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Expression of storage lipid biosynthesis transcription factors and enzymes in *Jatropha curcas* L. cell suspension cultures and seeds

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Abstract
The oleaginous *Jatropha curcas* has been proposed as a promising source for biodiesel production in seed or potentially by *in vitro* production in cell cultures. However, little is known concerning the optimal growth conditions and the transcription of key factors and enzymes involved in the biosynthesis of storage lipids in *J. curcas* cell cultures. Additional knowledge is also needed for these factors in seeds. Here, we assess target gene expression in endosperm cells *in planta* and endosperm-derived cell suspension cultures (EDCCs). Endosperm cells were taken from three representative seed developmental stages, and cell suspensions were grown from these samples. Glucose, nitrogen, and abscisic acid concentrations were varied in an attempt to optimize biomass growth and oil yield. Oil production in EDCCs reached a maximum of 5% (w/w) of total lipids. Although much lower than lipid production in seed, lipid profiles of EDCCs remain identical to those produced *in planta*. The expression levels of five major transcription factors (TFs), as well as *KAS1*, *accA*, *DGAT1/2* and *PDAT1* enzymes, and the O*LE1* protein, all key components of the lipid biosynthesis pathway were also measured. Significant expression of *LEC1*, *FUS3*, *ABI3*, and *WRI1* was found in endosperm cells throughout seed development, suggesting similar functions to their counterparts in *Arabidopsis* and providing a reference expression level for cell cultures. *J. curcas* EDCCs showed lower expression of most TFs compared with endosperm tissue, with the exception of *WRI1* which had comparable expression levels in the two systems. Conversely, the enzymes *KAS1*, *accA*, and *DGAT* had the same or higher expression levels in EDCCs *versus* endosperm cells. Interestingly, the genes that encoded for *DGAT1* and *DGAT2* enzymes were found preferentially expressed in endosperm cells and EDCCs, respectively. Contrary to other studies, our findings indicate that the addition of ABA does not result in increased expression of genes involved in storage lipid biosynthesis.

Keywords Storage lipids · Oil body · Amyloplasts · Cellular differentiation · Transcription factors · Cell suspensions · LAFLs

Introduction

Jatropha curcas L. (Euphorbiaceae) is currently undergoing domestication to improve agronomic quality and oil production so that it can be a profitable source for biodiesel (Yue *et al.* 2013). Although, previous studies in *J. curcas* have identified many of the genes involved in reserve lipid biosynthesis, including the production and storage of triacylglycerides (TAGs) (Costa *et al.* 2010; Xu *et al.* 2011; Gu *et al.* 2012; Jiang *et al.* 2012), the identification of the full set of transcription factors (TFs) regulating these pathways is still incomplete. So far, candidate genes controlling maturation processes in *Arabidopsis thaliana* (Arabidopsis) have served as a reference point for studying oil production in *J. curcas* and other plant species.

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In Arabidopsis several TFs are known to control embryogenesis, and in parallel primary metabolite production, during seed development. Among them are the LAFLs which consist of four genes: *LEAFY COTYLEDON1* (*LEC1*), *ABSCISIC ACID INSENSITIVE3* (*ABI3*), *FUSCA3* (*FUS3*), and *LEAFY COTYLEDON2* (*LEC2*) (Fatihi *et al.* 2016). The LAFLs have been identified as master regulators of the seed maturation phase, when storage compounds accumulate seed desiccation proceeds (Santos-Mendoza *et al.* 2008; Roscoe *et al.* 2015; Fatihi *et al.* 2016). During the maturation phase, these TFs directly or indirectly regulate pathways related to the biosynthesis of triacylglycerides (TAGs) which include carbon transport, glycolysis, fatty acids (FAs) synthesis, the Kennedy pathway, and oil body formation (Mu *et al.* 2008; Angeles-Núñez and Tiessen 2011; Elahi *et al.* 2015). For instance, storage lipid biosynthesis is controlled by the LAFLs *via* the activation of a downstream TF, *WRINKLED1* (*WRI1*), which controls key points in the glycolysis and fatty acid synthesis during seed maturation (Baud *et al.* 2007).

The involvement of the LAFLs in the regulation of TAGs content has been demonstrated by heterologous expression experiments in a broad range of species, resulting in the increase of TAGs in several cases. For instance, the constitutive expression of *BnLEC1* in *Brassica napus* caused increased transcription of the enzymes participating in oil biosynthesis which correlated with the increase of total seed oil without major changes in the FA profile (Elahi *et al.* 2016). However, the overexpression of *ZmLEC1* in *Zea mays* increased seed oil but pleiotropic effects were observed such as a reduction of seed germination rates and leaf growth (Shen *et al.* 2010). Overexpression of *AtFUS3* under the control of an inducible promoter activated oil accumulation in *A. thaliana* seedlings and in tobacco BY2 cells (Zhang *et al.* 2016). Likewise, the ectopic expression of *WRI1* homologs from different species in *Nicotiana benthamiana* leaves induced the upregulation of genes involved in carbon flux, FAs synthesis, TAGs assembly, and an increased oil content in vegetative tissue (Grimberg *et al.* 2015). The available data points to important roles of the LAFLs and downstream TFs in the regulation of storage lipids and therefore are target genes for oil yield improvement by manipulating their expression.

In comparison, comparable studies in oleaginous species including *J. curcas* are scarce. For instance, Jiang *et al.*, (2012) documented similar expression patterns for the *J. curcas* homologs of *LEC1*, *LEC2*, *FUS3*, *ABI3*, and *WRI1*, to those found in *A. thaliana.* This suggested that in *J. curcas* they are performing similar roles as master regulators of the maturation phase. More recently, Ye *et al.*, (2018) obtained an increase in lipid content and seed biomass by over-expressing *JcWRI1.* All available data suggest that *LEC1*, *LEC2*, *FUS3*, *ABI3*, and *WRI1* are master regulators of the seed maturation process in *J. curcas*. However, more detailed studies including different developmental stages and isolated cell cultures are necessary before targeting specific genes to modify this crop so that it can profitably produce oil for biodiesel.

The characterization of metabolic pathways in *J. curcas* has been done either in mature plants or from isolated organs, like seeds, derived from field-grown plants. However, analysis of field grown plants is difficult due to tissue complexity, the identification of specific developmental stages during which maturation occurs, and lack of control of environmental conditions. In contrast, *in vitro* cell suspension cultures offers a suitable alternative to study several complex processes in a more simplified manner by allowing strict control of cell differentiation and culture conditions (van Gulik *et al.* 2001; Mulabagal and Tsay 2004; Mustafa *et al.* 2011). Cell suspensions of *J. curcas* from different tissues (*e.g.*, endosperm) have been standardized (Demissie and Lele 2013; Solís-Ramos *et al.* 2013; Bernabé-Antonio *et al.* 2014; Carmona *et al.* 2018). However, storage lipid metabolism has been difficult to study in such systems because cells undergoing dedifferentiation processes and concomitant increases in cell division rates exhibit inhibition of many metabolic pathways including those involved with TAGs accumulation (Wen and Kinsella 1992; Tjellstrom *et al.* 2012).

For plant cultures grown *in vitro*, whether they are derived from calluses, cell suspensions, or somatic embryos, several strategies can be implemented to switch on or to increase the activity of a given metabolic pathway. Some hormones are known to control seed development, as Abscisic acid (ABA) playing a key role during seed maturation (Finkelstein 2010). For instance, the peak of ABA accumulation during seed development in *Ricinus communis* coincides with the accumulation of storage compounds in the embryo and the endosperm (Chandrasekaran and Liu 2014). *In vitro*, ABA is generally added to promote fatty acids accumulation in somatic embryos of different plant species and thus improve maturation (Finkelstein and Somerville 1989; Kim and Janick 1991; Attree *et al.* 1992). Also, there is an increase in total lipid content and a change in lipid profiles for cell suspension cultures of *Lesquerella fendleri* (Kharenko *et al.* 2011). Similarly, the presence of ABA triggers TAGs synthesis in *Arabidopsis* cotyledons (Yang *et al.* 2011). In addition to ABA, water availability controls the activation of genes that regulate maturation processes, dormancy, and desiccation tolerance in seeds (Angelovici *et al.* 2010). Thus, often media used in somatic embryogenesis protocols are supplemented with high concentrations of mannitol, sucrose, or polyethylene glycol in order to generate osmotic stress which triggers ABA biosynthesis and activates the accumulation of reserve compounds (Knox and Avjioglu 1989; Attree *et al.* 1992; Grigová *et al.* 2007).

An additional factor influencing storage lipids synthesis is nitrogen availability. For instance, in microalgae cultures nitrogen depletion significantly increases lipid synthesis (Zhu *et al.* 2016). For plant species, nitrogen depletion studies are scarce; however, it has been found that nitrogen limitation enhances gene expression associated with the biosynthesis

and accumulation of TAGs (Gaude *et al.* 2007; Yang *et al.* 2011). Neither the effects of ABA, osmotic agents, and nitrogen depletion have been tested in *J. curcas* cell suspensions, nor the expression of key TFs involved in the accumulation of reserve compounds (like TAGs), has been evaluated.

In this sense, it is essential to determine the factors that control cellular, molecular, and biochemical responses in *J. curcas* cell suspensions. Our long-term goal is to optimize the *in vitro* production of primary metabolites such as triacylglycerols for this oleaginous plant. Here, we have identified key developmental seed stages of *J. curcas* growing in the field in order to have a reference pattern for oil production in endosperm tissue *in vivo*. In order to test whether endosperm-derived cell suspension cultures (hereafter referred to as EDCCs) can reproduce the same lipid profile and content as observed in seed, we supplemented the growth media with different glucose and nitrogen concentrations, as well as with and without ABA. Finally, we measured the expression of five TFs (*LEC1, LEC2 FUS3*, *ABI3*, and *WRI1*), five enzymes (*accA*, *KAS1*, *DGAT1*, *DGAT2*, and *PDAT*), and the *OLE1* protein, in three selected endosperm developmental stages and in *J. curcas* EDCCs.

The *J. curcas* EDCCs presented identical lipid profiles but lower total lipid content when compared with the endosperm *in vivo*. Our results suggest that cellular dedifferentiation processes lead to important changes in gene expression that ultimately affect, but do not shut down, storage lipids biosynthesis in EDCCs. Although these results are somehow expected due to the reduction in overall biomass, we were able to target the reduction of lipid content mainly to changes in expression of early biosynthetic genes in the pathway such as *LEC1*, *LEC2 FUS3*, *ABI3*, and *WRI1*. In contrast, several enzymes and downstream proteins have similar expression levels *in vivo* and *in vitro*. We were also able to identify for the first time, preferential expression of transcript variants of *DGAT* in the endosperm *versus* EDCCs. Finally, in contrast to previous studies, the addition of ABA to *J. curcas* EDCCs does not improve lipid yield and rather results in a massive downregulation of target genes.

Materials and methods

Plant material, establishment, and maintenance of cell suspension cultures *Jatropha curcas* plant material was collected in March 2015, in the subregion of the Bajo-Cauca, Antioquia, Colombia. Cell suspensions were established from endosperm of the collected seeds following Carmona *et al.* (2018) and the *BRA*-2 cell line was used as it has high rates of cell division (Carmona *et al.* 2018). Endosperm derived cell suspension cultures (EDCCs) were subcultured every 8 d, in modified Murashige and Skoog (MS2; Murashige and Skoog 1962; see more details in Supplemental Table 1) culture medium. During subcultures, spent culture medium was removed, and 15 mL of pelleted cells were transferred to 85 mL of fresh medium in 250-mL Erlenmeyer flasks with cotton plugs to enable gas exchange. The EDCCs were kept in a rotary incubator shaker at 80 rpm and 29 ± 2°C in darkness.

Induction of storage lipids in EDCCs In an attempt to induce the accumulation of storage lipids in *J. curcas* EDCCs, culture media with different carbon levels (30, 60, or 80 g L^{-1} glucose, Phytotechnology ®, Shawnee Mission, KS), nitrogen (100 or 2500 mg L^{-1} KNO_3, EMSURE®, Darmstadt, Germany) and ABA (0 or 1 mg L^{-1}; Phytotechnology ®) were used. As inoculum, approximately 800 mL of a final exponential phase cell suspension (14 d after the previous subculture), with a cell aggregate size < 500 μm and cell viability close to 90% was used. The cells were washed five times with modified MS2 culture medium (supplemented with 30 g L^{-1} glucose and without hormones or a nitrogen source), using the Nalgene filtration system (500 mL) with a 20-μm nylon membrane (Spectra Mesh®, Waltham, MA).

For each experimental unit, 30 mL of washed cells were transferred to 30 mL of the MS2 culture medium (with different glucose concentrations) in a 250 mL Erlenmeyer flask. ABA was added from a stock solution of 500 mg L^{-1}, dissolved in 1 M KOH, to a final concentration of 1.0 mg L^{-1}. In the case of EDCCs control treatment without ABA, the same volume of sterile water containing 1 M KOH was added. For all assays, three replicates of each condition were kept in a rotary incubator shaker using orbital agitation of 80 rpm at 29 ± 2°C in darkness, for 9 d. At the end of incubation, the biomass of each experimental unit was analyzed for total lipids, sugars, and gene expression and observed microscopically.

Microscopy Microscopic observations were performed immediately after samples were collected. To determine cell viability, the cells were stained with 0.2-mg mL^{-1} fluorescein diacetate (FDA) in acetone (Sigma Aldrich, Saint Louis, MO; Carmona *et al.* 2018). To identify amyloplasts, cells were stained with 2% (w/v) Lugol (Sigma Aldrich). To identify oil bodies, 20-μg mL^{-1} Nile red in acetone (Sigma Aldrich) and Sudan IV (Sigma Aldrich) were used as was reported before (Carmona *et al.* 2018). Observation and cell analysis were carried out with a Nikon Eclipse 80i microscope (Nikon®, Kanagawa, Japan), using a phase contrast system for the × 40 objective, and fluorescence for Nile Red and FDA (Carmona *et al.* 2018). Three samples from the same experimental unit were observed in different fields (at least six fields, using the Neubauer chamber and covers/slides). Comparing the observations of each sample in optical field and fluorescence were used to determine the percentage of living cells and of cells with oil bodies or amyloplasts.

Lipid extraction and analysis of thin layer chromatography Lipid extraction was carried out following Xu *et al.* (2011) with some modifications (for more details see the complete protocol in supplementary material). The lipids obtained were separated by thin layer chromatography, for which 5 μL of each sample was loaded on the thin layer plates, 1.5 cm from the bottom (9 cm × 8 cm silica gel, thin layer chromatography (TLC) 60G F_{25}, EMD Millipore). The developing solvent consists of n-hexane (EMSURE®, Darmstadt, Germany), diethyl ether (EMSURE®), and acetic acid (glacial 100%, EMSURE®, Darmstadt, Germany) at a volumetric ratio of 90:10:1, respectively. The samples were dried with cold air for a few seconds and revealed with iodine vapors.

Total sugar quantification The carbon source that was not consumed by the cells during the kinetic growth studies was quantified. After centrifugation of the suspensions, 500 μL of supernatant from each sample was treated with 500 μL of a solution containing 1% (w/v) 3,5-dinitrosalicylic acid (Fisher Scientific, Geel, Belgium), 0.2% (w/v) phenol (EMSURE®) 1% (w/v) NaOH (Honeywell™, Morris Plains, NJ), 0.05% (w/v) Sodium Sulfite (Spectrum™, Germany), and 30% (w/v) Rochelle Salt (Sigma Aldrich). The samples were incubated in a water bath at 95°C for 7 min, placed in an ice bath for 5 min, and then dried for 3 min at room temperature. Distilled water (5 mL) was then added to each tube, and 200-uL aliquots from each sample were measured in triplicate by absorbance at a wavelength of 515 nm in a plate Spectrophotometer (Biotek, Power Wave XS2, Winooski, VT).

Histological analysis of the seeds Fruits were collected at different developmental stages and classified by color and size. The seeds were then dissected from the fruit and various seed attributes were recorded (*e.g.*, color, consistency, testa appearance, weight and size). Approximately 15 seeds were collected for each developmental stage (Supplemental Table 2). Finally, nine developmental stages were obtained (see results); three of them 2, 5, and 6 were selected and described as S1, S2, and S3 respectively.

Seeds without testa were fixed in FAA solution for 5 d and kept at 4°C. The tissues were rinsed with distilled water and dehydrated with in a standard ethanol series (70, 80, 90, 96, and 100% v/v). Each sample was maintained in each ethanol solution for 4 h at 4°C.The samples were then transferred to HistoChoice (Sigma Aldrich) and subsequently embedded in paraffin (Paraplast Plus, Sigma Aldrich). Serial sections with a thickness of 5 μm were prepared on a rotary microtome LEICA RM2125. Sections were stained with Safranin-Alcian blue (Tolivia and Tolivia 1987) or double stained first with periodic acid-Schiff reagent (PAS, Merck HX 106073) and then with Amido black (1% w/v Amido black 10B with 7% w/v acetic acid (glacial) 100% - EMSURE®). Slides were mounted in Entellan (EMD Millipore, Darmstadt, Germany). To identify lipids, Nile red staining of freehand sections was performed using a similar procedure as described above for EDCCs. Slides were examined under optical and fluorescence microscopy. The seeds used for the gene expression assays were dissected into embryo, inner integument, and endosperm, and were stored at − 80°C until processing.

Gene expression analyses by qRT-PCR Relative gene expression for *LEC1*, *LEC2*, *FUS3 WRI1*, *ABI3*, *Acca*, KASI, *DGAT1*, *DGAT2*, *PDAT*, and *OLE1*, in both seeds and EDCCs was evaluated using quantitative real-time qPCR (qRT-PCR). For the seed analysis, the testa was removed, and the endosperm was carefully dissected from the embryo ensuring that only endosperm tissue was retained, in selected developmental stages (*i.e.* S1, S2, and S3). EDCCs grown in 30 and 60 g L^{-1} of glucose were used in this study, for a total of eight culture media. Total RNA was extracted from EDCCs and seeds, using Trizol® (Invitrogen, Carlsbad, CA), following the manufacturer's instructions. RNA samples were treated with DNase I (Fermentas, Waltham, MA) to remove contaminating DNA and RNA was quantified by absorbance at 260 nm in a spectrophotometer (NanoDrop-1000, Thermo Scientific, Waltham, MA). First-strand cDNA was synthesized with AMV Reverse Transcriptase (A3500 Promega, Madison, WI), using 2 μg μL^{-1} RNA and following the manufacturer's protocol. The cDNA obtained was used for amplification by qRT-PCR using specific primers for each gene (Supplemental Table S3).

All PCR reactions were performed in a Rotor-Gene Q 5plex HRM thermocycler (QIAGEN, Valencia, CA), using the intercalation dye SYBR as a fluorescent reporter. Each reaction was performed in a 25-μL mixture containing, 2-μL diluted cDNA (concentration approximately 38 ng μL^{-1}), 12.5 μL of Master Mix SYBR Green with Rox (Fermentas, Waltham, MA), and 0.75 μL of each reverse and forward primer, (final concentration 300 nM). The PCR protocol consisted of an initial step of 10 min at 95°C, 44 cycles of 15 s at 95°C, 30 s at 53–57°C and 30 s at 72°C. Relative transcription levels were established using expression of the glycerol-3-phosphate dehydrogenase cytosolic (GPDHC) gene, as an internal control. Levels of expression were presented as $2^{-\Delta CT}$ where $\mathbf{\Delta C_T} = C_T$ (target gene)–C_T (reference gene) (Schmittgen and Livak 2008). The values represent the average of three biological replicates, each analyzed in triplicate. PCR controls were performed in the absence of cDNA. For each experiment, a two-way ANOVA was carried out along with Tukey's multiple comparison test (p value < 0.05), using the statistical program Prism 6.0 version 6.03.

Results

The accumulation of storage compounds in EDCCs EDCCs grown in different culture media (30, 60, or 80 g L^{-1} glucose; 100 or 2500 mg L^{-1} KNO_3; and 0 or 1 mg L^{-1} ABA) showed viability between 80 and 90% (Fig. 1*a–b*) with an increase of plasmolyzed cells at higher glucose concentrations. Exopolysaccharides were produced by the cells at the end of the growth kinetics, possibly in response to stress conditions. Double staining with Lugol and Nile red (Fig. 1*c–f*) showed the presence of cells with amyloplasts and possibly oil bodies (approximately 1 to 2 μm). To confirm these observations, additional staining was performed with Lugol and Sudan (Fig. 1*d–h*), revealing the presence of amyloplasts and oil bodies. These observations were recorded under all conditions; however, more cells with oil bodies were seen in cultures grown under nitrogen limiting conditions (100 mg L^{-1} KNO_3) than noted at the higher nitrogen level. Amyloplast accumulation was more pronounced in media supplemented with 60 or 80 g L^{-1} of glucose than with the lower (30 g L^{-1}) level of glucose.

Analysis of lipids and carbon consumption in EDCCs Total lipid concentration was measured for each of the culture conditions tested (Fig. 2). Statistical analysis (Tukey test, $p < 0.05$) showed that varying glucose concentration did not have an effect on total lipid levels. However, significant differences among the other variables were found. For EDCCs grown in 30 g L^{-1} glucose, total lipid levels were about 5% (w/w) when grown with 2500 mg L^{-1} of nitrogen and ABA. When cells were grown at the same glucose concentration (30 g L^{-1}) but with 100 mg L^{-1} of nitrogen and without ABA, a lower total lipid value was observed (3.5%, $p < 0.05$). EDCCs grown in 60 g L^{-1} glucose, exhibited different total lipid values in the four-culture media. In cells grown in 100 mg L^{-1} of nitrogen and without ABA, the lowest percentage of lipids was registered (3.2%; $p < 0.05$), compared with the highest value in those supplemented with 1 mg L^{-1} of ABA (5.5%, $p < 0.05$). Lipid accumulation in EDCCs was consistently lower (< 5% w/w) than observed in mature seeds (stage S3), for which 52.9% (w/w) of total lipids was found.

In order to assess the lipid species synthesized by *J. curcas* EDCCs, the lipid banding pattern by TLC was compared between treatments. Previous studies in *J. curcas* served as reference for the identification of the lipid types (Kim *et al.* 2014; Chaitanya *et al.* 2015b). We were able to identify bands (from bottom to top in Fig. S1) corresponding to the phospholipids (PLP) as the most polar molecules, followed by the bands of different neutral lipids, next to PLP are likely diacylglycerides (DAGs), followed by the bands of free fatty acids (FFA), which precede the triacylglycerides (TAGs) (Fig. S1). Finally, the uppermost band corresponds to sterol esters (SE), which is more visible in the lipids extracted from the cell suspensions when compared with seeds. The banding pattern of lipids is identical between EDCCs and seeds; however, lower production of total lipids is found in EDCCs (Fig. 2).

The consumption of carbon source by EDCCs was also evaluated by measuring glucose concentration at the beginning and the end of the culture growth (Fig. 3). Carbon consumption was not influenced by changes in nitrogen and ABA concentration when EDCCs were cultured in 30 g L^{-1} glucose. EDCCs cultured between 60 and 80 g L^{-1} glucose,

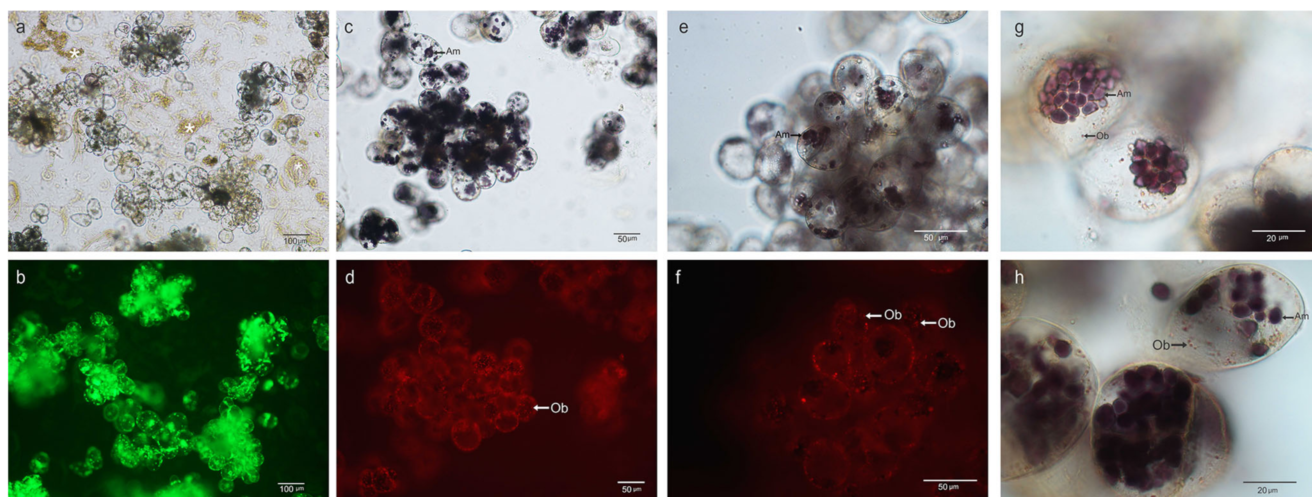

Figure. 1. Microscopy analysis of *Jatropha curcas* L., endosperm-derived cell suspension cultures (EDCCs) growing in different culture media, cells observed under optical and fluorescence microscope. (*a–b*) Cells after FDA stain growing in medium containing 80 g L^{-1} glucose, 100 mg L^{-1} KNO_3, and 1 mg L^{-1} ABA. (*c–f*) Double staining with a Lugol solution and Nile red; (*c–d*) Cells growing in medium containing 30 g L^{-1}glucose and 100 mg L^{-1} KNO_3; (*e–f*) Cells growing in medium containing 80 g L^{-1} glucose 2500 mg L^{-1} KNO_3 and 1 mg L^{-1} ABA. (*g–h*) Double staining with a Lugol solution and Sudan IV, cells growing in medium containing 60 g L^{-1} glucose, 100 mg L^{-1} KNO_3, and 1 mg L^{-1} ABA. *Black arrows* indicate cells storing starch in amyloplasts (Am), and *white* and *black arrows* indicate cell with oil body (Ob). *Scale bars a–b* 100 μm; *c–f* 50 μm; *g–h* 20 μm. *ABA* abscisic acid

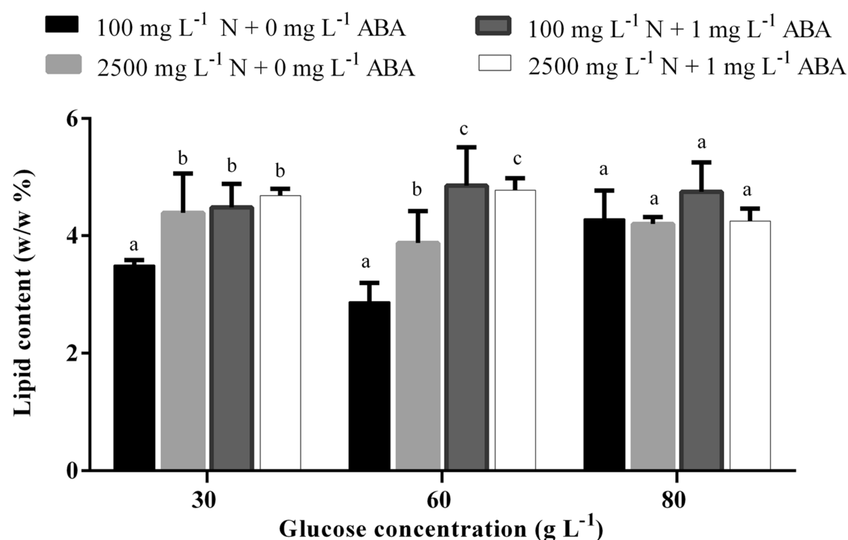

Figure 2. Percentage of total lipids in *Jatropha curcas* L., EDCCs growing in different culture media. Statistical analyses were performed for each glucose concentration, because there was no interaction between them. The different *letters* indicate significant statistical differences ($p < 0.05$, HSD Tukey test) in each group of samples. The data presented correspond to the average values ± the standard error of three replicates. *ABA* abscisic acid

consumed a greater proportion of the carbon source ranging from 35 to 45 g L^{-1}. Under these conditions, leftover glucose remains, possibly generating osmotic stress.

Morphological and histological characteristics of *J. curcas* seeds The fruits and seeds of *J. curcas* collected were classified into nine different developmental stages based on their external morphology and size (Fig. 4*a*). Fruits in early stages of development (stages 1 to 3) show a green pericarp, whereas a shift to yellowish pericarps marks the mid-developmental stages 4 to 6. Stages 7 to 8 were defined by a shift to patchy brown pericarps. The fruit is completely ripened when it is fully brown and dehydrated (stage 9).

Seeds change from having a soft white testa in early stages of development (stages 1 and 2) to yellowish-brown in more advanced stages (3 to 5), and then black and rigid by the end of their development (stages 6 to 9). Three of the nine developmental stages (Supplementary Table 2), were selected (stages 2, 5, and 6, hereafter, they are referred to as S1, S2, and S3, respectively.) for detailed analysis.

At stage S1 (Fig. 4*b–e*), the embryo is in intermediate cotyledonary stage (Brito *et al.* 2015) and is surrounded by endosperm (Fig. 4*b*), and the endosperm is surrounded by the internal integument (Fig. 4*c*). At this stage, the endosperm lacks accumulated storage compounds as indicated by (PAS) carbohydrate staining, the Amido black protein specific staining, and the Nile red lipid staining (Fig. 4*d–e*). At stage S2 (Fig. 4*f–i*), the embryo exhibits laminar cotyledons that extend throughout the seed (Fig. 4*f*). The radicle is fully differentiated at this stage and two very pronounced areas on each side which form the lateral buds composed of meristematic cells (Fig. 4*g*). At this stage, the endosperm occupies most of the seed and accumulates mainly protein

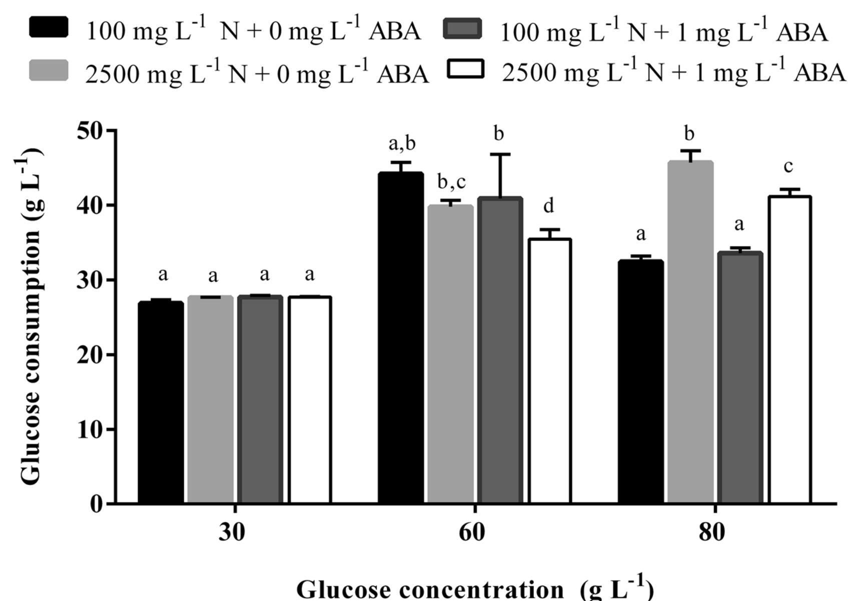

Figure 3. Glucose concentration consumed during *Jatropha curcas* L. growth kinetics. Statistical analyses were performed for each glucose concentration, because there was no interaction between them. The different *letters* indicate significant statistical differences ($p < 0.05$, HSD Tukey test) in each group of samples. The data presented are the average values ± the standard error of three replicates. *ABA* abscisic acid

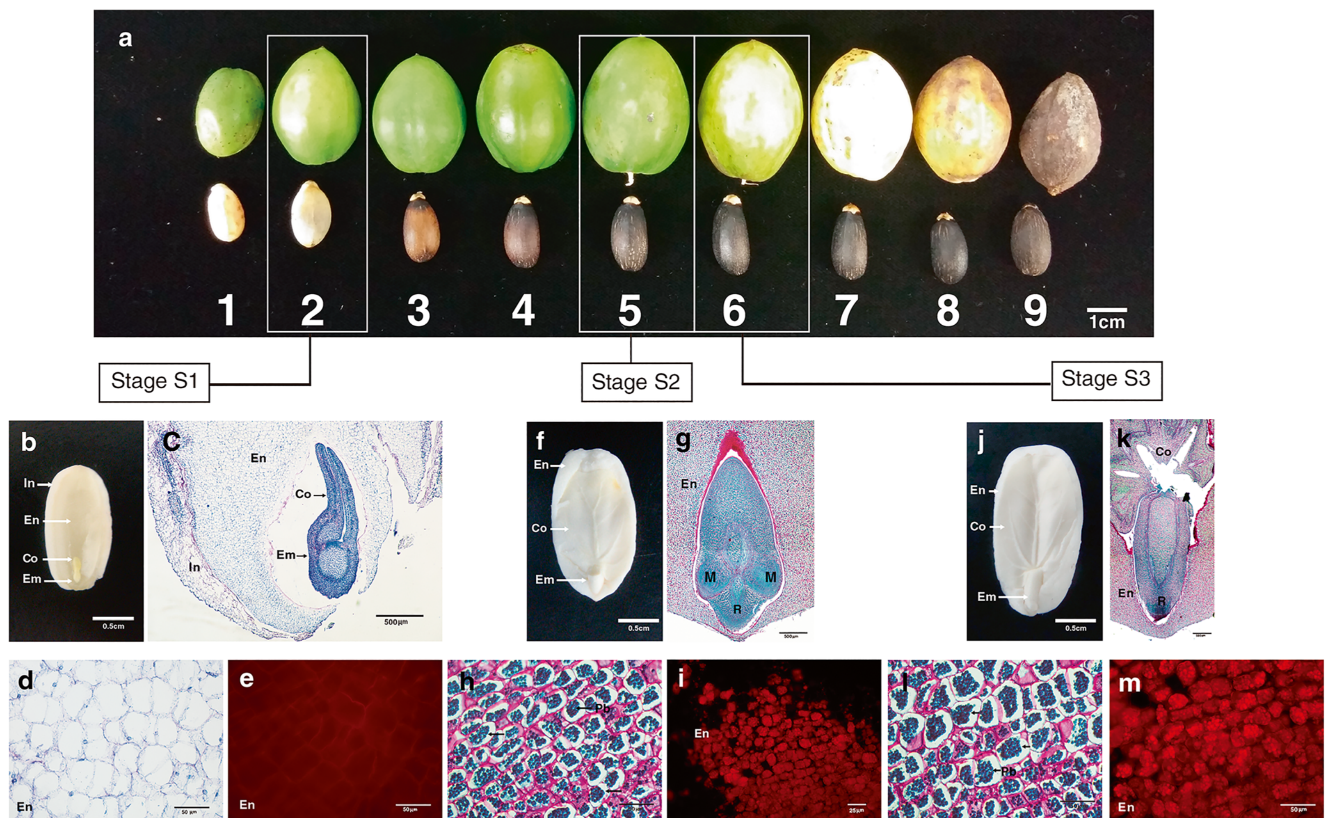

Figure 4. Morphological changes throughout fruit and seed development. (*a*) Classification of the fruits and seeds of *Jatropha curcas L.* in different stages of maturation based on the features described in Table S2. (*b–e*) Stage S1. (*f–i*) Stage S2. (*j–m*) Stage S3. All stages were selected as they represented key maturation stages in the complete series presented in *a*. (*b, f, j*) Fresh longitudinal section of the seed in which the inner integument (In), endosperm (En), embryo (Em), and cotyledons (Co) are evident. (*c, g, k*) Longitudinal semithin section of the seeds stain with PAS and Amido black, when is evident some tissues like En, Em, Co, and In. Also in *g* and *k* is evident in the embryo the radicular (R) and two islands of meristematic cells (M). (*d, e, h, i, m*) Longitudinal semithin section of the endosperm that consists of thin-walled cells, staining with PAS and Amido black and Nil red, in some of them are evident the proteins body, Pb (storage proteins) and oil body (storage lipids). *Scale bars*: *a* 1 cm; *b, f, i* 0.5 cm; *c, g, k* 500 μm; *d, e, h, j, m* 50 μm; *i*; 25 μm

bodies (Pb) (Fig. 4*h*) and small oil bodies (ob) (Fig. 4*i*). Finally, at S3 (Fig. 4*j–m*), anatomical features are very similar to the S2 stage, but the embryo has doubled in size, and the shoot apical and the root apical meristems are fully established. At this stage, accumulation of proteins and lipids was observed in the endosperm (Fig. 4*l–m*).

Gene expression of TFs that regulate development and metabolic processes in seeds and EDCCs Gene expression was compared between endosperm cells and EDCCs. In *J. curcas* seeds the *LEC1*, *LEC2*, *FUS3*, *ABI3*, and *WRI1* expression was higher at stage S1 and decreased during subsequent stages (Fig. 5*a*). *LEC1* expression level was significantly higher in comparison with the other TFs, showing its highest peak at the beginning of the development, while the transcription of *LEC2* was present at very low levels throughout seeds development. For *FUS3*, the expression level was similar across S1–S3, however in comparison with the other TFs expression of *FUS3* is much lower (thirty-fold less than *LEC 1*). Finally, *ABI3* and *WRI1* showed higher expression during stage S1, but decreased by half in later stages (S2 and S3).

Expression of the same TFs in EDCCs is much lower compared with seeds (Fig. 6*a*). For instance, *LEC1*, *LEC2*, *FUS3*, and *ABI3* expression showed very low levels in all cultures. Nevertheless, *WRI1* expression level was significantly higher (Tukey test, $p < 0.05$) and comparable with those observed for *FUS3* in seeds (Fig. 5*a*). Expression of *WRI1* increased when the cells were grown in 100 mg L^{-1} KNO_3 plus 60 g L^{-1} glucose and in the absence of ABA, when compared with all other media tested. This suggests that the nitrogen source and glucose have an effect in the regulation of *WRI1*.

Gene expression of the enzymes involved in the synthesis of reserve lipids in seeds and EDCCs The first reaction of *de novo* fatty acids biosynthesis in plants is catalyzed by acetyl-CoA carboxylase. This plastid-localized enzyme catalyzes the reaction between phosphoenolpyruvate and bicarbonate to form malonyl-CoA (Gu *et al.* 2011). The genes coding for the heteromeric acetyl-CoA carboxylase enzyme have been characterized in *J. curcas*. Each of the four subunits is encoded by

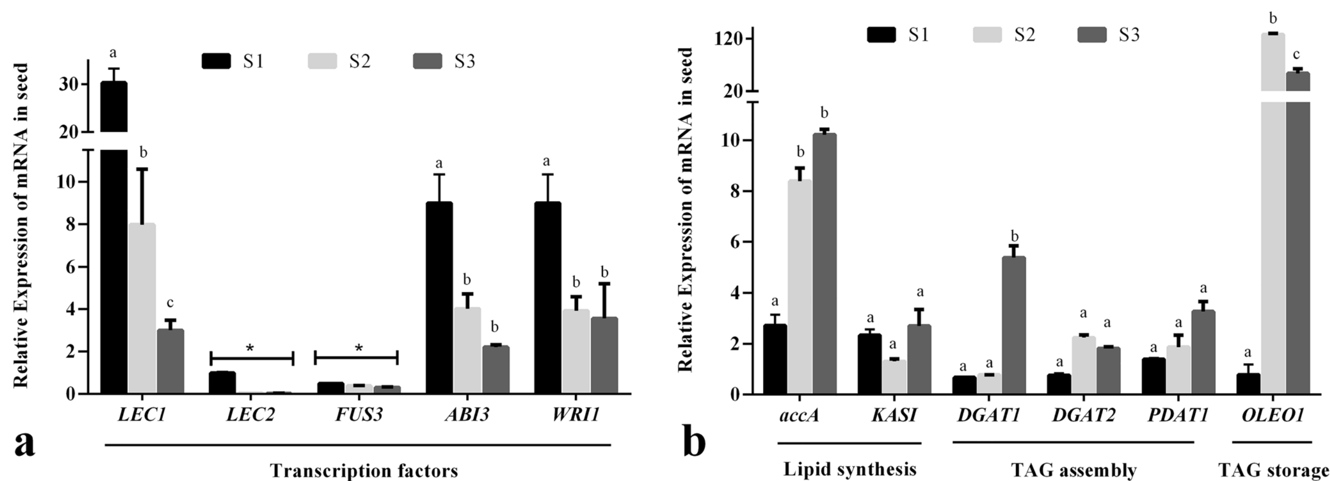

Figure 5. Relative expression of the transcription factors and enzymes in *Jatropha curcas* L. seeds in different stages of development: (*a*) relative expression of the transcription factors, *LEAFY COTYLEDON 1/2* (*LEC1/2*), *ABSCISIC ACID INSENSITIVE3* (*ABI3*), *FUSCA3* (*FUS3*), and *WRINKLED 1* (*WRI1*) in S1, S2, and S3 seed developmental stages. (*b*) Relative expression of enzymes *Acetil-CoA carboxilase subunit* α*-CT* (*accA*), *Phosphatidyl glycerol acyltransferase* (*KAS1*), *Diacylglycerol acyltransferase 1/2 (DGAT1/2)*, *Phosphatidyl glycerol acyltransferase* (*PDAT1*), and *Oleosin1* (*OLE1*) in S1, S2, and S3 seed developmental stages. Expression levels were analyzed by qRT-PCR, and the relative abundance of mRNA was normalized against the *GDAPHc* gene in the corresponding samples. The data represent averages of three biological replicates ± SD with three technical replicates. The different *letters* indicate significant statistical differences ($p < 0.05$, HSD Tukey test) in each group of samples. *TAG* triacylglyceride

a single copy gene (Gu *et al.* 2011). In this study, we assessed the expression of the *accA* gene which encodes for the β-carboxyltransferase subunit (β-CT) and found a fivefold increase in expression from stage S1 to stage S3 in seeds (Fig. 5*b*) correlated with a greater accumulation of reserve compounds as lipids (Fig. 4). Similar expression levels of *accA* were obtained in EDCCs grown in medium containing 2500 mg L^{-1} KNO_3 and 30 g L^{-1} glucose without ABA (Fig. 6*c*).

The KAS1 enzyme catalyzes the elongation of fatty acids from 4:0 carbons to 16:0 (Wu and Xue 2010). In seeds, *KAS1* showed two peaks during stages S1 and S3, with a higher expression level in S3 (Fig. 5*b*). For EDCCs, the highest expression of *KAS1* was found when cells were cultured in medium containing 2500 mg L^{-1} KNO_3 and 30 g L^{-1} glucose without ABA. This is the same culture medium in which *accA* registered higher values. For the rest of the culture media, the genetic expression was fourfold lower.

TAGs are synthesized in the endoplasmic reticulum through the Kennedy pathway that involves three sequential steps of acylation between glycerol-3-phosphate (G3F) with acyl chains. The last step to synthesize these molecules is catalyzed by DGAT1, DGAT2, and PDAT enzymes, which assemble a fatty acid in the *sn-3* position of a diacylglycerol (DAG), to form the TAG (Li *et al.* 2010). In this study, *DGAT1* was expressed predominantly in the seed S3, fivefold more than in the S1 and S2 (Fig. 5*b*), when the endosperm cells store reserve compounds as lipids (Fig. 4). *DGAT2* expression was lower in S1 and increased in S2 and S3 (Fig. 5*b*). However, comparing the expression of both enzymes for S3, *DGAT2* expression was threefold lower than *DGAT1*. On the other hand, in EDCCs, *DGAT1* expression did not change significantly, among cultures (Fig. 6*d*, Tukey test, $p < 0.05$), showing lower values than *DGAT2*. The later, surprisingly registered higher values than the ones evidenced in seeds (Fig. 5*b*). Furthermore, higher levels of *DGAT2* expression occur in EDCCs grown in absence of ABA while little change occurs under different KNO_3 or glucose shifts.

For PDAT, which also participates in the assembly of the TAGs (Li *et al.* 2010), we have recorded a threefold increase in expression from S1 to S3 (Fig. 5*b*). However, regarding EDCCs, the expression pattern of *PDAT* showed no clear association to glucose or KNO_3, but higher expression is seen in the absence of ABA (Fig. 6*d*).

Finally, biosynthesis and accumulation of TAGs in seeds concludes with the formation of oil bodies, which stores these molecules until they are used by the embryo during germination. These organelles are formed by a phospholipids monolayer, which has embedded a large number of proteins, including oleosins (Pyc *et al.* 2017). *Oleosin 1* (*OLE1*) expression was assayed and showed the highest expression level of all genes evaluated, with a significant change among S1, S2, and S3 stages increasing during development (Fig. 5*b*, Tukey test, $p < 0.05$). These findings confirm once again that in S2 and S3, stages maturation and accumulation process of storage compounds is highly active, and that *OLE1* gene expression could be used as a marker of the activation of the maturation process in plant cells. Comparatively, *OLE1* expression in EDCCs was very low and slight increases were only observed in the absence of ABA in media with 2500 mg L^{-1} KNO_3 and 30 g L^{-1} glucose or 100 mg L^{-1} KNO_3 and 60 g L^{-1} glucose (Tukey test, $p < 0.05$). Low expression of *OLE1* coincides

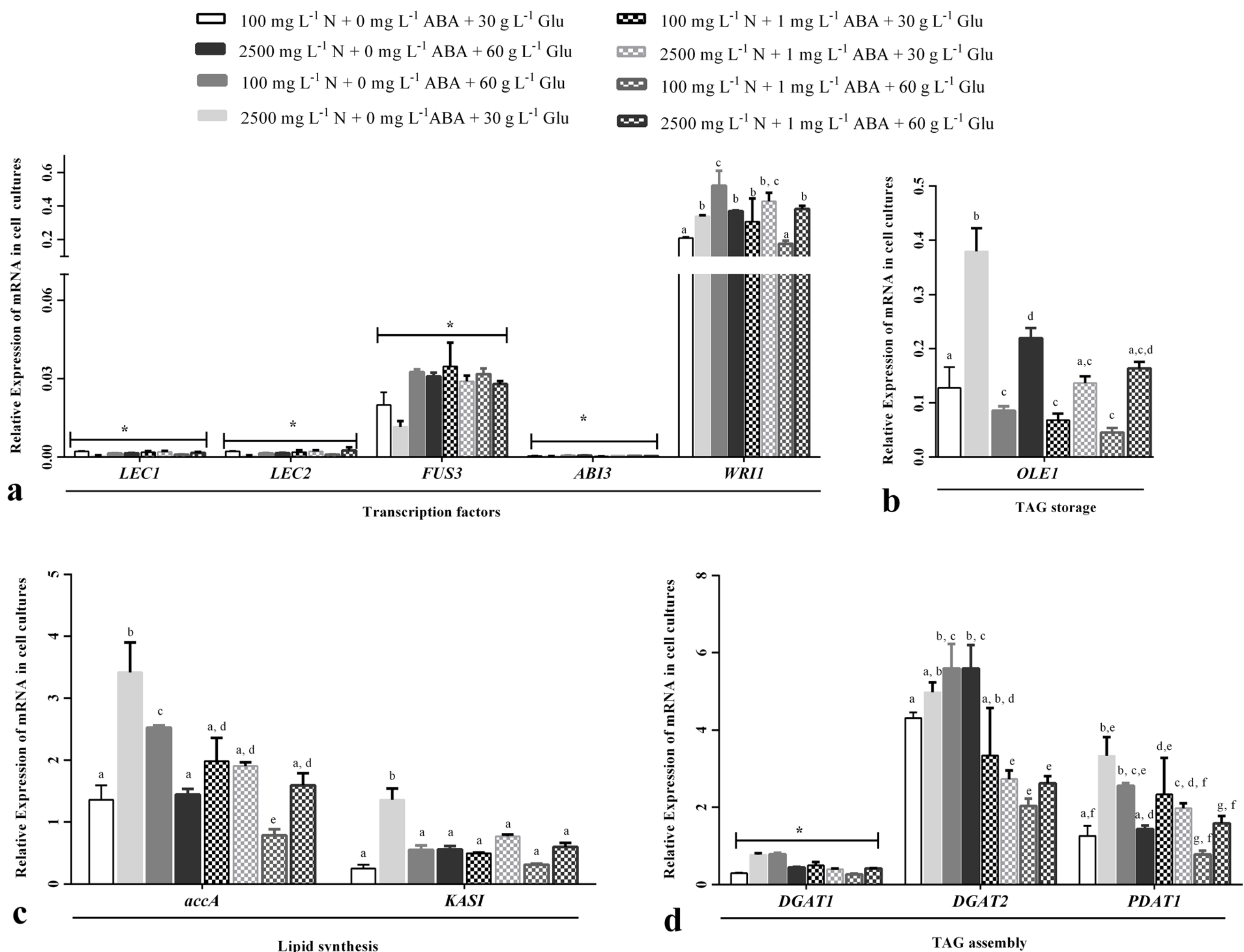

Figure 6. Relative expression of the transcription factors and enzymes in *Jatropha curcas* L., EDCCs grown in different culture media: (*a*) relative expression of the transcription factors *LEAFY COTYLEDON 1/2* (*LEC1/2*), *ABSCISIC ACID INSENSITIVE3* (*ABI3*), *FUSCA3* (*FUS3*), and *WRINKLED 1* (*WRI1*). (*b*) Relative expression of *Oleosin1* (*OLE1*). (*c*) Relative expression of *Acetil-CoA carboxilase subunit α-CT* (*accA*), *Phosphatidyl glycerol acyltransferase* (*KAS1*). (*d*) Relative expression of *Diacylglycerol acyltransferase 1/2* (*DGAT1/2*) and *Phosphatidyl glycerol acyltransferase* (*PDAT1*). Expression levels were analyzed by qRT-PCR and the relative abundance of mRNA was normalized against the *GDAPHc* gene in the corresponding samples. The data represent averages of three biological replicates ± SD with three technical replicates. The different *letters* indicate significant statistical differences ($p < 0.05$, HSD Tukey test) in each group of samples. *ABA* abscisic acid, *Glu* glucose

with low fractions of oil bodies in the cellular cytoplasm, rather rich in amyloplasts.

Discussion

The aim of this study was to assess whether *J. curcas* EDCCs are able to produce lipids *in vitro* and to test target gene expression patterns of the major lipid biosynthetic TFs, enzymes, and proteins and comparing these to expression levels *in vivo* in endosperm. Despite the fact that EDCCs undergo cellular dedifferentiation processes triggering the downregulation of several metabolic pathways, lipid production is not halted completely. In fact, lipids are produced and the lipid profile is similar as to what was observed in seeds. Interestingly, TFs such as *WRI1* and some of the enzymes retain similar expression levels in seed and cultures. We discuss the most important results in the light of optimization for *in vitro* oil productivity in this promising oleaginous plant.

The accumulation of storage compounds in EDCCs is affected by carbon: nitrogen ratio Although plant suspensions have the capacity to produce storage compounds such as lipids and carbohydrates, a wide range in accumulation levels have been observed, which are strongly dependent on culture conditions (Wen and Kinsella 1992; Hampp *et al.* 2012; Tjellstrom *et al.* 2012). In this study, the change in carbon:nitrogen ratio in EDCCs had an important effect on storage compounds accumulation. Our first report showed the presence of amyloplasts in the BRA-2 line of *J. curcas*, but they were not a

predominant organelle. In addition, cells containing oil bodies were not found when they were grown in multiplication culture medium (Carmona *et al.* 2018). In this study, the BRA-2 line was grown in different culture media in an attempt to stimulate storage compound accumulation. It was found that the addition of ABA, increasing the sucrose concentration from 30 to 80 g L^{-1} and the supplementation of nitrogen source with 100 mg L^{-1} KNO_3 (instead of 2.500 mg L^{-1}) stimulated the formation of oil bodies and amyloplasts. The number of amyloplasts far exceeded the number of oil bodies. A similar response has been found in other species. For instance, when the microalgae *Chlamydomonas reinhardtii* is grown under limiting nitrogen conditions, storage lipids synthesis only occurs when a carbon source is supplied in higher levels (Fan *et al.* 2012). Different responses to nitrogen limitation have been reported in *Arabidopsis* seedlings. In some cases, there is a decrease in total fatty acids, but in others, especially with added sucrose, there is an increase of TAGs (Gaude *et al.* 2007; Yang *et al.* 2011). Knox and Avjioglu (1989) used somatic embryos as a model system to study oil accumulation. Addition of sucrose increased TAGs accumulation to levels that were higher than observed in mature seeds (Knox and Avjioglu 1989). On the other hand, supplementing media with sugars can also promote starch synthesis (see review by Rook *et al.* 2006). For EDCCs of *J. curcas*, a similar response was observed, when the cell cultures were grown in the presence of a higher concentration of glucose (60 or 80 g L^{-1}) a higher presence of amyloplasts was found. Thereby, it seems that the preference for the biosynthesis of different storage compounds (lipids, proteins, and carbohydrates), is regulated by multiple factors, including cell type and culture conditions. However, it is clear that for *J. curcas* EDCCs, there is an effect of the carbon:nitrogen ratio that strongly influences the relative accumulation of starches and lipids.

The LEC1, LEC2, FUS3, ABI3, and WRI1 TFs regulate the development and the maturation process in *J. curcas* seeds In this study, *LEC1*, *LEC2*, *FUS3*, *ABI3*, and *WRI1* expression was compared during three stages endosperm development. These factors are considered as the master regulators of embryogenesis and maturation processes in seeds for various species (Fatihi *et al.* 2016). Highest expression for these TFs occurred during the S1 stage (Fig. 4*d–h*). Expression of *LEC1*, *FUS3*, *ABI3*, and *WRI1* was also observed in subsequent stages of development (S2 and S3). Previous studies for *J. curcas* seeds showed that the expression of *LEC1* and *LEC2* take place at early developmental stages (Jiang *et al.* 2012), similarly to what was found in our study. However, *FUS3*, *ABI3*, and *WRI1* were expressed later, just before the beginning of the maturation stage (Jiang *et al.* 2012), in contrast to what was found here. These differences between the two studies in expression levels may have been caused by differences in genotype, stage classification, or analytic techniques. However, despite of these differences, both studies coincide in the participation of these TFs in the regulation of the development processes and the accumulation of storage compound in *J. curcas* seeds.

In *Arabidopsis*, *AtLEC1*, and *AtLEC2* are expressed exclusively during seed development, are known to control early embryonic differentiation, and are key regulators of storage compound accumulation during the maturation phase (Braybrook *et al.* 2006; Wang *et al.* 2007). Other TFs critical to seed development include *AtFUS3*, *AtABI3* (Kagaya *et al.* 2005; Wang *et al.* 2007), and *AtWRI1* (Baud *et al.* 2007). For *J. curcas* seeds, the accumulation of storage compounds is compartmentalized and occurs mainly in the endosperm (Chaitanya *et al.* 2015a). The high levels of *LEC1* expression during early stages of the endosperm development appear to play a role in cell division, growth, and membrane lipid biosynthesis possibly even cell identity in the endosperm. During later stages, *LEC1* could control carbon flow to support synthesis of storage compounds such as TAGs, as is its function in *A. thaliana* (Mu *et al.* 2008) and *B. napus* (Elahi *et al.* 2016). On the other hand, interaction analysis for these TFs in *Arabidopsis* has allowed proposing a regulatory network, in which *AtLEC1* acts at the highest level in the hierarchy of regulation, with some functional redundancy with *AtLEC2*, which together activate the expression of *AtFUS3*, *AtABI3*, and *AtWRI1*. In Jatropha, *LEC1* is expressed at a higher level than *LEC2* at all stages of development. This suggests a more important function of *LEC1* on the regulation of *FUS3*, *ABI3*, and *WRI1* than *LEC2*. In addition, *LEC1* and *LEC2* may have less functional redundancy than is present in other species. However, later studies will be necessary to confirm these hypotheses.

Two other TFs, ABI3 and FUS3, are critical for seed development. *AtFUS3* is expressed and *AtLEC2* are expressed early in development and regulate the hormonal environment necessary for the establishment of embryo identity. Expression of *AtLEC2* is maintained during the seed maturation phase, and controls the synthesis of storage compounds (Roscoe *et al.* 2015). *AtABI3* is expressed from the globular embryo stage to the maturation phase, with highest levels observed at the end of the development. *AtABI3* is known to control the acquisition of desiccation tolerance and dormancy (Roscoe *et al.* 2015). Moreover, recent studies show that ABI3 plays an important role in the accumulation of storage proteins, while FUS3 is a critical regulator of TAGs synthesis (Roscoe *et al.* 2015). In this study, we found levels of *FUS3* significantly lower than those obtained for *LEC1*, *ABI3*, and *WRI1* (Fig. 5*a*), which could suggest that in *Jatropha FUS3* is not as important as other TFs for maturation processes. In turn, the relatively higher expression of *ABI3* over *FUS3* and *LEC2* at all stages of development suggests that ABI3 together with LEC1 could regulate endosperm cells identity during early

stages of development. During the maturation stage, ABI3 could exert a high degree of control of the synthesis of proteins and lipids, as well as in the participating in the activation of desiccation tolerance and dormancy.

In different plant species, WRI1 interacts with a large number of proteins to form a network that regulates expression of genes involved in the glycolysis pathway and lipid synthesis (Baud *et al.* 2007; Li *et al.* 2015; An *et al.* 2017). Recent work in *J. curcas* shows that *WRI1* endosperm–specific expression is observed from intermediate stages of development (from the third week after fertilization) and is maintained during the maturation phase. In addition, ectopic expression of *JcWRI1* in *Arabidopsis* mutants restores the lipid accumulation (Ye *et al.* 2018). In our study, *WRI1* was also expressed from intermediate stages of development and remained during the maturation phase. This expression pattern corresponds to expression of the genes that encode for the enzymes *KASI* and *DGAT1*. These results agree with those found by *Ye et al.*, (2018) who also demonstrated that JcWRI1 binds specifically to promoter regions of these enzymes. In *J. curcas*, WRI1 functions to control enzymes involved in fatty acids and TAGs synthesis during seed development.

EDCCs express differentially the TFs that regulate lipid biosynthesis in seeds In the previous section, the regulation of several TFs during seed development and the expression of these genes in EDCCs was discussed. The endosperm cells of *J. curcas in planta* are part of a mature tissue composed of quiescent cells, with a cytoplasm occupied mainly by protein and oil bodies (Fig. 4*i–m*). When cell suspensions were established from this tissue, the cells exhibited a change, forming cells with features similar to meristematic cells, becoming smaller, with high cell division rates and little or no accumulation of reserve compounds (Carmona *et al.* 2018). These clear differences between cells *in vitro* and *in vivo* suggest a strong change in the cellular program. Expression levels of the TFs in EDCCs were remarkably low (Fig. 6*a*) compared to those found in seeds (Fig. 5*a*). However, the expression of these TFs was not completely absent for all genes, and a significant expression level was found for *WRI1* and for enzymes involved in fatty acids synthesis. High expression level for some enzymes related to TAGs assembly was also observed. These results indicate that storage lipid synthesis in *J. curcas* EDCCs is not completely absent. In fact, storage lipids did accumulate in EDCCs with concentrations reaching 5% (w/w). It might be possible to further boost lipid levels in EDCCs by manipulating culture conditions (*i.e.*, by altering hormone, carbon, or nitrogen levels) or by changing expression levels of transcription factors by gene manipulation.

The ABA induces a change in gene expression of the enzymes involved in synthesis and assembly of TAGs Culture medium was supplemented with ABA in an attempt to promote production of storage lipids (TAGs) in *J. curcas* EDCCs. However, addition of ABA did not stimulate lipid production and actually resulted in reducing expression of genes related to fatty acids syntheses (*accA*, *KASI*, *DGAT1*, *DGAT2*, and *PDAT*) (Figs. 2 and 6*c–d*). ABA plays a critical role in many processes during seed development and is involved with embryo and endosperm maturation, reserve compounds biosynthesis, desiccation tolerance, and dormancy (Finkelstein 2010; Chandrasekaran and Liu 2014; Maia *et al.* 2014). In *Arabidopsis* seedlings, ABA switches on TAGs synthesis in vegetative tissue by controlling genes related to regulation and synthesis of TAGs (Yang *et al.* 2011; Kong *et al.* 2013). For cell suspensions of *Lesquerella fendleri*, ABA presence doubled the percentage of total lipids (Kharenko *et al.* 2011). However, this was not the case in this study.

EDCCs and seeds have different preference to express one or another isoform of the DGATs enzymes EDCCs were also analyzed for the expression of the genes that encode for enzymes involved in fatty acids and TAGs biosynthesis. Indeed, *accA*, *KASI*, *DGAT1*, *DGAT2*, and *PDAT* were all expressed in EDCCs (Fig. 6d), although at levels lower than observed in seed (Fig. 5b). *DGAT2* expression was high in EDCCs, approaching similar levels to those found by *DGAT1* in seeds. Expression patterns in seed were similar to those previously reported for this species (Xu *et al.* 2011; Gu *et al.* 2012). This leads to ask for the reason why is there a preference to express one or another isoform in both seeds and EDCCs considering that both cells have the same origin.

In plants, isoform recruitment for a particular protein can vary across developmental stages in space and time, as well as between species (Li *et al.* 2010; Misra *et al.* 2013). In *J. curcas*, the DGAT1 and 2 forms use preferentially oleic acid and palmitic acid, respectively (Chaitanya *et al.* 2015b). On the other hand, in *R. communis*, DGAT2 is the preferred isoform, expressed at higher levels, and assembles ricinoleic acid (Kroon *et al.* 2006). The same preferential usage of DGAT2 occurs in other oleaginous species to process unusual fatty acids (Li *et al.* 2010). However, in *J. curcas*, unusual fatty acids have not been reported in seeds, and thus, it is likely that preferential usage of a specific isoform in EDCCs is triggered by culture conditions resulting in diverse fatty acid substrates.

Conclusion

The analysis of gene expression in *Jatropha* seeds suggests that the genetic regulatory network encompassed by *LEC1*, *FUS3*, *ABI3*, and *WRI1* plays a pivotal role in seed development and maturation, as well as primary metabolite production especially in the endosperm. We used expression levels in seed as reference for expression in endosperm derived cell

suspension cultures (EDCCs). Under our growth conditions, although synthesis of storage carbohydrate, such as starch was favored, some TAGs production occurred. This finding is supported by gene expression studies that found that EDCCs have significant expression levels of the *WRI1* TF, as well as, *accA*, *KAS1*, and *DGAT2* enzymes. Altogether, our data highlights the importance of future efforts to further test and optimize growth conditions to enhance oil production in EDCCs. Additionally, our study lays the foundation for additional studies aimed at establishing *in vitro* productive cell cultures of *J. curcas* by employing genetic engineering approaches focused on manipulating expression of transcription factors and biosynthetic genes to overcome limiting factors and provide oil production levels comparable with those observed in endosperm tissue found in seed.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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