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Proteomic analysis reveals different responses to drought between the *Cleome spinosa* (C3) and *Cleome gynandra* (C4)

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Abstract: The global emergence of low water availability causes extensive damage to crops in many regions. Although the responses of plants to drought stress have been extensively investigated, molecular studies on plants with different carboxylation pathways are limited. Therefore, we aimed to identify quantitative differences in proteins functioning in differential drought tolerance of C3 (*Cleome spinosa*) and C4 (*C. gynandra*) species. Proteomic analysis functionally characterized 33 differentially accumulated proteins in *C. spinosa* and 15 proteins in *C. gynandra* leaves. The identified proteins were involved in multiple aspects of leaf metabolism such as photosynthesis, energy metabolism, protein metabolism, and stress defense. The up-regulated accumulation of RuBisCO proteins may have contributed to carboxylation in stressed *C. spinosa*, but RuBisCO activase proteins were severely down-regulated. Additionally, down-regulation of ferredoxin-nicotinamide adenine dinucleotide phosphate (NADP) reductase and oxygen-evolving enhancer proteins was only found in *C. spinosa*, which possibly related to the inhibition of electron flow. The up-regulation of enolase may contribute to the energy requirement in *C. gynandra*, while down-regulation of glycolytic enzymes, such as fructose-bisphosphate aldolase and triosephosphate isomerase, was found in *C. spinosa* suggesting the impaired energy metabolism under drought stress. The proteomic analysis suggests different adaptive strategies between *C. spinosa* and *C. gynandra* against drought stress.

Key words: *Cleome spinosa*, *Cleome gynandra*, C3 and C4 plants, drought stress, proteomics

1. Introduction

Abiotic stresses including high temperature, drought, and salinity seriously impede crop productivity and agricultural sustainability. Considering the low water availability worldwide, drought is one of the most critical threats in terms of agricultural productivity (Ashraf et al., 2011). Hot climates and increasingly rare summer rains increase the severity of drought (Stuart et al., 2011). Nevertheless, the decline in the quality of agricultural lands has been increasing over the years with the effect of global climate changes (Peters et al., 2011). Drought stress leads to multiple plant responses by activating multiple signalling pathways (Zandalinas et al., 2018). These activated signals modulate stress-inducible genes, contributing to the adaptation to drought stress (Casaretto et al., 2016). To increase plant adaptation to drought, the molecular basis of plant responses to water deficiency must be understood (Faghani et al., 2015). Production of agricultural plant species resistant to biotic stresses has been provided by omics technologies including transcriptomics, genomics, metabolomics, and proteomics (Roy et al., 2011; Weckwerth, 2011). Proteomics is a powerful method for identifying proteins in a cell under control and stress conditions, determining expression levels, understanding protein-protein interactions, and revealing post-translational modifications (Mertins et al., 2013; Wang and Komatsu, 2018).

The responses of plants to drought stress depend on the severity and duration of stress, the plant species, and the developmental stage (Chaves et al., 2003). C3 plants are generally better adapted to moderate climates while C4 plants are usually found in hot dry climates (Ward et al., 1999). To study the C4 photosynthesis, some species belonging to the genera *Flaveria* and *Amaranthus* were used (Patel et al., 2004; Uzilday et al., 2014). However, important knowledge regarding the developmental process of C4 photosynthesis was obtained from Arabidopsis a C3 plant (Brown et al., 2005). To understand how C4 photosynthesis develops in plants, it is necessary to clarify the regulation of common genes in C3 and C4 species (Brown et al., 2005). Therefore, comparative studies of C3 and C4 species close to *Arabidopsis* will be important (Marshall et al., 2007). *Cleome spinosa* (C3 species) and *C. gynandra* (a NAD–malic enzyme type C4 species)

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represent an ideal pair for a comparative analysis of the complex trait of C4 photosynthesis (Bräutigam et al., 2011). Therefore, *Cleome* species may provide a model system for studying the molecular and genetic basis for economically and ecologically important pathway such as photosynthesis. *Cleome* species are also well known for medicinal importance (Silva et al., 2016; Moyo et al., 2018). The differences in the level of oxidative stress and the antioxidative defence system in *C. spinosa* and *C. gynandra* species subjected to drought stress were compared (Uzilday et al., 2012), but there was no proteomic study comparing the leaf proteome. Therefore, in this study, proteome changes in the leaves of *C. spinosa* and *C. gynandra* species exposed to drought stress were evaluated by gel-based proteomic approach.

2. Materials and methods

2.1. Plant materials and stress treatments

After surface sterilization in 0.5% NaClO solution for 10 min, the seeds of *Cleome spinosa* (C3) and *Cleome gynandra* (C4) were rinsed with distilled water several times. The seeds were then germinated in sterile petri dishes lined with two layers of wet filter paper. The seeds of both species were maintained at 20/30 °C in 18/6 h darkness/light for 5 days (Ochuodho et al., 2006). Uniformly sized seedlings were transferred to pots containing the mixture of peat moss soil:vermiculate:perlite (7:2:1). These pots were placed in a growth cabinet (23 °C, photosynthetic photon flux density of 260 μmol m^{-2} s^{-1}, 16:8 h photoperiod and 60% relative humidity) for 2 months. The pots were watered with Hoagland's solution every two days. Seedlings with 10–11 leaves were subjected to drought stress. For application of drought stress, irrigation was withheld for 10 d. C3 species (*C. spinosa*) displayed wilting symptoms more than C4 species (*C. gynandra*) at the end of stress treatment. After the control and drought treatments, leaves of seedlings were harvested from 3–4 plants, pooled, and stored at −80 °C until proteomic analyses.

2.2. Extraction of soluble proteins

Proteins were isolated from the leaves of *Cleome* species as described previously (Hurkman and Tanaka 1986). Briefly, portions (2 g) of samples were thoroughly ground to powder in liquid nitrogen. The powders were transferred to 20 mL of extraction buffer consist of 0.7 M sucrose, 0.5 M Tris-HCl (pH 8.3), 2% β-mercaptoethanol, 2% NP-40, 1 mM PMSF, and 20 mM $MgCl_2$, and homogenates were incubated for 10 min on ice. Proteins were extracted with 20 mL Tris-HCl-saturated phenol solution. After centrifugation, the phenolic phase was recovered and mixed with four volumes of 0.1 M ammonium acetate in methanol. The mixture was kept at −20 °C overnight to precipitate the proteins. The precipitated proteins were collected by centrifugation at 3500 ×g for 10 min and then washed 3 times with cold methanol containing 0.1 M ammonium acetate. Protein pellets were dried in a desiccator and stored at −20 °C until use. The pellets were dissolved in lysis buffer (4% CHAPS, 2 M thiourea, 7 M urea, 0.2% Ampholyte pH 3-10, and 40 mM DTT), and the protein concentration was estimated according to Bradford (1976).

2.3. 2-DE and image analysis

Isoelectric focusing (IEF) was performed with IPG strips (17 cm, pH 4–7) by using a Protean i12 IEF System (Bio-Rad, USA) in triplicates for each treatment. For analytical gels, IPG strips were passively rehydrated overnight with 80 μg proteins in 300 μL of rehydration buffer. Five hundred microgram of protein sample was loaded onto IPG strips for preparative gels. IEF process was performed with a total of 70,000 Vh. Following the two-step equilibration using dithiothreitol and iodoacetamide, SDS-PAGE in the second dimension was performed using 12% polyacrylamide gels. Protein spots in analytical gels were detected with silver staining (Sinha et al., 2001), whereas those in preparative gels were stained with coomassie brilliant blue (CBB) (Candiano et al., 2004).

Images of the silver-stained gels, which were acquired with the ChemiDoc MP system (Bio-Rad), were used for analysis. PDQuest software (Version 8.0; Bio-Rad) was used for spot detection, matching, quantification of differences in spot intensities between treatments. Spot quantity was normalized as a relative volume to compensate possible staining differences between gels. Proteins exhibiting at least 1.5-fold reproducible abundance changes between compared samples were subjected to statistical analysis ($p < 0.05$). Relative comparison of the significant changes between spot intensities between treatments was carried out using Student's t-test ($p < 0.05$).

2.4. Sample preparation and mass spectrometry analysis

Destaining and in-gel digestion of the protein spots, which were excised manually from CBB stained gels were performed using an in-gel tryptic digestion kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). The tryptic peptides were extracted with 0.1% trifluoroacetic acid (TFA) in 60% acetonitrile (ACN). The solutions in the tubes were with vacuum concentrator (Eppendorf, Germany), and the dried peptides were re-suspended in 10 μL of 0.1% TFA. Samples were desalted with using ZipTipC18 (Millipore, USA). Peptide mixtures were loaded on the MALDI target together with 2 mg/mL a-cyano-4-hydroxycinnamic acid as matrix. Mass spectra (*m/z* 800-3000) were acquired on an AB Sciex TOF/TOF 5800 mass spectrometer (Applied Biosystems, USA). Database searching for protein identification was carried out by MASCOT program (http://www.matrixscience.com) using Swiss-Prot databases. The search parameters were green plants database, one missed cleavage site,

trypsin as the digestion enzyme, variable modifications of Oxidation (M), fixed modifications of Carbamidomethyl (C), ±0.4 Da for fragments tolerance, and 50 ppm for mass accuracy. Proteins with high MASCOT score confidence intervals above 95% were considered as a credibly identified protein. The sequences of the identified proteins were searched against the UniProt database to predict their functions. The protein-protein interactions were established using STRING 11.0 against *Arabidopsis thaliana* TAIR10 database (Szklarczyk et al., 2011). Gene ontology enrichment analysis was carried out by BiNGO 3.0.3 (Maere et al., 2005) a plugin for Cytoscape. Venn diagram was used to compare the proteins from different samples.

3. Results

To determine the protein alterations in the leaf tissues of *C. spinosa* (C3) and *C. gynandra* (C4) plants in response to drought treatment, a comparative proteomic analysis was performed. Proteomic analysis revealed the up- or down-regulated protein spots under drought stress in both species (Figure 1, Tables 1 and 2). Of these proteins, 96 showed differential abundances under drought stress. Protein identification through the mass spectrometry and bioinformatics analyses resulted in identification of 48 proteins (Tables 1 and 2), with 33 from *C. spinosa* and 15 from *C. gynandra*. Differentially expressed proteins were classified into six functional group (Figure 1b, c). The identified proteins in *C. spinosa* were mainly classified into photosynthesis (45.4%), energy (18.2%), and stress defence (12.1%). In *C. gynandra*, the identified proteins were mainly related to metabolism (33.3%), photosynthesis (20%), and protein metabolism (20%). The number of photosynthesis related proteins was 15 in *C. spinosa* and 3 in *C. gynandra* (Table 1). Several photosynthesis related proteins were found to be differentially expressed between the two species including RuBisCO, carbonic anhydrase (CA), ferredoxin-NADP (nicotinamide adenine dinucleotide phosphate) reductase (FNR), oxygen-evolving enhancer proteins (OEE), RuBisCO activase (RCA), glyceraldehyde-3-phosphate dehydrogenase A (GAPA), ribulose-phosphate 3-epimerase (RPE), malate dehydrogenase

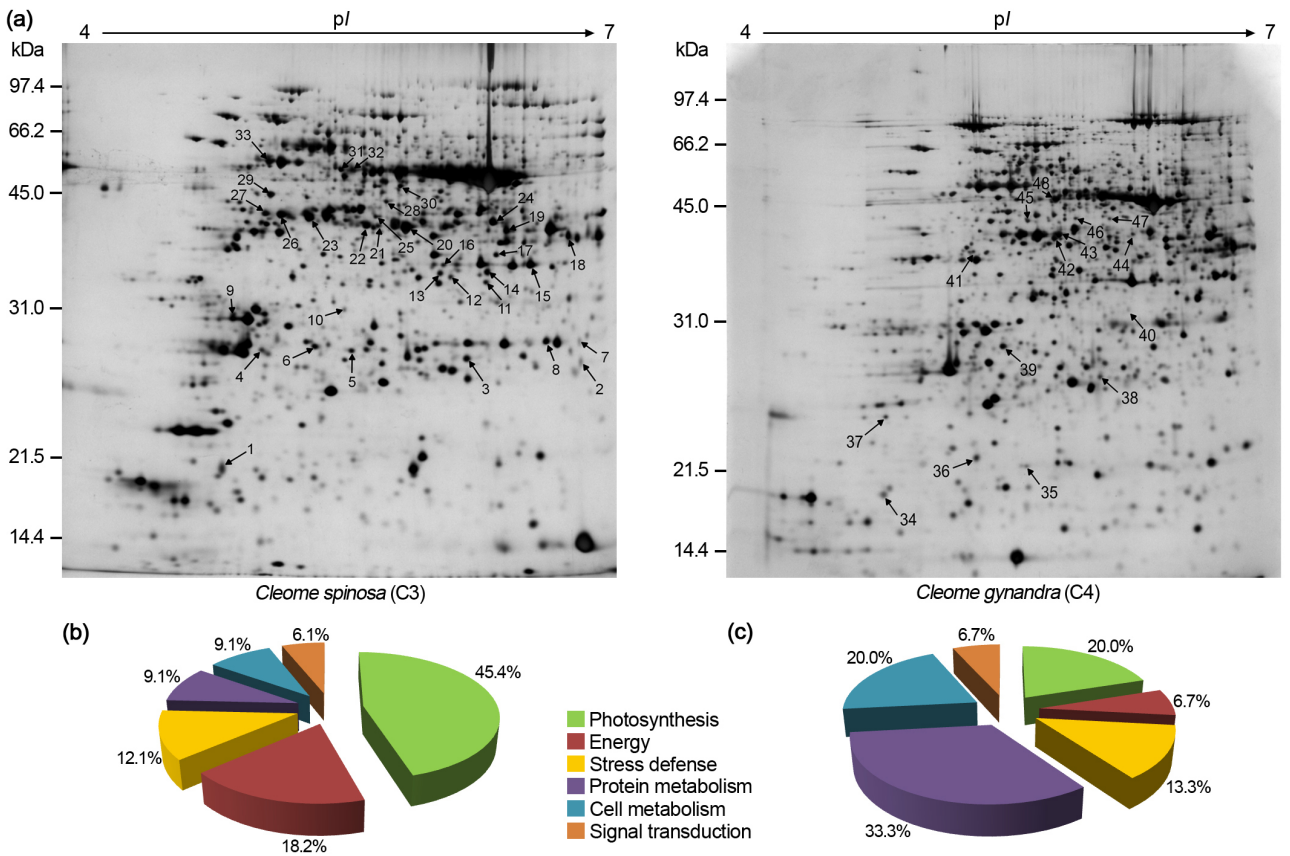

Figure 1. The representative 2-DE gels of *C. spinosa* (C3) and *C. gynandra* (C4) under drought stress (a). Numbered arrows (1-48) indicate the spots that were identified by MS and significantly regulated between control and drought stress. Functional characterization of identified proteins in *C. spinosa* (b) and *C. gynandra* (c).

Table 1. Differentially accumulated proteins in leaf tissues of *Cleome spinosa* (C3) seedlings exposed to drought stress.

Spot	Accession no	Protein	Score	MW/pI	Cover.	MP	Fold change
Photosynthesis							
2	RBS1_FLAPR	RuBisCO small chain 1 Chloroplastic *Flaveria pringlei*	128	T:19.6/8.93 E:27.4/6.85	33%	10	+3.24
3	RPE_SOLTU	Ribulose-phosphate 3-epimerase Chloroplastic *Solanum tuberosum*	110	T:29.9/7.61 E:27.7/6.25	31%	7	-1.61
7	RBS1_FLAPR	RuBisCO small chain 1 Chloroplastic *Flaveria pringlei*	176	T:19.6/8.93 E:29.0/6.85	33%	11	+1.60
8	CAHC_PEA	Carbonic anhydrase Chloroplastic *Pisum sativum*	200	T:35.4/7.01 E:29.1/6.66	12%	10	-6.67
9	PSBO1_ARATH	Oxygen-evolving enhancer protein 1-1 Chloroplastic *Arabidopsis thaliana*	246	T:35.1/5.55 E:30.4/5.01	23%	15	-2.22
11	FNRL2_ARATH	Ferredoxin-NADP reductase, leaf isozyme 2 Chloroplastic *Arabidopsis thaliana*	138	T:41.1/8.51 E:35.0/6.34	24%	19	-2.86
13	FENR1_PEA	Ferredoxin-NADP reductase, leaf isozyme Chloroplastic *Pisum sativum*	238	T:40.2/8.56 E:35.6/6.11	37%	23	-1.61
14	FENR1_PEA	Ferredoxin-NADP reductase, leaf isozyme Chloroplastic *Pisum sativum*	262	T:40.2/8.56 E:36.0/6.35	36%	19	-1.50
19	G3PA_ARATH	Glyceraldehyde-3-phosphate dehydrogenase A Chloroplastic *Arabidopsis thaliana*	63	T:42.5/7.62 E:41.1/6.45	3%	2	-2.17
20	RCA_ORYSJ	RuBisCO activase Chloroplastic *Oryza sativa* subsp. *japonica*	244	T:51.4/5.43 E:41.0/5.93	26%	20	-2.00
21	RCA_ORYSJ	RuBisCO activase Chloroplastic *Oryza sativa* subsp. *japonica*	101	T:51.4/5.43 E:41.0/5.77	15%	17	-2.70
22	RCA_ARATH	RuBisCO activase Chloroplastic *Arabidopsis thaliana*	90	T:51.9/5.87 E:41.0/5.71	11%	9	-2.17
23	PGKH_SPIOL	Phosphoglycerate kinase Chloroplastic *Spinacia oleracea*	287	T:45.5/5.83 E:42.1/5.42	27%	15	-1.89
24	RBL_AREDR	RuBisCO large chain Chloroplastic *Arenaria drummondii*	60	T:52.6/6.13 E:42.1/6.38	29%	18	+1.60
29	RCA_ARATH	RuBisCO activase Chloroplastic *Arabidopsis thaliana*	101	T:51.9/5.87 E:45.0/5.20	13%	8	-1.50

Table 1. (Continued).

Energy							
6	TPIS_COPJA	Triosephosphate isomerase Cytosolic *Coptis japonica*	84	T:27.1/7.67 E:28.3/5.44	22%	7	-1.56
15	ALFC_ORYSJ	Fructose-bisphosphate aldolase Chloroplastic *Oryza sativa* subsp. *japonica*	220	T:41.9/6.38 E:37.3/6.58	23%	11	-1.75
18	ALF2_PEA	Fructose-bisphosphate aldolase Cytoplasmic isozyme 2 *Pisum sativum*	73	T:38.4/6.77 E:40.5/6.77	17%	8	-1.69
30	UGPA2_ARATH	Probable UTP-glucose-1-phosphate uridylyltransferase 2 *Arabidopsis thaliana*	181	T:51.7/5.80 E:49.7/5.88	26%	14	+2.09
32	ATPB_WHIBI	ATP synthase subunit beta Chloroplastic *Whiteheadia bifolia*	216	T:53.7/5.28 E:52.0/5.64	40%	27	-5.00
33	ATPA_EUCGG	ATP synthase subunit alpha Chloroplastic *Eucalyptus globulus*	349	T:55.5/5.15 E:55.7/5.20	29%	27	-1.89
Stress defense							
4	FRI1_SOYBN	Ferritin-1 Chloroplastic *Glycine max*	72	T:28.0/5.73 E:27.5/5.16	18%	6	+1.51
10	SDR1_ARATH	(+)-neomenthol dehydrogenase *Arabidopsis thaliana*	96	T:32.8/5.38 E:31.2/5.60	17%	10	+1.80
17	P2_ARATH	Probable NADP-dependent oxidoreductase P2 *Arabidopsis thaliana*	73	T:37.9/8.09 E:37.9/6.39	9%	8	+1.50
25	MDAR3_ARATH	Probable monodehydro ascorbate reductase Cytoplasmic isoform 3 *Arabidopsis thaliana*	79	T:46.5/6.41 E:42.3/5.76	5%	4	+1.79
Cell metabolism							
26	GLNA2_ARATH	Glutamine synthetase Chloroplastic/mitochondrial *Arabidopsis thaliana*	129	T:47.4/6.43 E:42.4/5.27	33%	12	-1.61
27	GLNA2_ARATH	Glutamine synthetase Chloroplastic/mitochondrial *Arabidopsis thaliana*	114	T:47.4/6.43 E:42.4/5.18	20%	12	-1.61
28	METK2_ELAUM	S-adenosylmethionine synthetase 2 *Elaeagnus umbellata*	330	T:43.1/5.50 E:44.6/5.82	37%	21	-1.64
Protein metabolism							
1	RK123_ARATH	50S ribosomal protein L12-3 Chloroplastic *Arabidopsis thaliana*	92	T:19.7/5.51 E:19.7/4.95	20%	5	-2.17

Table 1. (Continued).

5	CH10C_ARATH	20 kDa chaperonin Chloroplastic *Arabidopsis thaliana*	174	T:26.8/8.86 E:29.5/5.64	22%	9	-2.78
31	AMPL2_ORYSJ	Leucine aminopeptidase 2 Chloroplastic *Oryza sativa* subsp. *japonica*	146	T:61.8/8.29 E:52.0/5.57	24%	19	+1.69
Signal transduction							
12	ANXD6_ARATH	Annexin D6 *Arabidopsis thaliana*	149	T:36.5/7.72 E:35.5/6.16	16%	9	+2.61
16	ANXD1_ARATH	Annexin D1 *Arabidopsis thaliana*	88	T:36.2/5.21 E:37.0/6.12	17%	7	+2.71

(MDH), and phosphoglycerate kinase (PGK). On the other hand, levels of some drought stress-related proteins showed similar trend between the two species including glutamine synthetase (GS) and S-adenosylmethionine synthase (SAMS).

In *C. spinosa*, drought stress strongly induced the accumulation of stress-responsive proteins, and to a less significance, proteins in generation of precursor metabolites and energy, and photosynthesis (Figure 2a). Similarly, in *C. gynandra*, stress-responsive proteins remained highly enriched, but there were more proteins related to cellular and biosynthetic processes (Figure 2b). The protein-protein interaction network revealed well-connected networks among different proteins (Figure 3). Good interactions were found for proteins including GAPA and PGK suggesting the importance of carbohydrate metabolism in drought-stressed *C. spinosa* (Figure 3). The comparison of *Cleome* species by Venn diagrams has showed that more proteins decreased in *C. spinosa* with respect to *C. gynandra* (Figure 4).

4. Discussion

Plants subjected to drought stress displayed suppressed photosynthesis through destabilization of RuBisCO and damage to photosystems (Nishiyama and Murata, 2014). Moreover, suppression of photosynthetic machinery under drought stress can be varying depending on the plant species. Uzilday et al., (2012) reported that drought stress did not cause a significant effect on photosynthesis of *C. gynandra* (C4) while it had a slight effect on photosynthesis of *C. spinosa* (C3). Here, abundance of RuBisCO proteins was markedly increased in *C. spinosa* under drought stress while RCA proteins were decreased. Similarly, the accumulation of RuBisCO protein was increased in drought-sensitive fennel genotype (Khodadadi et al., 2017). Moreover, down-regulation of RCA has been shown in drought-sensitive cultivars of barley (Kausar et al., 2013), rapeseed (Urban et al., 2017), and wheat (Michaletti et al., 2018). It has been also demonstrated that the decrease in RCA protein is related to the inhibition of photosynthetic activity under drought stress (Michaletti et al., 2018). Down-regulation of other carbon fixation enzymes (RPE, GAPA, and PGK), besides RCA, may contribute to diminished photosynthetic activity in drought-stressed *C. spinosa*. NAD-dependent isoform of MDH catalyses the conversion of oxaloacetate to malate in chloroplasts, and it could be involved in malate valve. The malate valve in chloroplast was seen to play an important role in regulating of ATP/NADPH ratio in response to metabolic demands (Scheibe, 2004). In our study, MDH protein was up-regulated in *C. gynandra* under drought stress suggesting its important role in C4 photosynthesis.

Carbonic anhydrases were markedly down-regulated in drought-stressed *C. spinosa*. Although the role of carbonic anhydrase in C4 plants is known, its role in C3 plants is less understood. Recent studies have provided increasing evidences that CA proteins participate in a wide range of physiological processes such as regulation of stomatal movements to modulate gas-exchange between plants and the atmosphere (Rowlett, 2010) and promotion of water-use efficiency (Cui et al., 2012). There is increasing evidence that the stromal CAs have a role in plant stress defence. Over-expression of rice CA rice in transformed *Arabidopsis* induced tolerance to salinity stress (Yu et al., 2007).

Oxygen-evolving enhancer proteins are the subunit of photosystem II (PSII), which are involved in the light-dependent reactions. Drought stress decreased the accumulation of oxygen-evolving enhancer 1 (OEE1) protein in *C. spinosa*, whereas the abundance of OEE2 was increased in *C. gynandra* under drought stress. The OEE2 was identified as core protein of PSII, and it functions in the catalysing water splitting (Yi et al., 2005). It has been demonstrated that accumulation of OEE1 and OEE2

Table 2. Differentially accumulated proteins in leaf tissues of *Cleome gynandra* (C4) seedlings exposed to drought stress.

Spot	Accession no	Protein	Score	MW/pI	Cover.	MP	Fold change
Cell metabolism							
40	PDX1_HEVBR	Probable pyridoxal biosynthesis protein PDX1 *Hevea brasiliensis*	406	T:33.1/5.79 E:33.4/6.21	33%	26	-1.72
43	GLNA2_ARATH	Glutamine synthetase Chloroplastic/mitochondrial *Arabidopsis* thaliana	176	T:47.4/6.43 E:45.0/5.79	39%	15	-2.17
44	GSA_BRANA	Glutamate-1-semialdehyde 2.1-aminomutase Chloroplastic *Brassica napus*	207	T:50.2/6.43 E:44.4/6.24	28%	15	-2.04
46	METK_MESCR	S-adenosylmethionine synthetase *Mesembryanthemum crystallinum*	353	T:42.9/5.43 E:49.7/5.88	33%	16	-1.96
47	METK2_ELAUM	S-adenosylmethionine synthetase 2 *Elaeagnus umbellata*	381	T:43.1/5.50 E:49.7/6.10	47%	26	-1.89
Photosynthesis							
36	PSBP_BRAJU	Oxygen-evolving enhancer protein 2 Chloroplastic *Brassica juncea*	52	T:23.3/4.91 E:22.3/5.28	17%	4	+2.00
41	CHLI_ARATH	Magnesium-chelatase subunit chli Chloroplastic *Arabidopsis thaliana*	189	T:46.2/6.08 E:42.1/5.25	26%	20	-2.22
42	MDHP_MEDSA	Malate dehydrogenase [NADP] Chloroplastic *Medicago sativa*	229	T:47.8/6.43 E:44.4/5.75	22%	13	+1.73
Protein metabolism							
34	RK123_ARATH	50S ribosomal protein L12-3 Chloroplastic *Arabidopsis thaliana*	130	T:19.7/5.51 E:19.1/4.72	18%	6	+1.58
35	IF5A2_SOLLC	Eukaryotic translation initiation factor 5A-2 *Solanum lycopersicum*	107	T:17.5/5.78 E:21.6/5.59	18%	9	+2.18
45	IF4A1_ORYSJ	Eukaryotic initiation factor 4A-1 *Oryza sativa* subsp. *japonica*	316	T:47.1/5.37 E:49.6/5.59	43%	32	+1.50
Stress defense							
38	SODF_ARATH	Superoxide dismutase [Fe] Chloroplastic *Arabidopsis thaliana*	66	T:23.8/6.06 E:27.2/6.05	17%	5	+1.71
39	PAP6_ARATH	Probable plastid-lipid-associated protein 6 Chloroplastic *Arabidopsis thaliana*	102	T:30.4/5.82 E:29.9/5.44	20%	8	+1.50
Energy							
48	ENO2_HEVBR	Enolase 2 *Hevea brasiliensis*	400	T:47.9/5.92 E:57.4/5.75	33%	24	+1.55
Signal transduction							
37	TCTP_MAIZE	Translationally-controlled tumor protein homolog *Zea mays*	194	T:18.7/4.52 E:24.8/4.73	25%	8	+2.09

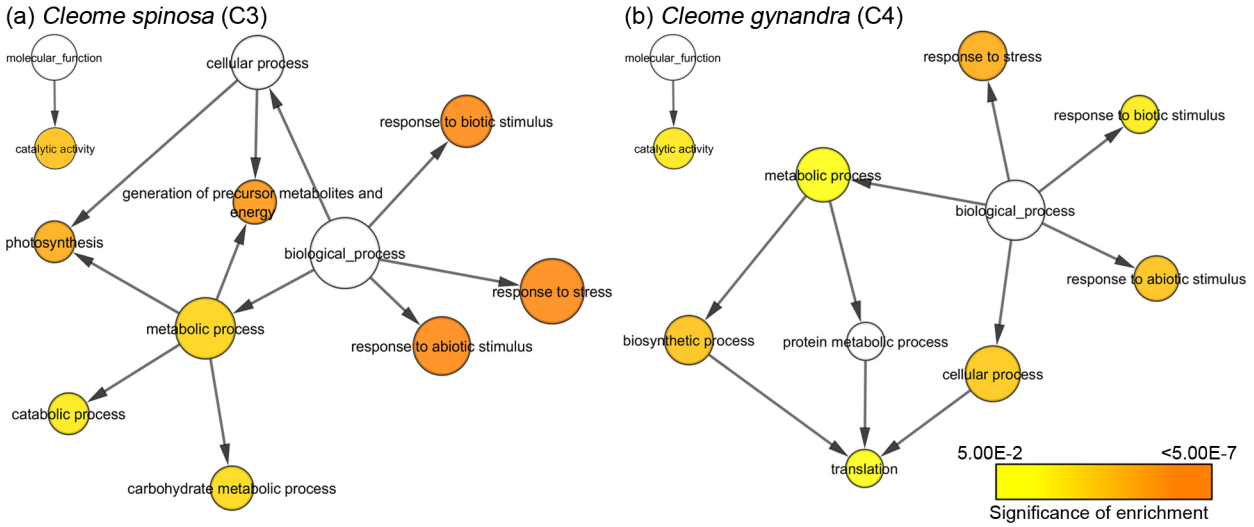

Figure 2. Biological pathway and molecular function networks of *C. spinosa* (a) and *C. gynandra* (b) generated by BiNGO.

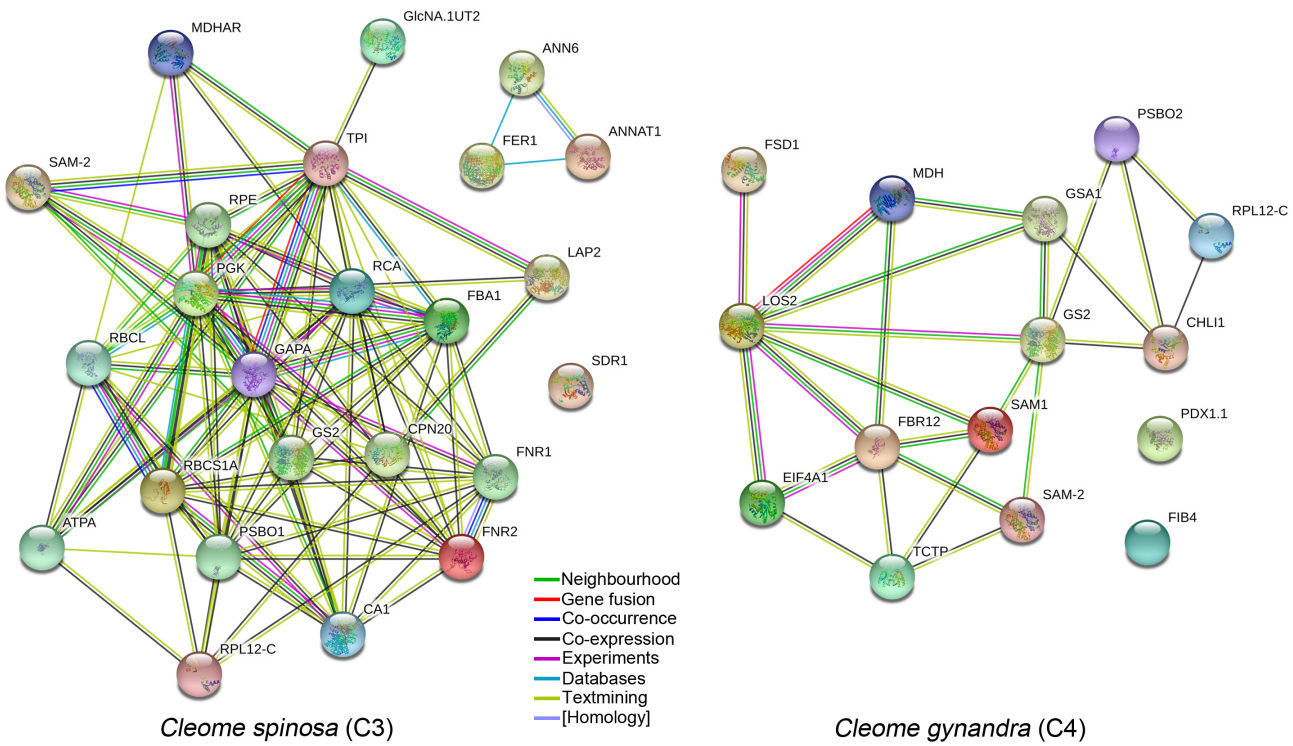

Figure 3. Analysis of a functional network by STRING 11.0 (http://string-db.org). In the evidence view, the links between proteins represent possible interactions.

proteins increase in drought tolerant barley and bean genotypes but decrease in sensitive genotypes (Kausar et al., 2013; Zadražnik et al., 2013). Moreover, Xin et al., (2018) suggested that up-regulation of OEE1 in drought-stressed maize plants is crucial for the continuity of photosynthetic activity. The increased expression of OEE2 in *C. gynandra* might be another adaptation mechanism to stabilize oxygen-evolving complex under drought stress. We also observed that the accumulation of FNR proteins decreased significantly in drought-stressed *C. spinosa*. FNR transfers electron from the ferredoxin to reduction of $NADP^+$ to NADPH, which is participating in pathways of carbon fixation (Hanke and Mulo, 2013). Down-regulation of these proteins might suppress linear electron

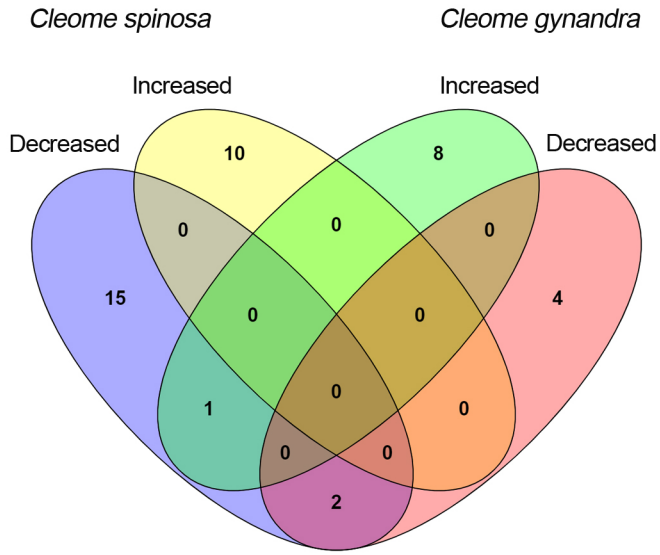

Figure 4. Venn diagram indicates the number of decreased or increased proteins in *C. spinosa* (C3) and *C. gynandra* (C4).

flow, which would inhibit the photosynthetic efficiency. Additionally, decreased FNR activity may disturb NADPH homeostasis in drought-stressed *C. spinosa* by delaying NADPH production (Chintakovid et al., 2017).

Plants adapted to abiotic stresses by triggering changes in expression levels of enzymes associated with energy metabolism. Since drought stress significantly reduces carbon dioxide assimilation through a net reduction in ATP (Tezara et al., 1999), enrichment of energy metabolism is suggested to help abiotic stress tolerance (Zhang et al., 2008). In our study, the beta and alpha subunits of ATP synthase protein were significantly decreased in drought-stressed *C. spinosa*. ATP is also produced by glycolysis and the tricarboxylic acid cycle. Our results showed that cytoplasmic and chloroplastic isozymes of triosephosphate isomerase and fructose-bisphosphate aldolase proteins were decreased significantly in *C. spinosa* under drought stress. Down-regulation of these enzymes may supress the glycolytic pathway and related intermediate metabolism. Additionally, drought stress significantly increased the accumulation of enolase 2, which may help to *C. gynandra* to alleviate the negative effects of drought stress by providing enough energy.

Activation of ROS-metabolizing enzymes is a universal response to different abiotic stresses. In our study, four proteins in *C. spinosa* and two proteins in *C. gynandra* were found to be involved in ROS metabolism. Among them, drought stress led to increase in accumulation of ferritin-1 and monodehydroascorbate reductase (MDHAR) in *C. spinosa*. Briat et al. (2010) have suggested that the ferritin family is strongly regulated under stressful conditions, and it plays a role in the sequestering of intracellular iron to limiting the formation of hydroxyl radicals. The enhanced accumulation of ferritin 1 under drought stress may facilitate the regulation of free iron levels in *C. spinosa*. The MDHAR enzyme catalyses the conversion of monohydroascorbate to ascorbate using NAD(P)H (Lisenbee et al., 2005). Transgenic tomato seedlings over-expressing a *MDHAR* gene displayed an enhanced tolerance to salinity and PEG-induced osmotic stress (Li et al., 2012). However, expression levels of chloroplastic Fe-superoxide dismutase and plastid-lipid-related protein 6 were up-regulated in drought-stressed *C. gynandra*. Enhanced accumulation of these proteins under drought stress has the potential to protect plants from oxidative stress.

Drought stress decreased the levels of GS proteins in both species. GS is an ATP-dependent enzyme, which is involved in assimilation of ammonia generated by photorespiration. Down-regulation of GS proteins has been reported in several plant species (Zadražnik et al., 2013; Wang et al., 2016; Michaletti et al., 2018). We also detected S-adenosylmethionine synthetase (SAMS) that functions in nitrogen metabolism, and its accumulation decreased in both species under drought stress. SAMS is a key enzyme catalysing the formation of S-adenosylmethionine (SAM) from ATP and L-methionine. SAM is utilized as precursor of polyamines, ethylene, and lignin (Chiang et al., 1996; Lee et al., 2007). Our results indicated that drought stress severely affected the nitrogen and amino acid metabolisms in the leaves of two species.

50S ribosomal subunit catalysis the peptidyl transfer reaction in chloroplasts (Kotusov et al., 1976). Drought stress decreased the accumulation of 50S ribosomal

protein L12-3 in *C. spinosa,* whereas, increased in *C. gynandra*. It has been reported that accumulation of 50S ribosomal L12 protein in drought tolerant increased in the drought tolerant peanut genotype (Katam et al., 2016). Enhanced accumulation of this protein in *C. gynandra* may be associated with a mechanism of resistance to the negative effect of drought stress on protein synthesis. Additionally, increased accumulation of annexin proteins was determined only in *C. spinosa*. Annexins, which are Ca^{2+} dependent membrane binding proteins, are key parts of Ca^{2+} signalling pathways (Mortimer et al., 2008). Definite annexins have been announced to be linked with plant tolerance to drought stress (Konopka-Postupolska et al., 2009). The increased accumulation of annexin D1 and D6 during stress in this research affirmed that the Ca^{2+} signal plays a role in drought responses of plants.

In conclusion, gel-based proteomic analysis was carried out to reveal the drought-responsive proteins of *C. spinosa* (C3) and *C. gynandra* (C4) species differing in carboxylation pathway. Proteins related to the organization of photosynthesis and energy metabolism have been mainly affected in drought-stressed *C. spinosa*. Although the expression level of RuBisCO small subunit proteins was up-regulated in *C. spinosa*, the level of RCA proteins which enabled RuBisCO to convert to active form decreased. Additionally, the expression level of OEE1 and FNR were decreased in *C. spinosa,* whereas the expression level of OEE2 was up-regulated in *C. gynandra*. On the other hand, large-scale analysis of the transcriptome can help to improve our understanding of the expression patterns of the genes varied between C3 and C4 plants. Transcriptomic studies focusing on target genes controlled by transcription factors will also provide important information on the mechanism of drought-induced alterations in C4 photosynthesis.

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