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Expression profiles of genes involved in fatty acid and triacylglycerol synthesis in developing seeds of Jatropha (*Jatropha curcas* L.)

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ABSTRACT

Jatropha (*Jatropha curcas* L., Euphorbiaceae), a potential biodiesel plant, has created tremendous interest all over the world for the use of its seed oil as a commercial source of biodiesel. Due to the unreliability of oil content in its seeds and low economic returns planting of jatropha in agriculture was restricted. Investigating the molecular basis of storage lipid accumulation during seed development is an immediate need to understand genetic factors regulating storage lipid biosynthesis in jatropha seeds. In this study, we characterized the seed development and lipid accumulation from female flowers pollinated to mature seeds, and investigated temporal expression profiles of 21 lipid genes involved in different steps of the pathways leading to fatty acid and TAG synthesis within jatropha developing seeds using quantitative real-time PCR technology. Concomitantly, 17 genes increased their expression levels in developing seeds compared to their expression in leaf, but showed various temporal expression patterns and different relative-maximum ratio ranging from 2.8 to 1,919,280-fold in developing seeds. Five gene groups with distinct temporal patterns were identified by clustering analysis of expression data. Two gene groups including 15 genes presented up-regulated expression patterns correlated with storage lipid accumulation in developing seeds. This study provided not only the initial information on promoter activity for each gene, but also a first glimpse of the global patterns of gene expression and regulation, which are critical to understand the molecular basis of lipid biosyntheses, identifying the rate-limiting genes during seed development and to create improved varieties by genetic engineering.

1. Introduction

Jatropha (*Jatropha curcas* L., Euphorbiaceae) is a great potential biodiesel crop, commonly known as purging nut or physic nut, and native to Mexico and Central America. Due to its wealth seed oil in the form of triacylglycerol (TAG) that serves as biodiesel feedstock and its growth property such as drought hardiness, small gestation period and wide adaptation to soil conditions, jatropha has created tremendous interest all over the world for the use of its seed oil (storage lipids) as a commercial source of biodiesel [1–4]. Currently, jatropha has been planted as a biodiesel crop in India, China, South

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America and Africa [5]. However, jatropha is still an undomesticated plant with unreliable seed yield and seed oil content and low economic returns in agriculture. The available germplasm growing in the wild lacks genetic information and has low genetic diversity [5]. There is an immediate need for genetic enhancement and metabolic engineering of jatropha which could create improved varieties to serve as its application in agriculture [6]. Investigating the molecular basis of storage lipid (i.e. TAG) accumulation during seed development is an essential prerequisite to understand genetic factors regulating storage lipid biosynthesis in jatropha seeds and to create improved varieties by genetic engineering.

Although molecular mechanism of storage lipid biosynthesis in higher plants may be variable, in general, the pathway of storage lipid biosynthesis mainly includes two conceptually simplified systems: fatty acid (FA) synthesis and TAG assembly. The step of FA synthesis produced a common and unique system intermediate, cytosolic acyl-CoA, using direct or indirect products of photosynthesis in plasmids. The TAG assembly sequentially consumed the acyl-CoA produced in the front system using substrate Glycerol 3-phosphate (G-3-P) in Endoplasmic reticulum (ER) [7]. The latter step was called as the Kennedy Pathway [8]. In developing seeds, quality and quantitative of the storage lipids synthesized depends on the composition of its constituent TAGs and a number of enzymes which participated in FA synthesis and transfer during photosynthesis in leaf and TAG assembly during the Kennedy Pathway [9–11]. Such control is exerted at the level of *de novo* FA synthesis in the plastids, in subsequent modification reactions on these FAs and at the level of microsomal acyltransferases in ER. We had known much about the individual enzymes and of genes coding their activities [11], and useful changes in TAG quality or yield have been made by genetic manipulation [12–15]. However, little is known about the overall expression and regulation profiles of multiple genes involved in lipid biosynthesis from *de novo* carbon flux to TAG during seed development [16,17]. This aspect of storage lipid accumulation is important for attempts to identify the key rate-limiting enzyme genes which regulate storage lipid accumulation during seed development in crops. In particular, raising overall oil yields from oleaginous seeds for providing feedstock for biodiesel production by genetic manipulation technology is a critical issue to facilitate the development of biodiesel in industry. Jatropha is a potential woody biodiesel plant, but the main problem associated with jatropha seeds is its oil content is unreliable [5]. Little is known about its lipid biosynthetic enzymes or how FA and TAG biosynthesis is regulated in jatropha seed [18]. Investigating the molecular basis of storage lipid accumulation during seed development is necessary to understand the physiological reasons for affecting unreliability of oil content in seeds and identify the rate-limiting enzyme genes which regulate storage lipid biosynthesis from *de novo* carbon flux to TAG during seed development.

Current efforts on genetic enhancement and metabolic engineering of oilseeds have been shifted towards searching for additional genes or general transcription factors that may up-regulate multiple activities or entire pathways leading to oil biosynthesis [13,19]. Therefore, knowledge of the expression overview of multiple genes and their regulation during Jatropha seed oil biosynthesis is needed to further understand the regulatory mechanisms controlling storage lipid accumulation. In this study, we obtained 21 genes involved in different steps of the pathways leading to fatty acid and TAG synthesis, and characterized their expression profiles during the time-course of seed development. Our results provided not only the initial information on promoter activity for each gene, but also a first glimpse of the global patterns of gene expression and regulation, which are critical to understand the molecular basis of lipid biosyntheses and identify the rate-limiting genes which regulate storage lipid biosyntheses during seed development in jatropha.

2. Materials and methods

2.1. Plant material and chemicals

The four-year-old jatropha trees (XTBG-JC0032) collected from southern Yunnan by seeds were grown at Xishuangbanna Tropical Botanical Garden (21°56′N, 101°15′E, 600 m asl), Chinese Academy of Sciences, Yunnan, China under natural climat conditions. We observed the development process of jatropha seeds from female flowers pollinated to mature seeds in July–October, 2009. Mature female flowers were tagged and man-pollinated by the time when the stigma was fully expanded, and the tagging dates were recorded as 0 day after pollination. Capsules of atleast six stages of 12, 18, 24, 30, 40 and 50 days after pollination (DAP) were harvested, dissected, and the corresponding seeds were collected for seed weight measurement, lipid extraction, or immediately frozen in liquid nitrogen and stored at −80 °C for RNA extraction. Leaf tissue was collected from a fully expanded young leaf and root tips were collected, washed and dissected from the same individuals. All chemicals used for lipid extraction and GC analysis were of the highest purity available.

2.2. Seed weight measurement and lipid extraction

Seed samples collected at six stages of seed development were weighed (fresh weight, FW) into Eppendorf tubes and dried under vacuum centrifugation over night. The dried samples were weighed (dry weight, DW). For lipid extraction, five replicate samples of dried seeds were accurately weighed, and frozen in liquid nitrogen for a few minutes; the frozen samples were ground to fine powder in a mortar, and the fine powder was transferred into 10 mL glass tubes for lipid extraction. Lipids were extracted using a mixture of 600 dm^3 hexane and 400 dm^3 isopropanol to determine total lipids (TL) gravimetrically [20]. The TL was then dissolved in hexane to give a concentration of 1 mg/mL. TAG was isolated by silica cartridges method, as described by Quartacci et al. [21]. Briefly, A 50 mm^3 aliquot of TL was transferred to silica cartridges, and TAG was eluted using 1 cm^3 of a 10 dm^3 addition of acetic acid to 1 m^3 of $CHCl_3$ to a vial. 2 mm^3 pentadecanoic acid was added to TAG samples as an internal standard and the aliquot were dried under nitrogen. Fatty acid methyl esters (FAMEs) were prepared by heating the dry TAG materials at 85 °C for 60 min in 2% (v: v) sulfuric acid in dry methanol, as described by Pomeroy et al. [22]. The resulting FAMEs were dissolved in 1 mL of dichloromethane (0.01% BHT) for GC analysis (GC-2104, Shimadzu).

2.3. Gene acquisition and primer design

To obtain the promising target genes associated with storage lipid accumulation from Jatropha seed, we searched the Gene bank database (http://www.ncbi.nlm.nih.gov/) and focused on genes directly participated in pathway from *de novo* Fatty acid biosynthesis to TAG assembly. We obtained 12 genes with cDNA sequence, which are tightly associated with lipid biosynthesis in jatropha developing seeds from gene bank database. We also constructed an EST library for developing seeds of jatropha, and cloned nine genes involved in storage lipid biosynthesis, based on the EST sequence available by Rapid Amplification of cDNA Ends (data not published). Totally, 21 lipid genes were obtained (see Table 1), including the nine genes cloned by our laboratory in this study. The 21 lipid genes involved 11 genes associated with FA biosynthesis and desaturation, i.e., two Acetyl-CoA Carboxylase genes (HomoACC and HeteroACC), three Pyruvate Dehydrogenase genes (PDAE1α, PDAE1β and PDAE2), three KAS genes (KAS I, KASII, KASIII), and three fatty acid desaturase genes (Chloω6, Chloω3 and Mitoω3); eight genes associated with TAG assembly during Kennedy Pathway (i.e, pGPAT, pGPDH, cGPDH, LPAT, PAP, PDAT, DGAT1 and DGAT2); and two oleosin genes (Ole1 and Ole2). We respectively predicted the subcellular location of functional enzyme proteins encoded by the 21 gene investigated by using protein BLAST program in NCBI homepage (http://blast.ncbi.nlm.nih.gov/) and WOLFPSORT online programs (http://wolfpsort.org/). The gene-specific primers were designed using the Primer Express software for Real-Time PCR version 3.0 (Applied Biosystems), and all parameters kept defaults. Length of PCR products varied from 50 bp to 100 bp. Information of optimized primer pairs is listed in Table 1.

2.4. Real-time PCR quantification

Total RNA was extracted from leaves, roots, and the developing seeds at six developmental stages respectively, using the silica particles-phenol/chloroform extraction method, as described by Ding et al. [23]. After extraction, RNA samples were treated with DNase I (Fermentas) to remove contaminating DNA, and then were cleaned by using RNA Clean Kit (Tiangen, China) with manufacturer's protocol. The clean RNA was quantified by absorbance at 260 nm in a spectrophotometer (NanoDrop ND-2000) and checked for quality by Goldview™ (Bioteke, China) agarose gel electrophoresis. First-strand cDNA was synthesized with the Prime Script™ RT-PCR Kit (Takara Bio. INC.), using equal amounts of Oligo dT primer with the manufacturer's instructions. The first-strand cDNA was used as template for Real-time PCR reaction. All Real-time reactions were performed an Applied Biosystems 7500 Real-Time PCR System using the intercalation dye SYBR as a fluorescent reporter. PCR reactions were performed in triplicate in a standard reaction mixture (25uL) contained 1uL cDNA template, 1×FastStart Universal SYBR Green Master mix with Rox (Roche), and 300 nM forward and reverse primer. Reactions were performed in MicroAmp 96-well plates (Applied Biosystems) covered with optical adhesive covers (Applied Biosystems). The PCR protocol consisted of initial step 50 °C for 2 min and 95 °C for 10 min according to instructions of the manufactures, followed by 40 repeats of 95°C for 15s, 59 °C for 15s and 72 °C for 32s. PCR product specificity was confirmed by melting-curve analysis and by electrophoresis on 4% agarose gel to ensure that PCR reactions were free of primer dimmers and non-specific amplicons. The jatropha Actin gene (a house-keeping gene) was used as internal reference to normalize the relative amount of mRNAs for all samples. Data acquisition and analysis of the real-time PCR assay were performed using the 7500 System SDS software version 1.4 (Applied Biosystems). Each fluorescent reporter signal was measured against the internal reference dye (ROX) signal to normalize for non-PCR-related fluorescence fluctuations between wells. The ratio of gene-specific expression to Actin signal was defined as relative expression. PCR controls were performed in the absence of added reverse transcriptase to ensure whether RNA samples were free of DNA contamination.

2.5. Gene clustering analysis and visualization

To partition the lipid genes into distinct groups such that genes assigned to the same cluster should have similar expression patterns, the quantitative expression data of each gene at various developmental stages were subjected to gene expression clustering analysis using the k-mean clustering method [17,24] provided within the software Expression Analyzer and DisplayER (EXPANDER) [25]. Input data were first standardized with mean 0 and variance 1, fixed norm and then pursued clustering with k-mean method. The k-mean numbers from 4 to 10 were tested (data not shown), and the clustering into five groups was selected because it gave the best scores for clustering quality. To view the expression patterns of clusters by a graph, the option of mean patterns with error bars operated by the EXPANDER was chosen that allows each cluster to be displayed in a separate panel with error bars representing standard deviations.

3. Results

3.1. Developmental changes in seed weight and oil content

Our observation showed that the developing seed grew gradually at the early stage of seed development before ca. 15 DAP, and the fresh weight of seeds increases rapidly after ca. 15 DAP, throughout the middle and late stages of development up to its maximum at ca. 45 DAP, and then seed fresh weight declined slightly during the natural desiccation at the late stage of seed development (Fig. 1). The increase of dry weight at the early stage of seed development (ca. 20 DAP before) is limited and lags the increase of fresh weight, suggesting the early seeds consisted mostly of water. After ca. 20 DAP the dry weight of seeds increases rapidly and reaches its maximum 710 mg at ca. 55 DAP. The total lipid accumulation is similar to the increase of dry weight, i.e. it develops gradually before 20 DAP, and rises rapidly throughout the middle and late stages of development and reaches its maximum (accounting for 27% of dry weight) at ca. 55 DAP. The content of total lipids dropped slightly at seed natural maturation desiccating period. However, the accumulation of TAG lags the increase of total lipids at the early stage of seed development, i.e., TAG is undetectable before ca. 24 DAP, consistent with the increase

Table 1 – Selection of lipid genes, primer sequences, size of Amplification products .

Lipid gene cellular location and activity [a]	Abbreviation	GenBank ID	Forward/Reverse primer	Amplificon Size (bp)
Plastid location				
Plastidial Glycerol-Phosphate Acyltransferase	pGPAT	FJ952147	5′-CCTTATCTTCAAAGCTCTGTTTGGT 5′-CCTT CGCGCACTCGATTTAA	77
Plastidial Glycerol-3-Phosphate Dehydrogenase	pGPDH	HQ827791[b]	5′- GATGTTGGCAAAGAACTAACACATTG 5′- GAGGATGATATATGGGAAACAGAAGA	91
Plastidial 1-Acylglycerol-Phosphate Acyltransferase	LPAT	HQ827792[b]	5′- GTCAGGTCTTCAGCGGCTAAA 5′- TGCCTGTGTAAAGCGAGTTCCT	82
β-ketoacyl-acp synthaseI	KASI	DQ987699	5′- GCCCTCCAATCCCCATCTAT 5′- TTTTAGTTGGAGGTTTCGTTGCA	85
β-ketoacyl-acp synthaseII	KASII	DQ987700	5′-ACGTGCGCCAAGGAGAAC 5′-GCCCAGCGACTGAGTCTGTT	77
β-ketoacyl-acp synthaseIII	KASIII	DQ987701	5′-GCGGTTCGAAGTGGGAAA 5′-CCCAAGTTAGACCAGCTCCAAA	72
Homomeric Acetyl-CoA Carboxylase	HomoACC	DQ987702	5′-AGCCACAGAGGCTTGTATTTTCTGA 5′-CATGCTATCATGCCAATGTCATT	102
Heteromeric Acetyl-CoA Carboxylase	HeteroACC	FJ952146	5′-GGAAGTGATGGGTTGCTACAGAA 5′-GTCCTCCACCACCTGCTGTT	100
Pyruvate Dehydrogenase E1 Subunit	PDAE1	FM894614[b]	5′- CCGAATAGAACGCAAATTTATAACC 5′- GGCAGCTGAGATGCTTGCTAA	64
Pyruvate Dehydrogenase E1 Subunit	PDAE1	FM894873[b]	5′-CAAACTTCGTTTCAATTCTGTTCCTAA 5′- CGGCTTCAGATACAGCAACAAT	83
Pyruvate Dehydrogenase E2 Subunit	PDAE2	FM893832[b]	5′- CATTCTGCCCCCTGGAACT 5′- CGTCCTTAGTAGCCACGACAGTAG	74
Chloroplastal –6 Fatty Acid Desaturase	Chlo 6	EU106889	5′-CGAGATCCTCTGTCATGATATCAATG 5′-GCCGCCCTTAGATTATAACTTGGT	81
Chloroplastal –3 Fatty Acid Desaturase	Chlo 3	EU106890	5′-GGAATAGAAGCCCGGGAAAA 5′-CTTCCTTTCATTCGGCAAGAA	74
Cytoplasm location				
Glycerol-3-Phosphate Dehydrogenase	cGPDH	HQ827793[b]	5′-TGTTGGCAGAAGAACCTGAGAA 5′- CATGCATTACGACCTTTCAACAAG	82
Endoplasmic reticulum location				
Phosphatidate Phosphatase	PAP	HQ827794[b]	5′-GGACCTCATGCATATTTTCAGATG 5′-CCGATTGCTGCACATTAAGAGA	91
Acyl-CoA : Diacylglycerol Acyltransferase 1	DGAT1	EU391591	5′-TCGTGGGAGGTCGGATATTAA 5′-CCCTGAGCGCTCGATGAG	65
Acyl-CoA : Diacylglycerol Acyltransferase 2	DGAT2	HQ827795[b]	5′-TTGACCATATTCAAATCCAGAGAGAT 5′-GAAATGGATAGAGCCAAGCCATA	90
Oleosin 1	Ole1	EU234462	5′- GCACAAAGGAGCCCTAAAGGA 5′- GCAAAAGAGTGACAACCGCTAA	71
Oleosin 2	Ole2	EU234463	5′-ACAGCCACACCAAGTTCAAGTG 5′-ATGGACCTTTTTGTTGACCTTTG	73
Microsomal 3 Fatty Acid Desaturase	Mito 3	EU267121	5′-TTTTTGTAATGTGGCTGGACTTTG 5′-CCCGGTACCAGGGAAGTTTT	75
Phospholipid: Diacylglycerol Acyltransferase	PDAT	HQ827796[b]	5′-TGGAGTTCCCAAGTCATAGTTTGA 5′-AGCAATACATAACGACAGAGAAAGAAAT	93
Constitutive control gene				
Actin	Act	GQ256649	5′- TGGTTCCACTATGTTCCCTGGTA 5′- CTTCATGCTGCTTGGAGCAA	76

a putative assignments of the gene name and cellular activity.
b these genes were cloned first-time at our laboratory.

of total lipids after 24 DAP, and reaches its maximum (accounting for 26% of dry weight) at ca. 55 DAP.

3.2. *Expression profiles of lipid genes*

Using reverse transcript PCR, we firstly examined the general expression differentiations of 21 lipid genes among jatropha different tissues including leaves, root tips and developing seeds, and found that the all 21 genes were expressed within all tissues examined, though the expression levels of the genes DGAT1 and DGAT2 is relatively low in root tips (data not shown). Further we examined the expression profiles of the 21 lipid genes at the time-course of seed development by using the quantitative real-time PCR. Using the leaf expression data as a reference, we detected that all 21 lipid genes presented various temporal expression patterns during the time-course of seed development. The expression patterns of 21 lipid genes within developing seeds were summarized in Fig. 2. The

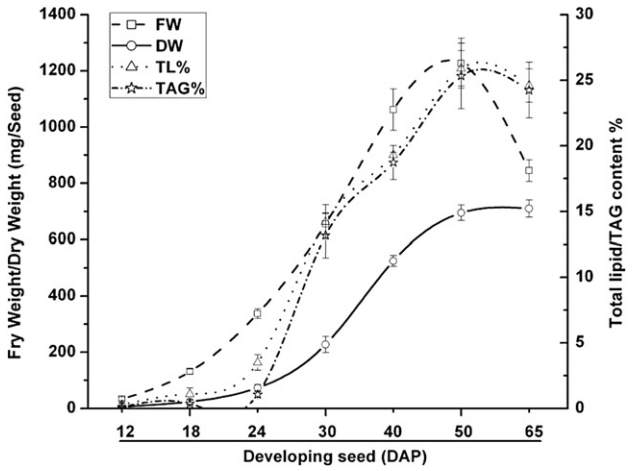

Fig. 1 – Changes in fresh weight, dry weight, total lipid content and TAG content during jatropha seed development (each data point represents the mean ± SD of five replicates).

17 genes were higher expressed within developing seeds, including cGPDH, DGAT1, heteroACC, HomoACC, KASI, KASII, KASIII, LPAT, Mitoω3, Ole1, Ole2, PAP, PDAT, PDCE1α, PDCE1β, PDCE2 and pGPDH than their expression level in leaf with the relative maximum expression ratio (seed vs leaf) from 1.7 to 239,910 folds (see Table 2). In particular, the expression levels of Ole1 and Ole2 were extremely higher within developing seeds than their expression in leaf. The four genes Chloω3, Chloω6, DGAT2 and pGPAT had a lower expression within developing seeds than their expression in leaf with the relative maximum expression ratio (seed vs leaf) from 0.2 to 0.4 (see Table 2). During developing seeds, the relative maximum expression ratio (seed vs seed) of 21 genes varied from 2.8 (pGPDH) to 1,919,280 (Ole1), suggesting the expression of these genes presented strong differentiation temporally at the time-course of seed development. The 13 genes including cGPDH, DGAT1, HomoACC, KASIII, Ole1, PDAT, PDCE2, KASI, LPAT, Ole2, PDCE1α, heteroACC and KASII were high and concentratively expressed at the middle-late stage of seed development (i.e. at 40 or 50 DAP), while their expression level was low at the early stage of seed development, i.e. before 30 DAP (see Fig. 2 A, D, G, J, M, P, S, H, K, N, Q and F). The genes Mitoω3 and PAP were high expressed at the early stage of seed development, i.e. before 24 DAP, while their expression level was low at middle-late stage, i.e. after 40 DAP (see Fig. 2 L and O). The genes PDCE1β and pGPDH were high expressed both at the early and late stages (see Fig. 2 R and U).

3.3. *Lipid gene clusters*

To examine relationships among the temporal expression patterns of the lipid genes, we performed clustering analysis and identified five groups with their own temporal patterns by using k-mean clustering method. The visualization results were described in Fig. 3. The genes located within a same cluster meant they had same or similar expression patterns during the development of seeds. On the whole, Cluster 3 (covering 13 genes) showed a major flat-rise pattern, together with Clusters 2 (including 2 genes) presented an up-regulated pattern; and Clusters 1 and 4 represented a down-regulated pattern, including 2 and 2 genes respectively, while Cluster 5 presented an S-shaped pattern, containing 2 genes.

4. Discussion

TAG usually is mostly accumulated within oleaginous seeds (specifically in endosperms or cotyledons) in plants. We characterized a time-course for seed development and lipid accumulation during jatropha seed development. The whole course of seed development from a female flower pollinated to a mature seed takes about 55 days. After ca.50 DAP jatropha seeds seem to be completely mature and begin desiccation. Our observation is consistent with previous studies [26,27]. During the first 24 DAP, the accumulation of dry material in developing seeds is limited, including small amounts of total lipids (less than 2.0% of dry weight). However, TAG was undetectable during this period, suggesting the composition of total lipids mainly be membrane lipids (such as glycosylglycerides or phosphoglycerides) which were associated with rapid cell growth, for fresh weight of seeds had started to rise rapidly since ca. 15 DAP. The small amounts of total lipids in dry materials, the lag of TAG accumulation comparing to the accumulation of other dry materials (such as proteins) at the early stage of developing seeds, and the rapid accumulation of TAG starting from 24 DAP to 52 DAP confirmed the general observation that biosynthesis of storage lipids usually occurred at the middle-late stage during the development of seeds [28].

Investigating the instantaneous expression profiles of genes associated lipid biosynthesis within the developing seeds contributes to understand the molecular basis of storage lipid accumulation in plant, though the biosynthesis of storage lipids is complex. Unsurprisingly, the all 21 lipid genes were expressed within different tissues examined in jatropha, since TAG usually could be found in all tissues in plant. Moreover, only two genes DGAT1 and DGAT2 are specifically associated with the accumulation of TAG [11,29]. Real-time quantitative PCR technology is one of the most sensitive and quantitative methods for measuring gene's expression at mRNA levels and has been widely used to examine the instantaneous expression of various genes in molecular biology [17,30]. We optimized all assay conditions of Real-time quantitative PCR during our experiments. Our results allowed simultaneous expression analyses of multiple genes and accurate comparisons of relative copy numbers among genes during seed development.

Within developing seeds the 21 lipid genes presented various temporal expression patterns with the relative maximum expression ratio (seed vs seed) ranging from 2.8 to 1,919,280 folds, suggesting their functional complex and specificity temporally at the time-course of seed development. Similarly, Chen et al. [17] reported 12 lipid genes which exhibited various temporal expression patterns at the different stages of developing seeds in castor bean. Among the 17 lipid genes highly expressed within developing seeds, there were 13 genes located in Clusters 2 or 3, including four genes (HomoACC, heteroACC, PDCE2 and PDCE1α) associated with *de novo* FA synthesis, three genes (KASI, KASII and KASIII) associated with

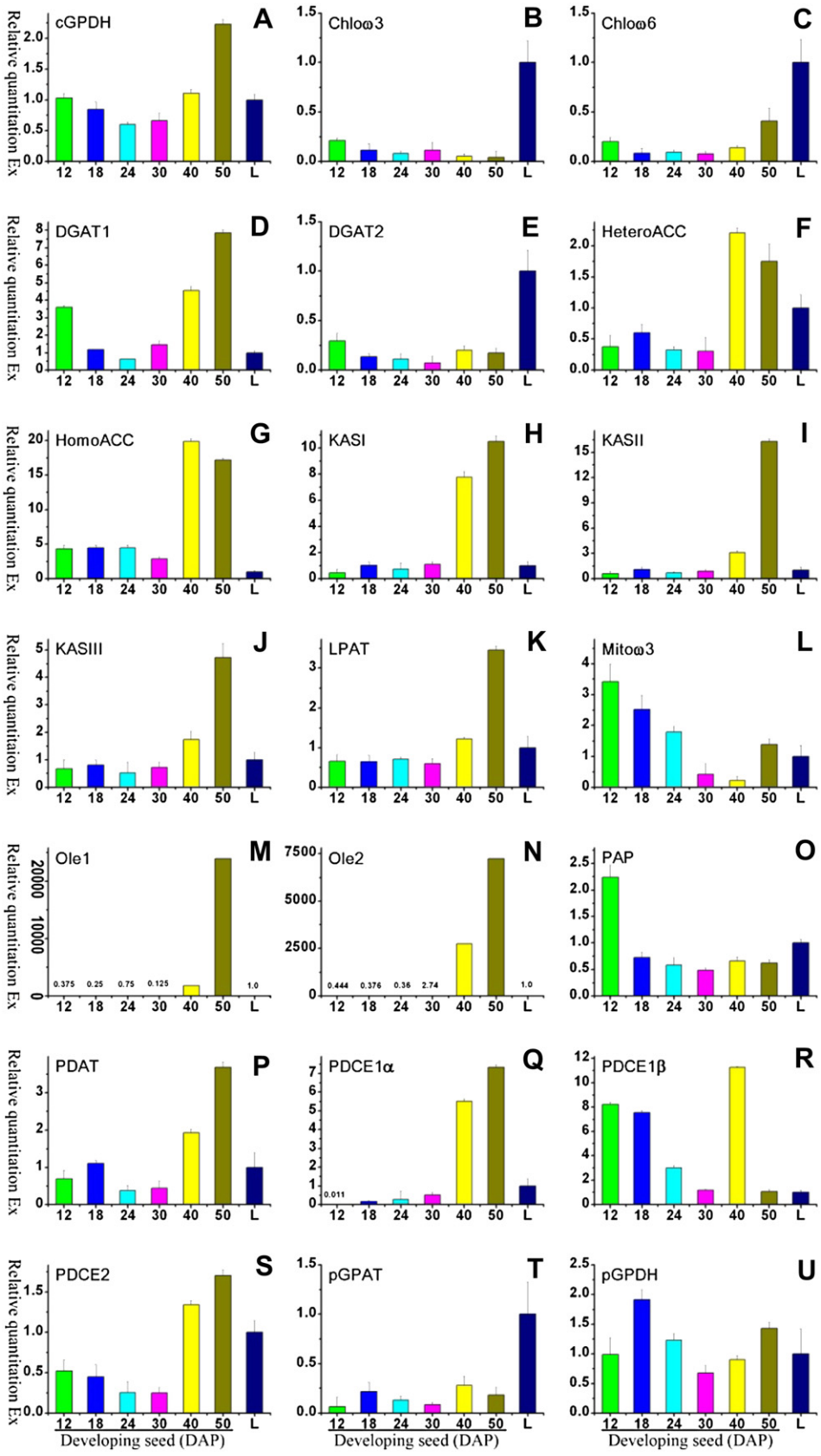

Fig. 2 – Expression pattern of lipid genes by real-time PCR in developing jatropha seeds and leaves. (abbreviated names for the genes are described in Table 1; each data point represents the mean ± SD of three replicates; values in graph indicate relative expression fold; DAP days after pollination; L denotes leaf tissue; Ex denotes expression fold).

Table 2 – Summary of transcript profiles of lipid genes.

Patterns	Gene[a]	Relative maximum expression ratio	
		Seed vs Seed[b]	Seed vs Leaf[c]
Cluster1	DGAT2	4	0.3
	PAP	4.6	2.2
Cluster2	DGAT1	12.8	7.8
	PDCE2	6.7	1.7
Cluster3	LPAT	5.8	3.5
	PDAT	9.9	3.7
	PDCE1α	667	7.3
	pPGAT	4.3	0.3
	HeteroACC	7.3	2.2
	HomoACC	6.9	20
	KASI	23	11
	KASII	27	16
	KASIII	9.2	4.7
	Ole1	1919280	239910
	Ole2	20047	7237
	Chloω6	5.3	0.4
	cGPDH	3.7	2.2
Cluster4	PDCE1β	10.6	11
	Chloω3	4.9	0.2
Cluster5	pGPDH	2.8	1.9
	Mitoω3	15.6	3.4

a abbreviated names for the genes are described in Table 1.
b Ratio of the maximum to minimum expression in seed.
c Ratio of the maximum expression in seed to the expression in leaf.

FA condensation in the late period of FA synthesis, three genes (DGAT2, LPAT and PDAT) associated with TAG assembly in Kennedy pathway, one genes cGPDH associated with the biosynthesis process of G-3-P which provides *de novo* substrate for TAG assembly in Kennedy pathway, and two gene (Ole1 and Ole2) specifically responsible for producing oleosin which packages oil body (see Fig. 3 and Table 2). These genes located in Clusters 2 and 3 presented similar temporal expression patterns and were highly and intently expressed at the middle-late stage of seed development (i.e. at 40 or 50 DAP), when TAG were rapidly accumulated during this period. This meant that the expression of these genes at the transcription level was closely correlated with TAG accumulation within jatropha developing seeds. Probably the high transcripts of these genes would be the mechanism for maintaining or meeting the growing demand of TAG synthesis in developing seeds. In Clusters 1 and 4, however, the two genes (including PDCE1β associated with *de novo* FA synthesis, and PAP associated with TAG assembly in Kennedy pathway) highly expressed in developing seeds exhibited a down-regulated expression pattern with development of seeds, i.e. they were mainly expressed at the early stage of seed development (before 24 DAP). The possible reasons included 1) there was a gene post-transcriptional regulation occurred, or 2) functionally their expression was not inevitably associated with TAG biosynthesis in jatropha developing seeds (probably, their function was associated with the biosynthesis of membrane lipids). In Cluster 5, the other two genes (including Mitoω3 associated with FA desaturation, and pGPDH associated with the biosynthesis of G-3-P) highly expressed in developing seeds exhibited an S-shaped expression pattern, suggesting their functional differentiation during lipid biosynthesis at different stages of seed development, i.e. their function might be associated with the biosynthesis of membrane lipids at the early-middle stage, and be switched to TAG biosynthesis at the late stage of seed development. In addition, the four genes (including Chloω3, Chloω6, DGAT2 and pGPAT) lowly expressed in developing seeds compared to their expression in leaf with the relative maximum expression ratio (seed vs leaf) from 0.2 to 0.4 (see Table 2). Probably, the transcript accumulation of these genes at the mRNA level has no necessarily links with TAG biosynthesis in jatropha developing seeds.

As mentioned above, among the 21 lipid genes examined, the genes DGAT1 and DGAT2 were specifically associated with biosynthesis of TAG. In the Kennedy pathway Diacylglycerol acyltransferase catalyzes the final step in TAG synthesis that acylates *sn*-1, 2- diacylglycerol (DAG) at the *sn*-3 position using an acyl-CoA substrate to form TAG [11]. Gene DGAT encoding Diacylglycerol acyltransferase had been proposed to be the rate-limiting enzyme in plant storage lipid accumulation [15,31–34]. Two heterogonous genes DGAT1 and DGAT2 were cloned in various plants such as *Arabidopsis* [35,36], tung tree [29], castor [37,38], soybean [39] *Tropaeolum* [40]. Current investigation showed that DGAT1 was high expressed within developing seeds and tightly associated with TAG accumulation at the time-course of developing seeds, suggesting DGAT1 be the major gene encoding Diacylglycerol acyltransferase and probably be one of rate-limiting factors for up-regulating TAG accumulation in jatropha seeds. Further investigating the over-expression of DGAT1 and its consequential influence on seed oil content by genetic engineering means is needed to determine whether DGAT1 is molecularly the main sensitive factor causing the unreliable oil content in jatropha developing seeds. Li et al. [41] examined the temporal expression patterns of genes DGAT1 and DGAT2 within developing seeds in *Arabidopsis*, soybean, *Vernonia*, *Stokesia*, *Euphorbia* and castor, and concluded DGAT2 preferentially incorporated unusual FA (such as ricinoleate, and Vernolic acid) into TAG, while DGAT1 was responsible for usual FA (such as oleate, palmitate) into TAG within oleaginous seeds. The TAG from jatropha seed oil comprised of usual FA and there was no production of the unusual FA [42], our results conformed Li et al.'s conclusion [41]. For all plants examined, DGAT1 appears to be a major gene for the accumulation of TAG in oleaginous seeds. However, function of DGAT2 appears to be differential in plants. DGAT2 was mainly expressed in jatropha leaf and poorly expressed in root tips and developing seeds. The function of DGAT2 appears to be redundant in jatropha developing seeds. However, the main function of DGAT2 in these plants which does not produce unusual FA is still unknown.

Plant storage lipids are usually stored in a specific organelle oil body after TAG is biosynthesized and detached from ER in oleaginous tissues particularly such as endosperm or cotyledon. The oleosin, a plant-specific structural oil-body-membrane-protein on oil bodies, has an important role in the formation of oil bodies and regulates the size of oil bodies in cells [43–47]. Studies had showed that oleosin accumulation in developing seeds such as rapeseed, sunflower and coriander could be detected at the early stage of seed development, but it only reached a maximum at the later developmental stages that coincide with seed dehydration [46]. The peak of oleosin

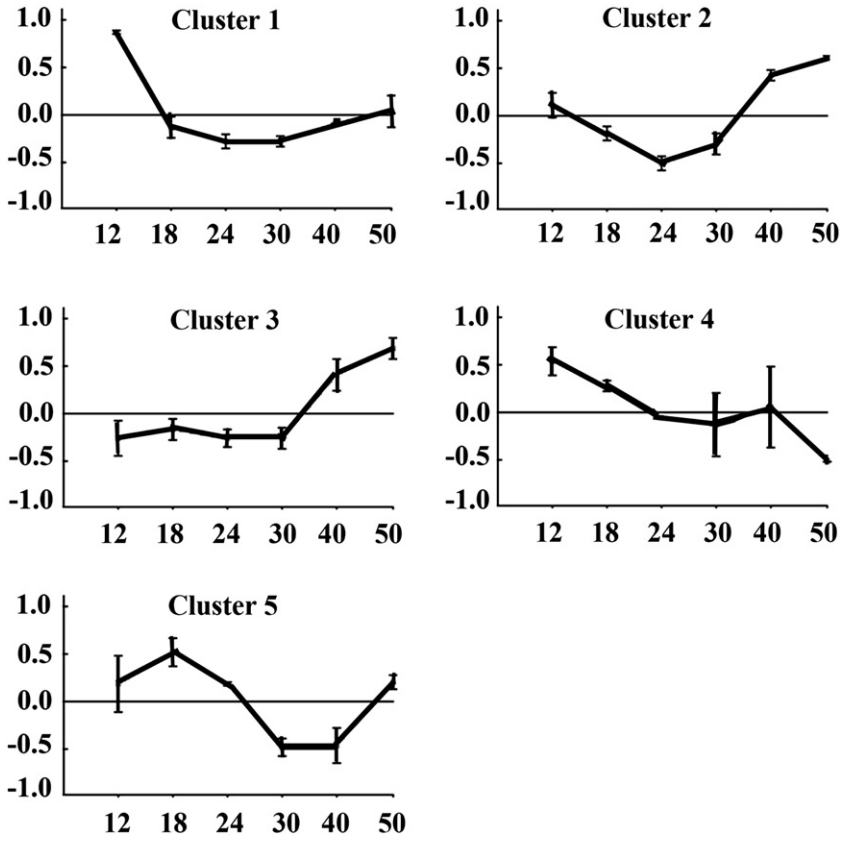

Fig. 3 – Expression patterns of lipid gene clusters during seed development (each cluster is represented by the mean expression pattern over all the genes assigned to it; Error bars denote ±SD; DAP days after pollination).

accumulation occurred somewhat later than that of storage lipid, indicating that the two processes of oleosin and TAG accumulation were not necessarily linked. Oleosins appear to be present in all desiccation-tolerant seeds, while they are absent in recalcitrant seeds [48]. Thus the physiological role of oleosins in seeds was thought to be closely associated with the nature of seed desiccation tolerance. In contrast, oleosin accumulation in developing maize embryos was closely correlated with TAG biosynthesis [44]. Moreover, expression of oleosin genes was strongly up-regulated and tightly correlated with TAG biosynthesis in developing seeds of sesame and castor [17,49], suggesting that the two processes of oleosin and TAG accumulation were closely linked. In most plants oleosins are encoded by multi-gene families and some of them are co-expressed [50]. Our current studies showed that genes Ole1 and Ole2 concentatively and strongly co-expressed at the late stage of seed development (i.e. at the 50 DAP) with the relative maximum expression ratio (seed vs seed) of 1,919,280 and 20,047 folds, respectively (see Table 2 and in Fig. 2 J, K). At this period, seeds had started to dehydrate (see Fig. 1). Meanwhile, the overall expression profile of gene Ole1 and Ole2 at the time-course of seed development presented an up-regulated pattern, correlating with TAG accumulation. These observations meant that the high transcript accumulation of oleosin genes at the mRNA level were associated with both TAG biosynthesis and seed desiccation tolerance in jatropha seeds. Correspondingly, the physiological roles of oleosins in developing seeds may participate in both the regulation of TAG biosynthesis and the evolving of seed desiccation tolerance in jatropha.

In short, our current investigation provided not only the initial information on promoter activity for 21 lipid genes, but also a first glimpse of the global patterns of gene expression at the time-course of seed development in jatropha. These results would be critical to understand the molecular basis of lipid biosyntheses and identify the rate-limiting enzyme genes which might regulate storage lipid biosyntheses during seed development and give rise to the unreliability of oil content in jatropha seeds. Further studies on investigating the function of these genes and their consequential influence on seed oil content by genetic engineering means are needed to create the improved germplasm and to serve as exploitation and utilization of biodiesel.

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