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MARISLANE CARVALHO PAZ DE SOUZA

TRANSCRIPTÔMICA DE *Jatropha curcas* L. EM RESPOSTA AO ESTRESSE SALINO COM DESENVOLVIMENTO DE MARCADORES MOLECULARES FUNCIONAIS PARA USO NO MELHORAMENTO GENÉTICO

RECIFE

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Tese apresentada ao Programa de Pós-Graduação em Biotecnologia, área de concentração Biotecnologia em Agropecuária, da Universidade Federal de Pernambuco, como requisito parcial para obtenção do título de Doutora em Biotecnologia.

Área de concentração: Biotecnolgia em Agropecuária
Orientador: Prof°. Dr. Éderson Akio Kido

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“Suba o primeiro degrau com fé. Não é necessário que você veja toda a escada. Apenas dê o primeiro passo. ”

Martin Luther King

RESUMO

A salinidade do solo é uma das principais restrições de produção para as culturas agrícolas, especialmente em *Jatropha curcas* (pinhão-manso). Analisar o mecanismo molecular sob estresse salino é fundamental para o desenvolvimento de plantas tolerantes ao estresse. O sistema radicular tem papel importante no enfrentamento da mudança osmótica impactada pela salinidade e poucos estudos de transcriptoma relacionados ao estresse salino em *J. curcas* foram relatados anteriormente. No entanto, pouco se sabe sobre os genes responsivos a esse estresse em pinhão-manso. O presente trabalho analisou o transcriptoma da raiz de dois acessos de *J. curcas* afim de identifcar genes associados à tolerância a salinidade utilizando 150 mM NaCl por três horas. Em seguida, gerou-se o conjunto de dados do transcriptoma baseado em RNA-Seq com raízes de *J. curcas* tratadas com 150mM NaCl por três horas, juntamente com controles não tratados em condição de solo, usando os acessos Jc171 e Jc183. Valores restrigentes de *p-value* < 0,0001, Log_2 FC ≥ 1 or ≤ -1 FDR ≤ 0,005 foram usados como limiares para avaliar a significância da expressão gênica diferencial. Identificamos 145.422 transcritos, desses, 4.646 foram Unigenes diferencialmente expressos (DEGs) no acesso sensível ao sal, Jc171 [2.781 induzidos (UR) e 1.865 reprimidos (DR)] e 57 no acessos tolerante, Jc183 (40 UR e 17 DR). OS DEGs foram categorizados por MapMan em diversos processos metabólicos, inclusive em vias com significativo impacto na resposta salina, incluindo: metabolismo de fitohormônio, metabolismo de carboidrato, metabolismo de aminoácido, metabolismo de lipídeo, metabolismo redox e processo de metabólito secundário. Usamos RT-qPCR para confirmar os padrões de expressão de nove DEGs relacionados ao sal de Jc171 e Jc183. Mudanças na expressão gênica também são causadas por fatores de transcrição (FTs), que são proteínas que regulam a ativação / supressão da expressão gênica, e desempenham papéis cruciais na resposta das plantas ao estresse salino. Neste estudo, um conjunto de 148 DEGs de FTs (78 UR e 70 DR) foram identificados. Entre eles, oito genes TFs (RAP2-3, RAV1, ERF9, DREB1H, ZAT12, PTI5, BZIP4 e MYB) tiveram a resposta confirmada por RT-qPCR. Em conclusão, nossa análise global do transcriptoma identificou genes envolvidos na resposta precoce a salinidade em *Jatropha*. Os DEGs identificados serão úteis no desenvolvimento de marcadores funcionais para uso do melhoramento genético da espécie.

Palavras-chave: Pinhão-manso. Salinidade. Bioinformática.

ABSTRACT

Soil salinity is one of the main production constraints for agricultural crops, especially in *Jatropha curcas* (Physic nut). Analyzing the molecular mechanism under saline stress is fundamental for the development of stress tolerant plants. The root system plays an important role in coping with salinity-impacted osmotic change, and few studies of transcriptome related to saline stress in J. curcas have been previously reported. However, little is known about the genes responsive to this stress on jatropha curcas. The present work analyzed the root transcriptome of two J. curcas accessions in order to identify genes associated with salinity tolerance using 150 mM NaCl for three hours. Next, the RNA-Seq-based transcriptome dataset with J. curcas roots treated with 150mM NaCl was generated for three hours, along with untreated controls in soil condition, using accessions Jc171 and Jc183. Stringent values of p-value <0.0001, Log2 FC $\geq$ 1 or $\leq$ -1 FDR $\leq$ 0.005 were used as thresholds to assess the significance of differential gene expression. We identified 145,422 transcripts, of which 4,646 were differentially expressed Unigenes (DEGs) in salt sensitive access, Jc171 [2,781 induced (UR) and 1,865 suppressed (DR)] and 57 in tolerant accesses, Jc183 (40 UR and 17 DR). DEGs have been categorized by MapMan into several metabolic processes, including pathways with significant impact on saline response, including: phytohormonium metabolism, carbohydrate metabolism, amino acid metabolism, lipid metabolism, redox metabolism, and secondary metabolite process. We used RT-qPCR to confirm the expression patterns of nine Jc171 and Jc183 salt-related DEGs. Changes in gene expression are also caused by transcription factors (TFs), which are proteins that regulate activation / suppression of gene expression and play crucial roles in the response of plants to salt stress. In this study, a set of 148 TF FGs (78 UR and 70 DR) were identified. Among them, eight TFs genes (RAP2-3, RAV1, ERF9, DREB1H, ZAT12, PTI5, BZIP4 and MYB) had the response confirmed by RT-qPCR. In conclusion, our global transcriptome analysis identified genes involved in the early response to salinity in Jatropha. The identified DEGs will be useful in the development of functional markers to use the genetic improvement of the species.

Keywords: *Jatropha curcas*. Salinity. Bioinformatics.

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LISTA DE ABREVIATURAS E SIGLAS

cDNA	DNA complementar
DEG	*Differentially Expressed Gene* (gene diferencialmente expresso)
DNA	Ácido desoxirribonucleico
EST	*Expressed Sequence Tag* (Etiqueta de sequência expressa)
Jc	*Jatropha curcas*
miRNA	microRNA
mM	Milimolar
NaCl	Cloreto de sódio
p / v	Peso/volume
PCR	*Polymerase chain reaction* (reação em cadeia da polimerase)
RNA	Ácido ribonucleico
RNASeq	Sabordagem para analisar o transcriptoma utilizando sequenciamento de nova geração
RTqPCR	*Real Time Quantitative PCR (PCR quantitativa em tempo real)*
dS/m	DeciSiemens por metro
ton ha^1	Tonelada por hectare
cm	Centímetro
K^+	Potássio
KUP	Família de transportadores de potássio (K +)
HAK	Família de transportadores de potássio (K +)
KT	Família de transportadores de potássio (K +)
HKT2	Transportador K + de alta afinidade
Na^+	Sódio
Cl	Cloreto
ROS	Espécies reativas de oxigênio
CO_2	Dióxido de carbono
Mbp	Mega pares de base
µm	Micrometro
Mb	Mega base
RNAi	RNA interferente
Kpb	kilo pares de bases
N50	Estatística que define a qualidade da montagem em termos de *contigs*
SSR	*Simple Sequence Repeats*
QTL	*Quantitative trait locus*
NCBI	*National Center for Biotechnology Information*
SNP	*Single nucleotide polymorphism*
CEes	Condutividade elétrica do extrato de saturação do solo

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1 INTRODUÇÃO

Jatropha curcas L. (pinhão-manso), é um arbusto/pequena árvore, de uso múltiplo, pertencente à família Euphorbiaceae a mesma da mamona (*Riccinnus communis* L.) e mandioca (*Manihot esculenta* Crantz). Possui características atraentes como a sua capacidade de resistir à seca e crescer em solo árido, além do alto teor de óleo de suas sementes, o que a torna uma das oleaginosas mais promissoras para a produção de biodiesel. Em regiões áridas e semiáridas, marcadas pela escassez de chuvas e elevada evaporação, torna o uso da irrigação predominante em áreas de cultivo agrícola. No entanto, o uso indiscriminado da irrigação com água de baixa qualidade, com altos teores de sais, incluindo o cloreto de sódio, leva ao acúmulo de sais no solo. O efeito adverso do excesso de sais como Na^+ e / ou Cl^- é chamado de estresse salino. Esse estresse afeta todos os principais processos das plantas, como germinação, crescimento, pigmentos fotossintéticos e fotossíntese, relação de água, desequilíbrio de nutrientes, estresse oxidativo e produtividade.

Para continuar desenvolvendo, as plantas implementam uma série de adaptações para aclimatar à salinidade, incluindo alterações fisiológicas e metabólicas para maior tolerância, de acordo com repertório genômico, e principalmente, da maneira como os genes são ativados. *J. curcas* apesar de resistente a seca, não produz sob estresse salino, principalmente devido aos efeitos fitotóxicos na germinação de sementes e no crescimento inicial das plântulas, limitando o seu crescimento e produtividade. Neste contexto, o melhoramento genético de *J. curcas* requer novas metodologias que permitam explorar a variabilidade genética disponível para os programas, assim como identificar genótipos elite e seus genes favoráveis para produção em condições desfavoráveis. Assim, esse estudo tem como objetivo identificar genes associados à tolerância ao estresse salino em *J. curcas* por meio da tecnologia de RNA-Seq para o desenvolvimento de marcadores moleculares funcionais, com uso melhoramento da espécie.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Analisar o perfil global de genes associados à tolerância ao estresse salino em *Jatropha curcas* L. através da técnica RNA-Seq para o desenvolvimento de marcadores moleculares funcionais, com uso melhoramento da espécie.

2.2 OBJETIVOS ESPECÍFICOS

- Gerar perfis de expressão (*in silico*) para transcriptomas RNA-seq de dois acessos de *J. curcas*, comparando duas situacões: sob estresse salino de NaCl (150 mM/ 3h) e condição controle;
- Identificar genes associados à tolerância ao estresse salino, através da análise comparativa de padrões de expressão de transcritos RNA-seq, dos acessos em resposta ao sal;
- Validar a expressão, a partir de uma segunda técnica (RT-qPCR), de um conjunto de genes associados à expressão diferencial *in silico* de transcritos RNA-seq;

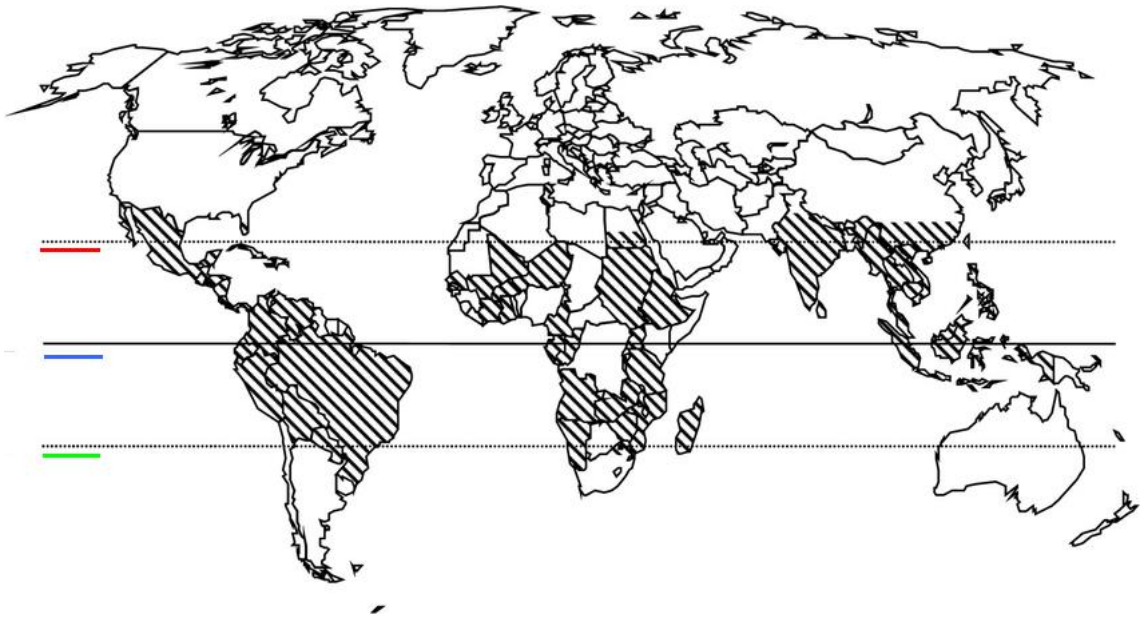
3 REVISÃO BIBLIOGRÁFICA

3.1 INFORMAÇÕES SOBRE *Jatropha curcas* L.

3.1.1 Aspectos gerais: origem, distribuição geográfica e taxonomia

Jatropha curcas L. (pinhão - manso) foi descrita inicialmente pelo botânico sueco Carl Linnaeus, no ano de 1753. O nome do gênero *Jatropha* deriva do grego *iatrós* (médico) e *trophé* (comida). É membro da grande e diversificada família Euphorbiaceae, que inclui aproximadamente 170 espécies conhecidas, parte delas (Euphorbia*)* reconhecidas pela produção de fitotoxinas (estéres de forbol) e seiva branca (látex) (HELLER, 1996). É uma oleaginosa nativa do México e da América Central, e centro de origem ainda indefinido. Acredita-se que tenha sido disseminada para outros países por navegadores portugueses através das Ilhas de Cabo Verde e Guiné Bissau (VIRGENS et al., 2017). É amplamente distribuída na América Latina, também sendo encontrada na África, Índia e Sudeste Asiático (**Figura 1**; PANDEY et al., 2012). No Brasil, *J. curcas* é encontrada nas as regiões Norte e Nordeste até o Estado do Paraná (ARRUDA et al., 2004).

Figura 1 - **Mapa da distribuição global de *Jatropha curcas***. Extensões hachuradas apresentam as localidades onde a espécie pode ser encontrada. Linhas vermelha, azul e verde representam o Trópico de Câncer, a linha do Equador e o Trópico de Capricórnio, respectivamente.

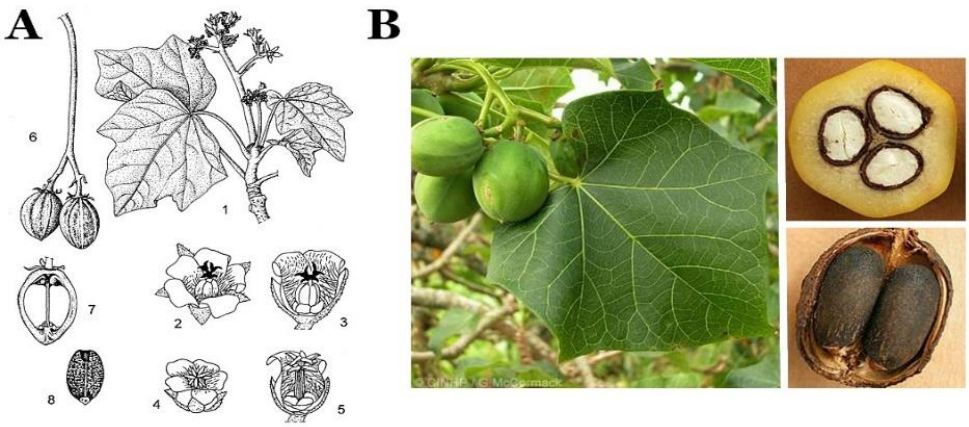


Fonte: Adaptado de King et al., 2009.

A literatura cita três variedades de *J. curcas*: (1) Nicarágua: apresenta poucos frutos por planta, porém grandes, possui baixa ramificação, folhas largas e grandes; (2) Cabo Verde: é a variedade mais comum, produz maior número de sementes e é encontrada em diversos países, provavelmente levada pelos portugueses para a África e Ásia, onde é comumente encontrada; (3) Mexicana: essa variedade tem alguns genótipos que não apresentam estéres de forbol sendo consumida pela população em algumas regiões do México, após processo de torra (HELLER, 1996; HENNING, 2004; BRITTAINE e LUTALADIO, 2010). As variedades recebem o nome das respectivas regiões onde foram cultivadas (Nicarágua, Cabo Verde e Mexicana). O tipo nicaraguense possui baixa ramificação, folhas largas e grandes; enquanto a de Cabo Verde produz maior número de sementes; o tipo mexicano apresenta baixa toxidez, sendo consumida pela população em algumas regiões do México, após processo de torra (HELLER, 1996).

3.1.2. Informações botânicas: da morfologia a características reprodutivas

A planta adulta de *J. curcas* é uma pequena árvore de crescimento rápido, cuja altura pode variar de dois a três metros, podendo alcançar até cinco metros em condições ideais de cultivo. Apresenta crescimento desuniforme, morfologia descontínua em cada internó e dormência induzida por flutuações térmicas e chuvas (HELLER, 1996). As mudas dessa espécie geralmente formam uma raiz pivotante central, quatro raízes laterais e muitas raízes secundárias. Suas folhas (**Figura 2A**) são dispostas alternadamente e variam de seis a 15 cm de comprimento e largura, sendo o tamanho e a forma da folha variável de uma variedade para outra. Além disso, os tecidos vasculares das hastes e ramos contêm látex. Os ramos e caules são vazios e a madeira maciça tem pouco valor (BRITTAINE e LUTALADIO, 2010).



Fonte: Adaptado de Heller, 1996.

Figura 2 - Características morfológicas de *Jatropha curcas*, apresentando ilustração botânica (A); 1. ramo florido; 2. flor feminina; 3. flor feminina aberta; 4. flor masculina; 5. flor masculina aberta; 6. fruto; 7. fruto em corte longitudinal; 8. semente) e **(B)** foto de planta adulta e seu respectivo fruto in natura e em corte transversal.

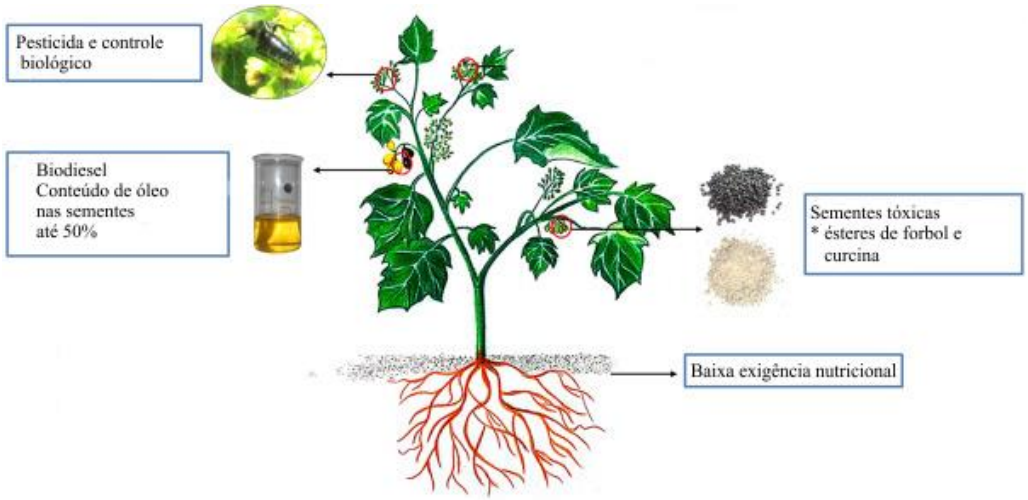
J. curcas é uma planta monoica, possui flores masculinas (**Figura 2A**) e femininas (**Figura 2A**) na mesma planta, sendo as flores masculinas de coloração amarelada, em maior número, localizadas nas extremidades das ramificações, e as femininas, logo abaixo das masculinas. Apresenta polinização cruzada, primordialmente entomófila, entre diferentes flores da mesma planta ou de plantas diferentes, sendo parcialmente autocompatível (VIRGENS et al., 2017; BRITTAINE e LUTALADIO, 2010). O fruto (**Figura 2B**) é seco, deiscente, capsular, ovóide, com diâmetro entre 1,5 cm a 3 cm, trilocular, com uma semente por cavidade. É formado por um pericarpo ou casca dura e lenhosa, inicialmente verde, que passa a amarela, castanha e, por fim, preta, quando atinge a maturação (PESSOA et al., 2012).

3.1.3 *J. curcas* e sua empregabilidade: importância de um vegetal multiuso

J. curcas é considerada uma espécie multiuso podendo ser utilizada como planta de cobertura do solo (mantendo a umidade do solo e diminuindo as perdas por evaporação), na fabricação de sabão e na medicina popular, onde é frequentemente utilizada no tratamento de doenças da pele e reumatismo (FAIRLESS, 2007). Além disso, *J. curcas* produz látex nos ramos que ao ser extraído, assemelha-se a Goma-laca branca, utilizada como tinta de marcação para asfalto, suas estacas são utilizadas como barreira de proteção contra animais de campo (bovinos e caprinos), devido a madeira ser macissa e oca e não servir para lenha (BRITTAINE e LUTALADIO, 2010).

J. curcas, por ser uma espécie multiuso, tem atraído os pesquisadores a explorar seu papel como agente pesticida e como controle biológico de pragas pela atividade dos inimigos naturais (**Figura 3**). Como alternativa viável aos insecticidas químicos, o potencial inseticida do extrato de folhas de *J. curcas* tem sido relatado contra larvas de *Aedes aegypti* e *Culex quinquefasciatus* (RAHUMAN et al., 2008). Djimmy et al. (2016) sugeriram recentemente o controle de pragas de insetos de *J. curcas* explorando inimigos naturais do inseto, *Calidea panaethiopica* (**Figura 3**), um heteróptero polífago da família Scutelleridae, que se alimenta de flores, frutos e sementes de *J. curcas*. As sementes apresentam compostos tóxicos (ésteres de forbol) e fatores antinutricionais de ação tóxica como tripsina, curcina e saponinas (BRITTAINE e LUTALADIO, 2010).

Figura 3 - *Jatropha curcas*: importância de um vegetal multiuso.



Fonte: Adaptado de Mazumdar et al. (2018)

Apenas as sementes da variedade Mexicana não são tóxicas (caracterizada como isenta ou baixo conteúdo de éster de forbol), e podem ser consumidas por humanos após o processamento de torra (*roasting*) (BRITTAINE e LUTALADIO, 2010). *J. curcas* se qualifica como potencial candidato para a produção de biocombustível, pois suas sementes apresentam expressivo conteúdo de óleo (40 - 50%) (**Figura 3**), com uma quantidade relativamente alta de ácidos graxos insaturados, os preferidos pelas indústrias de biodiesel (MAZUMDAR et al., 2018). Além disso, é um espécie de baixa exigência nutricional podendo ser cultivada em solos pouco nutritivos (**Figura 3**).

3.2 SALINIZAÇÃO DOS SOLOS

3.2.1 Alta salinidade: causas, expansão e consequências

A alta salinidade do solo é caracterizada por uma alta concentração de sais solúveis, no qual o NaCl é o sal mais solúvel e difundido. Os solos são classificados como salinos quando a condutividade elétrica (EC) é $\geq$ 4 dS/m (40mM de NaCl) (ACOSTA-MOTOS et al., 2017). Nessa concentração de sal no solo, o crescimento e rendimento da maioria das culturas são significativamente reduzidos. A salinidade do solo é uma das principais restrições ambientais à produção agrícola, afetando milhões de hectares de terra em todo o mundo e custando bilhões de dólares todos os anos (GHEYI et al., 2010). Além das causas naturais (intemperismo de minerais e do solo), a salinização do solo é comumente associada ao desmatamento pela

remoção da vegetação causando problemas de drenagem e consequentemente elevando os níveis de água salgada subterrânea (PEDROTTI et al., 2015).

A área de solos degradados por salinidade e sodicidade ocorre principalmente em consequência do uso inadequado de terras marginais e do manejo inadequado da irrigação e do solo (PEDROTTI et al., 2015). A salinidade é uma condição do solo que ocorre principalmente nas regiões áridas e semiáridas do mundo. Nessas regiões, a precipitação pluviométrica limitada associada à baixa atividade bioclimática, menor grau de intemperização, drenagem deficiente e a utilização de água de má qualidade, condu0zem à formação de solos com alta concentração de sais (HOLANDA, 2007). Estima-se que aproximadamente 7% de toda superfície terrestre apresenta-se salinizada, devido a processos naturais intrínsecos do próprio solo da região e pela atividade antrópica (PEDROTTI et al., 2015).

No Brasil, solos salinos e sódicos ocorrem no Rio Grande do Sul, na região do Pantanal Matogrossense e na região semiárida do Nordeste (RIBEIRO et al., 2003). Em Pernambuco, aproximadamente 20% da área total dos perímetros irrigados encontra-se salinizados (BARROS et al., 2004). A maior parte do perímetro irrigado de Custódia-PE, cessou as atividades agrícolas devido aos problemas de salinidade (OLIVEIRA et al., 2002). A salinização do solo pode provocar inúmeros efeitos ambientais, incluindo a degradação das propriedades físicas e químicas do solo com consequente compactação, e redução no crescimento das plantas (QADIR et al., 2013). Especificamente na região Nordeste, há grandes áreas com solos salinizados, devido ao déficit hídrico e à elevada taxa de evaporação, com maior incidência do problema nas terras mais intensamente cultivadas com o uso da irrigação, nos pólos de agricultura irrigada (SILVA et al., 2011).

As propriedades físicas e químicas do solo, como estrutura, estabilidade dos agregados, dispersão das partículas, permeabilidade e infiltração, pH, teor de nutrientes, capacidade de troca iônica, condutividade elétrica e matéria orgânica são influenciadas pelos tipos de cátions trocáveis (Al^{3+}, Ca^{2+} e Mg^{2+}) presentes no solo. O acúmulo de sais solúveis torna o solo floculado, friável e permeável, enquanto que o aumento da porcentagem de sódio trocável (PST) causado pela substituição dos íons cálcio (Ca^{+2}) e magnésio (Mg^{+2}) presentes no complexo de troca pelo sódio (Na^{+}), pode tornar o solo adensado, compactado em condições de seca, disperso e pegajoso quando encharcado (DIAS e BLANCO, 2010). A predominância de sódio no solo, aumenta a expansão e dispersão das partículas de argila, formando camadas impermeáveis, dificultando o movimento de ar e água (FASSBENDER e BORNEMISZA, 1987). A Percentagem de Sódio Trocável (PST) >15% (CEes > 4 dS/m; PST > 15%; pH < 8,5),

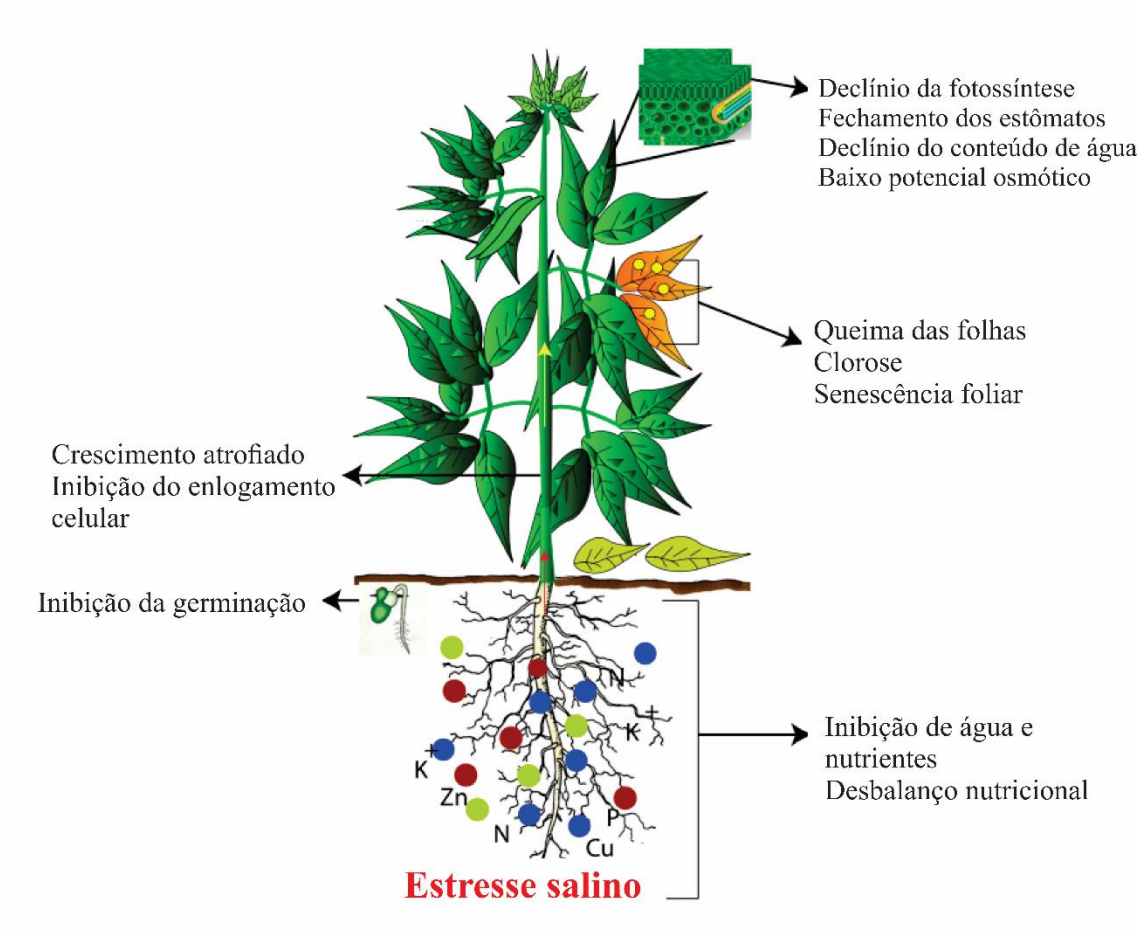
torna a atividade agrícola quase impraticável e antieconômica em solos salino-sódicos, devido ao difícil manejo e por sua recuperação ser bastante dispendiosa (SILVA et al., 2018).

3.2.2 Efeito da alta salinidade nas plantas: impactos e mecanismos de tolerância

As plantas quando submetidas a estresse salino desenvolvem uma série de respostas a fim de reestabelecer a homeostase. Essas respostas podem ser percebidas por alterações a nível fisiológico, molecular e metabólico (PANDEY et al., 2015). Fisiologicamente, as plantas são afetadas de duas maneiras: a primeira é o efeito osmótico, os sais dissolvidos na solução do solo reduzem o seu potencial hídrico. Quanto mais salino for um solo, maior será a energia gasta pela planta para absorver água e com ela os demais nutrientes (**Figura 4**) (ROY et al., 2014); a segunda é o efeito iônico, causado pela toxicidade de determinados elementos principalmente sódio (Na^+) e cloreto (Cl^-), que em alta concentração causa distúrbios fisiológicos e bioquímicos nas plantas, como por exemplo, absorção de água e assimilação de nutrientes (BATISTA et al., 2002; ROY et al., 2014).

Os estresses osmótico e iônico são responsáveis tanto pela inibição quanto pelo atraso na germinação, absorção de água e nutrientes devido ao aumento das concentrações de Na^+ e Cl^- na rizosfera (região onde o solo e as raízes das plantas entram em contato) causando desbalanço nutricional nas plantas (**Figura 4**) (RAHNESHAN et al., 2018). Esses íons interferem na absorção de alguns nutrientes, incluindo nitrogênio (N), fósforo (P), potássio (K), boro (B), cálcio (Ca), zinco (Zn), cobre (Cu), magnésio (Mg) e ferro (Fe) (**Figura 4**) (NADEEM et al., 2019), como também no elongamento celular, na fotossíntese [(fechamento dos estômatos e redução dos pigmentos fotossintéticos (carotenóides)], fluorescência da clorofila e dano estrutural na membrana, queima das folhas, manchas avermelhadas com posterior amarelamento das folhas mais velhas, queima das bordas e queda das folhas em estágios mais avançados e senescência (**Figura 4**) (NADEEM et al., 2019).

Figura 4 – **Representação da resposta da planta ao estresse salino**. As setas indicam os principais efeitos negativos induzidos pela sanilidade. Os círculos nas cores vermelho, azul e verde indicam os nutrientes presentes na rizozfera e não assimilados devido ao excesso de sal no solo. Os nutrientes incluem nitrogênio (N), fósforo (P), potássio (K), boro (B), cálcio (Ca), zinco (Zn), cobre (Cu), magnésio (Mg) e ferro (Fe)



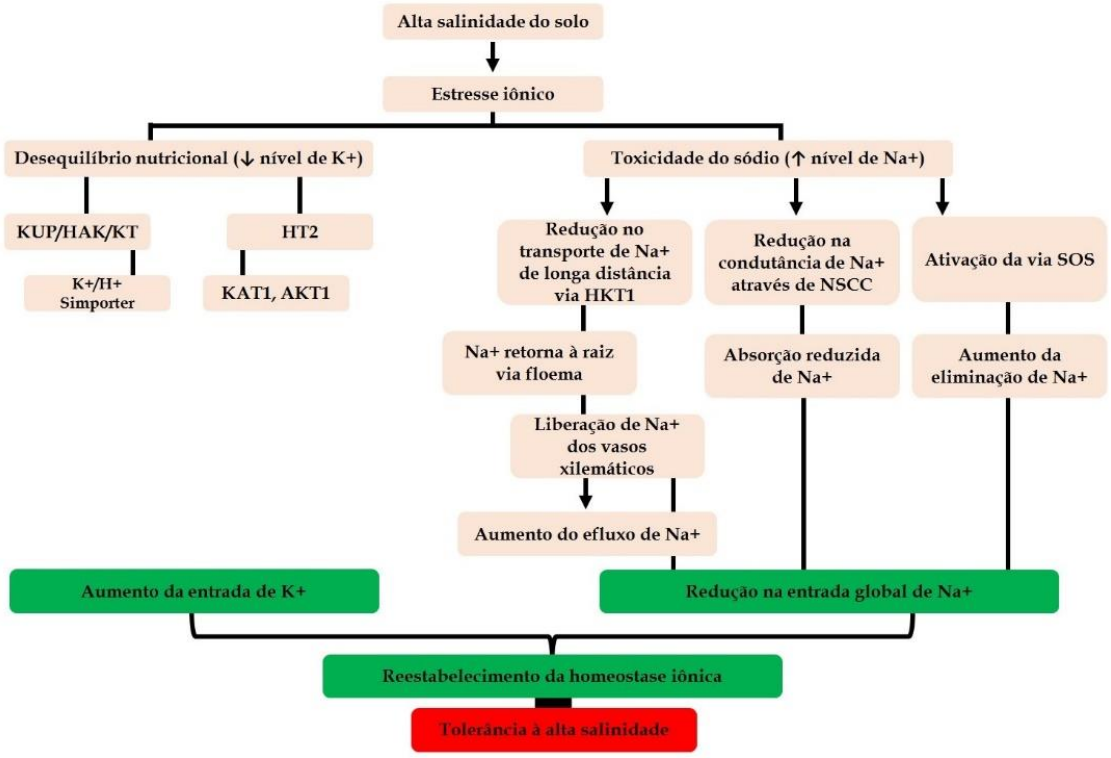
Fonte: Adaptado de Nadeem et al. (2019).

O estresse de alta salinidade causa desequilíbrio na homeostase dos íons dentro da célula da planta que por sua vez produzem um sinal de estresse iônico, esse estresse é decorrente do acúmulo de íons, em particular o Na^+, responsável por inibir a absorção e a translocação de nutrientes, especialmente o K^+, levando ao desequilibrio nutricional e a toxicidade por sódio (aumento do nível de Na^+) (**Figura 5**) (ASSAHA et al., 2017). Sob alta salinidade, os níveis de K^+ diminui no interior da célula, isso acontece pois, o Na^+ citoplasmático compete pelos mesmos sítios de ligação do K^+ e portanto, dificulta os processos metabólicos que dependem crucialmente do K^+ (**Figura 5**) (WANG et al., 2018).

Na tentativa de manter a homeostase iônica sob estresse salino, alguns transportadores de potássio são requeridos incluindo os da familia KT/KUP/HAK. Esses transportadores são responsáveis por mediar o efluxo de K^+ do vacúolo (via simporter K^+/H^+) para o citoplasma sob condições de estresse salino e manter a homeostase iônica (**Figura 5**) (GUPTA et al., 2008). Essa capacidade das plantas em manter a relação de K^+/ Na^+ no citosol é provavelmente um dos

principais mecanismos de tolerância das plantas ao sal (GRABOV, 2007). Por sua vez, os transportadores HKT em especial o da subfamilia HKT2, também está envolvido na homeostase iônica e na adaptação das plantas ao estresse salino (**Figura 5)**. Nas raízes, os transportadores HKT1 descarregam o Na^+ do xilema limitando a quantidade desse íon na célula, isso faz com que ocorra a liberação de Na^+ dos vasos xilemáticos aumentando o efluxo do sódio e reduzindo a entrada desse íon na célula (HENDERSON et al., 2018). Além disso, esses transportadores atuam como um co-transportador de potássio e sódio, que medeia o aumento da absorção de potássio sob o acúmulo externo de sódio e contribui para a tolerância ao sal (HORIE et al., 2011).

Figura 5 - Estratégias adaptativas visando tolerância à alta salinidade.



Fonte: A autora, 2018.

Os canais seletivos de K^+ da família *Shaker* são conhecidos como KAT1 e AKT1, são altamente seletivos e transportam íons de potássio, mesmo sob condições de alta salinidade (OBATA et al., 2007) (**Figura 5**). O KAT1 é fortemente expresso em células – guarda onde a absorção de K^+ ocorre durante a abertura estomática e o AKT1 é expresso principalmente na raiz e também é importante para a absorção de K^+ e nutrição da planta, especialmente sob condições de estresse (**Figura 5**) (WANG et al., 2018). AKT1 é um dos mais importantes

transportadores de K^+. Esses canais medeiam o crescimento contínuo pela absorção de K^+ pelas raízes das plantas através de várias concentrações exógenas de K^+ e promove a captação de K^+ mesmo sob baixas concentrações desse nutriente (ZHANG et al., 2018).

Sob alta salinidade, a toxicidade de sódio promove uma redução na condutância de sódio através dos canais não seletivos de cátions (NSCC), envolvidos na captação e movimentação de sódio através da membrana plasmática e na redistribuição desse íon a longas distâncias, mediando e reduzindo o influxo de Na^+ tóxico para a célula (**Figura 5**) (DEMIDCHIK e TESTER, 2002). O sinal de estresse de salinidade também é percebido por um receptor ou sensor de sal presente na membrana plasmática da célula, que geralmente é regulada pela ação coordenada de várias vias de sal super sensível (*SaltOverlySensitive* - SOS) que resulta no efluxo de íons em excesso (**Figura 5**) (ZHANG et al., 2018). Por essa via, a percepção e a transdução de sinal do sal resulta na modulação da expressão de genes associados a proteínas que estão envolvidas na exclusão de Na^+ do citosol (**Figura 5**) (ZHU, 2003). Em conjunto, esses mecanismos operam para reduzir a entrada global de Na^+ na célula vegetal, e promover o reestabelecimento da homeostase iônica e a tolerância ao estresse salino (**Figura 5**).

3.3 ÔMICAS: INFORMAÇÕES GERAIS

O desenvolvimento das tecnologias de sequenciamento de ácidos nucleicos de alto rendimento, deu início a uma nova era na biologia molecular. As tecnologias “Omics” incluem genômica, transcriptômica (expressão de genes), proteômica e metabolômica, que são conhecidas como tecnologias de alto rendimento (SHARAFI et al., 2019). Essas ciências são capazes de gerar quantidades enormes de dados sobre microorganismos, genes, elementos reguladores, RNAs, proteínas, metabólitos e vias em um curto período de tempo e a custos substancialmente mais baixos (DEBNATH et al., 2010). Essas novas tecnologias “ômicas” permitem a análise e a caracterização de sistemas biológicos em detalhes até então desconhecidos no qual milhares de genes e proteínas podem ser detectados simultaneamente (ULRICH-MERZENICH et al., 2007). As “ômicas” são nominalmente conhecidas como:

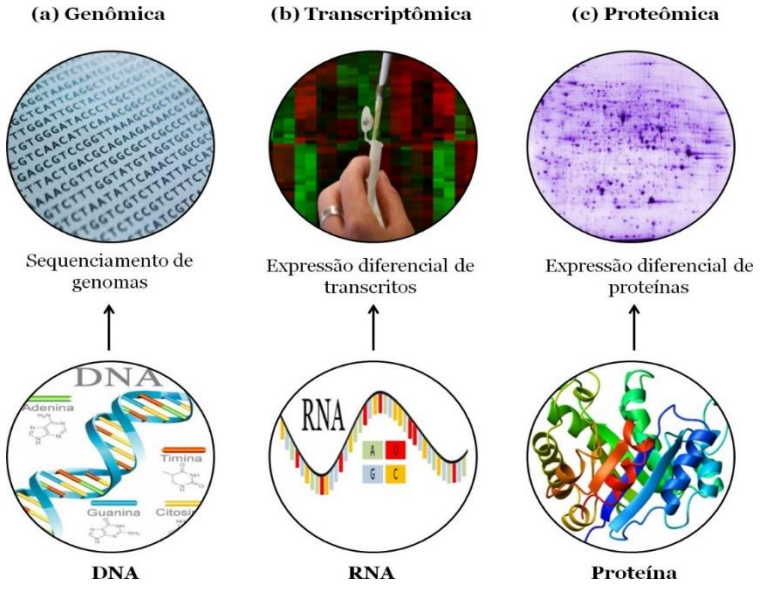
(a) **Genômica**: estudo em larga escala de grupos de genes. Esses grupos podem representar todos os genes de um organismo ou de vários organismos. Tal área pode ser dividida em três sub-áreas (SHARAFI et al., 2019);

(I) Genômica comparativa: envolve a comparação de genomas de espécies diferentes, podendo se inferir sobre similaridades, diferenças e/ou conservação evolucionária de sequências codificantes e motivos reguladores das mesmas (ULITSKY, 2016);

(II) Genômica estrutural: envolve o estudo da natureza física dos genomas e inclui o sequenciamento e mapeamento dos mesmos. A partir dessas atividades, pode-se designar *loci* a cromossomos específicos, bem como indicar ligação entre os mesmos. O conhecimento de um genoma particular é importante para a manipulação de genes e segmentos de DNA em uma dada espécie. Por exemplo, genes podem ser clonados com base no conhecimento de sua posição no genoma (processo denominado de clonagem posicional) (VARSHNEY et al., 2018).

(III) Genômica funcional, descreve as funções e interações de genes e proteínas, fazendo uso de abordagens genômicas. Combina dados derivados dos vários processos relacionados à sequência de DNA, expressão gênica e função da proteína, como transcrição, tradução de proteínas, interações proteína-DNA, proteína-RNA e proteína-proteína. Isso levou a anotações aprimoradas dos genes e de seus produtos e possibilitou estudos em todo o genoma com o objetivo de entender as interações e os processos moleculares na célula e modelar redes interativas e dinâmicas que regulam a expressão gênica (BUNNIK e ROCH, 2012).

Figura 6 - Apresentação das Ômicas fundamentais, indicando suas moléculas alvo e alguns de seus desdobramentos. **(a)** Genômica (sequencimento de genomas); **(b)** transcriptômica (*heat map* com a expressão de transcritos analisados); **(c)** Proteômica [eletroforese bidimensional em gel de acrilamida (2DPAGE), apresentando o perfil proteico de um determinado tecido sob dada condição].



Fonte: Ferreira Neto, 2018.

(b) **Transcriptômica**: estudo de todo um grupo de RNAs expresso em uma célula, pertencente a um dado tecido, sob uma determinada condição ou estágio de desenvolvimento (DONG e CHEN, 2013). Tal área de estudo é de suma importância uma vez que indica informações fundamentais sobre a fisiologia molecular da célula perante diferentes situações.

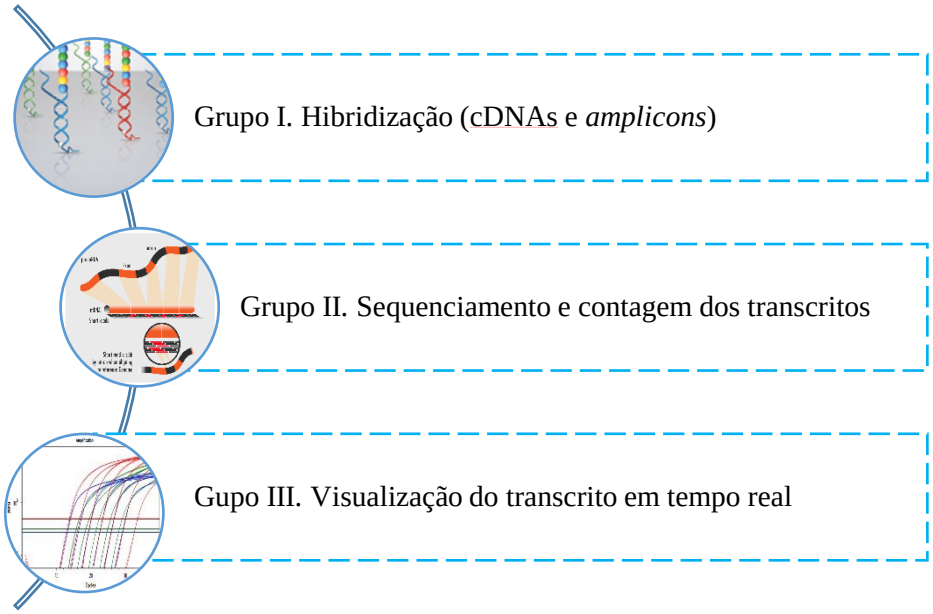
(c) **Proteômica**: apresenta grande relevância, tal como a transcriptômica. Segundo Graves e Haystead (2002), há duas definições para essa área: a clássica, a qual restringe tal área à análise em larga escala de proteínas e suas funções na célula; e a segunda, considerada mais inclusiva, define tal área como uma combinação de estudos de proteínas com análises que têm um *approach* genético, tais como análises da expressão de RNAs mensageiros, genômica e o estudo de duplos híbridos em leveduras.

Essas áreas de conhecimento permitem o estudo sistemático de um organismo vivo, entendendo os mecanismos moleculares envolvidos em processos de desenvolvimento, sobrevivência e adaptação.

3.1 Métodos para análise de transcriptoma: apresentação do tema

O genoma das células eucariotas contém milhares de genes cuja expressão é precisamente regulada para mantê-las vivas e funcionais. A detecção de mudanças na atividade gênica é usada para definir a identidade, função e estado de proliferação das células, sendo o perfil de expressão dos genes frequentemente correlacionado com a presença ou ausência de seus RNAs mensageiros (RNAm) correspondentes nas células. Desta forma, métodos para a apuração dos níveis de RNAm revelam o padrão transcricional temporal e espacial de genes e permitem a correlação da atividade gênica com processos biológicos e doenças. Para análise da expressão gênica em larga escala (chamado também de transcriptoma) são descritas algumas metodologias que podem ser divididas em três categorias principais (**Figura 8**) (TERAUCHI et al., 2008).

Figura 7 – Metodologias para a análise da expressão gênica.



Fonte: A autora, 2019

O principal representante do primeiro grupo é a técnica de *Microarray* (SCHENA et al., 1995). Essa tecnologia consiste na utilização de um *slide* (lâmina ou microarranjo) no qual as sondas (amostras de DNA) são imobilizadas em quantidades e posições precisamente definidas (*spots*), para se fazer a hibridização com um *pool* de mRNAs extraídos de amostras biológicas (*targets*), que foram previamente marcados com fluoróforos (marcadores florescentes) (GHEYAS et al., 2013). Após o processo de hibridização, cada lâmina é lavada para remoção dos "alvo" excedentes (que não se ligaram às sondas) e, em seguida, exposta à ação de raios laser que excitam os fluoróforos que foram incorporados aos "alvos", fazendo com que estes emitam luz (fluorescência). Quanto maior for a expressão de um determinado gene, maior será a quantidade de "alvos" marcados com o fluoróforo e, consequentemente, maior será a intensidade da fluorescência do complexo alvo-sonda após a hibridização (HIENDLEDER et al., 2005). Assim, a tecnologia de *microarrays* fornece uma medida indireta do nível de expressão gênica, mediante quantificação da abundância dos RNAs transcritos.

Algumas desvantagens existem em relação a essa técnica, a citar: pobre correlação entre diferentes plataformas de *microarray*; resultado semiquantitativo; tem como pré-requisito a necessidade de grandes quantidades de RNA para a obtenção de respostas robustas; hibridização cruzada de sondas com diferentes alvos; detecção dificultosa de transcritos com

número de cópias abaixo de 50 unidades por célula; ambiguidade na análise de dados e interpretação dos resultados, dentre outras (MATSUMURA et al., 2010; MURPHY, 2002).

No que tange ao segundo grupo, os principais representantes são as análises de bibliotecas ESTs (*Expressed Sequence Tags*; ADAMS et al., 1991) e recentemente, RNA-seq (MORIN et al., 2008). EST é uma sequência curta de nucleotídeos (500 a 800 nucleotídeos) gerada a partir de um único transcrito de RNA, que é convertido em cDNA por uma enzima chamada *transcriptase reversa*. Os cDNAs utilizados para a geração de EST são clones individuais de uma biblioteca de cDNA. Como esses clones consistem em DNA que é complementar ao mRNA, os ESTs representam porções de genes expressos e podem ser representados em bancos de dados como sequência de cDNA / mRNA. Os ESTs podem ser usadas para identificar transcritos de genes e são importantes na descoberta de genes e na determinação de sequências genéticas (WOLFSBERG e LANDSMAN, 1997).

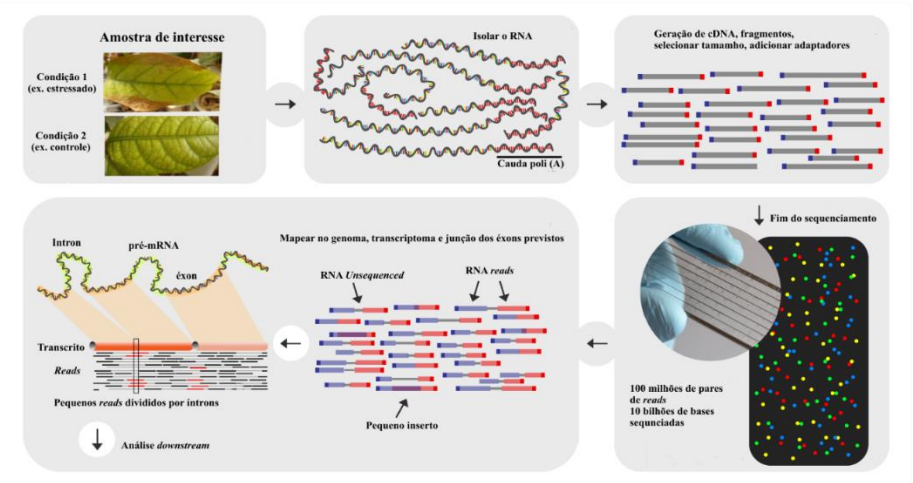
Apesar da ampla aplicabilidade dessa técnica em estudos genômicos, essa técnica apresenta limitações que impedem seu uso em certas aplicações. Devido ao seu protocolo de obtenção, sua qualidade é baixa, a porcentagem de ESTs com alto escore pode ser muito restrita. Adicionalmente, dependendo do tipo da biblioteca de cDNA utilizada para gerar essas sequências, a redundância pode ser alta reduzindo a possibilidade de encontrar transcritos raros (LORKOWSKI e CULLEN, 2003). Por sua vez, o Sequenciamento do RNA (RNA-seq), surgiu como uma ferramenta revolucionária, promissora na descoberta de novos transcritos e pequenos RNAs com alta especificidade (WU, 2013). As primeiras técnicas de RNA-seq usavam a tecnologia de sequenciamento Sanger, uma técnica que, embora inovadora na época, também era de baixo rendimento, cara e imprecisa (KUKURBA e MONTGOMERY, 2015).

Apenas recentemente, com o advento da tecnologia NGS (*Next Generation Sequencing*), associada a novas plataformas de sequenciamento de alto rendimento (*high throughput*) e de ampla utilização em todo o mundo [plataforma 454 FLX da Roche e a Solexa da Illumina (HiSeq2000/2500)] (BUERMANS e DUNNEN, 2014), pode-se aproveitar plenamente o potencial do RNA-seq (COSTA-SILVA, DOMINGUES e LOPES, 2017). O RNA-seq permite analisar transcriptomas com baixo custo quando comparada a outras técnicas de sequenciamento, é altamente sensível e precisa para medir a expressão de transcritos, detectar isoformas de genes, miRNA, transcritos raros e pode ser aplicada a qualquer espécie, com ou sem genoma de referência (CONESA et al., 2016).

Resumidamente, a técnica consiste em isolar o RNA, convertê-lo em DNA complementar (cDNA), preparar a biblioteca de seqüenciamento e sequencia-lo em uma plataforma NGS. (**Figura 9**). O primeiro passo no sequenciamento do transcriptoma é o isolamento do RNA de uma amostra biológica. O RNA deve ter qualidade suficiente para produzir uma biblioteca para sequenciamento. A qualidade do RNA é tipicamente medida usando um Agilent Bioanalyzer, que produz um RNA Integrity Number (RIN) entre 1 e 10, sendo 10 amostras da mais alta qualidade, mostrando a menor degradação. RNA de baixa qualidade (RIN < 6) pode afetar substancialmente os resultados do sequenciamento e levar a conclusões biológicas erradas. Portanto, o RNA de alta qualidade é essencial para experiências bem sucedidas de RNA-Seq (KUKURBA e MONTGOMERY, 2015).

Após o isolamento de RNA, o próximo passo no sequenciamento do transcriptoma é a criação de uma biblioteca de RNA-Seq, que pode variar pela seleção de espécies de RNA e entre plataformas NGS. A construção das bibliotecas de sequenciamento envolve principalmente isolar as moléculas de RNA desejadas [(selecionando RNAs poliadenilados (poli-A)], transcrever reversamente o RNA em cDNA, fragmentar ou amplificar moléculas de cDNA aleatoriamente e ligar a adaptadores de sequenciamento (**Figura 9**) (KUKURBA e MONTGOMERY, 2015). Esses adaptadores ligados ao cDNA são então amplificados e sequenciados para obter as *reads* (WANG et al., 2009) (**Figura 9**). Dependendo do tipo da plataforma de sequenciamento (Illumina, 454 Sequenciamento e SOLiD), os fragmentos sequenciados podem gerar de milhões a bilhões de fragmentos curtos (*reads*) (**Figura 9**) (HODZIC et al., 2017).

Figura 8 – Visão geral do fluxo de trabalho RNA-Seq.



Fonte: Adaptado de MacKenzie, 2019.

As *reads* obtidas pelo sequenciamento podem ser montadas por duas formas: (1) utilizando um genoma de referência; (2) montagem *de novo*. A montagem por referência utiliza informações de uma sequência conhecida (genomas ou transcriptomas completos) (LOGANANTHARAJ e RANDALL, 2016), por outro lado, a montagem "*de novo*" (do latim: "do início") é realizada através da identificação de sobreposição entre as *reads*, seguida da geração de transcritos montados pela identificação de uma, ou várias, sequência(s) consenso. A vantagem da montagem *de novo* é que pode ser utilizada quando um genoma de referência não está disponível ou está mal anotado (ROBERTSON et al., 2010). Entre as ferramentas de montagem *de novo* transcriptomas frequentemente utilizados estão o SOAP (XIE et al., 2014) e o Trinity (GRABHERR et al., 2011). Depois de montados, os transcritos são normalizados pelo método de FPKM (paired-end reads; *fragments per kilobase per million*) (TRAPNELL et al., 2010), e quantificados. Os modelos estatísticos mais utilizados para quantificar transcritos são: RSEM, edgeR ou DESeq.

O RNA-seq é uma técnica de alto rendimento e desenvoldida em múltiplas etapas, que são passíveis de erro. Assim, é importante a utilização de uma segunda ferramenta para confirmação dos resultados: a RT-qPCR (*Reverse transcription polymerase chain reaction quantitative real time*), considerada padrão ouro. A reação em cadeia de polimerase em tempo real combina a metodologia da PCR convencional a um mecanismo de detecção e quantificação por fluorescência (corante fluorescente: Sybrgreen ou Taqman), afim de monitorar a produção do produtos amplificados durante cada ciclo da qPCR. Nesse método, o corante liga-se as moléculas amplificadas e emitem um sinal de fluorescência quando excitados a cada ciclo. Assim, a intensidade da fluorescência é proporcional a quantidade do produto (NAVARRO et al., 2015).

3.1.2 Ômicas em *Jatropha curcas*: painel informacional geral

J. curcas (Euphorbiaceae, 2n = 2x = 22) têm sido investigada cientificamente quanto aos genes reponsivos aos estresses de sal, seca, frio e temperatura, composição de óleo nas sementes, reprodução e diversidade genética (**Tabela 1**). Estudos de genômica em *Jatropha* têm aberto uma nova era para o entendimento dessa planta não domesticada, onde a ligação de genes a características desejáveis (como tolerância a estresses abioticos) tem auxiliado os programas de melhoramento na seleção materiais produtivos e tolerantes a condições adversas do ambiente.

Tabela 1 - Principais estudos com tecnologias ômicas em *Jatropha curcas*.

Objeto de Estudo	Tecido	Estágio da Planta (dias)	Tecnologia	Referência
Óleo da semente	Endosperma	Semente (24, 36, 48 e 72h)	ABI PRISM 3700 DNA Analyzer	Costa et al., 2010
Desenvolvimento da semente	Semente	3 estágios de desenvolvimento	454 GLSFLX	King, Li e Graham, 2011
Identificação de transcritos em 5 diferentes tecidos	Raiz, Folha, flores, sementes e embriões	Raízes (2 meses), folhas maduras, sementes em desenvolvimento, embriões de sementes e flores	454 pyrosequencing FLX	Natarajan e Parani, 2011
Desenvolvimento da semente	Semente	Plantas com 4 anos (7 estágios de desenvolvimento)	Illumina GAII	Jiang et al., 2012
Metabolismo do óleo	Semente	4 estágios de maturação	Suppression Subtractive Hybridization (SSH)	Chandran et al., 2014
Descoberta de transcritos e desenvolvimento de SNPs	Meristema Apical	Folhas jovens	454 Genome Sequencer FLX	Laosatit et al., 2016
Diferenciação sexual das flores	Flores e inflorescência	6 estágios de desenvolvimento	Illumina HiSeq™ 2000	Xu et al., 2016
Diferenciação de tecidos reprodutivos	Brotos e inflorescência	Plantas com 2 anos	Ilumina HiSeq 2500	Govender et al., 2017
Desenvolvimento de SSR e SNPs	Folhas e calos		454 GSFLX	Tian et al., 2017
Genes relacionados ao aumento do número de flores femininas e produção de sementes	Flores	Plantas com 6 anos	RNAseq	Seesangboon et al., 2018
Descoberta de miRNAs	Folhas		RNAseq/Illumina	Prakash e Jadeja, 2018

Nesse sentido, para fornecer uma base para pesquisas futuras sobre *Jatropha* (como descoberta de genes, genômica funcional e desenvolvimento de marcadores), diversos grupos no mundo inteiro tem sequenciado seu genoma, e assim conseguido entender as bases moleculares dessa planta. Atualmente, três genomas de *J. curcas* foram sequenciados e estão disponíveis nos bancos de dados NCBI (https://www.ncbi.nlm.nih.gov/assembly/jatrophacurcas), Kazusa (https://www.kazusa.o g.jp/jatropha/) e *Jatropha curcas* Database (http://jcdb.xtbg.ac.cn/). A **Tabela 2**, apresenta as versões atualizadas e utilizadas em estudos de *J. curcas*. Além disso, foi criado um banco de dados com 46.947 ESTs, 217.142 sequências nucleotídicas, 77.761 proteínas e 25.943 genes. Esse esforço é essencialpara amplicar o conhecimento a respeito da espécie em questão. O banco de dados é continuamente atualizado com novas ESTs de diferentes partes da planta.

Tabela 2 - Genomas de *Jatropha curcas*.

Jatropha curcas L.				
Nome	Grupo de pesquisa	Tecnologia de sequenciamento	Nível	Referência
JatCur_1.0	Chinese Academy of Sciences	Illumina GAII; Illumina HS	*Scaffold*	Zhang et al. 2014
JAT_r4.5	Kazusa DNA Research Institute	454 GS FLX; Illumina GAII; ABI 3730xl	*Scaffold*	Sato et al. 2010; Hirakawa et al., 2012
RIL_Jcurcas_v1	Reliance Industries Ltd.	Illumina; PacBio	*Scaffold*	Kancharla et al. 2019

Através da transcriptômica foi possível entender a composição do óleo de *Jatropha* considerado o principal produto, a fenologia, teor de óleo, perfil lipídico e concentração de esteróis (COSTA et al., 2010), além dos genes envolvidos no metabolismo de ácidos graxos (Natarajan e Parani., 2011) (**Tabela 3**). King et al. (2011) encontraram proteínas de armazenamento (23,9%) como as mais abundantes no transcriptoma de *J. curcas*, com destaque para as oleosinas (2.8%) proteínas estruturais abundantes em óleos vegetais. A composição do óleo de *J. curcas*, é influenciada pelas diferenças nos perfis de expressão de enzimas relacionadas à síntese e degradação dos ácidos graxos, como o ácido graxo elongase (FAE), diferentes tipos de AcylACP Thioesterase e dessaturases. Os genes codificadores dessas enzimas constituem, portanto, alvos promissores para possíveis formas de otimizar as propriedades tanto do óleo de *Jatropha* como de outras oleaginosas (King et al., 2011).

Tabela 3 - Principais estudos com transcriptômica em *Jatropha curcas*.

Objeto de Estudo	**Tecido**	**Estágio da Planta (dias)**	**Tecnologia**	**Referência**
Óleo da semente	Endosperma	Semente (24, 36, 48 e 72h)	ABI PRISM 3700 DNA Analyzer	Costa et al., 2010
Desenvolvimento da semente	Semente	3 estágios de desenvolvimento	454 GLSFLX	King, Li e Graham, 2011
Identificação de transcritos em 5 diferentes tecidos	Raiz, Folha, flores, sementes e embriões	Raízes (2 meses), folhas maduras, sementes em desenvolvimento, embriões de sementes e flores	454 pyrosequencing FLX	Natarajan e Parani, 2011
Desenvolvimento da semente	Semente	Plantas com 4 anos (7 estágios de desenvolvimento)	Illumina GAII	Jiang et al., 2012
Metabolismo do óleo	Semente	4 estágios de maturação	Suppression Subtractive Hybridization (SSH)	Chandran et al., 2014

Descoberta de transcritos e desenvolvimento de SNPs	Meristema Apical	Folhas jovens	454 Genome Sequencer FLX	Laosatit et al., 2016
Diferenciação sexual das flores	Flores e inflorescência	6 estágios de desenvolvimento	Illumina HiSeq™ 2000	Xu et al., 2016
Diferenciação de tecidos reprodutivos	Brotos e inflorescência	Plantas com 2 anos	Ilumina HiSeq 2500	Govender et al., 2017
Desenvolvimento de SSR e SNPs	Folhas e calos		454 GSFLX	Tian et al., 2017
Genes relacionados ao aumento do número de flores femininas e produção de sementes	Flores	Plantas com 6 anos	RNAseq	Seesangboon et al., 2018
Descoberta de miRNAs	Folhas		RNAseq/Illumina	Prakash e Jadeja, 2018

A floração é uma das características agronômicas mais importantes para a melhoria do rendimento das culturas. Entretanto, são poucas as informações moleculares disponíveis a respeito de genes envolvidos no processo de desenvolvimento floral em *J. curcas*. Para explorar esse processo, Xu et al. (2016) realizaram o perfil transcricional do desenvolvimento floral e identificaram genes relacionados ao processo de diferenciação sexual e proteínas quinases ativadas por AMP (activated protein kinase) que contribuem para a diferenciação estaminal. Algumas proteínas como like ADP-ribosylation factor 6 (ARF6), RING-H2 finger protein ATL3J, CLV1 - Receptor protein kinase e fator de transcrição MYB contribuem para o desenvolvimento do saco embrionário em flores sem estames.

Govender et al. (2017) utilizaram a análise da expressão gênica diferencial para identificar genes chaves associados ao processo de transição da fase vegetativa para a fase reprodutiva. Alguns dos principais genes identificados foram proteínas de membrana e proteínas de transporte intracelular. Estudos envolvendo transcriptômica em *J. curcas* têm sido empregados também para o desenvolvimento de marcadores moleculares. Laosatit et al. (2016) desenvolveram 432 pares de iniciadores EST-SSR, dos quais 269 marcadores polimórficos mostraram transferibilidade em espécies de *Jatropha*. Além disso, identificaram 20 marcadores SNPs (*Single nucleotide polymorphism*) em quatro sequências codificantes, incluindo um gene relativo à síntese de ésteres de forbol.

Marcadores moleculares SSR (Simple Sequence Repeats) e SNPs (Single nucleotide polymorphism) foram testados em acessos de Jatropha. A taxa de polimorfismo utilizando o marcador SSR foi 19,8% e para marcadores do tipo SNP, a taxa de polimorfismo foi 13,1%. O resultado foi significativo, visto que os acessos de *Jatropha* apresentaram variação fenotípica significativa no campo, abrangendo grandes áreas geográficas, incluindo três continentes

(América Central, Ásia e África). Esses marcadores podem ser úteis para estudos em genética populacional, mapeamento e genômica comparativa (TIAN, et al., 2017).

Ao relacionar *J. curcas*, estresse abiótico e transcritptômica, observou-se que existem poucos trabalhos disponíveis na literatura envolvendo essa temática (**Tabela 4**).

Tabela 4 - Principais estudos sobre transcritptômica em *J. curcas* em resposta ao estresse abiótico.

Estresse abiótico	Tecido	Idade da Planta (dias)	Tecnologia	Autores
Frio	Folha	14	Illumina Hiseq 2000	Wang et al., 2013
Frio	Folha	14	Illumina Hiseq™ 2000	Wang et al., 2014
Salinidade	Raiz e folha	56	Illumina GAII	Zhang et al., 2014
Seca	Raiz e folha	56	Illumina GAII	Zhang et al., 2015
Seca	Raiz e folha	36	Illumina HiSeq™ 2000	Sapeta et al., 2013
Deficiência de Nitrogênio	Raiz e folha	56	Illumina GAII	Kuang et al., 2017
Inundação	Raiz	30	Ion Proton Sequencer	Juntawong et al., 2014
Resfriamento	Inflorescência e sistema apical	2 anos	Illumina HiSeq™ 2500	Liu et al., 2019

Wang e colaboradores (2013), avaliaram as mudanças na expressão gênica em *J. curcas* quando exposta à condição de frio (12 °C) por 12, 24 e 48 h. Os resultados mostraram que 3.178 genes foram significativamente induzidos e 1.244 foram reprimidos sob esse estresse. Os genes encontrados incluem àqueles envolvidos no transporte de elétrons e no equilíbrio redox, incluindo também fatores de transcrição (AP2 / ERF, ABC, MYB), transdução de sinal como a auxina e etileno e proteínas de estresse como quinases do tipo Serina. *Chaperone protein* DNAJ, *major allergen Pru arlike protein*, e genes comumente expressos relacionados à transferase como a metionina Smetiltransferase também foram encontrados. Esses resultados podem ajudar a melhorar a compreensão dos mecanismos de resistência ao frio em plantas e favorecer a triagem de genes cruciais para aumentar geneticamente a tolerância ao frio em *J. curcas.*

Zhang et al. (2014), estudaram o perfil transcricional de plântulas de pinhão-manso com um mês de idade durante 1, 4 e 7 dias sob estresse hídrico. Os genes induzidos foi o de síntese de ABA e síntese de rafinose. Os genes relacionados à biossíntese de ácidos graxos insaturados e poliinsaturados foram significativamente reduzidos nas folhas 7 dias após a irrigação. Com o aumento do estresse hídrico, genes relacionados à síntese de etileno, e degradação da clorofila foram regulados positivamente, e o teor de clorofila das folhas foi significativamente reduzido em 7 dias após a irrigação.

Kuang et al. (2017), estudaram os perfis transcricionais do genoma de pinhão-manso com um mês de idade analisados após 2 e 16 dias sob deficiencia de Nitrogênio (N). Os resultados mostraram que genes associados ao metabolismo do N, processamento e regulação do RNA, e transporte predominaram entre aqueles que apresentaram alterações na expressão. Os autores mostraram que quatro classes principais de genes, com papéis na absorção de N, reutilização de N, balanço da razão C / N, estrutura celular e síntese, foram particularmente influenciadas pela limitação de N a longo prazo. Essas descobertas podem oferecer caminhos sobre os mecanismos moleculares que regulam a realocação e reutilização de N, de modo a manter ou aumentar o desempenho das plantas mesmo sob condições ambientais adversas.

Jatropha é uma espécie altamente sensível ao alagamento, o que pode resultar em crescimento atrofiado e perda de peso. Nesse sentido, Juntawong et al. (2014), analisaram o transcriptoma de raízes de *Jatropha* por sequenciamento de RNA de alto rendimento (RNA-seq). Os resultados indicaram que 24 h de encharcamento causaram mudanças significativas na abundância de mRNA de 1.968 transcritos. O encharcamento promoveu respostas à hipóxia e à respiração anaeróbica. Por outro lado, o estresse inibiu a síntese de carboidratos, a biogenese da parede celular e o crescimento. Os resultados também destacaram os papéis do etileno, nitrato e óxido nítrico na aclimatação do encharcamento. Além disso, o perfil do transcriptoma identificou fatores de transcrição induzidos pelo encharcamento, incluindo membros das famílias AP2 / ERF, MYB e WRKY, implicando que a reprogramação da expressão gênica é um mecanismo vital para aclimatação do encharcamento. Essas descobertas revelaram respostas moleculares para o encharcamento em *Jatropha* e forneceram novas perspectivas para o desenvolvimento de uma cultivar tolerante a encharcamento no futuro.

Jatropha tem seu desenvolvimento afetado também por um dos principais estresses abióticos, o resfriamento. Liu e colaboradores (2019), analisaram plântulas de *Jatropha* submetidas a estresse de resfriamento de 4°C/24h. Neste estudo um grupo de fosfoproteínas (proteína + fosfato) foram identificadas, cuja função está relacionada a regulação da sinalização celular em resposta ao estímulo. Zhang et al. (2014), analisaram o transcriptoma de *J. curcas* em resposta ao estresse salino (100 mM de NaCl) em plantas com um mês de idade em três tempos de estresse: 2 horas, 2 dias e 7 dias. Em raizes, o maior número de genes foi regulado nos tempos de 2 h e 7 dias, enquanto em folhas houve um aumento gradual no número de genes regulados.

Entre os genes responsivos, destacam-se aqueles relacionados à via de sinalização do ABA, síntese da trealose e ROS. Em raizes, o maior número de genes modulados foi relacionado a síntese de trealose, enquanto que em folhas, o maior número de genes foi

relacionado à síntese de rafinose. O acúmulo deste soluto em raizes está relacionado ao mecanismo fisiológico em resposta ao sal, e tem papel primordial na estabilização das membranas celulares (KRASENSKY e JONAK, 2012), muito embora, a trealose também atue como osmoprotetor. Os genes relacionados à rafinose estão envolvidos na regulação osmótica e atuam como osmoprotetor em folhas de *J. curcas*, e podem servir como sinalizadores em resposta ao estresse abiótico (temperatura, seca e salinidade) (ZHANG et al., 2014).

No **quadro 1** estão sintetizados os principais genes responsíveis ao estresse salino. Esses genes estão relacionados com a biossíntese e transdução de sinal do ABA, regulação transcricional, ajuste osmótico, proteínas de choque térmico, enzimas antioxidantes, desidratação e estresse oxidativo.

Quadro 1 - Genes relacionados ao estresse salino em *J. curcas.*

Categoria	Símbolo	Função	Referência
Biossíntese e transdução de sinal do ABA	PP2C	Proteína fosfatase 2C (PP2C). Envolvida na dormência de sementes	Zhang et al., 2015
	SnRK	Serina/Treonina – proteína quinase. Envolvida na tolerância a salinidade	
	RD26	Fator de transcrição. Envolvido na resposta a desidratação	
Regulação transcricional	WRKY	Fator de transcrição	Zhang et al., 2014; Zhang et al., 2015
	basic helixloophelix (bHLH)	Fator de transcrição	
	MYB	Fator de transcrição	
	AP2/ERF	Envolvidos em múltiplas respostas de sinalização e desenvolvimento	Zhang et al., 2015
	AP2／EREBP	crescimento e desenvolvimento das plantas	Eswaran et al. 2012
	NAC	NAC proteins transcription factor	Zhang et al., 2014; Zhang et al., 2015
Ajuste osmótico	GOLS	Galactinol synthase 2	Zhang et al., 2015; Sapeta et al., 2016
	RS	Raffinose synthase	Zhang et al., 2014; Zhang et al., 2015; Sapeta et al., 2016
	CAO	Chlorophyll *a* oxygenase	Sapeta et al., 2016; Zhang et al., 2015
	PAO	Pheophorbide a oxygenase	Sapeta et al. (2016); Zhang et al. (2015)
Heat Shock Proteins: HSP20 e HSP70	HSP	Grande família de proteínas que tem a importante função de ajudar outras proteínas a dobrar e reparar o desdobramento.	Zhang et al. (2014)
Enzimas antioxidantes	APX		
Desidratação	LEA	Late embryogenesis abundant	Liang et al. (2013)
Estresse oxidativo			
Biossintese de fenilpropanóides, poliaminas e etileno	SAM-AdoMet-MTase;	Enzima constituintes estruturais da parede celular	Eswaran et al. (2012); Zhang et al. (2014)

Os trabalhos de transcriptômica em *Jatropha curcas* tem permitido identificar genes com potencial biotecnológico. Esses genes representam um recurso inestimável para a engenharia genética modificar tanto a composição como a quantidade do óleo, a produção de sementes e a tolerância a estresses abióticos, visando tornar *J. curcas* mais adequada para a produção de biodiesel e rentável para os agricultores. Além disso, todos esses estudos mecionados fornecem uma estrutura global que integra fatores reguladores que coordenam os mecanismos de tolerância das plantas sob estresse. Essas informações podem ser úteis para a identificação dos principais genes envolvidos na tolerância ao estresse salino em Jatropha e fornece um suporte importante para estudos futuros e melhoramento genético da espécie.

4 RESULTADOS

4.1 *De novo* RNA-Seq transcriptome analysis of *J. curcas* accessions under salt stress

Gmail **Ederson Kido <kido.ufpe@gmail.com>**

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De novo RNA-Seq transcriptome analysis of *J. curcas* accessions under salt stress

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Abstract

Jatropha curcas (physic nut) is an oleaginous, non-food, small tree that represents an excellent source of biodiesel, among other products, including some medicinal ones. In this way, *J. curcas* could be an option for farmers in tropical and semi-arid regions, especially where salinity can compromise production and yield of crops. We generated and analyzed the root RNA-Seq transcriptome of two *J. curcas* accessions (salt-tolerant Jc183, and salt-sensitive Jc171) after salt-treatment (150 mM NaCl/three hours). The *de novo* transcriptome covering 101 Mb assembled 145,422 transcripts (126,343 UniGenes), and around half of them encoded predicted proteins. Comparing the total of differentially expressed genes (DEGs) by

Jc183 (57) with that of Jc171(4,646), the intensive transcriptional effort of Jc171 stood out, probably trying to minimize the damages, some of them visually observed only in Jc171 leaves. The functional characterization of DEGs by the MapMan software, using *M. esculenta* genes as the reference, associated them with metabolic processes showing impacts on plant salt-stress responses. The associated processes covered the metabolisms of hormones, CHOs, lipids, amino acids, redox, and also secondary metabolites. Further, nine selected DEGs, including *S-adenosylmethionine synthase* (*SAM*), *carboxylesterase* (*CXE*), *Phenylalanine ammonia-lyase* (*PAL*), *homeobox-leucine zipper protein* (*HD-Zip*), *NAC TF* gene, *methionine-gamma lyase* (*MGL*), *S-adenosylmethionine-dependent methyltransferase* (*SAMe*), *peroxidase* (*PX*), and *xyloglucan endotransglucosylase* (*XTH*), were evaluated by RT-qPCR analysis, trying to validate their *in silico* expression. The developed functional molecular markers could be useful in marker-assisted selection process in breeding programs. Additionally, the data covering DEGs, provided as Supplementary information, may help to understand the molecular mechanisms involving *J. curcas* plants responding to salt stress, which is crucial for the development of salt-tolerant plants.

Keywords: Bioinformatic; Abiotic stress; Euphorbiaceae; Physic nut

1. Introduction

Physic nut (*Jatropha curcas* L.;) is an oilseed plant from Euphorbiaceae Family, with a widespread distribution, covering tropical and semiarid regions (Africa, Asia, and South America), and along all the Brazilian regions. Its occurrence combines different climates and soils conditions, including marginal agricultural production areas, and those relatively deficient in nutrients (Wang et al., 2018; Beltrão e Oliveira, 2008). *J. curcas* seeds present expressive oil contents (40 - 50%), with a relatively high amount of unsaturated fatty acids, which are the preferred by the biodiesel industries (Pramanik, 2003; Wang et al., 2018; Grover et al., 2014).

In Brazil, biodiesel production has increased in the last decade, mainly from soybean oil processing. However, soybean is a versatile crop, very important for human and animal nutrition. *J. curcas*, instead, not compete with the food market, and could be an option to be explored. Although, *J. curcas* is a relatively drought-tolerant plant, it is also considered a salt-sensitive one. Salinity in the soil is one of the leading causes of losses in agricultural production worldwide (FAO, 2015). It is estimated that globally 20% of all irrigated land are currently affected by salt stress (Taiz et al., 2017). Several factors, including soil composition, poor drainage, and inadequate irrigation could provoke salt stress. The combination of these factors became the Brazilian Northeast region potentially sensitive to salinity.

Plants in saline soils may grow irregularly due to osmotic stress caused by reduced water absorption, and the high concentration of ions, which interfere with the nutrient uptake, raising cytotoxicity (Munns, 2005). To understand the molecular mechanisms by which plants respond to salt stress is crucial to plant breeding programs. In this way, comparing the global expressed profiles of distinct accessions responding to salt NaCl is beneficial to uncover genes related to salt-tolerance mechanisms. Some transcriptomic approaches have been applied to *J. curcas* plants responding to abiotic stress, such as cold (Wang et al., 2013; Wang

et al., 2014), flooding (Juntawong et al., 2014), drought (Cartagena et al, 2015; Sapeta et al., 2015; Zhang et al., 2015), and also salinity (Zhang et al., 2014). However, in the present study, plants of two Brazilian *J. curcas* accessions were exposed (three hours) to salt NaCl (150 mM), and RNA-Seq libraries generated of their roots allowed the comparison of gene expression profiles, aiming to develop functional molecular markers potentially useful to assist selection steps in breeding programs.

1. Materials and Methods

2.1. Plant materials and NaCl treatment

Two *J. curcas* accessions (Jc183 and Jc171) from the seed bank of the Semiarid Tropical Agricultural Research Center (Embrapa Agroenergia, Brasília, DF - Brazil), initially collected in different Brazilian regions (Supplementary Table S1), were selected for the salt assay, based on previous studies (Lozano-Isla et al., 2018). The Jc183 accession was considered the salt-tolerant, concerning the accession Jc171. The conducted salt assay followed a completely randomized experimental design with two accessions, two treatments (without salt or with NaCl, 150 mM, three hours of salt exposure), and three biological replicates of each accession. Seeds of homogeneous sizes and weights were sown (March 2016) in pots (50 L) containing washed sand (20 kg), being cultivated in greenhouse at UFAL/CECA (Rio Largo, AL, Brazil; geodesic coordinates: 09º28'02" S; 35º49’43”W; altitude: 127 m; climate: humid, metathermic, moderate water deficiency in the summer (December - March), and water excess in winter, according to Thornthwaite and Mather method (1955). After the first eophylls (10 DAG, days after germination), seedlings were thinned to one plant per pot. Plants were irrigated every three days with Hoagland nutrient solution (Epstein, 1972) with one-fifth strength. Seven days before the salt application (65

DAG), plants were irrigated daily with Hoagland nutrient solution full strength. On the day before the salt application, plants were irrigated at 16 h. The salt was applied at 9-10 h and consisted of NaCl (150 mM) added to the Hoagland solution. After salt exposure (three h), roots were collected, immediately frozen in liquid nitrogen, and stored (-80 °C) until RNA extraction.

2.2. RNA extraction and RNA-Seq libraries

Total RNAs were extracted from roots using SV Total RNA Isolation kit (Promega, USA), following the manufacturer's instructions. RNAs integrities were verified in RNA agarose gel (1.5% w/v), and the RNAs concentrations estimated in NanoDrop 2000 spectrophotometer (Thermo Scientific ™). RNAs showing absorbance ratio 260/280 nm close to 2.0, and a minimum of 50 μL RNA solution (80 ng/μL) were sent to ESALQ - Genomic Center (São Paulo University, Piracicaba, SP, Brazil) for the RNA-Seq libraries generation and sequencing. All the RNAs integrities were re-evaluated, using the Agilent 6000 Bioanalyzer (Agilent Technologies, CA, USA). The total of 12 RNA-Seq libraries (two accessions x two treatments x biological triplicates) was generated following the Illumina TruSeq Stranded mRNA Sample Prep kit (Illumina Inc, CA, USA), "LS" Protocol. Libraries were sequenced on an Illumina HiSeq 2500 (100 bp, single-end reads), using flow Cell HiSeq run with the HiSeq SBS v4 chemistry.

2.3. De novo transcriptome assembly and DEGs identification

RNA-Seq raw sequence data were analyzed (FastQC v0.11.5) for reads qualities, before and after the initial filtering and trimming, using default parameters of the Trimmomatic tool (v.0.36; Bolger et al., 2014). Reads showing low quality, or unknown adapters and nucleotides, were excluded. Pairs of high-quality reads (Phred quality score, Q ≥

30 for all bases) were used for *de novo* transcriptome assembly performed with the Trinity software v.2.2.0 (Grabherr et al., 2011). A de Bruijn graph data structure represented the overlapping among the reads, and short reads with overlap regions were assembled into longer contigs. The longest transcripts in the cluster units were regarded as unigenes to eliminate redundant sequences. The alignment package Bowtie (v4.4.7; Langmead et al., 2009) was used to map reads back to unigenes. According to the comparison results, the expression levels were estimated employing RSEM (RNA-Seq by expectation maximization; Li and Dewey, 2011). Differences of an abundance of the unigene expression among the samples were represented using FPKM (Fragments Per Kilobase of transcript per Million mapped reads) method. Matrices of normalized FPKM values generated from the RSEM counts were used for the differential expression analyzes between the experimental conditions using the edgeR package (Robinson et al., 2010). Differentially expressed unigenes, henceforth, DEGs, for brevity, were determined based on *p-value* ≤ 0.0001, false discovery rate (FDR ≤ 0.005), and fold change (FC) based on Log_2 (FC) ≥ 1 (positive expression modulation) or ≤ -1 (negative modulation). FC is the ratio of the unigene abundance considering two RNA-Seq libraries.

2.4. Functional annotation of the assembled transcripts

Assembled transcripts (henceforth, transcripts, for brevity) were annotated using the BLASTx alignment (e-value $\leq 10^{-10}$) to various protein databases, including sets downloaded from *J. curcas* presented in the UniProtKB database (http://www.uniprot.org/), and those including the taxonomically related species *Manihot esculenta* and *Ricinus comunnis* from the *Phytozome* portal (v.12.1.6; https://phytozome.jgi.doe.gov/pz/portal.html). Annotation contributions based on each dataset were observed by Venn diagrams (Oliveros, 2015). Also, a second round of functional annotation using Trinotate pipeline (https://trinotate.github.io/)

were performed (BLASTx, e-value ≤ 10^{-5}), against several databases, including: NCBI (non-redundant) protein database (Nr) (ftp://ftp.ncbi.nih.gov/blast/db/), UniProt/SwissProt database, Kyoto Encyclopedia of Genes and Genomes (KEGG), GO (Gene Ontology), eggNOG and InterproScan. Trinotate also provided a web-based graphical interface to support local user-based navigation of annotations and differential expression data (Bryant et al., 2017).

2.5. Metabolic pathways, and heatmaps

Metabolic pathways associated with DEGs were identified applying MapMan software (v.3.6.0; Thimm et al., 2004), using *M. esculenta* best hits from BLAST alignments. Unigenes were hierarchically clustered by the Cluster 3.0 software (https://cluster2.software.informer.com/3.0/), based on the FC values modulated in the comparison stressed *versus* negative control (henceforth, S *vs.* C, for brevity), being the clusters visualized as heatmaps using the JavaTreeview v.1.1 software (Saldanha, 2004).

2.6. Expression validation by RT-qPCR

The expression analysis by RT-qPCR assay validated DEGs representing candidates selected based on the functional annotation and their expression modulated on the contrast *S vs. C*. To this end, cDNAs from RNAs pre-treated with DNase were evaluated on real-time PCR Thermocycler LineGene 9600 (Bioer, Hangzhou, China), in reactions including biological and technical triplicates for each experimental treatment, negative controls, and two reference genes [actin (Tang et al., 2016), and β-tubulin (Xu et al., 2016)], tested adequately for this purpose. The proposed *primer* pairs (Supplementary Table S2) were designed (Primer 3 tool; Rozen and Skaletsky, 2000) based on the *J. curcas* transcripts and the following parameters: amplicon size (70 - 200 bp), melting temperature [50 - 80ºC, 70ºC (optimum)],

and GC content (45 - 55%). *Primers* synthesized by Bioneer Corporation (South Korea) first amplified cDNAs in a conventional PCR test. After that, the RT-qPCR reactions (10 μL) included: 1 μL cDNA (sample diluted 1/5), 5 μL SYBR™ Green (GoTaq ® qPCR Master Mix, Promega), 0.3 μL of each *primer* (5 μM) and 3.4 μL ddH_2O. The reactions followed the settings: initial denaturation of 95°C for 2 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 60 s. The dissociation curves were obtained heating the amplicons from 65 to 95°C for 20 min after the RT-qPCR cycles. The LineGene software (v.1.1.10) estimated the Tm and Cq values, and the absolute and relative quantifications. Relative expression data were evaluated using the REST 2009 software (Relative Expression Software Tool v.2.0.13; Pfaffl et al., 2002), applying randomization test with 2,000 permutations, and testing the hypothesis of significant differences between the control and treatment groups. Also, the MIQE protocol was followed to increase the results reliabilities (*The Minimum Information for Publication of Quantitative Real-Time PCR Experiments*; Bustin et al., 2009).

3. Results and discussion

3.1. Visible damages on the J. curcas leaves

After three hours of salt exposure (150 mM NaCl), the salt-sensitive Jc171 accession presented visible damages on leaves, not observed in the salt-tolerant Jc183, which included, leaves slightly curved, wilted looking, and brown colored areas on the edges, progressing to necrosis (Fig.1). Walia et al., (2005) also noted visual damage of salinity stress on leaves of the sensitive rice cultivar IR29, in the form of necrosis at about one-third of leaf length. In the upland cotton (*G. hirsutum*) visible damage owing to salinity stress appeared on the leaves of salt-sensitive Nan Dan Ba Di Da Hua genotype, after 200 mM NaCl treatment (Peng et al., 2014); the same authors pointed that after 0.5 h, distinct wilting and dehydration were

observed on leaves of the analyzed genotypes, and after four h, both genotypes showed more severe wilting. All mentioned damages can significantly compromise photosynthesis, influencing the growth and development of plants and their yields directly.

A B

Fig. 1. Aspects of *Jatropha curcas* leaves after three hours of exposition to Hoagland solution plus NaCl solution (150 mM). A) Both accessions: the salt-sensitive Jc171 (blue ribbon) and the salt-tolerant Jc183(red ribbon) accession. B) Leaves (Jc171) showing visible damages: browning at the edges and brown spots.

3.2. The J. curcas transcriptome

The RNA-Seq libraries (12) generated with high-qualities RNAs (RIN > 8, Supplementary Fig. S1) after Illumina HiSeq 2500 sequencing provided for each accession (three biological replicates x two treatments) similar amounts of raw *reads* [113,668,090 (Jc183), and 124,618,733 (Jc171)]. Those *reads* showing good qualities (*Phred score* > 30), after filtering and trimming of adapters and low-quality bases, comprised 96,9 % (Jc183), and 96,3 % (Jc171) of the reads. The total of *reads* before and after the trimming step, concerning each library is shown in Supplementary Table S3. The *de novo* transcriptome generated based on the assembled transcripts covered 101 MB (based on unigenes, the size was 77 MB). The

transcriptome also covered 145,422 transcripts (126,343 unigenes) and presented a GC percentage of 41.55. The N50 for transcripts comprised 1308 bp (based on unigenes, 993 pb), which is the maximum length where at least 50% total assembled sequence resides in contigs of at least that length; further details of the generated transcriptome are shown in Supplementary Table S4.

3.3. Functional annotation of the assembled transcripts

The first round of transcript (145,422) annotation, exploring *J. curcas* proteins (UniProtKB, 27,650 deposited sequences) and those from taxonomic-related species [*M. esculenta* (41,381) and *R. comunnis* (31,221), *Phytozome* database], in individually BLASTx analysis (*e-value* $\leq e^{-10}$), identified 27,363 transcripts encoding predicted proteins observed in Euphorbiaceae family. Specifically, 25,668 transcripts associated directly with the *J. curcas* proteins, and other 1,695 transcripts with proteins from the two related species (Fig. 2A). Those transcripts encoding conserved proteins associated with the three species were 19,508 (Fig. 2A). From the set of 25,668 transcripts associated directly with the *J. curcas* proteins, only 9,288 transcripts (Fig. 2B) were properly annotated (protein name or gene function) based on those associated proteins. About the proteins properly annotated from the *J. curcas*-related species, another 14,642 transcripts were declared annotated (Fig. 2B). Considering only the sequence similarities with the *J. curcas* transcripts, the exclusive contribution of *R. cumunnis* exceeded that of *M. esculenta* (Fig. 2A), however, when considered the informative annotations, that position inverted (Fig. 2B).

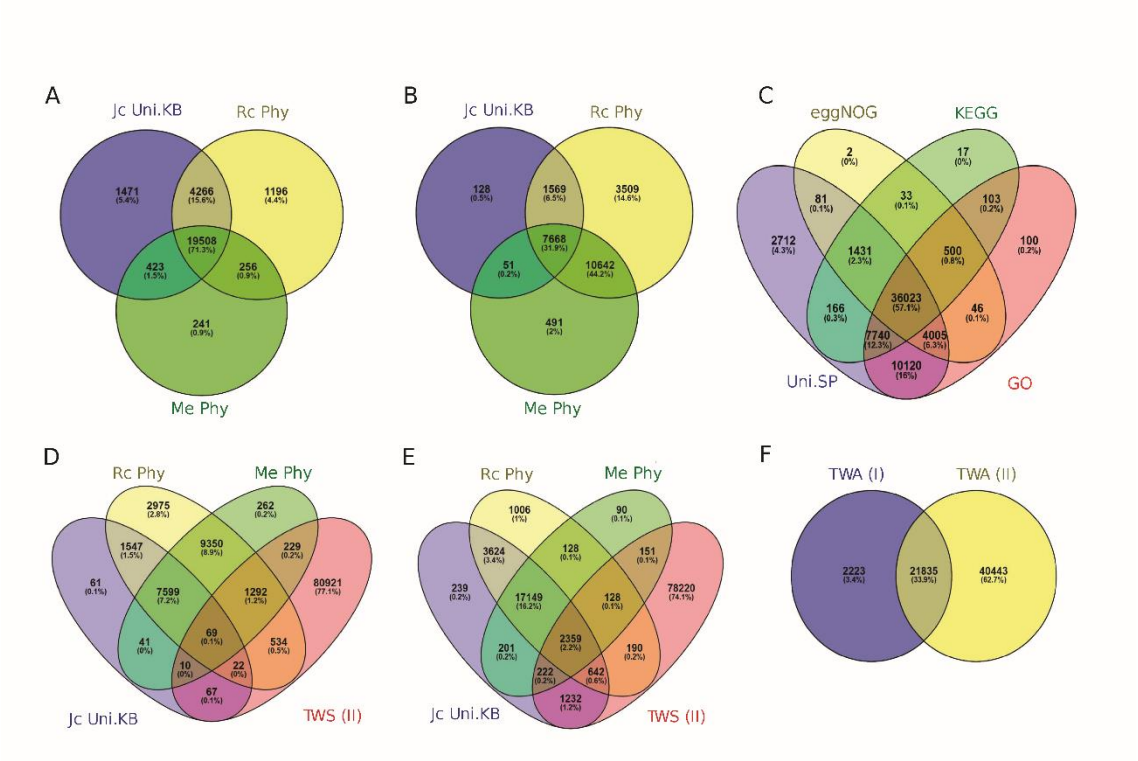

Fig 2. Venn diagrams comparing different results from the two annotation rounds of the RNA-Seq *J. curcas* transcripts: the first (I) round (BLASTx, *e-value* $\leq e^{-10}$) against proteins of *J. curcas*/UniProtKB (Jc UniP), *Ricinus communis*/Phytozome (Rc Phy) and *M. esculenta*/Phytozome (Me Phy), and the second (II) performing the Trinotate software (*e-value* $\leq e^{-5}$) against several protein databases (UniProt/SwissProt, KEGG, eggNOG, GO). A) The individual and shared contribution of the datasets used in I round, considering only similarities results. B) Comparison described before (A), but only considered the annotated results. C) The individual and shared contribution of the datasets used in the II round. D) Transcripts annotated by the I round compared with transcripts without similarities (TWS II) from the II round. E) transcripts encoding predicted proteins similar to those from the I round, regardless of whether there is functional annotation, compared with the transcripts without similarities (TWS II) from the II round. F) Transcripts annotated (TWA) by the two annotation rounds.

In short, concerning the 27,661 proteins from the UniProtKB database (January 2019), most of them (24,288) are annotated as *uncharacterized proteins*. The inclusion of the proteomes from *R. cumunnis* and *M. esculenta*, increased the annotation efficiency, especially *M. esculenta* (Phytozome database). The second round of annotation with the *Trinotate*

pipeline (Bryant et al., 2017), considering a less stringent analysis (*e-value* ≤ e^{-05}) and several databases, showed 63,079 transcripts encoding predicted proteins similar to those from UniProt/SwissProt (62,278 transcripts), eggNOG (42,121), KEGG (46,013), and Gene Ontology (58,637). The individual contribution of each database (Fig. 2C) highlighted the contribution of the Uniprot/SwissProt database, which is formed of manually cured sequences. However, 83,144 transcripts did not reach the required similarity threshold. Comparing the two annotation rounds, from the 83,144 transcripts failing to hit the II threshold (II round), some of them were previously annotated with *J. curcas* proteins or its related species (I round), but 80,921 remained non-associated (*transcripts without similarity* - TWS II, Fig. 2D). If discounting those transcripts showing acceptable similarities with the *J. curcas* and the related species (probably non-annotated), 78,220 transcripts remained without reaching the threshold (Fig. 2E).

In turn, the individual and overlapped contribution of the two annotation rounds are shown in Fig. 2F. Besides the 24,058 (21,835+2,223) transcripts annotated by I round analysis (Euphorbiaceae family members), another 40,443 transcripts were annotated by the II round analysis (Fig. 2F). In total, 64,501 transcripts were adequately annotated, while 80,921 were non-annotated (78,220 not reaching the similarity threshold after II rounds of annotation). Both sets are available for further researches.

3.4. The differentially expressed genes (DEGs) in response to the salt stimulus

The analysis of the expressed profiles of Jc183 and Jc171, comparing the respective contrast *S vs.C,* and considering the required thresholds (*p-value* ≤ 0.0001, FDR ≤ 0.005, Log_2 FC ≥ 1 or ≤ -1), identified 57 and 4,646 DEGs, respectively. The salt-sensitive Jc171 accession presented more transcriptional effort responding to the salt stimulus, trying to minimize the damages, as those visually observed in its leaves (Fig. 1). In a similar pattern,

286 the stress response of the salt-sensitive rice IR29 genotype was characterized by a relatively
287 large number of induced probe sets (in a GeneChip analysis, using the rice genome
288 Affymetrix array), when compared to the salt-tolerant FL478 (Walia et al., 2005). Another
289 assay (Walia et al., 2007) using the same GeneChip, including two *japonica* rice lines (Agami
290 and M103), besides the two *indica* lines mentioned above, revealed a strikingly large number
291 of induced genes, in response to the applied salinity stress, by the sensitive lines (IR29 and
292 M103) in relation to the tolerant ones.

293 From the Jc183 DEGs (40 UR and 17 DR; Supplementary Table S5), 23 (13 UR and
294 10 DR) were exclusively DEGs by Jc183 (five non-annotated, three URs and two DR), and 34
295 were DEGs also detected by Jc171, 33 of them presenting the same regulation by both
296 accessions (27 UR and six DR), and one divergence (DR/Jc183, and UR/Jc171 for DEG
297 encoding Galactinol synthase 1). From the set of 34 shared DEGs, five remained non-
298 annotated (four UR by both accessions, and one DR also by both accessions; Supplementary
299 Table S5). Details of the Jc183 DEGs (Trinity ID, annotation, regulation, log_2FC, and the
300 sequence in FASTA format), are available in the Supplementary Table S5. Concerning the
301 Jc171 profile, from the 4,612 identified DEGs (2,753 UR and 1,859 DR), 1,296 UR DEGs
302 remained non-annotated. The correspondent details covering the Jc171 DEGs were shown in
303 the Supplementary Table S6.

304 The two distinct *J. curcas* expressed profiles could contribute to identify useful salt-
305 related tolerance genes for plant breeding programs. A previous study conducted by plant
306 physiologists (UFPE, Brazil) showed differences between the same accessions analyzed here,
307 highlighting a better recovery capacity of Jc183 after saline stress (750 mM NaCl, 50 h) than
308 the Jc171 accession, inclusive showing an earlier emergence of the juvenile leaf (Real-Cortes,
309 personal communication).

310

3.5. Metabolic responses of the J. curcas accessions to the salt stimulus

A comparative MapMan analysis performed with 2,749 DEGs of both accessions, based on the correspondent *M. esculenta* best hits (BLAST alignments), covered 15 Jc183 DEGs (eight UR and seven DR), 2,711 Jc171 DEGs (1,338 UR and 1,373 DR), and 23 DEGs shared by both profiles, all of them presenting the same regulation (18 UR, and five DR). The analyzed DEGs, comprising more than half of all detected DEGs, identified MapMan bin codes (Thimm et al., 2004) associated to several metabolism pathways (CHOs, lipids, amino acids, phytohormones, and redox; Supplementary Table S7), as well as with secondary metabolites. The heatmaps represented by the expression modulated by each accession associated with the MapMan bin codes are presented in the Fig. 3. In general, the induced genes grouped and separated from the repressed genes; the induced genes exceeded the repressed ones, and the expression modulated by Jc171 in response to the salt-stimulus, was more intense than that of Jc183, since some of the corresponded genes not modulated its expression or was n.d. in the Jc183 profile (Fig. 3). Details covering each accession and MapMan category are discussed below.

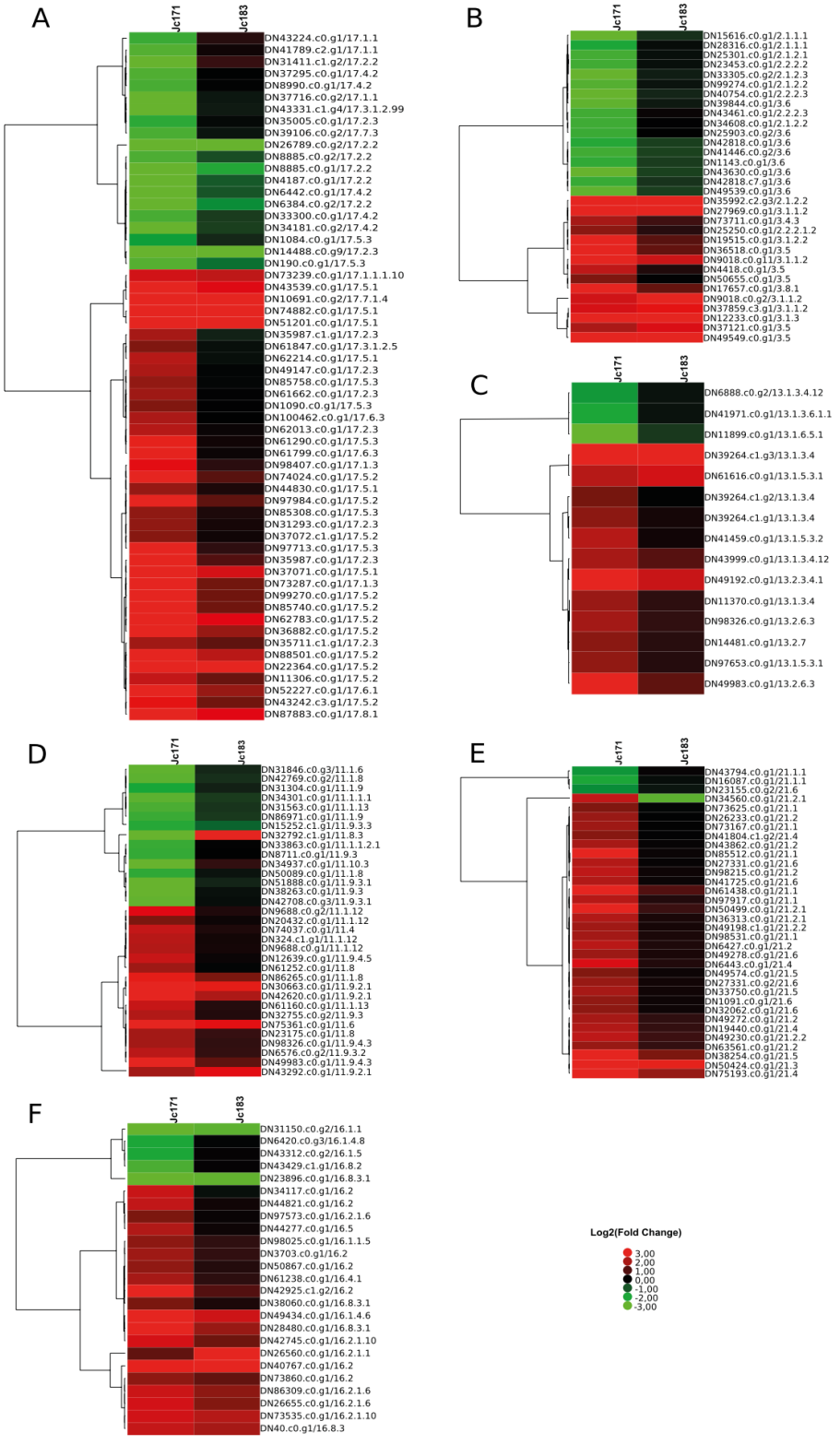

Fig. 3. Heatmaps representing the gene expression profiles of Jc171 and Jc183 accessions, generated by hierarchical clustering analysis, and involving different metabolisms: A) hormone; B) CHO; C) Amino acid; D) Lipid; E) Redox; F) Secondary metabolites. The columns represent the expression modulated by the accessions after three hours of salt exposure

(150 mM NaCl), in relation to the correspondent control without salt. The rows represent each *Jatropha curcas* RNA-Seq transcript and the annotated MapMan bin code. The clusters are on the left side. The up- and down-regulation of the transcripts are indicated in red and green, respectively, and the intensity of the colors increases with increasing expression differences based on the legend. The bin code description is provide in the Supplementary Table S7.

3.5.1. Phytohormone metabolism

The heatmap associated to the phytohormone metabolism (Fig. 3A) followed those general characteristics mentioned before: two groups; the induced group (Jc171) clustering more genes than the repressed one; expressed modulation by Jc171 more intense than those observed by Jc183.

Under normal conditions, the development of the root system architecture (RSA) of dicotyledonous plants, comprising the main root (MR) and the lateral roots (LRs), are under influence basically of auxin (AUX) and cytokinin (CK), two antagonistic phytohormones in some actions. However, under salt stress, the RSA development is influenced by abscisic acid (ABA), ethylene (ETH), jasmonic acid (JA), AUX, and brassinosteroids (BR) (Julkowska and Testerink, 2015). Also, under osmotic stress conditions, ABA regulates root growth via an interacting hormonal network with cytokinin (CK), besides ETH and AUX (Rowe et al., 2016). According to Fig. 3A, some induced genes were associated to ABA (bin codes 17.1.1.1.10, 17.1.3, and 17.2.3), ETH (17.5.1, 17.5.2, and 17.5.3), AUX (17.2.3), JA (17.7.1.4, 17.7.3), BR (17.3.1.2.5), gibberellin (GA; 17.6.1, 17.6.3), and salicylic acid (SA; 17.8.1).

After a saline stimulus, the endogenous ABA level increase due to the action of dioxygenases (9-cis-epoxycarotenoid-dioxygenase; EC 1.13.11.51), which correspond to the bin code 17.1.1.1.10 (Fig. 3A), cleaving carotenoid precursors (Julkowska and Testerink,

2015). The ABA accumulation assists the plant acclimatization under stress, including stomatal closure, growth modulation, and synthesis of protective metabolites, some of them (e.g., proline, sugars, myo-inositol, polyamines) are osmoprotectant compounds that present important roles in ionic adjustment.

A JA action model in response to saline stress was presented by Riemann et al. (2015). Briefly, during osmotic stress, phytohormones are affected, some in a positive way [ABA, JA, and 12-OPDA (JA-precursor 12-oxo-phytodienoic acid)] and others, such as GA, in a repressed way. These phytohormones interfere with regulatory proteins that are crucial in metabolism. When JA is produced, the JAZ repressor is degraded, releasing MYC2, which is a versatile transcription factor (TF), also activated by ABA, for acting. In turn, with the reduced level of bioactive GA, DELLA proteins (which are GA repressors) accumulate and interact with JAZ, also releasing MYC2 from repression. MYC2, in turn, activates metabolic pathways, such as the secondary compounds for flavonoids and terpenoids (isoprenoids), some of them are also osmoprotectant-related compounds, helping plants to saline-stress acclimatization. Concerning the repressed genes (Jc171), the correspondent counterpart in Jc183 was non-modulated or n.d. In this group, CK transcripts stood out (bin code 17.4.2, Fig. 3A). CK is closely related to ABA metabolism, acting antagonistically in certain situations (Guan et al., 2014). Arabidopsis CK-deficient showed enhanced salt (250 mM NaCl) and drought tolerance, and the observed CK-downregulation was associated with cell membrane integrity and ABA hypersensitivity, rather than stomatal density and ABA-mediated stomatal closure (Nishiyama et al., 2011).

3.5.2. CHO metabolism

The heatmap representing major and minor CHO metabolism presented the two groups mentioned before, with the induced genes grouping apart from the repressed ones, but

the induced genes numerically lower than the repressed ones (Fig. 3B). Some of the induced Jc183 genes modulated in a similar way of Jc171, while regarding the repressed Jc171 genes, the correspondent Jc183 almost not modulated its expression. Covering major CHO metabolism, the sucrose synthesis, represented by one of the main enzymes - Sucrose-phosphate synthase (bin code 2.1.1.1; DEG by Jc171; Fig. 3B), was repressed, reflecting the expected lower CO_2 fixation under saline stress. Still, in major CHO metabolism, the genes representing starch synthesis (2.1.2.1, 2.1.2.2; 2.1.2.3) was repressed (Fig. 3B), except by one induced *starch synthase* gene (both accessions). Starch is also an osmoprotective compound and thus protects macromolecules (membranes and proteins) from denaturing conditions (Singh et al., 2015). In turn, osmoprotectant compounds associated with the minor CHO metabolism [oligosaccharides of the raffinose family (3.1.1.2, 3.1.2.2; 3.1.3), and myo-inositol (3.4.3)], were induced by both accessions (Fig. 3B), probably helping to minimize salt-stress damages.

3.5.3. Amino acid metabolism

About amino acid metabolism (Fig. 3C), the heatmap followed the mentioned overall characteristics: induced and repressed genes (Jc171) grouped in independent clusters; the induced group clustering more genes; the Jc171 expression showing more expressive modulation than Jc183. From this set, the induced gene encoding methionine gamma-lyase (MGL; bin code 13.2.3.4.1, Fig. 3C) comprised one of the candidates selected to the RTq-PCR analysis. MGL converts methionine to 2-Ketobutyrate, a precursor in the Ile biosynthesis (Vijay and Jander, 2009), and studies covering drought-stress response pointed the amino acids Ile, Leu and Val increasing their abundances (Hildebrandt, 2018). Zhang et al. (2019) applying transcriptomic and metabolomic strategies to compare two contrasting sesame (*Sesamum indicum*) genotypes responding to salt stress (150 mM NaCl, different time points

406 up to 24 h) observed many free amino acids accumulating higher in ST (salt-tolerant) than in
407 SS (salt-sensitive) genotype, indicating this fact as a positive feature for withstanding salinity
408 stress. The studied amino acids included: alanine, asparagine, aspartate, glutamate, glutamine,
409 glycine, isoleucine, leucine, lysine, methionine, ornithine, phenylalanine, proline, serine,
410 threonine, tyrosine, and valine. Although substantial salt-induced accumulation was observed
411 to arginine, glutamine, glycine, methionine, ornithine, phenylalanine, and tyrosine, most of
412 the genes involved in the biosynthesis of these amino acids were up-regulated in both
413 genotypes under salt stress.

414 Based on the heatmap (Fig. 3C), an induced expression also involved genes, such as
415 *OASTL* (*cysteine synthase*, also *O-acetyl-serine* (*thiol*) *lyase*, bin code 13.1.5.3.1) and *SAT*
416 (*serine acetyltransferase*, 13.1.5.3.2). The respective enzymes are involved with the cysteine
417 biosynthesis. Transgenic plants overproducing SAT, OASTL or both enzymes present not
418 only elevated levels of the respective products (cysteine and O-acetyl-serine, OAS) but also
419 glutathione and other metabolites; in several cases, the transgenic plants were tolerant to the
420 abiotic stresses (Sirko et al., 2004).

421

422 ***3.5.4. Lipids metabolism***

423 About lipid metabolism, the heatmap followed most all of the general characteristic
424 mentioned before: two groups (induced and repressed grouping apart), but in this case, almost
425 the same amount of induced and repressed genes; more intense modulation by Jc171 when
426 compared with Jc183 (Fig. 3D). A clear divergence in regulation (repression by Jc171 and
427 induction by Jc183) involved the gene encoding SGT (UDP-glucose:sterol
428 glucosyltransferase; DN32792_c1_g1, bin code 11.8.3, Fig. 1D). The SGT enzyme catalyzes
429 the glycosylation of sterols to produce sterol glycosides. These glycosylated sterols play a
430 crucial role in modulating the properties and function of cell membranes (Ramirez-Estrada et

al., 2017). The mentioned authors correlated the expression of *SlSGT4* (tomato) with a marked increase in response to osmotic, saline and cold stress. Also, an induced *triacylglycerol lipase* gene (by both accessions) stood out (DN30663_c0_g1; bin code 11.9.2.1, Fig. 1D). In *A. thaliana*, a related gene (At2g31690) was among the most exclusively saline-stress induced in roots (Ma et al., 2006).

3.5.5. Redox metabolism

About the redox metabolism, the heatmap also presented the two primary groups, but almost all related genes were induced (Jc171), and again Jc171 modulated its expression more intensely than Jc183 (Fig. 1E). One divergence in gene regulation (induced by Jc171 and repressed by Jc183) was observed (DN34560_co_g1, Fig. 1E) involving the bin code 21.2.2, which is related to ascorbate. Ascorbate and glutathione are potent antioxidants that react directly with singlet oxygen and superoxide radical (reactive oxygen species, ROS), or by detoxification of hydrogen peroxide (H_2O_2) (Foyer and Noctor, 2011). At the cellular level, the salt-stress alter the ionic homeostasis causing an imbalance in redox status in the cell, with subsequent high production of ROS, which is perceived by the antioxidant systems related to ascorbate and glutathione (bin code 21.2 in Fig. 1E), ferredoxin-thioredoxin reductase (21.1), superoxide dismutase (21.6), and ascorbate peroxidase (Foyer and Noctor 2009). In *Eutrema salsugineum*, the induction of ascorbate-glutathione in response to saline stress (300 mM NaCl) prevented the harmful production of singlet oxygen in the photosystem PSII (Wiciarz et al., 2017). In the roots of Arabidopsis under salt stress (150 mM NaCl), salt-induced changes in the cell redox status affected the meristem root, impacting auxin transport (Jiang et al., 2016). Besides the importance of the antioxidant systems and the ROS scavenging, details of the generated profiles need further studies.

3.5.6. Secondary metabolites process

Concerning the secondary metabolites process (Fig. 1F), the heatmap also presented the general characteristics mentioned before: two groups, separating the induced genes from the repressed ones (Jc171); the repressed genes comprising a smaller set; the Jc171 modulating its expression more intensively when compared to Jc183. Induced genes covered phenylpropanoids metabolism (bin code 16.2, Fig. 1F). In this group, the bin code 16.2.1.1, corresponding to the *PAL* gene (*phenylalanine ammonia lyase*) was selected to the RT-qPCR validation assay. The PAL enzyme is the entry point into the phenylpropanoid pathway, being a crucial enzyme that catalyzes the first step in that pathway and leads not only to the accumulation of phytoalexins but also contributes to the development of plants and their responses to biotic stresses (Zhang et al., 2013).

Gruber et al. (2009) reported global transcriptional changes in the secondary metabolism, including phenylpropanoid, flavonoid, and isoprenoid pathways, in Medicago root apexes responding to salt stress (100 mM NaCl, one hour after salt treatment). Also, it should be noticed that isoprenoids and phenylpropanoids are part of the antioxidant defense, and their representatives are orchestrated daily by drought-stressed *Platanus acerifolia* plants during Mediterranean summers (Tattini et al., 2015). Additionally, salinity stress-induced genes involved in the flavonoid biosynthesis pathway in the salt-sensitive rice accession IR29 were observed but not in the salt-tolerant FL478 (Walia et al., 2005).

3.6. Selection of Jc183 DEGs and their expression validation by RT-qPCR

The hierarchical clustering of salt-tolerant Jc183 DEGs, and the corresponded expression by Jc171, allowed to select candidates to the RT-qPCR analysis (Fig. 4). The respective heatmap (Fig. 4) highlighted potential genes to be explored as a functional molecular marker for marker-assisted selection process in *J. curcas* breeding programs. From

481 the cluster with UR Jc183 DEGs almost not modulated by Jc171, two candidates were
482 selected [*S-adenosylmethionine-dependent methyltransferase* (*SAMe*), *Phenylalanine*
483 *ammonia-lyase* (*PAL*). Another six candidates included UR Jc183 DEGs also induced by
484 Jc171 [*S-adenosylmethionine synthase* (*SAM*), *peroxidase* (*PX*), *carboxylesterase* (*CXE*),
485 *homeobox-leucine zipper protein* (*ATHB*), *TF of NAC family*, *methionine-gamma lyase*
486 (*MGL*)]. Also, one DR Jc183 DEG (*xyloglucan endotransglucosylase*, *XTH*) was selected to
487 the RT-qPCR validation assay.

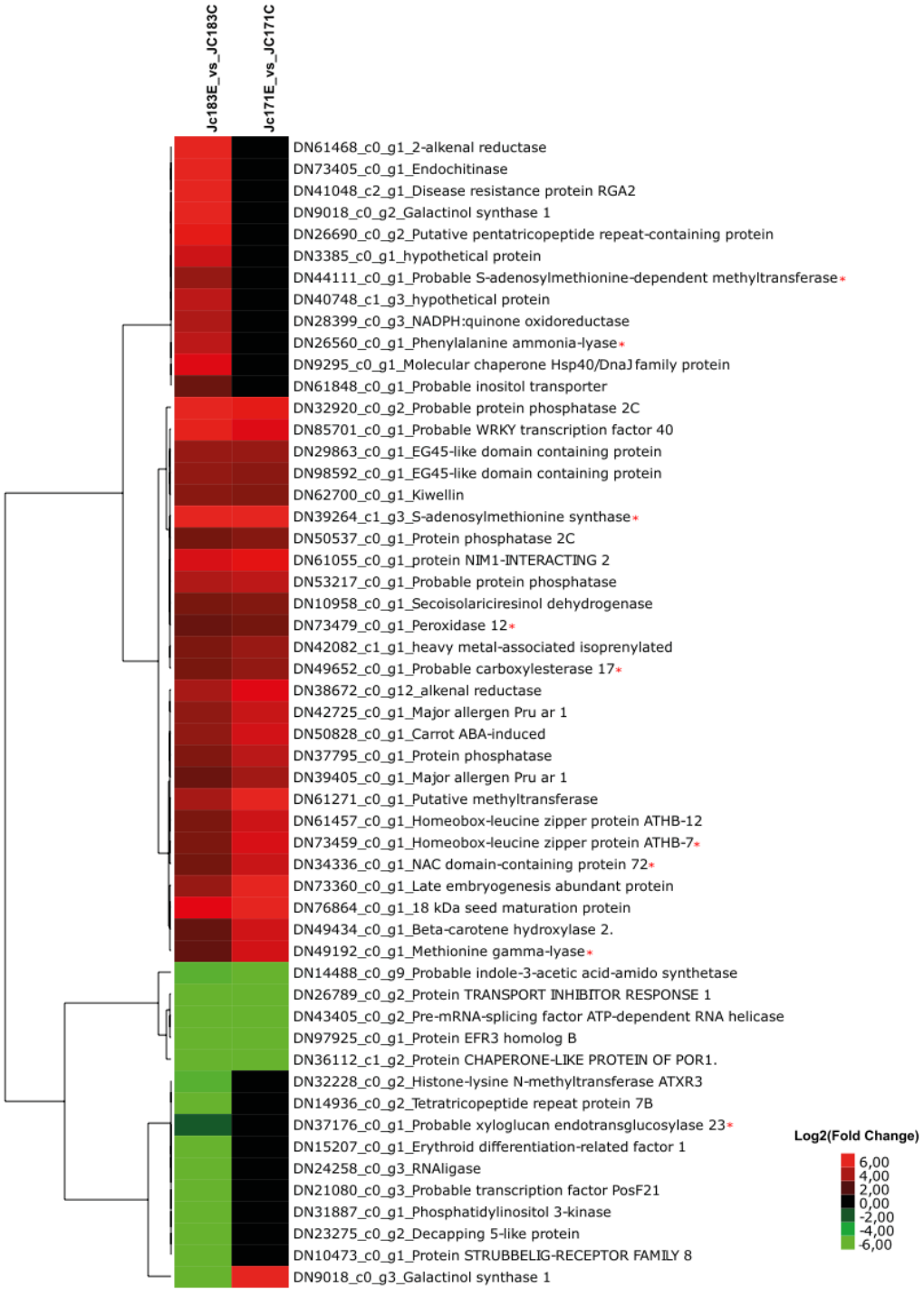

Fig. 4. Heatmap representing the hierarchical clustering analysis of the 57 DEGs of the salt-tolerant Jc183, and the correspondent expressions by salt-sensitive Jc171 accession, after three hours of salt exposure (150 mM NaCl), based on the ratio of Log_2FC (Fold Change) values, concerning the abundances of the transcript in the stressed library, in relation to the negative control library. DEG: differentially expressed gene [*p-value* ≤ 0.0001; false discovery rate, FDR ≤ 0.005; Log_2 (FC) ≥ 1 (up-regulation, red) or ≤ -1 (down-regulation, green). On the right,

495 the DEGs and its functional annotation. DEG with red asterisk were validated by RT-qPCR
496 assay.

497

498 The *in silico* data to be validated by the RT-qPCR analysis, from each selected
499 candidate gene, are shown in Table 1. The proposed primers pairs (candidates and references
500 genes, Supplementary Table S2) amplified cDNA samples (except PX *primers* with Jc171
501 cDNAs), presenting a unique amplicon (data not shown). RT-qPCR parameters [amplification
502 efficiency (E), *slope* (s), and correlation coefficient (R)], related to each *primer* pair and
503 target, based on standard curves from serial dilution of root cDNAs samples (accessions and
504 treatments), presented acceptable values (Supplementary Fig. S2), according the MIQE
505 protocol (Bustin et al., 2009), aiming to ensure reliable relative qPCR expression data
506 between samples. In general, the RT-qPCR results confirmed most of the *in silico* data, as
507 described below.

508

509 **Table 1**. Selected genes and respective expressions by the *J. curcas* salt-tolerant (Jc183) and
510 the salt-sensitive (Jc171) accessions, based on the *in silico* RNA-Seq analysis* and the RT-
511 qPCR results**.

Method **Accession**	**SAMe**	**PAL**	**SAM**	**PX**	**CXE**	**HD-Zip**	**NAC**	**MGL**	**XTH**
in silico									
Jc183	3.53/UR	4.44/UR	13.5/UR	2.49/UR	2.87/UR	2.95/UR	2.76/UR	2.36/UR	-2.08
Jc171	n.s	n.s	15.51/UR	2.76/UR	3.46/UR	5.09/UR	4.73/UR	4.99/UR	n.s
RT-qPCR									
Jc183	0.97/n.s	14.25/UR	12.30/UR	0.95/n.s	9.31/UR	5.03/UR	1.73/ n.s	15.71/UR	0.20/DR
Jc171	0.95/n.s	2.65/ UR	1.19/n.s	n.s	2.93/UR	18.69/UR	5.88/UR	8.16/UR	0.36/DR

512 UR: induced; DR: repressed; n.s: not significant at $p \leq 0.05$; SAMe: S adenosylmethionine-
513 dependent methyltransferase; PAL: Phenylalanine ammonia-lyase; SAM: S-
514 adenosylmethionine synthase; PX: Peroxidase; CXE: Carboxylesterase; HD (Zip):

Homeobox-leucine zipper; NAC: NAC transcript factor protein; MGL: Methionine-gamma lyase; XTH: Xyloglucan endotransglucosylase/hydrolase; * Log_2FC (FC: ratio of the abundances in the stressed library in relation to the respective control library);**Relative expression based on the REST software (v.2.0.13) (Pfaffl *et al*., 2002).

3.6.1. Carboxylesterase (CXE)

The induced DEG encoding CXE (EC 3.1.1.1), by both accessions (Table 1), confirmed its regulation in the RT-qPCR analysis (Fig. 5). CXEs are enzymes (α/β-hydrolase superfamily; Liu et al., 2014) that hydrolyze esters of short chain fatty acids. In plants, some of their biological functions are related to signal transduction and gene regulation (Lord et al., 2013). In plant signaling, CXEs activate phytohormones, such as SA and JA (Gershater and Edwards, 2007). The *ICME* (*isoprenylcysteine methylesterase*) gene, member of a small subfamily belonging to CXE family, encodes a protein involved in the *Arabidopsis* salt-response (200 mM NaCl), as a positive regulator of ABA signaling (Lan et al., 2010).

3.6.2. Homeobox-leucine zipper domain protein (HD-Zip)

The induced DEG (both accessions; Table 1) encoding ATHB-7 confirmed its UR regulation by RT-qPCR analysis (Fig. 5). The HD-Zip protein is a potential TF widely distributed in plants, playing roles in plant growth and response to abiotic stress (Shen et al., 2018). The overexpression of *ATHB-12* (*A. thaliana*) gene conferred salt tolerance (100 mM NaCl) in transgenic yeasts, regulating Na^+ exclusion and increasing NaCl tolerance (Shin et al., 2004). ATHB12 was involved in osmotic stress responses (Olsson et al., 2004). In transgenic tobacco, the expression of *CaHDZ12* (*Cicer arietinum*) transgene conferred salt stress tolerance (200 mM NaCl); in turn, the *CaHDZ12* silencing in chickpea plants led to a higher salt-sensitivity (Sen et al., 2017).

3.6.3. Methionine gamma-lyase (MGL)

The DEG encoding MGL (EC 4.4.1.11), induced by both accessions (Table 1), confirmed its expression by RT-qPCR analysis (Fig. 5). *Arabidopsis* plants responding to salt stress (100 mM NaCl), induced the *AtMGL* gene. MGL catalyzes the degradation of L-methionine to α-ketobutyrate, methanethiol, and ammonia. The α-Ketobutyrate is a precursor of isoleucine (Ile), an essential amino acid that also accumulates in plant cells under salt stress (Farhangi-Abriz and Ghassemi-Golezani, 2016).

3.6.4. Phenylalanine ammonia-lyase (PAL)

The induced Jc183 DEG encoding PAL (Table 1) confirmed its expression in the RT-qPCR assay (Fig. 5). PAL (EC 4.3.1.5) is a crucial enzyme in the phenylpropanoid pathway, catalyzing the deamination of L-phenylalanine (L-phe) to provide cinnamic acid, a precursor of secondary metabolites (Ibrahim et al., 2019). Phenolic acid, flavonoids, anthocyanins, lignins, and phytoalexins are derived from phenylpropanoids (Hsieh et al., 2010). Valifard et al. (2015) showed positive and significant correlations between the *PAL* gene induction, its enzymatic activity, and the phenolic content in *Salvia* species. The authors observed PAL activity increasing (42 - 45%) together to the phenolic content accumulation (35 - 43%) in the first six hours after the stress treatment (100 mM NaCl). Also, the induction of the *LjPAL* gene in *Lotus japonica* increased the PAL activity and the response to saline stress (150 mM NaCl) (Mrázová et al., 2017).

3.6.5. S-Adenosyl-L-methionine synthase (SAM)

The induced DEG encoding SAM (EC 2.5.1.6), by both accessions (Table 1), confirmed its expression by Jc183 (RT-qPCR; Fig. 5). SAM catalyzes the generation of S-adenosylmethionine (Lindermayr et al., 2006), which is a Polyamine (PA) precursor. PAs,

565 such as putrescine, spermidine, and spermine, are osmoprotectant compounds with relevant
566 contributions on the osmotic adjustment of the cells under salt stress (Chen et al., 2018;
567 Baniasadi et al., 2018). The positive correlation of PA accumulation with salt-tolerance of *A.*
568 *thaliana* plants (Kasinathan and Wingler, 2004), probably reflected their roles on proteins and
569 membranes stabilization, and the free radicals scavenging (Jang et al., 2012). Transgenic
570 tobacco plants overexpressing *SAMS2* gene (from *Suadea salsa*) also presented increased PAs
571 content, and salt-stress tolerance (200 mM NaCl) (Qi et al., 2010). A *SAM2* ortholog was
572 downregulated in *J. curcas* plants after two h/100 mM NaCl but upregulated at seven days
573 (Zhang et al., 2014). The authors highlighted the association of SAM with ETH biosynthesis
574 from methionine.
575
576 ***3.6.6. NAC transcript factor protein***
577 The induced DEG encoding the NAC TF (both accessions; Table 1) confirmed its
578 regulation only by Jc171 (RT-qPCR, Fig. 5). The NAC superfamily (**N**AM, **A**FAT, and **C**UC)
579 is one of the largest families of plant-specific TFs (Shao et al., 2015), playing crucial roles in
580 abiotic stress responses, including salt, drought, and cold (Mao et al., 2014; Sakuraba et al.,
581 2015). A *NAC TF* gene induced at two hours after the salt stress in *J. curcas* plants presented
582 downregulation at seven days (Zhang et al., 2014). Transgenic rice plants overexpressing the
583 *ONAC022* gene showed induction also two hours after salt-treatment (150 mM NaCl) (Hong
584 et al., 2016). The ONAC022 acts as a transcriptional activator stress-responsive and plays a
585 decisive role in drought and salt-stress tolerance modulating an ABA-mediated pathway.
586 Positive regulation of NAC was associated with the synthesis and accumulation of proline,
587 osmoprotective sugars, and LEA proteins (late embryogenesis abundant); all of these
588 compounds play relevant roles in abiotic stress tolerance (Song et al., 2011).
589

590 ***3.6.7. Peroxidase (PX)***

591 The induced DEG (both accessions, Table 1) encoding PX, not confirmed its
592 expression by Jc183 in the RT-qPCR assay, and no amplicon was amplified with the proposed
593 primers using Jc171 cDNAs (Fig. 5). PXs (EC 1.11.1.7) are oxidoreductase enzymes that
594 catalyze the reduction of peroxides, such as hydrogen peroxide (H_2O_2), and the oxidation of
595 organic and inorganic compounds (Chanwun et al., 2013). PX-ROS interactions are notable
596 against harmful by-products of oxidative metabolism, participating in cellular detoxification
597 and free radical scavenging (Schaffer and Bronnikova, 2012).

598

599 ***3.6.8. S-adenosylmethionine-dependent methyltransferase (SAMe)***

600 The gene encoding SAMe (Jc183 DEG, and n.s. by Jc171, Table 1) only confirmed the
601 n.s. expression of Jc171, according to the RT-qPCR results (Fig. 5). In *J. curcas* plants under
602 150 mM NaCl, the *SAMe* expression showed the highest expression in root tissue (by semi-
603 quantitative RT-PCR) after eight hours of salt exposure (Eswaran et al., 2012). SAMe is
604 synthesized from ATP and methionine, a reaction catalyzed by methionine
605 adenosyltransferase (SAM; Luka et al., 2009), also known as S-Adenosyl-L-methionine
606 synthase (synonyms; https://www.brenda-enzymes.org/). SAM-binding methyltransferases
607 utilize the methyl donor SAM as a cofactor to methylate proteins, small molecules, lipids, and
608 nucleic acids (Martin and McMillan, 2002). SAMes play an essential role in cellular
609 metabolism, transcription, signal transduction and detoxification (Hayashi et al., 2018). The
610 overexpression of *SAMe* in *Hibiscus cannabinus* plants responding to NaCl (200 mM, six
611 days) conferred salt-tolerance (Niu et al., 2016). The salt-induced *SAMe* gene (named
612 *IbSIMT1*) in sweet potato (*Ipomoea batatas*) under saline stress (86 mM NaCl/ four weeks)
613 showed induction in the first 12 hours of stress; the salt-tolerance was correlated with osmotic
614 balance adjustment, membrane integrity and photosynthesis protection, and ROS

615 detoxification (Liu et al. 2015). Probably, the three hours of salt-exposure time was not
616 enough to show the *SAMe* induction by Jc183 accession.
617
618 ***3.6.9. Xyloglucan endotransglucosylase/hydrolase (XTH)***
619 The gene encoding XTH was DR DEG by Jc183 (Table 1), and the DR expression was
620 confirmed by RT-qPCR assay, also by Jc171 (Fig. 5). The xyloglucans (hemicellulosic
621 polymers of dicotyledonous plants) bind to cellulose fibrils, whose interactions are modulated
622 by expansin enzymes and XTHs (Malinowski et al., 2004). Thus, XTH (EC 2.4.1.207) is
623 involved with wall remodeling and cell expansion. Under abiotic stress, XTH acts on the
624 flexibility of tissues (leaf and root), conferring greater cell wall extensibility, and better plant
625 adaptation (Tenhaken, 2015). In transgenic *Arabidopsis* plants responding to saline stress
626 (100 mM NaCl), the *CaXTH* gene (*Capsicum annuum*) was induced after six days of salt
627 treatment. Positive expression of the *CaXTH* gene in pepper roots conferred a low reduction
628 in root length of plants under salt stress (Cho et al., 2006). Depending on the abiotic stress,
629 the cell wall is a target to be affected. In the present study, the wall remodeling and cell
630 expansion in roots do not appear to be affected after three hours of J. *curcas* have been
631 exposed to salt (150 mM NaCl), based on the *XTH* gene expression.
632

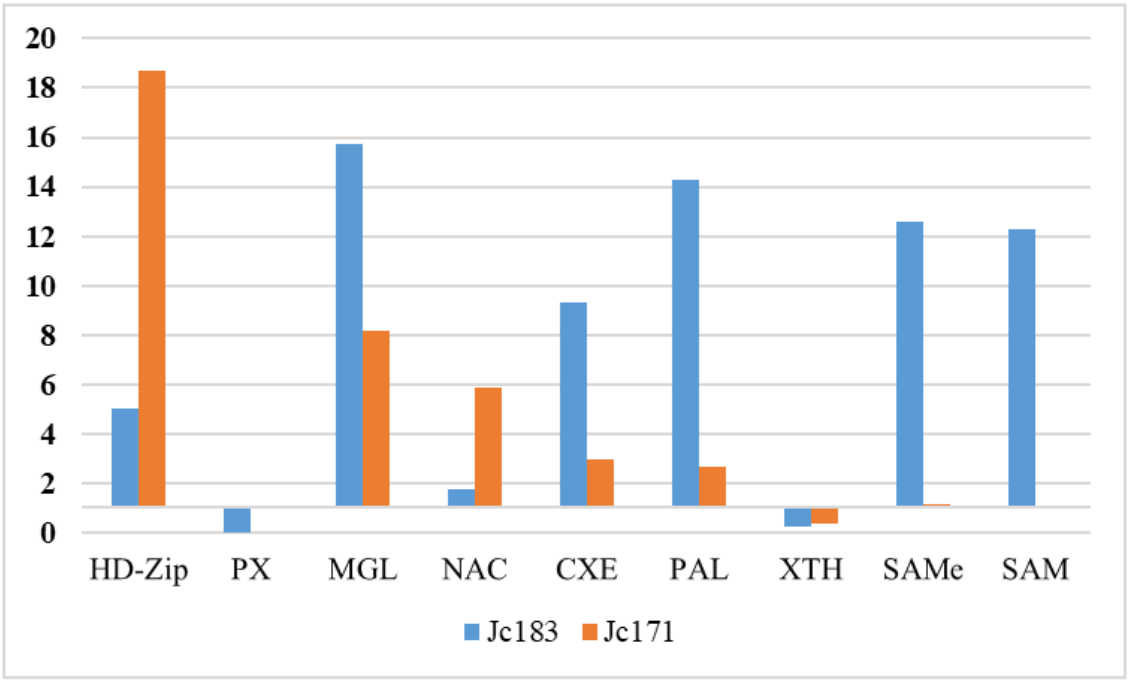

Fig 5. RT-qPCR results of selected Jc183 candidate genes, reference genes, and negative controls, performed with root cDNAs of *Jatropha curcas* Jc183 (salt-tolerant) and Jc171 (salt-sensitive) plants after three hours of salt-exposure. Expression values normalized by the reference genes *actin* and *beta-tubulin*, and the relative expression data calculated by the REST software (v.2.0.13) (Pfaffl *et al*., 2002). Genes: *HD-zip* (*homeobox-leucine*); *PX* (*Peroxidase*); *MGL* (*methionine-gamma-lyase*); *NAC Transcript factor*; *CXE* (*Carboxylesterase Probable 17*); *PAL* (*phenylalanine ammonia lyase*); *XTH* (*xyloglucan endotransglucosylase*); *SAMe* (*S-adenosylmethionine-dependent methyltransferase*); and *SAM* (*S-adenosylmethionine synthase 1*).

4. Conclusion

Sensitive plants in saline conditions use protection strategies, trying to stabilize photosystems, protect membranes and proteins, modulate the redox state, and provide detoxification of free radicals. The *de novo J. curcas* RNA-Seq transcriptome generated based on two accessions responding to salinity (150 mM NaCl, after three h), covered 101 MB and assembled 145,422 transcripts, around half of them encoding predicted proteins. Curiously, the salt-sensitive Jc171 accession induced more differentially expressed genes than the salt-tolerant Jc183. Based on Jc171 DEGs, the correspondent genes by Jc183, involving different

metabolisms (phytohormone, CHO, lipid, amino acid, redox), and some secondary metabolites, presented their expressions almost not modulated or even not detected. Based on the smaller number of Jc183 DEGs, the correspondent expressions by Jc171 was less similar. Although the intensive transcriptional effort of Jc171 inducing more DEGs in response to the salt stimulus, this effort was not enough to avoid the visible damages observed only in Jc171 leaves. In general, the upregulated genes numerically exceeded the downregulated ones, and despite some similar regulations presented by both accessions, the non-modulation by Jc183 suggest better control of the ionic homeostasis applying different salt-tolerance strategies from those observed in the Jc171 expressed profile. Some of the DEGs candidates involved in salt-stress tolerance with their expressions validated by RT-qPCR assays could be explored as functional molecular markers to be applied in marker-assisted selections, in *J. curcas* breeding programs. Thus, the present data not only uncovered genes related to salt-response but also promoted an overview of the molecular mechanisms underlying *J. curcas* salt-tolerance, providing potential functional molecular markers useful to the breeding programs. However, further studies are necessary to fully elucidate the molecular basis involving the development of plants under salt stress.

Conflict of Interests

The authors declare that they have no conflict of interests.

Authors contributions

LE, MFP, AMBI and EAK conceived and designed the experiments; MCPS, GALC, EB and MDS carried out the experiments; MCPS, GALC, MDS, and EAK analyzed the data; MCPS, and EAK wrote and revised the paper. All authors read and approved the final manuscript.

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4.2 Physic nut transcription factors: identification and transcriptional modulation under salt stress

Plant Molecular Biology Reporter - Submission Notification to co-author

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Full author list: George André de Lima Cabral; Eliseu Binneck; Marislane Carvalho Paz de Souza; Manassés Daniel da Silva; José Ribamar Costa Ferreira Neto; Marcelo Francisco Pompelli; Laurício Endres; Ederson A Kido, PhD

Dear M.Sc. Marislane Paz de Souza,

We have received the submission entitled: "Physic nut transcription factors: identification and transcriptional modulation under salt stress" for possible publication in Plant Molecular Biology Reporter, and you are listed as one of the co-authors.

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Physic nut transcription factors: identification and transcriptional modulation under salt stress

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Abstract:

Physic nut (*Jatropha curcas*), a small oleaginous tree spontaneously occurring in arid and semi-arid tropical regions, is a sustainable and renewable energy source for biodiesel. However, the *J. curcas* yield in such areas should consider soil salinity and its consequences. Transcription factor (TF) proteins recognize *cis*-regulatory elements in promoters of genes regulating their expression. In the present work, differentially expressed genes (DEGs) encoding putative TFs in physic nut plants after three hours of NaCl (150 mM) exposition covered 23 TF families. The expressed profiles of members from AP2/ERF and NAC families

basically presented induction after the salt treatment, while members of bHLH, FHY3-FAR1, and ARF families were repressed. The gene ontology (GO) enrichment analysis concerning the induced TF DEGs highlighted terms related to abiotic stress responses, while the repressed TF DEGs stood out terms highlighting the basal metabolism. In turn, the TF enrichment analysis predicted TFs over-represented targeting promoters of induced TF DEGs. The enriched TFs are good candidate as transgenes. Additionally, RT-qPCR analyses validated the up-regulation of six DEGs (*RAV1*, *ERF9*, *ZAT12*, *PTI5*, *MYB340*, and *BZIP4*) of eight candidates, suggesting the reliability of the expressed *J. curcas* TFoma after three hours of salt exposition (150 mM NaCl). The results help to understand the molecular basis of salinity stress response in physic nut plants. Also, provide valuable resources to select potential candidates for transgenic studies, as well as to develop functional molecular markers to assist selection steps in breeding programs.

Key words: *Jatropha curcas*, RNA-Seq, transcriptome, abiotic stress, salinity.

Introduction

Physic nut (*Jatropha curcas* L.), a perennial tropical plant belonging to the Euphorbiaceae family, has been an alternative source of renewable biofuel producer (Openshaw 2000). *J. curcas* presents some advantages over so-called oleaginous biodiesel plant producers, including the high (30–50%) oil content in the seeds allied to the relatively easily biofuel conversion (Deore and Johnson 2008), and no competition with human food destination. Since it is a spontaneous species occurring in arid and semi-arid tropical regions (Johnson et al. 2011), two problems need to be addressed: soil salinity and plant salt stress. Crop production in arid and semi-arid regions must consider natural saline/sodic soils, high plant evapotranspiration, low rainfall, and unfavorable physical/physicochemical soil

properties, which associated to irrigation problems leads to soil salinization, and its consequence in plant growth (Campos et al. 2012).

In plants growing in regions with low water availability, the high salt levels in soil solution reduce the osmotic potential in the root zone sufficiently to reduce water absorption (Dasgan et al. 2002), also the ions acting on protoplasm disturb the mineral plant nutrition (Munns 2002), limiting the plant growth (Gurgel et al. 2003).

Plants exposed to environmental stresses change their metabolisms according to the genes properly activated or repressed (Benko-Iseppon et al. 2005). Based on the set of transcription factor (TFs) activated by signal transduction in response to a stimulus, genes are expressed and the transcriptomes are reprogrammed. The TF proteins recognize the *cis*-regulatory elements (CRE) in the promoters of genes that will be expressed regulating that expression (Wang et al. 2009). In this way, TFs play a key role in biotic and abiotic stress responses, as well as in plant development (Riechmann et al. 2000), through the spatial and temporal regulation based on their targets (Zhang et al. 2011; Jin et al. 2014). Therefore, to characterize the TFoma after three hours of NaCl exposition (150 mM) helps to understand the transcriptional dynamics of *J. curcas* plants responding to the salt and also to improve the salt tolerance.

Materials and Methods

a) Plant material and the salinity assay

Two Brazilian physic nut accessions named Jc183 e Jc171 (Lozano-Isla et al. 2018) were carried out in a salt treatment assay with plants growing in greenhouse (March 2016) at the Agricultural Science Center/Federal University of Alagoas (UFAL/CECA, Rio Largo, AL, Brazil; geodesic coordinates 09º28'02"S; 35º49'43"W, altitude: 127 m). The classified

climate, according to Thorthwaite and Mather (1955), is wet, megathermic, with moderate water deficiency in the summer (December to March) and some excess of water in the winter (July to September).

Homogeneous seeds (size and weight) of both accessions were sown in pots (50 L) filled with 20 kg of washed sand. From the first eophiles (5-10 days after germination, DAG), the seedlings were thinned, leaving only the most vigorous plant per pot. The plants were sampled in a completely randomized design with three biological replicates (two accessions x two treatments (with and without salt) x three biological replicates). During their cultivation, plants were irrigated (4 p.m.) every three days with Hoagland nutrient solution (20% w/v) (Epstein, 1972). A week before the salt application (60 DAG), plants received Hoagland solution 100% (full strength) every day. To the salt application, a NaCl solution (150 mM) was added to the Hoagland solution, and plants were salt exposed (9 a.m.) for three hours. Plants irrigated only with the Hoagland solution comprised the negative control. After the NaCl exposure time, root samples were collected, immediately frozen in liquid nitrogen (N_2), being kept in - 80ºC until RNA extraction.

b) RNA isolation, the RNA-Seq libraries, and its sequencing

Total RNA was isolated from the root samples using the SV Total RNA Isolation System (Promega). The RNA concentration was estimated by NanoDrop spectrophotometer (Thermo Scientific NanoDrop 2000), and the RNA quality assessed by absorbance ratios (OD 260/280 nm ≥ 1.9 and OD 260/230 nm ≥ 1.9), and agarose gel electrophoresis, 1.5% (w/v). Re-analysis of the RNAs integrities by the Agilent Bioanalyzer 2100 system (Santa Clara, CA, USA) identified samples with RIN (*RNA Integrated Number*) ≥ 9.0. High-quality RNAs were used to generate RNA-Seq libraries (12: two accessions x three biological replicates x two treatment), at the Genomic Center of the "Luiz de Queiroz" College of

Agriculture (ESALQ/USP, Piracicaba, SP, Brazil). The RNA-Seq libraries were sequenced (2x100 bp *paired-end*) using the Illumina HiSeq2500 Platform (Eurofins MGW, Germany).

c) Transcriptome assembly and the transcript annotation

The quality data of the reads (base sequence quality and content) generated from the RNA-Seq *paired-end* libraries were visualized with the FastQC software (v.0.11.5), before and after adapter filtering and trimming (*paired-end*) steps using default parameters of the Trimmomatic tool (v.0.36; Bolger et al. 2014). After excluding reads showing low quality and those with unknown adapters and nucleotides, pairs of high-quality reads (*Phred* $\geq$ 30, all bases) were used for *de novo* transcriptome assembly performed with the Trinity 2.2.0 software (Grabherr et al. 2011). The expression levels of assembled transcripts and UniGenes were estimated by RSEM software (Li e Dewey 2011), and the alignment package Bowtie (v4.4.7; Langmead et al. 2009) was applied to map reads back to UniGenes. The normalized FPKM (*Fragment Per Kilobase of cDNA Per Million fragments mapped*) matrices were generated from the RSEM counts, which were used for the differential expression analyses performed by edgeR package (Robinson et al. 2010).

Potential transcripts encoding TFs were identified by BLASTx alignments (*e-value* $\leq$ e^{-10}) against proteins sets downloaded (August 2018) from the databases: NCBI (*J. curcas*; https://www.ncbi.nlm.nih.gov/), Phytozome v.12 (*Ricinus communis* and *Manihot esculenta*; https://phytozome.jgi.doe.gov/pz/portal.html), and UniProtKB/SwissProt (http://www.uniprot.org/).

d) Identification of the differential expressed genes (DEGs)

The gene expression analysis between experimental samples detected differentially expressed genes (DEGs) as those UniGenes showing *p-value* $\leq$ 0.0001, FDR (*False Discovery*

Rate) ≤ 0.005, and $Log_2FC \geq 1$ (classified as up-regulated, UR) or ≤ -1 (down-regulated, DR). Fold change (FC), based on Log_2FC values, was the ratio representing the modulation of the UniGene abundance in the stressed library compared to the negative control (henceforth, S *vs.* C, for brevity). The modulation of the gene expression data, after hierarchical clustering analysis performed by Cluster software (v.3.0; https://cluster2.software.informer.com/3.0/), generated heatmaps, visualized with the JavaTreeview software (v.1.1; http://jtreeview.sourceforge.net). The Venn diagrams were generated by online tool Venny (http://bioinfogp.cnb.csic.es/tools/venny/).

e) The GO and TF enrichment analyses

Together with the Gene Ontology analysis, a functional GO terms enrichment analysis identified those over-represented (Fisher's exact tests, *p-value* ≤ 0.01) based on the input file applied to the PlantRegMap tool (Plant Transcriptional Regulatory Map; http://plantregmap.cbi.pku.edu.cn; Jin et al. 2014). The input file corresponded to the set of genes encoding TFs, and individually covered the UR DEGs, the DR DEGs, and the non-DEGs (n.s.). A similar procedure was performed involving the TF enrichment analysis applying the respective tool also provided by the same database.

f) The gene expression validation by RT-qPCR assay

The gene expression of DEGs candidates encoding TFs [*RAP2*-3 (*ethylene-responsive transcription factor RAP2-3*), *RAV1*(*AP2/ERF and B3 domain-containing transcription factor RAV1*), *ERF9* (*ethylene-responsive transcription factor* 9), *DREB1H* (*dehydration-responsive element-binding protein 1H*), *ZAT12* (*Zinc finger protein ZAT12*), *PTI5* (*pathogenesis-related genes transcriptional activator PTI5*), *MYB340* (*Myb-related protein 340*), and *BZIP4* (*basic leucine zipper 4*); Table S1] were analyzed in RT-qPCR assays. *Primer* pair were designed

based on the correspondent RNA-Seq transcript using the *online* Primer 3 tool (Rosen and Skaletsky 2000), following some parameters: amplicon size (between 70 and 200 bp), melting temperature [50ºC (minimum), 70ºC (optimum) and 80ºC (maximum)], and GC content (45 - 55%). Proposed primers (**Supplementary Table S1**) were synthesized by Invitrogen Life Technologies (USA) and previously tested amplifying cDNAs in conventional PCR. After that, RT-qPCR reactions were performed in a real-time LineGene 9600 equipment (Bioer®, Hangzhou, China) using SYBR Green detection system. The PCR reaction (10 μL) included 5 μL of SYBR Green SuperMix (Applied Biosystems, Foster City CA, EUA), 1 μL of diluted cDNA (1/10), 0.3 μL of each *primer* (5 μM) and 3.4 μL ddH_2O. The reactions followed the settings: initial denaturation of 95°C for 2 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 60 s. All RT-qPCR reactions were performed in 96-well plates with three biological, and three technical replicates, the negative controls, and two reference genes adequately tested for the present assays [*β-tubulin* and *actin* (Ma et al. 2015)]. The dissociation curves were obtained, heating the amplicons from 65 to 95°C for 20 min after the RT-qPCR cycles. The LineGene software (v.1.1.10) estimated the Cq values (quantification cycles), and the absolute and relative quantifications. The relative expression data evaluated by REST 2009 software (Relative Expression Software Tool v.2.0.13; Pfaffl et al. 2002) applied randomization test with 2,000 permutations and considered the hypothesis of significant differences between the control and treatment groups. The MIQE (*The Minimum Information for Publication of Quantitative Real-Time PCR Experiments*; Bustin et al. 2009) protocol was followed to assure data reliabilities.

Results

a) The *J. curcas de novo* transcriptome and the DEGs encoding TFs

The high-throughput sequencing of the *J. curcas* RNA-Seq libraries (12) of roots exposed to NaCl (150 mM, three hours) generated 238,286,823 raw *reads*. After removing adapters and trimming low quality bases, 230,140,599 high-quality *reads* (*Phred* ≥ 30, all bases; 96.58% of the *reads*) allowed the *de novo* transcriptome assembly, with 145,422 transcripts (101 Mb) and 126,342 UniGenes (76 Mb), showing a GC% of 41.55, and the N_{50} comprising 1,308 bp for transcripts, and 993 pb for UniGenes. The global transcriptome will be not addressed in the presented report, only those transcripts encoding potential TFs.

Based on the BLASTx analysis (*e-value* e^{-10}) of the transcripts against protein databases from Euphorbiaceae species (Figure 1), 1,876 transcripts encoding TFs were identified. The inclusion of *R. communis* and *M. esculenta* (Phytozome database), both *J. curcas*-related species, besides increased the sequence similarities (**Figure 1**), also increased the efficiency of the annotation process.

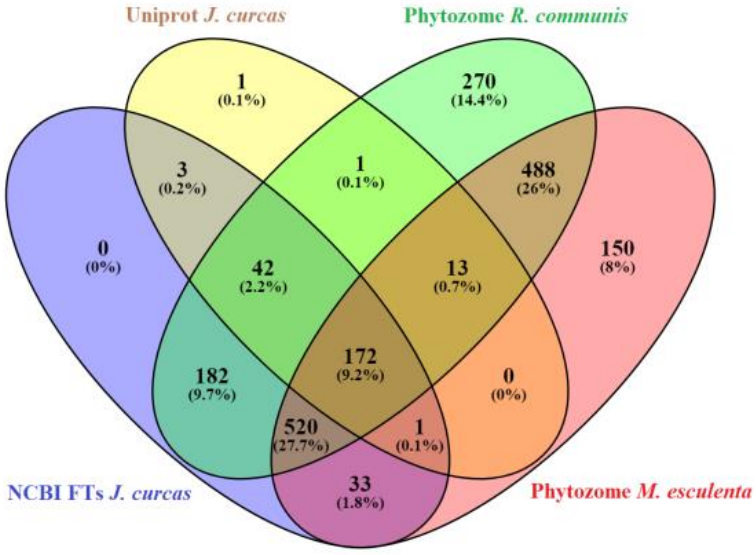


Figure 1. Venn diagram showing numbers of *Jatropha curcas* RNA-Seq transcripts (from roots of plants after three hours of NaCl exposition; 150 mM) encoding transcript factor proteins similar (e-value ≤ e-10) to those from different public proteins databases (NCBI, https://www.ncbi.nlm.nih.gov/.; Phytozome, https://phytozome.jgi.doe.gov/pz/portal.html; UniProt, https://www.uniprot.org/).

The declared DEGs from each accession [*p-value* ≤ 0.0001, FDR ≤ 0.005, $Log_2FC \geq 1$ (UR) or ≤ -1(DR)] in response to the salt-treatment was quite different (4,646 from Jc171, and 57 from Jc183), highlighting the great effort of the Jc171 trying to minimize visible damages only observed in Jc171 leaves (**Figure 2**). Based on that, the present investigation of TFs differentially expressed in response to the salt-treatment was restricted to the Jc171expressed profile.

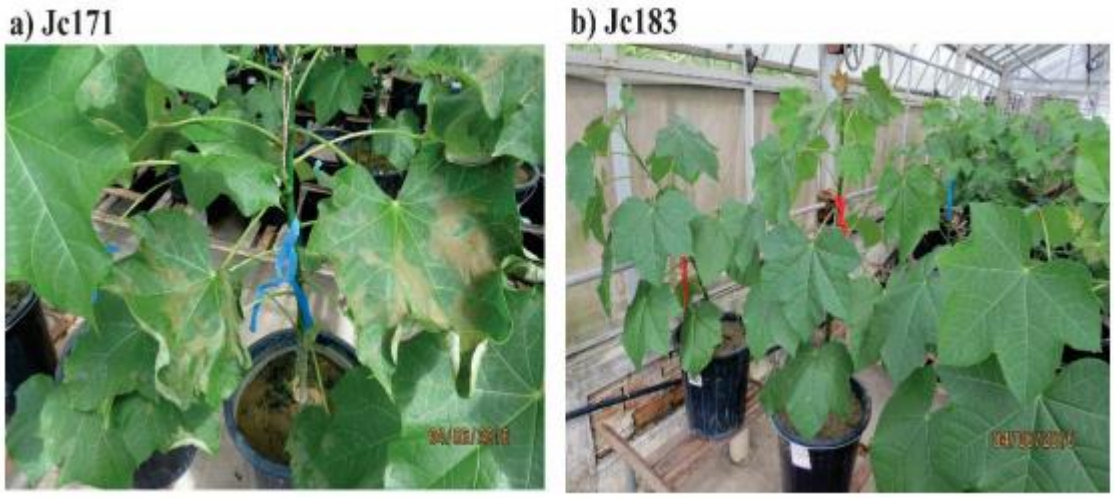


Figure 2. Visual damages on leaves only observed in Jatropha curcas Jc171 accession after three hours of NaCl exposition (150 mM).

From the Jc171 DEGs (4,646), 148 of them encoding TFs (78 UR and 70 DR; Table S4) encompassed 23 TF families (**Figure 3**).

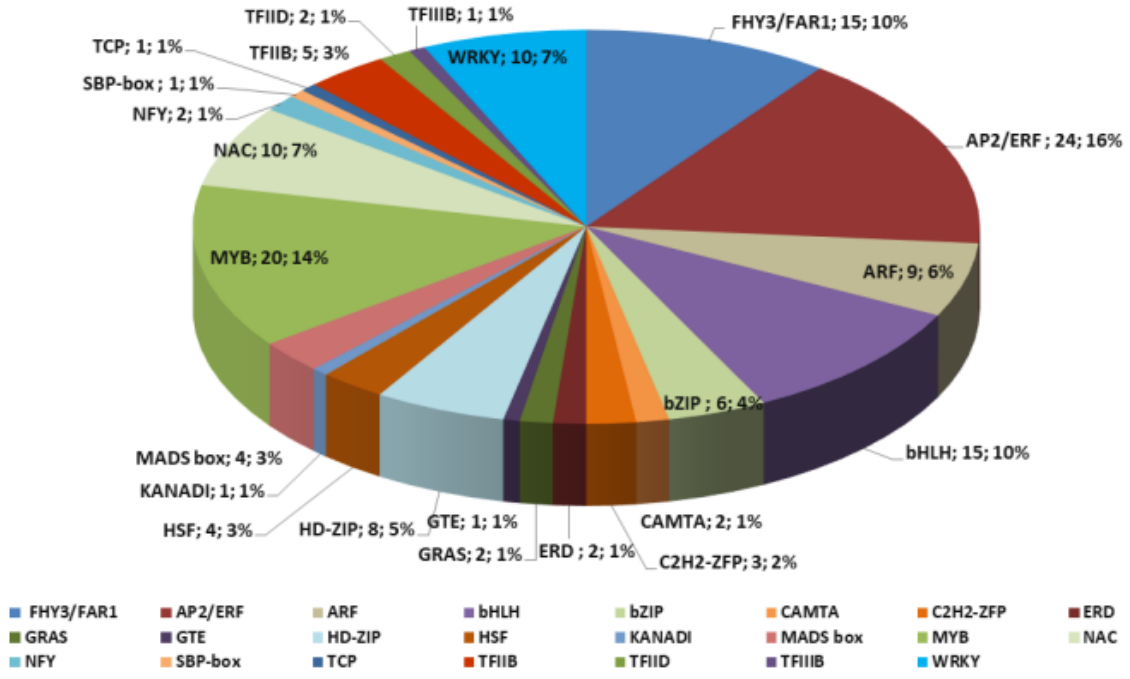


Figure 3. Transcription factor (TF) families associated with differentially expressed genes [DEG: p-value ≤ 0.0001, FDR ≤ 0.005, Log2FC ≥ 1 or ≤ -1] from *Jatropha curcas* Jc171accession after salt-treatment (150 mM NaCl). The TF family name is followed by the total of DEGs and the correspondent percentage.

The TF families outstanding in isoforms members were: AP2/ERF, MYB, bHLH, FHY3/FAR1, WRKY, NAC, ARF, HD-zip, and bZIP (**Figure 3**). Concerning a single TF family, the proportion of induced DEGs to the repressed DEGs was variable (**Figure 4**).

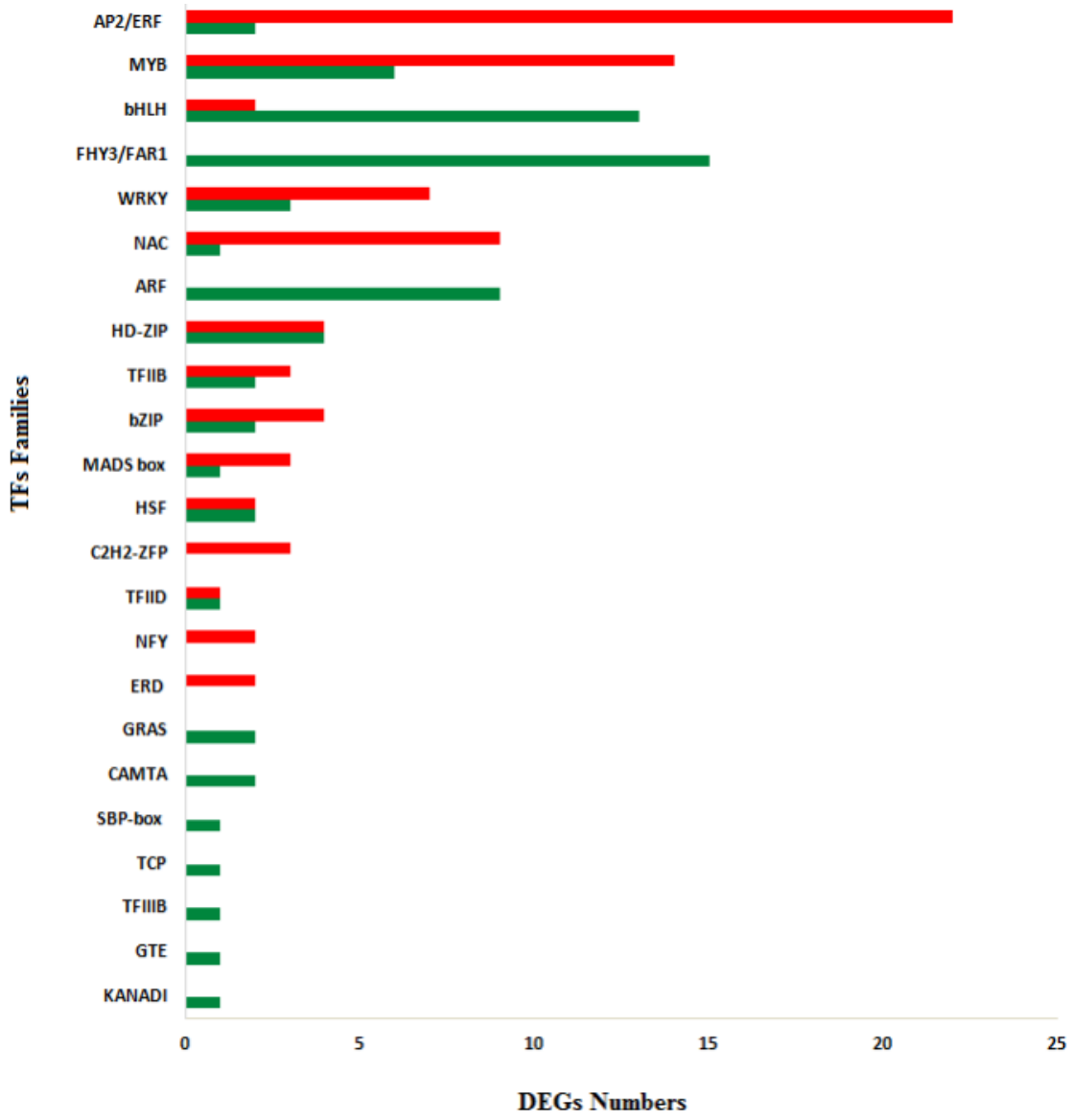


Figure 4. Families of transcription factors showing differentially expressed genes [DEG: p-value ≤ 0.0001, FDR ≤ 0.005, Log2FC ≥ 1 or ≤ -1] from Jc171accession after salt-treatment (150 mM NaCl): the induced DEGs are represented by red bars, and the repressed DEGs by the green bars.

TF families presenting more induced DEGs were AP2/ERF (22), MYB (14), NAC (9) and WRKY (7), while families showing more repressed DEGs were FHY3/FAR1 (15), bHLH (13), ARF (9) and MYB (6) (**Figure 4**). Also, some TF families only presented UR or DR isoforms members (**Figure 4**).

225

226 **c) The GO enrichment analysis**

227 From the 78 induced DEGs encoding TFs, 71 identified by the PlantRegMap tool in
228 the input gene list presented 128 enriched GO terms (*p-value* ≤ 0.01). From the 57 of 70
229 repressed DEGs (TFs) in the input gene list, the enriched GO terms were 143. When the input
230 gene list involved 958 TFs codified by non-DEGs, the enriched GO terms were 435. All the
231 enriched terms were distributed into the three main GO categories [Biological process (BP),
232 Molecular function (MF), and Cellular Component (CC); **Supplementary Table S2**].

233 Comparing the three sets of enriched GO terms in a Venn diagram, six terms
234 highlighted the UR DEGs codifying TFs (**Figure 5A**): *metabolic process* (GO:0008152),
235 *death* (GO:0016265), *heterocyclic compound binding* (GO:1901363), *response to wounding*
236 (GO:0009611), *cell death* (GO:0008219), and *organic cyclic compound binding*
237 (GO:0097159). Eight enriched terms outstood the repressed DEGs (**Figure 5A**): *single-*
238 *organism cellular process* (GO:0044763), *vegetative phase change* (GO:0010050), *cell*
239 *proliferation* (GO:0008283), *single-organism process* (GO:0044699), *negative regulation of*
240 *growth* (GO:0045926), *regulation of cell proliferation* (GO:0042127), *cell fate commitment*
241 (GO:0045165), and *regulation of circadian rhythm* (GO:0042752).

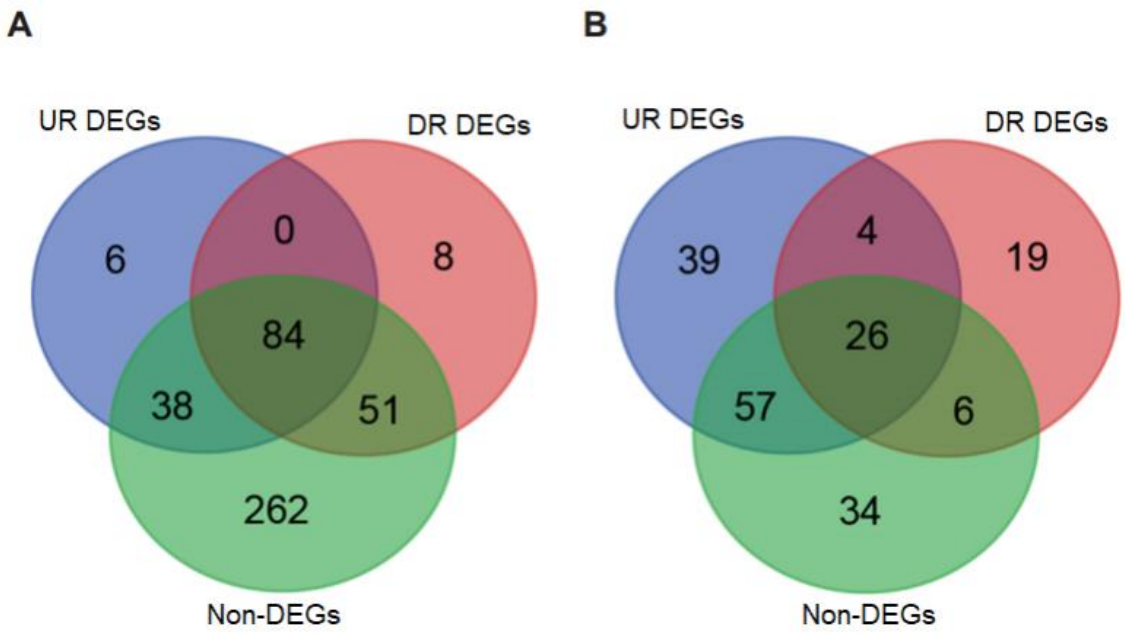


Figure 5. Venn diagram comparing the enriched GO terms (A) or the enriched TFs (B) identified by the respective PlantRegMap tool using individually different input gene list: the UR DEGs, the DR DEGs or the non-DEGs. DEG: differentially expressed gene (thresholds: p-value ≤ 0.0001, FDR ≤ 0.005, Log2FC ≥ 1 (UR, up-regulated) or ≤ -1 (DR, down-regulated).

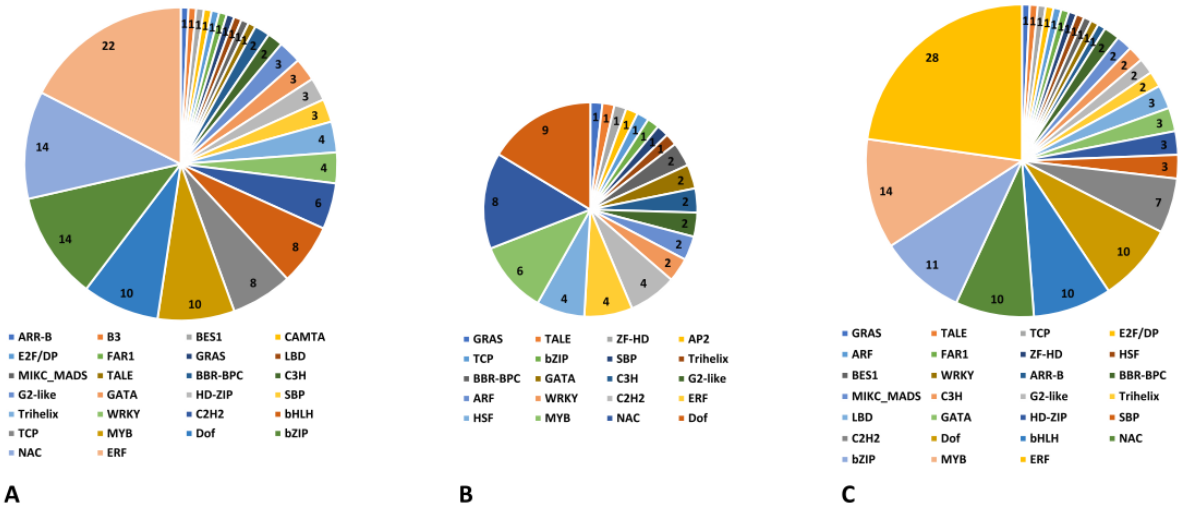
d) TF enrichment analysis

Defining 72 UR DEGs codifying TFs, as the target genes in the input list, the TF enrichment tool predicted 1,164 regulations by 245 TFs; those enriched TFs counted 126. When DR DEGs comprised the input gene list, 58 target genes predicted 185 TFs and 729 regulations; from the predicted TFs, 55 were considered enriched. When 1,000 TFs non-DEGs (n.s.) comprehended the input gene set, 11,936 predicted regulations involved 302 TFs, being 123 the enriched TFs. All the enriched TFs, their TF families, and number of predicted targets genes presented in the input gene list (UR, DR or non-DEGs) are presented in the **Supplementary Table S3**. Comparing the three sets of enriched TFs in a Venn diagram, those enriched TFs interacting exclusively with promoters of UR DEGs (as their targets) were

259 39, exclusively with the DR DEGs were another 19 TFs, and those with the non-DEGs were
260 another 34 TFs (**Figure 5B**).

261 ERF family members (22) comprised most of the enriched TFs predicted interacting
262 with the UR DEGs; also, ERF family (28) stood out with the non-DEGs; while Dof family
263 members (9) interacted most with the DR DEGs. The distribution of the TF families by
264 enriched FTs predicted targeting promoters of TF genes comprising the UR DEGs, the DR
265 DEGs, or the non-DEGs, all codifying putative TFs expressed in roots of *J. curcas* after the
266 salt exposition, is presented in the **Figure 6**, and the number or possible target genes in the
267 **Supplementary Table S3**.

268



269

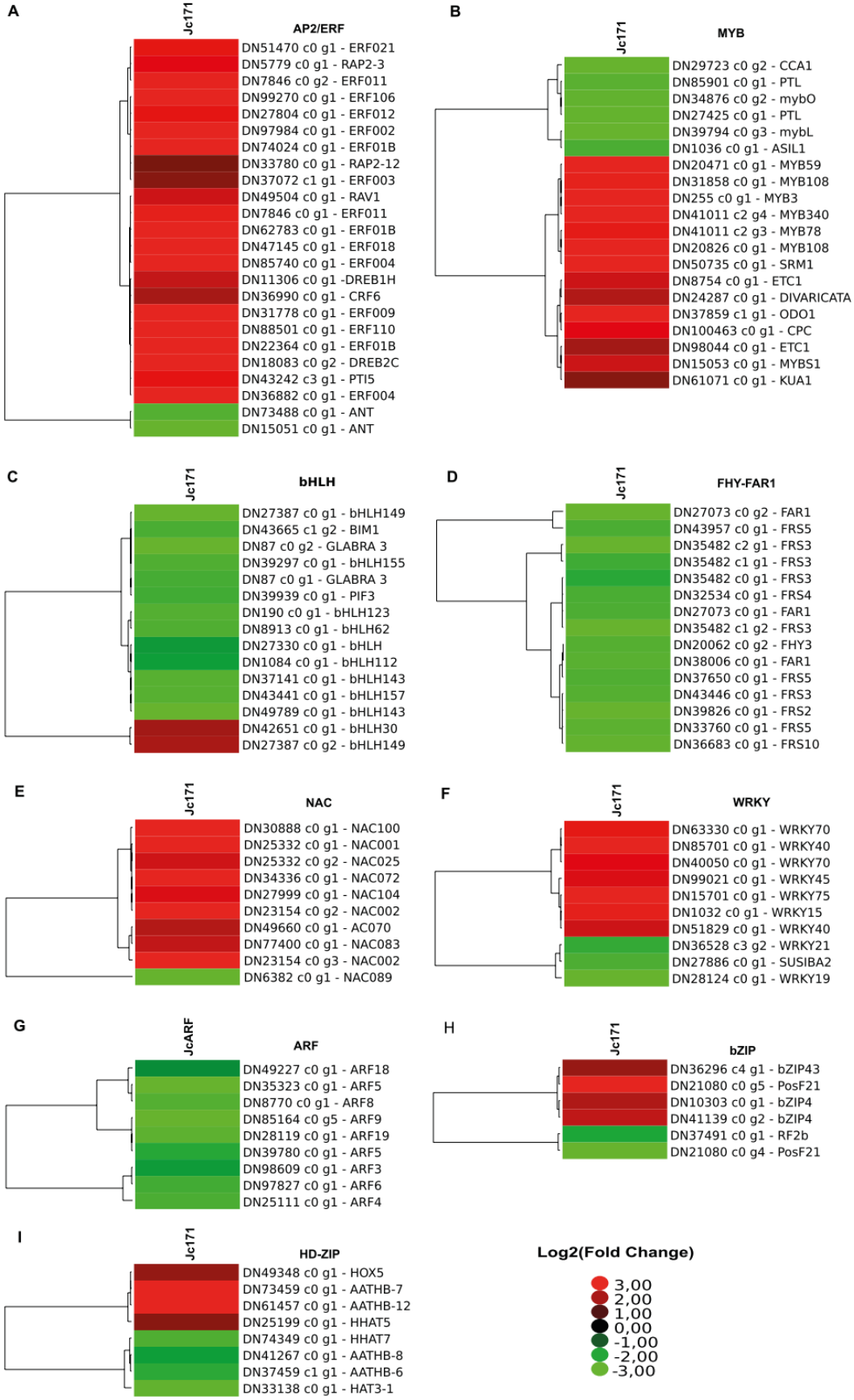
270 Figure 6. Distribution of enriched FT family members predicting targeting promoters of
271 different sets of TF genes: the up-regulated DEGs (A), the down-regulated DEGs (B), and the
272 non-DEGs (C) expressed in roots of J. curcas after salt-treatment (150 mM NaCl, three
273 hours). DEGs (threshold: p-value ≤ 0.0001, FDR ≤ 0.005, Log2FC ≥ 1 or ≤ -1.

274

275 **e) Expressed profiles of the most representative TF families associated to the salt**
276 **response**

The heatmaps with the expressed profiles of members of the most representative TF families are shown in **Figure 7**, and the respective expression data modulated, based on the ratio of Log_2FC values expressed by Jc171 after the salt-treatment in relation to the negative control is provided in the **Supplementary Table S4**, together with the sequence fasta format.

Almost all members of the AP2/ERF family were up-regulated after the salt stimulus (**Figure 7A**). A similar profile was observed with members of the NAC family (**Figure 7E**). Members of MYB (**Figure 7B**), WRKY (**Figure 7F**), and bZIP (**Figure 7H**) family showed more up-regulation than down-regulation. A balance with up- and down-regulated members comprised the HD-ZIP family (**Figure 7I**). In turn, almost all members of the bHLH family presented down-regulation (**Figure 7C**), and the totality of the FHY3-FAR1 (**Figure 7D**) and ARF (**Figure 7G**) members exhibited down-regulation after the salt-treatment.


A
Jc171
AP2/ERF
DN51470 c0 g1 - ERF021
DN5779 c0 g1 - RAP2-3
DN7846 c0 g2 - ERF011
DN99270 c0 g1 - ERF106
DN27804 c0 g1 - ERF012
DN97984 c0 g1 - ERF002
DN74024 c0 g1 - ERF01B
DN33780 c0 g1 - RAP2-12
DN37072 c1 g1 - ERF003
DN49504 c0 g1 - RAV1
DN7846 c0 g1 - ERF011
DN62783 c0 g1 - ERF01B
DN47145 c0 g1 - ERF018
DN85740 c0 g1 - ERF004
DN11306 c0 g1 -DREB1H
DN36990 c0 g1 - CRF6
DN31778 c0 g1 - ERF009
DN88501 c0 g1 - ERF110
DN22364 c0 g1 - ERF01B
DN18083 c0 g2 - DREB2C
DN43242 c3 g1 - PTI5
DN36882 c0 g1 - ERF004
DN73488 c0 g1 - ANT
DN15051 c0 g1 - ANT
B
Jc171
MYB
DN29723 c0 g2 - CCA1
DN85901 c0 g1 - PTL
DN34876 c0 g2 - mybO
DN27425 c0 g1 - PTL
DN39794 c0 g3 - mybL
DN1036 c0 g1 - ASIL1
DN20471 c0 g1 - MYB59
DN31858 c0 g1 - MYB108
DN255 c0 g1 - MYB3
DN41011 c2 g4 - MYB340
DN41011 c2 g3 - MYB78
DN20826 c0 g1 - MYB108
DN50735 c0 g1 - SRM1
DN8754 c0 g1 - ETC1
DN24287 c0 g1 - DIVARICATA
DN37859 c1 g1 - ODO1
DN100463 c0 g1 - CPC
DN98044 c0 g1 - ETC1
DN15053 c0 g1 - MYBS1
DN61071 c0 g1 - KUA1
C
Jc171
bHLH
DN27387 c0 g1 - bHLH149
DN43665 c1 g2 - BIM1
DN87 c0 g2 - GLABRA 3
DN39297 c0 g1 - bHLH155
DN87 c0 g1 - GLABRA 3
DN39939 c0 g1 - PIF3
DN190 c0 g1 - bHLH123
DN8913 c0 g1 - bHLH62
DN27330 c0 g1 - bHLH
DN1084 c0 g1 - bHLH112
DN37141 c0 g1 - bHLH143
DN43441 c0 g1 - bHLH157
DN49789 c0 g1 - bHLH143
DN42651 c0 g1 - bHLH30
DN27387 c0 g2 - bHLH149
D
Jc171
FHY-FAR1
DN27073 c0 g2 - FAR1
DN43957 c0 g1 - FRS5
DN35482 c2 g1 - FRS3
DN35482 c1 g1 - FRS3
DN35482 c0 g1 - FRS3
DN32534 c0 g1 - FRS4
DN27073 c0 g1 - FAR1
DN35482 c1 g2 - FRS3
DN20062 c0 g2 - FHY3
DN38006 c0 g1 - FAR1
DN37650 c0 g1 - FRS5
DN43446 c0 g1 - FRS3
DN39826 c0 g1 - FRS2
DN33760 c0 g1 - FRS5
DN36683 c0 g1 - FRS10
E
Jc171
NAC
DN30888 c0 g1 - NAC100
DN25332 c0 g1 - NAC001
DN25332 c0 g2 - NAC025
DN34336 c0 g1 - NAC072
DN27999 c0 g1 - NAC104
DN23154 c0 g2 - NAC002
DN49660 c0 g1 - AC070
DN77400 c0 g1 - NAC083
DN23154 c0 g3 - NAC002
DN6382 c0 g1 - NAC089
F
Jc171
WRKY
DN63330 c0 g1 - WRKY70
DN85701 c0 g1 - WRKY40
DN40050 c0 g1 - WRKY70
DN99021 c0 g1 - WRKY45
DN15701 c0 g1 - WRKY75
DN1032 c0 g1 - WRKY15
DN51829 c0 g1 - WRKY40
DN36528 c3 g2 - WRKY21
DN27886 c0 g1 - SUSIBA2
DN28124 c0 g1 - WRKY19
G
JcARF
ARF
DN49227 c0 g1 - ARF18
DN35323 c0 g1 - ARF5
DN8770 c0 g1 - ARF8
DN85164 c0 g5 - ARF9
DN28119 c0 g1 - ARF19
DN39780 c0 g1 - ARF5
DN98609 c0 g1 - ARF3
DN97827 c0 g1 - ARF6
DN25111 c0 g1 - ARF4
H
Jc171
bZIP
DN36296 c4 g1 - bZIP43
DN21080 c0 g5 - PosF21
DN10303 c0 g1 - bZIP4
DN41139 c0 g2 - bZIP4
DN37491 c0 g1 - RF2b
DN21080 c0 g4 - PosF21
I
Jc171
HD-ZIP
DN49348 c0 g1 - HOX5
DN73459 c0 g1 - AATHB-7
DN61457 c0 g1 - AATHB-12
DN25199 c0 g1 - HHAT5
DN74349 c0 g1 - HHAT7
DN41267 c0 g1 - AATHB-8
DN37459 c1 g1 - AATHB-6
DN33138 c0 g1 - HAT3-1
Log2(Fold Change)
3,00
2,00
1,00
0,00
-1,00
-2,00
-3,00

289

Figure 7. Heatmaps based on gene expression modulation of TFs family members identified in *Jatropha curcas* Jc171 roots after three hours of NaCl exposition (150 mM), in relation to the negative control without salt (ratio of Log_2FC values). The up- and down-regulation of the differentially expressed genes are indicated in red and green, respectively, and the intensity of the colors follow the legend.

f) Expression validation of TF DEGs by RT-qPCR analysis

Eight TF DEGs candidates were evaluated in RT-qPCR assays to confirm the *in silico* expressed profiles. The selected DEGs (*RAP2-3*, *RAV1*, *ERF9*, *DREB1H*, *ZAT12*, *PTI5*, *MYB340*, and *BZIP4*) and the reference genes (*β-tubulin* and *actin*) presented in the respective dissociation curves the unique expected amplicons (**Supplementary Figure S1**). The RT-qPCR parameters [amplification efficiency (E), *slope* (s), and correlation coefficient (R)] derived from standard curves generated using serial dilution of root cDNAs samples (accessions and treatments) and each *primer* pair presented acceptable values (**Table 1**), as those recommended following the MIQE protocol (Bustin et al. 2009), aiming to ensure reliable relative qPCR data. In general, most of the RT-qPCR results (75 %) confirmed the *in-silico* gene expression (except for *RAP2-3* and *DREB1H*), suggesting the reliability of the expressed TFoma (**Figure 8**, and **Table 2**).

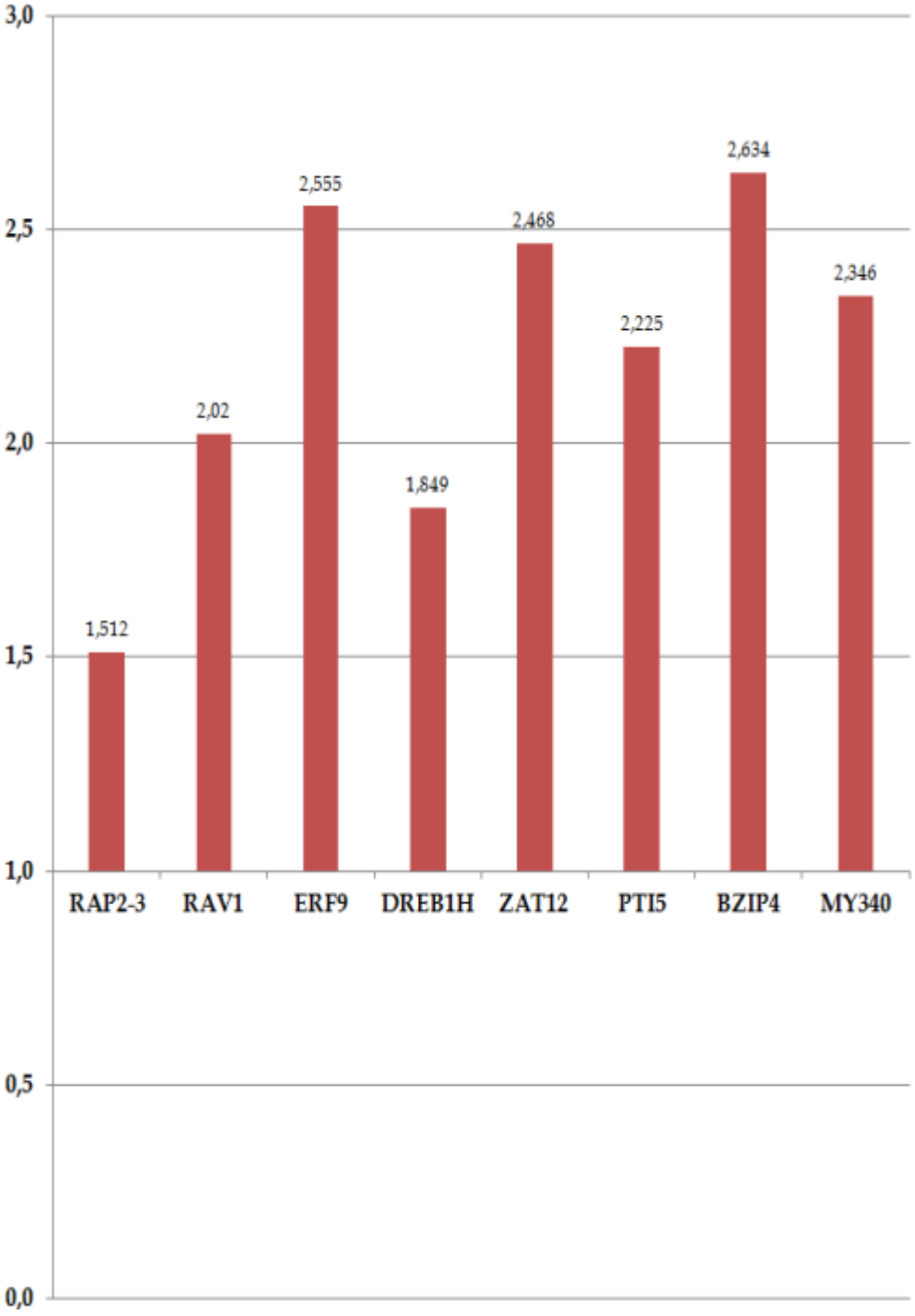


Figure 8. RT-qPCR results of eight candidate genes encoding TFs using cDNAs of *Jatropha curcas* root after three hours of NaCl exposition (150 mM). Expression data calculated by the REST software (v.2.0.13) (Pfaffl et al. 2002) considering biological and technical triplicates, and actin and β-tubulin as the reference genes.

316 **Table1.** RT-qPCR parameters [amplification efficiency (E), slope (S), correlation coefficient (R),
317 and Y intercept] derived from the standard curves using serial dilution of *Jatropha curcas* root
318 cDNAs samples (accessions and treatments) and each primer pair.

Gene (candidate/reference*)	E (%)	R	S	Y intercept
RAP2-3	91.51	-0.994	-3.54	27.78
RAV1	91.30	-0.998	-3.55	36.14
ERF9	105.45	-0.992	-3.20	34.68
DREB1H	98.69	-0.996	-3.35	33.64
ZAT12	104.71	-0.927	-3.21	33.88
PTI5	104.91	-0.915	-3.21	32.55
MYB340	109.78	-0.974	-3.11	34.12
BZIP4	91.03	-0.999	-3.56	32.59
*β-tubulin**	96.00	-0.986	-3.42	30.90
*Actin**	90.15	-0.998	-3.58	26.99

*reference gene: *actin* (Tang et al., 2016) and *β-tubulin* (Xu et al., 2016).

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321 **Table 2**. Selected putative transcript factor genes (DEGs) with the respective *in silico* expressions based
322 on RNA-Seq data and their expression by RT-qPCR analysis with *Jatropha curcas* cDNAs from roots
323 after three hours of NaCl exposition (150 mM).

Method	*RAP2-3*	*RAV1*	*ERF9*	*DREB1H*	*ZAT12*	*PTI5*	*BZIP4*	*MY340*
In silico*	2.665 (UR)	2.413 (UR)	3.390 (UR)	2.294 (UR)	3.659 (UR)	2.738 (UR)	2.072-2.260 (UR)	4.392 (UR)
RT-qPCR**	1.512 (n.s.)	2.023 (UR)	2.555 (UR)	1.849 (n.s.)	2.468 (UR)	2.225 (UR)	2.634 (UR)	2.346 (UR)

324 DEGs (differentially expressed genes: *p-value* ≤ 0.0001, false discovery rate (FDR ≤ 0.005), and fold
325 change (FC) based on Log_2 (FC) ≥ 1 (up-regulated, UR) or ≤ -1 (down-regulated, DR). * Log_2FC values
326 (FC: ratio of normalized transcript abundance observed in the stressed library in relation to the respective
327 abundance in the control library). **Relative expression by REST software (v.2.0.13) (Pfaffl *et al*., 2002),
328 UR ($p \leq 0.05$) considering biological and technical triplicates, and *actin* and *β-tubulin* as the reference
329 genes.

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Discussion

Plant transcriptomic studies have been performed identifying TFs associated with abiotic stresses and their interaction with the transcriptional reprogramming activation in living cells (Seki et al. 2002). Since many TFs described in plant abiotic stress responses play a crucial role in stress tolerance processes (Lata et al. 2011), the expression modulated by TFs usually results in dramatic metabolic changes (Liu et al. 1999). Here, a *de novo* RNA-Seq transcriptome analysis uncovered the TFoma differentially expressed in roots of a *J. curcas* Brazilian Jc171accession plants after three hours of salt exposition (150 mM NaCl). From the assembled transcripts encoding TFs (1,876), almost 8% were differentially expressed after the salt stimulus (78 UR and 70 DR; thresholds: *p-value* ≤ 0.0001, FDR ≤ 0.005, $Log_2FC \geq 1$ or ≤ -1). Jc171 seeds presented particular ability in germination, despite the presence of NaCl (50, 75, 100, and 150 mM) (Lozano-Isla et al. 2018).

a) The GO enrichment analysis

The enriched GO terms associated with UR or DR DEGs individualized the two distinct sets of genes encoding TFs.

The enriched GO terms based on the UR DEGs pointed out stress responses, as expected, but also cell death among others terms (Table S6). Programmed cell death (PCD) is a critical process in eukaryotic cells (Lam 2008), mediating adaptive responses of plants to environmental stresses (Shabala 2009). The Jc171 accession after three hours of the salt exposition presented visible damages in the leaves, probable progressing to necrosis (**Figure 2**). The ionic imbalance induced by salt stress also promotes PCD (Katsuhara and Shibasaka 2000; Huh et al. 2002). Otherwise, the transcription factor MYB108 (also named BOS1, botrytis sensitive1), despite suppressing PCD dissemination in *A. thaliana* injury sites (Mengiste et al. 2003), was associated with oxidative stress, salinity, and water deficit

359 responses. In the present work, two induced *MYB108-related* DEGs were identified
360 (DN31858_c0_g1 and (DN20826_c0_g1; **Supplementary Table S4**).

361 In turn, the enriched GO terms associated with the DR DEGs highlighted more plant
362 developmental activities, including *regulation of circadian rhythm* (Table S6), which is
363 involved in adaptive responses to stresses (Hotta et al. 2007; Legnaioli et al. 2009; Grundy et
364 al. 2015; Seo and Mas 2015). Metabolic and biochemical processes affected by circadian
365 rhythms include photosynthesis and respiration (Kreps and Kay 1997), stomatal opening
366 (McClung, 2001, water uptake by roots (Takase et al. 2011), and cellular Ca^{2+} levels
367 oscillations (Johnson et al. 1995). The MYB-related TF CCA1 (circadian clock associated1)
368 is a transcriptional activator strictly involved in circadian rhythm regulation, binding
369 promoters of at least two genes (*Lhcb, light-harvesting chlorophyll a/b-protein*) encoding
370 proteins related to the photosystem II (Wang and Tobin 1998). In the present work, the
371 putative *CCA1* codified by the DEG DN29723_c0_g2 (**Supplementary Table S4**) could
372 contributed with the expected photosynthesis inhibition of Jc171 after the salt stimulus. It is
373 known that saline stress causes disturbances in photosynthesis, leading to a decrease in plant
374 growth (Sudhir and Murthy 2004; Barhoumi et al. 2007), as salinity increases soil osmotic
375 potential, reducing water uptake by root, and compromising root growth rates (Munns and
376 Tester 2008) and growth of the leaves, reducing the photosynthesis (Julkowska and Testerink
377 2015).

378 **b) The TF enrichment analysis**

379 The enriched TFs predicted interacting with the UR DEGs codifying TFs were more
380 comprehensive (broadly distributed into TF families; **Figure 6A**) than those predicted based
381 on the DR DEGs (**Figure 6B**). The predicted TFs indicated an increased regulatory demand
382 for particular TFs in the Jc171 salt-stress response.

Two enriched TF from the BBR-BPC family [BPC1 (BASIC PENTACYSTEINE1) and BPC6 (BASIC PENTACYSTEINE6)] stood out probable inducing 29 – 41 TFs after the salt stimulus, 25 of them shared by the two enriched TFs (**Supplementary Table S5**). The BPC1 is the regulator of the floral homeotic *STK* gene (*seedstick*), which controls tissue identity through the regulation of a wide range of processes (Kooiker et al. 2005), while BPC6 is a transcriptional regulator of *LHP1* (*like heterochromatin protein1*) gene, which is associated with a PRC (polycomb-repressive complex) component involved in plant epigenetic control by histone methylation (Schuettengruber and Cavalli 2013; Hecker et al. 2015).

Enriched TFs members of Dof family also presented meaningful interactions with the UR DEGs. The enriched Dof zinc finger proteins DOF3.1 and DOF3.6, predicting targeting 28 - 33 DEGs, shared 22 DEGs as their targets (**Supplementary Table S5**). The DOF3.6 was associated with plant growth and development, targeting genes induced by salicylic acid, such as *ORG1*, *ORG2*, and *ORG3* (*OBP3-responsive genes*; Kang et al. 2003). OBP3 is also a Dof transcription factor. Other enriched TFs from Dof family were DOF5.6 and DOF3.4, each one regulating 23 UR DEGs (13 shared targets; **Supplementary Table S5**). DOF5.6 acts on the regulation of vascular tissue development (Guo et al. 2009), while DOF3.4 is involved in the cell cycle regulation (Skirycz et al. 2008).

Among the enriched TF members of the AP2/ERF family, ERF1B (Ethylene-responsive transcription factor 1B) and ERF5 (Ethylene-responsive transcription factor 5) stood out, regulating 15 and 16 UR DEGs, respectively (12 shared targets, **Supplementary Table S5**). ERF1B was related to salinity tolerance in *Avicennia officinalis* (Krishnamurthy et al. 2017), while ERF5 was previously associated with drought and salinity responses in *Solanum lycopersicum* (Pan et al. 2012).

Concerning the C2H2 family, the enriched transcription factor IIIA (TFIIIA) stood out, targeting 23 induced TF DEGs (**Supplementary Table S5**); the *TFIIIA* gene over expression was associated with salt-tolerance in *Medicago truncatula* (De Lorenzo et al. 2007). In turn, the three enriched TFs from the WRKY family predicted interactions targeting only two-three UR DEGs (**Supplementary Table S5**), e.g., the TF WRKY1, already associated with salinity and drought tolerance in *Triticum turgidum* (Mondini et al. 2012), were predicted targeting the promoters of *ERF1B* and *ZAT10* (Zinc finger protein ZAT10) DEGs (**Supplementary Table S5**).

c) The differentially expressed TFoma after the salt-treatment

Concerning almost 70 TF families identified in plants (Pérez-Rodríguez et al. 2010; Hong 2016), based on the DNA-binding domains (Riechmann et al. 2000), the identified *J. curcas* DEGs (148) encompassed 23 TF families with members displaying differential expression after the salt stimulus. Although the TF involvement in abiotic stress tolerance has been established (Reyes et al. 2004; Yanhui et al. 2006; Du et al. 2009; Yang et al. 2011; Cabello et al. 2012; Xie et al. 2012; Zhu et al. 2014; Zhang et al. 2014), and TFs families have been reported to orchestrate stress response pathways in plants, such as MYB, AP2/ERF, bZIP, MYC, NAC, HD-zip, and WRKY (Singh et al. 2002; Shameer et al. 2009), a comprehensive TFoma covering TFs differentially expressed in *J. curcas* roots after salt stimulus has not been presented. Until now, TF families presenting a *J. curcas* genome-wide analysis include WRKY (Xiong et al. 2013), NAC (Wu et al. 2015), MYB (Zhou et al. 2015), and AP2/ERF (Tang et al. 2016). In the mentioned reports, the authors exploring DGE (*Digital Gene Expression*) analysis identified TFs from those family in tissues (root, stem, leaf or seed) of plants under stress (drought, phosphate or nitrogen starvation, and salinity). The applied salt stress involved plants/seedlings under 100 mM NaCl for 2 hours or 2 - 7 days. Also, the gene expression of selected candidates when validated was performed using

semi-quantitative RT-PCR (Xiong et al. 2013; Zhou et al. 2015; Tang et al. 2016) or RT-qPCR analysis (Wu et al. 2015; Tang et al. 2016).

In the present work, despite the wide distribution covering 23 TF families, some families were not detected presenting DEGs. Those families included ABI3VP1, LFY, SBP, Alfin-like, CCAAT, LIM, Sigma70-like, CPP, LOB, SRS, CSD, TAZ, ARR-B, DBP, mTERF, TCP, BBR/BPC, E2F-DP, Tify, BES1, EIL, TIG, BSD, FHA, NOZZLE, TUB, G2-like, OFP, ULT, GeBP, SAP, VARL, Dof, PBF-2-like, VOZ, GRF, PLATZ, YABBY, RWP-RK, HRT, S1Fa-like, Zn-clus, and C3H.

Besides not represented in the present TFoma, Dof family members have been associated with abiotic stress tolerance (Li et al. 2016; Wen et al. 2016), including salinity (Ma et al. 2015). The same has been reported with TCP (salinity: Zhou et al. 2013; Yin et al. 2018), and CCAAT family members (drought: Nelson et al. 2007; Kuromori et al. 2014). A broad RNA-Seq analysis with *Hippophae rhamnoides* plants under drought stress presented repressed TF members from the families ABI3VP1, Dof, YABBY, CCAAT, FHA, G2-like, and C3H, while the induced TFs involved members from the families mTERF, PLATZ, TUB, LIM, and Orphans (Ye et al. 2018).

The main results involving TF family members encoded by the Jc171 DEGs are highlighted below.

c.1.) AP2/ERF family

Members of the AP2/ERF family play fundamental roles in plant development and biotic or abiotic stress responses (Tang et al. 2017). Some AP2/ERF possible encoded by UR DEGs were:

- ERF3 - Ethylene-responsive transcription factor 3 (DN37072_c1_g1; **Supplementary Table S4**): the gene over-expression was confirmed in plants under cold and drought

stress (Cao et al. 2006b; Trujillo et al. 2008); also, its over-expression in wheat (*Triticum aestivum*) transgenic plants, positively regulated physiological adaptive response to salinity and drought tolerance, through increasing proline content, chlorophyll accumulation, and cell redox homeostasis regulation (Rong et al. 2014).

- ERF11 - Ethylene-responsive transcription factor11 (DN7846_c0_g1; **Supplementary Table S4**): the gene expression was modulated by jasmonic (JA; Dombrecht et al. 2007), gibberellic acid (GA; Liu and Hou 2018), and abiotic stresses, including cold (Vergnolle et al. 2005); the TF protein contains a repressor domain that interacts with dehydration-responsive element (DRE) in the promoter of the *ACS2/5* (*1-Aminocyclopropane-1-carboxylic acid synthase - ACS*) gene, affecting the ETH biosynthesis under increasing ABA levels (Li et al. 2011).
- ERF21 - Ethylene-responsive transcription factor 21 (DN51470_c0_g1; **Supplementary Table S4**): the related TF binds to the promoter of *RD29A* gene (Mitsuda et al. 2010), which is recognized to regulate mechanisms of perception and fast induction in water deficit situations (Yamaguchi-Shinozaki et al. 1993).
- ERF12 or DREB26 - Ethylene-responsive transcription factor ERF12 (DN27804_c0_g1; **Supplementary Table S4**): it was highly responsive to salt treatment (200 mM NaCl), heat, and drought (Krishnaswamy et al. 2011); as a DREB subfamily member (Guo et al. 2005) has an amphiphilic repression motive (Zhao et al. 2014), which is characteristic of repressor proteins that inhibit the expression of stress-related genes (Kazan 2006).
- DREB1H - Dehydration-responsive element-binding protein 1H (DN11306_c0_g1; **Supplementary Table S4**): as a DREB subfamily member plays a crucial role in plant development and gene expression mediated by abiotic stresses (Zhao et al. 2014). However, the RT-qPCR analysis not confirmed the DEG up-regulation (**Table 2**).

- DREB2C - Dehydration-responsive element-binding protein 2C (DN18083_c0_g2; **Supplementary Table S4**): the related TF is a transcriptional activator of genes, such as *COR15A* (*cold-regulated 15a*; salinity tolerance; Song et al. 2014), *HsfA3* (*heat shock factor a3*; heat stress response; Chen et al. 2010), *NCED9* (*9-cis-epoxycarotenoid dioxygenase 9*; ABA biosynthesis; Je et al. 2014a), *CYS4 (phytocystatin 4*; thermotolerance; Je et al. 2014b).
- ERF1B - Ethylene-responsive transcription factor 1B (DN74024_c0_g1; **Supplementary Table S4**): this TF is related to the ETH signaling (Corbacho et al. 2013); the transcript up-regulation has been reported in plant responding to drought in soybean (Ferreira Neto et al. 2013), and tomato (Egea et al. 2018).
- RAV1 - AP2/ERF and B3 domain-containing transcription factor RAV1 (DN49504_c0_g1; **Supplementary Table S4**): the related TF presents roles in ABA signaling during seed germination, and the initial seedling development (Feng et al. 2014). The RT-qPCR analysis validated the DEG up-regulation (**Table 2**).
- ERF9 - Ethylene-responsive transcription factor 9 (DN31778_c0_g1; **Supplementary Table S4**): the gene is induced in leaves and roots at different stages of development under saline stress in tomato genotypes (Gharsallah et al. 2016). The RT-qPCR analysis confirmed the DEG up-regulation (**Table 2**).
- RAP2-3 - Ethylene-responsive transcription factor RAP2-3 (DN5779_c0_g1; **Supplementary Table S4**): the related TF modulates osmotic tolerance inducing genes like *PDC1* (*pyruvate decarboxylase1*), *SUS1* and *SUS4* (*sucrose synthases*) when associated to ABA signaling (Gibbs et al. 2015; Papdi et al. 2015). In this case, the RT-qPCR analysis not confirmed the DEG up-regulation (**Table 2**).
- PTI5 - Pathogenesis-related genes transcriptional activator PTI5 (DN43242_c3_g1; **Supplementary Table S4**): the related TF activates genes regulated by salicylic acid

(SA), such as *PR1* and *PR2* (pathogenesis-related genes) (Gu et al. 2002), which are involved in the systemic acquired resistance (SAR) process during phytopathogen infection (Ryals et al. 1996; Feys and Parker 2000). The RT-qPCR analysis confirmed the DEG up-regulation (**Table 2**).

c.2.) WRKY family

One of the primary TF groups involved in the control of biotic and abiotic stress responses (Ulker and Somssich 2004; Rushton et al. 2010). In the proposed TFoma, 10 members (7 UR and 3 DR) were identified, and some of them are presented below.

- WRKY40 - WRKY transcription factor 40 (DN51829_c0_g1, and DN85701_c0_g1; **Supplementary Table S4**): this TF acts primarily on plant defense susceptibility but suffering influence from previous stresses (stressors may have an antagonistic, synergistic or additive effect on plant; Anderson et al. 2004; Asselbergh et al. 2008); this TF negatively modulates the expression of repressors of the JA signaling pathway (JAZ7, JAZ8, and JAZ10), taking part in the defense systems (Glazebrook 2005).
- WRKY70 - WRKY transcription factor 70 (DN63330_c0_g1; **Supplementary Table S4**): this TF is a saline stress-response regulator interacting with another TF (Cys2/His2 zinc finger Zat7); both TFs presented involvement increasing salt tolerance (Ciftci-Yilmaz et al. 2007).
- WRKY45 - WRKY transcription factor 45 (DN99021_c0_g1; **Supplementary Table S4**): its expression is induced in ABA hormone-related response, and also in stress responses, including NaCl, dehydration, cold, heat, and pathogens infections (Yu and Qiu 2009).
- WRKY57 - WRKY transcription factor 57 (DN40050_c0_g1; **Supplementary Table S4**): this TF interacts with promoters of genes, such as *RD29A* (Yamaguchi-Shinozaki

and Shinozaki 1993) and *NCED3* (Chernys and Zeevaart 2000), assisting the plant adaptation to water stress tolerance, by increasing ABA levels (Finkelstein et al. 2002); the phytohormone ABA regulates essential processes (germination, seed dormancy, and stomatal behavior; Liotenberg et al. 1999); also, this TF affects *A. thaliana* germination under ABA influence and abiotic stress (osmotic, salinity and drought; Jiang et al. 2012).

c) MYB family

Members of the MYB family have been investigated in biotic and abiotic stress responses (Denekamp and Smeekens 2003; Seo et al. 2009). In the proposed TFoma, 14 MYB members were associated to the DEGs (8 UR and 6 DR), including:

- Transcription factor MYB108 (DN20826_c0_g1; **Supplementary Table S4**): MYB108 regulates abiotic stresses responses (e.g., salinity, drought and cold) by the JA pathway and the ROS-mediated cellular signaling (Mengiste et al. 2003; Schmid et al. 2005).
- Transcription factor KUA1 (DN61071_c0_g1; **Supplementary Table S4**): KUA1 is a transcriptional repressor of genes encoding peroxidases (PRXs; Lu et al. 2014); PRXs also promote ROS generation, such as H_2O_2, which can cleave the polymers of the cell wall, restricting plant growth (Passardi et al. 2004).
- Myb-related protein 340 (DN41011_c2_g4; **Supplementary Table S4;**): MYB340 activates the *PAL* gene (*phenylalanine ammonia-lyase*) transcription binding on its promoter (Moyano et al. 1996); the PAL enzyme is involved in the phenylpropanoid metabolism, and stresses (e.g., drought, and salinity) stimulating that metabolism (Cabane et al. 2012) generate precursors for lignin biosynthesis (Davin and Lewis

1992), which is also associated to stress tolerance (Liu et al. 2018). The RT-qPCR analysis confirmed the DEG up-regulation (**Table 2**).

- Transcription factor MYBS1 (DN15053_c0_g1; **Supplementary Table S4**): MYBS1 recognizes the TATCCA motif in promoters of genes (e.g., *α-amylase* gene), inducing its expression (Lu et al. 2002); however, during salt stress the α-amylase activity, degrading starch and releasing soluble sugar molecules, is reduced (Lin and Kao 1995; Othman and Al-Karaki 2006; Siddiqui and Khan 2011), affecting processes, such as germination and plant growth (Mei and Song 2008).
- Transcription factor SRM1 (Salt-Related MYB1; DN50735_c0_g1; **Supplementary Table S4**): SRM1 regulates the synthesis and signaling of ABA during germination and seed development in salinity conditions, activating the expression of the *NCED3*/*STO1* gene, which is a mediator of the ABA biosynthesis (Iuchi et al. 2001; Barrero et al. 2006).
- MYB-like transcription factor ETC1 (enhancer of try and cpc 1; DN98044_c0_g1; **Supplementary Table S4**): ETC1 acts as a negative regulator of trichome development, but also promoting an increase in the development of root hairs (Kirik et al. 2004).
- Transcription factor MYB59 (DN20471_c0_g1; **Supplementary Table S4**): TF involved in cell cycle regulation, and root growth (Mu et al. 2009); TF also responding to ETH, and JA (Razzaque et al. 2017).

d) HD-ZIP family

Members of the HD-Zip family play a significant role in plant growth and development, responding to several phytohormone stimuli, and stresses (Ge et al. 2015; Mao et al. 2016). In wheat (*Triticum aestivum*) plants, the salt-sensitive CS genotype

presented 21 induced HD-Zip genes, while the salt-tolerant DK presented 18 (Yue et al. 2018). In the present TFoma, eight HD-Zip members and DEGs were identified (4 UR and 4 DR), and some of them stood out:

- Homeobox-leucine zipper protein HAT5 (DEG DN25199_c0_g1; **Supplementary Table S4**): TF associated with salt stress tolerance in *Thellungiella halophila* (halophytic plant; Wang et al. 2004).
- Homeobox-leucine zipper protein ATHB-12 (DN61457_c0_g1; **Supplementary Table S4**): in transgenic plants under drought conditions, ATHB12 and ATHB7 act as negative plant development regulators in response to the ABA levels (Olsson et al. 2004); the salinity induces ABA biosynthesis (Mahajan and Tuteja 2005), in response to the osmotic and water deficit stresses (Popova et al. 1995; He and Cramer 1996).
- Homeobox-leucine zipper protein ATHB-7 (DN73459_c0_g1; **Supplementary Table S4**): in tomato, the ectopic expression of *ATHB7* conferred drought tolerance (Mishra et al. 2012); also, it was strongly induced by drought and by ABA (Söderman et al. 1996).

d) NAC family

Members of the NAC (**N**AM, **A**TAF, and **C**UC) family present crucial roles in plant development (Kunieda et al. 2008; Ohtani et al. 2011) and stress responses (Takasaki et al. 2015). From the induced DEGs, three NAC TFs stood out after the NaCl application.

- NAC domain-containing protein 72 (DN34336_c0_g1; **Supplementary Table S4**): the *AtNAC072* gene was induced by ABA (100 μM ABA), salinity (250 mM NaCl), and drought (Tran et al. 2004); NAC72 (*Poncirus trifoliata*) is the transcriptional repressor of *ADC* (arginine decarboxylase) gene (Wu et al. 2016), which enzyme is critical for the putrescine (Put) biosynthesis (Put is an osmoprotectant compound reducing

oxidative damages in roots; Zhang et al. 2014); *NAC72* induction has been associated with stress level (Wu et al. 2016); the related TF binds to the CATGTG motif in promoters of genes, such as *ERD1* (*early responsive to dehydration stress 1*) gene, which protein (ClpA, ATP-dependent CLP protease ATP-binding subunit clpA; Tran et al. 2004) is essential for the maintenance of the chloroplast enzymatic apparatus (Sjögren and Clarke 2011).

- NAC domain-containing protein 100 (DN30888_c0_g1; **Supplementary Table S4**): NAC100 binds promoters of cell expansion-related genes, such as *CESA2* (*cellulose synthase2*), and *PIP* (*Plasma Membrane Intrinsic Protein*) aquaporins (Pei et al. 2013), which are gateways for cell membrane water exchange (Yaneff et al. 2015).
- NAC domain-containing protein 2 (DN23154_c0_g2, and DN23154_c0_g3; **Supplementary Table S4**): *NAC2* gene induction in roots of *A. thaliana* plants responding to saline stress (200 mM NaCl) was reported (He et al. 2005).

e) bZIP family

Members of the bZIP family mediate several biological processes, including energetic metabolism (Baena-González et al. 2007), cell expansion (Fukazawa et al. 2000), tissue and organ differentiation (Silveira et al. 2007), seed maturation and embryogenesis (Lara et al. 2003). bZIP members also participate in biotic (Thurow et al. 2005), and abiotic stress responses (Ji et al. 2018), including drought and salinity (Ying et al. 2012; Liu et al. 2014). Two bZIP members associated with three induced DEGs stood out:

- Basic leucine zipper 43 (DN36296_c4_g1; **Supplementary Table S4**): bZIP43 is a positive regulator of *bHLH109* gene (Nowak and Gaj 2016), which was associated increasing LEA (late embryogenesis abundant) protein and enhancing plant stress tolerance (Nowak and Gaj 2016).

- Basic leucine zipper 4 [DN10303_c0_g1, and DN41139_c0_g2; **Supplementary Table S4**]: bZIP4 is also a positive regulator of the bHLH109 gene (Nowak and Gaj 2016), and the DEG (DN41139_c0_g2) up-regulation was confirmed by RT-qPCR results (**Table 2**).

f) C_2H_2-ZFP (C_2H_2 type Zinc Finger Protein) family

Members of the C_2H_2-ZFP family are involved in several biological processes (Gourcilleau et al. 2011), including growth mediation, plant development, and abiotic stress responses (Ding et al. 2016). In this case, the TF member stood out was:

- Zinc finger protein ZAT12 (DN26908_c0_g1; **Supplementary Table S4**): this TF regulate the expression of several oxidative-stress-response genes, including *APX* (*Ascorbate Peroxidase*), *CAT* (*Catalase*), *GR* (*Glutathione Reductase*), *POD* (*Guaiacol Peroxidase*) and *SOD* (*Superoxide Dismutase*) (Rizhsky et al. 2004; Davletova et al. 2005; Rai et al. 2012); the DEG up-regulation was confirmed the by RT-qPCR analysis (**Table 2**).

Conclusions

The present study represents the first TFoma differentially expressed in roots of *J. curcas* plants after salt stress (three hours of NaCl exposition, 150 mM), based on RNA-Seq de novo assembly strategy followed by gene expression validation in RT-qPCR assays. The proposed TFoma represents 148 DEGs (78 UR and 70 DR) codifying TFs encompassing 23 TF families. The GO enrichment analysis identifying those terms over-represented and exclusively associated with the UR or the DR DEGs, differentiated the two sets of DEGs. Enriched GO terms related to stress responses represented the UR DEGs, while GO terms more related to the basal metabolism represented the DR DEGs. The TF enrichment analysis

stood out the most representative TFs predicted interacting with the sets of genes encoding TFs (UR, DR, and the non-DEGs). Predicted TFs regulating cognate TFs genes (as their target genes) were identified, as well as enriched TFs predicting interactions with more than 40 UR DEGs; some of them sharing more than 20 targets. These TFs are promising transgene candidates. The RT-qPCR analysis confirmed the *in silico* gene expression of 75% of eight selected DEGs (from different TF families), and some of them could be functional molecular markers for marker-assisted selection on plant breeding programs, helping to develop *J. curcas* salt-tolerant accessions. The results help to understand the molecular mechanisms involved in *J. curcas* plants responding to salt-exposure.

Competitive interests

The authors declare that they have no competing interests.

Authors contributions

LE, MFP, EB, and EAK conceived and designed the experiments; GALC, MCPS, MDS, and JRCFN carried out the experiments; GALC, MCPS, MDS, and JRCFN analyzed the data; GALC and EAK wrote and revised the paper. All authors read and approved the final manuscript.

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