

Comparative analysis of salt-induced changes in the leaves proteome of two contrasting *Jatropha curcas* genotypes**Análise comparativa no proteoma, induzido por salinidade, em folhas de dois genótipos contrastantes de *Jatropha curcas***

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Natália Corte-Real

Doutora em Botânica

Universidade Federal Rural de Pernambuco

Centro de Biologia, Recife, PE, Brazil

E-mail: nataliacreal@gmail.com

Melquisedec S. Oliveira

Doutor em Biotecnologia

Laboratório de Genômica e Proteômica de Plantas

Departamento de Genética, Centro de Biociências

Universidade Federal de Pernambuco, Recife, PE, Brazil

E-mail: oliveirams@outlook.com

Alfredo Jarma-Orozco

Doutor em Ciências Agrícolas

Facultad de Ciencias Agrícolas

Universidad de Córdoba, Montería, Córdoba, Colombia

E-mail: jarma@fca.edu.co

Denise Fernandes

Doutora em Agronomia, Fisiologia Vegetal

Instituto Federal Catarinense, Rio do Sul, SC, Brazil

E-mail: denise.fernandes@ifc.edu.br

Marcos A. dos Santos

Mestre em Biotecnologia de Produtos Naturais

Instituto Pernambucano de Ensino Superior (IPESU), Recife, PE, Brazil

E-mail: marcosansan@yahoo.com.br

Laurício Endres

Doutor em Ciências Biológicas, Botânica

Laboratório de Fisiologia Vegetal

Centro de Agronomia

Universidade Federal de Alagoas, Maceió, AL, Brazil

E-mail: lauricioendres@hotmail.com

Tercílio Calsa Junior

Doutor em Ciências, Biologia na Agricultura e no Ambiente
Laboratório de Genômica e Proteômica de Plantas
Departamento de Genética, Centro de Biociências
Universidade Federal de Pernambuco, Recife, PE, Brazil
E-mail: terciliocjr@gmail.com

Marcelo F. Pompelli

Doutor em Agronomia, Fisiologia Vegetal
Laboratório de Ecofisiologia Vegetal
Departamento de Botânica, Centro de Biociências
Universidade Federal de Pernambuco, Recife, PE, 50670901, Brazil
E-mail: mfpompelli@gmail.com

ABSTRACT

The salt stress is one of the major abiotic stress factors limiting the productivity of many agricultural plant species. However, as a sessile organism, plant might adjust their metabolism reprogramming many different complex pathway aiming tolerate such different stresses by activating genes and transcriptional factors. Here we investigated the protein differential salt tolerance in two contrasting *Jatropha curcas* genotypes with a special emphasis on the proteomic changes in the leaves, contributing to the identification of candidate proteins for molecular markers in response to salinity tolerance. 6-months *J. curcas* plants were kept under 750 mM NaCl salt concentration. After 40 hours of stress, leaves were harvested and protein profile analyzed. Total proteins were extracted, purified and quantified. As results, we identify 110 salinity-responsive differently accumulated proteins in *J. curcas*, presumably associated with metabolic processes of ADP, ribonucleotides, carbohydrate and pyruvate derivatives, as well as ATP biosynthesis and response to metal ions as the main biological processes associated to tolerant-like *J. curcas* genotype. The comparative proteome revealed that 110 proteins were salt-responsive in both genotype, while 69 and 41 protein were salt responsive in the CNPAE183 and in CNPAE218, respectively. The tolerant-like genotype presented proteins from different pathways mainly for the salinity response, including proteins involved in signaling, antioxidant metabolism, as well as key enzymes from other metabolic pathways of energy production, such as photosynthesis and glycolysis, suggesting the maintenance of their function growth and development. Our results gave deeper insights into plasticity of salt tolerance responses of *J. curcas* cultivated under field-condition.

Keywords: purging nut, NaCl, salt stress, peptide, mass spectrometry, 2D electrophoresis

RESUMO

O estresse por salinidade é um dos fatores abióticos que mais limitam a produtividade de muitas espécies agricultáveis. Como um organismo sésil, as plantas devem ajustar seu metabolismo, reprogramando suas complexas e rotas metabólicas permitindo que a planta tolere as condições estressantes pela ativação de genes e fatores transcricionais. Neste estudo, investigamos a expressão diferencial proteica em dois genótipos contrastantes em relação a salinidade com especial ênfase nas mudanças proteômicas nas folhas, contribuindo para a identificação de proteínas candidatas para marcação molecular em resposta à salinidade. Plantas de 6 meses de idade foram crescidas sob 750 mM NaCl por 50 horas. Após 40 horas de estresse, folhas foram coletadas e a análise proteica foi executada. Proteínas totais foram extraídas, purificadas e quantificadas. Como resultado, identificamos 110 proteínas responsivas à salinidade diferencialmente acumuladas em *J. curcas*, presumivelmente associadas com processos metabólicos de ADP, ribonucleotídeos, carboidratos e

derivados do piruvato, bem como na biossíntese de ATP e respostas a íons metálicos como os processos biológicos associados a tolerância a salinidade. O proteoma comparativo revelou que 110 proteínas são responsivas à salinidade em ambos os genótipos testados, enquanto 69 e 41 proteínas foram responsivas somente no genótipo CNPAE183 e no CNPAE218, respectivamente. O genótipo tolerante apresentou proteínas de diferentes rotas metabólicas principalmente em resposta à salinidade, incluindo proteínas envolvidas na sinalização, metabolismo antioxidativo, bem como enzimas chave para outros processos metabólicos de produção de energia, tais como fotossíntese e glicólise, sugerindo a manutenção das funções de crescimento e desenvolvimento. Nossos resultados fornecem informações mais profundas e inéditas sobre a plasticidade das respostas de tolerância à salinidade em *J. curcas* cultivadas sob condições de campo.

Palavras-chave: pinhão-mando, NaCl, estresse por salinidade, peptídeos, espectrometria de massa, eletroforese

1 INTRODUCTION

As sessile organism, plants are continually subjected to numerous environmental stresses that can promote physiological and metabolic changes, generating responses that are dependent on genetic load and, especially, gene activation [1]. Together with water deficit, salinity is considered as one of the most severe and limiting stress conditions for large scale cultivation [1]. Salinity may interfere in several pivotal biological processes such as photosynthesis and protein synthesis, as well as fatty acid production and solute accumulation [2]. Plant response to salinity is a complex physiological, biochemical and molecular mechanisms to cope with osmotic and / or ionic stress, such as the synthesis of compatible solutes, antioxidant enzymes and metabolites, as well as exclusion control, compartmentalization and inclusion of ions by the root and their transport to the aerial part [1]. All of these responses to adverse conditions result from the regulation of specific gene expression, which may alter protein translation, as well as post-translational modification processes, leading to changes in regulation and cell signaling mechanisms [2].

Salinity-induced genes are often also drought-induced, showing a close relationship between these two conditions. In this sense, at least four independent signaling pathways act in the induction of genes under drought conditions: two ABA-dependent and two ABA-independent [3]. One of the dependent pathways requires action of *myeloblast* (MYB) and *myelocytomatosis* (MYC) transcription factors [4]. In addition to the transcriptional and post-transcriptional regulatory mechanisms, it has been shown that some mitogen-activated protein kinase (MAPK) acts at the post-translational level due to various types of stress, including salt stress, drought and cold [5]. Moreover, in response to salinity, pivotal proteins have been identified to be associated with the plasma membrane, typically receptors and kinases whose regulation is responsive to other stressors such as cold, drought, and ABA treatment [4]. In addition, some oxidative metabolism enzymes

(like, superoxide dismutase – SOD, ascorbate peroxidase – APX, and catalase – CAT) have been highly responsive to abiotic stresses alone or in combination [6]. Despite the relevance of the physiological responses associated with proteome, it have received meager attention in *Jatropha curcas* L. under salt stress. However, comparison studies of different genotypes of *J. curcas* from different provenance towards drought and their transcriptomic and physiological characterization has been elucidated to show different responses and adaptation strategies [7]. This further strengthens the importance of developing studies comparing genotypes under water deficit, to be able to understand the magnitude of the drought tolerance and screen genotypes with regard to both the tolerance to low availability of water and to responsiveness to irrigation, allowing an appropriate recommendation of cultivars for different cultivation systems [8].

The performance of *J. curcas* under salt stress has been the target of several physiological studies [9-14]. Recently, some studies [10-12] have been described *J. curcas* genotypes more tolerant do salinity than others. Although, *J. curcas* is a relatively drought-tolerant plant [15-17], it is also considered a salt-sensitive one [12, 18, 19], while others classify it as moderately tolerant to salt stress [13]. Such contrasting results can be explained by the fact that *J. curcas* is still in the domestication stage [20]. Several studies that examined the genetic variability of the species have been developed in the last few years [21, 22], providing promising results that interact to generate more productive cultivars that are more closely adapted to the edaphoclimatic conditions, this associated with its economic potential, can transform *J. curcas* as an efficient substitute to be used as fuel for diesel, its utilization as a new source of oil has tremendous scope in contributing to growing needs of the country for energy resources [23]. Thus, the objective of this work was to analyze the differential leaf proteome in two contrasting *J. curcas* genotypes using two-dimensional electrophoresis. The results obtained may contribute to the understanding of the molecular responses of salinity in *J. curcas*, as well as the identification of potential functional biomarkers for assisted breeding.

2. MATERIAL AND METHODS

2.1 PLANT MATERIAL AND GROWN CONDITIONS

All experiment was performed in the greenhouse at Federal University of Pernambuco (8°02'59" S; 34°56'55" W; 15 m a.s.l.). Originally, this study was designed to compare six different genotypes of *Jatropha curcas*. However, based in the previous study of our research group [10, 11], we selected two *J. curcas* genotype, such as CNPAE183, here referred as tolerant-like *J. curcas* genotype and CNPAE218, here referred as sensitive-like *J. curcas* genotype. So, seeds of these two

genotype was gently given us by Embrapa Agroenergy (Brasília, DF, Brazil). All seeds were storage at 4°C until their use [24], and then immersed in a solution of NaOCl 2% (v/v), plus two drops of Tween 20TM by 20 minutes and washed in deionized water three successive times. Subsequently, the seeds were transferred into polypropylene trays 50 x 30 x 10 cm for germination until the first-leaf expansion.

During the germination phase, all seedlings being irrigated every day with tap water. After germination, the seedlings were standardized and individualized in plastic pots (9 L), filled with 9 kg of washed sand, where the seedlings remained for at least 15 days, being fertiirrigated every two days with Hoagland nutrient solution [25] at 50% (pH 5.8) to acclimatization to the new environment. After them, the seedlings were fertiirrigated every day with full-strength Hoagland nutrient solution until 3-month old, when the experiment was started. The all experiments was performed under randomized block design containing two genotypes (CNPAE183, and CNPAE218), one saline concentrations (750 mM L⁻¹ of NaCl) added to Hoagland nutrient solution and 4 replicates. The saline concentration used in this study merged after two previously study of our research group [10, 11]. Corte-Real, Miranda [10] describes that while CNPAE183 readily recovered our net photosynthesis after salt stress alleviation, CNPAE218 never recovered our net photosynthesis, since even after 914 hours of salt alleviation its net photosynthesis were negative or almost zero. To effect of comparison, 750 mM L⁻¹ of NaCl, corresponding to 46.8 dS m⁻¹ of electrical conductivity, obtained after linear regression between [NaCl] and its respective electrical conductivity. Silva-Santos, Corte-Real [11] corroborating this findings, describes the leaf anatomy of both *J. curcas* genotype studied here. These authors describes that CNPAE183 morphoanatomically and physiologically more plastic than CNPAE218. In accord of Corte-Real, Miranda [10], under 750 mM L⁻¹ of NaCl, 3-month old *J. curcas* plants obtained maximum stress in about 48 hours after NaCl addition in the irrigation water. So, in this study, we consider 48 hours-plants as salt-stressed.

2.2 PROTEIN EXTRACTION AND QUANTIFICATION

A 3rd healthy leaf was harvested and flash-frozen in liquid nitrogen and then stored at -80°C until analysis. Proteins were extracted using phenol extraction [26]. About 0.2 g of frozen leaves was ground to fine powder with liquid nitrogen and then homogenized with 750 µl of the extraction buffer (pH 8.0) containing 700 mM sucrose, 500 mM Tris, 50 mM ethylenediamine tetraacetic acid (EDTA), 100 mM KCl, 2% (v/v) β-mercapto-ethanol, and 2 mM phenylmethanesulfonyl fluoride (PMSF) and then the mixture was incubated for 10 min in ice. Tris-

saturated phenol (750 μ l) was then added and the mixture was vortexed and shaken at room temperature for 30 min. Samples were then centrifuged (30 min, 11,300 g, 4°C). The upper phenolic phase was transferred to another tube. After several rounds of centrifugation, proteins were precipitated with 0.1 M ammonium acetate in methanol at -20°C over-night. The protein pellets were subsequently centrifuged (3 min, 17,600 g, 4°C) proteins, re-suspended in 1 mL of precipitation solution, rinsed with ice-cold acetone followed by a re-centrifugation, and finally dried at room temperature.

2.3 TWO-DIMENSIONAL GEL ELECTROPHORESIS (2-DE) AND MASS SPECTROMETRY

500 μ g of proteins from each extract were added with 0.005% (w/v) bromophenol blue and subjected to isoelectric focusing (IPG buffer, pH 3-10 nonlinear; GE Healthcare Life Sciences, Pittsburgh, PA, USA). The extracts were applied to dehydrated acrylamide impregnated tapes (IPG 13 cm, nonlinear pH 3-10 gradient; GE Life Healthcare Science), which were rehydrated in the Multiphor II system (GE Life Healthcare Sciences) for 7 h at 20°C. The IPG tapes were then equilibrated for 20 min in two disulfide bridge reducing solutions [27]. The second dimension was conducted in 12.5% vertical SDS-PAGE at 10°C. The resulting gels were impregnated with Coomassie G-250 blue colloidal dye according to the methodology described by Candiano, Bruschi [28].

J. curcas 2D gels were scanned in the ImageScanner III and their images processed in the LabScan 6.0 software (GE Healthcare Life Sciences). Differential protein accumulation was determined from gel images using the ImageMaster 2D Platinum v.7.05 software (GE Life Healthcare Sciences). Proteins (*i.e.*, spots) with statistically significant accumulation ($p \leq 0.05$) and change ratio up to 1.5-fold were selected and considered as differentially accumulated (DAPs), and subjected to identification by mass spectrometry. The selected proteins (spots) were excised from the gels and twice bleached with 25 mM ammonium bicarbonate, plus 50% (v/v) acetonitrile (ACN) by 30 min. The gel fragments were dehydrated with 100% acetonitrile for 5 min, and the gel fragments evaporated and rehydrated in a solution containing 20 mM DTT in 50 mM ammonium bicarbonate and incubated for 40 min at 60°C. The preparation of the selected differential peptides was performed according to the methodology described in Barbosa Neto, Pestana-Calsa [27]. The solutions containing the extracted peptides were dried at 30°C in a vacuum concentrator, followed by their resuspension in 1% (v/v) formic acid and transfer to new tubes. The MALDI-ToF/ToF mass spectrometer analysis were done in the Analytical Center of the Northeastern Strategic Technology Center (CETENE) using a mass spectrometer AutoFlex III (Bruker Daltonics, Inc. Karlsruhe,

Germany). The pellet was solubilized in 5 μ L 0.1% trifluoroacetic acid (TFA). For each reading cycle, 2 μ L of the sample were mixed with 2 μ L of α -cyano-4-hydroxycinnamic acid (Sigma-Aldrich Chemical Co, Darmstadt, Germany, part number C8982) in ACN and 3% TFA, and 2 μ L were twice applied to the metal plate cells.

2.4 PRESUMPTIVE IDENTIFICATION AND BIOINFORMATIC ANALYSIS

The presumptive identification of the mass spectra (MS) obtained in the peptide analysis was performed using the MASCOT platform (Matrix Inc. free available on http://www.matrixscience.com/search_form_select.html) through the peptide mass fingerprinting (PMF) method using the Viridiplantae and *Arabidopsis* sub-databases (Swissport and NCBIProt), in accord of following parameters: *i*) fixed modification: carbamidomethylation (C); *ii*) variable modification: oxidation (M); and *iii*) tolerance: 100 ppm at 1.2 Da. Subsequently, a complementary identification was conducted through a private version of the MASCOT software, kindly made available for access in collaboration with the Advancing Proteomics at University of Washington (Seattle, Washington, USA; <http://www.proteomicsresource.washington.edu/>), using the PMF method contrasted to Euphorbiaceae and *Jatropha curcas* databases, adopting the following parameters: *i*) fixed modification: carbamidomethylation (C); *ii*) variable modification: oxidation (M); and *iii*) tolerance: 200 ppm at 1.2 Da. Identifications with a score greater than to the cut-off value were considered significant. The considered score was $-10 \cdot \log(P)$, where P is the probability that the similarity found is random. Score values above the threshold value have statistical significance ($p < 0.05$).

Gene ontology (GO) analysis, was performed using the online tool Mercator (<https://plabipd.de/portal/mercator-sequence-annotation>), from the FASTA/Uniprot files of the identified proteins. After then, we performed a GO mapping related to biological processes, with *Arabidopsis thaliana* as reference. GO term enrichment analysis was performed on the Panther online platform (<http://www.pantherdb.org/>) for the *A. thaliana* genome and significant false discovery rate (FDR), $p < 0.05$.

3 RESULTS

Here, we describe that 2D gels showed that the isoelectric focusing stage provided adequate resolution, with relative protein diversity in different pI ranges, mainly between pH 4 and 7 (Fig. 1). After electrophoresis, gels with sample reproducibility between replicates of the same genotype were also obtained: correlation coefficient (r^2) of 0.9759 to tolerant-like *J. curcas* genotype and

0.9554 to sensitive-like genotype. These correlation coefficients allowed us to compare between genotypes under salinity and the identification of DAPs. As expected, we identified 145 DAPs, where it is possible to identify 110 DAPs (~76%) using PMF technique. Of these, 69 (~63%) proteins were exclusive and/or more accumulated in tolerant-like *J. curcas* genotype (CNPAE183; Table 1) and 41 (~37%) were exclusive and/or more accumulated in the sensitive-like *J. curcas* genotype (CNPAE218; Table 2).

Figure 1. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) showing leaf proteomic profile of CNPAE183 (A), and CNPAE218 (B) genotype of *Jatropha curcas* subjected to 48 hours of 750 mM NaCl.

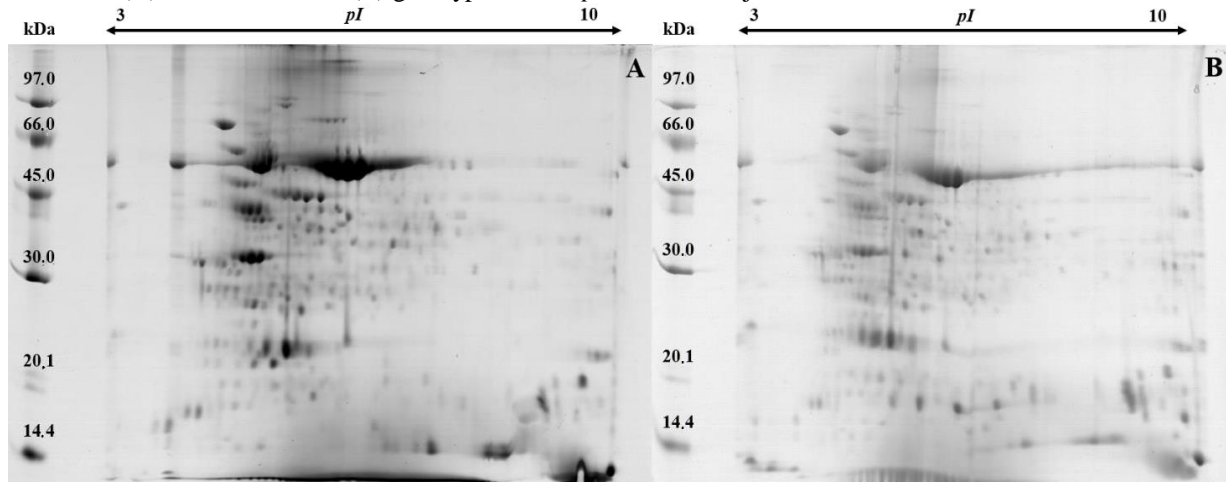


Table 1. Proteins identification of the CNPAE183 genotype of *Jatropha curcas* L. subjected to 48 hours of 750 mM NaCl contrasted to CNPAE218 as reference. Annotation from PMF;MALLDI-TOF-MS.

Spot	ANOVA	Proteins	ID	Score	M _{cal}	M _{Ano}	pI _{cal}	pI _{Ano}	Ortholog Specie	Ratio
Photosynthesis										
6	0.0463	Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase small subunit	D6BR54	88	14,689	20,473	7.39	9.06	<i>Jatropha curcas</i>	1.548
60	0.0078	Proteins in the Photosynthetic Oxygen-Evolving Complex, isoform 1	A0A067KA30	91	32,980	35,314	4.94	5.87	<i>Jatropha curcas</i>	1.313
84	0.0264	Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase isoform X1 Chl	A0A067L8Y9	84	41,785	52,211	4.83	5.56	<i>Jatropha curcas</i>	2.422
86	0.0333	Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase isoform X1 Chl	A0A067L8Y9	112	41,534	52,211	4.93	5.56	<i>Jatropha curcas</i>	2.834
100	0.0476	Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase large subunit	B1NWF7	183	51,464	53,087	6.23	6.09	<i>Jatropha curcas</i>	1.148
112	0.0072	Chloroplast ATP Synthase subunit beta	COLE81	119	55,182	53,278	4.98	5.10	<i>Jatropha curcas</i>	1.392
115	0.00074	ATP Synthase subunit alfa	COLE59	106	55,407	55,484	5.17	5.28	<i>Jatropha curcas</i>	1.704
118	0.0324	ATP Synthase subunit beta	COLE81	118	54,007	53,278	5.07	5.10	<i>Jatropha curcas</i>	1.761
143	0.0312	Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase large subunit	COLE82	204	56,097	53,087	5.65	6.09	<i>Jatropha curcas</i>	1.600
144	0.0342	Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase large subunit	COLE82	64	54,923	53,087	5.75	6.09	<i>Jatropha curcas</i>	1.693
146	0.0462	Photosystem II Assembly / Stability Factor (HCF136)	A0A067L4D0	58	38,351	43,076	5.14	7.08	<i>Jatropha curcas</i>	2.918
209	0.0139	Chloroplast ATP Synthase subunit alfa	COLE59	97	65,046	55,484	5.20	5.28	<i>Jatropha curcas</i>	*
216	0.0013	Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase small subunit	D6BR54	98	14,535	20,473	8.14	9.06	<i>Jatropha curcas</i>	*
227	0.0002	Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase large subunit	A0A199U950	70	29,612	37,029	5.09	8.50	<i>Manihot esculenta</i>	*
278	0.0025	Peroxisomal (S)-2-Hydroxy Acid Oxidases	A0A067L0G9	82	41,789	40,566	9.68	9.31	<i>Jatropha curcas</i>	*
279	0.0127	Peroxisomal (S)-2-Hydroxy Acid Oxidases	A0A067L0G9	81	36,257	40,566	9.16	9.31	<i>Jatropha curcas</i>	*
291	0.0000	Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase small subunit	D6BR54	89	14,833	20,473	7.17	9.06	<i>Jatropha curcas</i>	*
Glycolysis										
92	0.0067	Phosphoglycerate Kinase	A0A067JME5	71	43,274	50,648	5.86	8.41	<i>Jatropha curcas</i>	1.355
123	0.0105	Phosphoglycerate Kinase	A0A067JME5	68	43,411	50,648	5.51	8.41	<i>Jatropha curcas</i>	1.512
282	0.0106	Pyruvate Kinase	B9S7Y4	67	27,201	64,159	6.97	6.09	<i>Ricinus communis</i>	*
Mitochondrial Electron Transport Chain / ATP synthase										
111	0.0400	ATP Synthase subunit beta	A0A067KFH8	72	53,924	60,287	5.35	6.13	<i>Jatropha curcas</i>	2.747
131	0.0325	ATP Synthase	C1MNM6	81	23,614	60,369	6.23	6.99	<i>Micromonas pusilla CCMP1545</i>	1.365
211	0.0020	ATP Synthase subunit beta	A0A067KFH8	83	54,719	60,287	5.29	6.13	<i>Jatropha curcas</i>	*
Amino acid metabolism										
284	0.0021	Chloroplast Argininosuccinate Synthase	Q2QVC1	65	22,137	38,967	4.56	5.90	<i>Oryza sativa Japonica Group</i>	*
Secondary metabolism										
42	0.0095	1-Deoxy-D-Xylolose-5-Phosphate Synthase	Q38854	55	29,068	77,468	4.76	7.04	<i>Arabidopsis thaliana</i>	1.488
Plant pathogen signal										
80	0.0084	Defensin-like 257 Protein	Q2V3S8	56	40,253	9,329	7.38	5.21	<i>Arabidopsis thaliana</i>	2.349
94	0.0002	Defensin-like 257 Protein	Q2V3S8	56	43,963	9,329	5.37	5.21	<i>Arabidopsis thaliana</i>	4.398
145	0.0140	TMV Resistance Protein N, isoform 2	A0A067JI31	59	48,572	78,345	4.77	7.05	<i>Jatropha curcas</i>	1.900
236	0.0000	RING E3-E2-Ubiquitin Transferase	B9SBC6	61	80,171	116,067	6.19	5.68	<i>Ricinus communis</i>	*
Redox Protein										
231	0.0001	Catalase	A0A067L5U2	78	55,418	57,081	7.49	7.10	<i>Jatropha curcas</i>	*
Metabolism										

214	0.0001	Fold 2 Bifunctional Protein	A0A067K8J8	64	50,486	32,092	6.69	7.05	<i>Jatropha curcas</i>	*
Proteins evolved in RNA assembly or function										
107	0.0044	Maturase K	Q9AVL5	56	54,006	59,670	7.65	9.61	<i>Panax ginseng</i>	1.710
217	0.0072	Remorin	A0A067K8I1	59	16,811	24,708	3.96	9.78	<i>Jatropha curcas</i>	*
219	0.0000	DNA Cytosine-5-Methyltransferase 1A	Q7Y1I7	52	21,975	172,800	5.03	5.75	<i>Oryza sativa Japonica Group</i>	*
225	0.0001	TATA Box-Binding Protein	B9SWR1	63	28,096	21,950	6.60	9.94	<i>Ricinus communis</i>	*
234	0.0001	DNA-Directed RNA polymerase subunit beta	A0A327	56	80,466	120,937	5.80	8.74	<i>Coffea arabica</i>	*
241	0.0000	PHL5-MYB Transcription Factor Family	A0A067J9G2	58	28,834	29,597	6.03	9.18	<i>Jatropha curcas</i>	*
242	0.0223	GATA (19) Transcription Factor	B8AR30	52	29,544	29,479	9.64	6.14	<i>Oryza sativa Indica Group</i>	*
265	0.0033	AS1 Transcription Factor	O80931	56	65,895	42,559	6.50	9.16	<i>Arabidopsis thaliana</i>	*
272	0.0010	GATA (19) Transcription Factor	B8AR30	50	32,761	29,479	4.54	6.14	<i>Oryza sativa Indica Group</i>	*
281	0.0010	Nucleic Acid Binding Protein	B9RAN9	61	22,844	56,500	5.08	5.33	<i>Ricinus communis</i>	*
289	0.0020	GATA (19) Transcription Factor	B8AR30	49	29,485	29,479	5.93	6.14	<i>Oryza sativa Indica Group</i>	*
Proteins evolved in DNA assembly or function										
247	0.0004	FAR1 Protein, isoform X1	A0A067LFM0	61	36,634	99,952	9.36	6.22	<i>Jatropha curcas</i>	*
Signaling										
139	0.0488	BTB/POZ Domain At3g08570 Protein	Q9C9Z7	60	60,619	70,320	4.75	5.67	<i>Arabidopsis thaliana</i>	1.389
140	0.0098	Serine-Threonine Kinase Protein	B9S095	61	60,752	75,826	4.80	9.01	<i>Ricinus communis</i>	2.233
224	0.0001	Serine-Threonine Kinase Protein	B9S095	61	28,542	75,826	5.37	9.01	<i>Ricinus communis</i>	*
245	0.0001	Calcium-Binding Protein CML45	A0A067K9U4	60	35,574	23,016	6.56	5.71	<i>Jatropha curcas</i>	*
246	0.0033	Cell Wall-Associated Receptor-Like Protein Kinase 4	Q9S9M2	55	35,692	86,269	5.82	5.66	<i>Arabidopsis thaliana</i>	*
271	0.0015	Cell Wall-Associated Receptor-Like Protein Kinase 4	Q9S9M2	58	31,039	86,269	7.54	5.66	<i>Arabidopsis thaliana</i>	*
Cell Cycle Event										
222	0.0025	Cyclin-Dependent Kinase 2	A0A067KX42	59	27,069	57,085	5.37	9.35	<i>Jatropha curcas</i>	*
Development										
232	0.0007	Argonaute Protein I	O04379	57	80,069	117,201	5.89	9.38	<i>Arabidopsis thaliana</i>	*
286	0.0003	BPS1-Like Protein (DUF793)	Q9LMM6	46	20,482	38,787	4.62	8.98	<i>Arabidopsis thaliana</i>	*
Protein Translocation										
238	0.0002	Mitochondrial Inner Membrane Protein Translocation subunit TIM50	A0A067KLS5	58	15,579	41,995	4.69	7.72	<i>Jatropha curcas</i>	*
Other Proteins										
58	0.0002	Chloroplast 50 S Ribosomal L16 Protein	A6BM45	55	33,466	15,349	4.72	11.47	<i>Gnetum parvifolium</i>	1.565
88	0.0037	Mitochondrial 54 S Ribosomal L24 Protein	A0A067L255	62	43,113	24,550	4.34	9.74	<i>Jatropha curcas</i>	1.865
148	0.0462	RNF170 RING-HC Domain Protein	A0A067KTH4	60	28,509	28,318	5.22	8.80	<i>Jatropha curcas</i>	*
236	0.0000	RING E3 Ubiquitin Transferase	B9SBC6	61	80,171	116,067	6.19	5.68	<i>Ricinus communis</i>	*
43	0.0219	Hypothetical Protein	Q6K6N4	76	29,120	18,622	4.40	11.65	<i>Oryza sativa Japonica Group</i>	2.107
49	0.0204	Sulphotranferase tRNA	R9VQN8	74	31,060	55,070	4.78	6.24	<i>Enterobacter sp.</i>	1.606
62	0.0021	Golgi-Associated Kinesin-Like Protein with RAB6, subunit DUF662	F4IMV8	73	35,157	9,951	6.84	9.30	<i>Arabidopsis thaliana</i>	1.910
89	0.0186	Lola-Like protein	R9VWM1	59	41,545	30,415	5.04	9.00	<i>Enterobacter sp.</i>	1.764
95	0.0217	Domain of Unknown Function, Like DUF4408 and DUF761	B9RK72	63	44,698	27,280	5.44	6.88	<i>Ricinus communis</i>	1.757
130	0.0134	Epsin 3	A0A067L925	64	92,082	29,185	5.34	6.17	<i>Jatropha curcas</i>	4.036
220	0.0000	MATH-Coiled-Coil Domain At2g42460 Protein	F4IN32	54	21,967	34,353	5.28	9.29	<i>Arabidopsis thaliana</i>	*

240	0.0000	Cytochrome C Peroxidase	R9VWN0	63	27,336	40,002	5.15	8.59	<i>Enterobacter</i> sp. R4-368	*
243	0.0002	Type IV-secreted Rhs Protein	R9VJV2	58	32,021	165,495	5.30	5.87	<i>Enterobacter</i> sp. R4-368	*
250	0.0017	Chloroplast Stem-Loop 41 kDa-Binding Protein	A0A067JHM3	90	40,614	42,666	7.72	8.84	<i>Jatropha curcas</i>	*
254	0.0005	Recombinase Domain Protein	B9TDB5	62	42,016	33,549	5.17	9.86	<i>Ricinus communis</i>	*
275	0.0000	Cytochrome C Peroxidase	R9VWN0	62	99,310	40,002	5.54	8.59	<i>Enterobacter</i> sp. R4-368	*
295	0.0038	Protein MARD1	B6SU95	81	14,833	28,633	6.77	9.48	<i>Zea mays</i>	*
Unknown										
93	0.0469	No match	-	-	42,420	-	6.23	-	<i>Jatropha curcas</i>	2.592
106	0.0116	No match	-	-	54,106	-	7.86	-	<i>Jatropha curcas</i>	1.810
210	0.0001	No match	-	-	38,673	-	5.02	-	<i>Jatropha curcas</i>	*
215	0.0002	No match	-	-	14,983	-	4.84	-	<i>Jatropha curcas</i>	*
218	0.0001	No match	-	-	20,598	-	6.77	-	<i>Jatropha curcas</i>	*
221	0.0048	No match	-	-	23,440	-	6.13	-	<i>Jatropha curcas</i>	*
226	0.0277	No match	-	-	33,020	-	3.06	-	<i>Jatropha curcas</i>	*
228	0.0005	No match	-	-	29,536	-	5.26	-	<i>Jatropha curcas</i>	*
229	0.0005	No match	-	-	38,975	-	6.72	-	<i>Jatropha curcas</i>	*
230	0.0008	No match	-	-	41,782	-	5.69	-	<i>Jatropha curcas</i>	*
233	0.0000	No match	-	-	79,752	-	5.99	-	<i>Jatropha curcas</i>	*
235	0.0002	No match	-	-	79,752	-	6.08	-	<i>Jatropha curcas</i>	*
244	0.0019	No match	-	-	35,155	-	7.22	-	<i>Jatropha curcas</i>	*
252	0.0024	No match	-	-	41,151	-	6.45	-	<i>Jatropha curcas</i>	*
257	0.0413	No match	-	-	45,301	-	6.98	-	<i>Jatropha curcas</i>	*
258	0.0133	No match	-	-	44,682	-	6.11	-	<i>Jatropha curcas</i>	*
261	0.0002	No match	-	-	50,819	-	6.98	-	<i>Jatropha curcas</i>	*
264	0.0004	No match	-	-	65,776	-	6.60	-	<i>Jatropha curcas</i>	*
276	0.0094	No match	-	-	100,000	-	5.40	-	<i>Jatropha curcas</i>	*
277	0.0098	No match	-	-	99,894	-	5.48	-	<i>Jatropha curcas</i>	*
280	0.0001	No match	-	-	31,041	-	7.96	-	<i>Jatropha curcas</i>	*
283	0.0002	No match	-	-	22,093	-	4.41	-	<i>Jatropha curcas</i>	*
285	0.0000	No match	-	-	22,026	-	4.82	-	<i>Jatropha curcas</i>	*
288	0.0057	No match	-	-	34,277	-	7.23	-	<i>Jatropha curcas</i>	*
290	0.0024	No match	-	-	31,774	-	6.58	-	<i>Jatropha curcas</i>	*

Table 2. Proteins identification of the CNPAE218 genotype of *Jatropha curcas* L. subjected to 48 hours of 750 mM NaCl contrasted to CNPAE183 as reference. Annotation from PMF;MALLDI-TOF-MS through Mascot platform

Spot	ANOVA	Proteins	ID	Score	M _{cal}	M _{Ano}	pI _{cal}	pI _{Ano}	Ortholog Specie	Ratio
Photosynthesis										
119	0.0217	Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase small subunit	D6BR54	101	14,667	20,473	8.69	9.02	<i>Jatropha curcas</i>	3.096
179	0.0015	Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase large subunit	COLE82	140	56,131	53,087	3.16	6.09	<i>Jatropha curcas</i>	*
172	0.0016	Carbonic Anhydrase	A0A067LLS5	85	28,177	37,150	6.76	8.07	<i>Jatropha curcas</i>	*
Mitochondrial Electron Transport Chain / ATP synthase										
170	0.0058	ATP Synthase	C1MNM6	81	18,854	60,369	8.90	6.99	<i>Micromonas pusilla CCMP154</i>	*
Hormones										
204	0.0002	Cytochrome P450 Monooxygenase	Q0JL28	69	31,142	17,157	9.47	9.62	<i>Oryza sativa Japonica Group</i>	*
Plant Stress and Redox										
29	0.0022	Serine/Threonine Protein Kinase Like SD1-8, isoform XI	A0A199U9G4	58	23,530	36,962	4.94	8.76	<i>Manihot esculenta</i>	3.892
198	0.0022	Glutaredoxin C13	Q0IRB0	54	33,848	11,580	4.26	8.57	<i>Oryza sativa Japonica Group</i>	*
Proteins evolved in RNA assembly or function										
40	0.0449	Maturase K	Q8HQQ6	56	29,130	61,576	4.51	9.40	<i>Pinus pinaster</i>	1.598
77	0.0079	GATA (19) Transcription Factor	B8AR30	53	39,860	29,479	4.88	6.14	<i>Oryza sativa Indica Group</i>	1.544
162	0.0000	Manes.S021100_RPOL-1 Like Protein	A0A199UC42	59	33,550	29,201	4.57	9.64	<i>Manihot esculenta</i>	*
196	0.0012	Protein with a Conserved REM8-like B3 domain	Q8H2D1	58	27,481	52,448	6.57	6.25	<i>Arabidopsis thaliana</i>	*
197	0.0074	Remorin with a Conserved C domain	Q0JA18	71	28,098	31,637	6.49	8.14	<i>Oryza sativa Japonica Group</i>	*
Proteins evolved in DNA assembly or function										
28	0.0011	DNA Polimerase	A0A067JPG7	62	23,283	124,988	5.15	8.35	<i>Jatropha curcas</i>	2.593
Signaling										
16	0.0118	Calcium-Dependent Protein Kinase 20	Q84SL0	50	18,315	62,768	5.44	7.00	<i>Oryza sativa Japonica Group</i>	2.187
36	0.0003	Cell Wall-Associated Receptor-Like Protein Kinase 4	Q9S9M2	56	27,808	86,269	5.42	5.66	<i>Arabidopsis thaliana</i>	2.547
166	0.0004	Calcium-Binding Protein CML45	A0A067K9U4	60	20,108	23,016	5.00	4.71	<i>Jatropha curcas</i>	*
176	0.0007	BTB/POZ Domain At3g08570 Protein	Q9C9Z7	60	55,579	70,320	7.88	5.67	<i>Arabidopsis thaliana</i>	*
246	0.0033	Cell Wall-Associated Receptor-Like Protein Kinase 4	Q9S9M2	55	35,692	86,269	5.82	5.66	<i>Arabidopsis thaliana</i>	*
180	0.0005	Calcium-Binding Protein CML45	A0A067K9U4	62	62,111	23,016	4.56	4.71	<i>Jatropha curcas</i>	*
184	0.0032	Cell Wall-Associated Receptor-Like Protein Kinase 4	Q9S9M2	56	77,105	86,269	6.08	5.66	<i>Arabidopsis thaliana</i>	*
Cell Cycle Event										
206	0.0041	DUF1731 GH43 superfamily Protein	A0A067JUJ5	60	49,514	25,290	4.59	8.44	<i>Jatropha curcas</i>	*
Development										
17	0.0001	SHI Protein	Q9SJT8	55	18,362	20,314	5.82	9.39	<i>Arabidopsis thaliana</i>	5.233
27	0.0085	S-Adenosylmethionine-Dependent Methyltransferase	E5D1F9	67	23,449	24,924	5.55	5.35	<i>Jatropha curcas</i>	1.603
158	0.0011	CCT Protein Family	Q8LDM8	66	18,110	23,363	6.98	4.95	<i>Arabidopsis thaliana</i>	*
160	0.0032	CCT Protein Family	Q8LDM8	66	37,029	23,363	8.12	4.95	<i>Arabidopsis thaliana</i>	*
Other Proteins										
4	0.0185	Bark Storage Protein A	A0A067KNP8	61	13,300	35,198	9.84	7.82	<i>Jatropha curcas</i>	1.474
25	0.0020	Bark Storage Protein A	A0A067KNP8	63	23,115	35,198	5.40	7.82	<i>Jatropha curcas</i>	1.470
26	0.0009	Pentatricopeptide repeat-containing protein At1g32415, mitochondrial	POC7R0	56	23,878	86,159	4.39	6.01	<i>Arabidopsis thaliana</i>	2.292

54	0.0030	Ribosomal – RNA <i>N</i> -glycosidase	D0PWG0	70	33,249	32,940	5.59	8.54	<i>Jatropha curcas</i>	5.676
55	0.0173	ULP1 - Ubiquitin-like-specific protease 1	Q5JMY1	67	33,511	28,368	5.42	9.62	<i>Oryza sativa Japonica Group</i>	4.358
56	0.0246	dCTP pyrophosphatase 1 Similar to OSIGBa0111I14.3 Protein	Q01L63	66	33,093	18,852	5.19	5.29	<i>Oryza sativa Indica Group</i>	1.967
161	0.0009	2C 67 Protein Phosphatase	Q0J2R1	51	34,610	39,822	6.10	6.37	<i>Oryza sativa Japonica Group</i>	*
164	0.0123	UBX (10) Domain Protein	A0A067K7Q0	66	19,798	41,957	5.51	8.58	<i>Jatropha curcas</i>	*
165	0.0034	Chloroplast 50 S Ribosomal L16 Protein	P08528	56	32,946	15,635	5.28	11.56	<i>Zea mays</i>	*
159	0.0246	Ribosomal – RNA <i>N</i> -glycosidase	D0PWG0	66	32,589	32,940	6.10	8.54	<i>Jatropha curcas</i>	*
167	0.0030	Cytidine/Guanosine-2'-O)-Methyltransferase tRNA	A0A067JWU0	71	18,557	35,215	4.90	6.02	<i>Jatropha curcas</i>	*
168	0.0021	Receptors like Calcium/Calmodulin-Dependent Protein Kinase II	Q8VZJ9	57	16,324	46,374	6.82	9.26	<i>Arabidopsis thaliana</i>	*
171	0.0000	RNF170 RING-HC Domain Protein	A0A067KTH4	58	24,690	28,318	3.22	8.80	<i>Jatropha curcas</i>	*
173	0.0010	Plant Storage Protein, like 2	A0A067KXQ9	83	30,830	32,465	9.63	7.88	<i>Jatropha curcas</i>	*
175	0.0000	Protein without Conserved Domain	A0A067L4W9	59	36,439	45,564	4.86	4.72	<i>Jatropha curcas</i>	*
187	0.0000	Replication Factor C / DNA Polymerase III Gamma-Tau Subunit	B9S6E2	61	20,242	88,976	7.21	9.63	<i>Ricinus communis</i>	*
205	0.0067	Hypothetical Protein JCGZ_06028	A0A067JK77	61	39,174	22,787	5.29	5.69	<i>Jatropha curcas</i>	*
Unknown										
2	0.0302	No match	-	-	23,885	-	4.61	-	<i>Jatropha curcas</i>	2.663
3	0.0003	No match	-	-	13,508	-	3.26	-	<i>Jatropha curcas</i>	7.930
9	0.0019	No match	-	-	16,393	-	9.77	-	<i>Jatropha curcas</i>	6.736
45	0.0451	No match	-	-	29,870	-	5.75	-	<i>Jatropha curcas</i>	1.565
67	0.0027	No match	-	-	37,537	-	5.86	-	<i>Jatropha curcas</i>	2.043
69	0.0230	No match	-	-	37,454	-	5.71	-	<i>Jatropha curcas</i>	1.645
132	0.0131	No match	-	-	19,750	-	8.88	-	<i>Jatropha curcas</i>	2.831
150	0.0155	No match	-	-	36,537	-	5.27	-	<i>Jatropha curcas</i>	1.695
163	0.0441	No match	-	-	60,341	-	4.83	-	<i>Jatropha curcas</i>	*
169	0.003	No match	-	-	17,770	-	6.40	-	<i>Jatropha curcas</i>	*

Using gene ontology analyzes, it was possible to group the identified proteins into different categories related to biological processes (Fig. 2). Thus, PADs were grouped into 4 categories: primary and secondary metabolism, nucleus function & proteins and others. Proteins evolved in photosynthesis, electron transport rate / ATP synthesis, protein metabolism, redox, signaling, stress response, DNA and RNA assembly of function, cell cycle event, as well as development are common to both genotypes (Fig. 2). Protein related to the miscellaneous has been identified only in sensitive-like *J. curcas* genotype. On the other hand, processes involved with glycolysis, amino acid metabolism, carbon metabolism and protein translocation were exclusively identified in tolerant-like *J. curcas* genotype (Fig. 2).

Through enrichment analysis, the most accumulated proteins in tolerant-like *J. curcas* genotype were significantly enriched in ribonucleotide, ADP metabolic process, carbohydrate and pyruvate building blocks, as well as processes involved with ATP biosynthesis and metal family ion response (Table 3).

4 DISCUSSION

4.1 PHOTOSYNTHETIC METABOLISM

Generally, photosynthesis is the physiological processes most affected and sensitive to stressful environmental conditions, such as high saline concentration and water deficit. As expected, it was observed that both genotypes accumulated proteins involved with the photosynthetic process. Thus, the tolerant-like genotype had 19 most accumulated spots, including three copies of the RuBisCO small subunit (spots 6, 216 and 291; Table 1), four copies of the RuBisCO major subunit (spots 100, 143, 144 and 227, Table 1) and two RuBisCO activase (spots 84 and 86, Table 1). The latter is a key enzyme of photosynthetic regulation, acting on the activation and maintenance of RuBisCO catalytic activity [29], and may also act as chaperone during stress situations, ensuring some chloroplast functions [30]. The role in maintaining the correct structure of the RuBisCO complex is particularly important under stressful conditions [31], and may be related to the tolerant phenotype observed in the CNPAE183 genotype. In this context, the higher accumulation of photosynthesis proteins is associated with basal photosynthetic rate, even under deleterious conditions [30], a fact that may be fundamental for recovery after salt stress alleviation. In addition, the tolerant-like *J. curcas* genotype showed higher accumulation of proteins involved photosystem II assembly / stability factor, like HCF136 (spot 146, Table 1), and proteins involved in the photosynthetic oxygen-evolving complex, (spot 60, Table 1). The latter plays an important role in maintaining PSII activity [32]. Thus, the higher accumulation of these key proteins in the energy

production and integrity of the photosynthetic apparatus seems to contribute to a higher photosynthetic efficiency in the most tolerant genotype under saline conditions.

Through enrichment analysis, the most accumulated proteins in tolerant-like *J. curcas* genotype were significantly enriched in ribonucleotide, ADP metabolic process, carbohydrate and pyruvate building blocks, as well as processes involved with ATP biosynthesis and metal family ion response (Table 3).

Figure 2. Gene ontology of the proteins differentially accumulated in leaves of two different genotypes (CNPAE183, and CNPAE218) of *Jatropha curcas* subjected to 48 hours of 750 mM NaCl.

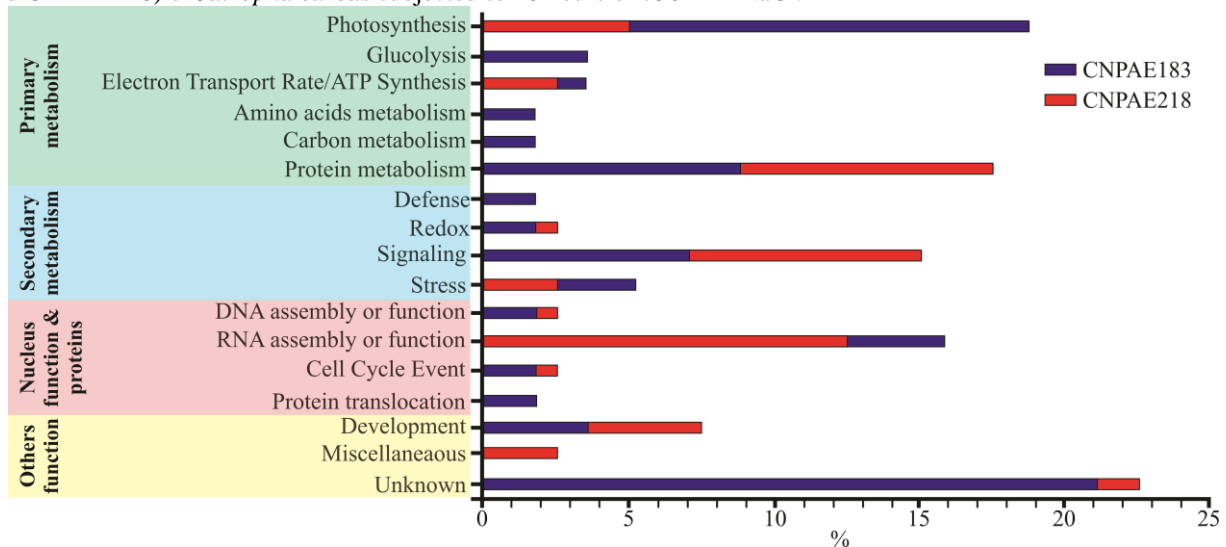


Table 3. Biological processes significantly enriched in *J. curcas* genotype CNPAE183 compared to model species *Arabidopsis thaliana*.

CNPAE183		
Biologic Process	Number of genes involved	<i>p-value_FDR</i>
Ribonucleotide Metabolic Process	4	9.30 E-06
ADP Metabolic Process	3	1.26 E-05
Metalloproteins Process	3	2.53 E-05
Carbohydrates Metabolic Pathway	5	6.41 E-05
ATP Synthase coupled to electron transport rate	2	1.98 E-04
Pyruvate Metabolic Pathway	2	1.98 E-04

In the sensitive-like *J. curcas* genotype, only two RuBisCO were identified and categorized in biological process, one large subunit (spot 179, Table 2) and one small subunit (spot 119, Table 2), plus one carbonic anhydrase (CA, spot 172, Table 2). The lastly is an enzyme that reversibly catalyzes CO₂ into carbonic acid (HCO₃⁻). The response of CA activity varies according to the genotype, duration and intensity of the stressful condition [33]. The higher accumulation of this enzyme in the sensitive-like *J. curcas* genotype, allows us to infer that this genotype needs to increase the [CO₂] near the RuBisCO carboxylation sites to obtain a similar result to tolerant-like *J.*

curcas genotype and this higher investment may be due to the lower carboxylative efficiency of RuBisCO in this genotype and lesser net photosynthesis when compared to tolerant-like *J. curcas* genotype [10]. Some scholars [34-36] have reported an increase in RuBisCO activity in salinity tolerant species/cultivars. This is supported by reports [37-39], which demonstrated that the oxygenase activity of RuBisCO (which carries out the first step of the photorespiratory pathway) gets significantly enhanced under conditions of salinity stress. In fine-tune with these scholars, Mansour, Fattoum [40] describes that independently on salinity dose, there was a decrease in RuBisCO activity in sensitive-like Rabiaa wheat-cultivar while there was an increase in RuBisCO activity in tolerant-like Karim wheat-cultivar. Gao, Cui [41] describes a down-regulation of RuBisCO activase indicated that decrease in RuBisCO activation of salt-stressed alfalfa seedlings, which results in inhibition of photosynthesis and overall growth. Similar conclusions were obtained by He, Yu [42] that describes a decrease of RuBisCO content or activity has been shown to cause low carboxylation efficiency in salt-sensitive. Wang, Cong [43] describes that after 3-hours of salt stress, the downregulated metabolic pathways in *Dunaliella salina* included not only those of primary, but also secondary metabolism. The majority of DAPs related to pivotal pathways, such as porphyrin and chlorophyll metabolism, and one carbon pool related to folate pathways, were downregulated after 24 h salt exposure. These findings indicate that salt stress fundamentally inhibited normal carbohydrate and energy metabolism in *D. salina* during the early stages of response. Both FTSY (a signal recognition particle docking protein) and *rbcL* (a Rubisco large subunit protein) were arrested in *D. salina* upon salt stress. This contradicts another study which showed upregulation of FTSY and *rbcL* and a strengthened glycolysis pathway, which could result in more energy for the generation of ATP and NADPH to resist salt stress [44].

Related to photosynthetic mechanism, the chloroplast ATP synthase are still involved, including the beta (spot 112 and 118, Table 1) and alpha (spot 115 and 209, Table 1) subunits, more accumulated in the tolerant-like *J. curcas* genotype. Salt stress may affect the photosynthetic process, altering the production capacity of ATP and NADPH [45]. It has been demonstrated that RuBisCO might show structural modifications, this could be the results of photodegradation, fragmentation and denaturation, active site modifications and solubility of membrane proteins [46]. Down-regulation in these enzymes suggest noticeable inhibition of photosynthetic dark reaction in *Anabaena* species and their inability to generate ATP, which is used for maintaining metabolic processes under stressful condition. Furthermore, inhibition in the activity of Krebs's cycle enzymes in all three studies *Anabaena* species not only lesser generation of ATP and NADPH but also reduce the production of many other metabolic precursors of different metabolic pathways [45]. While

reviewing the role of antioxidant potential in stress tolerance, Kaya, Ashraf [47] suggested that, under stress conditions, imbalance between generation of ATP and NADPH through the photosynthetic electron transport chain and their consumption in fixation of CO₂ in sugar causes the generation of ROS via the water-water cycle. Moreover, plants with better antioxidant potential are more tolerant to stress. However, enhancement in the amount and activities of antioxidant enzymes is energetically costly. In this sense, the higher physiological efficiency observed in tolerant-like *J. curcas* genotype, under saline stress, may be related to the higher accumulation of ATP synthase, indicating that the light reactions of photosynthesis in this genotype was less damaged through salinity. Higher translation of ATP synthase also was related to salt stress adaptation in *Paulownia fortunei* [48], *Dunaliella salina* [43], *Oryza sativa* [49], meeting energy demand during periods of recovery and plant development. The importance of ATP synthase in the most tolerant genotype in salt stressed plants is corroborated by the enrichment analysis of gene ontology terms, based on which significant variation was observed in two biological processes related to energy metabolism: ATP synthesis coupled to proton transport, ADP metabolic process ($p \leq 1.98E-04$ and $1.26E-05$, respectively). In addition, higher concentration of such proteins may also indicate less membrane degradation of the thylakoid, maintaining partial energy production, as well as adjustment of the pH of the thylakoid lumen. In acidic pH, there is a higher occurrence of protein aggregation and degradation [50], a fact that can be more efficiently mitigated in the tolerant-like *J. curcas* genotype.

4.2 GLYCOLYSIS

The carbohydrate metabolism is one of the major energy supply pathways. Salt-stressed plants generally have reduced translation of enzymes related to this carbohydrate metabolism [43, 51]. This contradicts our results; however, some studied gene expression have shown greater induction of genes related to pyruvate metabolism pathways, such as pyruvate kinase (PQ), in *Oryza sativa* [52] and *Saccharum* spp. [53] submitted to abiotic stresses, seemly as observed in this study in the tolerant-like *J. curcas* genotype (spot 282, Table 1). Pyruvate kinase is another enzyme evolved in the regulation of glycolysis, irreversibly catalyzing pyruvate formation, ensuring building blocks for the tricarboxylic acid (TCA) cycle, and consequently for electron transport chain and metabolite generation. Supporting the idea of the importance of these metabolisms in salt-stressed tolerant-like *J. curcas* genotype, significant enrichment was recorded for metabolic processes of pyruvate and carbohydrate derivatives ($p < 1.98E-04$ and $6.41E-05$, respectively). Thus, the greater accumulation of glycolytic enzymes suggests that the tolerant-like *J. curcas* genotype is able of dealing with the stressful condition by increasing proteins associated with energy metabolism, without

compromising the glycolytic pathway [54-56] and ensuring energy for metabolic reactions as well as carbon precursors for the formation of important metabolic compounds.

4.3 STRESS SIGNALING

Some genes are induced by saline stress, which encode and accumulate functional proteins that act on different metabolic pathways in response to stress signaling, as well as control and repair of cell damage [57]. In the present study, two different proteins evolved with stress signaling: ubiquitin transferase protein (UQE3) (RING-type E3; spot 236, Table 1) in tolerant-like *J. curcas* genotype and serine/threonine protein kinase SD1-8 isoform X1 in sensitive-like *J. curcas* genotype, involved in signaling in response to salinity. As observed in tolerant-like *J. curcas* genotype, a previous study reports that *A. thaliana* also presented higher expression of UQE3, by high saline concentration [58, 59]. According to these authors, the highest expression of this protein class is closely related to the ABA-mediated signaling pathway and is mainly involved in the protein degradation mechanism. Class E3 ligase enzymes transfer the polyubiquitin chain to the degradation target protein, which is subsequently recycled by the 26S proteasome. Hildebrandt, Nunes-Nesi [55] described that in salt-stressed leaves, proteins are degraded, and the complete oxidation of their amino acids produces the energy required to fuel the particular needs of certain organs. As in our study, the strong negative correlation between protein degradation and amino acids metabolism permit us to infer that protein degradation leads to amino acid synthesis representing building blocks for several other biosynthesis pathways and play pivotal roles during signaling processes as well as in plant stress response as previously reported by others [55, 60, 61].

In this study, we also reported a higher accumulation of two defensin-like proteins (spots 80, 94, Table 1) and one TMV resistance protein N (PNRTMV) (spot 145, Table 1) in tolerant-like *J. curcas* genotype. It is consensus that these proteins are mainly associated with biotic stress, considering that defensins are polypeptides with antimicrobial activity [62]. Other studies have reported the performance of these proteins under abiotic stress, such as water deficit and salinity [63, 64]. Kaya, Higgs [65], evaluating the expression of the *Capsicum annum* defensins in pepper leaves, reported greater tolerance to saline condition in plants that showed higher expression of defensin genes, demonstrating that this class of proteins plays numerous roles in plant defense, which promote greater adaptation of plants to adverse environmental conditions. Moreover, the higher translation of defensins-like system proteins in plants subjected to abiotic stresses may be related to the existence of cross signaling pathways, being common to both biotic and abiotic stresses [66].

4.4 REDOX METABOLISM

Saline and water stress are closely related to oxidative stress, causing the production of reactive oxygen species (ROS) such as superoxide radicals (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH^\bullet) whose may compromise cellular redox balance, resulting in oxidative damage and cell disruption [67]. In this sense, the production of antioxidant systems to combat these ROS is activated in the stressful condition in order to minimize the damage. The antioxidant system involved in this process includes the glutaredoxin system (Grx) and CAT. Through the result of this work it was possible to observe that the different genotypes presented different response pathways in redox metabolism. While sensitive-like *J. curcas* genotype showed exclusive accumulation of Grx (spot 198, Table 2), the tolerant-like *J. curcas* genotype accumulated CAT (spot 231, Table 1), both enzymes act on dismutation of H_2O_2 . CAT is an enzyme present in peroxisomes, acting on dismutation of H_2O_2 formed in these organelles, mainly as a result of alternative energy pathways such as photorespiration. Winter and Holtum [68] presented that *J. curcas* have CAM-like photosynthesis, but these authors show that C3 photosynthesis is the principal pathway of carbon fixation in *J. curcas*, corroborating the previous study of Fan, Li [69]. Unlike CAT, Grxs are proteins that are part of the glutathione cycle, which is a NADPH-dependent pathway [70]. Thus, decreased NADPH production under stress may compromise this antioxidant pathway, suggesting that the sensitive-like *J. curcas* genotype presents greater activation of a less efficient pathway in eliminating ROS when compared to CAT activity found in tolerant-like *J. curcas* genotype.

4.5 SIGNALING

Signaling to salt stress is related to the transduction of cellular signals, including ionic, osmotic, detoxification and coordination of cell cycle and expansion [2]. Ca^{2+} -dependent signaling network is an ionic pathway which may be induced by salt stress, and is closely related to cellular modulation of Na^+/K^+ ratio [71], as well as changes in intracellular $[Ca^{+2}]$, triggered by increased ABA concentration, playing an pivotal role in signaling pathways against pathogens, disease and stress responses [72]. This signaling pathway was activated in both *J. curcas* genotypes, in which calcium-binding proteins (CML45) were identified (spot 245, Table 1 and spot 166 and 180, Table 2). In fine tune of these, the sensitive-like *J. curcas* genotype presented higher accumulation of calcium dependent kinase (CDPK) 20 (spot 16, Table 2). In accord of Li, Wang [72], CDPK genes respond to high concentrations of ROS such as H_2O_2 . This response, together with the higher accumulation of CDPK in sensitive-like *J. curcas* genotype, may corroborate the hypothesis that the

tolerant-like *J. curcas* genotype presented higher accumulation of intracellular ROS due to saline stress, a concentration more efficiently controlled by the action of antioxidant enzymes such CAT. Both genotypes also presented type 4 cell wall-associated kinase receptors (spot 246 and 271, Table 1 and spot 36 and 184, Table 2), which are related to the first line of stress factor “sensors” located in the apoplast [73]. Its role in signaling against abiotic stress is not yet fully understood; however, in *A. thaliana* some cell-membrane-receptors may undergo a conformational change initiating signaling cascades through autophosphorylations when exposed to salt stress [74]. Changes in cell wall and membrane conformation are common events in cells under osmotic shock, and may be related to the perception of this type of stimulus, corroborating the experimental conditions of this study.

4.6 PROTEIN TRANSLOCATION

Regarding to protein translocation, the tolerant-like *J. curcas* genotype shows larger accumulation of import translocase protein, located in the mitochondrial inner membrane, TIM50 subunit (spot 238, Table 1). This subunit is part of a protein complex (TIM17:23), which is primarily responsible for translocation of most of the mitochondrial proteome [75].

Evidence indicates that these outer membrane (TOM) and inner membrane (TIM) proteins are also involved in other functions, such as maintaining mitochondrial morphology, regulation of fission and fusion of the organelle and recruitment of antiapoptotic and autophagy proteins [75]. In this sense, the efficiency in intracellular transport is an important factor in maintaining cellular structure and ensuring homeostasis under oxidative stress. Thus, the greater accumulation of these mitochondrial pathways in tolerant-like *J. curcas* genotype may indicate related metabolic efficiency, mainly regarding the transport of substrate for cellular respiration, presenting greater stabilization and integrity of mitochondria, contributing to homeostasis and cell cycle event. Higher protein-related gene expression from the mitochondrial import system following exposure to biotic and abiotic stress conditions has also been reported in tolerant *A. thaliana* plants [76]. Such possibility is remarkably important in plants under osmotic stress, since several adjustment routes such as synthesis of osmoregulators, osmoprotectors and amino acids may occur in mitochondria.

4.7 RNA ASSEMBLY OR FUNCTION

Some stress-mediated mechanisms are activated via metabolic pathways and coordinated by multiple signals involving plant hormones, protein kinases, phosphatases, as well as transcription factors [71]. The latter are mainly involved in the ability to alter gene expression, altering the

physiological status and may confer greater adaptability to environmental conditions [77]. In this sense, both *J. curcas* genotypes presented transcription factors responsive to salinity, but the phenotypic efficiency attributed to the tolerant-like *J. curcas* genotype may be related to the greater number and diversity of transcription factors responsive to ABA, favoring greater regulatory capacity in this genotype, as well as greater perception and adjustment to salt stress. In addition to transcription factors, other proteins were categorized into RNA metabolism and gene expression regulation in both genotypes. Among these, Maturase K (MATK) (spot 107, Table 1 and spot 40, Table 2) is an important plastid protein that is involved in the pre-mRNA splicing process for mature mRNA. The presence of MATK has already been mentioned to be involved in the response to saline condition [78], inducing tolerance for playing a pivotal role in post-transcriptional regulation. Despite the presence of this protein in both *J. curcas* genotypes, it is worth noting that the difference in molecular mass and isoelectric point between the MATKs identified in the genotypes suggests the formation of isoforms or modifications to these proteins, which may have favored the tolerant-like *J. curcas* genotype in the salinity responsibility. These data are corroborated by the significant enrichment found in the proteins involved in ribonucleotide metabolism processes.

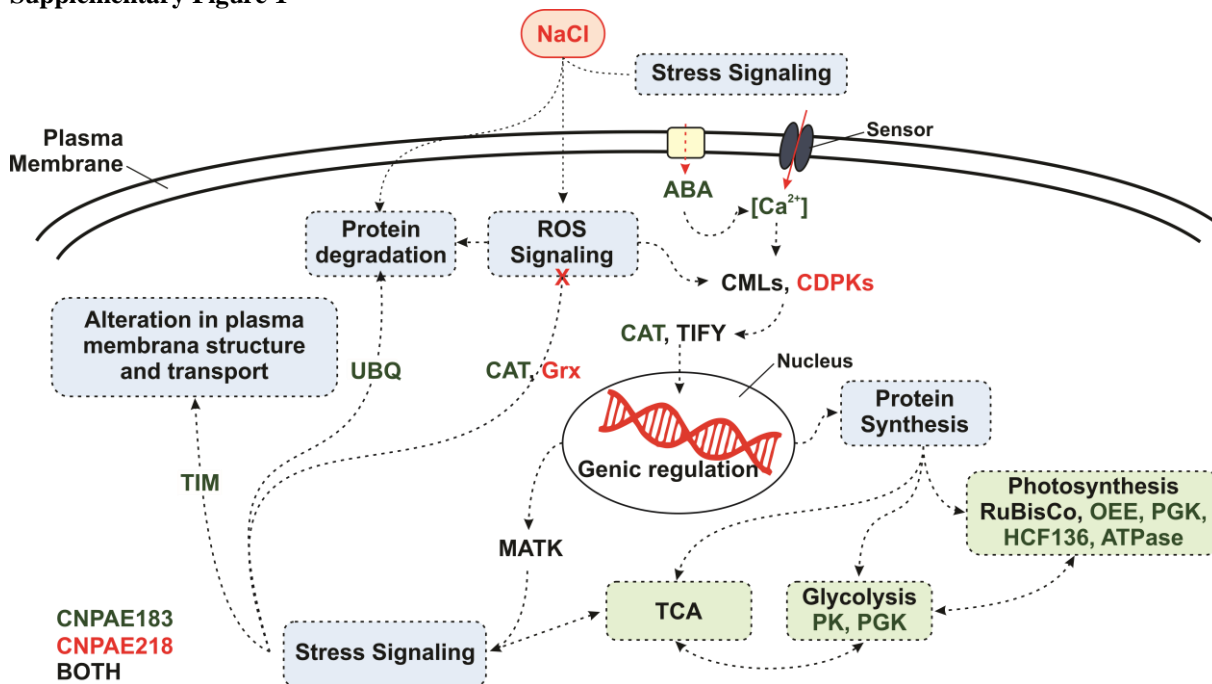
5 CONCLUSIONS

The *J. curcas* proteome reveals evidence of major molecular processes regulated by the salinity, which appear to be genotype-dependent. Thus, the differential proteomic analysis data revealed that the tolerant-like *J. curcas* genotype (*i.e.*, CNPAE183) showed proteins of different pathways related to the salinity response, including production of antioxidant enzymes, as well as signaling and stress regulation pathways, especially with ABA-responsive pathway (more details, see Supplementary Figure S1). In addition, the higher physiological efficiency of CNPAE183 is also due to its ability to produce pivotal enzymes from different energy and metabolic pathways, such as photosynthesis and glycolysis, ensuring its development. Among the key step-related proteins associated with increased tolerance of CNPAE183 are ATPase, protein kinase, and calcium signaling pathway-related proteins, whose metabolic role should be better studied. These results help to understand the physiological and molecular responses associated with the greater tolerance of *Jatropha curcas* to salt stress.

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Supplementary Figure 1



Supplementary Figure S1. Model proposed for gene modulation pathway in response to NaCl in two different genotypes (CNPAE183, and CNPAE218) of *Jatropha curcas* subjected to 48 hours of 750 mM NaCl. ABA, Abscisic acid. CMLs, Calcium-binding proteins. CDPKs, Calcium-dependent protein kinase. MYB, MYB Transcription factor family. TIFY, TIFY Transcription factor family. RuBisCo, Ribulose 1,5-Bisphosphatase Carboxylase-Oxygenase. OEE, Oxygen-evolving protein. PGK, Phosphoglycerate kinase. HCF136, Photosystem II Assembly / Stability Factor. ATPase, ATP Synthase. PK, Piruvate kinase. MATK, Maturase K. TIM, Translocase of the mitochondrial inner membrane. UBQ, Ubiquitin transferase. CAT, Catalase. Grx, Glutaredoxin

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