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Analysis of the oil biosynthesis transcripts of the *Moringa oleifera* Lam. mature seed embryos using RNA sequencing

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Abstract

***Moringa oleifera* seeds are capable of producing 40% edible oils that are gaining significance due to its nutritional advantages. Several studies have examined *M. oleifera* seed oil, nevertheless, these studies focused on the extraction of oil and methods of biodiesel production. There is a paucity of information on transcriptome level studies to determine the unigenes involved in oil biosynthesis metabolic pathways. The main objective of this study is to explore the transcriptome of the mature embryo of *M. oleifera* Lam. particularly the key genes related to oil biosynthesis. The transcriptome reflects the set of genes that are actively expressed at any given time produced in one or a population of cells in a given organism. Total RNA was extracted from 30 mature seed embryos obtained from 10 trees in Muñoz, Nueva Ecija, Philippines. RNA were pooled for cDNA library construction. Then, RNA-sequencing was done followed by de novo sequence assembly to provide a cost-effective and comprehensive means of transcriptome level information for *M. oleifera*. A total of 182,588 transcripts were generated in this study. Out of these transcripts, 3,556 unigenes are involved in oil biosynthesis. The most numerous group of unigenes are those involved in fatty acid biosynthesis with 1,009 unigenes, fatty acid catabolism with 982 unigenes and triacylglycerol catabolism with 608 unigenes. There are 33 unigenes encoding for transcription factors involved in regulating oil biosynthesis gene expression. This is the first transcriptome resource ever reported for *M. oleifera* mature seed embryo. These unigenes are unmatched in protein databases for *M. oleifera*. Hence, the transcriptome resource for the *M. oleifera* Lam. mature seed embryo generated in this study will be useful for the mapping of oil biosynthesis related genes and the understanding of metabolic pathways which could possibly be used to improve seed yield and oil content of *M. oleifera*.**

Keywords: RNA-sequencing, de novo sequence assembly, transcriptome, unigenes, oil biosynthesis

INTRODUCTION

Moringa oleifera Lam. is an oil producing plant. The seeds of *M. oleifera* are abundant in oil, identified as Ben oil which reportedly contains a high amount of oleic acid (73.22%), an 18-carbon long monounsaturated fatty acid. Since the oleic acid has good oxidative strength compared with polyunsaturated fatty acids, it is valuable in the food industry because it permits longer storage and high temperature frying processing. Ben oil is more stable than canola oil, soybean oil, and palm oil when used in frying (Abdulkarim et al., 2007). Mixing Ben oil with soybean oil and sunflower oil enhances the oxidative strength of the mixture. *M. oleifera* seed oil is considered equivalent to olive oil in terms of its chemical properties and is good for human consumption (Mani et al., 2007).

Other saturated fatty acids found in *M. oleifera* are palmitic acid (6.45%), stearic acid (5.50%), arachidic acid (4.08%) and behenic acid (6.16%). Also, small quantities of linoleic

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acid (1.27%), gadoleic acid (1.68%), and palmitoleic acid (0.97%) were detected (Palafox et al., 2012). *M. oleifera* oil is also potentially useful as biodiesel feedstock. The Philippines has vast and fertile lands, so *M. oleifera* could be grown on wide scale production as a potentially valuable crop, yielding useful oil with high-oleic content. Throughout the past years a number of studies have examined *M. oleifera* Lam. seed oil; conversely, these studies were dedicated on oil extraction and procedures of biodiesel production from the seed oil (Anwar and Bhanger, 2003; Abdulkarim et al., 2007; Mani et al., 2007). The transcriptome of some oil crops (Liu et al., 2013) have been analyzed using RNA-sequencing, however, there are no transcriptome level studies that have been attempted to determine the oil biosynthesis metabolic pathways which could be used to improve oil content in *M. oleifera*. The transcriptome is the set of all RNA molecules (mRNA, tRNA, rRNA and other non-coding RNAs) produced in one or a population of cells. It refers to a total set of transcripts (expressed genes) of a given organism present in a particular cell type. This study aimed to explore the transcriptome of the mature seed embryo of *M. oleifera* Lam. Particularly, the study discovered key unigenes in the *M. oleifera* mature embryo related to oil biosynthesis and pointed out their levels of expression.

MATERIALS AND METHODS

Collection of tissues for RNA extraction

Moringa oleifera Lam. is extensively distributed in the Philippines, so it has not been listed as an endangered or protected species. Ten pods each from ten trees were collected from Muñoz, Nueva Ecija, Central Luzon, Philippines. Thirty mature seed embryos were obtained and were pooled for RNA extraction. These samples were immediately frozen in liquid nitrogen and stored at -80°C in an ultralow freezer.

cDNA library construction, RNA sequencing and de novo sequence assembly

Total RNA was extracted from the seed embryos of *M. oleifera* Lam. using the Ambion mirvana Plant miRNA isolation Kit (Life Technologies, Inc., Carlsbad, CA, USA) following the manufacturer's protocol. Extracted RNA was qualified and quantified using a NanoDrop 1000 Spectrophotometer (Thermo Fisher, Waltham, MA, US). All the samples showed a 260/280 nm ratio from 1.9 to 2.1. A total of 30 mg of total RNA was used for cDNA library construction. cDNA library construction and normalisation were performed (Ambry Genetics, Aliso Viejo, CA, USA). The resulting library was sequenced using Illumina High Seq. and assembled de novo using the SOAP (Short Oligonucleotide Analysis Package) assembler (Ambry Genetics, Aliso Viejo, CA, USA).

Functional classification of unigenes

Annotations and identification of functions of the transcripts were done using top priority softwares such as the NCBI non-redundant database, BLASTx, Pfam, Swissprot, TREMBL and TAIR. In order to further assess the inclusiveness of the *M. oleifera* mature embryo transcriptome library and the efficiency of the annotation process, annotated unigene sequences were used to search for genes involved in Clusters of Orthologous Groups (COG) classifications, Gene Ontology (GO) assignments and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway assignments to predict and categorize their functions. The analysis included functional annotation of the transcripts, identification of unigenes that are involved in oil biosynthesis and metabolism.

Determination of FPKM (fragments per kilobase of exon per million fragments mapped) values indicating expression of the unigenes

FPKM values were obtained using the Cufflinks V. 2.1.1 software. Cufflinks tests for differential expression and regulation in RNA-Seq samples. It accepts aligned RNA-Seq reads and assembles the alignments into a parsimonious set of transcripts. Cufflinks then estimated the relative abundances of these transcripts based on how many reads support each one, taking into account biases in library preparation protocols.

RESULTS AND DISCUSSION

RNA sequencing reactions yielded 49,170 contigs with a total contig length of 33,890,616 bp. The mean contig length is 689 bp. On the other hand, the maximum contig length is 40,309 bp while the minimum contig length is100 bp. The N50 contig length is 1,500 bp. Total for the largest 10 contigs is 240,849 bp while the total for the largest 100 is 940,367 bp. A total of 182,588 transcripts were generated from the transcriptome (Table 1).

Table 1. Data of the *M. oleifera* RNA sequencing and transcriptome assembly.

Items	Number/size
Number of contigs	49,170
Total length	33,890,616 bp
Mean contig length	689 bp
Maximum contig length	40,309 bp
Minimum contig length	100 bp
N50 contig length	1,500 bp
Largest 10 total	240,849 bp
Largest 100 total	940,367 bp
Number of unigenes	182,588

Homology of the *M. oleifera* unigenes with plant species from top priority databases

Out of the 182,588 transcripts, 67,558 (37%) were described in at least one database with high homology with unigenes from *Theobroma cacao,* 34,692 (19%) from *Vitis vinifera*, 23,737 (13%) from *Ricinus communis*, 14,607 (8%), from *Jatropha curcas*, 12,781 (7%) from *Fragaria vesca*, 10,955 (6%) from *Glycine max,* and 9,129 (5%) from both *Cicer arietum* and *Solanum lycopersicum* (Figure 1). The E-values are from 0 to >1E-5 (Figure 2). The aligned lengths of unigenes are mostly from 45 to 200 bp (Figure 3).

Figure 1. Unigenes functional annotation result for the top-hit species distribution for BLASTx matches for *M. oleifera* unigenes using the following order of priority databases: NR, TrEMBL and Swiss-Prot.

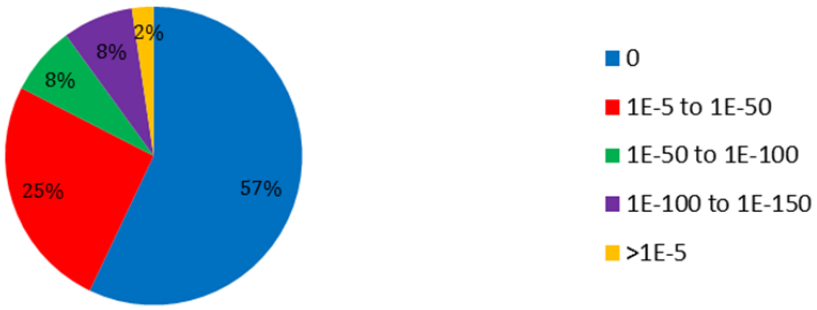

Figure 2. E-value distribution of top BLASTx hits for each unigene.

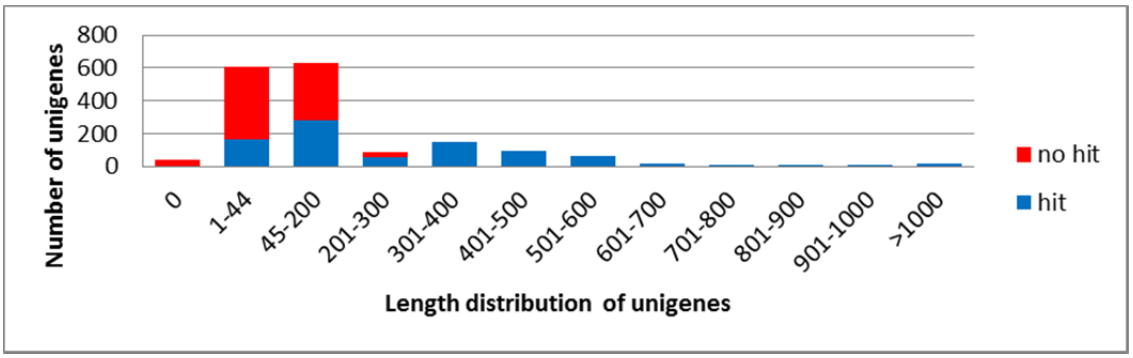

Figure 3. Distribution of unigenes in length with BLASTx hits compared with those without hits.

Functional classification of unigenes related to oil biosynthesis

The transcriptome of *M. oleifera* revealed 3,556 unigenes responsible for oil biosynthesis (Table 2). These transcripts are unigenes which are unmatched for *M. oleifera* in the databases because there was no transcriptome resource previously published for *M. oleifera* mature embryo. There are 1,009 (28.37%) unigenes linked to fatty acid biosynthesis, 379 unigenes (10.66%) involved in fatty acid elongation and 82 unigenes (2.30%) related to fatty acid desaturation. Additionally, there are 982 unigenes (27.61%) linked to fatty acid catabolism. On the other hand, there are 414 unigenes (11.64%) related to triacylglycerol (TAG) biosynthesis and 49 unigenes (1.37%) linked to acyl editing. Furthermore, there are 608 unigenes related to triacylglycerol (TAG) catabolism. A total of 33 unigenes encoding for transcription factors were also discovered in the transcriptome data (Table 2).

Table 2. Functional classification and number of unigenes related to oil biosynthesis.

Functional classification	No. of unigenes	Percentage (%)
Fatty acid biosynthesis	1009	28.37
Fatty acid elongation	379	10.66
Fatty acid desaturation	82	2.30
Fatty acid catabolism	982	27.61
Triacylglycerol biosynthesis	414	11.64
Acyl editing	49	1.37
Triacylglycerol catabolism	608	17.09
Transcription factors	33	.93
Total	**3556**	**100%**

Unigenes linked to fatty acid (FA) biosynthesis and elongation

In oil producing plants like *M. oleifera*, fatty acids are deposited as a form of triacylglycerol (TAG) and their biosynthesis route can be allocated into three phases (Hills, 2004). The first phase is de novo biosynthesis of fatty acids which occurs in plastids and is catalysed primarily by the fatty acid synthase complex (FAS). Corresponding to the KEGG pathway assignment, out of the 1009 unigenes involved in fatty acid biosynthesis, there are five upregulated enzymes involved in FA biosynthesis in the transcriptome, these are: Acyl carrier protein 1 (FPKM 525.27), Acetyl CoA isoform BC subunit (FPKM 845.56), Acetyl CoA Carboxylase Carboxytransferase alpha subunit (FPKM 1,364.87), Beta Ketoacyl ACP Synthase (11,372.86), 3-Ketoacyl carrier protein synthase 1 (FPKM 1372.86) (Table 3). Furthermore, from the 379 unigenes related to fatty acid elongation, there are specific unigenes that encode for enzymes such as Lipid Transfer Protein (FPKM 5,140.23) and Hydroxyacyl ACP dehydrogenase (FPKM 874.94) (Table 3) involved in FA elongation.

Table 3. Oil biosynthesis enzymes of *M. oleifera* by SOAP annotation of unigenes with FPKM values between 100-8,000.

Enzymes/proteins	Function	FPKM values
Acyl carrier protein 1	FA biosynthesis	525.27
Acetyl CoA isoform BC subunit	FA biosynthesis	845.56
ACC carboxytransferase alpha subunit	FA biosynthesis	1,364.87
Beta ketoacyl ACP Synthase 1	FA biosynthesis	1,372.86
3-ketoacyl carrier protein synthase 1	FA biosynthesis	1,372.86
Lipid transfer protein	FA elongation	5,140.23
Hydroxyacyl ACP dehydrogenase	FA elongation	874.94
Acyl (acyl carrier protein desaturase 6)	FA desaturation	7,164.82
Acetyl CoA acetyl transferase	FA catabolism	69.90
Acyl CoA oxidase 4	FA catabolism	138.92
Acyl CoA binding protein	TAG biosynthesis	911.72
Caleosin	TAG biosynthesis	1,100.27
Oleosin family protein	TAG biosynthesis	1,918.84
Stereoleosin	TAG biosynthesis	361.60
Phospholipase D alpha 1	Acyl editing	608.40

Unigenes associated to fatty acid (FA) desaturation

Several classes of enzymes play significant roles in fatty acid desaturation in plants, and can be categorized into two types. One type catalyzes the development of monounsaturated fatty acids from saturated fatty acids in plastids. This type contains only a soluble enzyme, acyl-ACP desaturase (Los and Mironov 2015). The other type is localized on the endoplasmic reticulum membranes and chloroplast and introduces double-unsaturated bonds at distinct positions (D12, D15 or D6) in fatty acids that are esterified to a glycerol backbone (Tasaka et al., 1996). Fatty Acid Desaturase 2 and Desaturase 6 are enzymes which desaturate oleic acid to form linoleic acid. Oleic acid and linoleic acids are major constituents of *M. oleifera* which is almost close to olive oil. Acyl carrier protein desaturase 6 which participates in the biosynthesis of polyunsaturated fatty acid is upregulated in *M. oleifera* mature embryo with an FPKM value of 7,164.82 (Table 3). Hence, fatty acid desaturase 6 are potential biotechnological targets for adjusting *M. oleifera* oil composition.

Unigenes linked to triacylglycerol (TAG) and oil bodies (OBs) biosynthesis

The synthesis of triacylglycerol (TAG) occurs in the endoplasmic reticulum (ER). Insights into the details of triacylglycerol biosynthesis, and information on the genes and enzymes involved in this process may lead to innovative strategies to modify the fatty acid composition of triacylglycerol and increase seed oil content. Out of the 414 unigenes linked to TAG biosynthesis in *M. oleifera*, a number of unigenes encode for Acyl CoA binding protein (FPKM 911.72) (Table 3). The biosynthesis of triacylglycerol involves acyl-editing of fatty acyl chains within the nitrogenous phospholipids of the endoplasmic reticulum. Another enzyme encoded by the TAG unigenes highly expressed in the *M. oleifera* mature embryo is the Phospholipase D Alpha 1 (FPKM 608.40). The next phase in TAG biosynthesis is the development of oil bodies (OBs), where TAG is merged alongside oleosin to form an oil body eventually released from the ER to the cytosol (Huang, 1992; Voelker and Kinney, 2001). A collection of TAGs can be stored as a form of OB enclosed by a membrane composed of a layer of phospholipids implanted with several proteins: oleosin, caleosin and steroleosin in mature seeds (Huang, 1992; Shimada and Hara-Nishimura, 2010). Oleosin is the most profuse structural protein in OBs; it supports stability of OBs through enlarged space bit resistance and charge repulsion, preventing merging of OBs (Huang, 1992; Frandsen et al., 2001). Oleosin is highly expressed in the *M. oleifera* mature embryo (FPKM 1918.84) (Table 3). Another protein, Caleosin is not only involved in the metabolism and synthesis of OBs, but may also be associated with plant drought tolerance (Frandsen et al., 2001; Naested et

al., 2000). Caleosin is also upregulated in the *M. oleifera* mature embryo (FPKM 1100.27) (Table 3). Stereoleosin (FPKM 361.60) (Table 3) is as well highly expressed in *M. oleifera* mature embryos. Steroleosin,-like proteins exemplifies a class of dehydrogenases/ reductases that are involved in signal transduction in plants controlled by a variety of sterols (Lin et al., 2002). The discovery of unigenes that are involved with caleosin, oleosin and steroleosin synthesis may influence functional studies in the oil production levels by metabolic engineering of *M. oleifera*.

Unigenes related to catabolism pathways for TAGs and FAs

The overall breakdown of TAGs can be distributed into two stages (Rismani-Yazdi et al., 2011). In the first stage, TAGs are metabolised to free FAs; lipases catalyse the hydrolysis of ester bonds that link fatty acyl chains to the glycerol backbone. In this research, one unigene encoding for mono/diacylglycerol lipase (FPKM 123.21) (Table 3) which liberates fatty acids and intermediate products (diacylglycerol or monoacylglycerol) from diacylglycerol, was found in the transcriptome library. In the next stage, fatty acids are broken down to acetyl-CoA, to be further broken down by oxidation or to follow other metabolic pathways, as well as re-esterification with glycerol, to form new acylglycerols (Jaworski et al., 1989). Based on KEGG pathway designation, two of the unigenes in the transcriptome encoding for enzymes related to fatty acid metabolism are highly expressed in the *M. oleifera* mature embryo. These are acyl-CoA oxidase 4 (FPKM 138.92) and acetyl-CoA acyltransferase (FPKM 169.90) (Table 3). Acetyl-CoA produced through fatty acid catabolism is subsequently used to generate energy for the cell through the tricarboxylic acid cycle or can contribute in the production of TAG. FA and TAG catabolism proceeds in a route opposed to that of their synthesis. Consequently, finding techniques to suppress the production of enzymes involved in FA and TAG catabolism can be an alternative approach of increasing the accumulation of lipids under settings that do not disturb plant development (Liu et al., 2013).

Transcription factors (TFs) involved in the oil synthesis of *M. oleifera*

There are 33 unigenes that encode for transcription factors found in the *M. oleifera* mature embryo. The transcription factors identified from the transcriptome data belonged to the following family of transcription factors: Apetala 2/AP2, C2H2 Zinc Finger, DOF, FUS3, Homeobox Lucine Zipper, L1B or LEC1, LIL (Leafy Cotyledon), MYB, Trihelix and AB13. Table 4 lists three of the most upregulated transcription factors encoded by the unigenes in the *M. oleifera* mature embryo. There are salient features of plant transcription factors that have been implicated in the control of seed oil deposition. These transcription factors are generally considered as master regulators of embryogenesis and seed maturation, being situated at, or near, the top of transcriptional cascades. Mutation of the genes encoding these transcription factors can have severe detrimental effects on seed maturation, including reductions in seed oil content. Conversely, increasing levels of their mRNA can lead to increased seed oil accumulation. Especially convincing are researches demonstrating that these genes, normally expressed predominantly in seeds, can induce the deposition of seed oil in vegetative tissues when ectopically activated in seedling (Liu et al., 2013).

Table 4. Transcription factors with FPKM values between 100-700.

Transcription factors	FPKM values
L1B or LEC1/LIL	677.15
MYB transcription factor	204.24
Homeobox leucine zipper	229.73

CONCLUSIONS

The first *M. oleifera* mature seed embryo transcriptome effort is reported in this study. Transcriptome analysis identified 182,588 transcripts which can provide a strong basis for future transcriptomic research and can serve as a reference transcriptome for future *M.*

oleifera RNA-seq experiments. Furthermore, 3556 unigenes that code for key enzymes and transcription factors involved in the metabolic pathways for FA and TAG biosynthesis and catabolism were identified. Although traditional breeding approaches play an important role in crop improvement, genetic engineering techniques are faster and specific, and allow the clear-cut improvement of crops for specific characteristics. For an oil producing crop like *M. oleifera* in which insufficient molecular information is available, RNA sequencing has provided unparalleled prospects for generating transcriptomic information. RNA sequencing enables economical, fast and thorough analyses of transcriptomes due to the assemblage of enormous information used for gene expression profiling, gene discovery, comparative, evolutionary and functional genomics studies. The findings in this study will substantially contribute in the improvement of *M. oleifera* attributes pertaining to oil biosythesis and will advance the breeding of new *M. oleifera* cultivars. Further directions of this study will focus on the analysis of other attributes of *M. oleifera* mature seed embryo, such as stress response and antioxidant properties. A comparison of the SOAP assembler and other assemblers such as OASES and TRINITY for de novo transcriptome assembly will also be considered.

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