby stimulating its calcium dependent activity by two fold [66]. Further it was demonstrated that CPK-1 increases cellular 14-3-3 proteins by induction of gene expression under environmental stress which in turn activates CDPK signal transduction pathway in plants to adapt the stress condition [66]. In summary, in the present study proteins identified in the functional category signaling might play a major role in the cross talk between root and shoot in order to provide protection against the water deficit condition (Figs. 5 and 6). It is also suggested that hydraulic signals might trigger the production of the hormone abscisic acid (ABA) in leaves under severe drought condition [67] but production of ABA in roots and its transport to leaves provides the mechanism of chemical signaling to determine the status of water supply in soil. These mechanisms are generated either for the purpose of determining water status or increased production of ABA to maintain root growth under drought condition [68]. ABA is suggested to have a major role in root–shoot signaling and also controls stomatal conductance in leaves [69]. In our analysis we have identified proteins which are categorized under hormone metabolism which include synthesis of abscisic acid (ABA), jasmonate and auxin (Supporting Information Table 17). Interestingly, hormone metabolism was observed to be slightly downregulated in root under water deficit condition compared to control. According to a study performed by Henson et al. [70], the high levels of ABA content in leaves of Pearl millet (*Pennisetum americanum* (L.) Leeke) resulting from drought stress lead to stomatal closure during night, but re-watering dissolves the ABA from leaves. Another hypothesis suggests that root length strongly affects the signaling process, hence the drought sensed signaling from root to shoot is not same for all species [12].

4. Conclusion

P. glaucum (L.) R. Br. – commonly known as Pearl millet – is an important food crop widely cultivated in drought prone soils in semi-arid regions of Africa and the Indian subcontinents. It is highly tolerant against drought and salt in contrast to wheat and maize. In the present study a comparative proteomic analysis was performed considering root, seed and leaf of Pearl millet under water deficit and control conditions. The level of drought stress was determined by using sensors (ML3 Theta Probe provided by Delta — T Devices Ltd) for water potential measurement and measurements of stomatal conductance. It was observed that stomatal conductance in control conditions showed a diurnal rhythm which was completely disturbed under drought condition. Sparse partial least squares (sPLS) regression analysis led to the identification of protein marker in root, seed and leaf which are highly correlated with the harvest index under drought stress condition.

Several drought responsive proteins in root, seed and leaf including germin like protein family, LEA, chlorophyll a/b protein family, and enzymes involved in NADPH metabolism were detected. Leaf showed the strongest effect under drought stress compared to root and seed. Functional categorization of proteins demonstrated that cell wall synthesis, defense, redox and light reaction, LEA and signaling are most strongly affected by drought stress. Orthologs of *A. thaliana*, rice and sorghum are provided for the identified protein drought stress candidates. Calreticulin, calnexin, 14-3-3 protein, and phosphoinositides-specific phospholipase C (PI-PLC) proteins were binned together in the functional category of signaling which showed increased levels under stress

condition. In our analysis we have also identified heat shock proteins (HSPs), molecular chaperones, storage proteins and late embryogenesis abundant (LEA) with increased levels in seeds. These proteins are known to have protective function by stabilizing the folding and conformation of structural proteins (like cell membrane) as well as the functionality of enzymes [71]. This accumulation of protective proteins might be a major mechanism of *Pennisetum* for drought resistance leading to healthy seeds even under water deficit; indeed the productivity of plants does not get as severely affected as for other plants under drought conditions.

Interestingly, hormone metabolism was observed to be altered in root and leaf under water deficit conditions compared to control. A potential explanation might be that deep penetrating roots of *Pennisetum* under drought stress negatively affect hormone metabolism thereby impairing the root to shoot communication. Future studies will address these hypotheses.

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Conflict of interest

The authors declare that no competing interests exist.

Author contributions

Conceived and designed the experiments: WW, AG, PC
Performed the experiments: AG, PC, GB, WP, AS
Analyzed the data: WW, AG, PC, MN, VR, DL, ND, RKV
Written the manuscript: AG, PC, RKV, WW

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