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Oil Biosynthesis in Underground Oil-Rich Storage Vegetative Tissue: Comparison of *Cyperus esculentus* Tuber with Oil Seeds and Fruits

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Cyperus esculentus **is unique in that it can accumulate rich oil in its tubers. However, the underlying mechanism of tuber oil biosynthesis is still unclear. Our transcriptional analyses of the pathways from pyruvate production up to triacylglycerol (TAG) accumulation in tubers revealed many distinct species-specific lipid expression patterns from oil seeds and fruits, indicating that in *C. esculentus* tuber: (i) carbon flux from sucrose toward plastid pyruvate could be produced mostly through the cytosolic glycolytic pathway; (ii) acetyl-CoA synthetase might be an important contributor to acetyl-CoA formation for plastid fatty acid biosynthesis; (iii) the expression pattern for stearoyl-ACP desaturase was associated with high oleic acid composition; (iv) it was most likely that endoplasmic reticulum (ER)-associated acyl-CoA synthetase played a significant role in the export of fatty acids between the plastid and ER; (v) lipid phosphate phosphatase (LPP)-δ was most probably related to the formation of the diacylglycerol (DAG) pool in the Kennedy pathway; and (vi) diacylglyceroltransacylase 2 (DGAT2) and phospholipid:diacylglycerolacyltransferase 1 (PDAT1) might play crucial roles in tuber oil biosynthesis. In contrast to oil-rich fruits, there existed many oleosins, caleosins and steroleosins with very high transcripts in tubers. Surprisingly, only a single ortholog of WRINKLED1 (WRI1)-like transcription factor was identified and it was poorly expressed during tuber development. Our study not only provides insights into lipid metabolism in tuber tissues, but also broadens our understanding of TAG synthesis in oil plants. Such knowledge is of significance in exploiting this oil-rich species and manipulating other non-seed tissues to enhance storage oil production.**

Keywords: Biosynthesis • *Cyperus esculentus* • Expression pattern • Fatty acid • Metabolism • Oil • Transcript level • Tuber.

Abbreviations: ACCase, acetyl-CoA carboxylase; ACP, acyl carrier protein; ACS, acetyl-CoA synthetase; BCCP, biotin carboxyl carrier protein; COG, Clusters of Orthologous Groups; CPT, cytidine-5'-diphosphocholine: DAG cholinephosphotransferase; DAG, diacylglycerol; DAS, days after sowing; DGAT, diacylglycerol-acyl-CoA acyltranferase; DGTA, diacylglyceroltransacylase; EAR, enoyl-ACP reductase; ER, endoplasmic reticulum; FA, fatty acid; FAD, fatty acid desaturase; FAME, fatty acid metyl ester; FATA/B, fatty acyl-ACP thioesterase A/B; FPKM, fragments per kilobase of exon model per million mapped reads; GO, Gene Ontology; G-3-P, glycerol-3-phosphate; GPAT, glycerol-3-phosphate acyltransferase; HAD, hydroxyacyl-ACP dehydratase; KAR, keto-ACP reductase; KAS, keto-ACP synthase; KEGG, Kyoto Encyclopedia of Genes and Genomes; LACS, long-chain acyl-CoA synthetase; LPAAT, lysophosphatidyl acyltransferase; LPCAT, lysophosphatidylcholine acyltransferase; LPP, lipid phosphate phosphatase; MCMT, malonyl-CoA: ACP malonyltransferase; PA, phosphatidic acid; PAH, phosphatidic acid phosphohydrolase; PAP, phosphatidate phosphatase; PDAT, phospholipid:diacylglycerol acyltransferase; PDCT, phosphatidylcholine:diacylglycerol cholinephosphotransferase; PDHC; pyruvate dehydrogenase complex; PC, phosphatidylcholine; PLC, phospholipase C; qRT-PCR, quantitative real-time PCR; SAD, acyl-ACP desaturase; TAG, triacylglycerol; TAGL, triacylglycerol lipase; WRI1, WRINKLED1; 16:0, palmitic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid.

Introduction

As the world population expands and vegetable oil consumption increases, the huge demand for plant oil has put pressure on the supply of natural oil-rich crop resources. Furthermore, the worldwide high demand for economically viable biodiesel requires the plant oil production to be greatly increased as for biodiesel feedstocks. Efforts to improve the oil production of oil crops have been underway for many years (Thelen and Ohlrogge 2002, Lu et al. 2011, Xu and Shanklin 2016). Enhancing oil accumulation in plant non-seed vegetative tissues such as leaf, stem, root and tuber is considered a potential effective approach for increasing overall plant oil production (Durret et al. 2008, Xu and Shanklin 2016). In nature, there are several plants that accumulate abundant oil in leaves (20% of dry weight as acylated sugars in leaf of wild tomato species) (Fobes et al. 1985), stems (~10% oil in phloem tissue of *Tetraena mongolica* stems) (Wang et al. 2007) and tubers (26% oil in tubers of *Cyperus esculentus*) (Stoller and Weber 1975).

Cyperus esculentus L. (yellow nutsedge or tigernut) is a grass-like perennial plant of the sedge family Cyperaceae (Defelice 2002). It is widespread commonly in tropical and temperate

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zones and now also in cooler regions. *Cyperus esculentus* is used as a crop plant, but it also occurs in the wild and as a weed (Castro et al. 2015). Its tubers are rich in high amounts of up to 40% starch, 30% oil, 20% sugar, 9% protein and 6% fiber (Arafat et al. 2009, Bado et al. 2015). The tubers are a source of nutritional food for human health because they contain high levels of phosphorus, iron and potassium, and vitamins E and C (Belewu and Belewu 2007). They can be eaten whole raw or ground into flour, as well as being used to make a refreshing drink called Chufas or chufa milk which is a common commercially available product in Spain (Sánchez-Zapata et al. 2012). The tuber flavor is somewhat sweet and can be used as a flavoring agent for ice creams, biscuits, salads, soups, yogurt, sweets, baby foods, health bars, breads, cakes and other foods (Cantatejo 1997). It can be used medicinally since it was found to help boost blood circulation, reduce cardiovascular diseases and heart attacks, and prevent stroke and inflammation of the respiratory passages. Also, it helps cure flatulence and reduces the risk of suffering colon cancer (Arafat et al. 2009, Sánchez-Zapata et al. 2012). In China, *C. esculentus* is used as a liver tonic, heart stimulant, sedative and to cure stomach pain and promote normal menstruation (Defelice 2002).

The characteristics of *C. esculentus* growth and development, morphology and major storage nutrients are well documented (Gifford and Bayer 1995, Ransom et al. 2009, Arafat et al. 2009, Turesson et al. 2010). In recent years, additional research has been focused on the cultivation and breeding of *C. esculentus*, and the extraction technology of oil, starch and protein (Sánchez-Zapata et al. 2012, Ezeh et al. 2014)

Cyperus esculentus is a unique plant species that has a relatively high yield, ranging from 4.5 to 12 t ha^{-1} (Makareviciene et al. 2013), and accumulates rich oil in tubers; therefore, it is currently regarded as a new kind of economic oil crop with excellent potential for oil production in the underground vegetative tissues. Furthermore, as an oil-rich tuber plant, *C. esculentus* represents an ideal model system to study the effects of carbon reallocation and alterations in enzyme activities on lipid accumulation during the development of a non-photosynthetic underground storage organ.

However, the biological mechanism of oil biosynthesis in tubers still remains unclear. Specifically, still unknown is the extent to which lipid metabolism differs between tubers and other oil-rich tissues of seeds and fruits, whether there are tissue- or species-specific lipid-related genes and regulation factors for tuber oil biosynthesis and if there exists a special regulation mechanism for tuber oil biosynthesis. To address these questions, we present a comprehensive analysis of the global transcriptome profile of developing tubers, aimed at identifying the transcripts involved in the biosynthesis of select lipids. The temporal analysis of gene expression and differentially expressed genes during oil biosynthesis revealed some different features from oil-rich seeds and fruits for the lipid-related gene expression profile and their levels of expression, which can help to characterize their biochemical and physiological function. Our study not only provides insights into the lipid metabolism in tuber tissues, but also provides a foundation for broadening our understanding of oil synthesis in oil plants.

Results and Discussion

Oil accumulation during tuber development

Under our growth conditions, tubers started to sprout after approximately 2 d upon seed tuber sowing. The growth period of tubers spanned around 120 d. It was reported that the development of tubers is similar to that of potato tubers formed by swelling of the subapical underground stolons (Turesson et al. 2010), which is involved in the initial transformation of the rhizome tissue into a storage compartment and later massive accumulation of storage reserves. In our experiments, initial tubers were observed to form on rhizomes at around 40 days after sowing (DAS). We determined the fresh and dry weights of tubers during their development and found that the masses varied with tuber growth. In the early stage (40–50 DAS), tubers are small and appeared white. The fresh weight was no more than 100 mg per tuber (**Fig. 1A**). In the middle stage (50–85 DAS), tubers were light brown in appearance. This period marks a rapid increase in tuber weight, having a growth rate of approximately 34 mg d^{-1}, much greater than the early (~7 mg d^{-1}) and late stage (~7 mg d^{-1}, 85–120 DAS). In the late stage, tubers became brown and they slowly increased to their final fresh mass of around 1,500 mg. A similar trend was also observed for the dry tubers (**Fig. 1A**).

Oil (triacylglycerol; TAG) is one of main storage reserves in *C. esculentus* tubers. To examine the influence of the developmental process on oil accumulation in the tuber, we determined the oil content during tuber development. Oil content was calculated from the amount of total fatty acids (FAs) based on fatty acid methyl esters (FAMEs). Our results showed that oil content in tubers increased continuously throughout all developmental stages and the accumulation of oil had a similar trend to the change in total tuber weight (**Fig. 1B**). For example, there was rapid oil accumulation in the middle stage (50–85 DAS), where the accumulation rate reached 4.09 mg per tuber d^{-1}, much higher than that of the early and late stage of approximately 0.72 and 1.27 mg tuber d^{-1}, respectively. During the later stages, oil accumulated steadily, achieving a content of around 204 mg oil per tuber at 120 DAS (**Fig. 1B**), corresponding to a value of 25.5% of total dry weight. We noted that in the fast oil accumulation stage, the oil accumulation rate of tubers is expected to be larger than those of oil seeds of small size such as Arabidopsis (Focks and Benning 1998, Ruuska et al. 2002), rapeseed (Vigeolas et al. 2003) and castor bean (Houston et al. 2009, Chandrasekaran and Liu 2013), which had a rate of oil accumulation of 0.56, 33.89 and 3.66 μg per seed d^{-1}, respectively (**Table 1**). This oil accumulation rate of the tuber is obviously lower than that of oil fruit of large size such as oil palm which has a rate of 81.77 mg fruit d^{-1} at the rapid oil accumulation stage (Tranbarger et al. 2011).

We further examined the individual FAs in the lipids (**Fig. 1C**). Five FAs were detected in tubers, with the most abundant being oleic acid (C18:1), followed by linoleic acid (C18:2) and palmitic acid (C16:0), each making up > 15.0% of the total FAs throughout the tuber developmental stages. The total amount of these three components accounts for >95.0%

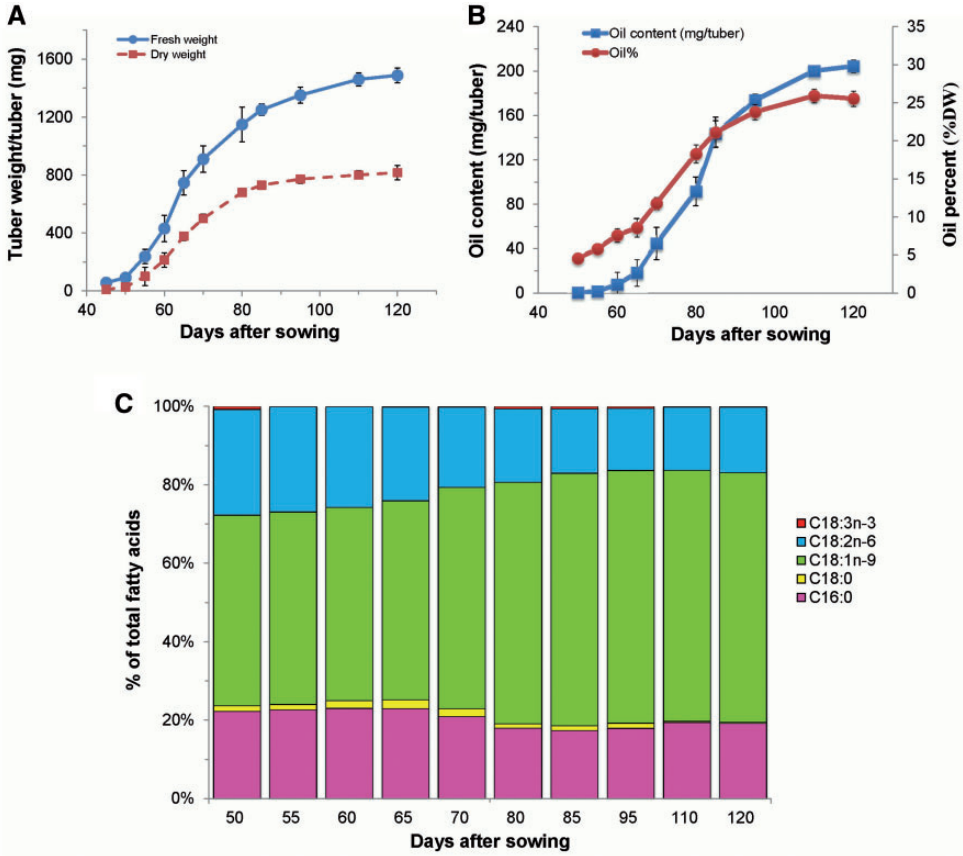

Fig. 1 Oil contents and fatty acid composition of developing tuber. (A) Fresh and dry weight of developing *C. esculentus* tubers from 45 to 120 d after seed tuber sowing. Tuber weights were based on 10–20 tubers. (B) Oil levels on the basis of tuber dry weight during tuber development. (C) Fatty acid composition of the developing tuber. Values represent means ± SD (n = 3).

Table 1 Lipid accumulation rate of plant oil-rich tissues at the stage of rapid lipid accumulation

Plant oil-rich tissues	Plant	Stage of rapid lipid accumulation	Rate of rapid lipid accumulation	Reference
Seed	*Arabidopsis thaliana* (Col)	9–13 DAF	0.56 μg FA seed^{-1} d^{-1}	Ruuska et al. (2002)
	Arabidopsis thaliana (Col)	9–13 DAF	0.46 μg oil seed^{-1} d^{-1}	Focks and Benning (1998)
	Arabidopsis thaliana (Ws)	7–17 DAP	0.78 μg FA seed^{-1} d^{-1}	Baud and Lepiniec (2009)
	Ricinus communis (Castor bean)	28–63 DAP	3.66 μg FA seed^{-1} d^{-1}	Chandrasekaran and Liu (2013)
	Glycine max (Soybean)	5–25 DAF	15.05 μg FA mg^{-1} DW d^{-1}	Collakova et al. (2013)
	Helianthus annuus (Sunflower)	10–36 DAF	12.23 μg oil mg^{-1} FW d^{-1}	Salas et al. (2006)
	Hordeum vulgare (Barley)	12–22 DAF	0.06 μmol lipid mg^{-1} FW d^{-1}	Neuberger et al. (2008)
	Brassica napus (Rape seed)	25–35 DAF	33.89 μg lipid seed^{-1} d^{-1}	Vigeolas et al. (2003)
	Brassica napus (Rape seed)	26–52 DAF	1.17% oil d^{-1}	Bhardwaj and Hamama (2003)
	Arachis hypogaea (Peanut)	18–53 DAF	1.03% oil d^{-1}	Yin et al. (2013)
	Carya cathayensis (Hickory)	82–97 DAP	3.55% oil d^{-1}	Huang et al. (2016)
Mesocarp	*Canarium album* (Olive)	159–180 DAFB	0.34% oil d^{-1}	Bellincontro et al. (2013)
	Elaeis guineensis (Oil palm)	80–160 DAP	81.77 mg oil fruit^{-1}d^{-1}	Tranbarger et al. (2011)
	Persea Americana (Avocado)	105–195 DAFB	0.75 mg FA g^{-1} FW d^{-1}	Kilaru et al. (2015)
Tuber	*Cyperus esculentus* (Tigernut)	7–42 DAI	2.04 mg FA tuber^{-1} d^{-1} 0.67% oil d^{-1}	Turesson et al. (2010)
	Cyperus esculentus (Tigernut)	50–85 DAS	4.09 mg FA tuber^{-1} d^{-1} 0.47% oil d^{-1}	This study

DAF, days after flowing; DAFB, days after full bloom; DAP, days after pollination; DAI, days after tuber initiation; DAS, days after seed tuber sowing; FA, fatty acid.

of total FAs. The largest component was oleic acid, which at the early stage accounted for almost half of the total FAs. As the tubers developed, oleic acid increased to its highest value of approximately 63%. The two detectable minor FAs were stearic acid (C18:0) and α-linolenic acid (C18:3), which each comprised <2.0% of the total FAs. Obviously, oil tubers contained a high proportion of unsaturated fatty acids, which were dominated by monounsaturated oleic acid.

These results further indicated that the tuber development and variations in its biomass were positively correlated with the changes in tuber oil accumulation and FA composition.

Transcriptome sequencing, de novo assembly and functional annotation of *C. esculentus* transcripts

A comprehensive and systematic transcriptome analysis will not only provide insights into lipid metabolism in tuber, but will also improve the understanding of plant lipid metabolism and provide a foundation for metabolic engineering to enhance storage oil production in vegetative tissues of diverse species. In order to explore genes associated with lipid metabolism in *C. esculentus*, total RNA was isolated from representative tubers from three developmental stages (i.e. the early stage 50 DAS, the middle stage 85 DAS and the late stage 120 DAS) and subjected to sequencing using the Illumina Hiseq4000 platform (maximum read length 2×150 bp). De novo assembly of the processed reads >200 bases was performed using the Trinity program (Grabherr et al. 2011). Overall, 99,558 assembled transcripts ranging from 201 to 20,000 bases were obtained, with an average length of 787 bp and N50 of 1,398 bp. The GC content of transcripts was 42.49%, lower than 57.51% of AT content. A total of 24,567 transcripts (24.68% of total) were found to be longer than 1,000 bp (**Supplementary Table S1**).

For functional annotation of the pooled transcripts, sequence alignments were conducted using BLASTX (E-value cut-off $\leq 1e^{-5}$) against the databases of NCBI non-redundant protein database (NR), Kyoto Encyclopedia of Genes and Genomes (KEGG), Swiss-Prot, Search Tool for the Retrieval of Interacting Genes (STRING), Clusters of Orthologous Groups (COG) and Gene Ontology (GO), which resulted in a total of 40,864 (41.05% of total 99,558) transcripts being annotated from all the databases (**Supplementary Table S1**). Based on NCBI NR annotation, 25,525 (33.21% of the otal 76,856) unigenes were successfully mapped according to the known sequences with the best match, which were categorized into 63 functional groups under three major biology function classes, i.e. molecular functions, cellular components and biological processes. The distribution of transcripts into various functional classes annotated by COG and GO databases is shown in **Supplementary Figs. S1 and S2**, respectively.

Most of the known lipid-related genes are detectable in *C. esculentus* tubers and display similar patterns of transcriptional regulation to oil-rich seeds and fruits

A total of 2.97% (1,214) of the annotated transcripts were identified to be involved in various lipid pathways, and >600 unigenes from the 1,214 annotated lipid-related transcripts were found to be expressed in the developing tubers of *C. esculentus*. The distribution of lipid-related unigenes that were categorized into different lipid biochemical pathways is shown in **Fig. 2A**. The majority of the lipid transcripts were involved in cutin, suberin and wax biosynthesis (23.61%). Phospholipid metabolism & signaling (15.12%), extra plastidial phospholipid synthesis & editing (10.34%), β-oxidation (7.10%) and fatty acid biosynthesis (6.94%) were other major categories. Thirty-two unigenes relating to biosynthesis of plastidial polar lipids (glycero, galacto and sulfo lipids) and 47 unigenes for TAG biosynthesis and TAG storage were also identified. It reflects that most of the known lipid enzymes were successfully detectable in tubers of *C. esculentus*.

We found that, among these metabolic pathways, the expression of genes related to FA synthesis were most abundant, with a value of transcript levels represented by FPKM (fragments per kilobase of exon model per million mapped reads) per enzyme being, on average, >2- to 10-fold higher than other pathways of lipid metabolism (**Fig. 2B**). This result might reflect that transcriptional regulation of FA synthesis rather than of TAG biosynthesis genes is an important factor associated with high oil accumulation in *C. esculentus* tuber, as is the case for other oil-rich tissues such as seed and mesocarp (Bourgis et al. 2011, Troncoso-Ponce et al. 2011, Kilaru et al. 2015).

In *C. esculentus* tuber, >200 genes were expressed in association with the metabolic pathways of de novo TAG biosynthesis starting from sucrose (**Supplementary Tables S3, S4**), which is involved in conversion of (i) sucrose to pyruvate including sucrose degradation and glycolysis; (ii) plastidial FA synthesis from pyruvate; and (iii) TAG synthesis and storage in the endoplasmic reticulum (ER). It was shown that, similar to oil seeds and fruits (**Fig. 3A**), the enzymes for carbohydrate metabolism from sucrose to pyruvate, in particular glycolysis, were highly expressed across all the developmental stages of the tuber, much more than those of plastid FA synthesis and membrane TAG synthesis. Nevertheless, the temporal expression pattern of carbohydrate metabolism displayed a trend similar to those of FA synthesis and TAG synthesis, indicating a coordinated expression of carbohydrate and lipid metabolism (**Fig. 3B**).

Overall, our data indicated that, as in oil-rich seed and non-seed tissues of fruit, abundant expression for genes involved in carbohydrate metabolism and subsequently FA synthesis with comparatively low transcripts for TAG synthesis also occurred in *C. esculentus* tuber. Our data further support the fact that the conservation exists in common enzyme stoichiometry and temporal regulation of transcripts associated with oil synthesis for different oil-rich tissues and in diverse species (Kilaru et al. 2015).

Transcriptional patterns for most enzymes involved in plastid FA synthesis in tubers were similar to those in oil seeds or fruits

In all plants studied, de novo FA synthesis in plastids is related to three main sequential enzymatic reactions (Ohlrogge and Browse 1995). The first one is the formation of malonyl-CoA from acetyl-CoA that is catalyzed by acetyl-CoA carboxylase (ACCase). The next is involved in a sequence of condensation reactions of malonyl-CoA with a growing acyl-acyl carrier protien (ACP) or acetyl-CoA catalyzed by multiple isoforms of fatty acid synthase (FAS), finally forming 16:0-ACP or 18:0-ACP. The next step is the desaturation of 18:0-ACP, which is catalyzed by stromal stearoyl-ACP desaturase (SAD) to form 18:1-ACP. Once

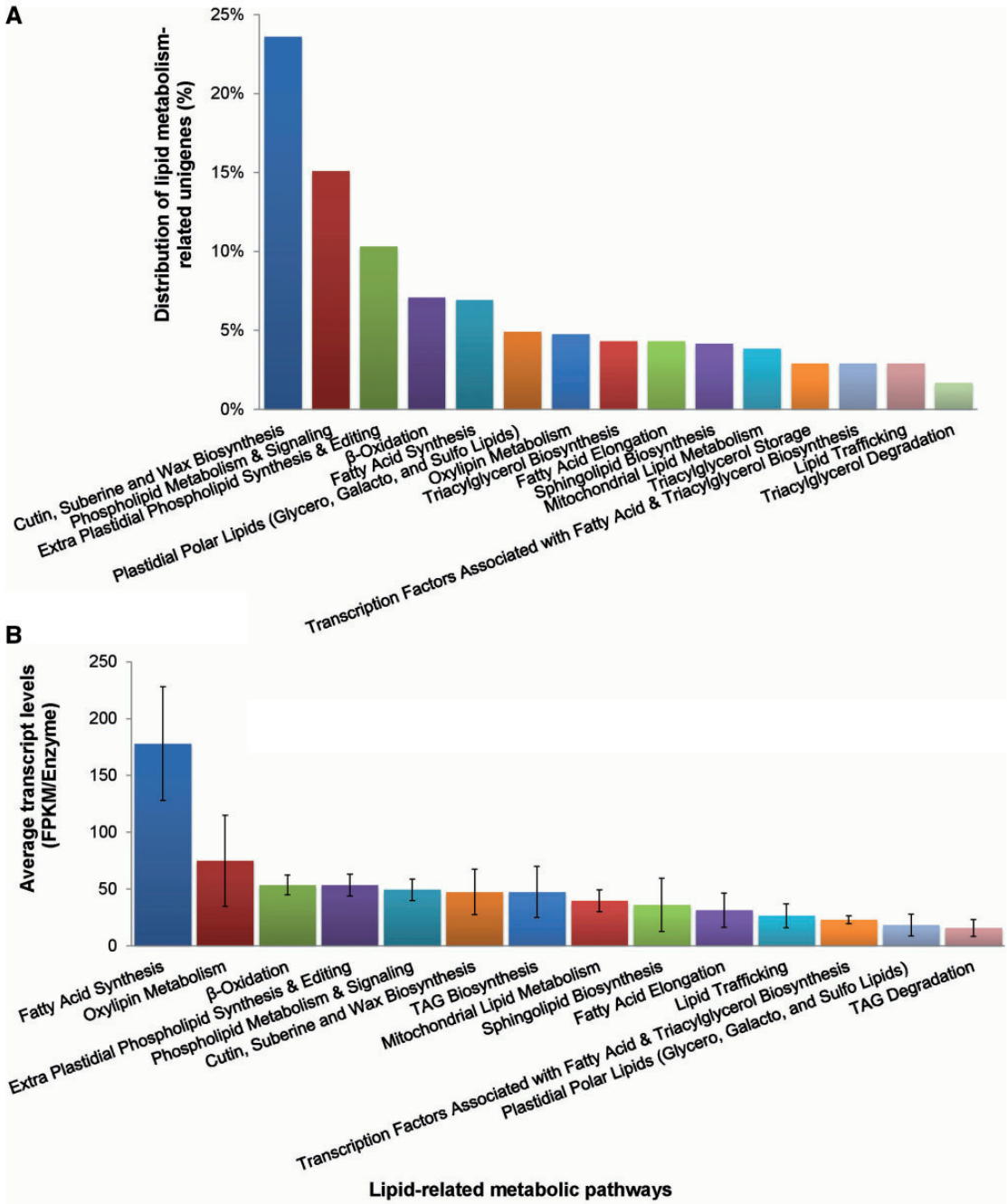

Fig. 2 Lipid-related unigenes identified in different lipid functional categories. (A) Distribution of unigenes in functional groups. (B) The transcript levels represented by FPKM (fragments per kilobase of exon model per million mapped reads) per protein in each lipid group. The data are averaged on three developing stages of tuber, with error bars indicating their SD. Isoforms or subunits of protein orthologs were regarded as a single ortholog.

synthesized, acyl-ACP is converted into free FA through the action of acyl-ACP thioesterase (FATA and FATB). Free FAs are reactivated by long chain acyl-CoA synthetase (LACS) and exported from plastids to enter the eukaryotic glycerolipid metabolic pathways.

In this study, >50 unigenes were identified to encode at least 15 enzymes and/or proteins which are involved in FA synthesis (**Fig. 4A**). Among these enzymes/proteins, the pyruvate dehydrogenase complex (PDHC), ACCase, ACP and SAD are transcribed more abundantly than any other enzymes (**Fig. 4B**), implicating the important roles of these proteins in FA synthesis. The overall transcript levels for these four proteins accounted for >65% of the total for FA synthesis at each stage of tuber development. Similar phenomena were also observed in developing oil seeds of *Ricinus communis*, *Brassica napus*, *Euonymus alatus* and *Tropaeolum majus*, and oil mesocarps of *Elaeis guineensis* and *Persea Americana* (Bourgis et al. 2011, Troncoso-Ponce et al. 2011, Kilaru et al.

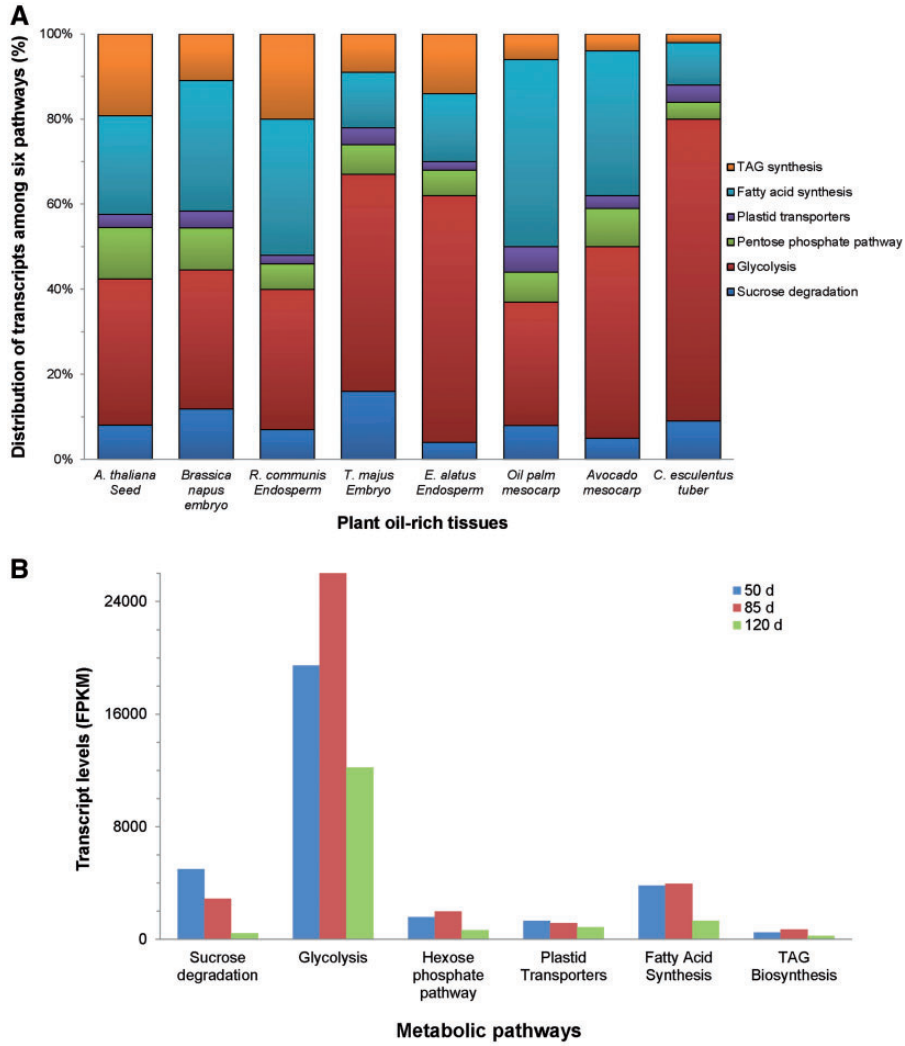

Fig. 3 Expression pattern for six pathways related to TAG synthesis. (A) The relative distribution of transcripts among the six pathways in diverse plant oil-rich tissues. The data are averaged on all developing stages of plant tissues. (B) Temporal transcriptional levels of six metabolic pathways from sucrose to TAG synthesis in developing tuber of *C. esculentus*.

2015). As in oil-rich seeds and fruits, expression levels for SAD in tubers were the most abundant, whereas those for malonyl-CoA:ACP malonyltransferase (MCMT) in all cases were relatively low. Similarly, the highest expression for the ortholog of SAD also occurred during all of the development of tubers (**Fig. 4B**).

In addition, we found that the transcripts for eight FA synthesis enzymes/proteins including PDHC, ACP, ACCase, ketoacyl-ACP synthase II (KASII), ketoacyl-ACP reductase (KAR), hydroxyacyl-ACP dehydratase (HAD), enoyl-ACP reductase (EAR) and MCMT showed down-regulation patterns during tuber development (**Fig. 5A**). A similar declining trend of the temporal expression pattern was also observed for these orthologs in oilseeds (Troncoso-Ponce et al. 2011). Whereas the transcripts for SAD, ACS, KASIII, FATA and FATB displayed 'bell-shaped' patterns (**Fig. 5B**), i.e. their transcriptional levels went up from the early stage of tuber development and reached a peak at the rapid growth period of tuber, and then continuously declined to low levels at tuber maturity. A similar pattern also occurred for several TAG synthesis genes (see **Fig. 10**). Such a 'bell-shaped' transcriptional pattern was also reported previously for Arabidopsis transcripts involved in seed FA synthesis (Ruuska et al. 2002, Ruuska et al. 2004). Our quantitative real-time PCR (qRT-PCR) analysis further confirmed the 'bell-shaped' expression patterns for some lipid-related genes (**Supplementary Fig. S3**).

In our study, several proteins related to plastid FA synthesis are encoded by only one gene in tubers (**Fig. 4A**). These proteins included MCMT, KASIII, EAR and LACS9. Interestingly, the corresponding protein orthologs were also observed to be encoded by a single gene in oil mesocarps of oil palm and avocado, as well as in oil seeds of Arabidopsis, rapeseed, castor and burning bush (Bourgis et al. 2011, Troncoso-Ponce et al. 2011, Kilaru et al. 2015), suggesting that these four orthologs are well conserved in FA synthesis in seed and non-seed tissues. A single ortholog in the tuber transcriptome was also noted for other enzymes such as an E3 subunit of dihydrolipoamide dehydrogenase (LPD), a biotin carboxyl carrier protein (BCCP) of

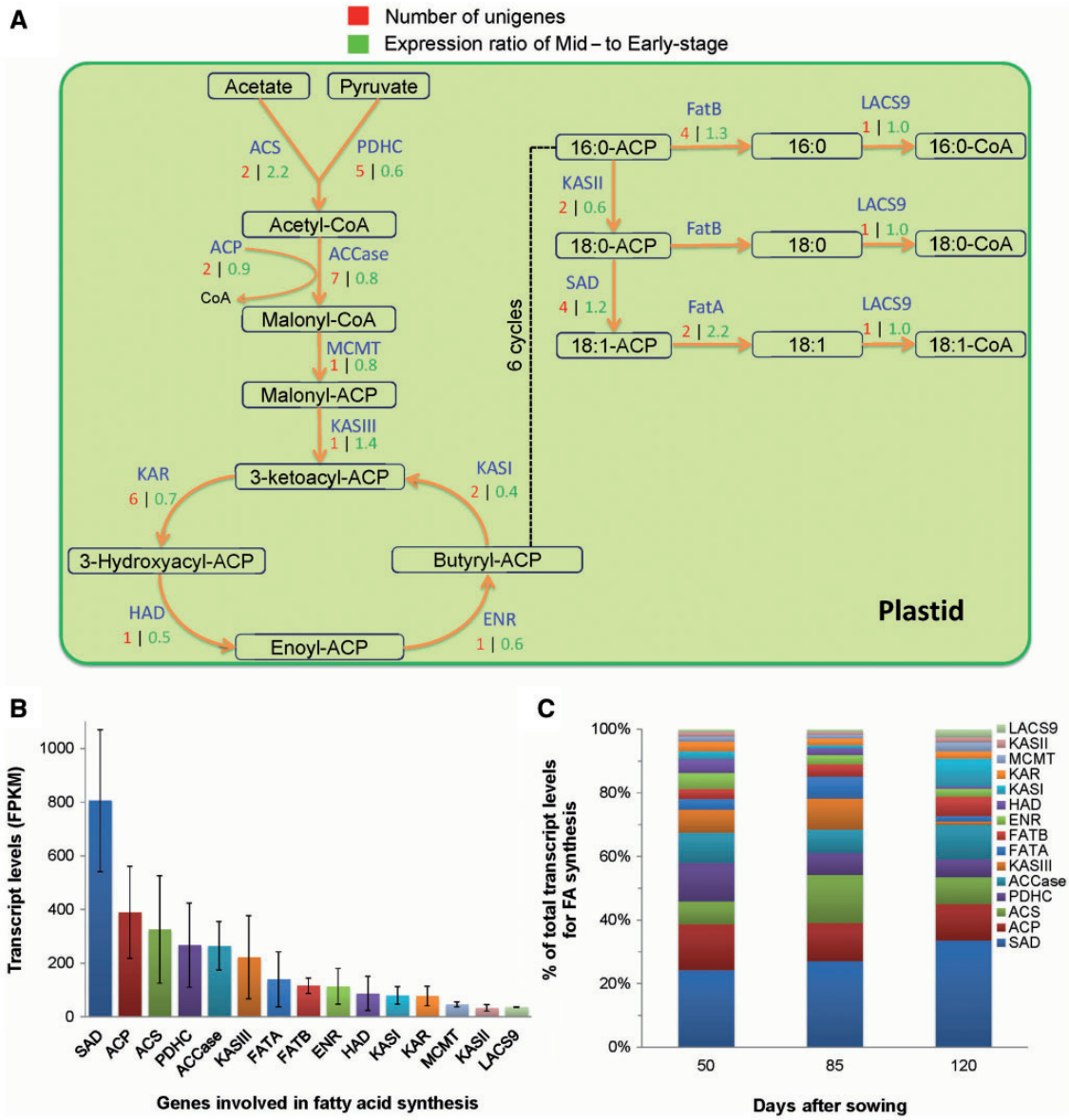

Fig. 4 Transcript levels of enzymes of fatty acid synthesis. (A) Schematic diagram of the fatty acid synthesis pathway. Enzyme or protein names are indicated in blue. (B) Average transcript levels (FPKM) for genes involved in plastidial fatty acid synthesis. The data are averaged on three developing stages of tuber with error bars indicating their SD. (C) Relative distribution of transcript levels for genes involved in fatty acid synthesis during tuber development. The FPKM values for subunits of a protein and for multiple isoforms were summed.

heteromeric ACCase and HAD (**Supplementary Table S3**), as is the case in oil mesocarp of avocado.

Together, the above comparative expression analyses indicate that control over FA synthesis transcript expression is highly conserved in different oil-rich tissues and in diverse species, and also during tissue development.

Acetyl-CoA synthetase may be an important contributor to acetyl-CoA formation for FA biosynthesis

For de novo FA biosynthesis, acetyl-CoA is the critical precursor (Roughan and Ohlrogge 1996). Four enzymes, PDHC, acetyl-CoA synthetase (ACS), ATP-citrate lyase and acetylcarnitine transferase, can produce acetyl-CoA for FA synthesis (Oliver et al. 2009). However, the last two enzymes were believed to be unlikely to have contributed to plastid acetyl-CoA formation. In the plastids, acetyl-CoA can be generated from pyruvate catalyzed by plastidial PDHC or from acetate associated with ACS (Roughan and Ohlrogge 1996, Nikolau et al. 2003).

As in other oil seeds or fruits, the *C. esculentus* transcriptome also presents orthologs of ACS and PDHC enzymes. PDHC, comprised E1, E2 and E3 subunits, and all these three subunits of PDHC displayed co-ordinated temporal expression with a down-regulation pattern during tuber development (**Fig. 6A**). Among the three enzyme subunits of PDHC, PDH-E1 in all cases was transcribed most abundantly, indicating the role of PDH-E1 to PDHC activity for FA synthesis conserved in diverse plant oil-

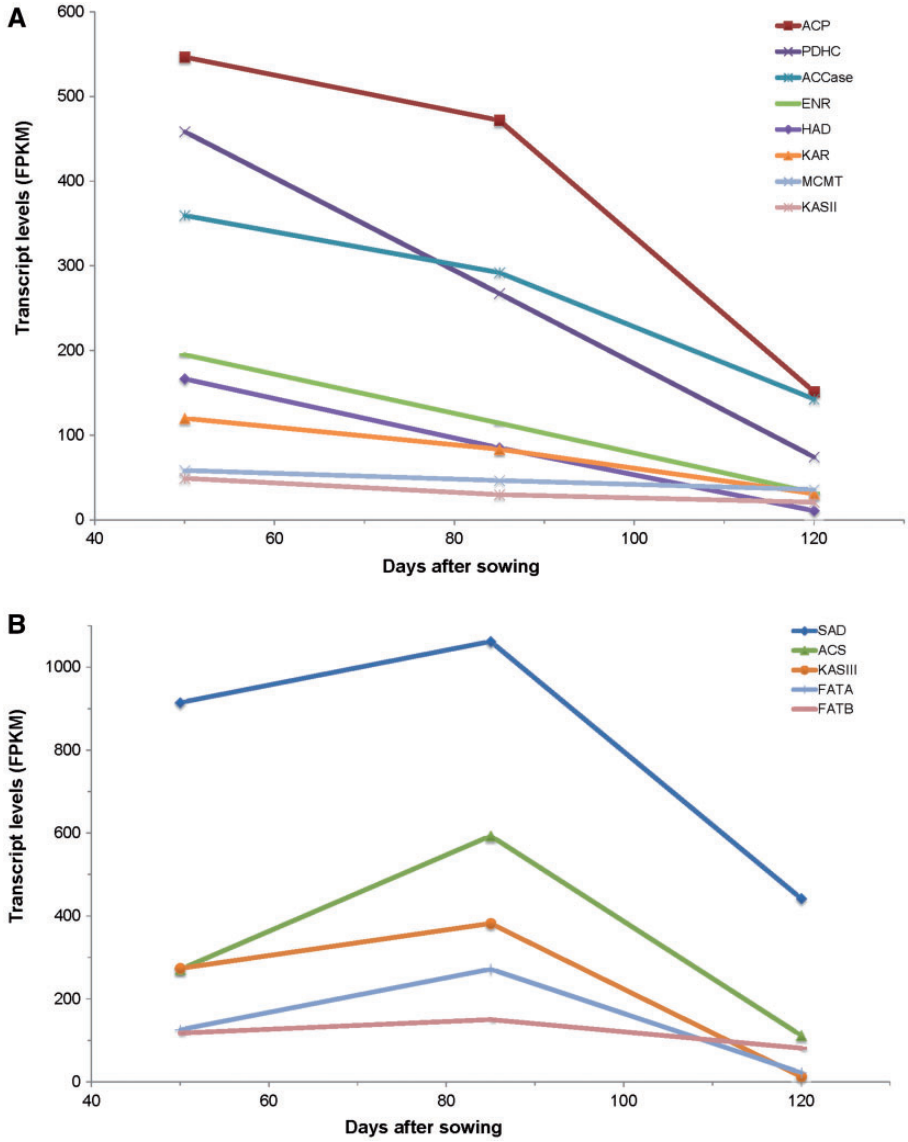

Fig. 5 Temporal changes in transcript levels for various fatty acid synthesis genes during tuber development.

rich tissues. The importance of PDHC for FA biosynthesis in plastids was previously confirmed by a lot of biochemical and molecular evidence that acetyl-CoA made from pyruvate by PDHC is the major source of plastid acetyl-CoA (Bao et al. 1998, Ke et al. 2000, Schwender et al. 2006, Lin and Oliver 2008).

The role of ACS in the plastidial acetyl-CoA formation is not well understood. ACS was found to be required for normal plant growth and was suggested to remove acetate substrate rather than supplying acetyl-CoA for FA biosynthesis (Lin and Oliver 2008). Other works showed that in *Chlamydomonas reinhardtii*, ACS was induced as part of the PDHC-bypass and seemed to serve as a major carbon assimilation pathway channeling acetate for FA biosynthesis (Avidan and Pick, 2015). We noted that the average transcripts for PDHC in oil-rich seeds and fruits were much more abundant, at least 10-fold higher, than those for ACS (Bourgis et al. 2011, Troncoso-Ponce et al. 2011, Kilaru et al. 2015). In stark contrast, however, the overall expression for PDHC in *C. esculentus* tubers was less than that of ACS, with the ratio being only 0.8 (**Fig. 6B**). Furthermore, in oil seeds and fruits, only one ortholog for ACS was represented, whereas in *C. esculentus*, ACS was encoded by two unigenes. In addition, our data showed that the transcription for these two ACS genes in tubers was correlated positively with plastidial FA synthesis (**Fig. 6A**). Together, our data imply that ACS was most probably an important contributor to the production of acetyl-CoA required for plastid FA synthesis in *C. esculentus* tubers.

Expression pattern of the stearoyl-ACP desaturase gene was associated with high oleic acid composition in tubers

Before entering the glycerolipid metabolism, FAs produced in the plastid undergo desaturation. FA desaturation is an enzymatic reaction in which a double bond is introduced into the FA chain (Shanklin and Cahoon 1998). The formation of monounsaturated FAs from saturated FAs takes place in plastids, while

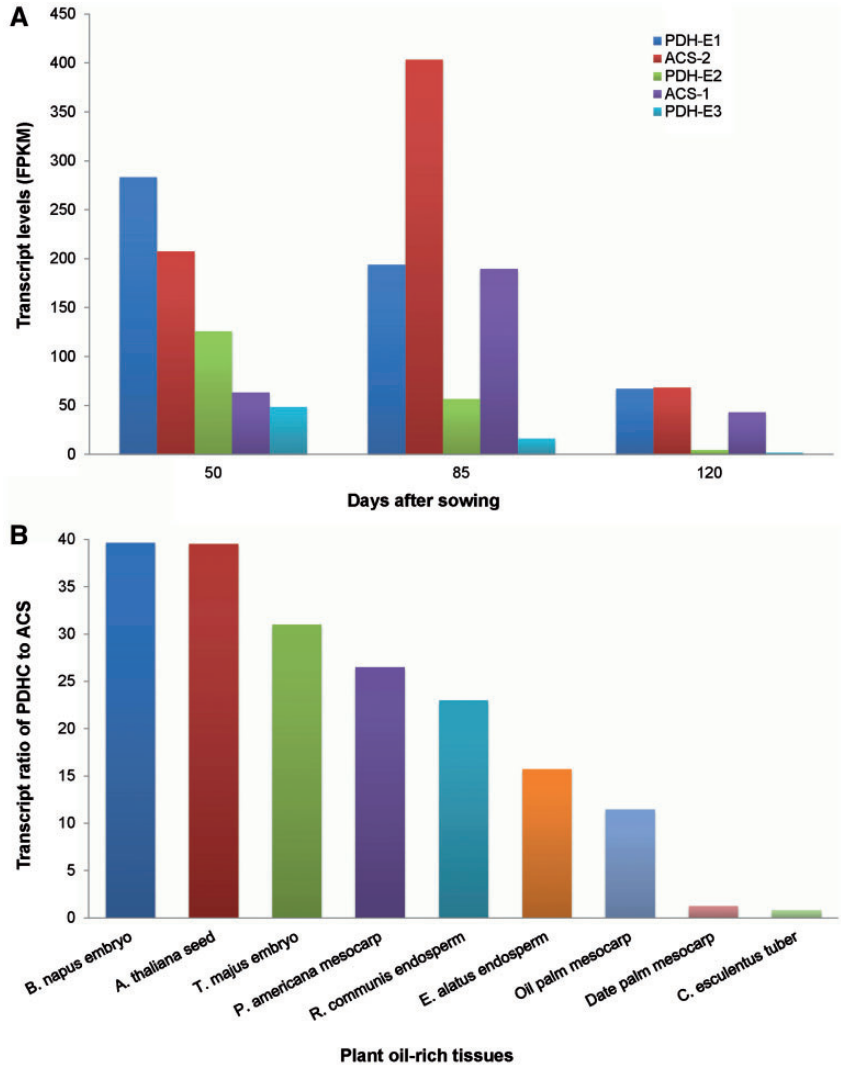

Fig. 6 Expression pattern for plastidial enzymes catalyzing acetyl-CoA formation. (A) Temporal changes in transcript levels for enzymes (PDHC and ACS) responsible for generation of plastid acetyl-CoA toward fatty acid synthesis during tuber development. (B) The ratio for average transcript levels of PDHC to ACS in different oil-rich tissues and in diverse species.

further desaturation of the monounsaturated FAs occurs in the ER or chloroplast. In plants, synthesis of monounsaturated FA is catalyzed by SAD, whereas the formation of polyunsaturated FAs is catalyzed by various specific desaturases, including Δ12-desaturase (FAD2 and FAD6) that converts oleic acid (C18:1) to linoleic acid (C18:2), and Δ15-desaturase (FAD3, FAD7 and FAD8), which catalyzes the desaturation of linoleic acid (C18:2) to form α-linolenic acid (C18:3) (Ohlrogge and Browse 1995). SAD catalyzes desaturation of stearoyl-ACP by introducing the double bond to the Δ9 position of the FA chain, therefore generating oleoyl-ACP, which is then further catalyzed by FATA to form free oleic acid (Moreno-Pérez et al. 2012). SAD is the key rate-limiting enzyme regulating the pools of oleic acid and determining the homeostasis between saturated FAs and unsaturated FAs, and thus plays an important role in the unsaturated FA biosynthetic pathway in higher plants.

As shown in **Fig. 4**, SAD ortholog was more highly expressed than any other enzymes during the development of tubers, and its expression constituted around a quarter of all the transcript levels for the plastidial FA synthesis enzymes, which was consistent with the fact that the majority of total FAs of tuber oils is dominated by unsaturated FAs (**Fig. 1C**). SAD exhibited >10-fold higher expression levels over MCMT with relatively low expression (**Fig. 7A**), as in other plant oil seeds and fruits rich in monounsaturated oleic acid (Troncoso-Ponce et al. 2011, Kilaru et al. 2015, Huang et al. 2016, Yao et al. 2016). In contrast, the SAD transcript levels in oil palm and date palm mesocarp (Bourgis et al. 2011), which contained a high content of saturated FA (>60% of total FAs), were not more than 2-fold higher than that of MCMT.

High transcript levels for SAD were related to its low catalytic efficiency at a turnover rate of 0.5 s^{-1} (Shanklin and Cahoon 1998, Rogge and Fox 2002). Previous biochemical and molecular evidence demonstrated that the activity of SAD significantly affected the ratio of saturated to unsaturated FAs and there was a positive correlation between the oleic acid level and the activity of SAD where the FAB2 isoform is a key

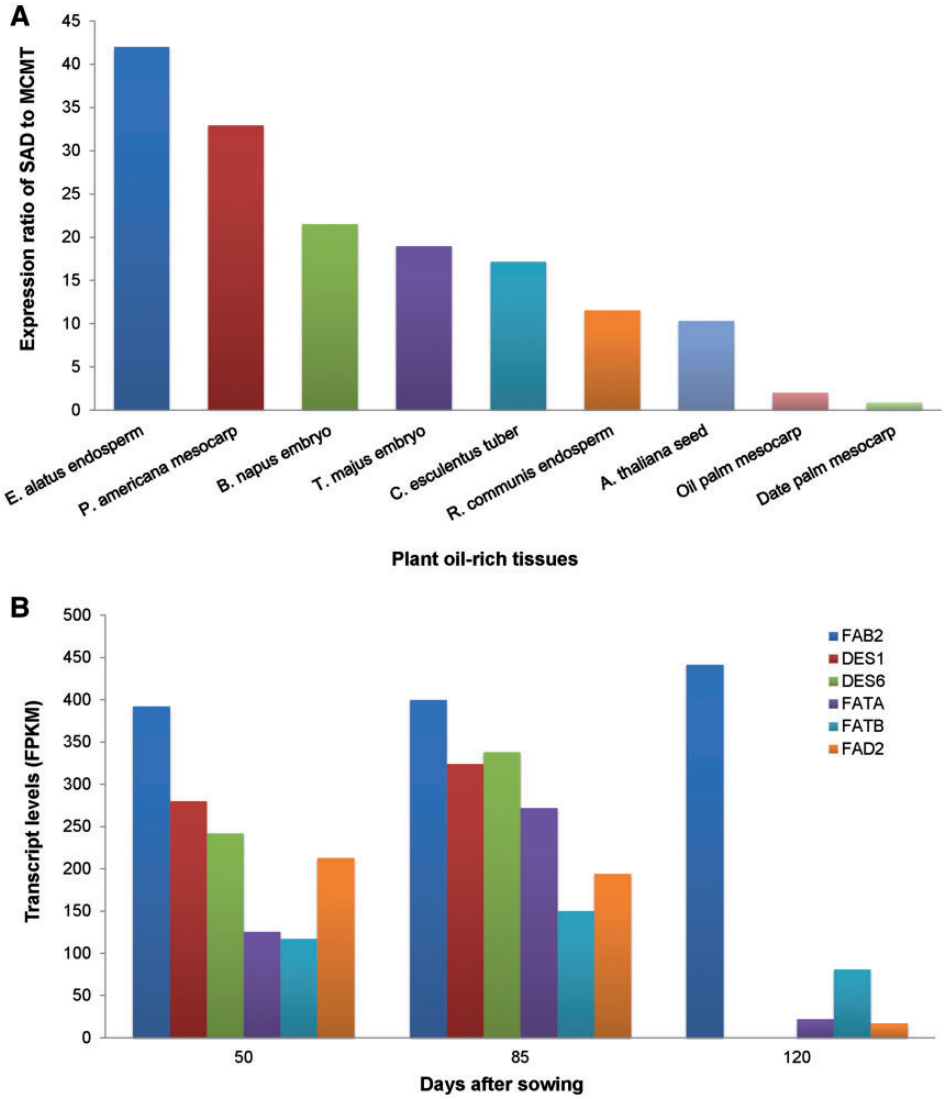

Fig. 7 Expression pattern for some desaturases and acyl-ACP thioesterases. (A) The ratio for average transcript levels of SAD to MCMT in different oil-rich tissues and in diverse species. (B) Temporal changes in transcript levels for enzymes of stearoyl-ACP desaturase isoforms (FAB2, DES1 and DES6), acyl-ACP thioesterase (FATA and FATB) and oleate desaturase FAD2 during tuber development.

determinant (Knutzon et al. 1992, Slocombe et al. 1992, Kachroo et al. 2007, Schluter et al. 2011, Klinkenberg et al. 2014, Zhang et al. 2015). In *C. esculentus* tubers, three isoforms of SAD including FAB2, DES1 and DES6 were detected, of which the transcript for the FAB2 ortholog is the most abundant (**Fig. 7B**). The highest transcript level for the orthologs of FAB2 was also noted for other oleic acid-rich seeds such as rape, nasturtium, hickory and camellia, as well as fruit mesocarp of avocado (Troncoso-Ponce et al. 2011, Kilaru et al. 2015, Huang et al. 2016, Yao et al. 2016), indicating a vital function conserved in the formation of oleic acid and also a conserved evolutionary relationship for this SAD isoform. In addition, transcripts for FAB2 showed a steady up-regulation pattern with higher levels than those of the orthologs of DES1 and DES6 during tuber development, which was consistent with the variation in oleic acid content (**Fig. 1C**), implying an important contribution of FAB2 to oleic acid synthesis.

High transcript levels of ER-associated acyl-CoA synthetase may indicate an important role in the export of fatty acids between the plastid and the ER in tubers

Newly synthesized free FAs need to be exported and converted to acyl-CoA by LACS so as to provide activated acyl groups as substrates for FA-derived metabolic pathways such as phospholipid and TAG biosynthesis occurring in the ER, indicating the critical roles of LACS in FA metabolism (Schnurr et al. 2002, Shockey et al. 2002). In Arabidopsis, a family of nine genes encoding LACS protein has been identified to play their roles in various aspects of lipid metabolism at different subcellular localizations. Among these LACS isoforms, LACS1, LACS2 and LACS3 are localized to the ER and involved in surface lipid synthesis (Shockey et al. 2002, Zhao et al. 2010, Yang et al. 2012). LACS1 also contributes to oil biosynthesis in developing

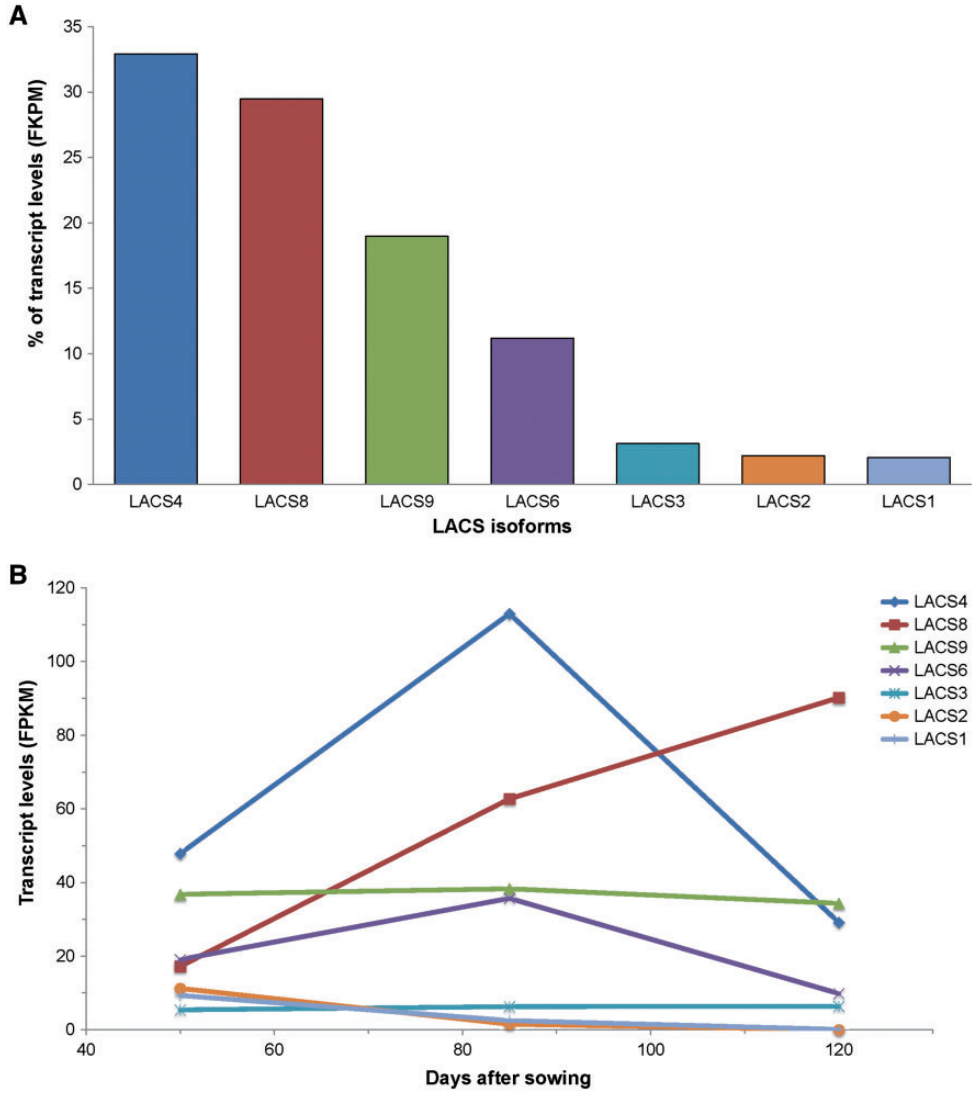

Fig. 8 Transcripts for long-chain acyl-CoA synthetase genes. (A) Relative distribution of average transcript levels for isoforms of long-chain acyl-CoA synthetases (LACSs) involved in fatty acid transfer. (B) Temporal changes in transcript levels for isoforms of LACS.

seeds. LACS5 was floral tissue specific (Shockey et al. 2002) while peroxisomal LACS6 and LACS7 were associated with FA β-oxidation (Fulda et al. 2002). Plastidial LACS9 is the most highly expressed and regarded as the major LACS isoform that is involved in export of FAs from plastids (Schnurr et al. 2002). ER-associated LACS4 or LACS8 in Arabidopsis was previously shown to have an overlapping function with LACS9, despite different subcellular localizations (Jessen et al. 2015).

In *C. esculentus* tuber, ER-associated LACS1, LACS2 and LACS3 orthologs were poorly expressed, while LACS5 was not detectable, suggesting less contributions of these isoforms to oil biosynthesis. The most transcribed is the LACS4 ortholog, which is followed by LACS8 and LACS9 (**Fig. 8A**). This was in contrast to other oil-rich seeds and non-seed mesocarps of oil palm (Bourgis et al. 2011; Troncoso-Ponce et al. 2011), where LACS9 transcripts were most abundant. In *C. esculentus* tuber, ER-associated LACS isoforms (LACS4 and LACS8) represented approximately 70% of the transcripts of LACS orthologs, while the plastidial LACS9 ortholog was only 19%. The much higher expression for ER-associated LACS than plastidial LACS also existed throughout tuber development (**Fig. 8B**), suggesting an important role for ER-related LACS in the export of FAs between the plastid and ER in tubers, similar to the case in oil mesocarp of avocado (Kilaru et al. 2015). The LACS8 ortholog was the exception that was continuously up-regulated and accounted for >50% at tuber maturity.

Transcriptional patterns for most enzymes involved in TAG biosynthesis in tubers are similar to those of oil seeds and fruits

TAG biosynthesis and assembly have been well documented in the oil seeds and fruits of oleaginous plants. TAG biosynthesis is involved in a series of acylations of glycerol-3-phosphate (G3P) with acyl-CoA and late dephosphorylation. This occurs mainly in the ER. In Arabidopsis, at least 36 enzymes and two proteins

are involved in oil biosynthesis (Beisson et al. 2003). Twenty-eight of these enzymes are encoded by single genes, and the remaining eight enzymes and the two proteins are encoded by small gene families.

In *C. esculentus* tuber, the essential proteins for TAG synthesis via the Kennedy pathway were identified and all were expressed during tuber development (**Fig. 9**; **Supplementary Table S3**). These protein orthologs included the glycerol-3-phosphate acyltransferase (GPAT), lysophosphatidicacid acyltransferase (LPAAT), phosphatidic acid phosphatase (PAP) and diacylglycerol acyltransferase (DGAT). Similar to other oil-rich tissues in diverse species, the expression levels for TAG synthesis enzymes were also relatively low in *C. esculentus* tuber, with on average 7-fold less than that of FA synthesis (**Figs. 2B**, **3**). Nevertheless, the temporal expression for TAG synthesis displayed patterns similar to those of plastidial FA synthesis, suggesting a co-ordinated temporal pattern between plastid FA and non-plastidial oil synthesis.

Other similar features were also noted in tubers as well as oil seeds and fruits. For example, the orthologs of DGAT2 and phospholipid:diacylglycerol acyltransferase 1 (PDAT1) were all detectable in diverse plant oil-rich tissues. DGAT ortholog was highly expressed while phosphatidylcholine:diacylglycerol cholinephosphotransferase (PDCT) was expressed at relatively low levels (**Fig. 9B**). The expression of GPAT9 was more highly transcribed than other isoforms of the family, i.e. GPAT1–GPAT8. Between the two LPAAT forms, the transcript for LPAAT2 was the most abundant (**Supplementary Table S3**). Several enzymes and/or proteins were represented by only a single ortholog, such as GPAT9, DGAT1, PDCT and FAD2 (**Fig. 9A**), suggesting that these orthologs are conserved for TAG synthesis in seed and non-seed tissues. Others encoded by a single gene included LPAAT5 and two isoforms of lipid phosphate phosphatase (LPP), LPP-β and LPP-δ.

Together, all these facts indicated that most TAG biosynthesis genes show similar expression patterns among diverse oil-rich tissues.

LPP-δ isoform is most highly expressed among ER phosphatidic acid phosphatases in *C. esculentus* tuber

PAP dephosphorylates phosphatidic acid (PA) by removing the phosphate group to form diacylglycerol (DAG), which is a precursor for both phospholipid and TAG biosynthesis (Bates et al. 2013). In Arabidopsis, two types of PAP were proposed for their role in de novo DAG synthesis. They belong to the soluble cytosolic phosphatidic acid phosphohydrolase (PAH) and membrane-bound LPP subfamily, respectively (Eastmond et al. 2010, Craddock et al. 2015). However, it is still unclear which isoforms of PAP contribute to TAG synthesis. A double knockout of two genes, PAH1 and PAH2, only resulted in a 15% reduction in seed FA levels. It was therefore implied that Arabidopsis PAH1 and PAH2 were apparently not involved in TAG synthesis, and other PAP isozymes were the major contributors (Bourgis et al. 2011, Bates et al. 2013). Two membrane-bound LPP isoforms, LPP-β and LPP-δ were suggested to be more probable candidates for generating DAG destined to be converted to TAG (Bourgis et al. 2011).

Among the various orthologs that code for PAP in *C. esculentus* tuber, PAH2 and LPP-β were transcribed at low levels and their levels were less changed or down-regulated during tuber development (**Fig. 10**). PAH1 had moderate transcript levels; however, it showed down-regulation during the development of the tuber. In contrast, LPP-δ showed a pattern that correlated with oil synthesis and its transcript levels on average were most abundant and were 2- to 10-fold higher than those of other PAP isoforms. That was quite different from oil seeds and fruits, where PAH2 or LPP-β was the predominantly transcribed isoform among PAP orthologs. Thus, it is likely that LPP-δ plays an important role in formation of the DAG pool for oil synthesis in *C. esculentus* tuber.

DGAT2 and PDAT1 may play crucial roles in tuber oil biosynthesis

TAG is biosynthesized de novo via two different pathways in the ER. One is acyl-CoA dependent that uses acyl-CoA as the acyl donor and DAG as the acceptor (Kennedy pathway) (Kennedy 1961). Another is acyl-CoA independent that uses phospholipids as acyl donors and DAG as the acceptor which is catalyzed by PDAT (Dahlqvist et al. 2000), or uses two DAGs as donors and acceptors catalyzed by diacylglyceroltransacylase (DGTA) (Lung and Weselake 2006).

In the conventional acyl-CoA-dependent Kennedy pathway, FAs are introduced into DAG at the *sn*-3 position catalyzed by DGAT. DGAT is regarded as a key enzyme and rate-limiting regulator for TAG biosynthesis (Lung and Weselake 2006). Two families of membrane-bound DGAT with no essential homology to each other, namely DGAT1 (type-1) and DGAT2 (type-2), are present in most oil plants. In this study, one copy of DGAT1 and two copies of DGAT2 orthologs were identified in *C. esculentus* tuber (**Supplementary Table S3**). Between the two DGAT isoforms, DGAT2 was transcribed most abundantly in the tuber, with a $>$ 3-fold increase within a narrow range, and showed a highly correlated temporal expression pattern with oil synthesis (**Fig. 10**). In contrast, DGAT1 was down-regulated at low transcript levels and was on average 7-fold less abundant than DGAT2. The relatively low transcript and expression pattern of DGAT1 in tubers were sharply in contrast to oil seeds and fruits, where DGAT1 orthologs were more abundantly expressed than DGAT2 and were up-regulated consistent with oil accumulation in developing oil-rich tissues, suggesting that DGAT1 is the predominant enzyme synthesizing TAG in oil seeds and fruits (Bourgis et al. 2011, Troncoso-Ponce et al. 2011, Kilaru et al. 2015).

DGAT2 homologs were usually abundant in oil seeds of epoxy and hydroxy FA-accumulating plant species (*Vernonia galamensis*, *Euphorbia lagascae*, *Stokesia laevis* and *Ricinus communis*) (Kroon et al. 2006, Burgal et al. 2008, Li et al. 2010) and in those of eleostearic acid-rich tung plants (*Vernicia fordii*) (Shockey et al. 2006), and also in oil-rich mesocarps of olive (*Olea europaea*) (Alagna et al. 2009, Banilas et al. 2011) and oil palm (Bourgis et al. 2011, Tranbarger et al. 2011) for producing

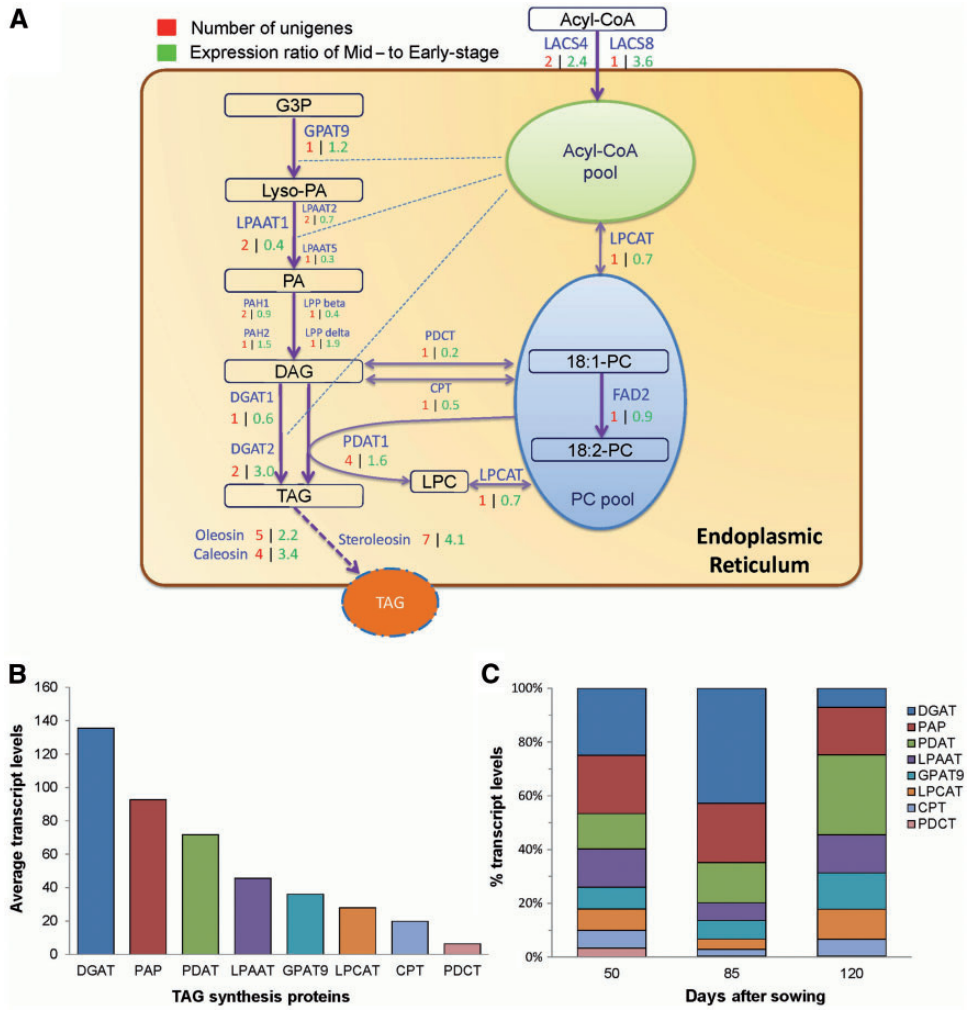

Fig. 9 Expression levels for genes associated with triacylglycerol (TAG) synthesis. (A) Schematic diagram of the fatty acid synthesis pathway. Enzyme or protein names are indicated in blue. (B) Relative distribution of transcript levels of enzymes of TAG synthesis in plant oil-rich tissues. The data are averaged on all the developing stages of seeds or fruits or tubers. The transcript values for subunits of a protein and for multiple isoforms were summed. (C) Relative distribution of transcript levels for TAG synthesis enzymes during tuber development. The FPKM values for subunits of a protein and for multiple isoforms were summed.

normal oil. DGAT2 orthologs in the oil tissues with highly accumulated unusual FAs were suggested to be required for incorporation of unusual fatty acids such as hydroxylated acyl chains and eleostearic acids into TAG.

The acyl-CoA-independent pathway for TAG synthesis is involved in either PDAT that uses phosphatidylcholine (PC) as the acyl donor where a fatty acyl moiety was directly transferred from PC to DAG (Dahlqvist et al. 2000), or DGAT that catalyzes the transfer of an acyl moiety between two DAG molecules to form TAG and monoacylglycerol (MAG) (Lung and Weselake 2006). Four unigenes encoding PDAT1-like protein (AT5G13640) were identified while no DGAT orthology was detectable. As shown in **Fig. 10**, the expression levels for PDAT1 were much higher than that of DGAT1 during tuber development and its transcription was correlated positively with oil synthesis, suggesting the important role that PDAT played in tuber TAG production. In contrast, in oil seeds and fruits, PDAT showed transcript levels that were much lower or comparable with the levels of the different DGATs (Bourgis et al. 2011, Troncoso-Ponce et al. 2011, Kilaru et al. 2015). In Arabidopsis, the role of PDAT in overall TAG synthesis remained unclear; however, evidence has indicated that PDAT and DGAT1 have overlapping functions in seed oil production (Zhang et al. 2009), suggesting a co-operation between PDAT and DGAT pathways in TAG synthesis in plant oil-rich tissues (Chapman and Ohlrogge 2012).

PC-mediated DAG production and acyl editing for TAG biosynthesis in *C. esculentus* tuber

Apart from donating fatty acyl directly for the DAG *sn*-3 position to produce TAG by the action of PDAT, PC has two other ways to channel FAs for TAG synthesis. One is the DAG production derived from PC as the substrate for TAG synthesis, which is through the reversible catalytic action of diacylglycerol cholinephosphotransferase (PDCT/ROD1) (Lu et al. 2009)

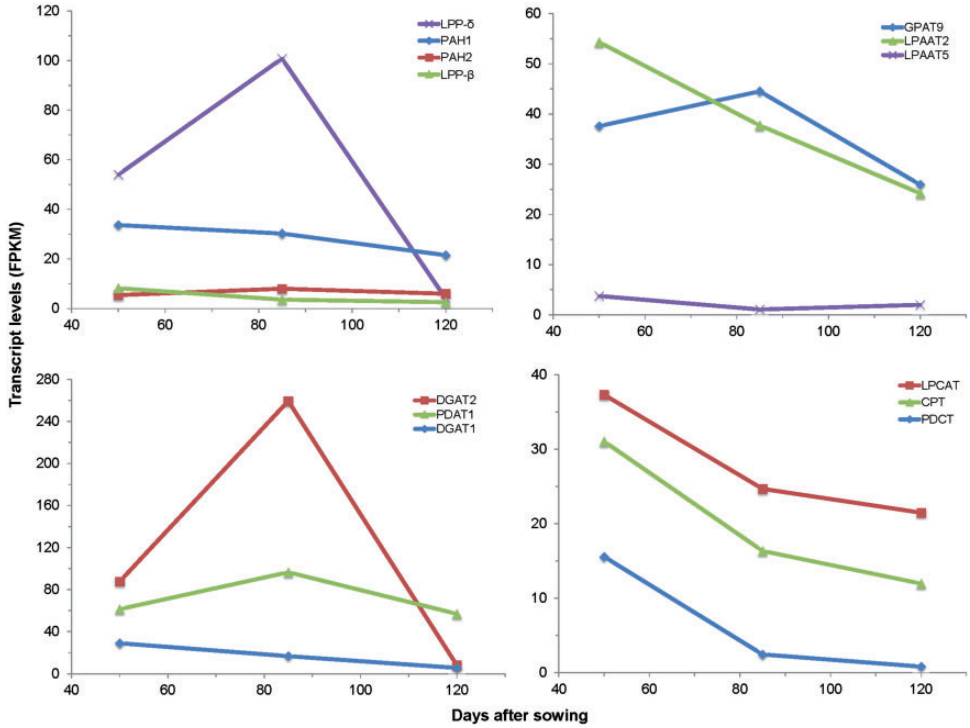

Fig. 10 Temporal changes in transcript levels for triacylglycerol (TAG) synthesis enzymes during tuber development.

and/or cytidine-5′-diphosphocholine:DAG cholinephosphotransferase (CPT) (Slack et al. 1983), or possibly by the irreversible action of phosphatidylcholine-hydrolyzing phospholipase C (PLC). The other way is acyl editing where PC exchanges its FAs with the acyl-CoA pool, which is catalyzed by lysophosphatidylcholine acyltransferase (LPCAT) (Bates et al. 2007).

In *C. esculentus* tuber, single copes of PDCT, CPT and LPCAT orthologs were identified (**Fig. 9A; Supplementary Table S3**). Similar to most oil-rich seeds and fruits, the ortholog of CPT was expressed more highly than that of PDCT. Both CPT and PDCT orthologs were down-regulated during the development of the tuber, which showed a different expression pattern from that of PDAT. Furthermore, the total transcript levels for CPT and PDCT were on average 3-fold less than that of PDAT. It is noteworthy that in *C. esculentus* tuber, the overall expression levels for PDAT together with CPT and PDCT were on average comparable with and slightly higher than those of PAP orthologs that generated a DAG pool via the Kennedy pathway, suggesting an important role for PC as an intermediate in TAG synthesis in *C. esculentus* tuber.

PC-mediated acyl flux can be also carried out by acyl editing, which is involved in a PC deacylation and lysophosphatidylcholine reacylation cycle which exchanges the modified FA on PC with the acyl-CoA pool without net PC synthesis or degradation (Bates et al. 2013). In *C. esculentus* tuber, a single ortholog of LPCAT was expressed in a pattern similar to that of CPT and PDCT (**Fig. 10**; **Supplementary Table S3**), i.e. it was down-regulated during the development of the tuber. Interestingly, FAD2 ortholog also showed a down-regulation transcript pattern. The transcript levels for LPCAT and FAD2 were decreased by >40% and 90%, respectively at tuber maturity, relative to the earlier stages of tuber development. Coinciding with the changes in transcripts for LPCAT and FAD2, the contents of polyunsaturated FAs dominated by C18:2 declined by almost 40% from 27.07% to 16.81% of total FAs (**Fig. 1C**). In contrast to most oil seeds and mesocarp of oil palm, where FAD2 transcript levels are >10-fold higher than that of GPAT9 (Bourgis et al. 2011; Troncoso-Ponce et al. 2011), the transcript levels for FAD2 ortholog in *C. esculentus* tuber were only on average four times higher than that of GPAT9, comparable with avocado mesocarp (Kilaru et al. 2015).

Taken together, our study showed that, as in other non-seed oil-rich tissues, PC-mediated acyl flux in *C. esculentus* tuber may also play an additional and important role in TAG biosynthesis.

High expression of TAG storage genes and low transcripts of TAG degradation genes are associated with high oil contents in *C. esculentus* tuber

Once synthesized, TAG is sequestered and stored in the form of oil bodies with a hydrophobic core mainly consisting of TAG that is surrounded by a monolayer membrane composed of phospholipid-containing proteins such as oleosin, caleosin and steroleosin (Huang 1996, Frandsen et al. 2001, Lin et al. 2002).

We identified six unigenes that encoded oleosin, four encoding caleosin and seven encoding steroleosin in *C. esculentus* tuber (**Fig. 9A; Supplementary Table S3**), as in most oil seeds where the orthologs of these three proteins were encoded by more than one gene. One unigene encoding a homolog of lipodystrophy protein SEIPIN was also detected. Three SEIPIN

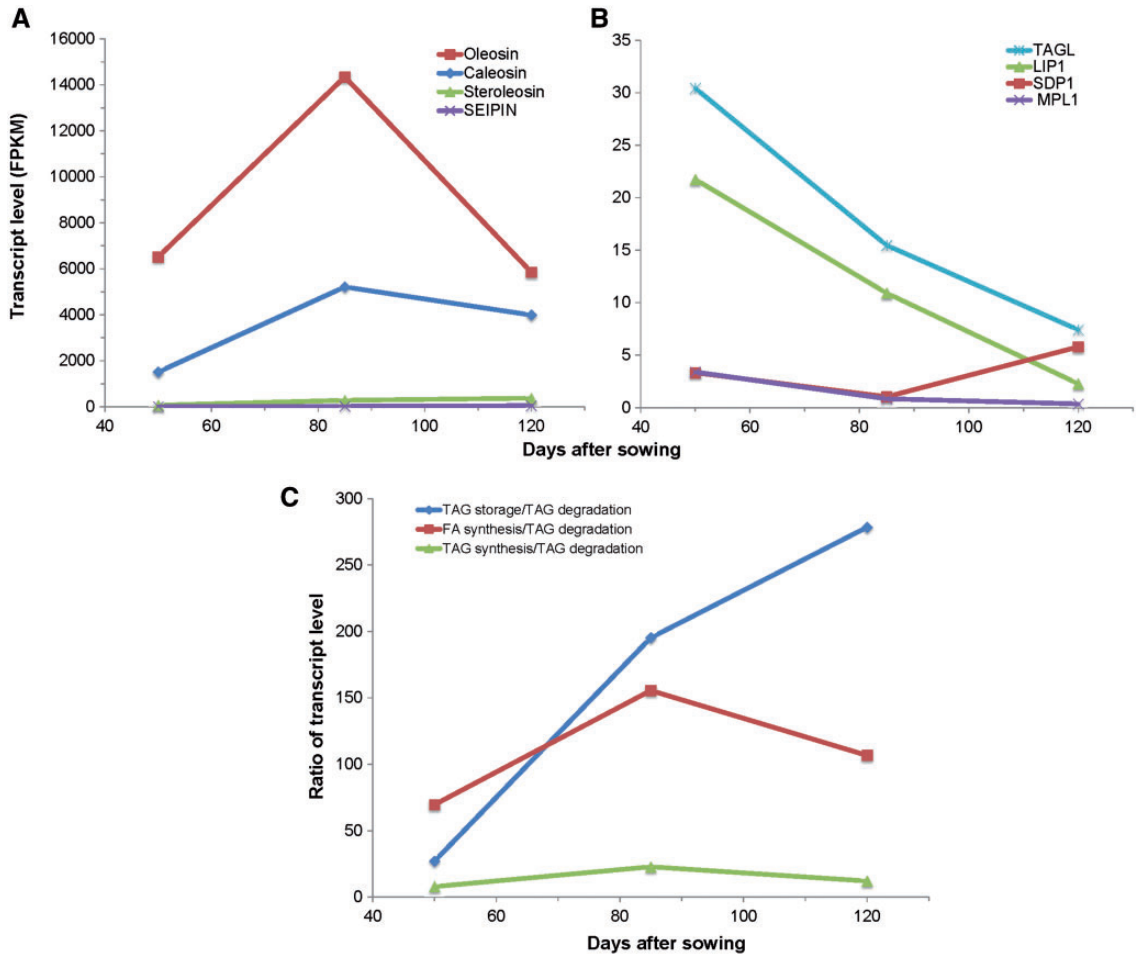

Fig. 11 Expression levels for genes associated with triacylglycerol (TAG) storage and degradation. Temporal changes in transcript levels for TAG storage (A) and degradation (B) enzymes. The ratio of expression of enzymes for fatty acid synthesis, TAG synthesis and TAG storage relative to TAG degradation is also displayed (C).

homologs were recently reported in *Arabidopsis thaliana* that might play an important role in modulating the number and sizes of oil bodies (Cai et al. 2015). The existence of so many different orthologs of oil body proteins in vegetative tissues is surprising, since it was thought for a long time that these proteins are expressed exclusively in seeds as well as pollen grains and they are almost entirely absent in vegetative tissue/cell types (Huang 1996, Levesque-Lemay et al. 2016). Indeed, several recent studies have shown that oil mesocarps of oil palm, olive and avocado had very low expression of seed-like oil body proteins (Banilas et al. 2011, Bourgis et al. 2011, Kilaru et al. 2015).

However, as in the oil seeds, the genes related to the oil body proteins in tuber were very highly expressed and up-regulated during tuber development (**Fig. 11A**), a pattern quite distinct from that of FA and TAG synthesis. In *C. esculentus* tuber, transcripts for the ortholog of oleosins were the most abundant, followed by caleosins and steroleosins in that order, similar to the case in oil seeds (Troncoso-Ponce et al. 2011, Huang et al. 2016). The expression levels of oleosins in tuber were on average >2- to 30-fold higher than those of caleosins or steroleosins (**Fig. 11A**). These data sugguested that the mechanism of oil storage in *C. esculentus* tuber was conserved and similar to that of plant oil seeds. This is, however, quite different from the oil-rich fruits of olive, oil palm and avocado (Banilas et al. 2011, Bourgis et al. 2011, Kilaru et al. 2015). In oil palm and avocado mesocarps, the transcripts for the orthologs related to oil body proteins decreased or remained constant at very low levels during development and, relatively, the caleosin ortholog was most highly expressed among oil body proteins, indicating that these seed-like oil body associated proteins are not essential for the stabilization of TAG in oil fruit tissues. It was suggested that other lipid droplet-associated proteins (LDAPs) were most likely to play a significant role in stabilizing TAG during fruit development (Kilaru et al. 2015).

Increasing evidence suggested that oil catabolism is also active during seed development (Graham 2008), and therefore oil content is regulated by the dynamic balance between both synthesis and breakdown. Indeed, several investigations have shown that suppression of TAG hydrolysis lipases such as patatin-like acyl-hydrolase SUGAR-DEPENDENT1 (SDP1) leads to a substantial accumulation of TAG (Eastmond 2006, Kelly et al. 2013). In *C. esculentus* tuber, orthologs of a large family of TAG lipases (TAGLs) were detectable, including one copy of SDP1 and lipase 1 (LIP1), four copies of MPL1 (*Myzus persicae-*

induced lipase 1) and other TAGLs (**Supplementary Table S3**). Furthermore, these orthologs of TAGLs were expressed at various levels during tuber development, suggesting that oil breakdown was also active in the developing tuber. However, these TAGL orthologs were down-regulated and transcribed at very low levels (<35 FPKM for each) relative to TAG synthesis or TAG storage throughout tuber development (**Fig. 11B**). The expression levels for TAGL orthologs were on average 12- and 100-fold less than that of TAG synthesis and TAG storage, respectively (**Fig. 11C**). Collectively, these results might indicate that the high expression of TAG storage genes together with very low expressions for TAG degradation genes could ensure the high oil content in *C. esculentus* tuber.

WRI1-like transcription factor is poorly expressed in *C. esculentus* tuber

In seed tissues, transcription factor WRINKLED1 (WRI1) is a member of the APETALA2-ethylene-responsive element-binding protein (AP2/EREBP) family and plays a vital role in oil accumulation by regulating glycolysis and FA biosynthesis (Cernac and Benning 2004, Baud and Lepiniec 2009, Marchive et al. 2014). WRI1 is directly or indirectly under the control of several embryogenesis and seed maturation master regulators such as LEAFY COTYLEDON (LEC) genes LEC1, LEC1-like (L1L), LEC2 and FUSCA3 (FUS3), and ABA-insensitive3 (ABI3) by binding to their promoter sequences (Baud et al. 2007, Baud and Lepiniec 2009).

Of > 200 putative transcription factors detected in the tuber transcriptome (**Supplementary Table S2**), a single gene-encoded transcription factor (AT3G54320) was found having a very high similarity to Arabidopsis WRI1 (E-value $<10^{-102}$) and one from maize (E-value $<10^{-96}$). Unexpectedly, the WRI1-like protein was transcribed at very low levels in oil tuber, with an FPKM value of <6 throughout the whole of tuber development (**Fig. 12**), suggesting that WRI1 transcriptional expression is not a prerequisite for oil synthesis. This is quite distinct from the oil-rich mesocarp of oil palm and avocado (Bourgis et al. 2011, Tranbarger et al. 2011, Dussert et al. 2013, Kilaru et al. 2015), where WRI1 homologs were highly expressed. The finding of low transcripts for WRI1 ortholog in tuber was comparable with that of oil seeds, in which the transcript levels for orthologs of WRI1 were not abundant in developing oil seed tissues, especially in the developing embryo of *Tropaeolum majus* and endosperm of *Euonymus alatus* in which WRI1 was expressed at very low levels (Troncoso-Ponce et al. 2011). All these results thus indicated that the relationships between WRI1 transcription and oil accumulation are complex and need to be further clarified in oil-rich seed and tuber.

It is noteworthy that in *C. esculentus* tuber, however, expression of the WRI1 ortholog was down-regulated during tuber development (**Fig. 12**), and, like in oil seeds, the temporal expression was well matched with the patterns of its putative target genes involved in FA synthesis and glycolysis such as MCMT, KAR, HAD, ENR, ACP, KASII and FAD2 as well as protein subunits PDH-α, BCCP1, plastid pyruvate kinase beta 1 (pPK-β1) and one isoform of fatty acid elongase (FAE, AT2G26640).

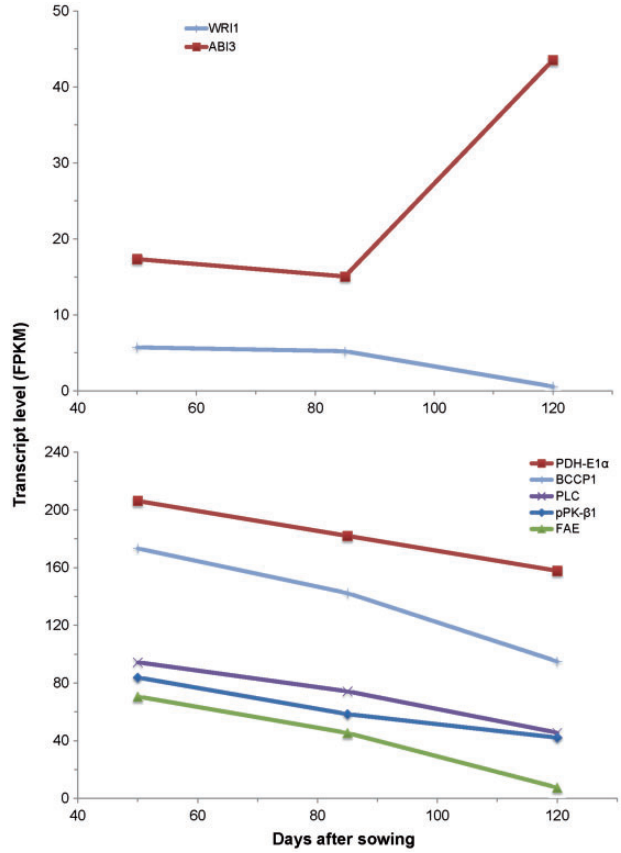

Fig. 12 Temporal changes in transcript levels for WRI1-like protein and its target genes during tuber development.

Interestingly, the expression of the WRI1 ortholog showed a temporal pattern similar to that of PDCT (**Fig. 10**) and a non-specific PLC (**Fig. 12**), which is important for the conversion of PC to DAG, supporting the evidence that PDCT is also a direct target of WRI1 (Adhikari et al. 2016). Previously, it was observed that transcript levels of PDCT in *wri1 wri3 wri4* triple mutant seeds were lower than in the wild type (To et al. 2012) and transient overexpression of WRI1 in *Nicotiana benthamiana* leaves greatly up-regulated the transcript levels of PDCT and PLC (Grimberg et al. 2015). All these results demonstrated that the role of WRI1 and its homologs in oil biosynthesis is highly conserved among diverse oil-rich tissues.

In contrast to seed tissues, where WRI1 is controlled by master regulators such as LEC1, LEC2 and FUS3, orthologs of these upstream regulators were either not found or were barely detectable in *C. esculentus* tuber, similar to oil mesocarp of oil palm and avocado (Bourgis et al. 2011, Kilaru et al. 2015). An ortholog of ABI3 (At3g24650) was identified in this study; however, the transcripts were much more abundant than WRI1 and displayed a different temporal pattern. These data strongly indicate that the WRI1-related regulatory networks in non-seed tissues are different from those in seeds, and are possibly tissue specific.

Other WRI1-like proteins including WRI2 (AT2G41710), WRI3 (At1g16060) and WRI4 (At1g79700) were reported in seeds of Arabidopsis (To et al. 2012). In Arabidopsis, WRI2 was observed to be unlikely related to FA biosynthesis, whereas WRI3 and WRI4 can complement the *wri1* mutant and are required for flower and stem cutin synthesis through control of FA synthesis (To et al. 2012). In the mesocarp of avocado, an isoform of WRI2 was also highly expressed in addition to WRI1, suggesting that the WRI2 ortholog may play an additional role in TAG accumulation in non-seed tissues (Kilaru et al. 2015). However, no obvious orthologs for WRI2, WRI3 and WRI4 were identified in *C. esculentus* tuber.

Taken together, our data provide a strong indication that the function of the WRI1 ortholog in FA biosynthesis is highly conserved between monocots and dicots, but distinguishable in different plant species. It is most probably that a specific regulatory mechanism of the WRI1 ortholog, or other transcription factors, in addition to WRI1, regulate oil accumulation in *C. esculentus* tuber.

Expression of the glycolysis pathway providing carbon for fatty acid synthesis in *C. esculentus* tuber is similar to that of non-green heterotrophic oil seeds

In plants, carbon assimilation that involves sucrose metabolism followed by glycolysis and transport of its intermediates to the plastids plays a crucial role in providing carbon for FA synthesis (Plaxton 1996, Fischer and Weber 2002). Glycolysis is a central metabolic pathway that provides energy and carbon in the form of pyruvate for FA synthesis. In plants, glycolysis occurs in both the cytosol and plastids (Plaxton 1996).

In *C. esculentus* tuber, orthologs associated with sucrose synthase (SuSy) were highly expressed, with around 2,500 FPKM/enzyme on average and >25-fold higher than neutral invertases (N-INVs) (**Supplementary Table S4**). In addition, the transcript levels for fructokinase (FK) were also higher than those for hexokinase (HXK) (**Supplementary Table S4**; **Fig. 13A**). This is quite similar to oil seeds (Troncoso-Ponce et al. 2011), but differernt from oil mesocarp of oil palm and avocado that exhibited relatively high transcriptional activity for invertase and hexokinase (Bourgis et al. 2011, Dussert et al. 2013, Kilaru et al. 2015).

Along with high transcript levels for SuSy, the transcripts for cytoplasmic orthologs of UDP-glucose pyrophosphorylase (UGP), FK and pyrophosphate-dependent phosphofructokinase (PFP) were more abundant than that of hexokinase (**Fig. 13**), suggesting that cytosolic glycolysis might use more fructose as a substrate. In plastid, the orthologs of HXK, glucose-6-phosphate isomerase (GPI) and ATP-dependent 6-phosphofructokinase (PFK) were expressed much less than in the cytosol, pointing to the fact that the early glycolysis in plastids most probably used fructose as the major substrate.

Our transcriptome analysis indicated that in *C. esculentus* tuber, orthologs of cytosolic glycolytic enzymes were much more highly expressed than the plastidial isoforms in every case (**Fig. 13**). The cytosolic glycolytic enzymes were expressed with transcript levels >5- to 60-fold higher than their counterparts in plastids, demonstrating that a complete glycolytic pathway may occur primarily in the cytosol. This is similar to the non-green heterotrophic oil seeds (Troncoso-Ponce et al. 2011), and different from the green photoheterotrophic seeds (such as rape and soybean) (Agrawal et al. 2008) and oil-rich mesocarps of oil palm and avocado where the distribution was more balanced between the two glycolytic compartments (Bourgis et al. 2011, Dussert et al. 2013, Kilaru et al. 2015). It was suggested that carbon sources produced mostly through the cytosolic glycolytic pathway were probably imported into plastids and converted into pyruvate for further FA synthesis (Dussert et al. 2013). Consistent with this hypothesis, a high level of transcripts for the orthologs of plastidial transporters for glucose (GLT), glucose-6-phosphate (GPT), triose phosphate (TPP), phosphoenolpyruvate (PPT), dicarboxylate (DiT), pyruvate (BASS) and adenine nucleotide transporter (NTT) with a total value of >1,000 FKPM, on average, was noted in *C. esculentus* tuber (**Supplementary Table S4**).

It is noteworthy that the transcripts for the orthologs of fructose-bisphosphate aldolase (FBA) and glyceraldehyde-3-phosphate dehydrogenase (GAP) were more abundant in both the cytosol and plastids (**Fig. 13A**), suggesting that the glycolysis involved in steps from fructose-1,6-bisphosphate to 1,3-bisphospho-d-glycerate, is highly active in the cytosol and plastids. The high abundance of transcripts for the orthologs of FBA and GAP were also observed in other seeds and fruits (Bourgis et al. 2011, Troncoso-Ponce et al. 2011, Dussert et al. 2013, Kilaru et al. 2015), implying an evolutionarily conserved role for these two enzymes in glycolysis among oil-rich tissues of dicot and monocots.

Conclusions

As a unique tuberous plant, *C. esculentus* accumulates significant amounts of oil and starch as major storage products in tuber, in contrast to common tuber and root crops such as potato and sugar beet that exclusively produce carbohydrates as the main storage reserves. Therefore, *C. esculentus* is an ideal model plant for studying carbon partitioning toward oil biosynthesis in underground non-seed tissues. For the first time, we have established a public transcriptomic framework and information for an oil-rich Cyperaceae plant with immense nutritional and health importance. In this study, we carried out comprehensive transcriptome analyses of lipid metabolism, with special reference to genes involved in TAG biosynthesis from sucrose degradation and leading up to TAG accumulation in oil tuber, and compared them with those of above-ground oil-rich tissues of seeds and fruits (oil palm, avocado and olive) to uncover the underlying mechanism for the regulation and biosynthesis of TAG in tuber plants.

Our results showed that the core metabolic pathways from pyruvate generation to TAG biosynthesis are similar in different oil-rich tissues and in diverse species, and the predominant genes related to these core pathways are functionally conserved, supporting the suggestion that a common set of gene isoforms is conserved for TAG biosynthesis throughout

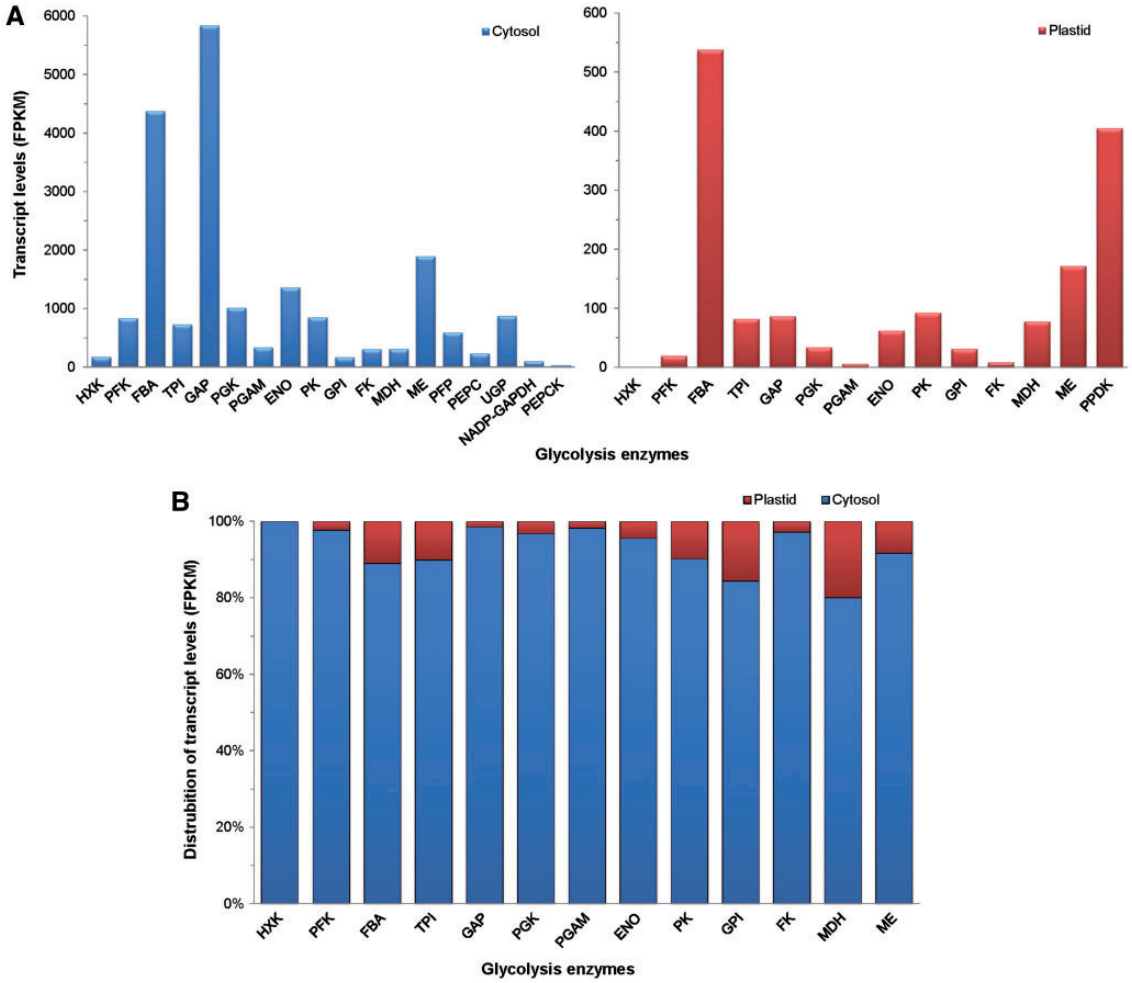

Fig. 13 Transcript levels for glycolysis enzymes in the cytosol (A) and plastid (B), and the relative distribution between them (C). The levels represented by FPKM are averaged based on the transcript levels of all developing stages of tuber. The FPKM values for subunits of a protein and for multiple isoforms were summed.

plant evolution (Bourgis et al. 2011, Troncoso-Ponce et al. 2011, Dussert et al. 2013, Kilaru et al. 2015). The high level of transcripts of carbohydrate metabolism followed by moderate expression for FA synthesis with relatively low transcripts for TAG synthesis in *C. esculentus* tuber are also similar to those of oil seeds and fruits. Nevertheless, the transcriptional patterns for these metabolic pathway genes in *C. esculentus* tuber resemble much more those observed in oil seeds, particularly in non-green heterotrophic oil seeds. For example, in both *C. esculentus* tuber and non-green heterotrophic oil seed are displayed much higher transcript levels for cytosolic glycolytic enzymes than the plastidial isoforms in every case, down-regulation for most FA synthesis genes and less change or a slight decrease in transcripts for most TAG synthesis genes, as well as up-regulation and abundant transcripts for TAG storage genes during the development of tissues. In addition, the poor expression along with down-regulation of the WRI1 ortholog associated with its target genes also occur in these two different developing tissues.

However, several distinct gene expression patterns that are likely to be tissue or species specific are also noted in *C. esculentus* tuber. The orthologs for plastidial ACS, ER-associated LACS isoforms (LACS4 and LACS8) and LPP-δ, where their function in TAG biosynthesis remains obscure in seeds, were abundantly transcribed in *C. esculentus* tuber, in sharp contrast to those of oil-rich seeds and fruits. Therefore, the oil-rich tuber of *C. esculentus* could serve as an ideal plant material to determine the roles that these three enzymes play in acetyl-CoA formation, transportation of FAs and generation of the DAG pool for oil synthesis, respectively. Since the orthologs for DGAT2 and PDAT1 were highly expressed more than DGAT1 in tubers, this oleaginous tissue is thus also suitable to determine the roles of DGAT2 and PDAT1 and to explore the possibility of their overlapping function in catalyzing TAG synthesis. Four copies of the PDAT1 ortholog were also noted for *C. esculentus* tuber, while there was only a single ortholog of PDAT1 in oil seeds and fruits (Bourgis et al. 2011, Troncoso-Ponce et al. 2011, Kilaru et al. 2015). The transcripts for orthologs of multiple PDAT1 isoforms were abundant compared with that of DGAT1 (**Supplementary Table S3**). Function analysis for various isoforms of PDAT1 ortholog can be expected to clarify the roles of PDAT1 isoforms in oil biosynthesis of tuber.

In conclusion, our comprehensive transcriptome analyses of *C. esculentus* tuber along with comparison with other oil-rich tissues of seeds and fruits revealed both conserved and distinct species-specific expression patterns for oil biosynthesis genes. Such knowledge is of significance in the exploitation and use of this oil-rich species and has implications for manipulating non-seed tissues especially vegetative tissue of diverse species to increase storage oil production.

Materials and Methods

Plant materials and growth conditions

Cyperus esculentus we used is the cultivated variety of *Cyperus esculentus* L. var. *sativus* Boeck. Mature and dried seed tubers were stored in a refrigerator at 4 °C. Prior to germination, the seed tubers were soaked in warm water at 30–40°C for 3 d. Tubers were kept moist at 22 ± 0.5 °C and 40–60% relative humidity for germination in a growth chamber under conditions of cool-white fluorescent lighting at a level of 130 μmol m^2 s^{-1} with a 16 h light/8 h dark cycle. After approximately 2 d, tubers started to sprout and were left to develop for a week. Then emerging plants were transferred to cultivation pots containing nutrient soil. Plants were grown in the growth chamber under the same condition and were watered once per week with nutrient Murashige and Skoog (MS) solution. Fresh and tender tubers at different development stages were collected and immediately stored in liquid nitrogen for later experiments.

Fatty acid determination via direct methylation

Direct transmethylation of FAs into FAMEs for GC analysis was performed according to the modified method as described previously (Yang and Ohlrogge 2009). The FAME extracts were analyzed using gas chromatography with a GC-9900 apparatus (Beijing Jiafen Analysis Instrument Technology Co. Ltd.) with a flame ionization detector on a FFAP (a type of nitroterephthalic acid-modified polyethylene glycol) column (30 m length × 0.32 mm i.d., 0.5 μm film thickness).

Total RNA extraction

Total RNA was extracted from the developing tubers at 50, 85 and 120 DAS with three biological replicates for each development stage, pooled from at least 10 tubers. RNA from each pool was isolated using a modified cetyltrimethylammonium bromide (CTAB)-based method (Gambino et al. 2008). In brief, the extraction buffer [50 mM EDTA, 2 M NaCl, 2% CTAB, 2% PVP, 3% β-mercaptoethanol (added just before use), 100 mM Tris–HCl (pH 8.0)] was pre-heated at 65 °C and added to powdered tuber sample (buffer : sample=5: 1, v/w). After incubation for 30 min at 65 °C, samples were extracted twice in an equal volume of chloroform : isoamyl alcohol (24 : 1, v/v) by centrifuging at 12,000 × *g* for 10 min at 4 °C and the supernatant fraction was withdrawn. A half volume of 4 M LiCl was added into the supernatant and incubated for 4 h at −20 °C for RNA precipitation. By centrifuging at 12,000 × *g* for 10 min at 4 °C, the pellet was dissolved in RNase-free water and mixed with 3 M NaAc (water : NaAc=9 : 1, v/v). After that, two and half of volume of cool absolute ethanol were added and incubated for 1 h at −20 °C. RNA was pelleted (12,000 × *g* for 20 min at 4 °C), washed in cool 75% ethanol, dissolved in RNase-free water and stored in liquid nitrogen for later experiments.

cDNA library construction and deep sequencing

The yield and quality of RNA were determined using a NanoDrop 2000 spectrophotometer and an Agilent 2100 Bioanalyzer. The samples showed a value of 260/280 nm of 1.8–2.2, OD260/230 of approximately 2.0 and RNA integrity number (RIN) of ≥ 6.6. RNA samples were submitted to the Shanghai Major Biomedicine Technology Co. Ltd. for cDNA library construction and sequencing. Through isolation and enrichment of mRNA by polyadenylation using oligo(dT)-attached beads, mRNA was fragmented and reverse transcribed to cDNA and then was PCR amplified after end repair and adaptor ligation. The cDNA library with equimolar concentrations of 150 bp paired-ends was constructed using Illumina's TruSeq RNA sample preparation kit (Illumina Inc.) and was sequenced using the Illumina Hiseq4000 sequencing platform.

De novo transcriptome assembly and annotation

The generated raw sequencing reads were first filtered to remove adaptor reads, ambiguous reads with 'N' bases (N% >5%) and low-quality sequences (Phred Score <20 bases), yielding a data set consisting of clean reads. High-quality filtered reads from all samples were pooled and de novo assembled by using the Trinity program with version trinityrnaseq_r20140413, which was specifically suitable for de novo assembly of full-length transcripts (Grabherr et al. 2011). The filtered 150 bp paired-end read data in the FASTQ format were deposited in the National Center for Biotechnology Information's (NCBI) BioProject database as a Short Read Archive (SRA) under accession numbers PRJNA320781, PRJNA320787 and PRJNA312713. A pooled data set of contigs was generated by combining the filtered reads from the sequenced libraries and subjected to de novo assembly. The final assembly with sequence lengths longer than 200 bp was subsequently used for annotation and functional quantification.

Annotation for all the sequences (>200 bp) was performed using a BLAST homology search (hits with E-value cut-off ≤ $1e^{-5}$) against the databases of the NCBI NR protein database (ftp://ftp.ncbi.nlm.nih.gov/blast/db/), KEGG (http://www.genome.jp/kegg/), Swiss-Prot (http://www.ebi.ac.uk/uniprot/), STRING (http://string-db.org/), COG (http://www.ncbi.nlm.nih.gov/COG/) and GO (http://www.geneontology.org/). GO and KEGG pathway information for the annotated transcripts was assigned based on the homologous proteins.

The transcript levels for unigenes were calculated based on the FPKM method (Trapnell et al. 2010). The FPKM method is reliable as an expression measure since it corrects for biases in total gene exon size and normalizes to the total number of short-read sequences obtained in each library (Mortazavi et al. 2008).

Quantitative real-time PCR (qRT-PCR) analysis

Total RNA was isolated as described above. Purified RNA was reverse transcribed by the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific) according to the manufacturer's protocol. Q-PCR was performed with SYBR® Green Realtime PCR Master Mix (Toyobo Co. Ltd.). The intensity of fluorescence was quantified using an RT-PCR detection system of Eppendorf Mastercycler ep realplex (Eppendorf Company). The following standard thermal profile was used for all PCR experiments: 95 °C for 2 min; 40 cycles of 95 °C for 15 s, annealing temperature of 55 °C for 15 s and an extension temperature of 72°C for 30 s. After that, the PCR products were heated to denaturation at 90 °C for 15 s, followed by cooling down to 60 °C for 15 s to facilitate the heteroduplex formation, and was then heated to 95 °C at 20 °C min^{-1} and incubated for 15 s. Fluorescence signals were captured at the end of each cycle, and the melting curve analysis was performed from 60 to 95 °C. Relative normalized expression was determined by the $\Delta\Delta C_T$ method (Schmittgen and Livak 2008) using 18S rRNA as internal control. Primer pairs used for qRT-PCR analyses are provided in **Supplementary Table S5**.

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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