



Expression analysis of genes involved in the synthesis of oleic and linoleic acids in *Jatropha cinerea* seeds from Northwestern Mexico

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ABSTRACT: Plants belonging to genus *Jatropha* has arisen interest because of its high oil content that could be used to produce biodiesel. It is also widely reported that the main fatty acids in *Jatropha* oilseed are oleic and linoleic acids. However, there are scarce studies related to native species of *Jatropha* from Northwestern Mexico which are adapted to arid conditions, and the expression of genes involved in fatty acid synthesis for these species is still unknown. Therefore, the aim of this study was to analyze the expression of five genes, *ACPI*, *KASII*, *D9SD*, *FAD2-1* and *FAD2-2*, which are involved in the oleic and linoleic acids synthesis in mature wild-seeds of *Jatropha cinerea*, a native species from Sonoran Desert, using semi-quantitative RT-PCR. The *J. cinerea* seeds were randomly collected in Bahía de Kino, Sonora (México) which is a region characterized by its harsh environments such as saline soils and extreme temperature changes and *J. curcas* mature seeds from a non-toxic variety from Veracruz, Mexico were used as a reference. The RT-PCR analysis of three biological replicates were considered to ensure data consistent. Our analysis showed a higher expression of *KASII* and *FAD2-1* genes in *J. cinerea* seeds compared to *J. curcas*, meanwhile the expression of *ACPI*, *D9SD* and *FAD2-2* were higher in *J. curcas*. Furthermore, *Actin* and *FAD2-1* genes sequences here obtained are the first reported for *J. cinerea*, thus providing information to develop further studies.

Key words: fatty acids biosynthesis, PCR, *KASII*, *FAD2-1*.

Análise de expressão de genes envolvidos na síntese de ácidos oleico e linoleico em sementes de *Jatropha cinerea* do noroeste do México

RESUMO: Plantas pertencentes ao gênero *Jatropha* têm despertado interesse devido ao seu alto teor de óleo que poderia ser usado para produzir biodiesel. Também é amplamente relatado que os principais ácidos graxos das oleaginosas da *Jatropha* são os ácidos oleico e linoleico. No entanto, existem poucos estudos relacionados a espécies nativas de pinhão-manso do noroeste do México que estão adaptados a condições áridas, e a expressão de genes envolvidos na síntese de ácidos graxos para essas espécies ainda é desconhecida. Portanto, o objetivo deste estudo foi analisar a expressão de cinco genes, *ACPI*, *KASII*, *D9SD*, *FAD2-1* e *FAD2-2*, que estão envolvidos na síntese de ácidos oleico e linoleico em sementes selvagens maduras de *Jatropha cinerea*, espécies nativas do Deserto de Sonora, usando RT-PCR semi-quantitativa. Sementes de *J. cinerea* foram coletadas aleatoriamente em Bahía de Kino, Sonora (México), região caracterizada por ambientes agressivos como solos salinos e mudanças extremas de temperatura, e sementes de *J. curcas* maduras de uma variedade não-tóxica de Veracruz, México, usado como referência. Análise RT-PCR de três repetições biológicas foram consideradas para garantir dados consistentes. Nossa análise mostrou uma maior expressão dos genes *KASII* e *FAD2-1* em sementes de *J. cinerea* comparado a *J. curcas*, enquanto a expressão de *ACPI*, *D9SD* e *FAD2-2* foi maior em *J. curcas*. Além disso, as sequências dos genes *Actin* e *FAD2-1* obtidas são as primeiras relatadas para *J. cinerea*, fornecendo, assim, informações para o desenvolvimento de novos estudos.

Palavras-chave: Biossíntese de ácidos graxos, PCR, *KASII*, *FAD2-1*.

INTRODUCTION

The genus *Jatropha* belongs to the Euphorbiaceae family and the plants of this genus are widely distributed around the world with approximately 186 species (FRESNEDO-RAMÍREZ & OROZCO-RAMÍREZ, 2013).

Jatropha species are succulents, shrubs and trees that are well known by deep roots, allowing them to adapt to arid conditions. Moreover, *Jatropha* plants are distinguished by their ability to survive in dry and saline soils and conditions considered marginal for agriculture; their presence in these environments benefits the soil health; providing an organic mulch

making the soil available for agricultural practices (BRITTAINE & LUTALADIO, 2010). The study of *Jatropha* plants has been motivated by its potential as ornamentals, medical applications and oil extraction from various species for the production of paints and solvents and recently as an input for the production of biodiesel, since it has been reported that the most abundant fatty acids which are present in its oil are oleic and linoleic acids (MARTINEZ-HERRERA et al., 2010; WANG et al., 2011; GÁMEZ-MEZA et al., 2013; KHALIL et al., 2013). To obtain high quality biodiesel in terms of stability, oil must have a higher oleic acid content than linoleic acid content because it is known that a higher polyunsaturated fatty acids (PUFAs) content will impact negatively not only in the stability of the oil but also in the number of cetane which, is an important parameter considered in biodiesel production (YE et al., 2013).

Jatropha curcas is the most studied species among the genus. However, recent studies have been published on wild native species from Northwestern Mexico (*J. cordata*, *J. cardiophylla*, *J. cuneata* and *J. cinerea*); which are considered an imprint source of oilseed (30-40% oil seed content), from which 30-50% consist in PUFAs (mainly linoleic acid). Therefore, studies using molecular markers based on its biochemical characteristics are done to improve the oil quality, developing new and non-toxic varieties and optimize the performance of the seeds (QU et al., 2012; OLIVEIRA et al., 2013). In Mexico, there are 48 species of *Jatropha*, of which 39 are endemic, representing 21% of all known species. Among the most representative species in arid regions and dry hot climates in the Northwestern Mexico are included *J. cordata*, *J. cardiophylla*, *J. malacophylla*, *J. cuneata* and *J. cinerea* (FRESNEDO-RAMÍREZ & OROZCO-RAMÍREZ, 2013).

Jatropha cinerea, a wild species relative close to *Jatropha curcas* (the most representative of the genus), is widely distributed along the coast of Sonora, the Baja California Peninsula and Sinaloa (TURNER et al. 2005). It grows in saline soils and is adapted to dry and subtropical climates and often exposed to long drought periods (HISHIDA et al., 2014). Besides, *Jatropha cinerea* mature seeds have an important oil content composed mainly by oleic and linoleic acid (SOSA-SEGURA et al., 2014; SOTO-LEÓN et al., 2014; LOVIO-FRAGOSO, et al., 2017). This information suggests that *Jatropha cinerea* may be an appropriate species for studying

as a biofuel source for its abundant seed production under harsh conditions, factors that limit the *Jatropha curcas* development in regions as the Sonoran Desert with temperatures around 45°C and scarce annual precipitation (HISHIDA et al., 2014). Conversely, the information related to genes involved in fatty acid synthesis in these species is limited. Therefore, the aim of this study was to analyze the level expression of genes involved in the synthesis of the oleic and linoleic acids in *J. cinerea* and *J. curcas* mature seeds and correlate the amount of these oils in the seeds.

MATERIALS AND METHODS

Plant material

Jatropha cinerea mature wild-seeds were randomly collected on October 2014 in Bahía de Kino, Sonora, México (28°49'52.1106" N 111° 44' 23.316" O and 28°49'45.6234" N 111°57' 12.1176" O). *Jatropha curcas* mature seeds from a non-toxic variety from Veracruz, Mexico were used as a reference. Seeds were manually shelled and dried under sun for 12h and stored in plastic containers at - 80°C prior to analysis.

Total RNA isolation and purification

Total RNA was isolated from ground seeds using TRIZOL (Invitrogen, Whaltman, United States of America) according to the manufacturer's protocol. The RNA samples were purified using RNEasy MinElute Cleanup Kit (Qiagen, Venlo, Netherlands) following the manufacturer's protocol. Then, total RNA was quantified with spectrophotometry using a NanoDrop 1000 (Thermo Scientific, Wilmington, United States of America) and the purity was assessed using the 260/280nm and 260/230nm absorbance ratios. Afterwards, the integrity of the RNA samples was determined by 1.0% agarose gel electrophoresis and GelRed™ (Biotium, Fremont, United States of America).

cDNA synthesis

For semiquantitative RT-PCR, the cDNA was obtained from 2µg of total RNA by reverse transcription using SuperScript™ III Reverse Transcriptase kit (Invitrogen, Whaltman, United States of America) and the Ilustra Ready-To-Go RT-PCR Beads kit (General Electric, Chicago, United States of America) according to the manufacturer's protocol; a DNase I

(Roche, Mannheim, Germany) treatment step was included prior cDNA synthesis. In the absence of information on *J. cinerea* sequences, primers sequences used were taken from existing information in *J. curcas* genome. The *Jatropha curcas Actin* gene was used as an internal control.

Semiquantitative RT-PCR was performed using the Illustra™ PuReTaq™ Ready-To-Go™ PCR beads kit (General Electric, Chicago, United States of America) following the manufacturer's instructions. The reaction was carried out in a SimpliAmp™ thermocycler (Applied Biosystems, Foster, United States of America). Amplified products were analyzed by electrophoresis on 1.0% gels containing GelRed™ (Biotium, Fremont, United States of America) and visualized with a transilluminator Bio-Imaging System MiniBis pro (Bio America Inc, Miami, United States of America). Relative intensities of the amplified PCR products were determined using Image J (Java) an image analysis software (version 1.7.0_51) and expressed in arbitrary units (AU). The ratio between the RNA sample to be determined and *Actin* was calculated to normalize for initial variations in sample concentration and as an efficient reaction control. Three biological replicates were performed for both species to ensure consistent data which was expressed as the average of fold-difference of expression levels.

Sequencing analysis

The gDNA (genomic DNA) was extracted from *Jatropha cinerea* leaves that were collected during the seed collection and stored at - 80°C prior to use. The gDNA extraction was performed using the Plant ZR/Seed Zymo Research Miniprep DNA kit (Zymo Research, Irvine, United States of America) following the manufacturer's instructions. Resulting gDNA was used to perform PCR reactions of each gene considered in this study. 100µl of PCR products amplified from gDNA of *Jatropha cinerea* were obtained for each gene and sent to Laboratorio de Servicios Genómicos, LANGEBIO CINVESTAV (Irapuato, Mexico) for analysis. Resulting sequences were analyzed using the program BioEdit version 7.2.5 (Hall, Ibis Biosciences, CA, USA) and used BLAST for comparison to the correspondence *Jatropha* sequences.

RESULTS AND DISCUSSION

RT-PCR

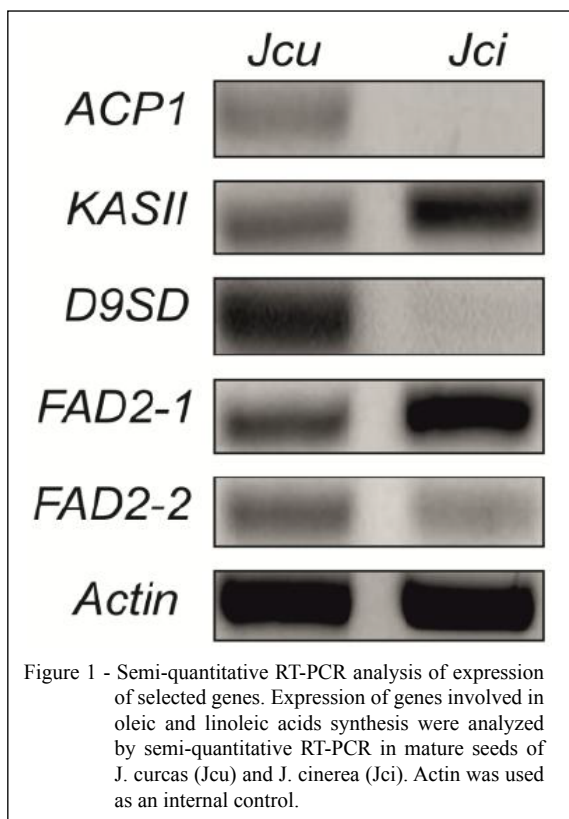
Genes involved in the oleic acid synthesis

ACP enzymes are cofactors responsible for acyl chain modification and chain-length

termination during lipid biosynthesis in living organisms, being the isoform I the one that is reported mainly in seeds (LI et al., 2010). The ACP1 gene analysis showed that this gene had a lower expression in mature seeds of *J. cinerea* (0.22-fold) compare with *J. curcas* (0.57-fold) (Figure 1). The LI et al. (2010) measured the ACP mRNA levels in developing peanut seeds, finding that at initial stages of seed development the levels of poly (A) mRNA per seed increased sharply and then decreased steadily as the seed matured. This coincides with the reported by GU et al. (2012), that during *Jatropha curcas* seeds development, the expression of this gene increases progressively as an indicator of fatty acids synthesis to cover its physiological needs; once the seeds reach maturity, this gene is practically downregulated.

The synthesis of fatty acids in plants occurs mainly in plastids by the condensation of malonyl-CoA by KAS enzymes, of which isoform II is responsible for final elongation step in the form of plastids C16:0 ACP to C18:0 ACP (MA et al., 2017). It has been reported that at mature stages of development of *Jatropha curcas* seeds, the expression of KASII gene tends to be highly induced (GU et al., 2012); once the fatty acid is elongated from C16 to C18, it can subsequently undergo desaturation for obtaining unsaturated fatty acids (WEI et al. 2012). In the present study, the expression analysis showed a higher induction of *KASII* gene from *Jatropha cinerea* of 0.80-fold than *Jatropha curcas* seeds 0.66-fold (Figure 1), suggesting that in *Jatropha cinerea* could be a mechanism that favors the elongation from C16:0 to C18:0 over *Jatropha curcas* but it is necessary to develop further studies to confirm this statement.

The *D9SD* gene is linked to the synthesis of oleic acid; according to the analysis performed in this study, *D9SD* was defined as a low expression gene. The *D9SD* expression analysis in *Jatropha cinerea* seeds showed no expression of this gene while in *Jatropha curcas* seeds this gene was expressed 0.72-fold higher (Figure 1). GU et al. (2012) reported that at mature stages of the development of *J. curcas* seeds, *D9SD* is downregulated. Moreover, it has been reported in *Jatropha curcas* that at filling stages of the seed's development, *D9SD* gene showed high expression and then it decreased abruptly as the seeds approach maturation (GU et al., 2012). These results suggested that at mature stages the oleic acid synthesis in *Jatropha cinerea* seeds is reduced or practically repressed. In spite of a high content of oleic acid, in both species, a slight relationship



was showed between the gene expression and its content for *J. curcas* (37.59g / 100g FAME) and for *J. cinerea* (34g / 100g FAME) according to LOVIO-FRAGOSO et al. (2017).

Genes involved in the linoleic acid synthesis

The *FAD2* gene family which encode the enzymes 1-acyl-2-oleoyl-sn-glycero-3-phosphocholine delta 12-desaturases (*FAD2*) are responsible for the production of linoleic acid from oleic acid in oilseeds plants. Whereas *Arabidopsis thaliana* has a single *FAD2* gene that is expressed in seed and vegetative tissues, in *J. curcas*, three putative genes have been identified that encoded to *FAD2* proteins (*JcFAD2-1*, *JcFAD2-2* and *JcFAD2-2m*) (QU et al. 2012). The *FAD2-1* gene is high expressed in seeds compared to other tissues, while *FAD2-2* is high expressed in seeds and not detectable in leaves (TANG et al. 2005); even though *FAD2-2* also contributes in the production of linoleic acid; however, *FAD2-1* (*JcFAD2-1*) gene has been of particular interest because it is considered the main gene responsible for the conversion of oleic acid to linoleic acid and because it has been

reported that its expression level is higher than that of *FAD2-2* during the period of maximal storage lipids biosynthesis of seed development (QU et al., 2012). In this context, our analysis showed that in *Jatropha cinerea* seeds the *FAD2-1* gene had a higher expression (0.66-fold) than *Jatropha curcas* seeds 0.51-fold (Figure 1). Instead, the *FAD2-2* gene showed a lower expression in *Jatropha cinerea* seeds (0.27-fold) while in *Jatropha curcas* seeds this gene showed a higher expression (0.66-fold, Figure 1). In addition, linoleic acid content differs slightly between *J. cinerea* (46.92g / 100g FAME) and *J. curcas* (42.81g / 100g FAME) (LOVIO-FRAGOSO, et al., 2017). These results showed a moderate relationship between linoleic acid content and the expression of *FAD2* genes, suggesting that the linoleic acid production is slightly favored in mature seeds of *J. cinerea* compared than *J. curcas* seeds, and *FAD2-1* is the main gene responsible of the linoleic acid synthesis. According to the study performed by GU et al. (2012), desaturases play a crucial role as responsible for the presence of polyunsaturated fatty acids. It has been reported in other studies that the *FAD2* transcript expression differed significantly from *D9SD* expression pattern. The *FAD2* expression increased sharply at the first stages of seed development and reaching levels that surpass anthesis and slowly decreases as seeds approach to maturation, agreeing to reports of GU et al. (2012) with RUUSKA et al. (2002) results in *Arabidopsis* and FOFANA et al. (2006) in flax, in which a bell-shaped pattern was also observed in *FAD2* expression. Moreover, it is important to mention that *FAD2* genes are related to environmental adaptation of plant vegetative growth and it is also reported its expression as response to abiotic stress, being evaluated its expression as consequence of the production of linoleic acid under low temperatures and its necessary presence under salt stress showing an increased at its expression levels in *Arabidopsis* (SCHERDER & FEHR, 2008; ZHANG et al. 2012). This suggested that *FAD2-1* expression in *Jatropha cinerea* seeds could also be related to salinity tolerance because it is growing in saline soils.

Conversely, there are other genes involved in the oleic and linoleic acid synthesis that are not considered in this study that could provide more information to comprehend the relationship between the levels of expression of the genes and the presence of fatty acids, specifically oleic and linoleic acids. The study of

these and other genes involved in the biosynthesis of fatty acids could provide a more complete insight between expression and content in seeds. Moreover, there are other factors that could have influenced in our results as the design of primers used in PCR reactions based on *Jatropha curcas* genome due to the absence of *J. cinerea* sequences in databases; therefore, it could affect the results when comparing gene expression along with the fatty acid profile (HERNÁNDEZ et al., 1994). It is worth to mention that this is the first study that analyzes the oleic and linoleic acid genes in *Jatropha cinerea* seeds and give valuable information to understand the relationship between the levels of expression of the genes and the presence of fatty acids as well as the suggestion to use this species as an alternative source for biofuel production in arid regions.

Sequencing analysis

PCR products from Actin, *ACPI*, *KASII*, *D9SD*, *FAD2-1* and *FAD2-2* genes of *Jatropha cinerea* were sequenced from gDNA to obtain the greatest coverage of these genes and thus get more information of each one as possible. Sequences were analyzed and compared in public databases such as GenBank, using the National Center for Biotechnology Information (NCBI) with the Basic Local Alignment Search Tool (BLAST), considering parameters as identity percentage, e-value (<1e-03) and sequence number to confirm if they correspond to the genus *Jatropha* and the specific gene examined. As we expected, our results showed a high identity of the *Jatropha cinerea* sequences with the *Jatropha curcas* sequences deposited in the GenBank (Table 1),

suggesting a close phylogenetic relationship between both species.

Results generated in the present study provided highly relevant information because to our knowledge, this is the first report on analyzing the expression of genes involved in the oleic and linoleic acid synthesis in *J. cinerea*. Moreover, here we submitted the first *Actin* and *FAD2-1* gene sequences of *Jatropha cinerea* to GenBank at NCBI with the accession numbers MF363000 and MF363001, respectively, which provided important information for further studies in this area.

CONCLUSION

The present study is the first one on providing results about the expression of linoleic and oleic acids synthesis genes in *Jatropha cinerea*. The high expression of the *KASII* and *FAD2-1* genes in mature seeds of *J. cinerea* and *J. curcas* suggests that the synthesis of linoleic acid is favored over the synthesis of oleic acid. Finally, the sequences of *Jatropha cinerea* submitted to the GenBank provide basic information to develop other studies to extent the knowledge and to comprehend the function, expression pattern and variability of genes involved not only in the synthesis of oleic and linoleic acids that will allow in a future to improve the content of oleic acid for species such as *Jatropha cinerea* that lives in harsh environments as the Sonoran Desert, characterized with high temperatures and saline soils but also to study the genes such as *FAD2* in *Jatropha cinerea*, that is suggested to play a role in the resistance to abiotic stress such as salinity and cold in other plant species.

Table 1 - *Jatropha curcas* sequences which showed homology with *Jatropha cinerea* sequences obtained in this study.

Gene name	GenBank ID	Description	-Sequence identity (%) -	
			<i>J. curcas</i>	<i>A. thaliana</i>
<i>ACPI</i>	JQ806272.1	<i>Jatropha curcas</i> plastid acyl carrier protein 1 mRNA	94	46
<i>KASII</i>	NM_001306021.1	<i>Jatropha curcas</i> 3-oxoacyl-[acyl-carrier-protein] synthase II, chloeo-plastic (LOC105638018), mRNA	99	31
<i>D9SD</i>	JQ806303.1	<i>Jatropha curcas</i> delta-9-estearoyl-acyl carrier protein desaturase mRNA	98	39
<i>FAD2-1</i>	JQ806297.1	<i>Jatropha curcas</i> fatty acid desaturase 2 mRNA	97	30
<i>FAD2-2</i>	JQ806301.1	<i>Jatropha curcas</i> delta-12-acyl-lipid desaturase mRNA	98	32
<i>Actin</i>	NM_001308728.1	<i>Jatropha curcas</i> actin-7 (LOC105645143), mRNA	97	34

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DECLARATION OF CONFLICTING INTERESTS

The authors declared no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

AUTHORS' CONTRIBUTIONS

JPL-F, LAM-J and CH-K conceived and designed experiments. JPL-F performed the experiments, JPL-F, LAM-J, NG-M, OM, MH-O and CH-K carried out the lab analyses. LAM-J and CH-K supervised and coordinated the experiments. JPL-F, OM and MH-O performed statistical analyses of experimental data. JPL-F, MH-O and CH-K prepared the draft of the manuscript. All authors critically revised the manuscript and approved of the final version.

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