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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 promissory plant source for biodiesel and in turn an alternative for in vitro oil production. Nevertheless, 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. Here we assess target gene expression in endosperm cells in planta and endospermderived cell suspension cultures (EDCCs). Endosperm cells were taken from three representative seed developmental stages. Cell suspensions were grown under different glucose, nitrogen, and abscisic acid concentrations to test for optimal biomass growth and oil yield. Production in EDCCs only reaches 5% w/v of total lipids likely due to the change in carbon:nitrogen ratio in vitro , which favors starch accumulation over lipid storage. However, lipid profiles of EDCCs remain identical to those produced in planta . We compared 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 involved in the lipid biosynthesis pathway. We found significant expression of LEC1, FUS3 , ABI3 , and WRI1 in endosperm cells throughout seed development, suggesting similar functions to their counterparts in Arabidopsis and providing a reference expression level for cell cultures. The J. curcas EDCCs showed lower expression of most TFs compared to the endosperm, with the exception of WRI1 , with comparable expression levels in the two systems. Conversely, the enzymes KAS1 , accA and DGAT had the same or higher expression ranges in EDCCs versus endosperm cells. Interestingly, the splicing variants DGAT1 and DGAT2 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 an increased expression of the genes involved in storage lipids biosynthesis Thus, of

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	the optimization of TAGs biosynthesis in J. curcas EDCCs requires strategies aimed at improving culture conditions different from those tested here or over-expressing early biosynthetic TFs that are not able to reach target levels in vitro .

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35 **Abstract**

The oleaginous *Jatropha curcas* has been proposed as a promising source for biodiesel production in seed or potentially by i*n 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 seed. 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*. We measured 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. We found significant expression of *LEC1, FUS3*, *ABI3*, and *WRI1* in endosperm cells throughout seed development, suggesting similar functions to their counterparts in Arabidopsis and providing a reference expression level for cell cultures. The *J. curcas* EDCCs showed lower expression of most TFs compared to 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 splicing variants *DGAT1* and *DGAT2* 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.

Key words: *storage lipids, oil body, amyloplasts, cellular differentiation,* LAFLs *transcription factors, cell suspensions.*

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 fuel (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.

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 TAG content has been demonstrated by heterologous expression experiments in a broad range of species, resulting in the increase of TAG 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, in overexpression of *ZmLEC1 in Zea mays* increased seed oil pleiotropic effects were observed as the result of the*,* which but reduced 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 up-regulation 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 point 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, similar studies in oleaginous species like *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 could offer 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 R 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 cultures of plants 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, with 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 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 to 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 *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 line cell was used as it has high rates of cell division (Carmona et al. 2018). Endosperm derived cell suspension cultures (EDCCs) were subcultured every 8 days (d), in MS2 culture medium (Supplemental Table 1). 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 levels of carbon (30, 60, or 80 g/L of glucose), nitrogen (100 or 2500 mg /L of KNO_3) and ABA (0 mg/L or 1 mg/L) 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 glucose and without hormones or a nitrogen source), using the Nalgene filtration system (500 ml) with a 20 μm nylon membrane (Spectra Mesh®).

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 (Phytotechnology) was added from a stock solution of 500 mg/L, dissolved in 1 M KOH, to a final concentration of 1.0 mg/L. In the case of EDCCs without ABA the same volume of sterile water + 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 days. 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 of fluorescein diacetate (FDA) in acetone (SIGMA ®) (Carmona R et al. 2018). To identify amyloplasts, cells were stained with lugol (2 % w/v). To identify oil bodies, 20 μg/mL of Nile red in acetone (SIGMA ®) and Sudan IV (SIGMA®) were used as was reported before (Carmona R et al. 2018). Observation and cell analysis were carried out with a Nikon Eclipse 80i microscope (Nikon®), using a phase contrast system for the 40X objective, and fluorescence for Nile Red and FDA (Carmona R et al. 2018). Three samples from the same experimental unit were observed in different fields of visualization (at least 6 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 (TLC)

Lipid extraction was carried out following Xu (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 x 8 cm silica gel, TLC 60G F_{254} 25, EMD Millipore). The developing solvent consists of n-hexane, diethyl ether, and acetic acid 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 growth kinetic studies was quantified. After centrifugation of the suspensions, 500 µL of each sample was treated with 500 µL of a solution containing 1% w/v of 3,5-Dinitrosalicylic acid, 0.2% w/v of Phenol, 1% NaOH 1 % w/v, 0.05 % w/v Sodium Sulfite 0.05% w/v and 30 % w/v Rochelle Salt. The samples were incubated in a water bath at 95 °C for 7 min, then placed in an ice bath for 5 min, and then dryed for 3 min at room temperature., Distilled water (5mL) was then added to each tube and 200 uL alliquotes from each sample were measured in triplicate by absorbance at a wavelength of about 515 nm in a plate Spectrophotometer (Biotek, Power Wave XS2).

Histological analysis of the seeds

Fruit were collected at different developmental stages and classified by color and size. The seeds were then dissected from the fruit and various seed attributes 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 denominated 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 %, 100 % v/v). Each sample was maintained in each ethanol solution for 4 h at 4°C.The samples were then transfered to HistoChoice ® (SIGMA) and subsquently embedded in paraffin (Paraplast Plus, SIGMA). 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 106 073) and then with Amido Black (1 % w/v Amido Black 10B + 7 % w/v Acetic acid). Slides were mounted in Entellan® (EMD Millipore). 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.

Analysis of gene expression 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 PCR (RT-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 30g/L and 60 g/L 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, USA), following the manufacturer's instructions. RNA samples were treated with DNase I (Fermentas) to remove contaminating DNA and RNA was quantified by absorbance at 260 nm in a spectrophotometer (NanoDrop-1000, Thermo Scientific). First-strand cDNA was synthesized with AMV Reverse Transcriptase (A3500 Promega, Madison, USA), using 2 µg/µl RNA and following manufactures protocol. The cDNA obtained was used for amplification by RT-PCR using specific primers for each gene (Supplemental Table S3).

All PCR reactions were performed in a Rotor-Gene Q 5plex HRM thermocycler (QIAGEN), using the intercalation dye SYBR as a fluorescent reporter. Each RT-PCR was performed in a 25 µl mixture containing, 2 µl diluted-cDNA (concentration approx.38 ng/uL), 12.5 uL of Master Mix SYBR Green with Rox (Fermentas), and 0.75 uL of each reverse and forward primer, (final concentration 300 nM). The PCR protocol consisted of an initial step of 10 min at 95 ºC, 44 cycles of 15 s at 95 ºC, 30 s at 53 ºC - 57 ºC and 30 s at 72 ºC. 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 added reverse transcriptase to ensure that RNA samples were free of DNA contamination. For each experiment, a 2-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 g/L, 60 g/L or 80 g/L glucose; 100 mg/L or 2500 mg/L KNO_3 and 0 mg/L or 1 mg/L ABA) showed viability between 80 % and 90 % (Fig 1a-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 1c-f) showed the presence of cells with amyloplasts and possibly oil bodies (approx. 1□m to 2 □m). To confirm these observations, additional staining was performed with Lugol and Sudan (Fig. 1d-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 KNO_3) than noted at the higher nitrogen level. Amyloplast accumulation was more pronounced in media supplemented with 60 g/L or 80 g/L of glucose than with the lower (30 g/L) 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 didn't have an effect on total lipid levels. However, significant differences among the other variables were found. For EDCCs grown in 30 g/L glucose, total lipid levels were about 5% w/w when grown with 2500 mg/L of nitrogen and ABA). When cells were grown at the same glucose concentration (30 g/L) but with 100 mg/L of nitrogen and without ABA, a lower total lipid value was observed (3.5%, $p < 0.05$). EDCCs grown in 60 g/L glucose, exhibited different total lipid values in the four-culture media. In cells grown in 100 mg/L of nitrogen and without ABA, the lowest percentage of lipids was registered (3.2 %; $p < 0.05$), compared to the highest value in those supplemented with 1 mg/L 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 figure 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 (DAG), followed by the bands of free fatty acids (FFA), which precede the triacylglycerides (TAG) (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 to 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 glucose. EDCCs cultured between 60- 80 g/L glucose, consumed a greater proportion of the carbon source ranging from 35 g/L to 45 g/L. 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. 4a). 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 is 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 4b-e) the embryo is in intermediate cotyledonary stage (Brito et al. 2015) and is surrounded by endosperm (Fig 4b) The endosperm is surrounced by the internal integument (Fig 4c). 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 4d-e). At stage S2 (Fig 4f-i) the embryo exhibits laminar cotyledons that extend throughout the seed (Fig 4f). 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 4g). At this stage the endosperm occupies most of the seed and accumulates mainly protein bodies (Pb) (Fig 4h), and small oil bodies (ob) (Fig 4i). Finally, at S3 (Fig 4j-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. 4l-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 5a). *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 to 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 to seeds (Fig 6a). 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 to those observed for *FUS3* in seeds (Fig 5a). Expression of *WRI1* increased when the cells were grown in 100 mg/L KNO_3 + 60 g /L Glu and in the absence of ABA, when compared to 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 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). We found a five-fold increase in expression from stage S1 to stage S3 in seeds (Fig 5b) correlated with a greater

accumulation of reserve compounds as lipids (Fig 4). Similar expression levels of *accA* were obtained in EDCCs grown in 2500 mg/L KNO3 + 30 g/L Glu without ABA (Fig 6c).

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 5b). For EDCCs, the highest expression of *KAS1* was found when cells were cultured in 2500 mg/L KNO_3 + 30 g/L Glu 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 four-fold 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 Diacyl Glycerol (DAG), to form the TAG (Li et al. 2010). In this study, *DGAT1* was expressed predominantly in the seed S3, five-fold more than in the S1 and S2 (Fig 5b), when the endosperm cells store reserve compounds as lipids (Fig 4). *DGAT2* expression was lower in S1 and increased in S2 and S3 (Fig 5b). However, comparing the expression of both enzymes for S3, *DGAT2* expression was three-fold lower than *DGAT1.* On the other hand, in EDCCs, *DGAT1* expression did not change significantly, among cultures (Fig 6d, Tukey test, $p < 0.05$), showing lower values than *DGAT2.*The later, surprisingly registered higher values than the ones evidenced in seeds (Fig 5b). Furthermore, higher levels of *DGAT2* expression occurs 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 three-fold increase in expression from S1 to S3 (Fig 5b).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 6d).

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 it showed the highest expression level of all genes evaluated, with a significant change among S1, S2, and S3 stages increasing during development (Fig 5b, 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 medium with 2500 mg/L KNO3 + 30 g/L Glu and in 100 mg/L KNO_3 + 60 g/L Glu (Tukey test, $p < 0.05$). Low expression of *OLE1* coincide 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 down regulation of several metabolic pathways, lipid production is not halted completely. In fact, lipids are produced and the lipid profile is the similar as is 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 and 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, the presence of oil bodies was not found when they were grown in multiplication culture medium (Carmona R 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 and decreasing KNO_3 from 2.500 mg/L to 100 mg/L stimulated the formation of oil bodies and amyloplasts. The number of amylopasts 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, the supplementing media with sugars can also promote starch synthesis (see review by Rook et al. 2006). This response that observed in EDCCs of *J. curcas* when the concentration of glucose was increased from 30 g/L to 60 or 80 g/L Thereby, it seems that the preference for the biosynthesis of different storage compounds (lipids, proteins, and carbohydrates), is regulated by multiple factors, such as including, cell type and culture conditions. However, it is clear that for *J. curcas* EDCCs, there is an effect in carbon:nitrogen ratio that strongly influences the relative accumulation of starch 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 4d-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 maturation stage (Jiang et al. 2012), in contrast to what was found here. These differences between the two studies in expression levels in may have been caused by differences in genotype, stage classification, or analytic technique. 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, and 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 appears to play a role in cell division, growth, 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 to propose a regulatory network, in which At*LEC1* acts at the highest level in the hierarchy of regulation, with some functional redundancy with At*LEC2*, which together activate the expression of At*FUS3, AtABI3* and At*WRI1*. 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 clarify 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 5a), which could suggest that in Jatropha this TF 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 lipids biosynthesis in seeds.

In the previous section, we discussed the regulation of several TFs during seed development., We also analyzed the expression of these genes in EDCCs. 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 4i-m). When cell suspensions were established from this tissue, the cells exhibited a change, forming cells with features similar to meristematic cells, becomings 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* suggests a strong change in the cellular program. Expression levels of the TFs in EDCCs were remarkably low (Fig 6a) compared to that found in seeds (Fig 5a). 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 (ie, 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 EDCCs of *J. curcas.* 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 6c-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 seed 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 acid and TAG 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). Which leads us to ask, 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 carbohydrates (such as starch) was favored, some TAG 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 future 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 to those observed in endosperm tissue found in seed.

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Authorship

All authors have made substantial contributions to the construction of this study. Laura Carmona, Aura Urrea, Natalia Pabón and Daniel Gil proposed and developed the original project, designed the experiments, acquisition of all the data, analysis and interpretation of them. Lucia Atehortua, as the manager of the laboratory facilitated the infrastructure resources y supervised the experiments.

Conflict of interest statement

The authors have no conflict of interest.

Figure captions

Fig 1: Microscopy analysis of *J. curcas* EDCCs growing in different culture media, cells observed under optical, and fluorescence microscope. **a-b** Cells after FDA stain growing in 80 g/L Glu + 100 mg/L KNO_3 + 1 mg/L ABA. **c-f** Double staining with a Lugol solution and Nile red; c-d: Cells growing in 30g/L Glu + 100 mg/L KNO_3; **e-f** Cells growing in 80 g/L Glu + 2500 mg/L KNO_3 + 1 mg/L ABA. **g-h** Double staining with a Lugol solution and Sudan IV, cells growing in 60 g/L Glu + 100 mg/L KNO_3 + 1 mg/L ABA. Black arrows indicate cells storing starch in amyloplasts (Am), and white arrows indicate cell with oil body (Ob). a-b: the bars show 100 µm. c-f: the bars show 50 µm g-h: the bars show 20µm.

Fig 2: Percentage of total lipids in *J. curcas* 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 Tuckey Test) in each group of samples. The dates are the averages ± the standard error of three replicates.

Fig 3: Glucose concentration consumed during 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 Tuckey Test) in each group of samples. The dates are the averages ± the standard error of three replicates.

Fig 4: Morphological changes in seeds and their components during development. **a** Classification of the fruits and seeds of *J. curcas* in different stages of maturation based on the features described in Table S2. **b-e** Stage S1, designated before as stage 2. **f-i** Stage S2, designated before as stage 5. **j-m** Stage S3, designated before as stage 6. **b,f,j** fresh longitudinal section of seed in which the inner integument (In), endosperm (En), embryo (Em), and cotyledons (Co) are evident, the bars show 0.5 cm. **c,g,k** longitudinal semithin section of the seeds stain with PAS + 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), the bars show 500µm**. d,e,h,I,m** longitudinal semithin section of the endosperm that consists of thin-walled cells, staining with PAS + Amido black and Nil red, in some of them are evident the proteins body, Pb (storage proteins) and oil body (storage lipids). **d,e,h, m** the bars show 50 µm. **i** the bars show 25 µm.

Fig 5: Relative Expression of the transcription factors and enzymes in *J. curcas* seeds in different stages of development: **a** Relative Expression of the transcription factors *LEC1, LEC2, FUS3, ABI3* and *WRI1* in S1, S2 and S3 stages of seeds. **b** Relative Expression of enzymes accA, *KAS1, DGAT1, DGAT2, PDAT1 and OLE1* in S1, S2 and S3 stages of seeds. Expression levels were analyzed by RT-qPCR 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 Tuckey Test) in each group of samples.

Fig 6: Relative Expression of the transcription factors and enzymes in *J. curcas* EDCCs grown in different culture media: **a** Relative Expression of the transcription factors *LEC1, LEC2, FUS3, ABI3* and *WRI1*. **b** Relative Expression of *OLE1.* **c** Relative Expression of accA and *KAS1.* **d** Relative Expression of *DGAT1, DGAT2* and *PDAT1*. Expression levels were analyzed by RT-qPCR 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 Tuckey Test) in each group of samples.

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Table S1: Composition of the culture media used in this research.

Component (mg/L)	**MS1***	**MS2****
Macro nutrients		
Sucrose	3000	3000
NH_4NO_3	800	------
$CaNO_3.4H_2O$	2361	------
KNO3	------	2500
$MgSO_4$	180	180
KH_2PO_4	170	170
K_2SO_4	990	990
Micro nutrients		
$CuSO_4.5H_2O$	0.25	0.26
$MnSO4.H_2O$	22.3	22.4
$ZnSO_4.7H_2O$	8.6	8.7
H_3BO_3	6,2	6.3
$Na_2MoO4.2H_2O$	0.25	0.26
Na_2EDTA	37.3	37.4
$FeSO_4,7H_2O$	27.8	27.8
$Na_3C_6H_5O_7$	0.5	0.6
$CaCl_2$	94.7	94.8
Vitamins and amino acids		
Thiamine HCl	5	5
Glycine	2	-----
Nicotinic acid	0.5	0.5
Pyridoxine HCl	0.5	0.5
Biotin (KOH)	1	1
Myo-Inositol	100	100

L-Glutamine	30	------
Hormones		
IAA	4	------
KINETIN	2	------
2,4D	------	0.5
BAP	------	0.5

*Culture media based on the basal composition of the Murashige and Skoog (MS) culture medium with modifications by (Atehortúa and Correa 2011). ** MS2 based on MS1 with modifications made during this research, data not shown. IAA: Indoleacetic Acid. 2,4-D: 2,4Dichlorophenoxyacetic acid. BAP: 6-Benzylaminopurine

Protocol 1: Extraction of lipids from cell suspension

The lipid extraction was carried out using as base protocol the one proposed by Xu *et al*., [8] making several modifications.

The biomass of each culture condition was collected in Falcon of 50 ml and centrifuge at 13,000 rpm for 15 min. After, 4 ml of the supernatant was taken for the quantification of sugars, and then the biomass was transferred to a Nalgene filtration system (500 ml) with a 20 µm nylon membrane, washing several times with deionized water to try to remove the largest amount of exopolysaccharides and remaining culture medium. Samples were stored at - 80 º C until processing.

For each sample, 3 g of fresh biomass was weighed and frozen in liquid nitrogen, the frozen samples were ground to fine powder in a mortar. The fine powder was transferred into 50 ml Falcon tubes with a mixture of 20 ml of hexane: isopropanol, 3:2 respectively,

vortexed for 2 min and centrifuged at 13,000 rpm for 10 min at room temperature. Then the samples differentiated in 3 phases, a pellet with biomass, an aqueous phase with proteins, carbohydrates and isopropanol and an apolar phase containing lipids and other hydrophobic metabolites that are retaining with hexane. The last one phase was transferred with a glass pipette to a round-bottom flask (previously dried at 45 °C for 12 h and weighed). To the remaining sample two extractions more were performed in the same way as described above, they were centrifuged under the same conditions, again taking the apolar phase and joined it with the previous supernatant in a flask. To remove the solvents from the samples, the flasks were placed into a rotary evaporator, with a water bath of 50 ºC, a rotation speed of 40 rpm and a vacuum pressure of 335 psi (poundforce per square inch). Once the solvent was evaporated from the samples, the flasks were placed into the convection oven at 40 °C for 20 min, completely evaporating the solvents. After the drying time, the flasks were cooled and weighed to determine total lipids (TL) gravimetrically. The remaining biomass and the aqueous phase were transferred to glass flask (20 ml, previously dried) and were placed into the convection oven at 70 °C for 2 d, recording their weight by gravimetry. This same protocol was used for lipids extraction from the seeds.

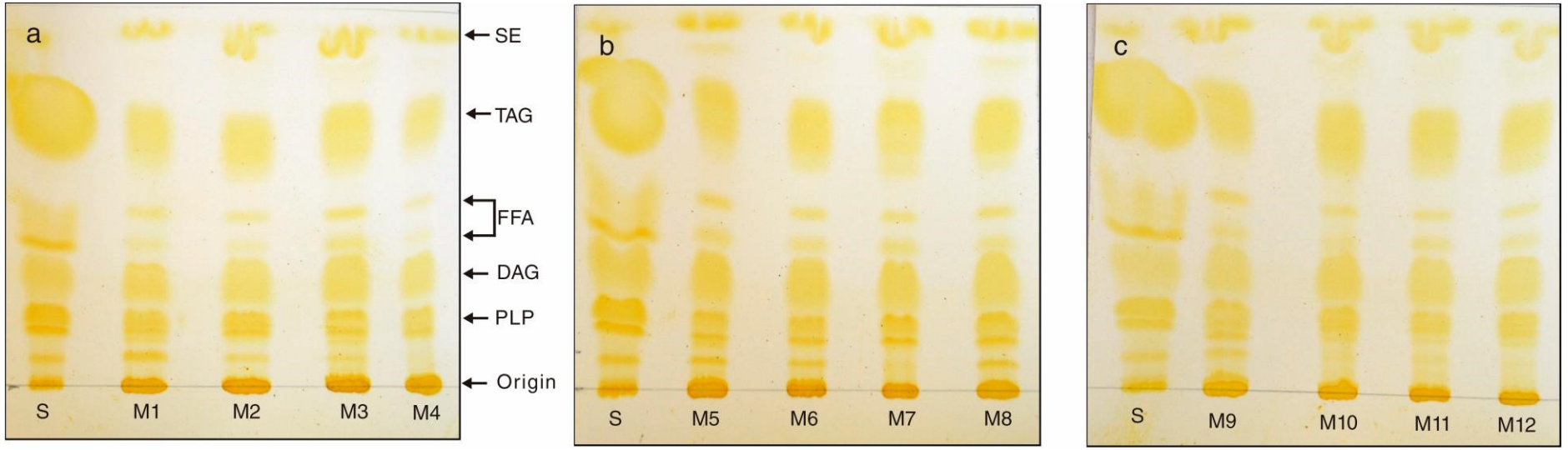

Fig S1: Thin layer chromatography (TLC) separation of total lipids from seed (S) and suspensions *J. curcas* growing in different culture media: **a** culture media: M1: 30g/L Glu + 100 mg/L KNO_3; M2: 30 g/L Glu + 2500 mg/L KNO_3; M3: 60g/L Glu + 100 mg/L KNO_3; M4: 60 g/L Glu + 2500 mg/L KNO_3. **b** Culture media: M5: 80 g/L Glu + 100 mg/L KNO_3; M6: 80 g/L Glu + 2500 mg/L KNO_3; M7: 30 g/L Glu + 100 mg/L KNO_3 + 1 mg/L ABA; M8: 30 g/L Glu + 2500 mg/L KNO_3 + 1 mg/L ABA. **c** Culture media: M9: 60 g/L Glu + 100 mg/L KNO_3 + 1 mg/L ABA; M10: 60 g/L Glu + 2500 mg/L KNO_3 + 1 mg/L ABA; M11: 80 g/L Glu + 100 mg/L KNO_3 + 1 mg/L ABA; M12: 80 g/L Glu + 2500 mg/L KNO_3 + 1 mg/L ABA. 5□l of total lipids was fractionated by TLC on silica gel plates. DAG, diacylglycerol, FFA, free fatty acid SE, sterol ester; TAG, Triacylglycerol; PLP, Phospholipid. Mobile phase: hexane: Ethyl acetate: Acetic acid, 90:10:1, respectively; developed with iodine vapors.

Table S2: Characteristics of *J. curcas,* in different seed development stages. The dates are the averages ± the standard error of between ten and fifteen measurements for each stage.

Stage	Testa color	Testa consistency	Stretch marks formation on the testa	Length of seed (mm)	Width of seed (mm)	Weight of complete seed (g)	Fresh weight of endosperm (g)
1	White	Soft	Not	10.84 □ 0.70	7.150 □ 1.07	0.648 □ 0.07	0.30 □ 0.01
2	Cream-Beige	Semi-hard	Not	13.82 □ 0.63	8.90 □ 0.87	0.919 □ 0.04	0.44 □ 0.02
3	Yellow-Brown	Semi-hard	Not	19.80 □ 0.94	10.37 □ 0.37	1.001 □ 0.08	0.52 □ 0.06
4	Black-Brown	Semi-hard	Yes	20.28 □ 0.91	10.66 □ 0.50	1.113 □ 0.08	0.58 □ 0.08
5	Black-Brown	Hard	Yes	21.77 □ 0.65	10.62 □ 0.38	1.175 □ 0.12	0.69 □ 0.06
6	Black	Hard	Yes	21.23 □ 0.65	10.52 □ 0.48	1.123 □ 0.11	0.63 □ 0.05
7	Black	Hard	Yes	20.86 □ 0.49	10.30 □ 0.29	1.081 □ 0.08	0.64 □ 0.06
8	Black	Hard	Yes	20.01 □ 0.73	9.250 □ 0.45	0.956 □ 0.09	0.59 □ 0.05
9	Black	Hard	Yes	20.21 □ 0.50	10.34 □ 0.49	0.824 □ 0.06	0.52 □ 0.05

Table S3: List of genes evaluated with the sequences of the primers designed for their amplification *.

Gen	Abbreviation	N° Accession	Primers Forward /Reverse	TM (ºC)
Transcription Factors				
ABA INSENSITIVE 3	*ABI3*	XM_012230451.1	5'-CCCGTTTGTTAATGGAGCTT-3' 3'-TTGAGCAGAATCAAGCATTTG-5'	54
FUSCA3	*FUS3*	XM_012212174.1	5'-CCGTAATGAGGTTGTTGCTG-3' 3'-AAGCAGAAGCAGCATTAGCA-5'	55
LEAFY COTYLEDON 2	*LEC2*	XM_012237019.1	5'-CACCAATGCTACCAAACCAA-3' 3'-ATGCTGTGATCTGGTGGAGA-5'	55
LEAFY COTYLEDON 1	*LEC1*	JF703667.1	5'-TGAAGCTGTCCGAGATGAAC-3' 3'-GCACTCATTGTCATCACCAGT-5'	57
WRINKLED 1	*WRI1*	NM_001035780.2	5'-GGTGGGAAGCTCGAATTG3'- 3'-TGGTTACTGCATTTGCTCCT-5'	55

Oil body proteins				
			5'-GCCTGGTGCAGATAAGATTG-3'	
			3'-AGCCTCTTGAGCCTTGTGTT-5'	
OLEOSINA 1	OLEO1	JQ806305.1		55
Enzymes involve in fatty acids synthesis and TAGs assembly				
Diacylglycerol acyltransferase			5'-TGATGGATCCAGGTATGGTG-3'	
	DGAT1	EU477378.1		
(DGAT) 1			3'-GGCCGGTACGTGAATTTAATA-5'	55
Diacylglycerol acyltransferase	*DGAT2*	HQ827795.1	5'-CAGACCATGTGGGTTTCTTG-3'	57
(DGAT) 2				
			3'-GCATCCAGGAGGGAAGTAAA-5'	
Acetil-CoA carboxilasa	*Acca*	HQ153095.1	5'-CATGGGCTTTAAGGACAGGT-3'	57
subunidad □-CT				
			3'-TGCAGAGAGGCCTTGAGTAA-5'	
Phosphatidyl glycerol			5'-ACGGAGACGAGACTGTTCCT-3'	
acyltransferase	*PDAT*	HQ827796.1		57
			3'-CGACCCTCCAGAAGATTAGC-5'	

β-Ketoacyl-[acyl carrier protein] synthase I	*KAS1*	DQ987699.1	5'-GATTACGGGTATGGGTTTGG-3' 3'-AATTTAGAGGCATCGAACCG-5'	55
Control				
Cytosolic Glyceraldehyde-3-Phosphate Dehydrogenase	*GDPHC*	NM_001306035.1	5'-GATCAATCGAGCAACTGGAA-3' 3'-ACATTGCCAAAGGTTTCCTC-5'	55

* These primers were designed using the online tool "GenScript Real-time PCR (TaqMan) Primer Design" (https://www.genscript.com).

A gene analysis, was carried out, to design the primers to amplify the transcription factors, by aligning the coding sequences for *J. curcas*, with the sequences of *Arabidopsis thaliana, Oryza sativa, Manihot esculenta and Ricinus communis L;* identified the conserved domains for each gene family, and the primers were designed outside of these regions. Alignments were performed in the online MAFFT version 7 (https://mafft.cbrc.jp/alignment/software/) and the BioEdit software. The sequences were obtained from the databases: Gene Bank (http://www.ncbi.nlm.nih.gov/) and Phytozome (https://phytozome.jgi.doe.gov).

Figure 1

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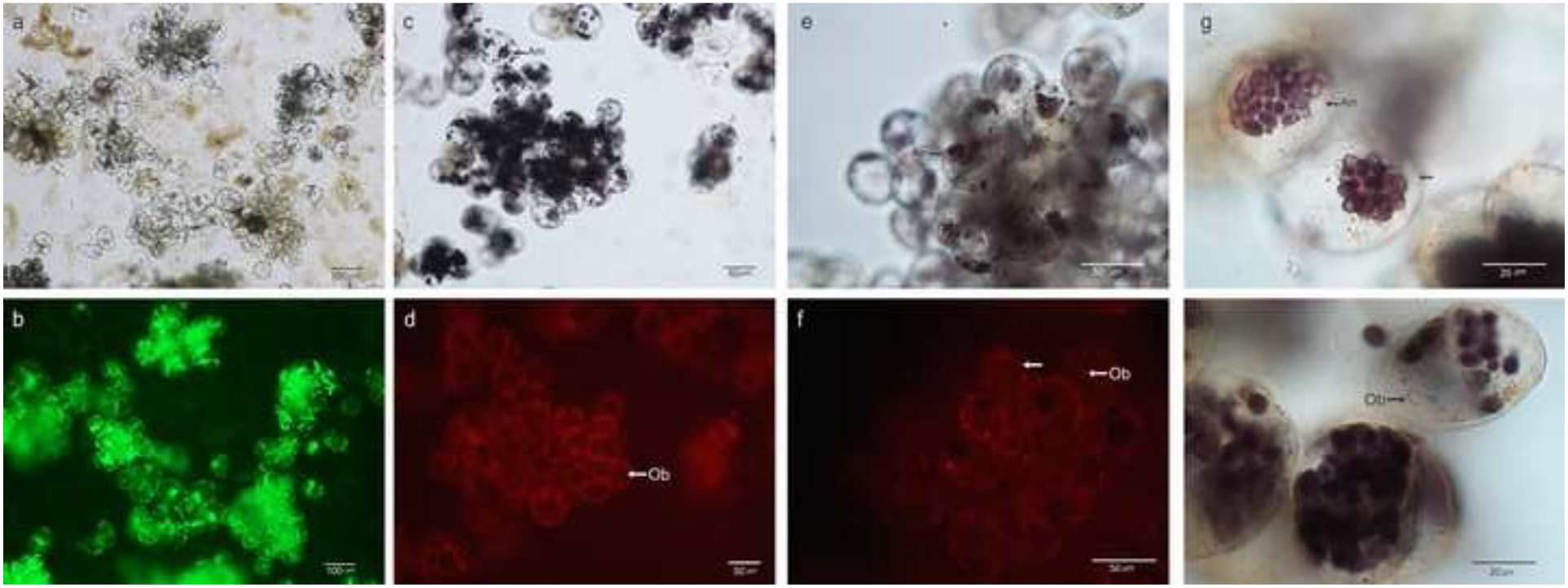

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Figure 2

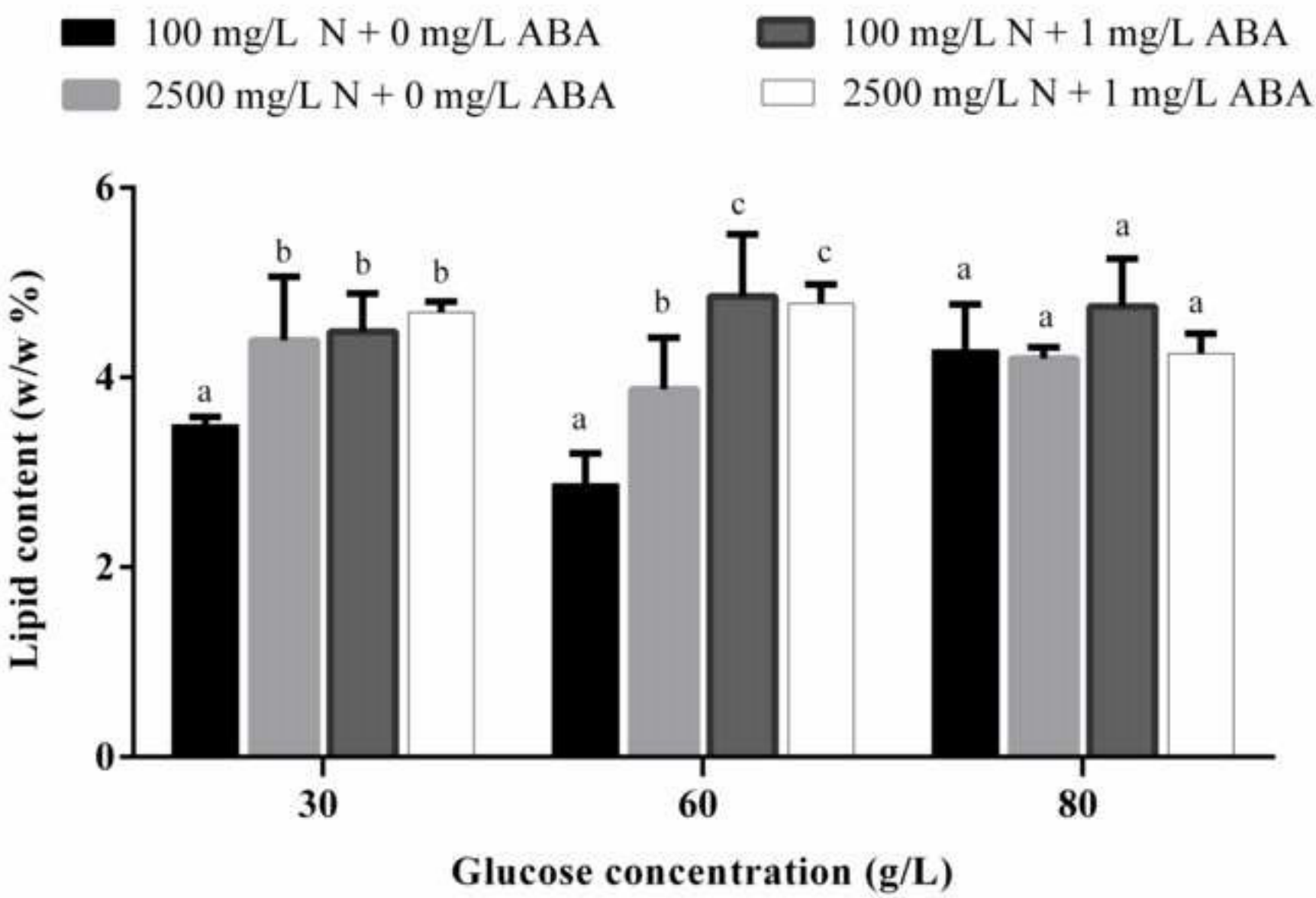

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Figure 3

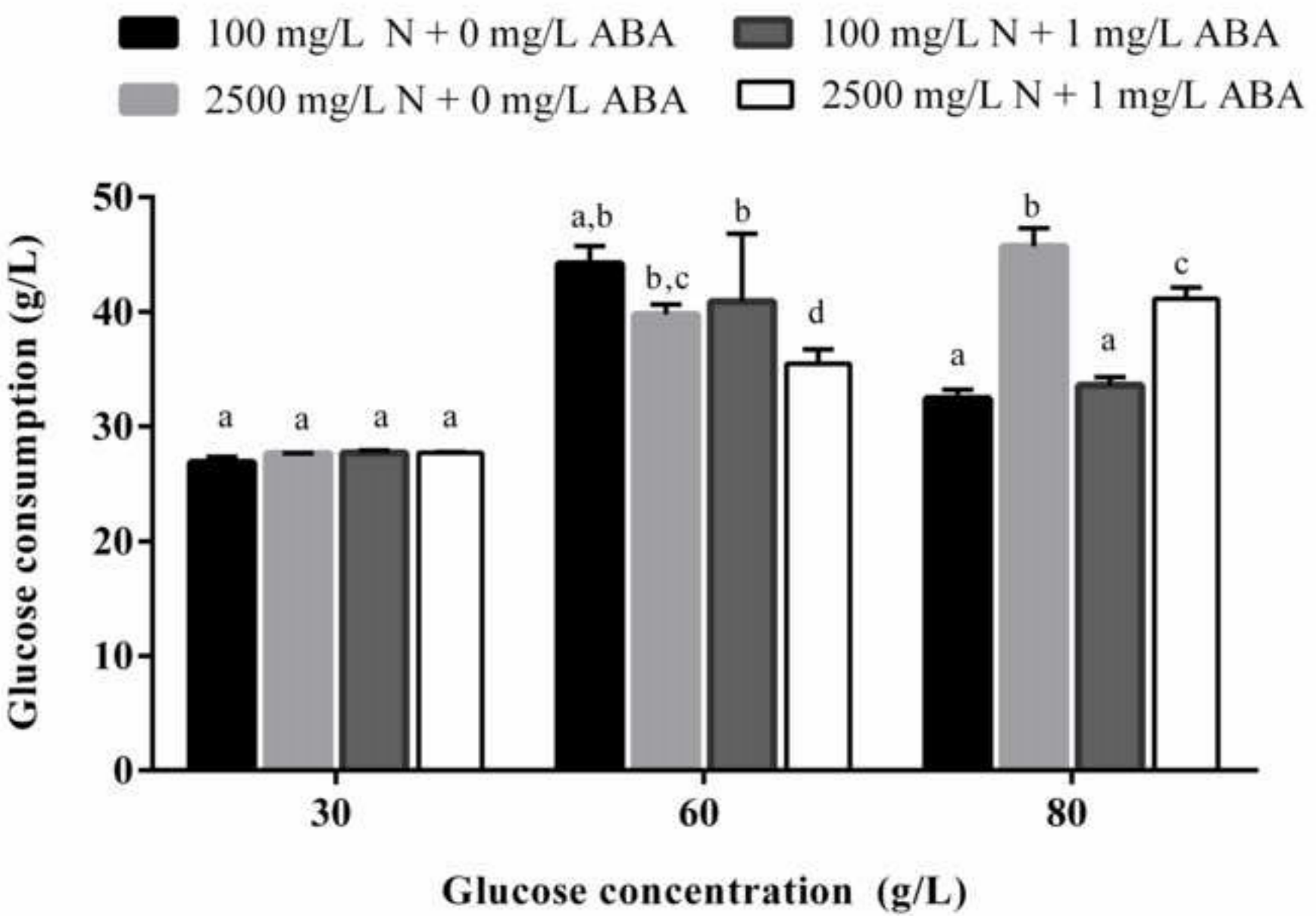

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Figure 4

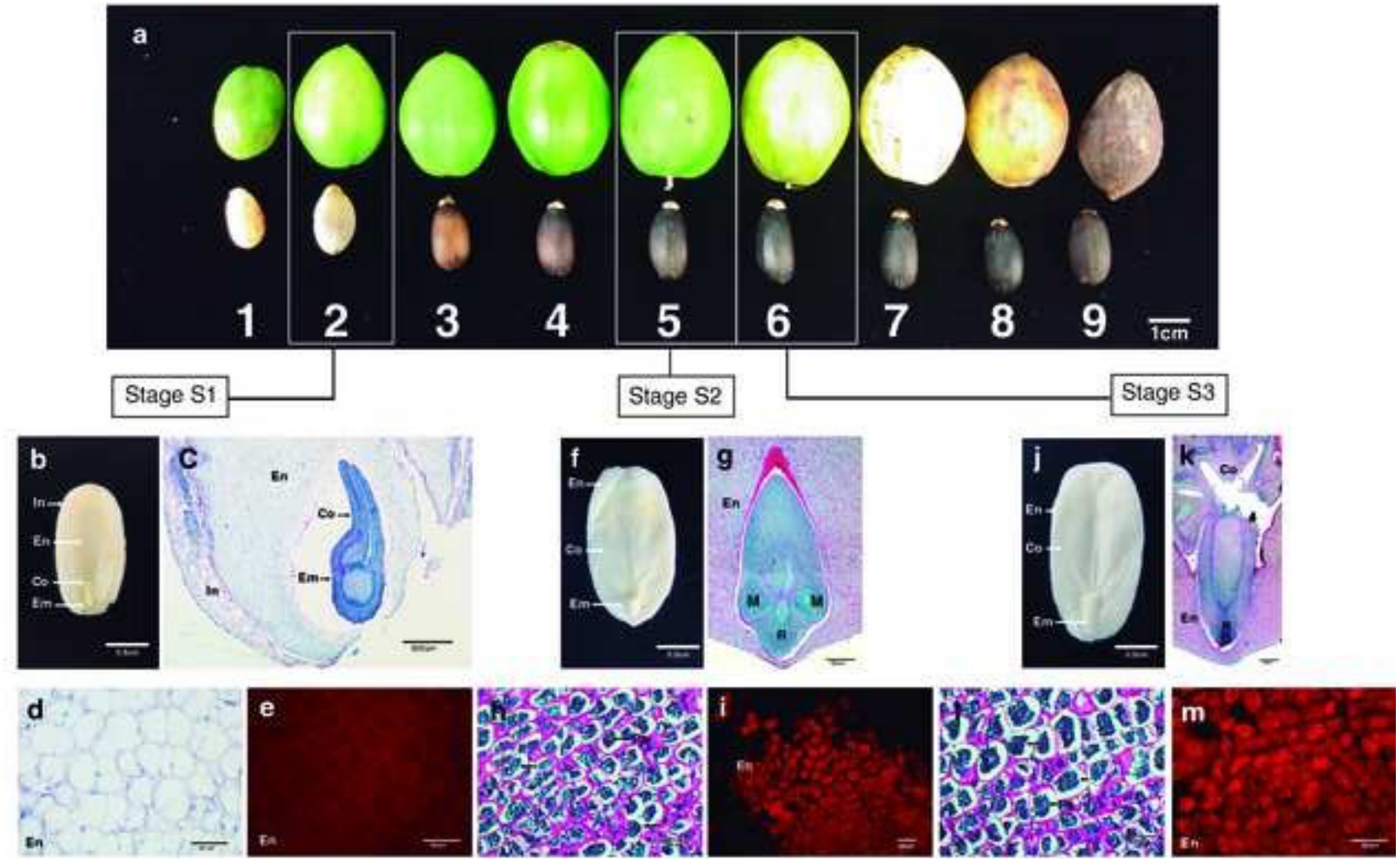
a
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Figure 5

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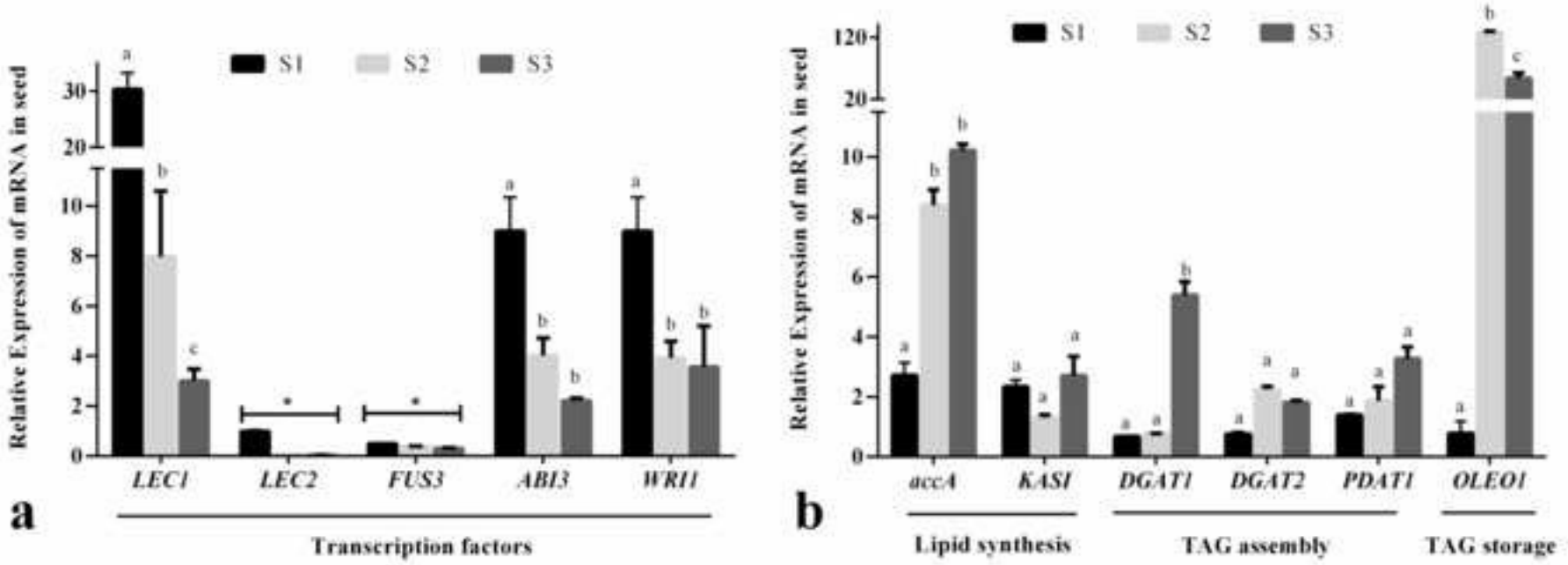

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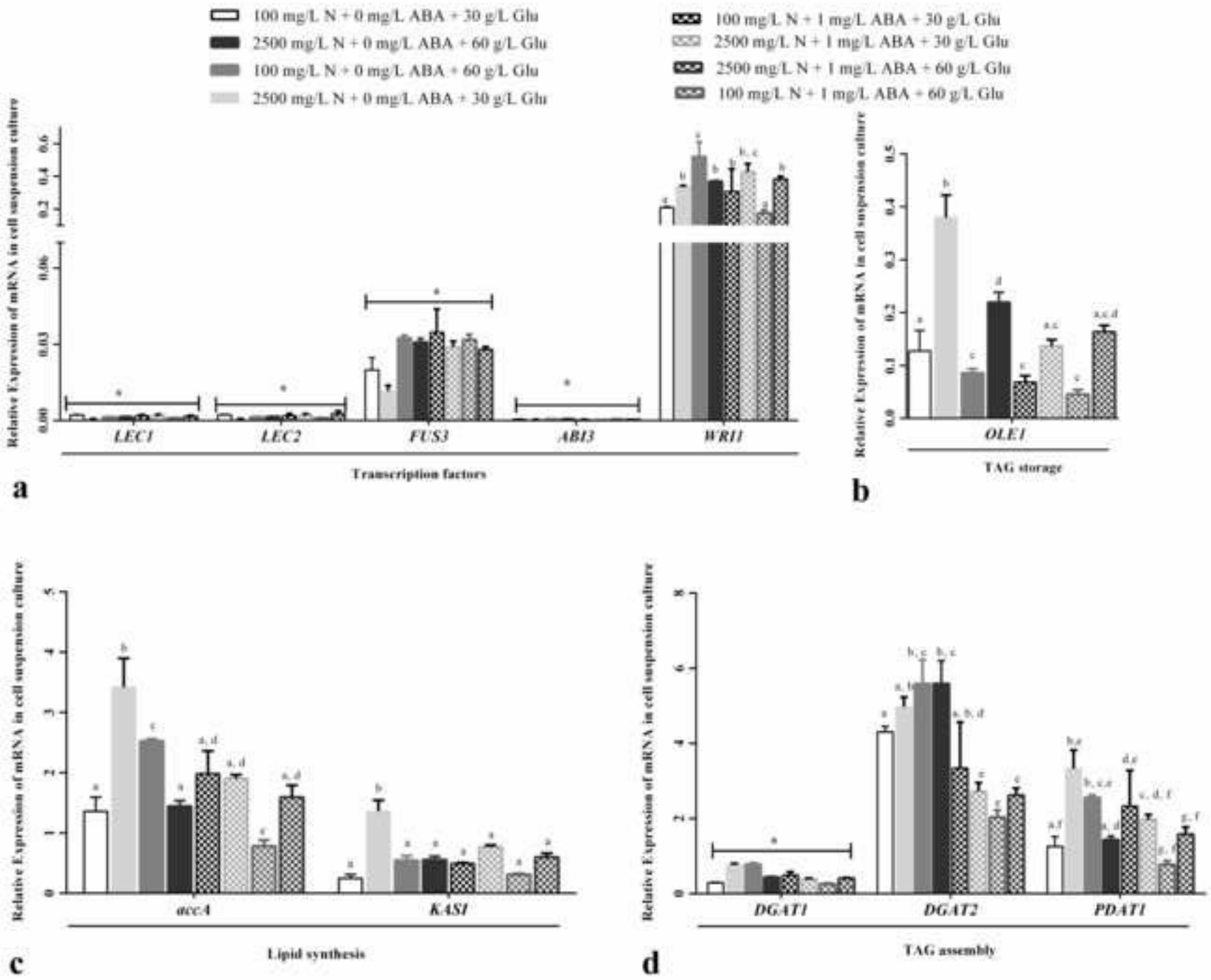
Figure 6


100 mg/L N + 0 mg/L ABA + 30 g/L Glu
2500 mg/L N + 0 mg/L ABA + 60 g/L Glu
100 mg/L N + 0 mg/L ABA + 60 g/L Glu
2500 mg/L N + 0 mg/L ABA + 30 g/L Glu
100 mg/L N + 1 mg/L ABA + 30 g/L Glu
2500 mg/L N + 1 mg/L ABA + 30 g/L Glu
2500 mg/L N + 1 mg/L ABA + 60 g/L Glu
100 mg/L N + 1 mg/L ABA + 60 g/L Glu
Relative Expression of mRNA in cell suspension culture
LEC1
LEC2
FUS3
ABI3
WRI1
Transcription factors
a
OLE1
TAG storage
b
accA
KASI
Lipid synthesis
c
DGAT1
DGAT2
PDAT1
TAG assembly
d