

Proteomic Analysis of PEG-Induced Drought Stress Responsive Protein in *TERF1* Overexpressed Sugarcane (*Saccharum officinarum*) Leaves

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Abstract Drought is the major abiotic stress limiting sugarcane growth and productivity. ERF proteins regulate a variety of stress responses in plant. Overexpression of *TERF1* can enhance the tolerance of transgenic sugarcane to drought stress. To improve the efficiency of sugarcane breeding, better understanding of the tolerance mechanism at molecular level is required. Two-dimensional gel electrophoresis (2-DE) coupled tandem mass spectrometry (MS/MS) analyses were conducted to compare the leaf proteome of the *TERF1* OE and WT sugarcane plants to PEG stress. Using statistical program, 50 significantly differential protein spots were detected, of which 36 spots were identified by PMF and MS/MS fragmentation. Most of the identified proteins corresponded to metabolism, energy, protein synthesis, and disease/defense. Results implicated that the involvement of different metabolic pathways that may be activated in the *TERF1* overexpressed transgenic sugarcane to cope with drought environment. Of the identified proteins, abundance of pentatricopeptide repeat (PPR) containing protein and peptidyl prolyl cis-trans isomerase (PPIase) were decreased, but the abundance of vital proteins, such as metabolism protein (14-3-3 like protein), photosynthetic protein (RuBisCO large subunit, PEP carboxylase),

ferredoxin, glyceraldehyde 3-phosphate dehydrogenase, elongation factor Tu, several small heat shock proteins, and peroxidases were increased. Analysis of protein properties showed that majority of the differentially abundant proteins associated with drought were stable, hydrophilic, and transmembrane proteins. Thus, the results of our study unravel the regulatory mechanism of *TERF1* for drought stress tolerance of transgenic sugarcane and provide new insight into adaptation to osmotic stress through altering the expression of particular proteins.

Keywords Proteomics · PEG stress · *TERF1* overexpression · Sugarcane · 2-DE · Mass spectrometry

Abbreviations

TERF1	Tomato ethylene responsive factor 1
TCA	Trichloroacetic acid
2-DE	Two-dimensional gel electrophoresis
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
OE	Overexpressed
MOWSE	Molecular weight search
WT	Wild-type
IEF	Isoelectric focusing
PEG	Polyethylene glycol
SDS	Sodium dodecyl sulfate
ERF	Ethylene responsive factor
PMF	Peptide mass fingerprinting
RWC	Relative water content
MS	Mass spectrometry
DTT	Dithiothreitol
NCBI	National center for biotechnology information
MALDI-TOF-TOF-MS	Matrix-assisted laser desorption/ionization-time of flight/time of flight tandem mass spectrometry
ROS	Reactive oxygen species

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Introduction

Sugarcane is the major sugar-producing crop as well as one of the most renewable energy crop widely grown under rainfed condition in many countries (Laclau and Laclau 2009). Drought stress severely affects the growth and development of sugarcane (Prabu et al. 2011). After planting, bud germination and establishment of crop depend on soil moisture. Thus, the plant growth and survival largely depend on the ability of the plant to cope with the drought stress during dry period. Due to the complex polyploidy nature of sugarcane, its improvement via traditional breeding is limited. Thus, transgenic approach becomes an important feature for improving sugarcane crop. Overexpression of stress responsive proteins through generation of transgenic plants is one of the main practical approaches in developing tolerant plants. Transcription factors (TFs) play an important role in maintaining the expression of functional protein. The TFs of candidate genes enhance or repress the expression of proteins in response to biotic or abiotic stimuli. ERF proteins are a subfamily of the AP2/ethylene responsive element binding protein (EREBP) transcription factor family which is unique to plants (Singh et al. 2002). The AP2/ERF transcription factors regulate various response processes in plant including abiotic and biotic stress responses (Licausi et al. 2010; Sharoni et al. 2011). Overexpression of *ERF* improves tolerance of transgenic plants against drought, salt, and freezing (Xu et al. 2008; Zhu et al. 2010). *TERF1* is an ERF gene identified in tomato, and overexpression of this gene improves tolerance of transgenic rice to drought and high salinity (Gao et al. 2008). Overexpression of *TERF1* increases the abundance of proline and decreases loss of water in transgenic rice lines, which helps plants to maintain osmotic equilibrium and membrane stability that provides plant tolerance under stress condition (Gao et al. 2008). The previous reports elucidate gene expressions at the transcriptional level. However, the underlying mechanisms of drought stress on growth and development as well as changes of metabolic processes remain unclear.

Proteomic study focuses on protein abundance changes, modifications, and analysis of the protein components; proteomics is a powerful tool for investigating the molecular mechanisms of the plant stress responses (Kim et al. 2009; Swami et al. 2011). To elucidate the molecular mechanisms of the *TERF1* transcription factor, identification of the proteins induced in response to drought stress in transgenic sugarcane is necessary. Proteomic study with two-dimensional gel electrophoresis (2-DE) is an efficient method for analyzing the differential expression pattern of proteins in plant (Agrawal et al. 2009; Ding et al. 2011; Falvo et al. 2011; Kim et al. 2011). There are few reports about proteomic study of sugarcane in response to drought stress. Different proteins' abundance from drought-tolerant sugarcane cultivar exposed to drought stress at natural condition was detected by

Jangpromma et al. (2010). Zhou et al. (2012) found four differentially-induced proteins from sugarcane leaves which are exposed to osmotic stress. In another report, Ngamhui et al. (2012) identified small heat shock proteins, CuZn-SOD, APX, and ATP synthase protein from sugarcane plant in response to drought stress. In this study, *TERF1* overexpressed transgenic sugarcane was investigated through comparing differential protein abundance between *TERF1* overexpressed and wild-type sugarcane plant in response to polyethylene glycol (PEG) stress.

The object of this study was to identify the proteins that altered due to overexpression of *TERF1* gene to improve drought tolerance in transgenic sugarcane.

Materials and Methods

Generation of Transgenic Plant and Drought Stress Treatment

In our previous work, *TERF1* overexpressed transgenic sugarcane plants were obtained through *Agrobacterium*-mediated method. Full length of *TERF1* complementary DNA (cDNA) was introduced into sugarcane cultivar Guitang 281 under the control of CaMV35S promoter, through *Agrobacterium*-mediated transformation. Wild-type (WT) plants were regenerated from non-transformed callus of the same cultivar under the same condition. Subsequently, plantlets were placed in the plastic pots with 25-cm width and 30-cm depth. Single plant of OE and WT lines were placed in each pot with three replications and kept in greenhouse. Drought-tolerant OE sugarcane lines were selected and the ectopic expression of *TERF1* was checked by (RT)-PCR (Fig. S1).

For drought treatment, 7-month-old OE and WT sugarcane plants were exposed to polyethylene glycol (PEG 8000; 20 % w/v) by flushing 1-L PEG solutions to each pot every 5-day interval. Control pots were maintained under well-watered condition. After 21 days of treatment, the second leaves of OE and WT lines from drought stress condition and normal condition were harvested in three replicates, stored at -70°C and used for protein extraction. Leaves for measuring chlorophyll and RWC were collected under drought stress condition at the 7th, 14th, and 21st day from the second leaf of the same plant under drought stress condition and normal condition.

Determination of Chlorophyll Content

Leaf chlorophyll content was estimated following the method of Moran (1982). Briefly, second fully expanded leaves as described above were selected. Leaf disks with an area of 1 cm^2 were cut by cork borer from the leaf blade. Three independent replicates of each treatment were selected and two disks of each leaf were made. The leaf disks were incubated in the dark at 4°C for 24 h in vials containing 5 mL

N, N-dimethylformamide (DMF). Three-milliliter chlorophyll extraction was taken out for chlorophyll quantification. The chlorophyll concentration was quantified using a UV-Visible spectrophotometer (BioMate 3S, Thermo Scientific) at wavelengths of 647 and 664 nm (OD_{664} and OD_{647} , respectively). Total chlorophyll content was calculated by the equation below and expressed in the unit microgram per square centimeter ($\mu\text{g}/\text{cm}^2$).

$$\text{Chlt} = 7.04 \text{ OD}_{664} + 20.27 \text{ OD}_{647}$$

Estimation of RWC

Leaf disks of 1 cm^2 for relative water content (RWC) estimation were obtained as mentioned above. Three independent replicates of each treatment were selected and five disks of each leaf were made. Fresh weight (W_f) of all the disks was determined immediately. The leaf disks were then hydrated in deionized water at room temperature for 24 h in the dark to obtain turgid weight (W_t). Finally, the leaf disks were dried for 48 h at 70°C to determine dry weight (W_d). RWC was calculated with the equation by Matin et al. (1989).

$$\text{RWC} = [(W_f - W_d) / (W_t - W_d)] \times 100$$

Protein Extraction and Two-Dimensional Gel Electrophoresis

Total protein was isolated using trichloroacetic acid (TCA)/acetone extraction method (Amalraj et al. 2010). Leaf samples (1 g) from three independent replicates of OE and WT lines in each treatment were collected and grinded to fine powder using liquid nitrogen in mortar and pestle. The fine powder was taken to an 8-mL tube and then a 6-mL extraction buffer (10 % w/v TCA in acetone with 0.07 % dithiothreitol (DTT)) was added. The tubes were incubated overnight at -20°C for precipitation of proteins, after that, centrifuged at $12,000g$ for 20 min at 4°C . The pellets were re-suspended in 6-mL wash buffer (0.07 % w/v DTT in ice-cold acetone), mixed by vortexing, incubated at -20°C for 1 h, and followed by centrifugation for 20 min at $12,000g$ at 4°C (this step was repeated three times). Finally, the pellets were dried into powder by vacuum dryer and stored at -70°C . Powdered pellet (100 mg) was added in 800- μL lysis buffer (7 M w/v urea, 2 M w/v thiourea, 4 % w/v 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 2 % w/v IPG buffers, pH 4–7, and 2 % w/v DTT), incubated for 3 h at 37°C , and vortexed every 30-min interval and centrifuged at $12,000g$ for 20 min at 4°C . Total protein content was estimated following the method of Bradford (1976), using bovine serum albumin as the standard.

For first dimensional isoelectric focusing (IEF), IPG DryStrips (GE Healthcare, Amersham, UK), 24 cm, pH 4–7 linear gradient were used. Total soluble protein (1,000 μg)

was dissolved in rehydration buffer (7 M urea, 2 M thiourea, 2 % (w/v) CHAPS, 2 % DTT, 0.5 % (v/v) IPG buffer, and 0.002 % bromophenol blue (BPB)) and made to volume of 450 μL . The IPG strips were rehydrated for 18 h at room temperature with increasing linear voltage as follows: 3 h at 30 v, 1 h at 100 v, 1 h at 500 v, 3 h at 1,000 v, 1 h at 3,000 v, 1 h at 8,000 v, 12 h at 8,000 v, and 1 h hold at 500 v. Working temperature was maintained at 20°C for IEF and electric current was set as 50 mA per strip. IEF strips were equilibrated with 10-mL reduction buffer (urea 6 M, sodium dodecyl sulfate (SDS) 2 %, BPB 0.002 %, glycerol 30 %, 50 mM Tris-HCl (pH 8.8), 1 % DTT) and 10-mL alkylation buffer (instead of DTT of reduction buffer, 2.5 % iodoacetamide was used) for 15 min with gentle shaking. Then, the IPG strips were washed with running buffer (0.025 M Tris, 0.192 M glycine, and 0.1 % w/v SDS).

The strips were then placed on vertical slab of 12.5 % polyacrylamide gel. Electrophoresis was started with electric current at 15 mA/strip, and it was increased to 30 mA/strip after front edge of BPB reached to the gel. Low molecular weight (LMW) maker (GE healthcare, Amersham, UK) containing Phosphorylase b (97.4-kDa), BSA (66.2-kDa), Actin (43-kDa), Carbonic anhydrase (43-kDa), Trypsin inhibitor (20.1-kDa), and lysozyme (14.4-kDa) was loaded beside the strip. After the completion of running, the gel was taken out and rinsed in fixation solution (40 % ethanol and 10 % acetic acid) overnight with gentle shaking for fixing the protein spots in analytical gel and then washed three to four times with ultrapure water, each for 15 min. Staining of gels was performed overnight with gentle shaking using Coomassie brilliant blue (CBB) solution (0.12 % of CBB G250, 10 % of ammonium sulfate, 10 % of phosphoric acid, and 20 % of methanol).

Image Acquisition and Statistical Analysis of Data set

Images of gels were obtained using image scanner III (GE Healthcare). Gel images were analyzed by the software of ImageMaster 2D Platinum 7.0 (GE Healthcare). Four sets of gels, two from control WT and OE plants and another two from stressed WT and OE plants, were prepared for protein spot matching and statistical analysis. The differences between WT versus OE plants under control and WT versus OE plants under stressed conditions were observed. For this, protein spots in WT and OE gels under control and stressed conditions were matched and mean volume was compared. Statistical analysis was performed by Student's *t* test for comparing significant differences among the spots of two groups. Hence, abundance of protein spot volume ratio at 10 % increased/decreased was considered as increased or decreased abundance and differentially expressed as they showed a significance difference ($p < 0.05$) and displayed reproducible change patterns.

Protein Identification by Mass Spectrometry and Database Search

Protein spots of interest were picked manually by 200 μL PCR tube. The bottom of PCR tube was cut according to the size of the spot, pressed it on spot, taken out, and the spots were removed by micropipette and cut into pieces. The spots pieces were washed with destaining buffer (50 % ACN, 25 mM) and dried in a freeze drier and incubated with 3–5 μL trypsin (15 ng/ μL , 25 mM NH_4HCO_3) for 18 h at 37 °C. The spots were then immersed for 1 h in 50 μL of 2.5 % TFA and 50 % ACN at 37 °C. The supernatant was combined and vacuum-dried.

The resulted peptides were dissolved in 2 μL of 0.5 % TFA and 50 % ACN, and spotted onto a laser desorption/ionization (MALDI) target plate with CHCA as matrix. The samples were analyzed using Applied Biosystems 4800 Proteomics Analyzer. A combined PMF tandem mass spectrometry (MS plus MS/MS) search was conducted using GPS Explorer V2.0 software (Applied Biosystems) over Swiss-Prot database employing the MASCOT search engine (Matrix Science, London; <http://www.matrixscience.com>), and protein spots were searched from Viridiplantae in the Swiss-Prot database. The search parameters were as follows: 50 ppm mass tolerance; trypsin digestion with two missed tryptic cleavage sites; MH^+ and monoisotopic; and carbamidomethyl as a fixed modification for cysteines, oxidation as a variable modification for methionines. We did not download the database, and the search of database was conducted online. Proteins were identified based on the score of molecular weight search, sequence coverage percentage, and matched peptide numbers, respectively. As indicated by the Mascot search engine, the score was higher than 56 (probability value of $p < 0.05$), and at least one tandem mass spectrometry (MS/MS) sequence matched was accepted as identified.

Bioinformatics Analysis of Differentially Expressed Proteins

Basic Local Alignment Search Tools (BLAST) of NCBI (<http://ncbi.nlm.nih.gov/blast/>) was used to know the sequence of MS/MS-analyzed protein. Identified proteins were divided into functional class as described by Bevan et al. (1998), using Database for Annotation, Visualization and Integrated Discovery (<http://david.abcc.ncifcrf.gov>), ProtFun server (www.cbs.dtu.dk/services/ProtFun/), and UniProt database (www.uniprot.org) search. Physical and chemical properties, hydrophobicity, and transmembrane analysis of differentially-expressed proteins were done by ProtParam, ProtScale, and TMpred tools of the Swiss Institute of Bioinformatics (www.expasy.ch/tools).

Results

Analysis of RWC and Chlorophyll Content Changes

The extent of the PEG-induced drought stress was monitored through measuring the relative water content and chlorophyll content of OE and WT sugarcane plants. Under normal condition, RWC between the OE and WT was similar. The RWC declined gradually during PEG stress treatment (Fig. 1), and the OE transgenic lines have higher RWC than the WT lines as measured from the 7th day to the 21st day under drought stress. At the 21st day, the leaves of WT plants were observed to be wilted and dead, but OE lines remained almost normal. At the 21st day, the RWC of WT plants reduced to 54 %, whereas it was 85 % in OE lines when measured.

Chlorophyll contents of OE and WT sugarcane plants were similar under normal condition. Under PEG stress treatment, the chlorophyll content of OE lines was significantly higher than that of the WT lines as measured from the 7th to the 21st day (Fig. 2). After 21 days of PEG stress treatment, leaf chlorophyll content of WT reduced to 2.5 $\mu\text{g}/\text{cm}^2$, while it was 4.4 $\mu\text{g}/\text{cm}^2$ in OE. These results indicated that the photosynthetic activity was higher in OE line than in WT line under drought stress condition. Thus, results of our present study implicated that TERF1 can stabilize the photosynthetic activity and decrease water loss in sugarcane under drought stress.

Analysis of Total Protein Content Changes

Under PEG stress condition, total protein content in sugarcane leaves of OE lines was higher than that of WT lines (Fig. 3). There is no significant difference of protein content in WT plants between drought stress condition and normal condition.

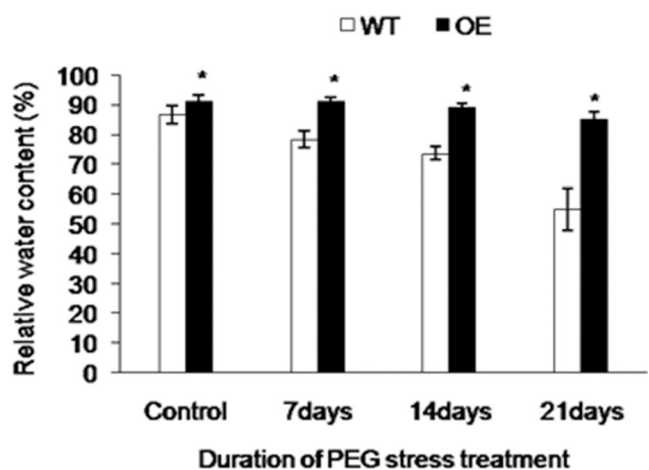


Fig. 1 Leaf relative water content of wild-type (WT) and *TERF1* overexpressed (OE) line in response to PEG stress. Data are mean \pm SD with three replicates. Asterisks indicates statistically significant difference at $p < 0.05$

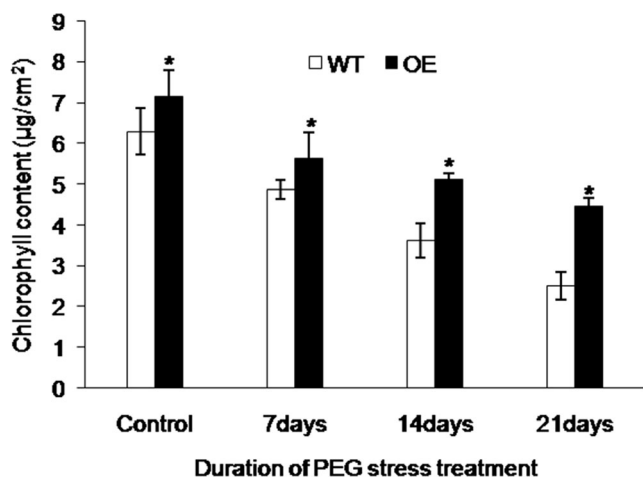


Fig. 2 Leaf chlorophyll content of WT and OE sugarcane in response to PEG stress. Data are mean±SD with three replicates. Asterisks indicate statistically significant difference at $p<0.05$

Under normal condition, protein contents of OE and WT sugarcane leaves were statistically similar.

Image Analysis and Identification of Differentially-Expressed Proteins

Molecular mechanism of drought tolerance in the OE sugarcane was examined through detecting abundance changes of protein(s) and statistically analyzing the protein spots between OE lines and WT plants. Three biological replicated gels of OE and WT sugarcane leaves protein under both normal and PEG stress conditions were conducted. Under normal condition, protein abundance between OE and WT plants was similar. While under drought stress condition, 50 statistically significant ($p<0.05$) differentially-expressed protein spots were detected using *t* test. All the 50 differential protein spots were subjected to MALDI-time of flight (TOF)/TOF-MS analysis and MS spectra was used to screen Viridiplantae

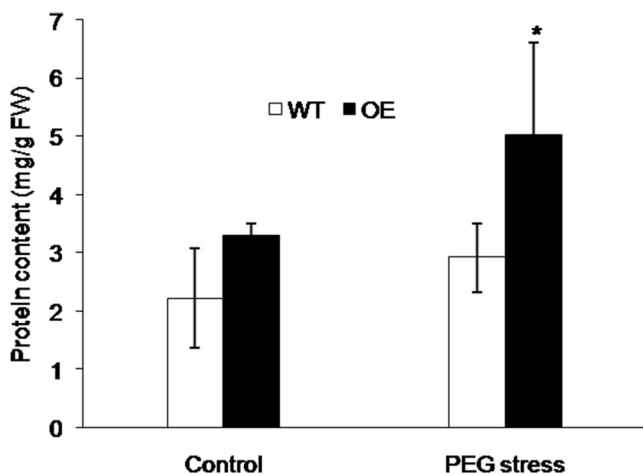


Fig. 3 Protein content changes in leaves of WT and OE sugarcane under control and PEG stress conditions. Data are mean±SD with three replicates. Asterisks indicate statistically significant difference at $p<0.05$

index of the Swiss-Prot database. Thirty-six spots were successfully identified with a high probability score, and the position of identified protein spots were marked on the gels (Fig. 4). Under drought stress condition, expression levels of identified spots were calculated by volume percentage (Table S1). The expression patterns of some of these spots were predicted by ImageMaster 2D Platinum software (Fig. 5).

Under stress condition, among the 36 protein spots, abundance of 26 spots were increased, 2 were decreased, and 8 were newly found in OE sugarcane lines (Table S1). Some proteins were found in multiple spots, which were phosphoenolpyruvate carboxylase (spots 11, 29, and 30), ribulose biphosphate carboxylase large chain (spots 13, 21, 35, and 36), ferredoxin-NADP reductase (spots 19 and 20), probable fructose-biphosphate aldolase (spots 23, 24, and 25), malate dehydrogenase (spots 26 and 27), glyceraldehyde-3-phosphate dehydrogenase (spots 28 and 32), and L-ascorbate peroxidase (spots 16 and 17). Most of these proteins spots were differed in both of their experimental MW and *pI* values on 2-DE gel except probable fructose-biphosphate aldolase. The spots of this protein were differed only in their *pI* values (Table 1). There were several possible reasons for the presence of a protein with multiple spots. For one protein, there may be several subunits and these subunits will be separated when 2DE is conducted. And furthermore, post transcriptional modifications (PTM) maybe existed in some proteins which may cause this phenomenon.

Classification of Identified Proteins and Analysis of Their Abundance Level

Identified proteins were grouped into functional categories as described by Bevan et al. (1998). They were assigned to seven groups (Table 1, Fig. 6): energy (52.7 %), disease/defense (25 %), metabolism (8.3 %), protein synthesis (5.5 %), transcription (2.7 %), intracellular traffic (2.7 %), and signal transduction (2.7 %).

NAD (P) H-quinone oxidoreductase subunit M (spot 10), cellulose synthase-like protein D2 (spot 14), and 14-3-3-like protein 1 (spot 18) were metabolism proteins, which showed abundance increased in OE lines after PEG stress in the present study. It has been previously reported (Nouri and Komatsu 2010) that quinone oxidoreductase protein abundance was increased, and 14-3-3-like protein D abundance was decreased in soybean under PEG stress. In our study, the majority of the differentially expressed proteins were energy metabolism-related proteins. Energy metabolism function group comprises 19 proteins (Table 1), and decreased abundance was observed only in peptidyl prolyl cis-trans isomerase (spot 2). However, in another work, abundance of this protein was increased in leaf and root tissues of sorghum under drought stress (Sharma and Singh 2003). Other protein spots showed increased abundance in transgenic lines compared

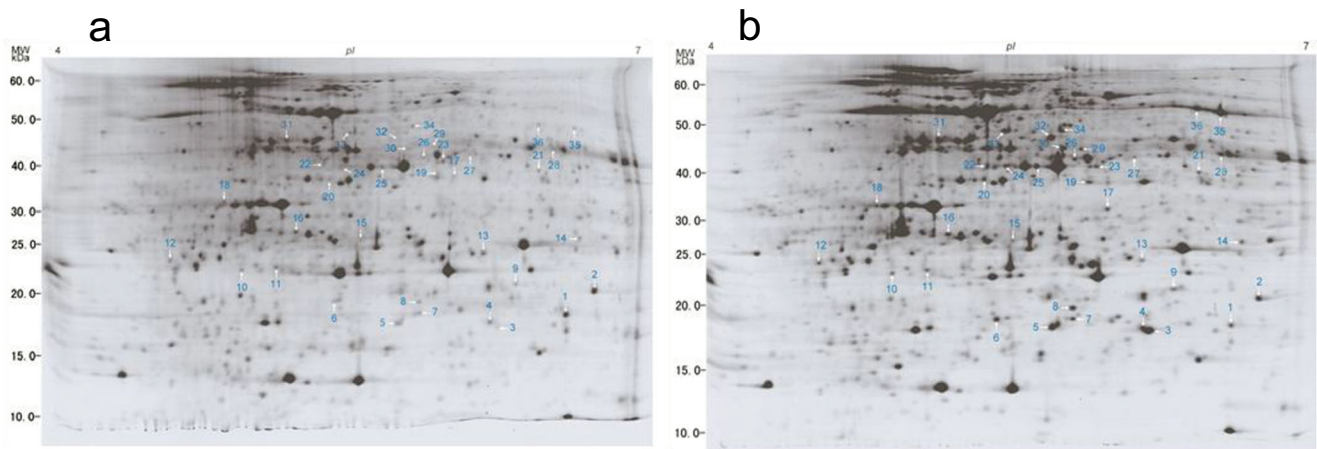


Fig. 4 Representative Coomassie blue stained 2-DE gels of wild-type (a) and *TERF1* overexpressed (b) transgenic sugarcane leaves in response to PEG stress. The proteins identified are marked with *arrows* and *numbers*

with WT lines under PEG stress condition. They were, phosphoenol pyruvate carboxylase (spots 11, 29, and 30), ribulose biphosphate carboxylase (RuBisCO) large chain (spots 13, 21, 35, and 36), ferredoxin-NADP reductase (spots 19 and 20), probable fructose-biphosphate aldolase I (spots 23, 24, and 25), malate dehydrogenase (spots 26 and 27), glyceraldehyde-3-phosphate dehydrogenase A (spot 28), glyceraldehyde-3-phosphate dehydrogenase B (spot 32), ATPase 3, plasma membrane-type (spot 31), and phosphoglycerate kinase (spot 34). Increased abundance of photosynthetic protein RuBisCO large subunit was observed in transgenic bentgrass leaves in response to drought stress, while in the leaves of non-transgenic plant this protein's abundance was decreased or remained unchanged under the same condition compare to the control plants (Merewitz et al. 2011). Plomion et al. (2006) reported that, under drought stress, the abundance of ferredoxin-NADP reductase was increased in the leaves of poplar. Castillejo et al. (2008) observed that the abundance of glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase were decreased in susceptible variety of sunflower under drought stress.

In the present study, the abundance of protein synthesis function group includes the 60S acidic ribosomal protein (spot 22) and elongation factor Tu (spot 33) (Table 1). Ngamhui et al. (2012) reported that the increasing abundance of elongation factor Tu in tolerant sugarcane cultivar resulted from water deficit condition. Transcription, intracellular traffic, and signal transduction function proteins (Table 1) were translationally controlled tumor protein homolog (spot 12), pentatricopeptide repeat-containing protein (spot 1), and Phytochrome A type 4 (spot 15), respectively. The abundance of pentatricopeptide repeat-containing protein (spot 1) was decreased, and the other two were increased in transgenic sugarcane lines compared with the WT lines under drought stress.

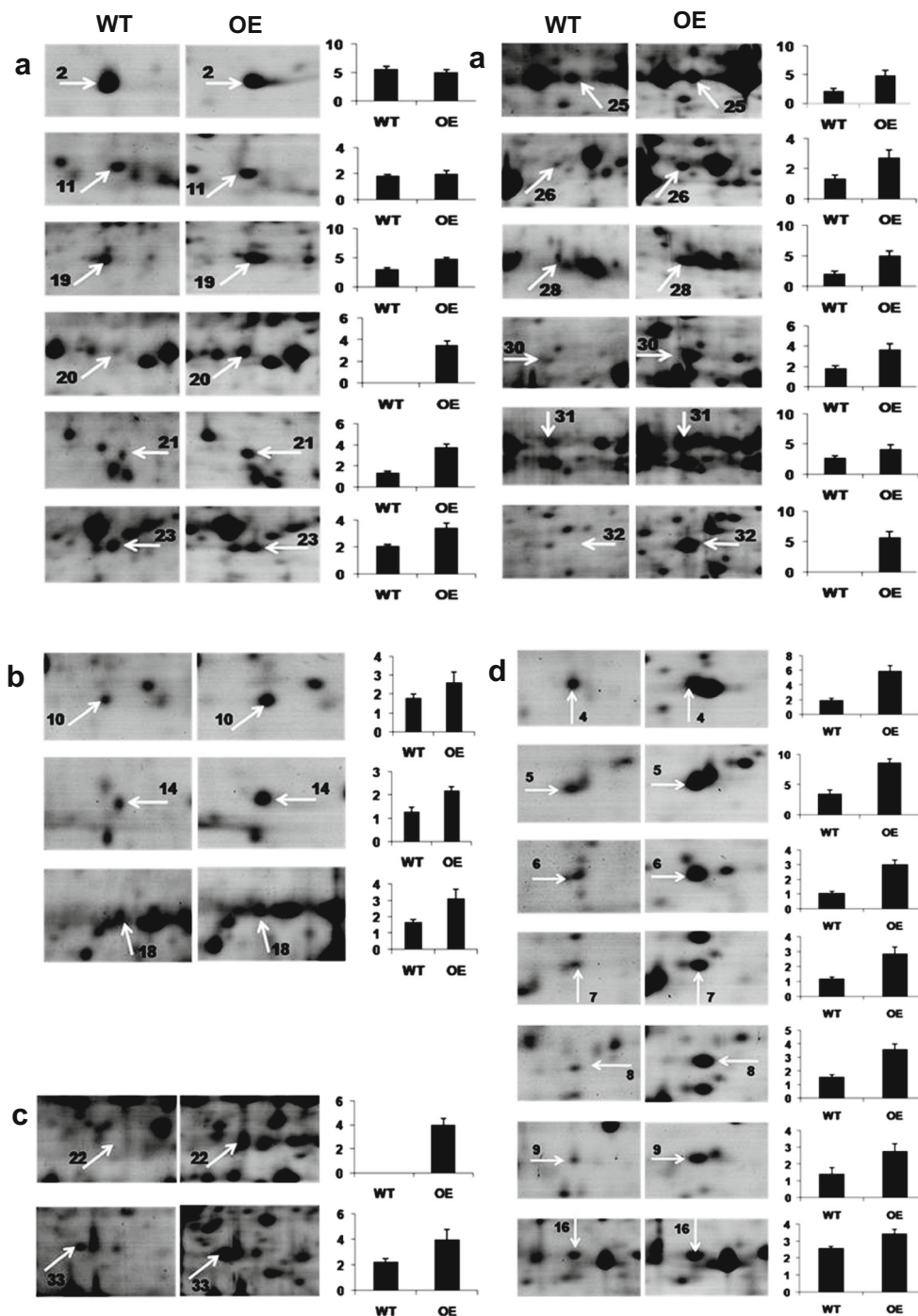
Our present study found the abundance of small heat shock proteins (sHSP), and peroxidase proteins were increased under drought stress. These proteins were found to work in defense mechanism. These proteins comprise, 17.6 -kDa class I heat shock protein 3 (spot 3), 18.1 -kDa class I heat shock protein (spot 4), 17.5 -kDa class I heat shock protein (spot 5), 17.4 -kDa class I heat shock protein (spot 6), 17.8 -kDa class I heat shock protein (spot 7), 18.0 -kDa class I heat shock protein (spot 8), probable phospholipid hydroperoxide glutathione peroxidase 6 (spot 9), L-ascorbate peroxidase (spot 16), and L-ascorbate peroxidase T (spot 17). It was reported that high abundance of small heat shock proteins and ascorbate peroxidase were also observed in tolerant maize compared with susceptible ones under drought stress condition (Benesova et al. 2012). Xu and Huang (2010) also found an increased abundance of several heat shock proteins in drought-tolerant Kentucky bluegrass cultivar.

Bioinformatics Analysis of Identified Proteins

Physical and chemical properties of differentially expressed proteins were analyzed by Protparam and ProtScale tools of Swiss Institute of Bioinformatics (www.expasy.ch/tools) (Table 2). Abundance of hydrophobic amino acid, such as alanine, valine, and leucine was observed in about 61 % of proteins. Thus, the core of the most identified proteins was made up with these hydrophobic amino acid residues. About 72 % of the proteins have higher number of negatively charged residues than their positively charged residues, which indicate that most of the differentially-expressed proteins were acidic protein.

Transmembrane analysis exhibited that majority of the identified proteins were transmembrane except 14-3-3 like protein, translationally-controlled tumor protein homolog, and small heat shock proteins (Table 2). Hence, the transmembrane proteins may be the main functional protein which provides membrane stability as well as serves as a receptor for signal sensing under drought stress in *TERF1* overexpressed transgenic sugarcane.

Fig. 5 Enlarged view of representative protein spots of WT and OE sugarcane showing differential expression patterns in response to PEG stress. Energy function proteins (a), metabolism function proteins (b), protein synthesis function proteins (c), and defense proteins (d). *Left and right bar* indicate spot volume of WT and OE, respectively. Values are mean spot volume of three gels \pm SD



This result is consistent with some previous result reported by Zhou et al. (2012) in sugarcane, Nouri and Komatsu (2010) in soybean, and Breton et al. (2003) in wheat.

Discussion

Plant growth and development are severely affected by drought stress. Sugarcane is an important economic crop due

to its utilization for sugar and ethanol production. Drought stress is an important factor for sugarcane like other arid and semiarid dryland crops, which reduces their yield and quality markedly.

The ability of water retention in plant is widely used as an indicator for drought tolerance (Dhanda and Sethi 1998). Plants with high water retention capacity can survive under low water potential of drought due to their ability to stay green for maintaining the canopy of crop (Long and Ort 2010). Water deficit stress changes diverse growth and physiological

Table 1 Transgenic sugarcane PEG stress responsive leaf proteins identified by MALDI-TOF-MS and database searching

Spot no. ^a	Protein name and species	NCBI accession no. ^b	Swiss-Prot accession no. ^c	Protein score ^d	Theo.MW/ pI ^e	Exp.MW/pI ^f	Abundance level (%) ^g	Spot volume ratio (OE/WT) ^h	Matching Peptides	Sequence coverage (%) ⁱ	Location ^j
Metabolism											
10	NAD (P) H-quinone oxidoreductase subunit M (<i>Vitis vinifera</i>)	gi 209573123	A7NVJ4	66	24.32/6.84	22.00/4.67	Up (45)	1.45	2	23	Chloroplast thylakoid membrane
r14	Cellulose synthase-like protein D2 (<i>Arabidopsis thaliana</i>)	gi 75174138	Q9LFL0	61	129.61/ 7.50	25.00/6.46	Up (72)	1.72	3	8	Plasma membrane
18	14-3-3-like protein 1 (Fragments) (<i>Pseudotsuga menziesii</i>)	gi 205686166	P85938	97	9.02/4.88	30.21/4.59	Up (85)	1.85	2	67	–
Energy											
2	Peptidyl prolyl cis-trans isomerase CYP20-2, (<i>Arabidopsis thaliana</i>)	gi 32130267	Q9ASS6	85	28.52/9.39	19.32/6.54	Down (12)	0.88	1	11	Chloroplast thylakoid membrane
11	Phosphoenolpyruvate carboxylase (<i>Sorghum bicolor</i>)	gi 115578	P15804	134	108.94/ 5.91	22.36/4.85	Up (19)	1.19	4	18	Cytoplasm
13	Ribulose biphosphate carboxylase large chain (<i>Clarkia xantiana</i>)	gi 131935	P28392	282	53.31/6.04	23.19/5.95	Up (18)	1.18	5	15	Chloroplast
19	Ferredoxin–NADP reductase, leaf isozyme, chloroplastic (<i>Pisum sativum</i>)	gi 119905	P10933	96	40.45/8.56	34.00/5.67	Up (54)	1.54	4	19	Chloroplast stroma, thylakoid membrane
20	Ferredoxin–NADP reductase, chloroplastic (<i>Mesembryanthemum crystallinum</i>)	gi 729477	P41343	73	41.32/8.54	35.00/5.14	Up ^T	nd	2	21	Chloroplast
21	Ribulose biphosphate carboxylase large chain (<i>Saccharum officinarum</i>)	gi 75290174	Q6ENV5	194	53.33/6.33	36.00/6.23	Up (183)	2.83	5	17	Chloroplast
Energy											
23	Probable fructose-bisphosphate aldolase 1 (<i>Arabidopsis thaliana</i>)	gi 75313518	Q9SIU4	93	43.07/6.18	37.00/5.71	Up (65)	1.65	2	13	Apoplast, chloroplast thylakoid
24	Probable fructose-bisphosphate aldolase 1 (<i>Arabidopsis thaliana</i>)	gi 75313518	Q9SIU4	132	43.07/6.18	37.00/5.25	Up ^T	nd	3	15	Apoplast, chloroplast thylakoid
25	Probable fructose-bisphosphate aldolase 1 (<i>Arabidopsis thaliana</i>)	gi 75313518	Q9SIU4	121	43.07/6.18	37.00/5.41	Up (128)	2.28	2	13	Apoplast, chloroplast thylakoid
26	Malate dehydrogenase (<i>Zea mays</i>)	gi 18202485	Q08062	60	35.91/5.77	38.00/5.60	Up (101)	2.01	3	21	Cytoplasm
27	Malate dehydrogenase (<i>Zea mays</i>)	gi 18202485	Q08062	57	35.91/5.77	39.00/5.90	Up ^T	nd	2	23	Cytoplasm
28	Glyceraldehyde-3-phosphate dehydrogenase A (<i>Zea mays</i>)	gi 120657	P09315	146	43.18/7.00	39.06/6.34	Up (141)	2.41	4	27	Chloroplast
29	Phosphoenolpyruvate carboxylase 3 (<i>Sorghum bicolor</i>)	gi 115578	P15804	69	108.94/ 5.91	41.00/5.64	Up (70)	1.70	3	8	Cytoplasm
30	Phosphoenolpyruvate carboxylase 3 (<i>Sorghum bicolor</i>)	gi 115578	P15804	210	108.94/ 5.91	41.00/5.55	Up (96)	1.96	5	28	Cytoplasm
31	ATPase 3, plasma membrane type (<i>Arabidopsis thaliana</i>)	gi 114339	P20431	59	104.90/ 6.73	43.41/4.91	Up (52)	1.52	3	12	Plasma membrane
Energy											
32	Glyceraldehyde-3-phosphate dehydrogenase B (<i>Pisum sativum</i>)	gi 120663	P12859	72	48.58/7.57	43.00/5.48	Up ^T	nd	3	32	Chloroplast
34	Phosphoglycerate kinase, cytosolic (<i>Triticum aestivum</i>)	gi 129916	P12783	78	42.15/5.64	45.00/5.53	Up (185)	2.85	3	16	Cytoplasm
35	Ribulose biphosphate carboxylase large chain (<i>Saccharum officinarum</i>)	gi 75290174	Q6ENV5	358	53.33/6.33	49.00/6.35	Up (656)	7.56	6	31	Chloroplast
36	Ribulose biphosphate carboxylase large chain (Fragment) (<i>Erythrina cristata-galli</i>)	gi 17367566	Q33438	141	50.84/6.22	50.00/6.22	Up (328)	4.28	4	17	Chloroplast
Transcription											
12		gi 75244796	Q8H6A5	147	18.73/4.52	23.00/4.30	Up (18)	1.18	3	28	–

Table 1 (continued)

Spot no. ^a	Protein name and species	NCBI accession no. ^b	Swiss-Prot accession no. ^c	Protein score ^d	Theo.MW/ pI ^e	Exp.MW/pI ^f	Abundance level (%) ^g	Spot volume ratio (OE/WT) ^h	Matching Peptides	Sequence coverage (%) ⁱ	Location ^j
Translationally controlled tumor protein homolog (<i>Zea mays</i>)											
Protein synthesis											
22	60S acidic ribosomal protein P0 (<i>Zea mays</i>)	gi 6094102	O24573	79	34.59/5.20	37.10/5.16	Up ^T	nd	2	17	Ribosome
33	Elongation factor Tu, (<i>Pisum sativum</i>)	gi 6015084	O24310	87	53.13/6.62	45.00/5.25	Up (78)	1.78	4	17	Chloroplast
Intracellular traffic											
1	Pentatricopeptide repeat-containing protein A13.g54980 (<i>Arabidopsis thaliana</i>)	gi 75210358	Q9SV46	61	95.92/5.66	17.45/6.40	Down (30)	0.69	5	15	Mitochondria
Signal transduction											
15	Phytochrome A type 4 (<i>Avena sativa</i>)	gi 130182	P06594	64	126.32/ 5.75	25.47/5.29	Up ^T	nd	4	7	Mitochondria
Disease/defense											
3	17.6-kDa class I heat shock protein 3 (<i>Arabidopsis thaliana</i>)	gi 21264442	P13853	119	17.59/5.36	16.00/5.99	Up ^T	nd	4	21	Cytoplasm
4	18.1-kDa class I heat shock protein (Fragment) (<i>Medicago sativa</i>)	gi 123554	P27879	108	16.45/5.20	16.50/5.94	Up (208)	3.08	4	46	Cytoplasm
5	17.5-kDa class II heat shock protein (<i>Zea mays</i>)	gi 123549	P24631	92	17.55/5.56	16.83/5.49	Up (144)	2.44	4	38	Cytoplasm
6	17.4-kDa class I heat shock protein (<i>Arabidopsis thaliana</i>)	gi 21431764	P19036	64	17.42/5.21	17.43/5.20	Up (186)	2.86	2	23	Cytoplasm
7	17.8-kDa class I heat shock protein (<i>Daucus carota</i>)	gi 123548	P27396	57	17.76/7.90	17.00/5.59	Up (144)	2.44	2	17	Cytoplasmic
8	18.0-kDa class I heat shock protein (<i>Daucus carota</i>)	gi 123552	P27397	145	18.02/6.93	18.25/5.59	Up (128)	2.28	2	11	Cytoplasmic
9	Probable phospholipid hydroperoxide glutathione peroxidase 6, mitochondrial (<i>Arabidopsis thaliana</i>)	gi 47117812	O48646	61	25.74/9.38	20.00/6.10	Up (94)	1.94	1	12	Apoplast, chloroplast, cytosol, mitochondria
16	L-ascorbate peroxidase (<i>Pisum sativum</i>)	gi 1351963	P48534	86	27.23/5.52	26.00/4.95	Up (32)	1.32	3	6	Cytoplasm
17	L-ascorbate peroxidase T, chloroplastic (<i>Arabidopsis thaliana</i>)	gi 118572831	Q42593	64	46.29/6.81	30.00/5.77	Up ^T	nd	4	12	Chloroplast thylakoid membrane

^a Spot numbers correspond to the spots in 2-DE gel in Fig. 4

^b Accession number in the National Center for Biotechnology Information (NCBI) database

^c Swiss-Prot accession number

^d Mascot score probability for the entire protein

^e Theoretical molecular weight (MW) and isoelectric point (pI) calculated from EXPASY (www.expasy.org) database

^f Molecular weight and isoelectric point of protein spots estimated from 2-DE gel

^g Expression changes of protein spots measured from spot volume of OE and WT, analyzed by image master 2D platinum software. Values in brackets indicate the percentage change of protein expression under PEG stress. T indicates that the spots were present only in transgenic plant

^h Spot/volume ratio of OE and WT, calculated from the mean spot volumes, nd indicates that spots were not detected in WT, but appeared in OE

ⁱ Sequence coverage of the identified proteins

^j Subcellular location of the proteins as predicted by online bioinformatics data source (<http://www.cbs.dtu.dk/services/TargetP/>; www.uniprot.org)

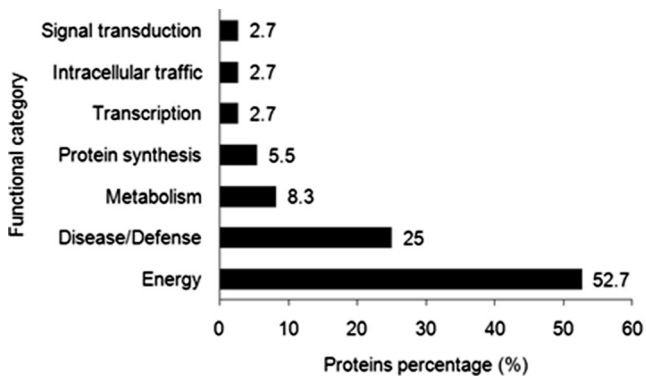


Fig. 6 Functional categories of the transgenic sugarcane PEG stress responsive leaf proteins identified by mass spectrometry

processes in sugarcane. Various studies demonstrated that drought causes a decline of leaf chlorophyll level in susceptible varieties of sugarcane, but tolerant cultivars maintain higher chlorophyll level than susceptible ones (Silva et al. 2007; Jangpromma et al. 2010; Zhao et al. 2010). Chlorophyll degradation in sugarcane exposed to osmotic stress has a negative effect on photosynthetic capacity (Cham et al. 2012). Photosynthetic rate is related with chlorophyll content and stomatal conductance. Under water deficit condition, it is observed that tolerant cultivars of sugarcane showed high photosynthetic rate due to high chlorophyll and relative water content (Zhao et al. 2010). It is previously reported that ERF transcription factors TERF1 can maintain high water content under drought and salt stress (Gao et al. 2008). TERF2 can also retain chlorophyll content at normal level under cold stress (Tian et al. 2010) when they were overexpressed in rice plant.

On one hand, drought stress inhibits protein synthesis and, on the other hand enhances synthesis of stress-responsive proteins through altering the expression of genes in plant. Quantitative and qualitative changes of protein synthesis occur in plants under water deficit stress. In this study, altered protein expressions associated with the enhanced drought tolerance in transgenic sugarcane have been examined.

Abundance of Metabolism-Related Proteins in Response to Stress

Drought stress induces significant alterations in several metabolic pathways. Proteins with increased abundance, such as NAD (P) H-quinone oxidoreductase, cellulose synthase-like protein D₂, and 14-3-3-like protein 1, were observed in transgenic sugarcane lines following drought stress. NAD (P) H-quinone oxidoreductase is a membrane protein in the thylakoid membrane of chloroplast, which is involved in the processes of oxidation-reduction, and biosynthetic, and root hair elongation. Oxidoreductases are ubiquitously found in the plasma membrane of plants which were observed to be involved in proton pumping, nutrient uptake, signal

transduction, and growth regulation (Lüthje et al. 1997). The cellulose synthesizing genes in higher plants are different from the genes in bacteria and plant cellulose synthase proteins which are integral membrane proteins (Richmond 2000). The 14-3-3 proteins are found in eukaryotic organisms as regulatory proteins which regulate primary metabolism, ion transport, cellular trafficking, and gene expression in plants (Sehnke et al. 2002). The 14-3-3 proteins play a vital role against abiotic stresses such as drought, cold, and osmotic stresses through maintaining ion homeostasis. In *Arabidopsis thaliana*, potassium channel and H⁺ATPase transport are regulated by these proteins which implicate that 14-3-3 proteins have a crucial role in regulating plant drought tolerance (Fuglsang et al. 2007; van den Wijngaard et al. 2005).

Abundance of Energy-Related Proteins and Their Functions

Drought stress has a great impact on energy metabolism of plants. Stomata closure and CO₂ availability under osmotic stress depend on the tolerance mechanism of the plant. In this study, peptidyl-prolyl cis-trans isomerase protein was identified as decreased abundance in transgenic sugarcane plant in response to drought stress. Peptidyl-prolyl cis-trans isomerase (PPI) enzyme catalyzes the peptidyl-prolyl band from cis- to trans- in the folding of proteins. Due to their protein folding, chaperonic properties, and responsiveness to stress, the peptidyl-prolyl cis-trans isomerase has been suggested to play an important role for tolerance to stress. It was observed that PPI activity was increased in sorghum seedlings in response to osmotic stress. Water stress significantly increases PPI activity in leaf and root tissues of tolerant genotype, while PPI activity in two tissues of susceptible cultivar decreases remarkably under drought stress (Sharma and Singh 2003).

Ribulose-1, 5-Biphosphate Carboxylase (RuBisCO) is the main enzyme for carbon metabolism in leaves. During photosynthesis, CO₂ is combined with Ribulose-1, 5-Biphosphate (RuBP) by RuBisCO and producing 3-phosphoglyceric acid. RuBisCO and ATPase beta chains play a vital role to maintain chloroplast as well as whole cell function of plant under stress (Huo et al. 2004). In higher plants, RuBisCo has eight small and eight large subunits (Parry et al. 2008). In our study, RuBisCO large subunit was detected as increased abundance. This result is consistent with previous results in sugarcane under drought stress (Jangpromma et al. 2010; Zhou et al. 2012), transgenic bentgrass under drought stress (Merewitz et al. 2011), tolerant variety of sunflower under drought stress (Castillejo et al. 2008), and *Sorghum bicolor* under salt stress (Ngara et al. 2012). In contrast, decreased abundance of RuBisCO have been reported in the proteomic analysis of rice under drought stress (Ali and Komatsu 2006), potato under salt stress (Aghaei et al. 2008), sugar beet under drought stress (Hajheidari et al. 2005), and rice under chilling stress (Yan et al. 2006).

Table 2 Analysis of physical and chemical properties of differentially expressed proteins under PEG stress

Spot no.	No. of amino acids	The most abundant amino acid	No. of negatively charged residues	No. of positively charged residues	Grand average of hydrophobicity (GRAVY)	No. of possible transmembrane helices	
						Inside-outside	Outside-inside
1	850	Leu 98	105	93	-0.169	4	5
2	259	Ser 30	23	31	-0.303	1	2
3	157	Glu 18	27	23	-0.578	0	1
4	143	Glu 17	28	23	-0.790	0	0
5	161	Val 18	27	25	-0.333	0	0
6	156	Val 19	27	22	-0.569	0	0
7	157	Lys 19	26	27	-0.551	0	0
8	159	Glu 18	24	24	-0.628	0	0
9	232	Ser 30	23	32	-0.277	1	1
10	208	Leu 16	26	26	-0.628	1	1
11	960	Leu 109	134	121	-0.309	3	3
12	167	Lys 18	31	19	-0.307	0	0
13	475	Gly 46	61	53	-0.290	2	3
14	1,145	Leu 99	127	128	-0.220	11	13
15	1,129	Leu 120	145	118	-0.160	8	9
16	250	Ala 26	36	28	-0.332	2	1
17	426	Ser 45	50	49	-0.284	2	3
18	80	Leu 10	13	8	-0.064	0	0
19	360	Lys 36	45	49	-0.280	3	3
20	365	Lys 38	47	51	-0.392	3	3
21	476	Gly 46	59	54	-0.249	2	3
22	319	Val 32	46	38	-0.072	1	2
23	399	Ala 51	40	38	-0.120	3	1
24	399	Ala 51	40	38	-0.120	3	1
25	399	Ala 51	40	38	-0.120	3	1
26	332	Val 36	36	31	0.024	4	3
27	332	Val 36	36	31	0.024	4	3
28	403	Val 42	42	42	-0.042	1	2
29	960	Leu 109	134	121	-0.309	3	3
30	960	Leu 109	134	121	-0.309	3	3
31	949	Ala 95	108	105	0.052	11	13
32	451	Val 50	49	50	-0.047	1	3
33	488	Leu 44	56	54	-0.121	4	3
34	401	Ala 49	53	48	0.155	1	2
35	476	Gly 46	59	54	-0.249	2	3
36	455	Gly 46	57	51	-0.265	3	4

Photosynthesis is the key metabolic process of plants, which is often negatively affected by stress conditions. Efficiency of photosynthesis is regulated by Ribulose-1, 5-bisphosphate carboxylase/oxygenase. Initially, CO₂ is fixed with PEP in mesophyll cells by phosphoenolpyruvate carboxylase (PEPC) and producing C₄ compound which is transported to bundle sheath cells and provides CO₂ to

RuBisCO (Sage 2004; Edwards et al. 2004). Drought, chilling, and salt stresses repress cell growth, photosynthesis, and activate respiration (Shinozaki and Yamaguchi-Shinozaki 2007; Soussi et al. 1998). It is reported that overexpression of PEPC in maize increases the tolerance against drought (Jeanneau et al. 2002), but it is still unclear whether overexpressed PEPC confers more stress tolerance to plant.

Plant ferredoxins (Fds) are involved in photosynthetic activity and transport electrons from photosystem I (PS I) to ferredoxin-NADP⁺-oxidoreductase (FNR), where NADPH is produced for CO₂ assimilation (Fukuyama 2004). The abundance of the ferredoxin-NADP⁺-oxidoreductase (FNR) increased under drought stress. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) is involved in ROS scavenging and stress tolerance mechanism (Hancock et al. 2005). Under drought stress condition, ROS detoxifying proteins' abundance is increased in tolerant plants, such as GDPDH and protect photosystem II (PSII) which are against the production of ROS. Fructose-1, 6-biphosphate aldolase is a vital enzyme in plants, which is involved in glycolysis and gluconeogenesis in the cytoplasm as well as the Calvin cycle in plastids.

Malate dehydrogenase is involved in the malate/oxaloacetate shuttling system and plays a pivotal role in many metabolic pathways including the tricarboxylic acid cycle, glyoxylate bypass, amino acid synthesis, gluconeogenesis, and facilitation of exchange of metabolites between cytoplasm and subcellular organelles. ATPase 3, plasmamembrane protein is involved in phosphoenolpyruvate carboxylase activity, ATP biosynthetic process, and ATP hydrolysis with proton transport. For instance, plasma membrane H⁺-ATPase provides an electrochemical H⁺ gradient across the membrane to prepare the energy needed for secondary transport and for the regulation of cell turgor and intracellular pH (Michelet and Boutry 1995). Activation of this protein is modulated by signaling molecules such as 14-3-3 protein and protein kinases (Xu and Shi 2006). Phosphoglycerate kinase is involved in the Calvin cycle, and its abundance is found to be increased in maize under drought stress (Tai et al. 2011). It is also previously found to be overexpressed in *Arabidopsis* under cold stress (Bae et al. 2003).

Abundance of Transcription Proteins Under Stress

In the present study, translationally controlled tumor protein (TCTP) homolog was detected as transcription factor protein; the increased abundance of which was observed under drought stress. Calcium-binding activity of TCTP is reported as its prime molecular function (Bommer et al. 2002). Moreover, signals from extracellular stimuli such as calcium stress and heat shock etc. regulate TCTP levels (Xu et al. 1999; Bommer et al. 2002). Changes of plant TCTP's at transcript or protein level are associated with various physiological conditions, such as cold stress, high temperature, and salt stresses (Cao et al. 2010; Li et al. 2010), which clearly suggests that TCTP is involved in growth controlling as well as stress responses in plants.

Abundance of Synthesis-Related Proteins and Their Functions

In our study, 60S acidic ribosomal protein and elongation factor Tu were detected as involved in protein synthesis and

were found overexpressed. For protein synthesis, elongation factor Tu plays a crucial role for polypeptide chain elongation and is involved in the regulation of translation. In the present study, increased abundance of EF-Tu might provide a way of protection to transgenic sugarcane from drought. Similarly, increased abundance of EF-Tu is found in sugarcane (Ngamhui et al. 2012), transgenic bentgrass (Merewitz et al. 2011), maize (Benesova et al. 2012), and bermudagrass (Zhao et al. 2011). However, the role of 60S acidic ribosomal protein in the tolerance mechanism of transgenic sugarcane is not clearly understood.

Abundance Changes of Intracellular Traffic Proteins

In this functional group, pentatricopeptide repeat (PPR)-containing protein was detected as decreased abundance in transgenic sugarcane line under drought stress. The PPR proteins are only found in eukaryotes with high abundance in terrestrial plants (Fujii and Small 2011). It has been proposed that PPR proteins participate in gene expression regulation such as RNA cleavage, splicing, and translation at posttranscriptional level. In our research, decreased abundance of PPR protein in transgenic sugarcane line under drought stress indicated that RNA translation in transgenic sugarcane might be degraded at some extent due to the effect of stress.

Function of Signal Transduction Proteins and Their Relation with This Study

Phytochrome A type 4 was detected as increased abundance which belongs to the functional group of signal transduction. Signal sensing and processing of external stimuli is mediated by signal transduction cascades. Detection, amplification, and integration of external signals are done by this molecular circuit in order to generate responses including gene expression and enzymatic activity. Light is the primary source of energy as well as an environmental signal which influences growth and modulate developmental processes of plant. Light-dependent responses in plant are regulated by a variety of photoreceptors, such as phytochromes, cryptochromes, and phototropins (Quail 2002). Vital plant responses including chloroplast development, leaf senescence, and leaf abscission are controlled by the phytochromes. In our study, increased abundance of Phytochrome A type 4 may contribute to stabilize the chlorophyll content of transgenic sugarcane against drought stress.

Role of Defense Proteins in Response to Stress

Osmotic stress is the secondary stress which accompanies with drought, and plant under drought stress also shows oxidative stress symptom. For these stresses, plants respond

in complex ways, which may lead the same group of proteins to be activated in response to diverse stresses (Levitt 1980). Molecular chaperones, detoxifying enzymes, and osmoprotectants are products of the stress-inducible genes which protect plant cells from stresses directly (Seki et al. 2003; Shinozaki et al. 2003).

In the present study, the changes of nine disease/defense-related proteins (Fig. 6) were detected in the transgenic sugarcane under drought stress. Among them, seven small heat shock proteins (sHSP) all showed increased abundance in transgenic sugarcane line following stress. Many reports revealed that plant sHSPs are induced under diverse abiotic stresses such as drought, salinity, and oxidative stress and confer increased tolerance to plant against these stresses. High abundance of sHSPs in drought-tolerant Kentucky bluegrass cultivar is observed by Xu and Huang (2010). In another report, it was found that sHSPs showed strongest respond in maize in respond to drought stress (Benesova et al. 2012). Increased abundance of sHSPs is also observed in tolerant cultivar of rapeseed in response to drought stress (Bandehagh et al. 2013). It is also reported by Heckathorn et al. (2002) that the level of ROS is decreased by sHSPs and thereby photosystem II reaction is protected during stress. Glutathione has multifunctional roles in cellular metabolisms, such as reductants and radical scavenging agents (Noctor et al. 1997; Foyer et al. 1997). It also functions as an intracellular signaling agent, responsive to changes in the extracellular environment (S'anchez-Fern'andez et al. 1997).

Reactive oxygen species (ROS) are considered as the main source of cell damage under abiotic stresses including drought stress (Mittler et al. 2004). Plants in response to stress produce antioxidant enzymes, namely superoxidase dismutase, catalase, ascorbate peroxidase, and glutathione peroxidase for scavenging the ROS (Xiong and Zhu 2002). Phospholipid hydroperoxide glutathione peroxidase (PHGPx) is an antioxidant enzyme that catalyzes hydrogen peroxide, lipid peroxides, and organic hydroperoxide for protecting cells and enzymes from oxidative damage (Dixon et al. 1998). We have identified two ROS-removing proteins, glutathione peroxidase and ascorbate peroxidase, which conferred drought tolerance to transgenic sugarcane.

In conclusion, this work provides new insight on transgene function underlying the defense mechanism of *TERF1*-overexpressed transgenic sugarcane in response to PEG-induced drought stress. However, further investigation for validating the proteins is needed to provide more information about the regulatory function of this transcription factor gene.

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