Comparison of Leaf Proteomes of Cassava (*Manihot esculenta* Crantz) Cultivar NZ199 Diploid and Autotetraploid Genotypes

Feifei An¹, Jie Fan¹, Jun Li², Qing X. Li³, Kaimian Li¹*, Wenli Zhu¹, Feng Wen⁴, Luiz J. C. B. Carvalho⁵, Songbi Chen¹*

1 Tropical Crops Genetic Resources Institute, Chinese Academy of Tropical Agricultural Sciences/Key Laboratory of Ministry of Agriculture for Germplasm Resources Conservation and Utilization of Cassava, Hainan, China, 2 Analysis and Testing Center, Jiangsu University, Jiangsu, China, 3 Proteomics Core Facility, Department of Molecular Biosciences and Bioengineering, University of Hawaii at Manoa, Manoa, Hawaii, United States of America, 4 Guangxi Sub-tropical Crop Research Institute, Nanning, China, 5 Genetic Resources and Biotechnology, Embrapa, Brazil

Abstract

Cassava polyploid breeding has drastically improved our knowledge on increasing root yield and its significant tolerance to stresses. In polyploid cassava plants, increases in DNA content highly affect cell volumes and anatomical structures. However, the mechanism of this effect is poorly understood. The purpose of the present study was to compare and validate the changes between cassava cultivar NZ199 diploid and autotetraploid at proteomic levels. The results showed that leaf proteome of cassava cultivar NZ199 diploid was clearly differentiated from its autotetraploid genotype using 2-DE combined MS technique. Sixty-five differential protein spots were seen in 2-DE image of autotetraploid genotype in comparison with that of diploid. Fifty-two proteins were identified by MALDI-TOF-MS/MS, of which 47 were up-regulated and 5 were down-regulated in autotetraploid genotype compared with diploid genotype. The classified functions of 32 up-regulated proteins were associated with photosynthesis, defense system, hydrocyanic acid (HCN) metabolism, protein biosynthesis, chaperones, amino acid metabolism and signal transduction. The remarkable variation in photosynthetic activity, HCN content and resistance to salt stress between diploid and autotetraploid genotypes is closely linked with expression levels of proteomic profiles. The analysis of protein interaction networks indicated there are direct interactions between the 15 up-regulation proteins involved in the pathways described above. This work provides an insight into understanding the protein regulation mechanism of cassava polyploid genotype, and gives a clue to improve cassava polyploidy breeding in increasing photosynthesis and resistance efficiencies.

Introduction

Cassava (*Manihot esculenta* Crantz) is a perennial shrub of the Euphorbiaceae family. It is a major calorie source for sub-Saharan Africans and is ranked the sixth most important source of caloric in human diet worldwide [1]. In south China, cassava mainly provides raw materials to produce starch and biofuel. It is a potential crop to grow in semi-arid lands if a suitable cultivar is bred [2]. However, cassava breeding faces several limitations such as (1) its heterozygous genetic makeup which makes it time consuming to breed efficiently and (2) low tolerance to salinity and cold which makes it difficult to grow in north China and saline soils [3]. Because of its importance, a number of important studies regarding genome, proteome and transcriptome have been performed. A draft genome sequence and limited proteome identification have been generated to provide new clues for cassava breeders to overcome the limitations [3–6].

One of the potential approaches to increase stress resistance is to produce polyploidy (whole-genome duplication) [2]. The polyploid crops, influenced by nuclear genome size, have much larger cells than the diploid ones [7]. Several studies showed that the significance of the proportional increase in cell volume with increase in DNA content [8,9]. This cell volume increase along with DNA content increase was observed in secondary xylem radial and vasicentric axial parenchyma cells, and radial parenchyma and sieve elements of secondary phloem [2,3,9]. Additionally, DNA content increase in the synthesized polyploids led to the description of structural changes such as chromosomal rearrangements and gains or losses of DNA sequences, demonstrating the occurrence of modifications at the level of gene expression. The studies from Brassica, potato and cotton polyploids have shown that some genes are silenced after polyploidization, while others are derepressed [10–12], which suggests that functional and phenotypic evolution may be driven by these genomic changes.
Although these have been significant recent advances on the genomic and transcriptomic consequences of genomic merger and doubling, the fate of translated gene products, i.e., the proteome, remains poorly studied in the context of polyploidization [12,13]. Using two-dimensional electrophoresis (2-DE) combined mass spectrometry (MS), it is often possible to visualize, quantify, and identify hundreds or even thousands of proteins in a given tissue or cell sample. Proteome analysis is increasingly used in functional plant studies. Proteomic analysis has the potential to provide a broad view of plant responses to stress at the level of proteins [14,15]. Some reports have been focused on proteomic analysis of plant polyploids including cabbage [16], wheat [17–19], cotton [12], potato [20], banana [21], Arabidopsis [22] and Tunggagogan [23]. However, little is known about the effect of polyploidy on the cassava global protein networks that underlie key physiological or developmental processes.

In the present study we investigate and validate the changes of proteome patterns between cassava NZ199 polyploid genotype and its diploid plants using 2-DE combined MALDI-TOF-MS/MS. All differential proteins were clustered into cohesive groups based on their biochemical functions. The biological network of protein-protein interaction was established to describe the polyploid photosynthetic activity tolerance against stresses. Our data will provide crop breeders a set of protein database involved in cassava polyploidy and would be useful to understand the polyploidy mechanism involved in the key physiological or developmental processes.

Materials and Methods

Analysis of different ploidy levels in cassava

Cassava cultivar NZ199 autotetraploid genotype was polyploidized artificially by the use of colchicine applied as a solution of 0.001% to lateral buds over a period of 72 h. The emerging shoots were screened for the formation of chimeras or total tetraploids with the chimeras eliminated. To identify autotetraploids, buds were observed for standard chromosome counting and flow cytometric analysis (CellLab QuantaTM SC MPL, Beckman, USA) [24] in addition to observing leaf shape. The shoots were propagated vegetatively and grown at the Cassava Germplasm Pool, Tropical Crops Genetic Resources Institute, Chinese Academy of Tropical Agricultural Sciences. Functional leaves on the top of cassava diploid and autotetraploid plants grown for 180 d at CGB were sampled for protein extraction. One leaf was collected from one cassava plant, and three leaves were collected.

Protein extraction and 2-DE separation

Collected leaves were washed with distilled water and the central nervure was removed. Leaves were cut into pieces and 0.5 g pieces were weighed for protein extraction. Leaf proteins were extracted with phenol according to the procedure of Chen et al. [25]. Protein pellets were dissolved in sample buffer [9.5 M urea, 2 M Thiourea, 4% (w/v) CHAPS, 1% (w/v) DTT, 2.5 mM EDTA, 2.5 mM EGTA], and the protein content was then measured with the protein assay according to the instruction manual (Bicinchoninic acid protein assay kit, product code BCA-1 of rehydration stock solution l of 100 mM lo of 25 mM ammonium bicarbonate, 50% acetonitrile) with recognition as the top ranking match in the Mascot Standard scoring system [3].

Differential proteins were identified using MALDI-TOF-MS/MS at Analysis and Testing Center, Jiangsu University. After 1 μl peptide extract produced by the in-gel digestion was placed on an anchor chip and air-dried, it was covered with 1 μl solution of 0.4 mg/ml α-cyano-4-hydroxycinnamic acid in a mixture of acetonitrile and 0.1% trifluoroacetic acid (TFA) (70:30) and then air-dried. The mass spectra were acquired on an Ultraflex-TOF mass spectrometer (Bruker, German). Spectra were internally calibrated with trypsin auto-digestion products. Data captured by MALDI-TOF-MS/MS were matched via Mascot v2.2.03 (http://www.matrixscience.com) against NCBI [Taxonomy: Viridiplantae (Green Plants)]. Carbamidomethyl (Cys) and oxidation (Met) were considered as variable modifications. A single missed cleavage was permitted. Peptide mass tolerance was set as 3.0 Da and MS/MS ion mass tolerance was set at 1.5 Da. Peptide charge states (+1, +2, +3) were taken into account. Routine protein identification required sequence-confirmed data for a minimum of one peptide with recognition as the top ranking match in the Mascot Standard scoring system [3].

Western blot analyses

Leaves of cassava cultivar NZ199 diploid and autotetraploid genotypes were homogenized. The protein extraction and Western blot were performed according to the method previously reported [3]. Proteins detected by immuno-staining with antiRubisco-polyconal antibody (AS07218), anti-APX antibody (AS08368) and follows: 300 V for 0.05 h, then, increased from 300 V to 3500 V as a gradient over 1.5 h, and finally 3500 V for 4.20 h. The focused strips were equilibrated twice for 15 min each first in 1% (w/v) DTT and then 2.5% (w/v) iodoacetamide prepared in equilibration buffer (50 mM Tris-HCl (pH 8.8), 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS). The second-dimension electrophoresis was performed by SDS-PAGE in a vertical slab of 12% acrylamide using an SE 600 Series Vertical Slab Gel Unit (Hoeter Scientific Instruments, San Francisco, CA, USA). Preparative gels were stained with Colloidal Coomassie brilliant blue G-250 [26]. Three independent biological replications were carried out.

Image and data analysis

Gel matching for protein quantification was performed by Image Scanner III (GE healthcare) and Delta 2D (DECODON GmbH, Greifswald, Germany) software, and spot pairs were confirmed visually. The differentially expressed spots were determined by using Scheffe’s test at P<0.05. The abundance of each protein spot was estimated by the percentage volume (% Vol). Only those with significant and reproducible changes were considered to be differentially accumulated proteins.

Tryptic in-gel digestion

Briefly, the differential spots were excised from 2-DE gels and cut into ~1 mm², and then washed twice in MilliQ water for 10 min. The washed gel pieces were subjected to destaining solution (25 mM ammonium bicarbonate, 50% acetonitrile) followed by sonication for 5 min. The gel pieces were again washed twice in MilliQ water for 10 min, followed by washing twice in 50% acetonitrile for 10 min. After dehydration with 50 μl acetonitrile for 2–3 min, the gel pieces were digested overnight at 37°C in 15 μl of sequencing grade trypsin (Promega) according to the manufacturer’s instructions (1 μg in 100 μl of 25 mM ammonium bicarbonate). The supernatants were transferred to a fresh tube and stored at 4°C until analysis.
anti-PrxQ antibody (AS05093) from Agrisera. Western blots were developed according to the method of NBT/BCIP from Roche (11681451001).

Photosynthetic activities measurement by imaging pulse amplitude modulation

The Maxi-version of the Imaging Pulse Amplitude Modulation (Imaging PAM) and the software Imaging WIN version 2.39 (both Heinz Walz GmbH, Effeltrich, Germany) were used to determine the photosynthetic activities according to Behr et al. [27]. The selected leaves attached to cassava cultivar NZ199 diploid and autotetraploid genotypes were adjusted in the dark for 20 min prior to measurement. The leaves were detached at 10:00 a.m (ambient full sunlight). The detached leaf was clamped onto the holder at a distance of 7 cm between the leaf and the CCD camera. The other variables were as follows: light intensity at 2, frequency at 1, damping at 2; saturation pulse with intensity at 10 and width at 8. Each recording started by determining the dark fluorescence parameter F0. A saturating light pulse was employed to determine the maximal fluorescence parameter Fm. The maximal quantum yield of PS II was calculated as \( \frac{F_v}{F_m} = \frac{F_m - F_0}{F_m} \). In the presence of light intensity \( 185 \text{ mE m}^{-2} \text{ s}^{-1} \), the current fluorescence yield (\( F_t \)) and the maximum light adapted fluorescence (\( F_{m9} \)) were measured to calculate the effective PS II quantum yield (\( \Phi_{PSII} = \frac{F_{m9} - F_t}{F_{m9}} \)). Additionally, the nonphotochemical quenching [NPQ = (\( F_m - F_{m9} \))/\( F_m \)] was determined to show the proportion of absorbed light energy that is not used for photosynthetic electron transport. NPQ/4 is NPQ divided by 4 in order to allow for a display in a color code ranging from 0.0 to 100%. For quantitative analyses of the whole leaves, the software used 5 areas of interest of 25 mm\(^2\) that were randomly distributed over each leaf segment measured. For each variant, three individual plants were used and the respective results were averaged [27].

Salt-stress detection

The stems (length about 1.0–1.5 cm with one axillary bud) of cassava NZ199 diploid and autotetraploid genotypes, obtained from in vitro tissue culture at CGB, were cultured on MS [0.03 mg l\(^{-1}\) naphthylacetic acid (NAA), 3% sucrose, 0.3% Gelrite, pH5.8–6.0] and salt stress medium (50 mM NaCl, 0.03 mg l\(^{-1}\) NAA, 3% sucrose, 0.3% Gelrite, pH5.8–6.0), respectively. Three stems were cultured in one tissue-culture bottle and incubated in the tissue culture room at 26–28°C under a 12-h photoperiod. The length and fresh weight of 12 plantlets of both genotypes in salt-stressed medium were measured after 50 days.

Determination of cyanogenic glucosides (CGs) by HPLC

Five plantlets, grown for 2 months in vitro, were selected from each cassava cultivar NZ199 diploid and autotetraploid genotypes. A leaf disc was sampled from the first unfolded leaf of each plant by snap-closing a 2 ml-Eppendorf lid around one of the leaf fingers. Five replicates were carried out in each diploid and autotetraploid genotypes. To determine the content of CGs directly, the leaves samples were immersed into 300 ml pre-warmed 85% (v/v) methanol and boiled at once in a water bath at 100°C for 3 min, then, cooled on ice. The methanol extract was transferred to a new tube and filtered through a 0.45 μm filter [28].

CG content was determined on a Hitachi LC 2130 series coupled with autosampler L-2200, UV detector LC 2030 and chromatography workstation \(^7\) 2100P (Hitachi, Japan). The HPLC column was a Kromasil 100-5SIC8 column (250×4.6 mm, 5 μm). The mobile phases consisted of water (A) and acetonitrile (B). The flow rate was 0.8 ml/min. The gradient program was as follows: 0 to 30 min, linear gradient 10% to 100% (v/v) B, 30 to 33 min, linear gradient 100% to 10% B, 33 to 38 min. The UV detection wavelength was set at 215 nm. The column temperature was
The injected volume of samples was 20 μl. The concentration of hydrogen cyanide (HCN) standard sample is 0.5 ppm collected from the National Institute of Metrology of China. The retention time for CG was 28.6 min.

Generation of protein interaction networks

All proteins identified in cassava cultivar NZ199 autotetraploid were compared with those in diploid genotype and were used to generate a wider protein interaction map by employing a Pathway Studio software program (www.ariadnegenomics.com) [25].

Results

Determination of cassava polyploid genotypes

The autotetraploid status was validated under light microscope. Examination using the leaves of confirmed diploid (Fig. 1A and C) and autotetraploid (Fig. 1B and D) cassava NZ199 plants showed that the chromosome number from diploid genotype was 36 (2n = 2x = 36) (Fig. 1E), whereas that of autotetraploid genotype was 72 (4n = 4x = 72) (Fig. 1F). The fluorescence peak of diploid nuclei was located at channel position 200 (Fig. 1G), whereas that

Figure 2. 2-D gel protein profiles of leaves from cassava NZ199 diploid (A) and autotetraploid genotypes (B) and wrapped 2-DE map from diploid and autotetraploid genotypes (C). The white and black arrows in pane C indicated proteins that showed detectable changes (>2.0-fold of the normalized volume) in abundance compared with those observed in the control; white indicated a down-regulated match, and black indicated an up-regulated match. Small boxes indicated the gel regions to be amplified to highlight clearly detectable spots in Fig. 3. doi:10.1371/journal.pone.0085991.g002
of autotetraploid nuclei was located at channel position 400 (Fig. 1H), consistent with the expected DNA relationship of the two types of plants.

Protein profiles of cassava autotetraploid and diploid genotypes

A total of approximately 500 protein spots were detected by digital image analysis, and at least 300 spots gave reproducible staining patterns for all samples as judged by eye and by spot intensity ranking [Fig. 2A and 2B, Figure S1]. Using a spot-to-spot comparison and statistical analysis, a total of 65 stained spots (Fig. 2C) from autotetraploid genotype were found to have significant changes (p < 0.05) with greater than 2.0-fold altered intensity compared with diploid genotype. The differential spots were determined using Scheffe’s test at P < 0.05. Of these, the expression of 14 spots was down-regulated and the remainder up-regulated (Fig. 2C). As shown in Fig. 3, the detectable spots with differentially abundant expression in Fig. 2C were clearly highlighted (a, diploid genotype; b, autotetraploid genotype). 3-D images of these spots were generated using Delta2D software to detect the significant changes between diploid and autotetraploid genotypes as shown in Figure S2.

Functional grouping of identified proteins

Sixty-five spots with differential expression were isolated from 2-DE gels and identification performed using MALDI-TOF-MS/MS, of which fifty-two protein spots were identified. Other 13 spots, however, remained unidentified. Forty-seven identified proteins were up-regulated and 5 were down-regulated in autotetraploid genotype compared with diploid genotype. Functions of 52 differentially expressed proteins were annotated via the survey of gene banks (Table 1, Fig. 4). Fifteen proteins are associated with carbohydrate and energy metabolism, of which 4 proteins (spot 35, spots 47–49) were down-regulated. The 32 up-regulated proteins were associated with photosynthesis (6 spots, 11%), defense system (12 spots, 23%), HCN metabolism (2 spots, 4%), protein biosynthesis (6 spots, 11%), chaperones (3 spots, 6%), amino acid metabolism (2 spots, 4%) and signal transduction (1 spot, 2%). Other 5 were function-unknown proteins, including 1 down-regulated protein. The characteristics of 4 down-regulated proteins, spot 35 and spots 47–49, relate with energy metabolism. Protein spot 35 was identified as NAD(P)-binding Rossmann-fold-containing protein and spots 47–49 were putative ATP-binding proteins (Table 1). To ensure reliability of differential proteins on 2-DE gels, the protein expressions of Rubisco, APX and PrxQ in leaves of cassava diploid and autotetraploid genotypes were detected by immunoblotting to validate the proteomic analysis (Fig. 5). For example, the result from western blot showed that the expression of Rubisco small subunit in autotetraploid genotype was more than that in diploid genotype, which is similar with that seen on 2-DE analysis (Figs. 3 and 5).

Photosynthetic activities in cassava diploid and autotetraploid genotypes

Imaging-PAM studies were performed with the leaves from 50 d-old in vitro cassava plants of diploid and autotetraploid genotypes to understand the effects of up-regulated proteins
| Spot Number* | Identification                                      | Fold changesb (Mean±SE) | Accession no | Theoretical pl/ Mw(kDa) | Score⁴/No. of Unique peptides matched⁵ |
|-------------|-----------------------------------------------------|--------------------------|--------------|-------------------------|--------------------------------------|
| 13          | ATP synthase subunit beta, mitochondrial            | 2.02±0.05 (+)            | P17614       | 5.95/59.86              | 617/4                                |
| 14          | ATP synthase subunit beta, mitochondrial            | 2.34±0.11 (+)            | P17614       | 5.95/59.86              | 156/2                                |
| 15          | ATP synthase subunit beta, mitochondrial            | 2.09±0.06 (+)            | P17614       | 5.95/59.86              | 156/2                                |
| 16          | ATP synthase subunit beta, mitochondrial            | 2.82±0.09 (+)            | P17614       | 5.95/59.86              | 185/2                                |
| 26          | Phosphoglycerate kinase - Arabidopsis thaliana      | 2.27±0.10 (+)            | AAB60303     | 4.93/41.51              | 69/1                                 |
| 28          | alcohol dehydrogenase, putative - Ricinus communis | 4.86±0.11 (+)            | XP_002525379 | 8.61/41.58              | 92/1                                 |
| 30          | ATP synthase beta subunit - Gunnera manicata       | 2.26±0.13 (+)            | ABV65134     | 5.23/34.10              | 74/1                                 |
| 35          | NAD(P)-binding Rossmann-fold-containing protein - A. thaliana | 2.06±0.06 (-)            | NP_565868    | 8.37/34.88              | 263/2                                |
| 43          | putative triosephosphate isomerase - A. thaliana   | 2.52±0.14 (+)            | AAD29799     | 7.67/33.35              | 63/1                                 |
| 46          | Triose phosphate isomerase, cytosolic - Oryza sativa subsp. japonica | 3.53±0.11 (+)            | P48494       | 5.38/27.06              | 63/1                                 |
| 47          | Putative ATP-binding protein - Stenotrophomonas maltophilia K279a | 2.08±0.07 (-)            | CAQ46869     | 5.87/30.40              | 88/1                                 |
| 48          | Putative ATP-binding protein - S. maltophilia K279a | 2.02±0.06 (-)            | CAQ46869     | 5.87/30.40              | 88/1                                 |
| 49          | Putative ATP-binding protein - S. maltophilia K279a | 2.12±0.08 (-)            | CAQ46869     | 5.87/30.40              | 88/1                                 |
| 64          | ATP synthase CF1 epsilon subunit - Spinacia oleracea | 2.19±0.12 (+)            | NP_054942    | 6.59/14.70              | 124/1                                |
| 65          | ATP synthase epsilon chain - Androa decaryi        | 2.08±0.05 (+)            | CAD22407     | 5.87/14.28              | 349/4                                |
| 4           | Nuclear encoded precursor to chloroplast protein - Pisum sativum | 2.37±0.10 (+)            | AAA33680     | 6.55/102.71             | 67/2                                 |
| 8           | Rubisco large subunit-binding protein subunit beta, chloroplastic | 2.57±0.11 (+)            | P08927       | 5.85/62.98              | 77/1                                 |
| 22          | Oxygen evolving enhancer protein 1 precursor - Bruguiera gymnorrhiza | 2.48±0.10 (+)            | Q9LR4_9ROSI  | 35.11/6.48              | 94/3                                 |
| 31          | Putative Rubisco activase protein - Zantedeschia hybrid cultivar | 4.71±0.10 (+)            | AAT12492     | 5.08/27.69              | 43/1                                 |
| 55          | Cytochrome b6-f complex iron-sulfur subunit, chloroplastic | 3.71±0.15 (+)            | P26291       | 8.63/24.24              | 170/2                                |
| 60          | Ribulose 1,5-bisphosphate carboxylase small chain precursor - M. esculenta | 8.36±0.16 (+)            | AAF06098     | 8.33/20.41              | 120/3                                |
| 36          | Peroxiredoxin - Phaseolus vulgaris                | 4.58±0.06 (+)            | CAC17803     | 5.18/28.62              | 123/1                                |
| 39          | Ascorbate peroxidase APX2 - M. esculenta           | 2.85±0.08 (+)            | AAX84679     | 5.31/27.67              | 169/2                                |
| 44          | Ascorbate peroxidase APX2 - M. esculenta           | 2.26±0.04 (+)            | AAX84679     | 5.31/27.67              | 169/2                                |
| 45          | ascorbate peroxidase APX2 - M. esculenta           | 2.52±0.11 (+)            | AAX84679     | 5.31/27.67              | 484/4                                |
| 58          | Chain A, Prx D - Populus Tremula                   | 2.17±0.09 (+)            | 1TP9_A       | 5.56/17.43              | 130/2                                |
| 59          | Glutaredoxin                                       | 2.06±0.05 (+)            | O81187       | 6.05/11.13              | 63/1                                 |
| 9           | Beta-glucosidase - M. esculenta                   | 2.05±0.07 (+)            | CAA64442     | 5.80/63.10              | 71/1                                 |
| 10          | Beta-glucosidase - M. esculenta                   | 2.11±0.12 (+)            | CAA64442     | 5.80/63.10              | 71/1                                 |
| 24          | Chloroplast latex aldolase-like protein - M. esculenta (Manioc) | 2.28±0.07 (+)            | Q5PYQ2_MANES | 33.78/6.22              | 146/3                                |
| 29          | Metacaspase-9-A. thaliana                         | 3.43±0.08 (+)            | AED90710     | 5.81/35.51              | 72/1                                 |
| 33          | CDSP32 protein - Solanum tuberosum                | 6.13±0.16 (+)            | CAA71103     | 8.07/33.46              | 101/1                                |
| 61          | Thioredoxin-like protein - A. thaliana            | 8.58±0.18 (+)            | AAF04439     | 7.62/16.89              | 64/1                                 |

**Table 1.** Identification of differential proteins in cassava cultivar NZ199 leaves from autotetraploid and diploid genotypes.
involved in leave photosynthesis metabolism on the photosynthetic activities of cassava polyploid plants. In the present study, we chose the sensitive photosynthesis parameters Fv/Fm, ΦPSII and NPQ/4 to determine the changes of photosynthetic activities. Fig. 6 and Table 2 showed that autotetraploid induction could produce significant effects on the efficiency of excitation energy capture by open Fv/Fm, ΦPSII and NPQ/4, suggesting that an increase in maximal and effective quantum yield and a concomitant increase in NPQ/4 processes are sensitive markers for polyploid genotypes. These data imply up-regulated proteins associated with photosynthesis will result in increase of photosynthetic activities in cassava autotetraploid genotype.

Effects of salt stress on cassava diploid and autotetraploid genotypes

Plantlets of cassava diploid and autotetraploid genotypes grown on the MS medium and salt-stressed medium for 30 d and 50 d were examined to observe the effects of up-regulated proteins on salt stress tolerance (Fig. 7). The length and fresh weight of 50 d-old cassava plantlets grown on salt-stressed medium showed inhibitory effects of high salt concentrations on the growth of both cassava genotypes. However, length and fresh weight of cassava plantlets from diploid genotype in the presence of salt were significantly lower than those from autotetraploid genotype (Table 3). These data imply association of the up-regulated proteins on the increase of salt stress tolerance.

| Spot Number | Identification | Fold changesb (Mean±SE) | Accession no | Theoretical pl/ Mw(kDa) | Scorec/d No. of Unique peptides matchedd |
|-------------|----------------|--------------------------|--------------|------------------------|--------------------------------------|
| 7           | hsp70 -Petunia × hybrida | 2.66±0.11(+)            | CAA31663     | 5.06/70.78             | 42/1                                 |
| 34          | SHOOT1 protein - Glycine max | 2.26±0.10(+)            | AAP33012     | 5.26/40.24             | 91/1                                 |
| 63          | HSP19 class II - Citrus × paradisi | 2.02±0.07(+)            | 8.01/11.14   | 67/1                   |
| 27          | Glutamate ammonial ligase, cytosolic- A. thaliana | 2.24±0.10(+)          | S18603       | 5.40/40.73             | 57/1                                 |
| 56          | mitochondrial glycine decarboxylase complex H-protein - Populus tremuloides | 2.27±0.08(+)          | ABO61731     | 4.78/17.62             | 168/2                                |
| 6           | Linamarase- M. esculenta | 4.99±0.23(+)            | AAB22162     | 5.42/61.37             | 260/2                                |
| 32          | acetone-cyanhydrin lyase- M. esculenta | 2.27±0.08(+)          | S45682       | 6.15/29.50             | 179/3                                |
| 37          | 14-3-3 protein- M. esculenta | 4.73±0.06(+)            | ADD92154     | 4.79/29.81             | 68/1                                 |
| 21          | predicted protein-Physcomitrella patens subsp. patens | 2.25±0.06(+)          | EDQ53885     | 6.76/46.38             | 89/2                                 |
| 38          | unnamed protein product - S. oleracea | 2.21±0.10(+)          | CAA29062     | 5.58/35.04             | 314/3                                |
| 40          | unnamed protein product - M. esculenta | 2.38±0.11(+)          | CBC70131     | 5.31/27.67             | 112/1                                |
| 52          | Predicted protein-Populus trichocarpa | 3.26±0.16(−)         | XP_002356568 | 9.02/26.95             | 56/1                                 |
| 54          | forkhead-associated domain-containing protein - Arabidopsis lyrata subsp. lyrata | 2.16±0.06(+)          | XP_00287556  | 8.46/22.23             | 88/1                                 |

The spots showing differential expression (>2.0-fold of the normalized volume) were counted after gel analysis and manual editing with Delta2D software. Each value represents the mean ± SE of triplicates. Protein spots whose abundance increased (+) or decreased (−) after polyploidy were shown. The numbers corresponded to the 2-DE gel in Fig. 2.

a. The numbers corresponded to the 2-DE gel in Fig. 2–3.

b. Expression change level in tetraploid genotype compared with diploid genotype.

c. NCBI accession number.

d. Probability-based MOWSE (molecular weight search) scores.

e. The number of unique peptides identified by MS/MS, and individual ions scores are all identity or extensive homology (p<0.05).

doi:10.1371/journal.pone.0085991.t001

Table 1. Cont.
Variation in CG content of cassava autotetraploid and diploid genotypes

Variations of CG content in the leaves collected from 50 d-old in vitro cassava plants of diploid and autotetraploid genotypes were studied to validate the effects of up-regulated proteins in relevance with HCN metabolism (spots 6 & 32) in autotetraploid genotype. The CG content of autotetraploid genotypes significantly increased in comparison to diploid genotypes (Fig. 8I–IV). These data imply that an increased conversion of HCN from cyanide containing compounds may be related with up-regulated proteins (linamarase and acetone-cyanhydrin lyase) which are involved in HCN synthesis.

Protein interaction networks

All differential proteins identified in cassava autotetraploid genotypes were used to generate a wider protein interaction map by employing a Pathway Studio software program (Fig. 9). The relationships of binding and regulation were established for 15 differential proteins, responding to plant photosynthesis, yield, adaptation and stresses. CDSP32 is localized in the chloroplast. There are direct interactions between 15 up-regulated proteins, including ATP synthase subunit beta, alcohol dehydrogenase, beta-glucosidase, phosphoglycerate kinase, triose phosphate isomerase (TPI), Rubisco activase (RCA), Rubisco, APX2, CDSP3, peroxiredoxin (PrxQ), thioredoxin, translation elongation factor,
glutamate-ammonia ligase, chaperone and 14-3-3 proteins, whereas RCA can establish relations with other proteins through regulating the processes of photosynthesis and plant yield (Tables S1, S2, S3).

Discussion

Sheffield et al. (2006) compared proteomes between cassava fibrous and tuberous roots and found 292 differentially expressed spots on gels. Of those, 232 proteins were identified [6]. Li et al. (2010) identified 383 proteins from cassava somatic embryos, plantlets and tuberous roots using LC-ESI-MS/MS [3]. These data were helpful to understand proteome patterns between cassava different tissues. To further understand the mechanism of cassava whole-genome duplication induced by colchicine, we first investigated the significant changes in the proteome patterns of cassava diploid and autotetraploid genotypes and assess the potentially cassava-polyploid breeding. Differential protein spots that were found to contribute to this variation included proteins of eight functional categories (Table 1 and Fig. 4), in which there are direct interactions among 15 up-regulated proteins (Fig. 9).

Effects of polyploid induction on the proteome were considerably pronounced. Statistical analysis showed clear differences in protein patterns between autotetraploid and diploid genotypes. In the present work, the proteins associated with plant defense system including defense proteins [beta-glucosidase (spots 9, 10), chloroplast latex aldolase-like protein (spot 24), metacaspase (spot 29), CDSP32 (spot 33), thioredoxin-like protein (spot 61)], detoxifying and antioxidant proteins were highly accumulated in cassava NZ199 autotetraploid genotype compared with diploid plants. Beta-glucosidase is a key enzyme regulating the abscisic acid (ABA) pool in plants under stresses [29,30]. Chloroplast latex aldolase-like protein plays a role in defense and stress responses [31]. CDSP32 (a chloroplastic drought-induced stress protein) and thioredoxin-like protein, induced by environmental stress conditions are known to participate in the response to oxidative and drought stresses [32–34]. It is reported that plant metacaspases, a family of cysteine proteases structurally related to caspases, play important roles in biotic and abiotic stress-induced programmed cell death [35,36]. PrxQ (spot 36) and APX (spots 39, 44, 45) are reported to participate in the protection against oxidative damage [25,32]. Moreover, the evidences of salt stress on cassava diploid and autotetraploid genotypes showed that up-regulated proteins associated with plant defense could result in the increase of cassava plantlet tolerance to salt stress (Fig. 7 and Table 3).

Table 2. Photosynthetic parameters collected from cassava cultivar leaves of NZ199 diploid and autotetraploid genotypes.

| Cassava genotypes | Fv/Fm (Mean±SE) | ΦPSII (Mean±SE) | NPQ/4 (Mean±SE) |
|-------------------|-----------------|-----------------|-----------------|
| NZ199 diploid     | 0.753±0.012 A    | 0.525±0.003 A    | 0.098±0.012 A   |
| NZ199 autotetraploid | 0.828±0.007 B   | 0.587±0.009 B    | 0.201±0.019 B   |

Values were means ± SE. Different capital letters in the same column indicated statistically significant differences according to Duncan test (P<0.01). doi:10.1371/journal.pone.0085991.t002
was up-regulated by more than 4.73-fold in autotetraploid genotype compared to diploid (Table 1). Previous studies revealed that multiple members of the 14-3-3 regulatory protein family act as mediators in ABA signaling through direct interaction with ABA-responsive element binding factors, and then alter the expression of related genes to enhance plant resistance against salinity or drought [37]. In addition, the previous studies described that the anatomical alterations of cassava polyploid plants, showing differences in density, compaction and thickness of parenchyma cells, may confer upon high tolerance to drought in tetraploid plant. The large number of vessel groupings in the tetraploid type may also maintain a larger quantity of water than in case of fewer ones in diploid plants [2,38]. All above described results provide evidences at proteomic and anatomical levels to support polyploid genotype owning high tolerance to stresses.

Polyploids consistently exhibit larger mesophyll cells with more chloroplasts and greater photosynthetic capacities per cell than their diploid progenitors [39,40]. The causes of these differences at

| Salt stress to cassava NZ199 genotypes | Shoot Height (Mean±SE) | Root Length (Mean±SE) | Root Weight (Mean±SE) | Aboveground Weight (Mean±SE) |
|----------------------------------------|------------------------|-----------------------|-----------------------|-----------------------------|
| Diploid control                        | 10.43±0.25 A           | 10.43±0.21 A          | 0.16±0.02 B           | 0.21±0.01 B                 |
| Diploid salt stress                    | 1.3±0.10 D             | 5.53±0.29 C           | 0.05±0.02 C           | 0.04±0.01 C                 |
| Autotetraploid control                 | 6.83±0.42 B            | 10.57±0.21 A          | 0.27±0.03 A           | 0.42±0.02 A                 |
| Autotetraploid salt stress             | 3.43±0.32 C            | 7.80±0.53 B           | 0.17±0.02 B           | 0.22±0.04 B                 |

Values were means ± SE. Different capital letters in the same column indicated statistically significant differences according to Duncan test (P<0.01).

doi:10.1371/journal.pone.0085991.t003

Figure 8. Chromatograms of cyanogenic glucoside of cassava cultivar leaves from NZ199 diploid and autotetraploid genotypes. I, HCN standard sample (0.5 ppm); II, NZ199 diploid genotypes; III, NZ199 autotetraploid genotype; IV, Extraction yield of cyanogenic glucoside from diploid and autotetraploid genotypes. Chromatographic conditions were: Kromasil 100-5C18 column (250×4.6 mm, 5 μm), gradient elution with aqueous acetonitrile, flow rate of 0.8 ml/min, UV detection at 215 nm, and column temperature at 30 °C.

doi:10.1371/journal.pone.0085991.g008
the level of underlying proteins are unknown. In the present study cassava polyploid genotype could increase the expressed levels of 6 photosynthesis related proteins including nuclear encoded precursor to chloroplast protein (spot 4) [41], Rubisco large subunit binding protein subunit beta (spot 8), oxygen evolving enhancer protein (spot 22) [25], RCA (spot 31), cytochrome b6-f complex iron-sulfur subunit (spot 55) [42] and rubisco (spot 60) (Table 1). These proteins are very likely involved in enhancement of photosynthesis, carbohydrate and energy metabolism in cassava leaves [3]. Rubisco serves as the main gateway for inorganic carbon to enter metabolic pathways in most ecosystems and hence is unique in its importance to support life. It catalyzes the key reaction in the photosynthetic assimilation of CO2 [13]. RCA could regulate the processes of plant photosynthesis and then affect the plant yield (Fig. 9). These proteins are very likely involved in enhancement of photosynthesis, carbohydrate and energy metabolism in cassava leaves [3]. Rubisco serves as the main gateway for inorganic carbon to enter metabolic pathways in most ecosystems and hence is unique in its importance to support life. It catalyzes the key reaction in the photosynthetic assimilation of CO2 [13]. RCA could regulate the processes of plant photosynthesis and then affect the plant yield (Fig. 9).

The previous studies showed that glutamate-ammonia ligase (GLUL or GS, previous name: glutamine synthetase) (spot 27), a key enzyme in the GS–glutamate synthase cycle, plays a pivotal role in the recycling of NH4\(^+\) that is released during photorespiration by generating glutamate from NH4\(^+\) and glutamine [49]. There are two isoforms of GS in higher plants, cytosolic GS1 and chloroplastic GS2, with recycling of photorespiratory NH4\(^+\) depending on GS2 [50]. The present study demonstrated that cytosolic GLUL at a higher expression in tetraploid genotype than diploid plants.

In the present study, cassava tetraploid genotype increased the expressed levels of HCN metabolism-related proteins including linamarase (spot 6) and acetone-cyanhydrin lyase (spot 32) (Table 1). Linamarase is an enzyme found in the cell walls of cassava. The major cyanogen in cassava is linamarin stored in the

---

**Figure 9. Biological networks generated for combination of twelve differential proteins.** Fifteen differentially up-regulated proteins including ATP synthase subunit beta, alcohol dehydrogenase, beta-glucosidase, phosphoglycerate kinase, triose phosphate isomerase, RCA, Rubisco, APX2, CDSP3, peroxiredoxin, thioredoxin translation elongation factor, glutamate-ammonia ligase, chaperone and 14-3-3 in cassava autotetraploid genotypes were used to generate a protein-protein interaction network through Pathway Studio analysis. Regulation is marked as an arrow with R, Chemical Reaction as an arrow with C and Binding as an arrow without any marks. The entity table, relation table and reference table data were presented in in Tables S1, S2, S3.

doi:10.1371/journal.pone.0085991.g009

---
vacuole. Upon tissue disruption linamarin is deglycosylated bylinamarase, producing acetone cyanohydrin. Acetone cyanohydrin can spontaneously decompose at pH>5.0 or temperature >35°C, or is enzymatically broken down by acetone cyanohydrin lyase, which is hydroxynitrile lyase involved in the catalysis of cyanogenic glucosides [51], to produce acetone and free cyanide [52]. The evidences of HCN detection by HPLC showed that upregulated proteins associated with HCN metabolism could result in the increase of HCN content in cassava autotetraploid genotype in comparison to diploid genotype (Fig. S1–IV).

The autopolyploid genotypic variation is obvious as 65 proteins were detected showing significant differences in the present study. The proteomic study based on iTRAQ showed that the levels of protein divergence were relatively high between Arabidopsis autopolyploid and allopolyploid plants [22]. Proteomics is a powerful tool to characterize varieties of autopolyploids and allopolyploids [21]. Allopolyploidy can accelerate evolution through rapid and reproducible genomic changes in the first generation of nascent polyploids, including elimination of DNA sequences, gene silencing, alteration of cytosine methylation and generation of nascent polyploids, including elimination of DNA.

Conclusions

The results suggest that 2-DE combined MS and bioinformatics techniques are a valid method to detect the changes of cassava polyploidy genotypes in proteome levels. The present study differs from the previous genomic-level studies of polyploidy in which investigate the differentially expressed proteins in relation with cassava autotetraploid genotype. The functional classification and expression levels of differential proteins showed that polyploid formation would be a complicated process of polyploidization, in which polyploidy provides a reservoir of duplicate genes as substrate for crop improvement. Following cassava polyploidization, new gene copies may undergo modifications allowing functional diversification in plants through the biological network of protein-protein interaction and the functional analyses of differential proteins. A useful proteome data set we provided would be helpful to predict the tolerance mechanism at a protein level of cassava polyploidy plants against environmental stresses. Further analysis of the diversity levels and the proteome patterns of duplicate gene not only traces variation of polyploids, but also provide insights into improvement of cassava polyploidy breeding in increasing photosynthesis and resistance efficiencies.

Supporting Information

Figure S1 Scatter plot showed the ratios of the relative volumes. A, Uses spots for normalization, detected spots~500, reproducible spots~300; B, Scatter plots of reproducible protein spots on two 2-DE images from diploid and autotetraploid genotypes, respectively.

Figure S2 3-D maps of 65 differential proteins generated by Delta2D software based on the abundance of spots in 2-DE maps. a, differential spots from diploid genotype; b, differential spots from autotetraploid genotype. Blue circles indicated the location of protein spots from diploid genotype; yellow circles indicated spots from autotetraploid genotype. White letters indicated down-regulated; black letters indicated up-regulated. The numbering corresponded to the 2-DE gel in Fig. 2.

Table S1 Entity table views of protein-protein interactions in biological networks generated for cassava polyploid genotypes.

Table S2 Relation table views of protein-protein interactions in biological networks generated for cassava polyploid genotypes.

Table S3 Reference table views of protein-protein interactions in biological networks generated for cassava polyploid genotypes.

Acknowledgments

We thank Ms Ruili Xu (TCGRI, CATAS) for providing the cassava cultivar NZ199 from Cassava Germplasm Bank, TCGRI, CATAS. We also thank Ms Qinfei Wang (TCGRI) for skillful technical assistance in HPLC analyses.

Author Contributions

Conceived and designed the experiments: FA JF JL QXL KL SC. Performed the experiments: FA JF JL KL WZ FW SC. Analyzed the data: FA JF JL QXL KL WZ FW SC. Contributed reagents/materials/analysis tools: FA JF JL KL WZ FW LJBC SC. Wrote the paper: FA QXL KL SC.

References

1. Raji AA, Anderson JV, Kolade OA, Ugwu CD, Dixon AG, et al. (2009) Gene-based microsatellites for cassava (Manihot esculenta Crantz): prevalence, polymorphisms, and cross-taxa utility. BMC Plant Biol 9: 116.
2. Nassar NMA, Graciano-Ribeiro D, Fernandes SD, Araujo PC (2008) Anatomical alterations due to polyploidy in cassava, Manihot esculenta Crantz. Genet Mol Res 7(2): 276–283.
3. Li K, Zhu W, Zeng K, Zhang Z, Ye J, et al. (2010) Proteome Characterization of cassava (Manihot esculenta Crantz) somatic embryos, plantlets and tuberous roots. Proteome Sci 8: 10.
4. Carvalho LJ, Lippolis J, Chen S, de Souza CR, Vieira EA, et al. (2012) Characterization of Carotenoid-protein Complexes and Gene Expression Analysis Associated with Carotenoid Sequestration in Pigmented Cassava (Manihot Esculenta Crantz) Storage Root. Open Biochem J 6: 116–130.
5. Prochnik S, Marri PR, Desany B, Rahnivandiz P, Kodira C, et al. (2012) The Cassava Genome: Current Progress, Future Directions. Trop Plant Biol 5(1): 80–94.
6. Sheffield J, Taylor N, Faquett C, Chen S (2006) The cassava (Manihot esculenta Crantz) root proteome: Protein identification and differential expression. Proteomics 6(5): 1508–1598.
7. Jeglings AJ, Leech RM (1984) Anatomical variation in 1st leaves of 9 Triticum genotypes and its relationship to photosynthetic capacity. New Phytol 90: 371–392.
8. Nassar NMA (2006) The synthesis of a new cassava-derived species Manihot xerii Nassar. Genet Mol Res 5(3): 536–541.
9. Cavalieri-Smith T (1985) Cell volume and the evolution of eukaryotic genome size. In: Cavalieri-Smith T, editor. The evolution of genome size. Chichester, UK: John Wiley and Sons, pp. 105–184.
10. Albertin W, Ballau T, Brabanti P, Chevre AM, Eber F, et al. (2006) Numerous and rapid nonstochastic modification of gene products in newly synthesized Bacillus subtilis Autotetraploids. Genetics 173(2): 1101–1113.
11. Cai D, Rodriguez F, Teng Y, Ané C, Bonierbale M, et al. (2012) Single copy nuclear gene analysis of polyploidy in wild potatoes (Solanum section Petota). BMC Evol Biol 12: 70.
12. Hu G, Houston NL, Pathak D, Schmidt L, Thelen JJ, et al. (2011) Genomically biased accumulation of seed storage proteins in allopolyploid cotton. Genetics 189(3): 1103–1115.
13. Bomharry A, Edwards KD, Sanchez-Tamburrino J, Mueller LA (2012) Deciphering the complex leaf transcriptome of the allotetraploid species Nicotiana tabacum: A phylogenomic perspective. BMC Genomics 13: 406.
14. Lehesranta SJ, Davies HV, Shepherd LV, Nunnan N, McNicol JW, et al. (2005) Comparison of tuber proteome of potato variety landraces, and genetically modified lines. Plant physiol 138(3): 1600–1609.
15. Chen M, Thelen JF (2010) The essential role of plastidial triose phosphate isomerase in the integration of seed reserve mobilization and seedling establishment. Plant Signal Behav 5(3): 5583–5585.
16. Albertin W, Brabant P, Catrice O, Eber F, Jenczewski E, et al. (2005) Autopolyploidy in cabbage (Brassica oleracea L.) does not alter significantly the proteomes of green tissues. Proteomics 5(8): 2131–2139.
17. Amiceur N, Merlino M, Leroy P, Brandlard G (2003) Chromosome mapping and identification of amphipathic proteins of hexaploid wheat kernels. Theor Appl Genet 108(1): 62–72.
18. Islam N, Tsujimoto H, Hirota H (2003) Proteome analysis of diploid, tetraploid and hexaploid wheat: towards understanding genome interaction. Proteomics 3(4): 549–557.
19. Merlino M, Leroy P, Chambon C, Brandlard G (2009) Mapping and proteomic analysis of albumin and globulin proteins in hexaploid wheat kernels (Triticum aestivum L.). Theor Appl Genet 117(12): 1321–1337.
20. Hoehenwarter W, Larchimi A, Hummel J, Egelhofer V, Selbig J, et al. (2011) MAPA distinguishes genotype-specific variability of highly similar regulatory protein isoforms in potato tuber. J Proteome Res 10(7): 2979–2991.
21. Carpentier SC, Panis B, Renaut J, Samyn B, Vertommen A, et al. (2011) The use of 2D-electrophoresis and de novo sequencing to characterize inter- and intra-cultivar protein polymorphisms in an allotetraploid crop. Phytochemistry 72(10): 1243–1250.
22. Ng DW, Zhang C, Miller M, Shen Z, Briggs SP, et al. (2012) Proteomic divergence in Arabidopsis autopolyploids and allopolyploids and their progenitors. Heredity 108(4): 419–430.
23. Koh, J., Chen S, Zhu N, Yu F, Sohls PS, et al. (2012) Comparative proteomics of the recently and recurrently formed natural allotetraploid Brassica rapa (Asteraceae) and its parents. New Phyol 196(1): 292–305.
24. Pfosser M, Amos A, Lelley T, Heberle-Bors E (1995) Evaluation of sensitivity of flow cytometry in detecting aneuploidy in wheat using dioscin and diclofenac wheat ear addition lines. Cytometry 21(4): 307–393.
25. Chen S, Gollop N, Heuer B (2009) Proteomic analysis of salt-stressed tomato (Soluna lycopersicum) seeds: effect of genotype and exogenous application of glycitein}. J Exp Bot 60(7): 2005–2019.
26. Neuhold V, Arold N, Taube D, Elhardt W (1988) Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Comassie Brilliant Blue G-250 and R-250. Electrophoresis 9(6): 255–262.
27. Behr M, Humbeck K, Hause G, Deising HB, Wirsol SGR (2010) The hemirotrophi clostrotrichia graminicola locally induces photosynthetically active green islands but globally accelerates senescence on aging maize leaves. Mol Plant Microbe In 23(7): 879–892.
28. Jørgensen K, Bak S, Busk PK, Sorensen C, Olsen CE, et al. (2005) Cassava plants with a depleted cyanogenic glucoside content in leaves and tubers. Distribution of cyanogenic glucosides, their site of synthesis and transport, and blockage of the biosynthesis by RNA interference technology. Plant physiol 139:363–374.
29. Harada E, Kim J, Meyer AJ, Heil R, Clemens S, et al. (2010) Expression profiling of tobacco leaf trichomes identifies genes for biotic and abiotic stresses. Plant Cell Physiol 51(10): 1627–1637.
30. Arve LE, Terfa MT, Gislerød HR, Olsen JE, Torre S (2013) High relative air humidity and continuous light reduce stomata functionality by affecting the ABA control of photorespiration. J Exp Bot 46: 1397–1414.
31. Pfosser M, Amos A, Lelley T, Heberle-Bors E (1995) Evaluation of sensitivity of flow cytometry in detecting aneuploidy in wheat using dioscin and diclofenac wheat ear addition lines. Cytometry 21(4): 307–393.
32. Broin M, Cuine S, Peltier G, Rey P (2000) Involvement of CDSP32, a drought-resistant protein of Arabidopsis thaliana. J Biol Chem 275(2): 4770–4774.
33. Dorion S, Clendenning A, Jeukens J, Salas JJ, Parveen N, et al. (2012) A large decrease of cytosolic trisphosphate isomerase in transgenic potato roots affects the distribution of carbon in primary metabolism. Planta 236(4): 1177–1190.
34. Bhattacharyya M, Upadhyay R, Vishweshwara S (2012) Interaction signatures stabilizing the npl15-binding rosmanin fold: a structure network approach. Plos ONE 7(12): e51676.
35. Leegood RC, Lea PJ, Adcock MD, Hasler RE (1995) The regulation and control of photosynthesis. J Exp Bot 46: 1397–1414.
36. Husén SS, Stotheth JT, Yamashita E, Cramer WA (2013) Induced conformational changes within the cytochrome bfi complex of oxygenic photosynthesis. Biochemistry 52(15): 2649–2654.
37. Romanov MV, Smith JAC, Filatov DA (2012) Rubisco evolution in C4 eudicots: An analysis of amaranthaceae Senus Lato. PLOS ONE 7(12): e52974.
38. Guido PE, Christen L, Brown SV, Williams N (2013) Three distinct isoforms of ATP synthase subunit β are expressed in Tbrucei and assembled into the mitochondrial ATP synthase complex. PLOS ONE 8(1): e54039.
39. Tsukamoto Y, Fuyukishima Y, Hara S, Hishiri T (2013) Redox control of the activity of phosphorylase kinase in Synechocystis sp. PCC6803. Plant cell physiol 54(4): 484–491.
40. Shi J, Chen S, Gollop N, Goren R, Goldschmidt EE, et al. (2008) Effects of anaerobic stress on the proteome of citrus fruit. Plant Science, 175: 478–498.
41. Karlin-Neumann GA, Tobin EM (1986) Transist peptides of nuclear-encoded plastoprotein share a common amino acid framework The EMBO Journal 5(1): 9–13.
42. Hasan SS, Stoleth JT, Yamashita E, Cramer WA (2013) Induced conformational changes within the cytochrome bfi complex of oxygenic photosynthesis. Biochemistry 52(15): 2649–2654.
43. Karlin-Neumann GA, Tobin EM (1986) Transit peptides of nuclear-encoded plastoprotein share a common amino acid framework The EMBO Journal 5(1): 9–13.
44. Gulde PE, Christen L, Brown SV, Williams N (2013) Three distinct isoforms of ATP synthase subunit β are expressed in Tbrucei and assembled into the mitochondrial ATP synthase complex. PLOS ONE 8(1): e54039.
45. Kumagai E, Araki T, Hamaoka N, Ueno O (2011) Allopolyploidy-a shaping force in the evolution of barley. Ann Bot 107(1): 1381–1386.
46. Trummler K, Wajant H (1997) Molecular cloning of acetone cyanohydrin lyase from cassava roots elevates protein and free amino acids while decreasing the cyanogenic glucoside content in leaves and tubers. Mol Plant Microbe In 23(7): 2005–2019.
47. Leegood RC, Lea PJ, Adcock MD, Hasler RE (1995) The regulation and control of photosynthesis. J Exp Bot 46: 1397–1414.
48. Kumagai E, Araki T, Hamaoka N, Ueno O (2011) Allopolyploidy-a shaping force in the evolution of barley. Ann Bot 107(1): 1381–1386.
Copyright of PLoS ONE is the property of Public Library of Science and its content may not be copied or emailed to multiple sites or posted to a listserv without the copyright holder's express written permission. However, users may print, download, or email articles for individual use.