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Research article

The chloroplast proteome response to drought stress in cassava leaves

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ABSTRACT

Cassava is an important tropical crop with strong resistance to drought stress. The chloroplast, the site of photosynthesis, is sensitive to stress, and the drought-response proteins in cassava chloroplasts are worthy of investigation. In this study, cassava leaves were collected for ultra-structure observation from plants subjected to different drought stress conditions. Our results showed that drought stress can promote starch accumulation in cassava chloroplasts. To evaluate changes in chloroplast proteins under different drought conditions, two-dimensional electrophoresis was performed using purified chloroplasts, which resulted in the identification of 26 unique chloroplast proteins responsive to drought stress. These drought-responsive proteins are predominantly related to photosynthesis, carbon and nitrogen metabolism, and amino acid metabolism. Among them, most photosynthesis-related proteins are downregulated, with decreases in photosynthetic parameters upon drought stress. Several proteins associated with carbon and nitrogen metabolism, including rubisco and carbonic anhydrase, were upregulated, which might promote drought tolerance in cassava by enhancing the carbohydrate conversion efficiency and protecting the plant from oxidative stress. Our proteomic data not only provide insight into the complement of proteins in cassava chloroplasts but also further our overall understanding of drought-responsive proteins in cassava chloroplasts.

1. Introduction

Cassava, an important tuber plant worldwide (Xue et al., 2005; Wang et al., 2016a), is a drought-resistant tropical crop. As a drought-tolerance mechanism, the base leaves of cassava can rapidly shed to reduce transpiration (Li et al., 2001; El-Sharkawy, 2004; Zhang et al., 2009), with growth rapidly resuming after water balance is restored. In general, further analysis of drought resistance mechanisms in cassava can provide the theoretical basis for the cultivation of drought-resistant varieties of cassava as well as supply important genetic resources.

When cassava plants are subjected to drought stress condition, leaf expansion and development nearly halt (Alves and Setter, 2004). Photosynthetic parameters in these leaves are reduced (Liu et al., 2011; Li et al., 2013), and the contents of proline, abscisic acid and malondialdehyde, as well as the activities of peroxidase and superoxide dismutase are increased (Wang et al., 2010; Yu et al., 2012). To clarify the molecular mechanisms of drought resistance in cassava, many drought-related genes have been identified by cDNA library (Lokko et al., 2007; Sakurai et al., 2007) and transcriptomic (Utsumi et al., 2012; Fu et al., 2016) analyses. Furthermore, many microRNAs and long noncoding RNAs associated with drought tolerance have been found in cassava (Ballen-Taborda et al., 2013; Li et al., 2017). Cassava genome sequencing (Wang et al., 2014) has also revealed a large number of genes related to drought resistance, including ethylene response factors (Fan et al., 2016), basic leucine zipper (bZIP) factor (Hu et al., 2016), a homeodomain-leucine zipper (HD-Zip) gene family (Ding et al., 2017), and myeloblastosis (MYB) transcription factors (Ruan et al., 2017).

In general, proteins directly perform many cellular functions, though a large difference between the expression levels of genes and their corresponding proteins may exist (Hajduch et al., 2010). Therefore, it is necessary to explore the mechanism of the cassava response to drought stress at the proteomic level. Differential proteomic analysis of two cassava cultivars in response to drought stress at the proteomic level has been performed using the iTRAQ (isobaric tags for relative and absolute quantification) technique, and the results showed that many drought-responsive proteins are mainly involved in ion transport, the antioxidant system and secondary metabolism, signal transduction and

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gene regulation. Among them, linamarin might play an important role in nitrogen reallocation in cassava under drought conditions (Zhao et al., 2015). Chloroplasts are specialized photosynthetic organelles that are vulnerable to damage by many environmental stresses, yet proteomic analysis of cassava chloroplasts in response to drought stress has not been reported to date. In this study, comparative proteomics of cassava chloroplasts under drought stress was performed. Our results provide useful data for further revealing the mechanism of drought resistance in cassava.

2. Materials and methods

2.1. Plant materials and drought treatment

The drought-tolerant cassava cultivar South China 8 (SC8) was used in this study. Cassava plants were propagated clonally from cuttings of parental stems with at least two nodes and approximately 8 cm in length. The plants were grown in plastic pots (9 cm height, 11 cm diameter) containing a 1:1 mixture (V/V) of potting soil and vermiculite mixture. One plant was grown in each pot. The pots were placed in a non air-conditioned glass house in the Chinese Academy of Tropical Agricultural Sciences (Haikou, China). The glass house was covered with a glass roof, and the wall was half open and half sealed with glass to allow air flow. The temperature in the glass house ranged from 20 °C to 38 °C. No supplementary light was used other than sunlight received in the glass house. The plants were watered with 0.5 L of tap water per pot at 3 days interval, and fertilized with 1/2 Hoagland nutrient solution.

When the plants were 40 days old after planting of the cuttings, thirty plants with equal growth were selected for drought treatments. Twenty plants were water stressed withholding water for 5 days (ten plants) and 15 days (ten plants), respectively. Ten plants kept receiving water as control. The leaves from the control and different drought treatments were respectively collected at the same day for ultra-structural observation, and chloroplasts isolation.

2.2. Transmission electron microscopy

The ultra-structure of chloroplasts from cassava plants subjected to different drought treatments has been described elsewhere (Wang et al., 2013). A 0.2 cm × 0.5 cm long slice from the middle part of each type of experimental leaf was fixed in 5% (v/v) glutaraldehyde and 2% formaldehyde in 0.1 M phosphate buffer (pH 7.2) for 24 h at 4 °C, followed by 6 h of fixation in 2% OsO_4, rinsed with 0.1 M phosphate buffer (pH 7.2) for three times. Then, the samples were dehydrated by graded ethanol solutions and embedded in Epon812. The blocks were sectioned with a glass knife using an ultra-microtome and were collected onto copper grids, which were then sequentially stained with uranyl acetate followed by lead citrate and examined under a JEOL JEM-1230 transmission electron microscope (TEM, JEM 1230, Hitachi, Tokyo, Japan). Five biological replicates from each drought treatment were performed for TEM analysis.

2.3. Isolation and purification of cassava chloroplasts

Chloroplasts were isolated from cassava leaves under different drought treatments as described (Chang et al., 2015). The chloroplast integrity was examined using an Axio scope microscope (Carl Zeiss, Jena, Germany), and the purity of chloroplast proteins was assessed by measuring the enzyme activity of catalase, which is an indirect cytosolic marker in plants (Fan et al., 2009a). In brief, soluble proteins from crude extracts and chloroplasts were extracted in 50 mM potassium phosphate buffer (pH 7.5). Protein concentrations were determined using the Bradford assay with BSA as a standard (Bradford, 1976). The reaction mixture was prepared by adding 30 μL of protein extraction containing 10 μg protein to 960 μL of potassium phosphate buffer. The reaction was initiated by the addition of 10 μL of H_2O_2 (3% v/v), and the decrease in absorbance at 240 nm was followed for 5 min. The absorbance value of the purified chloroplast fraction showed no significant changes during the detection time. The assay was performed in triplicate, and the absorbance values were plotted against time.

2.4. Chloroplast protein extraction and two-dimensional electrophoresis analysis of chloroplast proteins

To analyze drought-response mechanisms at the protein level, the purified chloroplast proteins of cassava plants under different drought treatments were extracted using the BPP protocol (Wang et al., 2007a). The washed protein pellets were air-dried and dissolved in Lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 30 mM Tris-HCl, pH 8.5). Protein concentrations were determined using the Bradford assay with BSA as a standard (Bradford, 1976).

Approximately 1200 μg of chloroplast proteins were loaded onto an immobilized pH gradient strip holder that contained 24-cm strips with linear gradients that ranged from pH 4 to 7 (GE Healthcare, Uppsala, Sweden). The strips were hydrated for 18 h at room temperature, and then were placed into an Ettan IPGphor isoelectric focusing system. The program of isoelectric focusing was performed as following: 250 V, 3 h; 500 V, 2 h; 1000 V, 1 h; 8000 V, 3 h; 8000 V, 110 kV h; 1000 V, 18 h (Wang et al., 2011). The proteins were separated in the second dimension as described (Wang et al., 2009). Each 2-DE separation was repeated at least three times for each sample to ensure reproducibility of the protein patterns. The gels were visualized by the GAP staining method as described (Wang et al., 2012a) and were then scanned at a resolution of 600 dots per inch. The images were analyzed with the ImageMaster 2D Platinum Software (version 5.0, GE Healthcare). Spots were automatically detected by the software, and when necessary edited manually. Data were normalized by expressing protein abundance as relative spot volume. One-way Anova and Student's *t*-test were performed with 5% level of significance to validate the differential spots. Only spots present in two out of three replicates were considered for the differential abundance analysis.

2.5. Protein identification via MALDI TOF/TOF MS

Differential protein spots were manually incised in prepared gels and digested in-gel with bovine trypsin (Modified Trypsin, Sequencing Grade, Roche, Cat. 11418025001) as described (Wang et al., 2007b). Samples were identified using 5800 TOF/TOF MS (AB SCIEX, USA). All peptide mass fingerprint spectra were internally calibrated with trypsin autolysis peaks, and all known contaminants were excluded during this process. A total of 15 main peaks in each MS spectrum were sequentially selected for MS/MS analysis with ProteinPilot Software (version 4.5), and all MS/MS data from TOF/TOF spectra were combined with the corresponding MS data for Mascot Algorithm (version 2.3) and searched against a self-constructed database derived from cassava genome (BioProjects ID: PRJNA394209), which includes 43,286 gene sequences. The searched parameters were: 200 ppm mass tolerance for precursor ions and 0.3 Da for fragment ions, MH^+ monoisotopic mass values, oxidation of methionine allowed, one missed cleavage, and allowed fixed modification of carbamidomethylation. Protein with a score confidence interval above 95% (total protein score usually higher than 45, $p < 0.05$) was considered as a confident identification. The identified proteins were categorized to specific processes or functions by searching Gene Ontology (GO, http://www.geneontology.org/).

2.6. Protein functional classification and hierarchical clustering analysis

To determine functions, the identified proteins were searched against UniProt (http://www.ebi.uniprot.org) and the NCBI database (http://www.ncbi.nlm.nih.gov). SOTA (Self-organizing tree algorithm) hierarchical clustering of expression profiles was performed using

Cluster software (version 3.0). GO analysis was performed with Blast2GO as described (Conesa and Gotz, 2008), using the GO annotation search tool with the data from NCBI (http://www.ncbi.nlm.nih.gov). Finally, the differentially expressed proteins identified were analyzed using KEGG pathway analysis with KOBAS (http://kobas.cbi.pku.edu.cn/), and protein-protein interactions were assessed using the STRING V.10.5 database (http://string-db.org/). A high confidence view (score 0.9) was selected to present protein-protein networks.

2.7. qRT-PCR analysis

Approximately 1 μg of isolated total RNA was used to generate cDNA using Reverse Transcriptase Kit (Thermo, USA). The primer pairs used for quantitative real-time PCR (qRT-PCR) are provided in Supplementary Table S1. Triplicate quantitative assays were performed with the SYBR Green PCR Master mix (Thermo, Tokyo, USA) and an Mx3005P sequence detection system according to the manufacturer instruction. A gene fragment encoding *Manihot esculenta* actin (accession No. XR_002490310.1) was used as an internal control to normalize the amount of template cDNA (Supplementary Table S1).

3. Results

3.1. Ultra-structural observation of mesophyll cells in cassava leaves under different drought conditions

Ultra-structural changes of chloroplasts in cassava leaves were determined by transmission electron microscopy, and the results indicated that drought treatment can substantially affect the chloroplast shape and promote accumulation of starch granules (Fig. 1). Chloroplasts from control plants were typically fusiform and contained only a few small starch granules (Fig. 1A and D), whereas drought treatment for 5 days caused the shape of chloroplasts to expand and become more rounded (Fig. 1B). Swollen chloroplasts were observed after 15 days of drought treatment (Fig. 1C), and more starch granules accumulated under drought conditions (Fig. 1E and F). These results indicate that the ability of cassava to accumulate starch granules might increase the energy supply to resist the damaging effects of drought stress.

In many cases, thylakoid membrane disorganization was accompanied by disrupted grana stacking. Compared to control plants, the grana lamellae (GL) gradually became incompact and thinner with increasing drought treatment time, whereas severe drought stress (drought for 15 days) did not cause disruption of the thylakoid granum structure (Fig. 1G–I). These observations indicate that chloroplasts might play crucial roles in cassava drought adaptation.

3.2. Identification of drought-responsive chloroplast proteins in cassava leaves

As maintaining the integrity and high purity of chloroplasts is key for chloroplast proteomics, the purified chloroplasts from cassava were examined with regard to these aspects. The chloroplasts isolated were highly pure, with no appreciable level of other cytoplasmic contamination (Fig. S1). Altogether, the chloroplasts isolated from cassava leaves by percoll density gradient centrifugation in this study were suitable for our two-dimensional electrophoresis (2-DE) analysis. Only protein spots with at least two significant peptide sequences and a 1.5-fold change in intensity (confidence above 95%, $p < 0.05$) were characterized as drought-responsive chloroplast proteins.

Compared with the control, 45 protein spots were reproducibly detected as drought-responsive proteins (Fig. 2) and positively identified by MALDI TOF/TOF MS. These proteins represent 26 unique proteins; the protein identities are listed in Table 1. After 5 days of drought treatment, the abundances of 16 protein spots were significantly different: 2 protein spots were upregulated and 14 downregulated. Moreover, the abundances of 43 protein spots were markedly changed after 15 days of drought treatment, with 14 increased and 29 decreased (Fig. 2; Table 1). These results indicate that the abundance of more proteins might change significantly to adapt to the drought environment increasing time of drought stress.

3.3. KEGG pathway and changes in abundance of the identified drought-responsive chloroplast proteins

To determine the molecular interaction and relationship networks of the 26 unique chloroplast proteins, KEGG pathway analysis was performed using KOBAS software. According to the results, the proteins are enriched in seven metabolic pathways (Fig. 3A), including photosynthesis, antenna protein of photosynthesis, carbon fixation, carbon metabolism, glyoxylate and dicarboxylate metabolism, biosynthesis of amino acids and nitrogen metabolism. The major pathway is photosynthesis, and 15 protein spots (representing 7 unique proteins) are involved in this pathway, including ATP synthase α (ATPSα, spots 1, 2, 29, 32), β (ATPSβ, spots 18, 25) and γ subunit (ATPSγ, spot 15), oxygen evolving enhancer protein 1 (OEE 1, spots 5, 6) and 2 (OEE 2, spots 12, 13), photosynthetic NDH subunit of lumenal location 3 (pNDHL, spot 14), ferredoxin-NADP reductase (FNR, spot 31), and cytochrome *b*6-f complex iron-sulfur subunit 2 (ISP2, spot 42). Except for ISP2 and ATP synthase α subunit (spot 32), the abundance of these proteins decreased after drought treatment (Fig. 3B, Table 1). The second pathway is antenna protein of photosynthesis with the ability to capture light energy (7 proteins), including chlorophyll *a*/b-binding protein 6 (CAB6, spots 40 and 41), 8 (CAB8, spots 7, 11, 17, 26), and 13 (CAB13, spots 8 and 10), and chlorophyll *a*/b-binding protein of LHCII type 1-like (LHCII, spots 24, 27, 38). These proteins were mostly downregulated by drought stress, though CAB6 and LHCII (spot 38) were upregulated after drought treatment for 15 days (Fig. 3B, Table 1).

Many of the identified proteins are involved in multiple metabolic pathways (Fig. 3B), including ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit (RBCL, spot 33) and small chain (RBCS, spots 3, 44, 45), participating in carbon fixation, carbon metabolism, and glyoxylate and dicarboxylate metabolism. Although their abundance did not change obviously after 5 days of drought treatment, increases were significant after drought treatment of 15 days. Fructose-bisphosphate aldolase 1 (FBA1, spots 4, 22) and phosphoglycerate kinase (PGK, spot 19) are related to carbon fixation, carbon metabolism, and biosynthesis of amino acids, and the abundance of these proteins was decreased by drought stress, especially after 15 days (Fig. 3B, Table 1). These results suggest that these pathways might interconnect with each other to regulate drought adaptation in cassava.

3.4. Protein-protein interactions among drought-responsive chloroplast proteins

Subsequently, to elucidate the regulatory networks of these chloroplast proteins in response to drought conditions, the STRING database was employed to evaluate protein-protein interactions among the 26 unique proteins. Because there is no cassava or closely related species data in the STRING database, we utilized the corresponding *Arabidopsis thaliana* protein sequences with the *A. thaliana* database. In this study, we selected a high confidence view (score 0.9) to present the protein-protein networks. Based on the results, 14 of the 26 unique proteins are involved in protein-protein interactions, which were classified into 4 clusters (Fig. 4). Among them, cluster II interacts with cluster I and cluster III. Four proteins are present in cluster I, including FBA, PGK, RBCL and RBCS, which are key enzymes of carbon fixation (Fig. 3B). Cluster II contains four proteins, ATPSα, ATPSβ, ATPSγ and ISP, which chiefly participate in photosynthesis (Fig. 3B). Five unique proteins, OEE1, OEE2, CAB6, CAB8 and CAB13, are found in cluster III. Among them, OEE1 and OEE2 play important roles in maintaining the stability of photosynthesis system II (PS II), and CAB6, CAB8 and CAB13 are involved in capturing light energy. Only two proteins are grouped into

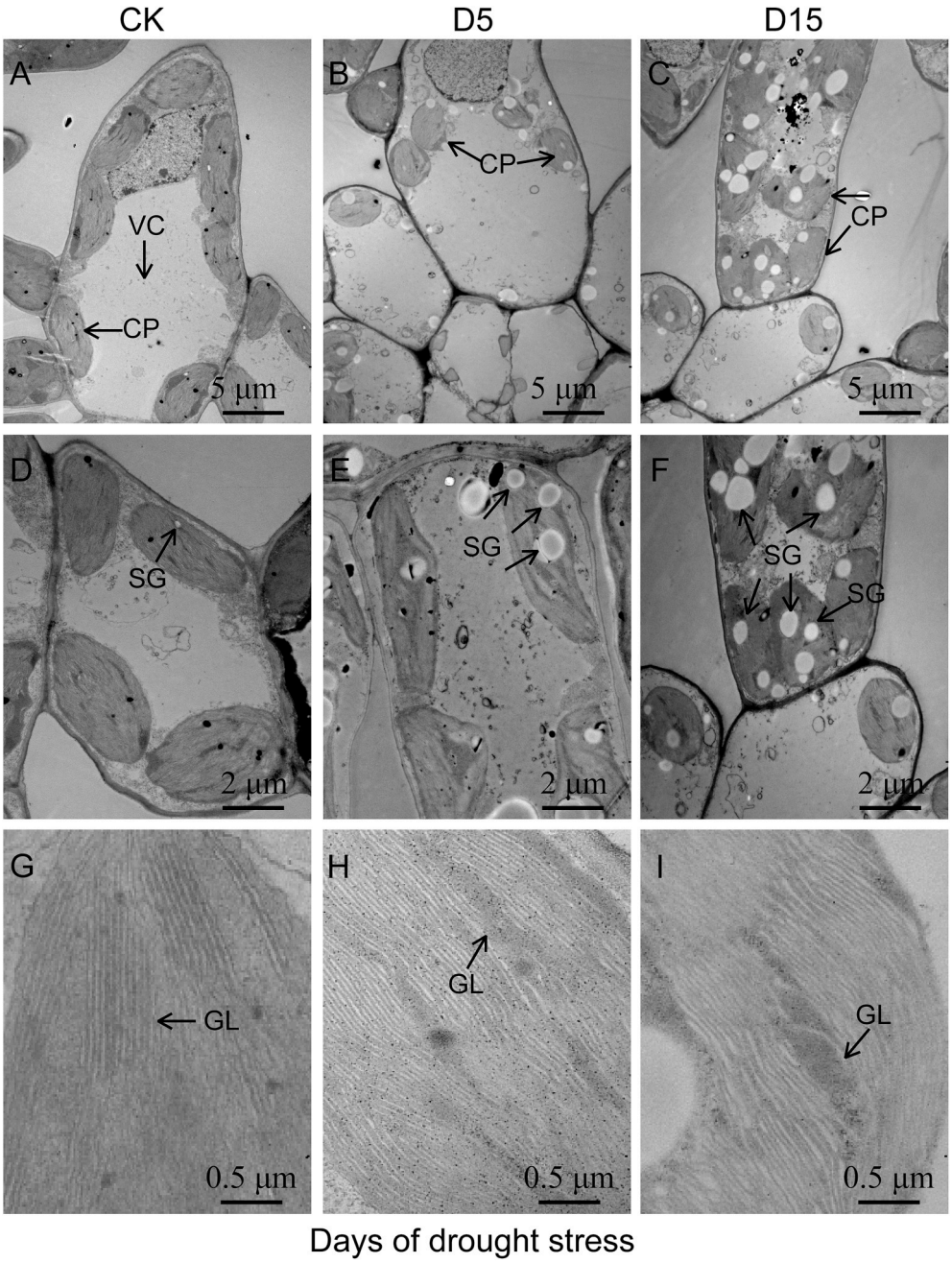

Fig. 1. Structural changes of mesophyll cells in cassava under different drought conditions. Typical shapes of chloroplasts of cassava after 0 (A), 5 (B) and 15 (C) days of drought treatment are presented. Starch granules (D–F) and granum lamellae (G–I) from drought-treated plants are highlighted. CP, chloroplast; GL, granum lamellae; VC, vacuole; SG, starch granule.

cluster IV: thylakoid lumenal 16.5 kDa protein (TLP, spot 30) and peptidyl-prolyl cis-trans isomerase CYP38 (spot 36). These protein-protein interactions reveal that proteins related to photosynthesis interact with each other to regulate photosynthesis in cassava.

3.5. Expression level analysis of selected protein-encoding genes

To further evaluate correlations between the abundance of proteins and their cognate transcripts, qRT-PCR was carried out to examine the mRNA levels of nine drought-responsive proteins: CYP38, PGK, FNR, OEE1, OEE2, FBA, TLP, GSL and carbonic anhydrase (CA) (Fig. 5). The results showed obvious increases in the expression levels of *CYP38*, *GSL* and *FBA*, whereas the levels of *PGK*, *OEE*, *CA* and *TLP* were significantly reduced after at least one drought treatment. Conversely, *OEE1* and *FNR* levels did not change significantly under drought stress (Fig. 5).

Significantly, the patterns of change in mRNA level for 4 genes (CYP38, GSL, PGK and OEE2) were similar to the abundances of the proteins. Although the protein abundances of CA and TLP were increased and those of FBA, OEE1 and FNR were decreased when cassava plants were subjected to drought treatment, the mRNA expression patterns differed from their protein abundances (Fig. 5). Drought-induced changes in the abundances of certain proteins were very different from the changes in the levels of their cognate transcripts (Fig. 5), suggesting that post-transcriptional regulation, translation and post-translation regulation might play key roles in controlling the final functions of these genes.

Based on our proteomics data and ultra-structural observation, possible mechanisms of drought tolerance in cassava chloroplasts are

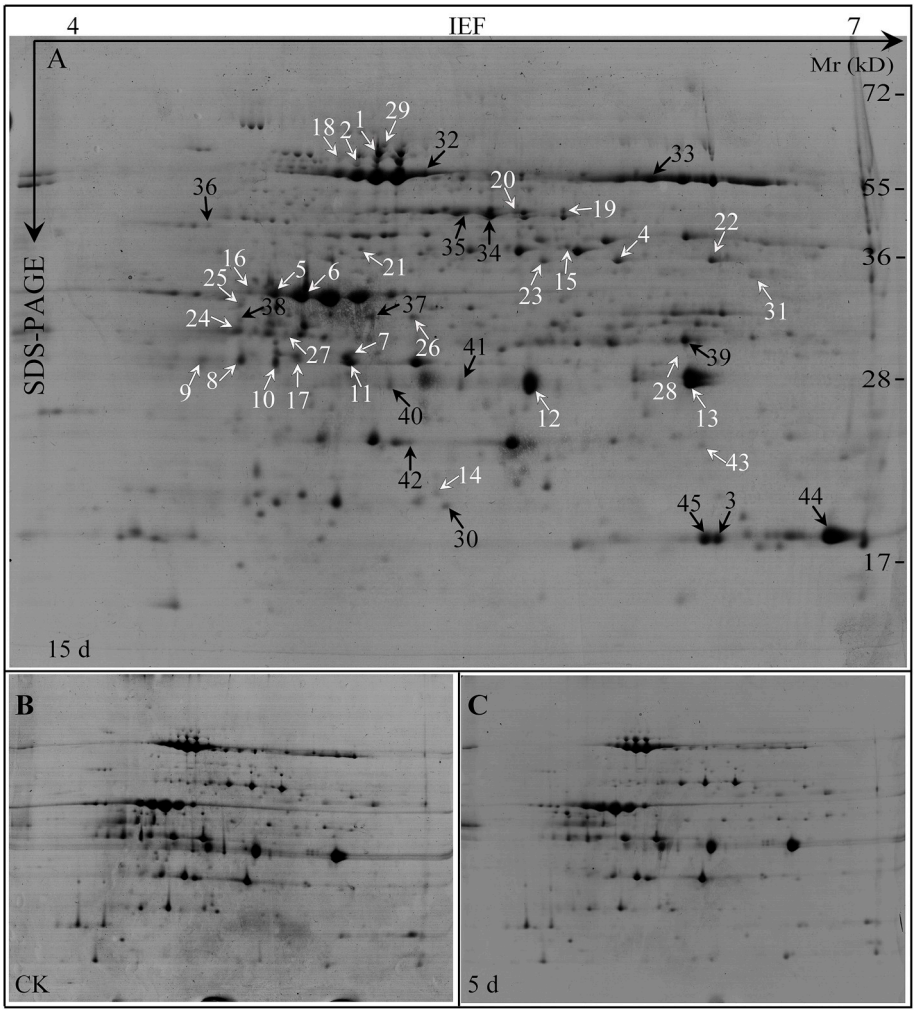

Fig. 2. Typical 2-DE gels of chloroplast proteins from cassava under drought stress. 2-DE gels of chloroplast proteins of cassava leaves after 0 (B), 5 (C) and 15 (A) days of drought conditions are presented. Differentially expressed protein spots under drought stress are marked in A. Black arrows indicate upregulated protein spots after drought stress; the white arrows represent downregulated spots.

proposed (Fig. 6). Drought-responsive chloroplast proteins isolated from cassava leaves mainly participate in photosynthesis, carbon and nitrogen metabolism, and amino acid metabolism, and most proteins related to carbohydrate and energy metabolism were significantly altered by drought stress. Many photosynthesis-related proteins were downregulated by drought stress, which might contribute in reducing the photosynthetic parameters of cassava under drought stress. In addition, the increase in starch accumulation in chloroplasts might increase the supply of energy for cassava to resist the damaging effects of drought stress. Overall, the cassava chloroplast proteome data agreed with the ultra-structural findings for mesophyll cells. Several proteins associated with carbon and nitrogen metabolism, including rubisco and CA, were upregulated by drought stress, which might improve the drought tolerance of cassava by enhancing the carbohydrate conversion efficiency, protecting this plant from oxidative stress damage.

4. Discussion

Drought stress is a major abiotic stress limiting crop yield (Chaves and Davies, 2010). As a unique species between C3 and C4 plants, cassava can not only make full use of light energy for efficient photosynthesis to accumulate starch (Mitko et al., 1993) but also has a strong drought ability to survive in harsh environments (Anna et al., 2010). In this study, the protein accumulation characteristics and regulatory mechanism of chloroplasts from cassava during drought tolerance were analyzed, and new insight into the drought tolerance mechanism in cassava chloroplasts was obtained.

4.1. Changes in chloroplast shape and starch accumulation patterns might be important for cassava to adapt to drought stress

The chloroplast, a unique organelle for photosynthesis and the most important organelle for energy conversion in plants, is highly sensitive to stress conditions (Endress and Sjolund, 1976). Under slight drought stress, the structure of the chloroplast changes mildly, the granum lamella is disturbed, and the thylakoid lumen is slightly enlarged. However, deformed chloroplasts, damaged membrane structures, and destroyed granum lamellae are found when plants are subjected to severe drought conditions (Wang et al., 2012b). Our results showed that the shape of chloroplasts in cassava changed from an ellipse to a near-round or one-sided irregular shape under drought stress. Additionally, chloroplast granum lamellae became thinner and looser but were not destroyed under severely drought stress, indicating that photosynthesis was affected. These results are supported by previously reported significantly decreased maximum photosynthesis rate, apparent quantum yield and light saturation point in cassava under severe drought stress (Liu et al., 2011; Li et al., 2013). Therefore, the altered chloroplast shape might reduce the damage caused by drought stress.

Starch is synthesized in chloroplasts in many higher plants, and the distribution and presence of starch granules in chloroplasts can

Table 1
MS identification of drought-responsive proteins from the cassava chloroplasts.

Protein spot[a]	Accession No.[b]	Protein name	Exp. *p*I/M*r* (kDa)[c]	Sequence coverage (%)[d]	Score[e]	Changed ratio[f] D5/CK	D15/CK
1	YP_001718422.1	ATP synthase CF1 alpha subunit	5.22/55.6	14	239	1.04	0.25
2	YP_001718422.1	ATP synthase CF1 alpha subunit	5.22/55.6	15	206	1.00	0.20
3	XP_021629607.1	ribulose 1,5-bisphosphate carboxylase small chain precursor	8.33/20.6	29	285	0.68	3.71
4	XP_021609330.1	fructose-bisphosphate aldolase 1	8.14/43.0	27	303	0.94	0.33
5	XP_021630622.1	oxygen-evolving enhancer protein 1	5.76/35.4	35	210	0.98	0.33
6	XP_021630622.1	oxygen-evolving enhancer protein 1	5.76/35.4	19	244	0.88	0.27
7	XP_021631529.1	chlorophyll *a-b* binding protein 8	6.85/29.6	24	178	1.14	0.20
8	XP_021606144.1	chlorophyll *a-b* binding protein 13	5.26/28.5	40	258	0.9	0.3
9	XP_021620491.1	2-Cys peroxiredoxin BAS1	7.66/29.6	13	97	0.93	0.35
10	XP_021606144.1	chlorophyll *a-b* binding protein 13	5.26/28.5	47	239	0.86	0.23
11	XP_021631529.1	chlorophyll *a-b* binding protein 8	6.85/29.6	32	179	1.02	0.47
12	XP_021605404.1	oxygen-evolving enhancer protein 2	8.65/28.5	34	187	0.7	0.31
13	XP_021605404.1	oxygen-evolving enhancer protein 2	8.65/28.5	38	237	0.87	0.22
14	XP_021593277.1	photosynthetic NDH subunit of lumenal location 3	9.37/25.2	15	123	1.34	0.32
15	XP_021597648.1	ATP synthase gamma chain	6.07/41.7	20	140	1.27	0.54
16	XP_021630622.1	oxygen-evolving enhancer protein 1	5.76/35.4	21	182	1.24	0.46
17	XP_021631529.1	chlorophyll *a-b* binding protein 8	6.85/29.6	32	148	1.20	0.24
18	YP_001718444.1	ATP synthase CF1 beta subunit	5.11/53.8	18	174	0.62	0.19
19	XP_021633936.1	phosphoglycerate kinase	8.26/50.3	16	117	0.38	0.56
20	XP_021608054.1	glutamine synthetase leaf isozyme	6.53/48.2	16	166	0.43	0.34
21	XP_021606204.1	peptidyl-prolyl cis-trans isomerase CYP37	6.05/51.9	19	225	0.59	0.24
22	XP_021609330.1	fructose-bisphosphate aldolase 1	8.14/43.0	27	358	0.65	0.34
23	XP_021624183.1	cysteine synthase	5.67/34.3	20	72	0.31	0.26
24	XP_021599266.1	chlorophyll *a-b* binding protein of LHCII type 1-like	5.29/28.3	15	140	0.64	0.37
25	YP_001718444.1	ATP synthase CF1 beta subunit	5.11/53.8	29	298	0.55	0.16
26	XP_021631529.1	chlorophyll *a-b* binding protein 8	6.85/29.6	14	48	0.66	0.59
27	XP_021599266.1	chlorophyll *a-b* binding protein of LHCII type 1-like	5.29/28.3	15	136	0.6	0.14
28	XP_021595497.1	carbonic anhydrase	7.04/36.7	14	73	0.61	0
29	YP_001718422.1	ATP synthase CF1 alpha subunit	5.22/55.6	17	325	0.14	0.65
30	XP_021615066.1	thylakoid lumenal 16.5 kDa protein	9.57/24.9	37	343	1.29	1.73
31	XP_021601666.1	ferredoxin–NADP reductase, leaf isozyme, chloroplastic isoform X1	8.90/40.8	16	74	0.65	0.34
32	YP_001718422.1	ATP synthase CF1 alpha subunit	5.22/55.6	17	331	1.64	4.42
33	YP_001718445.1	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit	6.18/53.2	10	120	1.16	4.01
34	XP_021608054.1	glutamine synthetase leaf isozyme	6.53/48.2	13	229	0.18	6.50
35	XP_021592164.1	elongation factor Tu	6.05/53.0	23	298	0.94	3.76
36	XP_021594363.1	peptidyl-prolyl cis-trans isomerase CYP38	5.07/49.9	18	84	0.8	2.20
37	XP_021593464.1	plastid-lipid-associated protein 6	6.60/30.9	27	298	1.08	2.86
38	XP_021599266.1	chlorophyll *a-b* binding protein of LHCII type 1-like	5.29/28.3	15	74	1.46	2.18
39	XP_021595497.1	carbonic anhydrase	7.04/36.7	32	388	1.12	2.70
40	XP_021617482.1	chlorophyll *a-b* binding protein 6	5.96/26.7	8	72	0.79	1.94
41	XP_021617482.1	chlorophyll *a-b* binding protein 6	5.96/26.7	13	78	0.96	2.85
42	XP_021592948.1	cytochrome *b*6-f complex iron-sulfur subunit 2	8.73/29.4	14	148	1.95	0
43	XP_021617166.1	rhodanese-like domain-containing protein 14	9.38/26.5	16	78	0.82	0.31
44	XP_021629607.1	ribulose 1,5-bisphosphate carboxylase small chain	8.33/20.6	47	387	0	5.54
45	XP_021629607.1	ribulose 1,5-bisphosphate carboxylase small chain	8.33/20.6	39	391	0.85	2.67

Note.
[a] Assigned spot number as indicated in Fig. 2.
[b] Database accession numbers according to NCBInr.
[c] Experimental isoelectric point (c, *p*I) and molecular weight (M*r*, kDa) of the identified proteins were produced by ImageMaster.
[d] Protein sequence coverage.
[e] The Mascot searched score (M. S.) against NCBInr.
[f] The relative abundance changed ratios on 2-DE gels from plants after 5 and 15 days of drought treatments compared with control plants were presented.

indirectly reflect the environmental conditions of plants (He et al., 2005). Evidence has shown that the activity of enzymes in mitochondria is reduced and respiratory metabolism and ATP production are decreased under low-temperature conditions, which inhibits the ability to hydrolyze starch and blocks the transport of photosynthetic products. As a result, starch accumulates in chloroplasts (He et al., 2005). At the same time, increases in starch granules might help to maintain a high sugar concentration near the thylakoids to contribute to thylakoid stability and normal photosynthetic phosphorylation (Wang et al., 2016a; He et al., 2005). Therefore, we speculate that the swollen morphology of chloroplasts and increased number of starch granules in cassava under drought stress (Fig. 1) might maintain these characteristics to provide energy when plants are subjected to drought conditions.

4.2. Chloroplast proteins involved in photosynthesis, carbon and nitrogen metabolism and amino acid metabolism might contribute to drought tolerance in cassava

The metabolic balance in chloroplasts is easily perturbed by environmental stresses (Zhu, 2016), and drought has significant effects on chloroplast protein expression. Our proteomic analysis showed that drought-responsive chloroplast proteins isolated from cassava leaves are mainly those of which participate in photosynthesis, carbon and nitrogen metabolism, and amino acid metabolism, with 14 of the 26 drought-responsive proteins being involved in photosynthetic-related processes, including photosynthesis-antenna protein CAB, and others such as OEE, FNR, ATPS, ISP, pNDHL, Rubisco, FBA, and PGK (Fig. 3; Table 1). Inhibition of absorption capacity, reduced light energy conversion efficiency and obstructed electron transport in chloroplasts can reduce the photosynthetic rate in drought-stressed plants (Li, 2001).

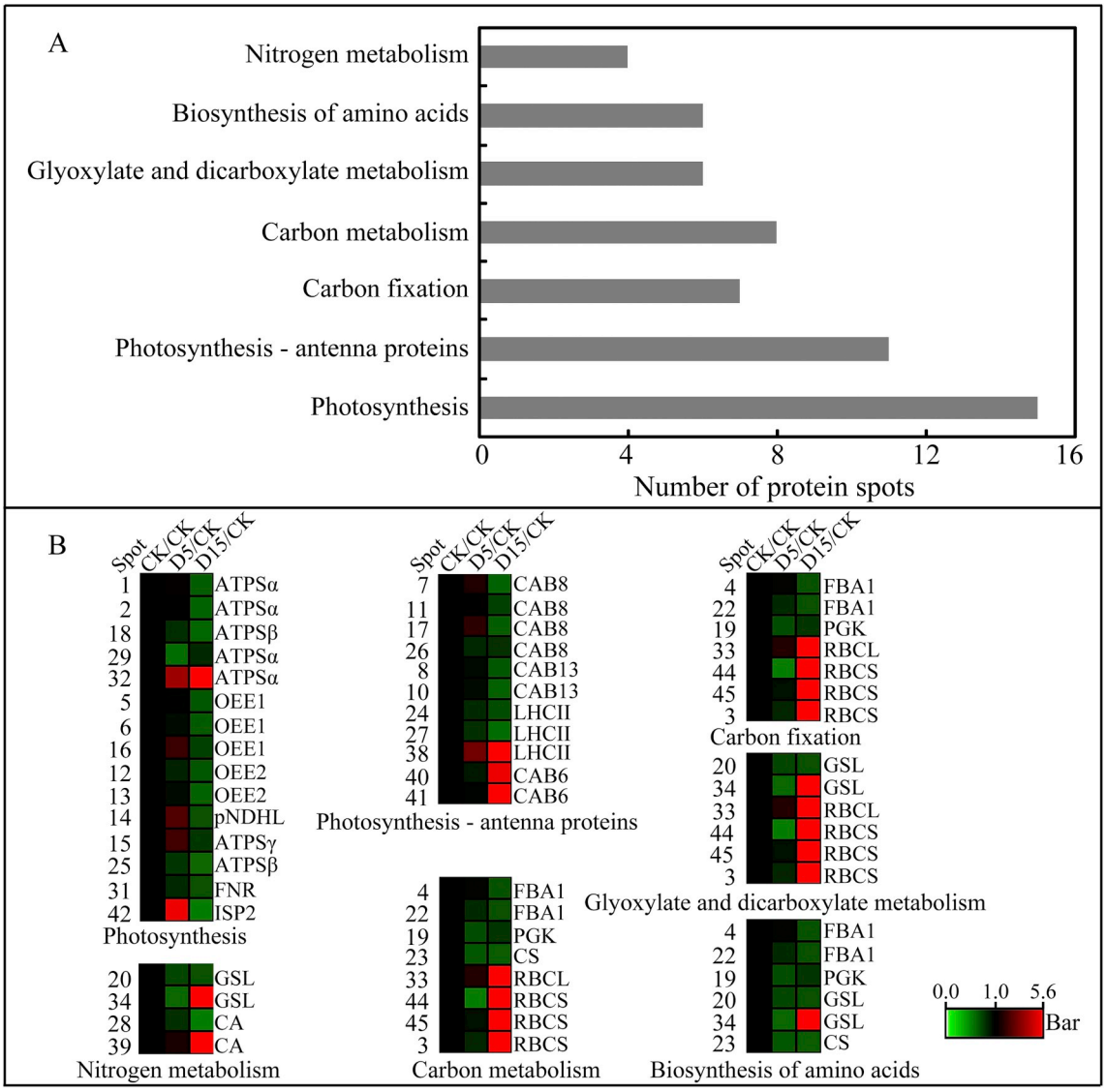

Fig. 3. KEGG pathways and altered abundance of the identified drought-responsive proteins in chloroplasts KEGG pathways (A) and changes in abundance (B) of the 26 unique drought-responsive chloroplast proteins are highlighted. Red color represents upregulation; green color shows downregulation. CK indicates the control group; D5 and D15 represent 5 and 15 days of drought treatment, respectively. Abbreviations: ATPS, ATP synthase; CA, carbonic anhydrase; CAB, chlorophyll *a*/b-binding protein; CS, cysteine synthase; FBA1, fructose-bisphosphate aldolase 1; FNR, ferredoxin-NADP reductase; GSL, glutamine synthetase leaf isozyme precursor; ISP2, cytochrome *b*6-f complex iron-sulfur subunit 2; LHCII, chlorophyll *a*/b-binding protein of LHCII type 1-like; OEE, oxygen-evolving enhancer protein; pNDHL, photosynthetic NDH subunit of lumenal location 3; PGK, phosphoglycerate kinase; RBCL, ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit; RBCS, ribulose 1,5-bisphosphate carboxylase small chain. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

CAB mainly functions in collecting and transporting light energy in PS I and PS II reaction centers, maintaining an optimal photosynthetic system (Shi et al., 1995). The reduced abundance of CAB8, CAB13 and LHCII due to drought treatment (but not CAB6, Table 1) in this study might limit absorption capacity and light energy conversion efficiency in cassava leaves. OEE is an early drought-response protein involved in repair of damaged proteins and in maintaining oxygen release, thereby preserving the stability of photosynthesis (Gazanchian et al., 2007). Accumulation of OEE2 is enhanced in response to stress induced by salt and ABA treatments (Abbasi and Komatsu, 2004). Furthermore, OEE2 was upregulated to a greater extent in drought-tolerant wheat than in drought-sensitive wheat (Cheng et al., 2015). FNR has been reported to bind peripherally to PS I and is implicated in generating NADPH for reducing power (Hanke et al., 2008). ISP is an important component of the cytochrome *b*6-f complex, which participates in electron transfer between PS I and PS II (Staeheun and Arntson, 1983). ATP synthase catalyzes production of the energy storage molecule adenosine triphosphate (ATP) in photosynthetic phosphorylation. In this study, accumulation of OEE1, OEE2, ISP, FNR, as well as the α, β and γ subunits of ATP synthase CF_1, was notably decreased by severe drought stress, indicating that the photosynthesis and photosynthetic phosphorylation processes in cassava might be affected in a severe drought environment. In summary, many photosynthetic-related proteins were downregulated when plants were subjected to drought stress (Fig. 3, Table 1), suggesting that the photosynthetic parameters of cassava might be inhibited by reducing the abundance of these photosynthesis-related proteins under drought stress. Our proteomic results are supported by previous reports of reduced chlorophyll fluorescence parameters (Yu

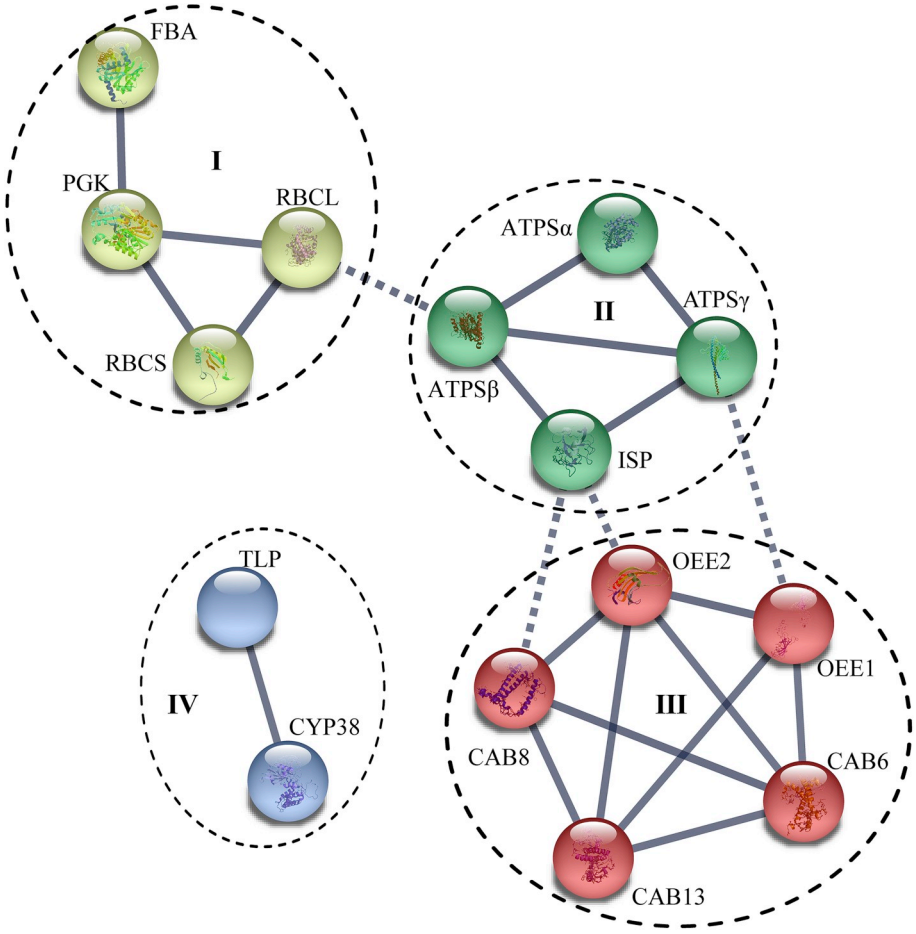

Fig. 4. Protein-protein interaction analysis of the identified drought-responsive chloroplast proteins. The solid lines and dashed lines indicate that interaction between proteins were strong and weak, respectively. Abbreviation: ATPS, ATP synthase; CAB, chlorophyll *a*/b-binding protein; FBA, fructose-bisphosphate aldolase; ISP, cytochrome *b*6-f complex iron-sulfur subunit; OEE, oxygen-evolving enhancer protein; PGK, phosphoglycerate kinase; RBCL, ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit; RBCS, ribulose 1,5-bisphosphate carboxylase small chain; TLP, thylakoid lumenal 165 kDa protein.

et al., 2012; Li et al., 2013) and are consistent with other findings in cassava (Zhao et al., 2015).

Rubisco is a key enzyme determining the carbon assimilation rate in photosynthesis, and its level is regulated by various abiotic stresses, such as salinity (Chang et al., 2015), water logging and temperature stress (Wang et al., 2017; Perdomo et al., 2017). In tobacco, the leaf water content is closely related to rubisco activity (Parry et al., 2002). As a key enzyme in plants, FBA is involved in both glycolysis and gluconeogenesis in the cytoplasm and also in the Calvin cycle in plastids. Most FBA genes in *Sesuvium portulacastrum* (Fan et al., 2009b) and *Arabidopsis* (Lu et al., 2012) were found to be significantly induced in roots by drought stress. In addition, increased protein abundance of FBA during water deficit was detected in the orphan crop *Eragrostis tef*, even though FBA enzymatic activity only increased at 60–65% of the relative water content (Kamies et al., 2017). However, a successive decline in the enzymatic activity of FBA was observed in both drought-tolerant and sensitive *Cicer arietinum* under water-deficit conditions (Khanna et al., 2014). These results suggest that FBA might exhibit different patterns in different plants when subjected to drought. PGK catalyzes the phosphorylation of 3-PG, producing 1,3-BPG and ADP, as part of the reactions that regenerate ribulose-1,5-bisphosphate to function in the Calvin cycle. Under drought conditions, the abundance of PGK is increased in drought-tolerant fennel but decreased in drought-sensitive fennel (Khodadadi et al., 2017), whereas PGK activity declined in both drought-tolerant and -sensitive *C. arietinum* (Khanna et al., 2014). In this study, the abundances of RBCL and RBCS were significantly induced by drought stress, though accumulation of FBA and PGK decrease markedly (Fig. 3). Combined with the accumulation of starch granules in the cassava chloroplast after drought treatment (Fig. 1), we suggest that the increased abundance of rubisco might cooperate with FBA and PGK to improve drought resistance by enhancing the conversion efficiency of starch. CA is a zinc-containing metalloenzyme, and specific association between CA and rubisco enables CO_2 to interact with the latter to maintain its functional machinery (Badger and Price, 1994). It has been reported that higher CA gene expression increases resistance to cytotoxic concentrations of H_2O_2 (Raisanen et al., 1999). In soybean, increased CA accumulation in cells might enhance their drought tolerance to become more resistant to cytotoxic concentrations of H_2O_2 (Das et al., 2016). In our study, the mRNA expression level of *CA* decreased, while the abundance of CA protein increased after 15 days of drought treatment, which might due to the modification of CA protein to reduce the process of protein degradation (Wang et al., 2016b). Thus, it is possible that the increase in expression of CA in cassava confers resistance to toxic levels of cellular H_2O_2 and protects the plant from oxidative stress under drought.

It is well known that increased levels of amino acid, especially proline, are closely related to decreased water potential in plant leaves (Boggess and Steward, 1976). By catalyzing the condensation of glutamate and ammonia to form glutamine accompanied by ATP consumption, glutamine synthetase (GS) plays an essential role in the metabolism of nitrogen. Several reports indicate that expression of GS is regulated by osmotic stress but with variability among different plants. For example, GS activity is reduced in rice seedlings under salinity (Lutts et al., 1999), whereas GS expression in sunflower cells (Santos et al., 2004), cashew leaves (Silveira et al., 2003), and rice roots (Yan et al., 2005) is induced by salt or osmotic stress. In addition,

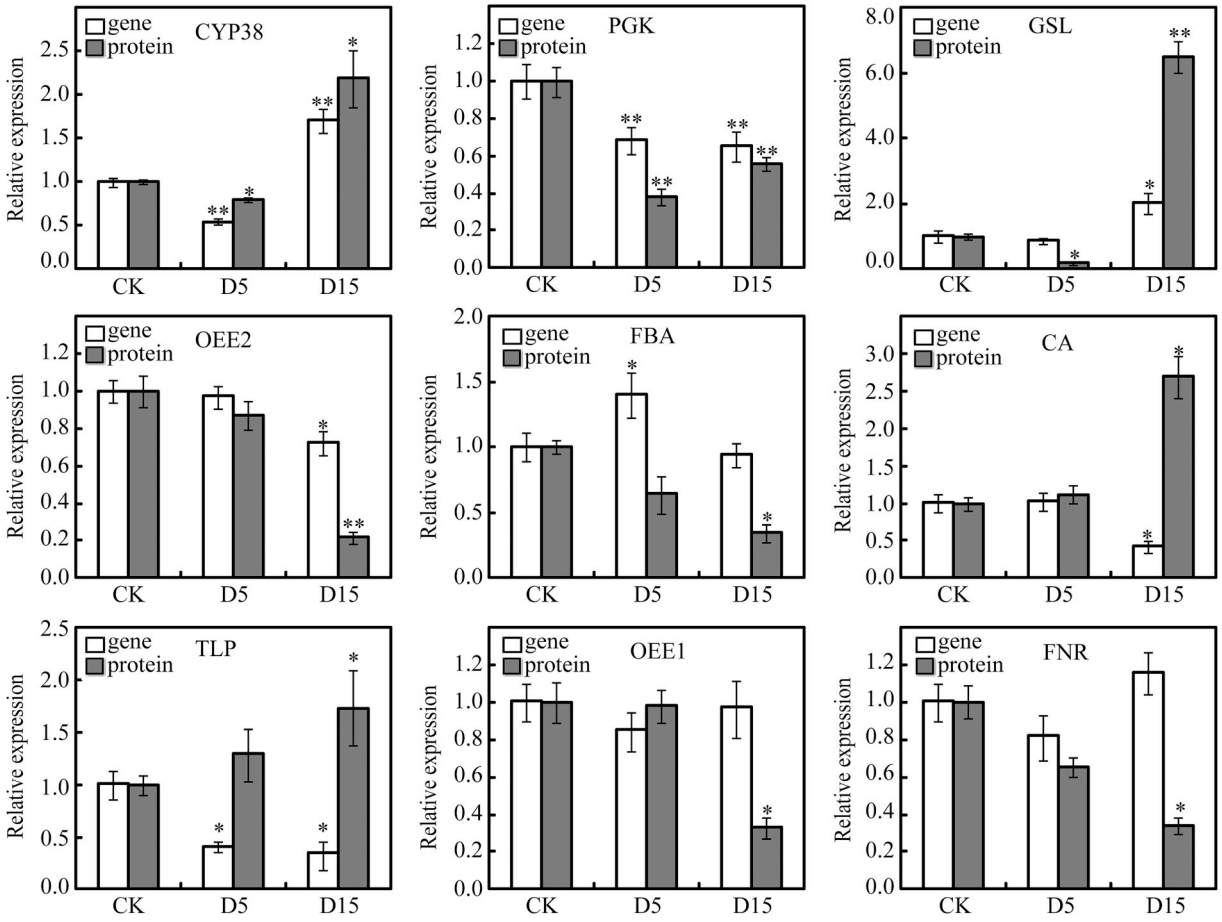

Fig. 5. Comparison of changes in gene and protein patterns under drought stress The expression patterns of 9 drought-responsive genes (white bars) were determined by qRT-PCR. Actin was used as an internal standard. Error bars represent the ± SD of three independent qRT-PCR reactions. The asterisks indicate that the differences were significant (*, $P < 005$; **, $P < 001$; ***, $P < 0001$). The accumulation patterns of 9 drought-responsive proteins (gray bars) were measured from 2-DE gels. The abbreviations are as follows: CA, carbonic anhydrase; CYP38, peptidyl-prolyl cis-trans isomerase CYP38; FBA, fructose-bisphosphate aldolase; FNR, ferredoxin-NADP reductase; GSL, glutamine synthetase leaf isozyme precursor; OEE, oxygen-evolving enhancer protein; PGK, phosphoglycerate kinase; TLP, thylakoid lumenal 165 kDa protein.

overexpression of GS can enhance the salt tolerance of transgenic rice plants (Hoshida et al., 2000). Overall, upregulated expression of the GS gene might increase the drought tolerance of cassava by promoting glutamine production. CYP38, localized in the thylakoid lumen, is reported to play a critical role in the assembly and maintenance of the PS II supercomplex in *Arabidopsis* (Fu et al., 2007), and the upregulation of CYP38 observed in this study might increase the stability and biogenesis of PSII, similar to the results of cadmium stress responses in *Cakile maritima* (Taamalli et al., 2015).

5. Conclusions

Based on our proteomics data and the observed ultra-structural alterations, we identified a comprehensive list of chloroplast proteins in cassava and investigated possible related mechanisms of drought stress tolerance. Our results showed that compared with control plants, drought stress inhibited the photosynthetic parameters of cassava through the downregulated expression of mainly photosynthesis-related proteins, altered chloroplast shape and granum lamellae structure disorder. Moreover, increased starch granule accumulation in chloroplasts, as well as enhanced levels of expression of several proteins associated with carbon and nitrogen metabolism, might promote drought tolerance in cassava by increasing the carbohydrate conversion efficiency. Proteins related to photosynthesis, carbon and nitrogen metabolism, and amino acid metabolism are synergistically expressed to regulate biological processes in cassava in response to drought stress. Further study will focus on the specific biological functions of drought-responsive proteins contributing to the resistance in cassava.

Author contribution statement

XCW and APG conceived and designed research. LLC and LMW conducted experiments of proteomics experiments. CZP and ZT conducted the protein extraction and analyzed the proteomics data. DW, GHD and JHX assisted the data analysis. LLC and XCW wrote the manuscript. All authors have read and approved the final manuscript.

Conflicts of interest

The authors have declared that there is no conflict of interest in the present investigation.

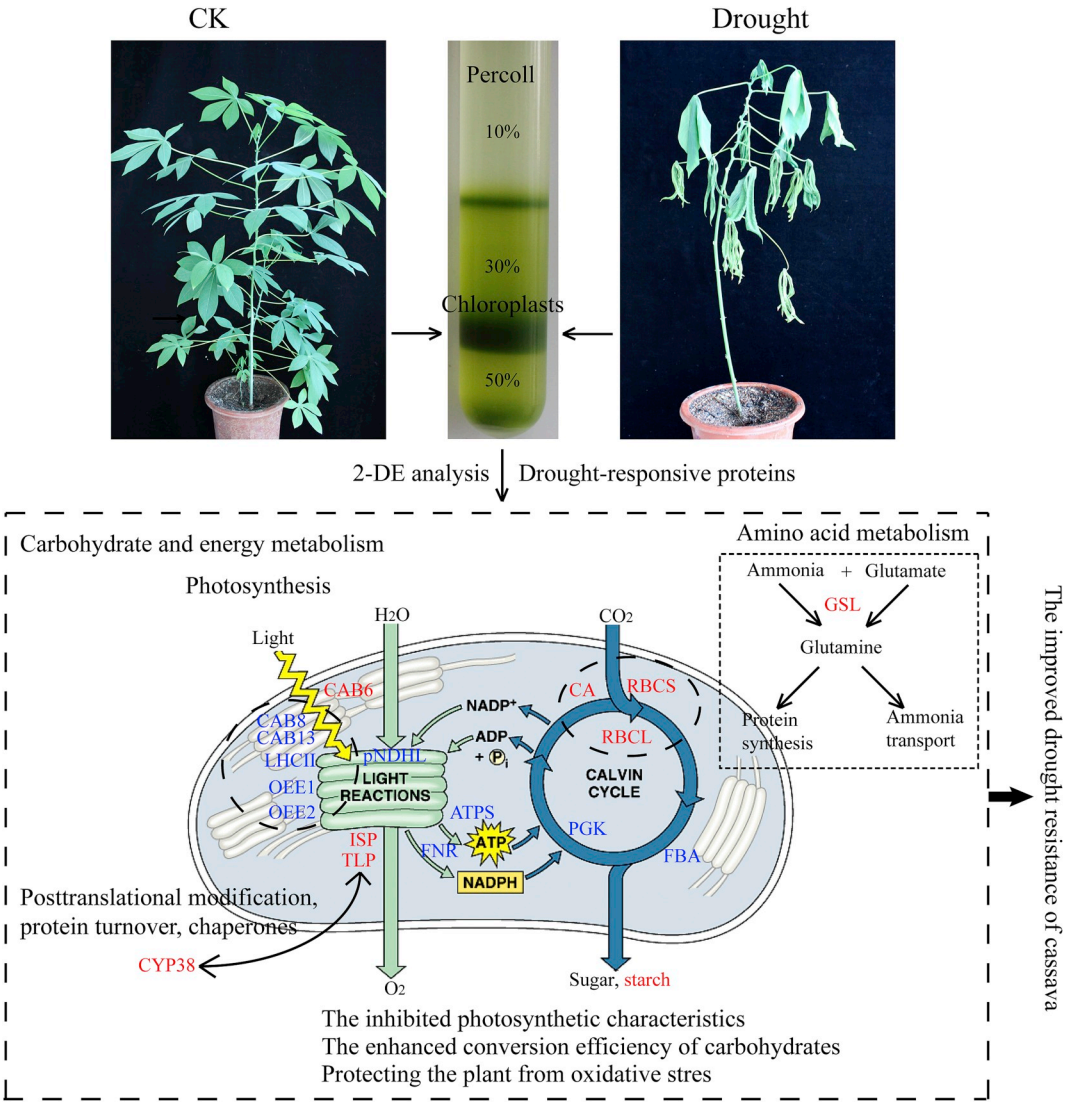

Fig. 6. A schematic diagram of the drought tolerance mechanism in cassava chloroplasts. Red color represents upregulated proteins after at least one drought treatment, and blue color shows downregulated proteins. Abbreviation: ATPS, ATP synthase; CA, carbonic anhydrase; CAB, chlorophyll *a*/b-binding protein; CS, cysteine synthase; CYP38, peptidyl-prolyl cis-trans isomerase CYP38; FBA1, fructose-bisphosphate aldolase 1; FNR, ferredoxin-NADP reductase; GSL, glutamine synthetase leaf isozyme precursor; ISP2, cytochrome *b*6-f complex iron-sulfur subunit 2; LHCII, chlorophyll *a*/b-binding protein of LHCII type 1-like; OEE, oxygen-evolving enhancer protein; pNDHL, photosynthetic NDH subunit of lumenal location 3; PGK, phosphoglycerate kinase; RBCL, ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit; RBCS, ribulose 1,5-bisphosphate carboxylase small chain; TLP, thylakoid lumenal 165 kDa protein. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.07.025.

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