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Method for Label-Free Quantitative Proteomics for *Sorghum bicolor* L. Moench

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Abstract

Sorghum (*Sorghum bicolor* L. Moench) is a rapidly emerging high biomass feedstock for bioethanol and lignocellulosic biomass production. The robust varietal germplasm of sorghum and its completed genome sequence provide the necessary genetic and molecular tools to study and engineer the biotic/abiotic stress tolerance. Traditional proteomics approaches for outlining the sorghum proteome have many limitations like, demand for high protein amounts, reproducibility and identification of only few differential proteins. In this study, we report a gel-free, quantitative proteomic method for in-depth coverage of the sorghum proteome. This novel method combining phenol extraction and methanol chloroform precipitation gives high total protein yields for both mature sorghum root and leaf tissues. We demonstrate successful application of this method in comparing proteomes of contrasting cultivars of sorghum, at two different phenological stages. Protein identification and relative quantification analyses were performed by a label-free liquid chromatography tandem mass spectrometry (LC/MS-MS) analyses. Several unique proteins were identified respectively from sorghum tissues, specifically 271 from leaf and 774 from root tissues, with 193 proteins common in both tissues. Using gene ontology analysis, the differential proteins identified were finely corroborated with their leaf/root tissue specific functions. This method of protein extraction and analysis would contribute substantially to generate in-depth differential protein data in sorghum as well as related species. It would also increase the repertoire of methods uniquely suited for gel-free plant proteomics that are increasingly being developed for studying abiotic and biotic stress responses.

Keywords Abiotic stress · molecular method · quantitative proteomics · sorghum · total protein extraction

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Abbreviations

TCA	Trichloroacetic acid
2DE	2-Dimensional Gel Electrophoresis
2-D DIGE	2-Dimensional Difference Gel Electrophoresis
MALDI-TOF-MS	Matrix Assisted Laser Desorption/ Ionization – Time of Flight - Mass Spectrometry
CBB	Coomassie Brilliant Blue Stain
ABC	Ammonium Bicarbonate
2-DLC/MS-MS	2-Dimensional Liquid Chromatography Mass Spectrometry
DAG	Days After Germination
SDS	Sodium Dodecyl Sulphate
Bis	N,N-Methylbisacrylamide
DTT	Dithiothreitol
TEMED	N-N-N'N' Tetramethylethylenediamine
PMSF	Phenylmethanesulfonylflouride
DMSO	Dimethylsulfoxide
APS	Ammonium Persulphate
BPP	Bromophenol Blue

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PVPP	Poly Vinyl Poly Pyrrolidone
1-D SDSPAGE	1-Dimensional SDS Polyacrylamide Gel Electrophoresis
GO	Gene Ontology
EDTA	Ethylene Diamine Tetra Acetic Acid
PLGS	Protein Lynx Global Server
CC	Cellular Component
iTRAQ	Isobaric Tags for Relative and Absolute Quantitation

Introduction

Sorghum (*Sorghum bicolor L.* Moench) is a widely cultivated staple crop in the arid and semi-arid areas of the world (www.fao.org). Its high biomass production potential in a short growth span of four months, high holocellulose levels and sugary stem stalks also make sorghum a dedicated biofuel feedstock (Thomasson et al. 2009). Its cultivation has alleviated the pressure on traditional biofuel crops like corn and sugarcane, leading to increased food security (Shoemaker and Bransby 2010). In majority of its global cultivation areas, the sorghum crop faces drought, high salinity and other abiotic as well as biotic stresses, exacerbated by climate change. These factors adversely affect both biomass and grain yields in sorghum. Engineering stress-tolerance is therefore of prime importance to sustain the agriculture and livelihoods revolving around sorghum.

Plant protein expression levels are directly correlated to the developmental/physiological state of the plant. Proteomic studies have contributed to unravel important relationships between protein abundance and plant stress acclimatization (Yin et al. 2014). Quantitative proteomics has allowed rapid identification of proteins, their expression dynamics and post-translational modifications (Barkla et al. 2013). This has important implications in development of stress tolerant crops through biomarker selection and transgenic strategies.

In sorghum, comparative proteomics has been explored to study abiotic stress response and to successfully identify prominent protein groups as being drought or salinity responsive (Ngara et al. 2012; Sharma et al. 2012; Jedmowski et al. 2014; Roy et al. 2014). However, majority of these studies so far have relied on conventional approaches for protein extraction (Damerval et al. 1986; Méchin et al. 2007)and gel-based protein abundance studies including 2-dimensional gel electrophoresis (2-DE) and 2D-difference gel electrophoresis (2-D DIGE) followed bymatrix assisted laser desorption/ionization – time of flight - mass spectrometry(MALDI-TOF-MS) technique for individual protein identification. However, it is difficult to obtain consistent and reproducible results for quantitative proteomic analyses using 2DE. Another concern is poor resolution of integral membrane proteins (Wu et al. 2003; Westermeier and Marouga 2005) and possibility of identifying multiple proteins in a single protein spot from a gel (Campostrini et al. 2005). Additionally, sensitivity issues with coomassie brilliant blue (CBB)/silver gel-staining procedures, labour-intensive protein processing for MALDI-TOF/MS based identification and insufficient data for thorough statistical analysis make accurate interpretation of comparative in-gel experiments all the more challenging (Campostrini et al. 2005; Malcevschi and Marmiroli 2012).

Therefore, gel-free approaches in plant proteomics are increasingly being adopted (Abdallah et al. 2012; Malcevschi and Marmiroli 2012). In these approaches, complex peptide fractions, generated after proteolytic/tryptic digestion are resolved using fractionation strategies, which offer high-throughput analyses of the proteome providing a snapshot of the major protein constituents. Some of these fractionation procedures include 2-D liquid chromatography (LC), ion exchange chromatography, reverse phase chromatography, OFFGEL Electrophoresis (Pirondini et al. 2006; Tuli and Ressom 2009; Abdallah et al. 2012) and more recently isobaric tags for relative and absolute quantitation (iTRAQ) (Xie et al. 2016; Shi et al. 2017). It is followed by *denovo* sequencing of the peptide fragments by MS/MS and computational processing to identify the proteins in the sample. Given the rising interest in label-free quantitative plant proteomics, it is important to develop whole-tissue protein sample extraction techniques that are uniquely suited to produce samples of high purity for these downstream processing and analyses.The purpose of this study was to develop a method for efficient protein extraction from sorghum and further a gel free, quantitative proteomics approach for in-depth coverage and analysis of the *S. bicolor* proteome, as applicable to its varieties and tissue-types at different maturity levels. Through gene ontology analysis, we demonstrate that the method developed is broadly applicable to sorghum tissues from mature plants and generates thorough, accurate tissue specific protein abundance data for hundreds of proteins. This method will be immensely useful to study the proteome of this important biofuel crop and use the comparative proteomic information generated to identify candidate proteins/genes to improve desired traits in sorghum cultivars.

Methods

Plant Material and Chemical Reagents

Leaf samples were collected from sorghum genotypes IS 18542, IS 23143 (Fig. 1b), ICSV01 and ICSV700 grown in greenhouse conditions at ICRISAT, India, during December 2014 – mid-February 2015 with temperatures around 28-30^{o}C and an average 12-hour photoperiod/day. Root samples were collected from IS 18542 and IS 23143 only, more from the middle of the whorl. Each genotype was through 8 plants. The

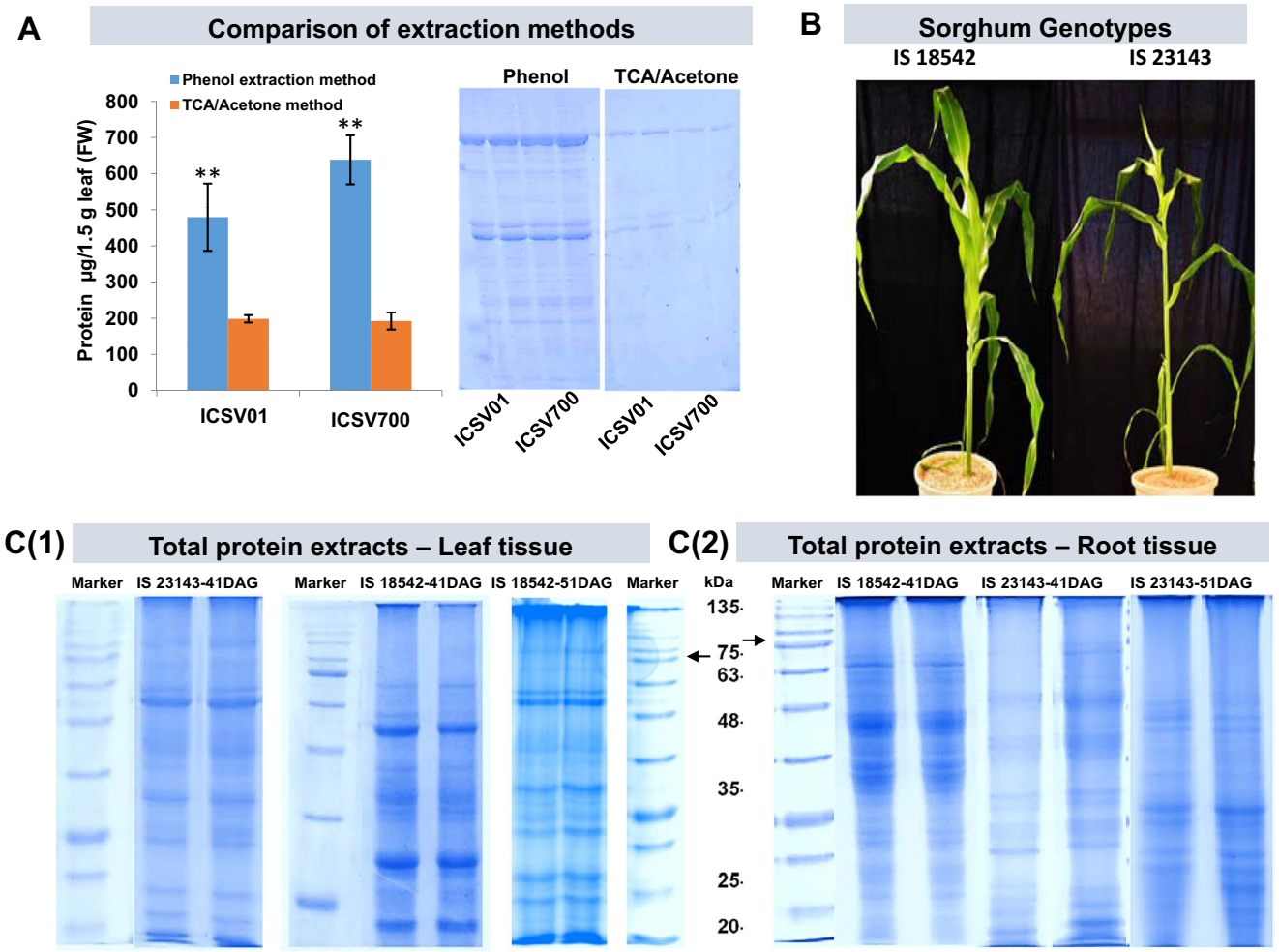

Fig. 1 **a** Quantitative and qualitative comparison between developed protein extraction method and well established TCA-Acetone method for leaf tissues of varieties ICSV01 and ICSV700 **b** Representative images of Sorghum varieties IS 18542 and IS 23143. **c** Comparison of leaf, root proteins at two harvest time points in Sorghum varieties IS 18542 and IS 23143 (1) Comparison of total leaf protein extracts (2) Comparison of total root protein extracts

pots were arranged in complete randomized design. Two Data loggers (EasyLog DATAQ) were installed in the glasshouse along the length gradient to record temperature and humidity at an interval of 1hr.

Sampling was done at 41 days after germination (DAG) and 51 DAG. Two mature leaves and roots were sampled per plant. Tissue collected was flash frozen in liquid nitrogen and stored at -80°C until protein extraction. All protein extractions were conducted in triplicate for each genotype for each of the tested methods.

All chemical reagents used were of analytical grade. Tris-saturated phenol, 2-mercaptoethanol and MBT092-100LN pre-stained protein ladder were procured from HiMedia (Mumbai, India). Dithiothreitol (DTT), N-N-N'N' tetramethylethylenediamine(TEMED) and phenylmethanesulfonylflouride(PMSF) were purchased from Sigma-life sciences (Bengaluru, India) and dimethylsulfoxide(DMSO), ammonium persulphate (APS), acrylamide from Sigma-Aldrich (Bengaluru, India). CBBand bromophenol blue (BPB) were purchased from Bio-Rad (CA, USA). Polyvinylpolypyrrolidone (PVPP) was purchased from Merck Millipore (Darmstadt, Germany) and RapiGest^{TM}SF Surfactant from Waters (MA, USA).

Protein Extraction Protocols

Total Protein Extraction

The total protein extraction protocol has been modified in part from the phenol extraction method described in Isaacson et al. (2006). Suitable amount of frozen sorghum leaf or root tissue was ground in liquid nitrogen to a fine powder with addition of PVPP to ensure removal of polyphenolic impurities. The powder was suspended in required volume of phenol extraction buffer (Table 1), an equal volume of phenol saturated with Tris-HCl was added and the phenolic phase was recovered. The mixture was gently shaken on ice (30 min) followed by centrifugation (5000 *g*, 30 min, 4°C) and collection of the

Table 1 Recommended amounts of plant tissues, reagents and estimated protein yield for the protein isolation method developed

Genotype	Tissue	PVPP (mg)	Extraction buffer (mL)	Estimated protein yield range (mg/mL)	Final protein yield range (μg/g tissue)	Potential key contaminants
IS 18542, IS 23143,	Leaves (2g)	40	10	1.5-2	225-300	Polyphenols
ICSV01, ICSV700	Leaves (1.5 g)	30	7.5	1.6-2.2	320-430	Polyphenols
IS 18542, IS 23143	Roots (4g)	400	40	1.2-3	90-225	None

phenolic phase. To the phenolic phase an equal volume of extraction buffer was added and the phenolic phase was recovered as described above. This extraction was repeated two more times. The volume of the finally collected phenolic phase was noted and 2.5 volumes of ice-cold precipitation solution (0.2 M ammonium acetate in methanol) was added to it and it was left for protein precipitation at -20^{0}C, overnight. The protein pellet was obtained on the following day after centrifugation (5000*g*, 30 min, 4^{0}C). The protein pellet was washed successively with 100% methanol and 100% acetone (chilled) until it was well decolorized. The pellets were dried under laminar flow, transferred to micro-centrifuge tubesand re-solubilized in minimal volume of urea solubilization buffer (7 M Urea, 2 M Thiourea,40 mM DTT and 1 mM PMSF) by repeated vortexing. This solution was centrifuged (15000*g*, 5 min., 4^{0}C) and 100 μL of the clear supernatant was taken in a fresh 1.5mL micro-centrifuge tube. Methanol-chloroform precipitation of the solubilized protein was performed as per the method developed by Wessel and Flugge (Wessel and Flügge 1984) to obtain purified protein pellet. Briefly, 4 volumes (400 μL) of 100% methanol was added to the protein solution followed by rapid vortexing. Then 1 volume of chloroform (100 μL) was added and vortexed followed by addition of 3 volumes (300 μL) of Milliq water and vortexing. This solution was spun for 1 min. at 14000g at room temperature and the top aqueous layer was carefully removed. To this 4 volumes methanol was added and mixed by vortexing. Centrifugation was repeated and methanol carefully removed. The pellet recovered was dried under laminar flow and finally solubilized in 0.1% Rapigest^{TM}solution with intermittent vortexing and sonication in hot water bath (60-65^{0}C). The clear protein solution obtained after centrifugation was checked for quality, concentration and stored at -80^{0}C, till further use.

TCA-Acetone Protein Extraction

Sorghum leaf was ground in liquid nitrogen (1 gm) and mixed with 8 ml 100% ice-cold acetone and 1 ml 100% trichloroacetic acid (TCA, w/v) in a centrifuge tube. After thorough mixing, the proteins were precipitated at -20°C for 1 hr. Protein pellet was recovered by centrifugation at 5000 *g*, 30 min at 4^{0}C. The supernatant was discarded and the pellet was washed with 1 mL ice-cold acetone to ensure complete removal of TCA. This was followed by centrifugation as mentioned above to recover the protein and plant debris in pellet. The pellet was dried at room temperature and dissolved in an appropriate volume of 2-D rehydration buffer (7 M Urea, 2 M Thiourea, 40 mM DTT and 1 mM PMSF). To ensure complete solubilisation of protein, the sample was left at room temperature (22-25^{0}C) for 1 hr, vortexing it after every 10 min, followed by centrifugation (5000 *g*, 10 min at 4^{0}C), to remove the plant debris. The dissolved protein (supernatant) was transferred to a micro-centrifuge tube and centrifuged again (5000 *g*, 10 min at 4^{0}C), to pellet out any un-dissolved components. The clear dissolved protein solution was used for qualitative/ quantitative analysis and stored at -80°C till further use.

Protein Quantification and SDS-PAGE Profiling

The total protein concentration for each of the biological triplicates was determined using the Bradford assay (Bradford 1976) with bovine serum albumin standards (1-10 μg/mL) prepared in 50 mM ammonium bicarbonate (ABC) from 1 mg/mL or 100X concentrated solution.Assays were done in a total volume of 1mLwith 2-5 μL of the sample. Absorbance was measured at 595 nm after 5 min incubation in the dark at room temperature. The protein quality was checked using 1-D SDS-PAGE. The protein quantification data obtained from sorghum tissues was analyzed by students' T test, and considered significantly different at $p<0.01$ (marked by ** in Fig. 1a). A total of 50 μg of protein denatured with 1D-SDS PAGE gel loading buffer was loaded per well. The protein profiles as obtained after CBB staining were documented using BIO-RAD Gel Doc^{TM}EZ imager. The yields and quality of the proteins obtained with phenol extraction method and TCA/Acetone extraction method were recorded. From the biological triplicates for each genotype used for protein extraction, 100 μg of two samples with the best protein profiles as observed through 1-D SDS PAGE were further processed for high throughput expression analysis using liquid chromatography tandem mass spectrometry (LC-MS/MS) to provide proof of suitability of method for gel-free downstream processing.

Proteomics Analyses

The proteomic profiling and relative quantification analysis were performed by LC-MS/MS as detailed below at the Rajiv Gandhi Centre for Biotechnology, India.

In-Solution Trypsin Digestion

Approximately 120 μg of proteins from each sample, normalised to a concentration of 1 μg/μL, was subjected to in-solution trypsin digestion to generate peptides. Disulfide bonds were reduced by incubation of proteins with 100 mM DTT in 50 mM ABC for 30 min at 60^0C. After cooling at room temperature for 5 min, 200 mM iodoacetamide in 50 mM ABC was added, and the reactions were incubated in dark for 30 min to bring about alkylation. Proteins were then digested using sequencing grade modified trypsin (Sigma) in 50 mM ABC at a unit trypsin: total protein mass (in μg) ratio of ~1:25 and incubated for 17 h at 37^0C. The digestion was stopped by addition of formic acid to a final concentration of 1.0% and incubating at 37°C for 20 min. The digested peptide solutions were centrifuged at 20,817g for 12 min, and the supernatant was stored at -20^0C until LC-MS/MS analysis

LC-MS/MS

The tryptic peptides obtained from each sample as mentioned above, were separated using a nanoACQUITY UPLC® chromatographic system (Waters, Manchester, UK) by reversed-phase chromatography. Instrument control and data processing was done with MassLynx4.1 SCN781 software. The peptide sample was injected in partial loop mode in 5 μL loop (injection volume 3.0 μL). Water was used as solvent A and acetonitrile was used as solvent B. All solvents for the UPLC system contained 0.1% formic acid. The tryptic peptides were trapped and desalted on a trap column (Symmetry® 180 μm x 20 mm C18 5 μm, Waters, MA, USA) for 1 min at a flow rate of 15 μL/min. The trap column was placed in line with the reversed-phase analytical column, a 75 μm (internal diameter) X 200 mm HSS T3 C18 (Waters, MA, USA) with particle size of 1.8 μm. Peptides were eluted from the analytical column with a linear gradient of 1 to 40% solvent B over 55.5 min at a flow rate of 300 nL/min followed by a 7.5 min rinse of 80 % solvent B. The column was immediately re-equilibrated at initial conditions (1% solvent B) for 20 min. The column temperature was maintained at 40^0C. The lock mass, [Glu1]-Fibrinopeptide B human (Sigma, Bengaluru, India) (positive ion mode [M+2H] 2+ = 785.8426) for mass correction was delivered from the auxiliary pump of the UPLC system through the reference sprayer of the NanoLockSpray^{TM}source at a flow rate of 500 nL/min. Each sample was injected in triplicate with blank injections between each sample.

MS analysis of eluting peptides was carried out on a SYNAPT® G2 High Definition MS™ System (HDMS^E System (Waters, MA, USA). The instrument settings were: nano-ESI capillary voltage – 3.4 KV, sample cone - 40 V, extraction cone - 4 V, IMS gas (N_2) flow - 90 (mL/min). To perform the mobility separation, the IMS T-Wave™ pulse height is set to 40 V during transmission and the IMS T-Wave™ velocity was set to 800 m/s. The travelling wave height was ramped over 100% of the IMS cycle between 8 V and 20 V.

All analyses were performed in positive mode ESI using a NanoLockSpray^{TM}source. The lock mass channel was sampled every 45 s. The time of flight analyzer (TOF) of the mass spectrometer was calibrated with a solution of 500 fmole/μL of [Glu^1]-Fibrinopeptide B human (Sigma, Bengaluru, India). This calibration set the analyzer to detect ions in the range of 50 - 2000 m/z. The mass spectrometer was operated in resolution mode (V mode) with a resolving power of 18,000 FWHM and the data acquisition was done in continuum format. The data was acquired by rapidly alternating between two functions – Function-1 (low energy) and Function-2 (high energy). In Function-1, only low energy mass spectra (MS) was acquired and in Function-2, mass spectra at elevated collision energy with ion mobility (HDMS^E) was acquired. In Function-2, collision energy was set to 4 eV in the Trap region of mass spectrometer and is ramped from 20 eV to 45 eV in the transfer region of mass spectrometer to attain fragmentation in the HDMS^E mode. The continuum spectral acquisition time in each function was 0.9 seconds with an interscan delay of 0.024s.

MS/MS Data Analysis

The acquired ion mobility enhanced MSE spectra was analysed using Protein Lynx Global SERVER™ v2.5.3 (PLGS, Waters, MA, USA) for protein identification as well as for the label-free relative protein quantification. Data processing includes lock mass correction post acquisition. Processing parameters for PLGS were set as follows: noise reduction thresholds for low energy scan ion – 150 counts, high energy scan ion - 50 counts and peptide intensity - 500 counts (as suggested by manufacturer). The protein identifications were obtained by searching against the Sorghum database downloaded from UniProt (http://www.uniprot.org/proteomes/UP000000768). During database search, the protein false positive rate was set to 4%. The parameters for protein identification were made in such a way that a peptide was required to have at least 1 fragment ion match, a protein was required to have at least 3 fragment ion matches and a protein was required to have at least 1 peptide match for identification. Oxidation of methionine was selected as variable modification and cysteine carbamidomethylation was selected as a fixed modification. Trypsin was chosen as the enzyme used with a specificity of 1 missed cleavage. The protein data set was filtered by considering only those identified proteins which have at least 2 peptides. Data sets were normalized using the 'auto-normalization' function of PLGS and label-free quantitative analyses was performed by comparing the normalized peak area/intensity of identified

peptides between the samples. Furthermore, fold changes higher than 30% difference (ratio of either <0.70 or >1.30) were considered to be indicative of significantly altered levels of expression. Apart from this analysis (including the PLGS inbuilt statistical analysis) no other statistics was applied in the present analysis as the objective was to assess whether the protein quality and processing was suitable for *in solution* proteomics. However, when dealing with biologically relevant samples additional statistics should be separately applied on the data obtained after PLGS analysis.

Protein quantitative analysis, led to the identification of proteins unique to, up/down regulated and/or unchanged in the leaf and root tissue. The gene ontology analysis of the sorted proteins was carried out using online panther tools (http://pantherdb.org/). The proteins up-regulated in root and leaf were analyzed for molecular function, biological process and cellular components using *S. bicolor* as the organism. The fraction of unique proteins in leaf and root tissues was analyzed for cellular localization using the tool (http://geneontology.org/page/go-enrichment-analysis).

Data Availability The datasets during and/or analysed during the current study available from the corresponding author on reasonable request.

Results

Comparison of Developed Extraction Method with Conventional Techniques

Two different methods namely (i) phenol (ii) TCA-acetone extraction methods were used for protein isolation from mature leaf tissues from sorghum varieties ICSV01 and ICSV700 for experimentally establishing the efficiency of the developed method over a conventional technique. The phenol extraction method outperformed the latter in terms of both quantity and quality of proteins extracted as observed in Fig. 1a. These results clearly indicate the advantage of phenol extraction method in obtaining high yields of protein from mature tissues of sorghum. The quality of obtained proteins justifies the longer processing times needed for this method. This result agrees well with literature findings of efficient use of phenol-based extraction protein methods over TCA-acetone (Isaacson et al. 2006). Although the latter is the most common protein extraction protocol, it has been found not to be necessarily good for more complex plant tissues and works better with younger tissues (Saravanan and Rose 2004; Carpentier et al. 2005). Phenol extraction has been used successfully with wood, olive leaves and other recalcitrant tissues such as tomato, banana and avocado fruits and orange peel (Pavoković et al. 2012; Wu et al. 2014). As observed with our results, phenol based extraction generates samples of a higher purity, as possibly compounds such as polysaccharides and other water-soluble contaminants are partitioned into a discrete aqueous phase away from the protein-enriched phenolic layer(Isaacson et al. 2006). This method also shows promise for wide-scale application in sorghum proteomics as it has been tested on four distinct sorghum cultivars in our study.

Application of Developed Method for Protein Isolation in Root/Leaf Tissues from Different Sorghum Varieties

The sorghum genotypes IS 18542 and IS 23143 (Fig. 1b) were selected based on their differing biomass production potential and contrasting characteristics towards drought resistance for proteomics studies. This method was applied to different tissue types from the cultivars at varied maturity stages to test effect of these biological factors on protein from extraction. Total proteins were obtained from mature leaf and root tissues of both genotypesusing the developed method. The protein yield (mg/mL) ranged from 225-300 μg proteins per gram leaf tissue and 90-225 μg proteins per gram root tissue respectively for sorghum varieties IS 18542 and IS 23143 while the yields from mature leaf tissue of other varieties ICSV01 and ICSV700 ranged from 213-300 μg per gram leaf tissue (Table 1). Protein profiles were analyzed using 1-D SDS-PAGE (Fig. 1c, 1-2). Biological triplicateswere included for both tissue types (leaf and root) to emphasize reproducibility of developed method and the protein profiles of biological replicates showed distinctive similarity. The distinction in the protein profiles for both tissue types was clearly visible (Fig. 1c, 1-2). Noticeably different protein profiles in leaf/root tissues enable confidence to map distinct stress-related changes in these tissues at a molecular level through downstream LC-MS/MS analysis and better understand the role of different plant tissues in stress-acclimatization.

Application of the Method to Sorghum Genotypes at Different Growth Stages

For sorghum genotypes IS 18542 and IS 23143, sampling was done at two distinct growth stages (41 and 51 DAG) (Fig. 1c (1-2)). Analysis of the 1-D SDS PAGE protein profiles outlined the differences in protein expression and accumulation within the genotypes, arising due to growth. This method therefore shows great promise in assisting differential proteomic studies aimed at understanding the stress-resisting molecular mechanisms acting at different development stages in plants.

Label-Free Proteomic Identification and Quantification of Sorghum Leaf and Root Tissue Specific Proteins

Conventional protein extraction techniques often involve use of common denaturing and solubilisation reagents like SDS, 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), urea and DTT. These reagents have been shown to commonly interfere with LC based quantitative proteomics techniques (Isaacson et al. 2006). iTRAQ analysis has been used to study aluminium tolerance and drought tolerance in sorghum (Zhou et al. 2016; Handakumbura et al. 2017). However, the protein extraction and preparation method cited therein still uses reagents that can interfere with downstream analysis. The method developed in this study is uniquely compatible with downstream LC-MS/MS based analysis. Through repeated steps of re-solubilisation, protein precipitation and washing, the final protein is free of these reagents and suitable for LC-MS/MS analysis. The protocol described here also involves the unique use of sonication for re-solubilisation of protein pellet in the required buffer for proteolytic treatment. Usually, sonication has been primarily used in protein extraction for preliminary cell lysis steps and applied for bacterial or mammalian single cell systems but not commonly reported for plant systems (Brown and Audet 2008). Tryptic peptides generated from proteins extracted by this method were directly introduced to LC/MS-MS for high-throughput proteomics.

The protein identification results from LC-MS/MS were obtained in the form of protein data for three technical replicates, for two biological replicates per genotype. Data from technical replicates was pooled and normalized using PLGS software, representing the proteins for each biological replicate. Results for proteins identified in leaf and root samples are presented in Table 2. Of the 1238 proteins, 271 and 774 proteins were unique for leaf and root samples respectively, while 193 proteins were common in both tissues Several proteins were found to be more in leaf than root tissues or vice-versa.

Gene Ontology (GO) Analysis

GO analysis is a method to outline genes and gene product properties unified across species. This was used in this study for corroborating the unique functions of the identified and differentially expressed leaf/root tissue-specific proteins with their expected functions and establishs in-silico validation of the developed method. Online PANTHER (Protein ANalysisTHrough Evolutionary Relationships) tools were used for the same. The PANTHER Classification System has been designed to classify proteins (and their genes) to facilitate high-throughput analysis. These tools enabled protein classification under (i) evolution and function, (ii) families and subfamilies, (iii) molecular functions, (iv) biological processes, (v) cellular compartment and (vi) pathways (http://pantherdb.org/). The sorghum root and leaf specific proteins were further successfully and finely corroborated with their leaf/root specific functions (Table 3).

The cellular component (CC) analysis of up-regulated proteins from root showed proteins involved in proton-transporting V-type ATPase, V1 domain (GO:0033180), proton-transporting two-sector ATPase complex, catalytic domain (GO:0033178), proton-transporting two-sector ATPase complex (GO:0016469), membrane protein complex (GO:0098796) enriched by greater than 5 folds. The proteins up-regulated in leaves by several folds were, proton-transporting ATP synthase complex, catalytic core F(1) (GO:0045261), proton-transporting ATP synthase complex (GO:0045259), chloroplast part (GO:0044434), chloroplast stroma (GO:0009570) and thylakoid (GO:0009579) (Table 3). Comparison of the GO Slim CC analysis of the leaf and the root proteins indicated differential distribution across categories (Fig. 2a) and further sub-components of organelle proteins clearly indicated the plastid proteins in leaf and absence of the same in root (Fig. 2b).

The gene ontology of the unique proteins in root and leaves outlined proteins which were tissue specific. Cellular component analysis of the same resulted in detection of proteins from chloroplast thylakoid membrane (GO:0009535), plastid stroma (GO:0009532), photosystem (GO:0009521), chloroplast

Table 2 Quantitative summary of proteins identified from sorghum root and leaf tissue and PLGS comparison

Tissue	Leaf		Root	
	Biological replicate 1	Biological replicate 2	Biological replicate 1	Biological replicate 2
Total proteins (common across all 3 technical replicates)	247	299	606	357
Common across biological replicates	226		327	
PLGS Comparison Data				
Total number of proteins identified with high confidence	1238			
Unique proteins	271		774	
Equally detected	193			

Table 3 Complete Gene Ontology (GO) cellular component analysis results for sorghum leaf and root tissue samples

GO cellular component complete	Sorghum bicolor – Ref list (32965)	Input (103)	Input (expected)	Over/under	Fold Enrichment	P-value
Proteins up-regulated in sorghum leaf						
Proton-transporting ATP synthase complex, catalytic core F(1) (GO:0045261)	9	3	0.03	+	> 100	1.49E-03
Proton-transporting ATP synthase complex (GO:0045259)	22	4	0.07	+	58.76	3.47E-04
Proton-transporting two-sector ATPase complex, catalytic domain (GO:0033178)	22	3	0.07	+	44.07	2.11E-02
Proton-transporting two-sector ATPase complex (GO:0016469)	45	4	0.14	+	28.73	5.76E-03
Apoplast (GO:0048046)	81	7	0.25	+	27.93	3.53E-06
Chloroplast stroma (GO:0009570)	189	8	0.58	+	13.68	6.88E-05
Plastid stroma (GO:0009532)	196	8	0.61	+	13.19	9.05E-05
Mitochondrial part (GO:0044429)	212	6	0.66	+	9.15	2.44E-02
Thylakoid (GO:0009579)	250	7	0.77	+	9.05	6.16E-03
Chloroplast part (GO:0044434)	411	10	1.27	+	7.86	2.95E-04
Plastid part (GO:0044435)	416	10	1.29	+	7.77	3.29E-04
Chloroplast (GO:0009507)	727	17	2.25	+	7.56	4.83E-08
Plastid (GO:0009536)	754	17	2.33	+	7.29	8.40E-08
Cytosol (GO:0005829)	703	13	2.18	+	5.98	1.30E-04
Extracellular region (GO:0005576)	505	9	1.56	+	5.76	1.28E-02
Mitochondrion (GO:0005739)	526	9	1.63	+	5.53	1.75E-02
Cytoplasmic part (GO:0044444)	3087	30	9.55	+	3.14	3.95E-06
Cytoplasm (GO:0005737)	4247	41	13.14	+	3.12	2.25E-09
Intracellular (GO:0005622)	7432	45	23	+	1.96	4.87E-04
Intracellular part (GO:0044424)	7141	42	22.1	+	1.9	3.13E-03
Cell part (GO:0044464)	8809	48	27.26	+	1.76	3.79E-03
Cell (GO:0005623)	8880	48	27.48	+	1.75	4.78E-03
Unclassified (UNCLASSIFIED)	19929	51	61.66	-	0.83	0.00E+00
Intrinsic component of membrane (GO:0031224)	5462	3	16.9	-	< 0.2	6.44E-03
Integral component of membrane (GO:0016021)	5349	1	16.55	-	< 0.2	1.29E-04
Chloroplast part (GO:0044434)	411	58	3	+	19.3	5.63E-53
Plastid part (GO:0044435)	416	58	3.04	+	19.07	1.10E-52
Chloroplast envelope (GO:0009941)	156	20	1.14	+	17.54	4.06E-16
Plastid envelope (GO:0009526)	161	20	1.18	+	16.99	7.38E-16
Chloroplast (GO:0009507)	727	70	5.31	+	13.17	7.39E-54
Plastid (GO:0009536)	754	70	5.51	+	12.7	8.24E-53
Membrane protein complex (GO:0098796)	356	33	2.6	+	12.68	2.88E-23
Organelle envelope (GO:0031967)	362	21	2.65	+	7.93	2.58E-10
Envelope (GO:0031975)	364	21	2.66	+	7.89	2.86E-10

Table 3 (continued)

Intracellular organelle part (GO:0044446)	2367	73	17.3	+	4.22	5.27E-24
Organelle part (GO:0044422)	2375	73	17.36	+	4.2	6.46E-24
Cytoplasmic part (GO:0044444)	3087	93	22.57	+	4.12	1.83E-31
Protein complex (GO:0043234)	1557	38	11.38	+	3.34	3.81E-08
Cytoplasm (GO:0005737)	4247	102	31.05	+	3.29	4.53E-27
Macromolecular complex (GO:0032991)	2177	47	15.92	+	2.95	1.03E-08
Intracellular membrane-bounded organelle (GO:0043231)	5220	89	38.16	+	2.33	6.98E-13
Membrane-bounded organelle (GO:0043227)	5228	89	38.22	+	2.33	7.66E-13
Intracellular part (GO:0044424)	7141	115	52.21	+	2.2	1.54E-16
Intracellular organelle (GO:0043229)	5691	91	41.61	+	2.19	1.42E-11
Organelle (GO:0043226)	5700	91	41.67	+	2.18	1.57E-11
Intracellular (GO:0005622)	7432	116	54.33	+	2.13	1.17E-15
Cell (GO:0005623)	8880	126	64.92	+	1.94	3.25E-14
Cell part (GO:0044464)	8809	124	64.4	+	1.93	1.46E-13
Membrane (GO:0016020)	6532	79	47.75	+	1.65	6.41E-04
Cellular_component (GO:0005575)	13036	135	95.3	+	1.42	7.61E-05
Unclassified (UNCLASSIFIED)	19929	106	145.7	-	0.73	0.00E+00
GO cellular component complete	Sorghum bicolor – Ref list (32965)	Input (53)	Input (expected)	Over/under	Fold Enrichment	P-value
Proteins up-regulated in sorghum root						
Proton-transporting two-sector ATPase complex, catalytic domain (GO:0033178)	22	4	0.04	+	92.21	5.64E-05
Proton-transporting two-sector ATPase complex (GO:0016469)	45	4	0.09	+	45.08	9.55E-04
Mitochondrial part (GO:0044429)	212	5	0.42	+	11.96	2.86E-02
Membrane protein complex (GO:0098796)	356	6	0.7	+	8.55	3.30E-02
Mitochondrion (GO:0005739)	526	7	1.04	+	6.75	3.54E-02
Unclassified (UNCLASSIFIED)	19929	36	39.3	-	0.92	0.00E+00
GO cellular component complete	Sorghum bicolor – Ref list (32965)	Input (271)	Input (expected)	Over/under	Fold Enrichment	P-value
Unique proteins in leaf						
Nitrite reductase complex [NAD(P)H] (GO:0009344)	2	2	0.01	+	> 100	4.58E-02
Photosystem II oxygen evolving complex (GO:0009654)	18	8	0.13	+	60.79	7.69E-10
Photosystem I (GO:0009522)	27	11	0.2	+	55.73	1.29E-13
Thylakoid lumen (GO:0031977)	21	8	0.15	+	52.11	2.59E-09
Photosystem II (GO:0009523)	40	14	0.29	+	47.87	8.76E-17
Photosystem (GO:0009521)	75	26	0.55	+	47.42	2.72E-32
Plastoglobule (GO:0010287)	33	10	0.24	+	41.45	5.36E-11
Thylakoid membrane (GO:0042651)	180	54	1.32	+	41.04	3.04E-66
Chloroplast thylakoid membrane (GO:0009535)	158	47	1.16	+	40.69	4.84E-57
Plastid thylakoid membrane (GO:0055035)	158	47	1.16	+	40.69	4.84E-57
Photosynthetic membrane (GO:0034357)	203	57	1.48	+	38.41	1.54E-68
Chloroplast thylakoid (GO:0009534)	182	49	1.33	+	36.83	1.59E-57
Plastid thylakoid (GO:0031976)	182	49	1.33	+	36.83	1.59E-57
Thylakoid part (GO:0044436)	216	58	1.58	+	36.73	1.03E-68
Thylakoid (GO:0009579)	250	62	1.83	+	33.92	1.13E-71
Organelle subcompartment (GO:0031984)	279	49	2.04	+	24.02	1.12E-48
Extrinsic component of membrane (GO:0019898)	48	8	0.35	+	22.8	1.63E-06
Oxidoreductase complex (GO:1990204)	60	9	0.44	+	20.52	4.23E-07
Chloroplast stroma (GO:0009570)	189	28	1.38	+	20.26	7.30E-25
Plastid stroma (GO:0009532)	196	29	1.43	+	20.24	8.49E-26

Table 3 (continued)

Chloroplast part (GO:0044434)	411	58	3	+	19.3	5.63E-53
Plastid part (GO:0044435)	416	58	3.04	+	19.07	1.10E-52
Chloroplast envelope (GO:0009941)	156	20	1.14	+	17.54	4.06E-16
Plastid envelope (GO:0009526)	161	20	1.18	+	16.99	7.38E-16
Chloroplast (GO:0009507)	727	70	5.31	+	13.17	7.39E-54
Plastid (GO:0009536)	754	70	5.51	+	12.7	8.24E-53
Membrane protein complex (GO:0098796)	356	33	2.6	+	12.68	2.88E-23
Organelle envelope (GO:0031967)	362	21	2.65	+	7.93	2.58E-10
Envelope (GO:0031975)	364	21	2.66	+	7.89	2.86E-10
Intracellular organelle part (GO:0044446)	2367	73	17.3	+	4.22	5.27E-24
Organelle part (GO:0044422)	2375	73	17.36	+	4.2	6.46E-24
Cytoplasmic part (GO:0044444)	3087	93	22.57	+	4.12	1.83E-31
Protein complex (GO:0043234)	1557	38	11.38	+	3.34	3.81E-08
Cytoplasm (go:0005737)	4247	102	31.05	+	3.29	4.53E-27
Macromolecular complex (go:0032991)	2177	47	15.92	+	2.95	1.03E-08
Intracellular membrane-bounded organelle (GO:0043231)	5220	89	38.16	+	2.33	6.98E-13
Membrane-bounded organelle (GO:0043227)	5228	89	38.22	+	2.33	7.66E-13
Intracellular part (GO:0044424)	7141	115	52.21	+	2.2	1.54E-16
Intracellular organelle (GO:0043229)	5691	91	41.61	+	2.19	1.42E-11
Organelle (GO:0043226)	5700	91	41.67	+	2.18	1.57E-11
Intracellular (GO:0005622)	7432	116	54.33	+	2.13	1.17E-15
Cell (GO:0005623)	8880	126	64.92	+	1.94	3.25E-14
Cell part (GO:0044464)	8809	124	64.4	+	1.93	1.46E-13
Membrane (GO:0016020)	6532	79	47.75	+	1.65	6.41E-04
Cellular_component (GO:0005575)	13036	135	95.3	+	1.42	7.61E-05
Unclassified (UNCLASSIFIED)	19929	106	145.7	-	0.73	0.00E+00
GO cellular component complete	Sorghum bicolor – Ref list (32965)	Input (764)	Input (expected)	Over/under	Fold Enrichment	P-value
Unique proteins in root						
Proteasome core complex, alpha-subunit complex (GO:0019773)	12	8	0.28	+	28.77	2.91E-07
Nuclear proteasome complex (GO:0031595)	12	6	0.28	+	21.57	2.16E-04
Proteasome core complex (GO:0005839)	26	13	0.6	+	21.57	5.01E-11
Cytosolic proteasome complex (GO:0031597)	16	7	0.37	+	18.88	5.86E-05
Proteasome complex (GO:0000502)	54	23	1.25	+	18.38	6.54E-19
Proteasome regulatory particle, base subcomplex (GO:0008540)	20	8	0.46	+	17.26	1.47E-05
Proteasome accessory complex (GO:0022624)	26	10	0.6	+	16.6	4.15E-07
Proteasome regulatory particle (GO:0005838)	26	10	0.6	+	16.6	4.15E-07
Eukaryotic 48S preinitiation complex (GO:0033290)	16	5	0.37	+	13.48	1.84E-02
Translation preinitiation complex (GO:0070993)	16	5	0.37	+	13.48	1.84E-02
Cytosolic small ribosomal subunit (GO:0022627)	73	21	1.69	+	12.41	8.37E-14
Cytosolic part (GO:0044445)	224	50	5.19	+	9.63	1.37E-29
Plant-type cell wall (GO:0009505)	135	29	3.13	+	9.27	3.62E-16
Cytosolic ribosome (GO:0022626)	198	42	4.59	+	9.15	8.71E-24
Small ribosomal subunit (GO:0015935)	100	21	2.32	+	9.06	3.48E-11
Cytosolic large ribosomal subunit (GO:0022625)	117	21	2.71	+	7.74	6.52E-10
Ribosomal subunit (GO:0044391)	258	44	5.98	+	7.36	2.75E-21
Large ribosomal subunit (GO:0015934)	155	23	3.59	+	6.4	2.54E-09
Cytosol (GO:0005829)	703	97	16.29	+	5.95	1.89E-41

Table 3 (continued)

Ribosome (GO:0005840)	353	47	8.18	+	5.74	1.67E-18
Extracellular region (GO:0005576)	505	63	11.7	+	5.38	7.66E-24
External encapsulating structure (GO:0030312)	365	43	8.46	+	5.08	6.45E-15
Cell wall (GO:0005618)	365	43	8.46	+	5.08	6.45E-15
Mitochondrial envelope (GO:0005740)	140	15	3.24	+	4.62	6.91E-04
Mitochondrial membrane (GO:0031966)	124	12	2.87	+	4.18	1.97E-02
Vacuolar membrane (GO:0005774)	158	13	3.66	+	3.55	4.86E-02
Whole membrane (GO:0098805)	248	20	5.75	+	3.48	1.06E-03
Ribonucleoprotein complex (GO:1990904)	676	54	15.67	+	3.45	5.21E-12
Intracellular ribonucleoprotein complex (GO:0030529)	676	54	15.67	+	3.45	5.21E-12
Chloroplast stroma (GO:0009570)	189	15	4.38	+	3.42	2.22E-02
Plastid stroma (GO:0009532)	196	15	4.54	+	3.3	3.31E-02
Mitochondrion (GO:0005739)	526	40	12.19	+	3.28	6.35E-08
Mitochondrial part (GO:0044429)	212	16	4.91	+	3.26	2.27E-02
Vacuole (GO:0005773)	368	25	8.53	+	2.93	1.28E-03
Cytoplasm (GO:0005737)	4247	268	98.43	+	2.72	1.04E-52
Intracellular non-membrane-bounded organelle (GO:0043232)	1020	64	23.64	+	2.71	7.83E-10
Non-membrane-bounded organelle (GO:0043228)	1020	64	23.64	+	2.71	7.83E-10
Organelle membrane (GO:0031090)	591	37	13.7	+	2.7	4.37E-05
Cytoplasmic part (GO:0044444)	3087	192	71.54	+	2.68	2.99E-34
Organelle envelope (GO:0031967)	362	22	8.39	+	2.62	2.55E-02
Envelope (GO:0031975)	364	22	8.44	+	2.61	2.75E-02
Bounding membrane of organelle (GO:0098588)	457	25	10.59	+	2.36	4.29E-02
Intracellular organelle part (GO:0044446)	2367	126	54.86	+	2.3	1.69E-15
Organelle part (GO:0044422)	2375	126	55.04	+	2.29	2.20E-15
Macromolecular complex (GO:0032991)	2177	113	50.45	+	2.24	6.84E-13
Cell periphery (GO:0071944)	1724	78	39.96	+	1.95	1.03E-05
Intracellular (GO:0005622)	7432	334	172.24	+	1.94	7.49E-36
Intracellular part (GO:0044424)	7141	320	165.5	+	1.93	1.81E-33
Cell (GO:0005623)	8880	384	205.8	+	1.87	5.47E-40
Cell part (GO:0044464)	8809	380	204.16	+	1.86	4.36E-39
Intracellular organelle (GO:0043229)	5691	236	131.9	+	1.79	1.08E-17
Organelle (GO:0043226)	5700	236	132.1	+	1.79	1.32E-17
Protein complex (GO:0043234)	1557	63	36.09	+	1.75	8.15E-03
Intracellular membrane-bounded organelle (GO:0043231)	5220	186	120.98	+	1.54	3.56E-07
Membrane-bounded organelle (GO:0043227)	5228	186	121.16	+	1.54	4.02E-07
Cellular_component (GO:0005575)	13036	436	302.12	+	1.44	4.96E-20
Membrane (GO:0016020)	6532	108	151.39	-	0.71	1.14E-02
Unclassified (UNCLASSIFIED)	19929	328	461.88	-	0.71	0.00E+00
Membrane part (GO:0044425)	5695	70	131.99	-	0.53	4.98E-08
Intrinsic component of membrane (GO:0031224)	5462	53	126.59	-	0.42	7.87E-13
Integral component of membrane (GO:0016021)	5349	49	123.97	-	0.4	8.94E-14

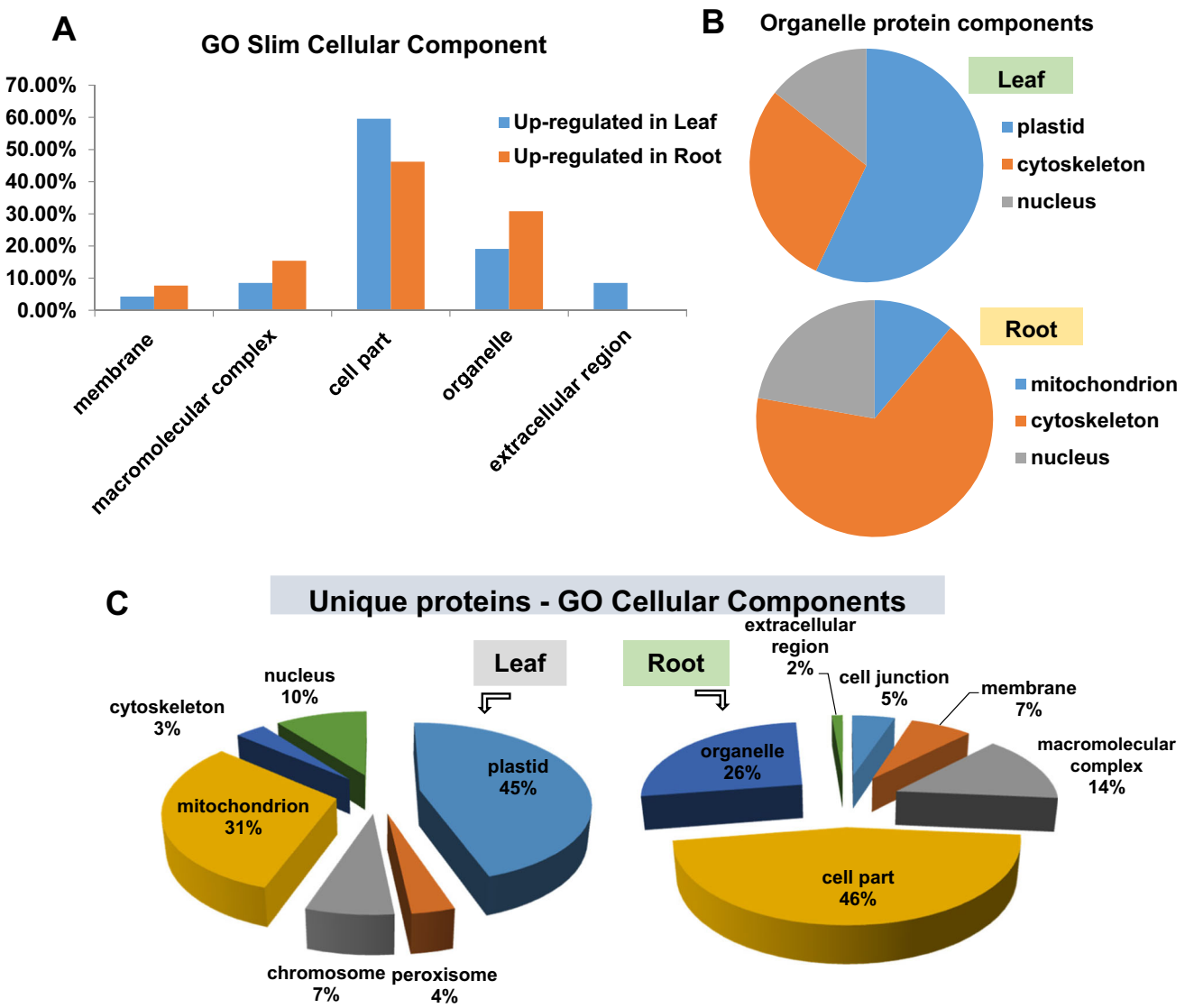

Fig. 2 Gene ontology cellular component (GO-CC) analysis of sorghum leaf and root proteins as performed using the http://pantherdb.org/ (**a**) comparison of up-regulated proteins in leaf and root (**b**) Elaborations of the organelle component from leaf and root up-regulated proteins (**c**) comparisons of unique protein components from leaf and root

stroma (GO:0009570), photosystem II (GO:0009523), photosystem I (GO:0009522), photosystem II oxygen evolving complex (GO:0009654), NAD(P)H dehydrogenase complex (plastoquinone) (GO:0010598), light-harvesting complex (GO:0030076), thylakoid light-harvesting complex (GO:0009503) and others as unique to sorghum leaf tissue. On the other hand, sorghum root unique proteins were specific to cytosol (GO:0005829), ribosomal subunit (GO:0044391), cell-cell junction (GO:0005911), proteasome core complex (GO:0005839), alpha-subunit complex (GO:0019773), golgi-associated vesicle (GO:0005798), golgi-associated vesicle membrane (GO:0030660), plasmodesmata (GO:0009506) as detailed in Table 3. Further, comparing the GO slim CC analysis clearly represented protein categories unique from leaf (plastid) and root (organelle - mostly other than plastids) (Fig. 2c).

The GO analysis data for differentially regulated as well as unique proteins indicates that the method developed for protein isolation, proteomics and data analysis yields protein data which is tissue specific and/or treatment specific. This method based on GO enrichment analysis - performing enrichment analysis on gene sets to identify the set of genes that are over-represented (or under-represented) under certain conditions (http://geneontology.org/page/go-enrichment-analysis) - is hence able to selectively enrich information/identity of tissue-specific proteins and provide a good coverage of the tissue specific proteome. It is therefore a proof of quality of protein stability/integrity as isolated and processed by this method.

Discussion

We hereby report an efficient detergent-free method for total protein extraction from mature leaf and root tissues of sorghum. This phenol-based protein extraction method has been experimentally validated to be advantageous over conventional TCA-acetone based protein extraction method. This protein isolation method was found to be effective on different sorghum genotypes, varied mature tissue types at two distinct growth stages with consistent reproducibility in protein profiles/yields. The proteins isolated by the phenol method were successfully interfaced with gel-free LC/MS-MS for quantitative comparative proteomics. Protein expression profiling and gene ontology data was generated for the sorghum proteins identified using the downstream bioinformatics tools. The comparison of root and leaf proteome led to selective detection of proteins enriched in and/or unique to the tissues. The proteins further corroborated accurately to their tissue specific functions. The protocol developed here is efficient for label free quantitative proteomics to analyze the global proteome changes in sorghum. The method can be successfully applied to practically any sorghum tissue and further coupled with label-free proteomic analysis. It represents a powerful, novel tool for obtaining an in-depth, reproducible coverage of the proteomes of sorghum and related species.

Suggested avenues for fine-tuning of the developed protocol should be aimed at shortening the time for sample processing, minimizing protein losses and thereby increasing the overall yield further.This protocol can also lead to efficient fractionation of the complex total protein samples on multiple levels, including tissue, organelle, and membrane fraction as well as study of post-translational modifications for the discovery of additional and essential abiotic stress-responsive proteins from sorghum. While a complete description of the crop plant abiotic stress proteome may never be attainable, as different strategies are applied and integrated, a more comprehensive picture will be obtained, which will help further to dissect the metabolic and cellular pathways and mechanisms that are essential for abiotic stress tolerance in sorghum. This will ultimately allow for the translation of proteomic findings into the long-term goal of successful field applications.

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Authors' Contributions AAS conducted all experimental work at ICRISAT, India, quality control on proteomics analysis samples, preparation of raw proteomic data for bioinformatic analysis, has written major portion of the manuscript and formatting of the same. ANN conducted the experimental work at IBB Pune and a part of bioinformatics analysis on proteomic data and written relevant sections of the manuscript arising from this work. AJ and the technical team at RCGB conducted all LC-MS/MS based proteomic analysis, generated the raw differential proteomic data across the different sorghum cultivars in the study and written relevant sections of the manuscript arising from this work. VAT and SPR have planned, designed and supervised the experimental and bioinformatics works, as principal investigators at IBB, SPPU, Pune and ICRISAT, Patancheru respectively. They acquired funding for the work and edited and reviewed the manuscript for submission. All authors read and approved the final manuscript.

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Compliance with Ethical Standards

Ethics Approval and Consent to Participate Not applicable

Consent for Publication Not Applicable

Conflict of Interest The authors declare that they have no conflict of interest.

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