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# 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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**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 reprograming 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 séssil, 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, purificas 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

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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 interferes 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 (8º02'59" S; 34º56'55" 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 20$^{TM}$ 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 3$^{rd}$ healthy leaf was harvested and flash-frozen in liquid nitrogen and then stored at -80ºC 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 µl) 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 µg 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 µL 0.1% trifluoroacetic acid (TFA). For each reading cycle, 2 µL of the sample were mixed with 2 µL of α-cyano-4-hydroxycinnamic acid (Sigma-Aldrich Chemical Co, Darmstadt, Germany, part number C8982) in ACN and 3% TFA, and 2 µL 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.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

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0.9554 to sensitive-like genotype. These correlation coefficients allowed us to compared between genotypes under salinity and the identification of DAPs. As expected, we identified 145 DAPs, where is possible identified 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.

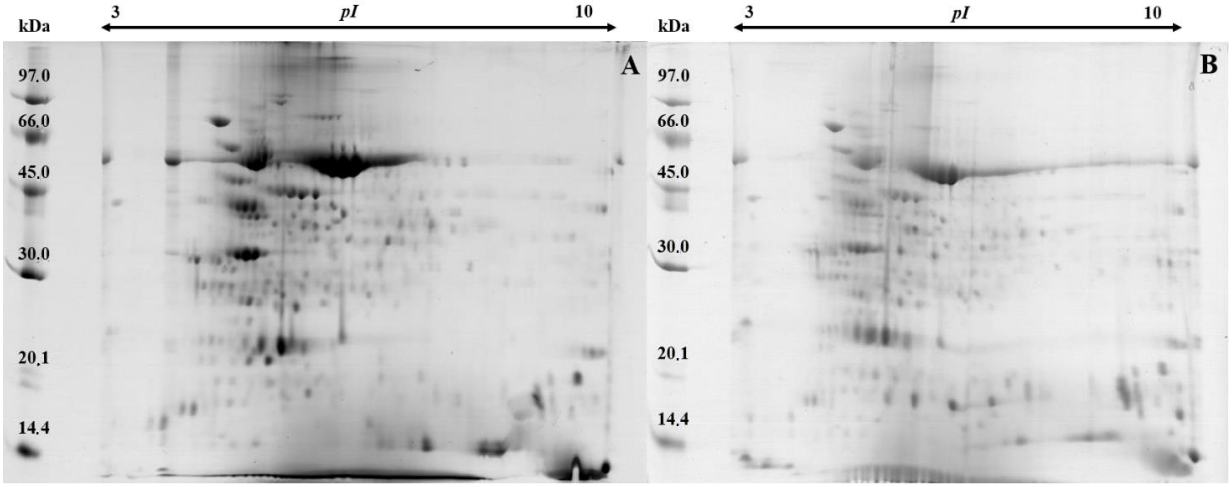

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Table 1. Proteins identification of the CNPAE183 genotype of *Jatropha curcas* L. subjected to 48 hours of 750 mM NaCl contrasted to CNPAE218 as reference. Annotation from PMF;MALLDI-TOF-MS.

| 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 | *Jatropha curcas* | 1.548 |
| 60 | 0.0078 | Proteins in the Photosynthetic Oxygen-Evolving Complex, isoform 1 | A0A067KA30 | 91 | 32,980 | 35,314 | 4.94 | 5.87 | *Jatropha curcas* | 1.313 |
| 84 | 0.0264 | Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase isoform X1 Chl | A0A067L8Y9 | 84 | 41,785 | 52,211 | 4.83 | 5.56 | *Jatropha curcas* | 2.422 |
| 86 | 0.0333 | Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase isoform X1 Chl | A0A067L8Y9 | 112 | 41,534 | 52,211 | 4,93 | 5,56 | *Jatropha curcas* | 2.834 |
| 100 | 0.0476 | Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase large subunit | B1NWF7 | 183 | 51,464 | 53,087 | 6.23 | 6.09 | *Jatropha curcas* | 1.148 |
| 112 | 0.0072 | Chloroplast ATP Synthase subunit beta | COLE81 | 119 | 55,182 | 53,278 | 4.98 | 5.10 | *Jatropha curcas* | 1.392 |
| 115 | 0.00074 | ATP Synthase subunit alfa | COLE59 | 106 | 55,407 | 55,484 | 5.17 | 5.28 | *Jatropha curcas* | 1.704 |
| 118 | 0.0324 | ATP Synthase subunit beta | COLE81 | 118 | 54,007 | 53,278 | 5.07 | 5.10 | *Jatropha curcas* | 1.761 |
| 143 | 0.0312 | Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase large subunit | COLE82 | 204 | 56,097 | 53,087 | 5.65 | 6.09 | *Jatropha curcas* | 1.600 |
| 144 | 0.0342 | Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase large subunit | COLE82 | 64 | 54,923 | 53,087 | 5.75 | 6.09 | *Jatropha curcas* | 1.693 |
| 146 | 0.0462 | Photosystem II Assembly / Stability Factor (HCF136) | A0A067L4D0 | 58 | 38,351 | 43,076 | 5.14 | 7.08 | *Jatropha curcas* | 2.918 |
| 209 | 0.0139 | Chloroplast ATP Synthase subunit alfa | COLE59 | 97 | 65,046 | 55,484 | 5.20 | 5.28 | *Jatropha curcas* | * |
| 216 | 0.0013 | Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase small subunit | D6BR54 | 98 | 14,535 | 20,473 | 8.14 | 9.06 | *Jatropha curcas* | * |
| 227 | 0.0002 | Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase large subunit | A0A199U950 | 70 | 29,612 | 37,029 | 5.09 | 8.50 | *Manihot esculenta* | * |
| 278 | 0.0025 | Peroxisomal (S)-2-Hydroxy Acid Oxidases | A0A067L0G9 | 82 | 41,789 | 40,566 | 9.68 | 9.31 | *Jatropha curcas* | * |
| 279 | 0.0127 | Peroxisomal (S)-2-Hydroxy Acid Oxidases | A0A067L0G9 | 81 | 36,257 | 40,566 | 9.16 | 9.31 | *Jatropha curcas* | * |
| 291 | 0.0000 | Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase small subunit | D6BR54 | 89 | 14,833 | 20,473 | 7.17 | 9.06 | *Jatropha curcas* | * |
| **Glycolysis** | | | | | | | | | | |
| 92 | 0.0067 | Phosphoglycerate Kinase | A0A067JME5 | 71 | 43,274 | 50,648 | 5.86 | 8.41 | *Jatropha curcas* | 1.355 |
| 123 | 0.0105 | Phosphoglycerate Kinase | A0A067JME5 | 68 | 43,411 | 50,648 | 5.51 | 8.41 | *Jatropha curcas* | 1.512 |
| 282 | 0.0106 | Pyruvate Kinase | B9S7Y4 | 67 | 27,201 | 64,159 | 6.97 | 6.09 | *Ricinus communis* | * |
| **Mitochondrial Electron Transport Chain / ATP synthase** | | | | | | | | | | |
| 111 | 0.0400 | ATP Synthase subunit beta | A0A067KFH8 | 72 | 53,924 | 60,287 | 5.35 | 6.13 | *Jatropha curcas* | 2.747 |
| 131 | 0.0325 | ATP Synthase | C1MNM6 | 81 | 23,614 | 60,369 | 6.23 | 6.99 | *Micromonas pusilla CCMP1545* | 1.365 |
| 211 | 0.0020 | ATP Synthase subunit beta | A0A067KFH8 | 83 | 54,719 | 60,287 | 5.29 | 6.13 | *Jatropha curcas* | * |
| **Amino acid metabolism** | | | | | | | | | | |
| 284 | 0.0021 | Chloroplast Argininosuccinate Synthase | Q2QVC1 | 65 | 22,137 | 38,967 | 4.56 | 5.90 | *Oryza sativa Japonica Group* | * |
| **Secondary metabolism** | | | | | | | | | | |
| 42 | 0.0095 | 1-Deoxy-D-Xylolose-5-Phosphate Synthase | Q38854 | 55 | 29,068 | 77,468 | 4.76 | 7.04 | *Arabidopsis thaliana* | 1.488 |
| **Plant pathogen signal** | | | | | | | | | | |
| 80 | 0.0084 | Defensin-like 257 Protein | Q2V3S8 | 56 | 40,253 | 9,329 | 7.38 | 5.21 | *Arabidopsis thaliana* | 2.349 |
| 94 | 0.0002 | Defensin-like 257 Protein | Q2V3S8 | 56 | 43,963 | 9,329 | 5.37 | 5.21 | *Arabidopsis thaliana* | 4.398 |
| 145 | 0.0140 | TMV Resistance Protein N, isoform 2 | A0A067JI31 | 59 | 48,572 | 78,345 | 4.77 | 7.05 | *Jatropha curcas* | 1.900 |
| 236 | 0.0000 | RING E3-E2-Ubiquitin Transferase | B9SBC6 | 61 | 80,171 | 116,067 | 6.19 | 5.68 | *Ricinus communis* | * |
| **Redox Protein** | | | | | | | | | | |
| 231 | 0.0001 | Catalase | A0A067L5U2 | 78 | 55,418 | 57,081 | 7.49 | 7.10 | *Jatropha curcas* | * |
| **Metabolism** | | | | | | | | | | |

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

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

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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 | *Jatropha curcas* | 3.096 |
| 179 | 0.0015 | Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase large subunit | COLE82 | 140 | 56,131 | 53,087 | 3.16 | 6.09 | *Jatropha curcas* | * |
| 172 | 0.0016 | Carbonic Anhydrase | A0A067LLS5 | 85 | 28,177 | 37,150 | 6.76 | 8.07 | *Jatropha curcas* | * |
| **Mitochondrial Electron Transport Chain / ATP synthase** | | | | | | | | | | |
| 170 | 0.0058 | ATP Synthase | C1MNM6 | 81 | 18,854 | 60,369 | 8.90 | 6.99 | *Micromonas pusilla CCMP154* | * |
| **Hormones** | | | | | | | | | | |
| 204 | 0.0002 | Cytochrome P450 Monooxygenase | Q0JL28 | 69 | 31,142 | 17,157 | 9.47 | 9.62 | *Oryza sativa Japonica Group* | * |
| **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 | *Manihot esculenta* | 3.892 |
| 198 | 0.0022 | Glutaredoxin C13 | Q0IRB0 | 54 | 33,848 | 11,580 | 4.26 | 8.57 | *Oryza sativa Japonica Group* | * |
| **Proteins evolved in RNA assembly or function** | | | | | | | | | | |
| 40 | 0.0449 | Maturase K | Q8HQQ6 | 56 | 29,130 | 61,576 | 4.51 | 9.40 | *Pinus pinaster* | 1.598 |
| 77 | 0.0079 | GATA (19) Transcription Factor | B8AR30 | 53 | 39,860 | 29,479 | 4.88 | 6.14 | *Oryza sativa Indica Group* | 1.544 |
| 162 | 0.0000 | Manes.S021100_RPOL-1 Like Protein | A0A199UC42 | 59 | 33,550 | 29,201 | 4.57 | 9.64 | *Manihot esculenta* | * |
| 196 | 0.0012 | Protein with a Conserved REM8-like B3 domain | Q8H2D1 | 58 | 27,481 | 52,448 | 6.57 | 6.25 | *Arabidopsis thaliana* | * |
| 197 | 0.0074 | Remorin with a Conserved C domain | Q0JA18 | 71 | 28,098 | 31,637 | 6.49 | 8.14 | *Oryza sativa Japonica Group* | * |
| **Proteins evolved in DNA assembly or function** | | | | | | | | | | |
| 28 | 0.0011 | DNA Polimerase | A0A067JPG7 | 62 | 23,283 | 124,988 | 5.15 | 8.35 | *Jatropha curcas* | 2.593 |
| **Signaling** | | | | | | | | | | |
| 16 | 0.0118 | Calcium-Dependent Protein Kinase 20 | Q84SL0 | 50 | 18,315 | 62,768 | 5.44 | 7.00 | *Oryza sativa Japonica Group* | 2.187 |
| 36 | 0.0003 | Cell Wall-Associated Receptor-Like Protein Kinase 4 | Q9S9M2 | 56 | 27,808 | 86,269 | 5.42 | 5.66 | *Arabidopsis thaliana* | 2.547 |
| 166 | 0.0004 | Calcium-Binding Protein CML45 | A0A067K9U4 | 60 | 20,108 | 23,016 | 5.00 | 4.71 | *Jatropha curcas* | * |
| 176 | 0.0007 | BTB/POZ Domain At3g08570 Protein | Q9C9Z7 | 60 | 55,579 | 70,320 | 7.88 | 5.67 | *Arabidopsis thaliana* | * |
| 246 | 0.0033 | Cell Wall-Associated Receptor-Like Protein Kinase 4 | Q9S9M2 | 55 | 35,692 | 86,269 | 5.82 | 5.66 | *Arabidopsis thaliana* | * |
| 180 | 0.0005 | Calcium-Binding Protein CML45 | A0A067K9U4 | 62 | 62,111 | 23,016 | 4.56 | 4.71 | *Jatropha curcas* | * |
| 184 | 0.0032 | Cell Wall-Associated Receptor-Like Protein Kinase 4 | Q9S9M2 | 56 | 77,105 | 86,269 | 6.08 | 5.66 | *Arabidopsis thaliana* | * |
| **Cell Cycle Event** | | | | | | | | | | |
| 206 | 0.0041 | DUF1731 GH43 superfamily Protein | A0A067JUJ5 | 60 | 49,514 | 25,290 | 4.59 | 8.44 | *Jatropha curcas* | * |
| **Development** | | | | | | | | | | |
| 17 | 0.0001 | SHI Protein | Q9SJT8 | 55 | 18,362 | 20,314 | 5.82 | 9.39 | *Arabidopsis thaliana* | 5.233 |
| 27 | 0.0085 | S-Adenosylmethionine-Dependent Methyltransferase | E5D1F9 | 67 | 23,449 | 24,924 | 5.55 | 5.35 | *Jatropha curcas* | 1.603 |
| 158 | 0.0011 | CCT Protein Family | Q8LDM8 | 66 | 18,110 | 23,363 | 6.98 | 4.95 | *Arabidopsis thaliana* | * |
| 160 | 0.0032 | CCT Protein Family | Q8LDM8 | 66 | 37,029 | 23,363 | 8.12 | 4.95 | *Arabidopsis thaliana* | * |
| **Other Proteins** | | | | | | | | | | |
| 4 | 0.0185 | Bark Storage Protein A | A0A067KNP8 | 61 | 13,300 | 35,198 | 9.84 | 7.82 | *Jatropha curcas* | 1.474 |
| 25 | 0.0020 | Bark Storage Protein A | A0A067KNP8 | 63 | 23,115 | 35,198 | 5.40 | 7.82 | *Jatropha curcas* | 1.470 |
| 26 | 0.0009 | Pentatricopeptide repeat-containing protein At1g32415, mitochondrial | P0C7R0 | 56 | 23,878 | 86,159 | 4.39 | 6.01 | *Arabidopsis thaliana* | 2.292 |

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| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 54 | 0.0030 | Ribosomal – RNA *N*-glycosidase | D0PWG0 | 70 | 33,249 | 32,940 | 5.59 | 8.54 | *Jatropha curcas* | 5.676 |
| 55 | 0.0173 | ULP1 - Ubiquitin-like-specific protease 1 | Q5JMY1 | 67 | 33,511 | 28,368 | 5.42 | 9.62 | *Oryza sativa Japonica Group* | 4.358 |
| 56 | 0.0246 | dCTP pyrophosphatase 1 Similar to OSIGBa0111I14.3 Protein | Q01L63 | 66 | 33,093 | 18,852 | 5.19 | 5.29 | *Oryza sativa Indica Group* | 1.967 |
| 161 | 0.0009 | 2C 67 Protein Phosphatase | Q0J2R1 | 51 | 34,610 | 39,822 | 6.10 | 6.37 | *Oryza sativa Japonica Group* | * |
| 164 | 0.0123 | UBX (10) Domain Protein | A0A067K7Q0 | 66 | 19,798 | 41,957 | 5.51 | 8.58 | *Jatropha curcas* | * |
| 165 | 0.0034 | Chloroplast 50 S Ribosomal L16 Protein | P08528 | 56 | 32,946 | 15,635 | 5.28 | 11.56 | *Zea mays* | * |
| 159 | 0.0246 | Ribosomal – RNA *N*-glycosidase | D0PWG0 | 66 | 32,589 | 32,940 | 6.10 | 8.54 | *Jatropha curcas* | * |
| 167 | 0.0030 | Cytidine/Guanosine-2'-O)-Methyltransferase tRNA | A0A067JWU0 | 71 | 18,557 | 35,215 | 4.90 | 6.02 | *Jatropha curcas* | * |
| 168 | 0.0021 | Receptors like Calcium/Calmodulin-Dependent Protein Kinase II | Q8VZJ9 | 57 | 16,324 | 46,374 | 6.82 | 9.26 | *Arabidopsis thaliana* | * |
| 171 | 0.0000 | RNF170 RING-HC Domain Protein | A0A067KTH4 | 58 | 24,690 | 28,318 | 3.22 | 8.80 | *Jatropha curcas* | * |
| 173 | 0.0010 | Plant Storage Protein, like 2 | A0A067KXQ9 | 83 | 30,830 | 32,465 | 9.63 | 7.88 | *Jatropha curcas* | * |
| 175 | 0.0000 | Protein without Conserved Domain | A0A067L4W9 | 59 | 36,439 | 45,564 | 4.86 | 4.72 | *Jatropha curcas* | * |
| 187 | 0.0000 | Replication Factor C / DNA Polymerase III Gamma-Tau Subunit | B9S6E2 | 61 | 20,242 | 88,976 | 7.21 | 9.63 | *Ricinus communis* | * |
| 205 | 0.0067 | Hypothetical Protein JCGZ_06028 | A0A067JK77 | 61 | 39,174 | 22,787 | 5.29 | 5.69 | *Jatropha curcas* | * |
| **Unknown** | | | | | | | | | | |
| 2 | 0.0302 | No match | - | - | 23,885 | - | 4.61 | - | *Jatropha curcas* | 2.663 |
| 3 | 0.0003 | No match | - | - | 13,508 | - | 3.26 | - | *Jatropha curcas* | 7.930 |
| 9 | 0.0019 | No match | - | - | 16,393 | - | 9.77 | - | *Jatropha curcas* | 6.736 |
| 45 | 0.0451 | No match | - | - | 29,870 | - | 5.75 | - | *Jatropha curcas* | 1.565 |
| 67 | 0.0027 | No match | - | - | 37,537 | - | 5.86 | - | *Jatropha curcas* | 2.043 |
| 69 | 0.0230 | No match | - | - | 37.454 | - | 5.71 | - | *Jatropha curcas* | 1.645 |
| 132 | 0.0131 | No match | - | - | 19,750 | - | 8.88 | - | *Jatropha curcas* | 2.831 |
| 150 | 0.0155 | No match | - | - | 36,537 | - | 5.27 | - | *Jatropha curcas* | 1.695 |
| 163 | 0.0441 | No match | - | - | 60,341 | - | 4.83 | - | *Jatropha curcas* | * |
| 169 | 0.003 | No match | - | - | 17,770 | - | 6.40 | - | *Jatropha curcas* | * |

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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 miscellaneaous 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.

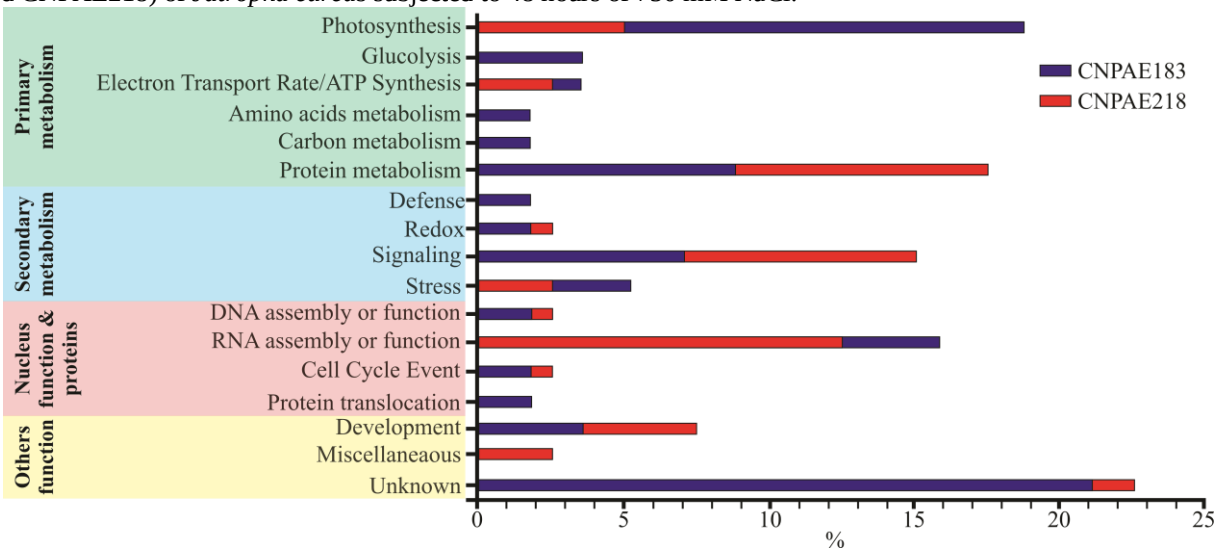


Table 3. Biological processes significantly enriched in *J. curcas* genotype CNPAE183 compared to model species *Arabidopsis thaliana.*

| **CNPAE183** | | |
|---|---|---|
| Biologic Process | Number of genes involved | *p-value*_FDR |
| Ribonucleotide Metabolic Process | 4 | 9.30 E-06 |
| ADP Metabolic Process | 3 | 1.26 E-05 |
| Metalloproteis 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 $CO_2$ into carbonic acid ($HCO_3^-$). 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 [$CO_2$] 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 Kreb'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 $CO_2$ 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* genoptype, 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].

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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 ($OH^•$) 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 glutarredoxin 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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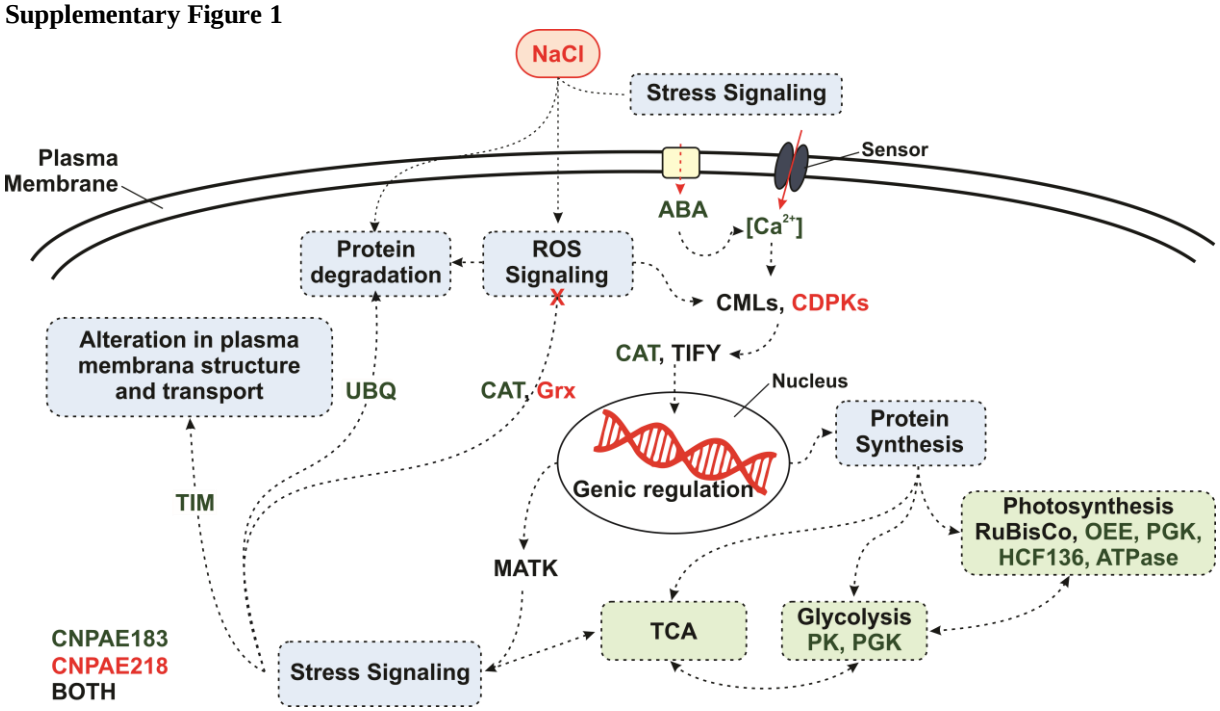
**Supplementary Figure 1**




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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