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Proteomic evaluation of free fatty acid biosynthesis in *Jatropha curcas* L. (physic nut) kernel development

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***Jatropha curcas* L. is one of the economic crops that are cultivated for biodiesel production. Here, the fatty acid and protein profiles of *J. curcas* kernels were evaluated during their development. The fruits were divided into eight developmental stages (stages I to VIII) based on their age and morphology. The fatty acid content was analyzed at each stage using gas chromatography after conversion to methyl esters. Fatty acid levels were found to differ between all eight developmental stages, although the major fatty acid in each stage was oleic acid followed by linoleic, palmitic and stearic acids, respectively, except in stage I where linoleic acid was more common than oleic acid. All fatty acids showed a maximum content at stage III, a rapid decline at stage IV and another peak at stage VII before declining. Significant changes were found in the relative abundance of 22 proteins during seed development, of which the expression levels for transcripts encoding for four of these proteins, acetyl CoA carboxylase, phosphoenolpyruvate carboxylase, mercaptopyruvate sulfurtransferase and 4-coumarate: coenzyme A ligase, as evaluated by quantitative RT-PCR, were altered between the developmental stages of the kernels in a broadly similar pattern as the level of most fatty acids.**

Key words: *Jatropha curcas* L., FAME, ACCase, PEPC, MST, 4CL, quantitative real time PCR.

INTRODUCTION

Jatropha curcas L. (Malpighiales: Euphorbiaceae) is cultivated for harvesting the unique oil contained in its seeds that can be used as raw material for the production of biodiesel. *J. curcas* is currently one of the most promoted oilseed crops since its seeds have a high oil content of up to 50% (Vollmann and Laimer, 2010), with low pest and disease levels and produces a high yield, from 540 to 680 L/ha on poor soil upwards. Moreover, since it can be grown on soils that other crop plants cannot and is itself non edible, it does not conflict ethically or economically with food crop production. Its major fatty acids are oleic acid (34.3 to 45.8%; 18:1), linoleic acid (29.0 to 44.2%; 18:2), palmitic acid (14.1 to 15.3%; 16:0) and stearic acid (3.7 to 9.8%; 18:0) (Gubitz et al., 1999), which are suitable for biodiesel production. Proteomic analysis, which involves the identification and characterization of expressed proteins, has become a powerful tool for determining the potential biological roles and functions of individual proteins. Liu et al. (2009) compared the embryo and endosperm proteomes from the seeds of *J. curcas* and found that the proteins in the endosperm were catabolism-related enzymes to utilize the stored reserves to

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provide the nutrition for seed germination, whilst the proteins in the embryo were inclined towards anabolism for utilizing the nutrition from the endosperm to develop the new life. Yang et al. (2009) investigated oil mobilization during and post seed germination in the development of *J. curcas* using a proteomic analysis, and revealed that several pathways, including the beta oxidation, glycoxylate cycle, glycolysis, citric acid cycle, gluconeogenesis and pentose phosphate pathways, were likely to be involved in oil mobilization. Although *J. curcas* seeds accumulate very high levels of fatty acids in the kernel, most research has so far focused on a single or a few genes, and so our understanding of the changes during seed development and the elucidation of the relationship between the mechanisms of seed development and fatty acid accumulation are far from complete.

Because of recent advances in the accuracy and sensitivity of mass spectrometry (MS), and the high reproducibility of liquid chromatography (LC) separation and simple prefractionation of complex proteins by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), the label-free SDS-PAGE-LC-MS approach has been established as a method for directly identifying and quantifying thousands of proteins between two or more (control and study case) groups in a complex sample without any labeling or biochemical modifications (Rezaul et al., 2005). Despite limited genome resources, proteomics research on selected oilseed crops has shown that global analyses of proteins is currently possible, especially in *J. curcas* (Yang et al., 2009; Liu et al., 2009). In this study, a proteomic approach, in combination with SDS-PAGE and LC-MS/MS analysis for a metabolomic approach, was undertaken to potentially accelerate the understanding of the biosynthesis, breakdown and accumulation of oil and their related mechanism(s) in *J. curcas* seeds.

MATERIALS AND METHODS

Plant material preparation

Eight potentially partially overlapping developmental stages of *J. curcas* fruits were defined by the age (in days since fertilization) and morphological appearance of the fruits (Table 1). Fruits were gifted from the Biofuels by Biocatalysts Research Unit in the Faculty of Sciences, Chulalongkorn University, Bangkok, Thailand. After collection, all fruits were stored at -80°C until used.

Fatty acid composition analysis

The seeds from the eight developmental stages of *J. curcas* fruits were cracked and the seed coats were carefully removed. The white seed kernels were collected and milled using a laboratory mortar and pestle and then suspended in 500 µL of hexane / 500 mg ground nut kernel with agitation by vortex overnight to extract the *J. curcas* oil. The extract was clarified of ground kernel by centrifugation (10,000 x *g*, 25°C, 10 min), the supernatant was harvested and the hexane was then removed from the extracted oil by incubating in a desiccator at room temperature for 6 to 8 h. The methyl ester derivatives of the fatty acids (FAME) present in the oil were prepared by treating 1 g of oil with 10 ml of 0.5 M sodium methoxide (in methanol), whereupon two phases formed. Complete separation of the bottom glycerol phase (triglycerides) from the upper FAME phase was obtained by centrifugation at 10,000 x *g* for 10 min and the FAME phase was then collected. The FAME composition of the oil; and from that the deduced fatty acid composition in the *J. curcas* kernel was determined using gas chromatography (GC) with a GC-2010A (Shimadzu, Japan) fitted with a capillary column (Omegawax^{TM} 320, 30 m length × 0.32 mm inner diameter × 0.25 µm film thickness, Sigma-Aldrich, USA) and a flame-ionization detector. The total analytical time was 12 min. From this, the relative fatty acid compositions were calculated from three independent biological replicates, and expressed as the (w/w) percentage of each fatty acid present in the total measured fatty acid content.

Total protein extraction

The *J. curcas* seed kernel tissues from the eight developmental stages were ground into a fine powder in liquid nitrogen with a precooled mortar and pestle. About 250 mg of each ground sample was dissolved / suspended in 1 ml of 0.1% (w/v) SDS. Each protein mixture was then precipitated with 4 volumes of cold acetone overnight at -20°C followed by centrifugation (10,000 x *g*, 15 min at 4°C). After removal of the supernatant, the protein pellet was resuspended in 0.15% (w/v) sodium deoxycholic acid and the protein concentration of each sample was determined by the Lowry method using bovine serum albumin (BSA) as a protein standard.

SDS-PAGE analysis

Each 10 µg protein sample, and the protein standard marker (Low Molecular Weight SDS Marker Kit, GE Healthcare Bio-Sciences AB, Sweden), were mixed with loading buffer [0.125 M Tris-HCl pH 6.8, 20% (v/v) glycerol, 4% (w/v) SDS, 0.2 M dithiotreitol (DTT) and 0.02% (w/v) bromophenol blue] and heated at 95°C for 10 min before loading onto and resolved through a 9-cm long 15% (w/v) acrylamide resolving SDS-PAGE. Gel electrophoresis was carried out at 30 V for the stacking gel and 50 V for the separating gel (8 cm wide × 1 mm thick gels) using an electrophoresis power supply (Electrophoresis Power Supply, GE Healthcare Bio-Sciences AB, Sweden). After electrophoresis, gels were silver stained according to the method of Blum et al. (1987).

Protein identification

Protein bands from the silver stained SDS-PAGE gel were excised manually according to the molecular mass range. Each selected protein band was cut from the gel and then the slice was cut into small pieces, dehydrated by immersion in excess 100% (v/v) acetonitrile for 5 min, reduced by immersion in excess 10 mM DTT in 10 mM ammonium bicarbonate solution at room temperature for 1 h and then alkylated at room temperature for 1 h in the dark in the presence of excess 100 mM iodoacetamide in 10 mM ammonium bicarbonate solution. After alkylation, the gel pieces were dehydrated and digested by trypsin solution (10 ng/µL in 50% (v/v) acetronitrile/10 mM ammonium bicarbonate) at room temperature for 3 h. After digestion, the peptides were extracted from gel plugs by immersion in 0.1% (v/v) formic acid in 50% (v/v) acetonitrile. The pooled peptide extracts were then injected into an Ultimate 3000 LC system (Dionex) coupled to ESI-Ion Trap MS (HCT ultra PTM Discovery System, Bruker Daltonik) with electrospray at a flow rate of 20 µL/min to the µ-precolumn (Monolithic Trap Column, 200 µm i.d. × 5 cm). The sample was separated on a nano column (Monolithic Nano Column, 100 µm i.d. × 5 cm) at a flow rate of 1 µL/min. The solvent gradient [solvent A: 0.1% (v/v) aqueous formic acid; solvent B: 49.95% (v/v) H_2O, 49.95% (v/v) acetonitrile, 0.1% (v/v) formic acid] was run from 10% (v/v) B to 70% (v/v) B from 0 to 13 min, and then at 90% (v/v) B from 13 to 15 min and 100% (v/v) B at

Table 1. Characteristics of the eight developmental stages of *J. curcas* fruits.

Developmental stage	Age[a]	Fruit color	Seed color	Fruit stalk color	Fruit texture
I 	14	Green	Brown	Green	Hard
II 	19	Green	Black	Green	Hard
III 	24	Greenish-yellow	Black	Green	Hard
IV 	29	Yellow	Black	Green	Hard
V 	34	Yellowish-black	Black	Green	Hard
VI 	39	Blackish-yellow	Black	Green	Soft
VII 	44	Black	Black	Green	Soft
VIII 	50 up	Black	Black	Black	Hard, dry

[a]Age in days post-fertilization.

Table 2. PCR primers, annealing temperature and expected amplicon sizes.

Gene	Primers (5' to 3')	Ta (°C)[a]	Expected size (bp)[b]
Ubiquitin	F: CACCAAGCCAAAGAAGATCAAGCAC R: GGTTGGCCATGAAAGTTCCAGC	60	148
ACCase	F: GAGACACTGGAAGGCTGGGC R: GCCAGCCTGAGGAACAACCC	60	162
PEPC	F: CCCTGGATCTTCGCATGGAC R: GGCAAACACCATCTCAACCAAGTC	60	157
MST	F: TCTGCTCTTGGAATTGAGAACA R: TGCACTAGATTCAACATCATATCC	60	174
4CL	F: GGATGACAGAAGCAGGGCCA	60	170

15 to 20 min.

The MS/MS spectra were analyzed by the DeCyder MS 2.0 differential analysis software (GE Healthcare) and then database searched against the NCBI nr database using the Mascot software (Matrix Science, London, UK) (Perkins et al., 1999). Viridiplantae (Green plants) was chosen and the conditions were fixed at carbamidomethyl (C) and oxidation (M), while peptide and MS/MS tolerances were 1.2 Da each. All proteins were functionality identified by GoCat (http://eagl.unige.ch/GoCat).

Total RNA isolation and cDNA synthesis

Total RNA was extracted from *J. curcas* kernels according to the modified method of Yu and Goh (2000). The homogenate was incubated in a water bath at 60°C for 15 min with occasional shaking and then centrifuged at 7,500 x *g* for 15 min at 4°C. After centrifugation, the aqueous phase was extracted at least twice with an equal volume of chloroform: isoamyl alcohol [24:1 (v/v)]. The RNA pellet was resuspended in 900 µL of diethylpyrocarbonate (DEPC)-treated water, precipitated and kept overnight at -20°C. After centrifugation (as aforementioned), the pellet was washed with 2.5 M lithium chloride and washed again with 70% (v/v) ethanol, dried and dissolved in DEPC-treated water. The RNA purity and concentration were determined by nanodrop (NanoDrop ND-1000 Spectrophotometer, USA). The integrity of RNA was evaluated by denaturing gel electrophoresis based separation and visualized by uv-transillumination after ethidium bromide staining (2 µg / ml for 20 min). The aforementioned total RNA extract was used as the template for the synthesis of first-strand cDNA using the *RevertAid*™ First Strand cDNA Synthesis Kit (Fermentas), as per the manufacturers' protocol. In brief, 1 g of total RNA, 0.5 µg oligo $(dT)_{12-18}$ and sterile distilled water were incubated at 70°C for 5 min and quick chilled on ice. Then, 4 µL of 5X reaction buffer, 2 µL of dNTPs mixture, and 1 µL of RNase inhibitor were added.

The mixture was then mixed gently and incubated at 37°C for 5 min. After adding 1 µL of RevertAID RT followed by incubation at 42°C for 60 min, the reaction was stopped by heating at 70°C for 10 min and chilled on ice. The cDNA was used as a template for amplification by PCR.

Real-time PCR analysis

The DNA sequences for ubiquitin, acetyl CoA carboxylase (ACCase), phosphoenolpyruvate carboxylase (PEPC), mercaptopyruvate sulfurtransferase (MST) and 4-coumarate: coenzyme A ligase (4CL) of *J. curcas* were obtained from the GenBank database (Accession numbers gi256427002, gi572606, gi2145479, gi224579301 and gi116055441, respectively). Gene-specific primers (Table 2) were designed manually and the sequence similarity was checked against the GenBank database using BLASTn (Altschul et al., 1990) to confirm their potential gene-specificity. The first stage reverse transcription (RT) reaction was carried out with the *RevertAid*™ First Strand cDNA Synthesis Kits (Fermentas), according to the vendor's suggested protocol. The second stage quantification of specific cDNA levels was then evaluated by quantitative real time PCR (qrt-PCR) using the iCycler IQ™ Real-Time PCR Detection System (Bio-Rad, CA, USA) in a total reaction volume of 10 µL containing a final concentration of 100 mM KCl, 40 mM Tris-HCl, pH 8.4, 0.4 mM of each dNTPs, 50 U/mL iTaq DNA polymerase, 6 mM $MgCl_2$, 20 nM SYBR Green I, and stabilizers, 50 ng of cDNA from the first stage RT step above and 10 pmol of each specific primer (iQ™ SYBR® Green Supermix).

The thermal cycling was performed at 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, and annealing (Table 2) for 30 s and 72°C for 30 s.

Relative standard curves and melting curve analysis

For construction of the standard curve for the qrt-PCR of each gene, the PCR product of the target genes and ubiquitin were amplified, whereas recombinant plasmids were used as the templates for construction of the standard curve. Plasmid DNA templates harboring the same gene fragment as the target amplicon of interest and that for ubiquitin as a housekeeping reference gene for cDNA loading standardization were diluted in the 10^1 to 10^9 copy number range and used as templates. Then, qrt-PCR on the cDNA template was performed and each standard point was run in triplicate. A graph of the cycle threshold and total copy number was constructed and used as the standard curve to calculate the copy number of each target gene.

RESULTS

Oil content and fatty acid profiles

Oil content and fatty acid profiles of *J. curcas* seeds were found to vary among the eight developmental stages (Figure 1 and Table 3). The net average oil content gradually but markedly (6.6-fold) increased in the seeds as

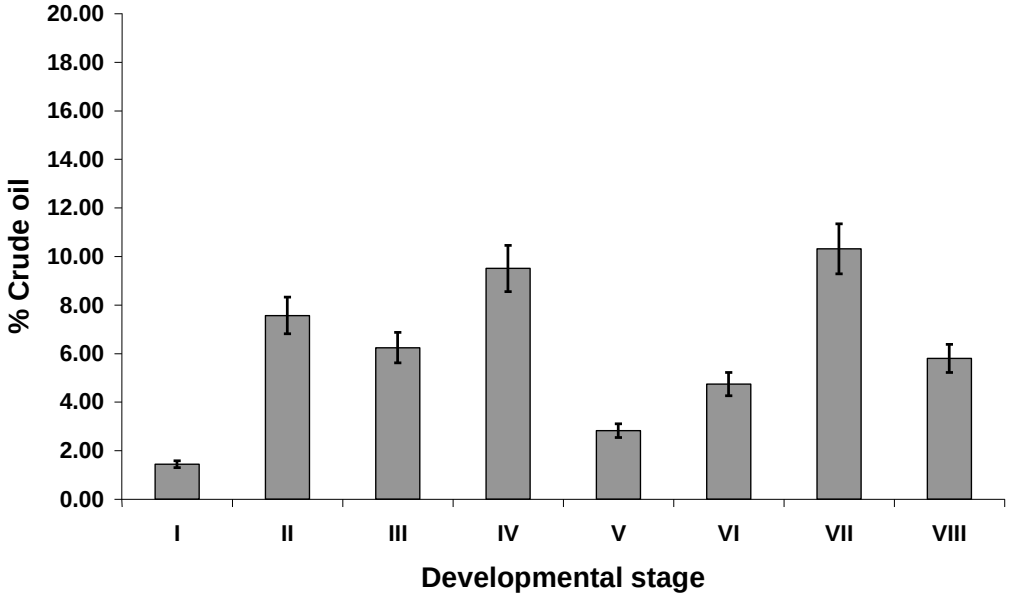

Figure 1. Changes in the average net level of crude oil [% (w/w) of the kernel] in the kernels from the eight different developmental stages of *J. curcas* fruits. Data are shown as the mean ± 1 S.D. and are derived from three independent samples. Means with a different lowercase letter are significantly different ($p < 0.05$; Mann-Whitney U tests).

as they developed from stage I (1.44 wt. %) to IV (9.50 wt. %), decreased in stage V (2.82 wt. %) and then increased again in stage VI (4.74 wt. %) up to 10.32 wt. % in stage VII before finally decreasing again in stage VIII to 5.80 wt. % (Figure 1). The fatty acid profiles, as monitored by GC analysis of the FAME derivatives are shown in Table 3. In total, 12 different fatty acids were detected (as FAME esters), but the major fatty acids were oleic acid followed by linoleic, palmitic and stearic acids, except for stage I kernels where linoleic acid was more common than oleic acid. Vaccinic acid and eicosapentaenoic acid were found only in stage III kernels, and docoshexaenoic acid only in stages IV and VI, but all three fatty acids were only found in these developmental stages at a very low level. Overall, each fatty acid level typically increased from developmental stages I to III, then decreased sharply in stage IV before increasing and decreesing slightly in stage V (exception is linoleic acid) and VI (exception is linoleic acid), respectively, and then increasing sharply in stage VII (except for palmitic, palmitoleic, linolenic and arachidic acids, which increased less dramatically) before declining slightly again at stage VIII. Thus, the highest concentration of fatty acids were found in kernels from developmental stages III and VII, and the lowest in stages I and VIII.

SDS-PAGE and protein profile analyses

The protein samples from the eight different seed developmental stages were analyzed by SDS-PAGE [15% (w/v) acrylamide resolving gel], and visualized by silver staining. The intensity of many kernel protein bands within a range of 20.1 to 30 kDa increased (up-regulated) while some protein bands with a molecular weight lower than 14.4 kDa decreased in intensity (down-regulated) during development of *J. curcas* fruits (Figure 2). From the protein expression profile throughout the eight stages of seed development (data not shown), a total of 20 to 22 putatively identified proteins (depending upon if the acetyl CoA carboxylase is one protein or three different isoforms) (Table 4) that are likely to be related to fatty acid biosynthesis were found to significantly change in their expression profile between the different developmental stages. Four of these 20 to 22 potential proteins have been reported to play a major role in fatty acid biosynthesis in other organisms, that is ACCase, PEPC, MST and 4CL, and so were selected for monitoring their respective transcript levels.

Real time PCR analysis

The level of the mRNA coding for ACCase, PEPC, MST and 4CL in the kernels were relatively low compared to that for ubiquitin, but during seed development they were upregulated and reached a maximum expression level at stage II (MST; 1.35-fold increase) or stage III (2.82-, 2.0- and 1.6-fold for ACCase, PEPC and 4CL, respectively), followed by a dramatic decrease to almost no detectable expression level for all four gene transcript levels at stage IV (Figure 3). These four transcripts then showed a significant increase in their expression level at stage V to almost that seen at stage II (MST) or III (ACCase,

Table 3. Fatty acid composition in the eight developmental stages of *J. curcas* kernels. The data (µg/ml FAME) are shown as the average ± 1 S.D. and are derived from three repeats. Means within a row followed by a different letter are significantly different (P<0.01; Duncan's MMT).

Fatty acid	I	II	III	IV	V	VI	VII	VIII
Myristate	8.17±0.16	12.55 ± 0.07	24.62 ± 0.14	11.12 ± 0.05	12.47 ± 0.07	9.32 ± 0.17	17.03 ± 0.01	8.63 ± 0.02
Palmitate	2042.7 ± 35.8	3762.8 ± 21.9	7773.4 ± 50.6	3465.9 ± 31. 5	3886.2 ± 23.9	3492.6 ± 33.6	5356.2 ± 4.7	2269.5 ± 16.9
Palmitoleate	143.1 ± 2.5	214.4 ± 1.1	285.9 ± 5.4	179.3 ± 1.20	197.1 ± 1.20	191.2 ± 1.9	241.5 ± 0.2	145.0 ± 1.2
Stearate	666.4 ± 10.9	2079.0 ± 12.8	5052.0 ± 28.3	1817.9 ± 16.4	2450.3 ± 16.0	1918.8 ± 19.0	3295.0 ± 4.4	1115.4 ± 7.70
Oleate	3456.7 ± 61.8	12425.0 ± 72.0	27338.5 ± 197.5	12514.6 ± 143.7	16447.2 ± 101.6	12666.2 ± 120.3	22152.6 ± 21.1	7821.9 ± 72.9
Vaccenate	0.00	0.00	23.4 ± 0.8	0.00	0.00	0.00	0.00	0.00
Linoleate	4135.7 ± 72.4	7765.2 ± 44.8	12709.3 ± 79.5	7495.2 ± 84.2	6690.8 ± 40.7	6224.0 ± 60.3	11818.6 ± 27.0	4319.5 ± 41.7
Linolenate	44.33 ± 0.90	75.6 ± 0.4	143.1 ± 0.9	51.15 ± 0.19	64.1 ± 0.4	64.4 ± 0.64	80.47 ± 0.17	43.77 ± 0.19
Arachidate	22.48 ± 0.44	60.7 ± 0.4	151.6 ± 0.9	48.97 ± 0.31	72.8 ± 0.5	61.5 ± 0.59	96.90 ± 0.19	19.12 ± 0.34
11-Eicosenoate	0.00	8.75 ± 0.15	35.18 ± 0.36	11.60 ± 0.21	12.5 ± 0.22	9.78 ± 0.18	23.65 ± 0.05	0.00
Eicosapentaenoate	0.00	0.00	13.75 ± 0.24	0.00	0.00	0.00	0.00	0.00
Docosahexaenoate	0.00	0.00	0.00	7.62 ± 0.26	0.00	18.63 ± 0.64	0.00	0.00

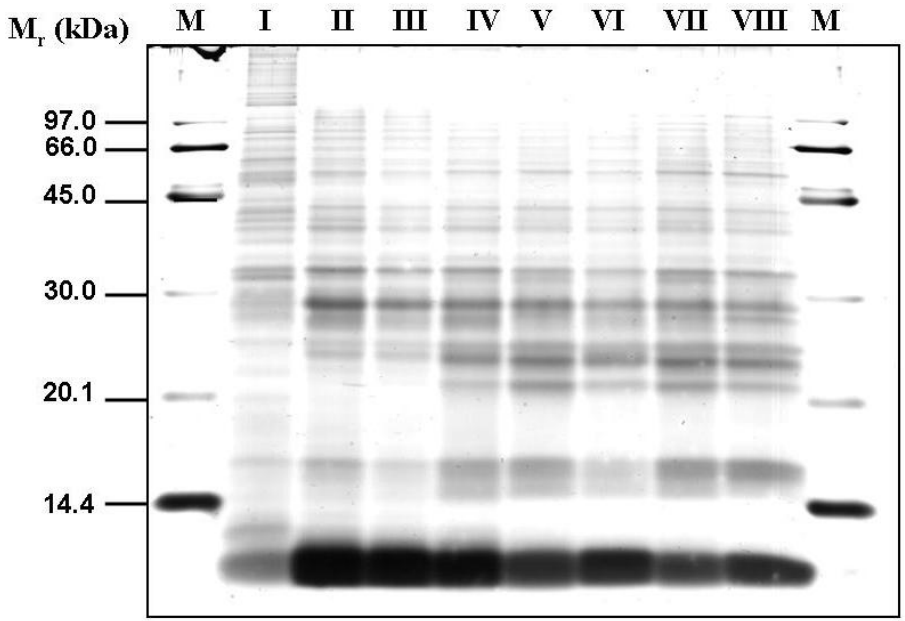

Figure 2. SDS-PAGE [15% (w/v) acrylamide resolving gel] resolved protein samples (10 g per lane) for the eight developmental stages of *J. curcas* seeds.

Table 4. Likely proteins with altered expression profiles during *J. curcas* kernel development that are related to fatty acid biosynthesis.

Accession number	Name of identified protein	Organism	MOWSE score	Peptide sequence
gi|162949342	4-Coumarate:coenzyme A ligase 1	*Physcomitrella patens* subsp. *californica*	12	KKSLQSEVRG
gi|11181943	Acetyl CoA carboxylase pRS1	*Brassica napus*	18	RKLLIVANDVTFKA
gi|572606	Acetyl-CoA carboxylase	*Brassica napus*	18	RKIIIVANDVTFKA
gi|11869927	Acetyl-CoA carboxylase 1	*Arabidopsis thaliana*	18	RKLLVIANDVTFKA
gi|116060125	Animal-type fatty acid synthase	*Ostreococcus tauri*	13	RTSGCEKG
gi|237682390	Branched-chain aminotransferase 4	*Brassica rapa*	11	KKWIPPPGRG
gi|108706991	Branched-chain-amino-acid aminotransferase 5	*Oryza sativa*	15	KAQMDAKSRG
gi|56606540	Dormancy-associated protein	*Codonopsis lanceolata*	16	KVVTGEGSGGKF
gi|15231611	Glycosyl hydrolase family 38 protein	*Arabidopsis thaliana*	14	KRGEAVDAEKL
gi|25992001	Lecithine cholesterol acyltransferase	*Medicago truncatula*	10	KLTFETAFKL
gi|116061081	Lipase	*Ostreococcus tauri*	25	RVDAVTFGQPRV
gi|224579301	Mercaptopyruvate sulfurtransferase	*Solanum lycopersicum*	10	KLVASVTCRM
gi|2145479	Phosphoenolpyruvate carboxylase	*Vanilla aphylla*	21	RKSAAELVKL
gi|54291440	Pr1-like protein	*Oryza sativa*	13	RSGAVDGVGGNTAKR
gi|116000579	Mitochondrial carrier protein	*Ostreococcus tauri*	17	KEAGVDLKSCRR
gi|116055441	Short chain alcohol dehydrogenase	*Ostreococcus tauri*	16	R.AVVDRT
gi|21553535	Ras-GTPase-activating protein	*Arabidopsis thaliana*	14	RGGGSS
gi|38228683	Receptor-like protein kinase	*Fagus sylvatica*	25	KGLNAKI
gi|255080050	Serine acetyl transferase	*Micromonas sp. RCC299*	17	RDHSLVKK
gi|27545461	S-related kinase 10.1A	*Arabidopsis lyrata*	11	KQLWYTPKD
gi|255547946	Steroid dehydrogenase	*Ricinus communis*	11	KKSQLQASQG
gi|2586125	□-Keto acyl reductase	*Arabidopsis thaliana*	12	KDVSDSIRS

PEPC and 4CL) before declining to a very low expression level by stage VII and essentially no expression by stage VIII. In terms of the expression levels, ACCase was by far the most significantly upregulated and abundant transcript, followed by 4CL and MST, with PEPC showing only a low expression level and a weak upregula-tion (Figure 3). The reactions catalyzed by these four enzymes in their respective pathways for fatty acid metabolism are shown in Figure 4.

DISCUSSION

Annarao et al. (2008) reported that triacylglycerol was the major lipid species in *J. curcas* seeds, while the level of fatty acids, sterol and moisture were low (Annarao et al., 2008). Because seed development may delay or interfere with the metabolism of triacylglycerol, fatty acids and sterols resulting in changes in the content of all lipids in the crude oil, then the level of fatty acids was not associated with crude oil content in Jatropha oil during seed development. In the *J. curcas* endosperm cell (kernel) crude oil, 12 fatty acids (myristic, palmitic, palmitoleic, stearic, oleic, vaccinic, linoleic, linolenic, arachidic, 11-eicosenoic, eicosapentaenoic and docosahexaenoic acids) were found, and it had a high free fatty acid content similar to that reported by Berchmans and Hirata (2008). The level of each fatty acid in the kernel varied significantly with respect to the developmental

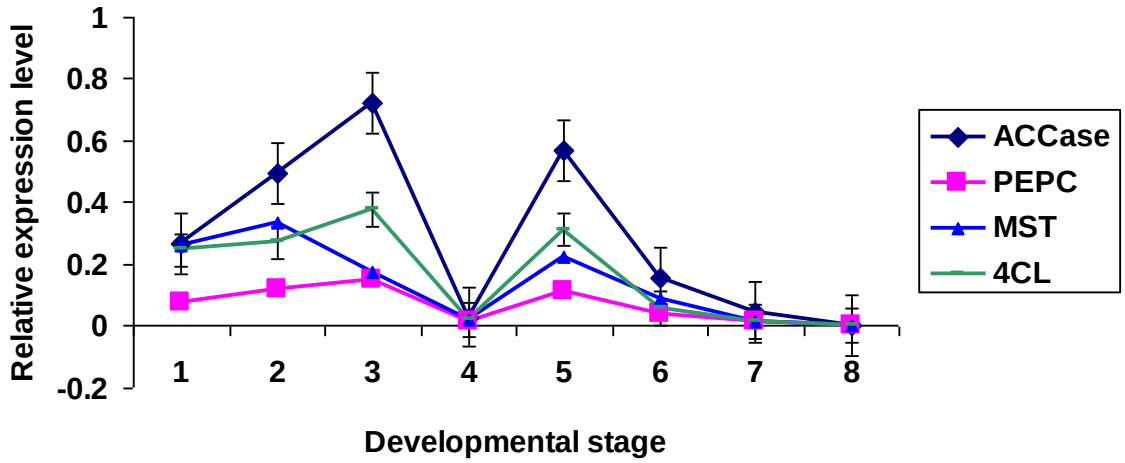

Figure 3. Transcript expression levels of the indicated genes in each of the eight developmental stages of *J. curcas* kernels, as measured using qrt-RT-PCR. The level of gene transcript expression was calculated after normalizing against the housekeeping ubiquitin transcript level in each sample, and so is presented as relative mRNA expression levels. Values are shown as the mean ± 1 S.D. and are derived from three repeats.

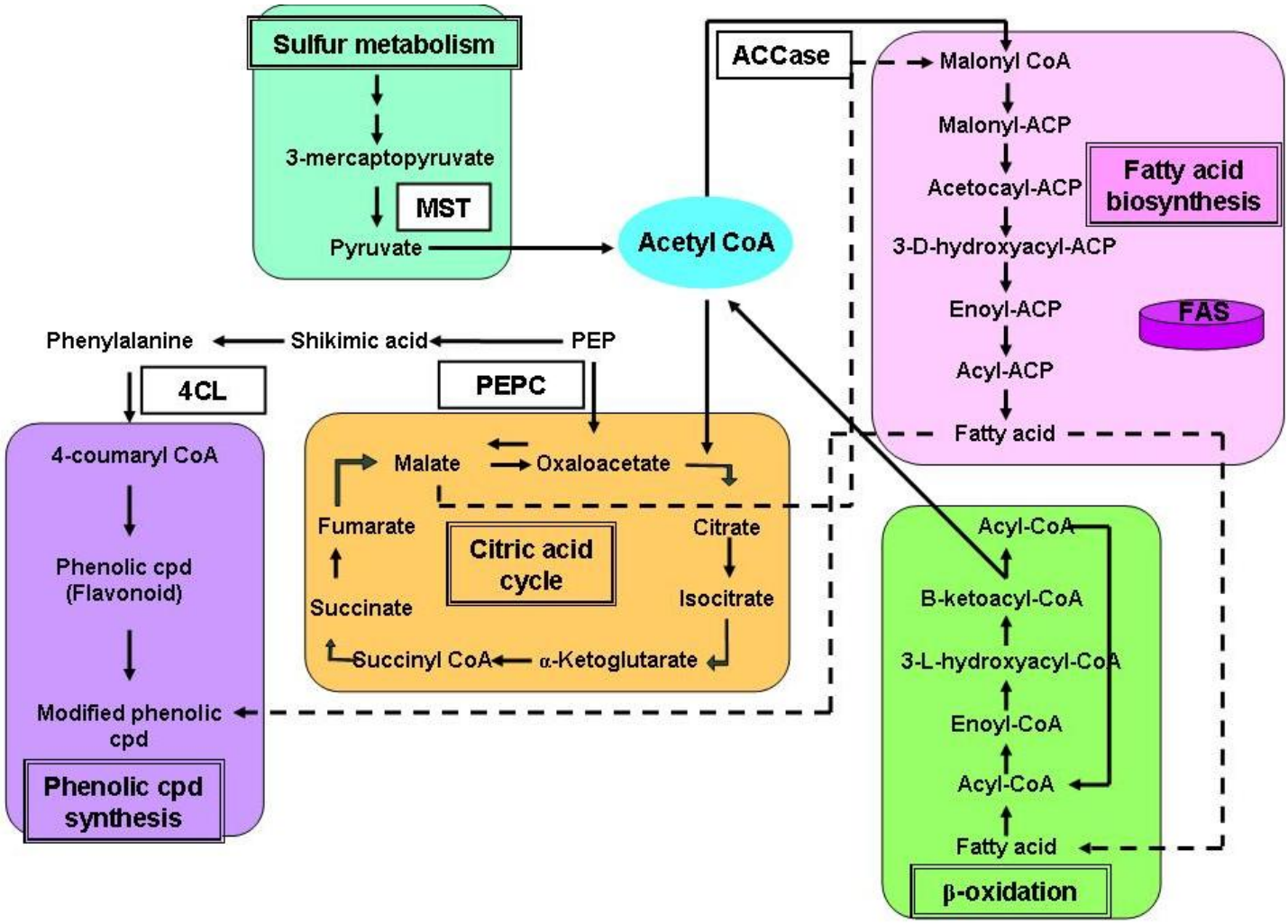

Figure 4. The proposed metabolic pathways of fatty acid biosynthesis and other related metabolism in *J. curcas* kernels showing the role of the ACCase, MST, PEPC and 4CL enzymes.

stage (age) of the kernel (Table 3), but the relative ranked proportions were almost constant with oleic acid (C18:1) in the highest amount (except for stage I kernels where it was linoleic acid), followed by linoleic (C18:2), palmitic (C16:0) and stearic (C18:0) acids. The high content of linoleic acid (C18:2), is an important characteristic of *J. curcas* seed oil for its use in biodiesel production (Goodrum and Geller, 2005). Most fatty acids in the kernel gradually increased in content from stages I to III (Table 3), similar to that reported by Annarao et al. (2008). Stearic, oleic and arachidic acids significantly increased by some seven- to eight-fold by stage III compared to stage I, and six of the other nine fatty acids increased as well but just not as dramatically.

The remaining three fatty acids were rare and only found at small levels in stage III (vaccenoic and eicosapentaenoic acids) or stages IV and VI (docosahexaenoic acid). This then suggests that, by and large, fatty acids synthesis and accumulation had started earlier than development stage III. Subsequently, 11 of the 12 fatty acids displayed a significant down-regulation from stages III to IV (only the rare docosahexaenoic acid perhaps increased, as in became detectable at a low level from undetectable before), and were then significantly raised again in stage VII (excepting linoleic acid which decreased slightly from stage IV to V). This was especially true for the oleic acid content that increased up to six-fold when compared to the level at stage I (Table 3). According to the fatty acid profile result, a turning point in seed development is likely to have occurred at stage IV because of the significant decrease in the level of all the fatty acids. Perhaps, by stage IV of seed development, the plant peroxisomes and glyoxysomes use acetyl CoA from β-oxidation as a biosynthesis precursor to synthesize glucose, sucrose and a wide variety of essential metabolites, as described by Nelson and Cox, (2008). Alternatively, the polyunsaturated fatty acids, especially the main polyunsaturated fatty acids of oleic and linoleic acids, may be mobilized in preference to other fatty acids. Thus, the mobilization or catabolism of fatty acids in the endosperm of *J. curcas* seeds is observed during development.

Based on the reasons aforementioned, the best developmental stages for harvesting kernels for optimal oil levels such as for biodiesel production were stages III and VII because of the high accumulation of oleic acid and linoleic acid, which are especially suitable fatty acids for biodiesel production. To date, comparative plant proteomics studies are still using traditional two dimensional polyacrylamide gel electrophoresis (2D-PAGE) with isoelectric focusing in the first and SDS-PAGE in the second dimension. Limitations of this method are sensitivity, dynamic range and reproducibility of protein quantification. One-dimensional (1D) gels, which separate proteins based on molecular mass, provide a low-resolution sepa-ration of proteins, but when coupled with tandem mass spectrometry can be used to identify proteins in moderately complex mixtures. In this work, the SDS-PAGE-LC-MS based proteomic analysis revealed 22 fatty acid biosynthesis related proteins whose expression levels changed during development. Total differentially expressed proteins from the eight different seed developmental stages were pre-separated by SDS-PAGE, spreading out the proteome over 27 gel slices that then reduces the overall sample complexity and resulted in a dramatically increased depth of analysis, and number of identified proteins. From this, 20 to 22 differentially expressed proteins that are likely to be involved in fatty acid biosynthesis as shown in MS/MS peak lists (that is their likely identification by peptide identity was to genes whose predicted proteins are annotated as likely to be genes whose function is known to be involved in fatty acid biosynthesis) were obtained (Table 4).

To elucidate mRNA expression levels and fatty acid biosynthesis in the different developmental stages of *J. curcas* kernels, the level of most fatty acids found in *J. curcas* oil such as oleic, linoleic, palmitic and stearic acids were partially associated with the transcript levels of the four target genes, ACCase, PEPC, MST and 4CL (Figure 3 and Table 3). That is the up-regulation of these gene transcripts in kernels at developmental stage II and especially stage III, their strong down-regulation at stage IV and then their upregulation at stage V followed by down-regulation to stage VIII closely matched the fatty acid levels in the kernels except for the increase in fatty acids seen at stage VII which does not have a corresponding increased gene transcript level, but rather they are still declining and already at very low levels. On the basis of these data, it can be suggested that the major control point in fatty acid accumulation during *J. curcas* seed development is the increase in the amount of mRNAs coding for the enzymes involved in fatty acid biosynthesis; although, this awaits genetic manipulation or pharmacological inhibition type approaches for confirmation. The first step of fatty acid biosynthesis is acetyl-CoA, which is carboxylated to malonyl-CoA by ACCase. This reaction is a key regulatory step in fatty acid biosynthesis in animals, bacteria and plants (Jackowski et al., 1991; Kim et al., 1989), and here the transcript expression level of ACCase was altered throughout seed development of *J. curcas* (Figure 3) in a similar manner to the fatty acid levels in the kernel (Table 3).

The mRNA expression pattern of PEPC in developing *J. curcas* seed kernels was similar to that reported by Sebei et al. (2006) during seed maturation of rapeseed (*Brassica napus* L.), where it increased during the lipid accumulation phase and was followed by a rapid decrease until the end of seed maturation in the same manner as the fatty acid profile. These findings suggested that PEPC could be involved in fatty acid biosynthesis during seed maturation of rapeseed and *J. curcas*, but again this awaits genetic or pharmacological manipulative approaches for confirmation. MST plays a physiological role in the regulation of many metabolic pathways, inclu-

ding co-factor biosynthesis, protection against biotic and abiotic stress, regulation of redox homeostasis, seed development, fatty acid biosynthesis, sulfur metabolism and secondary metabolism (Nagahara and Nishino, 1996; Nagahara and Katayama, 2005; Rajjou et al., 2008). Our results reveal, for the first time to our knowledge, that fatty acid accumulation in *J. curcas* kernels is potentially associated with an altered level of MST. The down-regulation of MST mRNA at stages II to IV might be a sign of the initiation of the mobilization or catabolism of fatty acids, whilst its up-regulation at stage V can facilitate the process involved in pyruvate synthesis. After oxidation to acetyl CoA, the pyruvate can be used as precursor for building up the fatty acids, leading to the observed increase in most of the fatty acids from stages V to VII. 4CL mediates activation of hydroxycinnamates into the phenylpropanoid pathways in which the high-energy intermediates can be used for biosynthesis of lignin, flavonoids, and various other protective, attractant and signaling metabolites (Hahlbrock and Scheel, 1989; Dixon and Paiva, 1995; Higuchi, 1997; Whetten et al., 1998).

The up-regulation of the lipid catabolism-associated transcript, 4CL, in kernels before developmental stage III matched the sharp decrease in the fatty acid content observed in the kernels at stage IV, potentially indicating oil in the endosperm was mobilized and the intermediate substances from β-oxidation process may be used in flavonoid or lignin biosynthesis. The potential correlation between the label free SDS-PAGE-LC-MS obtained proteomics data, including that for the ACCase, PEPC, MST and 4CL enzymes, and the intermediary fatty acid levels found in the kernels during seed development was shown. The potential fatty acid biosynthesis activation at the metabolomics and transcriptional level, as evident by the correlation between the increase in the fatty acids and the upregulation of the mRNA transcripts coding for these four important key genes was revealed but it is not perfect, possibly suggesting other mechanisms play a role as well. The first significant peak for the transcript expression of ACCase, PEPC, MST and 4CL mRNA occurred at stage III concurrent with the large increase in fatty acid levels. A rapid decrease of both fatty acid and transcript levels at stage IV could indicate delayed fruit development. Hence, the regulation of fatty acid biosynthesis at the transcriptional level following fruit development correlated more closely with the observed phenoltypic changes and fatty acid metabolism.

The results provide not only complementary information on the potential free fatty acids present in *J. curcas* kernels, but also information on potential biomarkers to clarify mechanisms involved in fatty acid metabolism.

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